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Tesi di dottorato

Genetic screening for SACS, ABHD12 and PRICKLE1 mutations in ataxia patients from Southern Italy

Relatore: Tutore: Prof A. Filla

dott.ssa Maria Fulvia de Leva Coordinatore: Prof. L. Annunziato

INTRODUCTION

AUTSOMAL RECESSIVE ATAXIAS

According to a pathogenic classification, the hereditary ataxias (HA) can be divided into five main categories: 1) mitochondrial; 2) metabolic; 3) defective DNA repair; 4) abnormal protein folding and degradation; 5) channelopathies (De Michele et al., 2004). Autosomal recessive ataxias fall in the first four groups, but the pathogenic mechanisms have not been defined in several of them. Mitochondrial ataxias are caused by defects in mitochondrial proteins, encoded by either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA). Mutations in nDNA are responsible for Friedreich ataxia (FRDA), mitochondrial recessive ataxia syndrome (MIRAS), and infantile-onset spinocerebellar ataxia (IOSCA). Point mutations in mtDNA cause different diseases such as myoclonic epilepsy with ragged-red fibres (MERRF) and neuropathy, ataxia, and retinitis pigmentosa (NARP). Large mtDNA deletions cause Kearns–Sayre syndrome (KSS). The mtDNA group has matrilinear inheritance or sporadic occurrence. Ataxia may also be a main

feature in partial muscle coenzyme Q10 (CoQ10) deficiency. Metabolic ataxias can be intermittent or progressive. The main causes are hereditary disorders of urea cycle, amino acid, pyruvate, vitamin E, or lipid metabolism, and storage and peroxisomal diseases. Ataxias associated with defective DNA repair comprise the HA caused by mutations in genes involved in sensing, excising, and repairing DNA damage. Two major groups may be distinguished: defects of either double-strand break (DSB) or single-strand break (SSB) DNA repair. Ataxia–telangiectasia (A-T) and ataxia–telangiectasia-like disorder (A-TLD) fall into the first group, where chromosomal instability, sensitivity to ionizing radiation, and tumors may also be present. Ataxia with oculomotor apraxia type 1 (AOA1), spinocerebellar ataxia with neuropathy 1 (SCAN1), xeroderma pigmentosum (XP), and Cockayne syndrome (CS), and possibly ataxia with oculomotor apraxia type 2 (AOA2), belong to the second group (Paulson and Miller, 2005).

Ataxias associated with abnormal protein folding and degradation comprise autosomal recessive spastic ataxia of Charlevoix–Saguenay (ARSACS) and Marinesco – Sjo¨gren syndrome (MSS). A chaperone-like activity has been proposed for sacsin, the product of the SACS gene, mutations of which are associated with ARSACS, and for the protein

codified by the SIL1 gene, mutated in MSS.

Other autosomal recessive ataxias whose pathogenesis does not fall into the previous categories include congenital ataxias, early-onset cerebellar ataxia with retained tendon reflexes (EOCA), polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract (PHARC), PRICKLE1 - progressive myoclonus epilepsy-ataxia syndrome, cerebellar ataxias with hypogonadism, with ocular features, with deafness, with myoclonus, and with extrapyramidal features.

The aim of our research was to study of three kind of recessive ataxias in patients referring to our center for neurodegenerative diseases: spastic ataxia of Charlevoix – Saguenay; polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract (PHARC); PRICKLE1 - progressive myoclonus epilepsy-ataxia syndrome.

Spastic ataxia of Charlevoix – Saguenay

Spastic ataxia phenotype is characterized by genetic heterogeneity as it is demonstrated by reports of patients with spastic ataxia who harbor mutations in *SACS* gene, FRDA gene and of patients who are linked to the SPG30, SAX1, SAX2, and ARSAL loci.

