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CLINICAL AND GENETIC STUDY OF SPINOCEREBELLAR ATAXIA TYPE 1 & 2

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ABSTRACT

Spinocerebellar ataxias are a heterogeneous group of autosomal dominant genetic disorders. They are caused by the variable degrees of degeneration of the cerebellum, spinocerebellar tracts and brain stem neurons. This study involves the clinical and genetic study of spinocerebellar ataxia type 1 and type 2 affected individuals. Both these types are caused by expansion of CAG repeat in their respective gene beyond a certain threshold. They are caused by dynamic mutation and demonstrate genetic instability and anticipation.The study involves 18 patients and 9 healthy controls from different regions of India. A noninvasive method of sample collection,i.e,buccal wash method is followed and PCR based tests using mutation targeted primers are used to diagnose for SCA1 and SCA2. Clinical features and neuronal history of patients were recorded and analyzed.

Keywords: Heterogenous, Spinocerebellar, Anticipation, PCR, SCA and Neuronal.

1. Introduction

Spinocerebellar ataxias are dominantly inherited, heterogenous group of neurological disorders characterized by variable degrees of degeneration of the cerebellum, spinocerebellar

tracts and brain stem neurons[1]. They are characterized by slowly progressive incoordination of gait and often associated with poor coordination of hands, speech, and eye movements. Frequently, atrophy of the cerebellum occurs. There are several types of SCA and the clinical features shared among these SCAs are ataxia, dysarthria, and eventual bulbar dysfunction but there are variable features that includes ocular dysfunction, extrapyramidal signs, pyramidal signs, peripheral neuropathy, intellectual impairment and seizures.The incidence of SCA is considered to be 3 cases per 100 000 people; [2] with SCA3 as the most common subtype in the world[3]. The prevalence of SCA however varies with geographical regions[4].

In India the most common subtype is SCA 2; but studies done is insufficient to understand the prevalence in Indian population as a whole[5].Among the different subtypes of SCA, 6 of them (SCA1, SCA2, SCA3, SCA6, SCA7, SCA17) and DRPLA are caused by CAG trinucleotide expansion in the respective genes. These CAG repeats encode polyglutamine tracts and therefore these disorders are also referred to as polyglutaminedisorders [6].Normal SCA1 alleles range from 6 ± 39, while clinical symptoms have been reported in individuals carrying 39 ± 81 CAG repeats[4]. Intermediate alleles from 36 ± 41 CAG repeats in the SCA1 gene show reduced penetrance[7]. It was found that, the age of onset is inversely correlated to the repeat length with large alleles being predominantly found in juvenile cases [7].Instability is a major characteristic of mutations caused by trinucleotide repeat expansions resulting in an increase in the mean size of the expansion over successive generations[8].

2. Experimental

The clinical disorders are characterized by wide range of phenotypes depending on the respective locus, size of the repeat and disease duration. These disorders manifest above certain threshold of CAG repeats. Clinical characterization of the SCA type is difficult as symptoms are varied and overlap [9]. However genetic diagnosis and confirmation of the SCA type is possible [10]. Molecular analysis also help to understand CAG instability, anticipation and inverse size correlation between repeat size and age of onset of symptoms.[11]The present investigation aims to confirm patients genetically positive for SCA1 and SCA2. Analyze whether the expanded CAG repeat correlates with earlier age of onset of the disease and greater severity of the disease. Analyze neuronal history of the individual family, through pedigree analysis and study anticipation of the disease gene. The study also aims to utilize non-invasive means of sample collection from patients for genetic testing. Therefore buccalmouth wash sample was taken from

patients instead of blood sample. Effective genetic testing would imply use of buccalmouth wash sample for routine diagnosis of patients by genetic testing. The study also aims to establish PCR based tests for diagnosis of SCA1 and SCA2.

In this method the individuals suspected for SCA TYPE 1 and TYPE 2 were assessed and selected. 18 affected individuals and 9 age matched healthy controls from different regions of India were taken for study. A detailed study proforma incorporating neurological history, pedigree chart andneurological examination results was structured. The age of onset would be based on historical information from the affected individual. The severity of disease would be measured by the age of death minus the age of onset during documenting of family history.A comprehensive neurological examination have been done and ascertaining the involvement of domains outside cerebellum and it include cognition, cranial nerves, motor system, extra pyramidal and peripheral nervous system. A detailed pedigree chart would help in discerning the pattern of inheritance of the disorder.

