RELATIONSHIP BETWEEN FMRP EXPRESSION IN CELL FROM HAIR ROOTS AND BLOOD SMEARS WITH COGNITIVE FUNCTIONING IN FRAGILE X FULL MUTATION FEMALE

HUBUNGAN ANTARA EKSPRESI FMRP DI SEL AKAR RAMBUT DAN HAPUSAN DARAH DENGAN FUNGSI KOGNITIF PADA WANITA FRAGILE X FULL MUTASI

A Thesis
Submitted to fulfill the assignment and fit out requisite in passing S2 program

Magister of Biomedical Science

Lantip Rujito
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The thesis:

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has been defend in front of thesis committee
in 19th January, 2009.

Supervisor I	Supervisor II
Prof. Sultana MH Faradz, MD, PhD	Rob Willemsen, PhD
NIP: 130 701 415	

Recognized by
Head of Magister of Biomedical Program
Post Graduate Program Diponegoro University

DR.dr.Winarto SpMK,SpM
NIP. 130 675 157

Declaration

I hereby declare that this thesis 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 acknowledgment has been made in the text.

Semarang, January 2009

Lantip Rujito

CURRICULUM VITAE

I. Personal Information

Full Name : Lantip Rujito
Mail address : Jl Dr.Gumbreg No 1 Purwokerto 53146, Jawa Tengah, Indonesia
Telephone : 081548803168
Office telephone : 0281641522
Facsimile : 0281631208
Date of Birth : 04 November 1978
Married status : married
Offspring : 2
E-mail : rujitoku@yahoo.com
Place of Birth : Kebumen
Sex : male
Nationality : Indonesia
Ethnicity : Javanese

II. Educational background

School	Name of school	enterance	completion
Elementary	SD III Candirenggo	1985	1991
Junior High School	SMP N Ayah	1991	1994
Senior High School	SMU N Gombong	1994	1997
University	FK UNS	1997	2001
	Physician internship	2001	2003

III. Employment record

Name of organization	Year of employment	Period of employment	Position	Type of work /field
Medical Faculty of Jenderal Soedirman University.	2004 - now	4 years	Lecturer	Education

AKBID Paguwarmas	2005	1 years	Lecturer	Education
AKPER Harapan Bangsa	2005 -2006	2 years	Lecturer	Education
AKPER PKU Gombong	2004 - 2006	2 years	lecturer	Education
Clinic of Fatimatuzzahra	2004	1 years	Physician	Health Service

IV. Research activities

1. Pola Finger Print pada penderita Acut Miocard Infarct, *Mandala of Health.Vol 3* (2005)
2. Hibridisasi Anopheles barbirostris Koloni Flores dan Jawa Tengah, Presented in National Seminar of Parasitologi, Purwokerto, (2005)
3. Kadar MDA (Malondialdehid) Penderita Jantung Koroner di RSUP dr Sarjito Yogyakarta, *Mandala of Health Vol 3*.(2006).
4. Efectivity of Mahkota Dewa Boiling Water for Blood Glucose Level Documented in library of Jenderal Soedirman University.
5. Pola Finger Print pada penderita Schizophrenia, presented in PPAI,(2007)

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Semarang, January 2009

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List of Abbreviations

aa	: amino acid
AGG	: Adenin Guanin Guanin
ADHD	: attention deficits hyperactivity disorders
AMPA	: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
CGG	: Citosin Guanin Guanin
DNA	: Deoxyribonucleid Acid
dCTP	: deoxycytidine Triphosphate
Eag I	: *Enterobacter agglomerans*
EDTA	: ethylenediamine tetra acetic acid
FMR1	: fragile X mental retardation 1
FMRP	: fragile X mental retardation 1 protein
FXTAS	: fragile X-associated tremor/ataxia syndrome
FraX A	: fragile X A
Hind III	: *Haemophilus influenzae* Rd
IQ	: Intelligence Quotient
Kb	: Kilo basepair
KH domains	: K homology Domain
kDa	: kilo Dalton
LTD	: long term depression
LTP	: long term potentiation
MAP1B	: microtubule-associated protein
MR	: mental retardation
mGluR5	: metabotropic glutamate receptors 5
mGluR1	: metabotropic glutamate receptors 1
mRNA	: messenger RNA
mRNP	: messenger Ribonuclei Protein

PCR	: Polymerase Chain Reaction
POF	: Premature Ovarian Failure
PBS	: phosphate buffered saline
RNA	: Ribonucleid Acid
rRNA	: ribosom RNA
tRNA	: transport RNA
UTR	: Untranslated Region
uL	: micro Liter

Abstract

Introduction: Fragile X syndrome is the most common form of inherited mental retardation in humans, caused by an expansion of the (CGG) repeat in the *FMR1* gene located on X chromosome. Males carrying a full mutation are always mentally retarded, whereas females carrying a full mutation show mild to moderate mental impairment in only approximately 60% of cases, due to the X-inactivation process. The absence of the *FMR1* gene product, FMRP, in the brain is hypothesized as causative for the observed mental retardation in fragile X patients. Antibody tests have been developed to identify fragile X patients, based on the presence or absence of FMRP in both lymphocytes and hair roots. It has been proposed that FMRP expression in hair roots from females carrying a full mutation may be used as a prognostic value for the mental capacities. Hair roots originate from the ectoderm, like brain tissue, and may therefore show a better correlation between FMRP expression and cognitive functioning than FMRP expression in lymphocytes, which originate from the mesoderm.
Objectives: This study focussed on the relationship of the antibody test for cognitive functioning in females carrying a full mutation using either hair roots or bloodsmears.
Method: Thirty samples from females carrying either a normal, premutation or full mutation were included in this study. All subjects were selected from an Indonesian fragile X family. The analyses were performed in a blind fashion. All subjects were tested for FMRP expression in lymphocytes and hair roots using the antibody test. Classification of the genotypes of the subjects was obtained by Southern blot analysis. IQ equivalent were determined using Standard Progressive Matrices (SPM) from Raven, a non-verbal IQ-test. Statistical analysis to show the correlation of FMRP expression and IQ equivalent was developed using Pearson correlation.
Result: We report a significant relationship between FMRP expression in hair roots and cognitive functioning in female carriers of the fragile X full mutation ($r=0.64$, $p=0.015$) whereas in lymphocytes no significant relationship could be established ($r=0.31$, $p= 0.281$). We also reported that FMRP expression in blood smear was relatively higher than hair roots. We also demonstrated in our study group that IQ equivalents were about 14 % in normal range, 7 % in the borderline range and 79 % in mild mental impairment range.
Conclusion: Although the size of our study group was limited the results suggest that FMRP expression in cell of hair roots has stronger correlation for cognitive functioning in females carrying a full mutation than FMRP expression in bloodsmears.

Keywords: fragile X syndrome, females full mutation, FMRP, cognitive functioning

Abstrak

Pendahuluan: Sindrom *Fragile X* adalah bentuk retardasi mental diturunkan yang paling sering ditemukan pada manusia, yang disebabkan oleh perpanjangan kodon CGG di gene *FMR1* yang berlokasi di kromosom X. Pria pembawa alel full mutasi umumnya akan menderita retardasi mental sedangkan wanita pembawa full mutasi menunjukan gejala retardasi mental dari tingkat rendah sampai sedang sekitar 60 % dari kasus, karena proses inaktivasi kromosom X. Ketidakadaan produk gen *FMR1* yaitu FMRP, dipercaya sebagai penyebab dari kejadian retadasi mental pada pasien-pasien sindroma fragile X. Tes antibody untuk mengidentifikasi pasien sindrome fragile X telah dikembangkan berdasarkan pada ada atau tidak adanya ekspresi protein FMRP pada limposit dan akar rambut. Ekspresi protein FMRP pada akar rambut dari wanita pembawa alel full mutasi telah dilaporkan dapat digunakan sebagai nilai prognostik untuk menilai kapasitas mental. Akar rambut yang berasal dari jaringan ektoderm seperti halnya jaringan otak diharapkan menunjukan korelasi yang lebih baik antara ekspresi FMRP dan fungsi kognitif dari pada ekspresi FMRP di limposit yang berasal dari jaringan mesoderm.

Tujuan: Studi ini memfokuskan pada hubungan test antibodi dengan fungsi kognitive pada wanita fragile X pembawa kelainan full mutasi dengan menggunakan jaringan akar rambut atau hapusan darah.

Metode: Tiga puluh sample baik yang normal, pembawa kelainan premutasi atau fullmutasi diikutkan dalam studi ini. Semua subjek diseleksi dari keluarga yang diketahui mempunyai riwayat sindrom *fragile X*. Analisis dilakukan dengan metode blind fashion. Semua subjek dilakukan tes untuk menunjukan ekspresi protein FMRP pada limposit dan akar rambut menggunakan tes antibody. Klasifikasi genotip dari subjek didapat dengan menggunakan analisis Southerb blot. IQ equivalent dari subjek ditentukan dengan menggunakan Standard Progressive Matrices (SPM) dari Raven, yang merupakan tes IQ non-verbal. Analisa statistik untuk menunjukan korelasi antara ekspresi FMRP dan IQ equivalent digunakan korelasi Pearson.

Hasil: Studi menunjukan korelasi yang signifikan antara ekspresi FMRP pada akar rambut dan fungsi kognitif pada wanita pembawa kelainan full mutasi ($r=0.64$, $p=0.015$), sedangkan pada ekspresi FMRP di limposit tidak menunjukan korelasi yang signifikan ($r=0.31$, $p= 0.281$). Studi ini juga menunjukan bahwa ekspresi FMRP di darah apus relatif lebih tinggi dari pada akar rambut. Distribusi IQ equivalent pada keseluruhan subjek adalah kurang lebih 14 % di kisaran normal, 7 % di kisaran borderline dan 79 % di kisaran sedang.

Kesimpulan: Meskipun jumlah subjek dari penelitian ini sedikit, hasil penelitian menunjukan bahwa eksresi FMRP pada sel akar rambut menunjukan nilai korelasi yang lebih kuat untuk fungsi kognitif pada wanita pembawa kelainan full mutasi dari pada ekspresi FMRP di hapusan darah.

Kata kunci: Sindroma fragile X, wanita pembawa full mutasi, FMRP, fungsi kognitif

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CHAPTER I

INTRODUCTION

1.1 Background

The fragile X syndrome is the most prevalent inherited form of mental retardation in human. The prevalence of this disease is approximately 1/4000 for males and 1/6000 for females in Western countries[1]. In Indonesia the population of fragile X syndrome is approximately 2 % of the mental retardation population, and in Central Java thus far more than 50 patients have been identified [2,3]. Causative location of fragile X syndrome is in fragile X mental retardation 1 (*FMR1*) gene which is located on the long arm tip of X chromosome. The vast majority of mutations is an amplification of a trinucleotide (CGG) repeat in the 5' non-coding region of the *FMR1* gene. Normal people have between 5 and 55 CGG repeats, carriers of the premutation have between 55 and 200 repeats, and full mutation subjects have more than 200 CGG repeats in their *FMR1* gene [4]. Full mutation expansion causes hypermethylation of the *FMR1* promoter and expanded repeat itself resulting in no *FMR1* protein (FMRP)[5].

The absence of FMRP in the neurons is responsible for the observed mental retardation in fragile X patients. Recent studies suggest that fragile X syndrome is caused by exaggerated response upon signaling through group 1 metabotropic glutamate receptors (mGluR1 and mGluR5)[6]. A basic mechanism underlying learning and memory is the ability of neurons to respond to signals by local translation of new

proteins at the connections between neurons (synapses), thus fine-tuning these connections. In a normal brain, increases in the rate of protein synthesis caused by synaptic activation of either mGluR1 or mGluR5 are balanced by FMRP (repressor). Since the fragile X patients lack FMRP, target mRNAs of FMRP are translated massively.

Males carrying the full mutation are ussually cognitively affected, whereas females carrying a full mutation show mild to moderate mental impairment in only approximately 60% of cases. Females carrying a full mutation are characterised by cells with and without FMRP expression, which can be explained by the presence of two X-chromosomes and random inactivation of one of the X chromosome (Lyonisation). Thus, in cells without FMRP expression the normal *FMR1* allele is inactivated, whereas in cells with FMRP expression the mutant *FMR1* allele is inactivated. It has been suggested that an insufficient number of FMRP expressing neurons in the brain in affected females causes the learning deficits as a result of the proportion of mutant *FMR1* alleles on the active X chromosome[7].

There are several methods to identify patients with fragile X syndrome. Molecular based diagnostics have been developed to obtain the reliable methods [8]. This DNA test allows the accurate determination of the length of the CGG repeat. PCR analysis can be used to amplify normal and premutation repeats, however it fails to amplify repeat sizes in the full mutation range. PCR-based diagnostics is also not suitable for the diagnosis of fragile X females as they are heterozygous and would be scored as normal, because of amplification of the normal-sized allele. Mozaic patients

with both premutation and full mutation alleles can also cause a false negative. Southern blot allows the accurate determination of the length of the CGG repeat. Methylation sensitive enzymes are used to determine whether the *FMR1* gene consists of methylated or unmethylated normal, premutation, or full mutation alleles either in male or female patients.

