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REVIEW

Molecular diagnosis of autosomal recessive cerebellar ataxia in the whole exome/genome sequencing era

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Abstract

Autosomal recessive cerebellar ataxias (ARCA) are a clinically and genetically heterogeneous group of rare neurodegenerative disorders characterized by autosomal recessive inheritance and an early age of onset. Progressive ataxia is usually the prominent symptom and is often associated with other neurological or additional features. ARCA classification still remains controversial even though different approaches have been proposed over the years. Furthermore, ARCA molecular diagnosis has been a challenge due to phenotypic overlap and increased genetic heterogeneity observed within this group of disorders. Friedreich's ataxia and ataxia telangiectasia have been reported as the most frequent and well-studied forms of ARCA. Significant progress in understanding the genetic etiologies of the ARCA has been achieved during the last 15 years. The methodological revolution that has been observed in genetics over the last few years has contributed significantly to the molecular diagnosis of rare diseases including the ARCAs. Development of high throughput technologies has resulted in the identification of new ARCA genes and novel mutations in known ARCA genes. Therefore, an improvement in the molecular diagnosis of ARCA is expected. Moreover, based on the fact that many patients still remain undiagnosed, additional forms of ataxia are expected to be identified. We hereby review the current knowledge on the ARCAs, focused on the genetic findings of the most common forms that were molecularly characterized before the whole exome/genome era, as well as the most recently described forms that have been elucidated with the use of these novel technologies. The significant contribution of whole-exome sequencing or whole-genome sequencing in the molecular diagnosis of ARCAs is discussed.

Key words: Autosomal recessive cerebellar ataxia; Whole-exome sequencing; Whole-genome sequencing; Homozygosity mapping; Next generation sequencing

Core tip: Molecular diagnosis of autosomal recessive cerebellar ataxias (ARCA) is challenging due to clinical overlap and increased genetic heterogeneity. Although use of traditional techniques led to the identification of causative mutations in the past, the recent employment of novel technologies in this field, has initiated a new era in the molecular diagnosis of ARCA. Limitations such as small sized families, large numbers of candidate genes within mapped intervals and large sized genes hindered the timely discovery of ARCA genes using conventional Sanger sequencing. ARCA gene discovery and molecular diagnosis should be achievable at a much faster rate through the use of whole-genome or whole-exome sequencing technologies.

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INTRODUCTION

Autosomal recessive cerebellar ataxias (ARCA) constitute a subgroup of the hereditary cerebellar ataxias[1]. They are clinically and genetically heterogeneous neurodegenerative disorders characterized by autosomal recessive inheritance and usually a clinical onset before the age of 20. Both the central and the peripheral nervous systems may be involved, as well as other systems and organs in rare cases[1,2]. In most cases the cerebellum, the spinocerebellar tract and/or the sensory tracts of the spinal cord are primarily affected[3]. Therefore, progressive ataxia is the main symptom and is often associated with other neurological or extra-neurological signs.

This group encompasses many rare diseases; Friedreich's ataxia and ataxia telangiectasia being the most frequent forms[2-4]. Their prevalence has been estimated to be 7 in 100000 inhabitants[5] with considerable variability observed in different geographic regions[3,6,7]. Many classifications have been proposed using different criteria and this issue still remains controversial. The first clinical classification of inherited ataxias, also encompassing ARCA, was proposed by Harding in 1983 and was based on the age of onset and pathological mechanisms[8,9]. Through the next decade, genetic studies led to new insights and thus additional criteria were used in some classifications. Koenig[10] proposed a classification of ARCA based on topographical and pathophysiological criteria, while in the next year the group of Filla proposed a pathogenic classification of the hereditary ataxias[11]. More recently, Palau and Espinos proposed a classification of ARCA that is based on inheritance patterns and natural history of the clinical symptoms[1].

Through the past 15 years, significant progress has been made in improving our understanding of the genetic etiology of the ARCA. Currently, more than 30 genes have been associated with ARCA (Table 1) and many more are expected to be discovered since the genetic causes still remain unknown, for a large number of patients/families. The identified ARCA genes are involved in variable cellular processes such as mitochondrial energy generation, DNA repair, transcription, RNA processing, protein folding and ion channels[12].

Despite the existence of different classification schemes and the significant number of already identified genes, diagnosis of ARCA remains a challenge due to their vast clinical variability and genetic heterogeneity[13-15]. The methodological revolution of genetics observed over the last years contributed significantly to the proper diagnosis of ARCA. The recently developed next-generation sequencing (NGS) technologies, whole-exome sequencing (WES)/whole-genome sequencing (WGS), or the strategy of using homozygosity linkage mapping combined with NGS, proved to be efficient for the identification of novel genes associated with rare diseases. In comparison to the traditional methods widely used in the past for gene identification, the new techniques are easier, cost and time-efficient since they offer the opportunity to screen the whole-genome or the coding part of the genome in one experiment[14]. Limitations such as locus heterogeneity, small family size and existence of numerous candidate genes within a mapped region are no longer an obstacle[16]. Although relatively new, these technologies proved to be powerful methods for gene identification in rare neurological disorders such as ataxia; 7 new genes were identified in the past 3 years. Moreover, exome sequencing is gradually used as a screening tool thus enabling the identification of mutations in already known genes[17-21].

