Altered expression of fragile X mental retardation-1 (FMR1) in the thymus in autoimmune myasthenia gravis

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Abstract
Predisposition to autoimmunity and inflammatory disorders is observed in patients with fragile X-associated syndromes. These patients have increased numbers of CGG triplets in the 5’ UTR region of FMR1 (Fragile X Mental Retardation 1) gene, that affects its expression. FMR1 is decreased in the thymus of myasthenia gravis (MG) patients, a prototypical autoimmune disease. We thus analyzed the number of CGG triplets in FMR1 in MG, and explored the regulatory mechanisms affecting thymic FMR1 expression. We measured the number of CGGs using thymic DNA from MG and controls, but no abnormalities in CGGs were found in MG that could explain thymic decrease of FMR1. We next analyzed by RT-PCR the expression of FMR1 and its transcription factors in thymic samples, and in thymic epithelial cell cultures in response to inflammatory stimuli. In control thymuses, FMR1 expression was higher in males than females, and correlated with CTCF (CCCTC-binding factor) expression. In MG thymuses, decreased expression of FMR1 was correlated with both CTCF and MAX (Myc-associated factor X) expression. Changes in FMR1 expression were supported by western blot analyses for FMRP. In addition, we demonstrated that FMR1, CTCF and MAX expression in thymic epithelial cells was also sensitive to inflammatory signals. Our results suggest that FMR1 could play a central role in the thymus and autoimmunity. First, in relation with the higher susceptibility of females to autoimmune diseases. Second, due to the modulation of its expression by inflammatory signals that are known to be altered in MG thymuses.

Keywords: Fragile X syndromes, Autoimmunity, FMR1, Thymus, Myasthenia gravis, Inflammatory cytokines, Thymic epithelial cells, CTCF, Max

Introduction
Regulation of the fragile X mental retardation-1 (FMR1) gene is dependent on CGG triplets in its 5’ UTR region. In most individuals the number of CGG triplets is around 30 ranging from 26 to 32 depending on studies [1]. However, this region can become unstable leading to CGG triplet expansion associated with diseases such as the fragile X syndrome (FXS, above 200 CGGs), fragile X-associated tremor/ataxia syndrome (FXTAS) or fragile X premature ovarian insufficiency syndrome (FXPOI) (from 55 to 200 CGGs) [1]. Individuals with 41 to 54 CGGs are considered in the “gray zone” genotype and seem also more at risk to have FXPOI [2]. Recent studies also define a new category of individuals with a lower number of CGGs, usually below 24–25. This genotype is also associated with health problems [3, 4] and a lower FMR1 expression [5]. FMR1 codes for the fragile X mental retardation protein (FMRP), a RNA binding protein with many functions. However, its role has been especially investigated...
in the nervous system [6], despite the fact that FMRP is highly expressed in other adult tissues, such as in the thymus [7].

Immune-mediated disorders, in particular autoimmune diseases, have been reported in premutation carriers, such as autoimmune thyroid disorders, rheumatoid arthritis, systemic lupus erythematosus, Sjögren’s syndrome [8, 9]. Myasthenia gravis (MG) is a rare autoimmune disease characterized by invalidating muscle weaknesses. It is caused by autoantibodies targeting components of the neuromuscular junction, mainly the acetylcholine receptor (AChR) [10]. We previously demonstrated that FMR1 expression is decreased in MG thymus [11]. The thymus plays a key role in central tolerance mechanisms avoiding the escape of autoreactive T cells and autoimmunity [12]. In addition, in MG, the thymus is often abnormal, and is characterized in AChR+ MG by ectopic germinal centers developing in an inflammatory environment [10]. These observations prompted us to further investigate the potential role of FMR1 in MG.

We hypothesized that MG patients could have an abnormal number of CGGs that would affect thymic FMR1 expression. This genotype could predispose to MG and even autoimmunity. Here, we analyzed the number of CGG triplets in the thymus of MG patients as compared to controls, and investigated the mechanisms regulating FMR1 expression in the thymus.

