A Study of the Characterization New Hemoglobin Variant: Hb Hachioji (β62 E6 c.187C>T, alanine-valine), Coincidentally Found in a Japanese Subject with Hemolytic Anemia of Unknown Origin

（原因不明の日本人溶血性貧血患者において偶然発見された新規の異常ヘモグロビン：Hb Hachioji（β62 E6 c.187C→T、アラニン→バリン）の特性解析）

By

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Dedication

To my beloved Grandmother

My beloved family

and

My beloved husband, Chafid N. Arachman

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CHAPTER I
INTRODUCTION

Frequently abnormal hemoglobin (Hb) arises from a point mutation in the globin gene that substitutes another amino acid residue for the original amino acid and this may affect the structure and function of the Hb molecule **[1].** Since hemoglobinopathy is inherited as Mendelian codominant trait **[2]**, an abnormal Hb may cause clinical manifestations. The severity of the phenotype or symptoms of abnormal Hbs varies in the degree. It should be noted that most of clinically important Hb variants have an amino acid substitution that is important in function and stability of the Hb molecule itself, depending on the substituted amino acid and location where it has occurred **[1]**. For example, residues that reside in subunits contact, heme pocket or 2-3 DPG binding sites may affect oxygen affinity. In the other hand, a carrier of a clinically silent variant may have less effect on the Hb molecule and exhibits normal phenotype. Clinically important phenotypes by the variants are; the most common sickle trait, Hb M or cyanosis, erythremia by the higher oxygen affinity variant and thalassemia by super unstable variant. However, many abnormal Hbs are silent or reveal no abnormal phenotype. Silent variant sometimes escapes from detection since it is asymptomatic.

Sixty-nine percent of abnormal Hb in Japanese people exhibits normal phenotype and no clinical manifestation **[3]**. Many of them are found on HbA1c measurement by high-performance liquid chromatography (HPLC) by inappropriately reduced or occasionally increased HbA1c level **[1, 4-6]**. Thus, clinically silent Hb variants may remain unnoticed. Even if it is suspected on the HbA1c measurement, it may escape correct diagnosis when the diagnosis is based only on screening tests such as isoelectric focusing and the isopropanol test **[7]**. Therefore, performing a confirmatory test by DNA analysis is mandatory. Our proband had hemolytic anemia that was first considered to be caused by 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 the heterozygous mutation at codon 62 of the *β-globin* gene, GCT to GTT, replaced alanine for valine. 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 could lead to misdiagnosis and even inappropriate treatment **[8]**. This scenario points to a shortcoming of characterization of a new abnormal Hb.

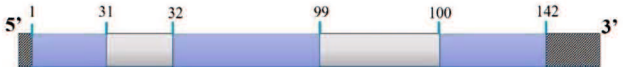
In this study, we report a new stable Hb variant, Hb Hachioji (β62 [E6] c.187 C>T, Ala-Val) which was coincidentally detected in a 32-year-old male proband with chronic hemolytic anemia and in his father who, however, was asymptomatic and healthy. Despite the investigation into the cause of the hemolytic involvement of the proband, it remained undetermined. We designed a study that includes the initial investigation and further testing **[9]**. The initial investigation includes the complete blood count and quantification of HbA_2 and HbF. Further testing is more specialized tests that include Hb electrophoresis by isoeletric focusing (IEF), heat and isopropanol stability test, test for identifying Hbs with altered O_2 affinity and confirmatory test by DNA analysis and reverse phase HPLC (RP-HPLC) and/or mass spectrometry.

CHAPTER II
LITERATURE REVIEW

1. Normal Structure and Genetic Basic of Human Hemoglobin

Human Hb is a heterotetramer protein and functions to deliver oxygen from the lung throughout out the body. It fills up to 90% of total volume of the red blood cell (RBC). Hb composed of four polypeptides, two alpha globin chains, designated α, and two non-alpha globin chains; beta, delta or gamma, designated γ, δ, β, respectively. Each globin subunit binds heme molecule, and the presence of iron makes the red color of Hb. The non-alpha globin subunits will pair with their homolog as a homodimer, then to work functionally, it will pair with the other type of homodimer, the α globin, therefore creates an assemble of heterotetramer molecule.

