Determination of SMN2 Copy Numbers in Iranian Spinal Muscular Atrophy Patients Using Multiplex Ligation-Dependent Probe Amplification

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Abstract
Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by homozygous deletion of the survival motor neuron gene 1 (SMN1) in more than 90% of the patients. According to the age of onset and severity of the disease, SMA is classified into three groups: type I (severe), type II (intermediate), and type III (mild). As reported, the SMN2 gene, centromeric copy gene, is correlated with the severity of the disease.

To determine the genotype-phenotype correlation, we studied 45 Iranian patients (15 SMA I, 10 SMA II, and 20 SMA III) using multiplex ligation-dependent probe amplification (MLPA) assay. Fourteen out of 15 SMA I patients (93.3%) carried two copies of SMN2 while the remaining 6.7% carried three copies. Among the type II and type III, 30% of the type II and 10% of the type III SMA patients carried two copies of the gene, while 70% of the type II and 90% of the type III carried three or four copies of SMN2, respectively.

This study showed that SMN2 copy number could affect the survival in SMA type I and ambulation conservation or loss in type III. Thus, investigation of SMN2 copy number could be an appropriate predictor for SMA disease type.

Key words: Spinal Muscular Atrophy; SMN2 Protein, Human; Gene Dosage; Multiplex Polymerase Chain Reaction; Iranian Population.

Introduction
Proximal spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by the destruction of α-motor neurons of the anterior horn cells, which leads to symmetrical muscle weakness and atrophy. The estimated incidence rate is 1/10, with an approximate carrier frequency of 1 in 50 (1, 2). Patients with SMA have been classified into three types on the basis of age of onset, motor development milestones, and severity of clinical symptoms: type I (SMA I; Werdnig-Hoffmann disease; severe SMA; children are never able to sit unaided; MIM 253300), type II (SMA II; intermediate SMA; children sit but never walk; MIM 253550), and type III (SMA III; Kugelberg–Welander disease; mild SMA; weakness develops in patients who were previously able to walk unassisted; MIM 253400) (3-6).

All the mentioned forms of SMA are associated with mutations of the survival motor neuron gene 1 (SMN1) on chromosome 5q11.2-13.3 (MIM 600354; GenBank: NM_000344) (1,2). Deletion and gene conversion events result in a 95% rate of homozygous loss of the SMN1 gene in patients with classic SMA (7-10), and about 5% of the diseased alleles are due to intragenic point mutations of SMN1 (4,11).

The gene is duplicated and located in a complex region of chromosome 5q13 also containing the...
Neuronal Apoptosis- Inhibitor Protein (NAIP) gene (4,9,12). The SMA-determining gene group includes 2 highly homologous genes, telomeric SMN1 and centromeric SMN2 (MIM 601627; GenBank NM_022875) (4,12,13). There is only one difference between SMN1 and SMN2 which is a change of C to T in exon 7 (13, 14). This change (c.840C>T) disrupts an exonic splicing enhancer (ESE) or creates an exon silencer element (ESS), which results in the majority of transcripts lacking exon 7.

As a result, we can find more incomplete transcripts of SMN1 which leads to an unstable SMN protein (13-15). Previous studies have shown that variations in the number of the copies of the SMN2 gene contribute to the severity of the SMA phenotype. The severity of the disease alleviates in patients with complete deletion of SMN1 copies because SMN2 can compensate for the deficiency of SMN1. Compared with SMA I patients, a higher proportion of SMA III patients have four copies of SMN2 (as a consequence of gene conversion events) (4,10,12,15-19). However, the influence of the number of SMN2 copies is not implicit, e.g. three SMN2 copies have been observed in both SMA I and SMA III. Therefore, this correlation does not seem to be absolute but SMN2 can be considered a phenotypic modifier gene (5,12,15,20).

Many different methods have been reported for determining the copy number of SMN genes including polymerase chain reaction (PCR)–restriction-fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), denatured high-performance liquid chromatography (DHPLC), real- time quantitative PCR, and multiplex ligation-dependent probe amplification (MLPA) [4, 12, 20-29].

