First report of inherited protein S deficiency caused by paternal *PROS1* mosaicism

Inherited protein S (PS) deficiency is a thrombophilic disorder with an autosomal dominant mode of inheritance.[1] In plasma, 60% of PS exists in a complex with C4b binding protein and the remaining 40% in free form; only free PS functions as a cofactor for the activated protein C. Inherited PS deficiency is classified into types I, II, and III according to the level of PS activity or free and total PS antigen.[2] Types I and III have different phenotypes but they are considered to be caused by the same genetic abnormality. According to the Human Gene Mutation Database Pro (*https://my. qiagendigitalinsights. com/bbp/view/hgmd/pro/all/php*), 453 variants localized in the protein S gene (*PROS1* gene) have been reported worldwide as of January 2021. Among these, missense/nonsense variants account for 61% (277 variants). However, genetic variants have been reported as undetectable in approximately half of the families with PS deficiency.[3] Based on the Suita study, which examined the prevalence of PS deficiency in the Japanese general population, the prevalence is 1.12%;[4] about 5-10 times higher than that in Europe and America (0.16–0.21%).[5] PS deficiency is the most common inherited thrombophilia in Japan.[6,7] In this study, we identified a novel *PROS1* variant and paternal mosaicism in a family with inherited PS deficiency in which both parents are asymptomatic. This is the first report of inherited thrombophilia due to parental mosaicism and highlights the importance of including information about potential mosaicism in genetic counseling.

The proband was a 34-year-old Japanese woman who was hospitalized with histiocytic necrotizing lymphadenitis. On admission to the hospital, total PS antigen (NSauto total protein S; NASCA Co., Ltd., Yokohama, Japan), free PS antigen (NSauto free protein S; NASCA Co., Ltd.), and PS activity levels (HemosIL PS-clot; I.L.Japan Co.,Ltd., Tokyo, Japan) were 55%, 25%, and <10%, respectively (Figure 1A). In the proband, acute inflammation caused by histiocytic necrotizing lymphadenitis may have increased vascular permeability, resulting in a decrease in free PS and a significant decrease in PS activity. The D-dimer concentration was significantly increased to 25.3 μg/mL (reference range, <1 μg/mL), but contrast-enhanced computed tomography showed no obvious thrombosis. In addition, the proband's sister had already been diagnosed with PS deficiency after developing deep vein thrombosis in her leg at 25 years old and is on warfarin therapy (Figure 1A). Based on the above, the proband was diagnosed with type I PS deficiency. Both parents have normal levels of PS activity and antigen (Figure 1A), and the father had suffered a stroke at 62 years old and was taking edoxaban but the mother had no history of thrombosis.

This study was approved by the Ethics Committee for Human Genome and Gene Analysis Research at Kanazawa University (approval no. 450-5) and by the National Cerebral and Cardiovascular Center (approval no. M14-026). After obtaining informed consent from the proband and her family members, genomic DNA was

A

	Total PS antigen (%) (RR: 65–135)	Free PS antigen (%) (RR: 60–150)	PS activity (%) (RR: 56–126)	Type of PS deficiency
Father	87	85	91	NA
Mother	97	86	75	NA
Proband	55	25	< 10	I
Sister	22*	14*	< 10*	I

