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High frequency of *GJA12/GJC2* mutations in Turkish patients with Pelizaeus-Merzbacher disease

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Introduction

Pelizaeus-Merzbacher disease (PMD; #312080) is a leukodystrophy associated with dysmyelination in the central nervous system (CNS) that usually segregates as an X-linked recessive trait. Clinical features include nystagmus, stridor, hypotonia, spasticity, ataxia, tremor, and deterioration of speech and mental function (1). The clinical severity, age of onset, and rate of progression in PMD vary widely, primarily depending on the nature of the causative mutation.

PMD can result from mutations in the highly conserved *PLP1* gene that encodes an extremely hydrophobic, tetraspan, integral membrane protein (2). About 80% of PMD patients carry *PLP1* gene duplications or missense mutations whereas deletions are extremely rare (3, 4). The *PLP1* duplications range in size between 100 kb to 4.6 Mb with heterogeneous distal and proximal breakpoints; no correlation could be found between the size and position of the duplications and the severity of the disease (5, 6). In general, duplications convey a milder clinical phenotype than point mutations in the *PLP1* gene, whereas patients with complete loss of *PLP1* appear to be less severely affected, but have also peripheral neuropathy (7, 8).

Patients with the PMD phenotype but without mutations of the *PLP1* gene are considered to have Pelizaeus-Merzbacher-like disease (PMLD; #608804). PMLD is a genetically heterogeneous and usually autosomal recessive trait. In a minority of PMLD cases, it is caused by mutations in gap junction protein $\alpha 12$ gene (*GJA12/GJC2*; #608803) coding for Connexin 47 (Cx47) (9). To date, 25 *GJA12/GJC2* mutations have been reported in PMLD

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Conflict of interest Dr. Lupski owns JRL, which is a paid consultant for Athena Diagnostics, Inc., has stock ownership in 23andMe and Ion Torrent Systems, Inc., and is a co-Inventor on multiple United States and European DNA diagnostic patents.

The Medical Genetics Laboratories (MGL) of the Dept of Molecular and Human Genetics at Baylor College of Medicine derives revenue from molecular diagnostic testing including genome-wide assays by Chromosomal MicroArray Analysis (CMA). MGL, <http://www.bcm.edu/geneticlabs/>

The other authors declare no conflict of interest.

patients, including a point mutation in the promoter region that is characterized as a transcription factor SOX10 binding site in the mouse *Gjc2* promoter (10-15).

The presence of phenotypic similarities in spite of genetic heterogeneity suggests Cx47 together with other connexins and PLP1 are critical parts of the myelination program that requires correct expression and targeting to the myelin sheath (16).

We investigated the genetic heterogeneity of the disease in a cohort of 19 families that were clinically diagnosed with PMD. The patients were screened for mutations in the *PLP1* and *GJA12/GJC2* genes by conventional PCR and Sanger sequencing methods and the chromosomal rearrangements in the *PLP1* locus were investigated further by array CGH.

Patients and methods

Patients

Nineteen unrelated Turkish families with PMD phenotype, including seven consanguineous pedigrees, were analyzed. Among 22 patients studied 17 were males and 5 were females. In two families, there were at least two affected individuals. Informed consent was procured from all participating subjects.

The cases were followed during 1997-2010 in the Department of Child Neurology, Istanbul Medical Faculty. Pyramidal signs were classified as mild (only brisk reflexes and clonus, with/or without the increase in extensor tonus of the lower extremities), moderate (prominent spasticity), and severe (tendon contracture). Cerebellar signs were also evaluated as severe, moderate, and mild according to the Klockgether ataxia score (17). Screening tests for inherited metabolic diseases, Aryl-sulphatase-A activity, VEP, NCV, MRI, and MRS were studied. The involvement of white matter, U fiber, corpus callosum, brain stem, cerebellum, and basal ganglia were assessed by MRI. MRS studies were obtained by a 1.5 T clinical scanner using PROBE/SV software (GE, Milwaukee; WI).

Interphase FISH

FISH was performed using standard protocols (18). A cosmid clone, cU125A1, containing the *PLP1* gene and a control cosmid, cU144A10, mapping approximately 850 kb distal to the *PLP1* gene were used as probes.

