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(CAG)n 三聯核酸重複序列之基因轉殖動物模式建立及研究

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行政院國家科學委員會專題研究計劃成果報告

(CAG)n 三聯核酸重複序列之基因轉殖動物模式建立及研究(2/3)
Establishment and studies of transgenic animal models expressing (CAG)n trinucleotide repeat expansion (2/3)

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一、中文摘要

為研究非轉譯的 CAG 三聯核酸重複序列是否會造成活體內的病理作用，我們製造基因轉殖小鼠使其表現肌肉特異性的 EGFP 基因，並在其 3’端不轉譯區含有不同長度的 CAG 重複序列。EGFP RNA 在所有的基因轉殖小鼠系中的表現量接近，但是在表現 200 次 CAG 重複的小鼠中 EGFP 的蛋白質表現量顯著下降。肌肉的組織化學分析顯示表現 200 次 CAG 重複的小鼠其肌肉細胞型態異常，並且其琥珀醯酸去氫酶和 NADH 還原酶的活性也不正常。藉由抓力及籠內活動力測試，我們觀察到 CAG_{200} 的成鼠其肌肉衰弱。另由 MyoD, Myf5, myogenine and CHCR 等肌肉分化指標基因的過度表現，顯示 CAG_{200} 的成鼠其肌肉分化不正常。更進一步 CAG_{200} 的成鼠其肌肉在葡萄糖存在下會發生神經性的攣縮現象，在正常及對照組老鼠不會產生此現象。這些結果是首次證明，位於一個不相關基因的 3’ 不轉譯區的 CAG 重複序列擴增會造成小鼠的肌肉病理現象，此與 RNA gain-of-function 機制符合。這也暗示非轉譯的 CAG 三聯核酸重複序列可能在人類疾病扮演一個角色。我們將以此模式進一步研究三聯核酸重複序列擴增的致病機制。

關鍵詞：三聯核酸重複序列擴增、CAG、基因轉殖小鼠、肌肉分化

Abstract

To investigate whether an untranslated CAG repeat expansion has pathogenic effect *in vivo*, we generated transgenic mice expressing muscle-specific EGFP transcripts with different sizes of CAG repeats in its 3’-untranslated region. While the expression levels of the EGFP transcripts were comparable in all transgenic lines, the EGFP protein levels were significantly reduced in mice expressing 200 CAG repeats. Histochemical analysis of the muscle revealed atypical cell morphology, as well as altered activities of succinate dehydrogenase and NADH reductase in CAG_{200} mice. Muscle weakness, as assessed by grip strength and cage activity, and aberrant muscle differentiation, as assessed by up-regulation of MyoD, Myf5, myogenine and CHCR, were observed in adult CAG_{200} mice. Furthermore, CAG_{200} mice exhibited nerve evoked muscle contracture in the presence of glucose, which was not seen in normal or control mice. These results demonstrate, for the first time, that untranslated CAG repeat expansion in an unrelated mRNA can result in pathogenic effects in mice, consistent with an RNA gain-of-function mechanism. It also suggests a possible role for untranslated CAG expansions in human disorders.

Keywords: trinucleotide repeat expansion, CAG, transgenic mice, muscle differentiation

二、緣由與目的

A growing number of human neuromuscular disorders have been found to be caused by expansion of unstable trinucleotide repeats (1). According to the location of the expansion, these trinucleotide repeat disorders are grouped into two categories. In the first category the repeat expansion is located in the coding region of the affected gene, resulting in a mutant protein with long track of amino acid homopolymers, such as “polyglutamine” from CAG repeats. These include Huntington’s disease (HD), spinal and bulbar muscular atrophy (SBMA), dentatorubral pallidoluysian atrophy (DRPLA), and spinocerebella ataxias SCAs -1, 2, 3, 6, 7, etc (2). In the second category the expansion is in the non-coding, or untranslated region (UTR). Examples include CGG or CCG expansion in fragile X syndrome (FRAX), GAA expansion in Friedreich’s ataxia (FRDA), CTG expansion in myotonic dystrophy type 1 (DM1) and SCA8, and an unusual CAG expansion in the 5’UTR in SCA12 (3).

