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RESEARCH

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The prevalence of *SMN* gene deletion/duplication in spinal muscular atrophy families referred to neuro-genetic centers of Mashhad, Iran

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Abstract

Background Spinal muscular atrophy (SMA) is a group of motor neuron diseases. In 95% of SMA patients, the telomeric copy of the *SMN* gene (*SMN1*) is homozygously deleted. Due to the autosomal recessive pattern of SMA inheritance, individuals with a family history of SMA are at risk of being carriers. A total of 622 individuals from SMA families, including parents, siblings, and first, second, and third-degree relatives, were recruited to the neuro-genetic clinic of Ghaem Hospital, Mashhad University of Medical Sciences, Mashhad, Iran. SMA cases and suspected carriers were referred to the genetic laboratory. Pregnant women underwent amniocentesis and chorionic villi sampling at 12–14 gestational weeks. RFLP-PCR and real-time PCR were performed for symptomatic and asymptomatic individuals (possible carriers), respectively. RFLP and real-time PCR were performed for amniotic fluid and chorionic villi samples.

Results The study enrolled 622 subjects from SMA-affected families, including 159 fetuses and 463 non-fetuses. Two samples were missing. A total of 268 individuals (43.2%) were healthy (wild type), 187 individuals (30.1%) were heterozygous for exon deletion of *SMN1*, and 143 individuals (23%) were homozygous for exon deletion of *SMN1*. Four individuals (0.6%) showed three copies of the *SMN1* gene.

Conclusion The frequency of carriers with two *SMN1* copies on a single chromosome (cis) was estimated at 2.9% (18/622), and the total rate of carriers was approximately 21.8%. Considering the high rate of SMA carriers in this study, genetic counseling and definitive prenatal diagnosis are of utmost importance for reducing the psychosocial burden of the SMA disease among Iranian families.

Keywords Spinal muscular atrophy, *SMN* gene, Genetic counseling, PCR

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Background

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease defined by progressive deterioration of the spinal cord motor neurons [1]. SMA is the most prevalent cause of infant mortality [2]. In some populations, the incidence rate of SMA varies from 5 to 13 per 100,000 live births, and the carrier frequency of pathogenic mutations of *SMN1* ranges from 1:100 to 1:45, with significant variability among different ethnicities [3–5]. The main manifestations of SMA are progressive muscle weakness and atrophy caused by the destruction of the spinal cord anterior horn cells and motor nuclei of the lower brain stem [2, 6]. The SMA is categorized into five types (0 to 4) based on clinical course and age of onset [5]. SMA type 0 has prenatal onset [7–9]. These patients present with hypotonia, weakness, congenital heart disease, and facial diplegia during infancy [5, 6, 10–12]. Respiratory failure is the prevalent cause of death in patients with SMA type 0, which mainly occurs in one to six-month-old infants [13]. Presentations of SMA type 1, also known as Werdnig-Hoffman disease, consist of poor head control, hypotonia, disturbed deep tendon reflexes, and dysphagia. In this type of SMA, respiratory failure can occur in the early two years of life [14–16]. Manifestations of SMA type 2 mainly happen in 6 to 12 months old [17]. Patients with SMA type 2 suffer from poor muscle tone, absent or declined deep tendon reflexes, scoliosis, and respiratory issues [17–19]. Despite SMA type 2, patients affected by SMA type 3 have limited or no respiratory muscle weakness and scoliosis [16, 20]. SMA type 3, also known as Kugelberg–Welander disease, often presents after 18 months old, in which muscle weakness in the legs is more significant than in the arms, resulting in walking problems and wheelchair dependency [21]. SMA type 4 is the late onset and uncommon form of the disease [22]. The *SMN* locus comprises two paralogous copies of the survival motor neuron gene *(SMN*) called *SMN1* and *SMN2*. These two genes are highly identical, with differences in five nucleotides and C-to-T transition in exon 7. *SMN2* is a phenotype modifier for SMA, and its copy number can determine the disease severity [1, 23, 24]. The estimated copy number of *SMN2* equals one in SMA type 0, two or three in SMA type 1, three in SMA type 2, three or four in SMA type 3, and four to eight in SMA type 4 [6, 25–27]. Deletion or mutations in the *SMN1* gene on chromosome 5q13.2 may lead to SMA disease, and the most common cause is homozygous deletion of exon 7 of *SMN1*. In another form of SMA, exon 7 of *SMN2* can fuse with exon 8 of *SMN1*, which is called *SMN1* to *SMN2* conversion. Gene conversion can cause a deletion, resulting in a pathogenic variant. Additionally, the *SMN1* and SMN2 loci are highly predisposed to denovo mutations due to frequent repeated sequences. As a result, the copy numbers of *SMN1*and 2 vary among the population. Individuals generally have a single copy of *SMN1* on each chromosome [1 + 1], whereas specific individuals have a triplet [2 + 1]. A carrier can have two copies of *SMN1* on a chromosome in cis, while the other chromosome has no copies [2 + 0] [28–38]. According to the autosomal recessive pattern of SMA, parents with one SMA-affected child have a 50% chance of giving birth to the asymptomatic carrier, a 25% chance of having an affected child, and a 25% chance of making an unaffected child (not a carrier) in the following pregnancies. Interpretation of *SMN1* dosage testing can be normal in 6% of parents with an SMA-affected child caused by homozygous deletion of *SMN1* because 4% of carriers have two copies of *SMN1* on one chromosome [2 + 0 genotype], which results in false-negative results in *SMN1* dosage testing and misdiagnosis. Another reason for this is the denovo deletion of exon 7 of one *SMN1* allele, which accounted for 2% of SMA cases in which one parent is the carrier [5]. The complexity of SMA diagnosis sheds light on the importance of genetic counseling. As consanguine marriages are common in Iran, the incidence rate of recessive disorders is high [38, 39]. Focusing on the importance of genetic counseling for families suffering from SMA, we aim to evaluate the prevalence of *SMN1* deletion/duplication mutations in SMA families referring to neuro-genetic centers of Mashhad, Iran.