Studies in genetic aspect of globin gene revealed that *α-globin* gene is duplicated, designated as *α2* and *α1* (HBA1 and HBA2) and *β-globin* (HBB) gene, were evolved from the same ancestor **[10]**. The revolutionary process made them separated apart yet they still share some basic aspects; both *α-globin* and *β*-globin gene (Fig. 1) consist of three coding region (exon) and two intervening regions (intron). The *α* and *β globin* genes are located on the short arm of the chromosome 11 and 16, respectively. Interestingly, human *α-globin* gene is duplicated into two designated α2 and α1. These two genes have completely same amino acids sequence but have slight differences in the nucleotide sequence in their introns **[11]**.



α-globin gene

β-globin gene

Figure 1. The *α* and *β-globin* gene.
Both *α* and *β-globin* gene consists of 3 exons and two intervening sequences (IVS). The coding sequence is relatively same size, resulting the length of amino acids are 142 and 147 for the α and β-globin, respectively.

Over the lifespan, human Hb underwent switching process from embryonic Hb to adult Hb. Hb switching is one of the underlying mechanism that each organism has to deal to achieve the requirement of oxygen supply during their lifespan. Human has two Hb switching steps; the first is from embryonic to fetal, and secondly is from fetal to adult globin. As illustrated in

Figure 2, Hb switching is not only about switching embryonic to adult globin, but it is also switching the place of production Hb itself, from yolk sack to liver, and to bone marrow [12]. During embryonic and fetal development, Hb Gower I ($\zeta_2\varepsilon_2$), Hb Portland I ($\zeta 2\gamma 2$) and Hb Portland II ($\zeta_{2\beta 2}$) is the primary Hbs that are found in the embryonic stage. Then it is gradually switched to HbF ($\alpha_2\gamma_2$) until birth. The HbF remains high until baby turns one year and HbA ($\alpha_2\beta_2$) start raising and permanently produced. HbA is continuously dominating total Hbs in the red blood cells until adolescence. While HbF remains produced but at the low proportion. Besides that, there is a minor adult Hb, designated HbA_2 ($\alpha_2\delta_2$) which the presence is significantly elevated in β-thalassemia; therefore, HbA_2 is a useful marker for the presence of β-thalassemia. These three primary Hbs, HbA, HbA_2 and HbF comprise of total Hb in human's adult red blood cell, 96%, 2-3% and 1%, respectively. Besides those three Hbs, such as glycated HbA or known as HbA1c and other post-translational modification Hbs are also found in the red blood cells in a low amount **[13]**.

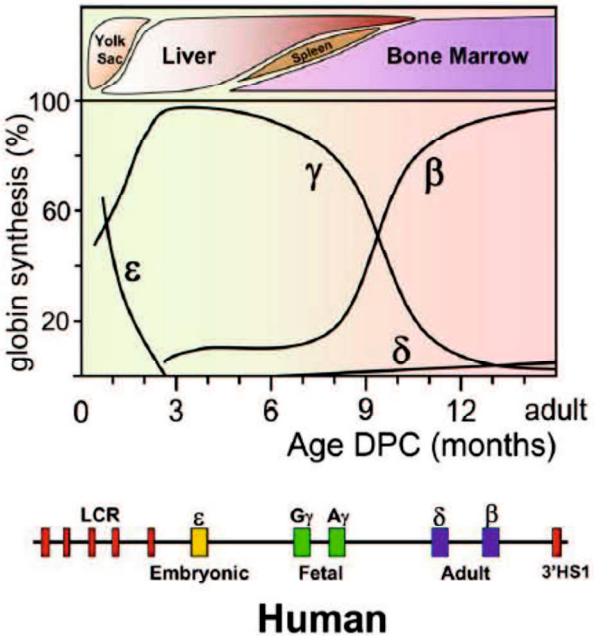


Figure 2. Schematic mechanism of human Hb switching

This illustration shows the switching from embryonic to adult Hb. Human embryonic globin to the adult hemoglobin takes place in various place depend on the developmental stage. The switching of each hemoglobin is different during the life span. The bottom illustration is the arrangement of gene order in the *β-globin* gene cluster with the enhancer, locus control region (LCR) located in the upstream region (From Sankaran *et al.*).

