Abstract

Objectives: Complement component 6 (C6) deficiency is a very rare genetic defect that leads to significantly diminished synthesis, secretion, or function of C6. In the current report, we demonstrate a previously undescribed, homozygous missense mutation in exon 17 of the *C6* gene (c.2545A>G p.Arg849Gly) in a 35-year-old Japanese woman with moyamoya disease and extremely low levels of CH50 (<7.0 U/mL).

Materials and Methods: The complement gene analysis using hybridization capture-based next generation sequencing was performed. CH50 was determined in patient's plasma mixed with plasma from a healthy donor or purified human C6 protein. Western blot was performed on patient's plasma using polyclonal antibodies against C6, with healthy donor's plasma and purified human C6 protein as positive controls while C6-depleted human serum as a negative control. The carriage of *ring finger protein 213* variant (c.14576G>A p.Arg4859Lys), a susceptibility gene for moyamoya disease, was examined by direct sequencing.

Results: CH50 mixing test clearly showed a deficiency pattern, being rescued by addition of only 1% healthy donor's plasma or 1 μg/mL purified human C6 protein

(1/50-1/100 of physiological concentration). Western blot revealed the absence of C6 protein in the patient's plasma, confirming a quantitative deficiency of C6. The *ring finger protein 213* variant was not detected.

Conclusions: Our data implies that unrecognized complement deficiencies would be harbored in cerebrovascular diseases with unknown etiologies.

Introduction

Complement component 6 (C6) deficiency is a very rare genetic defect that leads to significantly diminished synthesis, secretion or function of C6 (1). It is caused by a homozygous or compound heterozygous mutation of the *C6* gene and associated with susceptibility to severe recurrent infections, predominantly by *Neisseria meningitidis* (2).

Moyamoya disease (MMD) is a chronic and progressive cerebrovascular disease, characterized by steno-occlusive changes at the terminal portion of the internal carotid artery and an abnormal vascular network formation at the base of the brain. Although the pathogenesis of MMD remains to be elucidated, genetic, autoimmune, and infectious etiologies have been suggested (3).

We herein report a previously undescribed, homozygous mutation in exon 17 of the *C6* gene in a patient with MMD. We confirmed a quantitative deficiency of C6 in the patient by mixing test and Western blot analyses.

Materials and Methods

Genetic Analysis

Hybridization capture-based next generation sequencing on complement genes,

including *C1QA*, *C1QB*, *C1QC*, *C1R*, *C1S*, *C2*, *C3*, *C5*, *C6*, *C7*, *C8A*, *C8B*, *C9*, *CFB*, *CFI*, *CFP*, *MASP2* and *MBL2*, was performed at Kazusa DNA Research Institute (Kisarazu, Japan) and was covered by insurance as usual clinical practice. The carriage of *ring finger protein 213 (RNF213)* variant (rs112735431, c.14576G>A p.Arg4859Lys), a susceptibility gene for MMD, was examined by direct sequencing after the amplification of exon 61 of the *RNF213* gene by polymerase chain reaction using the following primers; 5'-CTGCATCACAGGAAATGACACTG-3' (forward) and 5'-TGACGAGAAGAGCTTTCAGACGA-3' (reverse). For family study, the carriage of *C6* variant (rs1235393519, c.2545A>G p.Arg849Gly) was examined by direct sequencing after the amplification of exon 17 of the *C6* gene by polymerase chain reaction using the following primers; 5'-AGTGAACACACTACATTGGCT-3' (forward) and 5'-GTACTAGCTGAGATGAAGGTT-3' (reverse) (Approved number: 18-002, Hokkaido University Certified Review Board).

Mixing Test Analysis

Patient's plasma was obtained by centrifugation at 2,000 x g for 15 minutes at room temperature with ethylenediaminetetraacetic acid as an anticoagulant and

mixed with plasma from a healthy donor at concentrations of 0, 1, 10 or 50% or with purified human C6 protein (Complement Technology, Tyler, Texas, USA) at concentrations of 0, 1, 10 or 50 µg/mL. CH50 in the mixed plasma was determined by the liposome-based immunoassay. Mean values of duplicate measurements are presented.