Methods and materials

Subject

The experimental study was performed on 622 subjects recruited to the neuro-genetic clinic of Ghaem Hospital, Mashhad University of Medical Sciences, Mashhad, Iran, from April 2009 to July 2021. Medical history, proper neurological examination, and workups such as electromyography (EMG) and nerve conduction velocity (NCV) tests were conducted by expert neurologists They are board-certified neurologists who have been involved in numerous research studies on SMA and other neuromuscular disorders. Any sign of SMA, such as muscle weakness, muscular atrophy, or decreased deep tendon reflexes, was carefully detected during the neurological examination. Inclusion criteria include individuals with abnormal EMG-NCV or other clinical findings favoring SMA, such as muscle weakness, muscular atrophy or decreased deep tendon reflexes. Therefore, any individuals who did not meet these criteria were not included in the study. Neurologist referred the eligible patients to the genetic laboratory of the Academic Center for Education, Culture, and Research (ACECR) for genetic counseling and diagnosis confirmation. Based on family history and pedigrees, suspected SMA carriers were also referred to the genetic laboratory. At the genetic laboratory, blood

samples were collected. RFLP polymerase chain reaction (PCR) and real-time PCR were performed for symptomatic and asymptomatic individuals (possible carriers), respectively. Pregnant women referred to the genetic laboratory for SMA screening underwent amniocentesis and chorionic villi sampling (CVS) at 12–14 gestational weeks. RFLP and real-time PCR were also performed on amniotic fluid and chorionic villi samples,. Detection of silent carriers was performed by selecting samples with two polymorphisms (g.27134T > G[NC_000005.9:g.70247901T > G, rs143838139] and g.27706_27707delAT [NC_000005.9:g.70248473_70248474del, rs200800214]) associated with duplication events in *SMN1*[40]. This study was performed in line with the principles of the Declaration of Helsinki. The review board of Mashhad University of Medical Sciences approved the study (IR.MUMS.MEDICAL.REC.1401.553), and the fetus's parents and other adult participants filled out the informed consent form.