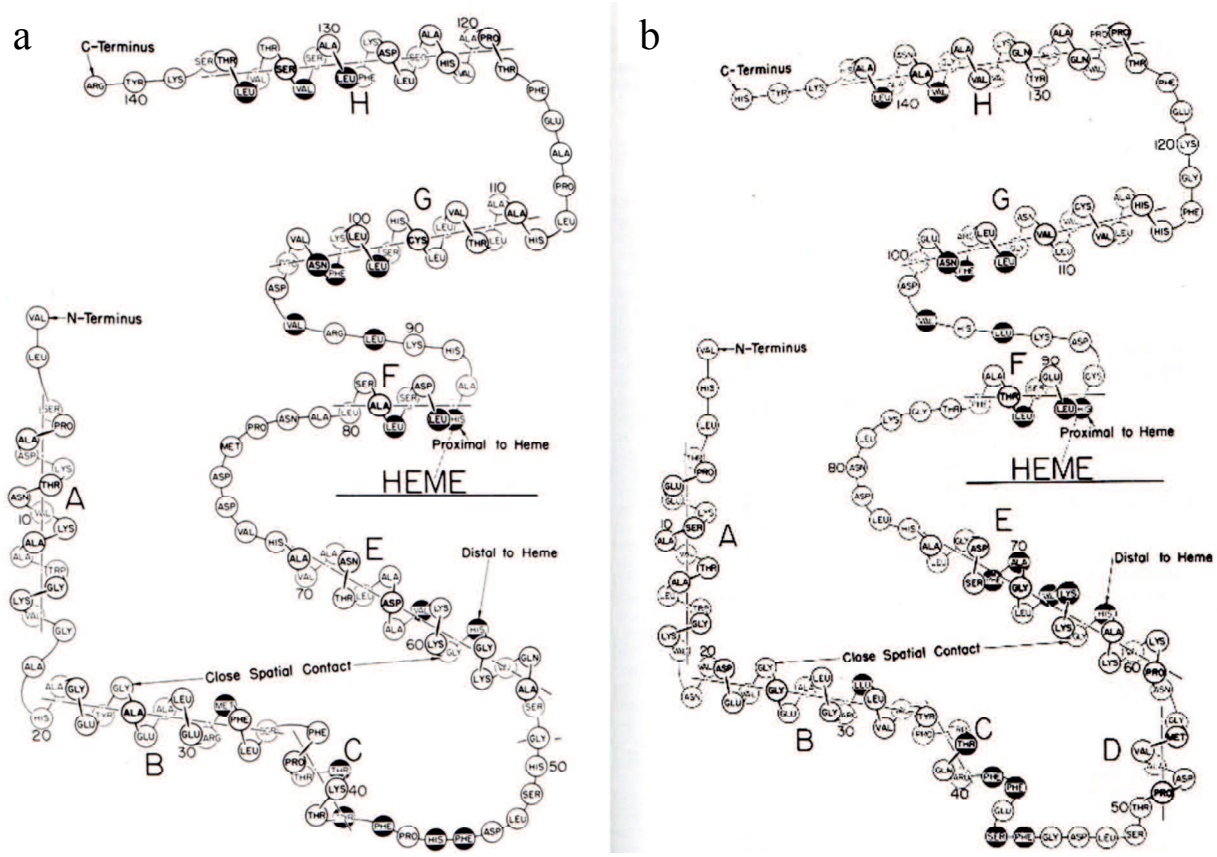


Figure 3. Primary structure of the α-globin (a) and β-globin (b)
The residues that constitute the α-globin and β-globin chain are illustrated in this figure. The α and β globin are 142 and 147 amino acids in length, respectively (From Huisman THJ and Schroder WA: New aspects of the structure, function, and synthesis of hemoglobins, Boca Raton, Florida, 1971, CRC Press).