Autosomal recessive spastic ataxia of Charlevoix–Saguenay (ARSACS; MIM 270550) is an early-onset neurodegenerative disorder showing pyramidal, cerebellar progressive involvement and peripheral neuropathy. This disorder, considered to be rare, was first described in the late seventies among French Canadians in the isolated Charlevoix–Saguenay region of northeastern Quebec, where the estimated carrier frequency is 1 of every 22 persons (Bouchard et al., 1998). Two founder mutations were identified in this population. Nowadays it is known that the disorder is not only limited to this region. After mapping of the ARSACS locus, one Tunisian (Mrissa et al., 2000) and two Turkish families (Gu¨cu¨yener et al., 2001) showed linkage to the same chromosomal region, and after gene identification, missense and nonsense mutations of the SACS gene have been found in Tunisia (El Euch-Fayache et al., 2003), Italy (Criscuolo

et al., 2004; Grieco et al., 2004), Japan (Ogawa et al., 2004; Hara et al., 2005; Shimazaki et al., 2005; Yamamoto et al., 2005), Turkey (Richter et al., 2004), and Spain (Criscuolo et al., 2005). The phenotype of these patients is comparable to that identified in Quebec, except for some minor differences: age at onset may be later, up to 20 years, retinal myelinated fibers are rare; cognitive impairment more frequent. Besides, Mrissa et al. (2000) reported early loss of ankle reflexes and Shimazaki et al. (2005) noted extensor plantar responses in the absence of spasticity and hyperreflexia.

Main clinical features include early-onset (1-5 years) progressive ataxia, dysarthria, spasticity, nystagmus, retinal striation, and distal amyotrophy. MRI Imaging shows cerebellar vermis atrophy, and peripheral nerve conduction studies reveal markedly decreased amplitude of the sensory potentials and reduced motor conduction velocities.

A postmortem study of a 21-year-old man (Richter et al., 1993) showed atrophy of the superior cerebellar vermis, especially in the anterior structures (central lobule and culmen), where Purkinje cells were absent. The molecular and granular layers were thin; in the spinal cord there was loss of myelin staining in the lateral corticospinal tracts and dorsal spinocerebellar tracts. Abnormalities were more pronounced in a 59-year-

old man, and extended to the hippocampus, neocortex, basal nucleus of Meynert, globus pallidus, thalamus, dentatus nucleus, and posterior columns (Bouchard et al., 2000). Sural nerve biopsy showed loss of large myelinated fibers; there was increased variability of intermodal length on nerve teasing.

The gene responsible for ARSACS, named *SACS*, maps to chromosome 13q11 and encodes the protein sacsin. This gene consists of nine coding exons including a gigantic exon spanning more than 12.8k bp. Sacsin is a 4,579-amino acid protein which includes a carboxy-terminus domain that harbors a higher eukaryotes and prokaryotes nucleotide-binding domain (HEPN) and an upstream 'DnaJ' motif, that has the potential to interact with members of the HSP70 family. Its N-terminus contains an UBQ region and has extensive homology for Hsp90. The presence of both UbQ and J-domains suggests that sacsin may integrate the ubiquitin–proteasome system and Hsp70 function to a specific cellular role. In addition, sacsin is characterized by the presence of two leucine zipper domains, three coiled-coil domains and seven nuclear localization signals (Engert et al., 2000; Grynberg et al., 2003). Sacsin is expressed in a variety of tissues, including skeletal muscles, skin fibroblasts and central nervous system, mainly the cerebral cortex, the granular cell layer of the cerebellum, and the

hippocampus (Engert et al., 2000).

Objectives of our work were the definition of *SACS* mutation prevalence in Italian cohort of early spastic ataxia patients; better characterization of the mutations which could improve our knowledge of sacsin normal function and pathophysiology of ARSACS, and finally the picturing of a finer genotype – phenotype correlation.

Methods. We have recruited 23 southern Italy patients (pts 1-23) with progressive early onset ataxia (≤ 16 years of age), pyramidal signs (two among: brisk tendon reflexes, spasticity and Babinski signs) and clinical (decreased or absent ankle reflexes and decreased vibration sense) or neurophysiologic signs of peripheral neuropathy and performed direct sequencing of PCR products of *SACS* nine coding exons and intron-exon boundaries. Friedreich Ataxia was previously excluded in all patients.