The mechanism by which expanded repeats lead to the pathogenic phenotype is complex and depends on the location of the repeat within a gene. Most CAG expansions are less than 150 repeats, located in coding regions and are translated into polyglutamine tracts within the corresponding protein. Recent lines of evidence suggest that aggregates of polyglutamine, protein misfolding and transcriptional dysregulation result in neuronal cell toxicity (2). In addition, loss of neurotrophic support due to reduced protein activity by polyglutamine expansions may also contribute to the pathogenesis of neurodegeneration (4). On the other hand, CTG expansions are usually very large and located exclusively outside coding sequences. The most intensively studied disorder in this category is DM1, which appears to involve a RNA gain-of-function mechanism.

DM1 is a multisystemic disorder characterized by skeletal muscle wasting and myotonia, cardiac conduction defects, insulin resistance and cataracts (5). It is caused by an expansion of CTG repeat in the 3’-UTR of the *DMPK* gene (6). This region overlaps the 5’ end of a neighboring homeodomain- encoding gene, *SIX5* (7). Haploinsufficiency of the DMPK protein and altered expression of *SIX5* have been demonstrated to contribute to partial DM1 phenotypes, for example, progressive myopathy, atrioventricular conduction abnormalities (8) and cataracts (9). However, absence of the major feature of DM1, myotonia, suggests that additional mechanisms must be involved. In support of this notion, mice expressing mRNA with long CUG repeats in the 3’-UTR of either *DMPK* or an unrelated transgene produce myotonia and myopathy (10,11), indicating that transcripts with expanded CUG repeats are pathogenic. Moreover, expansion of CCTG repeats in a second locus (DM2) also leads to a clinical presentation that is strikingly similar to DM1 (12). Together these findings point to an important role for RNA gain-of-function in DM pathogenesis.

Although current data strongly suggest that proteins with expanded polyglutamine tracts are key players in CAG repeat disorders, they do not rule out the possibility that expanded CAG RNA is contributing to the pathogenesis process. Indeed, CAG repeat RNAs are predicted to form stable secondary structures similar to those detected in DM1 (13), and RNA-binding proteins that specifically interact with CAG-repeat sequences have been reported (14). Genetic evidence that RNA-binding proteins are involved in polyglutamine disorders comes from a Drosophila model of SCA1 (15). Screening for genes that modify SCA1-induced neurodegeneration leads to the identification of genes that encode

RNA-binding proteins, suggesting that alteration of RNA processing is relevant to SCA1 pathogenesis (15).

To address whether untranslated CAG expansions are pathogenic *in vivo* in a trans-dominant manner, we generated transgenic mice expressing muscle-specific transcripts of the EGFP gene, of which no CAG repeat (CAG_0), 23 repeats (CAG_{23}) or 200 repeats (CAG_{200}) were inserted in the 3'-untranslated region. Our results demonstrate that expanded CAG RNA can result in pathogenic effects *in vivo* through a trans-dominant mechanism, suggesting a possible role for untranslated CAG expansions in human disorders.

三、結果與討論

To investigate the physiological effect of untranslated CAG trinucleotide repeat expansion, we first generated constructs containing the enhanced green fluorescent protein (EGFP) gene, either without any CAG repeats (designated as CAG_0), or with 23 or 200 CAG repeats (designated as CAG_{23} and CAG_{200}, respectively) inserted in its 3'-untranslated region. The *EGFP* gene is placed downstream of the gamma-sarcoglycan (*gsg*) promoter, which has been shown to direct strong gene expression in the skeletal muscle during mouse development (16). Three transgenic founder animals with CAG_0, seven with CAG_{23} and six with CAG_{200} transgenes were produced and were bred to establish independent lines.

RNA isolated from various tissues was subjected to RT-PCR analysis. The *EGFP* transcripts were present in heart, skeletal muscle, diaphragm, testis and ovary. All transgenic lines exhibited entirely the same patterns of tissue distribution. By Northern blotting using RNA isolated from the skeletal muscle, the length of the EGFP transcript from CAG_{200} lines is about 1.7 kb, which is 600 bp longer than that from CAG_0 lines as expected. There was no noticeable difference in the levels of these two transcripts. Using β-tubulin as loading controls, however, the EGFP protein levels were significantly reduced in tissue extracts of all CAG_{200} lines by Western blotting. This decreased protein expression also reflected in the low fluorescence intensity of EGFP in CAG_{200} lines under a fluorescent microscope. Thus, expression of the EGFP transcript with long CAG repeats in 3'UTR resulted in decreased protein expression.