Since 96% of total Hb composed of HbA, only the primary structure of α and β globin are illustrated in Figure 3. α and β globin are 142 and 147 amino acids in length, respectively. The assembly of amino acids in the polypeptide of globin monomer determine the fate of folding polypeptide. Most of the structure is helices shape, designated from A-H helices. However, the α globin lost the D helix. The heme pocket binding sites are located in E and F helices, distal and proximal sites, respectively. Regarding its function to deliver oxygen, an assemble heterotetramer molecule provide a remarkable machinery. As heterotetramer molecule (Fig. 4), four subunits work together to improve their maximum binding capacity to meet the oxygen requirement **[14]**.

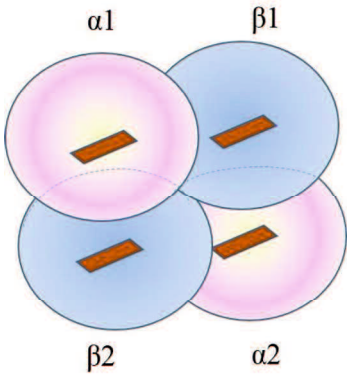


Figure 4. Schematic HbA tetramer

HbA consists of two α-globin chains and two β-globin chains. Heme molecules (brown color) at the specific residue at each subunit and give the distinctive red color. The α1β2 bonds and α1β2 bonds are located between the dimers while the α1β1 bonds are in the front and the α2β2 bonds are behind.

The contact of α1β1 is considered to promote the stability of the molecule, while the contact between α1β2 is also essential to maintain the cooperativity. Therefore, some of the abnormal Hbs that has a mutation in this site could profoundly alter the stability and affinity of the molecule [**13**]. Amino acids substitution, position, and location/contact, are crucial for the Hb molecule. Each globin subunits bind different subunits, called α1β1 and α1β2 contact. Each globin forms dimer bonding which has high affinity. In turn, globin monomer is unstable and tends to form intracellular precipitation that finally will harm red blood cells and cause hemolytic anemia. Therefore, the mutation that occurs in this position causes erythrocytes damage as a result of monomer accumulation. On the other side, α1β2 have lower affinity than α1β1 contact. This interaction plays a role in the process of tetramerization. Oxygen can lead to molecule instability from its "T" state (tense, which has low affinity and deoxygenated) to its "R" state (relaxed, which has high affinity and oxygenated). In this R state, oxygen is cooperatively bound by each subunit. This process mediates oxygen binding cooperativity which is described as the Hb-O_2 sigmoidal curve. Therefore, a mutation occurs at this location generally can alter the affinity of Hb molecule to oxygen **[15]**.

