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Original Article

MOLECULAR ANALYSIS OF THE CAMP- RESPONSE ELEMENT [CRE] ELEMENTS IN THE PROMOTER REGION AND EXON 1 OF THE SURVIVAL OF MOTOR NEURON 2 [SMN2] GENE IN MALAYSIAN SPINAL MUSCULAR ATROPHY PATIENTS; TO ELUCIDATE THEIR ROLE IN CIRCUMSCRIBING THE CLINICAL SEVERITY OF SMA

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ABSTRACT

Objective: In the Spinal muscular atrophy [SMA] genes [*SMN*1 and *SMN*2 genes]; the CRE-II elements at -400 bp in the promoter region of the *SMN* genes and CRE-I element at +108 bp in the exon 1 of the *SMN* genes, are reported to have a role in c-AMP induce expression of the *SMN* genes through its binding affinity to CREB-1. This study was designed to determine the role of CRE sites in the circumscribing the clinical severity of SMA.

Methods: Direct sequencing was performed for the PCR products of the promoter regions of the SMA patients with homozygous deletion of *SMN*1, different copy number of *SMN*2 and *NAIP* non deletion.

Results: No variation among the CRE-I and CRE-II sites was found in all the clinical types as compare to normal healthy control showing no role of CRE sites in circumscribing the clinical severity of SMA.

Conclusion: There was no sequence variation found in the CRE binding sites in the three different clinical types of SMA reflecting no role of CRE binding sites in circumscribing the clinical severity of SMA.

Keywords: SMA, CRE-II, CREB, Promoter of *SMN* gene.

INTRODUCTION

In some patients of Spinal Muscular Atrophy [SMA] with homozygous deletion of the *SMN*1 gene and 2 copies of *SMN*2 gene, the differences in the severity of SMA suggests that the production of the FL-SMN protein may be different in different clinical types even with the same number [2 copies] of the *SMN*2 gene [1]. Increased expression of the *SMN* genes through froskolin or BT2 which act through CRE sites in all clinical severities [2] of SMA reflects these compounds must have no effect if CRE binding site is mutated. This study aimed to analyze the variation in the CRE binding sites of the normal healthy individuals and patients of SMA from different clinical types. The study could be very significant in defining the difference in transcriptional control, variation among promoter of healthy compared to promoter of SMA patients and also variations among the promoter of *SMN*2 gene among SMA patients from different clinical types with CRE-I site at +108 bp and CRE-II element in at -400 bp upstream in the reported sequence for the promoter region of the *SMN*2 gene [3].

MATERIALS AND METHODS

Patients' recruitment

A total of 134 patients were included in this study by applying single proportion formula **[**$n > [z\ \alpha\ /\ \Delta]^2 x\ P\ [1-P]$**],** where n [96] is the minimum sample required, z α was set at 1.96 for allowing Type I error for 5% [0.05], Δ [0.1] is the estimation of having mutation in *SMN*2 promoter region and P [0.5] is the proportion of having mutation in *SMN*2 promoter region. The diagnosis was based on the clinical criteria as setup by the 59th and 93rd ENMC International Workshop on SMA in 1998 and 2001 respectively [4, 5]. The clinically summary of all the patients was reviewed at Hospital Universiti Sains Malaysia [HUSM] by the pediatric neurologist and 58 patients were excluded from the study as the criteria of SMA international consortium were not fulfilled by them. This study was conducted with the permission of USM ethical clearance and was funded by the grant from ministry [SAGA grant].

DNA extraction and SMA genes analysis

The samples of remaining 69 patients were received from different hospitals all over Malaysia. Informed consent was obtained prior to blood taking. Total of 69 normal healthy individuals were used as a negative control. DNA was extracted from whole blood using a DNA extraction kit, GeneAll® ExgeneTM Blood SV [GeneAll Biotechnology Co. Ltd., Korea]. The DNA was quantified using Eppendrof DNA spectrophotometer. The 42 patients with homozygous *SMN*1 deletion were analyzed for the copy number analysis of the *SMN*2 gene and *NAIP* deletion [6,7]. This part of the analysis has been reported previously by our group [8].