DNA extraction

Genomic DNA was extracted from whole blood, amniotic fluid, and chorionic villi samples using a DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

PCR

The extracted DNA was mixed with ten millimoles of dNTP-Mix, 10X PCR buffer, distilled water, Taq polymerase, and two sets of primers targeting exons 7 and 8 of *SMN1*. The PCR was performed in the following order: The first cycle was incubated at 95 °C for two minutes, followed by 30 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 45 s. The final cycle was incubated at 72 °C for 5 min—the limiting enzyme *Dra I* was utilized in RFLP-PCR to identify *SMN1* exons 7 and 8. The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide. The results of the PCR are displayed in Fig. 1.

Quantitative real-time PCR

Quantitative real-time PCR for detecting the *SMN1* and *SMN2* gene copy numbers was performed using SYBR green PCR Master Mix (PE Applied Biosystems) with an ABI PRISM 7700 thermal cycler (PE Applied Biosystems, Foster City, California, USA). Specific primers were designed for the amplification of the *SMN1* gene. Each reaction was carried out in a total volume of 20 µL. The reaction profile included 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for one minute. All experiments were conducted in triplicate. Of exon 12, albumin was used as a reference gene [41]. Our data were normalized to albumin expression by applying the comparative threshold cycle method (Ct). ΔCt and ΔΔCt were calculated, and the *SMN1* copy number was determined using the $2^{-\Delta\Delta Ct}$ method. ΔΔCt > 0.8 indicated no deletion of exons 7 or 8, ΔΔCt > −0.5 but < 0.5 showed heterozygous deletion, and ΔΔCt < −0.6 revealed homozygous deletion in exons 7 or 8 [41]. Real-time amplification plots of the *SMN1* gene exon seven and albumin $A = \pi r^2$ demonstrated in Figs. 2 and 3, respectively. Figure 4 shows a real-time quantitative analysis of *SMN1* gene copy numbers.

Data analysis was performed using the Statistical Package for Social Science (SPSS) for Windows version 22. The frequency of deletion/duplication mutations in *SMN1* and the copy number of *SMN2* were reported. The age distribution of subjects was calculated using mean ± SD and demonstrated by a circle diagram.

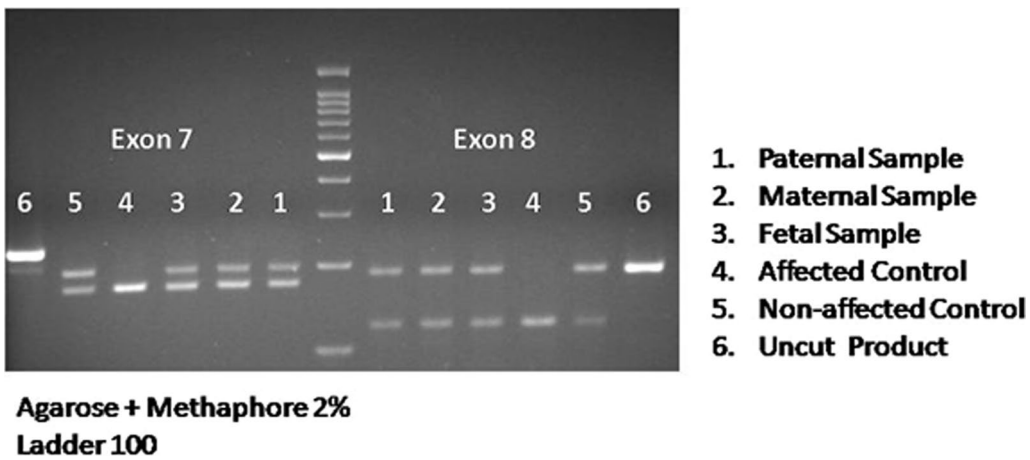

Fig. 1 Deletion of exons 7 and 8 in the *SMN1* gene using the polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) metho

