ORIGINAL ARTICLE

Molecular Genetic Analysis of Survival Motor Neuron Gene in 460 Turkish Cases with Suspicious Spinal Muscular Atrophy Disease

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Abstract

Objective

To describe 12 yr experience of molecular genetic diagnosis of Spinal Muscular Atrophy (SMA) in 460 cases of Turkish patients.

Materials & Methods

A retrospective analysis was performed on data from 460 cases, referred to Medical Genetics Laboratory, Ege University's Hospital, Izmir, Turkey, pre-diagnosed as SMA or with family history of SMA between 2003 and 2014. The PCR-restriction fragment length polymorphism (RFLP) and the Multiplex ligation–dependent probe amplification (MLPA) analysis were performed to detect the survival motor neuron (SMN)1 deletions and to estimate SMN1 and SMN2 gene copy numbers.

Results

Using PCR-RFLP test, 159 of 324 postnatal and 18 of 77 prenatal cases were detected to have SMN1 deletions. From positive samples, 88.13% had a homozygous deletion in both exon 7 and exon 8 of SMN1. Using MLPA, 54.5% of families revealed heterozygous deletions of SMN1, and 2 or 3 copies of SMN2, suggesting a healthy SMA carrier. Among patients referred for SMA testing, the annual percentage of patients diagnosed as SMA has decreased gradually from 90.62% (2003) down to 20.83% (2014).

Conclusion

Although PCR-RFLP method is a reliable test for SMA screening, MLPA is a necessary additional test and provide relevant data for genetic counseling of families having previously affected child. The gradual decrease in the percentage of patients molecularly diagnosed as SMA shows that clinicians have begun to use genetic tests in the differential diagnosis of muscular atrophies. Cost and availability of these genetic tests has greatly attributed to their use.

Keywords: SMA; SMN1; Retrospective; MLPA

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive inherited neuromuscular disease. The SMA incidence is 1 in 10.000 live birth, and a carrier frequency of 1/40-60 (1). SMA ranked second among inherited neuromuscular diseases within the Caucasian led to infant mortality (2). SMA is divided into four main categories according to the onset of the disease and the severity of the symptoms as follows. The Werding-Hoffman disease (type I), SMA type II, Kugelberg-Welander disease

(type III), and Adult type (type IV) (3). The survival motor neuron (SMN) gene located in 5q13 region is the responsible gene of SMA. Two copies of SMN gene are located on human chromosome 5q13, telomeric (SMN1) and centromeric (SMN2) copy. Mutation or deletion of exon 7 of the SMN1 gene is the main reason of SMA disease. Homozygous absence of SMN2 detected in 4.5% of healthy population suggests that the SMN2 gene is not SMA responsible gene directly, but the increased number of SMN2 copies can modify disease manifestations (4).

The purpose of this study was to present the results of postnatal and prenatal molecular genetic analysis of 460 cases referred to our medical genetics laboratory over a 12 yr period, to perform RFLP and MLPA analysis. The results of this study can improve genetic counselling of incurable SMA disease and prevent recurrence in families with SMA history in Turkey.

Materials & Methods

Patients

Data from 460 cases referred to Medical Genetics Laboratory, Ege University's Hospital, Izmir, Turkey from January 1st, 2003 to December 31th, 2014, for molecular genetic analysis of SMA were retrospectively evaluated. All patients signed the informed consent form for genetic testing routinely.

PCR-RFLP test was performed in 324 postnatal cases (180 males, 144 females), and 77 prenatal samples. The MLPA test, which has been available since 2013 in our lab, was performed in 59 cases (44 parents of affected child, 15 SMA patients). The ages of cases changed between 5 months and 63 yr.

PCR-restriction fragment length polymorphism (RFLP)

Genomic DNA was extracted from peripheral blood samples of suspected individuals, or from a chorionic villus biopsy (CVS), cultured amniocytes of the prenatal cases using QIAamp DNA mini kit (Qiagen, UK) according to manufacturer's protocol.

Homozygous deletions in exons 7 and 8 of the SMN1 gene were investigated by PCR-RFLP method (5). First, PCR was performed to amplify exon 7 and 8 of SMN gene using a forward 5′ - AGACTATCAACTTAATTTCTGATCA - 3′ and a reverse 5′- CCTTCCTTCTTTTTGATTTTGTCT -3′primer for exon 7 and forward 5′-GTAATAACCAAATGCAATGTGAA-3′ and reverse 5′-CTACAACACCCTTCTCACAG -3′ primer for exon 8. An acquired 187 and 189 bp PCR product of exon 7 and 8, was digested with DraI and DdeI restriction enzymes, respectively, according to manufacturer's protocol. The products were visualized by electrophoresis on 4% agarose gel.

