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A Coincidental Discovery of a New Stable Variant (Hb Hachioji or HBB: c.187C>T) in a Patient with Chronic Hemolytic Anemia of Unexplained Origin

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ABSTRACT
We report a new hemoglobin (Hb) variant, Hb Hachioji (HBB: c.187C>T), which was detected in a 32-year-old male with hemolytic anemia. The proband had undergone splenectomy in his childhood after being diagnosed with hereditary spherocytosis (HS) with no clinical improvement. A recent study showed that Heinz bodies were frequently observed in his red cells, however, no abnormal band was separated by isoelectric focusing (IEF), and the isopropanol (instability) test was negative. Direct sequencing revealed that the proband was a heterozygous carrier of a novel mutation (GCT>GTT) at codon 62 of the β-globin gene, leading to an alanine to valine substitution. This variant was named Hb Hachioji. Characterization at the mRNA level by cDNA sequencing detected βHachioji mRNA, as well as β0 mRNA. Subsequently, study of the proband’s family indicated that his father was a carrier of this Hb variant, although unexpectedly, the father was asymptomatic and clinically healthy. Oxygen affinity measurement of total Hb showed no alteration in the P50 and oxygen equilibrium curve. The presence of Hb Hachioji was confirmed by mass spectrometry (MS). Hb Hachioji comprised approximately 50.0% of the total Hb and was a stable variant. The phenotypic discrepancy between these two carriers suggested that Hb Hachioji may not be associated with the hemolytic involvement in the proband.

Introduction
Frequently, abnormal hemoglobin (Hb) arises from a point mutation that substitutes another amino acid residue for the original amino acid, which may affect the structure and function of the Hb molecule [1]. Sixty-nine percent of abnormal Hb in Japanese people exhibits a normal phenotype and no clinical manifestation [2], and many are found on Hb A1c measurement by high performance liquid chromatography (HPLC) by inappropriately reduced or occasionally increased Hb A1c levels [1,3–6]. Thus, clinically silent Hb variants may remain undetected. Even if this variant is suspected on the Hb A1c measurement, it may escape correct diagnosis when the diagnosis is based only on screening tests, such as isoelectric focusing (IEF), and an isopropanol test [7]. Therefore, DNA analysis is mandatory. Our proband had hemolytic anemia that was first thought to be caused by a highly unstable Hb variant because several Heinz bodies, or extremely denatured Hb, were detected, but no abnormal Hb was separated. However, DNA analysis disclosed the presence of Hb Hachioji. The Hb variant was named Hb Hachioji after the place where the proband resided. Subsequently, a follow-up family study demonstrated that the proband’s father was an asymptomatic carrier of the same variant. Thus, the hypothesis that Hb Hachioji was a highly unstable variant was abandoned. Without the family study, the initial consideration for the Hb Hachioji variant could lead to misdiagnosis and even inappropriate treatment [8]. This scenario points to shortcoming of characterization of a new abnormal Hb.

Here we report a new stable Hb variant, Hb Hachioji [β62(E6)Ala→Val, HBB: c.187C>T], which was coincidentally detected in a 32-year-old male proband with chronic hemolytic anemia and in his father who, however, was asymptomatic and healthy. In spite of investigation into the cause of the hemolytic involvement of the proband, it remained undetermined.

Materials and methods
The proband, a 32-year-old Japanese man, has been suffering from chronic hemolytic anemia since childhood. His attending doctor suspected hereditary spherocytosis (HS), which is the most common cause of congenital hemolytic anemia in Japan. The proband had subsequently undergone
splenectomy when he was 3 years old with no clinical improvement. His laboratory data were Hb 8.6 g/dL (reference 13.5‒17.5 g/dL), packed cell volume (PCV) 0.24 L/L (0.39‒0.52), reticulocyte count 8.1% (0.5‒1.5), total bilirubin 2.4 mg/dL (0.3‒1.2) and direct bilirubin 0.7 mg/dL (0.0‒0.4). The proband’s lactate dehydrogenase (LD) activity was increased by 448.0 IU/L (115.0–217.0), and his haptoglobin level was low (Table 1). The peripheral smears of the proband showed moderate anisocytosis and marked poikilocytosis, or macrocytosis with many target cells and spherocytes [Figure 1(a)]. In addition, a number of Heinz bodies were observed by supravital staining [Figure 1(b)]. Thus, the presence of hemolytic anemia was considered. The analysis of the proband’s parents revealed that they were clinically healthy. The parents’ laboratory data are also presented in Table 1. Ethical approval, including informed consent, was obtained.

