The localization and identification of novel SCA genes in the Dutch autosomal dominant cerebellar ataxia population

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The localization and identification of novel SCA genes in the Dutch autosomal dominant cerebellar ataxia population

De lokalisatie en identificatie van nieuwe SCA genen in de Nederlandse autosomaal dominante cerebellaire ataxie populatie

(MET EEN SAMENVATTING IN HET NEDERLANDS)

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Dineke Suzanne Verbeek

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PROMOTORS

Prof. Dr. P.L. Pearson[1]
Prof. Dr. H.P.H. Kremer[2]

CO-PROMOTOR

Dr. R.J. Sinke[1]

[1] Complex Genetics Section
DBG-Department of Medical Genetics
University Medical Center Utrecht
Utrecht, The Netherlands

[2] Department of Neurology
University Medical Center Nijmegen
Nijmegen, The Netherlands

BEOORDELINGSCOMMISSIE

Prof. Dr. J.H.J.Wokke, Utrecht
Prof. Dr. E. Bakker, Leiden
Prof. Dr. D. Lindhout, Utrecht
Prof. Dr. R.H.A. Plasterk, Utrecht

Voor Frank
&
Annemieke

LIST OF ABBREVIATIONS

ADCA: autosomal dominant cerebellar ataxia
SCA: spinocerebellar ataxia
CAG: glutamine
CTG: leucine
FHM: familial hemiplegic migraine
EA2: episodic ataxia type 2
DNA: deoxynucleic acid
SNP: single nucleotide polymorphism
LD: linkage disequilibrium
SHA: shared haplotype analysis
lod: logaritm of the odds
PCR: polymerase chain reaction
dNTP: deoxyribonucleoside triphosphate
$MgCl_2$: magnesium cloride
PRKCG/PKCγ: protein kinase C gamma
FGF: fibroblast growth factor
DAG: diacylglycerol
TPA: phorbol-12-myristate-13-acetate
Ca^{2+}: calcium

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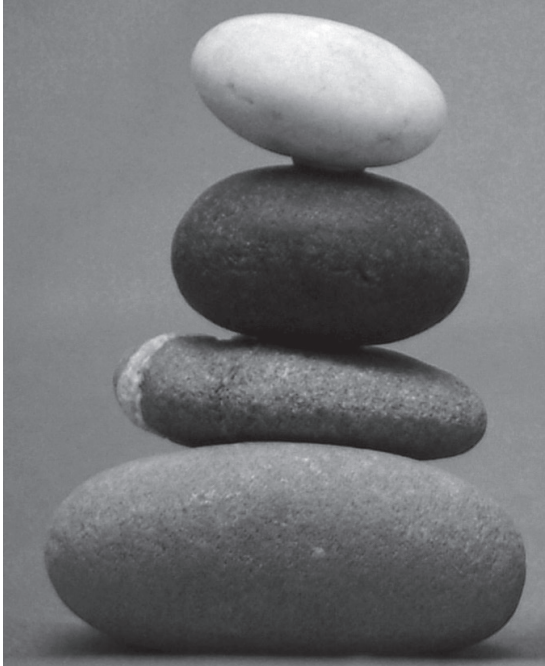
General introduction



NEURODEGENERATIVE DISORDERS

Hereditary neurodegenerative disorders are a clinically and genetically very heterogeneous group that includes, among others, Alzheimer's disease, Huntington's disease, Parkinson's disease, autosomal dominant cerebellar ataxias (ADCAs), Friedreich ataxia and other recessive ataxias, dentatorubropallidoluysion atrophy (DRPLA), multiple-system atrophy, amyotrophic lateral sclerosis (ALS), spinal and bulbar muscular atrophies, and familial spastic paraparesis. The disease processes are chronic and progressive, and mainly involve selective loss of neuronal cells in motor, sensory, and cognitive systems. The genetic defects that lead to neurodegeneration are complex and diverse. Disease genes have been identified that seem to play a role in a variety of biological pathways ranging from protein folding, recycling control, proteasome control and activation of caspases to transcriptional regulation, and RNA processing. Further research has yet to determine what the crucial underlying interactions and pathways are between these various disease genes.

AUTOSOMAL DOMINANT CEREBELLAR ATAXIAS

The ADCAs are a large group of neurodegenerative disorders. In general, ADCAs are characterized by gait and limb ataxia, disturbances in speech and oculomotor control. The ataxic syndrome is the result of neuronal loss in the cerebellum, brain stem and spinal cord, and is often accompanied by other variable signs and symptoms including myoclonus, tremor, epilepsy, cognitive impairment, and headache. The disease course is progressive, and the age of onset is mostly between 30 and 50 years, although the age of onset may vary between families of the same SCA-type or even within one family (see Table 1).

Mode of Inheritance

ADCAs, by definition, are inherited in an autosomal dominant mode and therefore the offspring of an ADCA patient has a 50% risk of inheriting the disease gene. Although most individuals diagnosed with ADCA have an affected parent, the family history may appear to be negative because of a failure to recognize the disorder in other family members. This may be due to the early death of the parent or other family members before the onset of symptoms, or due to the late onset of the disease. This phenomenon severely complicates genetic counseling of seemingly sporadic patients and their relatives.

In addition, the late age of onset of the disease complicates also family planning for childern of ADCA patients since they may be at risk to develop the disease in a later stage of life. The risk to sibs of a patient depends on the genetic status of the parents. If one parent has a diseased allele, the risk to each sib of inheriting this disease allele is 50%.

The risk to other family members also depends upon the genetic status of the patient's parents. If a parent is found to have the disease allele, his or her family members are at risk. But even when no disease allele is found, or when there is no indication that the disease is familial, the family members may still be at risk.

Furthermore, in the case of at-risk individuals, the age of onset, severity, specific symptoms, and progression of the disease are variable and cannot be predicted by the family history or by genetic testing. Children at risk for late-onset disorders should not be genetically tested, prior to age 16, in the absence of any disease symptoms. In general, the principal argument against testing at-risk children is that it removes their choice to know or not know this information. It increases the possibility of stigmatization within the family and in other social settings, could have serious educational and career implications and will complicate applying for health insurances in further life. On the other hand, at-risk testing before the age of 16 may occur in special occasions such as in diseases for which preventive therapy is available or when at-risk individuals are needed in a trial for drugs that have to be applicated before the onset of disease symptoms.

Clinical classification

Harding divided ADCAs into three major subgroups: types I, II and III [1]. This classification is based on the main phenotypic characteristics. ADCA type I comprises the most progressive form and is characterized by specific symptoms like opthalmoplegia, optic atrophy, extra-pyramidal signs and dementia, in addition to ataxia. Type II is always associated with progressive macular dystrophy, while type III is considered to be a "pure" cerebellar syndrome. A more detailed clinical overview per spinocerebellar ataxia (SCA) type is shown in Table 1. In recent years, this classification system has appeared to be of limited use because many ADCA families do not fit into one of the phenotypic categories defined by Harding. With the identification of disease-causing SCA genes, Harding's classification is gradually being replaced by a genetic categorization based on the gene or gene locus involved.

Genetic classification

The first SCA gene was cloned in 1993 and designated SCA1 [2]. Since then, ten additional SCA genes (SCA2, 3, 6-8, 10, 12, 14, 17 and FGF14) [3-10] have been cloned and at least another 13 SCA loci (SCA4, 5, 11, 13, 15, 16, 18-23, 25 and 26) have been identified by linkage analysis [11-27]. These numbers may increase in the future, and will expand the spectrum of genetic heterogeneity of the ADCAs, both in the Netherlands and worldwide.

Table 1
Summary of clinical characteristics of the different SCA types.

SCA locus	Mean age and range of onset	Clinical symptoms
SCA1	37 (4-74)	ataxia, dysarthria, nystagmus, extrapyramidal symptoms, spasticity, opthalmoparesis, slow saccades, axonal neuropathy, cognitive impairment
SCA2	32 (1-65)	ataxia, parkinsonism, hyporeflexia, spasticity, opthalmoparesis, slow saccades, axonal neuropathy
SCA3	36 (5-70)	ataxia, parkinsonism, extrapyramidal symptoms, spasticity (severe), opthalmoparesis, axonal neuropathy, restless legs
SCA4	? (19-72)	gait ataxia, dysarthria, axonal sensory neuropathy, pyramidal signs
SCA5	30 (10-68)	gait and limb ataxia, nystagmus, dysarthria, axonal sensorimotor neuropathy
SCA6	52 (30-71)	ataxia, dysarthria, nystagmus, tremor
SCA7	35 (0-70)	ataxia, retinal degeneration, slow saccades, pyramidal signs, hearing loss, no peripheral nerve pathology
SCA8	40 (1-73)	ataxia, dysarthria, nystagmus, tremor, spasticity, sensory neuropathy
SCA10	36 (26-45)	gait and limb ataxia, nystagmus, dysarthria and some have seizures (epileptic)
SCA11	25 (15-43)	"pure" ataxia, dysarthria, nystagmus, hyperreflexia
SCA12	35 (8-55)	gait ataxia, upper extremity tremor, dysmetria, hyperreflexia, in oldest subjects dementia, eye movement disorder
SCA13	childhood (<1-45)	slow progressive gait ataxia, dysarthria, nystagmus, hyperreflexia, mental retardation and mild developmental delays in motor acquisition
SCA14	27 (12-42)	pure cerebellar ataxia, myoclonus, dystonia, peripheral neuropathy, parkinsonism, cognitive problems
SCA15	26 (10-50)	slow progressive gait ataxia, dysarthria, nystagmus, some patients with hyperreflexia
SCA16	40 (20-66)	pure cerebellar ataxia, dysarthria, nystagmus, some patients with head tremor
SCA17	33 (6-48)	ataxia, hyperreflexia, dysarthria, nystagmus with dementia, seizures, psychosis and behavioral changes
SCA18	15 (12-25)	ataxia, dysarthria, nystagmus, sensory with neurogenic muscular atrophy
SCA19	34 (11-45)	mild ataxic syndrome, dysarthria, nystagmus, cognitive impairment, myoclonus, irregular tremor of the upper extremities, hypo/hyperreflexia
SCA20	47 (19-64)	slow progressive ataxia, dysphonia, palatal myoclonus, dentate calcification
SCA21	18 (7-30)	gait ataxia, akinesia, mild features of dysarthria, mild intellectual impairment, hyporeflexia, tremor
SCA22	? (10-46)	ataxia, dysarthria, nystagmus, slow progression, hyporeflexia
SCA23	50 (43-56)	slow progressive gait ataxia, dysarthria, hyperreflexia
SCA25	? (1-39)	ataxia, dysarthria, nystagmus, sensory neuropathy
SCA26	42 (26-60)	pure cerebellar ataxia, dysarthria, impaired visual pursuits
FGF14	34 (27-40)	ataxia, dysarthria, nystagmus, tremor, psychiatric episodes, axonal peripheral neuropathy

Table adapted from Schols et al. [50]. ? = data is not available

MUTATIONAL MECHANISM

Coding polyglutamine repeat expansions

Previously, ADCAs were acknowledged as polyglutamine disorders because the underlying molecular defect seemed to point to a common theme: CAG repeat expansions in the coding region of the SCA1, 2, 3, 6, 7 and 17 genes, leading to expanded polyglutamine tracts in the respective proteins. Noncoding CAG/CTG repeat tracts are not uncommon in the human genome, but are mostly not involved in any pathogenic mechanism [28; 29]. However, some repeat tracts located in particular genes are more unstable and prone to expand. When the repeat numbers exceed a certain threshold, which is gene dependent, they become pathogenic (see Table 2). In general, the disease threshold is above

Table 2
Overview of the genetic classification of the different SCA types.

SCA locus	Chromosome region	Mutation type	Range of repeat numbers/ allele		Gene/ Description and function
			wild type	mutant	
SCA1	6p23	$(CAG)_{n,coding}$	6-39	40-81	ataxin-1//Part of nuclear complex/gene transcription/RNA regulation
SCA2	2q23-q24.1	$(CAG)_{n,coding}$	14-32	34-59	ataxin-2/ Translational regulation and RNA processing
SCA3	14q24.3-q31	$(CAG)_{n,coding}$	12-40	55-86	ataxin-3/ Represses histone acetylation and transcription
SCA4	16q22.1				
SCA5	11p11-q11				
SCA6	19p13	$(CAG)_{n,coding}$	4-18	21-30	CACNA1A/ Calcium channel, P/Q type, alpha -1A subunit
SCA7	3p21.1-p12	$(CAG)_{n,coding}$	7-17	38-130	ataxin-7/ Subunit of GNC5 histone complex
SCA8	13q21	$(CTG)_{n,noncoding}$	9-91,>250	107-127	untranslated RNA/unknown
SCA9	reserved				
SCA10	22q13	$(ATTCT)_{n,noncoding}$	10-22	800-4200	SCA10/ unknown
SCA11	15q14-21.3				
SCA12	5q31-5q33	$(CAG)_{n,coding}$	7-28	66-78	PPP2R2B/ Protein phosphatase 2, regulatory subunit B
SCA13	19q13.3-q13.6				
SCA14	19q13.4	Missense			PRKCG/ Protein kinase C gamma
SCA15	3q24.2-3pter				
SCA16	8q23-24.1				
SCA17	6q27	$(CAG)_{n,\ noncoding}$	27-42	44-55	TBP/ TATA -box binding protein
SCA18	7q31-q32				
SCA19	1q21-p21				
SCA20	11p13-q11				
SCA21	7p21.3-p15.1				
SCA22	1q21-p21				
SCA23	20p13-12.3				
SCA25	2p21-p13				
SCA26	19p13.3				
FGF14	13q34	Missense			FGF14

30-40 repeats, with the exception of the SCA6 repeat that is already pathogenic at more than 20 repeats. The repeat length is also inversely correlated with age of onset [30; 31]. Thus, smaller repeat lengths are found in patients that display signs and symptoms at a later age. In contrast, longer repeat stretches are less stable than shorter ones, and accordingly tend to expand in subsequent generations. This leads to a decrease in age of onset and a more severe phenotype in successive generations; this phenomenon is referred to as anticipation. Striking examples of anticipation have been shown in SCA7 families, and to a lesser extent, in SCA2 families [32-34]. Infantile cases are characterized by a very rapid disease progression, and they die very young due to multi-systemic failure.

This phenotype is generally not recognized as a hereditary ADCA, because the child may become ill before one of the parents displays any disease symptoms. The disease phenotype is caused by extreme repeat expansions that only can be detected by Southern blot analyses. Interestingly, these enormous repeat expansions all appear to be paternally transmitted, suggesting a less stable SCA7 or SCA2 repeat during paternal meiosis than maternal meiosis.

The exact mechanism that induces the CAG repeats to expand is not known. Multiple mechanic models have been suggested, such as the formation of hairpin loops in the leading DNA strand [35], or primer slippages of the polymerase [36]. Furthermore, repeat expansions could occur as a result of recombination events or due to gene conversions [37]. In addition, several factors can influence the stability of the repeat, including the length of the repeat, the presence of interruptions in the repeat tract, aberrant CpG methylation, and acetylation [38; 39].

Non-coding repeat expansions and missense mutations

The idea that the only disease mechanisms possible in the pathogenesis of ADCAs were expanded polyglutamine tracts became unlikely after the identification of the disease-causing mutations in SCA8, SCA10, and SCA12 genes. Large, non-coding CTG repeat expansions were identified in SCA8 [13], but so far it is not certain whether this repeat expansion is the true disease-causing variation or just a polymorphism. Furthermore, SCA8 is unique in its kind because the predicted SCA8 gene appears to be an endogenous anti-sense RNA [40].

The functional implication of the non-coding ATTCT repeat expansion in intron 9 of the SCA10 gene and the untranslated CAG repeat expansion in the 5' promoter region of the *PPP2R2B* gene in the pathology of the ADCAs remains unknown [16]. In contrast to coding or non-coding repeat expansion mutations, missense mutations have been identified in the *FGF14* (no SCA symbol assigned) [41], *CACNA1A* (SCA6) [42], and *PRKCG* (SCA14) genes [9; 43-45].

PREVALENCE OF ADCA

Worldwide and in the Netherlands

There have been few epidemiological studies of ADCA and most do not reflect the true occurrence of the disorder. The studies were performed in specific, limited populations, and often included the entire range of hereditary ataxias. The heterogeneity of the disorder is reflected in the variation of the prevalence between different ethnic and continental groups. In general, prevalence estimates have been assumed to range from 0.3-3.0 per 100,000 inhabitants [46-48].

In 2000, a national survey was performed in the Netherlands, to estimate the prevalence of ADCA [31] (Figure 1B). At that time, 145 ADCA families with known

SCA mutations had been identified, but the total number of ADCA families was extrapolated to 227. This was because 70% of the families that display a phenotypically correct, dominant inherited ataxia are explained by mutations in the SCA1, 2, 3, 6 and 7 genes. The remaining 30% of the families must harbour yet unidentified mutations. Based on these numbers, the prevalence of ADCA was estimated to be 3.0 per 100,000 individuals. The SCA3 mutation occurs most frequently (28%) in the Dutch ADCA population, followed by CAG repeat expansions in the SCA6 gene (15%). Worldwide, SCA3 mutations display a frequency of 20-25%, but regional variations exist. Almost 65% of the Portuguese cases can be explained by SCA3 mutations [49]. SCA1, 2, 6 and 7, on average display a prevalence of approximately 2-5% [50]. However, the SCA1 mutation is frequently identified in Africa [51], and SCA2 accounts for most of the ADCA cases in India, Cuba and Italy [52; 53]. The remaining SCA types are considered to be rare mutations identified in singular families or specific populations [54-56]. The frequency of the SCA8 mutation in the Dutch ADCA population is about 3.0% (unpublished data, 2000); this has been observed in other populations and is shown in Figure 1A. Most of these numbers now need minor adjustments because the more recently identified SCA14 and SCA17 mutations were not included. In addition, founder effects clearly contribute to the population differences observed.

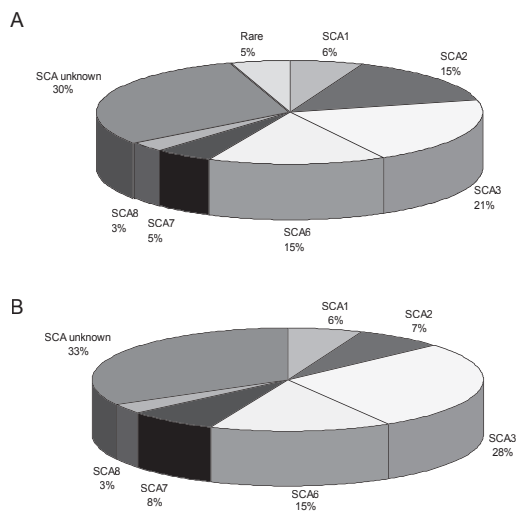


Figure 1

Prevalence of different SCA types. A) Prevalence worldwide: the numbers are slightly biased due to founder effects. The rare genotypes include mutations in SCA10, 12, 17, and FGF14 genes. B) Prevalence in the Netherlands based on figures from a national survey in 2000 [31]. The number of unknown SCAs needs to be adjusted with more recently identified SCA14 and FGF14 families that account for 4,5% of the total ADCA families. The SCA8 data have not been published.

In conclusion, since the more recently cloned SCA genes do not explain the 30% of patients in whom no mutation has been detected yet, it is likely that novel genes that contribute to the genetic heterogeneity of the ADCAs, worldwide and in the Netherlands, still need to be identified.

PATHOPHYSIOLOGY OF ADCA

Polyglutamine disorder

The SCA1, 2, 3, 6, 7, and 17 genes do not show any clear homology in function or structure, however, their only common underlying feature is their mutational mechanism: coding polyglutamine repeat expansions. The common link in pathology between these expanded polyglutamine proteins is the accumulation of protein aggregation complexes in the nucleus due to misfolding of the polyglutamine-containing proteins [57-60]. The resulting nuclear inclusions (NIs) contain at least the expanded polyglutamine-containing fragment of the protein, and sequester, among others, chaperone proteins such as heat-shock proteins, transcription factors such as CBP and ubiquitin-proteasome components [61-64]. Heat shock proteins have been found to prevent polyglutamine aggregation and can even reduce toxicity, cell death, and neurodegeneration [65; 66]. A major role for cellular stress and DNA damage response has been suggested in the pathology of polyglutamine-induced toxicity [67; 68].

The protective effects of chaperones on toxicity has been directly associated with suppression of caspase activity, and seems to be independent of polyglutamine aggregation [69]. Furthermore, the proteasome degradation of expanded polyglutamine-containing proteins seems to be very inefficient, even when directly targeted by proteasome components [70]. It has been suggested that these proteins have been kinetically trapped in the proteasome, and that the degradation process is directly influenced by post-translational modifications, and the sequestering of additional chaperones. Novel regulators of polyglutamine aggregation were identified through a genome-wide RNA interference screen in C. elegans [71]. Genes involved in RNA metabolism and protein synthesis, folding, trafficking, and degeneration were found to genetically modify the threshold of aggregation. This implies that different pathways are involved in the formation of aggregates. The primary role of the nuclear inclusions in the disease pathology remains a debate. Numerous reports have shown a positive correlation between increased aggregate formation, toxicity and cell death [72-74]. Others have found negative correlations and suggest that NIs are formed to protect the cells by enhancing the degradation of toxic polyglutamine-containing protein, and thereby reduce the risk of neuronal cell death [60; 75; 76].

Novel pathways and novel mutations

In addition to the "polyglutamine-induced toxicity and cell death hypothesis", results of micro-array expression studies in mouse models of Huntington's disease, another polyglutamine disease, and SCA1 have suggested that altered gene transcription may be an early consequence of polyglutamine-induced neurodegeneration [77; 78]. Research has shown that expanded polyglutamine proteins might alter gene transcription regulation via direct interactions with histone acetylases such as p300 and CREB proteins [79; 80]. Defective acetylating might be implicated as a cause of abnormal transcription and thus down-regulation of important genes.

The contribution of the non-coding SCA8, 10 and 12 repeat expansions in the pathology of ADCA remain to be established. Overexpression of either wild type or mutant SCA8 both induced similar neurodegeneration in the eye of *Drosophila*. Because both proteins have the same phenotypic outcome [81], this may just be the result of accumulation of the SCA8 protein in the cell, independent of the size of the repeat. How accumulation of the SCA8 protein might lead to neurodegeneration remains a question. In contrast, knockdown experiments of the SCA10 gene through sRNAi resulted in increased apoptosis in primary neurons, which might suggest a loss of function mechanism in the neuronal cells of SCA10 patients [82]. The SCA12 (*PP2R2B*) gene encodes a regulatory subunit of the serine threonine phosphatase, PP2A [16]. It has been suggested that the expression of PP2A is deregulated by the repeat expansion and may, in turn, alter the phosphorylation of particular PP2A targets such as PIP3 and ERK, which in turn may influence cell cycle or apoptosis events [83].

In contrast to the repeat expansion mutations, the most recent findings involve genes in which missense mutations were identified, i.e. the FGF14, a ligand for a tyrosine kinase receptor [41] and PRKCG (SCA14) proteins [9; 43-45]. Mice homozygous for disruption in the *Fgf14* gene develop ataxia and paroxysmal dyskinesia [84]. SCA14 mutations result in an increased kinase activity and altered membrane targeting in COS-7 cells [85]. These effects will probably lead to increased phosphorylation of downstream targets. Anomalous signal transduction appears to be at the core of SCA14 disease and, accordingly, other SCA mutations may also result in aberrant signaling.

MOUSE MODELS FOR ADCA

Suitable experimental in vivo models are required to study disorders that involve neuronal tissues. Some in vitro neuronal cell culture systems are available, like NGF-stimulated PC12 cells or isolated primary neurons from mice brain. However, rat or mouse neuronal precursor cells hardly resemble human neurons and primary neurons are very difficult to handle in culture, thus limiting their use in transfection studies. The experimental design of these cell lines is limited to RNAi knockdown systems or over-expres-

sion systems that often do not mimic the disease situation. In addition, brain material of deceased ADCA patients is rare, and when available it is often paraffin-embedded, which makes further protein or RNA work impossible. In the last decade, numerous transgenic or knockout mice have been generated for SCA1, 3, 7 and 14 that can be used as models to study cerebellar ataxias [86-90]. Mouse models are also available with mutations that affect the cerebellum. These Purkinje cell degeneration- (pcd), leaner-, weaner-, and stagger-mice display highly diverse pathohistological and motor-impaired phenotypes [91-94]. These mice provide a perfect opportunity to study the neuronal vulnerability of Purkinje cells and to examine therapeutic strategies that aim to reduce mutant transcript expression, decrease toxicity, and influence nuclear aggregate formation.

IDENTIFYING DISEASE GENES IN THE HUMAN GENOME

The accuracy and completeness of the human genome sequence is of crucial importance for all biomedical research. The Human Genome Project (HMG) was launched in 1990 with the goal of sequencing the complete euchromatic sequence of the human genome. In February 2001 the first draft of the human sequence was reported in Nature and Science by the International Human Genome Sequencing Consortium and Celera Genomics [95; 96]. Genes, or at least their coding regions, only comprise a very small fraction of the human genome, but are of major interest to biologists and geneticists. It is hoped that a list of all human genes and their encoding proteins will become available. The preliminary results of the HMG project reported a list of about 30,000-35,000 protein-encoding genes in the human genome, almost twice as many as in the fly or worm. However, the sequence was not completely finished and still contained ~150,000 gaps, and the order of many segments was unknown. So the draft was far from perfect but, just three years later, the human genome sequencing process was finished [97]. The overall human genome is ~3.08 Gb long, and now seems to contain only 20,000-25,000 protein-encoding genes. At least ~1,000 of these genes have already been associated with human diseases [98], but the remainder of the biologically relevant genes still have to be identified. The human gene map provides an excellent resource for identifying the disease genes from the SCA loci already identified.

Linkage analysis

Human disease gene mapping has been performed since 1937 [99], however, significant progress was really made in localizing disease genes when genetic maps of the human genome, containing DNA markers and restriction fragment length polymorphisms, became available in 1980 [100]. Since then, the common strategy to localize disease genes in extended families is to search genome-wide for marker alleles that co-segregate with the disease phenotype through the different generations of a pedigree. These marker locations point to the region(s) where the disease-causing gene(s) is located. The advantage of linkage analysis is that no prior knowledge about the function, location of the disease-causing gene, or the pathology of the disorder in question is needed. Unfortunately, the candidate intervals obtained through linkage analysis are usually large and contain too many interesting genes, so multiple strategies need to be used to refine a candidate interval. Testing of additional polymorphic markers or LD mapping strategies is not suitable when linkage is performed in only a few extended families. In addition, identifying more families that link to the same locus but share a smaller proportion of the disease chromosome may help to reduce the size of the candidate interval.