**Methods**

**Human samples**

For DNA analyses, thymic biopsies from AChR+ MG patients without thymoma (n = 58 females and 22 males, 18 to 50 years old) were collected after thymectomy and control thymic biopsies (n = 38 females and 10 males, 2 days to 50 years old) were collected from donors undergoing cardiovascular surgery at the Marie Lannelongue Surgical Center (Le Plessis-Robinson, France). In MG patients, peripheral mononuclear blood cells (PBMCs) (n = 8 females and 1 male) were also isolated from fresh whole blood, collected in EDTA tubes, using the Ficoll technique.

For RT-PCR analyses, thymic biopsies were also collected from AChR+ MG patients (n = 12 females, 15–35 years old) and control thymic biopsies (infant females n = 6, 3–12 months; adult females n = 6, 15–33 years old, and adult males n = 6, 15–44 years old). MG patients included had either a low-grade thymic hyperplasia (with 2 or fewer GCs per section, n = 6) or a high-grade thymic hyperplasia (with 3 or more GCs per section, n = 6). For western blot analyses, thymic biopsies were from AChR+ MG patients (n = 3 females, 31–37 years old) and control thymic biopsies (females (n = 2) and males (n = 2), 25–49 years old). All MG patients were only treated with cholinesterase inhibitors and had no other known diseases including thymoma.

Studies on blood and thymic samples were approved by local ethics committees (RCB 2006-A00164-47 and RCB 2010-A00250-39).

**DNA extraction and CGGs analysis**

DNA extraction was done from thymus biopsies or PBMCs using the all prep ADN/ARN mini kit from Qiagen (Courtaboef, France). Analysis of CGG repeat length and the number of AGG interruptions were determined using the AmpliPlex® FMR1 PCR kit from Asuragen (Theradiag, Marne la Vallée, France) according to the manufacturer instructions. Results were analyzed on GeneMapper software.

**RT-PCR**

RT-PCR analyses were done as previously described [11]. The primer sequences were from Eurogentec (Angers, France): FMR1 (F: 5’-CAGGGCTGAAGAAGATGG-3'; R: 5’-ACAGGAGTGAGGAAATCTGA-3'), CTCF (F: 5’-ACCATGGGAATTTGTTTCG, R: 5’-TCATGTCCTTTTCACCTTG-3'), MAX (F: 5’-ATGACATCGAGGTGGAGAGC-3'; R: 5’-CCTTTGGAGTGATGGGACTGA-3'). PCR were normalized to 28S (F: 5’-GGTAGGGACAGTGGGAATCT-3'; R: 5’-CGGGTAAACCGCGGGAGTAA-3') or GAPDH (F: 5’-CGACCATTGTCAAGCTCA-3'; R: 5’-AGGGGTCTACATGGCAACTG-3').

**Western blot**

Total thymic proteins were extracted in a solution containing 5% Tris–HCl 20 mM (pH 7.4), 0.1% Triton X100 supplemented with Halt™ Protease Inhibitor Cocktail (ThermoFisher Scientific, Villebon-sur-Yvette, France) using the fast prep apparatus. Extracts were cleared by centrifugation (13,000 g, 10 min. 4 °C). 20 µg of proteins were separated by 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked for 2 h in 5% dried milk in TBST before detection of the immune signal using ChemiDoc™ imaging system (Bio-Rad).
Thymic epithelial cell (TEC) culture  
Primary human TECs were cultured from infant thymus as previously described [11]. TECs were seeded (1.4 × 10^4 cells/cm^2) in RPMI-5% horse serum for 24 h and treated with Poly(I:C) (100 μg/ml; InvivoGen, Toulouse, France), IFN-I 1000 UI/ml (R&D Systems, Lille, France), IFN-II (IFN-γ (1000UI/ml; R&D systems) or IL-6 (10 ng/ml; R&D systems) in RPMI-0.5% horse serum for 24 h.

Statistical analyses  
For 2-by-2 comparisons, parametric (t-test), non-parametric (Mann–Whitney test) or paired (Wilcoxon test) tests were performed as specified in figure legends. Correlation analyses were performed using Spearman’s correlation coefficient for non-Gaussian distributed variables, with a p < 0.05 considered significant. For certain analyses, mean ± SEM were given in the text.