Sample Collection:In this study a non-invasive method of sample collection from the patients was adopted i.eBuccal wash sample collection. For this, 10ml of sterile water was taken in a 100ml centrifuge tube and the patient was asked to pour the water into mouth, rinse his mouth thoroughly for 60s. The patient was asked to pour the water from his mouth back into the centrifuge tube. Finally the sample was stored in 4˚C. The extraction of DNA from Buccalwash sample was carried out by HiMediaBuccalKit Extraction method. The water bath was set at 55˚C and the mouth wash was distributed into the 10 eppendorf tubes with each eppendorf having 1ml. The eppendorf tubes with the sample were centrifuged at 6000rpm for 5 mins and the supernatant was discarded and the pellet was retained. All the pellet was pooled into one eppendorf tube and 20µL of proteinase K was added and vortexed for 15s. 200µL of lysis solution was added to the sample and vortexed thoroughly to get a homogenous mixture.Theprepared sample was then incubated for 55˚ C for 10 mins and 200µL of ethanol (96- 100%) was added to the lysate obtained from the previous step and vortexed for 15s.

The lysate was then poured into HE lute Miniprep spin column and centrifuged at 10,000 rpm for 2 mins. The collection tube with the flow through liquid was discarded and the column was placed into new 2mL collection tube. 500µL of prewash solution (DS0011) was added and centrifuged at 10,000 rpm for 2 mins. The flow through liquid from the collection tube was discarded and the same collection tube was reused and 500µL of wash solutionwas added and

centrifuged at 13,000 rpm for 3mins to dry the column. The collection tube was discarded and a new 2mL collection tube was placed and 200µL elution buffer was added without the rim being touched and incubated at room temperature for 5 mins.After incubation the tube was centrifuged at 10,000rpm for 2 mins to elute the DNA. The column was discarded and the tube stored at -20˚ C.

The reagents used for the genetic detection of SCA 1 and SCA 2 are:-

Slno	**Reagents used**	**SCA 1**	**SCA 2**
1	2X PCR MasterMix[1.5Mm $MgCl_2$] 1.25 units 75mM 20mM 1.5mM 0.01% 0.2mM	Thermo scientific Thermoprime PlusDNApolymerase Tris-Hcl (pH 8.8 at 25˚C) $(NH_4)_4SO_4$ $MgCl_2$ Tween 20 each of dATP, dCTP,dGTP,dTTP	Thermo scientific ThermoprimeplusDNApolymerase Tris-Hcl (pH 8.8 at 25˚C) $(NH_4)_4SO_4$ $MgCl_2$ Tween 20 each of dATP, dCTP,dGTP,dTTP
2	$MgCl_2$ solution, 25 mM, 1.5 Ml	AppliedBiosystems	AppliedBiosystems
3	PCR water [sterile water]	nirlife healthcare	nirlife healthcare
4	Primers used Forward primer (rep2) Reverse primer (rep1)	sigma-aldrich CAACATGGGCAGTCTGAG CTGGAAATGTGGACGTAC	sigma-aldrich GGGCCCCTCACCATGTCG CGGGCTTGCGGACATTGG
5	Agarose	medox	medox
6	TBE	sigma	sigma
7	EtBr	medox	medox
8	Q Solution	-	Qiagen

Table. 1

After the extraction of DNA the quantification of DNA was carried out by NanoDrop technique to evaluate the efficiency of the extraction. To develop PCR technique for diagnosis of SCA1 and SCA2 from patient buccal wash sample was standardised. Primers used for amplification of SCA1 are Forward primer (rep2) CAACATGGGCAGTCTGAG, Reverse primer (rep1) CTGGAAATGTGGACGTAC. [12] Primers used for amplification of SCA2 are Forward primer (rep2) GGGCCCCTCACCATGTCG Reverse primer (rep1) CGGGCTTGCGGACATTGG.[13]