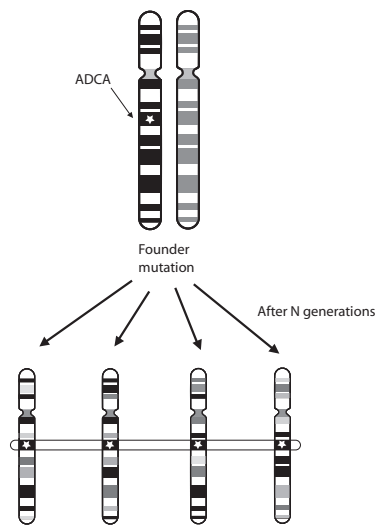


Figure 2
Schematic overview of occurrence and conservation of a founder mutation in a disease chromosome.

Shared haplotype analysis and founder mutations

Haplotype studies using closely linked microsatellites and/or intragenic single nucleotide polymorphisms (SNPs) have been used successfully to localize disease genes, and include genes identified in the Netherlands for benign familial cholestasis, diabetes type I, and infantile neuronal ceroid lipofuscinosis [101-103]. This strategy is based on the fact that seemingly unrelated or distantly related families will not only share a common founder mutation but also a conserved region surrounding the disease locus. A founder mutation is recognized when a disease mutation can be traced back to a common ancestor or founder (see Figure 2). Marker alleles surrounding the founder mutation will be in linkage disequilibrium (LD). LD describes a non-random distribution of alleles at different loci. In contrast, LD mapping can be used to identify genes without prior knowledge about the origin of the founder mutation [104]. The size of the LD interval resembles a time-window and helps to estimate when the mutation arose in a given population.

Founder mutations occur in many stable populations as well as in dynamic populations with a migratory history, such as the Netherlands. This, in combination with endogamy and a rapid population growth, may be the basis of founder mutations detected in the Dutch [105]. Founder mutations can easily be identified in religious, linguistic or geographic isolates, which mostly represent one extended family, and which may be county-specific or even European in extent. However, sometimes a mutation cannot necessarily be traced back to one common ancestor. Historical, regional and religious differences might have contributed to this effect.

In the past, the Netherlands was literally separated into two regions by its main rivers, the Maas and the Rhine that created a migration barrier between the north and south. The population in the eastern part of the Netherlands was also more homogeneous than in the cities in the west. This higher homogamy was probably the result of a less well-developed transport system and less influence from the immigrants who arrived in the harbors in the west. Religious groups were also important since religion determined education, friends, and partner choice. Provincial data from 1938-1941 showed that 89% of the Roman Catholics married a partner of their own religion [105]. As a result, the Dutch population has a relatively low genetic heterogeneity, which opens the opportunity to use the presence of shared chromosomal regions within small families for gene identification. Founders have already been identified for the SCA2, 3, 6 and 7 mutations in different ethnic populations, including the Dutch [52; 106-110]. Based on these results, there were probably founders for the other SCA loci for which no specific gene or associated mutation have yet been identified. This implies that independently referred ADCA families may actually be linked into larger families.

SCOPE OF THIS THESIS

Despite the fact that 11 SCA genes have already been identified, 30% of the ADCA families still remain undiagnosed. These so-called "SCA-negative" Dutch families are the basis of this research. The aim of this project was to collect genetic material from these Dutch families and to localize and identify novel SCA genes that cause ADCA. Linkage analysis was used to localize the disease-genes in two large Dutch ADCA families, in whom no mutations had been identified in the known SCA genes. After exclusion of the already identified SCA loci with two-point lod scores < -2, we performed genome-wide screens and identified two additional SCA loci in the Dutch ADCA population, SCA19 **(Chapter 2)** and SCA23 **(Chapter 3)**, respectively. However, the majority of the SCA-negative families are too small for traditional linkage analysis and an alternative approach was used to localize the disease genes in these families. This approach is based on the existence of founder mutations in the ADCA population, as illustrated for SCA3 and SCA6 **(Chapter 4)** and SCA14 **(Chapter 6)**. These results implied that independently referred ADCA families could actually be linked into larger families. To make use of this phenomenon, we set out to use a shared haplotype analysis (SHA) to localize and identify SCA genes from the known SCA loci, as shown for the SCA14 locus **(Chapter 5)**. In addition, we studied the implications of two SCA14 mutations on the cellular localization, translocation behavior, and activity of protein kinase C gamma in COS-7 cells. These results are described in **Chapter 7**. Finally, the implications of the results from these studies are discussed in **Chapter 8**.

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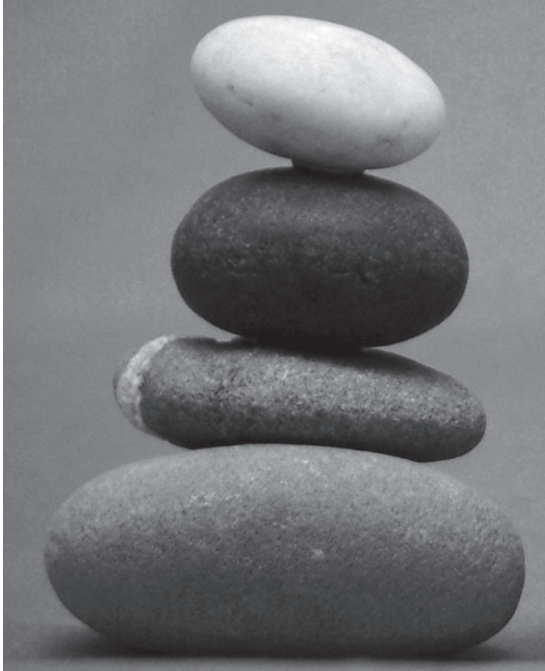
2

Identification of a novel SCA locus (SCA19) in a Dutch autosomal dominant cerebellar ataxia family on chromosome region 1p21-q21

Dineke Verbeek
Jurgen Schelhaas
Elly Ippel
Frits Beemer
Peter Pearson
Richard Sinke



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ABSTRACT

We present a linkage study in a four-generation autosomal dominant cerebellar ataxia (ADCA) family of Dutch ancestry. The family shows a clinically and genetically distinct form of ADCA. This neurodegenerative disorder manifests in the family as a relatively mild ataxia syndrome with some additional characteristic symptoms. We have identified a SCA19 locus, approved by the Human Genome Nomenclature Committee that can be assigned to the chromosome region 1p21-q21. Our mutation analysis failed to identify any mutations in the known spinocerebellar ataxia (SCA) genes and linkage analysis excluded the remaining SCA loci. We therefore performed a genome-wide scan with 350 microsatellite markers to identify the location of the disease-causing gene in this family. Multi-point analysis was performed and exclusion maps were generated. Linkage and haplotype analysis revealed linkage to an interval located on chromosome 1. The estimated minimal prevalence of ADCA in the Netherlands is about 3:100,000. To date, sixteen different SCA loci have been identified in ADCA (SCA1–8 and SCA10–17). However, mutation analysis has been commercially available only for the SCA1, 2, 3, 6 and 7 genes. So far, a molecular analysis in these SCA genes cannot be made in about one-third of the ADCA families. Thus, the identification of this new, additional SCA19 locus will contribute to expanding the DNA diagnostic possibilities.

INTRODUCTION

Autosomal Dominant Spinocerebellar Ataxia (ADCA) is one of the most frequently occurring subgroups of hereditary progressive neurodegenerative disorders. This serious and invalidating disorder is caused by a progressive neurodegeneration of the cerebellum, brain stem and spinal cord leading to a balance disturbance and loss of neurological functions. In addition to ataxia, various other symptoms such as tremor, epilepsy, retinal degeneration and mental retardation are seen. In general, the symptoms usually begin around the age of 40 years.

So far, a series of genes has been identified. Characterization of the first five spinocerebellar ataxia (SCA) genes, SCA1, 2, 3, 6, 7 [1-7] and the more recently identified SCA17 gene [8; 9], seem to point to a common underlying molecular defect, namely the involvement of a CAG repeat expansion in the coding region of the gene. However, these six genes do not show any homology in either structure or function, and the only common feature is the CAG repeat expansion. The CAG repeat expansions are translated into polyglutamine tracts leading to a so-called "polyglutamine" disease [10]. The initial CAG repeat number and the composition of the repeat (perfect or interrupted) [11] seem to be related to the expanded CAG repeat numbers. The repeat numbers of the

expansion are generally associated with the severity of the disease and the age of onset, often reported as intra-familiar anticipation.

Based on testing of the SCA1, 2, 3, 6 and 7 genes in all available Dutch ADCA families, we can conclude that mutations in these genes account for about 70% of the Dutch ADCA families. For the Netherlands, this indicates a minimal prevalence of the disorder of about 3:100,000, with the mutation in the SCA3 gene being found most frequently (44.1%), and followed by the SCA6 gene (23.4%) [12].The idea that the only causative component in the pathogenesis of ADCA is expanded polyglutamine tracts becomes more unlikely [13]. The analyses of SCA8, 10, and 12 [14-17] genes point to significant differences from the "polyglutamine" hypothesis. First, although the clinical characteristics between the different SCA genes show some overlap, there are a variety of additional neurological symptoms [18], including tremor and mental retardation.

Secondly, the mutations in these genes are also repeat expansions, but are non-coding CAG, CTG, and ATTCT repeats [14; 15; 19] instead of coding CAG repeats. How these non-coding repeat expansions contribute to the pathology of ADCA is not known. Thirdly, these mutations seem to be rare and have been described in only one or a few families. Some mutations seemed to be confined to specific populations [20] and new SCA genes still have to be identified to account for the remaining 30% of the Dutch ADCA families in which no mutation has been identified so far.

Here, we present a linkage analysis in a large Dutch ADCA family, in which no repeat expansion could be detected in the SCA1, 2, 3, 6, 7, 8, 12, and 17 genes. The remaining SCA loci (SCA4, 5, 10, 11, 13-14 and 16), for which mutations still have to be identified, [16; 17; 21-26] were excluded by 2-point linkage analysis [27]. We localised the disease gene involved in this family by performing a genome-wide screen. Significant evidence for linkage was found on chromosome region 1p21-1q21. The discovery of this novel locus, SCA19, adds to the extensive genetic heterogeneity exhibited by the ADCA phenotype.

MATERIALS AND METHODS

The patients

The family used in this study was described by Schelhaas et al. [27]. Briefly, clinical investigation showed a relative mild ataxic syndrome with some additional characteristic symptoms, including cognitive impairment, bad performance in the Wisconsin Card Sorting Test, myoclonus and postural irregular tremor of low frequency. Harding's classification [28] characterises the phenotype in this family as ADCA type I. There were too few affected individuals in this family to draw firm conclusions about anticipation. Although, a decrease in age of onset is seen, this is not accompanied by an increase in severity of the disorder [27]. Sixteen family members were included in the clinical inves-

tigation and 11 were diagnosed as affected. There were no asymptomatic obligate carriers. There was also no indication for sex-limited transmission since both male-to-male and male-to-female transmissions were observed.

Mutation analysis of known SCA genes

High molecular weight genomic DNA was extracted from peripheral blood leukocytes using a routine salting-out method. Diagnostic analysis failed to identify any mutation in one of the known SCA genes (SCA1-3, 6-8, 12, and 17) (data not shown). Subsequently, linkage analysis with specific markers excluded the remaining known SCA loci (SCA4, 5, 10, 11, 13, 14 and 16) [27]. Since point mutations have been described in the SCA6 gene [29], this locus was also excluded by linkage analysis using the intragenic marker D19S1150. The markers used in this linkage study were selected from the Marshfield database: http://www.marshmed.org/genetics_Markers.

Genome-wide genotyping analysis

In an attempt to localise the disease-causing gene, a genome-wide genotyping analysis was performed after excluding all the known SCA genes and loci. The linkage mapping set consisted of 350 microsatellite markers, covering all autosomes, with an average spacing of 10-15 cM. Additional markers were selected to narrow down possible candidate regions. All markers were 5'end-labeled with fluorescent-dyes: 6-FAM, TET or HEX. PCR reactions were performed in a total volume of 10 ml, containing 25 ng of genomic DNA, 1 ml PCR buffer II (Applied Biosystems, Foster City, CA, USA), 25 mM MgCl2, 2 mM dNTP's, 12.5 ng of each marker and 0.4 U of AmpliTAQ Gold (Applied Biosystems, Foster City, CA, USA). The PCR reaction started with a 10 min denaturation step at 94°C, followed by 33 cycles of 30 s denaturation (94°C), 30 s annealing (55°C) and 30 s extension (72°C), ending with a final extension step of 30 min at 72°C. The PCR was carried out in GeneAmp PCR system 9600 or 9700 machines (Applied Biosystems, Foster City, CA, USA). 0.9 ml of the PCR product was mixed with 1.6 ml loading buffer containing formamide, Blue Dextran, and GS500XL, the internal lane standard (Applied Biosystems, Foster City, CA, USA) and analysed on a 6% polyacrylamide gel (Longranger Single Packs, BioWhittaker Molecular Applications, Rockland, ME, USA) in an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA). Fragment analysis was performed using Genescan (v 3.1) and Genotyper (v 2.1) software.

Linkage and haplotype analysis

In the initial screen, two-point lodscores were calculated for each microsatellite marker using MLINK [30] of the FASTLINK (v 5.2) software package, assuming autosomal dominant inheritance, a disease frequency of 1:100,000, 95% penetrance. In the linkage analysis equal allele frequencies were used for all markers. All affected individuals (n=11) and the five unaffected individuals older than 50 years were included in the initial linkage analysis. Two-point lodscores of 1.0 and higher were considered to indicate regions of interest. These candidate regions were tested by additional markers and haplotype analysis for further fine mapping. Finally, a multi-point analysis was performed for all autosomes using Genehunter v 2.0 [31].

RESULTS

Linkage analysis

Five potential candidate regions, with two-point lodscores of 1.0 or higher, were obtained after testing the whole linkage screening set, involving loci on chromosomes 1, 7, 9, 10 and 12. Significant linkage of the disease (lodscore >3.0) was found only with the microsatellite marker D1S534 with a maximal two-point lodscore (Zmax) of 3.82 at a recombination fraction of 0.00 (Table 1). In addition, a multi-point analysis was performed for each autosome. Figure 1 shows the multi-point analysis for chromosome 1. The other possible candidate regions located on chromosomes 7, 9, 10 and 12 were excluded by both multi-point and haplotype analysis (data not shown).

Haplotype analysis of chromosome 1

Flanking markers of D1S534 were tested and although high, positive lodscores were obtained, none exceeded the Zmax of 3.82 (Table 1). The chromosome 1 candidate interval is defined by two recombination events in one affected person (III: 10) (Figure 2). These two recombination events, proximal at D1S1588 and distal at marker D1S1595, occurred on opposite sides of the centromere.

The marker order in this interval, based on the Marshfield database (October 2001), is: D1S1588 – 0 cM - D1S435 – 3.86 cM - GATA124C08 – 1.97 cM - GATA45B07- 5.54 cM - D1S1631 – 12.32 cM - D1S1675 – 2.68 cM - D1S534 – 1.71 cM - D1S2696 – 1.15 cM – D1S442– 6.31 cM - D1S1595. All the affected individuals share the same haplotype for the candidate interval flanked by D1S1588 and D1S1595, whereas none of the unaffected individuals showed this haplotype. The size of the interval, which harbours the disease gene in this family, is approximately 35 cM (Figure 2).

DISCUSSION

We performed a genome-wide screen in a four-generation Dutch ADCA family. The disease in this family manifests as a relatively mild ataxia syndrome with some additional characteristic symptoms [27]. Given the presence of the main clinical characteristics, including pyramidal signs, peripheral neuropathy and cognitive impairment, the phenotype in this family is classified as ADCA type I [28]. Our marker analysis reveals significant linkage with marker D1S534 with a maximal two-point lodscore (Zmax) of 3.82 at recombination fraction 0.00. The candidate interval is defined at the proximal boundary by marker D1S1588 and at the distal end by marker D1S1595. Multi-point and haplotype analysis of the chromosome 1 markers revealed a candidate interval of approximately 35 cM. This SCA19 locus, approved by the Human Genome Nomenclature Committee, can be assigned to the chromosome region 1p21-q21.

We used a haplotype sharing approach in an attempt to further narrow down the disease gene location of the SCA19 locus, and we are currently searching for additional ADCA families exhibiting linkage with the same region. However, at this moment, we only have two additional large Dutch ADCA families available for linkage studies. In these families no linkage could be detected (data not shown), indicating the presence of at least one other SCA locus in the Dutch population. In addition, we are searching for possible candidate genes within the candidate interval, by first focusing on CAG repeat containing genes. Mutation analysis is now being performed for the *KCNN3*, *TNRC4* and *KIAA0467* genes. Secondly, genes encoding ion channels may be

Table 1
Two-point lodscores between the disease locus and 13 chromosome 1 markers.

	Lodscore at recombination rate (theta)						
Markers	**0.00**	**0.10**	**0.20**	**0.30**	**0.40**	**Zmax**	**theta =**
D1S551	-0.29	-0.10	0.07	0.09	0.04	0.09	0.28
D1S435	-6.04	-0.38	-0.01	0.11	0.11	0.13	0.35
D1S1588	-3.91	0.17	0.28	0.26	0.15	0.29	0.22
GATA124C08	2.34	1.89	1.39	0.84	0.31	2.34	0.00
GATA45B07	3.02	2.46	1.83	1.14	0.41	3.02	0.00
D1S1631	2.10	1.68	1.23	0.73	0.23	2.10	0.00
D1S1675	1.94	1.60	1.22	0.79	0.31	1.94	0.00
D1S534	3.83	3.15	2.40	1.55	0.63	3.83	0.00
D1S2696	3.23	2.94	1.99	1.26	0.49	3.23	0.00
D1S442	1.46	1.19	0.89	0.56	0.22	1.46	0.00
D1S1595	-3.43	1.16	1.02	0.67	0.24	1.16	0.11
D1S1679	0.60	0.69	0.54	0.32	0.10	0.71	0.06
D1S1677	-4.78	0.27	0.34	0.28	0.16	0.34	0.19

of particular interest, because of the presence of myoclonus and tremor in this family. Other genetic disorders exhibiting myoclonus and tremor also carry mutations in ion channels, including epilepsy [32], Episodic Ataxia 2 (EA2) and SCA6 [33]. The SCA6 gene, encoding the a1A subunit of a voltage-dependent calcium channel, *CACNA1A*, was originally identified as being involved in familial hemiplegic migraine (FHM) [34]. Interestingly, distinct types of CACNA1A mutations have been identified in FHM and SCA6 [35]. A second familial hemiplegic migraine (FHM) locus has been localised to chromosome 1q21-q23 [36]. In parallel with the *CACNA1A* gene, candidate genes for FHM located on the long arm of chromosome 1 are also direct candidate genes for SCA19, although no migraine has been recorded in the affected individuals of the current family. Such candidates include the *KCNN3*, *KCNC4*, *KCNJ10*, *KCNA2* and *KCNA3* genes.

Following identification of SCA7, four novel SCA genes were defined (SCA8, 10, 12, and, 17). Approximately 200 individual Dutch ataxia patients have been tested but revealed no mutations (data not shown) in the SCA10, 12, or 17 genes. An exception was the SCA8 gene in which a repeat expansion was detected in several cases (data not shown). However, non-pathogenic polymorphisms in the SCA8 gene have also been de-

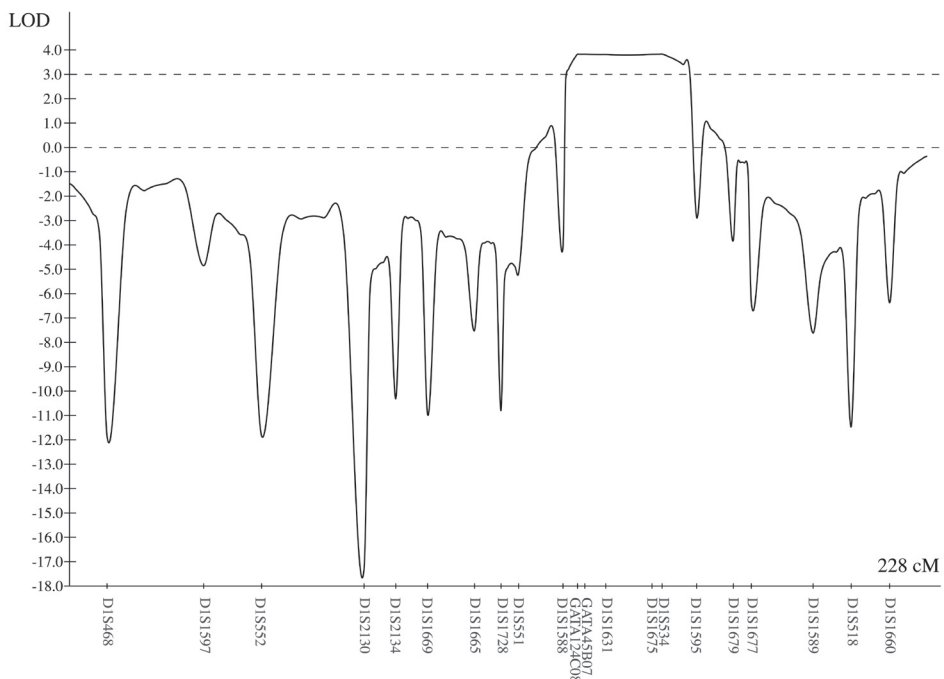

Figure 1

Multi-point linkage analysis for 21 chromosome 1 markers, covering the entire chromosome. Marker D1S1588 and marker D1S1595 flank the candidate interval.

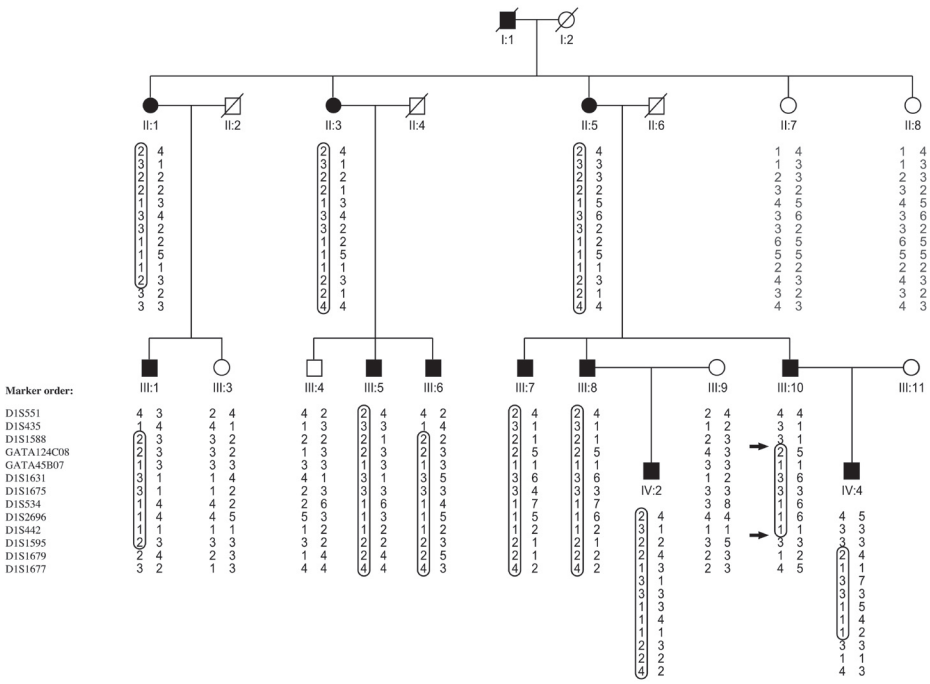

Figure 2

Haplotype analysis for 13 chromosome 1 markers. The putative disease haplotype is boxed. Arrows indicate the informative recombination events.

tected in normal controls and secondly, additional SCA8 expansions have been identified in patients carrying mutations in one of the other SCA loci [37]. The relation between SCA8 mutations and the etiology of ADCA must still be established. The frequency of these novel identified genes seems to be so rare that none can be the main causative mutation for the other 30% of Dutch ADCA families. However, our identification of the mutation involved in the SCA19 locus will improve the diagnostic possibilities and genetic counselling of some of the remaining ADCA families, and it will provide further insight into the controversy of whether expanded polyglutamine tracts are the main causation in the pathology of the ADCAs.

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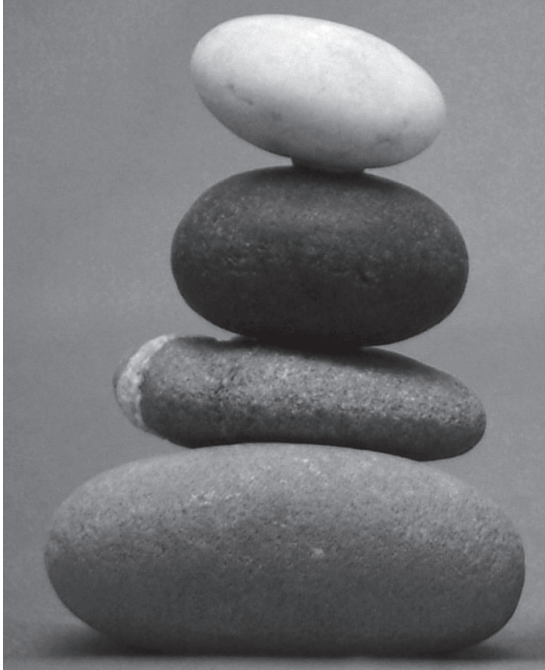
3

Mapping of the SCA23 locus involved in autosomal dominant cerebellar ataxia to chromosome region 20p13-12.3

Dineke Verbeek
Bart van de Warrenburg
Pieter Wesseling
Peter Pearson
Berry Kremer
Richard Sinke



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ABSTRACT

We report upon a Dutch autosomal dominant cerebellar ataxia (ADCA) family, clinically characterized by a late-onset (> 40 years), slowly progressive, isolated spinocerebellar ataxia (SCA). Neuropathological examination in one affected subject showed neuronal loss in Purkinje cell layer, dentate nuclei, and inferior olives, thinning of cerebellopontine tracts, demyelination of posterior and lateral columns in the spinal cord, as well as ubiquitin-positive intranuclear inclusions in nigral neurons that were considered to be Marinesco bodies. Data obtained from the genome-wide linkage analysis revealed a maximal lod score of 3.46 at theta = 0.00 for marker D20S199. This new SCA locus, on chromosome region 20p13-p12.3, was designated SCA23 after approval by the HUGO Nomenclature Committee. Currently, candidate genes are being screened for mutations within the SCA23 interval. In addition to the recently identified SCA14, SCA19, and FGF14 families, SCA23 is yet another novel SCA locus in the Dutch ADCA population, which further defines the genetic heterogeneity of ADCA families in the Netherlands.

