論　文　題　目

Combined deficiency of factors V and VIII by chance coinheritance of parahaemophilia and haemophilia A, but not by mutations of either *LMAN1* or *MCFD2*

（LMAN1 や MCFD2 の遺伝子変異でなく、極めて稀な

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【緒言】

血友病 A は血液凝固第 VIII 因子（FVIII）の欠損あるいは活性低下による血液凝固異常症である。血友病は治療が必要とされる先天性出血性疾患の中では最も頻度が高く、日本国内の血液凝固異常症の罹患調査で登録された患者数は約 4800 人とされている。X 連鎖劣性遺伝形式をとり、血友病 A では X 染色体長腕（Xq28）上の FVIII 遺伝子（*F8*）が責任遺伝子である。FVIII 活性により重症度が分類され、1%未満を重症、1～5%未満を中等症、5～40%以下を軽症としている。筋肉・関節内などの深部出血を特徴とし、重症例にその頻度が高い。軽症例（FVIII 活性 5%以上）では自然出血はほとんど見られず、手術・抜歯後・外傷後の際の止血困難で発見されることが多い。

血液凝固第 V 因子（FV）遺伝子（*F5*）の異常は、パラ血友病としても知られる常染色体劣性遺伝形式の FV 欠乏症で出血性素因となることがある。これは、外傷や手術の際に止血に時間を要する非常にまれな血液凝固障害であり、関節内出血、筋肉内血腫や脳内出血などの重症例もあるが、多くは軽症から中等度の症例であり無症候の場合もある。一方、FV 異常症では血栓性素因となることもある。その 1 つに、欧米で高率にみられる FV Leiden（FVL）があり、ヘテロ接合体でも血栓リスクとなるが、これまでその日本人での報告例はない。

また、FV と FVIII が同時に欠乏する第 V・第 VIII 因子合併欠乏症（combined FV/FVIII deficiency: F5F8D）も存在する。これは、遺伝性出血性疾患であるが、FV および FVIII の

合成分泌時に細胞内運搬に働く LMAN1（lectin mannose-binding 1）もしくは MCFD2（multiple coagulation factor deficiency 2）の欠損によって引き起こされるもので、*F5* および *F8* の異常によるものではない。この疾患の症状は月経過多や抜歯後の口腔内出血、術後・出産後の異常出血であり、一般的に出血症状は軽度である。

今回、当初 F5F8D として診断されていた患者において、遺伝子解析にて中等症の血友病 A かつ FV 欠乏症を合併するこれまでに報告例のない極めてまれな日本人家系を解析し、その原因遺伝子の同定とともに発症メカニズムを検討した。

【対象・方法】

対象は、当初 F5F8D として診断されていた患者とその兄弟およびその子ども（1 家系 3 症例）とした。インフォームドコンセントを得た後、発端者およびその家族の末梢血白血球分画よりゲノム DNA を抽出し、それぞれ *F5*、*F8*、LMAN1 遺伝子（*LMAN1*）および MCFD2 遺伝子（*MCFD2*）の塩基配列解析を行った。*F5* の全 25 エクソンを含む領域、*F8* の全 26 エクソンをイントロンとの境界領域およびプロモーター領域を含めて増幅させるプライマーを用いて PCR を行い、ダイレクトシークエンス法にてそれぞれの塩基配列を解析した。*LMAN1* および *MCFD2* も同様に解析した。また、検査所見と臨床症状との評価のために Thrombin Generation Assay（TGA）を実施し、発端者と同一の *F8* 変異を有する症例（比較症例）とを比較し検討した。なお、本研究は名古屋大学医学部生命倫理審査委員会の承認

を得て実施した。

【結果・考察】

F5F8D の原因遺伝子とされる *LMAN1* および *MCFD2* について、いずれもエクソン内に遺伝子異常を認めなかった。一方、*F5* と *F8* のそれぞれに原因と考えられる遺伝子異常を 3 症例すべてに同定した。同定した変異は、*F5* ではエクソン 10 にナンセンス変異（c.1600C>T, p.Arg534*）をヘテロ接合体で同定し、*F8* ではエクソン 23 に 1 塩基置換のミスセンス変異（c.6506G>A, p.Arg2169His）を同定した。*F5* では、その他に先行研究やデータベースで SNP と報告されている 1 塩基置換が数か所認められた。