Preparation of reaction mixture				**PCR Condition**		
Sl No	for20µl/PCR tube	SCA 1	SCA 2		SCA 1	SCA 2
1	PCR master mix	10.00µl	12.00µl	Initial denaturation temp	95.00˚C,5.OOmins	95.00˚C, 5.OOmins
2	PCR water/Q solution	5.25µl	3.00µl	cycling denaturation temp	94.00˚C, 0.30s	94.00˚C, 0.30s
3	$MgCl_2$ solution	0.25µl	-	annealing temp	60.00˚C, 0.30s	63.00˚C, 0.30s
4	Rep2 (10pmol/µl) Rep1 (10pmol/µl)	0.75µl 0.75µl	0.75µl 0.75µl	elongation temp	72.00˚C, 0.30s	72.00˚C, 0.30s
5	DNA	3.00µl	3.50µl	final extension temp	72.00˚C, 5.00mins	72.00˚C, 5.00mins
6	-	-	-	hold temp	04.00˚C	04.00˚C

Table. 2

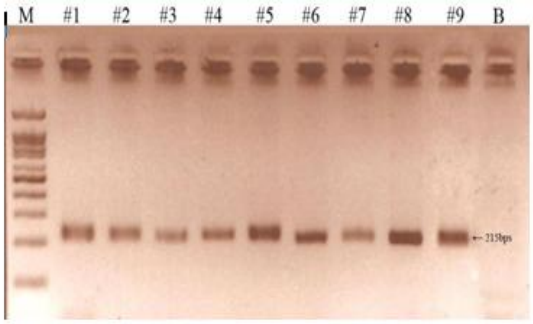
The PCR products were analyzed by agarose gel electrophoresis and amplicon size were determined with DNA ladder. The gel was imaged under Gel Doc UV transilluminator.Amplicon size of SCA 1 for the normal individuals rep1 and rep2 primers amplify a fragment of 211 bpswhile in SCA1 patients these amplify fragments ranging from 240 to 300 bps and for SCA 2 normal individuals SCA2A and SCA2B primers amplify a fragment of 136bps while in SCA1 patients these amplify fragments ranging from 190 to 250 bps.

3.Result and discussion

3.1 Genetic detection of SCA1 and SCA2

All the Samples were effectively collected by buccal wash method and DNA was extracted from the sample by Buccal kit extraction method and quantified by Nanodrop technique. The

minimum concentration of DNA obtained by this technique was 8.1 ng/µl and the maximum concentration was 47.8 ng/µl.In this study 18 affected individual samples and 9 healthy control samples were tested for SCA1 mutation by polymerase chain reaction using rep1 and rep2 primers for ATXN1 gene and SCA2 mutation was detected by SCA2A, SCA2B primers.



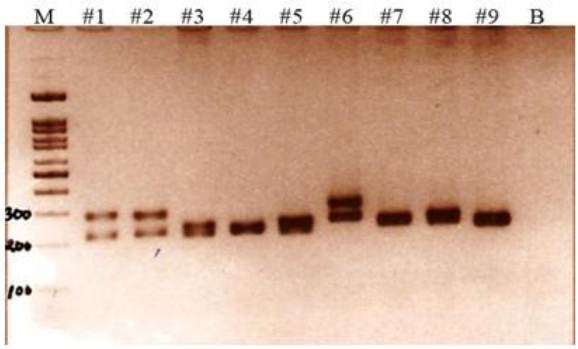
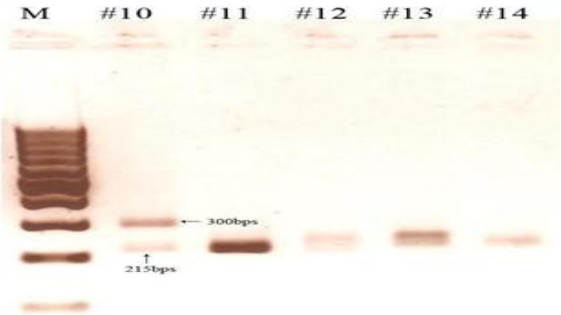


Fig.1 PCR products ofhealthy sample (SCA1) **Fig.2 PCR products of affected individuals sample (SCA1)**