INTRODUCTION

The autosomal dominant cerebellar ataxias (ADCAs) are a group of seriously invalidating disorders characterized by gait and limb ataxia, disturbances of speech and oculomotor control, in combination with variable other clinical features and usually with an adult age at onset [1]. Harding's classification of ADCAs into type I-III, based on the differential clinical features, is gradually being substituted by a classification based on molecular-genetic characteristics.

Since 1993, eleven spinocerebellar ataxia (SCA) genes have been cloned (SCA1-3, 6- 8, 10, 12, 14, 17 and fibroblast growth factor-14 (*FGF14*)) and an additional twelve SCA loci (SCA4, 5, 11, 13, 15, 16, 18- 21, 24 and 25) have been identified (HUGO nomenclature committee: http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl) [2-27]. In SCA1-3, 6, 7, and 17, the mutational mechanism is known to be a repeat expansion of coding CAG stretches leading to elongated polyglutamine tracts in the otherwise unrelated encoded proteins. However, in the SCA8, 10, and 12 genes, the mutation involves a non-coding CTG, ATTCT, and CAG repeat expansion, respectively [9; 11; 28]. Interestingly, the most recently identified mutations in the *FGF14* gene and the SCA14 (*PRKCG*) gene turned out to be missense mutations [19; 29-32].

Based on results of diagnostic testing of ADCA families, the estimated prevalence of ADCA in the Netherlands is about 3 per 100,000 individuals [33]. In approximately 30% of the Dutch ADCA families no repeat expansion is identified in the SCA1-3, 6, and 7 genes. So far, the SCA10, 12 or 17 repeat expansions have not been

reported in the Netherlands and the contribution of FGF14 mutations remains to be established. In addition, the contribution of the SCA14 mutations is currently being explored further in our laboratory. So far, the SCA14 mutations seem to be restricted to the Cys2 region of the C1 domain indicating a particular role for this part of the gene or the mutations that were identified. Still, we hypothesize that yet unidentified SCA genes are present in Dutch ADCA families.

Here, we present a Dutch ADCA family, clinically characterized by a late-onset (> 40 years), slowly progressive, isolated spinocerebellar ataxia. Linkage analysis localized the disease gene to chromosome region 20p13-p12.3, and this locus was designated SCA23 (approved by the HUGO Nomenclature Committee). The identification of SCA23 further adds to the genetic heterogeneity of Dutch ADCA families.

METHODS

The patients

The pedigree of the three-generation family that participated in this study is shown in Figure 1. In order to maintain confidentiality, some individuals are depicted with the symbol sex unknown. All participating family members signed an informed consent. The study was approved by the University Medical Center Utrecht (Utrecht) Medical Ethical Committee. DNA samples were available from 9 affected individuals and 5 presumptive unaffected relatives. Five of the affected family members were subjected to a thorough neurological examination. One asymptomatic carrier was detected.

Neuropathology and immunohistochemistry

The brain of subject II:11, who died in 1996 at the age of 80 years, was available for neuropathological examination and immunohistochemical studies. After formalin-fixation and external neuropathological examination, the brain and spinal cord were examined at cut surface and multiple samples were taken and embedded in paraffin for histology. The following tinctorial stainings were performed on 4 mm histological sections of these samples: hematoxylin and eosin (H&E), Bodian silver, and a combined Luxol Fast Blue and H&E (LFB-H&E) staining. In addition, on sections of relevant areas (brain stem, spinal cord, cerebellum, hippocampus) immunohistochemical stainings were performed for ubiquitin (DAKO Cytomation, Heverlee, Belgium), tau (Innogenetics, Gent, Belgium), Amyloid β (DAKO Cytomation, Heverlee, Belgium), a-synuclein (Neomarkers/Labvision, Fremont CA, USA), 1C2 (Chemicon International, Temecula, CA, USA), and ataxin-3 [34].

Mutation analysis of known SCA genes

High molecular weight genomic DNA was isolated from peripheral blood leukocytes using routine salting-out procedures. Direct mutational analysis of trinucleotide repeat expansions of the SCA1-3, 6-8, 12, and 17 genes was performed, but no repeat expansions were observed (data not shown). The other SCA loci (SCA4-6, 10, 11, 13, 14, 16, and 19) were excluded by two-point linkage analysis (lod score < -2.0). The polymorphic markers used were selected from the Marshfield database: http://research.marshfieldclinic.org/genetics/Map_Markers/maps/IndexMapFrames.html.

Genome-wide genotyping analysis

After excluding the known SCA loci as candidates, a genome-wide scan was performed. The screening set consisted of 350 polymorphic markers, covering the whole genome. The average spacing between two adjacent markers was 10-15 cM. The protocol used to amplify the polymorphic markers has been described previously [22]. Multiplex PCR products with HEX and FAM or HEX and TET labels were pooled. Finally, 1 ml of these pools were mixed with 4 ml HIDI including the internal lane standard ROX (Applied Biosystems Foster City, CA, USA), and run on an automated high-throughput capillary electrophoresis ABI3700 machine (Applied Biosystems Foster City, CA, USA). Subsequently, fragment analysis and genotyping were performed using Genescan (v3.5 NT) and Genotyper (v2.1) software. Two raters scored all genotypes independently.

Linkage analysis

Two point lod (logarithm of the odds) scores were calculated for each marker with the program MLINK [35] of the software package FASTLINK (v5.2), using an affected-only strategy assuming an autosomal dominant mode of inheritance, a disease frequency of 1:100,000, and equal allele frequencies of the markers. Two-point LOD scores higher than 1.0 were considered to indicate regions of interest. These genomic regions were further fine mapped by testing additional markers and haplotype analysis. With the program LINKMAP of the LINKAGE (v5.2) package, a multi-point analysis was performed to confirm the size of the candidate region.

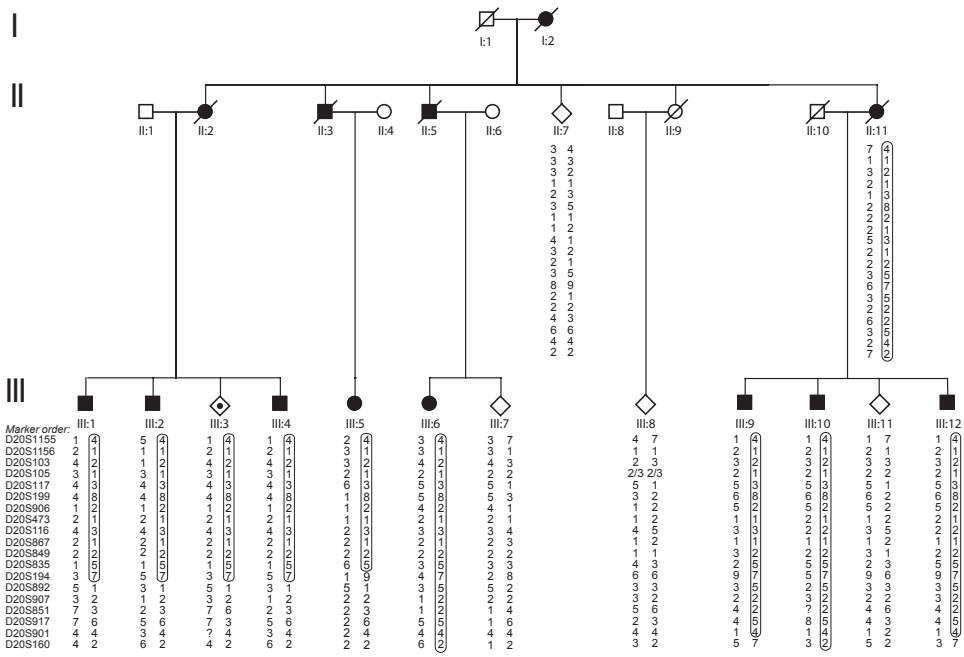

Figure 1

The pedigree of the SCA23 family. Haplotype analysis is shown for 19 chromosome 20 markers. In order to maintain confidentiality, some individuals are depicted with the symbol sex unknown. The disease haplotype is boxed. Open figures = unaffected; closed figures = affected; dotted figures = asymptomatic carrier; square = male; circle = female; diamond = unknown sex; / = deceased.

RESULTS

Clinical characteristics

In the five family members examined, the age at onset ranged from 43 to 56 years (mean 50.4 ± 4.9 years) and disease duration varied from 1 to 23 years (mean 10.2 ± 8.4 years) (Table 1). The presence of clinical anticipation could not be studied because we were only able to examine affected subjects from the second generation. The age at onset of individual II:11, of whom blood and brain tissue was preserved, could not be accurately extracted from the clinical records. Gait difficulties were the presenting feature in three, while cycling difficulties and a simultaneous deterioration of gait and speech were the presenting signs in two other subjects. After onset of disease, all displayed a slowly progressive, isolated spinocerebellar ataxia. In addition to gait and/or limb ataxia, which were present in all subjects, the neurological examination revealed dysarthria in three, slowing of saccades and ocular dysmetria both in three, decreased vibration sense below the knees in three, hyperreflexia in four, and Babinski's sign in two affected subjects.

Clinical severity grossly correlated with the duration of the disease; subject III:4, with a disease duration of 23 years, was wheelchair-bound outdoors. Cognitive deterioration or mental retardation, epilepsy, extrapyramidal features, signs of peripheral nerve involvement, or sphincter abnormalities were not present. Neurophysiological studies were not conducted. An MRI-scan of the brain in subject III:4 at the age of 60 years showed severe cerebellar atrophy, normal brainstem structures and basal ganglia, no cerebral cortical atrophy, and multiple small subcortical white matter lesions in the cerebral hemispheres that resembled vascular disease.

Neuropathology

The brain weight was 930 g (normal for age 1100-1400 g). Macroscopically, the cerebrum, cerebellum, brain stem, and spinal cord showed generalized, moderate to severe atrophy. The atrophy was most pronounced in the frontotemporal region of the cerebrum, the vermis of the cerebellum, the basis pontis, and the spinal cord. On cut surface, marked dilatation of the cerebral ventricles was found with moderate, generalized atrophy of the central nuclei. The substantia nigra and locus ceruleus showed normal pigmentation.

Microscopically, pronounced neuronal loss was present in the Purkinje cell layer, especially in the vermis (Figure 2A and B), and in dentate nuclei and inferior olives, accompanied by variable gliosis and myelin loss in the surrounding white matter. In the basis pontis, both the longitudinal and transverse white matter tracts were well myelinated, but the latter (i.e. cerebellopontine) tracts were relatively small. No neuronal loss was observed within the pontine nuclei. The substantia nigra and locus ceruleus contained an ample number of pigmented neurons with sparse loose neuromelanin pigment and an occasional Lewy body in the substantia nigra. In the spinal cord, especially in the posterior and lateral columns moderate, dispersed loss of (staining for) myelin was found without inflammatory infiltrate or gliosis; there was no clear loss of motorneurons.

In the tau-staining, some micro- and astroglial cells in and around central nuclei, dentate nuclei, and substantia nigra were positive. Vascular damage was present in the form of moderate to severe atherosclerosis of the circle of Willis and its major branches, with large recent ischemic necrotic areas in the left insular region (max. 8cm; territory of the left medial cerebral artery) and left occipital lobe (max. 5 cm; territory of left posterior cerebral artery), and dispersed old ischemic necrotic lesions (cerebral white matter, hypothalamus, cerebellar cortex and white matter, basis pontis; max. 1 cm). General autopsy revealed severe cachexia, emphysema, contractures of especially the lower limbs, and severe atherosclerosis of the large and middle-sized arteries with dispersed old infarcts in kidneys and spleen, myocardial hypertrophy, and atheromatous plaques in the pulmonary artery (the latter indicating pulmonary hypertension).

Table 2

Clinical characteristics of affected family members (M = male, F = female, AO = age-at-onset, G = gait difficulties, C = cycling difficulties, S = speech difficulties, - = absent, ± = subtle, + = present or mild, ++ = moderate, +++ = severe, = increased, N = normal).

Patient	Sex	Age (yrs)	AO (yrs)	Disease duration (yrs)	Presenting symptom	Gait ataxia	Limb ataxia	Dysarthria	Saccade slowing	Ocular dysmetria	Disturbed distal vibration and position sense	Tendon reflexes	Babinski's sign
III:6	F	62	53	9	G	++	++	-	+	-	++	↑	-
III:1	M	61	56	5	G/S	+	+	+	+	+	++	↑	+
III:2	M	64	51	13	G	+	+	+	-	-	-	N	-
III:4	M	66	43	23	G	+++	+++	+++	+	+	++	↑	+
III:12	M	50	49	1	C	-	±	-	-	±	-	↑	-

Linkage analysis

The SCA loci were excluded in the initial analysis with two-point lod scores (data not shown). Next, a genome-wide screen was performed and only two genomic regions showed two-point lod scores of >1.0, including regions on chromosome 20 and 22. In addition, analysis of our data might be hampered by the presence of possible asymptomatic mutation carriers, an affected-only strategy was performed. This resulted in a maximal lod score (Zmax) of 3.46 at theta = 0.00 for marker D20S199 (Table 2). The order and distance between the markers are based on the Marshfield database from April 2002 (http://research.marshfieldclinic.org/genetics/).

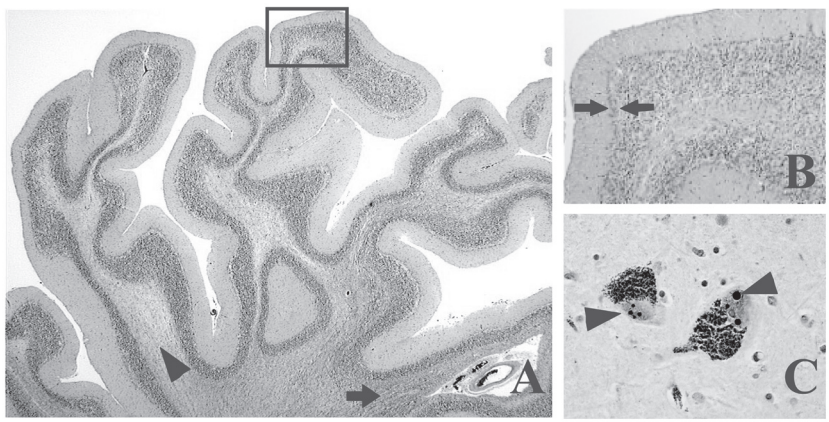

Figure 2

Neuropathological findings. The cerebellar folia, especially of the rostral vermis (A,B), show severe atrophy with (sub)total loss of myelin in some parts (lighter staining, loosely textured area of white matter, indicated by arrowhead in A) and relative preservation of myelin in other parts of the white matter (darker staining for myelin in more compact area, indicated by arrow in A). Panel B represents a higher magnification of the area indicated by the rectangle (A) illustrates severe Purkinje cell loss, accompanied by Bergmann gliosis in the affected cerebellar cortex (arrows in b). Occasional neurons in the substantia nigra contain up to four round, intranuclear inclusions in (arrowheads in c, max. diam. 5 mm), these are strongly ubiquitin positive but negative in the 1C2 and ataxin-3 staining and therefore interpreted as Marinesco bodies. No pathological neuronal intranuclear inclusions were encountered in the CNS of this patient. A,B: LFB-H&E staining; C: Ubiquitin staining; Original magnification x 12.5 (A), x 100 (B) and x 400 (C).

Haplotype and multi-point linkage analysis

Haplotypes were constructed by minimizing the number of possible recombination events (Figure 1). The candidate interval directly starts from the tip of the short arm of chromosome 20, because no recombination events were detected with the most proximal marker D20S1155. A recombination event was observed in affected individual III:5 with marker D20S194 located at 18.2 cM which determined the lower boundary of the interval. Multi-point linkage analysis (Figure 3) based on the affected-only strategy was performed and resulted in a Zmax of 3.56. Due to computer restraints the multi-point analysis was calculated with three markers at the same time.

The overlapping parts of the interval showed identical values, indicating that the analysis was performed correctly. The 95% candidate interval (max lod –1) from the multi-point analysis is flanked by marker D20S1155 and marker D20S835, and spans around 15 cM.

Candidate gene analysis

The SCA23 interval is located between the tip of the short arm of chromosome 20 and D20S194 and is approximately 18.2 cM in size, corresponding to ~6.1 Mb, and contains around 100 genes. Our first attempt focused on candidate genes that contain coding triplet repeats. This resulted in the identification of two coding (CTG)5/6 stretches in the GFR receptor alpha 4 (*GFRA4*), and the Attractin (with dipeptidylpeptidase IV activity, (*ATRN*)) gene. Only one coding CAG repeat was identified, that was located in the ubiquitin conjugating enzyme 7 interacting protein 3 gene. Four other noncoding intronic CAG repeats were located within the boundaries of the genes including Beta-Neoendorphin-Dynorphin precursor (*PDYN*; (CTG)7), KIAA1442 protein (*Q9P2A6*;

Table 2

Two-point lodscores between the disease locus and 11 chromosome 20

	Lodscore at recombination rate (theta)						
Markers	**0.00**	**0.10**	**0.20**	**0.30**	**0.40**	**Zmax**	**theta =**
D20S1155	2.22	1.82	1.36	0.84	0.3	2.22	0.00
D20S103	1.48	1.19	0.85	0.49	0.15	1.48	0.00
D20S117	2.49	2.06	1.56	0.99	0.38	2.49	0.00
D20S199	3.46	2.82	2.1	1.32	0.5	3.46	0.00
D20S473	0.39	0.34	0.25	0.14	0.04	0.39	0.00
D20S867	1.66	1.2	0.75	0.36	0.09	1.66	0.00
D20S835	2.02	1.68	1.25	0.76	0.27	2.02	0.00
D20S194	-4.57	0.53	0.46	0.26	0.07	0.53	0.10
D20S907	-0.08	-0.04	-0.02	-0.01	-0.01	-0.01	0.30
D20S901	1.27	1.08	0.8	0.48	0.16	1.27	0.00
D20S160	-6.50	-1.01	-0.45	-0.17	-0.04	-0.04	0.40

(CTG)5), ring finger protein 24 (*RNF24*; (CTG)5), FK506 binding protein peptidyl prolyl cis trans isomerase (*FKBP1A*; (CAG)7 , and signal-regulatory protein beta-1 precursor (*SIRP-beta-1*; (CAG)6). Four other CAG and CTG repeats with a maximal length of 5 repeats were identified, but it remained unclear whether these repeats are coding or non-coding sequences. However, no repeat expansions for any of these genes were observed (data not shown).

Secondly, only three other genes were selected interesting for missense mutation screening including neuronal cell death inducible kinase (*SKIP3*), major prion protein precursor (*PRNP*), and the prion gene complex, downstream (*PRND*). Since, over-expression of Doppel (Dpl) the paralog of the mammalian prion protein (*PrP*) is correlated with ataxia and death of cerebellar neurons in mice. All three genes were excluded as candidates based on our sequence data results.

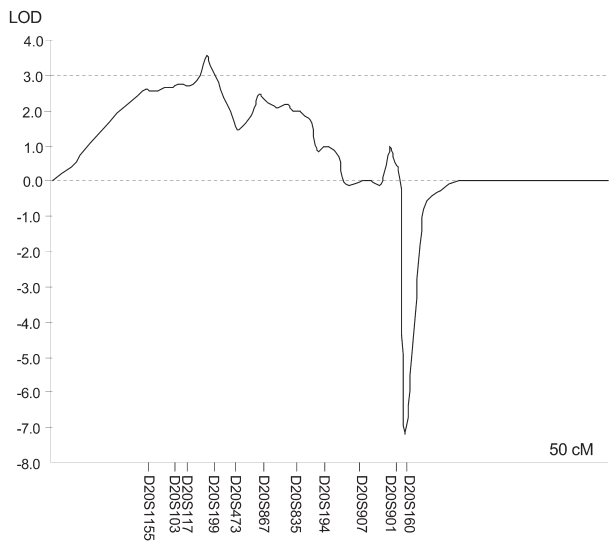

Figure 3
Multi-point analysis with eleven chromosome 20 markers.

DISCUSSION

The genome scan in this 3-generation Dutch ADCA family resulted in the identification of a novel SCA locus, designated SCA23, on chromosome region 20p13-p12.3. Clinically, the affected family members displayed a late-onset (> 40 years), slowly progressive, isolated spinocerebellar ataxia. Features mainly included gait and limb ataxia, disturbance of oculomotor control, dysarthria, and hyperreflexia. As distinctive extracerebellar disease features are absent, the SCA23 phenotype is clinically indistinguishable from other SCA subtypes, because relatively pure cerebellar syndromes have also been described in SCA5, SCA6, SCA11, SCA14, SCA15, SCA16, and SCA22 [36]. Genome-wide linkage analysis revealed a maximal two-point lod score (Zmax) of 3.46 at theta = 0.00 with marker D20S199. The SCA23 interval is located between the tip of the short arm of chromosome 20 and D20S194 and is approximately 18.2 cM in size, corresponding to ~6.1 Mb, and contains around 100 genes. Our candidate genes analysis was unable to identify the SCA23 disease-causing mutation. We focused on candidate genes that contain coding triplet repeats, since this is the most common mutation in the ADCAs worldwide. We searched for missense mutations in genes that correlated with ataxia and death of cerebellar neurons in mice, considering the fact that the two most recently identified SCA mutations were point mutations.

The neuropathological findings in subject II:11 grossly correspond to previous observations in this group of disorders, although we did not observe the neuronal intranuclear inclusions typically encountered in polyQ-associated ADCAs [36; 37] Some nigral neurons were found to contain ubiquitin-positive intranuclear inclusions, very much reminiscent of Marinesco bodies. In addition, Lewy bodies were occasionally present in nigral neurons. Substantia nigra involvement has been reported in SCA2 and SCA3 patients [38; 39]. Unfortunately, whether subject II:11 displayed additional parkinsonian features is unknown. Marinesco bodies are non-specific ubiquinated intranuclear inclusions in nigral neurons, observed in for example normal ageing and Alzheimer's disease. Interestingly, wild-type ataxin-3, the mutated protein in SCA3, was recently found to be recruited to Marinesco bodies in both nonhuman primates and in myotonic dystrophy patients [40; 41]. We did not find the Marinesco bodies in our case to be immunopositive for ataxin-3, but this may be due to the fact that different antibodies were used. How this finding of Marinesco bodies relates to the pathogenic processes involved in SCA23 and whether this also indicates failure of protein homeostasis mechanisms remains to be established, mainly by identifying the mutated gene product.

In conclusion, the SCA23 locus is a novel locus identified in the Dutch ADCA population. The candidate interval is located on chromosome region 20p13-12.3 and is approximately 18.2 cM. In an attempt to refine the size of the candidate interval, we are currently trying to identify additional ADCA families that also link to the SCA23 locus.

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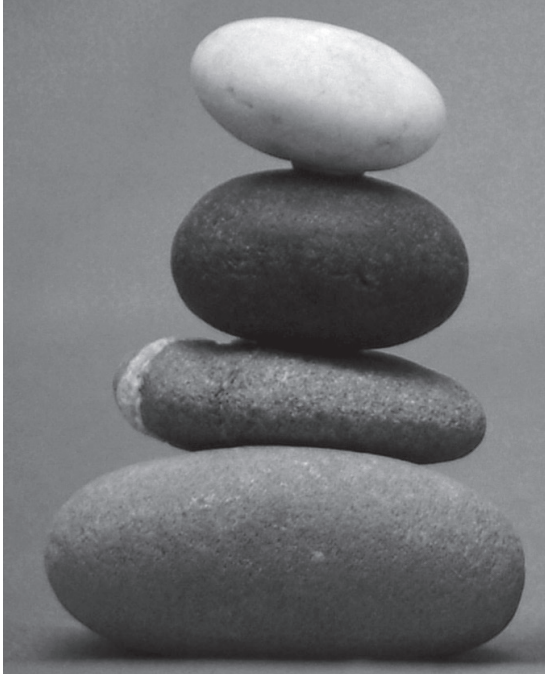
4

Haplotype study in Dutch SCA3 and SCA6 families: evidence for common founder mutations

Dineke Verbeek
Sytse Piersma
Eric Hennekam
Elly Ippel
Peter Pearson
Richard Sinke



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ABSTRACT

This pilot study was initiated to show the existence of founder effects in the Dutch autosomal dominant cerebellar ataxia population. The ADCAs comprise a clinically heterogeneous group of neurodegenerative disorders and the estimated prevalence in the Netherlands is approximately 3:100 000 individuals. Here, we focused on the SCA3 and SCA6 genes because mutations in these genes occur most frequently in the Netherlands. We were able to determine a common origin of the CAG repeat expansions in the majority of Dutch SCA3 and SCA6 families. Haplotype analysis and linkage disequilibrium (LD) studies with polymorphic markers revealed shared haplotypes surrounding the SCA3 and SCA6 genes. These results strongly suggest that ADCA families can be traced back to common ancestors in particular parts of the Netherlands.

INTRODUCTION

The autosomal dominant cerebellar ataxias (ADCAs) comprise a clinically heterogeneous group of neurodegenerative disorders, characterized by a progressive ataxic syndrome often accompanied by a wide range of additional symptoms including tremor, headache or epilepsy. The disorder is genetically heterogeneous: so far 10 SCA genes (SCA1-3, 6-8, 10, 12, 14 and 17) [1-9] have been isolated and characterized, while an additional 9 SCA loci (SCA4, 5, 11, 13, 15, 16, 18, 19, 21 and 24) [10-18] have been identified by linkage analysis. The estimated prevalence of ADCA in the Netherlands is approximately 3:100 000 individuals [19]. The ADCA cohort in Utrecht comprises families in whom in 70% of the cases the clinical diagnosis for SCA1, 2, 3, 6, or 7 could be confirmed by molecular genetic analysis.