Multiplex ligation–dependent probe amplification (MLPA) analysis

Estimation of SMN1 and SMN2 gene copy numbers on 5q13 locus was investigated by MLPA analysis. All MLPA steps were performed according to the manufacturers' protocol of the SALSA MLPA kit (MRC-Hollyland, Netherlands).

Data analysis was done using ABI-3130 genetic analyzer (Applied Biosystems, California, USA), and Genemapper 3.0 (Applied Biosystems, California, USA).

Results

PCR-RFLP results

From 324 postnatal samples, 159 cases (49.07%) had SMN1 deletions by PCR-RFLP test. From 159 deletion positive cases, 140 (88%) had deletion in both exon 7 and exon 8, remaining 19 (12 %) had only exon 7 deletion (Table 1). Types of the disease in patients with either exon 7 deletion or exons 7 - 8 deletions are given in Table 1.

From 2003 to 2014, the rates of postnatal patients detected with homozygous deletion have gradually decreased. While this rate was 90.62% in 2003, it was 20.83 % in 2014. During the period of 12 years, the average rate of patients had homozygous deletion with PCR-RFLP test was 49.04%. 63.82% of postnatal SMA patients had family history of SMA.

SMN1 deletion was shown in 18 (23.38%) samples from 77 prenatal cases carrying SMA type I risk. Among affected fetuses, sixteen (88.89%) fetuses had homozygous deletion in both exon 7 and exon 8 and only 2 (11.11%) fetuses had homozygous deletion in exon 7 of SMN1 gen. All families having prenatal test

Table 1. Postnatal SMN1 PCR-RFLP test results.

SMA subtype				SMN1		Total	Percentage (%)
I n = 81 (50.94%)	II n = 23 (14,46%)	III n = 49 (30.82%)	IV n = 6 (3.78%)	Exon 7	Exon 8	n =159	
8	3	7	0	Del	no del	19	12
73	20	42	6*	Del	del	140	88

del means deletion and no del means without deletion

* Age of cases: 32, 33, 35, 36, 39, 63 years old

had a history of previous affected child with SMA type I or type II, and termination of pregnancy was preferred by all families whose fetuses were diagnosed as a SMA. The babies with normal prenatal test results were normal healthy live-born babies at term and they did not show any features of SMA during the 1-yr follow-up period.

Information about parental consanguinity was available in 95 cases with SMA disease. Sixty-five patients out of 95 (68%), with SMA disease, were offspring of couples with consanguineous marriages

MLPA Results

We used MLPA test as a carrier screening test for 44 parents (22 couples) with a history of child loss due to SMA but without available DNA materials of aborted or death child and 15 patients who did not show enzymatic digestion of SMN2 PCR products in RFLP test. Test results showed that 12 couples (54.5%) were heterozygous for SMN1 gene deletion and had 2 or 3 copies of SMN2 gene pointing out a healthy SMA carrier.

Eleven of 15 postnatal cases, which were resistant to enzymatic digestion, showed homozygous deletion of SMN2 gene and 1 or 2 copies of SMN1 gene with MLPA test.

In one of the cases (10 yr old boy) with very mild SMA symptoms, MLPA test for SMA revealed a homozygous deletion of SMN1 and 3 copy of SMN2 gene. Segregation analysis showed that mother had 1 copy of SMN1 and 2 copies of SMN2 gene, father had the same genotype as his son with homozygous deletion of SMN1 and 3 copies of SMN2 gene. The father was 42 yr old and presented with mild SMA symptoms. He was then diagnosed as SMA type IV.

One case (1-yr-old female) had 2 copies of SMN1 gene and homozygote deletion of SMN2. Mild hypotonia was observed in this case, she could sit at the age of one. It was decided to follow-up further SMA findings and perform sequencing before diagnosed to have definite SMA disease. Remaining 2 cases (1 yr old and 7 yr old) had homozygous deletion of SMN1 and 2 copies of SMN2 and, clinically diagnosed as SMA I and SMA III disease, respectively.

Discussion

In our study, from totally 401 patient (324 postnatal and 77 prenatal) suspicious for SMA 177 (44.14 %) cases were determined as SMA by PCR-RFLP method, in accordance with another (6). From 159 postnatal cases with SMA, 81 (50.94 %) cases were determined as a SMA type I, 23 (14.46%) cases as a SMA type II, 49 (30.82%) as SMA type III, and 6 (3.78%) as SMA type IV, similar to Godinho et al. (7).

In this study, from 177 patients (159 postnatal and 18 prenatal cases) diagnosed as a SMA with PCR-RFLP method, 88.13% had homozygote deletion of both exon 7 and 8 of SMN1 gene, while 21 (11.86%) cases had homozygote deletion of exon 7 but the presence of exon 8 of SMN1. The last one indicates the possibility of conversion event (8). It means the exon 7 of SMN1 was converted to the exon 7 of the SMN2 while the exon 8 of SMN1 was still un-changed. The converted gene is called as 'Hybrid SMN gene' (9). Therefore, in PCR-RFLP results of these cases, the exon 7 of SMN1 could not be detected while the exon 8 of SMN1 was present. We found 18 (23.38 %) fetuses with SMN1 gene deletion

Table 2. The annual percentage of patients diagnosed with SMN1 deletion using PCR-RFLP method among suspicious postnatal cases referred to medical genetics laboratory for SMN1 genetic testing.