We hereby provide a review focused on the molecular diagnosis of ARCA with more emphasis on the recent discoveries observed in the whole exome/genome sequencing era.

MOLECULAR DIAGNOSIS OF AUTOSOMAL RECESSIVE CEREBELLAR ATAXIAS WITH THE CONVENTIONAL METHODS

The most common ARCA forms that have been molecularly diagnosed through the use of traditional methods as well as the most common mutations identified are outlined below. If an affected individual is clinically suspected of having a specific type of the following ARCA, then investigation could begin with targeted mutation analysis.

Degenerative ataxias

Friedreich ataxia: Friedreich ataxia is the most frequent inherited ataxia in the Caucasian population[1] and the most common form of degenerative recessive ataxia with an estimated prevalence of 1-2/50000[22-24]. The disease has been associated with mutations in the *FXN* gene located on chromosome 9q13[25-27]. A homozygous GAA repeat expansion within the first intron of the gene is the causative mutation for 95% of the cases[28], while the rest 5% of the cases are compound heterozygous for a GAA expansion and a point mutation in the *FXN* gene[29]. The expansion mutation leads to an impairment of transcription and further to a significant reduction in the expression of the encoded protein, frataxin. Although the function of frataxin has not been fully elucidated, its role in cellular iron homeostasis has been established. The deficiency caused by the expansion mutation results in mitochondrial dysfunction and oxidative damage[24,30].

Friedreich ataxia is considered as the first candidate for genetic testing of patients with ARCA in most populations. Specific polymerase chain reaction (PCR)-based molecular tests for the GAA repeat expansion are available in many laboratories[31-33]. Suspected compound heterozygote cases may be further investigated by Sanger sequencing of the *FXN* gene coding regions.

Mitochondrial recessive ataxia syndrome: Mitochondrial recessive ataxia syndrome is the most frequent juvenile- or adult-onset type of ataxia in Finland[34]. The disease has been associated with mutations in the *POLG* gene, encoding polymerase γ which is implicated in

Table 1 Autosomal recessive cerebellar ataxias

Type	Gene/protein	Gene OMIM#	Gene locus
Degenerative ataxias			
Friedreich's ataxia	*FXN*/Frataxin	*606829	9q13
SCAR9	*ADCK3*/aarF domain-containing protein kinase 3	*606980	1q42.13
MIRAS	*POLG*/Polymerase γ	*174763	15q25
IOSCA (MTDPS7)	*C10orf2*/Twinkle	*606075	10q24
Marinesco-Sjögren syndrome	*SIL1*/BiP associated protein	*608005	5q31
Charlevoix-Saguenay spastic ataxia	*SACS*/Sacsin	*604490	13q12
EOCARR (EOCA)	-	-	13q11-12
Ataxias with DNA repair defects			
AOA1	*APTX*/Aprataxin	*606350	9p13.3
AOA2 (SCAR1)	*SETX*/Senataxin	*608465	9q34
AOA3	*PIK3R5*/Phosphoinositide 3-kinase regulatory subunit 5	*611317	17p13.1
AT	*ATM*/ATM	*607585	11q22.3
ATLD	*MRE11A*/MRE11	*600814	11q21
SCAN1	*TDP1*/Tyrosyl DNA phosphodiesterase Ⅰ	*607198	14q31-q32
Congenital ataxias			
JBTS1	*INPP5E*/Phosphatidylinositol polyphosphate 5-phosphatase type Ⅳ	*613037	9q34.3
JBTS2	*TMEM216*/transmembrane protein 216	*613277	11p12-q13.3
JBTS3	*AHI1*/Jouberin	*608894	6q23.3
JBTS4	*NPHP1*/Nephrocystin-1	*607100	2q13
JBTS5	*CEP290*/Nephrocystin-6	*610142	12q21.3
JBTS6	*TMEM67*/Meckelin	*609884	8q22.1
JBTS7	*RPGRIP1L*/Nephrocystin-8	*610937	16q12.2
Cayman ataxia	*ATCAY*/Caytaxin	*608179	19p13.3
Metabolic ataxias			
AVED (VED)	α-TTP/α-tocopherol transfer protein	*600415	8q13.1-q13.3
ABL	*MTP*/Microsomal triglyceride transfer protein	*157147	4q22-24
CTX	*CYP27*/Sterol 27-hydroxylase	*606530	2q33-qter
Refsum's disease	*PHYH*/Phytanoyl-CoA hydrolase	*602026	10pter-p11.2
	PEX7/Peroxin 7	*601757	6q22-q24
Metachromatic leucodystrophy	*ARSA*/Arylsulfatase 1	*607574	22q13.31-qter
Niemann Pick disease type C	*NPC1*/NPC1 protein	*607623	18q11-q12
GM1 gangliosidosis	*GLB1*/Beta-galactosidase	*611458	3p21.33
GM2 gangliosidosis	*HEXA*/Hexosaminidase A	*606869	15q23-24
Wilson disease	*ATP7B*/ATPase Cu transporting beta-polypeptide	*606882	13q14.3
Aceruloplasminemia	*CP*/Ceruloplasmin	*117700	3q23-q24
CHAC	*VPS13A*/Chorein	*605978	9q21
Other recently identified types			
SCAR8	*SYNE1*/Syne-1	*608441	6q25.1-q25.2
Rundataxin-related ataxia	*KIAA0226*/Rundataxin	*613516	3q27.3-qter
Novel types identified by the use of next-generation sequencing			
SCAR10	*ANO10*/Anoctamin 10	*613726	3p22.1
SCAR11	*SYT14*/Synaptotagmin XIV	*610949	1q32.2
SCAR7	*TPP1*/tripeptidyl-peptidase 1 enzyme	*607998	11p15.4
Autosomal-recessive cerebellar ataxia with spasticity	*GBA2*/β-glucosidase 2	*609471	9p13.3
SCAR13	*GRM1*/Metabotropic glutamate receptor 1	*604473	6q24.3