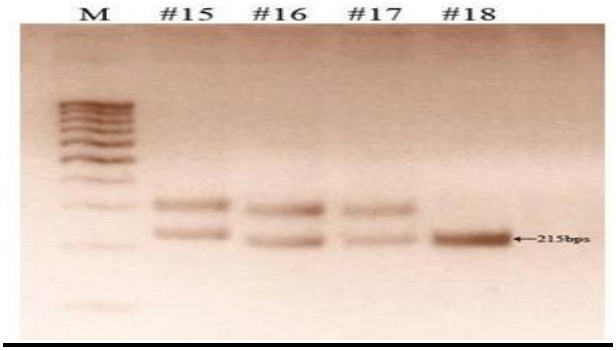
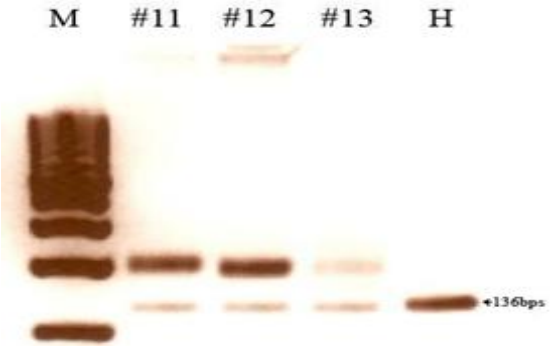


Fig.3 PCR products of affected individual sample (SCA1) Fig.4 PCR products of affected individual sample(SCA1)

In the figure 1 PCR product of the healthy sample for SCA1 showed the band at 215bp and all the 9 healthy controls showed band at the normal level of 215 bps for SCA1. From the figure 1, M is the 100bps DNA ladder to determine amplicon size of the PCR product and #1 to #9 are the nine healthy controls. Finally B is blank. From the figure 2 to figure 4 totally 18 affected individual samples are tested for SCA1, among the 18 individuals with clinical symptoms CAG repeat expansion in atleastone of the alleles was detected in 7 samples. Therefore in these seven

affected individuals one band was detected at the normal 215bps level and the other band around 300bps.

In the figure 2, M is the 100 bp DNA ladder to determine amplicon size of the PCR product and the test samples sample #1, sample#2, sample# 6shows two bands; one band at the normal 215bps and theother band at 300bps indicating one allele with the CAG expansion.These are SCA1 positive. Finally B is blank. Rest of the samples are SCA1 negative, as it shows only one band at the normal 215 bps level.In the figure 3, M is the 100 bp DNA ladder and the test sample #10shows two bands; at 215 bps and at 300bps indicating SCA1 positive. But the sample #11, sample# 12, sample #13and sample #14shows only one band at 215 bps level; these are SCA1 negative.In the figure 4, M is 100bps DNA ladder and sample#15, sample#16, sample #17shows 2 bands at 215bps and 300bps indicating theseare SCA1 POSITIVE. But the Patient #18 is SCA1 negative with band at normal 215bps level.Therefore samples #1, #2, # 6, #10, #15, #16 and #17 are SCA1 positive.



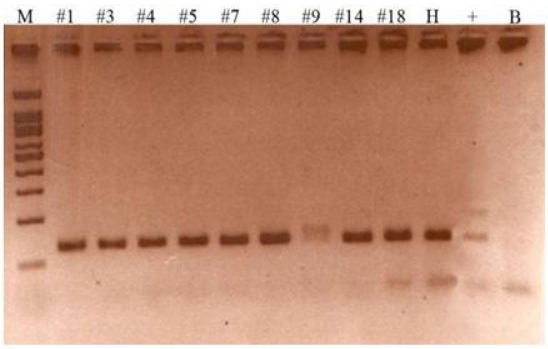


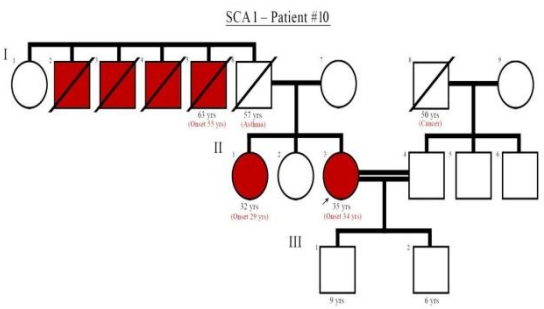
Fig.5 PCR products of affected individuals sample (SCA2)Fig.6 PCR products of affected individuals sample (SCA2)

