行政院國家科學委員會專題研究計畫成果報告

易脆 X 染色體症候群 FMRP 蛋白轉譯後調控研究

Study of the posttranslational modification of the FMRP protein of the fragile X syndrome

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一、中文摘要

易脆 X 染色體症候群為最常見之遺傳性智障。FMR-1 基因功能喪失似為導致此疾病之成因，故此基因在智能的發展上可能扮演一重要角色。由 Western blot 分析我們找到四位易脆 X 染色體病人有不同之罕見 FMRP 表現型態；一可能性為這些病人中 *FMR-1* 基因出現特殊突變而使 FMRP 被異常處理，另有可能為 FMR-1 基因雖正常但 FMRP 之修飾系統為異常；本計畫中將此四人之 FMR-1 基因序列全部定序分析後未發現有異常情形。是否有其他變異影響其穩定性仍待探討。而 FMRP 有 RNA 結合蛋白特有之 KH 和 RGG domain 且於試管內可和 RNA 結合。以此系統分析四株來自不同病人之 lymphoblastoid cell 中 FMRP 和 poly(U) 之結合能力也不同。

關鍵詞: 易脆 X 染色體症候群、 FMRP蛋白、 轉譯後調控、 淋巴母細胞

Abstract

Inadequate expression of functional *FMR-1* gene product (FMRP) leads to the most-frequent inherited mental retardation fragile X syndrome. We have found four fragile X patients with unusual FMRP expression pattern by Western blots. It is likely that certain intragenic mutations occur in the *FMR-1* gene of these patients and thus the mutated FMRP is processed differently. The other possibility is that in the system to study the methylation. patient the FMR-1 gene is normal but the posttranslational modification system (including the degradation system) of FMRP is abnormal. We sequenced DNA fragments obtained from RT-PCR or genomic–PCR products amplified the DNA from lymphoblastoid cells derived from the four patients. However, we could not identify any mutations in the FMR-1 gene. FMRP contained two KH domains and an RGG box known to be present in RNA binding proteins. In vitor FMRP had been shown to be able to bind RNA. We prepared lymphoblastoid cell extracts and the FMRP in different extracts appeared to bind RNA differntailly. It is thus possible other genes are involved in the FMRP modification in the patinets that leads to fragile X syndrome.

Key Words: Fragile X syndrome, FMRP protein, posttranslational regulation ; Lymphoblastoid cell

二、緣由與目的

Fragile X syndrome is the most frequent diagnosis in patients with inherited mental retardation (Sherman et al., 1984). Cytogenetically a fragile site at chromosome Xq 27.3 of patients can be induced by deprivation of folate (Sherman et al., 1984) and an *FMR-1* gene at the fragile site with polymorphic trinucleotide CGG repeats in the 5' untranslated region of the gene has been identified (Verkerk et al., 1991; Kremer et al.,

1991; Orberle et al., 1991). Expansion of triplet repeats to more than 200 copies in the fragile X patients results in hypermethylation of the CpG island promoter of *FMR-1* and thus shut down the transcription of the gene (Piereti et al., 1991). Patients with deletions (Wohrle et al., 1992; Gedeon et al., 1992), a missense point mutation (De Boulle et al., 1993), and other intragenic mutations (Lugenbeel et al., 1995) of the *FMR-1* gene have also been identified. Thus the insufficient expression of the *FMR-1* gene or inability to produce functional *FMR-1* protein products (FMRP) result in the abnormal phenotypes. Thus the physiological function of FMRP must be important for the development of intelligence.

The *FMR-1* protein contains two KH (hnRNP K homology) domains and one arginine and glycine rich RGG domain characteristic of RNA binding proteins (Siomi et al., 1993; Ashley et al., 1993b) and binding of FMRP to certain RNA molecules have been shown in vitro (Siomi et al., 1993; Ashley et al., 1993b; Siomi et al., 1994). The protein products are located predominantly in cytoplasm and rarely in nucleus as illustrated by transient expression experiments (Verheij et al., 1993). A heterogeneous set of *FMR-1* proteins can be detected in various tissues (Khandjian et al, 1995; Verheij et al., 1995) which might reflect the alternatively-spiced products. Combination of the alternatively-spliced sites can lead to at least 12 different isoforms of FMRP (Ashley et al., 1993a). Isoforms of FMRP lacking exon 14 appears to be nuclear-localized while others remain in cytoplasm (Sitter et al., 1996). FMRP have been shown to be associated with ribosome (Khandjian et al., 1996) probably via the interaction of RNA (Eberhart et al, 1996). Recently Eberhart et al. suggested that FMRP is a ribonucleoprotein with both nuclear localization and nuclear export signals (1996). Two FMRP homologue FXR1 and FXR2 also interact with FMRP (Zhang et al., 1995) and might mediate the FMRP and FXR association with 60S ribosomal subunits (Siomi et al., 1996).