SCAR7: Spinocerebellar ataxia autosomal recessive type 7; SCAR8: Spinocerebellar ataxia autosomal recessive type 8; SCAR9: Spinocerebellar ataxia autosomal recessive type 9; SCAR10: Spinocerebellar ataxia autosomal recessive type 10; SCAR11: Spinocerebellar ataxia autosomal recessive type 11; SCAR13: Spinocerebellar ataxia autosomal recessive type 13; MIRAS: Mitochondrial recessive ataxic syndrome; IOSCA: Infantile-onset spinocerebellar ataxia; AOA: Ataxias with oculomotor apraxia; AT: Ataxia telangiectasia; ATLD: Ataxia-telangiectasia-like disorder; SCAN1: Spinocerebellar ataxia with axonal neuropathy; CTX: Cerebrotendinous xanthomatosis; CHAC: Chorea-acanthocytosis; JBTS: Joubert syndrome; AVED: Ataxia with isolated vitamin E deficiency; ABL:Abetalipoproteinemia.

mtDNA replication and repair. The most common mutation identified thus far is the c.2243G > C [p.Trp748Ser] associated with the c.3428A > G [p.Glu1143Gly] mutation which is probably a polymorphism with a modifying role. Another frequent mutation is the c.1399G > A [p.Ala467Thr]. Homozygous or compound heterozygous patients for the three mutations have been reported[34-36]. The presence of the three mutations may be molecularly diagnosed by PCR and solid-phase minisequencing while a Real time PCR assay has been proposed for identifying the presence of the c.1399G > A mutation[34].

Infantile onset spinocerebellar ataxia: Infantile onset spinocerebellar ataxia is a rare disorder manifesting at a very early age and was first described in Finland. It has been associated with mutations in the *C10orf2* gene on chromosome 10q24 that encodes Twinkle, a mtDNA specific helicase and Twinky, a rarer splice variant[37]. The most com-

mon mutation identified is c.1523A > G [p.Tyr508Cys]. Mutations in this gene may be detected either by direct sequencing or by solid-phase minisequencing.

Autosomal recessive spastic ataxia of Charlevoix-Saguenay: Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is an early childhood onset disease characterized by spastic gait ataxia. It was first described in families from the Charlevoix-Saguenay region of northeastern Quebec and for many years it was thought to be restricted there. It has been mapped to chromosome 13q11 and causative mutations have been identified in the *SACS* gene[38] that encodes sacsin, a chaperone protein. Almost 96% of the molecularly diagnosed individuals from this region are homozygote or compound heterozygote for the two founder mutations c.8844delT [p.Pro2948fs] and c.7504C > T [p.Arg2502*] (originally reported as 6594delT and 5254C > T) identified by sequence-based analyses[39,40]. More than 100 other pathogenic mutations in individuals outside Quebec were identified since mutation analysis became available, demonstrating that mutations in SACS are a frequent cause of the disease worldwide[41-43]. Molecular genetic testing is usually performed by targeted mutation analysis, by direct sequencing of all coding exons and their flanking intronic sequences and by deletion/duplication analysis using the multiplex ligation-dependent probe amplification method[44].

Marinesco-Sjögren syndrome: Marinesco-Sjögren syndrome is a rare disorder with onset in infancy or childhood. Homozygosity mapping in two large consanguineous families of Turkish and Norwegian origin mapped the disease gene on chromosome 5q31[45]. Causative mutations have been identified in patients of different ethnic origin, in the *SIL1* gene encoding a protein that acts as a nucleotide exchange factor for the heat-shock protein 70 chaperone the 78-kDa glucose-regulated protein, mainly located at the endoplastic reticulum[46-48]. Molecular genetic testing is performed by direct sequencing of all coding exons and their flanking intronic sequences.

Spinocerebellar ataxia autosomal recessive type 9: Spinocerebellar ataxia autosomal recessive type 9 (SCAR9), also known as autosomal recessive cerebellar ataxia type 2 (ARCA2) refers to ARCA associated with muscle coenzyme Q_{10} deficiency[49]. Homozygosity mapping (GeneChip Human Mapping 10K 2.0 Xba Array, Affymetrix) in a large consanguineous Algerian family mapped the disease gene to chromosome 1q41-q42. *ADCK3* gene mutations have been identified by direct sequencing in this family, in additional families and also in sporadic patients[49-51]. The ADCK3 protein is a mitochondrial ancestral kinase which is involved in CoQ10 synthesis[49].

