EXPLORATION OF REAL TIME PCR AS A NEW DIAGNOSTIC METHOD OF FRAGILE X SYNDROME

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Thesis submitted in fulfillment of the requirements of the degree of Master of Science

Universiti Sains Malaysia

2010

ACKNOWLEDGEMENT

In the name of Allah, the Most Generous and the Most Merciful. All praises are devoted to Allah for His guidance and peace that give me the ingredients of success.

I wish to acknowledge USM Short Term Grant (304/PPSP/6131490 and 304/PPSP/6131471) and Post Graduate Research Grant Scheme (1001/PPSP/8132004) for the financial support throughout the study as well as several people who were involved directly or indirectly in this research project.

First of all, I would like to thank my supervisor, Associate Prof. Dr. Zilfalil Alwi for his support, excellent guidance and supervision throughout the experimental work and also during the manuscripts and thesis writing. His guidance and encouragement are greatly appreciated.

I would also like to convey my gratitude to my co-supervisor, Prof. Ravindran Ankathil and Dr. Salmi Razak for their encouragement, kind supervision and assistance during this study. Without them, this research will never be a success.

A very special thanks to Dr Pornprot Limprasert, Wanna Sudhikaran and all the staff and students from the Molecular Division, Pathology Department, Prince of Songkhla University, Thailand for their training (Southern Blot and MS-PCR analysis) and very warm hospitality during the 2 weeks that I spent for training in their laboratory.

My thanks also to the Immunology Department and Radiology Department of HUSM for facilitating and allowing me to use their equipment for completing my research.

I would like to take this opportunity to express my thanks to Dr. Hoh Boon Peng, Wati Hayati and Nurshafawati for their assistance and teaching me the molecular techniques during the early stage of my project. Not forgetting, lots of thanks to Nurazwana and

Munirah, MSc. students from Immunology department, also Atif Amin for their help and guidance on cell culturing.

I would like to extend my gratitude to my friends from Haematology Department, PPSP, Noor Adzha, Nik Mohd Khuzaimi, Mohd Anuar and Nurul Ain Fathma, my friends from molecular lab of PPSK, Khaizil Amylia and Siti Shuhadah for their precious help during the difficult period of my experimental works. Thanks also to all my friends, colleagues and staffs at the Human Genome Centre.

Finally I wish to acknowledge the greatest support, love and encouragement from my beloved parents, Elias Taib and Rokaida Sardar and my siblings for their love, patience, support and their faith in me.

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LIST OF ABREVIATIONS

5' UTR	5' Untranslated region
A_{260}/A_{280}	Ratio of 260 absorbance over 280 absorbance
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complimentary Deoxyribonucleic acid.
Ct	Cycle threshold
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH_20	Deionised distilled water
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphatase
DTT	Dithiothreital
dTTP	Thymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EEG	Electroencephalography
FISH	fluorescent in situ hybridization
FMR1	Fragile X Mental Retardation 1
FMRP	Fragile X Mental Retardation Protein
FRAXA	Fragile X Site A
FRAXE	Fragile X Site E
FUDR	5-fluorodeoxyuridine
FXS	Fragile X Syndrome
FXTAS	Fragile X-associated Tremor/Ataxia Syndrome
HCl	Acid hydrochloride
IQ	intelligence quotient

kb	kilobase
KCl	Potassium Chloride
kDA	Kilo Dalton
KH	K homology
LB	Lithium Boric Acid Buffer
LNAs	Lock Nucleic Acids
$MgCl_2$	Magnesium Chloride
min	minute
ml	milliliter
mM	millimolar
MR	Mental Retardation
mRNA	Messenger ribonucleic acid
MTX	Methotraxate
$Na_2H_2PO_4$	Monosodium phosphate
Na_2HSO_3	Sodium bisulfite
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng/µl	Nanogram per microliter
NMR	Nuclear Magnetic Resonance.
NS-XLMR	Non-syndromic X-linked mental retardation
NT	normal transmitters
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pH	Puissance de Hydrogen
PHA	Phytohaemagglutinin
pmol	Pico mole
qPCR	Quantitative Polyerase Chain Reaction
RBC	Red blood cell
RE	Restriction Enzyme
RNA	Ribonucleic acid

rpm	Rotation per minute
RT	Room temperature
RT-PCR	Real Time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SSC	saline sodium citrate
S-XLMR	Syndromic X-linked mental retardation
SYBR® Green I	SYBR® Green I Nucleic Acid gel stain
Taq	*Thermuphilus aquaticus*
TBE	Tris Base EDTA
T_m	Melting temperature
U	unit
UV	Ultra-violet
V	voltage
WBC	White blood cell
WHO	World Health Organization
XIST	X Inactive-Specific Transcript
XLMR	X-linked mental retardation

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PENEROKAAN ‘REAL TIME PCR’ SEBAGAI KAEDAH PENDIAGNOSAN BARU BAGI SINDROM ‘FRAGILE X’.