Blood was taken by venipuncture with EDTA as an anti-coagulant. The blood was subjected to routine hematological analysis and measurement of half glycerol lysis time GLT50 [9,10]. Hb F was quantitated by cation exchange HPLC for HbA1c (HPLC 723 G8; Tosoh, Tokyo, Japan). Hb A2 was measured by cellulose acetate membrane electrophoresis [11]. Hemoglobin instability was determined by the isopropanol test [12]. Hemoglobin was separated in by IEF [13]. Hb H inclusion and Heinz bodies in red cells were evaluated by supravital staining with brilliant cresyl blue and brilliant green/nude red, respectively.

DNA was extracted from peripheral blood by conventional phenol-chloroform extraction. The β-globin gene was amplified by polymerase chain reaction (PCR) using the forward primer (5’-AGT AGC AAT TGT TAC TGA TGG TAT GG-3’) and reverse primer (5’-TCC CCC AAG GTT TGA ACT AGC TCT T-3’). Subsequently, the 1.8 kb PCR product was subjected to agarose gel electrophoresis, and the pertinent band was excised and purified using a QIAquick gel extraction kit (Qiagen, Tokyo, Japan). The PCR product was subjected to automatic capillary sequencing [Applied Biosystems 3100 genetic analyzerTM (Avant Genetics, Tokyo, Japan)]. To determine the presence of mRNA coding for this novel variant, cDNA sequencing analysis was performed. Total RNA was extracted from 100 μL of packed red cells using Trizol (Invitrogen, Tokyo, Japan). The RNA was subjected to reverse transcription PCR (PrimeScriptTM RT-PCR Reagent Kit; TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer’s directions. The cDNA was amplified by using a set of primers (forward: 5’-ACA TTG GGT TCT GAC ACA ACT GTG TT-3’, reverse: 5’-TTG AGG TTG TCC AGG TGA GCC AG-3’) that amplified exons 1 and 2 of the β-globin coding region. The product (270 bp) was processed and subjected to sequencing analysis. Furthermore, in order to investigate the presence of glucose-6-phosphate dehydrogenase (G6PD) deficiency and P4.2 abnormalities (P4.2Nippon), the promoter area, 5’ untranslated region (5’UTR), 13 exons, and 3’UTR of the G6PD gene and exon 3 of the P4.2 gene were studied by PCR and DNA sequencing using the primers described by Adhiyanto et al. [10].

Functional properties were studied using the Hemox AnalyzerTM (TCS Scientific Corporation, New Hope, PA, USA). Unfortunately, functional analysis of the proband’s father was unfeasible. The proband’s erythrocytes were washed with saline and centrifuged at 1600 g for 5 min., which was repeated three times. The packed red cells were lysed by adding two volumes of cold distilled water and stored at 4 °C overnight. The stroma was removed as far as possible by centrifugation at 13,000 g for 20 min. The oxygen equilibrium curve (OEC) for whole blood was measured at pH 7.4 at 37 °C using Hemox buffer (TCS Scientific Corporation), while the Bohr effect was measured for the hemolysate using 0.05 M Bis-Tris buffer (containing 0.1 M NaCl), pH 7.2 and pH 7.4, respectively. Desalting as a pretreatment was followed by mass spectrometry (MS) measurement by the double layer method. The hemolysate was diluted with 0.1% of trifluoroacetic acid (TFA), and 10 μL (final concentration 0.167 g/dL) was bound to Millipore ZipTip C18 (Merck KgaA, Darmstadt, Germany) and eluted by 5 μL of a mixture of 0.1% TFA and 75.0% acetonitrile. The matrix thin-layer was prepared on a target ground steel TF (Bruker Daltonics, Bremen, Germany). The desalted sample was diluted five-fold with a matrix solution [sinapinic acid, formula weight (FW) = 224.22] in TA30 (saturated solution, final concentration, 0.067 g/dL). One microliter of the sample (0.67 μg) was applied to the thin-layer, dried, and subjected to the MS measurement using Ultraflextreme® tandem time of flight (TOF; Bruker-Daltonics, Billerica, MA, USA). Spectra were acquired in a linear positive ion mode and at a scan range of 840–20137 Da.