Haplotype studies using closely linked microsatellites and intragenic single nucleotide polymorphisms (SNPs) provided evidence for the existence of founder mutations for the CAG repeat expansion mutations in the SCA2, 3, 6 and 7 genes in different ethnic populations [20-24]. So far, no such extensive haplotype study has been performed in the Dutch ADCA population. We focused on the SCA3 and the SCA6 genes to identify the presence of possible founder effects in the Dutch ADCA population. The SCA3 mutation occurs most frequently (44.1%) in the Dutch ADCA population followed by mutations in the SCA6 gene (23.4%) [19]. The strategy is based on the fact that seemingly unrelated or distantly related ADCA families will not only share a common mutation but also a conserved region surrounding the disease locus in which marker alleles will be in linkage disequilibrium (LD).

We were able to identify a common origin of the CAG repeat expansions in the majority of Dutch SCA3 and SCA6 families and these results implies that independently referred ADCA families may actually be linked to larger families.

SUBJECTS AND METHODS

The patients

Twenty-one apparently independent SCA3 families and 12 SCA6 families, as well as 32 isolated SCA3 patients and 25 isolated SCA6 patients were selected from the Utrecht ADCA cohort. High molecular DNA was isolated from peripheral blood by a routine salting out procedure. The clinical diagnosis was confirmed by a positive mutation analysis (data not shown). In order to be able to generate haplotypes, we genotyped at least one affected individual with both parents (when available) to determine for phase. We created simulated haplotypes with unknown phase for the isolated patients. In total we typed 77 SCA3 patients and 50 SCA6 patients. Control samples from healthy, anonymous non-related individuals and parents (total 80 haplotypes) were used to estimate the allele and haplotype frequencies of the markers.

Polymorphic markers

Polymorphic markers flanking the SCA3 and SCA6 genes were selected from the Marshfield database http://research.marshfieldclinic.org/genetics/ (see Figures 1a and 1b), with an average spacing of 1-2 cM. If no markers were available in the database to provide optimal spacing, we identified additional polymorphic repeats using a tandem repeats finder program available on the web [25]. The primer sequences of those markers are given in Table 1. The protocol used to amplify the polymorphic markers has been described previously [17]. The different marker alleles were annotated using the CEPH 133101. The alleles that characterize the SCA3 core haplotype are represented by: D14S997 allele 4, 263 base pair (bp); D14S617 allele 6, 161 bp/allele 7, 165 bp; D14S1015 allele 2, 252 bp; D14S1015 allele 6, 138 bp; D14S973 allele 3, 150 bp and D14S977 allele 11, 120 bp. For SCA6 the relevant alleles are: D19S906 allele 3, 158 bp; D19S1165 allele 3, 158 bp; D19S558 (134701) allele 3, 150 bp; D19S1150 (134701) allele 6, 152 bp; D19S840 allele 3, 206 bp.

After amplification the markers were pooled in equal volumes according to their respective size ranges and the color of the fluorescent label. The markers were run on a 3700 ABI machine 96 capillary automated sequencer (Applied Biosystems, Foster City, Ca). The pooled and diluted marker sample was added to a mixture of HiDi and the internal lane standard Rox 500 (ratio 100:1). Analysis, sizing and calling of the fluorescent fragments were performed with Genescan v3.1 and Genotyper v2.1 software. Haplotypes were generated with the program Genehunter v2.1 and checked manually. In total, we used 21 SCA3 and 12 SCA6 haplotypes with known phase and 32 SCA3 and 25 SCA6 haplotypes with unknown phase in our analysis.

Single nucleotide polymorphisms (SNPs)

We typed three intragenic SNPs; 669A/G, 987C/G and 118A/C using the SNaP-Shot method (Applied Biosystems, Foster City, CA) in the 21 SCA3 families. Amplification of the SNPs was carried out with a standard PCR and primers as described elsewhere [21]. The templates were purified by a post-PCR Shrimp Alkaline Phosphatase (SAP) and endonuclease 1 enzyme treatment. After inactivation of the enzymes used in the purification, an allele-specific PCR was performed and the products were purified once more. The resulting products were analyzed on the ABI 3700 and Liz 120 (Applied Biosystems, Foster City, Ca) was used as an internal standard.

Linkage Disequilibrium (LD)

We searched for differences in the overall distribution of alleles between pairs of markers within the SCA3 and SCA6 regions. LD was calculated with an extension of Fisher's exact probability test using the Arlequin v2.0 program. P-values less than 0.05 were considered statistically significant.

RESULTS

Haplotype analysis of SCA3

In total, fourteen markers from the SCA3 locus region were typed in 21 SCA3 families (Figure 1a). We observed a highly conserved core haplotype in 17 of the 21 SCA3 families between markers D14S995 and D14S973; 4-6/7-2-6-3 corresponding to an approximately 1.4 Mb genomic region surrounding the SCA3 gene. In addition, we observed a truncated form of this haplotype; 6/7-2-6-3 in four additional families. The marker D14S617, located in the middle of the core haplotype, showed a 6-allele or a 7-allele (ratio 1:1) on the SCA3 chromosome. This core haplotype was never observed in 80 control chromosomes of unrelated individuals with known phase. Interestingly, extended haplotypes, up to 6.2 Mb in size, (see Figure 1a: depicted in white, black and gray boxes) were observed in three groups of families. Significant LD ($p < 0.05$) was observed involving a block of markers flanked by markers D14S995 and D14S977 (see Table 2).

We able to create the core haplotype; 4-6/7-2-6-3 in 31% of the constructed alleles of 32 isolated SCA3 patients. Truncated haplotypes derived from this core haplotype could be observed in an additional 33.7% of the chromosomes (data not shown). We used isolated SCA6 patients to control and explore the specificity of this SCA3 haplotype in alleles with unknown phase. The core haplotype; 4-6/7-2-6-3 could only be constructed in 2% of the SCA6 chromosomes. Genealogical research was performed to assist in defining the origin of the core haplotype and was able to link ten independently referred SCA3 families into four clusters (see Figure 1a). Interestingly, the SCA3 families that showed the 6-allele for marker D14S617 were clustered in the eastern part of the

Netherlands (province of Drenthe) and the families with the 7-allele were clustered in the western part (province of South Holland, data not shown). Significant LD ($p < 0.05$) was observed involving a block of markers flanked by markers D14S995 and D14S977 (see Table 2).

SNP haplotype analysis in SCA3

In order to confirm the existence of the worldwide intragenic SNPs haplotype, described by Gaspar et al. (2001), in the Dutch SCA3 population, we typed the SNPs 669A/G, 987C/G and 118A/C in 22 SCA3 families. The A-C-A haplotype is highly conserved throughout all Dutch SCA3 chromosomes (data not shown).

Haplotype analysis in SCA6

Haplotypes were constructed for 12 SCA6 families with 13 markers (see Figure 1b) surrounding the SCA6 gene. Eight families showed a shared haplotype between the markers D19S1165 and D19S840; 3-3-6-22-3 including the SCA6 gene. This core haplotype was not observed in 80 control chromosomes. Three additional families did not show the core haplotype. Two of the three families had an identical extended haplotype and one did not show any similarity at all.

Genealogical research showed only that families F068 and F071 were related and that most of the SCA6 families are concentrated in the province of North Holland (data not shown). Significant LD results were observed around the SCA6 gene for intragenic marker D19S1150 with marker D19S840 ($p=0.024$) (Table 3). The core haplotype could be created in 36% of the putative disease chromosomes of 25 isolated SCA6 patients. Truncated haplotypes derived from the initial shared haplotype were also detected in 44% of the chromosomes (data not shown). The core haplotype; 3-3-6-3 was never observed in the sporadic SCA3 patients that were used as controls.

Table 1
Additional SCA6 markers.

Forward primer	Reverse primer
CCAGACTGGTGCTCTGAC	GTGACCAACTTGCCTCTCT
CTGTAACCCCAGGAGTTTGT	ACGGAGTTTCGCTTTTGTA
GGAGGAGGTAGGAGAGAAGG	CACATTTCCAGGGAGAG

DISCUSSION

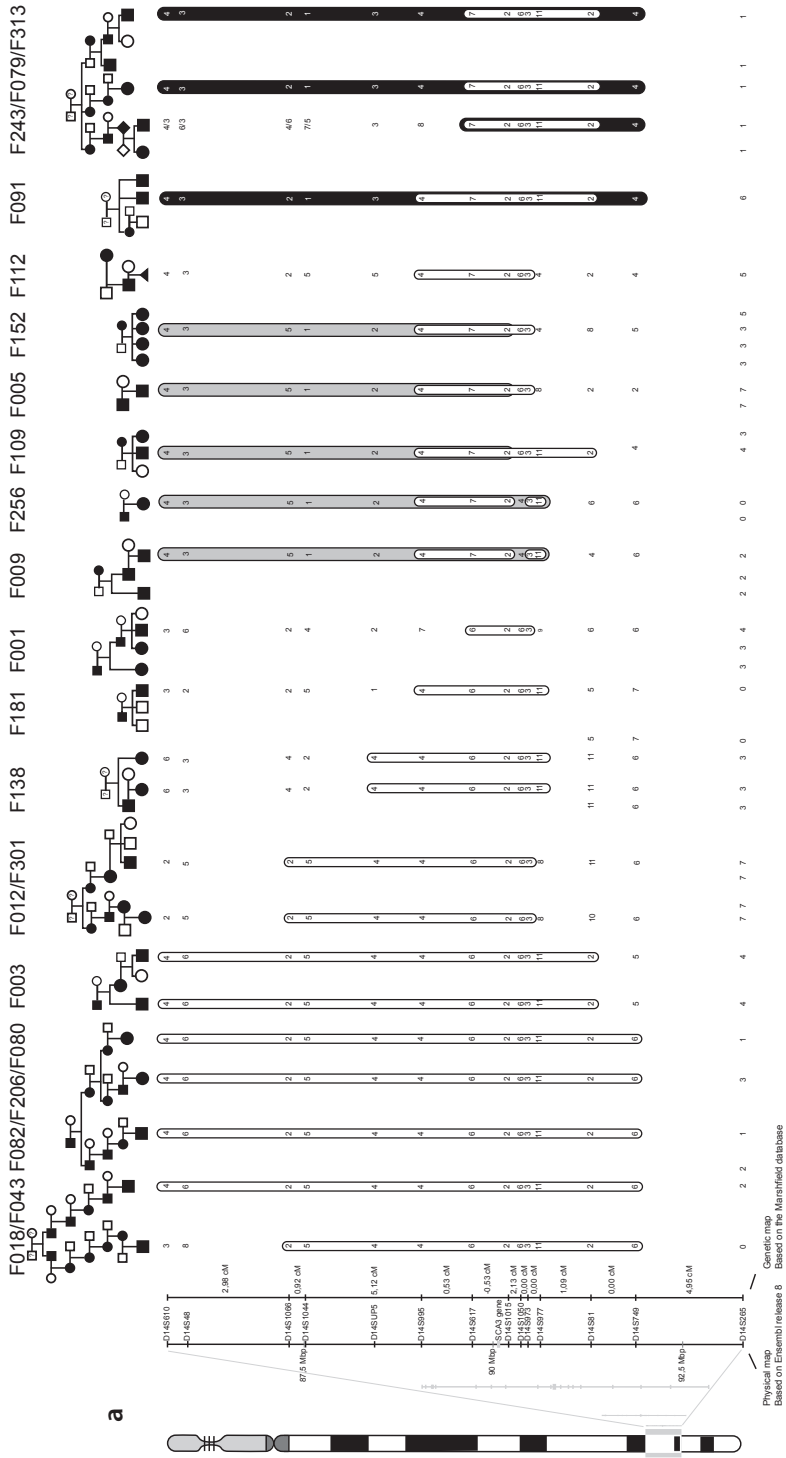
Founder mutations have been described previously in different ethnic ADCA populations. However, no such extensive shared haplotype study had been performed in the Dutch SCA3 and SCA6 families. We were able to demonstrate the presence of one major founder SCA3 mutation in the Dutch ADCA population. In addition, geographical clustering of most of these ADCA families in the similar province supports our findings and suggests that the ADCA families can be traced back to common ancestors in particular parts of the Netherlands

Interestingly, we noticed three groups of families with more extended haplotypes in the SCA3 region. This indicates that families within one haplotype group are more closely related than families from different groups, or than between families with less extended haplotypes. This was further confirmed by genealogical research; families from an extended haplotype group could be traced to a common link, whereas no link could be identified between families exhibiting haplotypes of different lengths. In addition, we were also able to detect the presence of the worldwide intragenic SNP haplotype in the Dutch SCA3 families. Not surprisingly, we observed significant LD between markers surrounding both the SCA3 and SCA6 genes. However, the LD data were less

Table 2

Linkage disequilibrium in SCA3 families between 14 pairs of markers. Significant LD (p = 0.05) is indicated by + and gray.

	D14S610	D14S1066	D14S1044	D14Sup5	D14S995	D14S617	D14S1015	D14S1050	D14S973	D14S977	D14S81	D14S749
D14S610	*				+		+					
D14S1066		*	+		+		+					
D14S1044		+	*			+						
D14Sup5				*		+						+
D14S995	+	+			*	+	+	+	+	+	+	
D14S617			+	+	+	*	+	+	+	+		+
D14S1015	+	+			+	+	*	+	+	+	+	
D14S1050					+	+	+	*	+	+		
D14S973					+	+	+	+	*	+		
D14S977					+	+	+	+	+	*		
D14S81	+				+		+				*	
D14S749				+		+						*


F018/F043 F082/F206/F080 F003 F012/F301 F138 F181 F001 F009 F256 F109 F005 F152 F112 F091 F243/F079/F313
a
D14S610
D14S48
D14S1096
D14S1044
D14SUP5
D14S935
D14S617
D14S1015
D14S1060
D14S977
D14S81
D14S749
D14S265
2.98 cM
0.92 cM
5.12 cM
0.53 cM
0.53 cM
2.13 cM
0.00 cM
0.00 cM
1.09 cM
0.00 cM
4.95 cM
87.5 Mbp
90 Mbp
SCA3 gene
92.5 Mbp
Genetic map
Based on the Marshfield database
Physical map
Based on Ensembl release 8

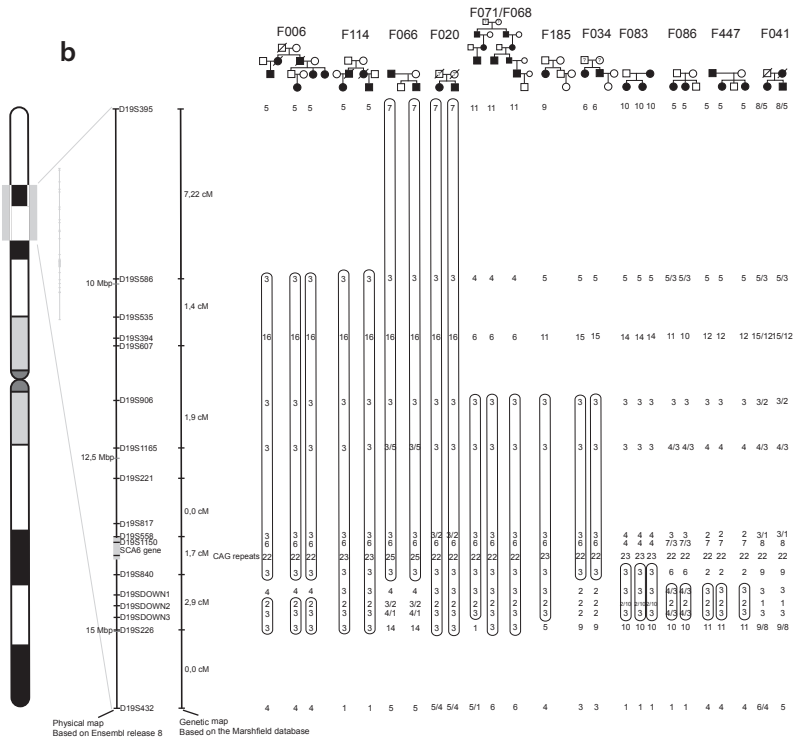


Figure 1

Shared haplotype analysis. a) Haplotype analysis SCA3 locus b) Haplotype analysis SCA6 locus.Thee markers were selected from the Marshfield database. The physical distances are based on the Ensembl release 8.0 and the genetic distances (Mb) are based on the Marshfield genetic map (cM). Only one affected chromosome per family is depicted in the figures. The shared haplotypes are boxed and the extended haplotypes are colored by white, gray and black.

convincing for SCA6, as a result of the small number of families, marker informativity and the more heterogenic background of the families. LD mapping within candidate regions can be a useful tool to localize diseas genes if it can be found, but in the absence of significant LD one can never be sure whether the disease locus has been missed. We observed multiple origins of the mutation for SCA6 in the Dutch population. However, one major founder haplotype accounted for approximately 70% of the SCA6 families. This finding was strengthened by regional clustering of most of the SCA6 families in the province of North Holland.

Intergenerational stability of the CAG repeat number has been considered to be a specific molecular feature of SCA6. To date, a limited number of reports have described meiotic instability of the SCA6 repeat [26; 27]. Nevertheless, we observed CAG repeat numbers ranging from 22, 23 and 25 repeats in families who belonged to the same haplotypic group. This might indicate that evidence for meiotic instability of the repeat can be found when there are sufficient numbers of generations between families. Genealogical research is an important tool in reconstructing the extended families needed to study meiotic instability.

Table 3

Linkage disequilibrium of 12 pairs of SCA6 markers. Significant LD (p = 0.05) is indicated by + and gray.

	D19S395	D19S586	D19S394	D19S906	D19S1165	D19S1150	D19S840	D19SDOWN1	D19SDOWN2	D19SDOWN3	D19S226	D19S432
D19S395	*	+	+									
D19S586	+	*	+									
D19S394	+	+	*		+						+	
D19S906				*	+							
D19S1165			+	+	*							
D19S1150						*	+					
D19S840						+	*					
D19SDOWN1								*		+		
D19SDOWN2									*			
D19SDOWN3								+		*		
D19S226			+								*	
D19S432												*

In conclusion, we have identified major common haplotypes for SCA3 and SCA6 families in the Dutch ADCA population. The combined use of both genealogical and genotype data allows linking of small pedigrees into extended families. This will be particularly useful for the identification of additional SCA-loci in the small ADCA families in which no mutation has been found yet.

ACKNOWLEDGEMENTS

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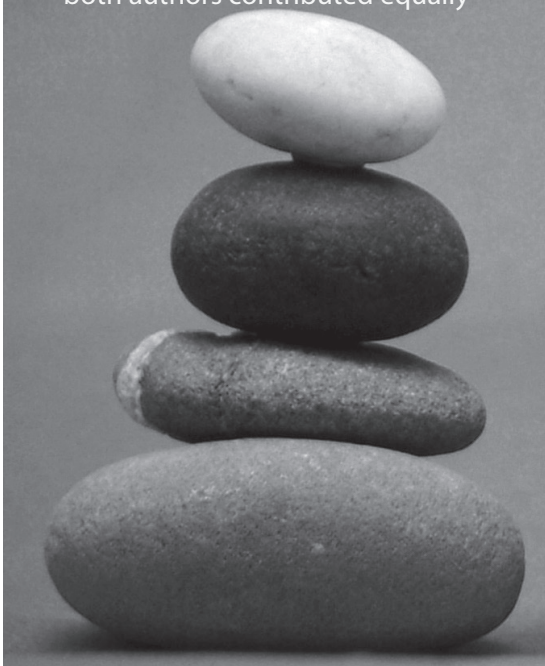
5

Identification of a novel SCA14 mutation in a Dutch autosomal dominant cerebellar ataxia family

Dineke Verbeek*
Bart van de Warrenburg*
Sytse Piersma
Eric Hennekam
Peter Pearson
Nine Knoers
Berry Kremer
Richard Sinke

* both authors contributed equally



Neurology (2003) 61: 1760-5

ABSTRACT

Objective To report a Dutch family with autosomal dominant cerebellar ataxia (ADCA) based on a novel mutation in the *PRKCG* gene. **Methods** We studied thirteen affected members of the six-generation family. After excluding the known SCA genes, a combination of the shared haplotype approach, linkage analysis, and genealogical investigations was used. Exons 4 and 5 of the candidate gene, *PRKCG*, were sequenced. **Results** Affected subjects displayed a relatively uncomplicated, slowly progressive cerebellar syndrome, with a mean age-at-onset of 40.8 years. A focal dystonia in two subjects with an onset of disease in their early twenties suggests extrapyramidal features in early-onset disease. Significant linkage to a locus on chromosome 19q was found, overlapping the SCA14 region. Based on the recent description of three missense mutations in the *PRKCG* gene, located within the boundaries of the SCA14 locus, we sequenced exon 4 and 5 of this gene and detected a novel missense mutation in exon 4, which involves a G->A transition in nucleotide 353 and results in a glycine-to-aspartic acid substitution at residue 118. **Conclusion** A SCA14-linked Dutch ADCA family with a novel missense mutation in the *PRKCG* gene was identified. The precise disease mechanism induced by these PRKCG mutations remains to be elucidated.

INTRODUCTION

A major part of the genetic background of autosomal dominant spinocerebellar ataxias (ADCAs) has been elucidated in the past decade [1]. Twenty-one genetic loci have been detected by linkage studies (SCA1-8, SCA10-19, and SCA21-23) and ten of the corresponding genes have been identified [2-21]. In approximately 60 to 70% of Dutch ADCA families, a mutation is found in the SCA1, SCA2, SCA3, SCA6, or SCA7 gene [22]. The other genes and loci seem to be rare or are confined to specific populations [23; 24]. Although the contribution of these genes to the Dutch ADCA population is unknown, novel mutations are likely present. We recently identified two novel loci in two Dutch ADCA families, SCA19 and SCA23 [18; 21]. In addition, a missense mutation in the *FGF14* gene on chromosome 13q34 was found in another Dutch family [25]. We reasoned that several of the Dutch mutation-negative ADCA families must have common origins and that, by combined linkage, haplotype, and genealogical analyses, we should be able to link some of the small ADCA families in larger clusters.

Here, we report a large six-generation Dutch ADCA family in which linkage to the SCA14 locus on chromosome 19q13.4-qter was found. Recently, missense mutations in exon 4 of the *PRKCG* gene that encodes protein kinase C γ (PKCγ) were reported in two ADCA families, one of which involved a SCA14-linked family reported

previously, and in one sporadic case [26; 27]. We therefore sequenced exon 4 and 5 of the *PRKCG* gene and identified a novel missense mutation (353G->A) in exon 4. This new family adds to the clinical and genetic characterization of PRKCG mutations in SCA14 patients.

SUBJECTS AND METHODS

After diagnostic screening, 24 Dutch ADCA families with affected individuals in multiple generations and without a mutation in the SCA1, SCA2, SCA3, SCA6, or SCA7 genes, were identified. By linkage, haplotype, and genealogical analysis (see below), three independently referred families (RF13, RF11, and RF17) were found to be linked and the pedigree of this combined large six-generation family is depicted in Figure 1 (only the affected individuals are shown). Of this extended family, seventeen family members from two generations were included in the study and all gave informed consent for additional research studies. In the past eighteen months, all the affected family members received a full neurological examination and a comprehensive case history was taken. When appropriate, neurological symptoms were graded as absent, mild, moderate, or severe. Blood samples (20 ml EDTA) were taken later.

Exclusion of the known SCA genes

Diagnostic screening was performed to exclude a mutation in the SCA1, SCA2, SCA3, SCA6, and SCA7 genes in the 24 families. High molecular weight DNA was obtained from leukocytes by a routine salting out procedure. No repeat expansions were found in the affected individuals. Further testing with the more recently identified SCA genes, including SCA8, SCA10, SCA12, and SCA17 was carried out and proved to be negative (data not shown). These 24 families were subsequently used in a combined linkage, haplotype, and genealogical approach.

Shared haplotype analysis

Because most of the 24 ADCA families were too small to perform traditional linkage analysis, we used an alternative strategy to localize the disease-causing genes in these families: the Shared Haplotype Analysis (SHA). This method is based on the assumption of a limited number of founder mutations in the Dutch ADCA families, which had already been observed in SCA3 and SCA6 families (unpublished data). For all 24 ADCA families, we focused on the remaining reported SCA loci, namely SCA4, SCA5, SCA11, SCA13, SCA14, SCA16, SCA18, SCA19, SCA21, and SCA23. Polymorphic markers within the candidate regions, with an average spacing of approximately 2.0 cM, were selected from the Marshfield database (see: http://research.marshfieldclinic.org/genetics/). The different marker alleles were defined using the CEPH 133101 control sample. Hap-

lotypes were constructed using the program GENEHUNTER (v1.2) and were corrected manually. We searched for identical haplotypes within the reported SCA loci in these small ADCA families. For each SCA locus, we created 100 control haplotypes from the genotypes of unrelated persons from a series of families collected for other purposes (data not shown). The alleles that characterize the SCA14-haplotype are represented by: D19S206 allele 7, 132 base pair (bp); D19S571: allele 1, 198 bp; D19S589: allele 2, 169 bp; D19S924: allele 3, 201 bp; D19S927: allele 2, 142 bp; D19S926: allele 4, 105 bp and D19S605: allele 2, 117 bp. Meanwhile, an exhaustive genealogical analysis was initiated to explore possible links between the apparently unrelated ADCA families that were included in the SHA, since other studies have shown that families in the Netherlands are often interrelated several generations back.

Linkage analysis

Two-point and multi-point linkage analyses were performed with the programs MLINK and LINKMAP programs of the LINKAGE package (v 5.2). In our model, we applied a disease frequency of 1.0 in 100,000 and equal allele frequencies. Both analyses were based on an affected-only strategy to avoid the problem with possible pre-symptomatic carriers.