Several reports have indicated that the MLPA assay is efficient in the detection of copy number changes in various genes. This technique provides the unique ability to hybridez several probes specific for SMN1 and SMN2 genes in a single experiment (11,12,26-29).

In this study, we evaluated SMN2 copy numbers in different types of SMA using the MLPA assay to establish a genotype–phenotype correlation in SMA patients based on the evidence that the SMN2 copy number relates with severity of the disease.

Materials and Methods

SMA patients were recruited from Kariminejad-Najmabadi Pathology and Genetics Center, Tehran, Iran. In all cases, the loss of both SMN1 copies was been previously established by PCR-RFLP. Medical history and clinical data were obtained by reviewing the reports of the neurological follow-up examinations and by questioning the referring clinician when necessary. Forty-five patients fulfilled the diagnostic criteria defined by the International SMA Consortium (30). Based on age of onset and clinical features, 15 patients were classified as SMA I, 10 as SMA II, and 20 as SMA III.

Genomic DNA was isolated from the peripheral blood using the salting out method (31) after written informed consent was obtained from each subject. MLPA analysis (32) was performed using the SALSA Probe Mix 021 according to the recommendations of the manufacturer (MRC Holland, Amsterdam, Netherlands), which detects copy number changes of both SMN1 and SMN2 genes. The kit contains 16 probes for several genes in the critical 5q13 region including SMN1, SMN2, BIRC1 (NAIP), GTF2H2, N-cadherin-like-gene, CDH6 (K-cadherin), RAD17, and SERF1A, as well as 22 control probes mapping to other autosomes.

Post analysis of MLPA included the use of ABI PRISM 3100 Genetic Analyzer and gene mapper Version 3.5 software. By comparing with five normal controls using cofialyzer version 2 software (MRC Holland), calculation of the relative peak area (RPA) was performed. SPSS software version 19 was employed for statistical analysis using the Fisher’s exact test.

Results

From a total of 45 patients, 15 cases (33.3%) were classified as SMA type I, 10 (22.3%) as SMAII, and 20 (44.4%) as SMA III. The demographic characteristics

<table>
<thead>
<tr>
<th>Type</th>
<th>Male</th>
<th>Female</th>
<th>Age (years) (Min-Max)</th>
</tr>
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<tbody>
<tr>
<td>Type I SMA</td>
<td>9</td>
<td>6</td>
<td>1-5</td>
</tr>
<tr>
<td>Type II SMA</td>
<td>8</td>
<td>2</td>
<td>3-11</td>
</tr>
<tr>
<td>Type III SMA</td>
<td>15</td>
<td>5</td>
<td>8-53</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>13</td>
<td>1-53</td>
</tr>
</tbody>
</table>
Table 2: SMN2 copy number in different types of SMA patients lacking SMN1 gene

<table>
<thead>
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<th></th>
<th>2 copies</th>
<th>3 copies</th>
<th>4 copies</th>
<th>Total</th>
</tr>
</thead>
<tbody>
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<td>Count</td>
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<td>1</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Type I SMA</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% within type</td>
<td>93.3%</td>
<td>6.7%</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>% within SMN2</td>
<td>73.7%</td>
<td>8.3%</td>
<td>0%</td>
<td>33.3%</td>
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<td>Count</td>
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<td>6</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Type II SMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% within type</td>
<td>30%</td>
<td>60%</td>
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<td>100%</td>
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<tr>
<td>% within SMN2</td>
<td>15.8%</td>
<td>50%</td>
<td>7.1%</td>
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<tr>
<td>Type III SMA</td>
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<td></td>
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<td>25%</td>
<td>65%</td>
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<tr>
<td>% within SMN2</td>
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<td>92.9%</td>
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<tr>
<td>% within type</td>
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<tr>
<td>% within SMN2</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 2: SMN2 copy number in different types of SMA patients lacking SMN1 gene

The results were analyzed according to the copy number of SMN2 for different types of SMA patients as demonstrated in Table 2. All cases had homozygous deletions of exon 7 and 8 (or exon 7 only) of the SMN1 gene. Results from MLPA revealed SMN2 copy numbers ranged from two to four copies with no copy of SMN1 in all types of SMA patients. Most of the type I patients (93.3%) had two SMN2 copies and none of them showed four copies whereas SMA III patients showed a majority of 3–4 copies (90%) and none showed one copy. SMA II patients predominantly showed 2–3 SMN2 copies (90%). Chart 1 shows the distribution of SMN2 copy numbers among SMA patients. Most of the patients had two copies of SMN2.