Willemsen and collegues, in 1995, have described alternative diagnostic tests to identify patients with fragile X syndrome on the basis of the absence of FMRP in lymphocytes and hair roots. This antibody test can identify persons with fragile X by means of only 1 or 2 drops of blood or 10-20 hair roots. Advantages of this antibody test, compared with the DNA test, include the following: (1) it can be performed in a single day and the costs are low; (2) it does not require radioactivity; and (3) it detects all loss-of-function mutations, including the prevalent CGG repeat amplification. Some males with fragile X show only a premutation *FMR1* allele and not a full mutation on Southern blots [9,] In these cases the antibody test is often used to identify patients with a mosaic pattern (both premutation and full mutation present) because individual cells or hair roots can be scored for the presence of FMRP expression.

The origin of hair roots from the ectoderm may explain why a better correlation exists between (the lack of) FMRP expression in hair roots from females carrying a full mutation and cognitive functioning compared to FMRP expression in lymphocytes [10]. Therefore, hair roots might be of value for predicting the mental capacities of females with a full mutation. It is likely that the X-inactivation pattern

within the ectoderm during early development will give rise to similar X-inactivation patterns in both brain and hair roots.

Based on previous research, Semin area is one of sub district in Gunung Kidul which has large population of fragile X. It is interesting to carry on further study related the FMRP and cognitive functioning of the female fragile X full mutation.

1.2 Research question

Is there a relationship between FMRP expression in cell of hair roots and blood smears with cognitive functioning in female fragile X full mutation?.

1.3 Objectives

1.3.1 General

These studies demonstrate a relationship between FMRP expression in hair roots and blood smears with cognitive functioning in the female fragile X full mutation.

1.3.2 Specific

1. To compare the magnitude of correlation between FMRP expression in hair roots and blood smears with cognitive functioning in the female fragile X full mutation.
2. To asses IQ-levels between female fragile X full mutation in an Indonesian cohort.

1.4 Benefits

1. To demonstrate diagnostic value of the antibody test for mental capacities in female carriers of the full mutation Fragile X.
2. To demonstrate the simply, rapid and inexpensive test for screening program of fragile X syndrome using non invasive materials from hair roots.

1.5 Originality of the Research

Researches	Publications	Year	Places	Result
Willemsen R, *et al*	Rapid antibody test for fragile X syndrome. *Lancet* 345: 1147–1148	(1995) .	Netherlands	The diagnostic power of the antibody test in blood smear is good for males, whereas the results are less specific for females.
Willemsen R *et al*	Rapid antibody test for diagnosing fragile X syndrome: a validation of the technique. *Hum Genet* 99:308–311	(1997)	Netherlands	The diagnostic power of the antibody test in blood smear is good for males, whereas the results are less specific for females
Tassone F *et al*	FMRP expression as a potential prognostic indicator in fragile X syndrome, *Am J Gen Genet*, 84:250-261	(1999)	USA	FMRP expression may have potential as a prognostic indicator in males with fragile X syndrome

Faradz SMH *et al*	A High rate of fragile X in a small district of Indonesia can be traced back to one common ancestor, The 8th International fragile X conference, Chicago, July 17-21	(2002)	Indonesia	53% of the male and female fragile X patients in an institution of isolated village in central Java could be retraced to one ancestor.
Willemsen R *et al*	Predictive testing for cognitive functioning in female carriers of the fragile X syndrome using hair root analysis, *Med Genet*;40:377–379	(2003)	Netherlands	FMRP test on hair roots might be strong prognostic indicator of cognitive functioning in female full mutation carriers and has great potential as a predictive test.

Chapter II

Literature Review

2.1 Introduction to The Fragile X Syndrome

Fragile X syndrome is the most common cause of mental retardation, after Down syndrome, worldwide. In 1943, Martin and Bell described the first extended kindred with mental retardation segregating in an X-linked manner. Fragile X is named after a site on the long arm of the X chromosome that is elongated and appears partly broken or ”fragile“ using cytogenetics analysis. The spectrum of fragile X syndromes ranges from normal development to developmental delay, mild to severe intellectual disabilities, autistic-like behaviour and attention problems.

Prevalence of fragile X syndrome in the world is not precisely known. Turner and colleagues reported a prevalence of 1/4000-1/6000 for males in Western countries [1]. Based on the best available evidence, the National Fragile X Foundation indicates that approximately 1 in 3600 to 4000 males in the world are born with the full mutation for fragile X syndrome. Approximately 1 in 6000 females in the world is born with the full mutation for fragile X syndrome. Approximately 1 in 800 men in the world are carriers of the fragile X premutation and approximately 1 in 260 women in the world are carriers of the fragile X premutation.[11] Using several studies, Hagerman estimated that current frequency of full mutation females is ~ 1/2500 [12]. In Taiwan it has been reported that in the screening program they found 1/1674 premutation allele, whereas in Singapore, 2.4 % cases from screening of mentally

retarded people was found [13,14]. In Indonesia the population of fragile X syndrome is approximately about 2 % from the total mental retardation population, and in Central Java so far more than 50 patients have been identified [2,3]. In the same study 53 % males and females with fragile X in institution of an isolated village in Central Java could be traced to have one ancestor [15]. Another study in a special school for mentally retarded individuals, revealed 3,9% from the total MR population [16.]

Dysmorphic features are variable in both males and females and are not always present. The classic features include elongated face with prominent jaw, which are more obvious after puberty, prominent and large ears, high arched palate, large testicles, which are mainly seen after puberty, recurrent ear infections, flat feet, loose joint, congenital hip dislocation, scoliosis, strabismus, and seizures [17].

Affected individuals may show psychiatric disorders, including attention deficits hyperactivity disorders (ADHD), autistic features [18] such as gaze avoidance (poor eye contact), shyness, hyperactivity, hand flapping, hand biting, and tactile defensiveness. Neurologic abnormalities that are occasionally seen include strabismus, nystagmus, and epilepsy. Seizure may be seen in ~ 17 % fragile X males based on electrophysiology data [17]

The clinical phenotype is highly variable which may reflect variable expression of the mutation (mosaic pattern). The hallmark of the fragile X phenotype is mental retardation. Males with a full mutation are always mentally retarded and may show typical physical and behavioural features. Females with fragile X are usually less severely affected than males, probably as a result of X inactivation. There are no

obvious physical characteristics of females with fragile X, and likewise mental impairment and behavioural problems are usually milder. Sixty percent of females carrying a full mutation have cognitive impairment varying from mild to borderline [17,19].

Premutation male and female carriers usually have no physical features and negative cytogenetics analysis, however , ~20% of female PM carriers manifest premature ovarian failure (POF) defined by menopause before 40 years. POF represents the final stage of a variety of diseases that result in the loss of ovarian follicles [20]. Older males carrying a PM (ranging between 71 and 135 CGGs, to date), may exhibit an unique neurodegenerative syndrome characterized by progressive intention tremor and ataxia named fragile X-associated tremor/ataxia syndrome (FXTAS). More advanced cases may be accompanied by memory and executive function deficits, anxiety, Parkinsonism, peripheral neuropathy, essential tremor and autonomic dysfunction [21].

2.2 *FMR1* gene mutations mechanism

In the vast majority of cases fragile X syndrome is caused by an unique form of dynamic mutation due to a stretch of CGG repeats in the 5' non coding region of the *FMR1* gene. This trinucleotide repeat is highly polymorphic and the expansion may occur as a multi-step process over many generations [4].

There are four class of alleles observed in the *FMR1* gene, according to the length of CGG repeat. The first group contains alleles ranging between 5 and 40

CGG repeat units. Repeats of this size remain stable upon transmission. A second class of alleles, largely overlapping with wild-type in size, show 40-55 CGG repeats and are called "gray zone alleles" or protomutations. Protomutations are only slightly unstable over many generations and never expand to a full mutation. They are transmitted silently across generations, sometimes adding (or subtracting) just a few repeats in length and creating a 'pool' of at risk alleles in the population that eventually can become premutation. The third class is called premutation. It may become unstable upon maternal transmission to the next generations, and can increase or decrease in length upon transmission to the next generations. The fourth class is called full mutation (>200 CGG) that usually leads to hypermethylation of both the promoter region and CGG repeats of the *FMR1* gene. The gene is transcriptionally silenced, and consequently, *FMR1* Protein (FMRP) is absent [22]

Alleles ranging between 55 and 200 CGG repeats are classified as premutations. Carriers with premutations alleles were usually considered of having normal gene function, including normal *FMR1* mRNA and FMRP levels, and normal cognitive abilities [23]. However, a number of reports have shown physical and psychological problems in males and females carrying premutations [17, 24]. Interestingly, carriers of a premutation show a consistent increase of *FMR1* mRNA levels (2-8 fold), despite reductions in FMRP levels, in their lymphocytes [25].

Expansion to a full mutation (over 200 CGG repeats) usually leads to an absence of *FMR1* Protein (FMRP). In males, the methylated full mutation is always associated with mental retardation, whereas approximately 60% of females with a full

mutation have only mild mental impairment. In females the situation is more complex because their cells contain two X-chromosomes. Dosage compensation in somatic cells of females is necessary for the expression of equal amounts of X-linked genes, which is achieved by inactivation of one of two X-chromosomes. The process of X-inactivation during embryonic development occurs shortly after blastocyst implantation [26]. Once X-inactivation is established, it is maintained during further cell proliferation and differentiation of the embryo. The choice of which X-chromosome is inactivated is a random process. Studies on monozygotic twins support this fact. At 2000, Willemsen and colleagues have reported about monozygotic twin sisters, who have a different phenotypic pattern (normal and learning disabilities). Both of them are full-mutation, based on DNA analysis, but one sister has normal FMRP expression and normal cognitive functioning, whereas the other sister show a lack of FMRP expression and presented with learning disabilities. It has been concluded that timing of X-inactivation process occurs earlier in development than inactivation of the full-mutation allele [27].

2.3 Mechanism of CGG repeat expansion

The exact mechanisms causing repeat instability is not clearly understood. All short tandem repeat undergo small changes over generations. These changes involve one or two repeat units and result in what is called a “polymorphism”. These variabilities are observed within the normal alleles. Premutations show more instability than other polymorphisms. Contractions and expansion involving 10-20

triplets occur quite frequently. Once a certain threshold is passed, the repeats behave very unstable, resulting predominantly in large expansion. In females carrying expanded CGG repeats, their length increases over generations. Once beyond a specific threshold the repeat becomes dramatically unstable, expanding rapidly up to a few thousand triplets. This phenomenon has given rise to the term 'dynamic mutation' [28].

The mechanisms which have been proposed to play a role in repeat instability involve meiosis as well as DNA replication, recombination and repair. During all these processes, duplication of the DNA occurs, allowing an opportunity for DNA mutations and secondary structures like hairpins or tetraplex to form, errors which must be removed by proof-reading and repair processes. These processes, which aim to keep mistakes in the copying of DNA to a minimum, are balanced by processes in meiosis such as recombination which generate maximum genetic variation for a species [29].

The presence of cryptic AGG interruptions within the CGG repeat led to suggestion that these interruptions might provide stability to the repeat and that instability might result from loss of AGG interruptions. Analysis of the AGG repeat interruptions showed that in most common normal alleles, AGG interruptions are found downstream of 9 or 10 CGG triplets. In longer repeat, relatively fewer AGG interruptions are found. Loss of these AGG interruptions always occurs at the 3' end of the repeat tract. The longest pure CGG tract found within a premutation or full

mutation is always located at the most 3' end. It is believed that a pure CGG tract at the 3' end of more than 38 repeats is enough to cause instability [30].

Another theory for the instability is focused about formation of d(CGG) secondary structures. Several structures of the CGG repeat, such as hairpins and tetrahelical structures have been suggested. The stability of the different structures seems to differ between both templates. Pausing of DNA polymerase at both hairpin and tetrahelical structures has been observed both in vitro and in vivo [31,32]. This stalling of the action of DNA polymerase might increase the probability of DNA polymerase slippage. According to the slippage model, this may result in expansion of the CGG repeat.

2.4 Timing of the CGG repeat expansion

Timing of the CGG repeat expansion is not known clearly; two time periods have been suggested to be implicated, that is, meiosis and early embryonic development. The anticipation in each succeeding generation of fragile X families indicates that expansion must occur in germ cells (either during premeiotic or meiotic division). Malter and colleagues have shown that a full mutation is present in oocytes from a female foetus with a full mutation. There is strong, albeit indirect, evidence against a postzygotic transition to a full mutation [33].

A kind of selection against full mutations is proposed for the male germ line. In mature sperm cells no FMRP is found, whereas in primordial germ cells there is clear expression of FMRP. Immunohistochemistry of the testes of full mutation foetuses

showed only a few primordial germ cells during early development that produces FMRP, which suggests the presence of a premutation in these FMRP-positive cells. During later development, the number of primordial germ cells expressing FMRP in full mutation male foetuses increases. This suggests that a selection process is present during embryonic spermatogenesis [33].