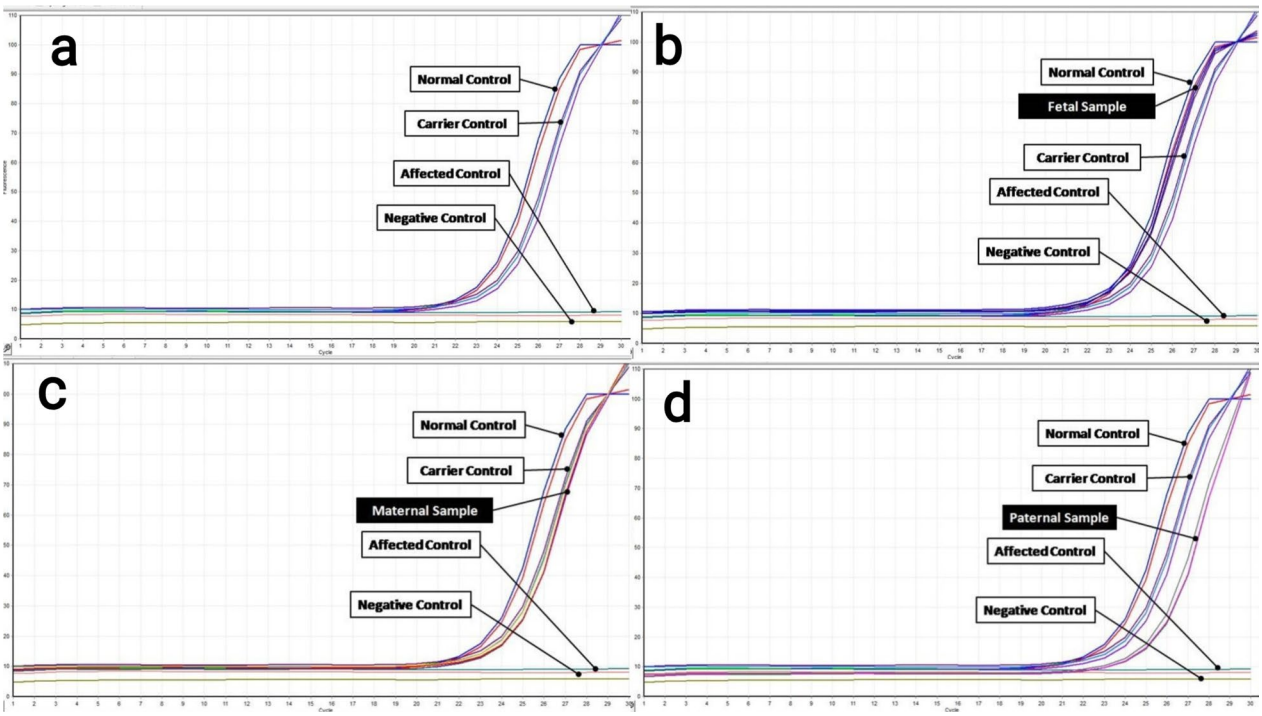

Fig. 2 Real-time amplification plots of the *SMN1* gene exon 7 for **a** normal control, carrier control, affected and negative control, **b** normal control, carrier control, affected control, negative control, and fetal sample, **c** normal control, carrier control, affected control, negative control, and maternal sample, **d** normal control, carrier control, affected control, negative control, and paternal sample

