SPINOCEREBELLAR ATAXIAS 8, 12 AND 14 IN TURKEY: MOLECULAR BASES AND GENETIC ANALYSES

by

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ABSTRACT

SPINOCEREBELLAR ATAXIAS 8, 12 AND 14 IN TURKEY: MOLECULAR BASES AND GENETIC ANALYSES

Spinocerebellar ataxias (SCAs) are a clinically and genetically heterogeneous group of neurodegenerative disorders that are inherited in an autosomal dominant manner. Since the clinical symptoms of SCA subtypes significantly overlap, and since there is a high clinical variation even in each SCA subtype, genetic analysis is required for differential diagnosis. The prevalence of SCA subtypes differs among populations, thus genetic analysis is directed based on the population-specific SCA prevalence. This study investigates the distribution of SCA8, SCA12 and SCA14 in Turkish patients, who were previously screened for the six more common SCAs (SCA1, 2, 3, 6, 7 and 17). Molecular analyses of SCA8 and SCA12 were performed both in SCA patients and in healthy controls by PCR, followed by GeneScan analysis. SCA8 analysis was performed only for scientific purposes because of its unclear molecular basis. SCA patients and healthy controls were shown to have no expanded SCA8 and SCA12 alleles. Trinucleotide repeat numbers of SCA8 could be determined in 59 SCA patients and in 60 healthy controls; SCA12 alleles were defined in 92 SCA patients and in 89 healthy controls. The repeat numbers of SCA patients and healthy controls at SCA 8 and SCA12 loci were found to be in the normal ranges. SCA8 analysis was also performed in PD, AD, HD, FA, SCA1 and SCA2 patients to assist in understanding the complex molecular basis of SCA8. In contrast to the above results, expanded SCA8 alleles were found in one PD, one AD, and one FA patient, including her heterozygous father and her two sisters. The finding of expanded SCA8 alleles in control patient groups questions the disease-causing character of CTG repeat expansion. Finally, exon 4 of the PRKCG, which is responsible for SCA14, was screened in SCA patients. DNA sequencing results revealed that the SCA patients examined within the framework of this thesis have no mutation or polymorphism in this region.

ÖZET

TÜRKİYE'DE SPİNOSEREBELLAR ATAKSİ 8, 12 VE 14: MOLEKÜLER TEMELLERİ VE GENETİK ANALİZLERİ

Spinoserebellar ataksiler (SCA) otozomal dominant geçiş gösteren, klinik ve genetik olarak heterojen yapıya sahip bir nörodejeneratif hastalık grubudur. SCA alt-tiplerinin klinik bulguları büyük oranda örtüştüğünden, ve her bir SCA alt-tipi içerisinde bile yüksek oranda klinik çeşitlilik gözlendiğinden, genetik analiz ayırıcı tanı için gereklidir. SCA alt-tiplerinin yaygınlığı toplumlar arasında değişir, dolayısı ile genetik analiz topluma özgü SCA dağılımına göre yönlendirilir. Bu çalışma, daha önce en yaygın altı SCA alt-tipi (SCA1, 2, 3, 6, 7 ve 17) için taranan Türk hastalarındaki SCA8, SCA12 ve SCA14'ün dağılımını incelemektedir. PCR ve GeneScan analizi kullanılarak hem SCA hastalarında hem de sağlıklı kontrollerde SCA8 ve SCA12 moleküler analizleri yapıldı. Henüz tam olarak bilinmeyen moleküler yapısı nedeniyle SCA8 analizi sadece bilimsel bir amaçla gerçekleştirildi. Sonuçlar, bu tez çerçevesinde incelenen SCA hastalarının ve sağlıklı kontrollerin tekrar artışı gösteren SCA8 ve SCA12 aleli taşımadığı yönündedir. SCA8 trinükleotid tekrar sayıları 59 SCA hastası ve 60 sağlıklı kontrolde, SCA12 trinükleotid tekrar sayıları 92 SCA hastası ve 89 sağlıklı kontrolde belirlenebildi. SCA hastalarının ve sağlıklı kontrollerin SCA8 ve SCA12 bölgelerindeki tekrar sayıları normal aralıklar içerisinde yer almaktadır. SCA8 tipinin karmaşık moleküler temelini anlamaya yardımcı olması için SCA8 analizi, Alzheimer, Parkinson, Huntington, Friedreich Ataksisi, SCA1 ve SCA2 hastalarında da yapıldı. Yukarıdaki sonuçların aksine, bir Parkinson, bir Alzheimer, bir Friedreich Ataksisi hastasında ve onun taşıyıcı babası ve kız kardeşlerinde tekrar artışı gösteren SCA8 alelleri bulundu. Kontrol hasta gruplarında tekrar artışı gösteren SCA8 alellerinin görülmesi, CTG tekrar artışının hastalığa yol açıcı karakterini sorgulamaktadır. Son olarak, SCA hastalarında SCA14 tipinden sorumlu PRKCG'nin 4. eksonu tarandı. DNA dizileme sonuçları çalışmada incelenen SCA hastalarının bu eksonda herhangi bir mutasyon ya da polimorfizm taşımadığını gösterdi.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

Ca^{2+}	Calcium
CACNA1A	α1A-subunit of a Ca^{2+} channel gene
DMPK	Myotonin Protein Kinase gene
FMR1	Fragile X Syndrome gene
FRDA	Friedreich Ataxia gene
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
PP2A	A serine/threonine phosphatase
PPP2R2B	Brain-specific regulatory subunit of the protein phosphatase PP2A
PRKCG	Protein Kinase Cγ gene
A	Adenine
AD	Alzheimer's Disease
ADCA	Autosomal Dominant Cerebellar Ataxia
AOA1	Ataxia with Oculomotor Apraxia type 1
AOA2	Ataxia with Oculomotor Apraxia type 2
Arg	Arginine
ARSACS	Autosomal Recessive Spasticataxia of Charlevoix-Saguenay
Asp	Aspartic acid
A-T	Ataxia-telangiectasia
AVED	Ataxia with Vitamin E Deficiency
bp	Base pair
BPB	Bromophenol Blue
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
CT	Computerized Tomography
CUG-BP	CUG-Binding Protein
Cys	Cysteine
DAG	Diacylglycerol

ddNTP	2', 3'-Dideoxynucleoside 5'-triphosphate
DM1	Myotonic Dystrophy type 1
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleoside 5'-triphosphate
DRPLA	Dentatorubropallidolysian Atrophy
EA1	Episodic Ataxia 1
EA2	Episodic Ataxia 2
EDTA	Ethylenedinitrilo-tetraacetate
EtBr	Ethidium Bromide
EtOH	Ethanol
FA	Friedreich Ataxia
FGF14	Fibroblast Growth Factor 14
G	Guanine
Gln	Glutamine
Gly	Glycine
HbS	Hemoglobin S
HD	Huntington’s Disease
His	Histidine
HSA	Hereditary Spastic Ataxia
HUGO	Human Genome Organization
ILOCA	Idiopathic Late-Onset Cerebellar Ataxia
IOSCA	Infantile-Onset Spinocerebellar Ataxia
kb	Kilobase
KLHL1	Kelch-like 1
Leu	Leucine
Lys	Lysine
M	Molar
MJD	Machado-Joseph Disease
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
OD	Optical Density
ORF	Open Reading Frame

PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
Phe	Phenylalenine
PKAAP	PKA Anchor Protein
PKC	Protein Kinase C
PKCγ	Protein Kinase C gamma
Pro	Proline
RED	Repeat Expansion Detection
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Real Time-Polymerase Chain Reaction
SBMA	Spinal and Bulbar Muscular Atrophy
SCA	Spinocerebellar Ataxia
SDS	Sodium dodecyl sulphate
Ser	Serine
SSCP	Single-Strand Conformation Polymorphism
T	Thymine
TBE	Tris-Boric acid-EDTA buffer
TBP	TATA-box Binding Protein
TE	Tris-EDTA buffer
Tyr	Tyrosine
U	Uracil
UTR	Untranslated Region
UV	Ultraviolet
Val	Valine

1. INTRODUCTION

1.1. Hereditary Ataxias

Hereditary ataxias are a clinically and genetically heterogenous group of neurodegenerative disorders. They are characterized by slowly progressive cerebellar atrophy and incoordination of gait and limbs associated with dysarthria and nystagmus (Bird, 2006).

The inherited ataxias can be classified into three subgroups by their mode of inheritance (Ruiz *et al*., 2002; Bird, 2006).

1. *Autosomal dominant cerebellar ataxias:*
   Spinocerebellar Ataxias (SCAs), Dentatorubropallidolysian Atrophy (DRPLA), Episodic Ataxia1-2 (EA1-2), Hereditary Spastic Ataxia (HSA)
2. *Autosomal recessive cerebellar ataxias:*
   Friedreich Ataxia (FA), Ataxia-telangiectasia (A-T), Ataxia with Vitamin E Deficiency (AVED), Ataxia with Oculomotor Apraxia type 1-2 (AOA1-2), Infantile-Onset Spinocerebellar Ataxia (IOSCA), Marinesco-Sjögren, Autosomal Recessive Spasticataxia of Charlevoix-Saguenay (ARSACS)
3. *X-linked cerebellar ataxias:*
   X-linked cerebellar ataxias are characterized in single families with other associated clinical findings.

1.2. Spinocerebellar Ataxias

SCAs are a genetically and clinically heterogenous group of neurodegenerative disorders, inherited in an autosomal dominant pattern. SCAs were first grouped according to Harding's classification, based on clinical findings as ADCAI (Autosomal Dominant Cerebellar Ataxias): cerebeller ataxias with extra-cerebellar symptoms; ADCA II: cerebellar ataxia with retinis pigmentosa, and ADCAIII: pure cerebellar ataxia. However, because the clinical findings of SCA subtypes are significantly overlapping and the clinical

variation is high even within a SCA subtype, the diagnosis of SCA patients, based on clinical features, becomes difficult and complex. Therefore, the identification of the genetic etiology of SCAs is required for understanding and classifying them (Manto, 2005). As more mutations and genes responsible for certain SCAs are defined, a more recent classification according to common pathological mechanisms is used.

1.2.1. Prevalence

Population studies in certain geographical areas throughout the world indicate that the prevalence of SCA is 3/100 000. However, because of the limited number of studies and the high number of undiagnosed SCA patients, this ratio may not be correct. The prevalence of SCA subtypes differ among populations. For example, SCA10 is the second most common SCA in the Mexican population, although it has not been seen in any other population yet. In addition, only 60-80% of clinically diagnosed SCA patients can be genetically diagnosed. Therefore, with the identification of new SCA subtypes, the prevalence ratios may change in future (Figure 1.1.) (Schöls *et al*., 2004).

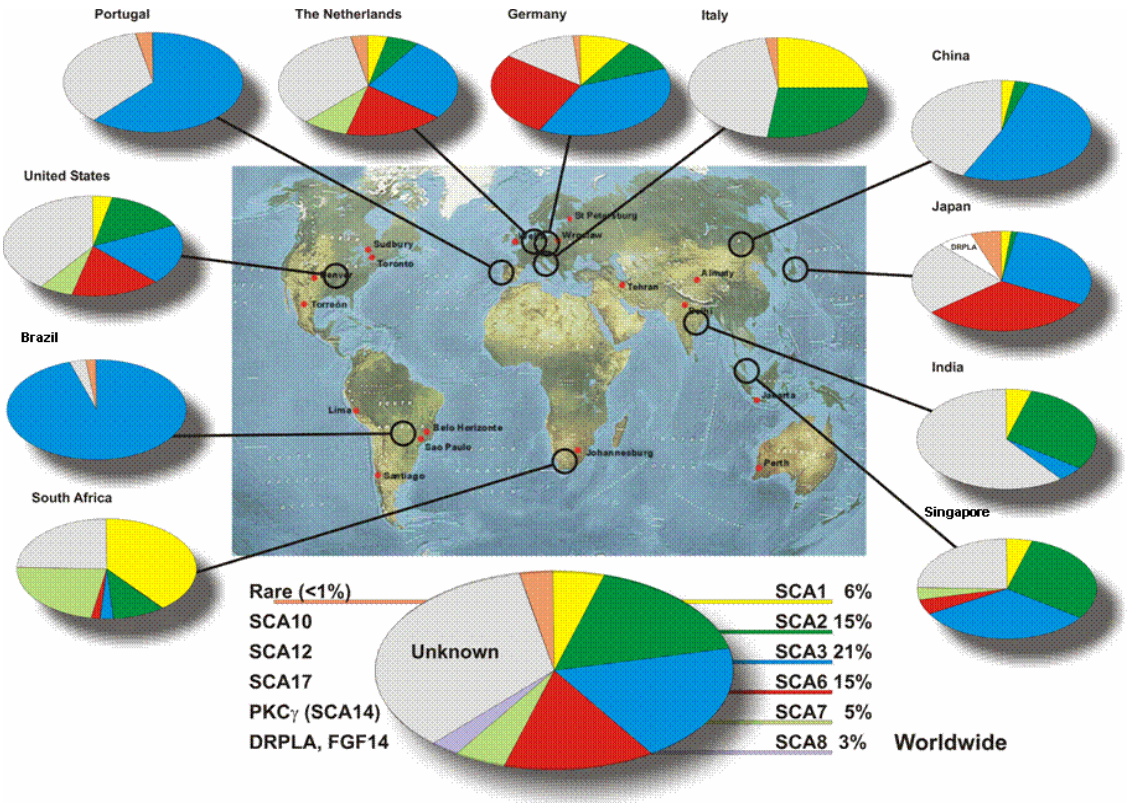


Figure 1.1. Prevalence of SCA subtypes throughout the world (Bird, 2006)

1.2.2. Phenotype

SCAs are characterized by loss of balance and coordination (Lima and Pimentel, 2004). Neurological symptoms of SCAs include oculomotor disturbances such as slow saccades, nystagmus, ophthalmoplegia, and pigmentary retinopathy; movement disorders such as Parkinsonism, dystonia, tremor, dyskinesisas, myoclonus, and chorea; pyramidal signs, cognitive impairment, seizures and peripheral neuropathy (Manto, 2005). SCAs are slowly progressive and late-onset disorders. Although the age at onset varies among different subtypes of SCAs, generally disease is seen at third or fourth decade of life, and death may occur in 10-20 years from disease-onset (Lima and Pimentel, 2004). Because different SCA subtypes affect different regions of the brain, Magnetic Resonance Imaging (MRI) is helpful in the diagnosis of SCAs in addition to the clinical findings. However, MRI may be normal in the early stages of the disease, and it is not used as a diagnostic tool. The observed neurodegeneration pattern by brain MRI in SCA patients is spinal atrophy, olivopontocerebellar atrophy, and cortical cerebellar atrophy (Schöls *et al.*, 2004).

1.3. Genetic Classification of SCAs

Until now, SCA1-8, 10-19, 21-23 and 25-27 subtypes were linked to a unique genomic locus and were given a number by the Human Genome Organization (HUGO). However, only genes that are responsible for SCA1-3, 6-8, 10, 12, 14, 17, 27 have been identified thus far. The remaining diseases are only linked to a specific locus, and large families are required for linkage studies to narrow down the locus of interest and to identify the potential genes (Table 1.1.).

Table 1.1. Genetics of spinocerebellar ataxias (adapted from Schöls *et al.*, 2004)

Disease name	Gene	Gene product	Locus	Mutation	Mutation region	Comments
SCA1	**SCA1**	**ataxin-1**	**6p23**	**CAG repeats**	**exon**	
SCA2	**SCA2**	**ataxin-2**	**12q24**	**CAG repeats**	**exon**	
SCA3	**SCA3/MJD**	**ataxin-3**	**14q24.3-q31**	**CAG repeats**	**exon**	**allelic to Machado-Joseph disease**
SCA4	**-**	**-**	**16q22.1**	**-**	**-**	**families in the US, Japan and Germany**
SCA5	**-**	**-**	**11p11-q11**	**-**	**-**	**families in the US (Lincoln Family) and Germany**
SCA6	**CACNA1A**	**α1A-subunit of a calcium channel**	**19p13**	**CAG repeats**	**exon**	
SCA7	**SCA7**	**ataxin-7**	**3p21.1-p12**	**CAG repeats**	**exon**	
SCA8	**SCA8**		**13q21**	**CTA/CTG repeats**	**3' UTR**	**caution in genetic testing**
SCA9	**Not assigned**	**-**	**-**	**-**	**-**	
SCA10	**SCA10**	**ataxin-10**	**22q13**	**ATTCT repeats**	**intron**	**all families of Mexican origin**
SCA11	**-**	**-**	**15q14-q21.3**	**-**	**-**	**one British family**
SCA12	**PPP2R2B**		**5q31-33**	**CAG repeats**	**5' UTR**	
SCA13	**-**	**-**	**19q13.3-q13.4**	**-**	**-**	**one French family**
SCA14	**PRKCG**	**Protein Kinase Cγ**	**19q13.4-qter**	**Missense mutation, deletion**	**exon**	
SCA15	**-**	**-**	**3p24.2-pter**	**-**	**-**	**one Australian family**
SCA16	**-**	**-**	**8q22.1-q24.1**	**-**	**-**	**one Japanese family**
SCA17	**TBP**	**TATA-box binding protein**	**6q27**	**CAG repeats**	**exon**	
SCA18	**-**	**-**	**7q22-q32**	**-**	**-**	**one Irish-American family**
SCA19	**-**	**-**	**1p21-q21**	**-**	**-**	**one Dutch family, might be allelic with SCA22**
SCA20	**-**	**-**	**Reserved**	**-**	**-**	
SCA21	**-**	**-**	**7p21-15**	**-**	**-**	**one French family**
SCA22	**-**	**-**	**1p21-q23**	**-**	**-**	**one Chinese family, might be allelic with SCA19**
SCA23	**-**	**-**	**20p**	**-**	**-**	**one Dutch family**
SCA24	**-**	**-**	**Reserved**	**-**	**-**	
SCA25	**-**	**-**	**12p13.31**	**-**	**-**	**one French family**
SCA26	**-**	**-**	**19p13.3**	**-**	**-**	**one Norwegian family**
SCA27	**FGF14**	**Fibroblast Growth Factor 14**	**13q34**	**Missense, frameshift mutation**	**exon**	**one Dutch and one German family**

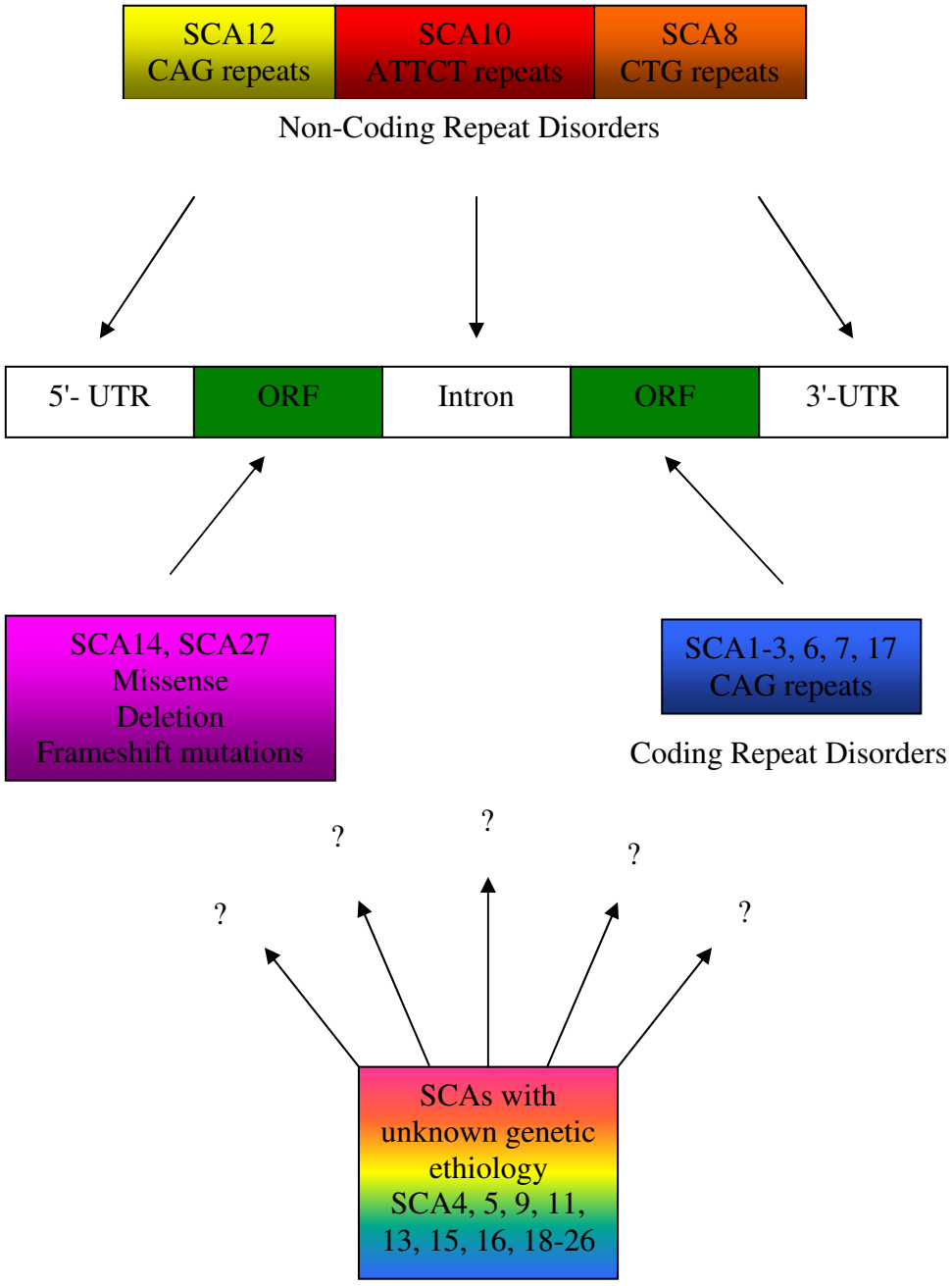


Figure 1.2. Genetics of spinocerebellar ataxias (adapted from Schöls *et al.*, 2004)

Spinocerebellar ataxias can be classified according to *the type* and *the location of the mutation in the gene*. All SCAs with known mutations are trinucleotide repeat disorders except for SCA10 with a pentanucleotide repeat expansion, and SCA14 and SCA27 with missense, deletion or frameshift mutations. Trinucleotide repeat disorders are divided into two large groups according to the location of the repeats in the gene as coding and non-

coding repeat disorders: SCA1, 2, 3, 6, 7, and 17 belong to the first group, SCA8 and SCA12 are in the second group (Figure 1.2.).