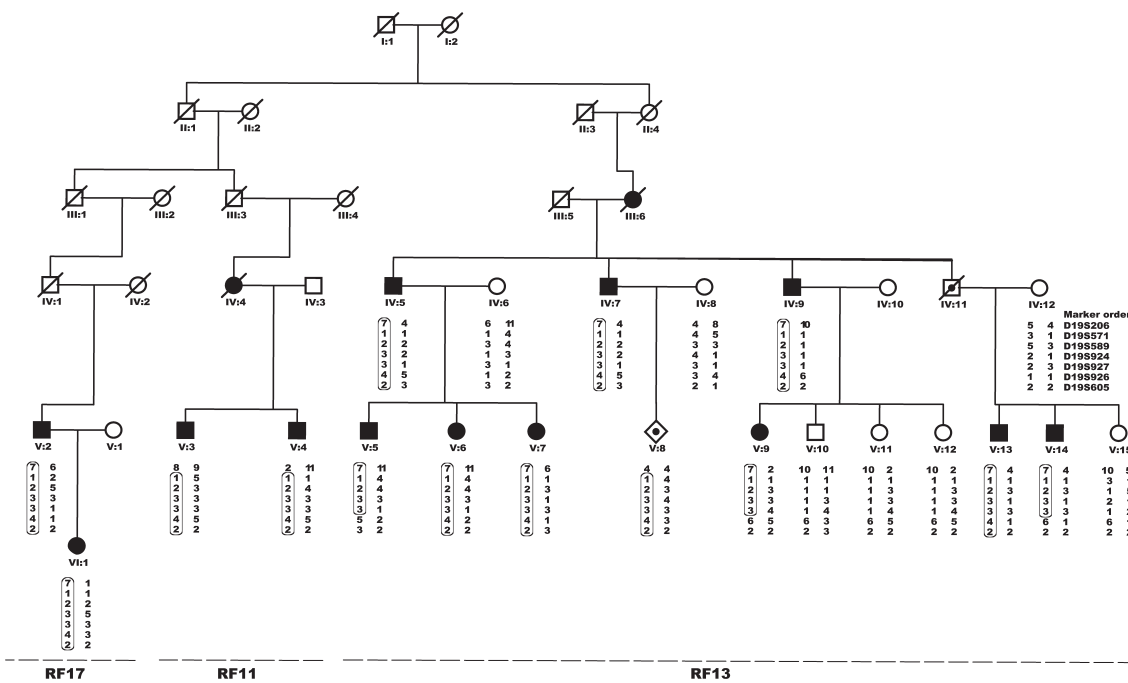


Figure 1

The pedigree of the combined family RF13, RF11, and RF17. Haplotype analysis for seven chromosome 19 markers is shown. The disease haplotype is boxed. Open figures = unaffected; closed figures = affected; dotted figures =obligate carrier; square = male; circle = female; diamond = unknown sex; / = deceased. In order to maintain confidentiality, unaffected family members are not shown.

Mutation analysis

Based on the recent report of three missense mutations in exon 4 of the *PRKCG* gene, we decided to screen exons 4 and 5 of this gene for mutations in affected individuals from all 24 families [26]. An additional 85 anonymous control individuals with no history of ataxia were also sequenced. Both exons were amplified using the primers described in the original report [26]. The template for the sequence reaction was amplified with Ampli Taq DNA polymerase, using a PCR mixture containing 10X Pol buffer (67 mM Tris-HCl, 6.7 mM MgCl2, 10 mM b-mercaptano-ethanol, 6.7 mM EDTA (pH=8.0) and 16.6 mM (NH4) 2SO4), 1.5 mM dNTP's, 0.15 mg/ml Bovine Serum Albumin, and 10% dimethylsulfoxide. The PCR protocol started with 4 minutes of denaturation at 94°C, followed by 35 cycles of 1 minute denaturation at 94°C, 1 minute of annealing at 56°C and 2 minutes of extension at 72°C, and ending with a final extension step of 7 minutes at 72°C.

PCR fragments were purified with Manu 30 Multiscreen PCR filter plate (Millipore, Danvers, USA.) and dissolved in 40 ul of water. DNA sequencing was performed using the Big Dyeä Terminator cycle sequencing ready reaction kit (Applied Biosystems, Warrington, UK). Sequence products were purified with the ManuN45 multiscreen sequence reaction filter plates (Millipore, Danvers, USA) and then were run on a 3100 ABI automated Sequencer (Applied Biosystems Foster City, CA, USA). Data were analyzed with Sequencing Analysis (v3.7) software.

RESULTS

Shared haplotype analysis

After all the markers were typed and haplotypes constructed, we identified a shared haplotype in three of the 24 ADCA families. In these families (RF13, RF17, and RF11), this shared haplotype covered almost the complete SCA14 locus. The genealogical investigations revealed that the RF17 and RF11 families could indeed be linked to the large RF13 family (see Figure 1). The closest common ancestor in these three families was identified four generations back. A partially shared haplotype, which corresponded only to the lower part of the SCA14 region between marker D19S924 and marker D19S605, was found in two other families (data not shown). However, the *PRKCG* gene turned out to be located outside this part of the haplotype. Therefore, this result was marked as false positive. The SCA14 haplotype was not observed in controls, where the allele frequencies were for marker D19S206: allele 7, 0.10; D19S571: allele 1, 0.38; D19S589: allele 2, 0.30; D19S926: allele 4, 0.18, allele; D19S927: allele 2, 0.41; D19S926: allele 4, 0.27 and D19S605; allele 2, 0.32, respectively.

Linkage analysis and haplotype analysis in the SCA14 locus

The positive results from the SHA were followed up and confirmed by multi-point linkage analyses in the extended six-generation pedigree (Figure 1). An affected-only strategy was used to perform the linkage analysis with seven markers in the SCA14 region. Significant linkage of 3.61 (two-point lodscore) at theta = 0.00 was found with marker D19S924 and a multi-point lod score (Zmax) of 4.56 was obtained. Finally, haplotype analysis determined the upper and lower boundaries of the SCA14 candidate interval by recombination events for markers D19S206 and D19S926 (see Figure 1). This resulted in a SCA14 candidate interval of approximately 9.54 cM and covering 10.2 Mb genomic DNA.

Mutation analysis

At the time we obtained our linkage results, three missense mutations in exon 4 of the *PRKCG* gene, located within the SCA14 region, were reported [26]. Accordingly, we sequenced exons 4 and 5 of the *PRKCG* gene in affected individuals from all 24 ADCA families. In the extended six-generation family, we identified a G–>A transition in nucleotide 353 resulting in a base pair substitution from glycine to aspartic acid, as depicted in Figure 2A, that replaces a non-polar for a charged polar amino acid. The Gly118Asp mutation is present in the C1B domain of the PKCγ protein. This C1B domain is highly conserved during evolution and the original glycine residue is present in many organisms (see Figure 2B). Subsequently, all related family members were sequenced and screened for the Gly118Asp mutation.

The mutation co-segregated completely with the disease phenotype in the family. In addition, two individuals were identified who also carried the mutation, which was in agreement with the haplotype analysis, but did they not show signs of the disease phenotype. Neither unaffected individual is depicted in Figure 1 in order to maintain confidentiality. No mutation was identified in any of the other 21 ADCA families. The Gly118Asp mutation was not detected in 85 control individuals (170 chromosomes), indicating that this change is unlikely to be a polymorphism.

Clinical characteristics

Thirteen family members of the extended six-generation family were found to be affected. The clinical features of affected family members are given in Table 1. The mean age-at-onset was 40.8 ± 10.7 years (range 21 to 59 years). One family member (V-8), with an age in the onset range, was thought not to have any symptoms, but on examination he was found to have difficulty in turning, an upper limb action tremor, and slight abnormalities in the heel-to-shin test. Findings were concluded to be too subtle to clearly indicate disease-onset. Life span appears to be unaffected, witnessed by the fact that three affected males are 80 years or older.

Striking age-at-onset variability was observed in two of the four parent-child groups studied. In one pair (V-2 and VI-1), the disease manifested 25 years earlier in the child. The other pair involves an asymptomatic male family member (IV-11) who died at the age of 56 years. His family did not recall any sign or symptom that suggested the presence of a cerebellar dysfunction at that age. However, the age-at-onset in his two sons (V-10 and V-11) was 21 and 24 years, respectively.

All subjects displayed cerebellar ataxia with a slowly progressive course, with a disease-onset after the age of 30 years in all but two subjects. The markedly earlier onset of the disease in subjects V-10 and V-11 was not accompanied by an increase in the rate of disease progression. A gait disorder was the presenting feature in all affected individuals. In addition to gait and limb ataxia, the neurological examination revealed cerebellar dysarthria in eleven subjects, slowing of saccadic eye movements in six and dysmetric saccades in seven subjects, and hyperreflexia in eight individuals. Two older subjects (IV-5 and IV-7) showed marked hyporeflexia (with absent Achilles tendon reflexes), as well

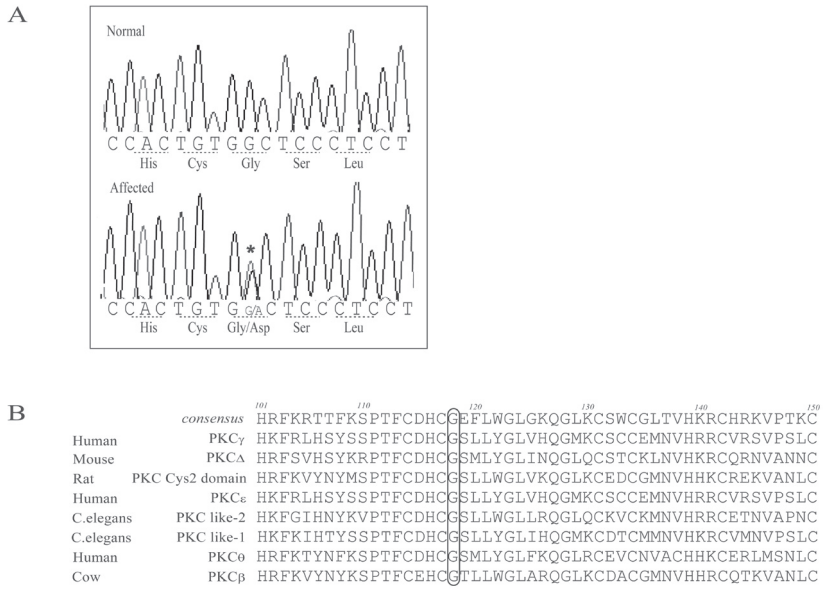


Figure 2

a) Sequence electropherograms for a part of exon 4 of the PRKCG gene. The upper panel shows the DNA sequence of a normal control. The DNA sequence of an affected individual (lower panel) shows a heterozygous mutation at base pair position 353G ->A (indicated by an asterisk). b) Evolutionary conservation of the Cys2/C1B domain at the glycine residue 118 (boxed) in different isozymes of PRKCG and different organisms.

as absent sense of vibration below the knees, which suggest the co-existence of a peripheral neuropathy, either age-related or disease-related. While hypotonia was not observed, individual V-7 showed rigidity of the upper limbs. A focal task-induced dystonia of the right hand (writer's cramp) was observed in the two brothers with relatively early-onset disease (V-10 and V-11). Individual IV-9 was very severely affected and completely immobilized, which prohibited a thorough neurological examination. However, bradyphrenia and the presence of frontal release signs could indicate additional diffuse involvement of the cerebral cortex. Cognitive decline or mental retardation was not encountered in other family members and no family member showed signs of autonomic disturbances, axial myoclonus, parkinsonism, tremor, or seizures. A MRI-scan had been made in three family members (V-2, V-6, and VI-1) and showed marked atrophy of cerebellar vermis and hemispheres (Figure 3), while no cortical atrophy, basal ganglia abnormalities, or white matter changes were observed.

DISCUSSION

The Dutch SCA14 family reported here, displayed a slowly progressive, relatively isolated spinocerebellar ataxia, with a mean age-at-onset of approximately 40 years. In addition to the cerebellar syndrome, hyperreflexia was frequently observed. In two older subjects, the clinical examination was suggestive of a peripheral neuropathy, but whether this represents a true disease characteristic or an age-related feature remains unclear. In two other subjects, the onset of disease in their early twenties and the presence of a focal task-induced dystonia of the right hand in both of them is noteworthy. Whether the dystonia is truly attributable to the disease itself remains to be established, but in this light it should be recalled that in the first SCA14 family reported by Yamashita et al., axial myoclonus was observed in five early-onset subjects [14]. Taking the upper limb rigidity in one of the family members reported here also into account, suggests that extrapyramidal features (in early-onset disease) could indeed be part of the phenotypic spectrum of SCA14. In addition, agu rats, which carry a homozygous nonsense mutation in the *PRKCG* gene that results in the complete absence of the catalytic domain of the PKCγ protein, display a parkinsonian phenotype [28]. Thus, *PRKCG* gene mutations appear to lead to pathology in both the cerebellar and the extrapyramidal pathways.

In this extended family, clinical anticipation was suggested in two of the four parent-child combinations. In addition, the family pedigree details of one of the other SCA14 families also suggest anticipation [14; 26]. However, caution is needed, because the number of patients for studying the anticipation phenomenon is too small, the recorded age-at-onset is known to be affected by a recall bias in older individuals, and, regarding this specific missense mutation in the *PRKCG* gene, the biological correlate of anticipation, commonly an expanded trinucleotide repeat, is lacking. The absence of

the usual indicator of anticipation also costs further doubts on the anticipation being present. We will therefore use the term 'onset age variability', which is remarkable in this SCA14 family, where the age-at-onset ranges from 21 to 59 years. The descriptive characteristics of the other SCA14 families reported also reveals onset ranges of 10 to 51 years and 12 to 42 years, respectively [14; 27]. This clinical variability may be a reflection of true (but unexplained) anticipation, or of problems in assessing the age-at-onset, or it may reflect modifying genes in a common genetic background instead of anticipation via the SCA14 locus.

Three ADCA families, originally believed to be independent, were found to be linked to a common ancestor four generations back and, with the shared haplotype analysis, a common haplotype covering the SCA14 locus was identified in these three families. Significant linkage in the combined family to the SCA14 locus was confirmed by multi-point linkage (Zmax = 4.56) and haplotype analysis. The boundaries of the SCA14 region were determined by recombinant events with the markers D19S206 and D19S926, providing a candidate interval of approximately 9.54cM. At that time, mutations in the *PRKCG* gene were reported to be found in two SCA families and in one sporadic case [26]. Interestingly, one of these families is partly of Dutch ethnicity. Based on these results, we screened exon 4 and 5 of the *PRKCG* gene and identified a novel

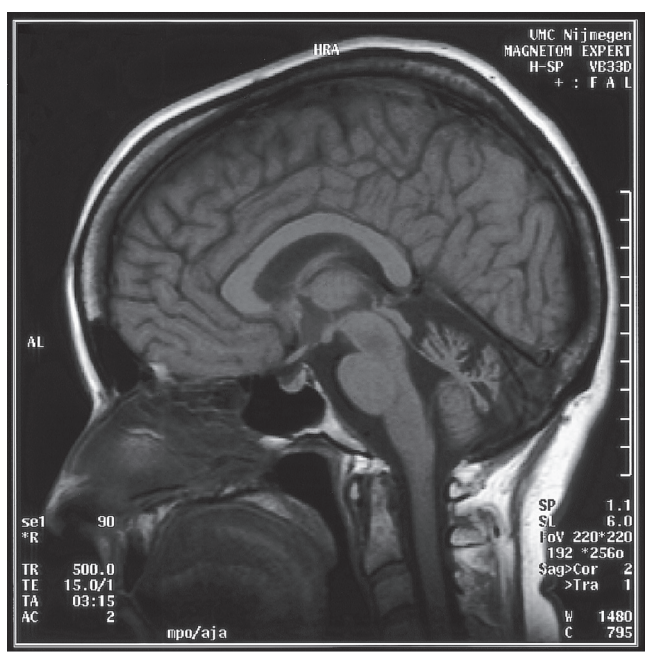


Figure 3
T1-weighted sagittal MRI scan of brain, showing marked atrophy of cerebellar midline structures.

Table 1

Clinical characteristics of affected family members (M = male, F = female, AO = age-at-onset, G = gait difficulties, C = cycling difficulties, - = absent, + = mild, ++ = moderate, +++ = severe, ND = not determinable, = increased, ¯ = decreased, N = normal.

Patient	Sex	Age (yrs)	AO (yrs)	Disease duration (yrs)	Presenting symptom	Gait ataxia	Limb ataxia	Dysarthria	Saccade slowing	Ocular dysmetria	Disturbed distal vibration and position sense	Tendon reflexes	Additional signs
IV-5	M	84	45	39	G/C	+++	+	+++	++	+	++	↓	
V-5	M	59	40	19	G	++	++	++	-	+	+	N	
V-6	F	56	45	11	G	++	+	-	++	++	+	↑	Rigidity arms
V-7	F	52	40	12	G	++	++	+	++	++	-	↑	
IV-7	M	80	45-50	30-35	G	++	++	++	+	-	+++	↓	
IV-9	M	81	47	34	G	+++	+++	+++	ND	ND	ND	↑	Frontal release signs
V-9	F	54	43	11	G	++	+	-	-	-	-	N/↓	
V-10	M	42	21	21	G	++	+	+	+	-	-	↑	Focal dystonia
V-11	M	45	24	21	G	++	+	+	-	-	-	↑	Focal dystonia
V-3	M	64	52	12	G	+	+	+	-	++	-	↑	
V-4	M	61	33	28	G	++	++	+	+	++	-	↑	
V-2	M	64	59	5	G	++	++	+	-	+	+	N	
VI-1	F	50	34	16	G	++	+/++	+	-	-	+	N/↑	

missense mutation in exon 4 that involves a G->A transition in nucleotide 353, which predicts a glycine-to-aspartic acid substitution (Gly118Asp) and replaces a non-polar for a charged polar amino acid. The mutation co-segregated with disease in all affected family members. The subject who showed too subtle cerebellar signs on examination to clearly indicate disease onset, also carried the mutation. In addition, the mutation was detected in two individuals, in whom the presence or onset of cerebellar disease was or could not be established.

The *PRKCG* gene encodes PKCγ, an isoform of protein kinase C, which is a member of the family of serine/threonine kinases. These kinases are intermediates in second messenger signaling pathways and are involved in various cellular processes. PKCγ, one of the classical isoforms, is built up of a regulatory and a catalytic domain. The regulatory domain comprises a C1 subdomain, containing two tandem repeat cysteine-rich regions (C1A and C1B), and a C2 subdomain [29]. The three missense mutations reported (His101Tyr, Ser119Pro, and Gly128Asp), as well as the Gly118Asp mutation we report here, are all located in the evolutionary conserved Cys2-region of the C1 regulatory domain, illustrating the important role that mutations in this specific region must play in cerebellar degeneration [26]. Two mutations seem to directly alter PKCγ function, as shown by protein-structure modeling studies, mainly by affecting zinc ion interaction and phorbol ester binding affinity [26; 30]. How this eventually results in the spinocerebellar degeneration remains to be elucidated, but the fact that PKCγ protein levels are reduced in Purkinje cells of SCA1 transgenic mice and that in one patient with the PRKCG mutation the majority of Purkinje cells did not show ataxin-1 staining, points to a potential role of PKCγ in the ataxin-1 pathway [26; 31]. To address this issue, we investigated whether the age-at-onset in the affected family members correlated with the length of the CAG repeat on both alleles of the SCA1 gene, but no significant correlation was found (data not shown). A mutation in the SCA1, SCA2, SCA3, SCA6, or SCA7 gene is found in 60 to 70% of Dutch ADCA families [22]. The identification of this SCA14 family will further contribute to the clarification of the genetic background of the remaining ADCA families.

It is of particularly interest that we were able to link three originally independent ADCA families by a combined linkage, haplotype, and genealogy analysis. This confirms our suspicion that there may only be a limited number of independent SCA mutations within the Dutch ADCA families. This assumption is reinforced by our unpublished results, which show a clear geographic distribution of given SCA mutations confirming close temporal and geographical links for many families. Although we were fortunate to be able to reconstruct such a large family as in this example of SCA14, the future challenge will be to obtain similar results with much smaller ADCA families.

While the mutations in the first SCA genes identified are characterized by a tri- or pentanucleotide repeat expansion that probably leads to a toxic gain of protein function, it is missense mutations that have been found in the two most recent SCA genes. This opens up a new perspective for neuroscientists in the further unraveling of the complicated disease mechanisms of dominantly inherited spinocerebellar degenerations.

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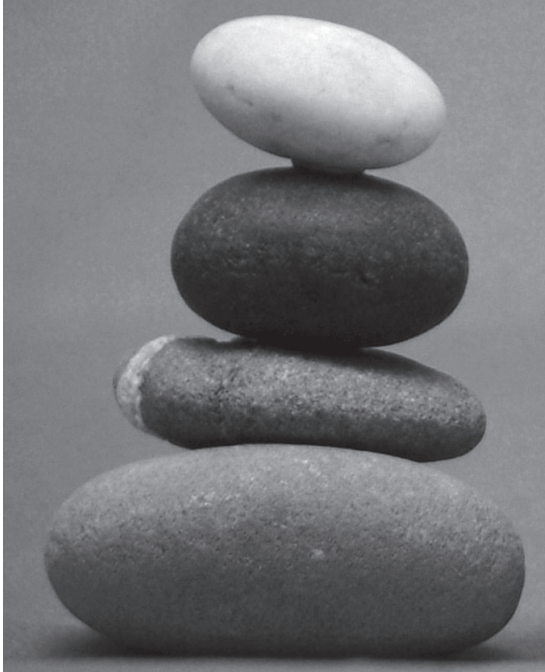
6

Gly118Asp is a SCA14 founder mutation in the Dutch ataxia population

Dineke Verbeek
Bart van de Warrenburg
Eric Hennekam
Dennis Dooijes
Elly Ippel
Corien Verschuuren-Bemelmans
Berry Kremer
Richard Sinke



Human Genetics (2005), in press

ABSTRACT

Missense mutations in the *PRKCG* gene have recently been identified in SCA14 patients; these include the Gly118Asp mutation that we found in a large Dutch ADCA family. We subsequently screened the current Dutch ataxia cohort (approximately 900 individuals) for SCA14 mutations in the Cys2 region of the *PRKCG* gene. We identified the Gly118Asp mutation in another eight individuals from five small families. Haplotype analysis identified a shared chromosomal region surrounding the SCA14 gene, and genealogical research was able to link all these ADCA patients to a single common ancestor. We therefore confirmed that the Gly118Asp mutation is a SCA14 founder mutation in the Dutch ADCA population.

INTRODUCTION

Spinocerebellar ataxia 14 (SCA14) is one of the autosomal dominant cerebellar ataxias (ADCAs) and is characterized by a slowly progressive, more or less isolated cerebellar syndrome that evolves after the age of 20 years. Interestingly, the first reports describe disease-causing missense mutations in the strongly conserved Cys2 region of the C1 domain of the protein kinase C γ (*PRKCG*) gene in families of Dutch/English, Dutch, and Japanese ancestry [1-4]. These findings demonstrate that SCA14 is present in different ethnic populations and displays a heterogeneous mutation spectrum. The effect of these mutations on the activity and function of the kinase is yet unknown.

In 2002, a national survey estimated the prevalence and frequency of the SCA1, 2, 3, 6 and 7 mutations in the Dutch population to be 3.0 per 100,000 inhabitants [5]. However, a molecular diagnosis could be made in only about two-thirds of the ADCA families, and the remaining one-third will therefore likely be due to novel or unknown SCA genes. After the identification of the SCA14 mutations, we decided to sequence the current panel of Dutch ADCA patients without a known genotype, for mutations in the Cys2 domain of the *PRKCG* gene, in order to reveal the frequency and heterogeneity of SCA14 mutations in the Dutch ADCA population. We were able to link eight new patients from five small families to the large family (RF13, RF11 and RF17) we had already identified [2]. Clinical characteristics were obtained for six of these new patients (Table 1).

MATERIALS AND METHODS

Mutation analysis

Because all SCA14 mutations reported, at that time, were located in the Cys2 region of the C1 domain, we sequenced exons 4 and 5 of the *PRKCG* gene for mutations as described previously [2].

Genealogical research

Subsequently, we performed genealogical research to see if we could cluster the SCA14 patients to particular regions of the Netherlands and whether we could link together some of the independently referred families. The data for the genealogical research included: (1) family data forms completed by patients, (2) population registries for 1853-1920, (3) Dutch civil registries of births, marriages and deaths (1811-2004, www.genlias.nl), (4) church archives with baptism, marriage and death registers (before 1811), (5) notary archives, and (6) citizen books for various towns (1500 – 1799).

Shared haplotype analysis

To estimate the size of the conserved SCA14 chromosomal region throughout the family (including some of the new patients), we typed the same seven polymorphic markers that were used to confirm linkage in our initial SCA14 family (RF11, RF17 and RF13) [2]. The different marker alleles were annotated using the CEPH 133101. The alleles that characterize the SCA14 core haplotype are represented by: D19S571 allele 1, 198 base pair (bp); D19S589 allele 2, 169 bp; D19S924 allele 3, 203 bp; D19S927 allele 3, 138 bp and D19S926 allele 4, 105 bp.

Clinical examination

The clinical data of our original SCA14 family (RF13, RF11 and RF17) has already been published [2]. Six of the eight new patients underwent a comprehensive neurological examination (Table 1). Age of onset data was obtained by structured history taking.

Table 1

Clinical characteristics of six of the affected family members.

Patient	Sex	Age (yrs)	AO (yrs)	Presenting feature	Gait ataxia	Limb ataxia	Dysarthria	Saccade slowing	Ocular dysmetria	Distal sensory disturbances	Tendon reflexes	Extracerebellar signs
VII:6	M	56	29	D	++	+/++	++	+	-	-	N	
VII:7	M	55	32	D	++	+	++	+	+	+	N/↓	
VII:8	F	52	43	G	+	+	-	-	-	-	↑	
VII:5	M	59	32	D/G/C	++	+	+	+	-	++	↑	
VII:9	M	52	30	G	++	++	+	+	+	-	N/↓	
VI:9	M	76	30	M	++	+	++	-	-	-	N	Action myoclonus limbs

M = male, F = female, AO = age-at-onset, D = dysarthry, G = gait difficulties, C = cycling difficulties, M = myoclonus, - = absent, + = present or mild, ++ = moderate, +++ = severe, ND = not determinable, ↑ = increased, ↓ = decreased, N = normal)

RESULTS

Mutational analysis

To identify additional SCA14 patients, we sequenced exons 4 and 5 of the *PRKCG* gene in our Dutch SCA-negative cohort, and identified another eight individuals who carried the Gly118Asp mutation. Of these eight patients, three were siblings while the other five were independent referrals (Figure 1).

Shared haplotype analysis

To determine the conservation of the Dutch SCA14 haplotype, we typed seven polymorphic markers surrounding the *PRKCG* gene, and found that patients carried identical haplotypes. The smallest shared chromosomal region still covered ~6.9cM (Figure 1). This SCA14 haplotype was not observed in controls, where the allele frequencies were for marker D19S206: allele 7, 0.10; D19S571: allele 1, 0.38; D19S589: allele 2, 0.30; D19S926: allele 4, 0.18; D19S927: allele 2, 0.41; D19S926: allele 4, 0.27; and D19S605; allele 2, 0.32.