Results  
Comprehensive analyses of CGG triplets in MG patients  
We analyzed the number of CGGs in 80 MG patients and 43 control donors. To compare the number of CGG triplets in males and females, the mean of CGGs on both alleles of the X chromosome was calculated for females. The control donor group had 2 premutated female carriers with 46/55 or 47/55 CGGs. The mean number of CGGs was 29.7 ± 0.8 (or 28.8 ± 0.5) without the premutated carriers. In MG patients, the mean number of CGG triplets was 28.4 ± 0.40. No significant difference was observed between controls and MG patients, and the distribution of the number of CGG triplets was similar with a peak of CGG triplets at 29–30 (Fig. 1A, B).

As females are more susceptible to autoimmunity, we analyzed separately female and male samples. We did not observe differences between MG and non-MG-donors in both groups (Fig. 1C). The number of CGG triplets can be different on each chromosome X inherited from the mother and the father. We analyzed separately the shorter (Fig. 1D) and the longer (Fig. 1E) CGG sequence of both chromosmes. However, we did not observe significant differences with this method of analysis. Variations of the number of CGGs for a given individual have been mentioned in different tissues [13]. To determine if variations could occur in the thymus, we compared the number of CGG triplets in DNA extracted from the thymus or PBMCs from the same donors. We showed that the number of CGGs was identical using DNA from the thymus or from PBMCs from MG patients (Fig. 1F).

The CGG repeat segment can be interrupted by AGG triplets, usually two AGGs in the general population [14]. In our samples, a few ones were difficult to interpret and were excluded. Analyzing 77 MG patients and 33 controls, the same proportion of AGG triplets were observed in both groups with 80% of the donors having two AGGs, 13% having one AGG and 7% three AGGs (data not shown). For patients with two AGG triplets, no difference in the number of CGG triplets was observed between controls and MG donors (data not shown).

Differential expression of FMR1 in the thymus in normal conditions and in MG patients  
First, we analyzed the expression of FMR1 mRNA in thymic samples from males and females. Primers used amplified all FMR1 transcript variants and we clearly observed a significant higher expression in males compared to females (Fig. 2A). Human FMR1 gene has 17 exons that can undergo alternative splicing, resulting in different FMRP isoforms [15, 16]. At the protein level, we explored for the first time FMRP expression in thymus extracts. We detected different FMRP isoforms (Fig. 2D). The most well-known, the full-length isoform, and two spliced isoforms (bands 1–3 (isoforms 6, 4 and 7) between 71 and 68 KDa) [17], and also three isoforms with a lower molecular weight (bands 4–6). Comparing male and female healthy donors, we observed a higher expression in males for the three isoforms with a high molecular weight (Fig. 2D, E). This could reflect the higher expression of FMR1 mRNA observed in males (Fig. 2A). The three other isoforms were expressed at the same level (Fig. 2D, E). We then analyzed the level of mRNA expression of transcription factors known to regulate FMR1 expression, such as Nrf-1 (nuclear respiratory factor 1), Sp1 (specificity protein 1), USF1 (upstream transcription factor 1), MAX (Myc-associated factor X), CTCF (CCCTC-binding factor) [18, 19]. In normal thymuses, only CTCF mRNA expression was differently expressed in males and females, and significantly correlated with FMR1 mRNA expression (Fig. 2B, C).

Our team previously showed a significantly decreased expression of FMR1 mRNA in the thymus of MG patients whatever the degree of follicular hyperplasia [11]. This decrease was confirmed on a larger number of individuals (fold-change decrease of 1.9 (p = 0.0102 for 12 controls and 12 MG patients)). At the protein level, comparing healthy and MG females, we surprisingly observed a decreased expression of the three isoforms with lower molecular weights but not of the isoforms with a high molecular weight. This suggests that the decreased expression of FMR1 mRNA observed in MG [11] could affect the expression of the FMRP isoforms with a low molecular weight (Fig. 2D, E). In MG thymuses, CTCF and MAX mRNA expression were decreased and their expression was strongly correlated with FMR1 expression (Fig. 2F, G). Nrf-1 and Sp1 mRNA expression were not
altered in MG thymuses (data not shown). **USF1** mRNA expression was increased in MG thymuses but not correlated to **FMR1** mRNA expression (data not shown). We previously demonstrated that **FMR1** decreased expression in MG thymuses was observed in thymic epithelial cells (TECs) [11]. We thus investigated the effects of