Results

As summarized in Table 1, the proband’s Hb A2 (3.1%, reference 2.0‒3.5%) was normal in quantity, and its electrophoretic profile was also normal. An elevated Hb F level (4.4%, reference <1.0%) was noted. Instability by the isopropanol precipitation test was normal. However, the proband’s

| Parameters | Proband (M-32) | Father (M-50s) | Mother (F-50s) | Reference range |
|------------|----------------|----------------|----------------|----------------|----------------|
| RBC (10^12/L) | 1.98 | 4.61 | 3.80 | 4.30–5.70 |
| Hb (g/dL) | 8.6 | 14.7 | 12.3 | 11.0–14.0 |
| MCV (fl) | 121.7 | 96.5 | 95.0 | 82.7–101.6 |
| MCH (pg) | 43.4 | 31.9 | 32.4 | 28.0–34.6 |
| MCHC (g/dL) | 35.7 | 33.0 | 34.1 | 31.6–36.6 |
| Total bilirubin (mg/dL) | 2.4 | 0.3 | 0.6 | 0.2–1.2 |
| Direct bilirubin (mg/dL) | 0.7 | 0.0 | 0.0 | <0.4 |
| LD (IU/L) | 488.0 | 501.0 | 313.0 | 120.0–230.0 |
| Reticulocytes (%) | 8.1 | 1.5 | 1.2 | 0.2–2.7 |
| Haptoglobin (mg/dL) | 14.7 | ND | ND | 19.0–170.0 |
| Hb F (%) | 4.4 | 0.9 | 0.5 | <1.0 |
| Hb A2 (%) | 3.1 | 4.2 | 3.0 | 2.5–3.5 |
| Isopropanol test | [-] | [-] | [-] | [-] |
| Heinz bodies | [-] | [-] | [-] | [-] |
| GLT50 (seconds) | 157 | 34 | 33 | 22–55 |
| IEF | [-] | [-] | [-] | [-] |
| C>T polymorphism | C>T | C>T | C>T | majority: C/C |

RBC: red blood cell count; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular Hb; MCHC: mean corpuscular Hb concentration; LD: lactate dehydrogenase; GLT50: glycerol lysis time; IEF: isoelectric focusing.
GLT<sub>50</sub> was extended by 157 seconds (reference value: 35–55). The IEF showed no abnormal band (Figure 2).

The sequencing analysis demonstrated a GCT＞GTT mutation at codon 62 of the β-globin gene that codes for alanine to valine [Figure 3(a)]. Common α-thalassemia (α-thal) mutations, including the most common −α<sup>3.7</sup> (rightward) deletion, were absent. The presence of the β<sub>Hachioji</sub> mRNA, as well as β<sub>A</sub> mRNA, was confirmed by cDNA sequencing [Figure 3(b)]. The peaks for β<sup>Hachioji</sup> (T) and β<sup>A</sup> (C) mRNAs were almost the same height, and it was surmised that a considerable amount of the β<sup>Hachioji</sup> mRNA was present. Thus, a sufficient amount of the transcript of the mutant allele was present, which was sufficient to result in β<sup>Hachioji</sup> production.

The P<sub>50</sub>, Hill’s coefficient (n), and the Bohr effect were 27.6 Torr (normal control: 28.7), 2.8 (2.6), and -0.5 (-0.5), respectively. These results showed that the function of the Hb as a whole hemolysate was normal and implies that Hb Hachioji results in normal function or that it is no longer present in the hemolysate.

