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Molecular basis of *SERPINC1* mutations in Japanese patients with antithrombin deficiency

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ABSTRACT

Background: Congenital antithrombin (AT) deficiency, which arises from various *SERPINC1* defects, is an autosomal-dominant thrombophilic disorder associated with a high risk of recurrent venous thromboembolism.
Patients/methods: We investigated *SERPINC1* defects in Japanese patients with congenital AT deficiency who developed venous thromboembolism or had a family history of deep vein thrombosis. We analyzed the full DNA sequences of *SERPINC1* exons and exon-intron junctions by PCR-mediated direct sequencing. If no mutation was found, multiplex ligation-dependent probe amplification (MLPA) was conducted for the relative quantification of the copy number of all exons in *SERPINC1*. If splice-site mutations were detected, mRNA splicing abnormalities were further investigated using an in vitro cell-based exontrap assay.
Results: We identified 19 different *SERPINC1* abnormalities, including 8 novel mutations, in 21 Japanese patients with AT deficiency. These abnormalities were distributed as follows: 9 missense mutations (42.9%), 3 nonsense mutations (14.3%), 1 splice-site mutation (4.8%), 2 small insertions (9.5%), 2 deletion mutations (9.5%) and 4 large deletions (19.0%). Cases with large deletions of *SERPINC1* included *Alu*-mediated gene rearrangements and non-*Alu*-mediated complex gene rearrangements; the latter could conceivably be explained using the fork stalling and template switching (FoSTeS) model.
Conclusions: We identified a variety of *SERPINC1* defects in Japanese patients with AT deficiency. The *SERPINC1* mutations detected in patients with type I AT deficiency included single nucleotide missense or nonsense mutations, small intragenic insertions or deletions, and large genomic structural deletions. Large deletions of *SERPINC1* were caused by various recurrent or non-recurrent complex genomic rearrangement mutations.

1. Introduction

Antithrombin (AT), a member of the serine protease inhibitor (SERPIN) superfamily, is a major circulating inhibitor of blood coagulation proteases such as factor (F) IIa (known as thrombin), FXa and, to a lesser extent, FIXa, FXIa and FXIIa. *SERPINC1*, which encodes AT in humans, is located on chromosome 1q25.1 and comprises 7 exons and 6 introns [1]. AT is synthesized in hepatocytes as a single-chain, 464-amino-acid precursor from which a 32-amino-acid signal peptide is cleaved to yield mature protein comprising 432 amino acids. AT contains two important functional domains: a heparin binding site domain and reactive site domain. Physiologically, this inhibitor circulates in a form with low anticoagulant activity; however, this activity can be strongly enhanced by conformational changes caused by the binding of heparin or heparin-like glycosaminoglycans (e.g., heparan sulfate). Thrombin-AT complex (TAT) formation can be accelerated by > 1000-

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fold in the presence of heparin [2].

Congenital AT deficiency is an autosomal-dominant thrombophilic disorder [3]. This condition is associated with a high risk of venous thromboembolism [4]. In the general population, the estimated prevalence of AT deficiency is 1:500 to 1:5000, without sexual, racial, or ethnic predilection [5,6]. Except in rare cases (e.g., AT Budapest 3 and AT Cambridge II), most AT-deficient patients exhibit heterozygosity for the *SERPINC1* mutation [7–9]. Studies in an experimental mouse model have shown that a homozygous null defect in *Serpinc1* resulted in embryonic lethality due to severe thrombosis and bleeding [10].

AT deficiency can be classified into type I and type II based on the AT antigen and activity levels [11,12]. Type I is characterized by a quantitative AT deficiency, with low levels of AT antigen and activity consequent to a lack of protein production or secretion. Type II is a qualitative AT deficiency characterized by a normal AT antigen level but low AT activity and can be further subdivided into 3 subtypes. Type II reactive-site defect (RS) is defined as a low level of serine protease inhibitory activity in both the presence and absence of heparin. Type II heparin binding site defect (HBS) is characterized by a decrease in heparin binding ability without impairment of serine protease inhibitory activity in the absence of heparin. Type II pleiotropic effect (PE) is attributed to an amino acid substitution mainly in the C-sheet of AT, resulting in a reduced plasma AT concentration and dysfunctional serine protease inhibitory activity.

In this study, our investigation of genetic mutations in 25 Japanese patients with AT deficiency led to the identification of 19 different *SERPINC1* abnormalities, including 8 novel mutations, in 21 patients. Herein, we report the distribution and molecular basis of *SERPINC1* mutations in 21 Japanese patients with AT deficiency.

2. Materials and methods

2.1. Patients and study design

During the 10-year period since December 2007, we have investigated *SERPINC1* defects in 25 Japanese patients with suspected congenital AT deficiency. AT deficiency cases were classified as type I or type II RS, -HBS or -PE based on the AT activity and antigen levels and genomic mutation type. After informed consent was obtained, a genomic DNA sample was isolated from the peripheral blood leukocytes of each patient. We analyzed all exons and exon-intron junctions of *SERPINC1* using PCR-mediated direct sequencing. If a single nucleotide substitution, small insertions or small deletions were not found, *SERPINC1* was subjected to relative quantification to detect large deletions. If a splice-site mutation was detected, the mRNA splicing abnormality was further investigated in vitro. This study was approved by the Ethics Committee of the Nagoya University School of Medicine. Gene and protein mutations are described according to the nomenclature of the Human Genome Variation Society.

2.2. Antithrombin assay

AT activity was measured by L-system ATIII Xa-based chromogenic assay (Sysmex, Japan) and CS-5100 (Sysmex). Coagtrol N plasma-based standard (Sysmex) was used as a calibrator for AT activity assay. AT antigen assay was outsourced to LSI Medience Co. Ltd (Tokyo, Japan). In this study, the reference range of AT activity and antigen were 80–120% and 65–134%, respectively.

2.3. SERPINC1-specific polymerase chain reaction (PCR)

We conducted polymerase chain reactions (PCRs) with KOD FX DNA polymerase (Toyobo, Japan) to amplify all *SERPINC1* exons and intron-exon junctions. PCR products were validated electrophoretically by amplicon size on 1.5% agarose gels and were extracted from gels using QIAEX II (QIAGEN, Japan). Direct sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit and ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). The primer sets for *SERPINC1*-specific PCR are listed in sTable 1.

2.4. MLPA analysis

In the case of a suspected exon or entire gene deletion in *SERPINC1*, the relative gene quantity of each exon was measured quantitatively according to multiplex ligation-dependent prove amplification (MLPA) method; here, the SALSA MLPA P227 SerpinC1 kit (MRC-Holland, Netherlands) was used according to the manufacturer's instructions.

2.5. Relative gene quantity mapping

For gene quantity mapping, we used PCR-mediated single nucleotide polymorphism (SNP) genotyping and quantitative PCR (qPCR) methods. SNP genotyping results were analyzed by PCR direct sequencing, and qPCR was performed using a SYBR Premix Ex Taq and Thermal Cycler Dice Real Time System II (TaKaRa Bio Inc., Japan) according to the manufacturers' protocols. The primer sets used for SNP genotyping and qPCR are shown in sTable 1.