* warfarin therapy

B

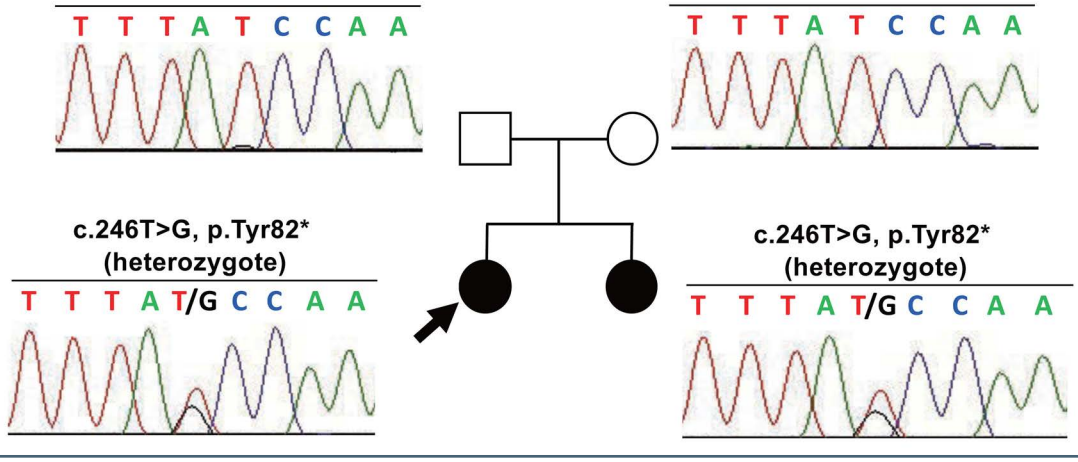

Figure 1. Protein S antigen and activity levels of a protein S-deficient family and the results of Sanger sequencing. (A) Levels of total protein S (PS) antigen, free PS antigen, and PS activity in PS-deficient family members. The type of PS deficiency of the proband and sister were shown. (B) Pedigree and sequencing results. Arrow indicates the proband, and filled symbols indicate affected patients. The proband and her sister were heterozygous for c.246T>G (p.Tyr82*) whereas the parents seemed to have no causative variants. Data from the father showed a very small peak of G (black) with a normal peak of T (red). Abbreviations: RR: reference range; NA: not applicable; *, warfarin therapy.

A

Forward Primer	TTGCATCTTTAATATAAAAACACATTATAA AATTAAGT
Reverse Primer	AGGTGGAGAGTTAGACAGGAACATATC
TaqMan MGB Probe (FAM)	ATTATTTTTAgCCAAAATAC
TaqMan MGB Probe (VIC)	GATTATTTTTAtCCAAAATAC

B

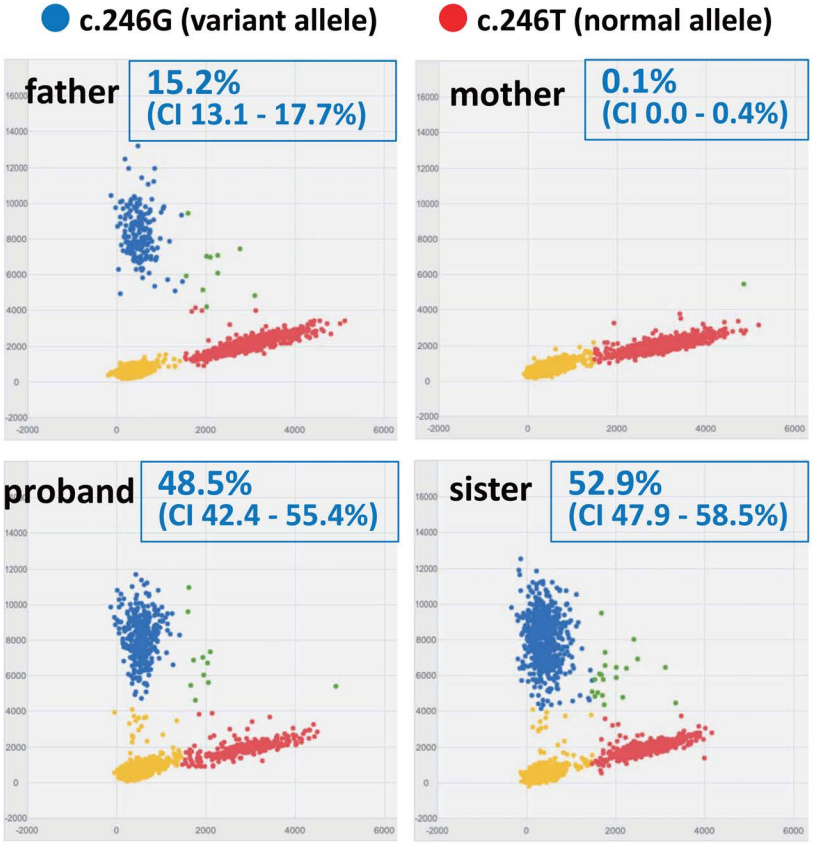

Figure 2. Quantification of the variant allele ratio of blood-derived DNA using digital PCR. (A) Sequences of the primers and probes. (B) Blue indicates FAM fluorescence for the target variant allele; red indicates VIC fluorescence for the normal allele; green indicates FAM+VIC; and yellow indicates no amplification.

extracted from peripheral blood leukocytes using a GENERATION Capture Column kit (QIAGEN, Tokyo, Japan). All exons and the exon-intron boundaries of *PROS1* were polymerase chain reaction (PCR)-amplified using GoTaq G2 Hot Start Master Mixes (Promega, Tokyo, Japan). PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Tokyo, Japan) and an Applied Biosystems 3500xL Genetic Analyzer (Thermo Fisher Scientific). Sanger sequencing indicated a novel heterozygous nonsense variant in exon 3 (NM_000313.4: c.246T>G; p.Tyr82*) in the proband and her sister. However, neither parent showed this variant by Sanger sequencing (Figure 1B). Since the probability of the same *de novo* variant occurring in the proband and her sister was considered virtually zero, this case was attributed to another cause such as the parents' genetic mosaicism. We therefore attempted to quantify the variant allele ratio. First, digital PCR (dPCR) was performed using QuantStudio 3D Digital PCR Master Mix v2 (Thermo Fisher Scientific) and the QuantStudio 3D Digital PCR system (Thermo Fisher Scientific) (Figure 2). The variant allele ratio was 48.5% for the proband, 52.9% for the sister, 15.2% for the father, and 0.1% for the mother (Figure 2B).