Molecular analysis of the CRE sites

A total of 10 patients were selected for further analysis. All these patients were checked for the presence of the *NAIP* gene to remove any clinical bias in the methodology and disease severity. Firstly, we included only the patients with 2 copies of the *SMN*2 gene. Later, nucleotide variation analysis was also performed on patients with 3 and 4 copies of the *SMN*2 gene in different clinical types considering the previous report stating that the patients with more than 2 copies of *SMN*2 and different clinical severity shows same levels of FL-SMN [1]. As previously reported [2], the CRE-II element was analyzed for the molecular variation in normal healthy individuals and SMA patients from different clinical types. The primers were designed manually using the *SMN*2 promoter sequence Gen Bank entry [GenBank accession number; AF187725]. Two primers were designed, forward P3932 [5'TGAGCTCAGGAGTTCGAGAC3'] and complementary reverse PCR [5'GGCGTGTATATTTTTCATTTCTC3'] for analyzing CRE-I site and for CRE-II we made use of primers for

exon 1 with slight modification in annealing temperature [9]. For CRE-I, The PCR was performed for a total of 30 cycles with the Ta of 58°C and an internal control of 200 bp. The PCR products were visualized on agarose gel electrophoresis with a concentration of 1.2% of agarose and 10mg/ml ethidium bromide. The image was captured using an Alpha Innotech image analyzer. The 720 bp amplified PCR products from normal healthy individual [n=2] and SMA patients were then cloned into pCR®2.1-TOPO® cloning vector [A] from Invitrogen. The supplied instructions were followed and the ligation mixture was prepared to transform into freshly prepared TOP10 *E. coli* competent cells. The average transformant efficiency of 8.2 - 8.5e-2 [transformants /µg DNA] was achieved against ampicillin [100µg/ml] and selected by blue white screening with IPTG concentration of 20% w/v. The transformants were confirmed further by PCR and the plasmid extraction was done using commercial plasmid extraction kit was used [Qiagen Inc. 28153 Avenue Stanford, Valencia, CA 91355, USA]. However, the protocol was based on an alkaline lysis method of plasmid extraction. The DNA concentration of the plasmid DNA was quantified using Eppendrof DNA spectrophotometer.

DNA sequencing and data analysis

The DNA sequencing for the CRE-II binding site was performed for the 10 patients and 3 normal healthy individuals [as a negative control] using direct sequencing of the PCR product; amplified with universal M13 reverse and M13 forward [-20] universal primers in the backbone of pTOPO2.1e. The plasmids with inserts were confirmed by digestion using *Dra*I site in the insert and pTOPO 2.1 e back bone. For the CRE-I site, no cloning was performed prior to sequencing and PCR amplified product of exon 1 was subjected directly to sequencing using specific primers [9]. The PCR products were subjected to direct sequencing after purification using Promega kit. The PCR products were sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction kit with Amplitaq DNA polymerase. FS [Perkin-Elmer, applied Biosyntech Division], on an automated ABI PRISM 3100 DNA sequencer [Perkin-Elmer, Applied Biosystem Division], following the manufacturer's instructions. In case of doubtful sequence [GT-rich or G-rich region, secondary structure, certain sequence context or motifs], sequencing reactions were performed with the dGTP Big Dye Terminator Ready Reaction kit [Perkin-Elmer, Applied Biosystem Division]. Sequences were aligned using the ClustalX program and Vector NTI Suite 9. In case of ClustalX, the alignment results were being stored as MSF files using Gene Doc software.

RESULTS AND DISCUSSION

All three forms of SMA are caused by loss or mutation of the telomeric survival motor neuron gene [*SMN1*], but the centromeric survival motor neuron gene [*SMN2*] is retained [10]. As we know now, that, molecules capable of inducing *SMN2* expression [11, 12] or altering the splicing of *SMN2* such that more full-length SMN transcript is produced have been identified [11, 13]. At the present time, there is limited information on the mode of action of these compounds as well as the protein complexes that interact with the *SMN* promoter. The CREB/ATF family of transcriptional activators consists of multiple protein species that recognize nearly identical binding sites [13]. It has also been reported that there is a novel cAMP response element, CRE-I [TGACGACA] in the promoter of the *SMN* gene at position -400 bp along with CRE-II binding site [TGACGACT] at +108 bp downstream of the *SMN* gene [1, 15] that can interacts with the cAMP response element binding CREB family of protein [1]. Even with the sequence similarity of 87.5%, Sarmila and colleagues reported CRE-II element to be involved in c-AMP induced over expression of the *SMN* genes under the effect of CREB-1 protein [1]. In 2003, Sumner and colleagues reported valproic acid to increase the SMN level in SMA patients [16] which was followed by the findings of Sarmila *et al., 2003,* which reported the increased over expression of CREB-1 protein and *SMN*2 in the presence of dibutyryl cAMP and froskolin [1].