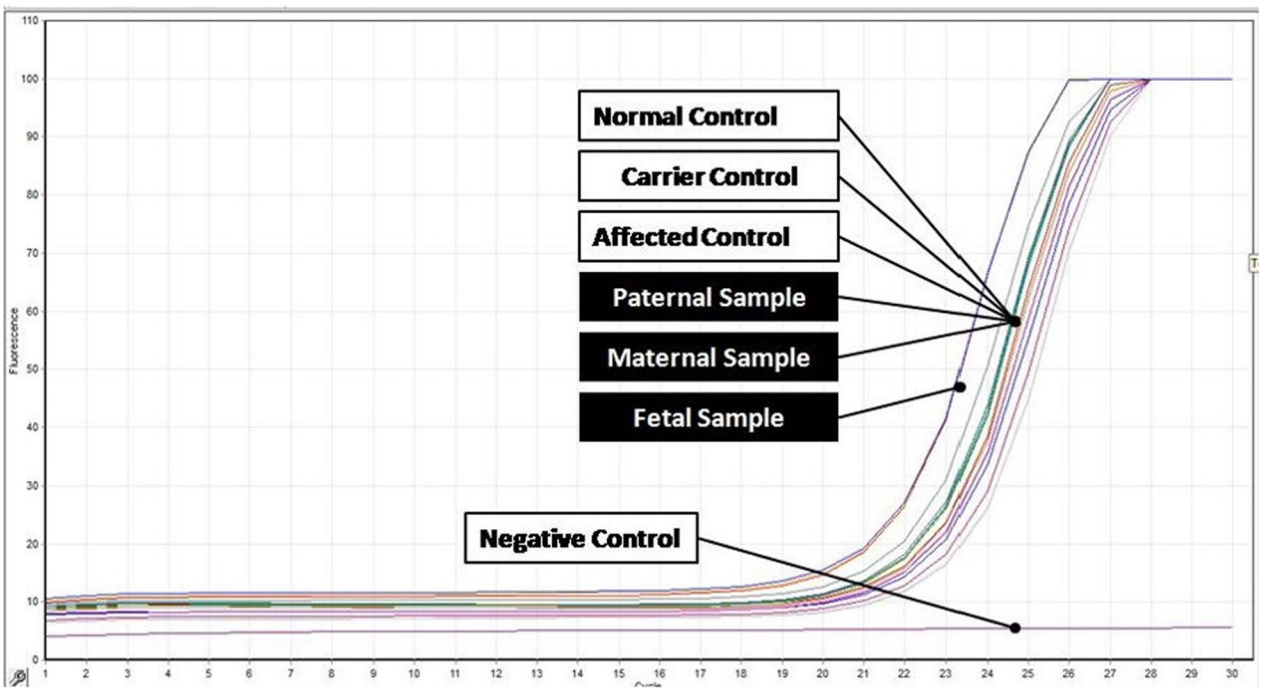

Fig. 3 Real-time amplification plots for albumin (control)