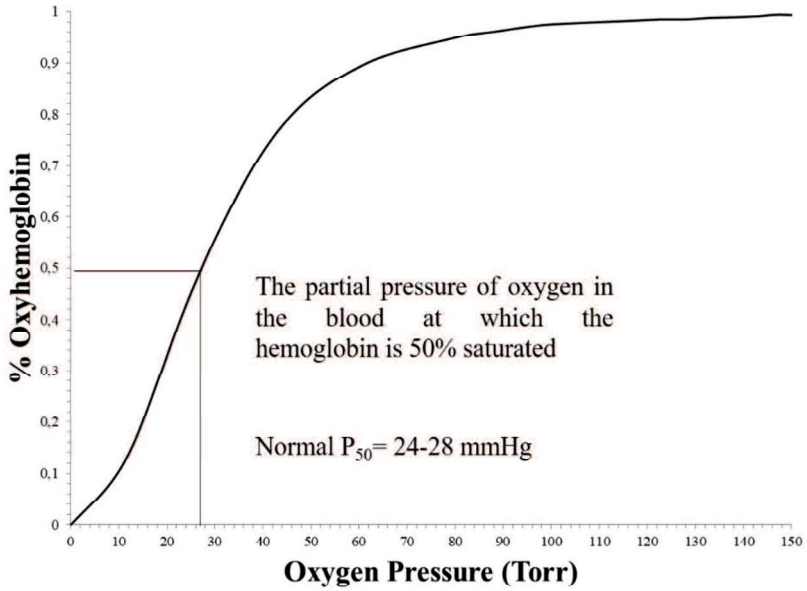


Figure 5. The Oxyhemoglobin curve.
P_{50} refers to the partial pressure of the oxygen in the blood at which hemoglobin is saturated and the Sigmoid shape of OxyHb represent the cooperativity binding of oxygen by the four subunits.

As illustrated in Figure 5, The oxygen equilibrium curve (OEC) of Hb is in sigmoid shape. This represents the four globin subunits have the cooperativity when binding the oxygen, therefore the curve drawn like a sigmoidal shape. As a comparison, myoglobin, another oxygen binding protein that has iron as well, its cooperativity is different with Hb, which has a hyperbolic shape. The Hb cooperativity binding that is in sigmoidal kinetic was studied by Hill. Hill's coefficient (n) represents the cooperativity of each subunit to bind oxygen. As we know, Hb consists of four subunits with heme attached to it. Therefore, four oxygen molecules are bound. However, n of Hb is 2.8. This value is derived from the Hill's equation, where S is the fraction of Hb that are filled (ranges from 0 to 1), PO_2 is the partial pressure of O_2 dissolved in the blood, and finally, P_{50} is the PO_2 at which S = 0.5.

The Bohr effect was first reported to influence the Hb's O_2 affinity. It was believed that is caused by the change in the amount of CO_2 was found to alter the Hb property by lowering the affinity for oxygen. However, in the more detailed study, it is revealed that this is primarily due to the reduction in pH. At the same time Hb bind O_2 and they reciprocally also unload the CO_2, and inversely happened in lung and tissue. This process is achieved because the reduction in pH triggers O_2 and CO_2 exchange [13]. In 1904, it was Christian Bohr that proved this theory. Tissue rich in CO_2, and its conversion into bicarbonate releases free H^+, which eventually will lower the pH in the tissue, this condition is reducing the affinity of Hb for oxygen. The term of

Bohr effect is to describe the heterotrophic influence by CO_2 and H^+ on the Hb property. In turn, inside the lung, low amount of CO_2 will raise the pH, then facilitate the Hb to favorably bind oxygen. At the physiological condition (pH 7.4), Bohr coefficient (d) of HbA is -0.6. Bohr coefficient reflects the number of protons per heme group that is bound to Hb when fully oxygenated. During the alkaline condition or high pH, d is negative. Therefore the H^+ is released during the oxygenation. Whereas d is positive at lower pH and H^+ are taken up. Thus, in any circumstances, releasing or binding H^+, changes in the pH may not alter the affinity of Hb to bind oxygen.

Table 1. The factors that affect the Hb affinity for oxygen

Factor	Right Shift (low affinity)	Left Shift (High affinity)
pH	↓	↑
CO_2	↑	↓
Temperature	↑	↓
2,3-DPG	↑	↓

Summarized in Table 1, temperature, pH, 2,3 DPG could alter the O_2 affinity that affects the oxygen affinity. Inside the red blood cells, there is 2,3 DPG; an abundant molecule resulted from the metabolism inside the red cells, that is also function as an allosteric molecule. In 1967, Reinhold and Ruth Benesch reported that 2,3 DPG decreased oxygen affinity to Hb. 2,3 DPG molecule bound to specific amino acids such as βHis2, βLys82, and βHis146. The bonding between 2,3 DPG to those amino acids interfere the relaxed state of Hb, therefore causing the lower affinity for oxygen **[15].**

