CHAPTER 2

LITERATURE REVIEW

2.1. FXTAS: THE *FMR1* GENE AND CLINICAL ASPECTS

The fragile X mental retardation 1 gene (*FMR1)* consists of a 38 kB region with 17 exons and is located at Xq27.3 (33). This gene is responsible for producing fragile X mental retardation protein (FMRP) which is an RNA binding protein that associates with polyribosomes (34). FMRP is highly present in adult human brain, especially in neurons (35). FMRP is involved in the transport and regulation of local mRNA translation at synaptic sites, which is important for neuronal function, including modulation of synaptic plasticity (36, 37). FMRP binds to specific mRNAs and mediates the targeting of these transcripts into the dendrite. Therefore, FMRP might have a role in transport and/or translational efficiency of specific mRNAs at the synapse (20).

There is a CGG repeat lying in the 5' UTR of *FMR1* with a variation in length among individuals. This CGG is transcribed but not translated. In the population, normal individual has 6-54 CGGs, with 29-30 CGGs on average (2, 38). These CGGs are vulnerable for dynamic mutation, which causes expansion of the repeat number. Expansion up to 55-200 CGG repeats is defined as premutation (PM). Individuals in premutation range exhibit elevated *FMR1* RNA levels and reduced protein levels. When the expansion reaches more than 200 repeats, defined as full mutation, the *FMR1* promoter region becomes hypermethylated

and is transcriptionally silenced, hence no FMRP is produced. This condition results in a mental retardation disease called fragile X syndrome. The prevalence of Caucasian individuals with fragile x syndrome is estimated about 1 in 4000 (4). In Indonesia Faradz *et al.* found that 5 of 262 male students (1.9%) with developmental disability in special schools were positive for fragile x syndrome (5).

Expansion to full mutation usually occurs from premutation and is restricted upon maternal transmission. In other words, mothers with premutation CGG size have a high risk to have children affected with fragile X syndrome (39). Sperm of full mutation males contains only premutation alleles, which explains why daughters of full mutation males are never affected with the fragile X syndrome (40). AGG interruption commonly appears every 9-11 CGG repeats of normal individuals, but it is absent in mostly premutation individuals. The presence of AGG is considered to be able to stabilize the repeat. Premutation females with an AGG interruption transmit the CGG repeat to the children more stable than without AGG interruption (38, 39). The presence of AGG in expanded premutation CGG does not influence either transcription or translation, according to *in vitro* and cell culture study (41). Study in general population of North America has shown that one in 813 males and one in 259 are premutation carriers (38, 42).

Premutation individuals were thought to have no clinical risk except that of transmitting more expanded repeats and a high risk to have fragile X syndrome children especially for woman. However it has become clear that 20% of the

premutation females manifest premature ovarian failure (POF), in which the menstruation cycle stops before the age of 40 (43). Moreover the premutation males have a chance to develop a disease called fragile X-associated tremor/ataxia syndrome (FXTAS). More than a third of premutation males older than 50 years develop FXTAS. The penetrance increases with age, becoming 50% at the age of 70-90. It has been suggested that the longer the CGG repeats the more increase in penetrance of FXTAS (9, 10). Allen *et al*. defined CGG repeats with more than 70 trinucleotides as a risk allele, while lower than 70 as low risk allele (44).

Patients with FXTAS develop some clinical features. They generally have cerebellar gait ataxia and intention tremor (8). Other features common seen are Parkinsonism, lower extremity neuropathy, autonomic dysfunction, and cognitive decline. The cognitive decline varies from mild frontal executive and memory deficits to global dementia (7, 8, 11). Besides the clinical features, individuals with FXTAS also display some psychological features. Psychiatric features present are anxiety, disinhibition, depression, and apathy (13).

It is very rare that females are affected with FXTAS. The reason behind this fact is probably caused by the presence of another normal allele which has normal expression and protects against the negative effect of premutation allele. A mix of the normal allele with the premutation allele in females perhaps explains the less severe outcome in female FXTAS compared with male FXTAS. It is considered that positive skewed X-inactivation in which normal allele express more than premutation allele, will lessen the toxic effect in FXTAS (45-47).