F5 に同定された変異は既報の変異で、その変異部は遺伝子変異のホットスポットとなる CpG 配列に存在した。また、同変異は血栓リスクとして有名な FVL 変異と同じ塩基でのナンセンス変異（未熟翻訳終了）で、このため本症例では FV 活性が低下したと考えられた。*F8* に同定されたミスセンス変異も既報の血友病 A 変異で、やはりその変異部が遺伝子変異のホットスポットの CpG 配列に存在していた。さらに、*F5* における SNP ハプロタイプ解析の結果、*F5* のナンセンス変異の対立アレルに APC Cofactor 活性が低下し血栓傾向になる FV R2 ハプロタイプの存在が明らかとなった。

また、TGA では、同一 *F8* 変異をもつ比較症例に比べ、発端者のトロンビン生成がより早期にかつ良好に起こり、出血症状の軽減につながっていることが示唆された。その理由

の 1 つとして、*F5* における FV R2 ハプロタイプにより血液凝固反応がより血栓傾向に働くために、発端者では出血傾向が極めて少ないことが考えられた。

【結語】

今回対象とした家系では FV および FVIII の活性が同時に低下しているが、その原因は F5F8D の原因遺伝子である *LMAN1* もしくは *MCFD2* の異常ではなく、*F5* および *F8* にそれぞれ異常をもつ家系であることが明らかとなった。FV と FVIII の両者の活性低下を認めた際には、F5F8D のみでなく個々の遺伝子異常も疑う必要性が示唆された。

Summary

Background: Parahaemophilia and haemophilia A (HA) are distinct hereditary bleeding disorders caused by gene defects of factor V (FV) and factor VIII (FVIII), respectively. Simultaneous deficiency of FV and FVIII is assumed to be combined FV/FVIII deficiency (F5F8D), that is an autosomal recessive bleeding disorder caused by mutations in *LMAN1* or *MCFD2*. We reported here a Japanese family with simultaneous FV/FVIII deficiency by coinheritance of FV deficiency and FVIII deficiency, but not by F5F8D.

Patients and Methods: Proband was 62-year-old man initially diagnosed as F5F8D, because his plasma levels of FV and FVIII activities were 30% and 2% of normal. Those of his younger brother and daughter were 33% and 19%, and 56% and 42%, respectively. We analysed their genomic DNAs by direct sequencing for *F5*, *F8*, *LMAN1* and *MCFD2*. In addition, we performed thrombin generation assay (TGA) to estimate whole blood clotting activity. This study was approved by the institutional committee for research ethics, and the written informed consent was obtained from all participants.

Results and Discussion: In all patients analysed, we identified a nonsense mutation in *F5* (c.1600C>T, p.R534*) and a missense mutation in *F8* (c.6506G>A, p.R2169H), both of which have been previously reported. There was no mutation causing F5F8D in *LMAN1* or *MCFD2*. We detected

the FV R2 haplotype which consisted of multiple nucleotide substitutions on one allele of P1 and P2, but not P3. In the TGA, P1 showed lower peak and lesser ETP values than normal, but shorter tt-peak, higher peak and larger ETP values than the comparative HA patient with the same *F8* mutation of P1, which may result in more rapid haemostasis than the comparative HA patients.

Conclusions: Mutations found in *F5* and *F8* were inherited independently in this family, resulting in an unprecedent disease of moderate HA combined with parahaemophilia. This is the first report of rare coinheritance of congenital FV and FVIII deficiencies.

