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Development of a Genetic Screening Method for Polyglutamine Expansion Spinocerebellar Ataxias in Universiti Kebangsaan Malaysia Medical Centre

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Abstract

Background and Aims: Spinocerebellar ataxia (SCA) is a progressive neurodegenerative disorder that encompasses a range of conditions characterised by the gradual deterioration of walking associated with poor coordination of hand movements, eye movements and speech. Currently, 40 types of SCA have been identified with different gene mutations. This study aims to develop locally a simple, easy and cost-effective genetic screening method to diagnose SCA types 1, 2, 3, 6 and 7 that are caused by a polyglutamine (polyQ) expansion. **Materials and methods:** Twenty-four subjects comprises 13 patients, 6 family members and 5 healthy controls from Universiti Kebangsaan Malaysia Medical Centre (UKMMC) gave informed consent to be included in this study. DNA samples were extracted from the blood samples of these subjects and the SCA genes, namely *ATXN1* (SCA 1), *ATXN2* (SCA 2), *ATXN3* (SCA 3), *CACNA1A* (SCA 6) and *ATXN7* (SCA 7), were amplified at the polyQ regions using previously published primers. The polymerase chain reaction (PCR) products for each SCA type were visualised by agarose gel electrophoresis and Sanger sequenced to validate the number of polyQ estimated. **Results:** The results obtained from PCR followed by gel electrophoresis confirmed that all five SCA genes were successfully amplified using the protocol developed. Using the protocol, pathological polyQ expansions were detected in *ATXN1* of one patient, in *ATXN2* of another patient, and in *ATXN3* in 3 patients. In addition, the protocol identified 2 children of patients genetically diagnosed elsewhere to have expanded polyQ in *ATXN3* and *CACNA1A*, and also have expanded polyQ mutations similar to their parents. Sanger sequencing results verified that the subjects had pathological polyQ expansion as visualized by gel electrophoresis. No subjects were found to have an expanded polyQ in *ATXN7* and no healthy controls were found to have a pathological expanded polyQ. **Conclusion:** In conclusion, the PCR method used in this study successfully amplified the polyQ regions in 5 SCA genes and Sanger sequencing confirmed clinically diagnosed SCA patients to have pathological polyQ expansion. Thus, the present study validates the PCR protocol developed to be used as a tool for screening SCA subtypes.

Keywords: spinocerebellar ataxia, polyglutamine, PCR, trinucleotide repeat, Sanger sequencing

Introduction

Spinocerebellar ataxia (SCA) is a group of neurodegenerative disorders that are inherited in an autosomal dominant manner (Wu *et al.,* 2021). SCA is characterized by chronic progressive cerebellar dysfunction and other neurological signs and symptoms. There are more than 40 genotypically distinct subtypes of SCA (Wu and Kapfhammer, 2022). The genetic causes of several SCAs, e.g. SCA1, 2, 3, 6, and 7, are due to the expansion of CAG (encoding for the amino acid glutamine, Q) trinucleotide repeats in different genes (Bird, 2016). The expanded CAG trinucleotide repeats subsequently lead to abnormal elongated polyglutamine (polyQ) tracts and the translated proteins became toxic to the

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neurons (Tan and Ashizawa, 2001). The estimation of SCA prevalence is between 0.3 to 3.0 per 100 000 people. However, this disease varies significantly according to race and country (Gan *et al.,* 2020; Soong, 2004).

With many overlapping clinical features between the subtypes of SCA, genetic testing is the only way to diagnose the SCA subtype. However, with over 40 genetic causes, it is a challenging decision to make as to which genes a physician should investigate. The cost for the genetic test will be expensive if all of the 40 genetic causes are tested; and if the cost is high, this could deter patients from having the genetic test (Tan & Ashizawa, 2001). Due to this, many SCA patients and their family members remain either undiagnosed or uncertain of the subtypes of SCA that they may have. In addition, evidence has shown that an expanded trinucleotide repeat is unstable, and thus could result in further expansion when passed on to the next generation, equating to an earlier age of onset, a more rapid rate of progression and an increase in severity of the disease in the subsequent generations (Paulson, 2018). This phenomenon, known as 'anticipation', makes it important to know the length of the polyQ in family members of patients diagnosed with SCA, even if they are asymptomatic (Jayadev and Bird, 2013).