Quantitative real-time PCR analysis

DNA samples were amplified using the primer pairs for *PLP1* exons 3 and 6 and *Periaxin* exon 6 as an autosomal reference with Light Cycler (Roche). A normal male and female were included as controls. PCR was performed according to the manufacturer's protocol.

aCGH

A tiling-path custom oligonucleotide microarray was designed for a 15.5 Mb genomic segment encompassing *PLP1* as described previously (19).

Screening of *PLP1* and *GJA12/GJC2* for point mutations

PLP1 and *GJA12/GJC2* were screened for mutations by SSCP and subsequent bidirectional sequencing. Direct sequencing has been performed for *GJA12/GJC2* promoter of the patients F7.3, F5.3, F11.3, F13.3, and F14.3. The primer sequences for *PLP1* and *GJA12/GJC2* promoter were as described previously (20, 21) and for *GJA12/GJC2* are listed in Supplementary 1. Segregation of the identified sequence alterations among family members and absence in at least 100 control chromosomes were investigated by appropriate

restriction enzyme analysis, SSCP analysis (p.G236R) or 8% denaturing polyacrylamide gels (p.D64fs214X).

Results

Clinical evaluation

Clinical and MRI findings were compatible with a PMD diagnosis in 22 subjects (Table 1, Supplementary 2). The age range of the patients was between six months-13 years at the time of admission (6.7 ± 5.9). The follow-up period was between two-14 years (7.4 ± 2.8), and at the end of follow-up, the ages were between 7-29 years (14 ± 6.6). Patient F17.4 died because of a lung infection during the follow-up. The most common clinical manifestations consisted of rotatory or pendular nystagmus within the first 2 years of life in all and ataxic gait within 5 years. All were socially interactive and appeared to have better receptive ability than expressive ability because of the cerebellar dysarthria and cognitive delay, but no polyneuropathy. Inherited metabolic diseases were ruled out by screening tests. All patients had cerebellar signs that contributed to their motor impairment as they aged. None of them walked independently at the end of follow-up except F9.3 (29 years old) with *PLP1* duplication and F23.3 (7 years old) with a 20 bp *GJA12* duplication. Five patients (F15.3, F16.3, F17.3, F17.4, and F21.3) never achieved a stable sitting position because of the severe hypotonia. The course of the disease was slowly progressive during a prolonged time interval.

MRI revealed bilateral diffuse superficial and deep white matter hyperintensity in T2 and PD weighted images with U fiber involvement in all and the brain had the appearance of a newborn in T1 (Fig. 1). N-acetyl-aspartate/creatinine (NAA/Cr) ratios were not significantly different from the normal variance found in control subjects ($p>0.05$) in MRS.

PLP1 gene rearrangements

PLP1 gene duplications were identified in patients F4.3, F9.3, F19.3, and F20.3 using FISH (data not shown) and quantitative real-time assays (Supplementary 3). The aCGH data provided further evidence for the presence of *PLP1* duplications, determined the duplication breakpoints, and revealed three further rearrangements in patients F6.3, F18.3, and F22.3 (Fig. 2). Because of unavailability of DNA samples from mothers the origin of these rearrangements could not be investigated. Four patients (F5.3, F11.3, F13.3, and F14.3) did not show any alterations within the resolution limits of our array.

Mutation analysis in *PLP1* and *GJA12/GJC2*

The *PLP1* *AhaII* polymorphism was observed in patients F7.3, F10.3, F14.3, F17.3, and F17.4. Patient F15.3 had the previously reported p.P215S mutation inherited from the mother (22). Patient F16.3 was hemizygous for the novel p.F232S mutation that was not observed in his mother (Fig. 3a and Supplementary 4).

