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計畫名稱：SR 蛋白質和脊髓性肌肉萎縮症的關係

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一. 中文摘要

脊髓性肌肉萎縮症（SMA）是一種體染色體隱性遺傳病，它是因脊髓前角的運動之細胞退化導致肌肉麻痺及肌肉萎縮，它的致病原因是 SMN1 基因缺損或突變造成。由於病人一定存在有一個以上 SMN2 基因，而此基因與 SMN1 只差兩個不改變胺基酸的鹼基，其中在 exon 7 上的鹼基會引起 alternative splicing 造成 SMN2 基因產生功能不佳的蛋白質。由於 alternative splicing 的產生常受 SR protein 的調控，因此。本研究先利用一些藥物來抑制 SMA 病人細胞株中的 SMN2 基因的 alternative splicing，然後再探討 SR protein 的變化，結果顯示在 25kd 及 30kd 左右的 SR protein 的增加，會抑制 SMN2 基因的 alternative splicing，而產生與 SMN1 相同的完整 SMN 基因產物。

結論: SMN2 的 alternative splicing 與 SR protein 有關，利用藥物調控 SR protein 將可能對 SMA 病人有治療的效果。

關鍵詞：脊髓性肌肉萎縮症，SR 蛋白質，SMN1 基因，SMN2 基因，alternative splicing

Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of the anterior horn cells of the spinal cord leading to muscular paralysis with muscular atrophy. The cause of SMA is due to mutations of SMN1 gene. There is a SMN1 homologous gene-SMN2, and two nucleotides difference between these two genes. These nucleotide differences results in an alternative splicing of exon 7 of SMN2, which is different from SMN1. The SMA patients at least one SMN2 gene, and we may use drugs to modify the alternative splicing of this gene to produce SMN1-like transcripts. The mechanism of alternative splicig is controlled by SR protein. In this study, we used hydroxyurea to treat SMA cell lines, and found the transcript of SMN2 gene changing to SMN1 gene-like transcript. We further used western blot method to analyze the expression of SR protein, the results showed both 25kd and 30kd SR protein were increased after hydroxyurea treatment, which is correlated with alternative splicing of SMN2 gene. From these results, we suggest that hydroxyurea may be a candidate drug for treatment of SMA disease.

Keywords: spinal muscular atrophy, SR protein, SMN1 gene, SMN2 gene. Alternation splicing

二. 緣由與目的

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of anterior horn cells of the spinal cord leading to muscular paralysis with muscular atrophy. Clinical diagnosis of SMA is based on findings of progressive symmetric weakness and atrophy of the proximal muscles. Affected individuals are usually classified into three groups according to the age of onset and progression of the disease. Children with type I SMA are most severely affected and usually have SMA symptoms before the age of 6 months and rarely live beyond 2 years. Type II and type III SMA are milder forms and the age of onset of symptoms varies between 6 months and 17 years. SMA is one of the most common

fatal autosomal recessive diseases in children with a carrier rate of 1-2% in the general population and an incidence of 1 in 10,000 newborns (1). No specific treatment is currently available for SMA patients.

Two survival motor neuron genes (*SMN*) are typically present on 5q13: *SMN1* (also known as SMN^{T}, *SMNtel*) and *SMN2* (also known as SMN^{C}, *SMNcen*). Loss of function mutations of both copies of the telomeric gene, *SMN1,* is correlated with the development of SMA (2-5). The nearly identical centromeric gene, *SMN2* appears to modify disease severity in a dose-dependent manner, as SMN protein levels from this gene are correlated with disease severity (6,7). However, the expressed amount of intact SMN protein from *SMN2* does not provide adequate protection from SMA (8).