ABSTRAK

Sindrom “Fragile X” (FXS) adalah di antara penyakit kerencatan mental dikalangan lelaki yang paling kerap di warisi. Penyakit ini disebabkan oleh gen *FMR1* yang tidak normal melalui pemanjangan jujukan CGG dan hipermetilasi pada bahagian promoternya. Alel termetilasi biasanya boleh menyebabkan kerencatan transkripsi dan menjurus kepada kehilangan penghasilan Protein ”Fragile X Mental Retardation” (FMRP). Modifikasi kimia dari sitosin kepada urasil dengan rawatan natrium bisulfit memberikan kaedah tambahan bagi kajian metilasi DNA yang boleh mengelakkan penggunaan analisis pemblotan Southern. DNA genomik diekstrak daripada darah periferal pesakit yang telah dikenalpasti secara klinikal sebagai pesakit FXS. Sampel-sampel tersebut dirawat dengan natrium bisulfit diikuti dengan amplifikasi menggunakan ”Real Time Multiplex Methylation Specific PCR” (RT-M-MSPCR) dengan gen *XIST* sebagai kawalan dalaman, diikuti dengan analisis graf lebur. Keputusan yang didapati menunjukkan semua sampel kawalan yang mana status metilasinya telah diketahui telah dapat dikesan dengan tepat menggunakan kaedah RT-M-MSPCR. Bagi tujuan pengesahan kaedah yang telah dilakukan, status metilasi bagi 45 orang pesakit lelaki telah dapat kesan dengan tepat berdasarkan perbandingan dengan keputusan pemblotan Southern. Tiga puluh sembilan sampel telah dikenal pasti sebagai tidak termetilasi, 4 sampel termetilasi penuh dan 2 sampel mengandungi kedua-dua alel termetilasi dan tidak termetilasi dan dikategorikan sebagai mozaik. Kaedah pengesanan

disahkan sebagai spasifik kerana suhu lebur bagi kedua-dua alel termetilasi dan tidak termetilasi masing-masing di dalam julat variasi yang kecil iaitu 84.91 ± 0.41ºC bagi alel tidak termetilasi, dan 90.57 ± 0.34 ºC bagi alel termetilasi. Peratusan metilasi bagi sampel mozaik juga boleh diperolehi dengan peratusan metilasi serendah 5%. Walaubagaimanapun, peng-di-aktifan rawak pada metilasi pesakit perempuan menyebabkan status pramutasi dan mutasi penuh pada perempuan tidak dapat dibezakan. Oleh kerana pesakit FXS lelaki adalah lebih kerap berbanding perempuan, maka kelemahan ini bukanlah penyumbang kepada masalah besar dalam saringan pesakit FXS. Kesimpulannya, hasil kajian ini menunjukkan RT-M-MSPCR adalah jitu, murah dan 100% sama sepesifik juga sensitif jika dibandingkan dengan kaedah pemblotan Southern. Kaedah pengesanan yang baru ini juga sangat mudah dilakukan bagi saringan FXS dalam jumlah yang banyak kerana ia cepat dan mudah dilaksanakan terutamanya bagi sampel yang mempunyai kuantiti yang sangat sedikit.

EXPLORATION OF REAL TIME PCR AS A NEW DIAGNOSTIC METHOD OF FRAGILE X SYNDROME

ABSTRACT

Fragile X Syndrome (FXS) is the most common form of inherited mental retardation in males caused by FMR1 gene abnormality associated with CGG repeats expansion and hypermethylation status of its promoter. Methylated alleles usually lead to transcriptional inhibition and consequently loss of Fragile X Mental Retardation Protein (FMRP) production. Chemical modification of cytosine to uracil by sodium bisulfite treatment has provided an additional method for the laboratory diagnosis of FXS, thus avoiding the use of the laborious Southern blot analysis, which is the gold standard test for FXS diagnosis. Thus, a study was done to explore a rapid, easy, reliable and cheap method for FXS diagnosis that can replace the laborious, time consuming and expensive Southern blot method. Genomic DNA was extracted from peripheral blood of 45 clinically diagnosed FXS patients. Samples were treated with sodium bisulfite followed by amplification using real-time multiplex methylation specific PCR (RT-M-MSPCR) with *XIST* gene as an internal control, followed by melting curve analysis. Our results showed all control samples with known methylation status were correctly diagnosed using Real Time Multiplex Methylation Specific PCR. For method validation purpose, the methylation status of other 45 male patients sample were also successfully diagnosed using our RT-M-MSPCR method and were concordance with the results of the Southern blot. Thirty nine samples were found to have unmethylated allele, 4 samples were fully methylated and 2 samples have both methylated and unmethylated alleles implying a

diagnosis of mosaicism. The method that was developed was confirmed to be specific as the melting temperature of both methylated and unmethylated *FMR1* promoter was found to be varied in only a small range of melting temperature differences of 84.91 ± 0.41℃ and 90.57 ± 0.34 ℃ for methylated and unmethylated promoter respectively. The methylation percentage of mosaic patients were successfully calculated with a detection ability as low as 5% methylation percentage. However, due to random inactivation, female methylation status cannot be identified thus premutation and full mutation females cannot be differentiated from normal females. Having said that, male patients are more commonly diagnosed with FXS, thus this is not a major limitation for FXS screening. In conclusion, our results showed that RT-M-MSPCR is reliable, inexpensive and have 100% equal sensitivity as well as specificity compared with Southern blot. This newly developed method is also very convenient in screening large number of male FXS patients as it is non-time consuming and easy to perform especially when there is a low quantity of samples (final concentration of genomic DNA) that need to be sensitively and accurately determined.