2.6. Exontrap analysis

If splicing mutations were detected, an exontrap analysis was performed to investigate abnormal splicing variants due to mutations. A DNA fragment containing exon/intron boundaries from mutant or wild-type (WT) *SERPINC1* was prepared by PCR with specific primers, digested with restriction enzymes, and inserted into an exontrap vector (MoBiTec GmbH, Germany). This vector comprised a 5′-exon, 600-bp intron containing multiple cloning sites, and 3′-exon. The constructed vector was then transfected into COS-7 cells using the $CaPO_4$-DNA precipitation method. After a 16-h culture period, total RNA was extracted from the cells using a RNeasy mini kit (QIAGEN) and subjected to reverse transcription using PrimeScript RT Master Mix (TaKaRa, Japan). Finally, messenger RNA derived from the constructed vector was analyzed by PCR amplification and direct sequencing.

3. Results

3.1. Genetic mutation profiles of AT deficiency in this study

We conducted genetic and genomic analyses of 25 Japanese patients with suspected congenital AT deficiency during the decade, 2007–2017, and identified 19 distinct *SERPINC1* abnormalities in 21 patients (84.0%). Based on the genotypes, AT activity and antigen levels and existing literature, 15 distinct mutations in 17 cases were classified as type I AT deficiency, while 4 different missense mutations in 4 cases were classified as type II AT deficiency (Table 1). All AT deficiency-causing *SERPINC1* mutations were heterozygous. The mutations were distributed as follows: 12 missense/nonsense mutations (57.1%: 9 missense and 3 nonsense), 1 splice-site mutation (4.8%), 2 small insertions (9.5%), 2 small deletions (9.5%), and 4 large deletions (19.0%).

3.2. Single base substitutions and small insertions or deletions

We identified 13 single base substitutions, 9 missense mutations, 3 nonsense mutations and 1 splice-site mutation (Cases 1–13 in Table 1). Three missense mutations—c.442T > C (p.Ser148Pro), c.1315C > A (p.Pro439Thr) and c.1370G > C (p.Arg457Thr)—were previously reported to cause type II AT deficiency (Cases 4, 6 and 7) [13–15]. We also identified a novel missense mutation, c.652A > T (p.Ile218Phe), that presumably caused type II AT deficiency. Interestingly, a mutation of the subsequent amino acid in the C-sheet, p.Asn219Asp, was previously reported to cause type II PE AT deficiency [12]. Three missense

Table 1
SERPINC1 mutations in 21cases of Japanese antithrombin deficiency.

Case	Age	Sex	AT activity (%)	AT antigen (%)	Genotype	Location	Type of mutation		Amino acid change	Type	CpG	Thrombosis	Onset age	Family history	Acquired risk factor	Reference
1	46	M	46	Not tested	c.269T > G	Exon 2	Single base substitution	Missense	p.Phe90Cys	Type I	No	VTE	46	Unknown	Unknown	This paper
2	30	F	32	34	c.374G > A	Exon 2	Single base substitution	Missense	p.Gly125Asp	Type I	No	DVT	30	Unknown	Pregnancy	[14]
3	32	F	49	Not tested	c.374G > A	Exon 2	Single base substitution	Missense	p.Gly125Asp	Type I	No	D-dimer: 1.55 mg/mL[c]	32	Sister (DVT)	Pregnancy	[14]
4	35	F	57	Not tested	c.442T > C	Exon 3	Single base substitution	Missense	p.Ser148Pro	Type II-HBS	No	None	–	Unknown	Pregnancy	[13]
5	62	F	60	71	c.652A > T	Exon 4	Single base substitution	Missense	p.Ile218Phe	Type II-PE	No	DVT	32	Unknown	Pregnancy	This paper
6	24	M	78[a]	109[a]	c.1315 C > A	Exon 7	Single base substitution	Missense	p.Pro439Thr	Type II-PE	No	VTE	22	None	Unknown	[13]
7	17	M	40	Not tested	c.1370G > C	Exon 7	Single base substitution	Missense	p.Arg457Thr	Type II-PE	No	VTE	17	Unknown	Pneumonia and myocarditis	[8]
8	26	F	56	Not tested	c.728C > T	Exon 4	Single base substitution	Missense	p.Thr243Ile	Type I	No	DVT	26	Unknown	Pregnancy	[24]
9	29	F	46	61	c.769T > C	Exon 5	Single base substitution	Missense	p.Trp257Arg	Type I	No	DVT	25	Unknown	Pregnancy	[13]
10	55	M	52	56	c.663G > A	Exon 4	Single base substitution	Nonsense	p.Trp221*	Type I	No	VTE	55	None	Smoking	This paper
11	42	F	63	Not tested	c.685C > T	Exon 4	Single base substitution	Nonsense	p.Arg229*	Type I	Yes	VTE	40	None	Unknown	[16]
12	23	M	46	53	c.1171C > T	Exon 6	Single base substitution	Nonsense	p.Arg391*	Type I	Yes	VTE	23	None	Smoking	[17]
13	38	M	62	58	c.1154-14G > A	Intron 5	Single base substitution	Splicing	p.Pro384_Gly385insValPheLeuPro	Type I or II	Yes	Screened by carrier diagnosis	–	Father (DVT)	–	[18,34]
14	32	F	45	43	c.462_464delCTT	Exon 3	Small deletion	Deletion	p.Phe155del	Type I	–	DVT	30	Unknown	Pregnancy	[19]
15	48	M	51	Not tested	c.462_464delCTT	Exon 3	Small deletion	Deletion	p.Phe155del	Type I	–	VTE	48	Unknown	Working style (desk work)	[19]
16	34	F	51	72	c.624+1dupG	Intron 3	Small deletion	Splicing	p.Glu209Glyfs*17	Type I	–	D-dimer: 7.11 mg/mL[c]	34	Father (DVT)	Pregnancy	This paper
17	21	F	53	41	c.907dupG	Exon 5	Small deletion	Frameshift	p.Glu303Glyfs*7	Type I	–	None	–	Yes (DVT)	Systemic lupus erythematosus	This paper
18	35	F	24	Not tested	c.881+(409_436)_1273-(741_714)del	Exon 5	Large deletion	Frameshift	p.(Gly255Valfs*23)	Type I	–	DVT	24	Unknown	Pregnancy	This paper
19	55	M	50	50	c.408+(523_534)_1153+(437_448)del	Exons 3–5	Large deletion	Frameshift	p.(Val137Valfs*23)	Type I	–	VTE	53	Unknown	Unknown (trauma?)	This paper
20	16	F	75[b]	62	g.173870507_173905610delins TGGAGTA (NC_000001.11)	Exon 7	Large deletion	–	–	Type I	–	DVT	16	Mother (DVT)	Pyelonephritis	This paper
21	43	F	49	Not tested	g.25326758_25437835delinsCGGATGG (NT_004487.19)	All exons	Large deletion	–	–	Type I	–	VTE	41	Unknown	Autoimmune-like symptoms?	[20]

[a] Under antithrombin replacement therapy.
[b] Under anticoagulant therapy with Apixaban.
[c] Cutoff: > 1.0 μg/mL.