Introduction

Factor V (FV) and Factor VIII (FVIII) are essential glycoproteins in blood coagulation acting as cofactors in prothrombin and factor X activations, respectively. Congenital FV deficiency (parahaemophilia) is a rare (1 in 1,000,000 births) autosomal recessive bleeding disorder caused by decrease in FV activity resulting from various mutations of the FV gene (*F5*) [1, 2, 3, 4]. The most common symptoms are mucosal and posttraumatic bleeding, whereas haemarthroses and muscle haematomas are less frequently observed and life-threating haemorrhages are rare [5].

Congenital FVIII deficiency (Haemophilia A: HA) is an X-linked recessive bleeding disorder caused by various mutations in the FVIII gene (*F8*) with estimated occurrence of 1 in 5,000 male births, which is the most common congenital bleeding disorder worldwide [6]. Accurate laboratory diagnosis and classification according to disease severity are critical prerequisites for therapeutic intervention [7]. HA is classified according to residual FVIII activity as severe (<1%), moderate (1-5%) and mild(<5-40%) [8, 9].

On the other hand, combined deficiency of FV and FVIII (F5F8D) is an autosomal recessively bleeding disorder caused by mutations in either *LMAN1* (lectin mannose-binding 1 [LMAN1] gene) or *MCFD2* (multiple coagulation factor deficiency 2 [MCFD2] gene) [10, 11], and is clinically distinguished from chance coinheritance of HA and FV deficiency. F5F8D is characterized by a

mild-to-moderate bleeding tendency with simultaneous decreases of FV and FVIII to 5 to 30% of normal in plasma [12, 13].

Prevalence of F5F8D is estimated to be rare (1 in 1,000,000 births) in the general population, but an increased frequency is observed in regions where consanguineous marriages is practiced [14]. LMAN1 is a mannose-selective lectin cycling between the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (ERGIC) [15, 16], and forms a Ca^{2+}-dependent complex with MCFD2, which is a small soluble protein having an EF-hand domain that interacts with LMAN1 [10, 17]. The LMAN1-MCFD2 complex is proposed as a cargo receptor that transports FV and FVIII from the ER to the Golgi [10, 18, 19]. Furthermore, Nishio et al. reported that MCFD2 but not LMAN1 may undergoes significant conformational alterations upon complex formation and provided a structural basis for the cooperative interplay between LMAN1 and MCFD2 in capturing FV and FVIII [20]. Thus, mutations in *LMAN1* or *MCFD2* are responsible for simultaneous deficiency of FV and FVIII. However, the possibility of additional locus heterogeneity and the involvement of a third F5F8D gene was also pointed out [21].

Coinheritance of parahaemophilia and HA is indistinguishable from F5F8D by laboratory coagulation tests and clinical symptom, but it is extremely rare due to the low frequency of both disorders in general population as mention above. Ultimate confirmation of F5F8D comes from

mutation identification in either *LMAN1* or *MCFD2*, whereas parahaemophilia and HA are confirmed by identification of abnormality in *F5* and *F8*, respectively. However, no routine genetic testing is currently available for these bleeding diseases, especially mutation analysis for F5F8D is done on a research basis in several medical centres. In this study, we performed genetic analysis of a Japanese family with simultaneous deficiency of FV and FVIII, and confirmed the decreases of FV and FVIII activities resulted from coinheritance of FV deficiency and FVIII deficiency not by F5F8D, in other word, this family is a rare case of moderate HA combined with FV deficiency.

Materials and Methods

Patients

Proband (P1) was 62-year-old man initially diagnosed as F5F8D, because his plasma levels of FV:C and FVIII:C were 30% and 2% of normal, respectively. He had clinical history of FVIII replacement therapy at tooth extraction bleeding and right upper arm hematoma, and also suffered from hepatitis C virus infection. His younger brother (P2) was 59-year-old man and showed similar low plasma levels of FV and FVIII (33% and 19%). He had liver cirrhosis, hepatocellular carcinoma, hypertension and esophageal varices. And proband's daughter (P3) was also seen deficiency of FV and FVIII activity (50% and 46%) (Fig.1). Later on, his maternal cousin (not included in this study) was found to be mild HA without FV deficiency, and we suspected that their FV/FVIII deficiency could be caused by abnormalities in both *F5* and *F8*, not F5F8D. Therefore, we analysed *F5, F8, LMAN1* and *MCFD2* to accurately diagnose this family disease. The study was approved by the Institutional Committee for Research Ethics, and all participants participate with written informed consents.