CHAPTER 1

LITERATURE REVIEW

1.1 Research Background.

1.1.1 Mental Retardation

Mental retardation is a term used when a person has a significant below average intellectual functioning accompanied with limitations in adaptive functioning in at least two of the skill areas such as communication, self care, home living, social/ interpersonal skills, use of community resources, self-direction, functional academic skills, work, leisure, health, and safety (American Psychiatric Association, 1994).

Some mental disorders have a definite etiological basis. It maybe due to environmental effects such as toxin, infections, trauma, perinatal anoxia, biochemical, chromosomal abnormalities or Mendelian genetic disorder. In 2005, J. Hamel reported that mental retardation is one of the main reasons for referral in pediatric, child-neurology and clinical genetic clinic.

The prevalence of mental retardation in developed countries is thought to be 2–3% (Leonard and Wen, 2002). Compared to female, more males were found to be mentally

retarded in a population (Herbst *et al*., 1980), probably as a result of X-linked mental retardation condition (XLMR).

In 2001, Chelly and Mandel reported that at least 23 of XLMR genes have been identified. Mutation in these 23 genes may lead to syndromic (S-XLMR) and non-syndromic (NS-XLMR), depending on whether other abnormalities in addition to mental retardation are found on physical examination, laboratory investigation and brain imaging. The most frequent example of syndromic XLMR is Fragile X Syndrome (Hamel, 2005).

1.1.2 Fragile X Syndrome

Fragile X Syndrome (FXS) is the most frequent type of inherited mental retardation. The prevalence of Fragile X Syndrome is approximately 1 in 4000 males and 1 in 8000 females (Crawford *et al.*, 2001). As reported, approximately 1 in 700 females are carriers and the frequency of premutation in general population is approximately 1 in 259 females and 1 in 813 males (Turner *et al*., 1996; Rousseau *et al*., 1995).

Fragile X Syndrome is caused by an unstable $(CGG)_n$ tri-nucleotide repeat located in the Fragile-X Mental Retardation 1 gene (FMR1) [MIM 309550] at the chromosomal locus Xq27.3 (Oberle´ *et al*., 1991; Verkerk *et al*., 1991; Yu *et al*., 1991).

In 1943, J. Purdon Martin and Julia Bell described the first family with Fragile X Syndrome. In this family, 11 male members were severely mentally retarded, and the

inheritance pattern appeared to be X-linked. X-linked traits are inherited on the X chromosome and are more common in males, who have only one X chromosome, than in females, who have two.

A constriction near the end of the long arm (q arm) of the X chromosome in four mentally retarded males and two of their mentally normal female relatives were reported by Herbert Lubs in 1969. This constriction made the X chromosome appear to be wrecked. Hence the syndrome was named "Fragile X Syndrome".

More families were identified as having mental retardation with the same chromosome fragile site in the late 1970s. Moreover, in 1977 Sutherland discovered that the ability to distinguish this fragile site relied on the chemicals used to study patients' chromosomes and this important observation helped develop the first diagnostic test for Fragile X Syndrome.

1.1.3 Hereditary pattern

Fragile X Syndrome has a complex inheritance pattern. Carrier females can pass their CGG repeats mutation to their children of either sex. However, carrier males, also referred to as normal transmitters (NT), can only transmit the CGG repeats mutation to their daughters. This is because in all X-linked disorders there is no male-to-male inheritance. Usually, carrier males show no physical or mental features of the syndrome and express no or little fragile site expression during the cytogenetic analysis.

It was estimated that 20% of males carrying the mutation have no phenotypic expression; they are called normal transmitting males. Among carrier females, 55% were found to express Xq27.3 fragile site, and one third of the carriers are mentally impaired. However, daughters of normal transmitting males appeared to have little or no phenotypic expression (Heitz and Mandel, 1992).

Fragile X premutation gene does not expand in its CGG repeat size when passed from an unaffected father to his daughters. However, in female, they are at risk for having children with a stretched full mutation. Thus, whenever one person in a family is found to have Fragile X Syndrome, the mutation of the FMR1 gene can almost constantly be traced back to previous generations, although many of the gene carriers in the family may be unaffected.

Although the location of this fragile site at X chromosome established that Fragile X Syndrome was indeed X-linked, inheritance of this disorder was obviously not distinctive for an X-linked disorder. In 1985, Sherman *et al.* verified that the risk of expressing mental retardation was dependent on the individual's position in a pedigree, with risk increasing in later generations. This observation is recognized as the "Sherman paradox" and crucial in understanding the genetic mutation that causes Fragile X Syndrome.

In Sherman paradox, it was hypothesized that the mutation arises in a two step process. The first mutation leads to a "premutation" state with no clinical symptoms and the second mutation alters the premutation state to a "full mutation". (Sherman *et al.,* 1985)

Sherman paradox also described the phenomenon where the low penetrance occurs at approximately 18% in brothers of normal transmitting males and more than 80% penetrance in brothers of affected males.