Western Blot Analysis

Patient's plasma, healthy donor's plasma, purified human C6 protein (Complement Technology, Tyler, Texas, USA), and C6-depleted human serum (Complement Technology, Tyler, Texas, USA) were treated with SDS sample buffer with 2-mercaptoethanol for 5 minutes at 95ºC, separated on gradient polyacrylamide gels (4-15%), and electroblotted onto polyvinylidene difluoride membranes (Merck, Darmstadt, Germany). Membranes were blocked for 30 minutes at 37º C in 5% nonfat milk in Tris Buffered Saline with Tween 20. After blocking, the membranes were probed with polyclonal antibodies against C6 (Complement Technology, Tyler, Texas, USA). As secondary antibodies, horseradish peroxidase-conjugated rabbit anti-goat (Sigma-Aldrich, Dorset, UK) was used. Signals were detected using ECL Western blotting detection reagents

(Cytiva, Buckinghamshire, UK) and LAS-4000 imaging system (FUJIFILM, Tokyo, Japan). The polyacrylamide gels with separated samples were also stained with Coomassie Brilliant Blue.

Results

Case Description

A 35-year-old Japanese woman experienced severe headaches two times with a one-month interval and visited a neurosurgery clinic. Physical examination showed no abnormal neurological findings; however, intracranial magnetic resonance angiography (MRA) revealed bilateral stenosis of the terminal portion of internal carotid artery and cisternal moyamoya vessels (Figure 1a, b). Further, three-dimensional constructive interference in steady state revealed decrease in the outer diameter of the involved arteries (Figure 1c), compatible with MMD (4, 5). These findings were again obtained by MRA repeated three months after the first visit, indicating a chronic cerebrovascular disease. She was referred to the rheumatology department for further diagnostic evaluation of autoimmune diseases that could underlie MMD. Blood tests revealed extremely low levels of CH50 in both serum and plasma in ethylenediaminetetraacetic acid despite

normal serum concentrations of C3 and C4 (Table 1). Although she had a history of acute hepatitis C, cryoglobulin was not detected in her serum. Among autoantibodies, only a low titer of antinuclear antibody was positive. Taken together, she was suspected to have complement deficiency and underwent genetic testing with written informed consent.

Genetic Findings

Genetic analysis using hybridization capture-based next-generation sequencing identified a heterozygous missense variant in exon 11 of the *MASP2* gene (rs144471433, c.1731A>C p.Gln577His) and a homozygous missense variant in exon 17 of the *C6* gene (rs1235393519, c.2545A>G p.Arg849Gly) (Figure 2a). The *MASP2* gene variant was unlikely to be pathogenic since its allele frequency is reported to be 0.065 in the Japanese database (public database by ToMMo). Conversely, the allele frequency of the *C6* gene variant (c.2545A>G p.Arg849Gly) is only 0.0006 in the Japanese database (public database by ToMMo) and has not been reported in any other database including ClinVar, gnomAD and HGMD. No other variants except one single nucleotide polymorphism (p.Asp627=, synonymous variant) were identified in exons of the *C6* gene. The *RNF213*

variant (c.14576G>A p.Arg4859Lys), a susceptibility gene for MMD, was not detected (Figure 3).

Significance of Homozygous c.2545A>G p.Arg849Gly in the C6 Gene

Since the genetic findings strongly suggested the pathogenicity of the homozygous *C6* gene variant (c.2545A>G p.Arg849Gly), we further analyzed its significance with mixing test and Western blot on the patient's plasma. Addition of only 1% healthy donor's plasma or 1 μg/mL purified human C6 protein (1/50-1/100 of physiological concentration (6)) rescued CH50 in the patient's plasma (1.08→17.15 and 1.08→29.49 U/mL, respectively) (Figure 2b). The rescue of CH50 reached a plateau by mixing with 10% normal plasma or 10 μg/mL purified C6, indicating a functional deficiency of C6 and the absence of autoantibodies against C6. To examine whether C6 protein is quantitatively deficient in this patient, Western blot was then performed with healthy donor's plasma and purified human C6 protein as positive controls while C6-depleted human serum as a negative control, revealing the absence of C6 protein in the patient's plasma (Figure 2c). These results confirmed the homozygous *C6* gene mutation (c.2545A>G p.Arg849Gly) as a cause of a quantitative C6 deficiency.