In the figure 5 and 6, 18 patient samples were tested for SCA2.Among 18 patient samples,CAG repeat expansion in one of the alleles was detected in 3 patients. Therefore in these 3 samples one band was detected at 136 bps and the other band around 200bps.In the figure 5, M is the 100 bps DNA ladder and H is the healthy control. In this resulttest samples #11, #12, #13shows two bands , one at normal 136bps level and theother at 200 bps level indicating one expanded allele at ATXN2. These individuals are SCA2positive. In the figure 6, M is the 100 bps DNA ladder, H is a healthy control, + is a positive control, B is blank and all the patients in the above fig are negative for SCA2 showing bands at only 136bps level. Therefore test samples #11, #12, #13 are

SCA2 positive.

3.2Pedigree Analysis

In the present investigation, among six SCA1 patients three of them showed a positive family history for ataxia while remaining seemed to be sporadic cases and the pedigree analysis of sample #10‘ with SCA1 mutation is as follows.



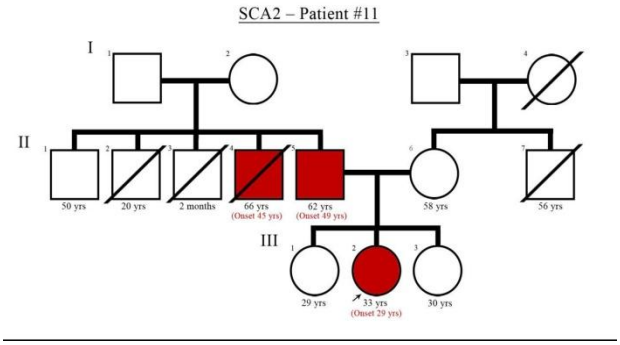


Fig.7 Pedigree of SCA1 positive individual Fig.8 Pedigree of SCA2 positiveindividual

In the figure 7, Proband II,3 is 35 yrs old and onset of the disorder is 29yrs and II,1 is also affected with an onset of 29 yrs.The earlier generation I, shows four members affected. Therefor it is observed that, the father of the proband did not show any symptoms and died of asthma at age of 57. His brothers were affected.I,5 died at the age 63 and had an onset of the disorder by 55 yrs. Anticipation or earlier age of onset with successive generation can be observed.Among these three SCA2 patients, two showed a positive family history of similar illness.In the figure 8, Proband III,2 is 33 yrs old and onset of the disease is 29 yrs and two other members of the family are affected i.e II,4 who showed an onset at 45 yrs and II,5 whose onset of the disorder is 49 yrs.Proband shows an earlier age of onset of the disorder indicating the phenomenon of anticipation.

SCA1 POSITIVE	#1	#2	#6	#10	#16	#17	#18
Sex, AGE	M,44	M,43	F,45	F,35	F,45	F,21	M,47
Age of onset	40	41	43	34	39	20	37
Family history	YES	NO	NO	YES	YES	YES	YES
Anticipation	YES	-	-	YES	YES	YES	YES
Neurological signs:							
Imbalance	+	+	+	+	+	+	+
Speech abnormality	-	+	+	+	+	+	+

Intellectual abnormality	-	-	-	-	-	-	-
Nystagmus	+	-	+	+	-	+	-
Dysarthria	+	-	-	+	+	-	+
Dysdiadochokinesia	+	+	+	+	+	+	+
Gait Ataxia	+	+	+	+	+	+	+
Limb ataxia							
Wasting	-	-	+	-	+	-	-
Dystonia	-	-	-	-	-	-	-
Tremor	-	+	+	+	+	-	+
Dyskinesia	-	-	-	-	-		-
MRI:					-		
Cerebellar atrophy	+	+	+	+		+	+
Brainstem atrophy		+	+				
Spinocerebellar tracts atrophy			+				
Impaired cranial nerves	-	-	+	+	-	-	+
Slow saccades	-	+	+	+	-	-	+
Broken pursit	-	-	+	+	-	-	+
Hyper reflex	-	-	-	-	-	-	+
Flexor plantar response	+	+		+	+	+	+
Extensor plantar response			+				
Diminished/abolished reflex	-	+	-	+	-	-	-
Spincter disturbances	-	-	-	-	+	-	+
Dementia	-	-	-	-	-	-	-
Tingling/numbness	-	-	+	-	-	-	-
Memory loss	-	-	-	-	-	-	-