Ataxias with DNA repair defects

ARCA with oculomotor apraxia: ARCA with oculomotor apraxia (OMA), known as ataxias with oculomotor apraxia (AOA), are characterized by cerebellar ataxia accompanied by ophthalmological and neurological symptoms[52,53]. Five distinct genetic forms have thus far been identified: ataxia-telangiectasia (A-T), ataxia-telangiectasia-like disorder (ATLD), ataxia with oculomotor apraxia type 1 (AOA1), ataxia with oculomotor apraxia type 2 (AOA2) and ataxia with oculomotor apraxia type 3 (AOA3)[54].

(1) Ataxia telangiectasia: Ataxia telangiectasia is the second most common ARCA after Friedreich ataxia. It presents in early childhood with progressive cerebellar ataxia, while later ataxia may be accompanied by a multitude of symptoms such as choreathetosis, dystonia, telangiectasia that is the guiding diagnostic feature, immunodeficiency, cancer predisposition, sinopulmonary infection, immunosensitivity and radiosensitivity[1,14]. The disease gene *ATM* is located on chromosome 11q23[55,56]. It is one of the largest genes, comprising 66 exons and encodes a member of the phosphoinositol-3 kinase family of proteins that are involved in cell cycle checkpoint control and the DNA repair through phosphorylation of substrates[1]. More than 500 mutations have been identified, that are positioned throughout all the coding exons of the gene[14]. Due to the large size of the gene, molecular diagnosis with the traditional methods has been difficult. A multiplex scanning method, detection of virtually all mutations-SSCP has been proposed however with reduced sensitivity[57]. The newly developed NGS technologies are promising for the diagnosis of diseases such as A-T with associated mutations in large genes.

(2) Ataxia-telangiectasia-like disorder: Ataxia-telangiectasia-like disorder (ATLD) is a very rare disorder that resembles A-T in its clinical phenotype[58] with absence of telangiectases and immune deficiency. It has been mapped to chromosome 11q21 and a small number of causative mutations have been identified in the *MRE11A* gene[59-61]. The encoded protein interacts with RAD50 and NBS1 thus forming the core of the MRN (MRE11-RAD50-NBS1) complex which is involved in DNA repair pathways, in DNA recombination and in multiple cell-cycle checkpoints control[60].

(3) Ataxia with oculomotor apraxia type 1: Ataxia with oculomotor apraxia type 1 (AOA1) was first described in Portuguese and Japanese families. The disease gene has been mapped to chromosome 9p13.3[62] and mutations in the *APTX* gene have been identified[63]. The encoded protein aprataxin, localizes to the nucleus[64] and the mitochondria[65] and it is involved in the nuclear and the mtDNA repair machinery. AOA1 is the most frequent type of ARCA in Japan and the second most frequent in Portugal after Friedreich ataxia. The insertion c.689insT and the missense c.617C > T [p.Pro206Leu] mutations, are more common in the Japanese and the nonsense c.837G > A [p.W279X] mutation is more common in Portuguese patients. More than 20 mutations including missense/nonsense or splice site mutations, small dele-

tions/insertions and gross deletions have been reported thus far in patients of different ethnicities, thus expanding the presence of AOA1 in other countries outside Japan and Portugal[3,66-70].

(4) Ataxia with oculomotor apraxia type 2: Ataxia with oculomotor apraxia type 2 (AOA2) is also referred to as autosomal recessive spinocerebellar ataxia 1 (SCAR1) since oculomotor apraxia is an occasional finding, not present in all the patients. It is the second most frequent recessive ataxia in Europe[4] and is usually (in 96% of cases) accompanied by a characteristic laboratory finding, the elevation of serum α-fetoprotein. AOA2 usually presents within the second decade of life and has a higher mean age of onset compared to AOA1 and A-T[52]. The disease gene has been mapped to chromosome 9q34 and later on mutations in the *SETX* gene were identified[71]. SETX is a large gene of 26 exons and encodes senataxin, a nuclear protein, which contains at its C terminus a classic seven-motif domain found in the superfamily 1 of helicases. The protein is involved in the response to oxidative stress[53]. Acting as an RNA/DNA helicase, senataxin might have a significant role in the DNA repair pathway, in the splicing machinery[71] and also in the processing of noncoding RNAs[52]. Even though the *SETX* gene cannot be easily analysed by routine sequencing due to its size, several patients have been studied with this method and more than 75 different mutations have been identified thus far worldwide[52,72,73] including missense, nonsense and splice site mutations, as well as small/gross deletions or insertions[74].

(5) Ataxia with oculomotor apraxia type 3: Ataxia with oculomotor apraxia type 3 has more recently been described in a consanguineous Saudi Arabian family with four affected individuals that present with a similar phenotype to AOA2. Elevated serum α-fetoprotein levels were identified in all the patients and 2 siblings had OMA. The homozygous missense c.1885C > T [p.Pro629Ser] mutation in exon 12 of the *PIK3R5* gene has been associated with the disease in this family[54]. *PIK3R5* is located on chromosome 17p12-p13. The encoded protein is a p101 regulatory subunit that interacts with class 1B PI3K which is involved in cell survival and growth as well as in metabolism, immune and cardiac functions. Even though it is registered as rs61761068 in db-single-nucleotide polymorphism (dbSNP), frequencies of the mutant allele in other populations indicate that it has only been detected in a heterozygous state, most likely representing non-affected carriers of the mutation[54].