Five different sequence alterations were identified in the *GJA12/GJC2* gene in six other families (Supplementary 4). A deletion of 17 basepairs (c.546-542del) was identified in *GJA12/GJC2* in patients F1.3, F2.3, and F3.3 from the same large consanguineous family. *HhaI* restriction analysis in the family revealed that the parents and the healthy siblings were heterozygous whereas all three affected individuals were homozygous for the mutation. Patient F7.3 and his father were heterozygous for the previously reported c.706G>C (p.G236R) mutation and the mother was homozygous for the normal allele (13). The residue affected was highly conserved among different species (Pan troglodytes, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*). A novel homozygous c.T284G (p.L95R) variation was identified in patient F12.3. The parents were from the same village and heterozygous for the

mutation. The mutant allele was absent in the two unaffected siblings. Patients F17.3, F17.4, and F21.3 had a 14 bp tandem duplication of nucleotides between 177 and 190 (c.177-190dup/p.D64fs214X). The parents in both families were heterozygous for the mutation. The last novel *GJA12/GJC2* variation identified in family F23 was a 20 bp tandem duplication (c.924-925ins20bp) that is expected to encode 37 more triplets in the 3' UTR of the wild type sequence. The segregation of the mutation could not be tested due to unavailability of DNA samples from parents (Table 2, Fig. 3b).

Discussion

We investigated 19 PMD families both clinically and genetically. *PLP1* gene duplications and missense mutations were observed in six families (32%); four families had *PLP1* gene duplications (21%) though with different clinical severity and two patients with *PLP1* missense mutations (11%) presented with the most severe phenotype among all patients analyzed. *GJA12/GJC2* gene mutations were identified in six other families (32%) three of which had consanguinity. aCGH analysis revealed chromosomal aberrations that did not include the *PLP1* gene in three additional families with an unknown effect on the phenotype.

The low frequency of *PLP1* duplications in our cohort compared to previous reports can be attributed to the high frequency of autosomal recessive cases, different ethnic backgrounds, and limitations in the detection systems or difficulty of distinguishing different myelin disorders. The severity of the disease did not show any correlation with the duplication size as found for other populations (5, 6). Distal breakpoints were either mapping within or spanning the low-copy repeat (LCR) positions providing further evidence that *PLP1* duplications may be stimulated by LCR-PMDs (23).

The small noncoding duplications downstream of *PLP1* might represent polymorphisms since copy number variations (CNVs) encompassing that region were reported, though the database resolution does not allow a correlation with the control samples. Another duplication downstream of *PLP1*, encompassing the region duplicated in our two patients, has been reported previously as the only genetic abnormality identified in one patient with spastic paraplegia type 2 (SPG2; #312920) which is an allelic disorder of PMD (24). Non-recurrent duplications upstream and exclusive of the peripheral myelin protein 22 gene (*PMP22*) have also been reported to be associated with Charcot-Marie-Tooth type 1A (CMT1A; #118220) peripheral neuropathy suggesting that CNV of regulatory regions may lead to pathogenicity via a position effect (25).

Clinical features of *GJA12/GJC2*-related PMD cases were variable, but milder in general than those observed with *PLP1* mutations. We could not perform brainstem auditory evoked potential (BAEP) studies although it was reported to allow separation of PMD from PMLD (26). VEP and MRI/MRS results were very similar in all patients. However, MRI contributes to recognition and evaluation of hypomyelination and may aid in differentiating PMD from other leukodystrophies or other neurodegenerative disorders.

The truncation *GJA12/GJC2* variations (p.V182fs257X/p.D64fs214X) identified in three families, hypothetically cause either proteins lacking the third and fourth transmembrane domains or loss of downstream domains after the first transmembrane domain and may lead to formation of non-functional proteins by disrupting their membrane insertion or stability. Alternatively, nonsense mediated decay may result in mRNA instability with no mutant protein being produced. Among those families with premature termination codon mutations, F17 and F21 showed the most severe clinical presentation almost comparable to those of patients with *PLP1* missense mutations. The p.P309fs477X mutation, that may cause production of a longer protein than the wild type, was associated with the mildest phenotype

in the cohort. This can be expected since the mutation affects only the intracellular carboxyl domain of the protein, possibly leading to a partially functional protein. The effect of truncation mutations were reported for another gap junction protein gene, *GJB1/Cx32*, that is known to be responsible for CMTX (#302800). Some of these mutations were shown to have decreased ability to form functional hemichannels and complete channels whereas for the ones that can form channels normally, channel activity is affected depending on length (27). Further studies are required to unravel the contribution of different domains of gap junction proteins to disease pathogenesis.

In one of our severely affected cases, only one mutant *GJA12/GJC2* allele could be identified. An autosomal recessive mode of inheritance was the most likely pattern since the patient's father was an unaffected carrier and the mutation was previously found in homozygous condition in another patient from Turkey (13). The second mutant allele may reside in either regulatory or intronic regions or in another connexin gene that forms heteromeric channels with Cx47.