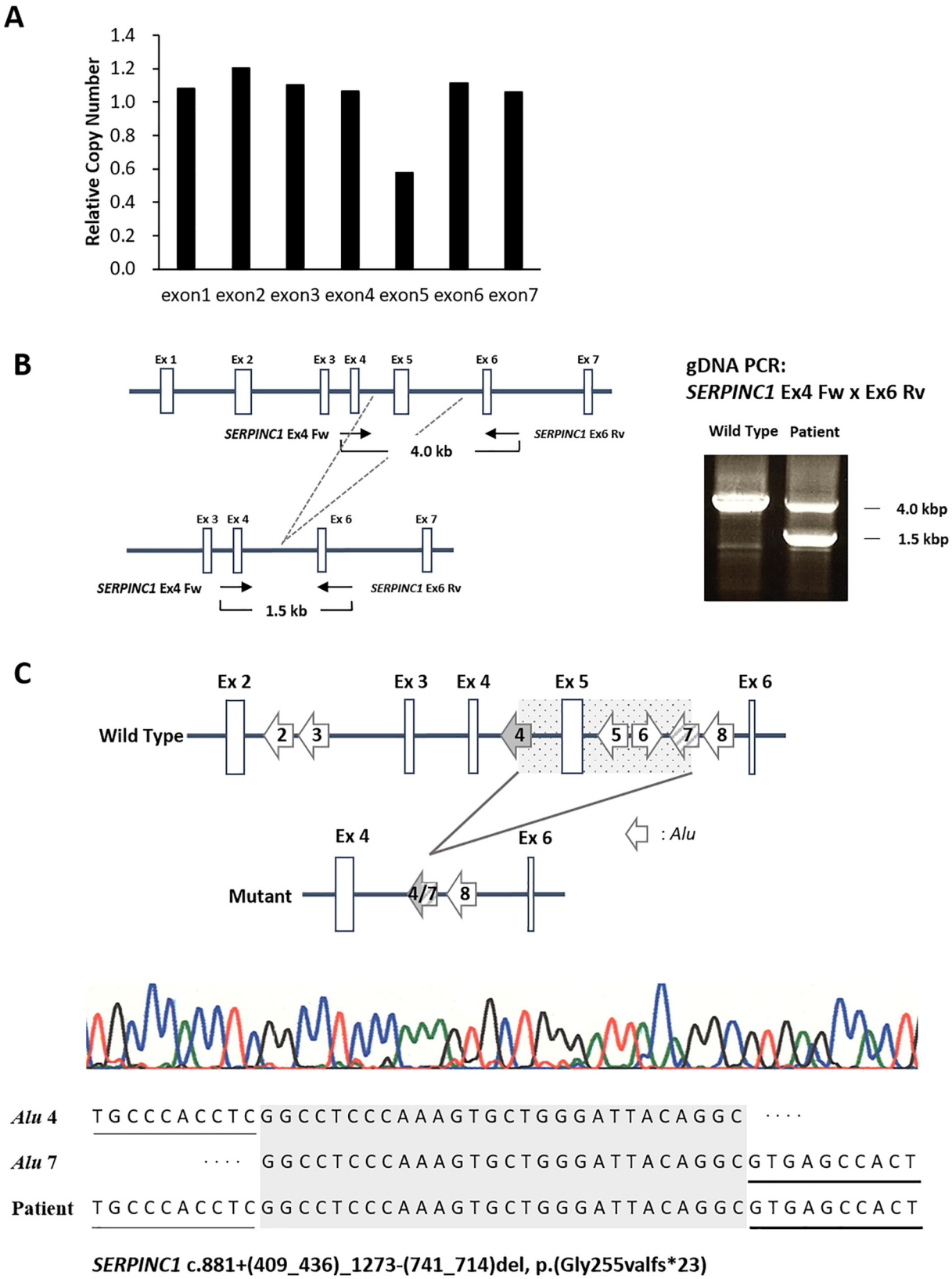

Fig. 1. Case 18: *SERPINC1* c.881 + (409_436)_1273-(741_714)del, p. (Gly255Valfs*23). A) Multiplex ligation-dependent probe amplification analysis demonstrating a heterozygous exon 5 deletion. B) Left panel: A scheme of the use of long-range PCR to investigate the *SERPINC1* intragenic rearrangement causing the exon 5 deletion. The primer set (Ex4 Fw/Ex6 Rv) was designed to yield a 4.0-kb amplicon product from the WT allele. Right panel: Results of long-range PCR. The abnormal product (1.5-kb amplicon) was clearly detected in the patient's genomic DNA. C) Upper panel: Diagram of an approximately 2.5-kb deletion, including exon 5 of *SERPINC1*. Lower panel: DNA sequence of the breakpoint junction. The highlighted alignment is a homologous sequence at the breakpoint junction. A direct sequencing analysis revealed that *Alu* 4 in intron 4 was connected to *Alu* 7 in intron 5 via a 28-bp identical sequence.