DNA sequencing analysis of F5, F8, LMAN1 and MCFD2

The genomic DNA samples were isolated from peripheral blood leukocytes by established

methods [22]. We amplified all exons and intron–exon junctions of *F5, F8, LMAN1* and *MCFD2* by polymerase chain reaction (PCR) using respective gene-specific primers and KOD FXneo DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) or AmpliTaq Gold Mix (Applied Biosystems, Thermo Fisher Scientific Inc) as described previously [23,24]. PCR products were analysed on 1.5% agarose gel electrophoresis with 1 μg/ml of ethidium bromide, purified using QIAEX II (QIAGEN, GmbH, Germany), and subjected to direct cycle sequence analysis using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, USA) and ABI PRISM 310 Genetic Analyzer.

Activity and antigen measurement of FV and FVIII, and thrombin generation assay (TGA)

We measured the FV and FVIII activities using one stage clotting assay. FV and FVIII antigens were quantified using enzyme-linked immunosorbent assay (ELISA) kit (Cedarliane Laboratories Ltd., Hornby, Ontario, Canada) and VisuLize^{TM} FVIII Antigen Kit (Affinity Biologicals Inc., Ancaster, Ontario, Canada) according to the manufacturers' instructions, respectively. In addition, we performed thrombin generation assay (TGA) to estimate whole blood clotting activity of P1, normal subject, and two other comparative HA patients with the same *F8* mutation as P1. The calibrated automated thrombography (CAT, Thrombinscope BV: Thermo Fisher Scientific, MA, USA) was used for TGA using citrated platelet poor plasma (PPP). Thrombin generation curves and

the area-under-the-curve (endogenous thrombin potential, ETP) were calculated using the Thrombinoscope TM software. Lag time, time to peak (tt-peak), peak thrombin and ETP were adopted by endpoint parameters.

Results and Discussion

Proband (P1) was initially diagnosed as F5F8D due to a decreased FV:C (30%) and FVIII:C (2%). His brother (P2) and daughter (P3) also had low FV:C and FVIII:C (Fig.1). We performed sequence analysis of all exons of *F5, F8, LMAN1* and *MCFD*, including exon/intron boundaries, by direct sequencing using genome DNAs. We did not find any causative mutations leading to F5F8D in *LMAN1* or *MCFD2*; however, we identified causative mutations in *F5* and *F8*, both of which were previously reported mutations [25, 26].

In *F5,* a heterozygous nonsense mutation (c.1600C>T, p.Arg534*) in exon 10, previously reported as a nonsense mutation located on the same amino acid position of FV Leiden (FVL) mutation (Arg506Gln: p.Arg534Gln, c.1601G>A), according to the nomenclature recommended by the Human Genome Variation Society) [25], was identified in all three patients (Fig. 2A). The codon of 534Arg (CGA) contained a CpG dinucleotide known as one of the hotspots for gene mutation; therefore, c.1600C>T and c.1601G>A mutations seemed to be relatively frequent to occur. This nonsense mutation appeared to be truncated, resulting in premature termination of FV translation into a non-functional protein. However, the mutated FV may not be present in plasma due to intracellular degradation of aberrant truncated FV and/or nonsense decay of mutant FV mRNAs.