1.4. Trinuclotide Repeat Disorders

SCA1-3, 6-8, 12 and 17 are caused by the expansion of unstable trinucleotide repeats in the genome. This new type of mutation, called dynamic mutation (Richards and Sutherland, 1992), is used to describe the non-Mendelian inheritance (Jakupciak and Wells, 2000a). The striking feature of the dynamic mutation is the expansion and intergenerational instability of tandem-arrayed nucleotide sequences. The repeat number is polymorphic in normal individuals, but when it exceeds the threshold size, it gains the pathology, and the pathological size is unique for each disorder (Lima and Pimentel, 2004). Because of the genetic instability mechanisms, as the original repeat number increases, the possibility of expansion in the successive generations increases. Therefore, the anticipation phenomenon - the severity of the disease increases and the age at onset decreases as the repeat number increases in the next generation- can be explained by genetic instability (Bowater and Wells, 2001). In addition, anticipation may be affected by the parental origin of the transmission. While most of the transmissions have paternal origin in polyglutamine disorders, the maternally inherited expansions are common in non-coding repeat disorders (Cummings and Zoghbi, 2000).

Replication, recombination, repair and transcription are counted among the processes that influence the genetic stability. Slippage of the complementary strands, occurring during deoxyribonucleic acid (DNA) synthesis, due to hairpin structure formation, in either the newly synthesized or template DNA may result in expansion or contraction of the repeats, respectively (Figure 1.3.) (Bowater and Wells, 2001). The proposed DNA recombination mechanism for the expansion of repeats is gene conversion instead of unequal crossing-over in humans. Gene conversion (recombinational repair) is described as the non-reciprocal transfer of genetic information from one DNA duplex to another with no exchange of flanking sequences. Therefore, it is consistent with the observation of no exchange of flanking sequences during the mapping of the genes responsible for neurological diseases (Jakupciak and Wells, 2000a; Jakupciak and Wells, 2000b). In

addition, the loss of interruptions within repeat tracts is thought to trigger genetic instability and increases the likelihood of repeat expansion (Richards, 2001).

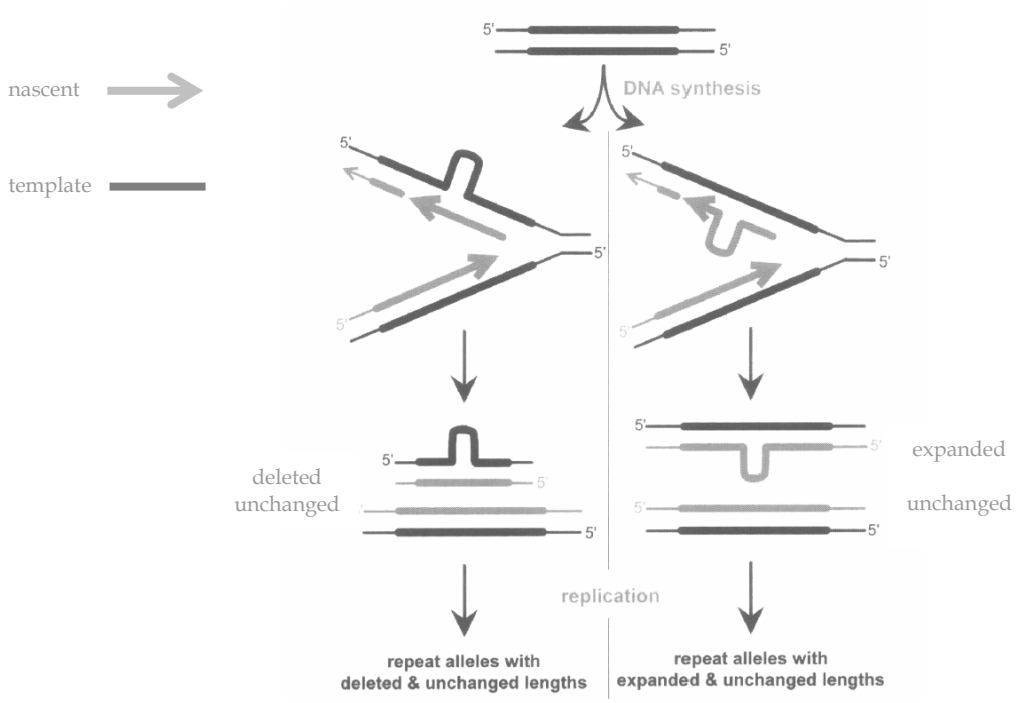


Figure 1.3. Occurrence of expansions and deletions at sites of DNA synthesis (Bowater and Wells, 2001)

1.4.1. Coding Repeat Disorders

Huntington's Disease (HD), Spinal and Bulbar Muscular Atrophy (SBMA), DRPLA and among SCAs, SCA1, 2, 3, 6, 7 and 17 are involved in this group of disorders. Because they share an exonic repeat sequence-CAG, and this repeat codes for glutamine, they are also called polyglutamine disorders. The size of the repeat expansions in this group is smaller than in noncoding repeat disorders. The intergenerational instability observed in polyglutamine disorders is more common during paternal transmission. The toxic gain-of-function mechanism is proposed as a model for the pathogenesis. Expanded polyglutamine tract confers new functions to the protein, which are toxic and lead to neuronal dysfunction and death. Although the genes resposible for this group of disorders are expressed in many other tissues outside of the brain as well, the pathology is restricted to certain subset of neurons (Cummings and Zoghbi, 2000; Lima and Pimentel, 2004).

1.4.2. Noncoding Repeat Disorders

Among SCAs, SCA8 and SCA12, and also Fragile X syndrome, Fragile XE syndrome, Friedreich Ataxia, and Myotonic Dystrophy type 1 (DM1) belong to this group of disorders. SCA10 is also a noncoding repeat disorder, but involves a pentanucleotide repeat (ATTCT). All of the disorders in this group arise from mutations in untranslated regions (UTR), 5' UTR, intronic region or 3' UTR of the gene. Although each disease may have unique pathological mechanisms, loss of function of the respective protein or gain of function of toxic triplet repeat transcript is proposed for this group of disorders. Noncoding repeat disorders differ from each other by the repeat sequence and the location of the repeat in the gene, which defines their unique pathological mechanism, but they have also several common features. Size and variation of the repeat expansions are greater in noncoding repeat disorders than in coding repeat disorders. The presence of intermediate-size expansions which result in a silent phenotype, but has the potential for being in the pathological range in successive generations, is also another common feature (Cummings and Zoghbi, 2000).

1. 5. Spinocerebellar Ataxias 8, 12 and 14

As it is shown in Figure 1.1., the genetic etiology of 62% of SCA patients throughout the world is accounted by CAG repeat expansions in SCA 1, 2, 3, 6 and 7 loci. Although the prevalence of SCA8, 10, 12, 14, 17, 27 and DRPLA is shown to be low throughout the world, screening of the populations for this group of disorders with known mutations is significant. The high prevelance of SCA12 in the Indian population and of SCA10 in the Mexican population display that rare SCA subtypes may not be as rare as expected in certain populations. Therefore, as a screening strategy, it is valuable to look for mutations responsible for this group of disorders after polyglutamine disorders.

1.6. Spinocerebellar Ataxia 8

SCA8 was first described by Koob *et al.* in 1999 in large seven-generation kindred called the MN-A family. SCA8 is the first SCA to be the result of a repeat expansion within a noncoding region in the responsible gene; and SCA8 is also the second disorder to

be caused by a CTG expansion in the 3' UTR, after the identification of Myotonic Dystrophy type 1, a multisystemic disorder.

1.6.1. Phenotype

Dysarthria, mild aspiration and gait instability are observed as initial symptoms among the affected members of the MN-A family. In addition, spastic and ataxic dysarthria, nystagmus, limb and gait ataxia, limb spasticity and diminished vibration perception are included in the clinical symptoms. SCA8 progresses slowly in comparison to other SCAs, but some severe cases are non-ambulatory at their fourties and fifties. Although age at onset varies between 18 and 65 years, the mean age at onset is 39 years (Koob *et al.*, 1999). No correlation between repeat numbers and age at onset and disease severity is apparent in this family. Magnetic resonance imaging shows atrophy of the cerebellar hemispheres and vermis (Day *et al.*, 2000). In general, the clinical features described in this family are consistent with other ataxia families (Mosemiller *et al.*, 2003). The effect of homozygote expanded alleles on the age at onset or phenotype varies according to different studies (Tazón *et al.*, 2002; Izumi *et al.*, 2003; Ikeda *et al.*, 2004)

1.6.2. Genetics

Although by repeat expansion detection (RED) analysis, a CAG repeat expansion is found in ataxia patients, further analysis displayed that the SCA8 gene is transcribed in the opposite direction, and the transcript contains combined repeats composed of CTA/CTG repeats. There are no significant open reading frames (ORF) that dictate translation of a polyglutamine tract. The mutation is located in the 3' terminal exon of a fully processed transcript and is mapped to chromosome 13q21.

CTA/CTG repeats are polymorphic in size, and CTA repeats preceeds CTG repeat tract. The combined repeat numbers observed among the ataxia patients in the MN-A family ranged between 110 and 130. On the other hand, molecular analyses of 1200 alleles of healthy individuals showed that the combined repeat numbers were between 16 and 91, but more than 99% of the alleles had 16-37 repeats (Koob *et al.,* 1999).

Although pathological and normal repeat number ranges were described in this large family and the control group, the strict borders of these ranges can not be determined. Screening of several different populations for SCA8 gives controversial results on pathological and normal repeat number ranges. The expanded SCA8 alleles described in ataxia patients with known genetic etiology, in patients with other disorders and healthy controls question the linkage between the expanded CTG tract and ataxia (Table 1.2.).

Table 1.2. Repeat numbers of expanded SCA8 alleles described in different patient groups and in healthy controls: literature survey

References	**Ataxia patients with unknown genetic etiology**	**Ataxia patients with known genetic etiology**	**Patients with other disorders**	**Healthy controls**
Ikeda *et al.,* 2000	89-155 (6 patients)	-	-	No expansion
Juvonen *et al.,* 2000	101- 345 (9 patients) 124- 800 (4 unaffected relatives)	-	-	86-675 (14 controls)
Silveria *et al.,* 2000	100-152 (11 patients) 128-170 (6 unaffected relatives)	-	-	>100 No expansion
Stevanin *et al.,* 2000	>91 (9 patients, 2 homozygous) (4 relatives)	No expansion (SCA1, 3, 7)	>91 (1 Lafora disease) (1 Family essential tremor)	107, 111, 123 (3 controls)
Worth *et al.,* 2000	141, 152, 208 (3 patients) 127, 127, 124 (3 unaffected relatives) 81, 95 (2 ILOCA)	-	-	100, 101, 103, 133, 174 (5 controls)
Celini *et al.,* 2001	90, 90, 110, 159, 320 (5 patients) 90, 105, 105 (3 unaffected relative)	No expansion (SCA1, 2, 3, FA)	75 (1 Bipolar disorder)	No expansion
Sobrido *et al.,* 2001	142, 155, 392 (3 patients)	97 (1 FA) 259 (1 SCA2)	91, 99 (2 AD)	-
Brusco *et al.,* 2002	89-97 (4 patients) 85, 94 (2 unaffected relative)	-	-	No expansion
Tazón *et al.,* 2002	111, 121, 121, 124 (3 patients, 1 homozygous) 116, 90 (2 unaffected relative)	No expansion (FA)	-	106 (1 control)
Izumi *et al.,* 2003	86-221 (15 patients, 4 homozygous)	82-144 (6 SCA6)	113, 90 (2 PD) 89 (1 AD)	~420, 450, 490 (3 contols)
Schöls *et al.,* 2003	106-200 (11 patients, 1 homozygous) 112, 260 (2 unaffected relative)	59 (1 FA) 71 (1 SCA1) 93, 113 (2 SCA6)	67, 82 (2 patients with dystonia) 93 (1 HD)	No expansion
Sulek *et al.,* 2003	79-150 (9 patients, 9 unaffected relatives)	53, 77, 113 (3 SCA1) No expansion (SCA2, 17)	91, 95, 100 (3 hyperlipidemia patients) No expansion (HD)	-

Table 1.2. Repeat numbers of expanded SCA8 alleles described in different patient groups and in healthy controls: literature survey (continued)

References	Ataxia patients with unknown genetic etiology	Ataxia patients with known genetic etiology	Patients with other disorders	Healthy controls
Sulek *et al.,* 2004	80-150 (5 patients)	-	-	91, 100 (2 controls)
Zeman *et al.,* 2004	>91 (6 patients)	-	>91 (2 suspected HD) No expansion (schizophrenia group)	>91 (3 controls)
Corral *et al.,* 2005	111/197, 158/101, 109/151, 110/145 (4 patients, homozygous) 86-126 (8 patients, heterozygous)	96 (1 FA)	-	No expansion

The debate is originated from the unique characteristics of SCA8 such as abnormal genetic instability, reduced penetrance, gender bias and variety in sequence configuration.

The genetic instability observed in SCA8 is higher, compared to other repeat disorders with autosomal dominant inheritance, but not as high as it is in DM 1(Koob *et al.*, 1999). Although the disease is transmitted both maternally and paternally, there is a maternal transmission bias among SCA8 families. In contrast to polyglutamine disorders, in which there is a paternal transmission bias through CAG repeat expansion, expansion of CTG repeats in maternal transmission and contraction of CTG repeats in paternal transmission is observed in SCA8. The maternal bias comes from the fact that the expansion of CTG repeats leads the allele to be in the pathogenic range with a higher penetrance rate. On the other hand, paternal contractions result in shorter CTG repeat tracts which are not in the pathogenic range (Moseley *et al.*, 2000a). Because both maternal and paternal SCA8 alleles are expressed in normal cerebellar tissue, imprinting is not a possible mechanism for maternal transmission bias (Day *et al.*, 2000).

Although the repeat size range among ataxia families described by Mosemiller *et al.* in 2003 is between 71 and 1000 CTA/CTG repeats; the disease penetrance has been mostly seen between 80 and 250 combined repeats. This is also supported by the high frequency of alleles with >91 combined repeats in ataxia patients than in the general population, and the high frequency of large alleles (600-800 combined repeats) in the general population than in ataxia patients (Moseley *et al.*, 2000a). The reduced penetrance observed above 250

CTG repeats may result from altered SCA8 gene expression, ribonucleic acid (RNA) processing and/or stability because of large repeats (Cummings and Zoghbi, 2000). However, the presence of unaffected relatives and healthy individuals with expanded repeats in the pathological range described by Koob *et al.* in 1999 in the MN-A family questions the validity of SCA8 expansion as a cause of cerebellar ataxia (Worth *et al.*, 2000; Stevanin *et al.*, 2000). Besides, the presence of intermediate and expanded alleles in ataxia patients with and without established genetic diseases or in patients with other neurodegenerative disorders supports the doubt about the disease-causing character of CTG expansions in SCA8 (Sobrido *et al.*, 2001; Schöls *et al.*, 2003). Somatic mosaicism, the presence of different CTG repeat numbers in peripheral blood and the affected tissues also present another perspective to incomplete penetrance (Silveria *et al.,* 2000; Ikeda *et al.*, 2000). In addition, the polymorphic CTA repeats which are in the range of 1-29 beyond the CTG repeat tract is suggested to cause difficulties in establishing a pathogenic range of allele sizes between ataxia families, since the SCA8 polymerase chain reaction (PCR) determines the overall length of the combined repeats (Day *et al.*, 2000).

Sequence configuration is another factor that may have a role in the penetrance of the disease. The triplet repeat interruptions observed in normal alleles of SCA1 gene, SCA2 gene, Fragile X syndrome gene (FMR1) and Friedreich Ataxia gene (FRDA) are known to stabilize the repeat tracts, but with the loss of these interruptions the repeat tracts predispose to expand (Moseley *et al.*, 2000a). According to the studies of Moseley *et al.* in 2000 and Tazón *et al.* in 2002, normal SCA8 alleles tend to have pure, uninterrupted repeat tracts while expanded SCA8 alleles are frequently observed to have interruptions. The origin of the interruptions is thought to be the point mutations which occur in CTG tracts. Duplications of the interruptions are also observed in the MN-A family. Both the variety of point mutations and duplication of interruptions may increase the number of distinct sequence configurations as observed among the MN-A family members (Moseley *et al.*, 2000a). However, triplet repeat interruptions are observed both in expanded and unexpanded CTG repeat tracts and also in polymorphic CTA repeat tracts in affected or unaffected individuals (Day *et al.*, 2000; Stevanin *et al.*, 2000; Sobrido *et al.*, 2001). So, the significance of triplet repeat interruptions in the penetrance of SCA8 is uncertain.

Finally, the linkage between CTG repeat expansion and ataxia can be discussed from two perspectives.

1. CTG repeat can cause ataxia, but other genetic modifiers such as repeat length and sequence configuration can affect the penetrance of the disease (Moseley *et al.*, 2000b).
2. The SCA8-CTG expansion is a non-pathogenic polymorphism linked to another ataxia locus (Stevanin *et al.*, 2000; Worth *et al.*, 2000).

The first perspective is based on five evidences: i) linkage data in the MN-A family with high lod score≅6.8; ii) the relationship between repeat length and ataxia; ataxia patients have longer CTG repeats (mean:117) than their unaffected relatives (mean:92); iii) the absence of pathogenic expanded SCA8 alleles in their control group; iv) high frequency of expanded SCA8 alleles among unrelated ataxia patients; v) the expression of SCA8 transcripts mainly in the central nervous system tissue (Moseley *et al.*, 2000a).

Recently described genetic differences between the MN-A family and small ataxia families are suggested to contribute to the increased penetrance of SCA8 in the MN-A family although the MN-A family and the small ataxia families share the same founder haplotype for the SCA8 expansion (Ikeda *et al.*, 2004).

Becuse SCA 8 has a complex inheritance pattern, and since the genetic etiology that gives rise to the disease is not clarified yet, diagnostic and predictive testing is not suggested for SCA8 (Worth *et al.*, 2000; Sobrido *et al.*, 2001; Schöls *et al.*, 2003; Ikeda *et al.*, 2004).

1.6.3. Pathogenesis

To understand the molecular pathology of SCA8, Nemes *et al.,* in 2000 defined the genomic organization of SCA8 transcripts. By defining splice donor sequences and splice acceptor sites, five exons (A, B, C1, C2, D or D`), spanning a genomic region over 32 kilo base (kb) can be determined on alternatively spliced SCA8 transcripts. In addition, RNA transcripts that lack the 3' terminal exon A containing CUG repeats are also observed. The lack of any ORF poses the question if SCA 8 transcript does not act as a messenger RNA

(mRNA). The pathogenecity of SCA1, 2, 3, 6, 7 and 17 is suggested to come from CAG repeat expansions that are translated into long polyglutamine tracts and exert toxicity on the corresponding proteins. In contrast, the SCA8 transcript is thought to act as an anti-sense RNA, since its 5' exon is trancribed through the first exon of another gene transcribed in the opposite site. The overlapping sequences include transcription, translation sites and the first splice donor sequence of the sense gene. The gene is composed of 11 exons and codes for a 748 amino acid protein which has a similar domain structure with an actin-binding Kelch protein from Drosophila (Figure 1.4.). Although the protein is not shown to bind actin and dimerize, its predicted domain structure resembles actin-binding proteins, so it is called as Kelch-like 1(KLHL1). Because subcellular localization of KLHL1 protein is shown to be cytoplasmic, it may have a role in organizing the actin cytoskeleton of the brain cells (Nemes, *et al.*, 2000).