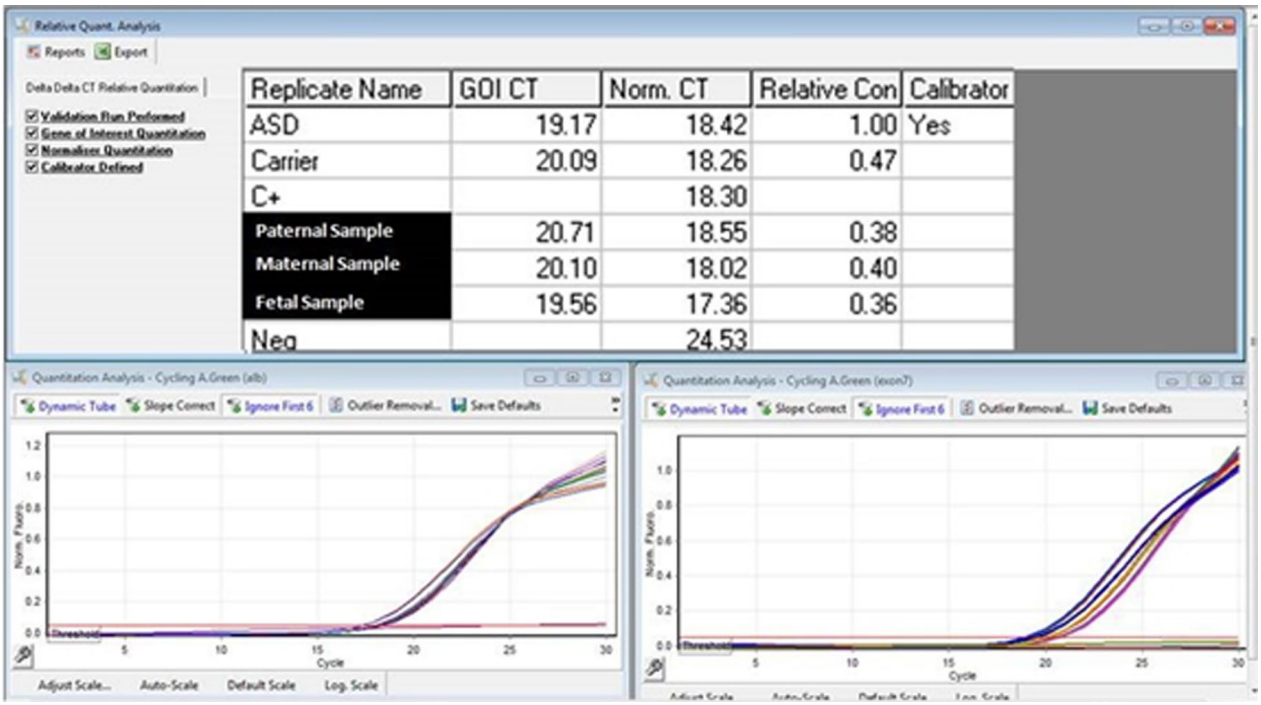

Fig. 4 Real-time quantitative analysis of *SMN1* gene copy number

Results

The study enrolled 622 subjects from SMA-affected families. SMA family refers to parents, siblings, and first, second, or third-degree relatives. Two samples were missing, and the dropout rate equals to 0.32%. The mean age of 463 non-fetus subjects (74.5%) was 13.8 ± 22.6 years old (one month to 85 years old). Age distribution of non-fetus subjects is shown in Fig. 5.

A total of 159 subjects were fetuses with a mean age of 13 weeks and one day (nine to 22 weeks). In total,

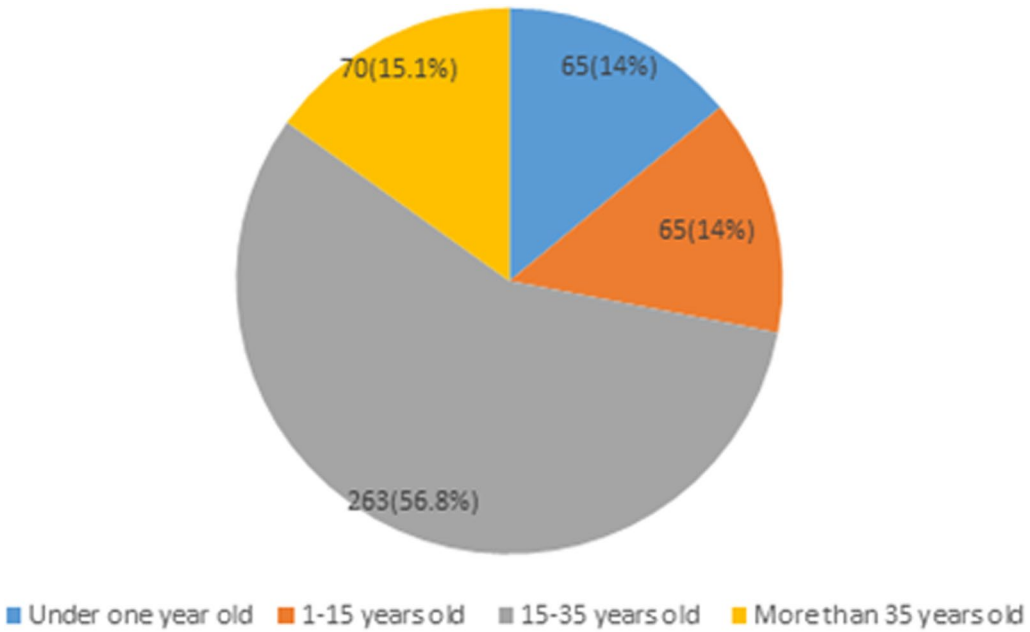

Fig. 5 Age distribution of non-fetus subjects

268 out of 620 subjects (43.2%) were normal (wild type for *SMN1:* 2 normal copies of the *SMN1*). One hundred eighty-seven subjects (30.1%) were heterozygotes for exon deletion in the *SMN1* gene, while 143 subjects (23%) showed homozygous exon deletion. Among individuals with homozygous deletion of *SMN1*, 52 patients (36.4%) showed deletion of exon 7, and 91 patients (63.7%) had deletion of exons 7 and 8 together. (Heterozygote for *SMN1:* one copy of the *SMN1* and homozygote for *SMN1*: 0 copy of the *SMN1*). Eighteen individuals (2.9%) showed two copies of *SMN1* on a single chromosome, and four subjects (0.6%) had three copies of *SMN1* on one chromosome. Heterozygous deletion of the *SMN2* gene was detected in four individuals (0.6%), and five individuals (0.8%) had more than two copies of the *SMN2* gene. Genetic testing results of the *SMN1* gene are summarized in Table 1. In genetic counseling, legal abortion was suggested to all families with homozygous deletion of *SMN1*.