Histological evaluation of the muscle morphology revealed no signs of fibrosis, inflammation or regeneration in muscle fibers ofn all mice. In the control mice, the shape and size of muscle fibers were homogeneous and most nuclei were situated peripherally. In CAG_{200} mice, muscle fibers of various size and shape, increased intermyofibril connective tissues, split fibers and centronucleated fibers were observed. The peripheral nuclei appeared larger and the relative nucleus/fiber ratio was increased (the nucleus/fiber ratio for NT and CAG_0 mice in a cross section were 1.049±0.025 and 1.023±0.025, respectively, versus 1.413±0.044 and 1.424±0.058 for two CAG_{200} lines, $p<0.001$).

By a modified SDH reaction using phenazine methylsulfate, fibers with heavy staining known as "ragged blue" fibers were observed in sections from CAG_{200} lines. These fibers reflected a marked excess of mitochondria and were not seen in the control muscle. The fibrillar network of NADHR reactivity in CAG_{200} muscle was irregular as compared to controls, and many fibers (both type I and type II) displayed an unusual "hollow" pattern where NADHR activity was lacking in one or more area in the fibers. These features are somewhat reminiscent of oligomitochondrial disease where focal lack of mitochondria results in so-called "moth-eaten" fibers. In addition, by ATPase staining, predominance of type I fibers and

type grouping were also observed in CAG_{200} muscle.

Mice carrying the CAG_{200} construct did not show overt phenotypic difference from the CAG_0 and NT mice. However, some of the CAG_{200} mice occasionally displayed bizarre postures and intermittent convulsions. When assayed by a locomotor activity test and a narrow bar hang assay (17), CAG_{200} mice showed reduced cage activity and muscle weakness, as they crossed less number of squares per minute and became fatigue and fell in less time than control animals, respectively. Mean litter size was close to control in all CAG_{200} lines (7.6 pups per litter for 20 litters versus 8.4 pups per litter for 31 litters in CAG_0), whereas breeding efficiency was greatly reduced. The time needed to get a litter born from heterozygous CAG_{200}-NT cross ranged from 6 to 8 weeks, compared to 3 to 4 weeks from CAG_0 or NT animal cross.

To examine muscle differentiation at the molecular level, we investigated the expression pattern of *myoD*, *myf-5* and *myogenin* in the skeletal muscle of neonatal and adult CAG_{200} and control mice. By RT-PCR, the expression of these genes was readily detected in neonatal tissues but was down-regulated in adult tissues normally. In CAG_{200} mice, however, expression of these genes remained high in adulthood even at 8 months of age. Recently a novel muscleblind-related gene, CHCR, was identified as an inhibitor of muscle differentiation (18). Expression of CHCR was low in adult muscle tissues of NT and CAG_0 mice, consistent with the previous report that CHCR mRNA is nearly undetectable or greatly reduced in differentiated NM14 and C2C12 cells (18), whereas its expression was dramatically increased in muscle of adult CAG_{200} mice. These results, together with the histological data, suggest that muscle differentiation in the CAG_{200} mice is impaired.

To determine if there were defects in muscle membrane conductance in CAG_{200} mice, sharp microelectrode recordings were performed on excised diaphragm muscle fibers. A single action potential was triggered in both NT and CAG_{200} fibers. There was no difference in the current threshold and latency, indicating that muscle membrane conductance was not impaired by untranslated CAG expansion. Because the patterns of mitochondrial enzyme activity were altered in CAG_{200} muscle, we then measured the muscle contracture by nerve-evoked stimulation (19). Contracture of isolated phrenic-nerve diaphragm was induced significantly by 5 Hz nerve-evoked stimulation for 20 min in CAG_{200} but not NT or CAG_0 mice. As a control, both CAG_{200} and NT mice produced muscle contracture by the same stimulation with glucose-free Krebs solution.

四、成果自評

The main goal of the second year of this project is to analyze the phenotypes of the transgenic mice and investigate the molecular mechanisms. So far the progress is good. We have gathered sufficient significant data. Because the results demonstrate for the first time that untranslated CAG repeat is pathogenic, these data are novel and highly competitive. A manuscript of this study is being prepared for publication and hopefully will be submitted by the end of July. We have begun the screening of possible downstream molecules involved in the pathogenesis process, which is part of the third year's work. We will keep progress in analyzing the possible candidate genes responsible for the phenotypes observed.

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