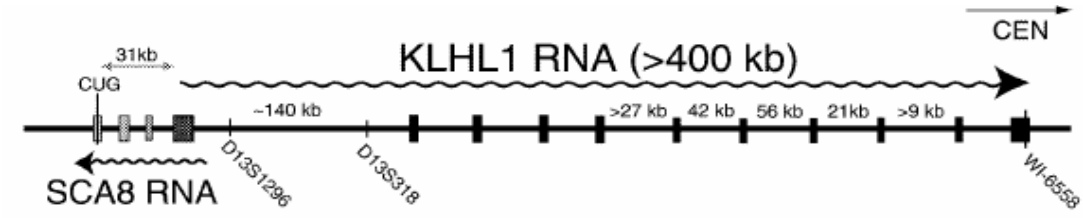


Figure 1.4. General genomic organization of the SCA8/KLHL1 region on chromosome 13 (Nemes *et al.*, 2000)

Northern and dot blot analyses show that human KLHL1 transcript is expressed primarily in cerebellum, substantia nigra, frontal lobe and medulla (Nemes *et al.*, 2000). The results of real time-polymerase chain reaction (RT-PCR) experiments displayed that the SCA8 transcript is expressed in brain tissue, whereas it is absent or only present at much lower levels in other tissues such as heart, placenta, liver, skeletal muscle, kidney and pancreas (Koob *et al.*, 1999). This increases the possibility of being an anti-sense RNA of KLHL1 transcript for the SCA8 transcript which is also expressed in cerebellum (Nemes *et al.*, 2000).

It is proposed that an anti-sense RNA can regulate gene expression in two ways in eukaryotes: by being expressed in competition with sense transcripts or by pairing with the homologous sense transcript and affecting post-transcriptional events such as splicing,

RNA transport, cytoplasmic stability and translation. Although it has not been shown if SCA8 transcript affects KLHL1 gene expression at post-transcriptional level, the genomic arrangement of SCA8 transcript in relation to KLHL1 supports its regulatory role on KLHL1 gene expression (Nemes *et al.*, 2000). In addition, to understand if SCA8 transcript is really an anti-sense RNA of KLHL1 and acts as regulator in KLHL1 gene expression, it is important to answer if both genes are co-expressed in any cells, if levels of KLHL1 are actually altered in SCA8 patients or if altered levels of KLHL1 protein could lead to cerebellar degeneration and ataxia (Mosemiller *et al.*, 2003). The possible pathological effects of the CUG expansion in the SCA8 transcript may be the accumulation of SCA8 transcripts and altered KLHL1 expression in a negative way through RNA-RNA interaction or prevention of SCA8 transcript from negatively regulating the expression of KLHL1 and the over-expression of KLHL1 protein which could be toxic for the cell (Nemes, *et al.*, 2000).

The pathogenesis of triplet repeat disorders can be explained by three different mechanisms (Jasinski *et al.*, 2003; Ranum and Day, 2004). Looking at SCA8 pathogenesis in the frame of these mechanisms would be helpful because of possible common pathological pathways:

- Loss-of-function mechanism
- Gain-of-function mechanism
- Gain-of-function mechanism at RNA level

The last mechanism is first described for an autosomal dominant disorder, myotonic dystrophy type 1 which is caused by an abnormal expansion of CTG repeats in the 3' UTR of myotonin protein kinase gene, DMPK. Three mechanisms are suggested in explaining the pathogenesis of DM1: i) Haploinsufficiency of the DMPK protein; ii) Local chromatin effects on the expression of neighbouring genes, including SIX5; iii) Pathogenic effects of the CUG expansion in RNA which acccumulates as nuclear foci and disrupts cellular function.

However, which mechanism contributes to the disease is not clear, thus all of them may explain the pathogenesis of the multisystemic disorder DM1. (Cummings and Zoghbi, 2000; Mosemiller *et al.*, 2003)

The similarities between the location and sequence of mutations in DM1 and SCA8 question if the pathological mechanisms of SCA8 and DM1 are common. It is proposed for DM1 that the repeat expansions in RNA transcripts alter the binding affinities and regulation of proteins that recognize CUG sequences. The alterations in one of these proteins, CUG-binding protein (CUG-BP) result in the abnormal regulation of their target transcripts (Mosemiller *et al.,* 2003). The expanded RNA transcripts are shown to accumulate as nuclear foci and co-localize with proteins of the muscleblind family as well, and the cellular consequences of the sequesteration of this group of proteins by the expanded CUG repeat hairpins is not known yet (Jasinska *et al.,* 2003). Although a third category of pathogenic mechanism, gain-of-function RNA mechanism is suggested for both diseases, the nature of these disorders- multisystemic (DM1) and neurodegenerative (SCA8)-differs. This difference is explained by the different expression patterns of DMPK and SCA8 genes. While DMPK is broadly expressed, the expression of SCA8 gene is confined to brain (Mosemiler *et al.*, 2003).

To understand the pathogenic mechanism of SCA8, the non-coding SCA8 transcript with the normal and expanded repeat tract is expressed in Drosophila retina. Both transcripts induce neurodegeneration in Drosophila eye. The neurodegenerative eye phenotype that results from overexpression of the expanded SCA8 allele is used as a sensitized genetic background in order to identify possible genetic modifiers. While mutations in *staufen*, *muscleblind*, *split ends* are found to enhance the SCA8 phenotype, the mutation described in *CG3249* which codes for a putative PKA anchor protein (PKAAP) suppresses the neurodegenerative phenotype. All of these proteins are expressed in neurons, have RNA binding abilities and, are conserved both in Drosophila and humans. None of the mutations in respective genes can exhibit a dominant phenotype on their own. Although the expression of both transcripts (with normal and expanded repeats) results in a similar phenotype, the mutations in certain genes affect the neurodegeneration in a different way in two genetic backgrounds which point to the impact of the CUG expansion. Finally, the interaction site of Staufen with the CUG-containing transcript is observed as

being the expansion part of the transcript, and this gives new clues on the pathogenesis of SCA8 (Mutsuddi *et al.*, 2004).

1.7. Spinocerebellar Ataxia 12

SCA12 is first described by Holmes, *et al.* in 1999 in a large pedigree of German descent by using RED analysis. SCA 12 is the first SCA described which arises from a repeat expansion in the 5' UTR of a gene.

1.7.1. Phenotype

The most striking clinical feature of SCA12 is action tremor of head or arms. Although the resemblance of this symptom with essential or intention tremor results in confusion, the characterization of the disease is clarified with the development of signs of mild cerebellar dysfunction that include gait ataxia, dysmetria of limb and eye movements, dysdidochokinesia, and dysarthria during the disease progression. Hyperreflexia, subtle parkinsonian features, and late-onset dementia are other distinguishing clinical signs of SCA12. The atrophy in the cerebellum and cerebral cortex with relative preservation of subcortical gray matter structures and the brainstem is observed by brain computerized tomography (CT) and MRI. Disease progression is slow compared to the other SCA subtypes. Age at onset ranges between 8 and 55 years with a mean of mid thirties in the large German descent family. The clinical symptoms defined in this German descent family are consistent with other SCA12 families (O'Hearn *et al.*, 2001; Holmes *et al.*, 2003).

1.7.2. Genetics

SCA12 is caused by CAG repeat tract which lies 133 nucleotides upstream of the 5' end of a complementary deoxyribonucleic acid (cDNA) of the gene PPP2R2B and maps to 5q31-33. The PPP2R2B gene encodes a brain-specific regulatory subunit of the protein phosphatase PP2A. Several evidence suggest that the CAG repeat tract lies in the 5' UTR of this gene that contains multiple transcription start sites (Figure 1.5.). This raises the possibility of being in an unidentified open reading frame of PPP2R2B gene or in open

reading frames of neighboring genes for CAG repeat tract. Western blots of proteins taken from lymphoblastoid cell lines of patients are stained with 1C2 antibody that detects long polyglutamine stretches, but no polyglutamine expansions are detected. In addition, Northern blots containing brain mRNA and mRNA from neuroblastoma cell line LA-N-1 give no signal for CAG repeat expansion. These data suggest that SCA12 pathogenesis does not result from the toxic effect of polyglutamine expansions as it is in SCA1, 2, 3, 6, 7 and 17. An alternative pathological mechanism is the altered gene expression as it is in Fragile X syndrome caused by CGG repeat expansion in the 5' UTR of FMR1. Because CAG repeat is within the putative promoter of the PPP2R2B gene, the repeat expansion may affect the gene expression. Preliminary data indicate that the mutation increases the PPP2R2B gene expression, which in turn, results in abnormal PP2A dephosphorylation activity (Holmes *et al.*, 2001).

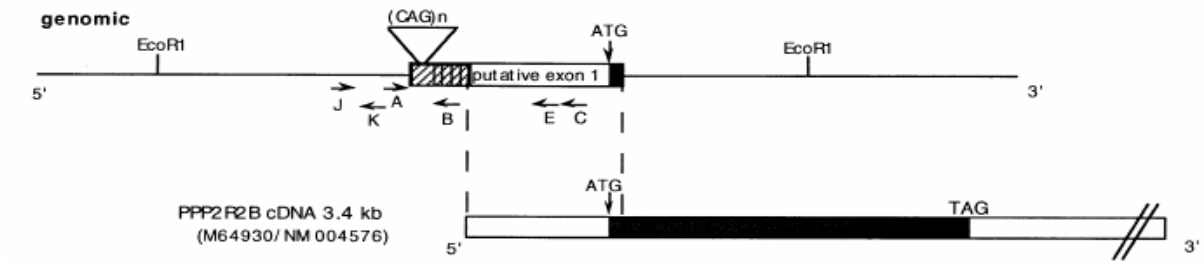


Figure 1.5. Location of the CAG repeat in the 5'UTR of the PPP2R2B gene (Holmes *et al.*, 2001)

According to population studies, SCA12 is defined only in one German, one Singaporean and 20 Indian families thus far (Cholfin *et al.*, 2001; Fujigasaki *et al.*, 2001; Srivastava *et al.*, 2001; Brusco *et al.*, 2002; Zhao *et al.*, 2002; Holmes *et al.*, 2003; Sulek *et al.*, 2004; Bahl *et al.*, 2005). While the normal alleles range between 7 and 32 repeats, the expanded alleles contain 51 to 78 repeats (Holmes *et al.*, 2003; Bahl *et al.*, 2005). However, the pathological and normal repeat ranges are yet to be determined. 49 triplets were found in an individual with SCA-12 like phenotype and Creutzfeld-Jakob disease (Hellenbroich *et al.*, 2003). The intermediate repeat lengths such as 40 and 41 triplets were found in two patients with idiopathic late onset cerebellar ataxia (ILOCA). In addition, 45 CAG repeat expansions were seen in a 28 year-old Indian individual with no family history of neurological disorder. Finally, an Iranian woman with unipolar depression and her two

monozygotic twin sons with schizophrenia were found to carry alleles of 53 triplets (Holmes *et al.*, 2003).

The vertical transmission of CAG repeats is modestly unstable in SCA12. In the German pedigree, while four maternal transmissions show slight contractions, one paternal transmission is stable. Three and five repeats expansions through paternal transmissions and stable maternal transmissions are observed in a large Indian pedigree. In five smaller Indian pedigrees, two of the three paternal transmissions show slight contractions (56 to 55 repeats and 67 to 60 repeats) and the other shows a slight expansion (67 to 69 repeats) (Srivastava *et al.*, 2001). No apparent correlation between repeat number and age at onset is observed. This can be explained by the small size of patients diagnosed as SCA12 until now, and the difficulty in determining the age at onset of this disorder (Holmes *et al.*, 2003).

Although most of the population screenings for SCA12 give negative results and SCA12 is described as a rare disease (Cholfin *et al.*, 2001; Brusco *et al.*, 2002; Sulek *et al.*, 2004), this is not the case for the Indian population. ~16% of 124 families with dominant cerebellar disease have the SCA12 mutation in Northern India (Bahl *et al.*, 2005). It is consistent with the observation that the greater proportion of normal alleles in the Indian population has more than 12 CAG repeats compared to the normal alleles of other populations. Because the larger alleles have more possibility to expand into the pathological range, the disease frequency can be correlated with the frequency of larger alleles in the normal range (Fujigasaki *et al.*, 2001).

1.7.3. Pathogenesis

PP2A, which is a serine/threonine phosphatase, has a role in several cellular events such as cell growth and differentiation, DNA replication, cellular morphogenesis and cytokinesis, regulation of kinase cascades, ion channel function, neurotransmitter release, microtubule assembly and apoptosis (Holmes *et al.,* 2001).

PP2A, which is a conserved protein, expressed in both plant and animal cells. It is composed of three subunits; structural (A), catalytic (C) and regulatory (B) subunits. While

the A and C subunits have two isoforms, there are at least 13 isoforms of the B subunit, encoded by distinct genes. The B subunit is recruited by the A subunit which forms a complex with the C subunit. Which isoform of the B subunit is going to be the regulatory unit differs according to tissue and cell types (Figure 1.6.) (Holmes *et al.,* 2001).

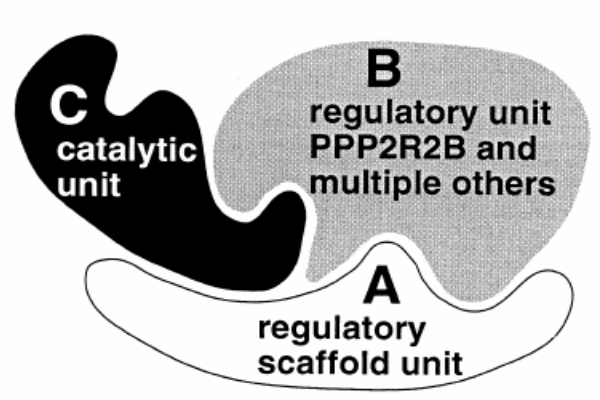


Figure 1.6. Schematic diagram of the trimeric holoenzyme protein phosphatase (PP2A) (Holmes *et al.*, 2001)

The B subunits affect the PP2A function through substrate specificity, tissue- and cell type-specificity and intracellular targeting. PPP2R2B is a brain-specific B subunit and is constitutively expressed in Purkinje cells of the cerebellar cortex. It is observed that the PPP2R2 class of regulatory subunits in which PPP2R2B takes place, regulates the dephosphorylation activity of the PP2A on specific substrates such as vimentin, histone-1 and tau. In addition, subcellular localization of the PP2A enzyme, containing the PPP2R2B regulatory subunit, is cytoplasmic and concentrates in neuronal cell bodies and dendrites (Holmes *et al.*, 2003).

Although altered gene expression of the PPP2R2B caused by the CAG repeat expansion is thought to be the most possible mechanism, other pathogenic mechanisms are also taken into account: i) the CAG repeat containing region may act as a splicing enhancer or inhibitor, so the repeat expansion may change the splicing pattern of the exons; ii) the CAG repeat expansion may exert a toxic effect at the RNA level as it is proposed for DM1; iii) the CAG repeat expansion may inhibit the transcription of the PPP2R2B gene as it is proposed for FRDA; iv) if PPP2R2B interacts with proteins other than the PP2A subunits,

then increased expression of PPP2R2B caused by CAG repeat expansion may cause toxic effects unrelated to PP2A in the cell (Holmes *et al.*, 2003).

1.8. Spinocerebellar Ataxia 14

SCA14 is the first SCA described that differs from other SCA subtypes in its mutational mechanism. Until now, tri- or pentanucleotide repeat expansions are found to cause SCAs with identified genes. Missense mutations and a deletion are observed to result in a nonepisodic SCA type, SCA14. Recent studies identified a missense and a frameshift mutation in Fibroblast Growth Factor 14 (FGF14) gene which leads to autosomal dominant cerebellar ataxia designated as SCA27 (Swieten *et al.*, 2003; Dalski *et al.*, 2005; Brusse *et al.*, 2006).

1.8.1. Phenotype

The phenotype of SCA14 is described as a slowly progressive cerebellar ataxia with varying range of age at onset. Although most of the SCA14 families show pure cerebellar ataxia (Yamashita *et al.*, 2000; Brkanac *et al.*, 2002; Alonso *et al.*, 2005; Verbeek *et al.*, 2005), some additional features such as dysarthria, nystagmus (Chen *et al.*, 2005), axial myoclonus (Yamashita e*t al.*, 2000), cognitive impairment (Stevanin *et al.*, 2004), focal dystonia (van de Warrenburg *et al.*, 2003), executive dysfunction, myorythmia, tremor or decreased vibration sense (Klebe *et al.*, 2005) are also observed. Life expectancy is not affected by SCA14. MRI studies display atrophy of the cerebellum (Yabe *et al.*, 2003; Verbeek *et al.*, 2004; Chen *et al.*, 2005; Alonso *et al.*, 2005).

1.8.2 Genetics

SCA14 is mapped to a region on chromosome 19q13.4-qter both in a Japanese and an American family of English and Dutch etnicity by linkage analysis (Yamashita *et al.*, 2000; Brakanac *et al.*, 2002). By screening of the PRKCG which is a strong candidate gene in the interval region, a missense mutation is found in the latter family (Chen *et al.*, 2003).

The PRKCG encodes the protein kinase C gamma (PKCγ) which is a serine/threonine kinase. PKCγ is a member of the protein kinase C (PKC) family and is involved in signal transduction, cell proliferation and differentiation, synaptic transmission and tumour promotion. PKCγ contains two main domains: The N-terminal regulatory domain and the C-terminal catalytic domain. The regulatory domain involves two regions: C1 and C2. While two cysteine-rich regions, Cys1 and Cys2 which are called C1, interact with two zinc ions separately and enable high-affinity diacylglycerol (DAG)/phorbol ester binding, C2 is a Ca^{2+} sensitive region. On the other hand, the catalytic domain comprises kinase and substrate-binding regions (Figure 1.7.).

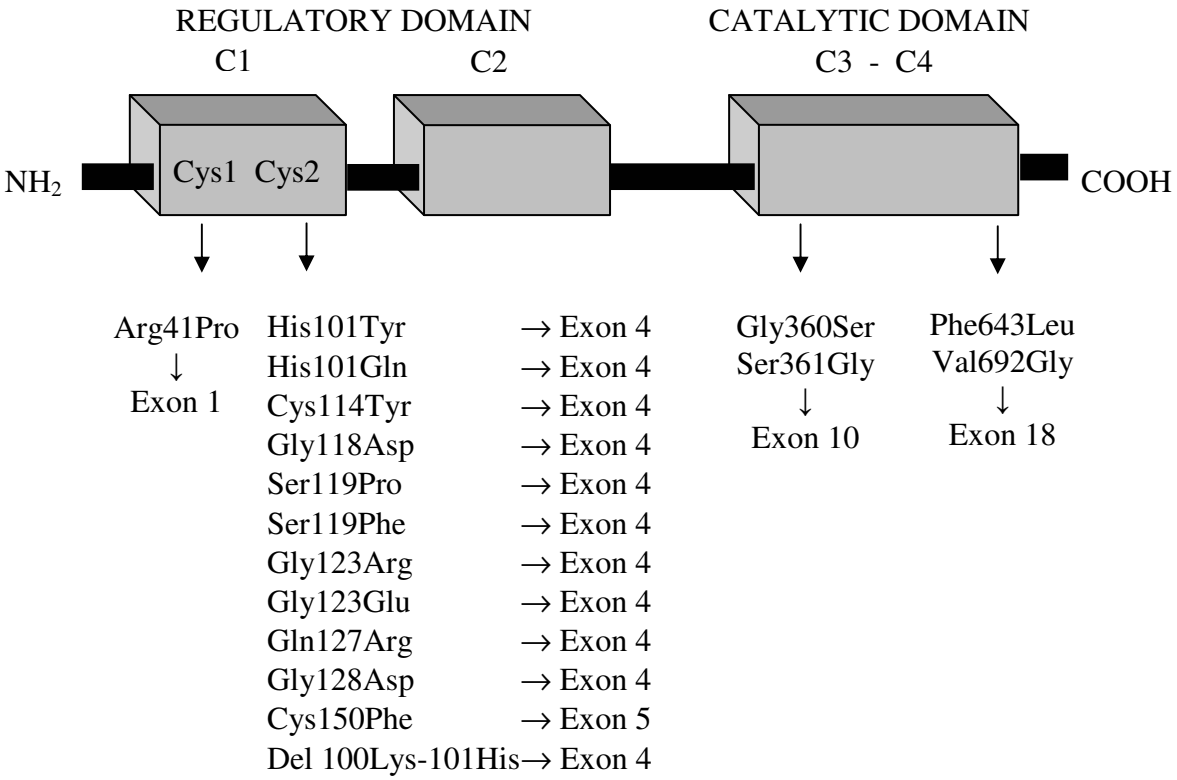


Figure 1.7. Primary structure of conventional PKC isozymes showing the regulatory and catalytic domains (adapted from Alonso et al., 2005)

Twelve missense mutations, one deletion in the regulatory domain and four missense mutations in the catalytic domain are defined in SCA14 thus far (Figure 1.7.). The high frequency of mutations in exon 4 suggests a screening strategy for SCA14. Although it is an efficient way to screen exon 4 in the first hand, all of the other exons should also be screened to put the sample apart as SCA14-negative.

