Expression changes of microtubule associated protein 1B in the brain of *Fmr1* knockout mice

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Abstract: Objective To explore the regulatory effect of fragile X mental retardation protein (FMRP) on the translation of microtubule associated protein 1B (MAP1B). **Methods** The expressions of MAP1B protein and MAP1B mRNA in the brains of 1-week and 6-week old fragile X mental retardation-1 (*Fmr1*) knockout (KO) mice were investigated by immunohistochemistry, Western blot, and *in situ* hybridization, with the age-matched wild type mice (WT) as controls. **Results** The mean optical density (MOD) of MAP1B was significantly decreased in each brain region in KO6W compared with WT6W, whereas in KO1W, this decrease was only found in the hippocampus and cerebellum. MAP1B in 6-week mice was much less than that in 1-week mice of the same genotype. The results of Western blot and *in situ* hybridization showed that MAP1B protein and MAP1B mRNA were significantly decreased in the hippocampus of both KO1W and KO6W. **Conclusion** The decreased MAP1B protein and MAP1B mRNA in the *Fmr1* knockout mice indicate that FMRP may positively regulate the expression of MAP1B.

Keywords: fragile X syndrome; fragile X mental retardation protein; microtubule associated protein 1 B; mice

1 Introduction

Fragile X syndrome is one of the most common inherited diseases due to the expansion of a CGG repeat sequence in the 5'-untranslated region of the fragile X mental retardation gene (*Fmr1*). The trinucleotide repeat expansion and the subsequent hypermethylation prevent the transcription of *Fmr1* and lead to loss or absence of the fragile X mental retardation protein (FMRP). FMRP is an mRNA binding protein and functions as a translational regulator. To date, more than 400 mRNAs associated with FMRP have been identified *in vivo*^[1-4]. In fragile X patient cells and the synaptoneurosomes isolated from the brain of *Fmr1* knockout (KO) mouse, the absence of FMRP leads to the abnormal polyribosome-association of several mRNAs, which normally bind to the FMRP. The dendritic spine maturation

was found delay in fragile X mental retardation patients, as well as in *Fmr1*-KO mice, indicating that FMRP may function in synaptic development. However, the biochemical link between FMRP deficiency and neuronal impairment during brain development has not been clarified.

Microtubule-associated protein 1 B (MAP1B), a neuronal microtubule-associated protein (MAP), is a predicted target of FMRP and plays important roles in neurite and synapse development^[5]. It was reported that MAP1B mRNA was upregulated in the absence of FMRP^[4,6,7]. Furthermore, the aberrantly elevated MAP1B protein was found abnormally increase the microtubule stability in *Fmr1*-KO neurons^[7]. However, another research showed that MAP1B decreased in the cerebellum of *Fmr1*-KO mice^[8]. Therefore, how FMRP regulates MAP1B translation in fragile X neurons and governs normal synapse development in the brain remains elusive. In the present study, we investigated the expressions of MAP1B protein and MAP1B mRNA in the *Fmr1*-KO mice and the age-matched wild (WT) mice.

2 Materials and methods

2.1 Experimental animals FVB inbred strain mice, includ-

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Article ID: 1673-7067(2007)04-0203-06
CLC number: R748
Document code: A
Received date: 2007-02-26

ing *Fmr1* KO and WT mice, were presented by Prof Oostra BA (Erasmus University of Netherlands). The experiments were conducted in accordance with the national legislation and associated guidelines. The genotype of the experimental animal was initially mapped by PCR^[9] and then confirmed by Western blot analysis for FMRP. One-week and six-week old KO mice and the age-matched WT mice were allocated to four groups: KO1W, WT1W, KO6W and WT6W. Animals were anesthetized with 10% chloral hydrate (250 mg/kg, i.p.) before experiments.