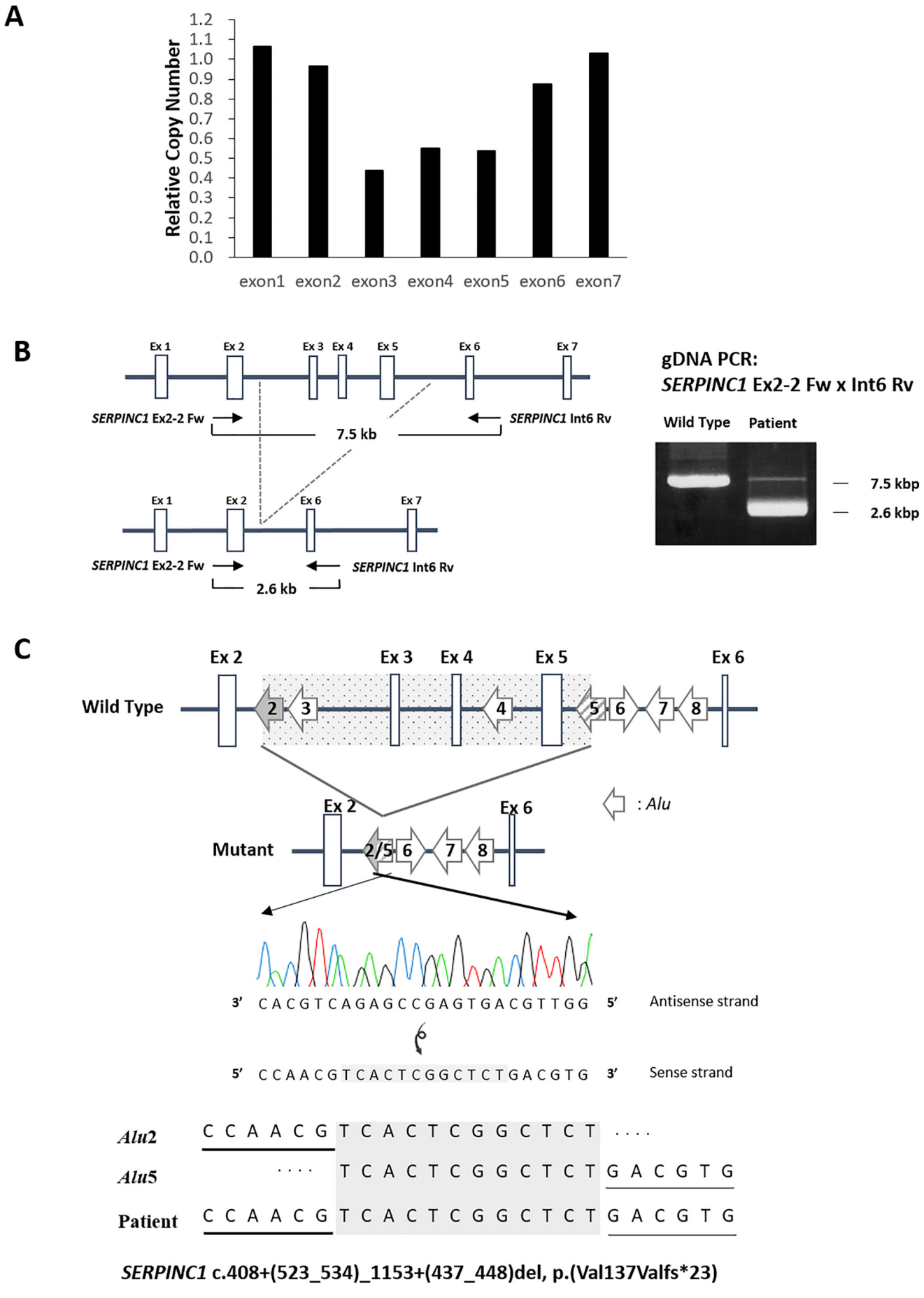

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Fig. 2. Case 19: *SERPINC1* c.408 + (523_534)_1153 + (437_448)del, p.(Val137Valfs*23). A) Multiplex ligation-dependent probe amplification analysis showing exons 3 to 5 deletion as a heterozygote. B) Left panel: A scheme of the use of long-range PCR to investigate the large deletion of *SERPINC1* spanning exons 3–5. The primer set (Ex2_2 Fw/Int6 Rv) was designed to yield a 7.5-kb amplicon product from the WT allele. Right panel: Results of long-range PCR. The abnormal PCR product (2.6-kb) was detected in the patient's genomic DNA. C) Upper panel: Diagram of the approximately 4.9-kb deletion, which included exons 3–5 of *SERPINC1*. Lower panel: DNA sequence of the breakpoint junction in the antisense strand. A reverse-complement alignment (sense strand sequence) is shown below. Lower panel: DNA sequence of the breakpoint junction. The highlighted alignment is a homologous sequence at the breakpoint junction. Direct sequencing revealed that *Alu* 2 in intron 2 was connected to *Alu* 5 in intron 5 via a 12-bp identical sequence.

mutations—c.374G > A (p.Gly125Asp), c.728C > T (p.Thr243Ile), and c.769T > C (p.Trp257Arg)—in 4 cases led to type I AT deficiency (Cases 2, 3, 8 and 9) and had previously been reported [15,16]. We also identified a novel missense mutation, c.269T > G (p.Phe90Cys), that appeared to cause type I AT deficiency (Case 1). Of the identified nonsense mutations, c.685C > T (p.Arg229*) and c.1171C > T (p.Arg391*) had been previously reported to cause type I AT deficiency (Cases 11 and 12) [17,18], whereas c.663G > A (p.Trp221*) was a novel mutation (Case 10). An intron substitution involving G-to-A at position −14 from the first nucleotide of exon 6 (c.1154-14G > A) had also been previously reported as a splicing mutation (Case 13) [19].

Within the coding region of *SERPINC1*, we identified 2 cases involving the same 3-nucleotide deletion (Cases 14 and 15) and 2 cases involving a single nucleotide duplication (Cases 16 and 17). Cases 14 and 15 harbored the same 3-nucleotide deletion in exon 3 (c.462_464delCTT, p.Phe155del), which was previously reported to cause type I AT deficiency [19,20]. In Case 16, a G duplication was detected at position +1 from the last nucleotide of exon 2; this novel mutation was located at the splice donor site of *SERPINC1* intron 3 (c.624 + 1dupG). The molecular pathogenesis of this *SERPINC1* c.624 + 1dupG is detailed in a subsequent paragraph (Molecular pathogenesis of splicing mutations). In Case 17, we identified a novel G duplication of c.907 in exon 4 (c.907dupG); this caused a change from Glu303 (GAG) to Gly303 (GGA) and caused a frameshift with a stop codon in the new reading frame at position 7 (p.Glu303Glyfs*7).

3.3. Large deletions

For 4 cases wherein no single nucleotide substitution or small insertion or deletion mutation was detected, we identified 4 types of *SERPINC1* genomic structural mutations through a genomic structural mutation analysis. One large deletion, a 111-kb deletion involving the whole *SERPINC1* (g.25326758_25437835delinsCGGATGG: NT_004487.19, Case 21), was previously reported by our group [21]. In the present report, we further describe the analytical results of the 3 unreported mutations in detail (Cases 18, 19 and 20).

In Case 18, a MLPA gene quantity analysis revealed a heterozygous deletion of exon 5 (Fig. 1A). To identify the breakpoints, we performed a long-range PCR and subsequent direct sequencing analysis. The primer set, which was designed to yield a 4.0-kb amplicon product from exons 4 to 6 of the WT allele, yielded an obviously abnormal 1.5-kb product from the patient's genomic DNA (Fig. 1B). Direct sequencing revealed a 2.5-kb deletion in which the *Alu* 4 in intron 4 was linked to *Alu* 7 in intron 5 via a 28-bp homologous sequence in *SERPINC1* (c.881 + (409_436)_1273-(741_714)del, p.(Gly255Valfs*23)) (Fig. 1C).

In Case 19, MLPA analysis revealed a large intragenic deletion ranging from exons 3 to 5 (Fig. 2A). We used a long-range PCR primer set spanning from introns 1 to 6 to obtain a 7.5-kb PCR product from the WT allele; however, an apparently abnormal 2.6-kb product was amplified from the patient's genomic DNA (Fig. 2B). Direct sequencing indicated that the *Alu* 2 of intron 2 had connected to *Alu* 5 of intron 5 via a 12-bp homologous sequence, resulting in a 4.9 kb deletion within *SERPINC1* (c.408 + (523_534)_1153 + (437_448)del, p.(Val137-Valfs*23)) (Fig. 2C).