Although *SMN1* and *SMN2* encode identical proteins, all three forms of proximal SMA are due to mutation in the *SMN1* gene, but not in the *SMN2* gene (2-5). The differences between these highly homologous genes are in their RNA expression patterns (9-12). Most *SMN2* transcripts lack exons 3, 5 or most frequently, exon 7, with only a small amount of full-length mRNA generated. On the other hand, the *SMN1* gene expresses mostly a full-length mRNA, and only a small fraction of its transcripts are spliced to remove exon 3, 5 or 7 (11,12). Recent studies have also shown that an AG-rich exonic splice enhancer (ESE) in the center of *SMN* exon 7 is required for constitutive inclusion of exon 7 (13). These findings also imply that the low levels of full-length SMN protein produced by *SMN2* are insufficient to protect against disease development (6,7). Clearly, the total amount of full-length oligomerization-competent SMN protein is a critical SMA determinant, and the amount of SMN protein correlates with the severity of pathologies (14). In addition, there is a strong correlation between the *SMN2* copy number and phenotype in human SMA and SMA-like mice (5,6,7,15,16).

We recently developed a SMA mouse model which genotypically and pheno-typically mimics human SMA (15). The severity of pathology in the knockout-transgenic mice is correlated with the amount of intact SMN protein. The difference between *SMN1* and *SMN2* gene expression is the number of full-length transcripts and the amount of SMN protein, and all 5q-linked SMA patients have at least a single intact copy of *SMN2*. Drugs that modify the pattern of *SMN2* transcript in SMA patients in order to increase full-length *SMN* mRNA expression and the amount of SMN protein may have a therapeutic effect on SMA patients. As a first step towards designing a therapeutic protocol for SMA patients, we used EBV transformed lymphoid cell lines from SMA patients to screen a series of drugs for their possible effect on the expression of the *SMN2* gene. Hydroxyurea which was found to be effective was then used to treat our SMA-like mice to determine its potential for the treatment of human SMA.

SR proteins (Ser-Arg proteins) constitute a family of pre-mRNA splicing factors that are highly conserved throughout the metazoa (17,18). These proteins have

multiple functions in splicing. Biochemical experiments have provided strong evidence that SR proteins play essential roles in general, or constitutive, splicing. They seem to be equally important in splicing regulation, through their ability to modulate selection of alternative splicing sites in a concentration-dependent manner which contributes to activation (and repression) of splicing through interaction with elements in the pre-mRNA known as splicing enhancers (or silencers) (27-29). Recently, Lorson and Androphy demonstrated that an AG-rich exonic splice enhancer in the center of *SMN* exon 7 is required for inclusion of exon 7 (13), and Hofmann et al further demonstrated that Htra2-β1, an SR-like splicing factor, promoted the inclusion of *SMN* exon 7, stimulating full-length *SMN2* expression. Htra2-β1 specifically functioned through and bound to an AG-rich exonic splicing enhancer in *SMN* exon 7 (30). In the present study, we have explored the relationship between drug's effect and SR protein.

三. 結果與討論

Hydroxyurea changes the processing of *SMN2* gene transcripts

EBV transformed lymphoid cell lines from all three types of SMA patients were established and used for drug screening. Several drugs were tested to investigate their potential effect on the expression of the *SMN2* gene using RT-PCR. Among them, hydroxyurea was able to change the expression pattern of the *SMN2* gene. The amount of exon 7-containing *SMN* mRNAs increased in lymphoid cells cultured with 5 ng/ml to 500 μg/ml of hydroxyurea (Fig. 1*a*). The maximal effect was found after 4 hours of stimulation (Fig. 1*b*). Hydroxyurea-treated lymphoid cells from different types of SMA patients showed an increased number of full-length *SMN* transcripts (Fig. 1*c*). To better understand the mechanism involved in this change in full-length *SMN* transcript levels, separate RT-PCRs were used to examine the patterns of alternative splicing in exons 3, 5 and 7. We found that the alternative splicing pattern of exons 3 and 5 was unchanged after hydroxyurea stimulation (Fig. 1*d* and *e*), but that the alternative splicing pattern of exon 7 of the *SMN2* gene changed to the *SMN1* pattern (Fig. 1*f*). Therefore, addition of hydroxyurea in the culture resulted in an increased number of full-length *SMN* mRNA transcripts.