Table. 3 Clinical Features among the individuals Positive For SCA1

SCA2 POSITIVE	**#11**	**#12**	**#13**
Sex	F,33	F,38	M,32
Age of onset	29	28	28
Family history	YES	YES	YES
Anticipation	YES	YES	YES
Neurological signs:			
Imbalance	+	+	+
Speech abnormality	+	+	+
Intellectual abnormality	-	-	-
Nystagmus	+	-	-
Dysarthria	+	-	-
Dysdiadochokinesia	+	+	+
Gait Ataxia	+		+
Limb ataxia		+	
Wasting	-	-	-
Dystonia	-	-	-
Tremor	-	-	+
Dyskinesia	-	-	-
MRI:			
Cerebellar atrophy	+	+	+
Brainstem atrophy	+	+	+
Spinocerebellar tracts atrophy	+	+	+
Impaired cranial nerves	+	-	+
Slow saccades	+	-	+
Broken pursit	+	-	+

Hyper reflex	+	-	-
Extensor plantar reflex			
Flexor plantar reflex	+	+	+
Diminished/abolished reflex	-	-	-
Spincter disturbances	-	-	-
Dementia	-	-	-
Tingling/numbness	-	-	-
Memory loss	-	-	-

Table. 4 Clinical Features of individuals Positive for SCA2

In this study, buccal wash sample was collected instead of blood sample. The concentration of DNA obtained from sample after DNA extraction ranged from 8.1ng/µl to 47.8ng/µl. The average concentration was 30ng/µl. The results from PCR indicate that even small amounts as 8.1ng/µl could effectively be used for PCR reactions and clear bands were obtained on agarose gel electrophoresis.The technique being non-invasive is a painless procedure and can easily be carried out and does not need the technical skill required for bloodsample collection. Use of sterile collection tube and sterile water makes the risk of infection to patient negligible.However, buccal wash sample collection method requires vigorous rinsing of mouth for 1 minute which patients with severe forms of ataxia and tremor or titubation may find difficult to perform. This could lead to low buccal cell counts in the sample eventually leading to lower concentration of DNA after DNA extraction. Nevertheless, considering the advantages of buccal wash sample collection over blood sample; the former can be effectively used for routine sample collection from SCA patients.Among 18 patients tested for SCA1 and SCA2, a total of 10 patients were detected with the CAG repeat expansion in SCA1/SCA2 gene which accounts for 52%. The patients negative for the test may be suffering from any other type of the many types of SCA.Earlier studies on different ethnic communities in India suggested that SCA2 was the most frequent form of SCA in India. However, this study shows that only 3 out of 10 patients detected were SCA2 positive i.e 16%, whereas SCA1 positive constitutes 38%. This suggests that SCA1 and SCA2 are the most common forms SCA in India and ,Genetic Testing for SCA1 and SCA2

should be the first line of tests to be carried out for diagnosis for suspected SCA patients until clinical features suggest some other form of SCA(other than types 1,2). Besides, more population based studies need to be carried out to study SCA in the Indian population.