Spinocerebellar ataxia with axonal neuropathy: Spinocerebellar ataxia with axonal neuropathy is a related syndrome to the AOA group. It is characterized by the co-occurrence of cerebellar ataxia and axonal neuropathy. A single locus on chromosome 14q31-q32 has been associated with the disease and a missense mutation (c.1478A > G [p.His493Arg]) in the *TDP1* gene has been described in a large Saudi Arabian family[75]. The encoded protein tyrosyl-DNA phosphodiesterase 1 is a member of the DNA single-strand break repair complex and plays a significant role in the DNA repair pathway[75,76].

Congenital and metabolic ataxias

Other ARCA are classified under the subgroups of congenital and metabolic ataxias (Table 1). Associated genes and mutations have been identified and molecular genetic testing by targeted mutation analysis or direct sequencing of the appropriate gene may be performed in ARCA patients based on the clinical picture. Moreover, in some cases of metabolic ataxias, biochemical analysis on specific available markers could precede and orientate the genetic testing.

Congenital ataxias: Congenital ataxias that have been associated with specific chromosomal loci and genes include the Joubert syndrome and the Cayman ataxia. Joubert syndrome has thus far been associated with seven loci (JBTS1, JBTS2, JBTS3, JBTS4, JBTS5, JBTS6, and JBTS7) and mutations have been identified in the seven *INPP5E*[77], *TMEM216*[78], *AHI1*[79], *NPHP1*[80], *CEP290*[81], *TMEM67*[82] and *RPGRIP1L*[83] genes respectively (Table 1). The Cayman ataxia has been described in an isolated population of the Cayman Island. A single locus on chromosome 19p13.3 has been associated with the disease and causative mutations in the *ATCAY* gene have thus far been identified[84].

Metabolic ataxias: The subgroup of metabolic ataxias includes disorders characterized by progressive ataxia, recurrent ataxia and ataxia as a minor feature. Progressive ataxia includes ataxia with isolated vitamin E deficiency, abetalipoproteinemia, cerebrotendinous xanthomatosis and Refsum disease[1]. Intermittent or minor ataxia includes: metachromatic leukodystrophy (*ARSA* gene)[85,86], Niemann-Pick type C 1 (*NPC1* gene)[87], GM1 (*GLB1* gene)[88,89] and GM2 (*HEXA* gene)[90,91] gangliosidosis, Wilson's disease (*ATP7B* gene)[92-94], aceruloplasminaemia (*CP* gene)[95] and Chorea-acanthocytosis (*VPS13A* gene)[96].

(1) Ataxia with isolated vitamin E deficiency: Ataxia with isolated vitamin E deficiency has a similar clinical picture with Friedreich ataxia. It is characterized by a biochemical abnormality, the very low plasma level of vitamin E and hence the low level of the α-tocopherol in the serum. The disease is caused by mutations in the α-*TTP* gene, encoding the α-tocopherol transfer protein which is responsible for the transfer of vitamin E to the nascent very low-density lipoproteins[97]. Diagnosis may include the finding of low serum vitamin E values in the absence of malabsorption[1] and further by performing genetic testing.

(2) Abetalipoproteinemia: Abetalipoproteinemia is characterized by deficiency of the liposoluble vitamin E, the low levels of cholesterol and the absence of the low-density lipoproteins. The clinical features comprise the

malabsorption syndrome, the pigmentary degeneration of the retina and progressive ataxic neuropathy. Moreover, patients show a characteristic deformation of their red cells called acanthocytosis[98]. The disease is caused by mutations in the *MTP* gene which encodes the microsomal triglyceride transfer protein catalyzing the transport of triglyceride, cholesteryl ester and phospholipid between phospholipid surfaces[99].

(3) Cerebrotendinous xanthomatosis: Cerebrotendinous xanthomatosis is a sterol storage disorder, characterized by abnormal accumulation of cholesterol and cholestanol in neural and other tissues, thus causing tendon xanthomas, premature atherosclerosis, cataracts and neurological dysfunction which is manifested as cerebellar ataxia, dementia and spinal cord paresis[100]. The disease gene has been mapped to chromosome 2q33-qter and is caused by mutations in the *CYP27* gene have been identified. *CYP27* encodes sterol 27-hydroxylase, a mitochondrial cytochrome P450 enzyme that has an important role in cholesterol and bile acid metabolism[100,101].

(4) Refsum's disease: Refsum's disease is caused by defective peroxisomal alpha-oxidation of phytanic acid, thus leading to its accumulation in blood and other tissues including neurons. It is characterized mainly by progressive retinitis pigmentosa, peripheral neuropathy and cerebellar ataxia. Other additional features include anosmia, deafness, cardiomyopathy, ichthyosis and skeletal abnormalities. The disorder is genetically heterogeneous since it has been mapped to two different loci. In most families, linkage was established to the first identified locus on chromosome 10pter-p11.2. Causative mutations have been found in the *PHYH* (or *PAHX*) gene[102,103] encoding the phytanoyl-CoA hydroxylase enzyme that is involved in the catabolic pathway of phytanic acid. In a subset of patients mutations in the *PAHX* gene have been excluded. Further linkage analysis of these families led to mapping of a second locus on chromosome 6q22-24. In this subgroup of patients causative mutations have been identified in the *PEX7* gene encoding the peroxin 7 protein which is implicated in the import of matrix proteins into the peroxisome[104].