Results. The complete sequencing of *SACS* in all patients detected three mutated patients and 12 different SNPs. In detail, we found a novel

compound heterozygous mutation (c.5719C>T plus c.12628_12633delTGAAA) in pt6 (Figure 1); a novel homozygous 5 bp deletion (c.7249_7254delCAGAA) in pt19 (Figure 2); a novel homozygous deletion (c.702_700delAA) in pt23 (Figure 3); and 12 different SNPs (rs17078720; rs3751368; rs4143768; rs9552929; rs2737700; rs17078605; rs2737701; rs2737699; rs2031640; rs41315020; rs1536365; rs17325713) in pts 2, 4, 5, 8, 10, 18; 20; 2-9, 10-12, 14-18, 21, 22; 6; 2,6,8,10,11; 4,5,9,18,20; 6,8,10,11; 2-5, 7-12, 14-18, 20-22; 2,6,8,11; 7,12,15,16; 3; 15,16,22 and 22 respectively (Table 1). Moreover we found a novel base pair change in pt10 (c.1310C>T) in heterozygous state (Figure 4).

All mutations causes premature truncation of sacsin and probably its loss of function. The compound heterozygous mutation in pt6 causes a new stop codon at p.1907 R>X and frameshift with new stop codon at p.4212 R>X; the homozygous deletion in pt19 produces frameshift with mistranslation and an early stop codon at p.2426 R>X; the homozygous deletion in pt23 causes frameshift with mistranslation and an early stop codon at p.235 K>X. We cannot confirm the pathogenicity of the novel base pair change of pt10 (p.T437M) for the Polyphen modeling analysis of the aminoacid change was “variant predicted to be benign” and for species alignment of sacsin showed that the residue at position 437 is not

conserved among different species. So we could only suggest that it is probably a novel SNP, not yet described in literature.

Perspectives for future research. Several studies have previously shown the ability of aminoglycosides to induce premature termination codon (PTC) readthrough and to restore a full-length protein synthesis. These antibiotics interact with the small ribosomal RNA subunit and decrease translational accuracy, leading to a deleterious protein synthesis in prokaryotes. The same mechanism is observed for the eukaryotic translation machinery but with a lower affinity for aminoglycosides, a feature possibly accounted for by two nucleotide divergences in the small ribosomal subunit. When the ribosome comes across a PTC, it may substitute an aminoacid to the stop codon and resume protein synthesis. Recessive genetic disorders caused by nonsense mutations are good candidates for aminoglycoside readthrough, as small amounts of functionally active protein may have a clinical impact. On this background we will test the effect of gentamycin on cultured skin fibroblasts of our mutated patients performing Western blot analysis of sacsin and RT-PCR of *SACS* mRNA.

Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract (PHARC)

Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract (PHARC) (MIM612674) is a neurodegenerative disease marked by early-onset cataract and hearing loss, retinitis pigmentosa, and involvement of both the central and peripheral nervous systems, including demyelinating sensorimotor polyneuropathy and cerebellar ataxia. The disease is slowly progressive, with recognition of the first symptoms typically in the late teens. In This Refsum-like disorder was first mapped to a 16 Mb region on chromosome 20 (Fiskerstrand et al., 2009), more recently it has been reported by the same authors that mutations in the a/bhydrolase 12 (ABHD12) gene cause PHARC disease (Fiskerstrand et al., 2010). Each of the four different ABHD12 mutations so far reported has been interpreted as a null mutation that would either abolish or severely reduce the activity of the encoding enzyme, a/bhydrolase 12. PHARC may, therefore, be considered a human ABHD12 knockout model. The question also arises whether less detrimental mutations may cause various incomplete phenotypes. The serious and progressive disease seen in