Genealogical research

Because we detected a shared haplotype surrounding the Gly118Asp mutation in our patients, we investigated their genealogy. Interestingly, all the grandparents of these patients originated from the Dutch provinces of Gelderland and North Brabant. Therefore, we were not very surprised when we identified a common ancestor born in 1722, who linked all the independently referred individuals together into a large seven-generation family (Figure 1). Not all the affected family members from our original SCA14 family (RF13, RF11 and RF17) are shown because this has already been published [2].

Clinical characteristics

Clinical characteristics from six of the new family members are summarized in Table 1. In the complete family of 22 individuals, the age of onset ranged from 21 to 59 years (mean 38.3 ± 9.9 years). Gait difficulties, speech disturbances, cycling difficulties, or myoclonic jerks (in one subject) were reported as initial symptoms. The core phenotype showed a slowly progressive, uncomplicated cerebellar ataxia. However, overt extrapyramidal features were observed in four patients: a focal task-induced dystonia of the dominant hand in two patients, and rigidity of the arms and myoclonic movements of the limbs, each in one patient. Other non-cerebellar signs included distal sensory disturbances in eight patients, primitive reflexes in one, hyperreflexia in ten, and hyporeflexia in five patients. MRI scans were available from seven patients and revealed: cerebellar atrophy in all, most prominently in the vermis; mild frontal or diffuse cerebral cortical atrophy in two; subcortical white matter lesions that were considered to be vascular in two; but no basal ganglia or brainstem abnormalities as is show previously [3].

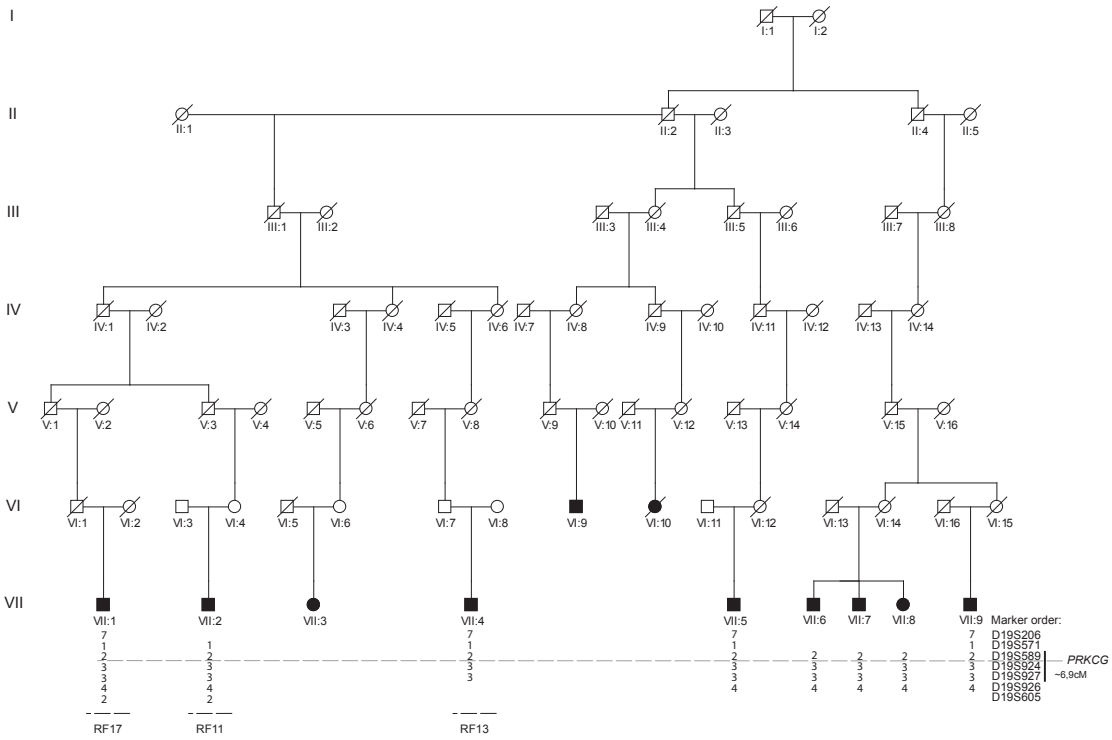


Figure 1

Pedigree of the seven-generation SCA14 family. Please note that, not all the affected family members from our original published SCA14 family (RF13, RF11 and RF17) are shown. Haplotype analysis for seven chromosome 19 markers is shown. Open figures = unaffected; closed figures = affected; dotted figures = obligate carrier; square = male; circle = female; / = deceased. In order to maintain confidentiality, unaffected family members are not shown.

CONCLUSIONS

We have demonstrated through a haplotype study and genealogical research that the Gly118Asp SCA14 mutation is a founder mutation in the Dutch population that originated from a common ancestor born in the early 18th century. Haplotype studies have already provided evidence for the existence of founder mutations in SCA2, 3, 6 and 7 in different ethnic populations [6-10]. Recently, we also showed that founder mutations for the SCA3 and SCA6 genes are present in the Dutch ADCA population and that ADCA families can be traced back to common ancestors in specific parts of the Netherlands [11].

Out of approximately 900 diagnostic requests that proved to be SCA-negative, we have identified a further eight individuals who carry the Gly118Asp mutation, which brings the total to 22 diagnosed SCA14 patients in the Netherlands. Furthermore,

genealogical research was able to link the independently referred patients and showed that the SCA14 ancestor originated from the Dutch province of North Brabant. These twenty-two SCA14 patients explain almost 2% of the ataxia patients in the SCA-negative cohort. Even more, these 9 (6 new identified families, and RF13, 11 and 17) originally independently referred families account for approximately 4% of the estimated total ADCA families (n= 227) in the Netherlands [5].

Clinical findings in the SCA14 patients reported here illustrate that, in addition to the slowly progressive cerebellar ataxia, SCA14 patients can display or even present with extrapyramidal features such as focal dystonia or action myoclonus, particularly in cases with a disease onset before the age of 30 years [3]. Interestingly, the SCA14 mutation spectrum seems not to be confined to the Cys2 region of the C1 domain. Only recently Stevanin et al. identified a missense mutation (F643L) in the catalytic domain of the *PRKCG* gene [4]. However, the phenotype of their SCA14 patients seems to differ since symptoms include also cognitive impairment, which was not detected in any of the other SCA14 families. Further studies will reveal whether a particular SCA14 phenotype with cognitive impairment indeed exists and, accordingly, to what extent mutations in the catalytic domain of the *PRKCG* gene contribute to the molecular genetics of ataxia patients.

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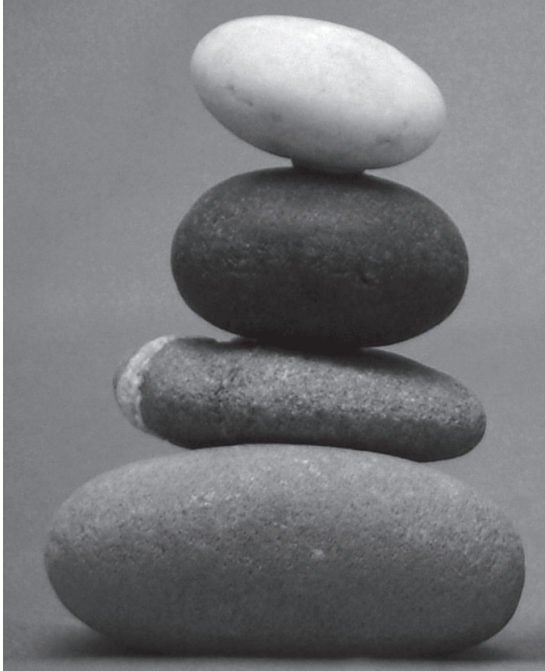
7

Spinocerebellar ataxia 14 mutations in protein kinase C gamma increase kinase activity and alter membrane targeting

Dineke Verbeek*
Melanie Knight*
George Harmison
Kenneth Fischbeck
Brian Howell

* both authors contributed equally



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ABSTRACT

The protein kinase C gamma (PKCγ) gene is mutated in spinocerebellar ataxia type 14 (SCA14). In this study, we investigated the effects of two SCA14 missense mutations, G118D and C150F, on PKCγ function. We found that these mutations increase the intrinsic activity of PKCγ. Direct visualization of labeled PKCγ in living cells demonstrates that the mutant protein translocates more rapidly to selected regions of the plasma membrane in response to Ca^{2+} influx. These results point to specific alterations in mutant PKCγ function that could lead to the selective neuronal degeneration of SCA14.

INTRODUCTION

SCA14 is an autosomal dominant neurodegenerative disorder characterized by slowly progressive cerebellar findings, including gait and limb ataxia, dysarthria, and abnormal eye movements, with an age of onset ranging from 33 to 43 years [1-3]. Atrophy of the cerebellum is apparent by MRI [3], and a reduction of cerebellar Purkinje cells has been observed in brain pathology [4]. Recently, *PRKCG*, the gene encoding the conventional protein kinase C (PKC) family member, PKCγ, was identified as the SCA14 disease gene [1]. In contrast to the repeat expansion mutations responsible for other forms of spinocerebellar ataxia, missense mutations cause SCA14. To date, the majority of mutations have been found in exons 4 and 5 of the *PRKCG* gene [1-3; 5], with one reported in exon 18 [6]. The effects of these mutations on the activity and function of PKCγ is not known. Furthermore, it is not clear how these mutations lead to loss of Purkinje cells and cerebellar dysfunction.

Purkinje cells are, however, known to be regulated by PKCs. For instance, the expression of cerebellar long-term depression (LTD), which likely regulates certain types of motor learning, requires PKC activity [7]. PKC appears to influence LTD by regulating the endocytosis of glutamate receptors on Purkinje cells [8-11]. Other synaptic functions are also regulated by PKC proteins. Polyinnervation of Purkinje cells by climbing fibers persists into adulthood in both PKCγ knockout mice and mice expressing a transgenic PKCγ inhibitor [12; 13]. This has been explained as a failure to eliminate redundant synapses during cerebellar development [14].

Recently, PKC has also been implicated in the regulation of the size and complexity of Purkinje cell dendritic arbors. In the presence of PKC inhibitors or in the absence of PKCγ, the dendritic trees are enlarged and the number of branch points is increased [15; 16]. In contrast, activation of PKC by phorbol-12-myristate-13-acetate (TPA) has a strong inhibitory effect on dendritic arbor growth in slice culture assays [16]. Thus, both positive and negative regulation of PKC kinases have profound effects on the Purkinje cell morphology and behavior.

The conventional PKC kinases are dependent upon 1,2 diacylglyerol (DAG) or phorbol esters, phospholipids, and Ca2+ for full activity [17]. DAG and phorbol esters bind to an amino-terminal cysteine-rich domain known as C1, which is composed of two similar subdomains known as Cys1 and Cys2 [17]. Six of the SCA14 mutations reported to date fall in the Cys2 subdomain [1-3; 5]. Calcium and phospholipid bind to another amino-terminal regulatory domain, referred to as Cys2. Interestingly the function of this domain appears to depend upon the phosphorylation status of a motif in the carboxy-terminal region that corresponds to T674 in PKCγ and is regulated by autophosphorylation [18]. Phosphorylation at this site leads an estimated 10 fold increase in affinity for Ca2+ and phosphatidylserine [18]. Carboxy-terminal phosphorylation and the amino5 terminal domains are important in regulating the intrinsic activity of the conventional PKCs and their affinity for components of the plasmid membrane. In turn, the cycling of conventional PKCs between the cytosol and membrane compartments likely regulates the access of these kinases to key substrates.

In this study, we investigated the effect of two SCA14 mutations; G118D [3] and C150F [5] on PKCγ function. These mutations that lie in the Cys2 subdomain increased the intrinsic kinase activity. By directly visualizing the subcellular localization of a PKCγ green fluorescent protein (GFP) fusion protein in living cells, we observed that mutant PKCγ has enhanced calcium-induced membrane translocation. The possible role of aberrant signal transduction in the selective loss of Purkinje cells in SCA is discussed.

RESULTS

PKCγ autophosphorylation in the presence of SCA14 mutations

In order to study the effects of SCA14 mutations on the kinase function and subcellular distribution of the PKCγ protein we generated expression constructs encoding an in frame fusion between PKCγ and green fluorescent protein (GFP).The GFP tag allowed us to distinguish the exogenously introduced kinases based on increased size relative to endogenous PKC isozymes, and by immunoreactivity with anti-GFP antibodies. The GFP tag also allowed us to analyze the subcellular distribution of the PKCγ kinases in living cells by florescence microscopy. We chose to study the effects of two distinct SCA14 mutations found within the Cys2 subdomain (Figure 1). The mutations G118D, identified in the Netherlands, and the C150F, identified in Australia, were introduced by site directed mutagenesis into the PKCγ coding region. These fusion proteins were used to compare the activity and subcellular distribution of the wild-type and mutant PKCγ kinases.

Since phosphorylation is known to play a central role in the regulation of PKC isozymes, we examined the major phosphorylation sites of PKCγ. Using phosphospecific antibodies, we were able to monitor phosphorylation at T514, T655, and T674.

COS-7 cells were transfected with the wild-type and the two mutant PKCγ constructs at approximately the same efficiency based on the percentage of green fluorescent cells (data not shown). Typically equal amounts of PKCγ (WT) and PKCγ (G118D) were recovered in cell lysates from transfected cells. However, lower amounts of PKCγ (C150F) were observed in total cell lysates, possibly reflecting a reduced protein stability of this mutant protein (data not shown). After normalizing PKCγ protein levels, we observed approximately equal phosphorylation status at T514, T655 and T674 (Figure 2). The mutations in the Cys2 domain therefore do not appear to alter the phosphorylation of PKCγ by PDK1 or alter the autophosphorylation of the kinase.

PKCγ mutants are distinguished from wild-type kinase by their response to Ca^{2+}

Previous characterization of PKCγ has shown that it translocates to the plasma membrane in response to treatment with a Ca2+ ionophore [19]. The calcium-induced translocation is reversible and may cycle a number of times during a one-hour period. We performed a time-lapse study with calcium-stimulated wildtype and mutant PKCγ-GFP transfected cells, to determine the influence of SCA14 mutations on this event. A calcium-induced translocation of both the wildtype and the mutant PKCγ-GFP proteins was observed in the first several seconds of Ca2+ stimulation (Figure 3). The PKCγ-GFP was released from the membrane approximately 15 seconds later, and returned to a diffuse cytoplasmic, predominantly perinuclear location. The earliest translocation that we detected differed between wild-type and mutant PKCγ. A higher porportion of the GFP-tagged mutant kinase translocated to the membrane than the wild type protein (Figure 3, 10 sec).

The majority of the wild-type PKCγ-GFP remained in the cytosol. In addition, we observed a qualitative difference in the distribution of membrane binding between wild-type and mutant PKCγ-GFP. While in the case of the wild-type protein, the dis-

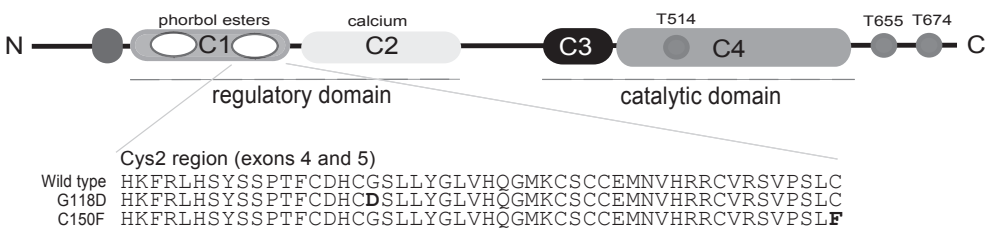

Figure 1

A schematic diagram illustrating the multi-domain PKCγ protein. The Cys2 region of the C1 domain is shown in the inset below and the G118D and C150F are indicated. The binding properties of the C1 and C2 domains and the relative positions of the autophosphorylation sites are revealed above the schematic.

tribution appeared to be uniform along the plasma membrane, the mutant kinases were clearly enriched in discreet membrane domains. These regions had the appearance of membrane ruffles and costaining with fluorescently tagged phalloidin indicated they were rich in filamentous actin (data not shown).

Since the C1 domain that harbors the SCA mutations has been shown to regulate membrane binding via DAG or phorbol esters, we studied the redistribution of PKCγ in transfected COS-7 cells in response to the phorbol ester, TPA. Wild-type and mutant PKCγ-GFP had similar patterns of cytosolic localization in unstimulated cells (Figure 4A; left panels). TPA-induced the redistribution of wild-type and mutant PKCγ-GFP from the cytosol to the membrane with similar kinetics and to a similar extent (Figure 4A). Unlike the Ca2+ response, the TPA induced-membrane targeting was not reversed over the 1 h time course of the study. After TPA stimulation, we observed co-localization of actin and the PKCγ-GFP protein (Figure 4B). Interestingly, membrane ruffling was induced by TPA treatment, and this effect was enhanced in the mutant PKCγ-GFP expressing cells as compared to cells expressing wild-type kinase (Figure 4B, arrow). After treatment, 45% of the wild-type PKCγ-GFP expressing cells displayed membrane ruffling (n=100) as compared to 82% of the mutantPKCγ-GFP expressing cells (n=100).

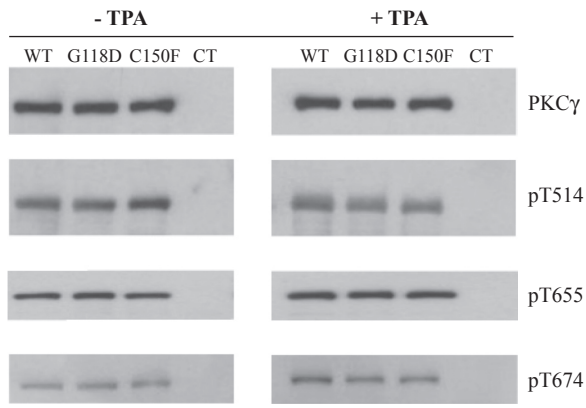

Figure 2

The autophosphorylation of mutant PKCγ is unaltered from the wildtype pattern. Total PKCγ-GFP levels of the wild type and mutant proteins from transfected COS-7 cells were normalized and detected by Western blotting with an anti-PKC (α, β, γ) antibody (top panel). Similar levels of phosphorylation were observed at T514, T655, and T674 of GFP-tagged wild-type (WT) and mutant (G118D, C150F) before and after TPA treatment, using phosphospecific antibodies for the Western blots. The amount of anti-PKCγ immunoreactivity was equivalent in the transfected samples, and the untransfected cells (CT) showed no immunoreactivity in the 110 kDa size range.

SCA14 mutations increase PKCγ kinase activity

Although the SCA14 mutations lie outside of the catalytic domain of PKCγ it is possible that they lead to a change in kinase activity by altering the protein's conformation. The activity of the wild type and mutant PKCγ-GFP proteins were therefore directly measured by in vitro kinase assays. The presence of the GFP tag provided a convenient handle to purify the recombinant proteins away from endogenous kinases. The activities of the kinases were assayed from unstimulated cells, and kinase activities were normalized for protein input by Western blotting against known quantities of GFP. The specific kinase activity of the PKCγ with the G118D and C150F mutations were increased in comparison to the wild-type kinase (Figure 5). Interestingly, the C150F mutation, which appears to reduce the stability of the kinase (data not shown), resulted in the greatest increase in activity.

DISCUSSION

The autosomal dominant spinocerebellar ataxias are generally caused by a repeat expansion mutation in otherwise unrelated disease genes. However, missense mutations in two genes encoding signal transduction proteins, FGF14 [20] and PRKCG [1-3], also lead to spinocerebellar ataxias. In this study, we found that two separate SCA14 mutations lead to increased PKCγ kinase activity, although they reside outside of the kinase domain. The mutant kinases showed a more pronounced redistribution to membrane domains than the wild-type kinase at early time points in response to Ca2+ treatment. We propose that SCA14 mutations in the regulatory C1 domain result in disinhibition of the PKCγ kinase and this initiates a change of events that ultimately leads to loss of Purkinje cells.

The C1 domain of the PKC family members mediates interactions with DAG and phorbol esters [21]. The translocation of PKCγ to the cellular membranes in response to TPA appears to be unaffected by these mutations. This is in line with a previous study that showed both the Cys1 and Cys2 domains of PKCγ bound to TPA and DAG with comparable affinities [22]. Therefore, even if the SCA14 mutations prevented TPA binding to the Cys2 domain, binding to the Cys1 domain would be predicted to compensate. While it is formally possible that the mutations elevated the affinity of the Cys2 domain for TPA, this seems unlikely.

Anomalies in PKCγ function, such as altered membrane targeting, were apparent in the absence of TPA. Therefore, the influences of the Cys2 domain mutations on TPA effects appear to be minimal. A similar rationale may extend to DAG stimulation of PKCγ; however, this was not directly tested in this study. It has been shown previously that the Cys1 domain of PKCγ is sufficient for translocation in response to TPA or a DAG analog [23]. This suggests that for some functions the Cys1 and Cys2 domains of PKCγ are redundant.

A pseudosubstrate, which is found at the beginning of the C1 domain corresponding to residues 19–31 of PKCγ, is thought to suppress the activity of PKC kinases [17]. Like PKC substrates it is rich in basic amino acids, however the pseudosubstrate contains an alanine in place of a serine or threonine. The pseudosubstrate is thought to block access to the active site of the catalytic domain leading to autoinhibition of the kinase. Upon binding to DAG or phorbol esters, conformational changes in the C1 domain are predicted to release the pseudosubstrate from the active site and allow access to substrates [24; 25]. Recently, physical interaction between the C1 and C2 domains of PKCγ have been demonstrated [26]. The SCA14 mutations and DAG or phorbol ester treatments may cause conformational changes in the amino-terminal regulatory domain by disrupting a similar interaction in PKCγ. Data presented in this manuscript are consistent with a model whereby mutations in the C1 domain result in a conformational change that reduces the ability of the pseudosubstrate to suppress PKCγ kinase activity. Structural analysis of the mutant kinases will be required to confirm this hypothesis.

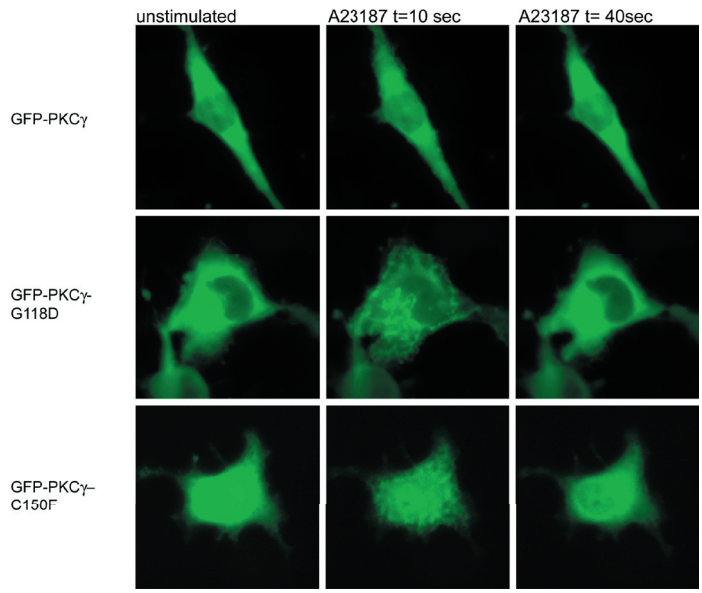

Figure 3

The Ca2+ induces a rapid translocation of mutant PKCγ to discreet regions of the plasma membrane. By 10 sec after the addition of the Ca2+ ionophore, A23187, wild-type and mutant PKCγ-GFP translocated to the plasma membrane. The fluorescent signal was enriched in membrane-ruffle like regions of the mutant PKCγ-GFP expressing cells and less fluorescence was detected in the cytosol of these cells as compared to the wild-type PKCγ-GFP expressing cells at the early time point. The wild-type and mutant fusion proteins reverted to the initial distribution by 40 sec.

Understanding of the SCA14 disease mechanism may be advanced by a better understanding of which substrate proteins are more highly phosphorylated in Purkinje cells from affected individuals or animal models of the disease. It is interesting that although the PKCγ isozyme is expressed broadly in the brain, the Purkinje cells appear to be particularly sensitive to increased kinase activity of PKCγ. It has recently been shown that PKC inhibitors support Purkinje cell survival in cerebellar slice cultures [27]. The mechanism for this protection is not clear. However, it has also been shown that activation of PKC can prevent the retrograde transport of neurotrophins [28]. And the retrograde propagation of neurotrophin signals is important for neuronal survival [29]. Therefore, increased PKCγ activity may reduce the viability of Purkinje cells by hindering the propagation of trophic signals.