Therefore, the development of a cheap and efficient screening method to find the genetic cause of SCA may play a clinical role in the management of the disease. SCA 1, 2, 3, 6 and 7 share a common pathology; the expansion of CAG trinucleotides in coding regions. In addition, these five types are the most common SCA subtypes globally. Hence, this study aimed to develop a simple, easy and cost-effective genetic screening test to identify SCA1, SCA2, SCA3, SCA6 and SCA7 in the local population at The National University of Malaysia (UKM) Medical Center (UKMMC), a referral hospital for SCA.

Materials and Methods

Subjects

The study was approved by the research ethics committee of the National University of Malaysia (UKM, JEP-2016-237). All subjects gave written informed consent to participate in the study and all experiments were conducted according to the institutional ethical guidelines. The subjects were either clinically diagnosed patients with SCA that have been followed up in the medical clinic in UKMMC from year 2007 to 2016, or asymptomatic family members of patients who had been genetically diagnosed with SCA, or healthy controls. In total, 13 SCA patients, 6 asymptomatic family members and 5 healthy controls were involved in the development of the protocol. Five mililitre of venous blood (5 mL) was collected from each subject in blood collection tubes (EDTA vacutainer tubes) and DNA sample extracted were tested for expanded CAG repeats in *ATXN1* (SCA 1)*, ATXN2* (SCA 2)*, ATXN3* (SCA 3)*, CACNA1A* (SCA 6) and *ATXN7* (SCA7).

Blood DNA extraction, amplification, and visualization

Genomic DNA was isolated from blood samples of all subjects using ReliaPrep Blood gDNA Miniprep System (Promega, USA) according to the manufacturer's protocol.

PCR amplification and visualisation

Genomic DNA of the regions of interest was amplified by PCR using either Roche FastStart™ Taq DNA Polymerase (Merck, Darmstadt, Germany) or AmpliTaq Gold™ Fast PCR Master Mix (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The composition of the different master mixes used are listed in Table 1 and Table 2 respectively.

Table 1. Reagents for the master mix using the Roche FastStart™ Taq DNA Polymerase

Reagents	40 µl reaction
Water, PCR Grade	20 µl
10x PCR Buffer + $MgCl_2$	5 µl
GC-RICH Solution (5x)	10 µl
PCR Grade Nucleotide Mix	1 µl
Forward Primer (2 µM)	5 µl
Reverse Primer (2 µM)	5 µl
Roche FastStart DNA Taq Polymerase	0.4 µl
Genomic DNA (>30ng/ul)	3.6 µl

Table 2. Reagents for the master mix using the AmpliTaq Gold™ Fast PCR Master Mix

Reagents	20 µl reaction
Water, PCR Grade	5 µl
Forward Primer (10 µM)	2 µl
Reverse Primer (10 µM)	2 µl
AmpliTaq Gold™ Fast PCR Master Mix	10 µl
Genomic DNA (>30ng/ul)	1 µl

The primers used for the PCR amplification were selected from previously published studies as listed in Table 3. PCR products were analysed by agarose gel electrophoresis (1.5-2%, TBE buffer) to estimate the length of the product using a 100 bp ladder. Sanger sequencing was then performed on excised PCR bands to precisely determine the number of CAG repeats. The previously reported range of full penetrance trinucleotide repeats expansion for SCA1, 2, 3, 6, and 7 patients were used to determine the cut-off for pathological trinucleotide expansion (Table 4). Thus, for the purpose of this study, the criteria for pathological polyQ were determined as $\geq$39 for *ATXN1*, $\geq$35 for *ATXN2*, $\geq$61 for *ATXN3*, $\geq$20 for *CACNA1A*, and $\geq$36 for *ATXN7*.