2.5 Fragile X Mental Retardation Protein (FMRP)

FMRP is the gene product of the *FMR1* gene on chromosome Xq 27.3, termed FraX A. The *FMR1* gene contains 17 exons spanning 38 kb. Splice donors and acceptors located in the 5' portion of the gene demonstrated greater adherence to consensus than those in the 3' end, providing a possible explanation for alternative splicing in the *FMR1* gene. The gene, expressed as a 4,8 kb mRNA, which translates into a 614 aa polypeptide of 69kDa [34]. FMRP expression is widespread throughout the body, however it has been associated with abundant expression in neurons and with testicular expression in spermatogonia [35].

FMRP contains RNA-binding sequence motifs, including two KH domains and an RGG box. The precise physiological function of FMRP is still not defined; however, a role in transport and/or translational efficiency of mRNAs has been suggested elsewhere [36]. Recently, a subset of mRNAs containing a G quartet has been identified as a potential target for FMRP, including those for important neuronal proteins like microtubule-associated protein MAP1B and semaphorin [37]. It has been suggested that the absence of FMRP in neurons results in misregulation or

mistrafficking of a subset of mRNAs and that this is the basis of the mental retardation in fragile X patients.

Recent evidence shows that mRNA transport translation in dendrites plays an important role in neuronal processes, including synaptic plasticity, which is essential for memory storage and learning processes [38]. Local protein synthesis at the synapse may be an important aspect of proper synaptogenesis because most dendritic polyribosomes are located within or at the base of spines. The presence of the protein machinery, postsynaptically, allows neurons to rapidly respond to signals at particular synapses through local translation of (specific) mRNAs. For this purpose, efficient transport of specific mRNAs, via mRNP particles, has to be established to this cellular location followed by efficient translation of mRNAs in the vicinity of the synapse. The dynamics of the transport of mRNP particles in neurons have been studied by different experimental approaches, and a supramolecular complex containing mRNAs, translational factors, and ribosomal subunits and motorproteins has been identified [39].

Recent studies suggest that fragile X syndrome is caused by exaggerated signaling through group 1 metabotropic glutamate receptors (mGluR1 and mGluR5) [6]. A basic mechanism underlying learning and memory is the ability of neurons to respond to signals by creating new proteins at the connections between neurons (synapses), thus fine-tuning these connections. In a normal brain, synaptic activation of mGluR5 is balanced by FMRP, similar to the way the accelerator and brake

balance the speed of a car. However, fragile X patients lack FMRP, leading to excessive protein synthesis at the synapse.

2.6 Cognitive functioning and Mental Retardation

Cognitive function refers to a range of high-level brain functions, including the ability to learn and remember information: organize, plan, and problem-solve; focus, maintain, and shift attention as necessary; understand and use language; accurately perceive the environment, and perform calculations. There are many factors that involve in development of cognitive functioning including genetics, health status either prenatal, antenatal, or postnatal, chronic disease, nutrition, level of education, social interaction, and many others factors. Cognitive functioning usually is represented by degree of intellectual quotient (IQ) or ranking of a person's mental abilities.[40]

Cognitive functioning itself was proposed that it is based on the general intelligence factor (abbreviated *g*), terms used in the field of psychology to quantify what is common to the scores of all intelligence tests. The two main components of general cognitive ability (g) were those identified by Spearman in 1923 [41] are respectively: (a) eductive ability (from the Latin educere, meaning ‘‘to draw out’’), the ability to make meaning out of confusion, the ability to generate high-level, usually nonverbal, schemata which make it easy to handle complexity; and (b) reproductive ability—the ability to absorb, recall, and reproduce information that has been made explicit and communicated from one person to another.

Cognitive disability that commonly termed as mental retardation (MR) is defined as significant subavarage intellectual function existing concurrently with deficits in adaptive behavior and manifested during the developmental period [42]. Etiology of mental retardation can be caused by many factors such as genetic, environmental and ecogenetic factors. The diagnostics process is considerably if the timing of development insult can be determined: prenatal, perinatal, and postnatal.

Table 1. Diagnostic categories of Mental Retardation[42]

Diagnostic categories	%
Chromosomal abnormalities	4 -28
Recognizable of syndromes	3 - 9
Nervous system abnormality	3 - 17
Complications of prematurity	2 - 10
Perinatal condition	8 - 13
Environmental/ teratogenic conditions	5 - 13
Metabolic/endocrine causes	1 - 5
Unknown	30 - 50

Mental retardation is usually referred by two conditions both of a person's level of intelligence measured by Intelligence Quotient (IQ) test and adaptive skills. The term "adaptive skills" means how well a person can deal with the tasks of everyday life. These tasks include the ability to speak and understand; home-living skills; use of community resources; leisure, self-care, and social skills; self-direction; basic academic skills (reading, writing, and arithmetic); and work skills [43]. Classification of the degree of Intelligence quotient (IQ) from mental-health

professionals has been used in different area. Table 2 and 3 below depict on both educational and psychiatry utility.

Table 2. IQ Classifications in Educational Use.[44]

Classification	IQ Score	Percent Included	
		Theoretical Normal Curve	Actual Sample
Very Superior	130 and above	2.2	2.1
Superior	120-129	6.7	8.3
High Average	110-119	16.1	16.1
Average	90-109	50.0	50.3
Low Average	80-89	16.1	14.8
Borderline	70-79	6.7	6.5
Extremely Low	69 and below	2.2	1.9

Table 3. IQ Classifications in Psychiatric Use [45]

Classification	IQ Score
Borderline Intellectual Functioning	IQ 71-84
Mild Mental Retardation	IQ 50-55 to approximately 70
Moderate Retardation	IQ 35-40 to 50-55
Severe Mental Retardation	IQ 20-25 to 35-40
Profound Mental Retardation	IQ below 20 or 25

2.7 Mental Retardation and FMRP

The molecular basis of the phenotypic variability in both males and females of fragile X patients is believed to be linked to the variable number of neurons in the brain that express FMRP. In males a methylated full mutation in their cells will always lead to mental retardation; however 13% of males with fragile X syndrome are high functioning with an Intelligence Quotient (IQ) score>70 [46]. Most high

functioning males show either partial methylation of the full mutation or a variable combination of premutation and full mutation alleles [47,48]. In contrast, approximately 60% of the full mutation females show cognitive deficits. The spectrum of phenotypic involvement varies from mental retardation to mild learning disabilities and/or emotional difficulties without notable physical involvement [17,19].

FMRP has been hypothesized as an RNA binding protein that is involved in the regulation of translation of many other transcripts in neurons [49]. Lack of FMRP in fragile X patients causes disregulation of the FMRP target transcripts. FMRP typically acts as a translation suppressor that is involved in synaptic plasticity through regulating local protein synthesis of specific target mRNAs in response to synaptic stimulation. FMRP binds to transcripts either in the cell soma or in the nucleus and subsequently these transcripts are transported to the synapse using a kinesin motorprotein[50]. After stimulation of the metabotropic glutamate system, FMRP mediates the translation of important transcripts for synaptic structural changes or synaptic plasticity [50,51].

One of the important pathways for cognitive development is the metabotropic glutamate receptor 5 (mGluR5) pathway (a member of group 1 metabotropic glutamate receptors) which leads to long term depression (LTD), weakened synaptic connections and eventually synaptic elimination. LTD is important in normal brain development so that unstimulated synaptic connections can be eliminated. Through long term potentiation (LTP) other synaptic connections are made stronger with stimulation. The mGluR5 pathway requires protein synthesis and FMRP normally

regulates this protein synthesis. However, in the absence of FMRP, like in fragile X syndrome, LTD is dramatically enhanced leading to more weak, immature and elongated synaptic connections [6].

A number of clinical features including epilepsy, MR, hypersensitivity to tactile stimuli, social deficits, and even loose stools have been hypothesized to be related to enhanced mGluR5 activity and LTD in fragile X syndrome. This is important for clinicians to understand because these findings have direct therapeutic implications. Both mGluR5 antagonists and ampakines that stimulate the AMPA receptors are in clinical trial stages of development and they have the potential to be successful in specific treatments for fragile X syndrome in the future.

2.8 Diagnosis of Fragile X syndrome

Methods to identify patients with fragile X syndrome have been rapidly developed. Cytogenetic methods which identified the fragile site at the long arm of the X-chromosome using specific media are not reliable as a diagnostic tool. Cytogenetic analyses show higher false positive rates due to expression of other fragile sites close to fragile X syndrome locus. False negative results can also occur due to repeat length, an age related decreased or technical issues [52].

DNA tests allow the accurate determination of the length of the CGG repeat [8]. PCR analysis can be used to amplify normal and premutation repeat, however, it fails to amplify full mutation repeats. PCR is also not suitable for the diagnosis of fragile X females as they are heterozygous and would therefore be scored as normal because

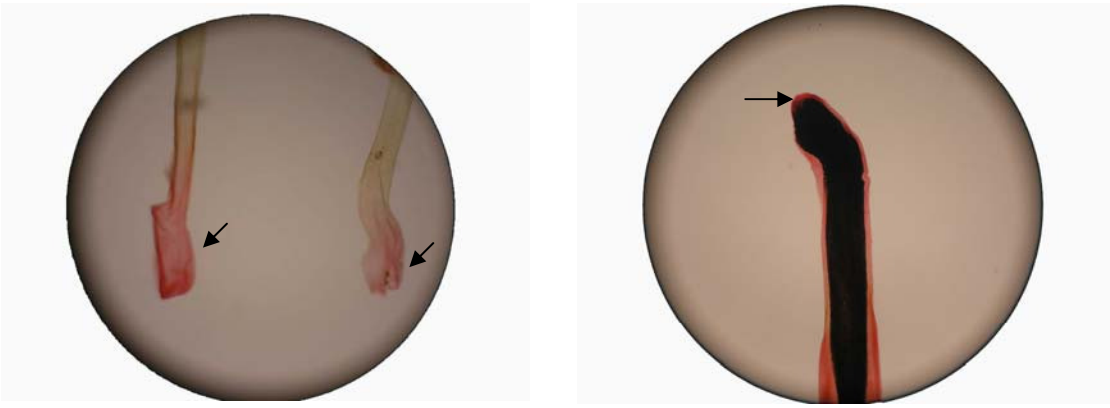
their notrmal allele will be amplified preferentially. Mozaic patients with normal or premutation alleles can also cause false negative results. Southern blot hybridization combined with methylation sensitive enzyme can be performed to determine whether the gene contains methylated or unmethylated alelles, either normal, premutation, or full mutation. Alternative diagnostic test for screening using immunohistochemistry method can also be performed to identify patients with fragile X syndrome on the basis of the absence of FMRP in lymphocytes and hair roots [9,10]

2.9 FMRP Investigation

It has been shown that high FMRP expression is present in the brain and testes. Low FMRP expression is present in blood cells; however FMRP can be detected in lymphocytes using specific enhancement steps in the immunocytochemical procedure. Willemsen and colleagues have described an alternative diagnostic test to identify patients with fragile X syndrome on the basis of the absence of FMRP in lymphocytes in fragile X patients. This antibody test can identify persons with fragile X by means of only 1 or 2 drops of blood [9,53].

Advantages of this antibody test, compared with the DNA test, include the following: (1) it can be performed in a single day; (2) it does not require radioactivity; and (3) it detects all loss-of-function mutations, including the prevalent CGG repeat amplification and other mutations. Nevertheless, a minority of fragile X patients still escape detection by DNA tests. Some males with fragile X show only a premutation allele and not a full mutation using Southern blot analysis. In this cases,

the antibody test is more reliable. The pictures of FMRP expresssion in hair roots is depicted in figures 1 below (courtesy from Rob Willemsen).