Two major peaks were observed for the proband with masses at 15868.70 ± 1.2 Da and 15896.17 ± 1.1 Da, corresponding to normal β and abnormal β globins, respectively (Figure 4). The molecular weight of the abnormal β-globin was higher than normal β-globin by 27.46 Da, almost conforming to the difference by the replacement from alanine to valine. Thus, substitution at codon 62 (C＞T) or β<sup>Hachioji</sup> was validated. The same result was obtained for the proband’s father. As expected, his mother did not share the β<sup>Hachioji</sup>. The intensity ratio of the abnormal/normal β-globin peak for the proband and his father were 0.99 and 0.91, respectively. Thus, β<sup>Hachioji</sup> comprised nearly half of their total β-globin, which also suggests that Hb Hachioji is a stable variant.

Discussion

Our study identified a new abnormal variant, Hb Hachioji, in a Japanese proband and his father. On initial analysis of the proband, it was surmised that Hb Hachioji was the cause of his hemolytic anemia. The proband had macrocytic anemia, probably due to reticulocytosis (Table 1). The proband’s elevated Hb F was probably related to the presence of hematopoietic stress by the hemolysis, and the proband had a polymorphism at -158 of the G<sub>c</sub> gene (C＞T), where a T polymorphism raises the Hb F level, resulting in hematopoietic stress [14,15]. Hb Hachioji was not separated on IEF. In addition, his instability (isopropanol) test was negative, while GLT<sub>50</sub>, which is prolonged by oxidative stress in thalassemia and unstable hemoglobinopathies, was prolonged [10]. At first, it was thought that Hb Hachioji presented as a highly unstable variant, which in erythrocytes prematurely denatured and precipitated, resulting in Heinz body formation with no Hb Hachioji remaining. Thus, it was thought that the proband’s Heinz body injuries to the erythrocyte membrane due to oxidative stress prolonged the GLT<sub>50</sub>. Fortunately, we had the opportunity to conduct a family study, and the proband’s father was also a carrier of Hb Hachioji but was asymptomatic without hemolytic anemia (Table 1). This unexpected finding changed our initial assumption.

To the best of our knowledge, the HbVar database (http://globin.cse.psu.edu/) presents two Hb variants that have a point mutation at β62(E6): Hb Duarte (Ala→Pro) and Hb J-Europa (Ala→Asp) [16]. Hb Duarte is an unstable variant probably due to the break of the α helix by the substituted proline and has increased oxygen affinity [17]. In contrast, Hb J-Europa is a stable variant and has a normal
Figure 3. Sequencing result of exon 2 of the β-globin gene of genomic DNA and cDNA. (a) A mutation of GCT (alanine) to GTG (valine) at codon 62(E6) in a sense strand is detected in the proband’s DNA. The result was confirmed by an antisense strand sequencing (not presented). (b) The proband is heterozygous for the GTG, or Hb Hachioji. The peak levels of C and T at codon 62 are almost the same. In addition, both peaks are lower than other single C or T peaks immediately after G, suggesting a half dosage each. Thus, although, the cDNA sequencing is not quantitative, a substantial amount of mRNA coding for Hb Hachioji [GTG (valine)], seems to be present.

Figure 4. Profile of MS. Analysis of the hemolysate demonstrates an abnormal β chain with a mass difference from the normal counterpart of 27.46 Da and 27.67 Da for the proband and his father, respectively (theoretical value 28.06 Da). The peaks of the βH and βHachioji are shown by the arrows. The 28 Da difference conforms to the mass change determined by DNA sequencing, or Ala→Val. The intensity of βHachioji and βH was comparable (the ratio 0.99 and 0.91 for proband and father, respectively) and approximately equal in amount, which also suggests that βHachioji is stable. Thus, Hb Hachioji comprises approximately 50.0% of the total Hb.
oxygen affinity, as does Hb Hachioji [18]. Hb Hachioji is the third variant at this position. The substitution of valine for alanine at β62(E6) is located at the x1β2 contact and is next to the distal histidine β63(E7) in the heme binding pocket. Thus, the amino acid replacement at the E6 helix was at first expected to render instability to the variant Hb molecule [3]. However, since alanine and valine are neutral amino acids with a similar structure, it seems unlikely that the replacement of alanine with valine would profoundly affect the conformation of the Hb molecule and deteriorate its stability to produce a clinically symptomatic phenotype. This lack of difference is also suggested by the normal oxygen affinity and \( P_{50} \) in whole blood.

