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SMA carrier testing using Real-time PCR as a potential preconception screening tool

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Background: SMA is a neuromuscular genetic disorder causing irreversible degeneration of the anterior horn cells of lower motor neurons. According to the age of onset and severity of the condition, it is classified into 5 subtypes. SMA carrier's frequency worldwide is 1:40–80. We used quantitative real-time PCR to determine the copy number of the disease-determining *SMN1* gene by rapid and reliable assays. We studied the *SMN1* gene copy number in Egyptian sample of 115 individuals, as well as in 10 SMA families.

Results: Our results showed that 57.4% of the couples with the previous history of an affected family members were carriers. On the individual level, carriers of single *SMN1* gene copy rate are much higher than the previously reported frequency rates. The effect of consanguineous marriages appears evident in SMA as an autosomal recessive disorder.

Conclusions: In conclusion, the carrier frequency detected in our cohort was high, which possibly corresponds with the worldwide report of SMA as a leading genetic cause of death among infants. Considering the high rate of consanguinity in developing countries confirms the importance of national SMA carrier screening in Egypt. The qPCR carrier screening test is a rapid-cost effective test that can detect approximately 90% of carriers. A population-based preconception prenatal screening for couples will also help reduce the disease burden.

Keywords: Spinal muscular atrophy, SMN1, SMA carriers, Real-time, PCR

Background

Spinal muscular atrophy (SMA) is one of the most common autosomal recessive neuromuscular disorders affecting infants and children. SMA incidence was estimated to be 1 in 6000–10,000 live births worldwide with a carrier frequency of 1:40–80 among different ethnic groups [5, 7]. The highest reported prevalence of disease-prone mutation carrier is among European Caucasoids with 1/47 frequency, followed by 1/52 in Asian Indians, 1/59 in Asians, 1/68 in Hispanics, and 1/72 in African Americans [32, 33].

SMA is a neuromuscular disorder resulting from the irreversible degeneration of the anterior horn cells of the α-motor neurons of the spinal cord, producing a proximal progressive muscles atrophy, and may lead to paralysis. Respiratory muscle weakness together with the thoracic cage deformity frequently results in respiratory failure and death, particularly in severe cases or with early-onset patients [3].

Clinical phenotypes of SMA have a heterogeneous range from a severe to a mild phenotype. It is now classified into five subtypes (types 0 to IV) based on the age of onset and severity of the condition. SMA type 0 is the most severe form with uterine onset, and death usually occurs before six months of age. Type I (Werdnig-Hoffmann disease, OMIM# 253300) represented the most common subtype in over half of the reported SMA cases with muscle weakness persisting at birth or before six months of age and patients usually die of respiratory failure within two years. Type II (OMIM# 253550), has an onset usually 18 months after birth, with patients able to sit but never walk by themselves and can survive beyond four years of age. The late-onset

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types are type III (Wohlfart–Kugelberg–Welander disease, OMIM#253400), in which the onset is delayed to more than 18 months and patients are able to walk but often become wheelchair-bound during youth or adulthood), [5, 33], while SMA type IV is the mildest late-onset form with normal life expectancy [32].

The SMA determining gene is called the "survival motor neuron" gene (SMN, OMIM #600354, #601627) located on 5q13. The large inverted duplication consists of two homologous genes arranged in tandem on each chromosome; *SMN1* (telomeric copy, the disease-causing gene) and *SMN2* (paralog centromeric copy). Both genes consist of nine exons and share more than 99% nucleotide identity with exon 8 remaining untranslated. They differ only by five single nucleotide variants (SNVs) within their 3′ ends; two SNVs are located in the coding region of exons 7 & 8, one in intron 6 and two in intron 7. These five unique SNVs are used as a diagnostic tool that allows the distinction between *SMN1* and *SMN2* genes [1, 7, 10].

Although both genes produce equal transcript amounts, almost 70–85% of *SMN2* derived transcripts are unstable, truncated and not fully functioning due to the exon 7 nucleotide exchange NM_000344.3:c.840C > T that interrupts a splicing enhancer and results in exon 7 skipping [33]. *SMN2* gene partially compensates the SMN protein with small amount of functional protein, thus an inverse correlation arises between the number of *SMN2* copies and the severity of the disease. SMA patients commonly have at least one *SMN2* copy. Carriers on the other hand are asymptomatic because they retain one functioning copy of *SMN1 gene* but can pass their nonfunctioning copy to their children [3, 4, 16].