Observation of equal frequencies for *PLP1* and *GJA12/GJC2* mutations in our cohort suggests that autosomal recessive *GJA12/GJC2* gene mutations may be more frequently found in association with PMD than expected in populations where consanguineous marriages are common. Absence of mutations in four of our families and previously reported cases presenting prototypical features of PMD suggests the presence of further genetic heterogeneity for PMLD. These patients can be screened for mutations in *HSP60*, *AIMP1/p43* and *MCT8* that were reported to be associated with PMLD presentation (28-30).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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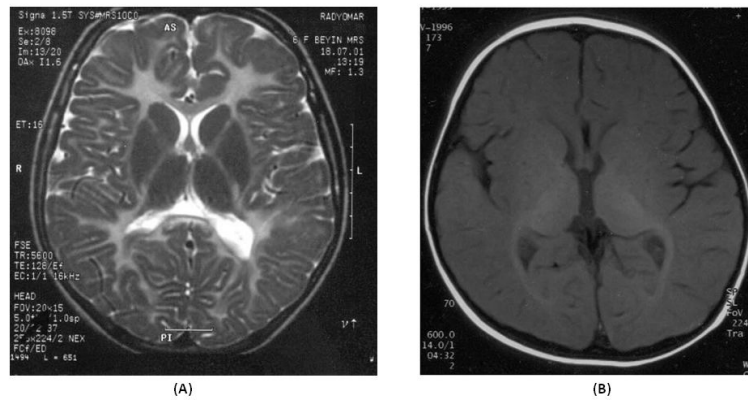


Fig. 1. Brain MRIs of two patients analyzed in this study. **a** Axial T2-weighted image from patient F3.3 (with p.V182fs257X mutation in *GJA12/GJC2*) shows diffuse white matter hyperintensities with U fiber involvement. **b** Axial T1-weighted image from patient F4.3 (with *PLP1* gene duplication) shows high signal intensity in the internal capsule, optic radiations like in a newborn indicating hypomyelination.

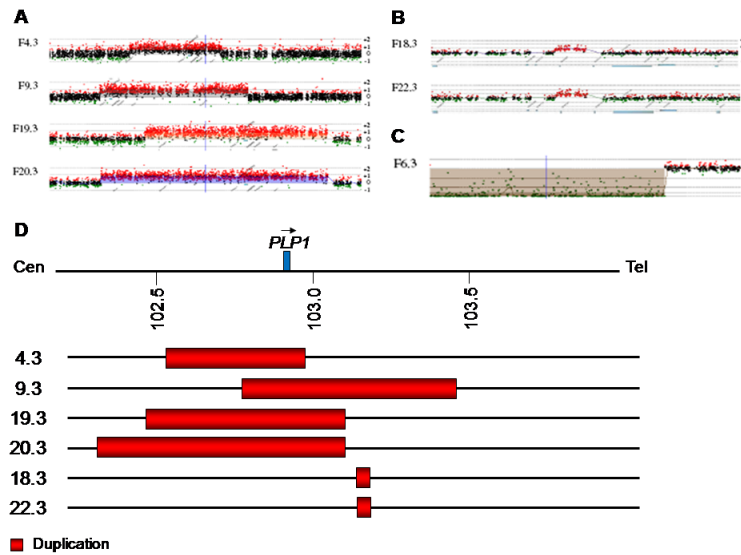


Fig. 2. Results of aCGH analysis of the *PLP1* gene rearrangements. The X-axis indicates the genomic positions of the probes. The positive and negative numbers on the Y-axis indicate gain and loss of genomic copy numbers, respectively. The vertical blue line indicates the position of the *PLP1* gene. **a** The four *PLP1* duplications identified in this study, **b** the two small duplications telomeric to *PLP1*, and **c** the deletion centromeric to *PLP1* are shown. **d** Breakpoints of the duplications identified in this study and the position of the *PLP1* gene is indicated.