The possible effects of the defined mutations are not clear yet, but the variety of the mutations results in a large spectrum of phenotypes. While in some SCA14 families anticipation is not observed and early-onset is not related to the more severe progression of the disease in the next generations (Klebe *et al.*, 2005), in the Portuguese SCA14 families, the clinical expression is more severe in the younger generation (Alonso *et al.*, 2005). Although the mutations co-segragate with the disease in most of the SCA14 family members, Chen *et al.* in 2005 described two families in which mutation carriers had no symptoms over the age of 60. This raises the possibility of reduced penetrance for SCA14.

Chen *et al.* in 2005 described a C to T change in the last nucleotide of exon 3 which does not result in an amino acid substitution. However, since it is a part of the consensus splice donor site, the nucleotide change is suggested to affect the efficiency of splicing. Although this nucleotide change is not found in their control chromosomes, Klebe *et al.* in 2005 detected the splice site mutation both in two ADCA families from Algeria and in 18 chromosomes from 70 North African controls. Therefore, the nucleotide change is suggested to be a polymorphism instead of a splice site mutation.

According to the population studies, SCA14 families in Netherlands account for approximately 4% of the total ADCA families (Verbeek *et al.*, 2005). In France, PKCγ mutations are found in seven families which represent 1.5% of all ADCA families. This number shows that SCA14 is a rare disorder as SCA6 is in France, but not as rare as SCA17 with 1% frequency. SCA14 is not represented in German ADCA families (Klebe *et al.*, 2005).

1.8.3. Pathogenesis

PKCγ is mostly expressed in brain and spinal cord, especially in the Purkinje cells of the cerebellum (Yabe *et al.*, 2003). While PKCγ-knockout mice do not develop progressive cerebellar atrophy, the dendritic tree of their Purkinje cells is abnormal and show excessive branches (Pandolfo *et al.*, 2005). Therefore, PKCγ is thought to play a role in dendritic development and may act as a negative regulator in dendtritic growth and branching (Yabe *et al.*, 2003).

Verbeek *et al.* in 2004 studied the effects of two PKCγ mutations, Glycine118Aspartic acid and Cysteine150Phenylalenine, on the kinase function and subcellular distribution of PKCγ. The results displayed that the kinase activity of mutant PKCγ increases compared to wild-type PKCγ although the mutations reside in the regulatory domain. The highly phosphorylated substrates of the mutant PKCγ in Purkinje cells of affected individuals may give a clue on the pathogenesis of SCA14. In addition, while the wild-type PKCγ was distributed uniformly along the plasma membrane in response to the treatment of Ca^{2+}, the mutant ones were enriched in discrete membrane domains.

Alonso *et al.* in 2005 investigated the influence of Histidine101Glutamine mutation found in a large Portuguese family on the expression of PKCγ and compared the expression levels in HEK293 cells transfected with both mutant and normal PKCγ. The data showed that there is no significant difference between the mRNA levels of wild-type and mutant PRKCG. However, the expression level of the mutant protein decreased which may be due to the degradation of the mutant protein or sequestration of the mutant proteins in cytoplasmic aggregates that result in neuronal death as it is in polyglutamine disorders. They concluded that SCA14 pathogenesis may be related to changes in the regulation of PKCγ which result in different phosphorylation patterns of its substrates.

The studies of Seki *et al.* in 2005 support the aggregation of mutant PKCγ in the cytoplasm. The insolubility of mutant PKCγ to the detergent Triton X-100 is higher and is correlated with the extent of aggregation in the cytoplasm. Both the high expression level and the mutant form of PKCγ induce cell death. Finally, the study points to the difference in susceptibility to aggregation and solubility to Triton X-100 of mutant PKCγs found in several SCA14 families. It is shown that the average age at onset of five families is negatively correlated with the insolubility of the corresponding PKCγ mutants which suggests a strong relation between insoluble PKCγ formation by missense mutations and the pathogenesis of SCA14.

An interesting point is also the reduction in PKCγ level in Purkinje cells of SCA1 transgenic mice and the lack of staining of ataxin-1 in the cerebellum of a patient with a

mutation in the PRKCG. The data increase the possibility of a common pathogenetic pathway for the neurodegeneration of SCAs (Chen *et al.*, 2003).

Most of the studies performed thus far, support the toxic gain of function, instead of loss of function mechanism to explain the pathogenesis of SCA14. Compared to the neurological phenotype of PKCγ knockout mice, a patient with a heterozygous mutation has more severe phenotype. This indicates that the mutant protein probably exerts toxic effects in the cells which is worser than the absence of the protein. The mutant protein may compete with the wild-type protein for attachment sites on the membrane or substrate-binding (Chen *et al.*, 2003). Increasing kinase activity of mutant PKCγ and the possible toxic effects of cytoplasmic aggregate formation also support the presence of toxic gain of function mechanism (Verbeek *et al.*, 2004; Alonso *et al.*, 2005; Seki *et al.*, 2005). This is consistent with the pathological mechanisms that are proposed for other polyglutamine disorders.

2. PURPOSE

Since the neurological phenotype of SCA subtypes are similar and overlapping, diagnosis based on clinical examination is not definitive. In this respect, genetic analysis gains importance in differential diagnosis. Population studies throughout the world indicate that the relative frequencies of SCA subtypes differ according to ethnic background. The frequencies of SCA1, 2, 3, 6, 7 and 17 in Turkey have been described elsewhere (Ersoy, 2005). Investigation of the Turkish SCA population to define the frequencies of SCA8, SCA12 and SCA14 is a necessary step to complement the future screening strategies. However, it is important to note that the analysis of SCA8 does not have a diagnostic aim because of its complex and unclear molecular basis. The framework and aims of this study can be described by the following points:

- Determining the repeat numbers at SCA8 loci in genetically undefined SCA patients and in healthy controls
- Screening the SCA8 loci of AD, PD, HD, FA, SCA1 and SCA2 patients
- Determining the SCA12 normal repeat range in healthy controls and screening genetically undefined SCA patients for SCA12
- Screening genetically undefined SCA patients for exon 4 of the PRKCG

3. MATERIALS

3.1. Blood and DNA Samples

3.1.1. Blood Samples of Spinocerebellar Ataxia Patients

Blood samples of spinocerebellar ataxia patients were provided by the Neurology Departments of the following centers: 22 samples from Cerrahpaşa Medical School, İstanbul University, İstanbul; 22 samples from İstanbul Medical School, İstanbul University, İstanbul; 18 samples from Marmara University, İstanbul; 14 samples from Çukurova University, Adana; 13 samples from Uludağ University, Bursa; nine samples from Bakırköy Mental Hospital, İstanbul; nine samples from Hacettepe University, Ankara; seven samples from İnönü University, Malatya; seven samples from Dokuz Eylül University, İzmir; five samples from Haydarpaşa Hospital, İstanbul; four samples from Ankara University, Ankara; four samples from Akdeniz University, Antalya; four samples from Haseki Education and Research Hospital, İstanbul; three samples from 19 Mayıs University, Samsun; two samples from Ege University, İzmir; two samples from Şişli Etfal Hospital, İstanbul; two samples from Celal Bayar University, Manisa; one sample from Buca Social Security State Hospital, İzmir; one sample from Süleyman Demirel University, Isparta; one sample from Maltepe University, İstanbul; one sample from Gazi University, Ankara; one sample from Mersin University, Mersin. SCA patients were previously screened for SCA1, 2, 3, 6, 7 and 17.

3.1.2. Blood and DNA Samples of Parkinson's Disease, Alzheimer's Disease, Huntington's Disease and Friedreich Ataxia Patients

Blood samples of Parkinson's Disease (PD) patients were provided by the Neurology Departments of the following centers: 36 samples from İstanbul Medical School, İstanbul University, İstanbul; 19 samples from American Hospital, İstanbul; one sample from Haseki Education and Research Hospital, İstanbul.

DNAs of Alzheimer's Disease (AD) patients were extracted from cheek epithelial cells that were collected in the framework of Turkish Alzheimer Prevalence Study.

Blood samples of Huntington's Disease (HD) patients were provided by the Neurology Departments of the following centers: seven samples from İstanbul Medical School, İstanbul University, İstanbul; seven samples from Ege University, İzmir; four samples from 19 Mayıs University, Samsun; three samples from Akdeniz University, Antalya; three samples from Dokuz Eylül University, İzmir; three samples Hacettepe University, Ankara; three samples from Gazi University, Ankara; three samples from Adnan Menderes University, Aydın; two samples from Celal Bayar University, Manisa; one sample from Cerrahpaşa Medical School, İstanbul University, İstanbul; one sample from Bakırköy Mental Hospital, İstanbul; one sample from Tepecik Social Security State Hospital, İzmir; one sample from Atatürk University, Erzurum; one sample from Kocaeli University, Kocaeli; one sample from Şifa Medical Center, İzmir; one sample from Adana Mental Hospital, Adana; one sample from Çukurova University, Adana; one sample from Karadeniz Technical University, Trabzon; one sample from Buca Social Security State Hospital, İzmir; one sample from Marmara University, İstanbul; one sample from İnönü University, Malatya; one sample from Şişli Etfal Hospital, İstanbul.

Blood samples of Friedreich Ataxia (FA) patients were provided by the Neurology Departments of the following centers: six samples from Çukurova University, Adana, two sample from Gazi University, Ankara; two samples from 19 Mayıs University, Samsun; two samples from Şişli Etfal Hospital, İstanbul; one sample from İstanbul Medical School, İstanbul University, İstanbul; one sample from Haydarpaşa Hospital, İstanbul; one sample from Kocaeli State Hospital, Kocaeli; one sample from Ege University, İzmir; one sample from Celal Bayar University, Manisa; one sample from Marmara University, İstanbul; one sample from Cerrahpaşa Medical School, İstanbul University, İstanbul.

3.1.3. DNA Samples of Healthy Controls

Conrol DNAs were extracted from cheek epithelial cells that were collected in the framework of Turkish Alzheimer Prevalence Study. The healthy controls used in this study were over 70 years old.

3.2. Oligonucleotide Primers

3.2.1. Primers for PCR Amplification and DNA Sequencing

Primers designed first by Koob *et al.* in 1999 and Holmes *et al.* in 1999 for the amplification of triplet repeat expansions located within SCA8 and SCA12 loci respectively, were used in this study. The primers for SCA8 and SCA12 were also labeled with fluorescent dyes 6-FAM and HEX for further GeneScan analysis. Primers for the amplification of exon 4 of the PRKCG that is responsible for SCA14 designed first by Chen et al. in 2003 and were used in this study. They were purchased from Iontek Ltd., İstanbul. The primer sequences used are given in Table 3.1.

Table 3.1. Oligonucleotide primer pairs used in PCR and DNA Sequencing

Primer Name	Primer Sequence
SCA8F4 SCA8F4-FAM	Forward : GTAAGAGATAAGCAGTATGAGGAAGTATG
SCA8R4	Reverse : GGTCCTTCATGTTAGAAAACCTGGCT
SCA12HEX-A SCA12HEX-A-HEX	Forward : TGCTGGGAAAGAGTCGTG
SCA12HEX-B	Reverse : GCCAGCGCACTCACCCTC
SCA14exon4F	Forward : CAAGGCAGGAGGAAAAGATA
SCA14exon4R	Reverse : ATTTCCCGGAACCCAGAC

3.3. Enzymes

Taq DNA Polymerase : 5u/µl, Promega, USA

ExTaq : 5u/µl, TaKaRa, Japan

3.4. PCR Purification Kit

PCR products were purified from excess primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge in order to get good quality results in DNA sequencing. QIAquick PCR Purification Kit was purchased from Qiagen, Germany.

3.5. Gel Extraction Kit

QIAquick Gel Extraction Kit which was used to extract and purify PCR products from two per cent agarose gels in TBE buffer, was purchased from Qiagen, Germany.

3.6. Buffers and Solutions

All chemicals and solutions were purchased from Merck (Germany), Sigma (USA and Germany), AppliChem (Germany), if it is not otherwise stated in the text.

3.6.1. DNA Extraction

Cell Lysis Buffer	:	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM Na_2EDTA (pH 7.4)
Nuclei Lysis Buffer	:	400mM NaCl 10 mM Tris-HCl (pH 8.0) 2 mM Na_2EDTA (pH 7.4)
Sodiumdodecylsulphate (SDS)	:	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	:	20 mg/ml in dH_2O, Promega, USA
Sodium Chloride (NaCl)	:	5 Molar (M) saturated stock solution

Ethanol (EtOH)	:	Absolute EtOH, Riedel-de Häen, Germany
TE Buffer	:	20 mM Tris-HCl (pH 8.0) 1 mM Na_2EDTA (pH 8.0)

3.6.2. Polymerase Chain Reaction

10X Buffer (Mg^{2+} free)	:	100 mM Tris-HCl 500 mM KCl 1 per cent Triton X-100 Promega, USA
10X Ex Taq Buffer (Mg^{2+} free)	:	TaKaRa, Japan
Magnesium Chloride ($MgCl_2$)	:	25 mM Promega, USA
2′-Deoxynucleoside 5′-triphosphate (dNTP)	:	100 mM of each dNTP Promega, USA
Dimethylsulphoxide (DMSO)	:	Sigma, Germany
5X Q-Solution	:	Qiagen, Germany

3.6.3. Agarose Gel Electrophoresis

10X TBE Buffer	:	0.89 M Tris-Base 0.89 M Boric acid 20 mM Na_2EDTA (pH 8.3)
Ethidium Bromide (EtBr)	:	10 mg/ml, Sigma, Germany

1 or 2 per cent Agarose Gel	:	1-2 per cent agarose (w/v) in 0.5X TBE Buffer, containing 0.5 µg/ml Ethidium Bromide
10X Loading Dye	:	2.5 mg/ml Bromophenol Blue (BPB) 1 per cent SDS in glycerol
DNA Ladder	:	100 base pair (bp), MBI Fermentas, Lithuania

3.7. Equipment

Autoclave	:	Model MAC-601, Eyela, Japan
Balance	:	GM 512-OCE, Sartorius, Germany
Centrifuges	:	Centrifuge 5415C, Eppendorf, Germany Universal 16R, Hettich, Germany
Deep Freezers (-20^{o}C)	:	Arçelik 2021D, Turkey
Documentation System	:	GelDoc Documentation System, BIO-RAD, USA
Electrophoretic Equipments	:	Horizon 58, Model 200, BRL, USA Thermo Minicell Primo E320 Electrophoretic Gel System, USA
Magnetic Stirrer	:	Chiltern Hotplate Magnetic Stirrer, HS31, UK

Ovens	:	EN 400, Nuve, Turkey 56^{o}C, LEEK, UK
Power supplies	:	EC 1000-90 Thermo, USA EC 250-90 Thermo, USA Model 200, BRL, USA
Refrigerator	:	4^{o}C Medicool, Sanyo, Japan Arçelik 4250T, Turkey
Spectrophotometer	:	CE 5502 Scanning Double Beam 5000 Series CECIL Elegant Technology, UK
Thermocyclers	:	Techne Progene, UK Techne Touchgene Gradient Progene, UK
Vortex	:	Fisons WhirliMixer, UK
Water Bath	:	Memmert, Germany
Water Purification	:	WaTech Water Technologies, Turkey

4. METHODS

4.1. DNA Extraction from White Blood Cells with NaCl Method

10 ml blood samples taken from the patients were collected into EDTA(K_3) containing tubes not to allow coagulation and stored at 4°C until DNA extraction. The samples were transferred to sterile Falcon tubes and 30 ml cold lysis buffer was added. After shaking the samples very well, they were kept at 4°C for 15 minutes in order to lyse the white blood cells. After calibrating for weight, the samples were centrifuged at 5000 revolutions per minute (rpm) for 10 minutes at 4°C to collect the nuclei. The supernatant was discarded carefully. The nuclear pellet was washed by adding 10 ml lysis buffer and resuspended by vortexing. The samples were again centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was discarded carefully. If the pellet was still dirty, then nuclear pellet wash and centrifugation steps were repeated. The nuclear pellet was resuspended in 3 ml nuclei lysis buffer by vortexing to lyse the nuclear envelope of white blood cells. After the addition of 400 µl dH_2O, 50 µl SDS (10%), 30 µl proteinase K (20 mg/ml), the samples were incubated at 37°C overnight, or at 56°C for three hours, for the degradation of cellular proteins. The samples were shaken well after the addition of 5 ml dH_2O and 5 ml of 5 M NaCl. This step is required to salt out the proteins. The samples were centrifuged at 5000 rpm for 30 minutes at room temperature. The supernatant was transferred into a sterile Falcon tube and DNA was precipitated by adding two volumes of cold absolute EtOH to the supernatant. The tube was inverted slowly several times. The precipitated DNA was fished out by the tip of a micropipette and transferred into an Eppendorf tube. After waiting for the ethanol to dry, DNA was dissolved in TE buffer; the amount of which depended on the amount of DNA, and it was stored at 4°C (Miller *et al.,* 1988). If there was no visible precipitated DNA after inverting the tube several times, the sample was stored at 70°C for two hours. The sample was centrifuged at 14000 rpm for 30 minutes at 18°C. The supernatant was discarded carefully taking care of the pellet. After waiting for the ethanol to dry, the DNA pellet was washed with 100 µl TE buffer.

4.2. Detection of DNA by Agarose Gel Electrophoresis

To test DNA quality and quantity, DNA samples were run on a 1 per cent agarose gel. 0.3 g agarose was dissolved in 30 ml 0.5X TBE buffer by boiling. The solution was allowed to cool to 55 ^{o}C. After addition of 1.6 µl EtBr (10 mg/ml), to visualize DNA under ultraviolet (UV) light, the gel was poured into a gel plate with one or two combs inserted in it, and allowed to solidify. Then the gel was placed in the electrophoresis tank filled with 0.5X TBE buffer. The combs were taken out from the gel, and the DNA sample which was mixed with 10X loading dye to a final concentration of 1X, was loaded into the slots by using a micropipette. The gel was run, approximately 10 minutes at 150V.

4.3. Spectrophotometric Measurement of DNA

The exact concentration of DNA was determined by spectrophotometric measurement. A 1:100 dilution of the stock DNA (with dH_2O) was prepared and dH_2O was used as blank. The optical density (OD) of DNA was read at 260 nm using a quartz cuvette. Since 50 µg/ml double stranded DNA has an OD of 1.0 at 260 nm, the following formula was used to calculate the concentration of the stock DNA:

Concentration (µg/ml): OD_{260} x 50 µg/ml x Dilution Factor

4.4. Polymerase Chain Reaction

The polymerase chain reaction was used to amplify the regions in the genome where the mutations were looked for. Because the regions of interest in this study have high GC-rich content, Hot-Start PCR was performed in order to improve the sensitivity and increase the specificity of the amplifications. In addition, since the same DNA samples were used to amplify SCA8 and SCA12 loci, multiplex PCR was performed. The SCA8 and SCA12 PCRs were multiplexed by combining the primer pairs of these loci in the same PCR tube. Although the lengths of PCR products of SCA8 and SCA12 overlapped, this problem was eliminated by labeling the primers with different fluorescent dyes, 6-FAM and HEX. Labelled PCR products were analyzed by GeneScan software to calculate the repeat numbers of the alleles. For all PCR reactions, 50-100 ng genomic DNA was used. After

mixing all PCR components and the DNA, the volume of the PCR sample was completed to 25 µl with dH_2O.