In this highly homologous region, a gene conversion between *SMN1* and *SMN2* can occur producing a hybrid *SMN* gene. Gene conversion mainly takes place by fusion of *SMN1* exon 8 with *SMN2* exon 7 converting *SMN1* into *SMN2* or vice versa. This results in variable copy numbers (CNs) of *SMN1* and *SMN2* [5, 27].

Approximately 95% of SMA patients are due to homozygous deletion of exon 7 of *SMN1* gene. The remaining 5% shows other pathogenic point mutations in compound either in homozygous form or in compound heterozygosity with *SMN1* deletion. Since the disease is autosomal recessive; de-novo variants are causative reasons in only 2% of the affected patients [21].

Three early treatments, Spinraza®(nusinersen), Zolgensma® (onasemnogene abeparvovec-xioi, OA), and Evrysdi® (risdiplam) received FDA approval for the amelioration of SMA symptoms and enhancing of long-term quality of life and survival [5, 11].

Spinraza® (Biogen, Cambridge, MA, USA) is the first SMA effective treatment and is a modified antisense oligonucleotide-based therapy that enhances the production of SMN protein by increasing the production of full-length SMN proteins [11, 21] .

Zolgensma® (onasemnogene abeparvovec "OA") is a gene replacement therapy that delivers a cDNA coding for the SMN protein using Adeno-associated virus 9 (AAV9) as a vector. It is systemically applied to children less than 2 years employed at two different doses [14].

Evrysdi® is an oral *SMN2* pre-mRNA splicing modifier recently approved for the treatment of SMA patients aged 2 months and older. Risdiplam directly promotes the generation of full-length SMN2 mRNA—which increases the production of functional SMN protein [23].

SMA treatment in pre-symptomatic infants increased the likelihood of survival and improved motor function. For an early diagnosis and the initiation of treatment, newborn SMA screening, carrier frequency of SMA, and *SMN2* copy number are important [21, 34].

The comparative Ct (threshold cycle) method can be used to determine the copy number of *SMN1* gene by simple quantitative real-time PCR assays. It detects the most common mutation in SMA and approximately 90% of carriers. This test can be used for Genetic counseling in such patients to reduce the likelihood of having an affected child in the future [1, 10].

This study is a pilot study aiming at throwing some light on the importance of SMA carrier detection in Egypt. Herein, we report the results of qPCR quantification of the *SMN1* gene in geographically heterogeneous Egyptian samples, as well as in SMA families seeking genetic counseling.

Subjects and methods

Subjects

The study is a case-series study conducted between 2018 and 2020 and included 115 adults, 10 SMA patients with homozygous deletion of *SMN1 gene*, and their parents (20 obligate heterozygotes of SMA parents), and 50 normal controls (for normalization of the results). All the subjects were seeking genetic counseling for a family history of a dead sibling with a provisional diagnosis of SMA or with reported SMA in another family member.

Six different families in which carrier status has been confirmed came later for fetal diagnosis. Amniotic fluid (AF) samples were withdrawn from the pregnant mothers. Herein, we offered SMA screening tests with adequate counseling about the disease, for all subjects included in the study (couples, pregnant women, or women seeking pregnancy).

The Medical Research Ethics Committee of the host research centre approved the study protocol and results.

Written informed consents were obtained from all participants according to the guidelines of the Medical Research Ethics Committee. All participants were seeking genetic counseling either premarital or before pregnancy.

Methods

For each participant, samples of 3–5 ml venous blood were withdrawn and transferred immediately to polypropylene tubes containing 0.5 M EDTA (pH 8.0, violet cap) and mixed thoroughly to prevent clotting and to stop nuclease activity.

Genomic DNA was extracted from peripheral blood leukocytes using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific, EU). DNA concentration and purity were determined using NanoDrop® 2000 (Thermo Scientific, EU).