In *F8,* we identified a causative missense mutation (c.6506G>A, p.Arg2169His) in exon 23, P1

and P2 were hemizygous for this missense mutation, and P3 was a carrier (Fig. 2B). This *F8* mutation occurred at CpG dinucleotide of the gene mutation hotspot, and many cases were previously reported as a mild HA [26]. The 2169Arg of FVIII is a surface-exposed residue located on C1 domain and a well-conserved amino acid among species. It has been reported that the C1 and C2 domains contribute to von Willebrand factor (VWF) binding for circulating FVIII stabilization and the p.Arg2169His mutation is associated with reduction of VWF binding [27, 28]. Liu et al. reported that Arg2169Cys decreased FVIII-VWF binding less than 1% of normal [27]. Currently, 103 cases with this mutation have been reported in the Leiden Open Variation Database 3.0 (https://databases.lovd.nl/shared/genes/F8) and it is one of the most frequent mutations causing mild or moderate HA worldwide. Thus, the reduction in FVIII activity in this family seemed to be caused by p.Arg2169His leading to instability of FVIII.

In contrast, there was no causative mutation in *LMAN1* or *MCFD2* leading to F5F8D. To date, extensive genetic analyses of F5F8D patients have revealed many causative mutations in *LMAN1* and *MCFD2*. It has been showed that about 70% of F5F8D patients have mutations in *LMAN1* and the other 30% in *MCFD2* [17, 24]. But in few cases, the genetic factor causing simultaneous deficiency of FV and FVIII is still unclear [21]. We presented here that the chance coinheritance of congenital deficiencies of FV and FVIII is quite rare but could be the third candidate for the

simultaneous deficiency of FV and FVIII. Thus, mutations found in *F5* and *F8* of the proband were independently inherited in this family, resulting in an unprecedented rare case with parahaemophilia and moderate HA. In our knowledge, this is the first report of such a rare case of simultaneous deficiency of FV and FVIII caused by coinheritance of parahaemophilia and moderate HA, not by mutations in *LMAN1* or *MCFD2*. In some of the cases of combined deficiency of coagulation FV and FVIII, it is necessary to analyse gene defects not only in *LMAN1* and *MCFD2*, but also in *F5* and *F8*. In a typical F5F8D patient due to mutation in *LMAN1* or *MCFD2*, both FV:C and FVIII:C are both mildly decreased. Taken together with our data, it was suggested that if there was a significant difference between FV and FVIII activities, independent mutations in *F5* and *F8* may be suspected.

To evaluate the haemostatic functions leading to the clinical symptoms, we compared several clotting assay parameters of P1 with those of comparative HA patients with the same *F8* mutation (Table 1). Although P1 had similar values of FVIII antigen and activity to the comparative HA patients, he showed very mild bleeding tendency and haemophilia arthropathy with an apparently better joint score [29], despite moderate HA. In TGA, P1 showed lower peak and lesser ETP values than the normal subject; however, he showed shorter tt-peak, higher peak and larger ETP values than the comparative HA patients, which may lead to more rapid haemostasis than the comparative

HA patients (Fig. 3). In addition, we performed haplotype analysis of *F5* and detected an FV R2 haplotype consisting of multiple nucleotide substitutions in the other allele without FV nonsense mutation in both P1 and P2, but not in P3 (Table 2).

FV R2 haplotype is a common genetic variation among several distinct populations [30] and the allelic frequency of FV R2 haplotype in the Japanese population was calculated to be 5.3% [31]. Plasma-based assays revealed that the FV R2 haplotype was associated with mild activated protein C (APC) resistance [32, 33]. APC resistance is the most common hereditary thrombotic disorder among Caucasians, especially the majority of these cases are caused by variant of the FV molecule, known as the famous FVL [34]. FVL mutation causes APC resistance by reducing the susceptibility of activated FV (FVa) to APC-mediated inactivation and additionally impairing APC cofactor activity of FV in activated FVIII (FVIIIa) inactivation, leading to thrombotic tendency. In our cases (P1 and P2), since FV expression of one allele was abolished by nonsense mutation, only FV derived from the other allele with a mild APC resistance R2 haplotype seemed to be present, alleviating the bleeding tendency. Similarly, a pseudo-homozygous patient for APC resistance exhibiting both nonsense and FV Leiden mutations, who did not suffer from any bleeding episodes, but reported one episode of thrombophlebitis, was reported [35]. However, further studies and long-term clinical evaluation are needed to elucidate the confident association between FV and FVIII abnormalities

and clinical symptoms in our cases.