SCA8 PCR components are shown in Table 4.1. and SCA8 PCR conditions are as the following:

Initial Denaturation	: 95 °C	5 minutes	
Hot Start	: 80 °C		
Denaturation	: 94 °C	30 seconds	30 cycles
Annealing	: 61 °C	30 seconds	
Extension	: 72 °C	2 minutes	
Final Extension	: 72 °C	10 minutes	

Table 4.1. PCR components of SCA8 analysis

Components	[Stock]	Volume (µl)	[End]
$MgCl_2$	25 mM	2.5	2.5mM
dNTP	5 mM	1.5	0.3 mM
SCA8F4	12.5 µM	1.0	0.5 µM
SCA8R4	12.5 µM	1.0	0.5 µM
Q-solution	5X	5	1X
ExTaq Buffer	10X	2.5	1X
ExTaq DNA Polymerase	5 u/µl	0.3	1.5 u

SCA12 PCR components are shown in Table 4.2. and SCA12 PCR conditions are as the following:

Initial Denaturation	: 95 °C	5 minutes	
Hot Start	: 80 °C		
Denaturation	: 94 °C	45 seconds	30 cycles
Annealing	: 60 °C	30 seconds	
Extension	: 72 °C	45 seconds	
Final Extension	: 72 °C	10 minutes	

Table 4.2. PCR components of SCA12 analysis

Components	[Stock]	Volume (µl)	[End]
$MgCl_2$	25 mM	2.5	2.5mM
dNTP	5 mM	1.5	0.3 mM
SCA12HEX-A	12.5 µM	1.0	0.5 µM
SCA12HEX-B	12.5 µM	1.0	0.5 µM
Q-solution	5X	5	1X
Ex Taq Buffer	10X	2.5	1X
ExTaq DNA Polymerase	5 u/µl	0.3	1.5 u

SCA8 and SCA12 multiplex PCR components are shown in Table 4.3. and multiplex PCR conditions are as the following:

Initial Denaturation	: 95 °C	5 minutes	
Hot Start	: 80 °C		
Denaturation	: 94 °C	45 seconds	} 30 cycles
Annealing	: 60 °C	30 seconds	
Extension	: 66.5 °C	2 minutes	
Final Extension	: 72 °C	10 minutes	

Table 4.3. Multiplex PCR components of SCA8 and SCA12 analyses

Components	[Stock]	Volume (µl)	[End]
$MgCl_2$	25 mM	2.5	2.5mM
dNTP	5 mM	1.5	0.3 mM
SCA8F4	12.5 µM	0.4	0.2 µM
SCA8R4	12.5 µM	0.4	0.2 µM
SCA12HEX-A	12.5 µM	0.3	0.15 µM
SCA12HEX-B	12.5 µM	0.3	0.15 µM
Q-solution	5X	5	1X
Ex Taq Buffer	10X	2.5	1X
ExTaq DNA Polymerase	5 u/µl	0.3	1.5 u

SCA14 (exon 4 of the PRKCG) PCR components are shown in Table 4.4. and SCA14 PCR conditions are as the following:

Initial Denaturation	: 95 ^{o}C	5 minutes	
Hot Start	: 80 ^{o}C		
Denaturation	: 94 ^{o}C	30 seconds	32 cycles
Annealing	: 56 ^{o}C	45 seconds	
Extension	: 72 ^{o}C	1 minute	
Final Extension	: 72 ^{o}C	10 minutes	

Table 4.4. PCR components of SCA14 analysis (exon 4 of the PRKCG)

Components	[Stock]	Volume (µl)	[End]
$MgCl_2$	25 mM	2.5	2.5mM
dNTP	5 mM	1.5	0.3 mM
SCA14exon4F	12.5 µM	1.0	0.5 µM
SCA14exon4R	12.5 µM	1.0	0.5 µM
DMSO	100%	2.5	10%
Taq Buffer	10X	2.5	1X
Taq DNA Polymerase	5 u/µl	0.2	1 u

The quality and quantity of the PCR products were determined by two per cent agarose gel electrophoresis.

4.5. PCR Purification and Preparation of Samples for DNA Sequencing

The QIAquick PCR Purification Kit Protocol was applied to purify the PCR products from excess primers, dNTPs, polymerases and salts prior to DNA Sequencing. Five volumes of Buffer PB was added to 40 µl PCR product and mixed. QIAquick spin column was placed in a two ml collection tube, provided by the kit. To bind DNA, the sample was applied to the QIAquick column and spinneded for one minute at 13 000 rpm. The flow-through was discarded and the QIAquick column was palced back into the same tube. After the addition of 0.75 ml Buffer PE to the QIAquick column for washing, the sample was spinned for one minute at 13 000 rpm. The flow-through was discarded and the

QIAquick column was palced back into the same tube. The column was spinned for an additional one minute at 13 000 rpm. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. 30 µl Buffer EB (10mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane and the column was spinned for one minute at 13000 rpm in order to elute the DNA. The quality and quantity of the purified PCR products were determined on a two per cent agarose gel.

4.6. Gel Extraction for DNA Sequencing

QIAquick Gel Extraction Kit Protocol was applied to extract and purify the PCR products loaded in to the gel. First, the DNA fragment was excised from the agarose gel with a clean, sharp scalpel, the gel slice was weighed in an Eppendorf tube and three volumes of Buffer QG were added to one volume of gel (100 mg~100 µl). To dissolve the gel slice completely, it was incubated at 50^{o}C for 10 minutes and mixed by vortexing. If the color of the mixture was yellow as Buffer QG, then it was proceeded to the next step. This checkpoint is required, because Buffer QG contains a pH indicator which is yellow at pH <7.5, the optimum pH for the adsorption of DNA to the QIAquick membrane. One gel volume of isopropanol was added to the sample and mixed to increase the yield of PCR products. The QIAquick spin column was placed in a two ml collection tube, provided by the kit. To bind DNA, the sample was applied to the QIAquick column and spinned for one minute at 13 000 rpm. The flow-through was discarded and the QIAquick column was placed back into the same tube. 0.5 ml of Buffer QG was added to QIAquick column and spinned for one minute at 13 000 rpm. The flow-through was discarded and the QIAquick column was placed back into the same tube. After the addition of 0.75 ml Buffer PE to the QIAquick column for washing, the column was allowed to stand 2-5 minutes and spinnned for one minute at 13 000 rpm. The flow-through was discarded and the QIAquick column was placed back into the same tube. The column was spinned for an additional one minute at 13 000 rpm. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. 30 µl Buffer EB (10mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane and the column was spinned for one minute at 13000 rpm in order to elute the DNA. The quality and quantity of the purified PCR products were determined on a two per cent agarose gel.

4.7. GeneScan Analysis

GeneScan Analysis Software is used to size and quantitate DNA fragments automatically through the data collected by ABI Prism 310 Genetic Analyzer which is an automated single-capillary genetic analyzer. During capillary electrophoresis, a camera detects the fluorescence, with which the PCR products were labelled. The fluorescence intensity that is recorded by ABI Prism 310 Genetic Analyzer, is displayed as peaks in the electropherogram. Electropherograms show fluorescence intensity as a function of fragment size or migration time. To increase the throughput by using ABI Prism multicolor fluorescent dye technology, it is possible to co-load the products of multiple PCR products or to load multiplex PCR products labelled with different fluorescent dyes in the same capillary injection. In each capillary injection, GeneScan Internal Lane Size Standard is loaded with PCR product not to allow measurement errors due to run-to-run variations. Because both the standard and the PCR product undergo the same electrophoretic forces in the same capillary tube, the experimental errors are supposed to be eliminated. From the fragment migration times of the internal lane standard, the GeneScan Analysis Software generates a *sizing curve* that is used to size the length of PCR products in base pairs as a function of migration time (Applied Biosystems, 2000). TAMRA-500bp ladder was used as a standard in this study. The PCR products of SCA8 and SCA12 were sent to Iontek Ltd., İstanbul for GeneScan analysis.

4.8. DNA Sequencing

Chain termination detection method is used for DNA sequencing. The PCR product is used as a template for the synthesis of new DNA strands by DNA polymerase. Oligonucleotide primers specific for the region of interest and smaller amounts of labelled 2′, 3′-dideoxynucleoside 5′-triphosphates (ddNTPs) are involved beside the regular 2′-deoxynucleoside 5′-triphosphates (dNTPs) in this new amplification reaction. As the ddNTPs are incorporated into the newly synthesized strand randomly, the DNA synthesis terminates due to the lack of 3' OH group that prevents the formation of a phosphodiester bond with the incoming dNTP. The lengths of the terminated fragments are determined by capillary electrophoresis using ABI Prism 310 Genetic Analyzer (Pulst, 2003). The SCA8

and SCA12 alleles extracted from two per cent agarose gels, and purified SCA14 PCR products were sent to Iontek Ltd., İstanbul for DNA sequencing.

5. RESULTS

5.1. Molecular Analyses of SCA8 and SCA12

5.1.1. Molecular Analyses of SCA Patients and Healthy Controls for the SCA8 and SCA12 Expansions

In the framework of this thesis, 142 SCA patients with different inheritance patterns (Table 5.1) and 100 healthy controls were analyzed for SCA8 and SCA12. Because the same DNA samples were used for the molecular analyses of SCA8 and SCA12, multiplex PCR was preferred which was found to be an efficient method for high-throughput analyses. Multiplex PCR amplifications of both loci displayed that none of the SCA cases and healthy controls carry an expanded SCA8 and SCA12 allele (Figure 5.1.).

Table 5.1. The heterogeneity of SCA patient panel for SCA8 and SCA12 analyses in respect to the inheritance pattern

Inheritance Pattern	Number of SCA Families/Patients
Autosomal dominant	10/20
Autosomal recessive	23/40
Sporadic	-/74
Unknown	-/8

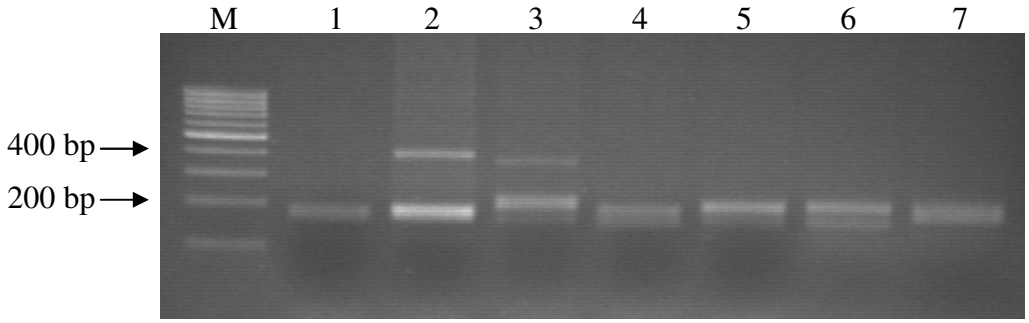


Figure 5.1. Amplification of both SCA8 and SCA12 loci by multiplex PCR
(M: 100 bp ladder, 1: healthy control, 2: SCA8 (+) control, 23 and 94 (CTA/CTG) repeats; 3: SCA12 (+) control, 13 and 78 CAG repeats; 4-7: SCA samples)

5.1.2. Determination of Trinucleotide Repeat Numbers at SCA8 and SCA12 Loci in SCA Patients and Healthy Controls

To determine the repeat numbers of the SCA patients and healthy controls at SCA8 and SCA12 loci, GeneScan analysis was performed. The disadvantage of overlapping ranges of SCA8 and SCA12 PCR product sizes was overcome by using primer pairs labeled with different fluorescent dyes, 6-FAM and HEX in multiplex PCR.

To confirm the accuracy of the GeneScan analysis for SCA8, a series of experiments were conducted. Ten normal heterozygote samples were chosen and SCA8 PCR products were run on a two per cent agarose gel. Both SCA8 alleles of these heterozygote samples were separately extracted from the agarose gel. All twenty extracted SCA8 alleles were sequenced in order to compare them with the results of the GeneScan analysis for the same ten samples. The results of DNA sequencing and GeneScan analysis for SCA-72 are given as an example for this comparison (Figure 5.2. and Figure 5.3.).

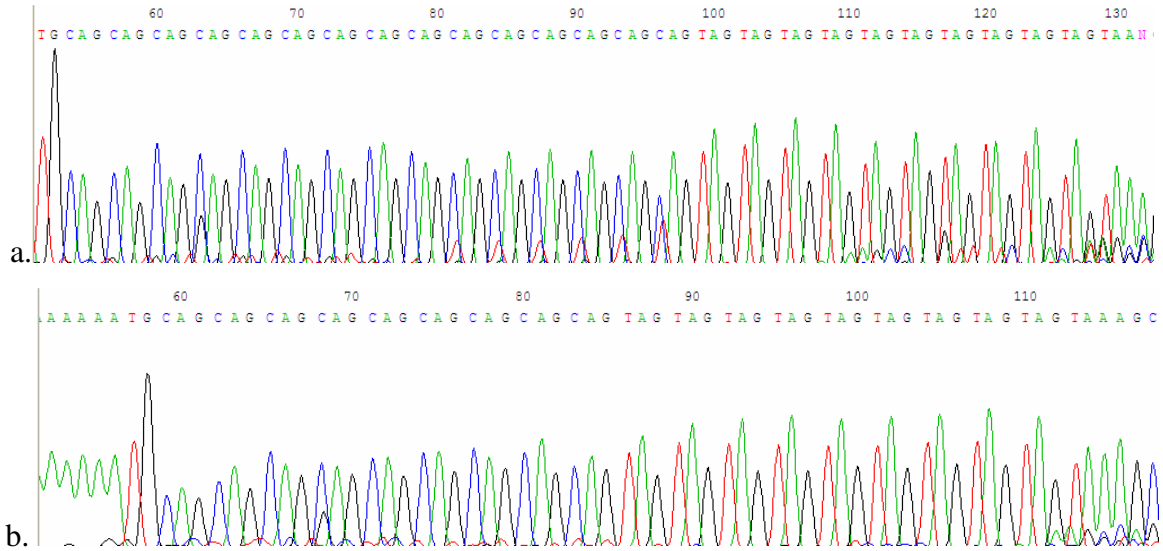
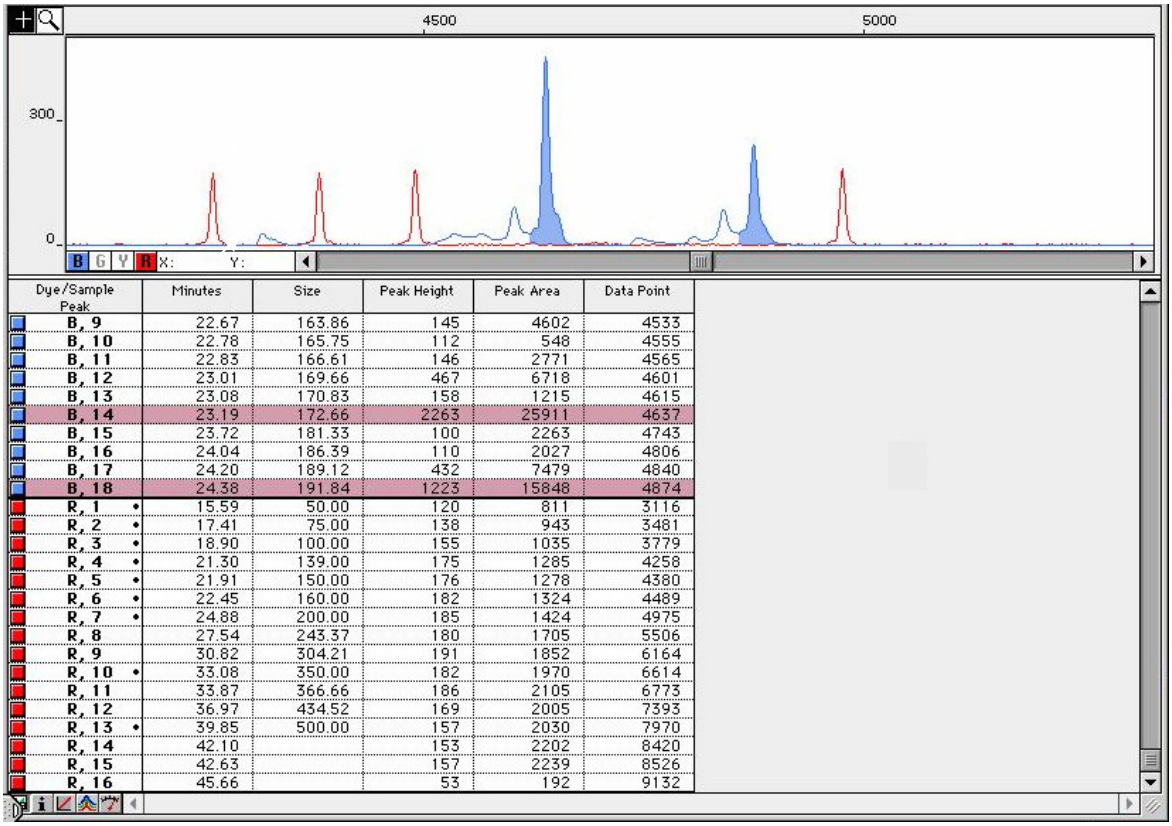


Figure 5.2. Sequencing results of SCA-72 for SCA8; SCA8 allele with 25 (10 CTA/ 15 CTG) repeats (a), SCA8 allele with 18 (9 CTA/9 CTG) repeats (b)



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
B, 9	22.67	163.86	145	4602	4533
B, 10	22.78	165.75	112	548	4555
B, 11	22.83	166.61	146	2771	4565
B, 12	23.01	169.66	467	6718	4601
B, 13	23.08	170.83	158	1215	4615
B, 14	23.19	172.66	2263	25911	4637
B, 15	23.72	181.33	100	2263	4743
B, 16	24.04	186.39	110	2027	4806
B, 17	24.20	189.12	432	7479	4840
B, 18	24.38	191.84	1223	15848	4874
R, 1 •	15.59	50.00	120	811	3116
R, 2 •	17.41	75.00	138	943	3481
R, 3 •	18.90	100.00	155	1035	3779
R, 4 •	21.30	139.00	175	1285	4258
R, 5 •	21.91	150.00	176	1278	4380
R, 6 •	22.45	160.00	182	1324	4489
R, 7 •	24.88	200.00	185	1424	4975
R, 8	27.54	243.37	180	1705	5506
R, 9	30.82	304.21	191	1852	6164
R, 10 •	33.08	350.00	182	1970	6614
R, 11	33.87	366.66	186	2105	6773
R, 12	36.97	434.52	169	2005	7393
R, 13 •	39.85	500.00	157	2030	7970
R, 14	42.10		153	2202	8420
R, 15	42.63		157	2239	8526
R, 16	45.66		53	192	9132

Figure 5.3. GeneScan analysis of SCA-72 for SCA8

The sizes of the SCA8 PCR products in bp were calculated by determining the repeat numbers obtained by DNA sequencing, and using the following formula:

SCA8 PCR product size = (repeat numbers X 3) + 120 (the region outside the repeats)

According to this formula, the expected SCA8 PCR product sizes of SCA-72 are 195 bp and 174 bp. However, as it can be seen from Figure 5.3., the product sizes are 191,84 bp and 172,66 bp according to GeneScan analysis. Because PCR products run faster during capillary electrophoresis and PCR product lengths were underestimated, a correction graph was required to obtain the real SCA8 PCR product sizes and repeat numbers of the samples. By comparing DNA sequencing results of the above twenty SCA8 alleles with the results of GeneScan analysis of the same ten samples, SCA8 correction graph was drawn (Figure 5.4.). By using the formula on the correction graph, the real SCA8 PCR product sizes were determined. The repeat numbers were calculated by using the corrected SCA8 PCR product sizes and the following formula:

$$\text{Repeat numbers} = \frac{\text{(the corrected SCA8 PCR product size - 120)}}{3}$$

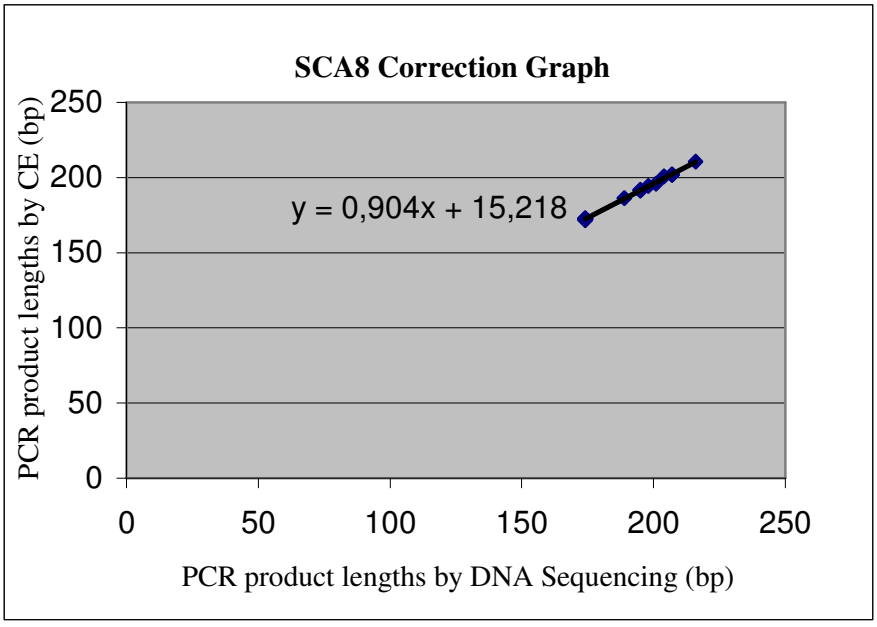


Figure 5.4. Correction graph of GeneScan analysis for SCA8

To confirm the accuracy of the GeneScan analysis for SCA12, the same series of experiments were conducted. Eight normal heterozygote samples were chosen and SCA12 PCR products were run on a two per cent agarose gel. Both SCA12 alleles of these heterozygote samples were separately extracted from the agarose gel. All sixteen extracted SCA12 alleles were sequenced in order to compare them with the results of the GeneScan analysis for the same eight samples. The results of DNA sequencing and GeneScan analysis for SCA-107 are given as an example for this comparison (Figure 5.5. and Figure 5.6.).