2.2 Immunohistochemistry The experimental animals ($n = 10$ for each group) were perfused transcardially with normal saline (0.9% NaCl), followed by 4% paraformaldehyde solution in 0.1 mol/L PBS (pH 7.4). Brains were removed and postfixed in 4% paraformaldehyde for 12 h at 4 °C, then cryopreserved in serial 10%, 20% and 30% sucrose-PBS. The samples were embedded in optimal cutting temperature compound (OCT) after their horizontal planes were adjusted parallel to the cutting plane. The brains were sequentially sectioned through the hippocampus and hypothalamus approximately at Bregma -2.51 to -2.60 mm, Interaural 1.26 mm, and at Bregma -5.68 mm, Interaural -1.88 mm, respectively, with a thickness of 30 μ m. Each section was categorized anatomically according to *The Mouse Brain in Stereotaxic Coordinates*^[10]. After being treated with 0.3% H₂O₂ in 0.01 mol/L PBS for 20 min to block the nonspecific antibody binding, sections were incubated in normal goat serum containing 0.1% Triton X-100 for 2 h, and then incubated with primary antibody (rat anti-mouse MAP1B monoclonal antibody MAP5, 1:1 000; Chemicon) containing 0.1% Triton X-100 and 1% normal blood serum overnight at 4 °C. On the following day, sections were processed with ABC kit (Vector Laboratories, USA), and the immunoreactivity was visualized by the three-step ABC technique with ImmunoPure Metal Enhanced DAB substrate kit (Pierce, Rockford IL, USA). Finally, sections were dehydrated with gradient ethanol and mounted with resina.

2.3 Western blot The hippocampi taken from the experimental animals of each group ($n = 6$) were processed with protein extracts under a normal procedure. Total protein in the samples was determined by Bradford method at 562 nm wavelength. For blotting, protein extracts were dissolved in 1 $\times$ loading buffer (250 mmol/L Tris-HCl (pH 6.8), 10% SDS, 0.5% BPB, and 50% glycerol), denatured at 99 °C for 5 min, and the same amounts of protein (10-30 μ g) were loaded onto a 12% SDS-polyacrylamide gel. Proteins were sepa-

rated in 1 $\times$ electrophoresis buffer and transferred to a nitrocellulose membrane by tank blotting in transmembrane buffer. Immunodetection was performed by using the MAP1B monoclonal antibody (1:10 000), and the β -actin monoclonal antibody (1:2 500) was set as loading control. The second antibody was a goat-antimouse antibody conjugated with horseradish peroxidase in a dilution of 1:2 000, detected with the enhanced-chemiluminescence (ECL) Western blotting detection reagents, and sequentially exposed to chemiluminescent film (10 s to 30 min).

2.4 In situ hybridization The cryosections (15 μ m) were fixed for 10 min in 4% paraformaldehyde at room temperature, then incubated in 0.25% acetic anhydride in 0.1 mol/L triethanolamine containing 0.1% active diethylpyro carbonate (DEPC) for 10 min. Sections were dehydrated in 70%, 80%, 90%, and 100% ethanol, then defatted twice in chloroform and rehydrated in 95% ethanol for 5 min. Subsequently, sections were prehybridized with the hybridization solution [50% deionized formamide, 5 $\times$ SSC (1 $\times$ SSC is 0.15 mol/L NaCl, 0.015 mol/L sodium citrate), 5 $\times$ Denhardt's solution, 10% dextran sulfite, and 100 μ g/mL salmon sperm DNA] for 1 h at 42 °C, and afterwards, hybridized with oligonucleotide probes (0.5 ng/mL) for 18 h at 42 °C. The probes were labeled with digoxin (Tianjin Haoyang Biological Company, Tianjin, China). The slides were then rinsed sequentially in 2 $\times$ SSC for 30 min at room temperature, in 0.5 $\times$ SSC for 30 min at 37 °C, and in 0.5 $\times$ SSC for 30 min at room temperature, followed by incubation with anti-Digoxin-biotin (diluted 1:50) for 2 h at room temperature. After washing with PBS for three times to remove the excess antibodies, the slides were incubated with Streptavidin-HRP for 30 min, followed by PBS rinses again. Color development was carried out with 3,3'-diamino benzidine solution. Sense probe hybridization was used as a background control.