Conclusions

We reported here the first report of a rare case of coinheritance of congenital FV and FVIII deficiencies. Mutations found in *F5* and *F8* were inherited independently in this family, resulting in an unprecedent disease of parahaemophilia combined with moderate HA. We proposed that a chance coinheritance of congenital FV and FVIII deficiencies could be the third mechanism for inherited F5F8D.

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Disclosures

I state that I have no conflict of no interest.

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Figure legends

Fig 1. Pedigree of cases suspected of combined FV/FVIII deficiency.

The proband (P1: black arrow) was suspected of combined FV/FVIII deficiency, due to his low values of both FV:C and FVIII:C. In addition, the brother (P2) and daughter (P3) of the proband also had low values of both FV:C and FVIII:C.

Fig 2. DNA sequences around mutations in *F5* and *F8*.

A. Sequence analysis of *F5*. Nucleotides and predicted amino acids sequences surrounding the mutation of exon 10 in the *F5*. Arrows indicate the site of mutation. The mutation was a C-to-T transition at nucleotide 1600, leading to a substitution of CGA (Arg) with TGA (Stop Codon) at codon 534 (c.1600C>T, p.Arg534*), which was heterozygous in all three patients.

B. Sequence analysis of *F8*. Nucleotides and predicted amino acids sequences surrounding the mutation of exon 23 in the *F8*. Arrows indicate the site of mutation. The mutation was a G-to-A transition at nucleotide 6506, leading to a substitution of CGT (Arg) with CAT (His) at codon 2169 (c.6506G>A, p.Arg2169His), which was hemizygous in P1 and P2, and heterozygous in P3.

Fig 3. TGA of the proband and comparative HA patients.

The thrombin generation assay (TGA) was performed using plasmas of the proband (P1) and the comparative HA patients (A and B) with the same mutation in *F8*. P1 showed lower peak and lesser ETP values than normal pooled plasma (NPP); however, he showed shorter tt-peak, higher peak and larger ETP values than comparative HA patients.

Table 1. Clinical characteristics and clotting assay parameters.

	Age	BMI	Joint Score*	PT (sec)	APTT (sec)	FVIII:C (%)	FVIII:Ag (%)
P1	62	32.7	0	12.4	72.9	2	1.8
Comparative Patient A	70	25.1	9	11.0	59.1	4	3.4
Comparative Patient B	63	16.9	7	10.9	49.9	7	0.9

*, Pettersson et al. [29]

We compared clinical characteristics and several clotting assay parameters of P1 with those of comparative HA patients with the same mutation in F8.

Table 2. Mutation and polymorphism analysis in *F5* including FV R2 haplotype.

Locate	Nucleotide	Amino acid	P1 alleles		P2 alleles		P3 alleles	
			*	R2	*	R2	*	
Exon 2	c.237A>G	p.Gln79=	G	A	G	A	G	G
Exon 4	c.405G>A	p.Ala135=	G	A	G	A	G	G
Exon 4	c.552G>T	p.Ser184=	G	T	G	T	G	G
Exon 8	c.1238T>C	p.Met413Thr	T	C	T	C	T	T
Exon 9	c.1380C>T	p.Asn460=	C	T	C	T	C	C
Exon 10	c.1538G>A	p.Arg513Lys	G	G	G	G	G	A
Exon 10	c.1600C>T	p.Arg534*	T	C	T	C	T	C
Exon 11	c.1716G>A	p.Glu572=	G	A	G	A	G	G
Exon 12	c.1926C>A	p.Thr642=	C	A	C	A	C	C
Exon 13	c.2289A>G	p.Glu763=	A	G	A	G	A	A
Exon 13	c.2450A>C	p.Asn817Thr	A	C	A	C	A	A
Exon13	c.3804T>C	p.Ser1268=	T	C	T	C	T	T
Exon13	c.3853C>A	p.Leu1285Ile	C	A	C	A	C	A
Exon13	c.3980A>G	p.His1327Arg	A	G	A	G	A	A
Exon13	c.4189C>T	p.Leu1397Phe	C	C	C	C	C	T
Exon16	c.5290A>G	p.Met1764Val	A	G	A	G	A	A

*, Nonsense allele; R2, FV R2 haplotype allele.