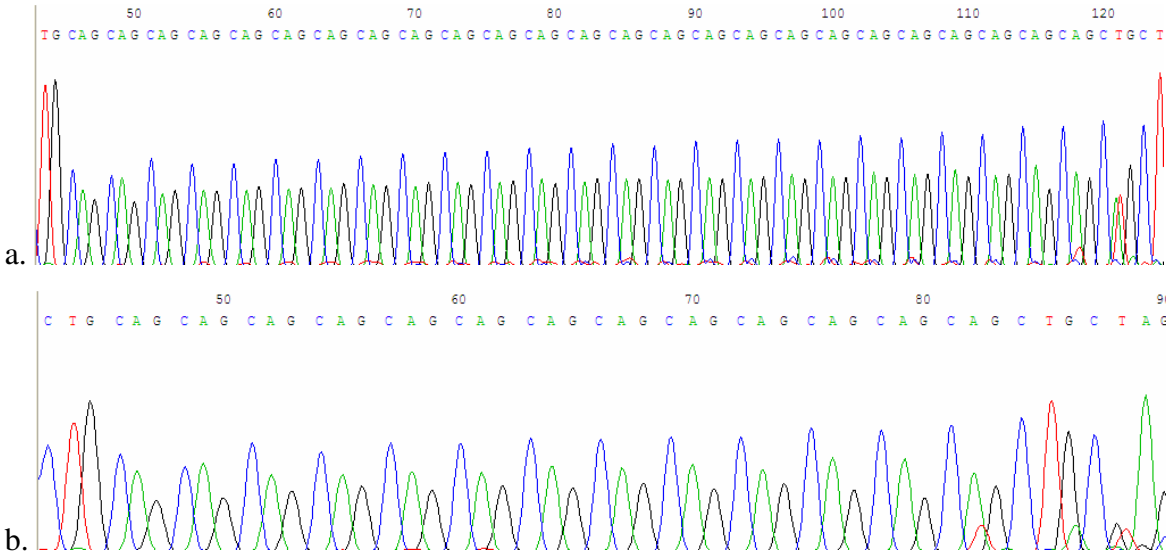
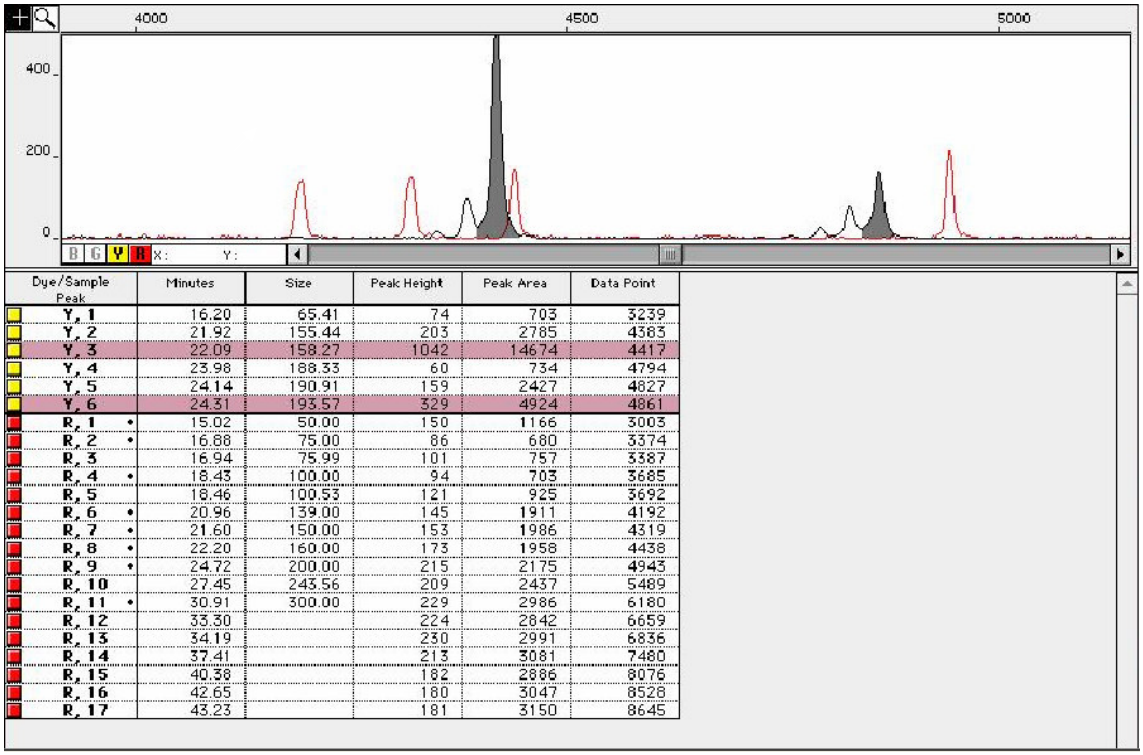


Figure 5.5. Sequencing results of SCA-107 for SCA12; SCA12 allele with 26 CAG repeats (a), SCA12 allele with 13 CAG repeats (b)



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
Y, 1	16.20	65.41	74	703	3239
Y, 2	21.92	155.44	203	2785	4383
Y, 3	22.09	158.27	1042	14674	4417
Y, 4	23.98	188.33	60	734	4794
Y, 5	24.14	190.91	159	2427	4827
Y, 6	24.31	193.57	329	4924	4861
R, 1 •	15.02	50.00	150	1166	3003
R, 2 •	16.88	75.00	86	680	3374
R, 3	16.94	75.99	101	757	3387
R, 4 •	18.43	100.00	94	703	3685
R, 5	18.46	100.53	121	925	3692
R, 6 •	20.96	139.00	145	1911	4192
R, 7 •	21.60	150.00	153	1986	4319
R, 8 •	22.20	160.00	173	1958	4438
R, 9 •	24.72	200.00	215	2175	4943
R, 10	27.45	243.56	209	2437	5489
R, 11 •	30.91	300.00	229	2986	6180
R, 12	33.30		224	2842	6659
R, 13	34.19		230	2991	6836
R, 14	37.41		213	3081	7480
R, 15	40.38		182	2886	8076
R, 16	42.65		180	3047	8528
R, 17	43.23		181	3150	8645

Figure 5.6. GeneScan analysis of SCA-107 for SCA12

The size of the SCA12 PCR products in bp was calculated by determining the repeat numbers obtained by DNA sequencing results and using the following formula:

SCA12 PCR product size = (repeat numbers X 3) + 122 (the region outside the repeats)

According to this formula, the expected SCA12 PCR product sizes of SCA-107 are 200 bp and 161 bp. However, as it can be seen from Figure 5.6., the product sizes are 193,57 bp and 158,27 bp according to GeneScan analysis. Again, because PCR products run faster during capillary electrophoresis and PCR product lengths were underestimated, a correction graph was also required to obtain the real SCA12 PCR product sizes and repeat numbers of the samples. By comparing DNA sequencing results of sixteen SCA12 alleles with the results of GeneScan analysis of the same eight samples, SCA12 correction graph was drawn (Figure 5.7.). By using the formula on the correction graph, the real SCA12 PCR product sizes were determined. The repeat numbers were calculated by using the corrected SCA12 PCR product sizes and the following formula:

$$\text{Repeat numbers} = \frac{(\text{the corrected SCA12 PCR product size} - 122)}{3}$$

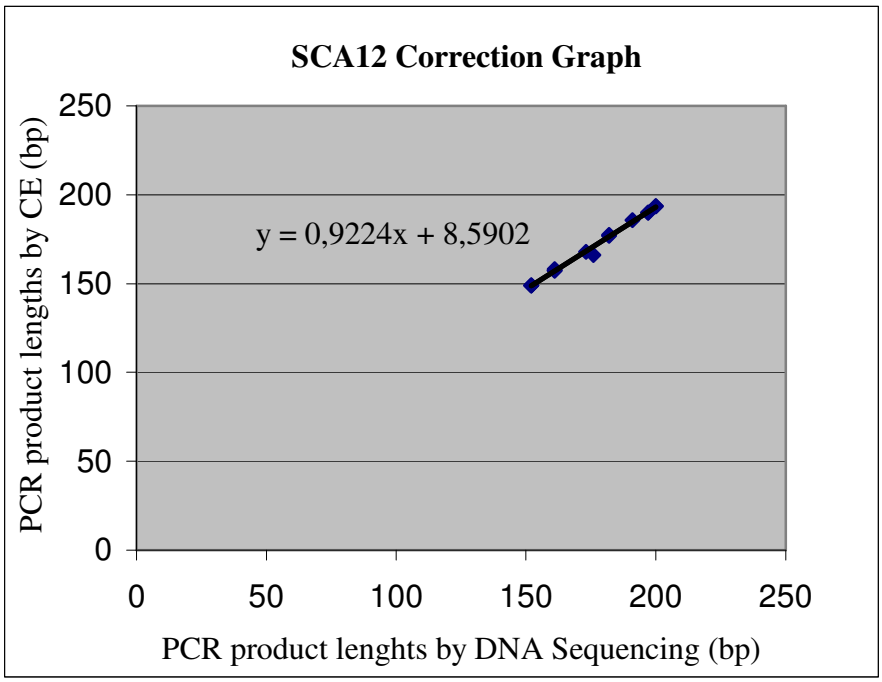
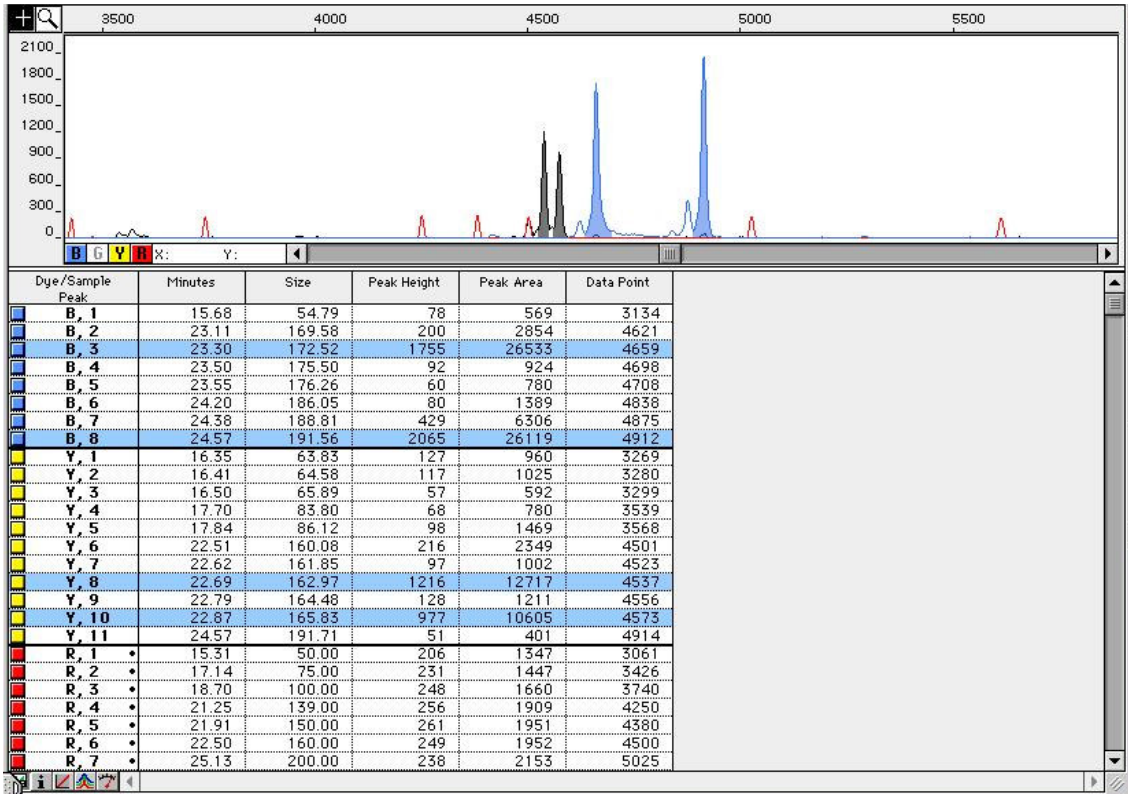


Figure 5.7. Correction graph of GeneScan analysis for SCA12

The correction formulas determined for SCA8 and SCA12, were also used for the multiplex PCR products, run by capillary electrophoresis and analyzed by GeneScan software (Figure 5.8.). The repeat numbers of the corrected SCA8 and SCA12 PCR product sizes were calculated by the formulas described above. Those fractional repeat numbers that were greater than 0.5 were rounded up to the next whole number.

"Stutter" peaks that represent the products with shorter repeat sizes (1-4 repeats) than the main PCR product, and artifact peaks were also observed in GeneScan analysis of some samples especially at the SCA8 locus. The dominant peak was chosen based on the peak height, size and area if it was distinguishable from the artifact peaks.

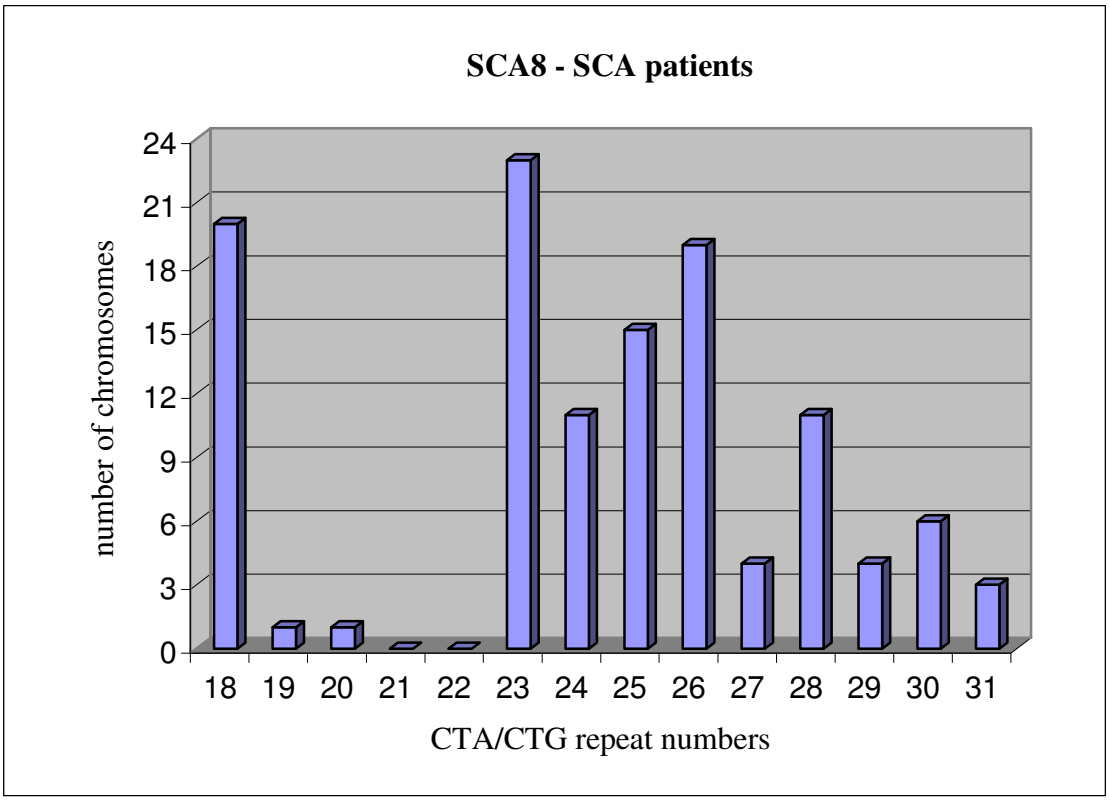


Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
B, 1	15.68	54.79	78	569	3134
B, 2	23.11	169.58	200	2854	4621
B, 3	23.30	172.52	1755	26533	4659
B, 4	23.50	175.50	92	924	4698
B, 5	23.55	176.26	60	780	4708
B, 6	24.20	186.05	80	1389	4838
B, 7	24.38	188.81	429	6306	4875
B, 8	24.57	191.56	2065	26119	4912
Y, 1	16.35	63.83	127	960	3269
Y, 2	16.41	64.58	117	1025	3280
Y, 3	16.50	65.89	57	592	3299
Y, 4	17.70	83.80	68	780	3539
Y, 5	17.84	86.12	98	1469	3568
Y, 6	22.51	160.08	216	2349	4501
Y, 7	22.62	161.85	97	1002	4523
Y, 8	22.69	162.97	1216	12717	4537
Y, 9	22.79	164.48	128	1211	4556
Y, 10	22.87	165.83	977	10605	4573
Y, 11	24.57	191.71	51	401	4914
R, 1 •	15.31	50.00	206	1347	3061
R, 2 •	17.14	75.00	231	1447	3426
R, 3 •	18.70	100.00	248	1660	3740
R, 4 •	21.25	139.00	256	1909	4250
R, 5 •	21.91	150.00	261	1951	4380
R, 6 •	22.50	160.00	249	1952	4500
R, 7 •	25.13	200.00	238	2153	5025

Figure 5.8. GeneScan analysis of both SCA8 and SCA12
(Blue and gray peaks show SCA8 and SCA12 alleles respectively)

5.1.2.1. Distribution of SCA8 Alleles among SCA Patients and Healthy Controls: The distribution of SCA8 alleles with different repeat numbers in 59 SCA patients and 60 healthy controls are shown in Figure 5.9. While the CTA/CTG repeat numbers range between 18 and 31 in genetically undiagnosed SCA patients, the numbers of repeats are in the range of 17-35 in healthy controls. SCA8 alleles show bimodal distribution both in SCA patients and healthy controls. The low frequency of SCA8 alleles with 27 combined repeats is not in perfect accordance with the bimodal shape of the histograms in SCA patients. SCA8 alleles with 18 and 23 repeats are the most frequent ones among both SCA patients and healthy controls (Table 5.2.). In addition, the samples, of which SCA8 repeat numbers could be determined, are heterozygotes except for one SCA patient, who is a homozygote.

a.



b.