1.1.4 Phenotypic Characteristics

People affected by Fragile X syndrome normally have a normal life span. It is because they do not suffer from any major medical problems and thus are generally healthy. However, there are some medical features which may be more common in Fragile X syndrome patients. Clinically, patients with Fragile X syndrome show wide variability of phenotype. The major clinical appearance in adult males is moderate mental retardation, with an IQ in the range of 40-70. However, mild mental retardation also been reported (Simola, 1984; Sutherland & Hecht, 1985; Fisch *et al*., 1991).

1.1.4.1 Intelligence Quotient (IQ)

Intelligence quotient (IQ) is widely used to define human general intellectual functioning (table 1.1). IQ less then 70 is classified as mental retardation. A person with mild mental retardation will have an IQ of 70-50 followed by moderate mental retardation with IQ range of 35-49. IQ range of 20-34 belongs to the severe mental retardation group and IQ less then 20, profound mental retardation. Approximately 2–3% of the population are mild to moderate intellectual disability and 0.5–1% of the population have from the moderate to severe mental retardation group (Raymond, 2005).

Table 1.1: Intelligence Quotient (IQ) Scores Reported by Different Authors in the normal population.

Level	Intelligence Quotient (IQ)				
	Sparrow et al., 1987*	Heber (AAMR, 1959, 1961)*	Grossman (AAMR, 1973)*	Grossman (AAMR, 1983)*	Ropers & Hamel, 2005)
Borderline	70 – 79	85 – 84	-	-	70-85
Mild MR	50 – 68	52 – 67	52 – 67	50 – 55~70	50-70
Moderate MR	40 – 49	36 – 51	36 – 51	35 – 40 to 50 - 55	35-50
Severe MR	30 – 39	20 – 35	20 – 35	20 – 25 to 35 – 40	20-35
Profound MR	< 20	< 20	20	< 20 or < 25	<20

*Source: Hussein, 1998.

1.1.4.2 Physical Characteristic

The physical characteristic in Fragile X patients include several dysmorphic features such as long and narrow face, prominent jaw, large ears, prominent forehead and macroorchidism. Affected females usually exhibit a similar but less severe phenotype than affected males.

Besides that, connective tissue abnormalities such as joint hypermobility also often arise. Joint hypermobility, also known as hyperlaxity or hyperextensibility is the term used for joints that stretch further than normal. For example, some hypermobile people can bend their thumbs backwards to their wrists, bend their knee joints backwards, or lay their leg behind the head.

Abnormalities of electroencephalography (EEG) are frequently found in Fragile X patients. However, epileptic seizures are rare (Opitz & Sutherland, 1984; Simola, 1984). In Fragile X patients, the EEG pattern is usually similar to rolandic epilepsy pattern. In this pattern of EEG, sharp waves with in the centrotemporal regions of the brain are discharge during sleep. Other EEG patterns that have been previously reported include theta rhythm, diffuse or multifocal spike waves, and a diffuse slowing background activity. Females with Fragile X Syndrome tend to present with unspecific EEG patterns. Thus, the frequency of epilepsy is lower (Feliciano, 2005).

In Fragile X Syndrome, it is hypothesized that the presence of EEG abnormalities such as excessive neuronal excitation and spiking could be caused by the dendritic spine

anomalies that can be observed in the brain of Fragile X Syndrome patients. Another theory proposed on GABAnergic system dysfunction due to the absence of FMRP (Feliciano, 2005).

Signs like hyperactivity, attention deficiency, learning disabilities and delay in learning language are often the first sign that lead to suspicion of Fragile X syndrome (Fryns *et al*., 1984, Sudhalter *et al*., 1991). Other behavioral abnormalities are also frequently observed in Fragile X males that include autistic-like behavior such as hand flapping, hand biting, rocking, poor eye contact and repetitive speech patterns, perseveration and echolalia. However, behavioral abnormalities occasionally happen in females (Reiss & Freund, 1992).

Usually, the clinical presentation of Fragile X Syndrome is variable and not all physical or behavioral symptoms are shown in every patient and the somatic signs are mostly apparent after puberty. Additionally, some patients do exhibit other unusual phenotypic profiles resembling Prader-Willi Syndrome (de Vries & Niermeijer, 1994) and Sotos Syndrome (Beemer *et al.*, 1986). Therefore, Fragile X Syndrome is often difficult to diagnose on a clinical basis alone especially prior to puberty.

1.1.5 Neurological abnormality

Recently, a progressive neurological abnormality has been identified, affecting male carriers beyond the age of 50 years old. The male carriers are generally healthy and have normal to above average intelligence until they reach their middle age. After the age of

50 years old, the men will have Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) and is characterized by tremors, balance problems and dementia that usually progress with age.

Due to the symptoms, persons with FXTAS were wrongly diagnosed as having Parkinson's disease, multiple systems atrophy-cerebellar type, sporadic olivopontocerebellar degeneration, or essential tremor. The correct diagnosis is however very crucial for appropriate treatment (Hagerman *et al*., 2001).

1.1.6 Structure and Function of FMR1 gene

A gene named FMR1 that caused Fragile X Syndrome was first cloned in 1991. The size of *FMR1* gene is about 40 kilobases (kb), encoding 3.9 kb of mRNA, consisting of approximately 0.2 kb of a 5' untranslated region, a 1.9 kb protein coding region, and a 1.8 kb 3' untranslated region. (Verkerk *et al*., 1991) The FMR1 gene is composed of 17 exons and the first exon is 9.9kb.