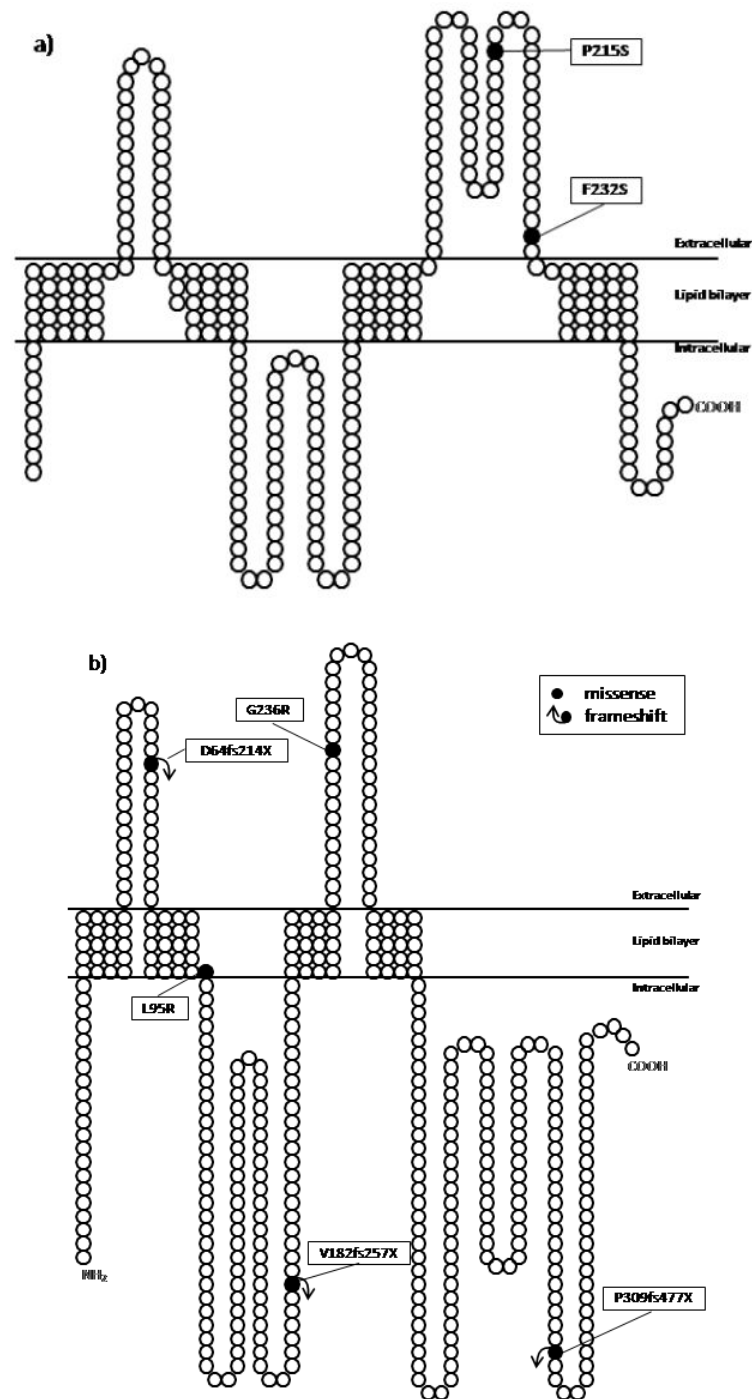


Fig. 3. Schematic representations of both **a** PLP1 and **b** GJA12/GJC2 proteins with their known domains and the locations of the mutations identified in this study. Black circles represent the amino acid changes along the proteins. The positions of the domains in PLP1 is based on the website (<http://www.uniprot.org/uniprot/P60201>) and for GJA12/GJC2 on the work of Yeager M and Nicholson BJ, 1996 (31).

Table 1

Clinical characteristics of patients analyzed in this study.