Other known ARCA types

The following ARCA forms have been reported more recently that have not been included in the classification scheme described by Palau and Espinos (2006).

Spinocerebellar ataxia autosomal recessive type 8: Spinocerebellar ataxia autosomal recessive type 8 (SCAR8), also known as autosomal recessive cerebellar ataxia type 1 (ARCA1) is an adult-onset slowly progressive cerebellar ataxia accompanied by dysarthria and other associated features[105]. It was first described and so far only reported in French-Canadian families most of them originating from defined regions of Quebec. A single locus on chromosome 6q25.1-q25.2 has been mapped and mutations in the *SYNE1* gene have been associated with the disease. Syne-1 is involved in anchoring specialized myonuclei underneath the neuromuscular junction[105] and plays a significant role in the development and maintenance of cerebellar functions. *SYNE1* is a large gene of 147 exons and this makes mutation screening by traditional methods expensive and complicated. Thus far direct sequencing of all the exons and flanking intronic sequences revealed a small number of missense and splice site mutations as well as a small scale deletion[105,106].

Rundataxin-related ataxia: A new form of pure recessive cerebellar ataxia accompanied by epilepsy and mental retardation named 'Salih ataxia' has been described in a large consanguineous Saudi Arabian family with three affected individuals[107]. Homozygosity mapping with SNP array (Gene Chip Human Mapping 10K 2.0 Xba Array, Affymetrix) and further linkage analysis with microsatellite markers, mapped the disease gene on chromosome 3q27.3-qter. Direct sequencing of the candidate genes enabled identification of the causative mutation in the *KIAA0226* gene. A single nucleotide deletion 2927delC [p.Ala943ValfsX146] frameshift mutation in this gene has thus far been identified. The encoded protein was named rundataxin based on its two conserved domains[107] and it is associated with vesicular trafficking and signaling pathways.

NEXT-GENERATION SEQUENCING AND MOLECULAR DIAGNOSIS OF AUTOSOMAL RECESSIVE CEREBELLAR ATAXIAS

NGS technologies provide powerful tools for the identification of new genes. During the last 3 years, use of homozygosity mapping in combination with WES or targeted sequencing using the NGS platforms, enabled the identification of 5 new genes associated with rare types of ARCA in small families. WES has also been used as a screening tool thus enabling the rapid identification of mutations in known genes and demonstrating that it can be an efficient diagnostic tool.

Identification of new genes

Spinocerebellar ataxia autosomal recessive type 10: A new form of pure recessive ataxia accompanied by downbeat nystagmus and lower motor neuropathy, first described in a Dutch consanguineous family and named spinocerebellar ataxia autosomal recessive type 10 (SCAR10), was the first recessive ataxia molecularly diagnosed with homozygosity mapping and NGS[108]. It is also known as ARCA3[2]. Homozygosity mapping with a 10K SNP array (Affymetrix) combined with linkage studies with short tandem repeat markers led to fine mapping of the disease gene to chromosome 3p21.32-p22.3. A large number of genes were included in the mapped region thus making identification of the causative mutation

with the traditional Sanger sequencing difficult. Use of Sanger sequencing to analyze 15 candidate genes failed to identify any mutation. Targeted NGS was subsequently performed that enabled identification of the causative mutation in the *ANO10* gene, thus demonstrating the power of this method towards the identification of new genes. A homozygous c.1529T > G [p.Leu510Arg] mutation was identified in the Dutch family. Additional mutations have been identified in patients originating from Serbia (a homozygous frameshift mutation c.1150_1151del [p.Leu384fs]) and France (compound heterozygous mutations c.1476 + 1G > T and c.1604del [p.Leu535X]) through direct analysis of the new gene by conventional Sanger sequencing[108]. More recently, a Japanese patient has been reported with a homozygous nonsense mutation (c.609C > G [p.Y203X]) identified by exome sequencing[19] indicating that this new method can be applied as a screening tool allowing the fast identification of mutations in known genes. The encoded protein *ANO10* is a member of the human anoctamin family and similar to the other proteins of this family it is a putative calcium-dependent chloride channel, thus representing a new pathway implicated in cerebellar ataxias. However, this function remains to be confirmed[108].

Spinocerebellar ataxia autosomal recessive type 11: A slowly progressive new type of ARCA accompanied by psychomotor retardation has been recently described in a small consanguineous Japanese family with two affected individuals and is known as spinocerebellar ataxia autosomal recessive type 11 (SCAR11). Homozygosity mapping using the Human SNP Array 6.0 (Affymetrix) combined with multipoint linkage analysis using extracted SNP array data revealed three candidate regions of homozygosity. WES was subsequently employed and data analysis focused on the regions of homozygosity. Sanger sequencing was used to further investigate filtered variants and the synaptotagmins 14 (SYT14) homozygous c.1451G > A [p.Gly484Asp] missense mutation has been associated with the disease. Further screening of all the coding regions of the entire gene in sporadic and familial ARCA cases failed to identify additional mutations. The encoded protein is Synaptotagmin XIV, a member of SYTs which are transmembrane proteins associated with exocytosis of secretory vesicles. Expression studies on SYT14 show that this protein plays a significant role in the cerebellum and support previous suggestions about the possible involvement of disrupted SYTs in neurodegeneration[12].