In most cases, Fragile X Syndrome occur due to elongation of a polymorphic repeat of $(CGG)_n$ stretch located at 5'- untranslated region of the FMR-1 gene (Oberlé *et al*., 1991; Fu *et al*., 1991; Kremer *et al*., 1991). FMR1 transcription is suppressed when the CpG island in its promoter region located 250 bp upstream from the CGG repeats is stretched and methylated (Eichler *et al*., 1993).

Repeat sequence of $(CGG)_n$ in normal individual is from 5 to 53 repeats per individual. In Fragile X premutation, the CGG triplet repeat number is from 60 to 200 repeats and become progressively unstable as the number of repeats increase. The bigger the extent of a woman's premutation CGG repeats, the higher the risk of expansion to a full mutation in her children (Sherman *et al*., 2005).

Even though the repeat range of normal and premutation individual has been identified, there is still no clear boundary between the upper limit of normal and the lower limit of the premutation range. Thus, alleles with 45-55 CGG repeats fall within the gray zone category. Some alleles in this category are unstable and can enlarge from generation to generation while others are stably inherited (Fu *et al*., 1991).

When the CGG repeats is 200 or more, the stretched repeat sequence and flanking CpG island are usually hypermethylated. This phenomenon will cause transcriptional silencing of the gene that is commonly referred to as the FMR1 full mutation (Reiss *et al*., 1995).

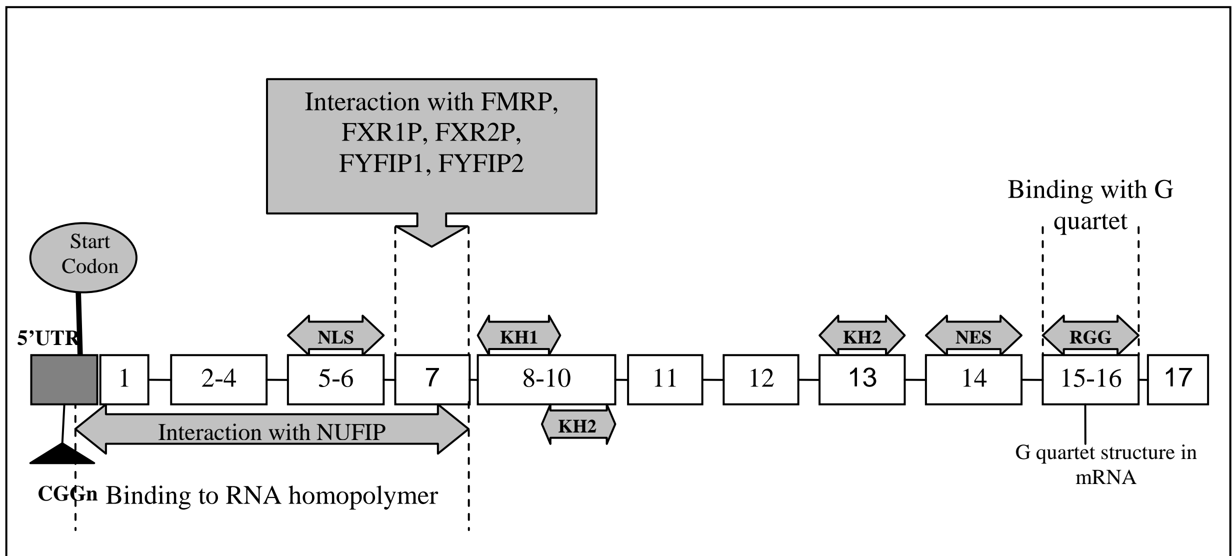
Normally, AGG interruptions of CGG triple repeat sequences take place once every nine or ten CGG repeats. The majority (64%) of normal alleles consisting of 5–50 CGG repeats have two or more AGG interruptions. However, most of the premutation alleles (50–200 repeats) have a single or no interruption (Eichler *et al*. 1995). In 1997, Chen *et al*. had revealed that the sequence organization of the normal Caucasian individual's alleles is 10A9A9 and Asians normally had the 9A9A9.

For Asians, the most frequent allele size in normal individuals was 29 (50%) followed by 30 repeats (22%) but for the Caucasians, the most common size of $(CGG)_n$ was 30 repeats followed by 29 repeats. For Native Americans from Pacific Northwest, the most frequent allele size was also 29 and 30 repeats with the remaining alleles of not more than 39 repeats (Chen *et al*., 1997).

1.1.7 Fragile X Mental Retardation Protein

Generally, FMR1 gene code for a protein called Fragile X Mental Retardation Protein (FMRP). This protein is a selective RNA binding protein which controls other mRNA, mainly in the central nervous system which probably plays an important function in brain development (Abitbol *et al*., 1993). FMRP is normally expressed in neurons, lymphocytes, fibroblasts, testes and placenta (Siomi *et al*., 1993). In a normal brain, FMRP is expressed in large amount in the cortex, hippocampus and cerebellar granule cell layer.