Discussion

Spinal muscular atrophy (SMA) is a neuromuscular autosomal recessive disorder with heterogeneous phenotypes caused by bi-allelic pathogenic variants in the *SMN1* gene. Homozygous deletion in exons 7 and 8 of the *SMN1* gene is responsible for 95% of SMA cases. In comparison, the remaining 5% have a heterozygous deletion in *SMN1* related to an intragenic *SMN1* rare inactivating pathogenic variant on the other allele. SMA's clinical phenotype and severity are associated with the number of *SMN2* copies, which are non-functional copies of the *SMN1* gene [42]. However, most of the SMA carriers have one copy of the *SMN1* gene on an allele; some of them can have two copies of this gene on a single chromosome (cis) with no copies on the other one [2+0]. These [2+0] carriers could be misdiagnosed with those who have one *SMN1* copy on each chromosome [1+1] due to false-negative results in genetic testing [43]. Some normal individuals may have three copies of the *SMN1* gene: two copies on a single chromosome and one copy on another [2+1] [42]. These genotype variations of the *SMN1* gene among normal and affected populations highlight the importance of genetic counseling. In our study, 268 out of 620 individuals (43.2%) were healthy (wild type), 187 individuals (30.1%) were heterozygous for exon deletion of *SMN1*, and 143 individuals (23%) were homozygous for exon deletion of *SMN1*. Four individuals (0.6%) showed three copies of the *SMN1* gene. The frequency of carriers with two copies of the *SMN1* gene on a single chromosome (cis) was estimated at 18 individuals (2.9%). Our study shows that the carrier rate in families with a history of SMA was approximately 1/5 (21.8%). Our highly estimated carrier rate can be attributable to evaluating carriers among family and relatives of SMA cases. In the study of M. Hasanzad et al. [44], the carrier frequency of SMA among the normal Iranian population was estimated at 1/20, which was higher than several ethnicities [45]. Among 200 normal Iranian population, 5% had one copy of the *SMN1* gene. In comparison, 75% and 15% had two and three copies of the *SMN1*, respectively, significantly higher than our results [44]. Molecular analysis of the *SMN* gene in 65 Spanish families showed that 87.7% of all SMA patients had a deletion in both exon 7 and 8 of the *SMN1* gene. Only 4.6% presented with a deletion in exon 7, which was far lower than our results (36.4%). This study also found the genotype of [*SMN1* exon 7, *SMN2* exon 8] in normal parents of SMA patients due to gene conversion to two normal chromosomes [46]. Another analysis of *SMN1*/*SMN2* copy numbers in 1712 newborn individuals and 25 core families of the Chinese population showed 41 newborns (2.39%) with heterozygous deletion in *SMN1* exon 7 as carriers, 1535 individuals with two copies of *SMN1* gene and 119 with three copies of *SMN1*. The genotype of [1+0] was predominant in newborn carriers and also carriers of SMA core families. Additionally, 44 obligatory

Table 1 Genetic testing results of *SMN1* gene

Type of sample	**Non-fetus**	**Fetus**		**Total**
Sampling method	**Peripheral blood**	**CVS***	**AF***	
Status of the SMN1 gene				
Wild type	163 (35.35%)	105 (66.87%)	0	268 (43.22%)
Heterozygote	186 (40.34%)	1 (0.63%)	0	187 (30.16%)
Homozygote	92 (19.95%)	49 (31.21%)	2 (100%)	143 (23.06%)
Two copies on a chromosome (cis)	17 (2.74%)	1 (0.63%)	0	18 (2.90%)
Three copies of the *SMN* gene	3 (0.65%)	1 (0.63%)	0	4 (0.64%)
Total	461	157	2	620