patients suggests that ABHD12 performs an essential function in the peripheral and central nervous systems and in the eye. This is supported by the high expression of ABHD12 in the brain, with a striking enrichment in microglia (Fiskerstrand et al., 2010). Currently, the only known substrate for ABHD12 is the main endocannabinoid 2-arachidonoyl glycerol (2-AG). This compound has important functions in synaptic plasticity (Makara et al., 2005; Straiker et al., 2009) and neuroinflammation (Zhang et al., 2008; Kreutz et al., 2009). In acute ischemia and/or excitotoxicity, 2-AG appears to have neuroprotective properties (Kreutz et al., 2009; Panikashvili et al., 2001; Di Marzo et al., 2008) but the effects of long-term increased levels of this metabolite have not been investigated. 2-AG represents the most abundant endocannabinoid and it is formed on demand from the membrane lipid diacylglycerol (by diacylglycerol lipase a or b). The endocannabinoid signaling system is the focus of increasing scientific interest, in part because of the potential for developing novel therapeutic agents. The system is tightly regulated and appears to be important for many physiological processes including neurotransmission, pain appreciation, appetite, mood, addiction behavior, body temperature, and inflammation (Di Marzo et al., 2008). Key players in these pathways are the G protein-

coupled cannabinoid receptors CB1 and CB2 and their endogenous ligands, endocannabinoids, as well as enzymes that synthesize or hydrolyze these ligands. Endocannabinoids act locally as lipid transmitters and are rapidly cleared by hydrolysis (Wang et al., 2009). Several enzymes are involved in 2-AG hydrolysis, and there is evidence that these enzymes are differentially expressed in various cell types and cellular compartments (Savinainen et al., 2011). In the mouse brain, monoacylglycerol lipase (MAGL) accounts for 85% of the hydrolase activity, with additional contributions from ABHD12 and a/b-hydrolase 6 (ABHD6). The apparent paradox of a purported minor role of ABHD12 in 2-AG hydrolysis versus the serious PHARC phenotype in the brain and eye suggests either that ABHD12 is of crucial importance only in certain cell types or that it is also acting on a hitherto unknown substrate other than 2-AG. The finding that microglial cells have a particularly high expression of ABHD12, but very low levels of MGLL (encoding MAGL) and ABHD6, indicates that the former alternative of differential cellular expression exists. Moreover, microglia dysfunction is known to be involved in neurodegenerative diseases (Landreth et al., 2009) as well as in retinal dystrophies (Eberth et al., 2009). Whether ABHD12 acts on more than one substrate is currently unknown, but many hydrolases have overlapping

functions, including MAGL, which is involved in lipolysis (Guzman, 2010) as well as in hydrolyzing 2-AG.

Interestingly, PHARC patients up to date reported did not show overt cannabinomimetic effects.

The finding of four different deleterious ABHD12 mutations in a total of 19 patients with PHARC disease from four countries (Norway, USA, United Arab Emirates and Algeria) clearly supports a causal genotype-phenotype relationship and a worldwide distribution. Besides, the screening of 190 Western Norway healthy blood donors have found two heterozygous carriers of this mutation, corresponding to a disease incidence of approximately 1/36,000 in this population, this indicates that the frequency of PHARC in is comparable to, or may be even higher than, relevant differential diagnoses like Friedreich ataxia and Refsum disease (Fiskerstrand et al., 2010).

On this behalf, we decide to screen a group of Southern Italy ataxic patients presenting a PHARC-like phenotype.

Methods. 11 patients were selected in according to the following inclusion

criteria: recessive inheritance, early onset ataxia (≤ 16 years old), and ocular impairment (retinitis pigmentosa, cataract or optic atrophy). Friedreich ataxia, ataxia oculomotor apraxia 1 and 2 were excluded in all patients. We performed direct DNA sequencing of sequencing of the 13 coding exons and the intron-exon boundaries of ABHD12 gene.

Results. We found no mutation and 10 validated SNPs (rs114038555, rs2274890, rs6107027, rs884613, rs2260197, rs746748, rs10966, rs2424708, rs1046073, rs11100): 3 coding, 3 intronic and 3 at 3'UTR (Table 2).

An heterozygous deletion in the 3'-UTR, c.*324delG, not reported in the database, was found in two patients, however it seems not to be pathogenic.