Anomalous signal transduction appears to be at the core of SCA14 disease. This begs the question of whether other SCA diseases are also caused by aberrant signaling. Mutations have been identified in the *FGF14* gene, that encodes a ligand for a tyrosine

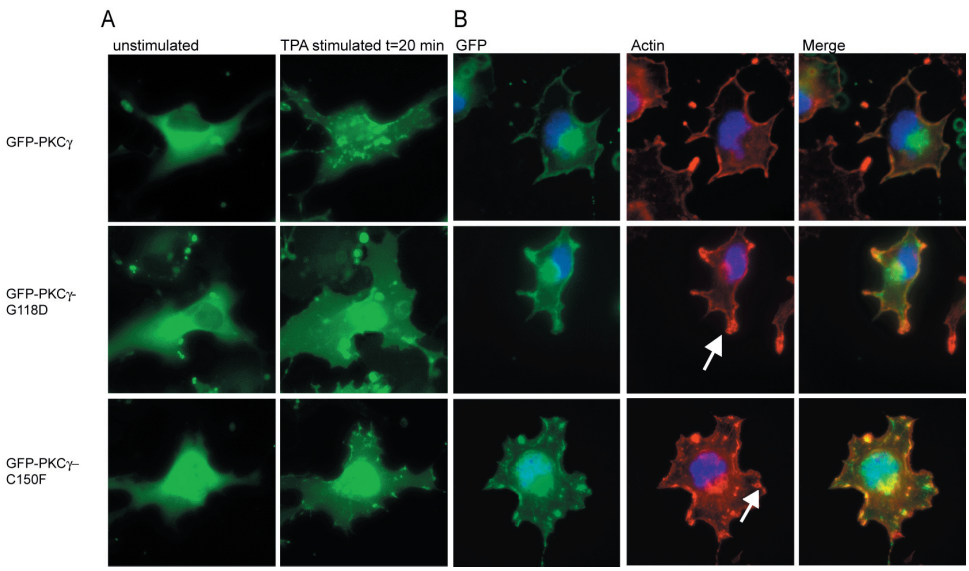

Figure 4

TPA-induced translocation of wild-type and mutant PKCγ is similar. (A) The subcellular distribution of wild-type and mutant PKCγ is indistinguishable prior to TPA stimulation (left panel) and 20 min after stimulation only subtle differences in membrane localization are apparent (right panel). (B) There is an apparent increase in the number of transfected cells (GFP positive; left panel) showing actin rich membrane ruffles detected by Alexa fluor 594-tagged phalloidin (red; middle panel). In cells that had apparent membrane ruffles, co-localization of GFP-tagged kinase and phalloidin-labeled actin filaments (yellow; left panel) was observed.

kinase receptor, in a spinocerebellar ataxia [20]. These mutations likely reduce the function of the protein since mice homozygous for disruption in the *Fgf14* gene develop ataxia and paroxysmal dyskinesia [30]. In SCA12, a non-coding triplet repeat has been identified in the *PP2R2B* gene, which encodes a regulatory subunit of the serine threonine phosphatase, PP2A [31; 32]. It appears that the triplet repeat expansion in this gene would regulate the expression of the regulatory subunit and may consequentially alter the phosphorylation of a subset of PP2A substrates. A serine phosphorylation site, S776 in ataxin-1, is required for full development of disease in an animal model for SCA1[33]. This serine is a target for the AKT or kinase and regulates binding to the scaffolding protein 14-3-3 [34]. It remains to be seen whether the expression of mutant forms of ataxin-1 itself leads to activation of AKT, other kinases or the suppression of phosphatases that regulate the phosphorylation of this site. PKCγ is predicted to phosphorylate S695 of ataxin-1, using the Scansite consensus program at low stringency (http://scansite.mit.edu/motifscan_seq.phtml), although a functional consequence of this is not known. Interestingly, ataxin-1 levels were lower in the Purkinje cells of an SCA14 patient and PKCγ levels were decreased in a mouse model for SCA1 [1; 35]. This suggests a functional interaction of the SCA1 and SCA14 gene products and a possible convergence in the disease mechanisms. The identification of signaling pathways that are compromised by SCA diseases offers the hope of therapeutic interventions for the treatment of these disorders.

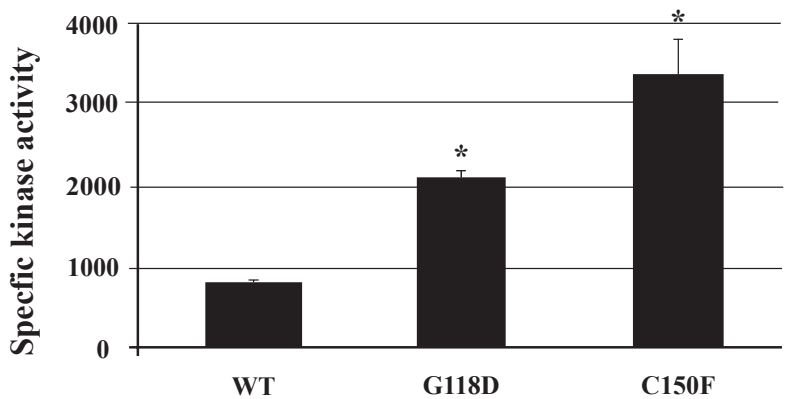

Figure 5

Mutations in the Cys2 region increase the phosphotransferase activity of PKCγ in vitro. Anti-GFP immunoprecipitates from cells expressing wild-type (WT), G118D, and C150F PKCγ were assayed for kinase activity against a PKC-peptide substrate.

MATERIALS AND METHODS

Vector construction

The PRKCG cDNA was amplified from IMAGE clone 5764695 by PCR using the following primers: forward 5'-GCC GAA TTC ACC ATG GCT GGT CTG GGC CCC-3' and reverse 5'-CGG GGA TCC CGC ATG ACG GGC ACA GGC AC-3', respectively. This introduced an EcoRI site at the 5' end and a BamHI site at the 3' end, which facilitated cloning into the respective sites of a pEGFP-N1 expression plasmid (Clontech). The Gly118Asp and Cys150Phe mutations were introduced into PRKCG cDNA with the Quikchange II Site-Directed Mutagenesis Kit (Stratagene) (Figure 1). This techniques involves amplifying a mutant version of the cDNA using the high fidelity polymerase, pfu. For the Gly118Asp mutant the following primers were employed: forward 5'-GCG ACC ACT GTG ACG CCC TCC TCT ACG G-3' and reverse 5'-CCG TAG AGG AGG GCG TCA CAG TGG TCG C-3' and for Cys150Phe: forward 5'-CGT GCC CTC CCT GTT TGG TGT CGA CCA CAC-3' and reverse 5'-GTG TGG TCG ACA CCA AAC AGG GAG GGC ACG-3'. In addition to the indicated mutations, translationally silent changes created restriction sites for HgaI and SalI in the Gly118Asp and Cys150Phe mutants respectively, to facilitate screening. All constructs were verified by sequencing.

Cell culture and transfection

COS-7 cells (ATTC) were cultured in DME high glucose medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and glutamate (100 µg/ml) in humidified atmosphere with 5% CO2 at 37°C. One day before transfection, 0.8 x 106 cells were seeded onto 60 mm culture dishes and transiently transfected with 3 µg of each plasmid with Lipofectamine 2000 reagent (Invitrogen) for 5 h in normal culture medium without antibiotics. After transfection, the cells were cultured at 37°C at least for 24 h before imaging.

Time-lapse study of expressing cells

Transfected cells (0.1 x 106 cells) were plated onto ΔT glass-bottom culture dishes (Bioptechs) and cultured for an additional 16 h before use. Before stimulation, the culture media was replaced by Hank's Balanced Salt Solution (5.33 mM KCl, 0.441 mM KH2PO4, 4.17 mM NaHCO3, 137.93 mM NaCl, 1 mM CaCl2 and 0.338 mM Na2H2PO4 supplemented with 5 mM Hepes). The cells were treated with either phorbol-12-myristate-13-acetate (TPA) (5 µM; Sigma), or calcium ionophore A23187 (10 µM; Sigma) at 21°C. Time-lapse experiments were performed for 50 min; every 30 sec an image was acquired digitally with a DeltaVision microscope (Applied Precision).

Immunostaining

Twenty-four hours after transfection, the cells were plated on glass coverslips coated with poly-L-lysine in 12-well culture dishes and grown for at least 16 hours before stimulation as described above. At times indicated, the cells were washed once with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After three washes with PBS, the cells were permeabilized with 0.3% Triton-X-100 for 10 min and washed again three times with PBS. The cells were stained with Alexa fluor 594 phalloidin (Molecular Probes) in 3% bovine serum albumin in tris buffered saline (TBS) for 60 min. The images were collected using a DeltaVision microscope and images were deconvolved using the Softworx program (Applied Precision).

Immunoblotting

Transfected cells were harvested and lysed in ice-cold RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 1 % sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 mM sodium phosphate [pH7.0], 2 mM EDTA, and 14 mM 2- mercaptoethanol) supplemented with complete mini protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail I (Sigma). Cell lysates were incubated for 20 min on ice, sonicated for 10 sec and centrifuged at 14,000 rpm for 20 min at 4°C. Where indicated transfected cells were stimulated with TPA (5 μM) for 50 min. The protein levels were quantified with the colorimetric BCA protein assay reagent kit (Pierce), and equal amounts were loaded on 12% Novex Tris-Glycine mini-gels (Invitrogen). After electrophoresis, the proteins were transferred onto nitrocellulose filters (Invitrogen) and blocked with 5% milk in preparation for western blotting. The following antibodies were used in the western blots as indicated in the legends: anti-PKC (α, β, and γ) (Upstate), anti-GFP Living Colors peptide (Clontech) antibodies, anti-PKC-phospho-T514, anti-phospho-T655 and phospho-T674 (Biosource) anti-phospho-Ser-PKCγ substrate, and anti-phospho-MARCKS (Cell Signaling).

PKCγ kinase assay

PKCγ-GFP protein was immunoprecipitated from cell lysate (200 μg) of transfected cells with 4 μg anti-GFP Living Colors peptide antibody, which was immobilized on 50 μl protein A/G PLUS-agarose beads (Santa Cruz). Immunoprecipitates were washed 3 times with ice-cold lysis buffer, followed by 2 washes with PAN buffer (100 mM NaCl, 10 mM Tris [pH=7.0], with the mini protease inhibitor cocktail), and finally resuspended in kinase assay dilution buffer II (Upstate). Immunoprecipitated proteins were assayed for PKCγ kinase activity by monitoring the incorporation of 32P into the substrate peptide [QKRPSQRSKYL] employing the PKC kinase system (Upstate). The amounts of PKCγ proteins used in the assays were determined by direct comparison with known amounts of GFP by western blotting. These values were used to determine the specific kinase activity of the wild-type and mutant PKCγ proteins.

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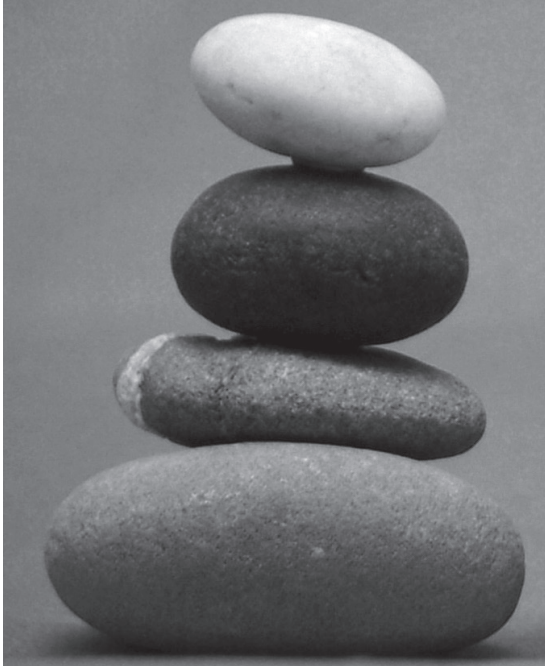
8

General discussion



GENETIC HETEROGENEITY OF THE ADCAs

The genetic heterogeneity of the ADCAs worldwide appears to be far larger than ever expected. Since 2000, six novel SCA genes and at least 10 additional SCA loci have been identified, adding to a total of 11 SCA genes, and 13 SCA loci. To date, mutation analysis of the SCA1, 2, 3, 6, and 7 polyglutamine repeats has revealed the disease-causing defect in ~70% of the cases. These genes do not show any clear homology in their function or structure, but they share their mutational mechanism: coding repeat expansions. The more recently identified SCA8, 10, and 12 genes show non-coding CTG/CAG/ATTCT repeat expansion mutations while in the latest SCA genes to be identified, *FGF14* and *PRKCG*, interestingly, it is missense mutations rather than repeat expansions that appear to be disease-causing. This suggests that the repeat expansions are not the common link between the different SCA types but that there may be another, as yet unrecognized, underlying pathway.

The contribution of the novel SCA genes (SCA8, 10, 12, 14, 17 and *FGF14*) to the Dutch ADCA population was unknown, or how far they would explain the remaining undiagnosed families. We therefore performed a screening in the SCA-negative panel. None of these families displayed SCA12 or SCA17 mutations, which was not surprising because these mutations have been found in specific populations only [1; 2]. The contribution of the SCA10 pentanucleotide repeat expansion is still unknown in the Netherlands, but will probably also be absent [3].

Screening for large SCA8 repeat expansions detected 11 cases in the Dutch cohort (unpublished data). Most patients seemed to be isolated cases within their family, and the ataxia appeared to be familial in only one case. Data from recent studies suggests that the untranslated SCA8 repeat expansions co-segregate as a polymorphism with the true (still unidentified) disease mutation. The finding that the SCA8 repeat expansions have also been identified in patients with other SCA types, and in control individuals strengthens this idea. Thus, with the ongoing debate about the implications of the untranslated SCA8 repeat expansion in the etiology of ADCA, screening for this variation has not yet been implemented in the DNA diagnostics.

So far, *FGF14* mutations have only been identified in one large Dutch ADCA family from Rotterdam [4]. This family displays a distinct phenotypic form of ADCA and there is only a small chance that additional mutational carriers will be present in the remainder of the SCA-negative panels in Rotterdam and Utrecht. The psychiatric episodes are used as an inclusion criterion for screening cases for mutations in the *FGF14* gene. Two small studies from France and Germany have also determined that *FGF14* mutations are not a major cause of ADCA in their ataxia populations, in fact no additional families have been identified [5; 6].

The frequency of SCA14 mutations worldwide is still unclear, but SCA14 is certainly present in ADCA families from several different countries including the United Kingdom, the Netherlands, Japan and France [7-10]. It seemed that the mutation spectrum was restricted to the highly conserved Cys2 region of the C1 domain. However, a recent French report described a missense mutation in the C4 domain of the *PRKCG* gene in one particular family [10]. Interestingly, besides the core ataxic syndrome, this family shows additional cognitive problems, which have not been described in any of the other SCA14 families. This suggests that the SCA14 mutational and phenotypic spectrum may be more complicated than initially anticipated. Further research is required to study these apparently distinct genotype-phenotype correlations.

We have set up a screen to identify SCA14 patients in the SCA-negative cohort (n=900), and so far, this has resulted in 22 genetically diagnosed SCA14 patients with the Gly118Asp mutation, from nine independently referred families. All these patients could be linked to a common ancestor originating from the province of Nort Brabant; see reference 8 and Chapter 6 [11]. More recently, a novel missense mutation, V138D, was identified in another Dutch SCA14 case in exon 5 of the *PRKCG* gene (unpublished data). The independently referred SCA14 families account for approximately 4% of the total ADCA families (n=227) in the Netherlands [12]. In conclusion, mutations in the SCA8, 10, 12, and 17 genes did not explain any of the SCA-negative families, in contrast to mutations in the SCA14 gene, which at least explained 10% of the true ADCA families in the SCA-negative cohort (n=65) [12].

To pursue the gene search in the remaining families, linkage studies were used to localize further SCA genes in the Dutch ADCA population. First, traditional linkage analysis in large families with multiple affected individuals was performed. Secondly, because most of the available families were too small for traditional linkage analysis, we chose an alternative approach to localize disease genes, i.e. a shared haplotype analysis.

LOCALIZING AND IDENTIFYING DISEASE GENES

At first glance there is no clear functional or structural relationship between the different SCA genes. It will therefore be too difficult, if not almost impossible, to use a direct candidate gene approach to identify novel SCA genes. Alternatively, the whole genome could be screened for trinucleotide repeat expansions using the Repeat Expansion Detection (RED) method [13] or the DIRECT method [14]. DIRECT has been used successfully to identify the SCA2 repeat expansion [14]. However, both methods have some significant limitations. First, the techniques detect all occurring repeats in the genome, without knowledge of the exact repeat location. Secondly, it is necessary to follow segregation of the repeat with the disease, because polymorphic repeat loci have been described, including ERDA-1 and SEF2-1 [15; 16]. Thirdly, not all the disease-causing

mutations turned out to be repeat expansions, and fourthly, missense mutations would not be detected. Finally, these techniques to localize disease genes have now become obsolete because automated repeat finder programs are available that can be used to search the human genome sequence for genes that contain particular repeat regions [17].

LINKAGE ANALYSIS

So far, the most successful strategy for localizing and identifying SCA genes has been traditional linkage analysis in large families, but linkage analysis also has limitations. Families with sufficient numbers of affected individuals are needed, and such families are rare. Obviously, this is further complicated by the generally late age of onset of ADCAs. Young and seemingly unaffected individuals can still develop the disorder at a later stage of life, but by then, the older generation is often deceased.

However, at the start of this study a large ADCA family was clinically characterized [18], and we were able to localize the disease-causing gene to chromosome region 1p21-q21, the SCA19 locus (Chapter 2) [19]. In the course of collecting novel ataxia families, which was combined with genealogy to try and link apparently independently families, a second large ADCA family was identified. The disease gene (SCA23) in this family was localized to chromosome region 20p13-12.3 (Chapter 3) [20].

SCA19

The SCA19 region has been studied intensively and contains a total of 486 transcripts. The search started for proteins that contained coding polyglutamine repeat stretches, because this is still the most frequent mutation identified. In addition, anticipation was observed in the SCA19 family, suggesting a repeat expansion mutation to be present. The most promising candidate gene, *KCNN3*, which harbors two coding CAG tracts, was quickly excluded because no repeat expansion was detected in the patients, and no additional missense mutations were identified through sequencing. Subsequently, all known CAG or CTG repeats located within the boundaries of the interval were screened for expansions. Most of these repeats were non-coding and not associated with any particular gene. Affected individuals that appeared to be homozygous carriers for a certain repeat were also screened via Southern blot analysis to exclude possible large expansions. No aberrant repeat fragments were identified in the patients.

Interestingly, the most distal part of the linkage region showed overlap with a second familiar migraine locus [21]. Since missense mutations in the SCA6 gene (*CACNA1A*) that encodes for an alpha-1A subunit voltage-dependant, P/Q type calcium channel can result in static or episodic ataxia, migraine, and epilepsy or a challenging combination of these phenotypes [22], we decided to screen all calcium and potassium channels located in the SCA19 region for missense mutations. No mutation

was detected, and we thereby excluded the *KCNC4*, *KCNA10*, *KCNA2*, *KCNA3*, and *KCNA4* genes. A case report appeared in the literature in which a balanced translocation t(1;19)(q21.3;q13.2) was described [23]. The patient showed mental retardation, ataxia and atrophy in the brain, which resembled the SCA19 phenotype. Two truncated proteins; the kinase CLK2 and PAFAH1B3 seemed to be involved. Sequence analysis excluded the *CLK2* gene that was located in the SCA19 region, as the disease-causing gene.

Ming-Yi Chung et al., 2003 [61] reported the identification of the SCA22 locus on chromosome 1p21-q23. There is significant overlap between the SCA19 and SCA22 regions: 26.9 cM between the markers D1S206 and D1S1595. Although it cannot be excluded that the genes lie in close approximation at this locus, it is more likely that the Dutch and Chinese families suffer from a mutation in the same gene, and that SCA19 and SCA22 represent an identical condition, which should be designated SCA19 as this was the first linkage assigned.

SCA23

The size of the SCA23 interval was considerably smaller than the SCA19 region, but still contained 101 genes. The same strategy was applied as for the SCA19 region and all coding and non-coding CAG/CTG repeats in the region [20] were screened for expansions. Again no repeat expansions were detected in the patients, and subsequently, three other genes were sequenced, including neuronal cell death inducible kinase (*SKIP3*), major prion protein precursor (*PRNP*) and the prion gene complex downstream (*PRND*). Prnp null mice display ataxia [24] and disruption of Doppel (Dpl = PRND in humans) prevents neurodegeneration and cell death of cerebellar neurons in mice with extensive Prnp deletions [25]. No mutations were detected in these genes. Pantothenate kinase 2 (*hPANK*) was also sequenced because mutations in this gene are associated with Hallervorden-Spatz disease that leads to pantothenate kinase-induced neurodegeneration with iron accumulation in the brain [26]. This disorder progressively affects the muscle tone and voluntary movements, without displaying ataxia. Again, no mutations were identified. Furthermore, the large attractin precursor protein was screened at the RNA level for mutations. Attractin null mice showed hypomyelination and vacuolation in the CNS that was associated with body tremor [27]. This suggests that attractin has a critical role in normal myelination in the CNS and might be a good candidate for SCA23. The sequence data did not reveal any potential disease-causing variation.

As the SCA23 disease gene was not identified, a different approach was used to select candidate genes. Recent studies showed that phosphorylation of a specific serine site in the ataxin-1 gene is required for full development of disease in an animal model for SCA1 [28]. This serine is a target for the AKT kinase and regulates binding to the scaffolding protein 14-3-3 [29]. 14-3-3 seems to be recruited into aggrosome-like inclusion bodies involved in Huntington's disease and Lewy bodies in Parkinson's disease,

while increased levels of 14-3-3 have been identified in the spinal fluid of Alzheimer and Creutzfeldt-Jakob disease patients. Studies have also shown that 14-3-3 can bind PRKCG, through interactions with the C1 domain that contains most of the SCA14 mutations [30; 31 2; 32]. Therefore, when we screened for the 14-3-3 binding motif in the other SCA genes using http://scansite.mit.edu/motifscan_seq.phtml. Similar consensus binding sites were identified in ataxin 2, CACNCA1, FGF14, PRKCG and TBP proteins. Certainly, just the presence of binding motifs will never prove any in vivo biological relevant interaction between proteins, and targeted functional studies will be required. Nevertheless, all proteins were analyzed that were encoded by genes located from the SCA23 region with the motif scanner and we identified four proteins that contained a similar site: syntaphilin, syntenin 2, protein C20orf75 precursor, and M-phase inducer phosphatase 2. In addition, these four genes were excluded as SCA23 candidates by performing sequencing analysis.

In conclusion, a major pitfall of linkage analysis is the size of the candidate region that often harbors too many interesting genes. In order to reduce the size of the interval and the number of genes, additional families are needed that link to the same locus. Unfortunately, a shared haplotype analysis in our Dutch cohort showed that no additional families link to the SCA19 or SCA23 locus. Future collaborations with other European ataxia research groups may help to overcome this issue.

SHARED HAPLOTYPE ANALYSIS

Most of the available patients are members of small families. We therefore used an alternative approach to localize and identify the disease genes involved. We reasoned that multiple ADCA families could carry identical disease-causing mutations that would have arisen in a common ancestor. Consequently, seemingly unrelated or distantly related ADCA families will not only share a common mutation but also a conserved region surrounding the disease locus in which marker alleles will be in linkage disequilibrium (LD). In this thesis, genetic evidence is provided for the existence of a founder for the SCA3 and SCA6 repeat expansion in the Netherlands (Chapter 4) [33]. In addition, geographical clustering of most of these ADCA families in particular provinces further supports our findings. This suggests that ADCA families can be traced back to common ancestors in particular parts of the Netherlands. Combining both genealogical and genotype data enables small pedigrees to be linked into extended families. This will be particularly useful for the identification of additional SCA loci in the small ADCA families in which no mutation has been found yet.

Twenty-eight small ADCA families with at least 2 affected individuals in a parents-child trio, with or without other affected and unaffected individuals, were collected from our Dutch SCA-negative cohort. Initially, we focused on the SCA loci that were

reported in the literature, i.e. SCA4, 5,11,13,14, 16, 18, 19, 21 and 23. We identified a shared haplotype in three of the 28 ADCA families that covered almost the complete SCA14 locus. Genealogical investigations revealed geographic clustering of these three families around Nijmegen. Moreover, they were eventually linked together as one large family (Chapter 5) [8]. Further analysis of the other 25 families revealed clustering of the grandparents of a large proportion of the families in the eastern part of Gelderland and North Brabant as was seen for the SCA14 family (see Figure 1). This effect might be biased because most ADCA families were recruited through the Nijmegen hospital. So far, we have not been able to link these 25 families by their genealogy. The genotyping results of the published SCA loci between the 25 families did not reveal any large shared haplotype (> 4 alleles) in the intervals. This might be a consequence of 1) the intermarker distance, which might still be too large and shared segments could have been missed due to larger generation-separation times; 2) the phenotype of the families. The cohort may not contain solely "pure" ADCA families, but may include families with episodic ataxia or any other dominant hereditary syndrome that also displays ataxia. From the results of the linkage and shared haplotype analysis we conclude that the 28 SCA-negative families comprise at lease one SCA19, three SCA14, and one SCA23. Recent data has shown that

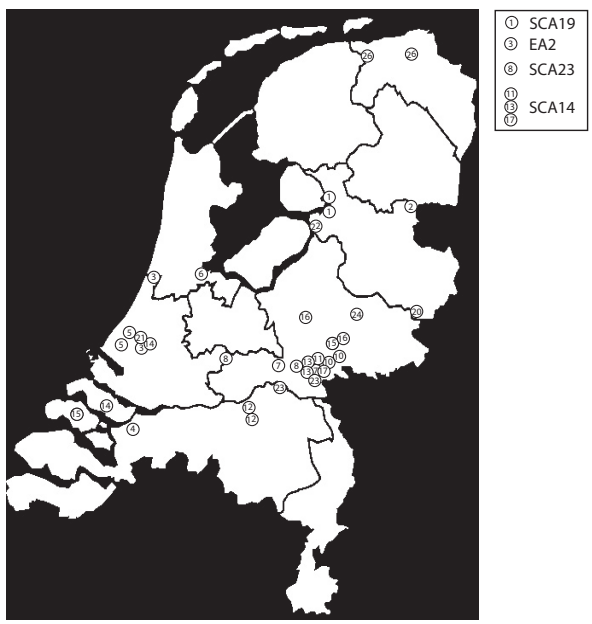


Figure 1
Geographic localization of ADCA-negative families collected for ADCA project between 2000-2003. Note the clustering of multiple families surrounding the city Nijmegen.

family 3 is significantly linked to the SCA6 locus, *CACNA1A*, with a max lod score of 4.1. Further clinical investigations revealed that some family members clearly displayed an episodic ataxia. Genome-wide linkage data of family 2 showed suggestive linkage with the SCA16 locus. However, the outcome of recent neurological examination of several familymembers did not support our haplotype data. Further research is needed to confirm our findings. The other 21 families remain genetically unclassified so far, but the geographic distribution of the families (Figure 1) suggests different mutational origins.

This study was not able to completely unravel the genetics of the smaller SCA-negative families using a shared haplotype analysis. This could be due to the large generation-separation time between the independently referred families. The ideal situation would be one where geographic clustering could still be detected when families have been separated for around 10 generations. This assumption might be too optimistic for every disease gene, as no large shared chromosomal segments were detected with the shared haplotype analysis. Furthermore, we expected each gene to have about equal contributions in the population. This turned out not to be true, as was shown by the estimated prevalence data of the known SCA genes [12]. More effort is now required to collect additional families and to perform extensive genealogical research to link independently referred families together. We still expect a limited number of disease-causing genes to be identified in the SCA-negative cohort, while the growing numbers of characterized families contribute to revealing the genetic heterogeneity of ADCA in the Netherlands.

CURRENT UNDERSTANDING OF THE DISEASE PATHOLOGY

Neurodegenerative disorders may share similar features: for example, late age of onset, progressive degeneration of specific subsets of neurons, aggregate formation in the case of polyglutamine disorders, and toxicity. These findings point to the possibility of a common pathogenic pathway. This idea is supported by the change in disease-causing mutation type, in contrast to the repeat expansion mutations that are observed in most of the SCA genes. Missense mutations are associated with novel forms of ADCA (SCA14 and FGF14).