In Case 20, MLPA analysis revealed the absence of exon 7 in *SERPINC1* (Fig. 3A). This deletion pattern was also detected in the patient's mother and younger brother. To search for internal and external deleted regions of *SERPINC1*, we performed genome quantity mapping based on genomic qPCR and PCR-mediated SNP genotyping. Genome quantity mapping suggested that the patient exhibited a large, heterozygous deletion of up to 38 kb that spanned from *ZBTB37* to *SERPINC1* exon 7 (Fig. 3B and Supplemental Table 2). To confirm this observation, a long-range PCR was designed to detect 4-kb amplicon in the mutant allele. The expected PCR products were detected in genomic DNA from the patient, mother and brother but not from a WT subject. Direct sequencing of the long-range PCR products revealed the linkage of an indel mutation (g.173870507_173905610delinsTGGAGTA: NC_000001.11) in *ZBTB37* exon 3 (g.173870507: NC_000001.11) to *SERPINC1* intron 6 (g.173905610: NC_000001.11) via a 7-bp sequence (TGGAGTA) inserted at the breakpoint. Interestingly, an identical 7-bp sequence was contained in a 10-bp alignment (CTTGGAGTAA) detected in *SERPINC1* intron 6 (g.173904556-173904565: NC_000001.11) (Fig. 3C).

3.4. Molecular pathogenesis of splicing mutations

We identified 2 cases harboring *SERPINC1* splicing mutations (Cases 13 and 16). We then conducted exontrap analyses to investigate the molecular pathogenesis of these splicing mutations.

In Case 13, we identified a G-to-A substitution at nucleotide position −14 from the first nucleotide of exon 6 (c.1154-14G > A) (Fig. 4A). We transfected COS-7 cells with an exontrap vector into which a WT or mutant allele fragment ranging from introns 4 to 6 of *SERPINC1* had been incorporated and subsequently analyzed the transcripts by RT-PCR (Fig. 4B). Notably, the sizes of the RT-PCR amplicons generated by the WT and mutant-derived transcripts were nearly identical. Direct sequencing of abnormal transcripts from the mutant vector revealed a 12-bp insertion (TCTTCCTTCCAG) into *SERPINC1* at the exon 5/6 junction (Fig. 4C).

In Case 16, a G duplication was detected at the splice donor site of *SERPINC1* intron 3 (c.624 + 1dupG) (Fig. 5A). Accordingly, PCR amplicons spanning introns 2 to 4 from WT and mutant *SERPINC1* were inserted into the exontrap vector (Fig. 5B left panel) and subsequently transfected into COS-7 cells. The RT-PCR amplicon sizes of both the WT and mutant exontrap vector transcripts were approximately 600 bp (Fig. 5B right panel); however, a subsequent direct sequencing analysis of transcripts from the mutant exontrap vector identified aberrant transcripts containing a G insertion at the splice junction of exons 3 and 4. This induced a frameshift mutation from the 209Glu codon, resulting in a termination codon at amino acid position 17 (p.Glu209Glyfs*17) (Fig. 5C).

4. Discussion

AT deficiency arises from a variety of *SERPINC1* mutations. In the present study, we investigated Japanese patients with suspected congenital AT deficiency and identified 19 different causative *SERPINC1* abnormalities. The distribution of AT deficient phenotype was 17 cases of type I and 4 cases of type II, respectively (case 13 was classified as type I deficiency). Type I AT deficiency was high frequent phenotype among VTE patients with suspected congenital AT deficiency. Mitsuguro et al. reported that patient with type I AT deficiency had more VTE events as compared to those with type II AT deficiency [22], which consisted with the present observation. In regard to *SERPINC1*