YEAR	PATIENT	SMA	PERCENTAGE
2003	32	29	90.62
2004	19	17	89.47
2005	18	12	66.66
2006	27	14	51.84
2007	27	15	55.5
2008	16	8	50
2009	21	10	47.61
2010	30	11	36.66
2011	42	19	45.23
2012	29	10	34.48
2013	39	9	23.07
2014	24	5	20.83
TOTAL	324	159	49.07

from 77 prenatal samples. This is compatible with the simple autosomal recessive Mendelian inheritance. Ogino et al. reported 14 (14%) samples out of 103 prenatal samples with SMN1 gene deletion. The reason for their lower frequency of deletion positive fetuses was that they performed prenatal test for both carrier parents with new partners whose genetic status was unknown, and parents with previous affected children without screening of SMN gene deletions. Early miscarriages of the fetuses with lethal mutations might have contributed to the lower frequency of positive results (10). We may say that SMA in the fetus does not usually lead to early miscarriages.

As shown in the Table 2, the annual percent of patients diagnosed with SMA among postnatal suspicious cases, referred to molecular genetics laboratory for SMA genetic testing using PCR-RFLP method, was decreased gradually from 90.62% in 2003 to 20.83% in 2014. Since molecular tests have become cheaper and more widely available, clinicians have increasingly used them in the differential diagnosis of muscular atrophies.

In our study, consanguinity rate was as high as 68% similar to the observations in other studies (11, 12). Average consanguinity rate is about 23% all over our country. As expected, it is much higher among the parents of patients with autosomal recessive diseases.

Detection of heterozygous deletion in SMN1 gene and screening of SMA carriers in general population can been investigated by MLPA. Determination of a homozygous deletion in SMN1 gene in a prenatal sample is a proof of disease in the fetus where no DNA is available from the previous affected child. Rarely SMA disease is caused by point mutations in the gene, for this reason, clinicians have to keep in mind that if the parents are not carrier by deletion analysis and a homozygous deletion was not identified in a fetus, we may still not exclude the risk of having a child with the disease (13, 14).

Among 44 healthy individuals (22 families), carrier status was confirmed for 12 (54, 5%) families (both mothers and fathers), in accordance with Marijana Miskovic et al. on Serbia patients (15).

While the SMN2 copy number relates to severity of the disease, the SMN1 and SMN2 copy number assessment using MLPA could be useful for the correlation of genotype-phenotype in SMA patients (16). As an example, in the case of a 42 yr old patient with homozygous deletion of SMN1 and 3 copies of SMN2 gene with mild SMA symptoms (SMA type IV), supporting the previous studies reported greater copy number of SMN2 may compensate the absence of SMN1 gene and cause to milder SMA phenotypes.

One of our cases (1 yr old female) had 2 copies of SMN1 gene and homozygote deletion of SMN2. Regarding her clinical findings, we consider that whether she has SMA or another disease causing hypotonia is controversial.

About 2% of SMA patients showed a de-nova mutation in one of the SMN1 alleles resulted from unequal crossing over (17, 18). We performed RFLP analysis in most of the patients (324 postnatal and 77 prenatal cases). RFLP analysis is not an appropriate test to determine the carriers. Therefore, we could not know the percentages of patients having de-nova mutations. Among 59 cases performed for MLPA test, we did not observe any case with de-novo mutations. For more accurate genetic counseling, in SMA families, carrier

testing with MLPA and sequencing to find the point mutations should be performed, when they are available. **In conclusion,** a simple, quick and an inexpensive PCR-RFLP analysis used as a reliable test for diagnosis of deletions in SMN1 gene in most laboratories, is not valid for carrier screening. For career screening of SMA, MLPA analysis should be used. Furthermore, sometimes SMN2 PCR products cannot been digested in RFLP tests because of low PCR amplification or homozygote deletion of SMN2 gene and leading to false negative test results, in this cases MLPA is a reliable test for diagnosis of SMA. For all these reasons and for SMA carrier screening, MLPA analysis should be used as a complementary test with PCR-RFLP.

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Author Contribution

Rashnonejad and Ozkinay: Concept/design, collected and evaluated the patients' data, and prepared the manuscript; Onay and Rashnonejad Performed molecular analysis; Atik, Gokben and Tekgul performed clinical works, had editing the manuscript; Atan Shahin researched literature and performed clinical works. All authors approved the final version of the manuscript.

All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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