PRICKLE1 - Progressive myoclonus epilepsy-ataxia syndrome

Progressive myoclonus epilepsies (PMEs) are a group of rare inherited disorders characterized by epilepsy, myoclonus, and progressive neurological deterioration, particularly ataxia and dementia. Several disorders with different patterns of transmission can cause PMEs, most of them being autosomal recessive. One rare cause of autosomal recessive PME has been recently identified and is due to mutations of PRICKLE1 (MIM 608500) (Bassuk et al., 2008). Two probably related families, previously reported in linkage to chromosome 12, and a further third family (Berkovic et al., 2005, El-Shanti et al., 2006, Straussberg et al., 2005) showed the same homozygous variant c.311G>A (R104Q) in PRICKLE1 (Bassuk et al., 2008). Two kindreds were from Northern Israel and one from Northern Israel and Jordan. All three pedigrees showed early onset ataxia (at 4-5 years of age) with later myoclonus and seizures, impaired upgaze, mild or absent cognitive decline, and normal MRI.

The finding of a shared haplotype and identical PRICKLE1 mutation in three separately ascertained families of the same ethnic group with PME suggests a founder effect.

First discovered in Drosophila (Goldschmidt, 1945), prickle proteins are highly conserved throughout evolution. Characterized by PET and LIM domains (Gubb et al., 1999) the prickle proteins function in the noncanonical WNT signaling pathway, which regulates intracellular calcium release and planar cell polarity (PCP) (Veeman et al., 2003).

Recently, mice lacking Prickle1 were shown to die early in gestation, confirming an essential role for Prickle1 in development. In vitro studies suggest that PRICKLE1 normally binds and translocates REST to the cytoplasm, thereby preventing REST from silencing target genes. The R104Q PRICKLE1 mutation lies within a known protein binding domain and thus disrupts REST binding, blocking the normal transport of REST out of the nucleus. These results suggest that tissues expressing mutant PRICKLE1 contain constitutively active REST which inappropriately downregulates REST target genes (Bassuk et al., 2008). This is significant because in addition to silencing neuronal genes in nonneuronal cells and neuronal precursors, REST also regulates target genes in mature neurons (Palm et al., 1998). REST targets include ion channels and neurotransmitters, and the PME-ataxia syndrome may occur when brain regions expressing mutant PRICKLE1 misexpress these target genes. Although Prickle function was implicated in the control of cell division and

morphogenesis during zebrafish neurulation and REST activity was recently described in fish and frogs, a role for the PRICKLE/REST interaction during neurogenesis has not yet been studied.

Prickle1 is expressed in multiple brain regions throughout mouse embryonic development, including regions such as the hippocampus, cerebral cortex, and thalamus, as well as the primitive cerebellum (Cromptom et al., 2007, Katoh et al., 2003, Okuda et al., 2007, Tissir et al., 2006). Similarly, in human adult thalamus, hippocampus, cerebral cortex, and cerebellum, PRICKLE1 is in neurons rather than glia. These findings demonstrate that PRICKLE1 is expressed in multiple areas of the brain thought to be involved in generating seizures (neurons of thalamus, hippocampus, and cerebral cortex) and ataxia (cerebellar neurons) (Bassuk et al., 2008).

Our aim was to search for PRICKLE1 mutations in a cohort of unclassified PME-ataxia patients from Southern Italy and to define their clinical phenotype.

Methods. Twenty index cases (11 females, 9 males) were selected

according to the following criteria: early onset sporadic or recessive ataxia, myoclonus, and/or tonic-clonic seizures. Mean age at onset ± SD was 13.6 ± 10.6 years (range, 1–38). Symptoms at onset were gait ataxia in seven patients; action myoclonus or tremor in five; epilepsy in six (tonic-clonic generalized seizures and/or myoclonic seizures); and peripheral neuropathy in two. Cognitive impairment was present in two patients. MRI showed cerebellar atrophy in seven patients. After informed consent, we excluded other forms of PME and recessive ataxia, such as MERRF, Unverricht–Lundborg disease, Lafora disease, Friedreich ataxia, and mithocondrial inherited ataxia syndrome in all patients.