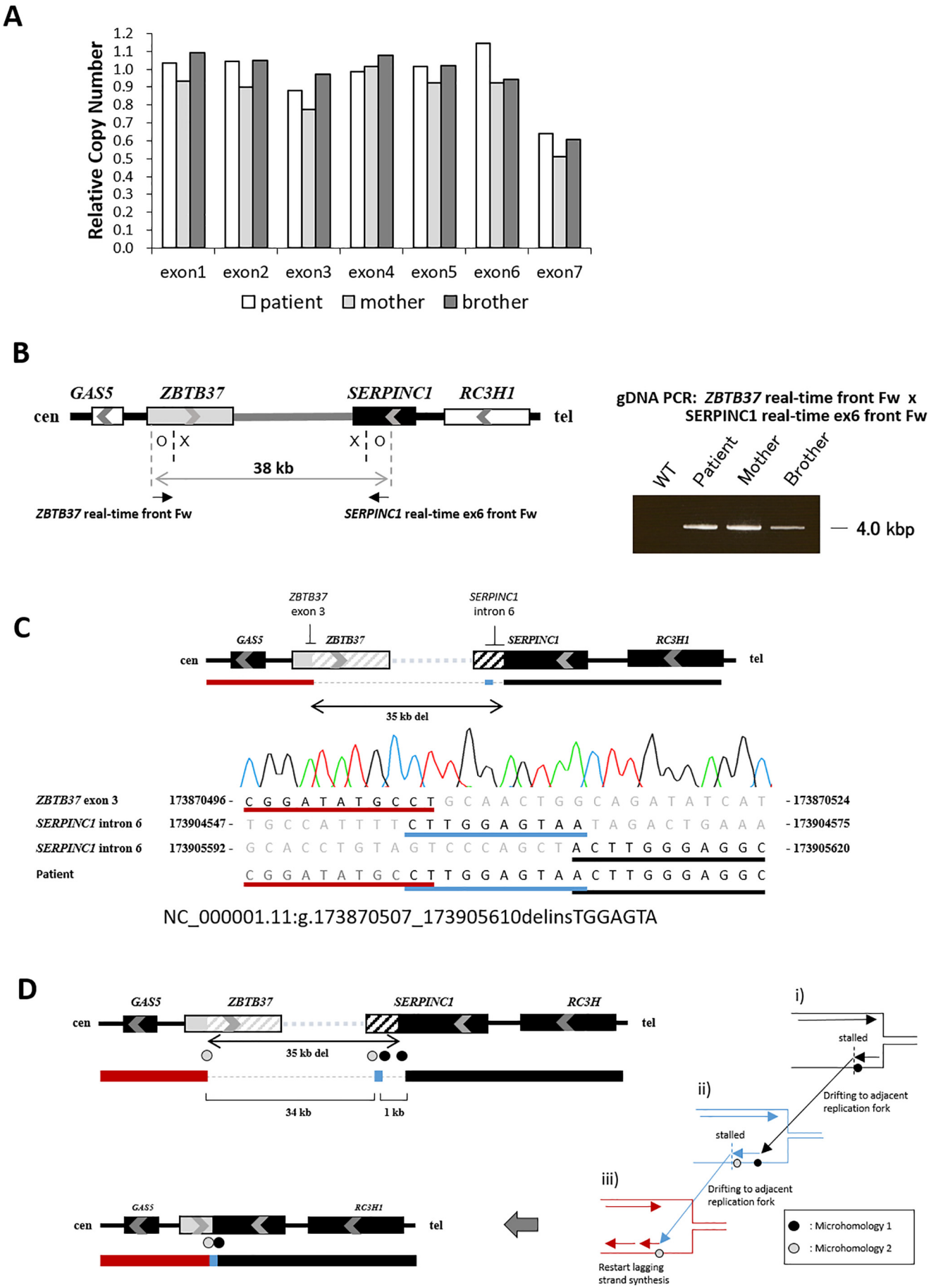

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Fig. 3. Case 20: g.173870507_173905610delinsTGGAGTA: NC_000001.11. A) Multiplex ligation-dependent probe amplification analysis revealed a heterozygous exon 7 deletion in the patient, his mother and brother. B) Left panel: Summary of the genome quantity mapping and scheme of the use of long-range PCR to investigate the large deletion from *ZBTB37* to *SERPINC1*, which spanned approximately 38 kb. The primer set (*ZBTB37* real-time front Fw/*SERPINC1* real-time ex6 front Fw) was designed to yield a 4-kb amplicon product from the mutant allele. Right panel: Results of long-range PCR. The abnormal PCR amplicons containing approximately 4 kb were detected in genomic DNA from the patient and his mother and brother, but not in WT genomic DNA. C) DNA sequence of the breakpoint junction. *ZBTB37* exon 3 was linked to *SERPINC1* intron 6 via a 7-bp insertion (TGGAGTA) at the breakpoint. A 10-bp alignment sequence containing the same 7-bp insert sequence (the inserted sequence + CT at 5′ side and A at 3′ side, respectively) was detected in *SERPINC1* intron 6 (g.173904556-173904565: NC_000001.11). The numbers to the right or left of each alignment indicate the genomic position in chromosome 1 (NC_000001.11). D) The proposed mechanism of genomic rearrangement in Case 20. This complex genomic rearrangement can be explained using the fork stalling and template switching (FoSTeS) model. (i) Specifically, the replication fork is stalled and temporarily paused at the DNA lesion in *SERPINC1* intron 6, g.173905610: NC_000001.11. (ii) The lagging strand disengages, invades the adjacent replication fork at *SERPINC1* intron 6 and anneals via microhomology 1 (g.173904565: NC_000001.11, shown as a black circle); this is followed by synthesis of the lagging strand at the invaded fork. (iii) The drifting lagging strand again disengages at the position of g.173904556: NC_000001.11, invades the other active replication fork at *ZBTB37* exon 3 and reinitiates strand synthesis at the microhomology 2 priming site (g.173870507: NC_000001.11, shown as a gray circle).

abnormality, all 4 patients with type II AT deficiency harbored distinct and previously reported missense mutations. In contrast, 15 different *SERPINC1* abnormalities, including 7 novel mutations, were identified among 17 patients with type I AT deficiency. The mutations detected in patients with type I AT deficiency included single nucleotide nonsynonymous missense or nonsense mutations, small intragenic insertions or deletions (< 50 bp), and large genomic structural deletions (≥50 bp). Additionally, some abnormalities involved splicing mutations.

We identified several novel nonsynonymous mutations and small insertions or deletions, including 2 missense mutation (c.269T > G, p.Phe90Cys: Case 1, and c.652A > T, p.Ile218Phe: Case 5), 1 nonsense mutation (c.663G > A, p.Trp221*: Case 10) and 2 G duplications (c.624 + 1dupG, p.Glu209Glyfs*17: Case 16, and c.907dupG, p.Glu303Glyfs*7: Case 17). We considered that the p.Phe90Cys missense mutation caused type I AT deficiency, because a different missense mutation involving the same amino acid position (p.Phe90Leu) was reported to cause type I AT deficiency [15]. In addition, the substitution of 90 Phe by Cys could devastate the conformation of the variant protein by interfering with existing disulfide bonds leading to instability. The p.Ile218Phe mutation may cause type II PE AT deficiency. Mutations of the subsequent amino acid, p.Asn219Asp and p.Asn219Lys, were previously reported to cause type II PE AT deficiency [12]. Type II PE AT deficiency has a tendency to slight reduction of AT antigen [11], which consistent with the laboratory data of the patient harboring p.Ile218Phe (Case 5 in Table 1). Nonsense and many frameshift mutations produce premature termination codons (PTCs), resulting in mRNA degradation by nonsense-mediated mRNA decay (NMD) or truncated protein synthesis. NMD is an intracellular surveillance mechanism used to eliminate aberrant mRNA and is the predominant mechanism underlying PTC-mediated genetic diseases [23]. Additionally, three mutations (p.Glu209Glyfs*17, p.Trp221*, p.Glu303Glyfs*7) could be considered to cause AT deficiency by PTC-mediated transcript destruction.

SERPINC1 is prone to structural variations because it contains a high proportion of intra/extragenic *Alu* repeat sequences [1,24]. In the literature, 24 cases involving large deletions of *SERPINC1* have been reported, and 8 of these cases were revealed to result from *Alu*-mediated non-allelic homologous recombination (NAHR) (Supplementary Table 3). The *Alu* element is classified as a short interspersed nuclear element (SINE) associated with a recurrent genetic copy number variation (CNV). In this study, we identified 2 novel patterns of *Alu*-mediated intragenic *SERPINC1* large deletion: exon 5 deletion via recombination between *Alu* 4 in intron 4 and *Alu* 7 in intron 5 (c.881 + (409_436)_1273-(741_714)del, p.(Gly255Valfs*23): Case 18) (Fig. 1), and a deletion spanning exons 3–5 via recombination between *Alu* 2 in intron 2 and *Alu* 5 in intron 5 (c.408 + (523_534) _1153 + (437_448)del, p.(Val137Valfs*23): Case 19) (Fig. 2). These *Alu*-mediated interstitial deletions could be configured via crossover recombination between repeated DNA sequences at non-allelic positions to yield inter-homologues, inter-sister chromatids, or intra-chromatids [25]. Interestingly, the 13-nucleotide (nt) sequence CCNCCNTNNCCNC was identified as an allelic recombination hotspot motif in > 40% cases involving NAHR [26]. This 13-nt hotspot motif is known to be present in *Alu* family elements, particularly *Alu*Y. Of the present cases of *Alu*-mediated NAHR, Case 18 possessed a sequence resembling CCTCCCA AAGTGC in the breakpoint region between *Alu* 4 and *Alu* 7 (Fig. 1C).

We further identified a non *Alu*-mediated genomic rearrangement lacking a sequence of approximately 35 kb, which spanned from *SERPINC1* exon 7 to the majority of *ZBTB37* (NC_000001.11: g.173870507_173905610delinsTGGAGTA, Case 20) (Fig. 3). This complex genomic mutation could be explained by FoSTeS/microhomology-mediated break-induced replication (MMBIR) [27]. Regarding the genomic rearrangement mechanism underlying Case 20, the FoSTeS model shown in Fig. 3D could be considered. Here, the replication fork is stalled and temporarily paused at the DNA lesion *SERPINC1* intron 6 (NC_000001.11: g.173905610) (Fig. 3D-i). The lagging strand then disengages to invade the adjacent replication fork at *SERPINC1* intron 6 and anneals via microhomology 1 (NC_000001.11: g.173904565, shown as black circle), followed by synthesis of the lagging strand at the invading fork (Fig. 3D-ii). The drifting lagging strand again disengages at the position of NC_000001.11: g.173904556, invades the other active replication fork at *ZBTB37* exon 3, and reinitiates strand synthesis from the microhomology 2 priming site (NC_000001.11: g.173870507, shown as gray circle) (Fig. 3D-iii). FoSTeS/MMBIR, a DNA-replication based genomic rearrangement mechanism, has been proposed to account for non-recurrent CNVs that cause genomic disorders (Supplementary Table 4). FoSTeS/MMBIR-based rearrangement likely occurs in regions of genomic instability resulting from unique architectural features such as non-B-DNA structures [28–30]. We previously reported another case in which a total *SERPINC1* deletion was attributed to FoSTeS (Case 21) [21]. These previous and present observations led us to hypothesize that the local genetic sequence of *SERPINC1* and its flanking region may possess an erroneous motif enabling the formation of non-B-DNA structures. Further studies are needed to investigate the potential involvement of FoSTeS/MMBIR in the total deletion of *SERPINC1*.