Numerous single nucleotide polymorphisms including FV R2 haplotype were observed in F5 of P1, P2 and P3 together with nonsense mutation (c.1600C>T, p.Arg534*). P1 and P2 had the nonsense mutation in one allele and FV R2 haplotype mutations in the other allele.

Fig 1. Pedigree of cases suspected of combined FV/FVIII deficiency.

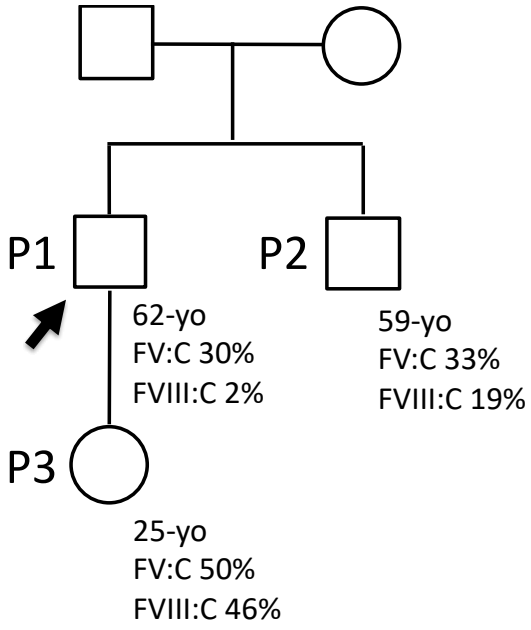
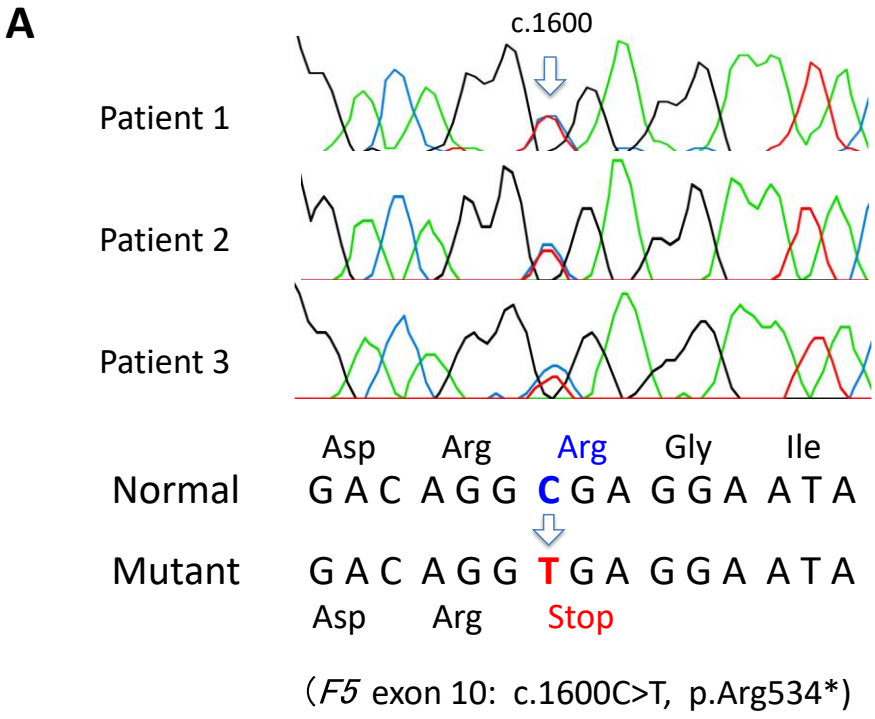


Fig 2. DNA sequences around mutations in *F5* and *F8*.