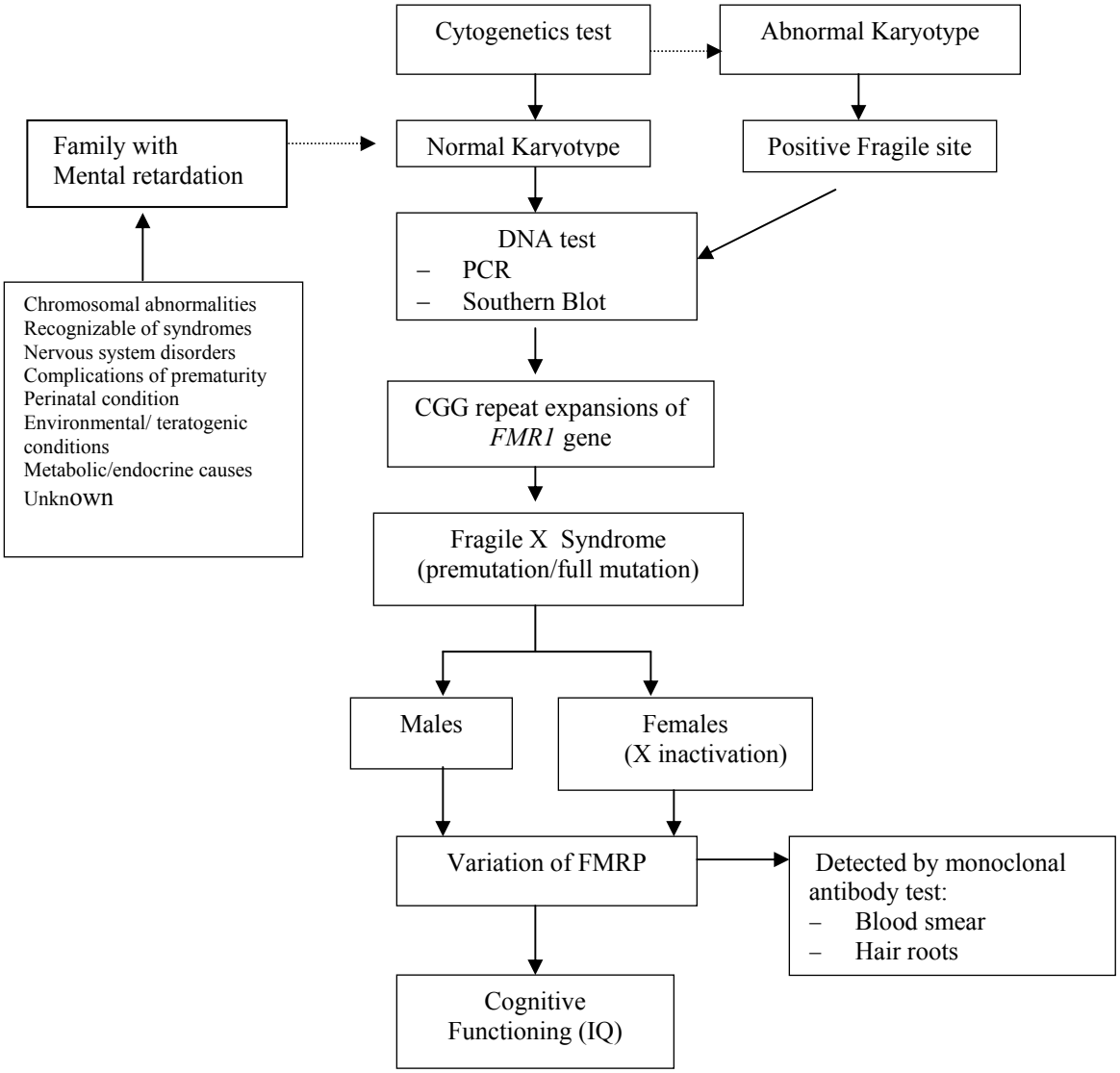


Figures 1. The difference of FMRP expression in cell of hair root follicle between blonde hairs and pigmented hairs**.**
Red appearance reflecting FMRP expression in the bulb of blonde hairs is more clearly visible than in pigmented hair follicles (arrow).

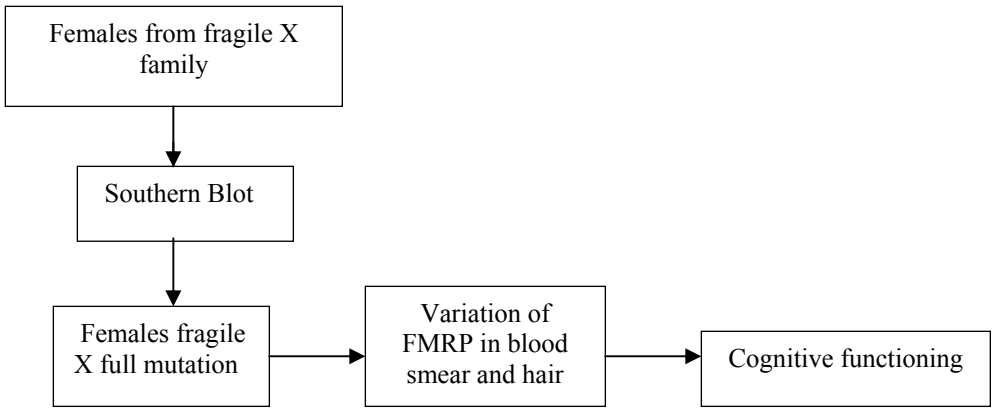
Testing hair roots allows the opportunity of testing a different tissue. Lymphocytes originate from connective tissue, which arises from the mesoderm germlayer. In contrast, both neurons and skin develop from the ectoderm. Most hair roots in affected males are devoid of FMRP. In cells from female full mutation carriers, one of the two X-chromosomes is inactivated and will not produce FMRP. As human hair roots are of clonal origin, they are labelled either positive or totally negative for FMRP expression, depending on which X-chromosome is active, the normal *FMR1* allele or the mutant *FMR1* allele. It has been suggested that hair root testing might be of value for predicting the mental functioning in female carriers of the full mutation, because like brain tissue, hair roots originate from the ectoderm during embryonic development. Thus, the X-inactivation pattern in hair roots might

be indicative of the X inactivation pattern in the brain and in this way reflects the number of neurones that express FMRP[10].

2.10 Theoritical Frame



2.11. Conceptual frame



2.12. Hypothesis

1. There is relationship between FMRP expression in hair roots and cognitive functioning in female carriers of the full mutation.
2. There is difference of correlation between FMRP expression in hair roots and blood smears with cognitive functioning in female carriers of the full mutation.
3. There is a variance value of IQ equivalents in female carriers of the full mutation.

Chapter III

Research Methods

3.1 Design

This research was conducted as cross sectional analysis.

3.2 Places and time

This research was conducted in three places as follow:

1. Semin village (Indonesia) as place of collecting samples
2. CEBIOR, Medical Faculty of Diponegoro University Semarang for DNA extraction and hair roots immuno-incubation/examination.
3. MGC Dept of Clinical Genetics, Erasmus University Rotterdam, The Netherlands as preservation study of hair roots, Southern blotting and blood smears examination. Period of research is from September 2007 until August 2008.

3.3 Subjects

3.3.1. Population

Population of this study is females from the fragile X family either affected or not affected.

3.3.2. Achievable population

Females of the fragile X family either affected or not affected in the District of Semin, Gunung Kidul regency, Yogyakarta, based on previous study [15] and one family in Purwokerto.

3.3.3. Females sample

We collected samples with the following criteria:

Inclusion: 1. Age range: 4 – 70 years old.

2. Agree to follow the research

Exclusion: 1. Malformation appearances

2. Down syndrome appearance

3. Refuse to follow the research

We then used total sampling method to include in this research.

3.4 Collecting materials.

After having informed consent we collected the venous blood and hair roots form the probands with the following:

1. Ten ml bloods were drawn and smears were immediately made from fresh blood. The remaining blood subsequently stored in the EDTA tubes for DNA extraction in the laboratory.
2. Hair roots were plucked smoothly from different area behind the ears using hands or specials pincet. It is due to better representative of limbic system and hippocampus area of the brain[40]. Hair roots then were placed in envelops with the name and code of patients.

3.5 Variable and Operational Definition

3.5.1 Variable definition.

1. FMRP: protein produced by *FMR1* gene on chromosome Xq27.3.

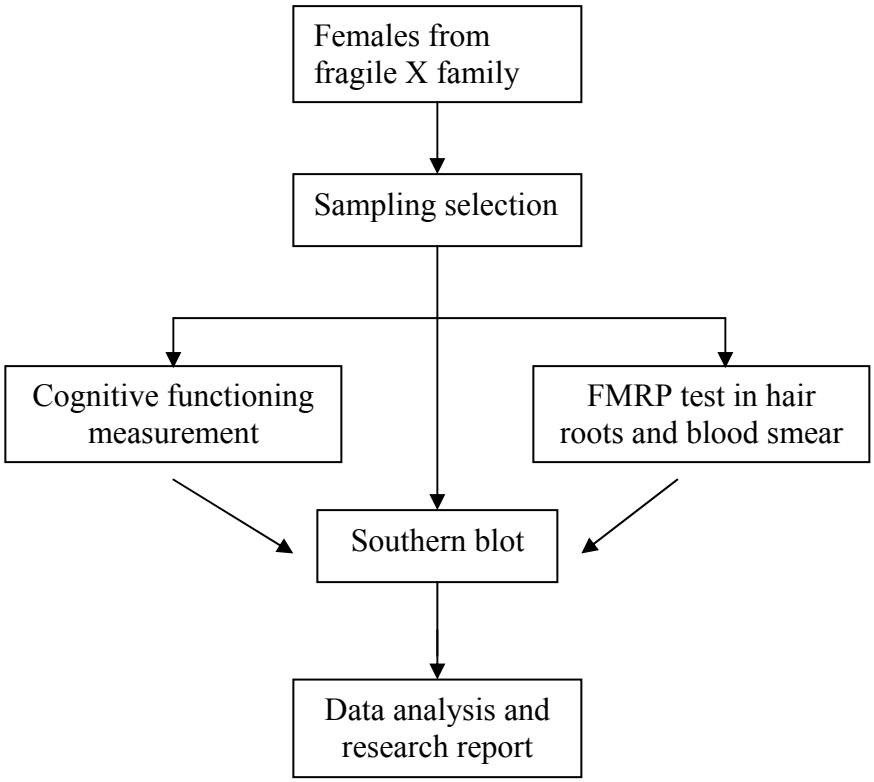
2. Cognitive functioning: basal intellectual function, which affects ability to think, reason, and learn.
3. Female fragile X full mutation: a condition that the 5' UTR region of *FMR1* gene of a female has over 200 CGG repeat expansions.

3.5.2. Operational definition

1. FMRP expression: percentage of positive-stained hair roots and blood smears using antibody test against FMRP. Blood smears and at least 20 hair roots were examined with Willemsen analysis procedure and counted by three different investigators who were unaware with the name of probands and the others result counting. Total counting from three different investigators was divided to get final value. Data is continuing scale.
2. Cognitive functioning: non-verbal Raven [54] test was applied to calculate IQ equivalent. Raven is International standard test to measure IQ equivalent, based on non-verbal intervention. Data is continuing scale.
3. Female fragile X full mutation: Southern blot analysis was implicated to determine genotypes of probands. This technique used a methylation sensitive enzyme (*Hind* III and *Eag* I) that allows us to discriminate between methylated and unmethylated *FMR1* alleles. Digoxigenine labelled probe pP2 and radioactive

labelled probe pAO365 were performed to show the genotypes of probands. Data is categorical scale.

3.6 Research Plot



3.7 Measurement Technique

3.7.1 General:

All persons have given written informed consent. After giving informed consent, individuals were visited at home or in school to measure cognitive functioning using Raven's Progressive Matrices. After completing the raven test, 10 ml heparinized peripheral blood was drawn from all subjects and subsequently bloodsmears were immediately made. After that, approximately

20 hair roots were plucked from different areas behind the ear on the scalp. Hair with visible bulbs and sheaths were selected and trimmed to just above the sheath. The hairs which were examined in Rotterdam were shipped directly after plucking. The hairs which were examined in Semarang were stored at room temperature after plucking day for next day experiment.

3.7.2 Preliminary study.

Preservation test for hair roots was done in Roterdam to test how long the hair roots can be stored at room temperature. Two series (periods) of hairs were completed from three different persons. The cut –off point was 75 % based on earlier report in literature [7] . Willemsen method was used to execute this test.

3.7.3 FMRP expression in Hair root.

Hair roots were fixed in 3% paraformaldehyde at room temperature (RT) for 10 min. Cells were permeabilized by treatment with 100% methanol at RT for 20 min. Hair roots were washed with phosphate-buffered saline, containing 0.15% glycine and 0.5% bovine serum albumin, and incubated as whole mount with mouse monoclonal antibodies against FMRP at 4 C degree for overnight. Visualization of antibody-antigen complexes was achieved by an indirect alkaline phosphatase technique, using α m-Powervision poly AP for 1 hr, followed by incubation in new fuchsin substrate–chromogen system (DAKO) for 10-15min. Levamisole was added in the substrate solution,

according to the guidelines of the manufacturer, to block endogenous alkaline phosphatase activity. Immunolabeled hair roots were examined with a stereo zoom microscope at a final magnification of 70 x. The number of FMRP-positive hair roots showing red color was expressed as a percentage of the total number of hair roots examined. Three investigators who were unaware of the phenotype of the individuals randomly numbered and scored all the hair roots for the presence or absence of FMRP. The mean of counting was used as a final data.

3.7.4 FMRP expression in blood smears

Blood smears were fixed in the 3% paraformaldehyde for 10 minutes followed by a permeabilization treatment with 100 % methanol for 20 minutes at RT. After washing in PBS– for 5 minutes, the endogenous peroxidase activity was blocked with PBS-Blocked (100 ml 0.1M PBS, 2 ml 30% H2O2, 1 ml 12.5% sodiumazide) for 30 minutes. Smears were washed in PBS+ containing 0,5 % BSA and 0,15 % glycin and incubated with mouse monoclonal antibody against FMRP at 4^{o} C for over night. Smears were rinsed in PBS+ for 3x5 minutes followed by biotinylated secondary antibody treatment for 10 minutes (Zymed-Kit "Reagent B). Subsequently, an incubation with peroxidase conjugated streptavidin for 10 minutes was performed after rinsed in PBS+ for 3x5 minutes. Finally, smears were rinsed in PBS+ for 4x5 minutes and PBS– for 5 minutes, respectively. As final step,

smears were incubated with DAB substrate (DAKO liquid DAB substrate-chromogen system) for 2x20 minutes and 1x10 minutes followed by Nuclear fast Red as counterstaining. A serial dehydration in ethanol and xylen is performed before mounted with Entellan. Smears were dryed for overnight at 37^{o} in an incubator. Immunolabeled smears were examined using a lightmicroscope at final magnification 1000 x. The brown precipitate in the cytoplasm of lymphocytes illustrated positive FMRP expression. The percentage of FMRP expression was achieved by counting hundred lymphocytes in different areas and scored them for FMRP expression.