The 4.4 kb full length mRNA from the FMR1 gene can code for a protein with a maximum length of 632 amino acids and 70–80 kDa of molecular mass (Verheij *et al*., 1993). However, from 20 different transcripts that might be produced by alternative splicing, only 4–5 of them and their corresponding protein products are actually detected in various tissues (Ashley *et al.,* 1993).



Source: Bardoni & Mandel, 2002.

Figure 1.1: The functional structure of FMRP.

To perform its role, FMRP binds to its own mRNA to a sequence containing a G quartet structure that is also found in some of the other mRNAs. As in figure 1.1, FMRP contains two types of RNA-binding motifs, 2 KH domains and an RGG box (Ashley *et al*., 1993). The importance of these domains was shown by discovery of a mentally retarded male with a point mutation that results in a second KH domain amino acid substitution (I304N) (De Boulle *et al*., 1993). In 2001, Schaeffer *et al*. reported that FMRP binds to its own mRNA via a purine quartet that is found at the C-terminal end of the part coding for the open reading frame.

At the subcellular level, FMRP is found in the cytoplasm and it is mainly associated with actively translating ribosomes (Khandjian *et al*., 1996). FMRP can dimerize either with itself or with the related proteins like FXR1P or FXR2P (Tamanini *et al*., 1999). These proteins have both a nuclear export signal at the C terminus and location signal within its N terminus. (Eberhart *et al*., 1996) The presence of these two patterns suggests that FMRP can move between the cytoplasm and the nucleus and might have a function in the transport of certain specific RNAs from the nucleus to the cytoplasm.

In 2001, Brown *et al*. identified that some of the FMRP-associated mRNAs were underrepresented in polysomes in patient cells but the mRNAs are equivalent in the cell itself. This alteration in polysome distribution of G quartet-containing mRNAs can be explained by assuming that FMRP is required to bring those mRNAs to the translating ribosomes. The misregulation of certain mRNAs might be one of the causes of mental retardation seen in Fragile X Syndrome.

1.1.8 Mutation that cause Fragile X Syndrome

Expansion of (CGG)n repeats was identified to be associated with hypermethylation of the region and may lead to the loss of activity of FMR1 gene in almost all fragile X cases. However, other alterations in the FMR1 gene can also be one of the origins of Fragile X Syndrome For example, intragenic point mutations (Lugenbeel *et al*., 1995) and deletions. In 1997, Wolff *et al*. observed that large deletion may eliminate the entire FMR1 gene and also the flanking sequence of the gene that may lead to Fragile X Syndrome. However, in 1992, Wöhrle *et al*. claimed that smaller deletions may also lead

to Fragile X Syndrome by removing the proximal part of the FMR1 gene from the 5' upstream region to exon one until exon eleven.

In 1993, De Boulle *et al.* characterized a point mutation of a single base pair transversion from T to A that changed an Ile codon 304 to an Asn codon (Ile304Asn). Although the altered protein differs by only one amino acid, location of this substitution in one of the KH domains appears to totally ruin the normal function of FMRP. So, in spite of normal mRNA levels, this mutation may lead to a particularly severe phenotype.

In 1995, Lugenbeel *et al*. reported other intragenic mutations in the FMR1 gene of a typical clinical phenotype fragile X patient. The mutation was a one base pair deletion in exon 5 resulting in a frameshift that may lead to a premature translational termination of the protein product. A two-basepair change in the splice acceptor site of exon 2 was also predicted, resulting in two transcripts of reduced size detected by RT-PCR.

1.1.9 FMR1 gene Hypermethylation Mechanism

Methylation of DNA mostly occurs at the carbon-5 position of cytosine residues within CpG island (Gardiner *et al*., 1987). CpG islands are the GC rich region of DNA. On average, the CpG islands are 0.5-4.0 kb in length and are unmethylated in normal tissues when initiated in the 5’ region of genes (Bird, 2002). This CpG islands are frequently located within the promoter region of human genes. Methylation within the CpG islands was shown to be associated with transcriptional inactivation and functional silencing of the corresponding gene due to chromatin compression (Jones & Takai, 2001).

DNA methylation is a major epigenetic modification of human genomic DNA. It is catalyzed by DNA methyltransferase enzymes that perform the addition of a methyl group to the carbon 5 of the cytosine ring in CpG nucleotides. This condition takes place only at cytosine bases that are located at 5' to a guanosine in each CGG trinucleotide repeats within a mammalian genome (Bird, 2002 & Gutierrez *et al*., 2003).

In the methylation mechanism, excessive expansion of the CGG repeat allows the formation of abnormal (non Watson-Crick) structures that most probably formed on the lagging strand during replication (Darlow and Leach, 1998). These abnormal structures may attract DNA methyltransferases (Kho *et al*., 1998). The hypermethylation of the CGG repeat would perhaps spread to the surrounding CpG island through indirect (MeCP2/MBD mediated) interference on transcription that may cause the 'de novo' methylation of the promoter followed by gene silencing.