Family study

Among her family, her father (I-1), a 63-year-old man with anxiety disorders, and her brother (II-3), a 31-year-old healthy man, agreed to undergo complement measurements and genetic analysis. Both of them had normal levels of CH50 (49.2 and 46.3 U/mL, respectively) and the heterozygous C6 gene variant (c.2545A>G p.Arg849Gly) (Figure 4). Whereas, according to the local guidelines (Guidelines for Genetic Tests and Diagnosis in Medical Practice. *Japanese Association of Medical Sciences*) her children (III-1,2), a five-year-old healthy boy and a two-year-old healthy boy, did not undergo genetic analysis, both of whom having normal levels of CH50 (47.8 and 37.6 U/mL, respectively) (Figure 4). Her mother (I-2), who had schizophrenia, unfortunately refused blood sampling. None of her family revealed a history of cerebrovascular disease.

Discussion

C6 is a part of the membrane attack complex (MAC) that forms a pore-like structure in cell membranes following the complement-mediated lysis. MAC plays a critical role in immune responses against infection with the genus *Neisseria*.

Individuals with deficiency in any of the MAC components, C5 through C9, are susceptible to meningococcal infections (7). C6 deficiency is a very rare condition with its prevalence reported to be 0.0027% (4 out of 145,640 healthy blood donors) in Japan (8) but remaining unknown in other regions or populations. The *C6* gene has 18 exons and translated C6 protein has 10 domains (9). The variant identified in the current case (c.2545A>G p.Arg849Gly) is located in the C-terminal domain (factor I modules; FIMs) of C6, whereas previously described variants are mostly in other domains including thrombospondin-like domain, membrane attack complex-perforin domain, and complement control protein modules (10-20). Although an *in vitro* study using FIMs-deleted recombinant C6 suggested the role of FIMs in the interaction with metastable C5b particularly in the complement classical pathway (21), it remains unclear how the missense variant in FIMs affect the synthesis or secretion of C6 *in vivo*.

MMD is sometimes associated with autoimmune diseases such as systemic lupus erythematosus (SLE) (22), whereas SLE develops in individuals with deficiencies of components of the complement classical pathway such as C1q and C4 (23). MMD developed in a patient with SLE and C1q deficiency (24). A case of SLE and C6 deficiency has also been reported (14). Although it cannot

be ruled out that MMD and C6 deficiency incidentally coexisted in the current case, their rarities would suggest the association between the two diseases. The absence of any other disease or genetic susceptibility would also support an impaired complement system as a cause of MMD in the current case. In addition, none of her family revealed complement deficiency or cerebrovascular diseases, further suggesting their association. On the other hand, MMD has been reported to be observed in patients with bacterial meningitis (25, 26), a common complication of deficiencies in the MAC components. Although these observations were explained with an autoimmune process triggered by infection, preexisting vascular disease cannot be excluded. It would also be speculated that unrecognized complement deficiencies underlay both MMD and bacterial meningitis.

In conclusion, we demonstrate a quantitative C6 deficiency caused by an amino acid substitution in FIMs in a patient with MMD. Our data implies an impaired complement system as one of the causes of MMD. Unrecognized complement deficiencies would be harbored in cerebrovascular diseases with unknown etiologies.

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Author Contribution

M Kato, Y Kudo, M Hatase, H Tsujimoto and Y Fukumori conducted the experiments. M Kato, N Tsuchida, S Takeyama, T Sugiyama, M Fujimura and I Yabe performed the clinical investigation of the patient. M Kato and T Atsumi wrote the manuscript with the contribution of N Inoue.

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Conflicts of Interest

M Kato has received research grants from AbbVie, Actelion, GlaxoSmithKline, Janssen, Nippon Shinyaku and Novartis and speaking fees from Eli Lilly. N Inoue has received research grants from Alexion pharmaceuticals and speaking fee from Alexion pharmaceuticals and Sanofi. T Atsumi has received research grants from Astellas, Takeda, Mitsubishi Tanabe, Chugai, Daiichi-Sankyo, Otsuka, Pfizer,

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Table 1. Laboratory data.