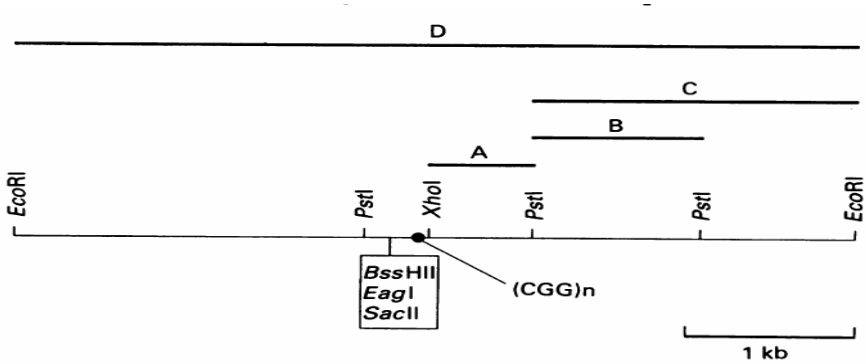
3.7.5 Salt Saturation Method for DNA extraction [55]

EDTA frozen blood was mixed with 5-10 ml NH4Cl lysis buffer for 10-30 minutes at RT. Solution was centrifuged for 5 minutes at 3000-3500 RPM and supernatant was discharged. NH4Cl lysis buffer was added followed by centrifugation for three times. White pellet was resuspended in 2 ml TE lysis buffer, Proteinase-K and 100 ul 10% SDS and incubated at 50^{o}C for 24 hours. Suspension was added with NaCl 6M approximately one third volume and centrifuged at 4000 RPM for 10 minutes. A new tube was used for supernatant and mixed with 100% ethanol twice the volume of the supernatant. DNA was washed with 70% ethanol, dried and subsequently blended with TE buffer.

3.7.6 Southern Bloot Analysis using digoxigenine labelled probe pP2

Seven mg of the genomic DNA (35ul of 200 ng/ul) was mixed well with 4.75 ul aquadest, 5 ul 10x SuRe/Cut Buffer H (Roche), 3 ul 50 mM spermidine, 1.25 ul *Hind* III which has recognition site AAGCTT (Roche cat #10656313001), 1 ul *Eag* I which has recognition site CGGCCG (Biolabs cat # R0505L). The mix was incubated for overnight at 37 ^{0} C to complete digestion. Five ul 10x Ficoll loading buffer was added to 45 ul of each digestion product and electrophoresed on a 0.7% agarose gel for overnight at 45 V. DNA was transferred to a nylon membrane (Hybond N1, Amersham) in Southern blotting apparatus for overnight followed by baking the membrane 20 minutes at 120 C. Prehybridization was performed in roller bottle with pre-heated DIG Easy Hyb Hybridization solution (Roche Cat # 11603558001) at least 1 hour at 42 C, 10 RPM. Prehybridization solution was replaced with hybridization solution contained denatured digoxigenine labelled probe pP2 (courtesy from Prof Ben Oostra, ErasmusMC, probe sequences and location of pP2 probe were noted in below) and leaved at 42 C overnight to complete hybridization. Membrane was washed 2x5 minutes with 50 ml 2xSSC/0.1 % SDS at room temperature. Detection procedure was achieved in clean trays with the following procedure; equilibrated membrane in 100ml washing buffer for 5 minutes followed by incubation in blocking solution for 60 minutes. Membrane was put in a piece of SaranWrap. 10 ul Anti-digoxigenine-AP-conjugate was added into blocking solution and membrane

was incubated in this solution for 30 minutes. After incubation, antibody solution was discarded and the membrane was washed in washing buffer for 2x15 minutes and detection buffer for 5 minutes. In the meantime 15 CDP star solutions (Roche Cat. no. 1685627) were diluted in 1500ul detection buffer. After washing procedure, membrane was placed in two sheets of cuted plastic bag and CDP star solution was pipette on top of the membrane. Bubbles present under the sheet were removed and membrane was incubated for 5 minutes. After incubation, liquid excess was removed and plastic bag was sealed. Membrane was exposured in film from 10 and 30 minutes. A normal band was shown by the presence of a 2,8 kb band illustrating normal X chromosome and 5,2 kb band reflecting methylated X chromosome.



Probes used in the diagnosis of fragile X syndrome. The restriction map of the 5·2 kb fragment containing the fragile site, the location of the CpG island (boxed), and the CGG repeat are given. The location and the names of the restriction fragments used to characterise the fragile X genotype are indicated.

Figure 2. Position of Probe pP2 in *FMR1* gene sequences

Sequences of pP2 probe totaling 1043bps were indicated in underlined figure below.

ggcgagcgcgggcggcggcggcggcggcggcggcggcggctgggcctcgagcgcccgcagcccacct
ctcgggggcgggctcccggcgctagcagggctgaagagaagatggaggagctggtggtggaagtgcggg
gctccaatggcgctttctacaaggtacttggctctagggcaggccccatcttcgcccttccttccctccctttcttc
ttggtgtcggcgggaggcaggcccggggccctcttcccgagcaccgcgcctgggtgccagggcacgctcg
gcgggatgttgttgggagggaaggactggacttggggcctgttggaagcccctctccgactccgagaggccc
tagcgcctatcgaaatgagagaccagcgaggagagggttctctttcggcgccgagccccgccggggtgagct
ggggatgggcgagggccggcggcaggtactagagccgggcgggaagggccgaaatcggcgctaagtga
cggcgatggcttattccccctttcctaaacatcatctcccagcgggatccgggcctgtcgtgtgggtagttgtgg
aggagcgggggcgcttcagccgggccgcctcctgcagcgccaagagggcttcaggtctcctttggcttctct
tttccggtctagcattgggacttcggagagctccactgttctgggcgagggctgtgaagaaagagtagtaagaa
gcggtagtcggcaccaaatcacaatggcaactgatttttagtggcttctctttgtggatttcggaggagattttaga
tccaaaagtttcaggaagaccctaacatggcccagcagtgcattgaagaagttgatcatcgtgaatattcgcgtc
ccccttttgttaaacggggtaaattcaggaatgcacatgcttcagcgtctaaaaccattagcagcgctgctactta
aaaattgtgtgtgtgtgtttaagtttccaaagacctaaatatatgccatgaaacttcaggtaattaactgagagtatat
tattactagggcatttttttttttaactgagcgaaaatatttttgtgcccctaagaacttgaccacatttcctttgaatttgt
ggtgttgcagtggactgaattgttgaggctttatataggcattcatgggtttactgtgctttttaaagttacaccattg
cagatcaactaacacctttcagttttaaaaggaagatttacaaatttgatgtagcagtagtgcgtttgttggtatgta
ggtgctgtataaattcatctataaattctcatttccttttgaatgtctataacctctttcaataatatcccaccttactaca
gtattttggcaatagaaggtgcgtgtggaaggaaggctggaaaatagctattagcagtgtccaacacaattctta
aatgtattgtagaatggcttgaatgtttcagacaggacacgtttggctataggaaaataaacaattgactttattctg
tgtttaccaattttatgaagacatttggagatcagtatatttcataaatgagtaaagtatgtaaactgttccatactttg
agcacaaagataaagccttttgctgtaaaaggaggcaaaaggtaaccccgcgtttatgttcttaacagtctcatg
aatatgaaattgtttcagttgactctgcagtcaaaattttaatttcattgattttattgatccataatttcttctggtgagtt
tgcgtagaatcgttcacggtcctagattagtggt

3.8.7 Southern Bloot Analysis using Radioactive labelled probe pAO365

Seven ug DNA (in 35 uL diluted solution with milliQ) was digested with restriction endonuclease enzyme (1,25 uL Hind III and 1,5 uL Eag I) in the buffer React H together with 20 mM Spermidine in a total volume of 15,75 uL for a minimum 5 hours at 37C.

Five uL loading buffer (bromophenol blue) was added to Digested DNA and subsequently 50 uL was loaded per lane. Thirty uL of 0,5% ethydium bromide

was added to the agarose gel. Electrophoresis of DNA was performed overnight in a 0,8% agarose gel at 38 volt in TAE buffer.

DNA in agarose gel was transferred into nylon membrane (Genescreen Plus Perkin Elmer) using a 0,4N NaOH+NaCl buffer solution for capillary blotting using filter membrane, paper stack and weighing mass (~ 500 grams) above the membrane for five hours. Then, the nylon membrane was washed in the phosphate buffer, dried and cross-linked into a 1200 UV Stratalinker.

Labelling beads were prepared by adding a tube Ready-To-Go DNA labelling beads (-dCTP) (Amersham) to 20 uL of water and incubating this solution for 5-60 minutes on ice. In the meanwhile the probe mix (pAO365) (sequence probe: ctcgagcgcccgcagcccacc. Position was indicated below) was denaturated at 95C for 5 minutes. Thereafter the Probe-mix was mixed with the labelling beads and 2,5 uL (alpha-32-P-dCTP) was added for incubation overnight. A sephadex G-50 column was used to purify the labelled probe. Oligonucleotide labeling products were denatured at 95 C for 5 minutes. The oligonucleotide labeling products were added to a hybridization buffer. Hybridization was performed overnight at 65 C. Tough cleaning was performed in 0,1 % SDS + phosphate buffer twice at 65C for 5 minutes and once at 65 C for 20 minutes. And finally only phosphate buffer was used to end the washing procedures.

Autoradiography was performed on X-ray film (Kodak ECL) for 1-3 days at -80 C using intensifications screens.

Sequence of pAO365 probe is indicated by underlined character.

agcgcggcggcggcggcggcggcggcggcggaggcggcggcggcggcggcggcggcggc
tgggcctcgagcgcccgcagcccacctctcgggggcgggctcccggcgctagcagggctgaagagaagat
ggaggagctggtggtggaagtgcggggctccaatggcgctttctacaaggtacttggct

3.8 Analysis of the data

All data was checked and determined correctly. Data then was coded, tabulated, and entered into the computer. Data was analyzed into description and hypothetical analysis. Before carrying out the hypothetical analysis, first the normality of the data was determined by Kolmogorov Smirnov test. Pearson correlation analysis was used at $\alpha = 0{,}05$ using SPSS 16 for Windows to determine significant correlation.

3.10. Research ethic

This research has been approved by Committee of Ethic, Faculty of Medicine Diponegoro University. The ethical clearance contains the statement that this research was conducted by the researcher with paying attention to Helsinki Consensus 1975 and PNEPK department of health of Indonesia. Informed consent explaining about the research, procedure, and benefits of the research was provided to all the subjects. It was also informed that any identities of the probands would not be published in general publication.

CHAPTER IV

RESULTS

4.1. Characteristic of probands

Thirty patients from a known fragile X family and three non fragile X syndrome based on previous research were included in this research. Most of them have been included in several previous researches. The characteristics of the probands are depicted in table 5, table 6, and table 7.

Table 4. The age distribution of the probands

Age	number	percentages
> 65	2	6.06
35-65	16	48.48
17-35	12	36.36
<16	3	9.09

Most of probands are in age range of 35-65 with 48.48 % and the lowest are in range <16; 9.09 %. Most of female patients of fragile X in this area are above 20 years old.

Table 5. The occupation distribution of the probands

Occupation	number	Percentages
Farmer labor	21	63.64
Traditional merchant	1	3.03
Student	7	21.21
No occupation	4	12.12

Occupational background of the probands is dominated as farmer labors, a non informal service, whereas traditional trader occupies the lowest endeavor. Seven students who are now studying, 6 of them were doing their study in special school (SLB) and 1 of them is in high school level (SMU), whereas non student status has a varying degree in educational background (table 6).

Table 6. The educational background of the probands

Education	number	percentages
No educational	20	60.61%
SD	3	9.09%
SMP	2	6.06%
SMU	2	6.06%
SLB	6	18.18%

Approximately, 61 % of probands are not educated and only about 6 % of probands who reach the high school level.

In general, physical health of the probands was in good condition. However, we could not report physical examination of the probands in whole context as we did not concern in physical features. All probands looked like displaying low nutritional status. It was figured by status of Body Mass Index (BMI) of the probands that 87 % of them showed underweight range. Most of them have lack in daily hygiene living such as self care and feeding. They were accustomed to be not quite aware to their personal hygiene.

4.2. Preliminary study.

Preservation of hair roots was performed before testing to the hairs from subjects. It would be valuable to know how long the hairs can be stored at room temperature. Test was applied using hair roots from normal individuals (man and woman). Two series (periods) of hairs were tested from three different persons. For each incubation, experiment was using a mixture of hair roots from these persons.