2.5 Image analysis and statistics disposal Immunostaining were observed under the German ZEISS Axintron microscope at 4 $\times$ 10 magnification, and pictures were collected with JVCky-F30b3-CCD colour image apparatus and analyzed with German Kontron IBAS 2.0 full automatic image analysis system. The optical density was analyzed at 5 sequential pieces of sections for each mouse and 3 fields for each section, and the mean value in each subregion was used for analysis. The mean optical density (MOD) from immunohistochemistry and *in situ* hybridization was corrected by background MOD value and negative controls.

Quantification of the immunoreactive bands of Western blot was performed with the image analysis system mentioned above. The final value for each band was then divided by the average “mean gray value” of β -actin band in the same lane.

All data were analyzed by paired *t*-test with SPSS11.0 for Windows. Results were expressed as mean $\pm$ SD and $P < 0.05$ was considered statistically significant.

3 Results

3.1 Distribution and expression of MAP1B in the brain

The immunoreaction was found in all regions of the brain,

some representative regions were shown in Fig. 1. The MOD of MAP1B immunoreactive staining was significantly decreased in each subregion of the brain in KO6W compared with WT6W, but only decreased in the hippocampus and cerebellum in KO1W compared with WT1W. The expression of MAP1B was lower in the 6-week mice than in the 1-week mice of the same genotype (Fig. 2). The result of Western blot also showed a significant decrease of MAP1B in the hippocampus of KO mice compared with its age-matched WT mice (Fig. 3).

3.2 The transcription level of MAP1B The result of *in situ* hybridization showed that MAP1B-positive cells in

Fig. 1 Immunohistochemical result of MAP1B. A-D: the hippocampus of WT1W (A), KO1W (B), WT6W (C) KO6W (D); E-H: the cerebellum of WT1W (E), KO1W (F), WT6W (G), and KO6W (H). Scale bar, 270 μ m.

Fig. 2 Mean optical density (MOD) value of MAP1B in different brain subregions by immunohistochemistry. The MOD decreased significantly in each subregion of the brain in 6-week *Fmr1* knock out mice (KO6W) compared with the corresponding age-matched wild type mice (WT6W). For 1-week mice, MOD decreased only in the hippocampus and cerebellum in KO1W compared with WT1W. * $P < 0.05$, ** $P < 0.01$. The MOD of 6-week mice, both WT and KO, was lower than the corresponding 1-week mice.

Fig. 3 Western blot analysis of MAP1B in the hippocampus. MAP1B decreased in both 1-week and 6-week KO mice compared with the corresponding age-matched WT mice. Values represent 6 independent experiments. ** $P < 0.01$.

Fig. 4 *In situ* hybridization analysis of MAP1B mRNA in the hippocampus. MAP1B mRNA decreased in both 1-week and 6-week KO mice compared with the corresponding age-matched WT mice. Values represent 6 independent experiments. ** $P < 0.01$. Scale bar, 270 μ m.

the hippocampus was significantly decreased in KO mice compared with WT mice, both in 1-week and 6-week. No significant difference of MAP1B mRNA expression was found between 1-week and 6-week mice for the same genotype (Fig. 4).

4 Discussion

MAP1B is the first microtubule associated protein to be expressed during brain development. It controls neurite extension and growth cone motility via modulating microtubule dynamics^[5,11,12]. MAP1B expresses at a higher level during embryogenesis. The expression reaches a peak on 2-3 d after the birth, and decreases to a stable level during 2-3 weeks after the birth^[13]. A gradual reduction of hippocampal MAP1B and MAP1B mRNA has been observed during the first two postnatal weeks^[7]. This study demonstrated a similar result that the expression of MAP1B was lower in adult mice than in neonatal mice. However, we failed to find the decline of MAP1B mRNA in adult mice. Our result did not support the idea that the decline of MAP1B

during the development was caused by reduced transcription^[14]. Since the down-regulation of MAP1B in the neonatal brain coincides with the completion of vigorous neuronal processes extension and the initiation of active synaptogenesis^[15], MAP1B was considered to play an important role in synaptic plasticity.