Table 3. Primer sequences

Target Gene	Forward	Reverse	References
ATXN1	AACTGGAAATGTGGACGTAC	CAACATGGGCAGTCTGAG	Lai *et al.,* 2009
ATXN2	CCCTCACCATGTCGCTGAAG	CAGGGCCTTCTAGCGTCG	Malandrini *et al.,* 1998
ATXN3	CCAGTGACTACTTTGATTCG	AAGTGTAGGTACACTTTCCGGT	Kawaguchi *et al.,* 1994
CACNA1A	CACGTGTCCTATTCCCCTGTGATCC	TGGGTACCTCCGAGGGCCGCTGGTG	Zhuchenko *et al.,* 1997
ATXN7	CACGACTGTCCCAGCATCACTT	TGTTACATTGTAGGAGCGGAA	David *et al.,* 1997

Table 4. Reference ranges for polyglutamine expansion SCAs (SCA 1, 2, 3, 6 and 7)

Ataxia	Repeat	Normal	Uncertain	Reduced Penetrance	Full Penetrance	References
SCA 1	(CAG)n (CAT)n (CAG)n	6-38; 39-44 CAT interrupted	-	-	39-44 CAG uninterrupted; 45-91	(Orr et al, 1993; Quan et al, 1995; Goldfarb et al, 1996; Zuhlke et al, 2002)
SCA 2	[(CAG)n CAA (CAG)n] n	14-31	32-34	-	35-500	(Imbert et al, 1996; Pulst et al, 1996; Sanpei et al, 1996; Cancel et al, 1997; Leggo et al, 1997; Costanzi-Porrini et al, 2000; Fernandez et al, 2000; Choudhry et al, 2001; Silveira et al, 2002; Mao et al, 2002; Kim et al, 2007)
SCA3 (MJD)	(CAG)2 CAA AAG CAG CAA (CAG)n	11-44	45-59	-	61-87	(Maciel et al; Takiyama et al, 1997; van Schaik et al, 1997; egan et al, 2000; Maciel et al, 2001; van Alfen et al, 2001; Padiath et al, 2005; Gu et al, 2004)
SCA 6	(CAG)n	4-18	-	19	20-33	(Ishikawa et al, 1997; Zhuchenko et al; Stevanin et al, 1997; Shizuka et al, 1998; Yabe et al, 1998; Katayama et al, 2000; Mariotti et al, 2001; Takahashi et al, 2004)
SCA 7	(CAG)n	4-19	28-33	34-35	36-460	(David et al, 1998; Stevanin et al, 1998; Koob et al, 1998; Benton et al, 1998; Johannson et al, 1998; Giunti et al, 1999; Nardacchione et al, 1999; Van de Warrenburg et al, 2001; Ansorge et al, 2004; Whitney et al)

Modified table from *Consensus and controversies in best practices for molecular genetic testing of spinocerebellar ataxias* (Sequeiros *et al.,* 2010)

Results

Optimization of PCR

The two master mixes were tested to see which were the best to amplify the trinucleotide expansion in *ATXN1, ATXN2, ATXN3, CACNA1A* and *ATXN7* according to the manufacturer's recommendation. Initially, AmpliTaq Gold™ Fast PCR Master Mix was used as it enables shorter PCR running time from a traditional 2 hours to approximately 40 minutes. Roche FastStart™ Taq DNA Polymerase was used when there was no amplification seen using AmpliTaq Gold™ Fast PCR Master Mix despite decreasing the annealing temperature to as low as 50°C. Thus, the trinucleotide repeats expansion region in *CACNA1A* and *ATXN3* were amplified using AmpliTaq Gold™ Fast PCR Master Mix whereas the trinucleotide repeat expansion region in *ATXN1*, *ATXN2*, and *ATXN7* were amplified using Roche FastStart™ Taq DNA Polymerase. Table 5 shows the optimized PCR thermal cycling conditions used with the different annealing temperatures for each target gene.

Table 5. Optimized PCR Thermal Cycling Conditions

Target gene	Temperature and Time				
	Denaturation	36 Cycles			Final Extension
		Denaturation	Annealing	Extension	
ATXN1	4 min at 95 °C	30 s at 95 °C	30 s at 55 °C	1 min at 72 °C	7 min at 72 °C
ATXN2			30 s at 63 °C		
ATXN7			30 s at 55 °C		
CACNA1A	10 min at 95 °C	3 s at 96 °C	3 s at 62.5 °C	5 s at 68 °C	10 s at 72 °C
		40 Cycles			
ATXN3	10 min at 95 °C	3 s at 96 °C	3 s at 50 °C	15 s at 68 °C	10 s at 72 °C

Screening of SCA 1, SCA 2, SCA 3, SCA 6, and SCA 7

The PCR screening results for SCA 1 (*ATXN1*), SCA 2 (*ATXN2*), SCA 3 (*ATXN3*), SCA6 (*CACNA1A*), and SCA 7 (*ATXN7*) are present in Table 6. Representative agarose bands are shown in Figure 1. The 5 healthy controls were detected to have single agarose bands with trinucleotide repeat expansion within the normal range.