Table 1: Characterization of the SMA patients used for the molecular analysis of the CRE-I and CRE-II elements, NA = Non applicable

Patients	SMA	Gender	Race	Consanguinity	Tongue fasciculation	Muscle biopsy
1	Type I	Male	Malay	No	Yes	Not done
2	Type I	Female	Malay	No	Yes	Not done
3	Type II	Female	Malay	NA	Yes	NA
4	Type II	Male	Malay	Adapted	No	Not done
5	Type II	Female	Malay	No	No	Neurogenic muscle atrophy
6	Type III	Female	Malay	No	NA	Not consented for second time
7	Type III	Male	Malay	No	Yes	Not done
8	Type III	Male	Malay	No	No	Selective group atrophy of muscle
9	Type III	Male	Malay	No	No	Not done
10	Type III	Female	Malay	No	No	Consistent with SMA
Patients	***SMN*1 exon 7**	***SMN*1 exon 8**	**NAIP**	***SMN*2 copy no.**	**Onset age**	**EMG**
1	deleted	deleted	non deleted	1	Since birth	Giant motor potential
2	deleted	deleted	non deleted	2	3 month	Not done
3	deleted	deleted	non deleted	2	NA	fibrillation potential, giant potential
4	deleted	deleted	non deleted	3	15 months	Consistent with SMA
5	deleted	deleted	non deleted	4	1 year	Nerve conduction normal
6	deleted	deleted	non deleted	2	18 months	spontaneous fibrillation at rest with denervation pattern
7	deleted	deleted	non deleted	2	Since 14 years old	Giant motor potential
8	deleted	deleted	non deleted	3	24 months	Giant muscle action potential
9	deleted	deleted	non deleted	4	Since 1 year	Not done
10	deleted	deleted	non deleted	4	8 months	Supportive of SMA

The differences in severity among SMA patients have prompted researchers to investigate the genomic variations that contribute to these phenotypes. Variation in the number of copies of the *SMN*2 gene contributes to the severity of SMA [17]. Furthermore, given that *SMN*2 is known to be transcribed, a difference in *SMN*2 copy number would also translate into a variation in the amount of functional protein produced. It was demonstrated that there is a correlation between disease severity and SMN protein levels and a higher ratio of *SMN*2/*SMN*1 gene dosage in the parents of SMA type II and III patients, compared with the parents of type I patients [17].

The *SMN2* promoter sequence of normal individuals submitted by Boda *et al*., [1999], has been a reference for all researchers and we made use of the same sequence for designing of primers for CRE-I element. We started our analysis with the total of 3 patients [one patient from each type], patient number 2, 3 and 6 [table 1].

The CRE-II binding site analysis was performed in these 3 patients followed by the variation analysis of the CRE-II binding site in promoter region in other 7 patients [type I = 1, type II = 2, type III =4]; as mentioned in table 1 [patient number 1, 4, 5, 7, 8, 9, 10]. All

these patients are with more than 2 copies of *SMN*2 gene except patient no. 1 which has only 1 copy of the *SMN*2 gene. All these patients are reported to be non deleted for *NAIP* gene which was analyzed to avoid any experimental and severity bias considering the SMA modifying genes [8].

Furthermore, the increased expression of the *SMN* genes through froskolin or BT2 which act through CRE sites in all SMA types reflects these compounds must have no effect if CRE site is mutated [1]. The absence of any mutation and positive effect of forskolin and Bt2 on CRE sites [2] in different clinical types of SMA confirms that the CRE site can only be used for c-AMP induced expression of *SMN* genes. However, the CRE sites might not be the cause of induction of SMA with no role in circumscribing the clinical severity of SMA. The absence of any variation in the CRE elements [fig. 1] in the promoter region and exon 1 of the *SMN*2 gene in SMA patients from different clinical types compare to the promoter region of the *SMN*2 gene in normal healthy individuals, suggested a role of other epigenetic factors and transcription factors which might be influential in controlling and coregulating the induction of *SMN* expression within 4.6kb promoter region of the *SMN*2 gene, hence, may influence the SMA severity in patients from different clinical severities with same copy number of *SMN*2 gene.

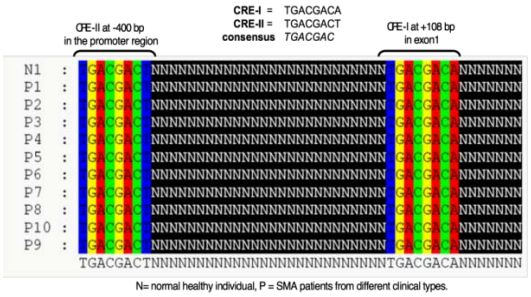

Fig. 1: The molecular analysis of the CRE-I and CRE-II binding sites in the SMA patients from different clinical types. N= Normal healthy individual, P[n]= SMA patient

CONCLUSION

There was no molecular variation found in the CRE-I and CRE-II sites in any of the clinical types of the SMA patients as compared to the normal healthy individuals in the expected promoter region of the *SMN*2 gene in SMA patients. The results suggested that the CRE-I and CRE-II elements have no role in severity of SMA in different clinical types.

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CONFLICT OF INTERESTS

Declared None.

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