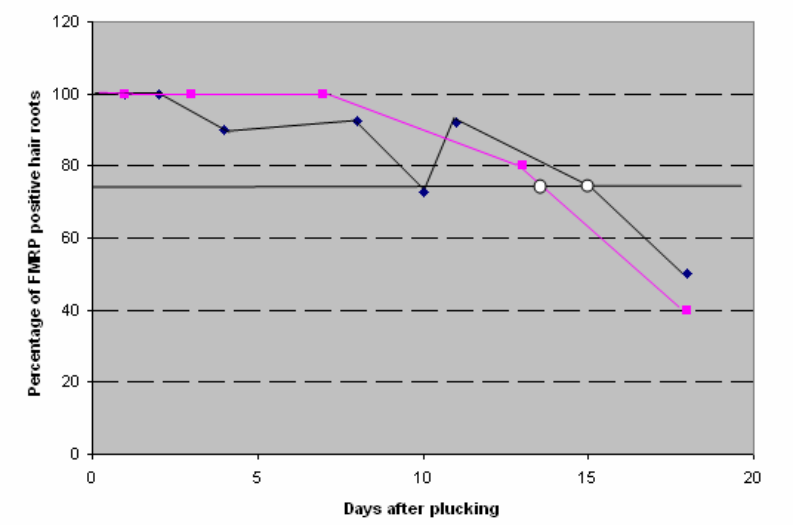


Figure 3. Diagram of period and percentages of hair roots expressing FMRP that was stored at room temperature (RT).
The color lines indicate the series and horizontal line indicate cut of point.

Figure 3 illustrated that the hairs can be stored at room temperature for 14 days before they reach the cut-off point (75 %). In our planning, this would allow us to pluck the hairs in Indonesia and ship them to Rotterdam because transport should take only one week.

In the first experiment, hair samples from Indonesia which were shipped by courier mail were tested for immunohistochemistry. The analysis was performed in a blind fashion and the hairs from normal persons were also included among these samples. The result was drawn in the table below:

Table 7 . Distribution of FMRP expression in hair roots that were examined in Rotterdam for preliminary study

Number	Code	% FMRP in hair roots
1	SM030	76
2	SM015	76
3	SM016	60
4	SM020	54
5	SM022	72
6	SM008	56
7	SM007	91
8	SM009	81
9	SM002	51
10	SM023	20
11	SM013	54
12	SM021	85
13	SM019	68
14	SM028	86
15	SM024	84
16	SM029	36
17	Normal 1	74
18	Normal 2	70
19	Normal 3	90

We found that FMRP expression from these samples ranges between 20 % and 91 %. However, two normal samples that were included in this examination showed low expression below the cut-off point of 75 %. Since the normal persons expressed too low percentages of FMRP, we then decided to perform the hair roots test in Indonesia.

4.3. FMRP Expression of the subjects.

In Indonesia we added three mentally retarded persons as comparator from non-fragile X syndrome to know whether FMRP also influence their cognitive functioning. In addition, the blood smears that were freshly made in Indonesia shipped to Rotterdam to perform immunohistochemistry test. Fragile X Mental Retardation Protein (FMRP) expression was shown in both blood smears and hair roots by using immunohistochemistry test introduced by Willemsen.

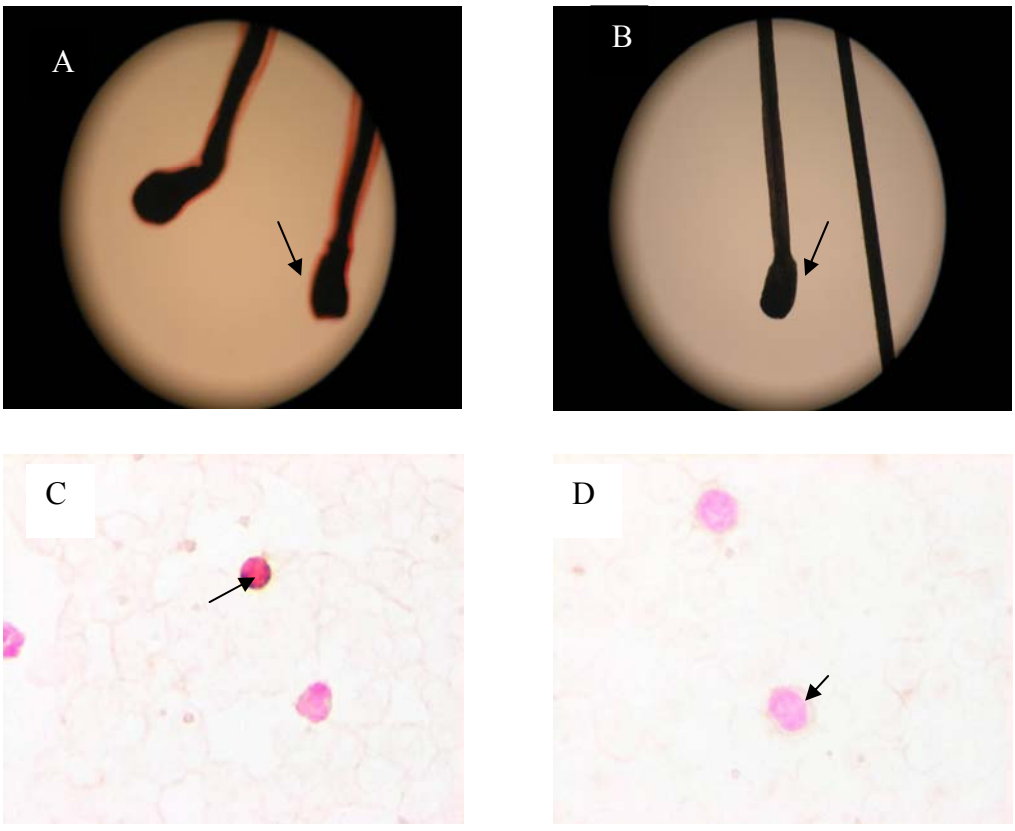


Figure 4. Fragile X Mental Retardation Protein (FMRP) expression in both blood smears and hair roots.
A: Positive appearance in follicle hair roots from normal probands, B: Negative appearance in follicle hair roots from full mutation probands, C: Positive appearance in lymphocytes cell from normal probands, D: Negative appearance in lymphocytes cell from full mutation probands

In Blood smears, FMRP expression was shown by brown precipitates in lymphocyte whereas FMRP expression in hair roots was shown by Red appearance in the sheaths and bulbs. Lymphocytes with negative expression were expressed by grey or white expression, as well as negative expression in hair roots (figure 4).

Table 8. Distribution of percentage of FMRP expression in hair roots and blood smear, IQ equivalent, and genotypes (alleles for fragile X).

No	Samples code	IQ equivalent	% FMRP in blood smear	% FMRP in hair roots	Genotypes (Southern blot)
1	SM001	60	98	85	MR Non Fragile X *
2	SM014	65	97	90	MR Non Fragile X *
3	SM003	70	95	86	MR Non Fragile X *
4	SM006	95	97	89	Normal
5	SM012	75	88	85	Normal
6	SM024	75	90	84	Normal
7	SM007	85	87	82	Normal
8	SM019	77	97	70	Normal
9	SM009	75	93	87	Normal
10	SM028	73	92	86	Normal
11	SM011	66	75	76	Normal
12	SM005	80	95	83	Normal
13	SM030	83	80	73	Premutation
14	SM021	75	94	85	Premutation
15	SM015	77	94	77	Premutation
16	SM004	75	97	80	Premutation
17	SM010	88	86	90	Premutation
18	PW001	101	94	85	Premutation
19	SM018	85	95	82	Premutation
20	SM008	60	65	50	Full mutation
21	SM020	65	65	50	Full mutation
22	SM002	60	44	64	Full mutation
23	SM022	60	76	40	Full mutation
24	SM023	63	63	41	Full mutation
25	SM016	70	55	60	Full mutation
26	SM025	65	61	54	Full mutation
27	SM017	50	97	75	Full mutation
28	SM027	85	88	87	Full mutation
29	SM026	50	45	31	Full mutation
30	SM029	50	58	25	Full mutation
31	SM013	55	62	50	Full mutation
32	PW002	85	76	69	Full mutation
33	SM031	65	67	60	Full mutation

* = based on previous research

Normal samples and premutation carriers showed high expression of FMRP both in blood smears and hairs roots with an average of 91,78 % and 82,89 %, respectively. Three samples of mentally retarded people from non-fragile X also showed a high FMRP expression (over 80%), whereas full mutation subjects showed on average 65.85 % in blood and 53,98 % in hair roots.

These findings show that in full mutation individuals the average FMRP expression in blood smears was higher than in hair roots. They also indicate that 15 samples show a different result for FMRP expression in blood smears and hairs over 10 %, which were mostly in the full mutation group. Comparison of FMRP expression in lymphocytes and hair roots is shown in figure 5.

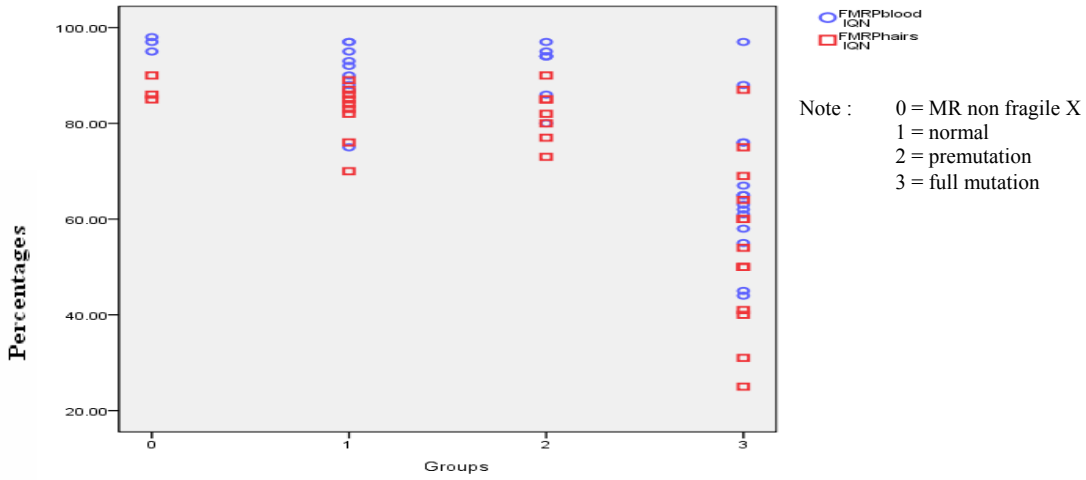


Figure 5. Diagram of percentage of FMRP expression in lymphocytes and hair roots in individual samples.

Non-full mutation groups show high expression of above 75 % except two samples, whereas the full mutation group varies from 25 % to 97 %. The hair roots FMRP is lower than in lymphocytes.

To determine whether a relationship exists between the percentage of FMRP expression in lymphocytes and hair roots, a correlation test was applied for these samples.

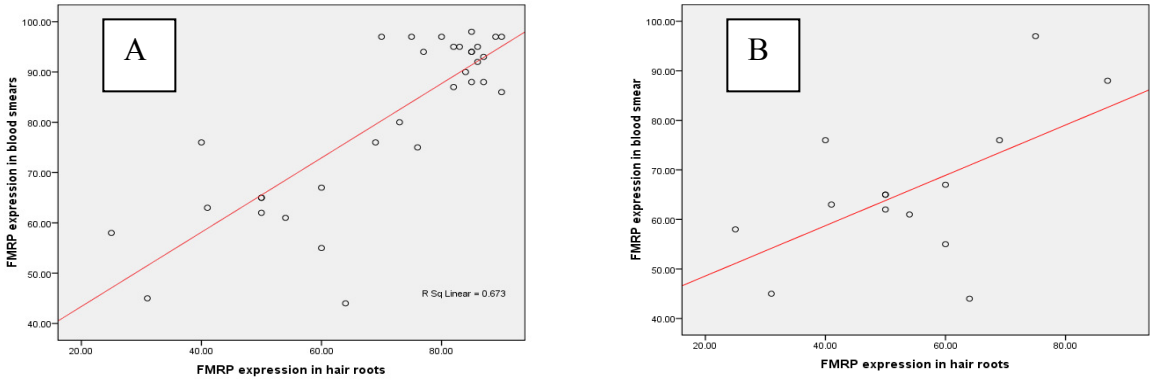


Figure 6. Correlation of FMRP expression between hair roots and lymphocytes in total sample (A) and only in full mutation group (B).

The Pearson correlation of total sample is highly significant at the 0.01 level ($r = 0.82$, p value < 0.001) and in full mutation group, correlation seems has lower value, however it still shows statistical significant at 0.05 level ($r = 0.58$, P value = 0.028). This finding informed us that in cells of individual normal, premutation or full mutation carriers, FMRP expression look like consistent between lymphocytes and hair roots, although more variation is seen in the full mutation group.

4.4. IQ equivalent of the probands

Raven Standard Progressive Matrices (SPM) was used to measure IQ equivalent of the subjects. A certified psychologist performed the test and also further analysis. Results then were depicted in table 8 above and distribution of IQ level among these groups was depicted in figure 7.