Spinocerebellar ataxia autosomal recessive type 7: Spinocerebellar ataxia autosomal recessive type 7 (SCAR7) was first described in a Dutch family in 2004 as a distinct ARCA type, based on its genetic locus, the onset of the disease and/or other clinical findings[109]. The disease gene has been mapped on chromosome 11p15 by genome-wide linkage analysis with microsatellite markers from the Marshfield genetic map. Identification of the causative mutation/gene was impossible for many years due to the large number (> 200) of genes within the candidate region. Recently, the use of WES enabled the association of the disease in the family with two *TPP1* compound heterozygous mutations (the splice site c.509-1G > C and the missense c.1397T > G [p.Val466Gly])[110]. These mutations were also detected in a sporadic SCAR7 patient. Mutations in the *TPP1* gene had already been associated with another disorder, the late infantile lipofuscinosis disease 2 (CLN2). Association of mutations in this gene with SCAR7 expands the phenotypes related to variants of the *TPP1* gene. Phenotype-genotype correlations proposed the hypothesis that CLN2 disease is due to variants causing the loss of enzyme activity, while SCAR7 is due to variants decreasing the enzyme activity. *TPP1* encodes the tripeptidyl-peptidase 1 enzyme, a member of the sedolisin family of serine proteases. This enzyme cleaves the N-terminal tripeptides from substrates and has a weak endopeptidase activity[110].

Autosomal-recessive cerebellar ataxia with spasticity: A distinct form of ataxia previously described in three consanguineous Tunisian families with 7 affected individuals has been recently molecularly characterized with the combined strategy of homozygosity mapping and WES thus providing further evidence about the potential of these methods[111]. Childhood or juvenile onset cerebellar ataxia was the main feature in all affected individuals of the families but later they developed pronounced limb spasticity. High density SNP genotyping was performed with the OmniExp-12, v1.0 DNA Analysis BeadChip (Illumina) and one large region of homozygosity on chromosome 9 was identified. The *APTX* gene mutated in AOA1 was included in the mapped region, so mutations in *APTX* were first excluded by Sanger sequencing. WES was then performed that revealed 2 causative mutations in the *GBA2* gene (the homozygous c.1018C > T [p.Arg340*] shared by two families and the homozygous c.2618G > A [p.Arg873His])[111]. Sanger sequencing confirmed segregation of the identified mutations with the disease in the families. Furthermore, WES was used to screen 21 molecularly undiagnosed Tunisian individuals with ataxia. Through this procedure an additional *GBA2* mutation (c.363C > A [p.Tyr121*]) was identified[111]. A second group associated 4 *GBA2* mutations with the spastic paraplegia type 46 (SPG46) phenotype[112].

GBA2 encodes the non-lysosomal β-glucosidase 2 protein that was first recognized as a microsomal bile-acid β-glucosidase, while more recently it was reassigned to sphingolipid metabolism[113], a critical process for the nervous system[114]. The role of the enzyme in the central nervous system development was confirmed through the validation of the functional phenotype of some of the identified mutations causing HSP *in vivo* thus demonstrating the connection of sphingolipid metabolism and neurodegeneration[112]. HSPs are another heterogeneous group of neurodegenerative disorders having common features with ataxias and as a consequence, many times it is difficult for the clinician to decide whether the patient has hereditary ataxia with spasticity or HSP with cerebellar features. Identification of mutations in the same gene

causing these two diseases demonstrates that a common pathway may be involved in neurodegeneration.

Spinocerebellar ataxia autosomal recessive type 13: A new form of congenital cerebellar ataxia identified in Roma patients has recently been described and molecularly characterized[115]. This new form is known as spinocerebellar ataxia autosomal recessive type 13 (SCAR13). WES was used both for linkage analysis and mutation detection. A subgroup of SNP genotypes was first selected from the exome sequence data and used to perform genome wide linkage analysis, thus emphasizing an additional application of this method. Linkage analysis mapped the disease gene to a single locus on chromosome 6q24. Data analysis was focused on the linked interval and finally resulted to the identification of two homozygous mutations in the *GRM1* gene (c.2652_2654del [p.Asn885del] and c.2660+2T > G). Sanger sequencing was used to verify the presence of mutations and their co-segregation with the disease within the families while PCR-based restriction-fragment-length-polymorphism assays and pyrosequencing were used to evaluate the mutation frequencies among control individuals. Both mutations interfere with a critical alternative splicing gene region. *GRM1* encodes the metabotropic glutamate receptor mGluR1 which is implicated in the modulation of intracellular Ca^{2+} levels and neuronal excitability. It is important for the development of the cerebellar cortex and the cerebellar and hippocampal synaptic plasticity, memory and learning[115].

Identification of mutations in known genes

The combined strategy of homozygosity mapping and WES has been recently applied for the investigation of a consanguineous Turkish family and revealed a novel homozygous missense mutation (c.1366C > G [p.Leu456Val]) in the *C10orf2* gene, mutations in which have already been associated with infantile-onset spinocerebellar ataxia (IOSCA)[16]. This rare type of ataxia was previously reported only in Finland. The recent identification of this novel mutation shows that IOSCA is not restricted to Finland.