Quantitative real-time PCR of SMN1 gene

Real-time PCR analysis of SMN1 gene copy number was performed in a total volume of 15 µl in each reaction using 0.5 µM of specific primers of SMN1 forward primer is 5'-CCTTTTATTTTCCTTACAGGGTTTC-3'; reverse primer is 5'-GATTGTTTTACATTAACCTTT CAACTTTT-3', 1 µM of GAPDH gene primers as a reference gene; GAPDH forward is 5'-CTACTGGCGCTG CCAAGGCTGT-3'; and reverse is 5'- GCCATGAGG TCCACCACCCTGT-3', 10 ng/µl DNA and 7.5 µl of SYBR Green I PCR Master Mix (Qiagen, Germany). All samples were analyzed in duplicate using StepOne real-time PCR system (Applied Biosystems, USA).

Multiplex ligation-dependent probe amplification (MLPA) analysis

MLPA analysis was done by SLSA MLPA P21-B1 (MRC-Holland, Amsterdam, Netherlands) according to manufacturer protocol (https://www.mlpa.com/WebForms). It was used for the analysis of amniotic fluid samples and one family.

Data analysis

Data were calculated by the comparative Ct method to detect the relative gene copy numbers. The copy number of the sample was determined by the following formula:

$\Delta\Delta Ct = [(\Delta Ct\ GAPDH - \Delta Ct\ SMN1)$ in calibrator sample]−[(ΔCt GAPDH − ΔCt SMN1) of unknown sample].

The relative gene copy numbers (RQ—Relative Quantification) were calculated by the expression: $2^{-\Delta\Delta Ct}$ (it is expected to be ≥ 1 in normal controls, about 0.5–1 in carriers ,and 0 in patients with SMA) [15].

Results

The study was conducted on 115 apparently normal individuals including 53 married couples. From the 53 families, both parents were carriers in 33 families (66 individuals) with RQ values from 0.326 to 0.93 (mean 0.65, SD 0.15, SE 0.019, CV% 23.41). These families were advised to consider prenatal diagnosis during any subsequent pregnancy. Prenatal diagnosis for 6 of these families was carried out using MLPA technique. Both exons 7 and 8 of *SMN1* gene were deleted in 2 samples confirming an SMA diagnosis. Carrier status was confirmed in 2 other samples with only one copy of *SMN1* gene exons 7 and 8; and 4 copies of exon 7 of *SMN2* gene. The other 2 samples showed normal copy numbers (Table 1).

The remaining 19 families were; 16 families with one carrier parent and the other partner showed normal copy number, and only 3 families both parents had normal copy number. The RQ values for the 16 families ranged from 0.48 and 0.93 (mean 0.7, SD 0.14, SE 0.035, CV% 19.85) for the carrier partner, and the other parent was non-carrier with RQ values between 1.0 and 4.0 (mean 1.57, SD 0.68, SE 0.17, CV% 43.39). For the 3 couples with both parents within the normal RQ values ranged from (1.03–2.4) (mean 1.5, SD 0.5, SE 0.2, CV% 31.9) (Table 1).

The last family (Family 53, not shown in Table1) was seeking genetic counseling due to a previously dead child, and a newborn with hypotonia and difficulty in breathing, suckling and swallowing. Real-time PCR has shown that the mother (19 years old) was the carrier with heterozygous deletion (1 copy) of exon 7 of *SMN1 gene*,

Table 1 SMA carrier results of 52 married couples ($n = 106$) and 10 obligate carries couples ($n = 20$) using real-time qPCR analysis

Subjects	Ratio of detected carriers	Range	Mean	SD	SE	CV%
Both Parents Carriers (*n = 66*)	57.4%	0.326–0.93	0.65	0.15	0.019	23.41
One partner Carrier (*n = 32*)	27.8%	0.48–0.93	0.7	0.14	0.035	19.85
		1.0–4.0	1.57	0.68	0.17	43.39
Both Parents Normal *(n = 6)*	5.2%	1.03 – 2.4	1.5	0.5	0.2	31.9
Obligate Carrier *(n = 20)*	0%	0.4–0.6	0.5	0.07	0.02	13.3

whereas the father had homozygous deletion of exon 7. The daughter was affected with homozygous deletion of exon 7 with type I SMA (confirmed by MLPA). The patient died at the age of 5 months. The real-time PCR results for the father were confirmed by MLPA showing homozygous deletion of exons 7 and 8 of *SMN1 gene*, 5 copies of exon 7 of *SMN2 gene*, 4 copies of exon 8 of *SMN2 gene* and heterozygous deletion (1 copy) of exon 5 of *NAIP gene*. The rest of the cases were 4 males and 5 females referred to our lab individually. One male and 4 females were carriers and the rest had normal copy number.