**Fig. 1** Analyses of CGG triplets in **FMR1** 5'UTR in the thymus of MG patients. Analysis of CGG repeat length using the AmplideX® **FMR1** PCR kit. DNA was extracted from thymic biopsies. A Comparison of MG patients (n = 80) and non-MG patients (n = 48). Here to compare the number of CGG triplets in males and females, the mean of CGGs on both alleles of the X chromosome was calculated for females. B Distribution of the number of CGG triplets in MG patients and non-MG patients. C Comparison of female and male MG patients (n = 58 females and 22 males) and non-MG patients (n = 38 females and 10 males). D, E Separate analyses of the shorter D or longer E CGG sequences on each chromosome X in females. F Comparison of the number of CGG triplets on DNA extracted from thymic and blood cells from a same MG patients. *p* values were assessed with an unpaired *t*-test (A), a Mann–Whitney test (C–E), and a Wilcoxon test (F).

(See figure on next page.)

**Fig. 2** Analyses of **FMR1**, **CTCF** and **MAX** expression in the thymus and thymic epithelial cells. RT-PCR analysis for **FMR1** (A) and **CTCF** (B) in the thymus of non-MG donors (infant females (n = 6, grey dots), adult females (n = 6, black dots) and adult males (n = 6, blue dots). Correlation between **FMR1** and **CTCF** mRNA expression in non-MG thymuses (C). Western blots for FMRF and GAPDH on thymic extracts. Six bands were recognized by the anti-FMRP antibody (D). Each band was quantified using Fiji and divided by the one corresponding to GAPDH. E for non-MG donors (female (n = 2) and male (n = 2) adults) and MG female patients (n = 3). RT-PCR analysis for **FMR1**, **CTCF** and **MAX** in the thymus non-MG female donors (infants and adults, n = 12, grey dots) and MG patients (n = 10–12, red dots). Correlation between **FMR1** and **CTCF** (F) or **MAX** (G) mRNA expression in MG thymuses. RT-PCR analysis for **FMR1** (H), **CTCF** (I) and **MAX** (J) in TECs from non-MG thymuses (n = 4–9 from different donors). TEC cultures were stimulated for 24 h with Poly(I:C) (100 μg/ml), IFN-I (1000 UI/ml), IFN-II (1000UI/ml) or IL-6 (10 ng/ml) in RPMI-0.5% horse serum for 24 h. PCRs with absolute quantification were performed for each gene analyzed and data were normalized to the GAPDH. For each experiment with a different TEC culture, the control values were set at 100. *p* values were assessed with a Mann–Whitney test and for correlation analyses a Spearman's correlation test was done.
Fig. 2 (See legend on previous page.)
inflammatory molecules on FMR1 expression in primary TEC cultures. We demonstrated that Poly(I:C) decreased FMR1 mRNA while IFN-I and IL-6 increased it, and IFN-II had no effect (Fig. 2H). As in MG thymuses, the decreased expression of FMR1 mRNA in TECs is associated with a decreased expression of CTCF and MAX mRNA induced by Poly(I:C). In contrast, the increase in FMR1 expression by IFN-I and IL-6 seemed more or less associated with CTCF and MAX mRNA increased expression (Fig. 2I, J).

Discussion
As FMR1 expression is decreased in MG thymuses, we hypothesized that MG patients may have an abnormal number of CGG triplets, potentially being in the “gray zone”. Analyzing thymic DNA, taking into account the sex, the different alleles of the X chromosome, and the interruption of CGGs by AGG sequences, we did not observe any differences between MG patients and control donors that could have been related to a predisposition to MG.