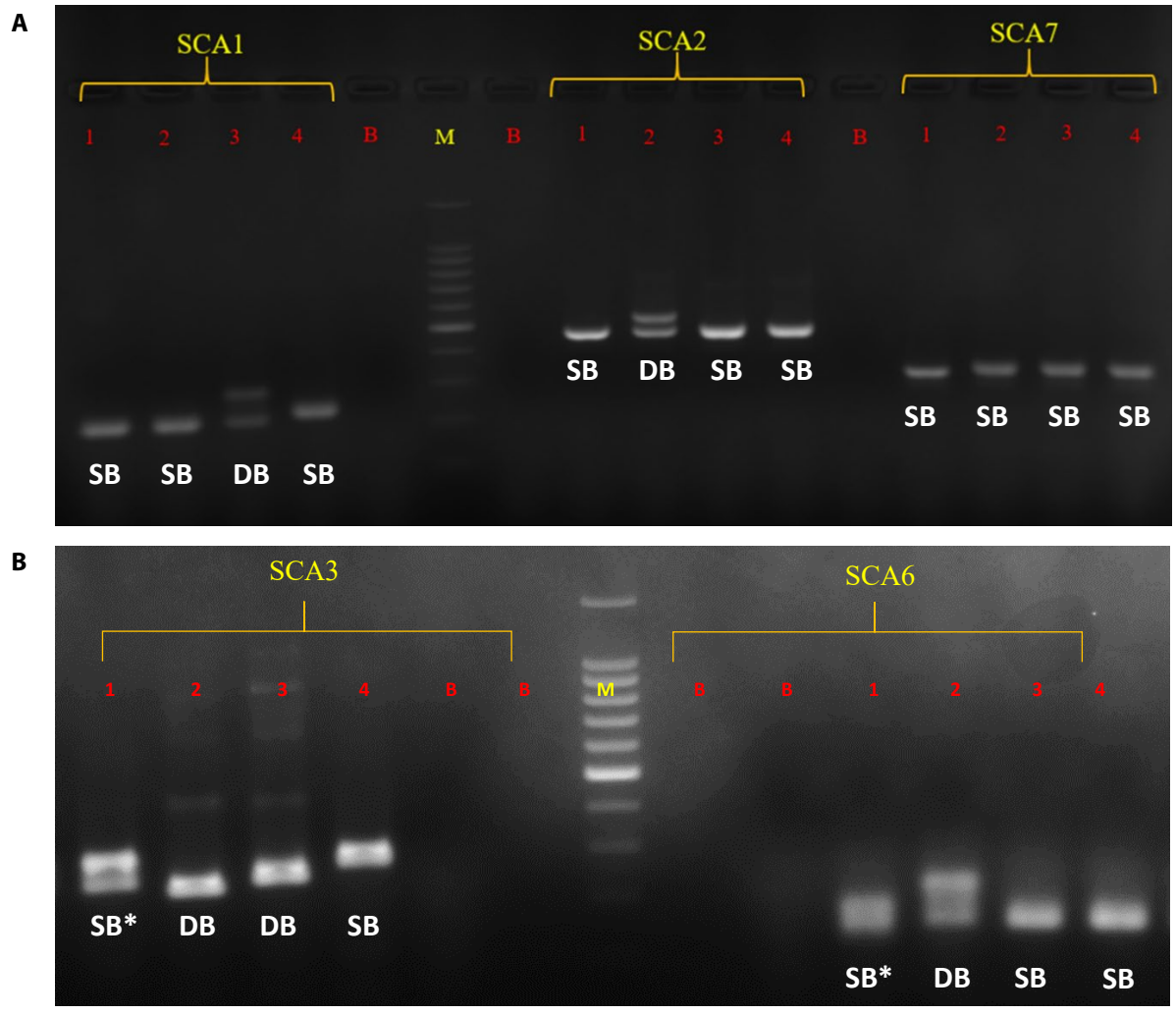

Figure 1. Screening for SCA1, 2, 3, 6, and 7.