MLPA was performed for 6 families for detection of SMA in amniotic fluid samples in pregnant mothers (Fig. 1). Results is shown in Table 2.

The amplification plots of the 50 normal controls showed almost identical Ct values (threshold cycle) of *SMN1* and *GAPDH genes*. In the 10 SMA patients, only *GAPDH gene* was amplified showing Ct values near to that of normal controls. Whereas in obligate carriers (SMA parents), the Ct values of *SMN1* increased by 2.2 (range 1.2–2.5) compared with Ct values of *GAPDH gene*.

The RQ ($2^{-\Delta\Delta Ct}$) for normal controls ranged from 1.0 to 2.4, in SMA patients the RQ were 0.0 indicating homozygous absence of *SMN1 gene* and RQ values of the 20 SMA parents (obligate carriers) were between 0.4 and 0.6 (mean 0.5, SD 0.07, SE 0.02, CV% 13.3).

Discussion

SMA is a severe neurogenetic disease with high mortality rate among infants and young children. It is a widely spread autosomal recessive disease. Reports of carrier frequencies were 1:40–80 among different ethnic groups worldwide, while the prevalence of disease-prone mutation carriers ranged from 1/47 for European Caucasoids, to 1/72 for African Americans [7, 33]. Due to the high frequency of SMA and the severe clinical outcomes of the disease in general population, the American College of Obstetricians and Gynecologists (ACOG) and American College of Medical Genetics and Genomics (ACMG) recommend universal SMA carrier screening and prenatal screening of SMA in couples regardless of race or ethnicity [6, 9].

Since SMA is an autosomal recessive disorder, consanguinity is expected to play a major role in its prevalence and incidence. Egypt is a home to many different ethnic groups. Consanguineous marriages rate reached 39% [26, 31].

We established quantitative real-time PCR to determine the carrier status of SMA in families where a history of SMA-like neurologically affected previously deceased family member exists. Neurologic disorders have the highest frequency among all genetic disorders in Egypt with a ratio of 31.38%. In a retrospective study conducted in Egypt from January 1966 to December 2009, SMA constituted 18.66% of neuromuscular disorders and 0.02% of all patients attending the Pediatric Hospital (17.7/ 100,000). Comparing this frequency with published data, Egypt showed higher SMA frequency than USA, Germany, Italy, Poland, England, Saudi Arabia, and Libya. Consanguinity among the studied group (47.8%) was higher than the reported rate among the general population in Egypt (38.9%) [12, 25].

Our results showed that 57.4% of the couples seeking genetic counseling were carriers.

On the individual level, SMA carriers represented 75.7% of the studied individuals. That is 87 of 115 apparently healthy individuals included in this study were carriers with only one copy of *SMN1* gene. This rate is much higher than the previously reported frequency rates [24], which could be explained by the selection criteria of the current study. As to validate the analysis, 50 healthy controls with no familial history of any neurogenetic disorders were tested showing normal copies of *SMN1 gene*.

The effect of the habit of consanguineous marriages appears evident in Arab populations. It raised the frequency of SMA carriers to reach 1 in 25 for morocco and 1 in 20 for both Iran and Saudi Arabia. Since India also practices consanguinity, in a group of 606 Indians, the carrier frequency was 1 in 38. SMA carrier frequency is heterogeneous among different ethnic populations. It was found to be higher among Caucasians and Ashkenazi Jews than Hispanics, Asians, and African American populations [20].