Mutation	Patient number	Consanguinity	Sex	Current age	Onset age of nystagmus	Course of nystagmus	Onset age of stridor	Dysphagia	Pyramidal signs	Cerebellar signs
PLP1 dup	F4.3	-	M	17	Congenital	Same	Absent	Absent	Moderate	Severe
PLP1 dup	F9.3	-	M	29	Absent	Absent	Absent	Absent	Moderate	Mild
PLP1 dup	F19.3	-	M	7	2,5 months	Same	Absent	Absent	Mild	Moderate/severe
PLP1 dup	F20.3	-	M	8	Congenital	Same	Absent	Absent	Moderate	Moderate/severe
PLP1 mut	F15.3	-	M	7	Congenital	Decreasing	2 years	Present	Severe	Severe
PLP1 mut	F16.3	-	M	8	2 months	Decreasing	Congenital	Present	Severe	Severe
CGH del	F6.3	+	M	17	3 months	Same	Absent	Absent	Severe	Severe
CGH dup, CNV?	F18.3	+	M	17	Congenital	Decreasing	Absent	Absent	Moderate	Severe
CGH dup, CNV?	F22.3	-	M	29	Absent	Absent	Absent	Absent	Mild	Moderate/Severe
GJA12/GJC2 mut	F1.3*	+	M	14	Congenital	Same	Absent	Absent	Severe	Severe
GJA12/GJC2 mut	F2.3*	+	M	18	1,5 years	Same	Absent	Absent	Severe	Severe
GJA12/GJC2 mut	F3.3*	+	F	14	2 months	Same	Absent	Present	Severe	Severe
GJA12/GJC2 mut	F7.3	-	M	22	1 month	Decreasing	Absent	Absent	Severe	Severe
GJA12/GJC2 mut	F12.3	Same village	F	18	3 months	Same	Absent	Present	Moderate	Severe
GJA12/GJC2 mut	F17.3	+	F	21	8 months	Decreasing	Absent	Absent	Moderate	Moderate/Severe
GJA12/GJC2 mut	F17.4	+	F	died at 14	9 months	Decreasing	Absent	Absent	Moderate	Severe
GJA12/GJC2 mut	F21.3	Same village	F	9	Congenital	Same	Absent	Absent	Moderate	Moderate/Severe
GJA12/GJC2 mut	F23.3	+	M	7	Congenital	Decreasing	Absent	Absent	Mild	Mild
-	F5.3	-	M	5	1 month	Decreasing	Absent	Absent	Moderate	Moderate
-	F11.3	-	M	14	1,5 years	Decreasing	Absent	Absent	Moderate	Severe
-	F13.3	+	M	28	Not known	Decreasing	Absent	Absent	Mild	Mild
-	F14.3	+	M	18	Congenital	Decreasing	Absent	Present	Moderate	Moderate/Severe

(*): Patients are from different branches of the same consanguineous family.

Table 2

Sequence variations identified in this study.

Patient number	Gene	Mutation	Putative effect on protein	Affected domain
F1.3 [*] , F2.3 [*] , F3.3 [*]	<i>GJA12/GJC2</i>	c.546-562del	p.V182fs257X	IC-1
F4.3	<i>PLP1</i>	<i>PLP1</i> gene duplication (438 kb)	Overexpression	-
F6.3	<i>PLP1</i>	325 kb deletion (~4.7 Mb centromeric to <i>PLP1</i>)	Unknown	-
F7.3 ^{**}	<i>GJA12/GJC2</i>	c.706G→C	p.G236R	EC-2
F9.3	<i>PLP1</i>	<i>PLP1</i> gene duplication (700 kb)	Overexpression	-
F12.3	<i>GJA12/GJC2</i>	c.284T→G	p.L95R	TM-2
F15.3	<i>PLP1</i>	c.643C→T	p.P215S	EC-2
F16.3	<i>PLP1</i>	c.695T→C	p.F232S	EC-2
F17.3, F17.4, F21.3	<i>GJA12/GJC2</i>	c.177-190dup	p.D64fs214X	EC-1
F18.3	<i>PLP1</i>	45 kb duplication (~212 kb telomeric to <i>PLP1</i>)	Unknown	-
F19.3	<i>PLP1</i>	<i>PLP1</i> gene duplication (640 kb)	Overexpression	-
F20.3	<i>PLP1</i>	<i>PLP1</i> gene duplication (795 kb)	Overexpression	-
F22.3	<i>PLP1</i>	46 kb duplication (~212 kb telomeric to <i>PLP1</i>)	Unknown	-
F23.3	<i>GJA12/GJC2</i>	c.905-924dup	p.P309fs477X	Cytoplasmic (carboxyl end)

^(*) Patients are from different branches of the same consanguineous family.

^(**) Patient was heterozygous for the c.706G→C mutation.