The presence of Hb Hachioji as a protein was later confirmed by MS. The identified mass, which differs by 28 Da from the normal \( \beta \) chain, suggested that \( \beta^{[\text{Hachioji}]} \) was present in the blood, and its coding mRNA seemed to be sufficiently present. The results of MS suggested that Hb Hachioji is an electrophoretically stable variant that overlaps with Hb A on IEF, and it may have caused no Hb A\(_{1c}\) reduction on cation exchange HPLC. In addition, the OEC study showed normal function. If Hb Hachioji was carried only by the proband’s father, it is unlikely that it would have been discovered. If the analysis had been carried out only in the proband without a family study, Hb Hachioji would have been misdiagnosed as a highly unstable variant. Therefore, this report highlights the importance of a family study in order to correctly define a new Hb variant.

The discrepancy of the phenotype between the proband and his father is an interesting issue. If the hemolytic anemia of the proband is not associated with Hb Hachioji, there must be another cause for his hemolytic involvement. Because the proband had already undergone a splenectomy, microspherocytes were no longer seen in the proband’s blood film [Figure 1(b)]. Therefore, the possibility of coexisting HS was genetically examined. The underlying cause of many Japanese HS cases is homozygous P4.2Nipp [19], and the presence of the P4.2Nippon in the proband’s parents was examined. However, the P4.2Nippon mutation was not detected. Since his splenectomy did not alleviate his hemolytic anemia, it is unlikely that his hemolytic anemia was caused by HS.

Heinz bodies are the final product of denatured Hb and lead to hemolytic involvement. Thus, the hemolytic anemia of our proband may be related to Heinz bodies. Since Heinz bodies are removed from the blood in the spleen, it tends to appear after splenectomy. Our proband had a splenectomy at 3 years of age after a diagnosis of HS. However, Heinz bodies, even after splenectomy, rarely appear in the blood without denaturation of Hb. The cause may be related to something that oxidizes and denatures normal Hb. Because the proband is male, we further suspected that he carried an X-linked genetic disorder that was passed to him from his mother. Hemolysis and Heinz bodies may also be seen in G6PD deficiency on exposure to an oxidant [1]. Although G6PD deficiency is relatively rare in the Japanese population (1/1000) [19], a number of cases have been reported [20,21]. We sequenced all of the exons of the G6PD gene in this patient and found no abnormalities. Therefore, a G6PD gene abnormality seems to be unlikely as the cause of hemolytic involvement in our proband. Another enzyme deficiency of erythrocytes, such as pyruvate kinase deficiency, which occurs second in frequency after G6PD in the Japanese population and causes hemolytic anemia but does not give rise to Heinz bodies; therefore, we did not test for this condition. The phenotype may become worse with coexisting red cell membrane abnormalities, as is seen in Hb Gunma [B127(H5)-β128(H6)Gln-Ala→0 and inserted Pro; codons 127/128 (→AGG)]; CAG GCT(Gln Ala)→C→→CT(Pro) (β\( \beta^\prime \)); HBB: c.383_385delAGG] with congenital elliptocytosis [22]. However, elliptocytosis was not present in our patient. A P4.2Nippon mutation that is asymptomatic in heterozygotes but symptomatic with \( \beta \)-thal minor was absent, as stated above [23].

The limitation of this study is the origin of the hemolytic syndrome that was observed in the proband and not in his father, who carries the same abnormal Hb, and that remains to be determined. To solve the question of the hemolytic anemia in the proband, a genome-wide association study was considered. This approach may provide a valuable approach to elucidate other mutations relevant to this hemolytic anemia and may be a powerful diagnostic tool [24,25]. However, due to sample limitations, we were unable to further investigate these possibilities. In conclusion, our study detected a new stable Hb in the Japanese population, Hb Hachioji, which was detected in two related subjects but have different phenotypes. It is interesting to note that Hb Hachioji may not have caused the hemolytic anemia in the proband. His symptoms are likely to be due to other factors that play a role in exacerbating clinical phenotypes. Moreover, our findings highlight the importance of conducting a comprehensive family study.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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