For the parent with *SMN1* exons 7 and 8 deletion and 5 copies of *SMN2* exon 7; two hypotheses arise. The first suggests that the father is an asymptomatic SMA type 4. Studies have shown that increasing copies of *SMN2* provide a protective effect against severe forms of SMA in a direct relationship manner. Also, the presence of one copy of *NAIP* gene was accompanied by an increased copy number of *SMN2* gene. Thus, individuals with homozygous *SMN1* deletion and five or more copies of the *SMN2* produce full-length SMN protein enough to compensate for the loss of the non-functioning *SMN1* protein till adulthood [18, 30]. Both *SMN2* and the *NAIP* are SMA-modifying genes and can ameliorate the disease severity while only *SMN1* is SMA causing gene [13, 19].

The second possible explanation for our family concerns with the limitations of MLPA technique. Although MLPA is accurate and reliable in detecting *SMN1 gene* deletion, it cannot detect both pathogenic point mutations and certain paralogous sequence variants between *SMN1* and *SMN2* genes e.g. c.859G > C and c.835-44A > G), these *SMN2* positive modifying variants are associated with better than expected phenotypes [2, 22].

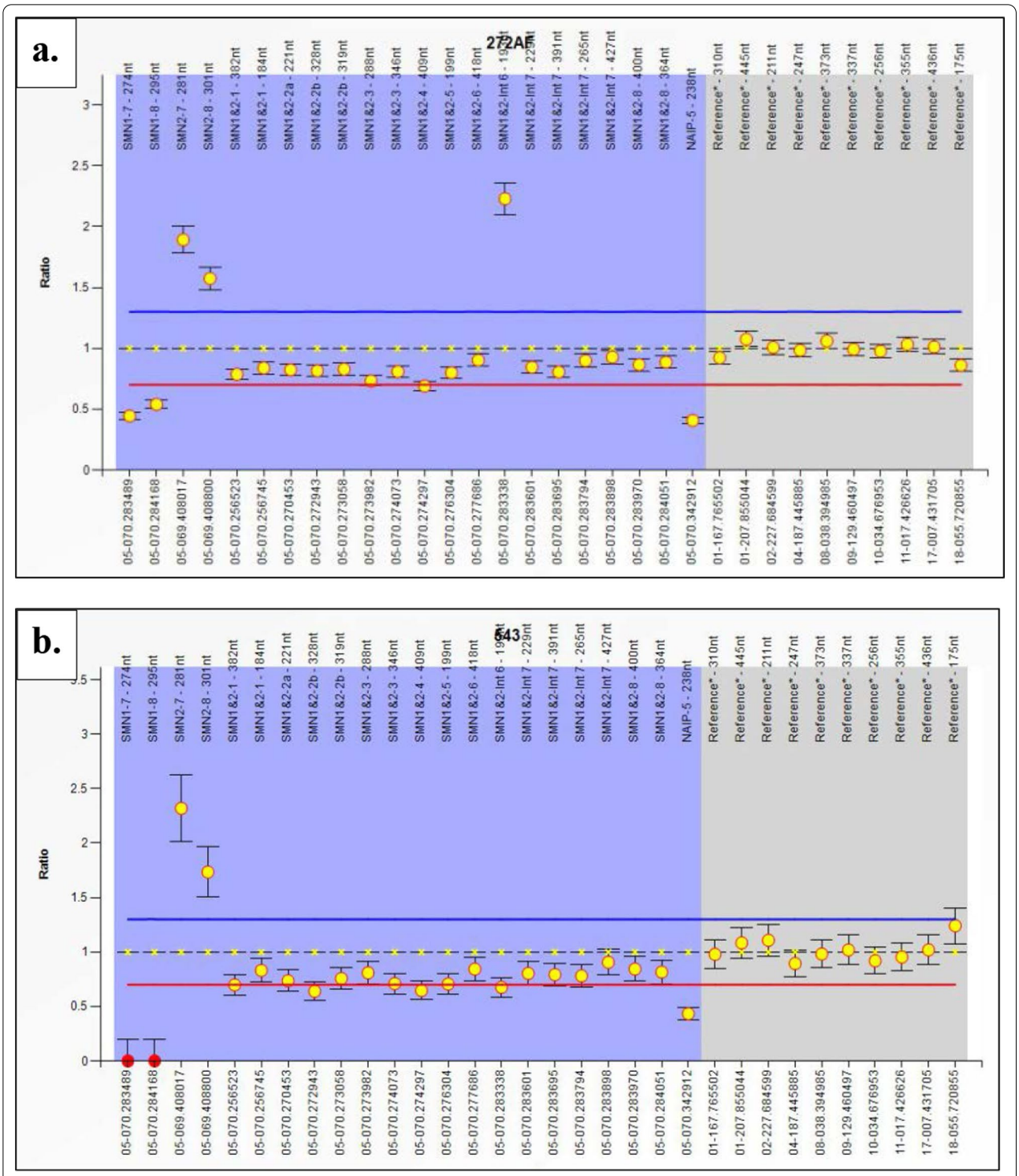

Fig. 1 MLPA analysis by Coffalyser® software showing the tested probes (32 probes for *SMN1*, *SMN2*, *NAIP* and reference genes) against the median values ratios detected in three amniotic fluid samples from different three families (Normal range: 0.7—1.3). **a** Heterozygous deletion of exons 7 and 8 of *SMN1* in amniotic fluid sample AF5. **b** Homozygous deletion of exons 7 & 8 of *SMN1* in asymptomatic parent of special case family (Family 53). **c** Normal copies of *SMN1* gene in amniotic fluid sample AF4

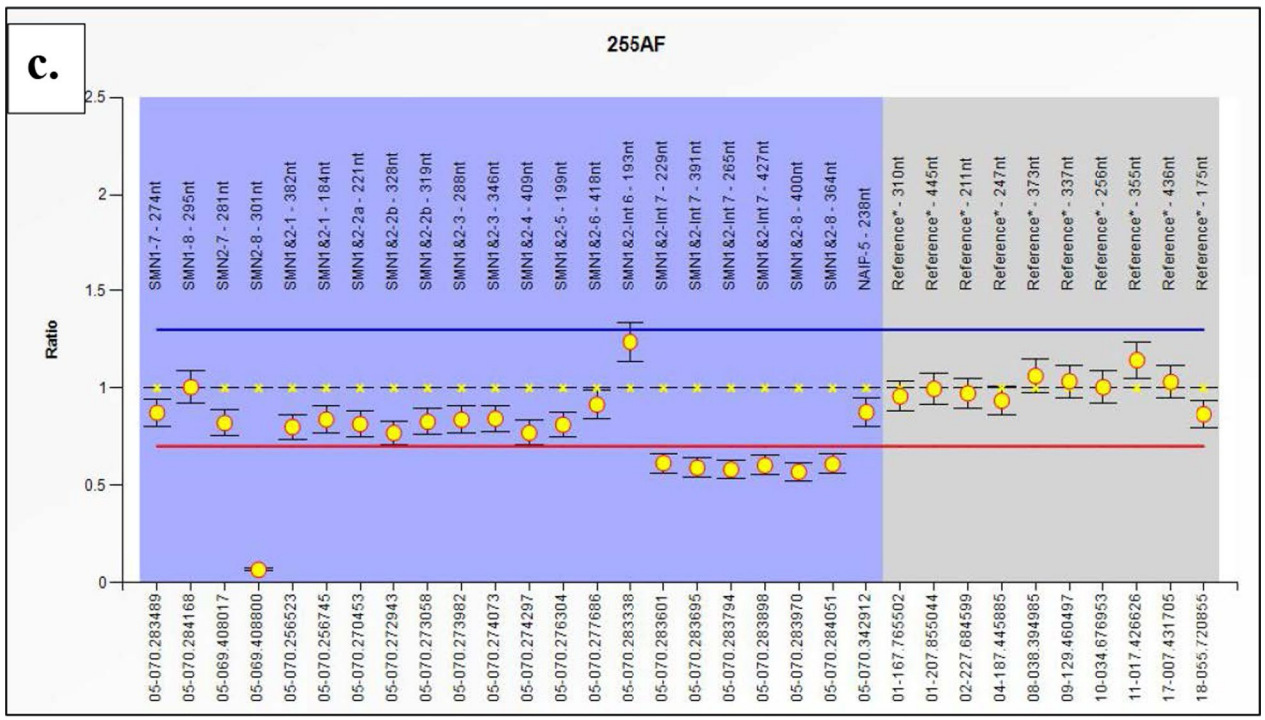

Fig. 1 continued

Table 2 Number of copies of *SMN1*, *SMN2* and *NAIP gene* detected by MLPA in 6 amniotic fluid samples