(A) DNA bands for amplified regions of SCA1, SCA2, and SCA7 using Roche FastStart™ Taq DNA Polymerase. DNA bands were visualized after electrophoresis at 110 V for 20 minutes.

(B) DNA bands for amplified regions of SCA3 and SCA7 using the AmpliTaq Gold™ Fast PCR Master Mix. DNA bands were visualized after electrophoresis at 110 V for 30 minutes. SB, single band. DB, double band. SB*, the single band had separated into two bands after an additional 10 minutes of electrophoresis at 110 V.

Table 6. Visualisation of amplified trinucleotide repeat (TNR) expansion in SCA genes.

DIAGNOSIS	**SUBJECT**	***ATXN1***		***ATXN2***		***ATXN3***		***CACNA1A***		***ATXN7***	
		Agarose	Sanger Seq	Agarose	Sanger Seq	Agarose	Sanger Seq	Agarose	Sanger Seq	Agarose	Sanger Seq
SCA3	Patient 1	Single		Single		Double	13 & 64	single		Single	10
UN	Patient 2	Single		Single		Single		single	13 & 14	Single	
SCA3	Patient 3	Single	29 & 32	Single		Double	8 & 72	single	11 & 13	Single	6 & 12
UN	Patient 4	Single		Single		Single		single	12 & 13	Single	
SCA2	Patient 5	Single		Double	21 & 42	Single	23 & 29	single		Single	9 & 10
UN	Patient 6	Single		Single		Single		single	13 & 14	Single	
UN	Patient 7	Single		Single		Single		single		Single	10
UN	Patient 8	Single		Single	20 & 21	Single	8	single		Single	10
SCA1	Patient 9	Double	27 & 57	Single	20 & 21	ND		single	7 & 12	Single	10
UN	Patient 10	Single	30	Single	19 & 27	Single		single	7 & 12	Single	6 & 10
UN	Patient 11	Single	28 & 29	Single		Single	8	single	11 & 13	Single	
SCA3	Patient 12	single	29 & 31	Single	20 & 23	Double	22 & 68	single		Single	
UN	Patient 13	Single	28 & 29	Single	18 & 22	ND		ND		Single	9 & 10
SCA6	Asymptomatic family member of patient 14 with SCA 6 (SCA 6)	Single	27 & 28	Single		Single		Double	14 & 25	ND	
NORMAL	Asymptomatic family member of patient 15 with SCA 3 (SCA 3)	Single		Single	20	Single		Single		ND	
NORMAL	Asymptomatic family member of	Single		Single	17 & 21	Single		Single		ND	

	patient 15 with SCA 3 (SCA 3)							
SCA3	Asymptomatic family member of patient 15 with SCA 3 (SCA 3)	Single	Single	Double	13 & 71	Single	ND	
NORMAL	Asymptomatic family member of Ppatient 15 with SCA 3 (SCA 3)	Single	25 & 28	Single	Single	15 & 21	Single	ND

*UN, unknown.

With Sanger sequencing, one patient (Patient 9) was determined to be heterozygote for a pathological TNR expansion in the *ATXN1* gene, hence diagnosed to have SCA 1. Similarly, one patient (Patient 5) was detected to have SCA 2 while 3 (Patient 1, Patient 3, and Patient 12) were detected to have SCA 3. To note, 2 asymptomatic family members of patients genetically diagnosed elsewhere to have expanded polyQ in *ATXN3* and *CACNA1A*, also had expanded polyQ mutations similar to their parents. No patients with SCA 7 were detected in this study.

Discussion

Polyglutamine tract expansion appears to be a common mechanism of hereditary neurodegenerative disease. The genes related to these types of SCA (polyglutamine expansion SCA) most often encode a protein called ataxin and the pathogenic mutation is the expansion of the CAG codon repeats that encode glutamine amino acid. According to La Spada and Taylor (2010), expansion mutations of CAG in the coding regions of the genes result in SCA because the mutant protein gains a new function that is toxic to neurons as the polyglutamine tract increase with more glutamine residues than normal. In this study, we only focused on and recommend initial screening for SCAs 1, 2, 3, 6 and 7 to be carried out as they make up the majority of dominantly inherited SCAs.