WES was applied in another recent study to investigate two affected non-consanguineous siblings with a multisystem neurological disorder of unknown genetic origin and revealed two novel heterozygous mutations in the known *SACS* gene. These patients had been previously investigated with conventional methods which failed to identify the associated mutations in contrast to this novel approach which rapidly led to molecular diagnosis. Both patients shared the compound heterozygous mutations c.2076delG [p.Thr692Thrfs*713] and c.3965_3966delAC [pGly1322Valfs*1343] in the *SACS* gene that have been associated with ARSACS. Another novel homozygous mutation in *SACS* (c.2439-2440delAT [p.Val815Glyfs*4]) has been identified in a Tunisian family with two affected individuals, by homozygosity mapping and WES[13]. A more recent study on a 4-year-old girl revealed an additional novel frameshift homozygous mutation in the same gene by WES[18].

WES also enabled the molecular diagnosis of the disease in an early onset spastic ataxia-neuropathy syndrome family. A homozygous missense mutation in the *AFG3L2* gene (c.1847G > A [p.Y616C]) has been identified[116]. Heterozygous mutations in the *AFG3L2* gene have previously been identified in autosomal dominant spinocerebellar ataxia type 28 patients[117,118]. The *AFG3L2* gene encodes a subunit of the m-AAA class of mitochondrial proteases which forms either a homo-oligomeric isoenzyme or a hetero-oligomeric complex with paraplegin, encoded by the *SPG7* gene[116]. Mutations in the *SPG7* gene have been associated with hereditary SPG7, characterized by adult-onset spasticity and weakness of the lower extremities[119]. In contrast to SCA28, this new syndrome associated with a recessive pattern of inheritance, is characterized by a much earlier onset and combines phenotypic features of SCA28 and *SPG7*[116].

SYNOPSIS

The diagnosis of ARCA is becoming progressively more complex and challenging since clinical and genetic heterogeneity is expanding. Mutations in more than 20 genes have been associated with ARCA through the last decade, some of them identified in single families. There exists a significant number of patients remaining without a molecular diagnosis thus leading to the hypotheses that many other novel ARCA genes remain to be discovered in the future and that a different gene might be associated with the disease in each family mainly in populations with frequent inbreeding. The development of NGS technologies has already contributed significantly towards the molecular diagnosis of these rare diseases and appears very promising towards the diagnosis of additional cases in the future.

Sequencing of known candidate genes with conventional methods based on clinical findings has proved to be time-consuming and costly, except in the case of metabolic ataxias for which biochemical test results can precisely guide the molecular diagnosis. Routine investigation of some already known genes such as *SYNE1*, *ATM*, *SETX*, *POLG* and *SACS* is prohibitive, due to their significantly large coding regions.

Homozygosity mapping in consanguineous families has proved to be a powerful tool and may precede sequencing thus contributing to a more targeted analysis and facilitating the identification of novel ARCA genes. High density genome wide SNP genotyping with SNP arrays is the most rapid and effective method applied in the last years for homozygosity mapping. However, further analysis with more informative markers is often required in order to confirm or exclude some of the identified regions. Moreover, instead of using a SNP array, extracted SNP genotypes from the WES data may be used for genome wide linkage analysis. If a small number of genes exists within a mapped interval, then investigation by conventional sequencing may be efficient. Otherwise, if a large number of candidate genes exists, conventional

sequencing proves to be difficult, time consuming and expensive compared to the new methodologies.

WES enables the rapid sequencing of all coding regions of the genome and its usefulness has already been demonstrated in research and diagnostic applications. Conventional sequencing remains useful as a complimentary method for the investigation of the segregation pattern of candidate variants previously revealed by WES and verification of the causative mutations. Moreover, investigation of families for mutations in a known gene within a mapped region could be performed either by conventional sequencing or by WES depending on the size of the coding regions that need to be examined. For small coding regions, the first method may be more cost effective while for large genes, WES is less expensive since costs have significantly decreased over the last year[14].

Although the application of NGS technologies enables the identification of novel ARCA genes and the characterisation of novel mutations in known genes thus providing molecular diagnosis in many cases, there are some limitations due to their current reduced ability to sequence repetitive DNA expansions[14]. The most common form of ARCA is caused by the GAA repeat expansion in intron 1 of the *FXN* gene. Additional forms of ARCA with unknown genetic etiology may also be associated with a similar type of mutation and will not be identified with the NGS approach.

CONCLUSION

Novel high-throughput sequencing technologies such as WES/WGS are ideal for simultaneously sequencing groups of candidate genes, whole-genome coding regions or the whole-genome and have brought a revolution in the molecular diagnosis of human genetic diseases. These technologies are capable of producing large amounts of data in one experiment and have been successfully used for the diagnosis of many rare and complex diseases including rare neurodegenerative diseases such as the ataxias. Their use for both research and diagnostic purposes has already contributed to the progress observed during the last years in the field of ataxias, including both the recessive and dominant forms. They are also very promising for the identification of many other new forms of ataxia in the near future since this group of disorders is very heterogeneous and a significant number of patients have already been excluded from many of the known genes. Determination of the genetic cause of each form is essential in order to initiate further investigation regarding the pathogenetic mechanisms and the potential therapies for these diseases.

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