1.1.10 Fragile X Mental Retardation 2 Gene (FMR2 gene)

FMR2 is a gene adjacent to the FMR1 gene. It consist of FRAXE which is a folate-sensitive fragile site situated in Xq28 that lay approximately 600kb distal to the FRAXA (Sutherland *et al*., 1992; Knight *et al*.,1993). Molecularly, individuals that express FRAXE had extension of a CCG repeat adjacent to a CpG island. Normal individuals show 4–39 copies of the polymorphic FRAXE CCG repeat, while individuals that express the fragile site usually have more than 200 copies and their CpG island was fully methylated (Knight *et a*l., 1993). Mutation of FRAXE may lead to a less specific and milder form of mental retardation.

Apart from the FRAXA and FRAXE, FRAXF (Parrish *et al*., 1994), FRA16A (Nancarrow *et al*.,1994) and FRA11B (Jones *et al*.,1995) were also identified to be able to undergo expansion and hypermethylation and are also associated with fragile sites. However, all these repeats are located in the untranscribed regions of the genome and have not been associated with any genetic disorder when 'fully' expanded and hypermethylated.

1.2 Gold Standard of Fragile X Syndrome Laboratory Diagnosis

1.2.1 Cytogenetic analysis

According to the Oxford Concise Medical Dictionary, cytogenetics is defined as a branch of biology that deals with the study of heredity and variation by the methods of both cytology and genetics. It concerned with the study of chromosomes and cell division that includes routine analysis of chromosomes such as G-banding, C-banding, Unbanded-Giemsa and other cytogenetic banding techniques, as well as molecular cytogenetics such as fluorescent in situ hybridization (FISH) and comparative genomic hybridization (CGH). Chromosomes were first observed in plant cells by Karl Wilhelm von Nägeli in 1842. Their behavior in animal (salamander) cells was illustrated by Walther Flemming, in 1882.

In cytogenetics technique, chromosomal analysis starts with white blood cells culture in a medium followed by treating cells in a hypotonic solution, which will swell the cells and spread the chromosomes. Mitosis is later arrested in metaphase by a solution of colchicines that is applied before squashing the preparation on the slide forcing the

chromosomes into a single plane. The analyses of the result are done by capturing the chromosomal image followed by karyotyping.

Fragile site at Xq27.3 (FRAXA) is one of the rare folate-sensitive fragile sites found in human chromosomes that can be identifed by cytogenetics technique (Sutherland, 1977). Since the detection of a unique type of X-linked mental retardation occurring in combination with a fragile site on the end of the long (q) arm of the X chromosome, efforts were made to demonstrate this oddity in lymphocytes (Lubs, 1969; Sutherland, 1977), lymphoblastoid cell lines and fibroblasts (Jacky and Dill, 1980; Tommerup *et al*., 1981). This cytogenetics test was done on affected males and female carriers as well as in amniotic fluid cells of carriers in order to perform prenatal diagnosis (Jenkins *et al*., 1981).

The expression of the fragile site on the X chromosome is well-known to be dependent on culture conditions, such as the content of folic acid and thymidine in the medium (Sutherland, 1977). Therefore, either a folic acid deficient medium such as TC 199 (Sutherland, 1977) or dihydrofolate reductase inhibitors like methotraxate (MTX) or thymidylate synthetase such as 5-fluorodeoxyuridine (FUDR) are used to demonstrate the presence of a folate sensitive fragile site at the X chromosome (Glover, 1981).

Although cytogenetic methods have improved tremendously, expression of fragile site at Xq 27.3 can never be detected in all metaphase spread of the patient’s cell. The expression usually varies from 2% to 60% of the metaphase examined. The frequency of X chromosomes with fragile site appears to be lower in heterozygous females compared

with affected males (Felix *et al*., 1998). For diagnostic purpose the minimum frequency of fragile X metaphases recommended was 4% (Jacky *et al*., 1991). However, even lower cut-off points are commonly used in diagnostic laboratories. The limitations of the cytogenetic test were obvious in detecting clinically normal carriers.

1.2.2 Direct mutation analysis (Southern Blot Analysis)

Southern blot technique is a technique of transferring deoxyribonucleic acid (DNA) to a solid membrane support for subsequent hybridization with a specific probe. This method was introduced by Edward M Southern in 1975. By using this technique, complex population of DNA molecules can be screened for the existence of sequences associated to a selected probe.

Methylated regions mapping in DNA using Southern blot are in fact based on the inability of methylation-sensitive restriction enzymes to cut sequences that contain methylated CpG site(s). This method can provide an estimation of the overall methylation status of CpG islands including some quantitative analysis (Issa *et al*., 1994). However, Southern blot requires large amounts of high molecular weight DNA (generally 5 μg or more).

Firstly in Southern blot technique, the DNA must be prepared and digested. Secondly, the fraction of DNA is separated according to size by agarose gel electrophoresis followed by transferring and fixation of the DNA onto the membrane. Hybridization of the membrane with a nucleic acid probe must then be done. After hybridization, the

membrane needs to be washed to remove the unspecific signal and lastly the hybridized probe will be subsequently detected.

In the diagnosis of Fragile X Syndrome, Southern blot only allows an estimated measurement of the size of CGG repeat segments but at the same time, an accurate assessment of the methylation status can be assayed. Even though Southern blot analysis accurately detects alleles in large size ranges, precise sizing is still not possible. Moreover, Southern blot analysis is more labor intensive than PCR and requires larger quantities of genomic DNA (Sherman *et al*., 2005).