Splicing mutation is a type of genetic defect that can cause a null phenotype of an inherited disorder, such as AT deficiency. To date, 24 *SERPINC1* splicing mutations have been reported (Supplementary Table 5). In this study, we identified a novel splicing mutation involving a G duplication at the splice donor site of intron 3 (c.624 + 1dupG, Case 16: Fig. 5). An in vitro exontrap analysis revealed that the mutation c.624 + 1dupG caused a frameshift from 209Gly that caused a stop codon at nucleotide position 17 (p.Glu209Glyfs*17) (Fig. 5C), suggesting that transcripts expressed from the mutant allele, like those from a nonsense mutation, could be degraded by NMD. We also identified a reported mutation involving a G-to-A substitution in the flanking region of the splice acceptor site in intron 5 (c.1154-14G > 4: Case 13) (Fig. 4). The mutation, c.1154-14G > A, is a common splicing mutation in *SERPINC1* [19,31–34]. The G-to-A substitution at position −14 from the first nucleotide of *SERPINC1* exon 6 was predicted to create a new potential (cryptic) splice acceptor site resulting in 4 additional amino acids (Val, Phe, Leu and Pro), with no later reading

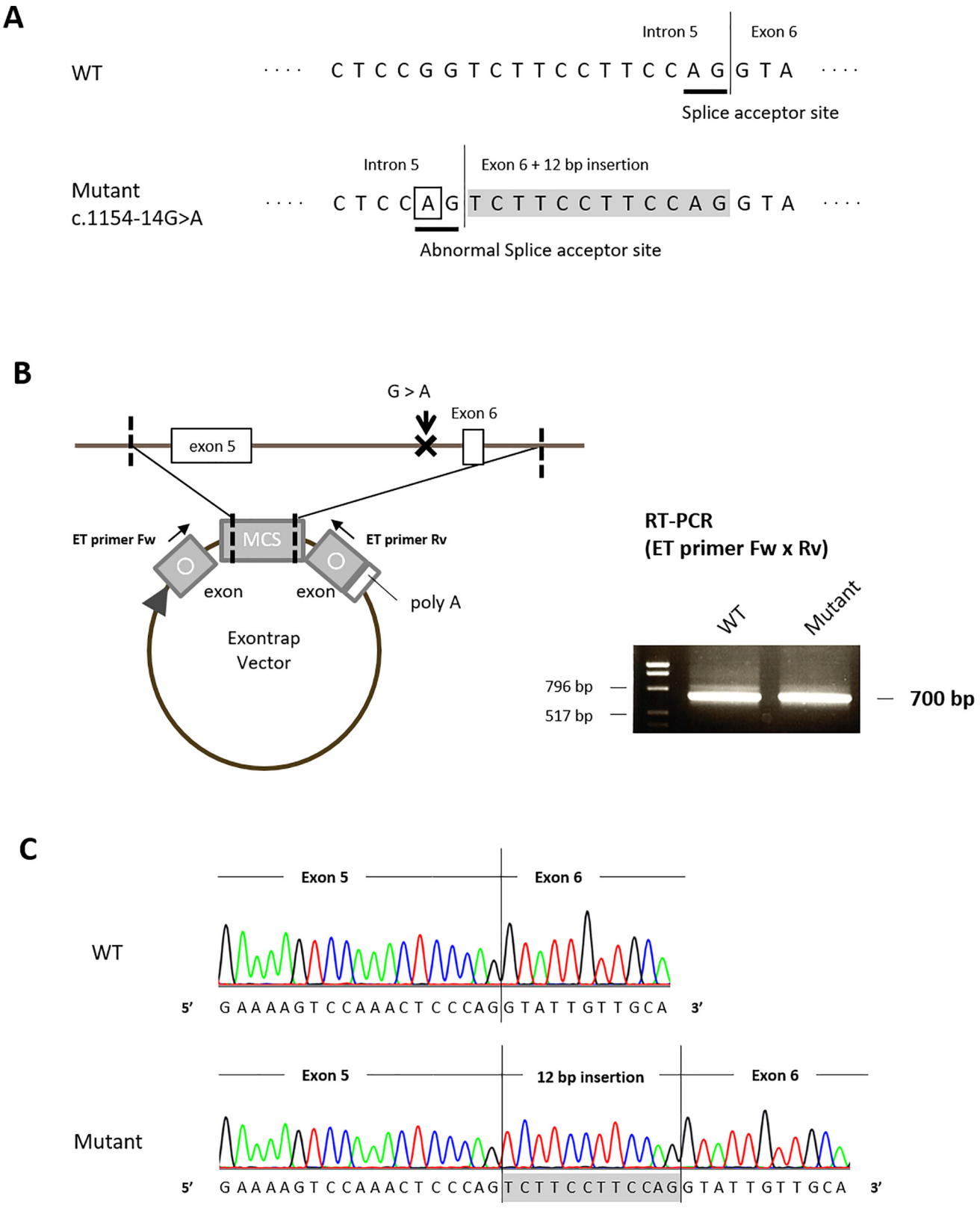
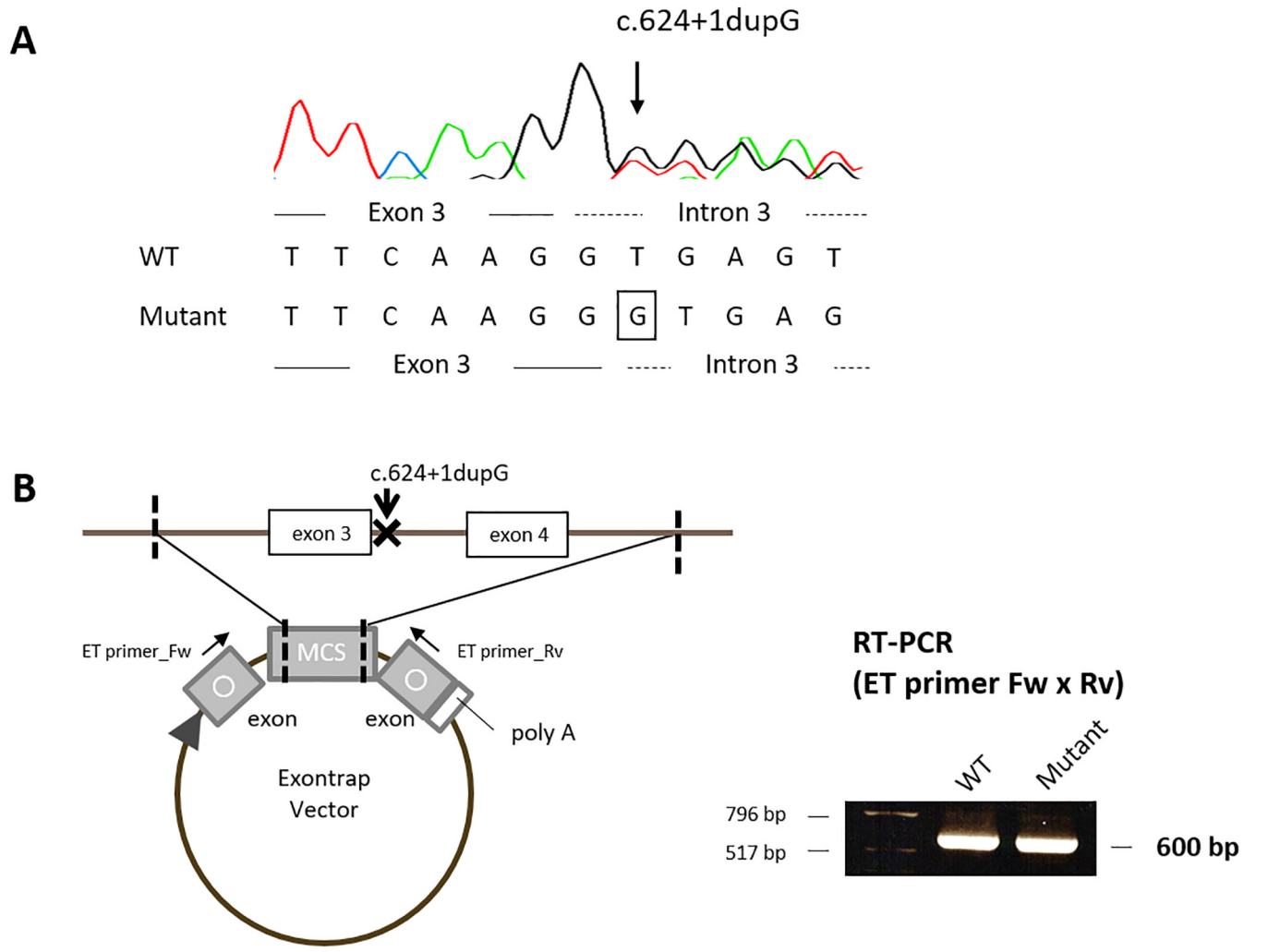