Direct sequencing of the seven coding exons and the intron-exon boundaries of PRICKLE1 has been conducted.

Results. No mutations were indentified. Five different SNPs were identified: three synonymous SNPs (rs3747562, rs3747563, rs34778200), one intronic (rs12230583) both in heterozygous and in homozygous state in the screened population, and one missense (rs3827522, c.2236 T>C) in heterozygous state in one patient. Notably, even if rs3827522 heterozygosity is 0.08 and is considered not pathogenic, proline 746 is

highly conserved among species and no homozygous c.2236C/C mutation has been reported from the HapMap-CEU and AFP_EUR_panel control DNA samples (http://www.ncbi.nlm.nih.gov/projects/SNP). Even not excluding the hypothesis of a pathogenic or a predisposing role of rs3827522, we could not find a second variation in our patient. PRICKLE1 dosage assay was not performed; therefore, gene copy-number alterations could not be excluded.

Conclusion

Aims of our research was to define the usefulness of genetic screening for three different kinds of autosomal recessive ataxia in undiagnosed ataxic patients.

Regarding ARSACS, our results confirm the worldwide diffusion, the uniform clinical presentation of the disease and the prevalence of loss of function mutations, further confirming the value of genetic screening for *SACS* mutation in case of early onset spastic ataxia. We also confirm the high frequency of SNPs throughout *SACS*.

As for PHARC we found no mutation in our selected patients, not confirming what already reported in literature, however our results are limited from the sample size of screened patients.

Finally, according to our analysis PRICKLE1 mutations are not a frequent cause of PME-ataxia in Southern Italy. So far, PRICKLE1 PME has been reported only in the three original Middle Eastern families, our data are in agreement with a recent survey showing no cases of PRICKLE1 mutations in 25 patients from different countries including Italy (Dibbens et al., 2009) and indicate that routine screening for these mutations in Italian patients is of limited clinical value.

TABLES AND FIGURES

Table 1. SNPs in SACS. Classification, position and significance are according ensembl database (www.ensembl.org).

SNP	Exon	Significance	Patients
rs17078720	2	Non Synonymous A/G	2, 4, 5, 8, 10, 18
rs3751368	3	Synonymous T/T	20
rs3751368	3	Non synonymous C/C	2, 3, 10, 11, 12, 14, 16, 18, 21, 22
rs3751368	3	Non synonymous C/T	4, 5, 7, 8, 9, 15, 17
rs2031640	8	Non Synonymous N/L	7,12,15,16
rs41315020	8	Synonumous A/A	3
rs1536365	8	Synonymous L/L	15,16,22
rs17325713	8	Non Synonymous A/T	22
rs4143768	10	Synonymous I/I	6
rs9552929	10	Synonymous V/V	2,6,8,10,11
rs2737700	10	Synonymous A/A	4,5,9,18,20
rs17078605	10	Non synonymous V/A	6,8,10,11
rs2737701	10	Synonymous Q/Q	2-5, 7-12, 14-18, 20-22
rs2737699	10	Synonymous L/L	2,6,8,11

Table 2. SNPs in ABHD12. Classification, position and significance are according ensembl database (www.ensembl.org).

SNP	Position	Significance
rs114038555	Intronic	
rs2274890	Intronic	
rs6107027	ex9	Synonymous R/R
rs884613	Intronic	
rs2260197	Intronic	
rs746748	ex12	Non Synonymous A/T
rs10966	ex12	Synonymous N/N
rs2424708	3'UTR	
rs1046073	3'UTR	
rs11100	3'UTR	