1.2.3 Direct mutation analysis (Polymerase Chain Reaction)

Polymerase chain reaction is a procedure that allows trace amounts of DNA to be quickly and repeatedly copied to produce a quantity sufficient to be analyzed using conventional laboratory methods.

A typical PCR programme usually consists of a denaturing step, an annealing step, and an extension step. Denaturation is normally carried out at 94 – 95 ºC but principally, it begins at 70ºC. During denaturing, two strands of DNA template are separated from each other. At the same time, polymerase enzyme is denatured, nucleotides are degraded, and primers are depurinated.

The temperature is then lowered to 55ºC so that oligonucleotide primer can hybridize on the single-stranded DNA template. Subsequent to that, the temperature is increased to

the optimal temperature for Taq polymerase which is 72ºC. As a consequence, the primer is elongated until a double-stranded DNA is formed, which is exactly equivalent to the original DNA template. For every cycle, the number of template DNAs will be doubled due to the complementation that occurs along both strands of the DNA templates.

1.3 Alternative potential methods on Fragile X Syndrome Diagnosis

1.3.1 Real Time PCR

Real Time Polymerase Chain Reaction (Real-time PCR) is a technique that is widely used recently. Real-time PCR system is based on the detection and quantification of a fluorescent reporter (Dorak, 2006). In real time PCR, the fluorescence emitted is monitored during the reaction as a marker of amplicon production during each PCR cycle (in real time) compared to the endpoint detection. This signal increases in direct proportion to the amount of PCR product in a reaction.

Real-time PCR that is also known as Quantitative Polymerase Chain Reaction (qPCR) is based on the revolutionary method of PCR. It is now has appear to be new and powerful application and widely used all over the world as the method of choice among scientist (Mark *et al.*, 2005). The potential of Real-time PCR is not only for better quantification of the starting DNA amount in the samples of an amplification but also for the detection of gene copy number and gene expression (Mark *et al*., 2005).

However, high GC content is always present in the genomic target sequence and as in conventional PCR, these GC rich sequences can inhibit the amplification process. High GC content promotes the formation of alternative DNA structure (Marco *et al*., 2006). It can also generate the polymerase pause site which will cause errors during the replication process (Mahmoud *et al*., 2008).

1.3.2 Taqman Chemistry

Presently, the popular detection method used for PCR products is the dye-based methods that utilize an intercalating dye (e.g., SYBR Green I). However, in real time PCR this intercalating dye is added to the PCR mix. This dye will bind and detect all double-stranded DNA that accumulates during the assay, regardless of the sequence of that DNA. Although this method offers great flexibility, it can unfortunately detect multiple amplicons that are generated by nonspecific priming as well as primer-dimers.

However, probe-based detection format can be used to replace the dye-base detection method. The probe-based method use labeled and sequence-specific probe that will anneal between the primer sites and will only generate a signal when correct amplicon is produced.

Taqman probe is one of the types of probe-based detection format. Taqman probe uses the hydrolysis probe format for real-time PCR detection. Technically, the hydrolysis probe format is a homogeneous 5´-nuclease assay, since a single 3´-non-extendable probe is cleaved during amplification. This single probe contains two labels near to each

other which are a fluorescent reporter dye at the 5´-end and a quencher label at or near the 3´-end.

When the probe is intact, the fluorescent signal is almost completely suppressed by the quenching label. When the probe is hybridized to its target sequence, it is cleaved by the 5´to 3´ exonuclease activity of the Taq DNA Polymerase, which “unquenches” the fluorescent reporter dye. During each PCR cycle, more of the released fluorescent dye accumulates, enhancing the fluorescent signal.

1.3.3 Lock Nucleic Acid

Lock Nucleic Acids (LNAs) are a novel nucleic acid analog and are used in probes and primers that require high oligonuleotide hybridization strength and specificity. Lock Nucleic Acid (LNA) Probe contains a 2’-O, 4’-C methylene bridge (figure 1.3). This bridge restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation that lead to enhance hybridization performance and excellent biological stability.

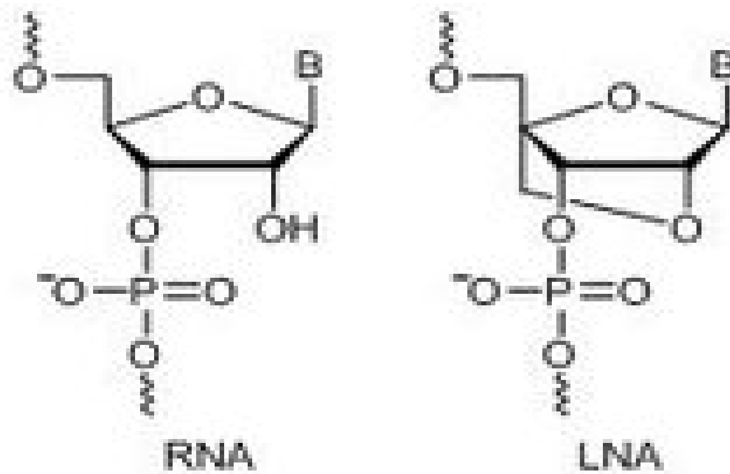


Figure 1.3: The differences in the structure between RNA and LNA monomer.