Second, we quantified the variant allele ratios using pyrosequencing with PyroMark Q24 (QIAGEN) to confirm the reproducibility of paternal mosaicism using a different principle of measurement (Figure 3). The results of pyrosequencing indicated that the variant allele ratios were 50% for the proband, 49% for the sister, 15% for the father, and 1% for the mother (Figure 3B). The variant allele ratios were consistent between dPCR and pyrosequencing for all family members, suggesting the presence of somatic mosaicism in the father. Based on this result, a careful review of the Sanger sequencing data of the father revealed the presence of a very small G peak with a normal T peak (Figure 1B).

Third, we evaluated the ratio of variant alleles in saliva-derived DNA to confirm the reproducibility of the somatic mosaicism presented in the blood-derived DNA of the father. The saliva-derived DNA was extracted and purified using Oragene Purifier (Kyodo International). The results of pyrosequencing showed that the variant allele ratios of the saliva-derived DNA were 50% for the proband, 50% for the sister, 17% for the father, and 2% for the mother (Figure 3C), confirming the reproducibility

of the father's mosaicism. Generally, the detection limits of dPCR and pyrosequencing are said to be more sensitive than that of Sanger sequencing: Sanger sequencing 15-20%;[8,9] pyrosequencing 5%;[8,10] dPCR 0.1-1%.[11,12] We performed pyrosequencing of five control subjects and an average variant allele ratio was 1% (*data not shown*). In addition, the detection limit of our pyrosequencing assay system calculated at 3δ was 2% (*data not shown*). However, we could not examine the control subjects with our dPCR assay system. Based on the above, the variant allele ratios detected in the mother, 0.1% for dPCR and 1-2% for pyrosequencing, were below the detection limits of their respective assay system and were, therefore, considered to be background. In this case, the paternal mosaicism was observed in the blood cells (mesoderm origin) and salivary gland cells (ectoderm origin). This may suggest that the genetic variant might have occurred early in the fetal development process. However, the variant allele ratio of the two types of DNA collected in this study happened to be about 15%-17%, but the variant allele ratios in other organs have not yet been verified; this makes it difficult to determine when the variant occurred from the variant allele ratios. It is also assumed that the father's germ cells