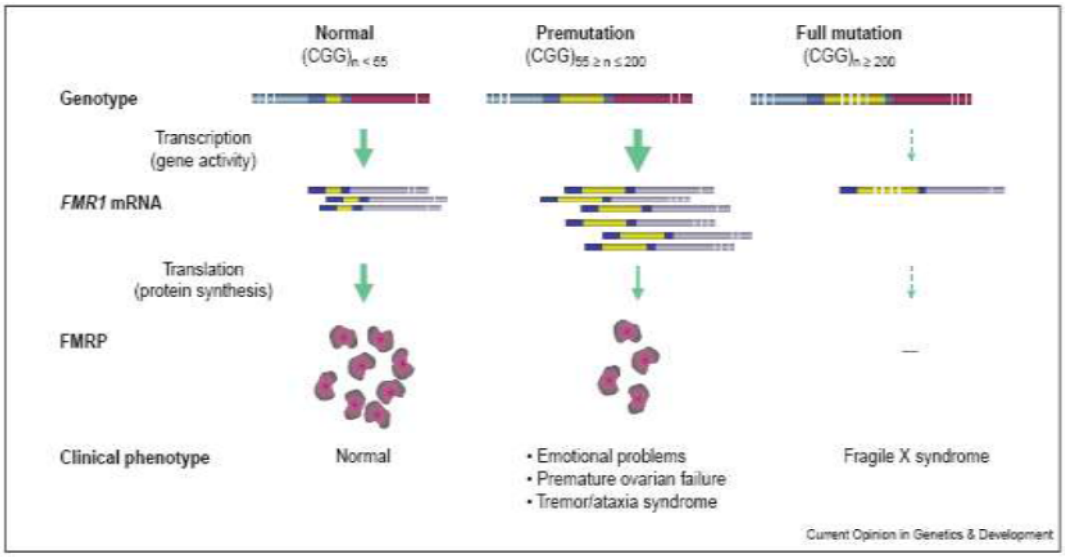

Figure 1. **CGG length, *FMR1* expression and the clinical outcomes** (48).

2.2. NEUROPATHOLOGICAL OF FXTAS

Neuroanatomical studies have shown Purkinje cell loss and Bergmann gliosis in premutation carriers. Global brain atrophy; white matter disease in subcortical, middle cerebellar peduncle (MCP), and periventricular region; and dilated ventricles are seen as MRI features of FXTAS (8, 49, 50). The MCP sign is a diagnostic criterion, since it is observed in 60% of premutation carriers with tremor and/or ataxia. Reduction in cerebellar volume, increased ventricular and white matter volume were also observed in a study of male premutation carriers (50).

The major neurohistological hallmark of FXTAS is the presence of eusinophlic ubiquitin-positive intranuclear inclusions in neurons and astrocytes (Figure 2) (14, 19). The inclusions are profound in the hippocampus and can be found in cerebrum and brain stem as well. The inclusions are not found in

Purkinje cells, although these cells are dropped out in FXTAS patient (15). Many proteins now have been identified to co-localize in the inclusions. Those proteins can be divided into eight categories: Ubiquitin-proteasome system, histone family, intermediate filament, microtubule, myelin associated protein, RNA-binding protein, stress-related protein, and chaperone (16, 17) (see table for detail). Recently another protein, sam68, was just identified to be present in human FXTAS brain (51).

Table 1. Proteins found in the human brain inclusions (16)

Proteins	**Category**
Ubiquitin	Ubiquitin-proteasome system
11S regulator to 20S proteasome	Ubiquitin-proteasome system
H2B histone family	histone family
Similar to H2A histone family, member Z	histone family
HIST I H4D protein	histone family
H2A histone family A (L)	histone family
H2A histone family, member Q (O)	histone family
Neurofilament 3	intermediate filament
Lamin A/C	intermediate filament
Vimentin	intermediate filament
Internexin neuronal intermediate filament	intermediate filament
NEFL protein	intermediate filament
Glial fibrillary acidic protein	intermediate filament

Beta5-tubulin	microtubule
Tubulin, alpha6	microtubule
Tubulin, alpha and ubiquitous	microtubule
Myelin/oligodendrocyte glycoprotein betha3 ‘	myelin associated protein
2’, 3’-cyclic nucleotide 3’-phospodiesterase (CNPase)	myelin associated protein
Myelin basic protein	myelin associated protein
Muscleblind-like1	RNA-binding protein
Heterogeneous nuclear ribonucleoprotein A2/B1	RNA-binding protein
Pur-alpha	RNA-binding protein
Alpha-beta crystalline	stress-related protein
HSP70	chaperone
HSP27	chaperone
HSP40	chaperone

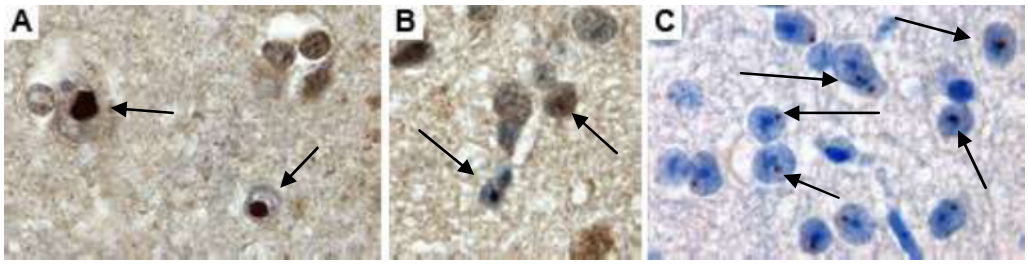

Figure 2. **Ubiquitin-positive intranuclear inclusions in human neurons (A), human astrocytes (B), murine neurons (C)** (52). Ubiquitin inclusions are shown with the arrow as a dark brown staining in the cell nucleus.