Fig. 4. Case 13: *SERPINC1* c.1154-14G > A p.Pro384_Gly385insValPheLeuPro. A) DNA alignment of the intron 5 splice acceptor site. The mutation caused a substitution of guanine to adenine at position −14 from the first nucleotide of exon 6 (c.1154-14G > A), which created a cryptic splice acceptor site. The resulting abnormal mRNA encoded 4 extra amino acids, Val, Phe, Leu, and Pro, but did not cause a later reading frame alteration. B) A cell-based transcription analysis using an exontrap vector. Left panel: Scheme of the exontrap analysis. A PCR amplicon spanning *SERPINC1* introns 4–6 was inserted into the multi-cloning site (MCS) of an exontrap vector. Total RNAs derived from COS-7 cells transfected with exontrap vectors containing the WT or mutant amplicon were subjected to RT-PCR. Right panel: RT-PCR results. Products approximately 700 bp in size were clearly detected in both COS-7 transfectants. C) RT-PCR direct sequencing of the WT- (upper) and mutant-transfectants (lower) products. The abnormal transcripts produced from the exontrap vector containing the mutant allele included a 12-bp insertion (TCTTCCTTCCAG, highlighted) into the *SERPINC1* exon 5/6 junction, consistent with previous reports.



(caption on next page)

Fig. 5. Case 16: *SERPINC1* c.624 + 1dupG, p.Glu209Glyfs*17. A) Results of DNA sequencing of the *SERPINC1* intron 3 splice donor site. B) Left panel: A scheme of the exontrap analysis. A PCR amplicon spanning *SERPINC1* introns 2–4 was inserted into the multi-cloning site (MCS) of an exontrap vector. Total RNAs derived from COS-7 cells transfected with exontrap vectors containing the WT or mutant amplicon were subjected to RT-PCR. Right panel: RT-PCR results. Products approximately 600 bp in size were clearly detected in both the COS-7 WT- and mutant-transfectants. C) RT-PCR direct sequencing of the WT- (upper) or mutant-transfectant (lower) products. The abnormal transcripts produced from the exontrap vector containing the mutant allele featured a G insertion at the splice junction between exons 3 and 4. This insertion led to a frameshift mutation wherein the first affected AA, glutamic acid-209 (GAA), was changed to glycine (GGA) and a new reading frame was terminated at position 17 (p.Glu209Glyfs*17).

frame alteration (p.Pro384_Gly385insValPheLeuPro). In this study, we performed a cell-based exontrap assay to examine the transcript alteration at c.1154-14G > A and observed a 12-bp insertion (TCTTCCT TCCAG) in the abnormal transcript (Fig. 4C), consistent with previous prediction. The variant protein with extra 4 amino acids, p.Pro384_Gly385insValPheLeuPro, had been considered to type I deficiency by decreasing its stability due to the tertiary structure aberration [19]. However, a recent report demonstrated that p.Pro384_Gly385insValPheLeuPro formed disulfide dimers in plasma and was categorized as type II deficiency [35]. Although plasma AT antigen of p.Pro384_Gly385insValPheLeuPro was apparently decreased (e.g., AT Ag: 58% in Case 13 of this study), its categorization have seemed to be controvertible.

Several cohort studies of the mutation prevalence among cases of AT deficiency have reported large deletion rates of 5.1–9.4% [15,36]. The present data suggest that large deletion is a relatively frequent event (19.0%), in contrast to previous reports. Although the first report of *SERPINC1* large deletion in a Japanese population was published by Sekiya et al. in 2011 [37], the prevalence of this genetic abnormality among Japanese patients with AT deficiency remains unknown. Castaldo et al. identified geographical differences in *SERPINC1* mutational profiles [38], and therefore the large deletions of *SERPINC1* resulting from recurrent or non-recurrent genomic rearrangements may be more frequent among Japanese patients with AT deficiency than previously reported. However, we are aware of the limited population scale of the present study and suggest that further investigation is needed to clarify the epidemiology of the genetic mutation(s) responsible for AT deficiency in the Japanese population.

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Author contribution

S. T. and E. H. designed and performed the research, analyzed data and drafted the manuscript. N. S. designed the project and collected and analyzed the clinical data. M. K., K. O., Y. H., M. T., S. S., A. S., performed the research and analyzed data. A. T. developed and supervised the project. A. K., F. H., S. O., T. Kanematsu., T. M., developed the project and collected and analyzed the clinical data. T. Kojima designed the project, analyzed data and wrote the manuscript.

Disclosure of conflict of interest

The authors state that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.04.004.

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