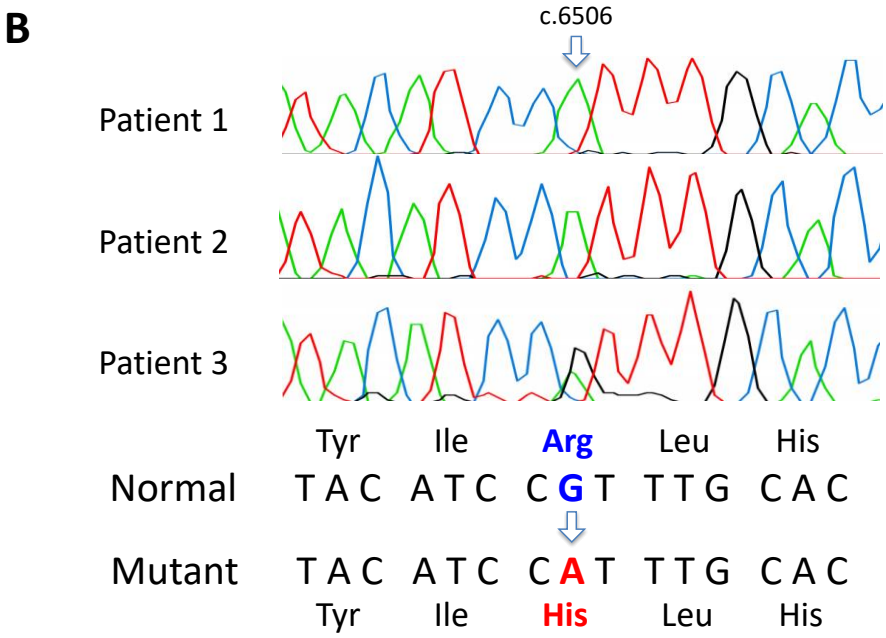
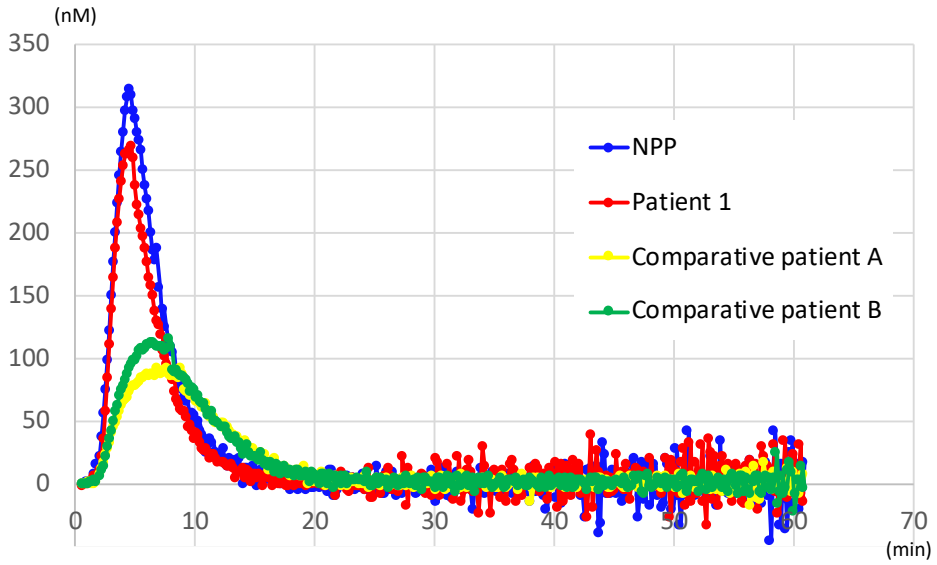


Fig 3. TGA of the proband and comparative HA patients.



	NPP	P1	Comparative patient A	Comparative patient B
Lagtime (min)	2.2	2.4	2.2	2.4
ETP (nM.min)	1476	1211	896	991
Peak (nM)	312	269	90	113
ttPeak (mim)	4.5	4.6	7.2	6.7
StartTail (min)	18.5	15.7	27.5	27.0