CVS chorionic villi sampling, *AF* amniotic fluid

carriers of core families showed two genotypes of [2+0] and two point mutations [47]. Simard et al. [48] investigated the *SMN1* mutations in 60 Canadian families and found that 87% have a deletion of exon 7 of the *SMN1* gene. The carrier parents of these families had a homozygous deletion of exon 7, 8, or both in the *SMN2* gene. The frequency of two *SMN1* copies in *cis* is higher in the Ashkenazi Jews and African populations. The research was conducted on 270 Spanish families to evaluate the presence of the c.*3+80 T>G and c.*211_*212del variants in the *SMN1* gene. They concluded that these variants are more prevalent in the SMA carriers with two copies of the *SMN1* in *cis* and those with hybrid *SMN2–SMN1* genes; however, [2+0] carriers do not necessarily have these variants [43]. The molecular analysis of the *SMN1* gene in 50 SMA patients in Khuzestan province, Iran, revealed that 90% of the SMA patients have homozygous deletion of exon 7 of the *SMN1* gene. In contrast, 70% have a deletion spanning exons 7 and 8 [49]. The study of Abbaszadegan et al. [50] on 150 individuals investigating the copy number of the *SMN1* gene by quantitative PCR showed that 33.33% of subjects had one copy of the *SMN1* gene, 58% of them had two copies and, 8.66% of individuals had no copy of the *SMN1* gene. The deletion ratio of the *SMN1 c*opies was 0.3 to 0.58. An investigation was performed on 460 individuals with a diagnosis or family history of SMA in Turkey using the RFLP-PCR and the multiplex ligation-dependent probe amplification (MLPA), and results were consistent with a homozygous deletion in both exons 7 and 8 of *SMN1* in 88.13% of SMA cases. 54.5% of families showed a heterozygous deletion of *SMN1* and two or three copies of this gene [51]. Huang et al. [52] evaluated 5200 pregnant women in the Guangdong province of China, in which SMA carrier frequency was 1.44%. Of 75 SMA carriers, 71 women showed both E7 and E8 heterozygous deletions, and four women only had an exon 7 heterozygous deletion of *SMN1*. Also, they all had one copy of *SMN1* but different *SMN2* copy numbers. Among 47 studied couples, three were carriers whose fetuses underwent genetic testing, and one fetus had a homozygous deletion of the *SMN1* exons 7 and 8. In contrast, the other two fetuses showed heterozygous deletion in both exons 7 and 8 of *SMN1*. In the study of Hasanzade et al. [53] for evaluation of the *SMN1* gene in the center and north of Iran with a 60% consanguine marriage rate, the frequency of homozygous deletion in exon 7 of the *SMN1* gene was estimated at 94%, which was consistent with China, Western Europe, Kuwait, and Japan and the prenatal diagnosis revealed 22.8% of fetuses were affected by SMA which was near our results for homozygote fetuses (32.07%), which was detected in CVS and AF samplings. American College of Obstetricians and Gynecologists (ACOG) and the American College of Medical Genetics (ACMG) highly recommend SMA screening to all pregnant and non-pregnant women to detect SMA carriers and help them make suitable choices before and after pregnancy. In conclusion, these recommendations shed light on the importance of genetic counseling [54, 55]. Taking into consideration subtle intragenic *SMN1* pathogenic variants, multiple copies of *SMN1* on a chromosome, denovo deletions of *SMN1*, and also germinal mosaicism, which could be missed in genetic testing, genetic counseling is complicated and necessitates diagnosis confirmation in index cases as well as determination of carrier status in parents[42]. Prenatal or pre-implantation genetic diagnosis (PGD) should be provided to all parents with at least one SMA-affected child. Prenatal diagnosis and PGD of SMA could be performed through CVS and amniocentesis at 11–14th and 16th gestational weeks, respectively[52, 56].

Conclusion

The estimated carrier rate in our families with a history of SMA was 1/5. In conclusion, we highly recommend genetic counseling and carrier detection to all families living in Khorasan province, Iran, and prenatal screening is also suggested for all carriers.

Acknowledgements

Not applicable.

Author contributions

Mohammad Shariati, Reza Boostani, Farah Ashrafzadeh, Mehran Beiraghi Toosi, Javad Akhondian, Narges Hashemi visited the patients and referred suspected cases of SMA and their family members to the genetic laboratory, and all of them read and approved the final manuscript. Alireza Davoudi collected clinical and genetic data of patients and analyzed them. Nafiseh Todarbarywrote and submitted the article under supervision of the corresponding author. Ariane Sadr-Nabavi supervised at genetic testing, analysis of the results and writing the article. She also took the grant from the Mashhad University of Medical Sciences.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. The review board of Mashhad University of Medical Sciences approved the study (IR.MUMS.MEDICAL.REC.1401.553), and the fetus's parents, as well as other adult participants, filled out the informed consent form.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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