SCA 1, 2, 3, 6, and 7 can account for almost 50% of autosomal dominant cerebellar ataxia. It is characterized by diffuse neurological dysfunction that can lead to death by brainstem failure (Jacobi *et al.,* 2012). The hallmark of these SCAs is genetic 'anticipation' whereby the diseases have an earlier age of onset in successive generations, with increasing severity of the symptoms (Jayadev and Bird, 2013). Age of onset is highly variable ranging from early childhood to the later ages of adulthood. Based on Tan (2003) review, the most common SCAs reported in Asian countries (Japan, China, India and South Korea) are polyglutamine expansion SCAs especially SCA 1, 2, 3, 6 and 7. However, there is a paucity of information on SCAs due to a lack of expertise and facilities in diagnosing SCAs in Asian countries. At present, DNA testing that can detect mutations in up to two-thirds of patients with dominantly inherited SCAs is commercially available overseas, but the cost is high.

As there was an extensive overlap between symptoms of different forms of SCA (and neurodegenerative disorders in general), and there was in addition to the association of 'anticipation' with polyglutamine expansion SCAs, genetic testing is needed to diagnose and classify the types of SCAs. Genetic testing is a great tool to confirm the diagnosis and to classify them into their subtypes. One molecular method used for classifying the subtypes of SCAs is next-generation sequencing. Recently clinical exome sequencing and multigene panel have been widely used to diagnose individuals with ataxia (Wallace and Bird, 2018). However, this genetic test generates a high error rate for insertion and deletion mutations (Chen *et al.,* 2018). Thus, nucleotide repeat expansions in hereditary ataxia are not suitable to be identified using this technique. Hence, alternative assays are required for classifying SCAs that are caused by nucleotide repeat expansions.

The latest molecular method for the diagnosis of polyglutamine expansion SCAs involves Southern blot analysis. Southern blot method has significantly higher accuracy than the next-generation sequencing method. Combinations of Southern blot and PCR methods allow reliable and sensitive testing for trinucleotide repeats (Jama *et al.,* 2013). However, in Malaysia, Southern blot analysis is not available as it requires high

technical expertise and is expensive and time-consuming. Therefore, we are proposing to use PCR and Sanger sequencing to screen for these five most common subtypes of SCA (SCA 1, 2, 3, 6 and 7), as PCR is a commonly used technique and widely used for molecular diagnosis.

PCR can amplify the short sequences of DNA within a few hours and requires only a small amount of DNA. Meanwhile, Sanger sequencing is good for sequencing single genes especially short DNA sequences (300 to 1000 base pairs). The protocol developed in this study had successfully amplified all the target regions of interest including the region containing pathologically expanded trinucleotide repeats. Moreover, Sanger sequencing was able to estimate the length of the abnormal repeats. However, the limitation of this study is that the method has yet to detect an individual with pathologically expanded trinucleotide repeats in *ATXN7* (SCA 7). This could be due to inability of our method to amplify the region when pathologically expanded trinucleotide repeats occur in *ATXN7*. Alternatively, it could be due to the fact that SCA7 is not as common as the other polyglutamine expansion SCAs, and therefore none of the patients screened had the SCA 7 subtype. In this current study, no mutation associated with SCA 1, 2, 3, 6, or 7 was found in patients 2, 4, 6, 7, 8, 10, 11, and 13. This could be as due to the patients do not have having these common SCAs but instead, the rarer subtypes.

In conclusion, this study validates a PCR and Sanger sequencing method that can be used as a screening test to identify SCA 1, SCA 2, SCA 3, and SCA 6. Our newly optimized thermal cycling conditions for PCR of polyQ expansions in *ATXN1*, *ATXN2*, *ATXN3*, and *CACNA1A* using primers previously reported is the first validated protocol that is simple and affordable. When the genetic test to diagnose SCA is made more affordable, this will be cost saving for the patient, leading to hopefully to a decrease in incidence of undiagnosed SCA patients nationwide. A cost-effective screening method is also important for early diagnosis in asymptomatic family members, in order to start disease monitoring and early intervention. By developing this simple methods of genetic screening for SCA at local healthcare centers in Malaysia, a cheap and fast alternative to screen for these common SCA subtypes could be made easily available.

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