2.3. MOLECULAR PATHOGENESIS OF FXTAS

A toxic RNA gain of function mechanism has been proposed as the mechanism underlying FXTAS (21, 22). The fact that premutation carriers have elevated levels of *FMR1* RNA as much as 8 fold over normal allele gives a clue to the proposed mechanism (6). The finding of *FMR1* RNA in the inclusions of premutation carriers also supports the toxic RNA gain of function mechanism. Another clue drives to this proposed mechanism is the fact that older full mutation carriers (which is not producing *FMR1* RNA) have totally different outcomes with FXTAS (18, 19).

Premutation carriers exhibit elevated levels of *FMR1* message with reduced levels of FMRP (6, 53). A feedback mechanism proposes that the elevated *FMR1* transcript levels in premutation carrier are caused by the increased transcription as an effort of the cells to compensate the low levels of FMRP (54). Based on study of Chen *et al.,* the elevated levels of *FMR1* mRNA is likely because of the CGG itself, in which the more CGG repeats will result in more open promoter leading to enhanced access for transcription factor (20, 55). While reduced levels of FMRP are caused by reduction of translational efficiency in alleles with long CGG tracts, in which this long CGG in the RNA will result in secondary structures, hairpins, causing an impediment of ribosome to effectively doing its work (41, 55, 56).

The mechanism leading to the pathology of myotonic dystrophy (DM) gives an insight into the mechanism underlying FXTAS disease, since both diseases are derived from the same genetic basic, an expanded repeat in non-

coding region. Myotonic dystrophy is caused by expanded CTG in 3' UTR of myotonic dystrophy protein kinase (*DMPK*) gene or by expanded CCTG in intron 1 of *ZNF9* gene (57). The expansions in DM produce nuclear foci similar to intranuclear inclusion seen in FXTAS. The nuclear foci contain the expanded repeat RNA as well as sequestered proteins especially RNA-binding protein such as MBNL1. Studies in DM conclude the toxic RNA gain of function as the mechanism leading to the development of this disease (57).

The toxic effect of expanded premutation CGG RNA was also found when human neural cells were transfected with *FMR1* 5'UTR with 88 CGGs and a GFP reporter. Expression of this expanded premutation CGG repeat reduced cell viability. Inclusions containing alpha-beta crystalline were observed, as well as an alteration in morphological and nuclear lamin structure. The cytotoxicity was indeed caused by the CGG RNA instead of the DNA, because cells transfected with expanded premutation CGG with deleted promoter, which means no expression, didn't show reduced cell viability (58). One study using microarray has shown the upregulation of genes required for apoptosis in cells transfected with construct expressing premutation CGG (59).

Expressing the expanded premutation CGG as a transcript in *drosophila melanogaster* flies displayed deterioration of neurons in the fly eyes. It also induced the formation of inclusions in the fly, which contained ubiquitin, Hsp70 chaperone and the proteasome (60). Evidences for the toxic effect of CGG RNA also come from studies using mice.

COS7 cells transfected with expanded 60 and 100 CGGs formed CGG RNA nuclear aggregates, according to studies using cell culture with fluorescence in situ hybridization (FISH) technique. The CGG RNA aggregates expanded over time and finally formed giant inclusions. The cells exhibited nuclear lamin A/C architecture disruption and cell death 72-96 hours after transfection. Cells transfected with only 20 CGGs did not exhibit those aggregates and disruptions, while transfection of 40 CGGs resulted in intermediate situation with rare and small intranuclear aggregates. Observation to the cells 24 and 72 hours after transfection demonstrated the presence of MBNL1 and hnRNP-G which are hardly seen at 24 hours, but increased after longer incubation. While sam68 was clearly seen starting from 24 hours, suggesting sam68 is an early inclusions marker (51).