	Patient	Normal range
WBC	5.2	3.3-8.6×10^3/μL
RBC	4.48	3.86-4.92×10^9/μL
Hb	13.5	11.6-14.8 g/dL
Hct	41.3	35.1-44.4%
Plt	231	158-348×10^3/μL
neutrophil	50.2	38.5-80.5%
lymphocyte	38.5	16.5-49.5%
CRP	<0.02	0.00-0.14 mg/dL
free T3	3.12	2.24-3.94 pg/mL
free T4	1.29	0.77-1.59 ng/dL
TSH	1.13	0.61-4.23 mIU/L
C3	91	73-138 mg/dL
C4	17	11-31 mg/dL
CH50 in serum	<7.0	31.6-57.6 U/mL
CH50 in EDTA plasma	<7.0	31.6-57.6 U/mL
Cryoglobulin	(-)	
HBsAg	<0.05	<0.05 IU/mL
anti-HCV	6.09	<1.00 S/CO
Autoantibodies		
RF	<4.5	<15.0 IU/mL
ANA	1:80 (homogenous, speckled)	<1:40
anti-CCP	1.8	<4.5 U/mL
anti-DNA	<2.0	<6.0 IU/mL
anti-Smith	<1.0	<10.0 U/mL
anti-Ro	<0.5	<10.0 Index
anti-La	0.7	<10.0 Index
lupus anticoagulant	1.17	<1.30
anti-CL IgG	7.7	0.0-20.0 U/mL
anti-CL IgM	1.3	0.0-20.0 U/mL
anti-β2GPI IgG	7.1	0.0-20.0 U/mL

anti-β2GPI IgM	<1.1	0.0-20.0 U/mL
Coombs test	(-)	
MPO-ANCA	<0.5	<3.5 U/mL
PR3-ANCA	3.2	<3.5 U/mL
Immune complexes		
C1q-IgG	1.7	0.0-3.0 μg/mL
monoclonal RF	2.2	0.0-4.1 μg/mL

ETDA, ethylenediaminetetraacetic acid; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; RF, rheumatoid factor; ANA, antinuclear antibody; CCP, cyclic citrullinated peptide; CL, cardiolipin; β2GPI, beta 2 glycoprotein I; MPO, myeloperoxidase; PR3, proteinase 3; ANCA, anti-neutrophil cytoplasmic antibodies; TSH, thyroid-stimulating hormone.

Figure 1. Time of flight magnetic resonance angiography (TOF-MRA) shows bilateral stenosis of the terminal portion of internal carotid artery (**a**) and cisternal moyamoya vessels (**b**). Three-dimensional constructive interference in steady state (3D-CISS) reveals decrease in the outer diameter of the involved arteries (**c**).

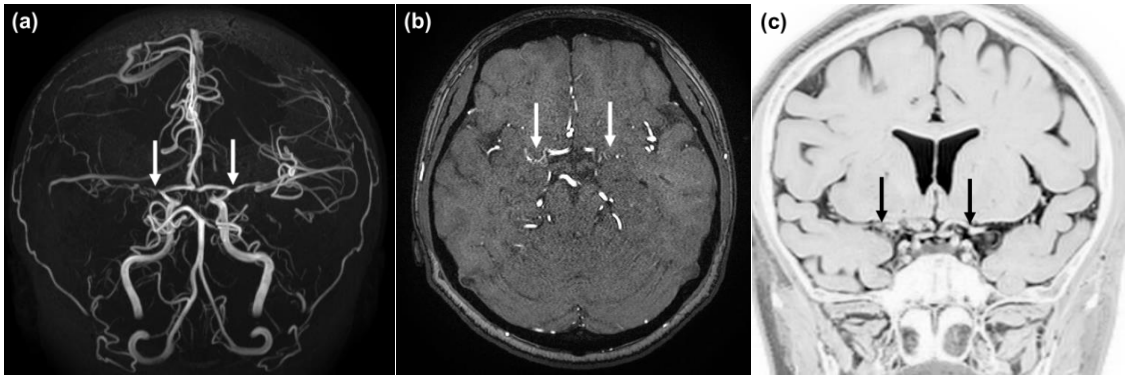
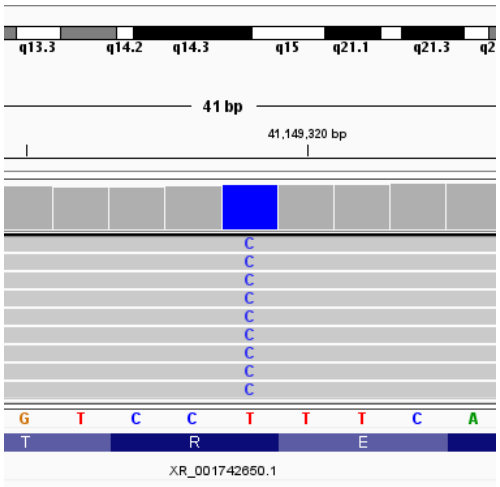
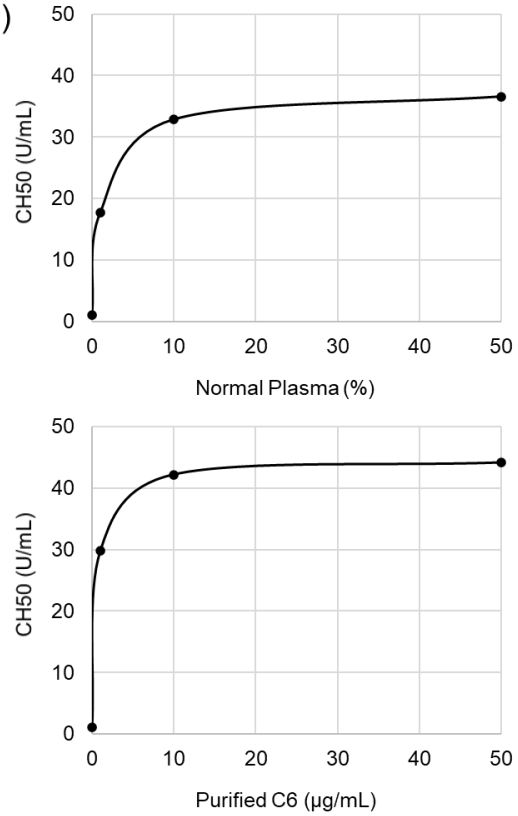