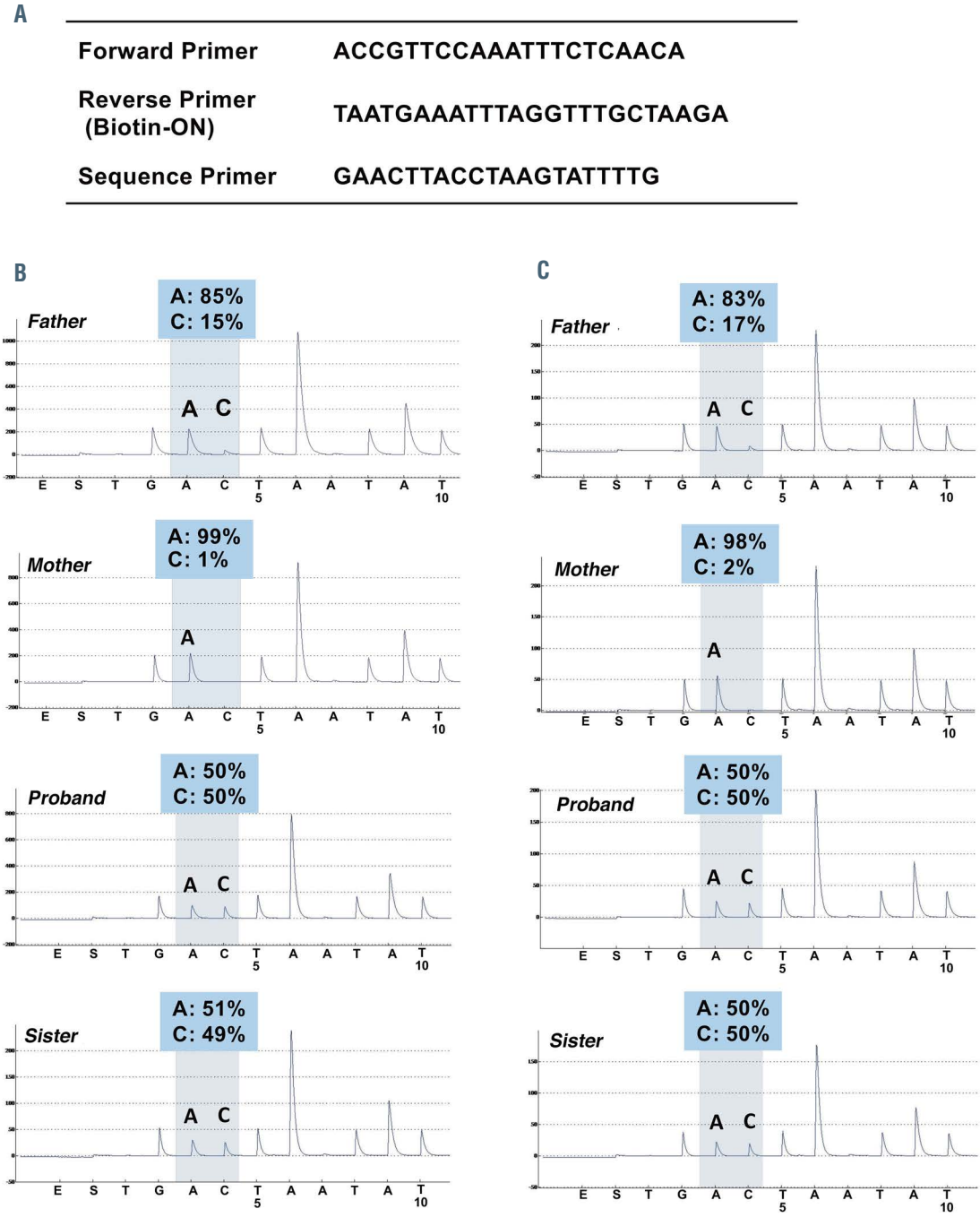

Figure 3. Quantification of variant allele ratios in blood- and saliva-derived DNA using pyrosequencing. (A) Sequences of the primers. The results of variant allele ratios quantified by pyrosequencing of blood-derived DNA (B) and saliva-derived DNA (C) from the proband and family members. The gray area shows the allele ratio of A and C at the variant locus. Since pyrosequencing was performed using reverse primers, the allele ratio of C represents the variant allele ratio.

carry the variant allele, but the semen could not be collected and analyzed due to lack of consent. For a definitive diagnosis of gonadal mosaicism, it is necessary to prove the presence of mosaicism in the father's semen, but in this case, since the two children are heterozygous for the same variant, it is probable that the father has somatogonadal mosaicism.[13]

In recent years, some cases of various diseases that were originally considered to be caused by *de novo* variants have actually been attributed to parental somatic or gonadal mosaicism.[13-15] Since the sensitivity of variation detection in Sanger sequencing, considered the standard for evaluating *PROS1* variants, is limited to approximately 15-20%,[8,9] heterozygous variants can be detected but mosaicisms are likely to be undetectable. Thus, another more sensitive method, such as dPCR or pyrosequencing, is needed for the genetic evaluation of parental mosaicism. In this study, we detected a mosaic variant in the father of two heterozygous sisters with PS deficiency. This is the first case of parental mosaicism in an inherited PS-deficient family, suggesting that parental mosaicism may exist among some patients who have been thought to have *de novo* variants of inherited thrombophilia. It has been reported that approximately 20% of sporadic cases of hemophilia A, as determined by traditional Sanger sequencing methods, have an asymptomatic mosaic mother.[15] The percentage of parental mosaicism present in sporadic cases of inherited PS deficiency needs to be determined by accumulating more cases in the future.

In conclusion, in cases of autosomal-dominant inherited thrombophilia, when the causative variant is detected only in the proband and a *de novo* variant is suspected, it is necessary to investigate the presence of parental mosaicism. For this purpose, it is very informative to quantify the variant allele ratio using multiple readily accessible specimens, such as blood, saliva, hair follicles, and urine to confirm the parental mosaicism. Furthermore, semen analysis should be performed as genetic screening for germline mosaicism in fathers of patients with *de novo* variants, and this analysis could be useful for prenatal counseling. In sporadic cases of inherited diseases, even if neither parent has the same variant using traditional sequencing methods, confirming the mosaicism of the parents is very important regarding the recurrence risk for the patient's siblings and is essential information for genetic counseling.