In spite of the above information, not too much is known about the stability and posttranslational modification of FMRP. It was suggested by Khandjian et al. that the FMRP is stable in quiescent cells and that the *FMR-1* gene expression is post-transcriptionally controlled (1993). In cooperation with Dr. Shuan-Yow Li, we have found four fragile X patients with unusual FMRP expression pattern by Western blots. In patient 268, besides the normal FMRP isoforms, some strong lower protein species can be detected. In patient 320, one lower band was detected. In patient 038, two to three lower faint bands was detected. In patient 237, reduced expression of normal FMRP species as well as two faint lower bands was detected. These patients are all phenotypically typical fragile X without (CGG)n expansion of *FMR-1* and thus might have other mutations of the *FMR-1* gene or other mutations that might reduce the FMRP function. By now only one point mutation (De Boulle et al., 1993) and two intragenic mutations (Lugenbeel et al., 1995) of *FMR-1* have been reported and thus the five special fragile X patients we identified are very unique and are very valuable resources for further analysis of the processing of FMRP. Since these patients are genetically independent individuals, it is possible that the mutations in these patients might be different. The most likely possibility for these patients expressing abnormal FMRP pattern is that certain intragenic mutations occur in the *FMR-1* gene of these patients and thus the mutated FMRP is processed differently. The other possibility is that in the patients the FMR-1 gene is normal but the posttranslational modification system (including the degradation system) of FMRP is abnormal. The results of this analysis will help to identify critical componants for the FMRP processing. Lymphoblastoid cell lines established from fragile patients appear to be a valuable system for the study of fragile X syndrome especially for patients with special FMR-1 mutations (non-typical 5'-(CGG)n

expansions of *FMR-1*, Verheij et al., 1995; Lugenbeel et al., 1995).

三、結果與討論

1. Mutation analysis of the FMR-1 gene in the four fragile X patients: Exon 1-10 and exon 15 of FMR-1 in the four patients had been analyzed by single strand conformational polymorphism previously. Total RNA from lymphoblastoid cells of the atypical fragile X patients were prepared. RT-PCR to amplify the coding region of FMR-1 covering exon 6-8 was performed following the method of De Boulle et al. (1993). The RT-PCR products were anlyzed by DNA sequencing. We further analyzed exons 9-17 by sequencing of the PCR products of each exon. However, no mutation in FMR-1 was identified in all four patients.

2. RNA binding analysis of FMRP: Whether the RNA binding activity and/or ribosome association of FMRP in patients with abnormal FMRP pattern are different from that of normal cells was investigated. RNA binding activity was determined by a poly(U)-binding assay at different salt concentration (Verheij et al., 1995). The poly (U) binding activity of FMRP in lymphoblatoid cells from normal individuals and patient 267 would not be affected by increasing salt concentration. The FMRP in lymphoblatoid cells derived from 038, 237 and 320 all showed decreased poly(U) binding activity upon the increase of the salt concentration.

3. Cellular localization of FMRP: The localization of FMRP in the lymphoblastoid cells from patients with abnormal FMRP expression pattern was investigated following the protocols of Corbin et al. (1997). Immunoblot analysis of subcellular fractions of lymphoblastoid cells derived from four atypical fragile X patient was performed. FMRP was present predominantly in the ribosomal fraction and slightly in the nucleus fraction. However, certain fraction of the FMRP in patient 320 appeared to be present in the soluble cytosolic fraction.

4. Examination of the FMRP modification systems: Recombinant FMRP with N-terminal (His)6 fusion can be obtained from Dr. Shuan-Yow Li‘s laboratory. Lymphoblastoid cell extracts from fragile X patients were incubated with the recombinant FMRP at 37℃ for 30 min. The level of the exogenous FMRP appeared to be normal after the incubation with all four lymphoblastoid cell extracts as analyzed by Western blot analysis using anti-FMRP as the first antibody

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四、計畫成果自評 ：

本計畫原預定對易脆 X **染色體症候群** FMR-1 **基因產物** FMRP **轉譯後調控進行探討，四位易脆** X **染色體病人有不同之罕見** FMRP **表現型態之** FMR-1 **基因序列全部定序分析後未發現有異常情形。是否另有** FMRP **之修飾系統為異常則不清楚；本計畫中原始目標雖未完全達成，但對** lymphoblastoid cells **其甲基接受蛋白質之分析則建立系統，整理其結果之論文** ”Protein N-arginine methylation in subcellular fractions of lymphoblastoid cells” **已整理投稿** *Biochemical Journal* **中。而此系統之建立，亦為後續實驗，奠立良好基礎。**

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