Figure 2. The *C6* gene variant in the case (**a**). Hybridization capture-based next-generation sequencing identified a homozygous missense variant at chromosome 5: 41,149,319 (GRCh38.p13) (c.2545A>G p.Arg849Gly). Results of mixing test (**b**). CH50 was determined in patient's plasma mixed with plasma from a healthy donor at concentrations of 0, 1, 10 or 50% (upper) or with purified human C6 protein at concentrations of 0, 1, 10 or 50 μg/mL (lower). Results of Western blot (**c**). Four samples, including C6-depleted human serum, patient's plasma, healthy donor's plasma, and purified human C6 protein (left to right), were applied. The Gel with separated samples was stained with Coomassie Brilliant Blue (CBB) and the electroblotted membrane with polyclonal antibodies against C6.

(a)


q13.3
q14.2
q14.3
q15
q21.1
q21.3
41 bp
41,149,320 bp
G T C C T T T C A
T R E
XR_001742650.1

(b)


CH50 (U/mL)
Normal Plasma (%)
CH50 (U/mL)
Purified C6 (μg/mL)
0
10
20
30
40
50

(c)

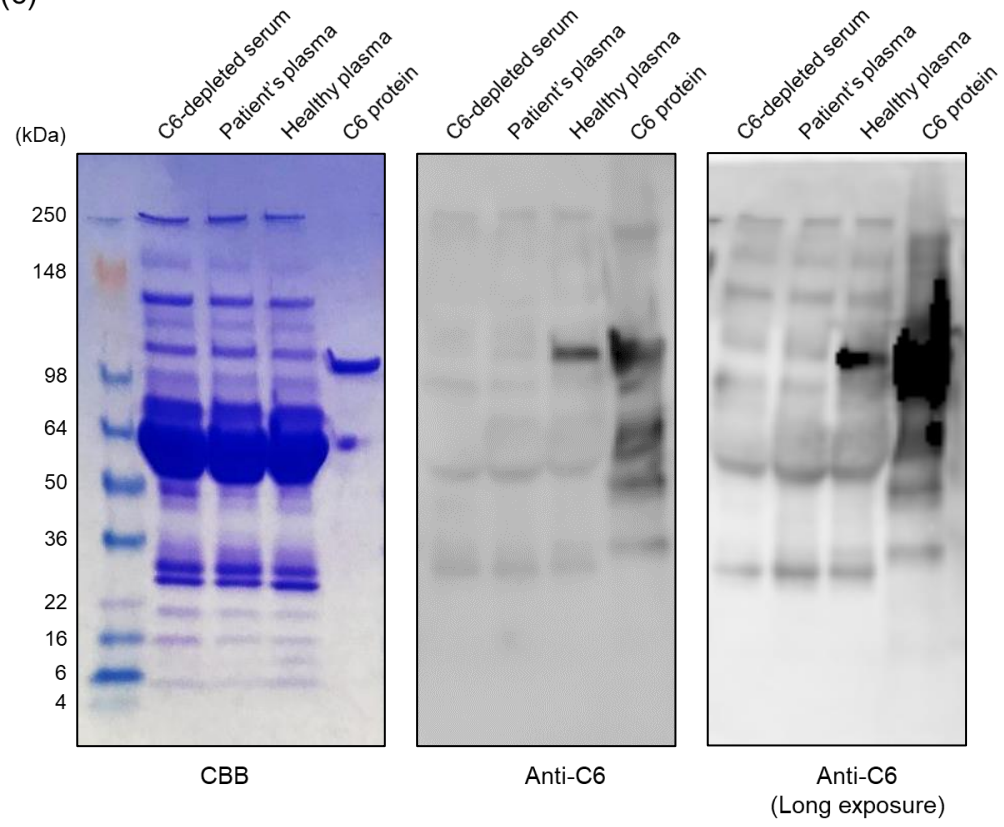

C6-depleted serum
Patient's plasma
Healthy plasma
C6 protein
(kDa)
250
148
98
64
50
36
22
16
6
4
CBB
Anti-C6
Anti-C6
(Long exposure)

Figure 3. The absence of the *RNF213* variant at chromosome 17: 80,385,145 (GRCh38.p13) (c.14576G>A p.Arg4859Lys), a susceptibility gene for moyamoya disease, in the case. The carriage of the variant was examined by direct sequencing after the amplification of exon 61 of the *RNF213* gene by polymerase chain reaction.

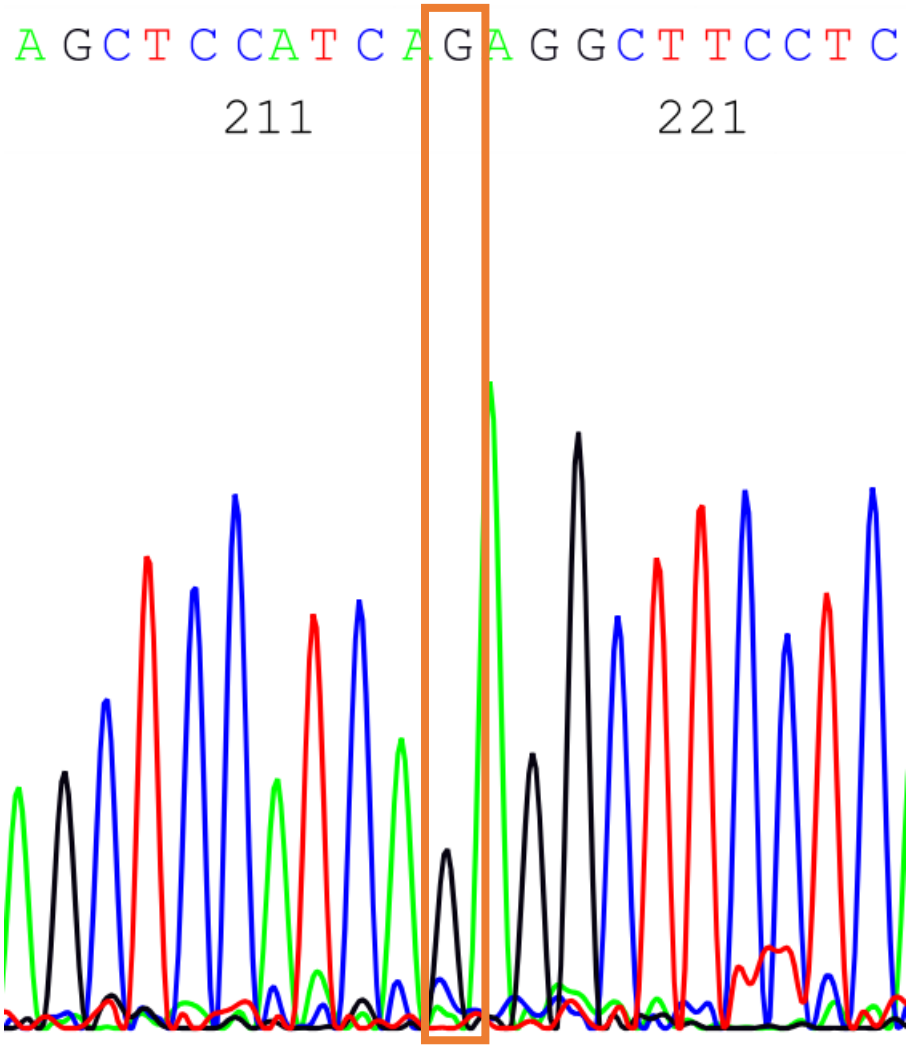


Figure 4. Family tree of the case including her father, mother, brother and children. P, proband; y.o., years old.