Drosophila models of SCA1, SCA3, and a polyglutamine disease model consisting of a polyglutamine tract of 127 repeats have been used to determine whether common pathways cause cell death, and if these diseasemodels were mediated by known modifiers of polyglutamine repeat disorders such as Hsp70, Hdji or UBB to rescue the disease phenotype in cells of the eye and brain of those flies [34]. The presence of such modifiers reduced aggregate formation and toxicity in these transgenic animals. In addition, TUNEL assays confirmed the role of apoptosis in these disorders [34]. Recent results showed that altered gene expression levels may underlie the early disease phase of SCA1 [35] before disease symptoms manifest. An extensive micro-array study was per-

formed to detect genes that showed differential expression levels in mice SCA1 cerebella of different disease stages [36]. A subset of genes appeared to show overlap in expression levels in two selected SCA1 disease stages. This might suggest that gene expression alteration in this set of genes could be central for the SCA1 disease process. Interestingly, these genes (*IP3R1*, *EAAT4*, *Homer3* and *G-substrate*) are all implicated in glutamate signaling. These results have been further supported by a very recent report, which showed that expanded polyglutamine peptides affect epidermal growth factor receptor signaling and change glutamate transporter expression in Drosophila [37]

Could abnormal MAPK signaling be a common theme in all SCA types leading to altered expression levels of important target genes? At least, aberrant RAS/MAPK signaling also seems to be a major problem in SCA14, SCA12 and FGF14. Furthermore, ataxin-1 levels were reported to be lower in the Purkinje cells of an SCA14 patient, and PKC gamma levels were found to be decreased in a mouse model for SCA1 [7; 38]. This suggests a functional interaction of the SCA1 and SCA14 gene products and a possible connection in the disease mechanisms. The SCA12 gene, *PP2R2B*, encodes a regulatory subunit of the serine threonine phosphatase, PP2A [39; 40]. Repeat expansions in PP2R2B might influence the expression of PP2A, and might consequently alter the phosphorylation, and thereby the activity of a subset of PP2A substrates in the signaling cascade. FGF14 is a ligand for a tyrosine kinase receptor, and mutations in this gene might alter the affinity of the ligand for its receptor influencing the remainder of the signaling cascade that has been activated by the receptor. Figure 2 shows a summary of our current understanding of ADCA disease pathology. Experimental therapies could target these underlying aberrant signaling mechanisms and transcriptional dysregulation.

THERAPIES

Currently, there is no therapy available to slow down or halt the ADCA disease progression, or to reverse the disease pathology in humans. Several symptomatic drugs are known to reduce some of the more specific manifestations, for example, myoclonus may be improved by using clonezepam or valproic acid, and Parkinsonian features in SCA2 and SCA3 can be effectively treated with levodopa or dopamine agonists for 10 years or longer [41; 42]. Tremor-controlling drugs, unfortunately, do not work well for cerebellar tremors. Two large screens have been performed to identify known drugs or chemical components that would counter the neurodegenerative process in polyglutamine diseases [43; 44]. Three drugs (approved by the US Food and Drug Administration) that are part of the cardiac glycoside class and known Na+/K+-ATPases protected against polyQ-induced toxicity and reduced caspase 3 activity and cell death in an in vitro system of the polyQ-expanded androgen receptor. However, further investigation is necessary in animal models before these drugs can be used in a human clinical trial.

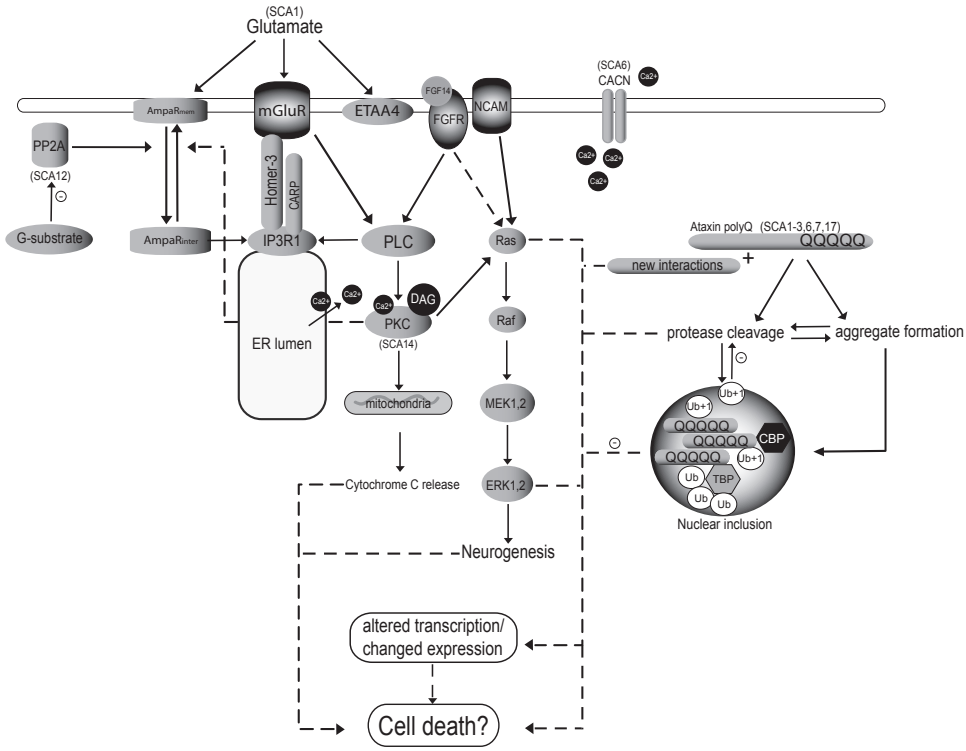


Figure 2

Possible common underlying pathway for the different SCA types leading to cell death and neurodegeneration

One major neuropathological feature of ADCA is the loss of Purkinje cells in the cerebellum. Cell replacement therapy using embryonic ventral mesencephalic tissue in the putamen has been reported to restore the dopaminergic neurotransmission and motor symptoms in Parkinson's disease patients [45]. However, the outcome of this therapy is highly unpredictable and variable in different patients, and 10-15% may develop severe dykinesia [46]. Futhermore, 3 out of 5 Huntington's disease patients that received intrastiatal implantation of fetal stiatal neuroblasts showed clinical improvements or at least stability of the motor and cognitive problems [47]. Without a better understanding of this effect, neuronal grafting is unlikely to be developed as a general therapy in human disorders of the brain. Grafting experiments have been performed using the mutant mouse pcd (Purkinje-cell degeneration), with cells of cerebellar primordium taken from mouse embryos [48]. The grafted cells invade the deficient cerebellar cortex of the host, proceed with their normal development and receive appropriate synaptic contacts. The use of neuronal precursor cells for neural transplantation and brain repair is limited since the cells are restricted in their mitotic competence [49]. A more recent report showed that bone-marrow stem cells could also differentiate into fully developed and function-

ally active Purkinje cells in the brain [50]. Findings from mutant SCA1 mice suggest that cell loss is not the primary cause of disease because ataxia has also been observed without significant Purkinje cell loss, although loss of Purkinje cell dendrites and spines was in fact seen in the early stages [51]. This long interval between the onset of neurological symptoms and the loss of Purkinje cells is a perfect time window to block mutant protein expression and to reverse the disease pathology. This was convincingly demonstrated using conditional SCA1 mice, in which the effect of the mutant polyQ was easily reversed in the early stages of the disease, whereas in later stages there was only partial recovery [52].

Additional experimental interventions such as upregulating chaperones [53], inhibiting caspases 1 and 3 [54] or administration of histone deacetylase inhibitors [55; 56] might potentially point to new approaches for an effective therapy. To date, the most promising studies addressed silencing of specifically the mutant SCA1 and SCA3 alleles in vitro [57], and even better, in an in vivo system [58]. Such specific silencing was accomplished using a technique that triggers the RNA interference mechanism by introducing small interfering RNAs (siRNA) (produced exogenously or expressed intracellularly) into cells or even into the brain area of interest [59; 60].

In conclusion, RNAi could become the method of choice, to be used as a gene therapy that directly inhibits the mutant allele, as demonstrated in a recent study that suppressed polyQ induced neurodegeneration in SCA1 transgenic mice. Direct siRNAs expression in SCA1 mutant Purkinje cells improved the cellular and behavioral characteristics of these mice [58]. The in vivo delivery, efficiency of the siRNA to silence the disease gene and the long-term effects of chronically triggering the RNAi pathway in humans remains to be tested in the future.

FINAL REMARKS AND CONCLUSIONS

We have tried to further unravel the genetic heterogeneity of ataxia patients who did not display mutations in the SCA1-3, 6 and 7 genes. Two novel SCA loci (SCA19 and SCA23) were identified using linkage analysis in two large ADCA families (Chapters 2 and 3). In addition, almost 10% of the SCA-negative families could be explained by the Gly118Asp missense mutation in the *PRKCG* gene (SCA14) (Chapters 5 and 6). Unfortunately, traditional linkage analysis requires large families with multiple affected individuals and our cohort mainly contained small families. As an alternative, a shared haplotype analysis was used to localize the disease-causing genes in these families, based on our findings that founder mutations are indeed present in the Dutch ADCA population (Chapter 4). We have shown that combining genotype information with genealogy is a very useful tool for linking independently referred families together into one large family. We need additional families and a denser genotyping method (like the Illumina SNP-arrays) to resolve the genetic background(s) of the remaining 24 Dutch ataxia families. Identifying the additional SCA genes involved, and their corresponding biological pathways, would improve the current diagnostic possibilities. It would also provide a better insight into the pathophysiology of the disease, which could eventually lead to therapeutic strategies.

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Summary

The autosomal dominant cerebellar ataxias are the largest subgroup of the neurodegenerative disorders. The disease is characterized by a progressive cerebellar ataxic syndrome and is often accompanied by variable symptoms such as myoclonus, tremor, mental retardation, headache, and epilepsy. The first clinical symptoms mostly manifest when patients are between 30 and 50 years old. The ataxic syndrome is the result of selective neuronal loss in the cerebellum, brainstem and spinal cord. Currently, there is no therapy to slow down or halt the disease progression, or to reverse the disease pathology in humans.

The ADCAs are genetically as well as clinically very heterogeneous. The disease is inherited in an autosomal dominant fashion, so that for the offspring of an ADCA patient, the risk of inheriting the disease gene is 50%. So far, 11 SCA genes have been cloned and an additional 13 SCA loci have been identified. Most of these genes do not show any clear homology in their function or structure, but 68% of the genes share their mutational mechanism: coding polyglutamine (CAGn) repeat expansions. However, some genes display non-coding CTG/CAG/ATTCT repeat expansion mutations, and in the latest SCA genes identified, missense mutations rather than repeat expansions appear to be disease-causing. This suggests that the repeat expansions are not the common link between the different SCA types but that there is still an unrecognized underlying pathway.

To date, mutation analysis of the SCA1-3, 6 and 7 genes reveals the disease-causing defect in ~70% of the cases. In this thesis, we have tried to further unravel the genetic heterogeneity of ataxia patients who did not display mutations in the SCA1-3, 6 and 7 genes. The most traditional way to localize and identify novel disease genes is to use linkage analysis, as shown in Chapters 2 and 3. Chapter 2 describes the identification of the novel SCA locus in a large Dutch ADCA family. This family displays a clinically and genetically distinct different form of ADCA. The family's phenotype shows additional symptoms such as cognitive impairment in addition to the relatively mild ataxia syndrome. Genome-wide linkage and haplotype analysis revealed significant linkage to chromosome region 1p21-q21. This novel locus was designated as SCA19. The candidate interval spans ~35 cM and is located between the markers D1S1588 and D1S1595. So far, the SCA19 disease gene has not been identified. A second large Dutch ADCA family did not reveal any mutations in the known SCA genes and a genome-wide linkage analysis in this family was therefore performed. The results are presented in Chapter 3. Significant linkage was observed with polymorphic markers located on chromosome 20. The SCA23 interval is flanked with the most proximal tip of chromosome 20 and marker D20S194, and is ~18.2 cM in size. The phenotype in this family is clinically

indistinguishable from the other SCA types. Neuropathological findings did not reveal positive nuclear or cytoplasmatic polyglutamine inclusions in the brain cells of a SCA23 patient, suggesting that a coding CAG repeat expansion mutation is not involved in the pathology of this SCA type. An extensive SCA23 candidate gene search and analysis has not yet been successful.

Unfortunately, traditional linkage analysis requires large families with multiple affected individuals and the Dutch SCA-negative cohort mainly contained small families. As an alternative, a shared haplotype analysis was used to localize the disease-causing genes in those families. This idea was based on the presence of founder mutations in the Dutch ADCA population, as illustrated in Chapter 4. We were able to show the existence of major founders for the SCA3 and the SCA6 repeat expansions in the Netherlands. Furthermore, these findings were strengthened by geographic clustering of most of these families in particular provinces. In this chapter we have shown that combining genotype information with genealogy is a very useful tool for linking independently referred families together into one large family. This will be particularly useful for identifying additional SCA loci in the small ADCA families in which no mutation has been found yet.

Chapter 5 describes such a combined analysis of three families in which we identified a shared haplotype that covered almost the complete SCA14 locus. Interestingly, genealogical research was again able to link these families together and geographic clustering showed a common ancestor in the Dutch province of North Brabant. Sequence analysis revealed a Gly118Asp (G118D) missense mutation in the highly conserved Cys2 region of the C1 domain of the protein kinase C gamma (*PRKCG*) gene in all patients of this family. The family displayed a slowly progressive ataxia syndrome with some additional features such as hyperreflexia, and focal dystonia in some individuals.

We screened the complete Dutch SCA-negative panel for mutations in the Cys2 region (exons 4 and 5) of the PKCγ (*PRKCG*) gene, reported in Chapter 6. This study was performed to determine the frequency of SCA14 mutations in the Dutch ataxia population. Almost 10% of the SCA-negative families (n=65) could be explained by the G118D mutation. Interestingly, genealogical research was able to link all independently referred families from the Utrecht and Rotterdam cohort to one common ancestor born in the early 18th century. We therefore confirmed that the G118D mutation is a SCA14 founder mutation in the Dutch ADCA population. Recently, a missense mutation in exon 18 of the *PRKCG* gene has been identified in a French SCA14 family, however, whether this mutation is indeed present in the Dutch SCA-negative cohort is still unknown.

In Chapter 7, we investigated the effects of two SCA14 missense mutations, G118D and C150F, on the function of PKCγ. Results showed that these SCA14 mutations cause an increase of intrinsic PRKCG kinase activity. The subcellular localization of PKCγ was directly visualized in living COS cells through the generation of a green fluo-

rescent protein (GFP) fusion protein. This experiment revealed that mutant PKCγ has an enhanced calcium-induced membrane translocation. In addition, a qualitative difference was seen in the distribution of membrane binding between wild-type and mutant PKCγ. Mutant PKCγ was clearly enriched in discreet membrane domains. These regions had the appearance of membrane ruffles, and were shown to be actin-rich. These specific alterations in mutant PKCγ function might lead to the selective neuronal degeneration seen in SCA14 patients.

In conclusion, the genetic heterogeneity of the ADCAs appeared to be larger than had been expected. In 28 Dutch SCA-negative families, we were able to explain 21% (6/28) of these families through significant linkage to the SCA19, SCA23 and EA2 (=SCA6) locus. In addition, a shared haplotype analysis revealed that another three independently referred families linked to the SCA14 region. The identification of these novel SCA loci and their respective genes will contribute to expanding the diagnostic possibilities in the future, and will provide novel insights for the development of potential therapies.

Samenvatting

De autosomaal dominante cerebellaire ataxieën (ADCAs) zijn de grootste subgroep van de neurologische degeneratieve aandoeningen. Deze ziekte wordt gekenmerkt door een progressief atactisch syndroom dat ontstaat vanuit de kleine hersenen en gaat vaak gepaard met variabele klachten zoals spierschokken in armen of benen, trillingen, mentale achteruitgang, hoofdpijn of epilepsie. De eerste klinische verschijnselen openbaren zich tussen het dertigste en veertigste levensjaar. De ataxie klachten zijn het resultaat van selectief verlies van zenuwcellen in de kleine hersenen, hersenstam en ruggenmerg. Momenteel is er geen therapie beschikbaar om het ziekteproces bij patiënten te verminderen of te stoppen of zelfs de klachten kan verhelpen. De ADCAs zijn zowel klinisch als genetisch erg verschillend. De kans om de ziekte door te geven aan het nageslacht is 50%. Dit noemt men een autosomaal dominant overerving van het ziektebeeld. Tot nu toe zijn er 11 SCA genen geïdentificeerd en 13 nieuwe SCA gebieden gevonden. De meeste van deze SCA genen laten geen enkele overeenkomst zien in hun genstructuur of werking, maar 68% van deze genen hebben hetzelfde mutatiemechanisme: coderende polyglutamine (CAGn) repeat expansies. Toch zijn er ook SCA genen die niet-coderende CAG/CTG/ATTCT repeat expansie mutaties bevatten en zelfs in de laatst gevonden SCA genen blijken punt mutaties in plaats van repeat expansie mutaties ziekteveroorzakend te zijn. Deze bevinding suggereert dat repeat expansies niet de gemeenschappelijke noemer zijn tussen de verschillende SCA types maar dat er waarschijnlijk nog een andere onbekende weg is die leidt tot dit ziektebeeld. Tot nu toe worden in 70% van de ADCA patiënten ziekteveroorzakende mutaties gevonden in de SCA1, 2, 3, 6 and 7 genen. In dit proefschrift wordt geprobeerd om de resterende 30% van de patiënten, waarin dus geen mutaties gevonden zijn in de boven genoemde genen, genetisch te ontcijferen.

De meest traditionele manier om nieuwe ziekte genen te lokaliseren en te identificeren is door gebruik te maken van koppelingsonderzoek zoals in hoofdstukken 2 en 3 is beschreven. Hoofdstuk 2 beschrijft de vondst van een nieuw SCA gebied in een grote Nederlandse ADCA familie. Deze patiënten in deze familie vertonen klinisch en genetisch een onderscheidende variant van ADCA. Het klinische beeld van deze familie laat naast het relatief milde atactische syndroom, cognitieve achteruitgang zien. Genoomwijd koppelingsonderzoek en DNA marker profiel analyse resulteerde in significante koppeling met chromosoom regio 1p21-q21. Dit nieuwe gebied wordt SCA19 genoemd. Het kandidaatgen interval is ongeveer 35 cM groot en bevindt zich tussen de markers D1S1588 en D1S1595. Tot nu toe is het SCA19 gen nog niet gevonden. Ook in een tweede grote Nederlandse ADCA familie waren geen mutaties te vinden in de bekende SCA genen. Daarom werd ook in deze familie genoomwijd koppelingsonderzoek uitgevoerd en de behaalde resultaten zijn beschreven in hoofdstuk 3. Significante koppeling

werd gevonden met DNA markers gelegen op chromosoom 20. Het SCA23 interval wordt begrensd door de meest proximale gelegen tip van chromosoom 20 en marker D20S194 en is ongeveer 18,2 cM groot. Het klinisch beeld in deze familie is niet te onderscheiden van de andere SCA typen. Neuropathologisch onderzoek toonde geen polyglutamine (CAGn) positieve kern of cytoplasmatische ophopingen aan, die meestal gevonden worden bij coderende CAG repeat expansie mutaties. Mogelijk heeft dit SCA type dus een andere oorzaak. Een uitgebreide zoektocht naar het SCA23 gen is tot nu toe nog niet succesvol gebleken.

Helaas zijn voor traditioneel koppelingsonderzoek grote families met meerdere aangedane personen nodig en het Nederlandse "SCA-negatieve" panel bevat grotendeels kleinere families. Als een alternatief werd een zoektocht naar gemeenschappelijke chromosoom stukken tussen patiënten gebruikt om de ziekte veroorzakende genen te lokaliseren in deze families. Dit idee was gebaseerd op de aanwezigheid van zogenoemde founder mutaties. Dit zijn mutaties die ontstaan zijn in gemeenschappelijke voorvaderen van de Nederlandse ADCA populatie, zoals hoofdstuk 4 laat zien. Het bestaan van zulke founders kon aangetoond worden voor de SCA3 en SCA6 repeat expansie mutaties in Nederland. Deze resultaten werden versterkt doordat de meeste ADCA families behorende bij een specifieke voorvader, geografisch gegroepeerd bleken te zijn in bepaalde provincies. Hoofdstuk 4 laat zien dat het combineren van DNA marker profielen in combinatie met genealogie een efficiënte methode is om oorspronkelijk onafhankelijk gerapporteerde families aan elkaar te koppelen tot een grote familie. Dit zou bijzonder geschikt kunnen zijn voor het vinden van andere, nieuwe SCA gebieden in de kleinere ADCA families waarin tot nu toe nog geen mutatie gevonden is.

Hoofdstuk 5 beschrijft zo'n gecombineerde analyse van drie families waarin gedeelde DNA marker profielen gevonden werden, die bijna het gehele SCA14 koppelingsonderzoek gebied bedekten. Interessant genoeg, bleken deze families aan elkaar verwant te zijn, gebruik makend van genealogisch onderzoek en geografische groepering. Dit leidde tot de lokalisatie van de gemeenschappelijke SCA14 voorvader in the provincie Noord Barabant. Gebruik makend van sequentie analyse, werd een Gly118Asp (G118D) missense mutatie gevonden, in de sterk geconserveerde Cys2 regio van het C1 domein van het PKCγ (*PRKCG*) gen in alle patiënten van deze familie. Het klinisch beeld van deze familie laat een langzaam progressief atactisch syndroom zien met in een aantal patiënten bijkomende verschijnselen zoals overgevoelige reflexen en een pijnloze handafwijking waarbij de vingers ongecontroleerd naar de handpalm krullen.

Verder wordt beschreven in hoofdstuk 6 hoe het gehele Nederlandse SCA-negatieve panel doorzocht werd op mutaties in de Cys2 regio (exonen 4 en 5) van het *PRKCG* gen. Deze studie werd uitgevoerd om de frequentie van SCA14 mutaties in de Nederlandse ataxie populatie te bepalen. Bijna 10% van de 65 SCA-negatieve families worden verklaard door G118D mutaties in PKCγ. Ook in dit geval hielp geneologisch onderzoek om alle onafhankelijk ingestuurde families vanuit de Utrechtse en het Rotterdamse co-

horten te herleiden tot een gemeenschappelijke voorvader, uit de vroege 18e eeuw. Met dit resultaat werd bevestigd dat de G118D mutatie een SCA14 founder mutatie is in de Nederlandse ADCA populatie. Recent is er een nieuwe missense mutatie in exon 18 van *PRKCG* gevonden in een Franse SCA14 familie. Of deze mutatie aanwezig is in het Nederlandse SCA-negatieve panel is nog niet bekend.

In hoofdstuk 7, werd het effect onderzocht van twee SCA14 missense mutaties, G118D en C150F, op de functie van het PRKCG eiwit. De resultaten lieten zien dat deze SCA14 mutaties een toename veroorzaken van de intrinsieke PRKCG kinase activiteit. De subcellulaire lokalisatie van PKCγ werd zichtbaar gemaakt in COS cellen door het PKCγ eiwit te fuseren met een groen fluorescent eiwit (GFP). Dit experiment toonde aan dat het mutante PKCγ eiwit een veranderde calcium-geïnduceerde membraantranslocatie heeft. Ook werd een kwalitatief verschil opgemerkt in the verdeling van membraan binding tussen de normale - en het mutante PKCγ eiwit. Het mutante PKCγ eiwit was duidelijk verrijkt aanwezig in specifieke membraan regio's, die qua uiterlijk leken op membraan rimpels en ook actine-rijk bleken te zijn. Deze specifieke veranderingen in de functie van het mutante eiwit leiden waarschijnlijk tot de selectieve neuronale degeneratie die gezien wordt in SCA14 patiënten.

Samengevat blijkt, de genetische heterogeniteit van de ADCA families groter te zijn dan in eerste instantie was verwacht. Van 28 SCA-negatieve families konden we 21% (6/28) verklaren door significante koppeling met de SCA19, SCA23 en EA2 (=SCA6) regio's. Daarnaast bleek dat nog eens 3 andere onafhankelijk ingestuurde families gekoppeld waren met de SCA14 regio met behulp van DNA marker profielen. Het vinden van nieuwe SCA gebieden en hun bijhorende genen, zullen in de toekomst bijdragen aan uitbreiding van de huidige diagnostische mogelijkheden en zullen nieuwe inzichten geven in de ontwikkeling van potentiële therapieën.

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Curriculum Vitae

Dineke Verbeek werd geboren op 18 juli 1977 te Leiderdorp. Na het eindexamen VWO aan het Christelijk Lyceum te Alphen a/d Rijn begon zij in 1996 aan de studie medische biologie aan de Universiteit van Utrecht. Haar hoofdvakstage vond plaats in 1998 bij de vakgroep Medische Genetica van het Universitair Medisch Centrum Utrecht, onder leiding van Dr. R.J. Sinke. Het doel van deze stage was het lokaliseren en identificeren van nieuwe spinocerebellaire ataxie genen in de Nederlandse ataxie populatie. Vervolgens deed zij in 2000 haar bijvakstage bij het Rudolf Magnus Instituut in Utrecht onder begeleiding van Dr. S. Lopez da Silva. Daar probeerde zij transgene D-Ptx1 fruit vliegen te maken om de rol van het D-Ptx1 eitwit te bestuderen in neuronale ontwikkeling. In december 2000 studeerde zij af en begon zij in januari 2001 aan haar promotie-onderzoek bij de afdeling Medische Genetica van het Universitair Medisch Centrum Utrecht, wat uiteindelijk heeft geleid tot dit proefschrift.

Dineke Verbeek was born on 18th July 1977 in Leiderdorp, in the Netherlands. She attended the Christelijk Lyceum secondary school in Alphen a/d Rijn from 1989 to 1996 and started in 1996 the study medical biology at the University of Utrecht. For her major practical training in 1999 she worked at the department of Medical Genetics of the University Medical Center Utrecht. The aim of this internship was to localize and identify novel spinocerebellar ataxia genes in the Dutch ataxia population. In 2000, she performed her minor practical training at the Rudolf Magnus Institute in Utrecht, and tried to generate transgenic D-Ptx1 fruit flies, in order to study its function in neuronal development. She graduated in December 2000, and started in Januari 2001 with her PhD-project in the department of Medical Genetics of the University Medical Center Utrecht, of which the results are described in this thesis.

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