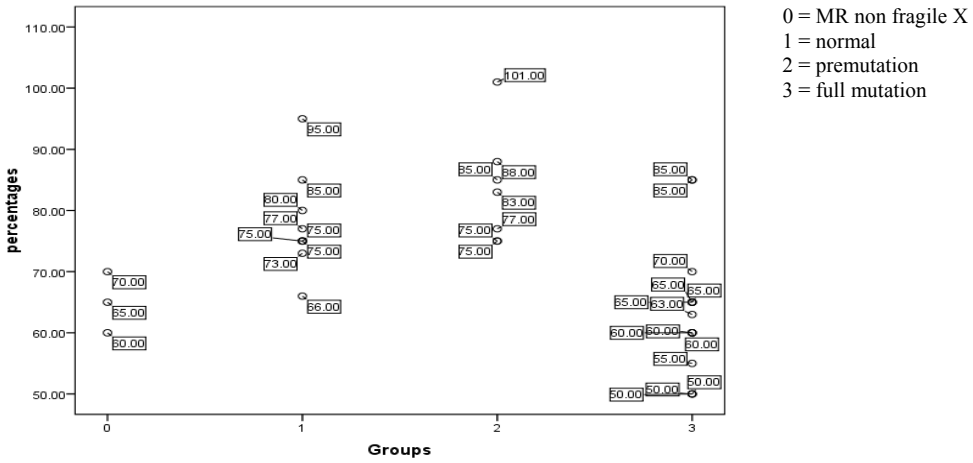


Figure 7. IQ equivalents (converted from Raven test) distribution in individual samples.

Normals and premutation carriers have an IQ range between 66 and 101, whereas full mutation carriers have IQ levels between 50 and 85. The average IQ value in Normals and Premutation carriers is 77.88 and 83.42 respectively, whereas full mutation carriers have 63.07. The highest IQ value is 101 in a premutation carrier and the lowest value is 50 in a full mutation carrier

Thirty percent of the sample shows IQ levels ≥ 80.00, whereas the other subjects were below 80. The average value was 77.88 in normal group, 83.42 in premutation carriers, and 63.07 in full mutation carriers. In the full mutation group there were about 14 % in normal range (≥85), 7 % in the borderline range (70-84) and 79 % showed mild mental impairment (50-69).

4.6. Genotypes of the probands.

To confirm genotyping of the probands, DNA test using Southern blot analysis was performed using a methylation sensitive enzyme that allows us to discriminate between methylated and unmethylated *FMR1* alleles. This analysis also showed us the classification of the alleles, whether it was normal, premutation, or full mutation alleles. Result was shown in figure 8 below. Figure A and B were using digoxigenine labeled probe pP2 and Figure C was using radioactive labeled probe pAO365.

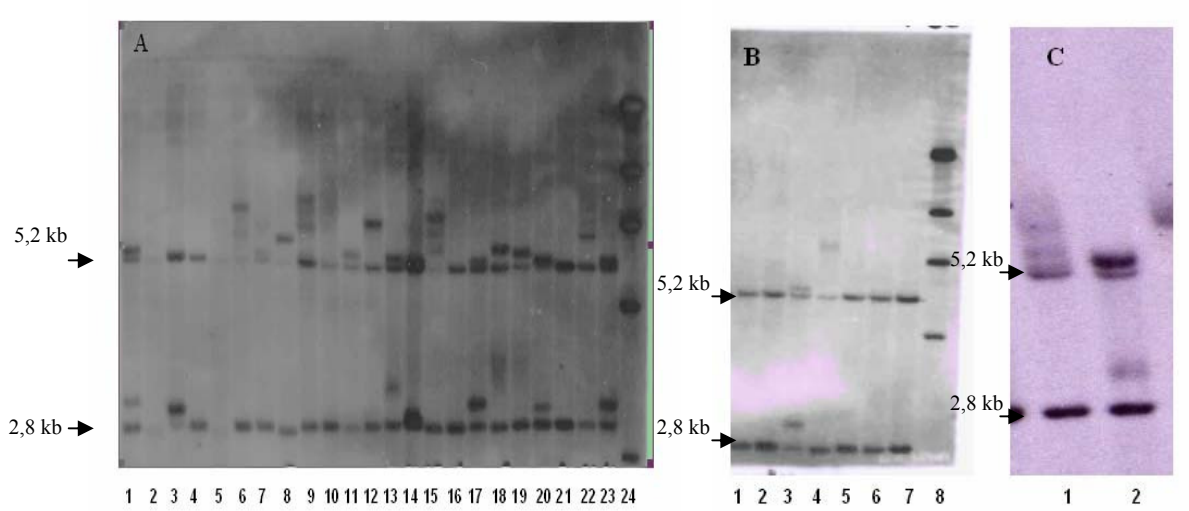


Figure 8. Figure of Southern blotting test from individual samples using double digestion with *Hind*III and *Eag*I.
A: Lanes 4,14,21 were normal samples. Lanes 1,3,13,20,23 correspondent to premutation samples whereas full mutation samples were shown in lanes 2,5,7,8,9,10,11,12,15,18,19, and 22. Lanes 6 and 17 indicated full mutation and premutation control, respectively. **B**: Lanes 1,2,5,6,7 were normal samples. Lane 3 correspondents to premutation sample and line 4 were full mutation sample. DNA ladder was indicated in lane 8. **C**: Lane 1 was full mutation and lane 2 was premutation.

Normal samples (figure 8A lines 4,14,21 and figure 8B lines 1,2,5,6,7) showed clear bands of 2,8 kb and 5,2 kb reflecting unmethylated normal allele and methylated normal allele due to X-inactivation process. Full mutation samples showed a high smear reflecting of high CGG repeat, except samples in figure 8A lanes 11 and 19 which were a little bit higher from premutation band. Figures 8A Lanes 7,9,10,15,22, and figure 8C lane 1 showed mosaic pattern with a premutation and high smear full mutation band. This Southern blotting experiment showed us a distribution of samples as follow; 14 samples were full mutation, 7 samples were premutation, and 9 samples were normal alleles.

4.7. Correlation between FMRP expression and IQ equivalents of Full mutation females.

In full mutation group, Pearson correlation test was carried out to determine whether a relationship exists between FMRP expression and IQ equivalent.

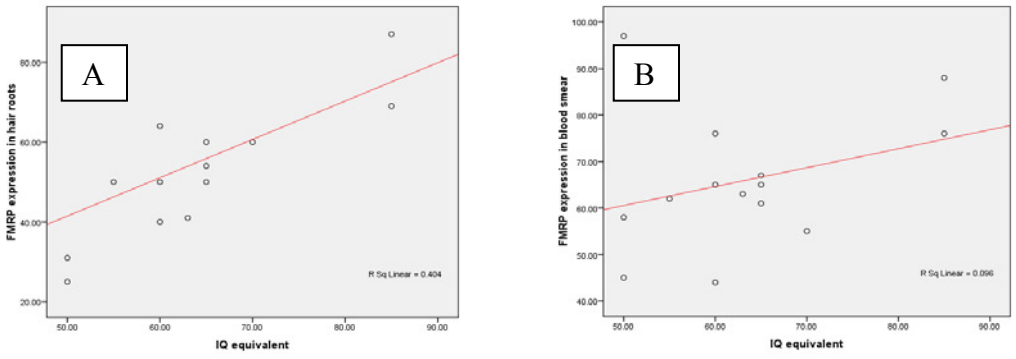


Figure 9. Correlation between FMRP expression and IQ equivalents in blood smear (A) and hair roots (B) in full mutation carriers.
The chart indicated a significant correlation between percentage of FMRP expression in hair roots and IQ equivalents in our group of full mutation carriers.

The P value of Pearson correlation test for FMRP in hair roots and IQ equivalent parameter shows high significance at level 0,05 (P= 0,015) however, the correlation of FMRP expression in lymphocytes and IQ equivalents statistically shows no significant relationship between these two parameters ($r = 0.31$, $p = 0.281$)

CHAPTER V

DISCUSSION

Mental retardation is one of professional health problem in the world that caused by many factors. Genetics, environment, health status of prenatal, perinatal, and postnatal can give rise to cognitive impairment. The absence of FMRP in neurons from fragile X patients is the cause of the mental retardation. Thus, FMRP expression studies in brain are important to understand the molecular mechanisms underlying the disease. For obvious reasons FMRP expression studies in brain are not realistic and will be limited to post-mortem material. However, immunocytochemical studies have been developed in a diagnostic setting to identify fragile X patients using FMRP expression in both lymphocytes from peripheral blood and hair roots.

In this study, subjects were obtained from peripheral area based on previous study. Semin area is one of district categorizing as minus region by local government. Characteristics of the land are bringing on the poverty condition. This is in line with several studies that poverty could give rise to low mental development [56]. All subjects varied from children to elderly people; however most of them were older than 20 years old. In the general population it was suggested that mental capability is relatively changed due to age increment [57], however an opposite study was proposed that age related cognitive decline could not be established [58]. There are many factors to explain about decreased cognitive functioning related to the age. We could also find that most of them were not educated well as 76 % of probands are not educated

and only one probands who reach the high school level. Definitely, they have no fix occupation since they were only farmer labor. It was represented by the fact that most of them were also having limitation in household income. Based Malanom report, it was understandable that in many studies, educational background and income opportunity also has effect in cognitive development [59].

Nutritional status may play a roll in cognitive development as most of them have low Body Mass Index. Some report told us that nutritional status represented by BMI is related with intelligence quotients, further study [60] showed that in healthy people, BMI also has correlation with cognitive functioning. However it could not be established well as in another study [61] showed that BMI had no influence on the IQ average to the cognitive functioning in general population. Instead of that, BMI is only about the anthropometrics measurement, it does not represent the inner brain status. Prominently, mental development looked like is influenced by micronutrients such as Fe, Zn, selenium, riboflavin, folat, and I odium [62, 63].

In the past, studies have been reported that FMRP expression in hair roots may serve as a predictor of the cognitive functioning of females carrying the fragile X full mutation [9, 10]. In contrast, FMRP expression in blood smears can only be used as a test to identify male fragile X patients. It has been reported that the test showed significant value to distinguish between males with fragile X and normal controls. But there was an overlapping result in the female group (full mutation versus controls).

In this study, first, we tested how long hair roots can be stored after plucking and still give reliable results in the hair root test. This is important because hair roots should be plucked in Indonesia followed by transport to The Netherlands for analysis. We found evidence that hairs can be stored for 14 days at room temperature. This is longer than has been reported before [7]. We hypothesize that this is caused by differences in the protocols that are used nowadays. Currently antibodies are used that show an enhancement of the immunolabeling. In this experiment we used α mouse powervision poly AP, whereas in the past biotinylated secondary antibody was used. Apparently the use of polyvision results in a more sensitive method.

This result suggested that the hair samples simply could be shipped to diagnostic center (Rotterdam) from peripheral area (Indonesia) using courier mail, which usually needs one week. In the first experiment we tested samples from Indonesia, which were shipped by courier mail, in a blind fashion. After analysis, we found that normal controls showed relatively low FMRP expression in hair roots, clearly below the cut-off point (table 7). This negative result may be caused by differences related to local circumstances, including humidity and temperature. In addition, conditions of shipment may also influence the quality of samples. This result prompted us to perform hair roots analysis in Indonesia with fresh hair roots.

This research found evidence that all the samples from female normal and premutation carriers expressed normal levels of FMRP (75 -100%) both in blood smears and hair roots, whereas full mutation female carriers showed a great variance in percentage of FMRP expression (figure 5). This finding is in line with the current

hypothesis that normal and premutation carriers show a normal translation of the *FMR1* mRNA. In contrast to carriers of the full mutation alleles that usually shows a methylation of the *FMR1* gene, resulting in lack of FMRP. A random lyonization process that occurred in the cells causes the variance of FMRP expression in female full mutation.

Normal and premutation subjects in this study show rather low IQ equivalents. A possible explanation for this observation is that they live in an isolated area where modern facilities are limited. Most of them are not educated and living in a simple environment. Also the diet may play a role because they eat more cassava than rice because of dry area. As a consequence they eat very less protein, which may harm the body because of cyanine in the cassava. In line, this finding represents the condition that they may have low micronutrient intake causing low support of brain normal development. It is also in line with previous literature mentioning that diet intake can influence in cognitive functioning development [64].Hypothyroidism is also one of problems in these areas [65]. Also we experienced that during completing the Raven test; we had to explain the test more frequently. In contrast, they finished their test very fast. This case relates with a previous study that mentioned alternative hypothesis to general ability (g) for why non-Western population score lower than Western population; such as test-wise, less interested, more anxious, work less efficiently, or give up sooner on items they find difficult [66].

Distributions of IQ equivalent in the full mutation group were about 14 % in normal range (≥85), 7 % in the borderline range (70-84) and 79 % showed mild

mental impairment (50-69). This finding is higher than previous research that mentioned about 60 % with mild mental retardation; however, in our studies the number of subjects was limited and should be expanded in the near future to draw final conclusions. This study also suggests that in this isolated area, less difference in mental capacities occurs between mentally retarded people and normal people. Figure 7 clearly informed us the difference in IQ equivalents of the full mutation, premutation, and normal groups.

In the full mutation group we found subjects that have relatively high IQ levels compared to others showing higher FMRP expression in their cells (compared with another samples). It can be described by the fact that females carrying a full mutation are characterized by cells with and without FMRP expression, which can be explained by the presence of two X-chromosomes and random inactivation of one of the X chromosome (Lyonisation). This finding also noticed that although human intelligence is influenced by many genetic factors, a single mutation in the *FMR1* gene resulting in lack of FMRP significantly reduces cognitive functioning in female full mutation carriers [10].