Satomi Nagaya,[1,] Keiko Maruyama,[2,*] Atsushi Watanabe,[3] Makiko Meguro-Horike,[4] Yuta Imai,[1] Yuki Hiroshima,[5] Shin-Ichi Horike,[4] Koichi Kokame[2] and Eriko Morishita[1,6]*

[1]Department of Clinical Laboratory Science, Division of Health Sciences, Graduate School of Medical Science, Kanazawa University, Kanazawa, Ishikawa; [2]Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Suita, Osaka; [3]Division of Clinical Genetics, Kanazawa University Hospital, Kanazawa, Ishikawa; [4]Advanced Science Research Center, Kanazawa University, Kanazawa, Ishikawa; [5]Department of Hematology, Nagano Red Cross Hospital, Nagano, Nagano and [6]Department of Hematology, Kanazawa University Hospital, Kanazawa, Ishikawa, Japan

**SN and KM contributed equally as co-first authors.*

Correspondence:

ERIKO MORISHITA - eriko86@staff.kanazawa-u.ac.jp

doi:10.3324/haematol.2021.278527

Received: June 23, 2021.

Accepted: October 1, 2021.

Pre-published: October 14, 2021.

Disclosures: no conflicts of interest to disclose.

Contributions: SN: investigation, visualization and writing, original draft; KM: investigation, visualization and writing, original draft; SN and KM are co-first authors. AW: methodology and supervision; MH: investigation; YI: investigation; YH: resources; SH: methodology, investigation, formal analysis; KK: investigation, formal analysis, funding acquisition, supervision; EM: conceptualization, funding acquisition, supervision, writing-review and editing.

Funding: this work was partially supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan to EM (grant number 20FC0201) and KK (grant number 20FC1024).

References

1. Dahlbäck B. The protein C anticoagulant system: inherited defects as basis for venous thrombosis. Thromb Res. 1995;77(1):1-43.
2. Mannucci PM, Valsecchi C, Krachmalnicoff A, Faioni EM, Tripodi A. Familial dysfunction of protein S. Thromb Haemost. 1989;62(2):763-766.
3. Johansson AM, Hillarp A, Säll T, et al. Large deletions of the PROS1 gene in a large fraction of mutation-negative patients with protein S deficiency. Thromb Haemost. 2005;94(5):951-957.
4. Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T. Prevalence of protein S deficiency in the Japanese general population: the Suita Study. J Thromb Haemost. 2004;2(6):1012-1013.
5. Beauchamp NJ, Dykes AC, Parikh N, Campbell Tait R, Daly ME. The prevalence of, and molecular defects underlying, inherited protein S deficiency in the general population. Br J Haematol. 2004; 125(5):647-654.
6. Miyata T, Kimura R, Kokubo Y, Sakata T. Genetic risk factors for deep vein thrombosis among Japanese: importance of protein S K196E mutation. Int J Hematol. 2006;83(3):217-223.
7. Kinoshita S, Iida H, Inoue S, et al. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. Clin Biochem. 2005; 38(10):908-915.
8. Harrington CT, Lin EI, Olson MT, Eshleman JR. Fundamentals of pyrosequencing. Arch Pathol Lab Med. 2013;137(9):1296-1303.
9. Cheng LY, Haydu LE, Song P, et al. High sensitivity sanger sequencing detection of BRAF mutations in metastatic melanoma FFPE tissue specimens. Sci Rep. 2021;11(1):9043.
10. Tsiatis AC, Norris-Kirby A, Rich RG, et al. Comparison of Sanger sequencing, pyrosequencing, and melting curve analysis for the detection of KRAS mutations: diagnostic and clinical implications. J Mol Diagn. 2010;12(4):425-432.
11. Carvalho TM, Dourado RM, Nakatani SM, et al. Digital PCR detection of EGFR somatic mutations in non-small-cell lung cancer formalin fixed paraffin embedded samples. Mol Cell Probes. 2021;58:101745.
12. Oshiro C, Kagara N, Naoi Y, et al. PIK3CA mutations in serum DNA are predictive of recurrence in primary breast cancer patients. Breast Cancer Res Treat. 2015;150(2):299-307.
13. Yamamoto K, Kubota T, Takeyari S, et al. Parental somatogonadal COL2A1 mosaicism contributes to intrafamilial recurrence in a family with type 2 collagenopathy. Am J Med Genet A. 2020;182(3):454-460.
14. Chesneau B, Plancke A, Rolland G, et al. Parental mosaicism in Marfan and Ehlers-Danlos syndromes and related disorders. Eur J Hum Genet. 2021;29(5):771-779.
15. Lannoy N, Hermans C. Genetic mosaicism in haemophilia: A practical review to help evaluate the risk of transmitting the disease. Haemophilia. 2020;26(3):375-383.