	SMN 1 Ex 7	SMN 1 Ex 8	SMN 2 Ex 7	SMN 2 Ex 8	NAIP Ex 5	Interpretation of results
AF 1	0	0	4	2	0	SMA- affected fetus
AF 2	2	3	2	1	2	Normal fetus
AF 3	1	1	4	3	1	SMA Carrier fetus
AF 4	2	2	2	0	1	Normal fetus
AF 5	1	1	4	3	1	SMA Carrier fetus
AF6	0	0	3	3	1	SMA- affected fetus

Carrier detection in our study was carried out by two techniques. Real-time qPCR represents the simple, rapid, cheap, screening tool. While MLPA is considered leveling up for more detailed insight into the genotype. Yet, their limitations in detecting rare genotypes ("2+0" or pathogenic point mutations) must be convoyed to families in whom non-carrier by MLPA testing would be reported [17, 28. 35].

Multiple treatment options become available. As a national response to the high number of both SMA cases and carriers in Egypt, the Egyptian National Drug Authority has approved in June 2021 the first spinal muscular atrophy (SMA) therapy, Risdiplam.

Studies in Egypt have shown that a great ratio of neurologically affected dead children does not have a definite diagnosis. Thus, a considerable ratio of SMA patients is missed undiagnosed. Lack of proper awareness of SMA carriers in Egypt is one of the main reasons behind that [8, 24].

Because of the cost of the treatment, the importance of carrier detection or preconception carrier screening and prenatal diagnosis grows up, as a countermeasure to tackle the burden of a disease with low to moderate outcome country.

Conclusions

In conclusion, the carrier frequency detected in our cohort was high, which signifies SMA as a leading genetic cause of death among the infants in our study. This corresponds with the literature reports of SMA as

a leading inherited cause of infant death [29]. Conjoining our results with the high prevalence of SMA worldwide and the high rate of consanguinity in developing countries confirms the importance of national SMA carrier screening in Egypt. Proper genetic counseling, carrier testing, and prenatal diagnosis all along with lowering the consanguinity rate are the SMA preventing scheme. This study highlights the importance of SMA carrier testing in Egypt. Thus, a large-scale population study to determine the SMA carrier frequency in Egypt is highly needed. As Egypt has a unique genetic pool, more population-specific studies on different geographical regions of the country with detailed demographical data would provide a more accurate insights about SMA carrier status. A population-based preconception carrier screening for couples will also help reduce the disease burden.

Abbreviations

AAV9: Adeno-associated virus 9.; ACMG: American College of Medical Genetics and Genomics.; ACOG: American College of Obstetricians and Gynecologists.; AF: Amniotic fluid; CNs: Copy numbers; DNA: Deoxyribonucleic acid; FDA: Food and drug administration; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.; MLPA: Multiplex ligation-dependent probe amplification; MREC: Medical Research Ethics Committee; NAIP: Neuronal apoptosis inhibitory protein; OA: Onasemnogene Abeparvovec-Xioi; PCR: Polymerase chain reaction; qPCR: Quantitative real-time polymerase chain reaction; RNA: Ribonucleic acid; RQ: Relative quantification; SMA: Spinal muscular atrophy; SMN: Survival motor neuron; SNVs: Single nucleotide variants.

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Authors' contributions

NE contributed to the molecular studies, statistical analysis, and writing and revising the article. HH contributed to molecular studies and writing the article. SS contributed to prenatal diagnosis and AF sample collection from pregnant mothers. HS contributed to data analysis and revising the article. ME contributed to the study design, molecular studies, and revising of the article. All authors have read and approved the manuscript, and all authors equally contributed to the study.

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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

The Medical Research Ethics Committee of the host research centre approved the study protocol and results. Written informed consents were obtained from all participants according to the guidelines of the Medical Research Ethics Committee. Reference number is not applicable.

Consent for publication

Written informed consents for publication were obtained from all participants according to the guidelines of the medical research ethics committee (MREC).

Competing interests

The authors declare that they have no competing interests.

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