In the Pedigree analysis of the SCA1 and SCA2 positive individuals clearly indicates the phenomenon of anticipation wherein earlier age of onset of SCA was observed with successive generations. Sporadic forms of SCA1 with no family history was also observed. This suggests reduced penetrance in earlier generation and genetic instability of CAG repeats causing expansion of the CAG repeat, leading to full penetrance of the expanded allele in the affected individual. Gonadal mosaicism in the parent could also be a cause of lack of disease symptoms in the parent but expression of disorder in the proband. Lack of knowledge about similar disorder in the family on part of the patient/person accompanying patient could have lead to wrong recording of family history.All 10 individuals detected with SCA1 and SCA2 were heterozygous for the alleles with CAG repeat expansion. In SCA1 patients the age of onset is by the third or fourth decade of life. [14] Age of onset was not statistically different in this study for SCA1 and SCA2.Patients with SCA1 shared classic clinical features of gait, limb ataxia, imbalance and speech abnormality but pyramidal signs of hyperreflexia and extensor plantar response was observed in only one patient each. Occulomotor dysfunction was common which includes nystagmus and slow saccades; seen in 4 out of 7 SCA1 positive individuals of this study. Motor and sensory deficits were rare. Individuals with SCA1 may experience decline in memory and intelligence, however no such feature was observed in any of the SCA1 patients involved in this study [15].The onset of the disorder in SCA2 patients were by the third decade as statistically reported earlier and the same was observed in this study [16].Hypoactive reflexes, slow saccades and tremor are more frequent in SCA2 [17] but hypoactive reflexes were not detected in any of the SCA2 positive individuals. Tremor was observed in one out of two SCA2 positive individuals while slow saccades was seen in two out of three individuals. Dementia was reported in 29% of the SCA2 individuals but no symptoms of dementia was observed in SCA2 positive individuals in this study[18]. Intellectual abnormality and memory loss was also not observed in any of the SCA2 positive individuals.

Earlier reports suggest pronounced olivoponto cerebellar atrophy in SCA2 patients and milder cerebellar atrophy in SCA1 patients[19]. Among SCA1 positive individuals severe OPCA was

seen in only one person, while in others cerebellar atrophy alone was detected. MRI result was unavailable for one SCA1 patient. All SCA2 positive individuals showed severeolivoponto cerebellar atrophy. Among these affected individuals none could be reliably diagnosed with SCA1/SCA2 with clinical features and MRI reports alone. Considering the wide variety of phenotype seen in SCA1 patients and overlap of SCA2 features with other types of SCA; diagnosis of patients by Genetic Testing becomes necessary. Among the 9 healthy controls, CAG expansion was not detected in anyone. Therefore PCR based diagnosis of SCA1 and SCA2 can be effectively used for diagnosis purpose. However, in individuals with intermediate alleles diagnosis by PCR alone is difficult to detect the expansion and understand its severity. Therefore DNA sequencing becomes the technique of choice for effective genetic counselling among these patients considering the phenomenon of anticipation in these group of disorders. Inverse size correlation between the size of the CAG repeat and age of onset could also be determined by DNA sequencing.

4. Conclusion

SCA are a heterogeneous, autosomal inherited group of disorders caused by degeneration of the cerebellumand its afferent efferent connections. There are several types of SCA, eight of which are caused by expansion of CAG repeat in their respective gene beyond a certain threshold. The expanded CAG repeat encodes a polyglutamine tract that forms cytotoxic inclusions in the neurons causing neuronal dysfunction and neuronal death. The study aims to develop PCR tests for diagnosis of SCA1 and SCA2, determine the effectiveness of noninvasive buccal wash method for sample collection and study clinical and genetic aspects of this disorder in the patients from Indian population. 18 affected individuals and 9 age matched and ethnic matched healthy controls were selected for this study and informed consent was obtained from them. Sample was collected by buccal wash method, DNA was extracted and quantified. The samples were tested for SCA1 and SCA2 using mutation specific primers by PCR technique.Buccal wash method yielded sufficient concentration of DNA to be effectively tested by PCR technique and clear bands were seen after agarose gel electrophoresis of the PCR products. 10 individuals were detected with SCA1/ SCA2 among which 7 were SCA1 positive and 3 were SCA2 positive. Both SCA1 and SCA2 constitutes 52% of the patients tested implying these two forms of SCA as the most common type in India. Genetic Testing for SCA1 and SCA2 should be the first line of tests to be carried out for diagnosis for suspected SCA patients. Earlier studies done on certain

communities suggest SCA2 as the most prevalent form in India but this study indicates SCA1 as the most frequent type. Therefore more population wide studies is required to be carried out in Indian population. The patients manifested classical clinical features of SCA1/ SCA2 but overlap of features and variety in the phenotypes makes genetic testing important for confirmation of the type of SCA. Therefore, sample collection by buccal wash method and PCR based tests can be effectively used to diagnose SCA1 and SCA2 positive patients.

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