Hydroxyurea increases exon 7-containing SMN protein in SMA lymphoid cells

To determine whether hydroxyurea-induced expression pattern changes of *SMN2* resulted in an increased amount of exon 7-containing SMN protein, we used different concentrations of hydroxyurea to treat the lymphoid cell lines (3 cases each for type I, II, and III respectively) established from different types of SMA patients. In both cytosolic and nuclear fractions, Western blot analysis indicated that hydroxyurea also increased the intact SMN protein after 4 hours stimulation with 0.5 ng/ml to 500 μg/ml of hydroxyurea (Fig. 2). However, a decreasing effect was found in the cytosolic fraction when more than 5 μg/ml hydroxyurea was used.

Hydroxyurea increases specific SR proteins in SMA lymphoid cell lines

SR proteins are known to play an important role in the processes of alternative splicing of genes (17,18), and previous studies have identified a splicing enhancer element in exon 7 of the *SMN* gene (10,13). To investigate hydroxyurea-induced expression pattern changes of *SMN2* involving the SR protein, we used different antibodies for SR proteins to detect SR protein expression patterns after hydroxyurea treatment. The results showed that two SR proteins of about 27 kDa were induced after treatment, which were detected using mouse anti-SR protein 16H3 antibody. However, no difference was found using the mouse anti-SRp20 antibody (Fig. 3). All (3 cases each for type I, II, and III) lymphoid cell lines, which were established from different types of SMA patients showed similar results.

Hydroxyurea has been shown to induce differentiation and apoptosis (19,20). There is evidence that hydroxyurea may act at the transcription level by increasing the acetylation of histones, thereby releasing constraints upon the DNA template and reactivating a number of genes (21,22). Hydroxyurea also increases the expression of fetal-globin genes in adult baboons, humans and other animals (23-25). These effects of butyrate may occur through the inhibition of histone deacetylase (21,22,25,26). In the case of *SMN*, hydroxyurea may acetylate nucleosomal DNA and release other factors that control alternating splicing of exon 7 of the *SMN2* gene.

We demonstrated that hydroxyurea induced two specific SR proteins involved in inclusion of exon 7 for full-length *SMN* expression of the *SMN2* gene. Our results strongly support that SR proteins are involved in *SMN2* exon 7 inclusion after hydroxyurea treatment.

Approximately 15% of all mutations that cause genetic diseases result from the defective splicing of pre-mRNA (31). A number of these mutations do not alter consensus splice sites or generate missense or nonsense mutations, yet do affect splice site selection (32,33). These mutations may cause skipping of exon(s) by disrupting the splicing enhancer(s). Our findings suggest that an approach similar to that used in our study may be effective in treating these kinds of genetic diseases as well.

Hydroxyurea has been used clinically to treat patients with sickle cell anemia and thalassemia for several years (34,35). The pharmacokinetics and toxicities of hydroxyurea are well documented; its toxicity is low and was well-tolerated in both human and animal studies (34-37). Our findings suggest that hydroxyurea is an excellent candidate for the treatment of human SMA.

In summary, SMA lymphoid cell lines were used to explore possible medication for the treatment of human SMA in this study. We found that hydroxyurea can change the expression pattern of *SMN2* and increasing the amount of full-length mRNA of *SMN2*. This is the first study to demonstrate a possible treatment for human SMA. The methods developed in this study may be useful in screening other candidate

drugs for SMA treatment. We also demonstrated that the mechanism of action of hydroxyurea involves a modification of the splicing of exon 7 of the *SMN2* gene under the regulation of SR proteins. Our methods may also provide a useful approach for the treatment of other splicing-defect-related diseases (31,38).

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