The result of FMRP expression in blood smears and hair roots also correlated with each other, although FMRP expression in blood smears seemed to be higher than in hair roots. This may be explained by differences of origin. Blood originate from mesoderm layer whereas hair roots like brain tissue from the ectoderm. Blood, especially lymphocytes, originate from a common lymphoid progenitor cell before differentiating into their distinct lymphocyte types. The formation of lymphocytes

which is known as lymphopoiesis will turn every day and will live for weeks till years [67].

It has also been suggested that there might be a selection against cells with a full mutation in dividing lymphocytes or that there is a bias toward inactivation of the X-chromosome in women during aging [68]. This may explain why there might be a better correlation between (the lack of) FMRP expression in hair roots and mental retardation. In addition, lymphocytes may show less reflection of the situation in brain tissue, because the rapid turnover may lead to high variation in clones that either carry the *FMR1* mutation on the active X chromosome or do not. Earlier studies using the FMRP test on lymphocytes did show a weak statistical correlation; however, the significance was not high enough to use this method as a reliable diagnostic or predictive test [10].

The benefit of hair roots as a diagnostic tool is shown in this study because we found, although in a small-sized study, a highly significant correlation between the percentage of FMRP expression in hair roots and IQ equivalent in female full mutation carriers. The statistical analysis showed us that level of cognitive functioning in female full mutation carriers typically are reflected by FMRP in hair roots than in blood smears. The origin of the tissue is important, however, methodological aspect may play a role as well and cannot be excluded from this research.

In summary we show that FMRP expression in hair roots is probably more useful to predict cognitive functioning in female full mutation carriers than blood,

however, the number of full mutation carriers tested in this study is too low to draw final conclusions.

CHAPTER VI

CONCLUSIONS AND SUGGESTIONS

6.1 Conclusions

1. There was strong relationship between FMRP expression in hair roots and cognitive functioning in female carriers of the fragile X full mutation.
2. FMRP expression in hair roots had stronger correlation for cognitive functioning than FMRP expression in blood smears.
3. IQ equivalent in female fragile X full mutation group were about 14 % in normal range, 7 % in borderline range and 79 % in mild mental impairment.

6.2 Future directions

1. Since sample size of this study is relatively limited, it is suggested to conduct next study using bigger sample to make statements about the reliability of the FMRP test and cognitive functioning in the female full mutation carriers.
2. Based on the result of this study that FMRP expression in hair roots has stronger correlation, non-invasive test, and can be performed in a single day with no harmful experience for probands, we can use it in screening programs.

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List of Appendixes

1. Statistical analysis

One-Sample Kolmogorov-Smirnov Test

		FMRPblood	FMRPhairs	IQ
N		33	33	33
Normal Parameters[a]	Mean	80.7879	70.6364	71.6061
	Std. Deviation	16.69348	18.53176	12.76700
Most Extreme Differences	Absolute	.198	.185	.094
	Positive	.151	.148	.094
	Negative	-.198	-.185	-.090
Kolmogorov-Smirnov Z		1.139	1.061	.540
Asymp. Sig. (2-tailed)		.149	.210	.933

a. Test distribution is Normal.

One-Sample Kolmogorov-Smirnov Test

		FMRPblood	FMRPhair	IQ
N		14	14	14
Normal Parameters[a]	Mean	65.86	54.00	63.0714
	Std. Deviation	14.696	16.893	11.18697
Most Extreme Differences	Absolute	.183	.121	.217
	Positive	.183	.094	.217
	Negative	-.087	-.121	-.121
Kolmogorov-Smirnov Z		.686	.452	.813
Asymp. Sig. (2-tailed)		.735	.987	.523

a. Test distribution is Normal.

Correlations

		FMRPblood	FMRPhairs
FMRPblood	Pearson Correlation	1.000	.820**
	Sig. (2-tailed)		.000
	N	33.000	33
FMRPhairs	Pearson Correlation	.820**	1.000
	Sig. (2-tailed)	.000	
	N	33	33.000

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		FMRPhair	FMRPblood
FMRPhair	Pearson Correlation	1.000	.584*
	Sig. (2-tailed)		.028
	N	14.000	14
FMRPblood	Pearson Correlation	.584*	1.000
	Sig. (2-tailed)	.028	
	N	14	14.000

*. Correlation is significant at the 0.05 level (2-tailed).

Correlations

		FMRPhair	IQ
FMRPhair	Pearson Correlation	1.000	.635*
	Sig. (2-tailed)		.015
	N	14.000	14
IQ	Pearson Correlation	.635*	1.000
	Sig. (2-tailed)	.015	
	N	14	14.000

*. Correlation is significant at the 0.05 level (2-tailed).

Correlations

		IQ	FMRPblood
IQ	Pearson Correlation	1.000	.310
	Sig. (2-tailed)		.281
	N	14.000	14
FMRPblood	Pearson Correlation	.310	1.000
	Sig. (2-tailed)	.281	
	N	14	14.000

2. Figures of FMRP expressions in follicles of hair roots

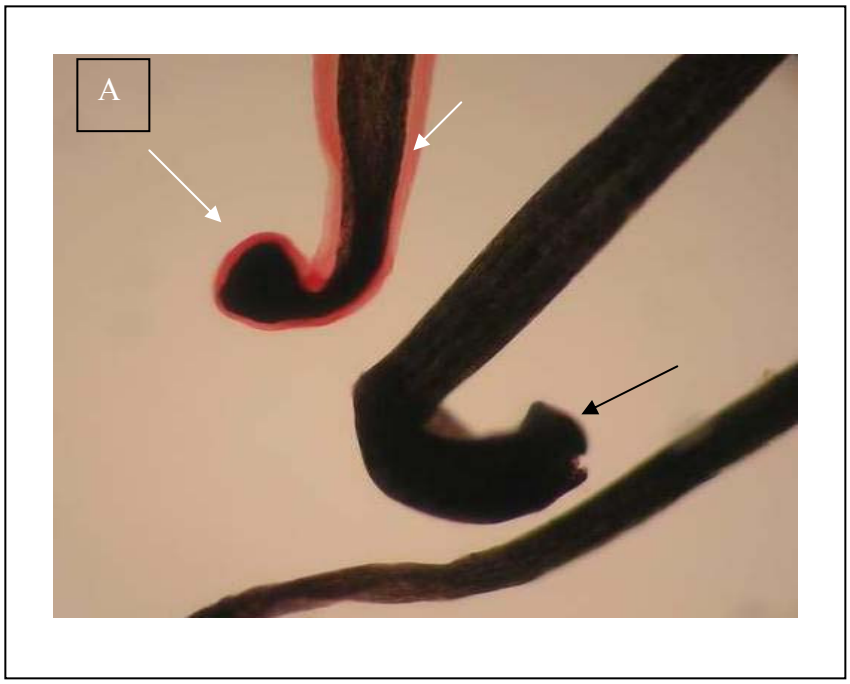

Figure A : Positives FMRP expressions in follicles of hair roots
Red appearance in the bulb of hair follicle and sheath reflects FMRP expression (white arrow), and black appearance showed negative FMRP (black arrow)

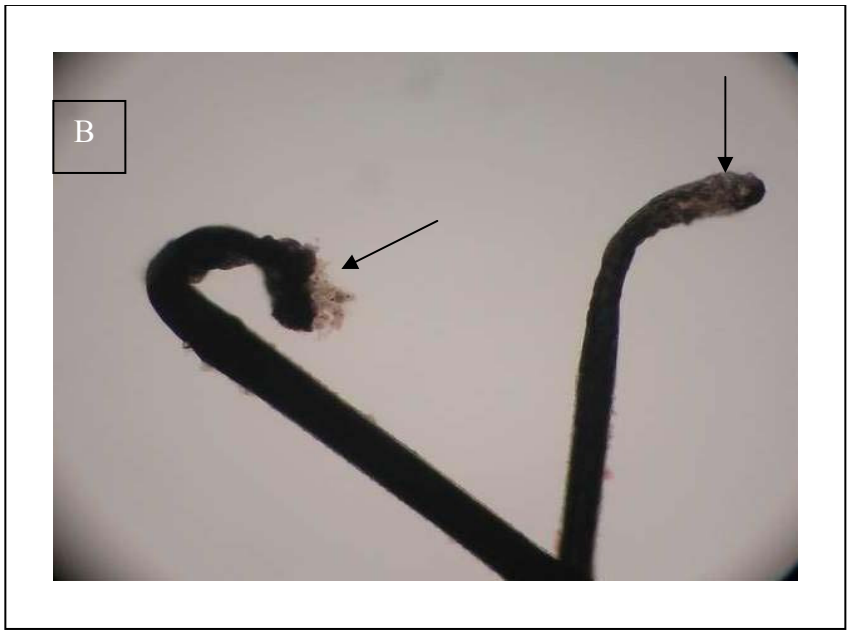

Figure B : Negative FMRP expressions in follicles of hair roots.
No red sign in the bulb of hair follicle reflects no FMRP expression (black arrow)

3. Figures of FMRP expressions in blood smears

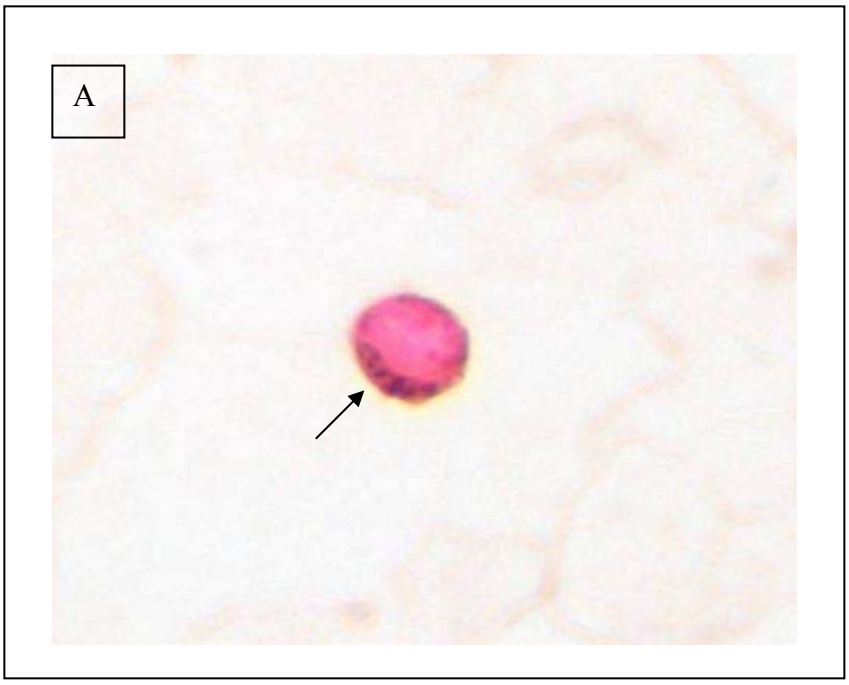

Figure A : Positives FMRP expressions in blood smears.
Brown precipitate in the cytoplasm shows FMRP expression in lymphocytes. (black arrow)

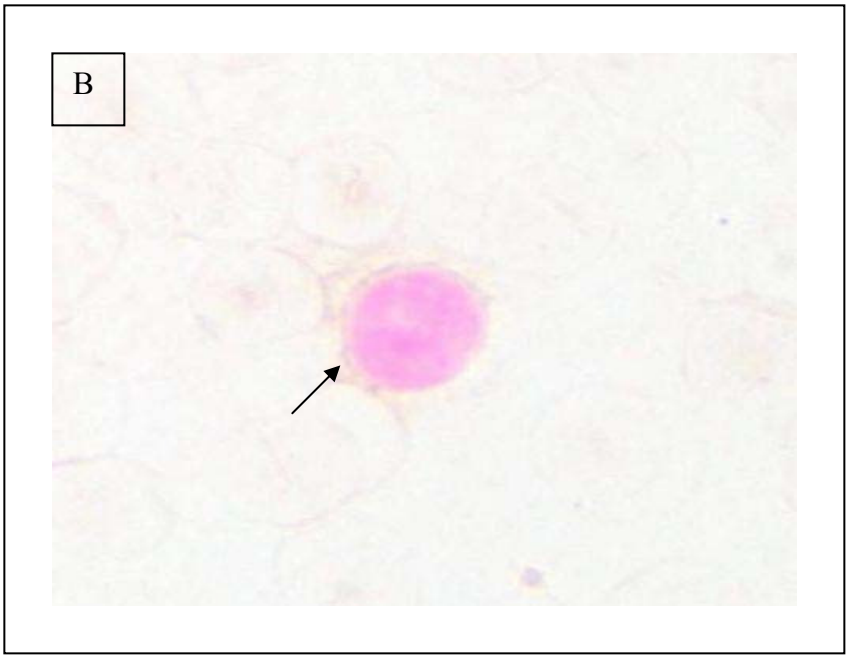

Figure B : Negative FMRP expressions in blood smears.
Grey appearance of cytoplasm indicates that there is no FMRP expression in lymphocytes (black arrow).