MAP mRNAs have been identified to have a recognizable purine G quartet structure, which, as a binding site for FMRP, has been shown to interact with FMRP directly *in vitro*^[16]. MAP1B mRNA is dendritically localized and colocalizes with FMRP granules in dendrites and develops synapses of cultured hippocampal neurons^[17]. Therefore, it was supposed that the translation of MAP1B mRNA was misregulated in absence of FMRP, causing the local regulation of cytoskeletal dynamics involved in synapse development to be altered. This hypothesis has been supported by the later study which MAP1B mRNA was inappropriately over-translated in both *Drosophila dFmr1 (dfxr)* null^[6] and mouse *Fmr1* KO models^[1,7]. Inversely, other report showed that MAP1B had a decrease tendency in the fron-

tal lobe and hippocampus of adult fragile X mice and got statistical significance in the cerebellum^[8]. In the present study, we found that MAP1B was significantly decreased in every brain subregion of adult KO mice and in the hippocampus and cerebellum of neonatal KO mice compared with the age-matched WT mice, which is in accordance with the report of Chen *et al.* Hinds HL *et al.* demonstrated that FMRP was high expressed in mouse brain during early embryogenesis period and gradually declined to a steady-state level during late embryogenesis period, and then it was high expressed again in adult^[19]. Although there are few data about FMRP expression in different brain region in mice, a strong *Fmr1* expression was found in the olfactory bulb and cerebellum of the tadpole brain. These results are helpful to explain why decreased MAP1B expression is more obviously in adult KO mice than in neonatal KO mice^[20]. Besides the down-regulated MAP1B as demonstrated by Chen *et al.* and us, some researches showed that other target-mRNAs and proteins were also decreased in absence of FMRP^[8,18]. Taken together, these results imply that the interaction between FMRP, polyribosome and mRNAs is extremely complex. Recent researches raised the possibility that the association of FMRP with its mRNA ligands may also involve micro-RNAs and other small noncoding RNAs^[21]. The decrement of MAP1B mRNA in our results also suggested that FMRP might be involved in transcription.

In conclusion, the results demonstrated that both MAP1B protein and MAP1B mRNA levels decreased in the *Fmr1* gene KO mice, suggesting that FMRP may positively regulate the expression of MAP1B. The interaction between FMRP and MAP1B, as well as the role of MAP1B in the abnormal synaptic plasticity in fragile X mental retardation, needs future investigation.

Acknowledgements: We thank Prof Oostra BA (Department of Clinical Genetics, Erasmus University of Netherlands) for his presentation of the *Fmr1* gene knockout mice and Prof Shu-Min Duan (Institute of Neuroscience, Chinese Academy of Science) for his advice on this study. This work was supported by the National Natural Science Foundation of China (No. 30370504) and the Natural Science Foundation of Guangdong Province, China (No. 04105429).

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微管相关蛋白 1B 在 *Fmr1* 基因敲除小鼠脑组织内的表达变化

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摘要: 目的 探讨脆性X智能低下蛋白(fragile X mental retardation protein, FMRP)对微管相关蛋白1B(microtubule associated protein 1B, MAP1B)是否具有调控作用。方法 应用免疫组化、免疫印记和原位杂交的方法, 对1周龄和6周龄的*Fmr1*基因敲除型(KO)和同龄野生型(WT)小鼠脑组织MAP1B及MAP1B mRNA进行分析。结果 免疫组化的结果显示: 6周龄KO小鼠各个脑区MAP1B的平均光密度值(MOD)值均显著低于同龄WT小鼠($P < 0.05$), 1周龄KO小鼠仅在小脑和海马显著降低($P < 0.01$); 各脑区MAP1B的MOD值在6周龄小鼠均比同基因型的1周龄小鼠显著降低($P < 0.05$)。免疫印记和原位杂交结果分别显示MAP1B及MAP1B mRNA在KO小鼠的海马组织均显著降低($P < 0.05$)。结论 MAP1B和MAP1B mRNA在*Fmr1*基因敲除小鼠脑组织的表达均显著减少, 提示FMRP可能正性调节MAP1B的表达。

关键词: 脆性X综合征; 脆性X智能低下蛋白; 微管相关蛋白1B; 小鼠