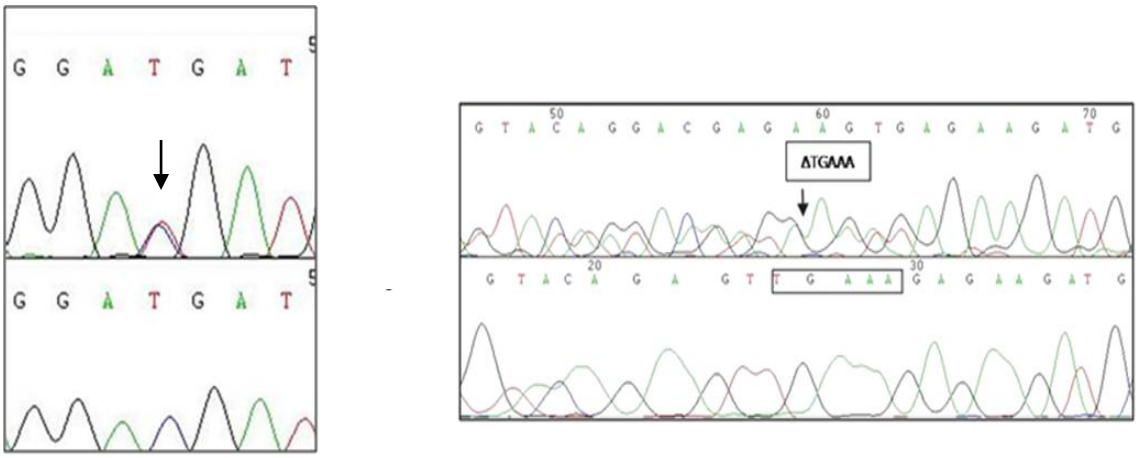

Figure 1. Electropherogram showing novel compound heterozygous mutation (arrows) (c.5719C>T plus c.12628_12633delTGAAA) in pt6 (top) compared to a normal control (bottom).

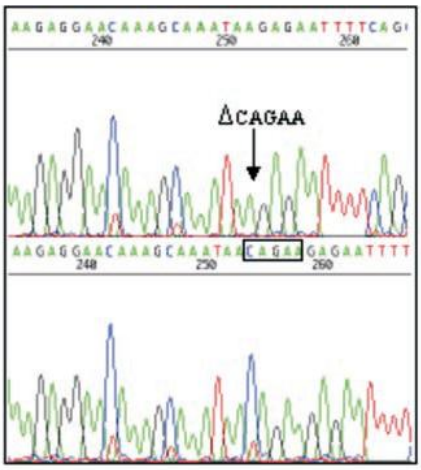

Figure 2. Electropherogram showing homozygous deletion (arrow) (c.7249_7254delCAGAA) in pt19 (top) as compared to a normal control (bottom).

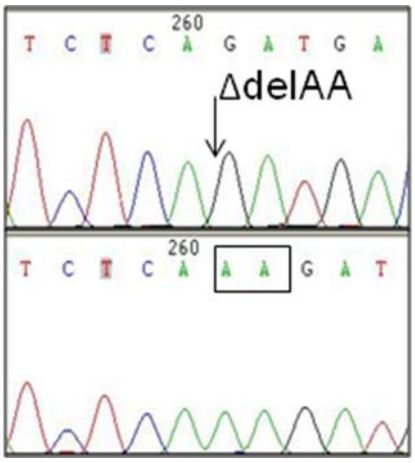

Figure 3. Electropherogram showing novel homozygous deletion (arrow) (c.702_700delAA) in pt23 (top) as compared to a normal control (bottom).

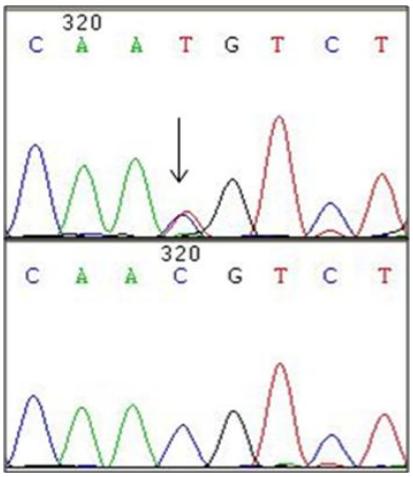

Figure 4. Electropherogram showing novel base pair change (arrow) (c.1310C>T) in pt10 (top) as compared to a normal control (bottom).

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