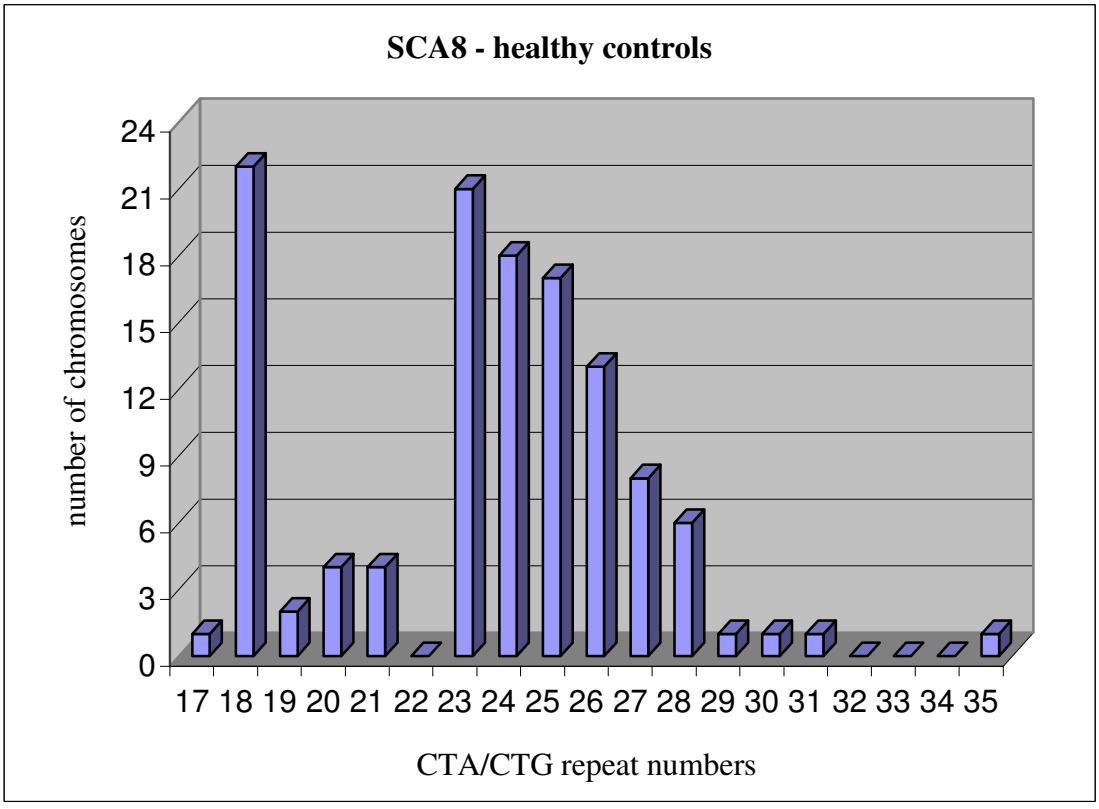


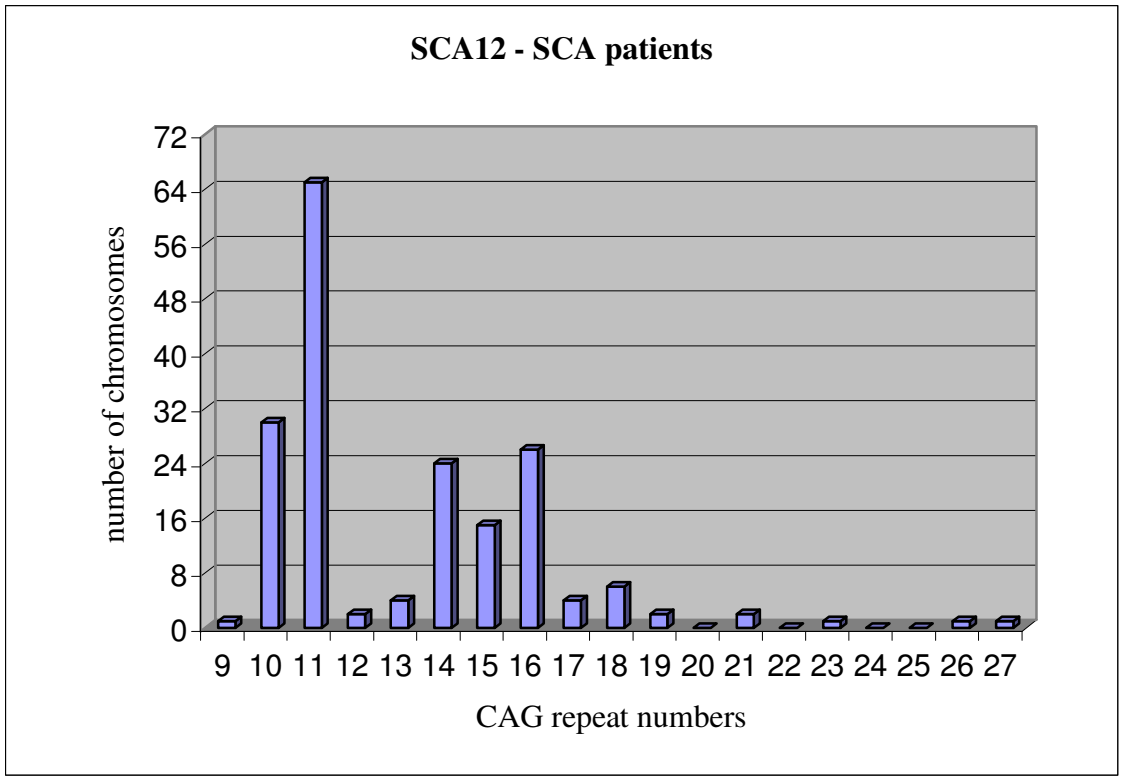
Figure 5.9. The distribution of SCA8 alleles with different CTA/CTG repeats among SCA patients (a) and healthy controls (b)

Table 5.2. The frequency of observed SCA8 alleles among SCA patients and healthy controls

SCA 8 alleles	SCA patients	Healthy controls
17 repeats	-	(<1.0%)
18 repeats	(16.9%)	(18.3%)
19 repeats	(<1.0%)	(1.7%)
20 repeats	(<1.0%)	(3.3%)
21 repeats	-	(3.3%)
22 repeats	-	-
23 repeats	(19.5%)	(17.5%)
24 repeats	(9.3%)	(15.0%)
25 repeats	(12.7%)	(14.2%)
26 repeats	(16.1%)	(10.8%)
27 repeats	(3.4%)	(6.7%)
28 repeats	(9.3%)	(5.0%)
29 repeats	(3.4%)	(<1.0%)
30 repeats	(5.0%)	(<1.0%)
31 repeats	(2.5%)	(<1.0%)
32 repeats	-	-
33 repeats	-	-
34 repeats	-	-
35 repeats	-	(<1.0%)

5.1.2.2. Distribution of SCA12 Alleles among SCA Patients and Healthy Controls: The distribution of SCA12 alleles with different repeat numbers in 92 SCA patients and 89 healthy controls are shown in Figure 5.10. CAG repeat numbers ranged between 9 and 27 in SCA patients, while the range of the repeats is 9-23 in healthy controls. SCA12 alleles show bimodal distribution both in SCA patients and healthy controls. The most frequently observed SCA12 alleles have 10 or 11 CAG repeats (Table 5.3.). In addition, the homozygosity rate is 29% in both SCA patients and healthy controls at the SCA12 locus.

a.



b.

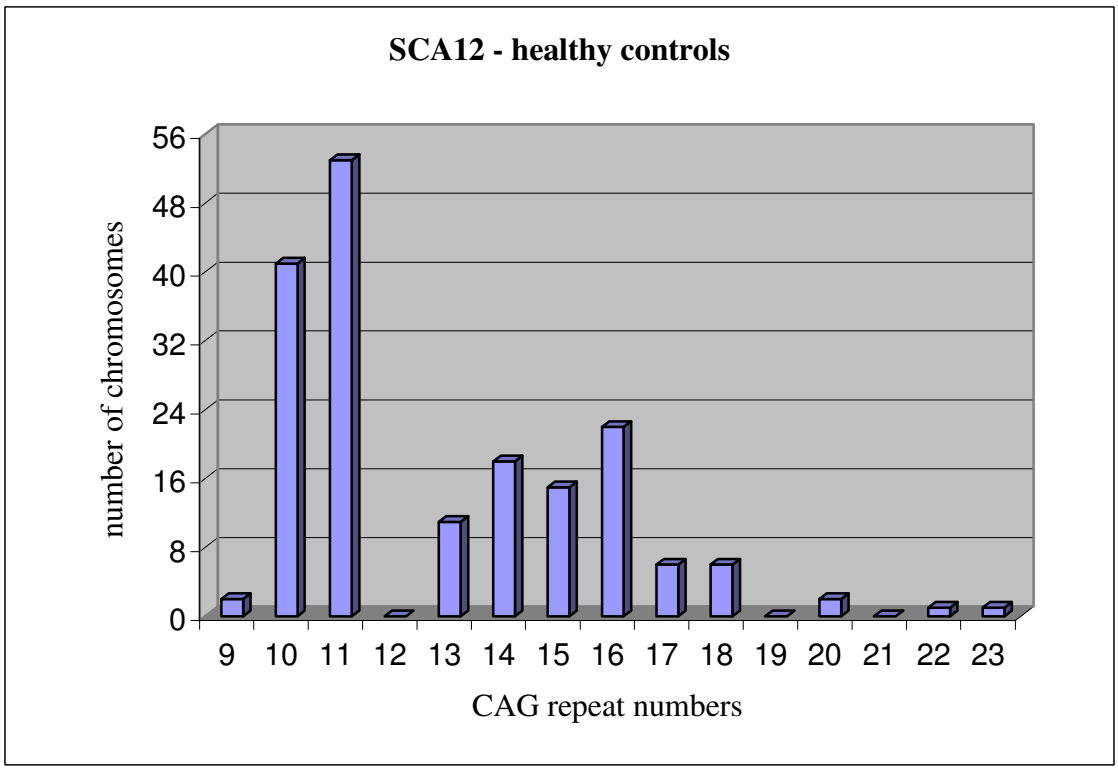


Figure 5.10. The distribution of SCA12 alleles with different CAG repeat numbers among SCA patients (a) and healthy controls (b)

Table 5.3. The frequency of observed SCA12 alleles among SCA patients and healthy controls

SCA12 alleles	SCA patients	Healthy controls
9 repeats	(<1.0%)	(1.1%)
10 repeats	(16.3%)	(23.0%)
11 repeats	(35.3%)	(29.8%)
12 repeats	(1.1%)	-
13 repeats	(2.2%)	(6.2%)
14 repeats	(13.0%)	(10.1%)
15 repeats	(8.2%)	(8.4%)
16 repeats	(14.1%)	(12.4%)
17 repeats	(2.2%)	(3.4%)
18 repeats	(3.3%)	(3.4%)
19 repeats	(1.1%)	-
20 repeats	-	(1.1%)
21 repeats	(1.1%)	-
22 repeats	-	(<1.0%)
23 repeats	(<1.0%)	(<1.0%)
24 repeats	-	-
25 repeats	-	-
26 repeats	(<1.0%)	-
27 repeats	(<1.0%)	-

5.1.3. Molecular Analyses of PD, AD, HD, FA, SCA1 and SCA2 Patients for the SCA8 Expansion

50 PD, 50 AD, 50 HD, 22 FA, seven SCA1 and six SCA2 patients who were all isolated cases, were also analyzed for the SCA8 expansion.

SCA8 expansion was observed in one PD, one AD and one SCA2 patient (Figure 5.11. and Figure 5.12.). While SCA2 patient has an intermediate SCA8 allele, DNA sequencing results display that PD patient has 23 (9 CTA/14 CTG) and 96 (8 CTA/88

CTG) repeats and AD patient has 26 (9 CTA/17 CTG) and estimated 124 (22 CTA/102 CTG) repeats. Sequence configurations of both expanded and normal SCA8 alleles of AD and PD patients show no triplet repeat interruption in CTA or CTG repeat tracts.

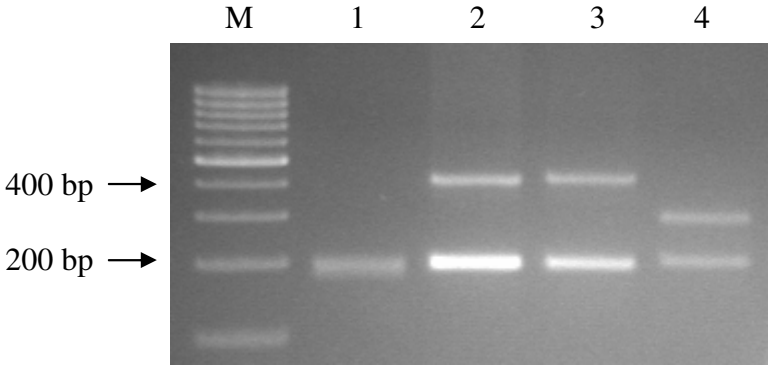


Figure 5.11. SCA8 PCR amplifications of PD and SCA2 patients (M: 100 bp ladder, 1: healthy control, 2: SCA8 (+) control, 23 and 94 (CTA/CTG) repeats; 3: PD patient, 23 and 96 (CTA/CTG) repeats, 4: SCA2 patient)

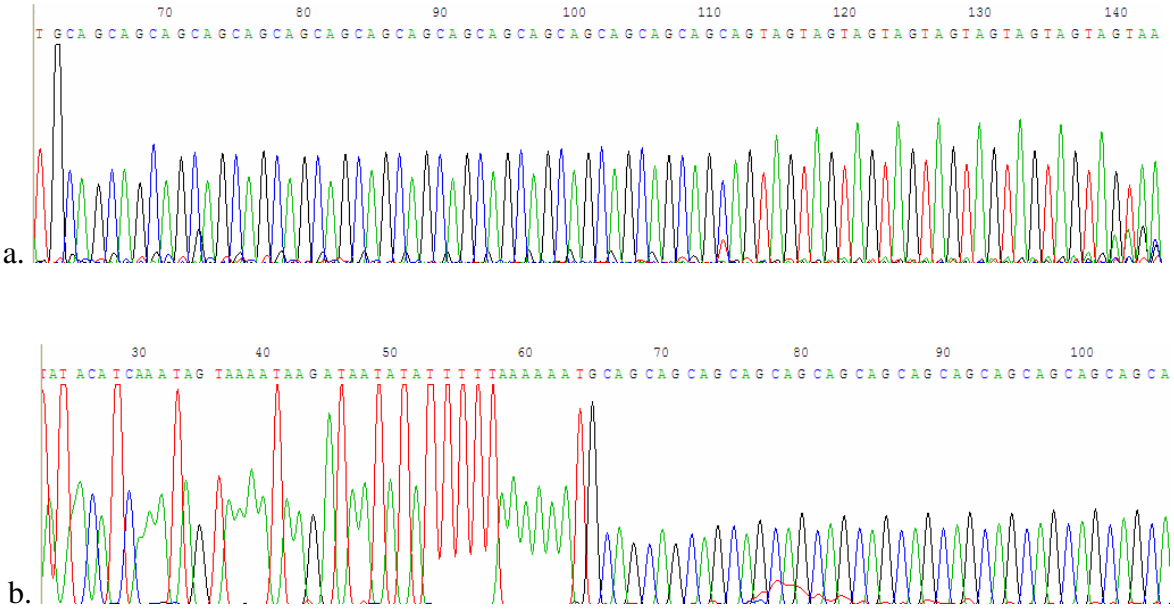


Figure 5.12. Sequencing results of AD patient for SCA8; SCA8 allele with 26 (9 CTA/ 17 CTG) repeats (a), SCA8 allele with estimated 124 (22 CTA/102 CTG) repeats (b)

b.

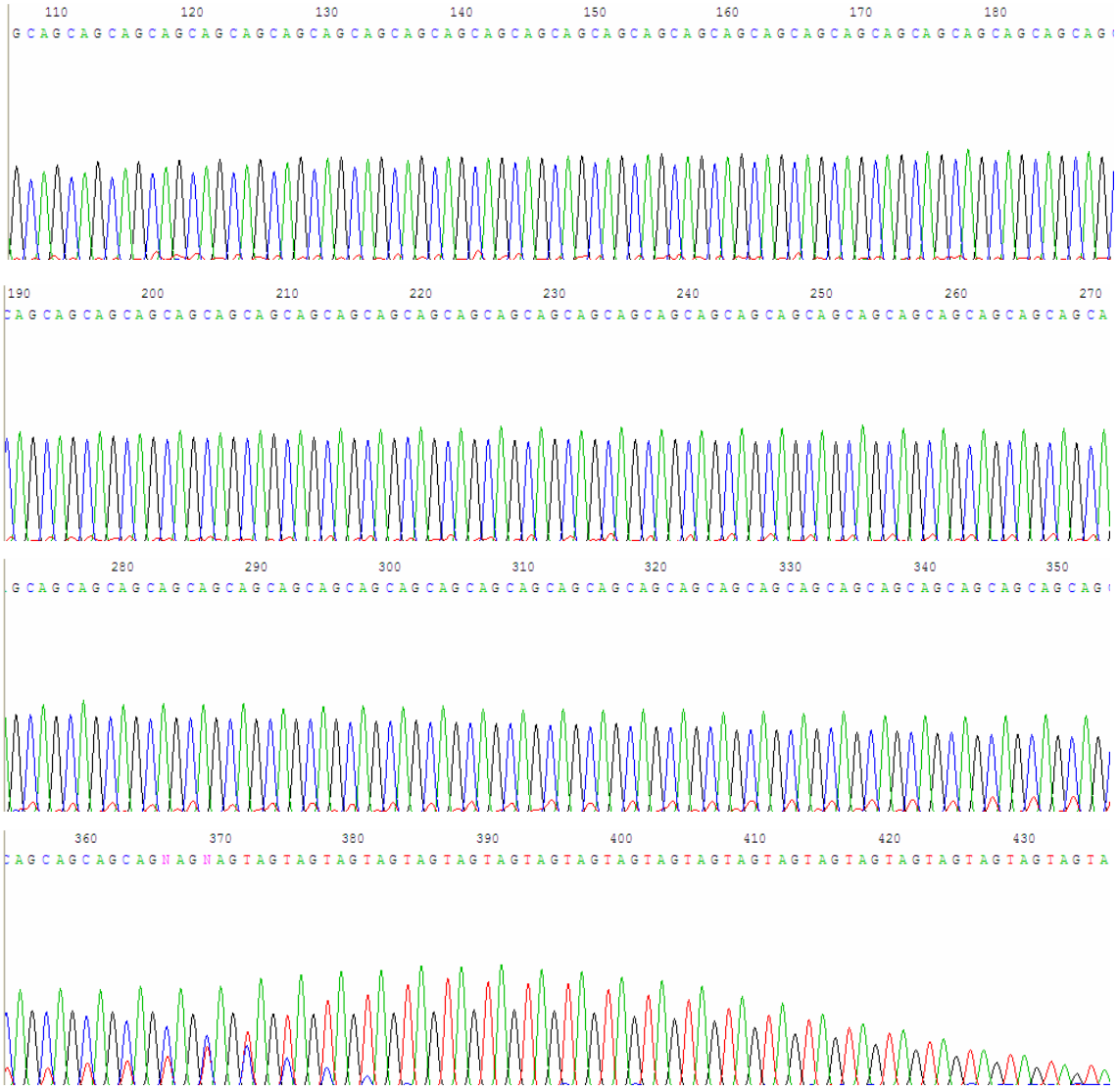


Figure 5.12. Sequencing results of AD patient for SCA8; SCA8 allele with 26 (9 CTA/ 17 CTG) repeats (a), SCA8 allele with estimated 124 (22 CTA/102 CTG) repeats (b) (continued)

In addition, one FA patient was observed to carry an expanded SCA8 allele like her father and two sisters who are also FRDA carriers (Figure 5.13. and Figure 5.14.). Since the brother is mentally retarded and it was difficult to obtain blood sample from him, he was not included in this study. CTA/CTG repeat numbers of examined members with expanded SCA8 alleles were obtained by DNA sequencing and are shown in Table 5.4. Three paternal transmissions of expanded SCA8 allele are modestly stable and two of them showed slight contractions. Sequence configuration of both expanded and normal SCA8

alleles of II.1, III.1 and III.5 showed no triplet repeat interruption in CTA or CTG repeat tracts. The sequence configuration of expanded SCA8 allele of III.3 will be confirmed.