The mean SMN2 copy numbers in SMA II–III patients was 3.2±0.7 (mean±SD), which is significantly higher than that observed in SMA I patients (2.1±0.2). The results of the Fisher's exact test (P<0.001) demonstrated a relationship between SMN2 copy number and severity of the disease. However, there were some exceptional cases: one of the 15 SMA I patients carried 3 SMN2 copies.

Discussion

Differences in genetic makeup and the environmental exposure of the affected individuals are due to difference in disease presentation. In Mendelian disorders, a single gene plays the predominant role in the disease phenotype. However, in most cases, the function of the gene mutation is not related to the phenotype. Thus, the effect of modifier genes should be considered (17,33). SMA patients show absence of the SMN1 gene or mutations in it and the influence of the SMN2 copy number –as a modifier in the SMA phenotype- has been reported in different studies (4-6, 12,15,16,18,22,28,34).

An inverse correlation between the SMN2 copy number and SMA severity was first suggested by Lefebvre et al. in 1995 who described the SMN locus (35). The identification of an SMA type III patient with a hybrid gene comprising SMN2 exon 7 and SMN1 exon 8 led to the hypothesis that gene conversion is a mechanism that results in less severe SMA phenotypes. The results of the largest cohort (375 patients) conducted by Feldkötter et al. revealed significant correlations between the SMN2 copy number, SMA type, and survival (4,20).

In this study, SMN2 copy numbers were analyzed in 45 SMA patients carrying homozygous deletion of the SMN1 gene. The outcomes were consistent with reported findings in other ethnic groups (4-6, 12, 14-16, 18, 26-29, 34) and support the fact that the disease phenotype is influenced by the number of copies of the SMN2 gene. In addition, patients carrying one SMN2 copy will not develop type III SMA, whereas...
patients with four SMN2 copies will not develop type I SMA but will probably evolve as a type III disease (4, 20, 26). In general, accumulating data indicates that the affected individuals with three or more copies of the SMN2 gene have milder phenotypes with a late onset of symptoms in comparison with those who have two copies of this gene (9, 18, 26, 36).

Therefore, we found a relationship between phenotype and SMN2 copy number. It was noteworthy that one patient with type I SMA had three copies and further studies should be considered. This discrepancy could be explained by two hypotheses: different levels of protein expression or the presence of modifying extrinsic factors or modifier genes (5, 17, 18, 37, 38).

According to a research by Harada et al., SMN2 copy is able to produce a trace of full-length mRNA species but a large amount of truncated mRNA species lacking exon 7 due to inactivation of the mechanism that splices exon 7 into the SMN2 mRNA species (10, 15). In fact, loss of function of the SMN protein lacking the exon 7-encoded domain has been reported to be a result of an impaired interaction with the proteins in RNA metabolism (13, 39, 40). A linear correlation between mRNA or protein expression level and SMN2 copy number has been demonstrated in lymphocytes (13, 39-41).

Taken together, these findings suggest that the SMN2 copy number should also be considered and analyzed in prognosis, with clinical criteria. In addition, the existence of equal SMN2 copy number in different phenotypes (3 copies in type I and III) suggests the presence of modifying factors which should be investigated.

Consequently, SMN2 copies are not functionally equivalent among SMA patients although the SMN2 copy number is correlated with disease severity in general. However, the splicing mechanism and some variants of SMN2 gene with positive or negative effects on its transcription may play a role in the genotypic and phenotypic discrepancies in SMA patients (13, 37, 38).

Identifying the genetic background of SMA patients will help not only to understand the pathogenesis of SMA, but also to develop new therapeutic strategies for the disease.

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**Conflict of Interest:**

The authors declare that there are no conflicts of interest.
References

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