It still remains a question why the expanded premutation CGG RNA and proteins sequestered in the nucleus and why the inclusions can produce FXTAS symptoms. One proposed mechanism explains that the cell might attract chaperones or elements of the ubiquitin/proteasome system to remove the excess of elevated expanded RNA. This CGG also recruits RNA-binding proteins and other proteins present in the inclusion. Those proteins sequestered in the inclusions might be important and the sequestrations can cause disorganization of cellular function regulated by those sequestered proteins, hence finally produce the neurodegeneration (16, 22). A possible cause of protein sequestrations are protein-protein interaction instead of directly recruited to the CGG RNA

aggregates. This is based on an observation if sam68 is not present in the inclusions, other proteins such as MBNL1 and hnRNP-G are absent as well (51).

2.4. MOUSE MODEL OF FXTAS

A KI mouse model was generated to study repeat instability by homologous recombination technique replacing the endogenous murine (CGG)8 with human (CGG)98. Cloning was performed with minimal changes made on the sequence. All known regulatory elements on the promoter were maintained. Although this long repeat is enough to produce repeat instability like in human case, these mice showed only minor instability of the CGG repeat and no methylation was observed even if the mice reach more than 200 repeats (24, 26, 27). After FXTAS was recognized and described, these mice then were subjected for neurohistology, biochemistry and molecular aspects of FXTAS (29). Elevated RNA levels were detected 3.5 fold compared with control mice (27).

Ubiquitin-positive intranuclear inclusions were also found throughout the brain. Appearance of the inclusions started at week 30 and the number expanded over time like in human FXTAS. Inclusions are present more than 55% in certain brain regions at 72 weeks of age. Proteins detected in inclusions were Hsp40, Rad23B, and 20S catalytic core complex of proteasome. Other proteins analyzed but not found in the inclusions were Fmrp, alpha-synuclein, poly-glutamine, and tau (29). The inclusions were not detected in the astrocytes, different with the human FXTAS findings (26, 29). This difference likely indicates lower/no expression of toxic RNA in the mice astrocytes. Other differences found in KI

mice compared to human FXTAS are the absence of neuronal loss, gliosis and Purkinje cell dropout. In behavioral tests, these mice didn't show a severe behavioral phenotype as seen in human patients. Only decreased performance in neuromotor tasks and mild age-dependent learning disturbances were seen in these mice (25).

Another KI mouse was generated by the Usdin group. These mice had an original size 118 of CGGs. They exhibited elevated levels of mRNA and showed positive correlation between the mRNA levels and CGG length. In some cases, large expansion to full mutation range was observed in these mice, however no methylation has been found. These KI mice exhibited similar genetic and pathophysiological performance to human FXTAS patients. These mice showed Purkinje cell loss with swollen axonal torpedos, which were not seen in the other KI mice (23).

Transgenic mice were also generated for FXTAS studies. These mice contained a transgene consisting of expanded premutation CGG and either *Fmr1* gene or GFP reporter gene. Using L7/Pcp2 promoter on the transgene, expression of this transgene was restricted to the Purkinje cells. Independent of the genes translated (Fmr1 or GFP), if expanded premutation CGG was transcribed in these transgenic mice, Purkinje neurons degeneration and axonal swellings were found. Again ubiquitin-positive intranuclear inclusions were present in the cells exposed to the toxic RNA, the Purkinje cells. Other proteins stained in the inclusions were 20S proteasome, Hsp40, and Rad23B. These mice demonstrated a decline in

motor-learning abilities in accelerating rotarod studies. This model has proven the toxicity of expanded premutation CGG RNA in mammals (61).

There is one sophisticated strategy to have a good mouse model for FXTAS. It is by using the tet-on-regulated inducible system and the specific driver promoter reverse tetracycline transactivator (rtTA). Inducible expression of an expanded premutation CGG repeat together with the use of specific driver promoters should give a good model to mimic the human FXTAS situation. The tet-on-regulated inducible system can control the expression by the induction of tetracycline (tet) or an analog such as doxycycline (dox) (30). This ability to control the expanded premutation CGG expression would be a great tool to study the reversibility of FXTAS. The specific reverse tetracycline transactivator (rtTA) drivers make it possible to restrict the place of toxic RNA transgene expression. The suitable driver promoters for FXTAS mouse model are PrP-rtTA which allows expression in all cell types in the brain except for the Purkinje cells (31); and GFA2-rtTA which restricts the expressions only in Bergmann glia and astrocyte (32). This restriction is a powerful tool to study the sufficiency and necessity of the cells in the development of FXTAS and allow us to know which cell types (neurons, Bergmann glia, astrocytes) contribute to FXTAS neuropathology.

Mice seem to be a good animal model to study FXTAS. There is a lot of information about FXTAS that can be collected using appropriate mouse model. The information will support a better understanding of FXTAS, hence opening possibilities for FXTAS therapies to cure this disease.