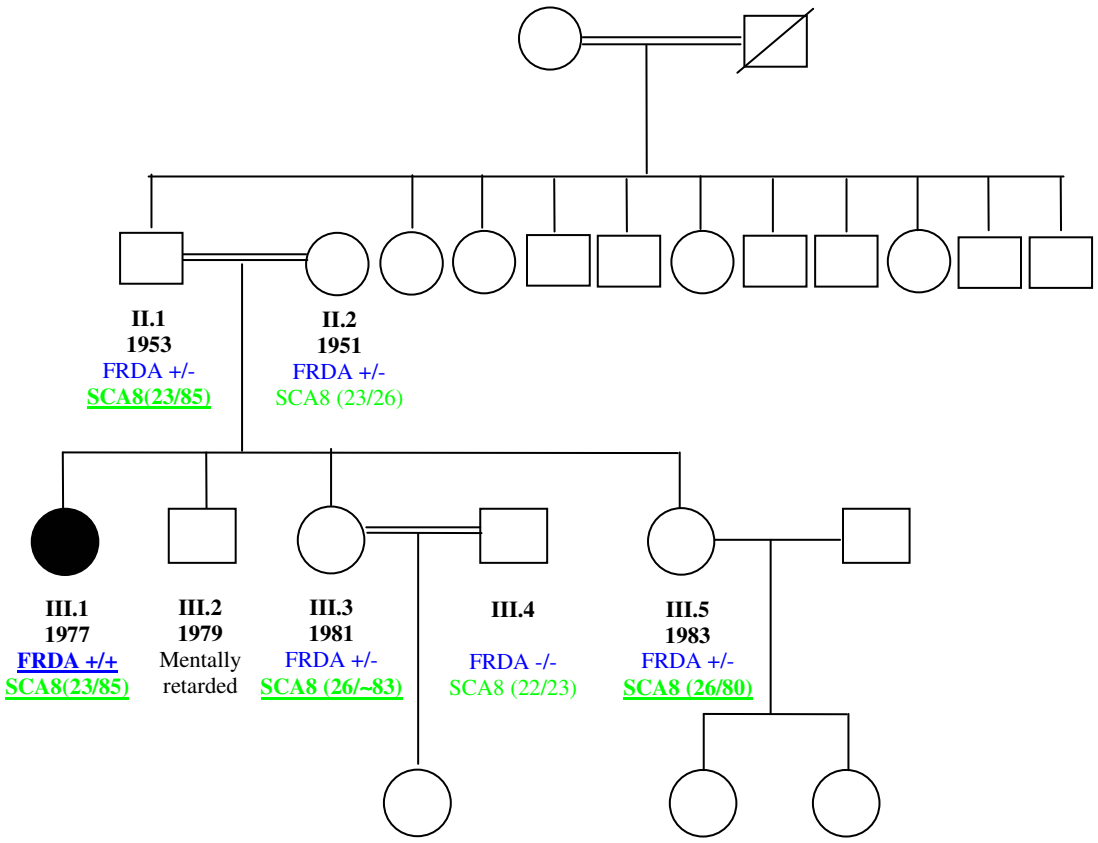


Figure 5.13. The pedigree of the FA family

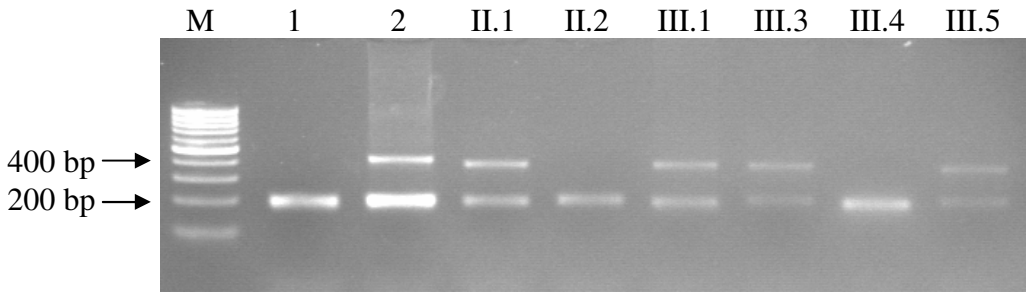


Figure 5.14. PCR amplification of the SCA8 locus for the members of the FA family (M: 100 bp ladder, 1: healthy control, 2: SCA8 (+) control; 23 and 94 (CTA/CTG) repeats)

Table 5.4. CTA/CTG repeat numbers of examined members with expanded SCA8 alleles in FA family

Family members	Normal allele	Expanded allele
II.1	11 CTA/12 CTG	13 CTA/72 CTG
III.1	11 CTA/12 CTG	12 CTA/73 CTG
III.3	9 CTA/17 CTG	~12 CTA/71 CTG
III.5	9 CTA/17 CTG	13 CTA/67 CTG

5.2. Molecular Analysis of SCA14

In the framework of this thesis, 125 SCA patients with different inheritance patterns (Table 5.5.) were analyzed for SCA14 by amplifying and sequencing exon 4 of the PRKCG (Figure 5.15.).

Table 5.5. The heterogeneity of SCA patient panel for SCA14 analysis in respect to the inheritance pattern

Inheritance pattern	Number of SCA families/patients
Autosomal dominant	9/17
Autosomal recessive	19/31
Sporadic	-/71
Unknown	-/6

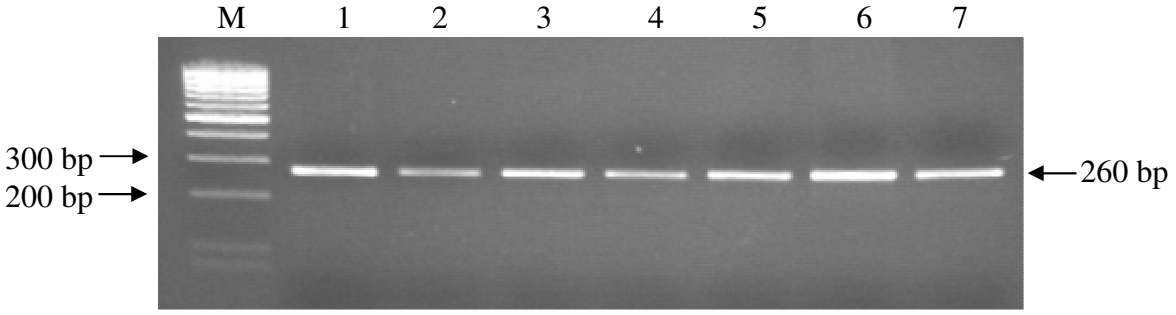


Figure 5.15. PCR amplification of exon 4 of the PRKCG for SCA14 analysis (M: 100 bp ladder, 1-7: SCA samples)

DNA sequencing results revealed that SCA cases have no mutation or polymorphism in exon 4 of the PRKCG.

6. DISCUSSION

6.1. Patient Panel

In the framework of this thesis, 142 SCA patients who were previously screened for SCA1, 2, 3, 6, 7 and 17, and 100 healthy controls were investigated for the presence of SCA8 and SCA12 mutations. 50 PD, 50 AD, 50 HD, 22 FA, seven SCA1 and six SCA2 patients were also analyzed for the SCA8 expansion. Finally, 125 SCA patients were screened for exon 4 of the PRKCG.

Although SCAs have an autosomal dominant inheritance pattern, our patient population included both sporadic cases and patients who display an apparent autosomal recessive inheritance. There are several reasons for including these patients in this study. In most cases, a well-established family history could not be obtained from neurologists or family members. The offspring may have developed ataxia prior to their parents because of anticipation; or parents may have died before clinical symptoms became apparent. False fatherhood, adoption, new dominant mutations and reduced penetrance are other reasons for the appearance of sporadic cases (Abele *et al.*, 2002; Margolis, 2003). Positive results obtained in similar patients in several other studies encouraged us to include them in our study.

6.2. Methodology: Advantages and Drawbacks

Determination of trinucleotide repeats in SCA patients and healthy controls is required for several SCAs caused by dynamic mutations to define the normal and pathological ranges of triplet repeats; since a growing number of SCAs result from expansion of trinucleotide repeats, efficient screening methods have been developed in order to shorten the testing time and reduce the testing costs. Instead of single SCA gene amplification and polyacrylamide gel electrophoresis (PAGE), multiplex PCR and capillary electrophoresis which present a semi-automated diagnosis of SCAs, are preferred. (Dorschner *et al.*, 2002; Margolis, 2003) In the framework of this project, multiplex PCR

of both SCA8 and SCA12 loci, capillary electrophoresis and GeneScan Analysis were applied.

The faster migration of PCR products compared to the internal size standards and underestimation of PCR product lengths are disadvantages of capillary electrophoresis. This can be explained by several reasons such as the significant differences of internal size ladder and PCR products in base composition, different sieving effects of capillary electrophoresis linear polymer, electro-osmotic effect and the extent of denaturation (Dorschner *et al.*, 2002). However, these disadvantages are overcome by drawing correction graphs to convert the raw PCR product size to the expected size. Dorschner *et al.* in 2002 generated the correction graphs for SCA1, 2, 3, 6 and 7 loci by comparing the lengths of PCR products that are run by PAGE and by capillary electrophoresis. In this thesis, the correction graphs were drawn for the SCA8 and SCA12 loci by comparing the lengths of PCR products that were sequenced and that were run by capillary electrophoresis.

The observation of multiple peaks, stutter and artifact peaks especially around the main SCA8 alleles in GeneScan analysis which were also seen in PAGE as multiple bands can be explained by polymerase slippage during elongation and somatic mosaicism (Applied Biosystems, 2000; Margolis, 2003). The main alleles were chosen based on the peak height, size and area if they were clearly distinguishable from the artifacts. If not, the experiments were repeated.

6.3. Molecular Analysis of SCA8

Because the role of CTA/CTG repeat expansions in the development of SCA8 is not clear yet, molecular analysis of SCA8 in SCA patients was not performed with the aim of diagnosis, but for merely research purposes. The SCA8 status of genetically undiagnosed SCA, AD, PD, HD, FA, SCA1, SCA2 patients and healthy controls were investigated in order to contribute to the debate ongoing, regarding the linkage between repeat expansions at SCA8 locus and its pathogenesis. Other factors such as sequence configuration and gender bias were also taken into account during analysis.

Although no CTG repeat expansions were observed in the SCA patients and healthy controls under investigation, expanded SCA8 alleles were found in one AD, one PD, and one FA patient and her family members. In addition, an intermediate SCA8 allele was observed in one SCA2 patient. The repeat size range among ataxia families described by Mosemiller *et al.* in 2003 is between 71 and 1000 CTA/CTG repeats; the disease penetrance were mostly seen between 80 and 250 combined repeats. According to the described repeat ranges, the AD patient with an estimated 124 repeats, the PD patient with 96 repeats, the FA patient and her carrier father with 85 repeats and her sisters with ~83 and 80 repeats fit to the ataxia group in which disease penetrance is mostly seen. Above all, expanded SCA8 allele size of the AD patient is well within the pathological range described in the MN-A family, in which SCA8 was first identified (Koob *et al.*, 1999).

Many studies on sequence configurations of SCA8 alleles also give controversial results like the studies on repeat sizes (Moseley *et al.*, 2000a; Tazón *et al.* in 2002; Day *et al.*, 2000; Stevanin *et al.*, 2000; Sobrido *et al.*, 2001). Because of these controversial observations, the role of sequence configuration in disease penetrance is uncertain. Sequence configurations of the expanded SCA8 alleles in our AD, PD and FA patients and the heterozygous father and sister showed pure CTA and CTG tracts without any triplet repeat interruption. In addition, sequencing results of twenty SCA8 alleles with repeat sizes in the range of 18-32 also showed no interruption both in CTA and CTG tracts. Therefore, our data present that normal alleles tend to have pure CTA and CTG tracts, like the expanded alleles that do not develop SCA8.

Except for the AD patient with an estimated 22 CTA repeats, SCA8 expanded alleles of the PD and FA patients (as well as her heterozygous father and two sisters) and twenty normal SCA8 alleles have CTA repeats in the range of 8-13. According to the studies of Day *et al.* in 2000, the MN-A family members have 3 CTA repeats, and the CTA repeats are in the range of 8-12 in other ataxia families. The presence of CTA repeat tract that range between 8 and 12 repeats both in ataxia families and normal individuals support the neutral polymorphism character of the CTA tract.

The paternal transmissions of expanded SCA8 allele, seen in the FA family, are modestly stable in contrast to the observation of paternal transmission bias through CTG repeat contraction in other studies (Moseley *et al.*, 2000a).

The presence of expanded SCA8 alleles in the FA patient and her heterozygous father as well as in her two sisters support the idea that the observed symptoms such as gait instability and balance problems in the FA patient, whose age at onset is 15, arise from two expanded FRDA alleles other than the expanded SCA8 allele.

The repeat numbers of normal alleles which were determined in SCA patients, who were found to be negative for SCA8 and in healthy controls, are in the range of 18-31 and 17-35 respectively. This is consistent with the normal range (16-37 repeats), described previously in 99% of 1200 control chromosomes (Koob *et al.*, 1999).

The studies going on for the last 10 years confirm that repeat expansions are the direct cause of SCA1, 2, 3, 6, 7, 10, 12 and 17. On the other hand, the reduced penetrance observed in SCA8, questions the direct linkage between CTG repeat expansion and the development of ataxia. It is supposed that the nature of the SCA8 allele contributes to the complexity of disease penetrance. The presence of two types of repeat tracts side by side has not been observed in any SCA until now. The pathological repeat ranges differ significantly among ataxia families. Controls and patients with defined genetic etiology also have expanded SCA8 alleles within the described pathological ranges. These observations show the uncertainty of upper and lower limits of normal and pathological ranges. CTA repeat tract seems to be a neutral polymorphism, but whether there is an effect of this tract on the pathogenesis has not been established yet. While the interruptions that are observed only in normal SCA1 and SCA2 alleles, are well-known to stabilize them, the role of the interruptions both in CTA and CTG repeat tracts are unknown. Although maternal expansion and paternal contraction bias are observed in general, some studies display contradictory results (Schöls *et al.*, 2003). In this respect, the data obtained in the framework of this study also question the disease-causing character of SCA8. The results show similarities with the studies of Sobrido *et al.* in 2001 with the findings of an FA patient with 97 repeats, SCA2 patient with estimated 259 repeats and two AD patients with 99 and 91 repeats. This study and the others show that the repeat expansion at the

SCA8 locus may be a rare polymorphism linked to other mutations in the SCA8 gene or another gene nearby (Stevanin *et al.*, 2000; Worth *et al.*, 2000, Schöls *et al.*, 2003). Therefore, before the points described above are clarified, molecular analysis of SCA8 should be done solely for scientific research purposes and not for genetic diagnosis.

6.4. Molecular Analysis of SCA12

Molecular analysis of SCA patients for SCA12 in this study did not give any positive result; this is consistent with the screening studies of several populations which display no expansion at the SCA12 loci of their SCA patients (Cholfin *et al.*, 2001; Sulek *et al.*, 2004; Brusco *et al.*, 2002). However, identification of 20 Indian SCA12 families indicates that SCA12 is not as rare in certain populations. Analysis of these families and ethnically matched normal unrelated individuals showed that one haplotype is significantly associated with the affected alleles which imply the presence of a common founder for SCA12 in the Indian population. Further analysis on the American pedigree of German descent in which SCA12 was first identified, revealed the absence of this haplotype. Therefore, the CAG repeat expansion at the SCA12 locus seems to have originated independently in an endogamous population in India and in the American pedigree (Bahl *et al.*, 2005).

The repeat numbers of SCA12 alleles are in the range of 9-27 in SCA patients and 9-23 in healthy controls in our population. The defined CAG repeat numbers both in SCA patients and healthy controls are in the normal ranges (7-32 repeats) described in European, French and Indian descent (Holmes *et al.*, 2003). Although the most common SCA12 allele observed in other populations has 10 CAG repeats (Fujigasaki *et al.*, 2001; Cholfin *et al.*, 2001; Sulek *et al.*, 2004; Brusco *et al.*, 2002), alleles of 10 and 11 CAG repeats together comprise the highest percentage of total SCA12 alleles in our population. It is an interesting point that although the general trend in our histograms, e.g. the distribution of SCA12 alleles are similar to the histograms of other studies, the peaks in our histograms seem to shift right by one CAG repeat. This slippage can be explained by the experimental error arising from capillary electrophoresis and GeneScan analysis. Inspite of a well-drawn correction graph, run-to-run variations might affect the results. However, because of the complex nature of trinucleotide repeat containing regions, ±1 repeat changes can be accepted within the error rate (Margolis, 2003).

Fujigasaki *et al.*, in 2001 remark that the number of larger alleles with more than 12 triplets was significantly greater in the Indian than in the French control subjects. Based on the observation that the higher rate of various SCAs paralleles the greater frequency of the large normal alleles in a certain population, this data support the appearance of SCA12 in India. Because the exact frequencies of SCA12 alleles in Indian population are not available, the comparison with our data cannot be established.

Since SCA12 is the only SCA in which action tremor of the upper extremities is the presenting and most common sign with later development of subtle cerebellar dysfunction, parkinsonian features, hyperreflexia, or cognitive dysfunction (Holmes *et al.*, 2001) and since CAG repeat expansion at the SCA12 locus among patients with ataxia is so rare, SCA12 is suggested to be considered only in cases with atypical phenotype (Cholfin *et al.*, 2001).

6.5. Molecular Analysis of SCA14

In contrast to other SCAs which are characterized by tri- or pentanucleotide repeat expansions, SCA14 is a nonepisodic ataxia resulting from missense mutations. This finding presents a new classification to SCAs according to the type of mutation and further complicates the disease mechanisms underlying SCAs.

Thus far, sixteen missense mutations and a deletion were described in exons 1, 4, 5, 10 and 18 of the PRKCG, responsible for SCA14 in English-Dutch, Dutch, Japanese, French, Portuguese and Australian families (Klebe *et al.*, 2005). Because eleven novel missense mutations are clustered in exon 4, screening of this hotspot region is suggested as the first step in genetic diagnosis. However, the presence of mutations in other exons as well, implies the requirement of sequencing the entire gene for SCA14 screening (Pandolfo *et al.*, 2005; Chen *et al.*, 2005).

Because screening for point mutations is complex, time-consuming and expensive, compared to repeat expansion disorders, which are caused by a single mutation in a single locus (Pandolfo *et al.*, 2005), screening of SCA patients for exon 4 of the PRKCG as a hotspot was anticipated. But no mutation or polymorphism was detected in our patient

population. However, before sequencing or single-strand conformation polymorphism (SSCP) analysis of other exons of the PRKCG, the SCA14 diagnosis cannot be excluded.

6.6. An Example for Molecular Heterogeneity in Turkey: Three Different Disease Genes Co-existing in the Same Family

During the screening of FA patients for SCA8, expanded alleles were found in the members of an FA family as discussed above. Further research on the family history showed that some of the members of this family were carriers of Hemoglobin S (HbS) (Figure 6.1.). The co-existence of mutations in three different loci in the same family is an interesting example for the molecular heterogeneity in Turkey, which is partly the result of consanguineous matings.

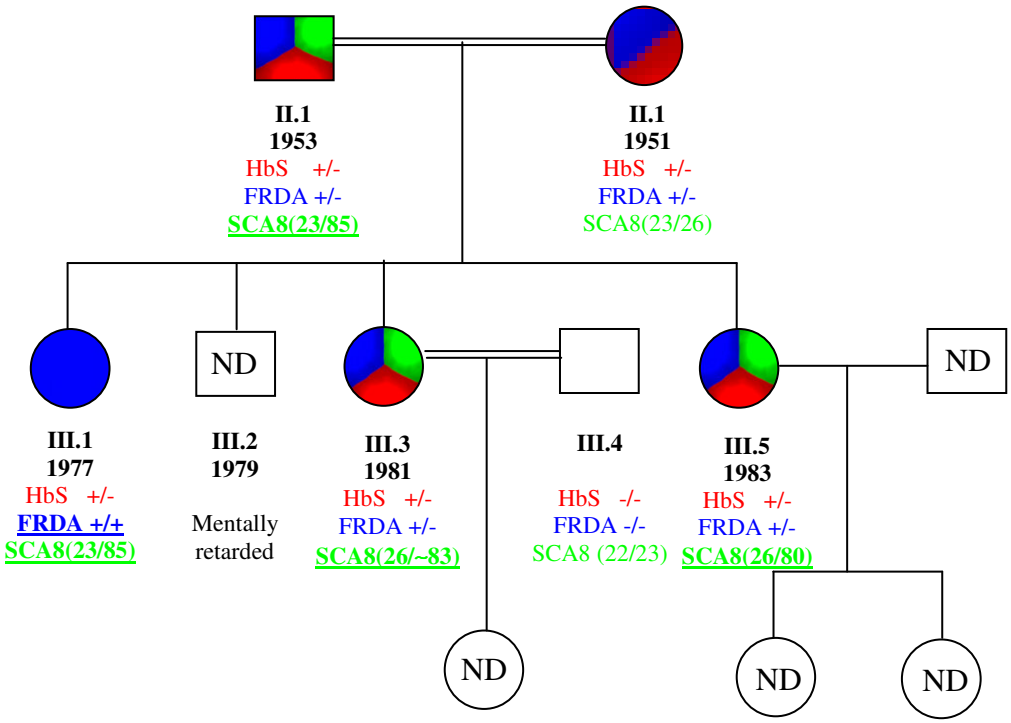


Figure 6.1. The pedigree of FA/HbS family

(ND: Not Determined)

6.7. Conclusions

Because SCAs are a genetically and clinically heterogenous group of neurodegenerative disorders, characterized by late-onset ataxia, various clinical features and autosomal dominant inheritance, the presence of ataxia, late age at onset, family history, clinical examination and confirmative genetic testing are required to define the exact disease type (Lau *et al.*, 2004). As a strategy, genetic analysis is suggested to be performed in respect to the distribution of SCA subtypes in the relevant ethnic background and the clinical features present (Schöls *et al.*, 2004). This study attempts to define the prevalence of SCA8, SCA12 and SCA14 in Turkey for the first time and designs a possible future screening strategy for SCA patients in Turkey.

Until now, the genetic studies on SCA have been conclusive which present an order for the classification of SCAs. Although major advances have been achieved in understanding the pathogenesis, especially in polyglutamine disorders, there has been no established therapy to prevent the progression of SCA yet. However, even the genetic diagnosis of SCAs is very significant to improve the quality of patient's life by developing direct treatments for disease-specific clinical symptoms. The great progression in the definition of genetic causes of SCAs in the last 10 years gives hope that the understanding of molecular pathogenesis of SCAs and the development of therapeutic strategies are not too far (Margolis, 2003; Schöls *et al.*, 2004).

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