So, how can we explain the decrease of FMR1 mRNA expression in the thymus of MG patients? While FMR1 expression has been studied in FXS and FX-related syndromes, little is known about the regulation of its expression in normal condition. Different transcription factors have been implicated in the regulation of FMR1 expression such as Nrf-1, Sp1, USF1/2, MAX, CTCF [18, 19]. In the thymus, we observed that FMR1 expression could be related to a predisposition to autoimmunity at two levels. First, in normal thymuses, CTCF controlled its expression in a sex-dependent manner. This observation is very important knowing that females are more prone to develop autoimmune diseases [20]. Second, in the inflammatory environment of MG thymus, CTCF and MAX were differentially expressed and could be involved in the decreased expression of FMR1. The implication of MAX in the regulation of FMR1 expression in MG thymuses was not clear, as MAX has been shown to repress Nrf1 and Sp1 activation of FMR1 [18]. CTCF is a widely expressed transcriptional regulator implicated in many important processes. In the thymus, CTCF could thus play a central role in regulating FMR1 expression in normal and inflammatory conditions [21, 22]. In addition, at the protein level, the analysis of FMRP expression is also complicated. The unique FMR1 transcript undergoes alternative splicing, resulting in potentially 20 different FMRP isoforms that have not all been yet characterized [15, 16]. The differences in FMR1 mRNA expression observed between males and females altered the expression of the most-well known isoforms with high molecular weight. In contrast, the decreased expression of FMR1 mRNA in MG patients seemed to modify the expression of low molecular weight isoforms. As the expression of thymic FMRP isoforms has never been studied before, our results open up many questions that would need to be investigated further.

Here, we demonstrated that FMR1 expression in TECs was sensitive to inflammatory signals. Indeed, Poly(I:C) decreased FMR1, while IFN-I and IL-6 increased it. These modulations of FMR1 expression were also associated with CTCF and MAX expression. Poly(I:C) is known to induce thymic changes, mediated by IFN-β (an IFN-I subtype), that can even lead to MG in mouse [23]. Poly(I:C) induces IFN-I and IL-6 expression in TECs [23]. Here, the opposite effects of Poly(I:C) and IFN-I were surprising, but suggest that Poly(I:C) could induce FMR1 decrease by a more potent independent signaling pathway or by interfering with the IFN-I and IL-6 signalization. The fact that FMR1 is well expressed in the thymus [7] and that its expression is influenced by environmental factors strongly suggest that it could play a critical role in the thymus and the immune system. However, the role of FMR1 in relation to immune functions has hardly been explored. Altered number and/or function of regulatory T cells (CD4+CD25+ T cells) are known to be associated with autoimmune diseases, including MG [24]. Careaga et al. showed that human premutation carriers have a decreased level of T cells expressing CD25+ upon stimulation in vitro [25]. PBMCs from human premutation carriers display a decreased expression of pro-inflammatory cytokines [25] and an increased expression of the anti-inflammatory cytokine, IL-10 [26]. However, the level of FMR1 expression has not been analyzed in these studies. In PBMCs from FXS patients, for which FMR1 expression is inhibited, an altered immune response is observed in response to the activation of the LPS and glutamate receptor pathways [27]. In a Drosophila model Fmr1 mutants exhibit reduced bacterial engulfment, an early step in phagocytosis, and delayed processes that require phagocytosis by glial cells. These data suggest that Fmr1 could regulate the activation of phagocytic immune cells [28]. In addition, gene expression analyses in the brain of fmr1 knockout mice reveal an over-representation of immunological signaling pathways [29]. The investigation of gene expression in this mouse model could be of interest to decipher the role of FMR1 in the human thymus.

There is clearly a link between FMR1 and autoimmunity depending on the number of upstream CGG triplets as observed in premutated carriers and FXPOI [8, 9]. Here, we suggest for the first time another link between FMR1 and autoimmunity at the thymus level. In particular, we showed that FMR1 expression is highly susceptible to the inflammatory environment. Nevertheless, further investigations are necessary to better understand its role in the immune system.
The datasets used and/or analyzed during the current study are available from the 
Availability of data and materials

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Consent for publication

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Competing interests

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