2. Hemoglobinopathy: Genetic Basic, Clinical, and Pathophysiological Aspect

Hemoglobinopathy is a group of Hb disorder that mainly divided into two diseases, the thalassemia, the imbalance of globin's production, and the abnormal Hb or Hb variant, the condition where structurally abnormal Hb is produced. Most of Hb variants is due to the mutation in the coding region, which cause the substitution or deletion of amino acids, while thalassemia is not only has a mutation in the exon, but also a broader range, caused by the mutation in the promoter area, intervening sequence (IVS) and untranslated region (UTR). Naturally, abnormal Hbs are rarely found due to clinically phenotype. Of 1200 Hb variants reported, about ¼ are having clinical manifestation. A carrier of a clinically silent variant may have less effect on the Hb molecule and exhibit normal phenotype. Most of abnormal Hbs that is widely reported has been able to be separated on IEF because it has an electrophoretic position that differs from HbA, HbF, and HbA_2. Therefore, they might be interesting to be

studied further since it may have different characteristics than other significant bands, mainly if they also have clinical manifestation **[16]** such as Hb S, Hb C, Hb D and Hb E. However, since determination of glycated Hb is now widely assessed by using HPLC, many abnormal Hbs are reported to be electrophoretically silent by IEF, are coincidentally found during HbA1c measurement, because it interferes the HbA1c peak on the HPLC. Since they create a different pattern or even incorrectly change HbA1c level, researchers are interested to study more those variants with an abnormal pattern.

The severity of the phenotype or symptoms of abnormal Hbs with the clinical manifestation is vary in the degree, and most symptomatic Hb variants in the adult are caused by the mutation of HbA because HbA is the major Hb component. It is interesting to note that, most of clinically important Hb variants have an amino acid substitution that is important in function and stability of the Hb molecule itself, depending on the substituted amino acid and location where it has occurred **[1].** For example, residues that reside in subunits contact, heme pocket or 2,3 DPG binding sites may affect oxygen affinity. Especially, when the oxygen binding properties are disrupted, the binding curve can be 'left shift" or increased oxygen affinity, and "right shift" or decreased oxygen affinity. The latter condition might profoundly enhance erythropoiesis in response to the demand for more oxygen release in the tissue.

Besides, reduction of the amount of the abnormal Hb depending on its stability. As discussed earlier, a mutation that occurs in an important position to the stability of the molecular will lead to intracellular precipitation, which may be visualized as Heinz bodies that are globular aggregates and detectable by supravital staining in the blood smear **[17]**. Heinz bodies impair the red cell membrane and alter the cell deformability or permeability. As a result, the erythrocytes are removed earlier from the circulation mainly at the spleen, and their lifespan becomes shorter, giving rise to the premature destruction of the erythrocytes, so-called hemolytic anemia. A mutation that occurs in the heme pocket could disrupt the heme environment which can cause Hb variants with cyanosis condition. The order of peptide is arranged to stabilize and introduce the favorable condition that maintains the hydrophobic condition and protect iron (Fe^{2+}) from oxidation (Fe^{3+}).

3. Laboratory Testing for Hemoglobinopathy

Hemoglobin is the most widely studied protein. Further, together with thalassemia, abnormal Hbs are a global genetic disease that still requires particular care and awareness, especially when it comes as a co-existence with other mutation that exacerbate the condition. Therefore, in more frequent thalassemia cases countries, the study of Hb is always conducted as a part of the regional project. The screening of thalassemia is commonly done by combining

several laboratory screening tests because only single testing might not give an accurate result. As suggested by the International Committee for Standardization in Hematology, the study must consist of the initial investigations and further testing included IEF, heat and isopropanol stability tests, and nowadays, the genetic testing is also mandatory **[9]**. Among most used methods of hemoglobinopathy, Hb electrophoresis and HPLC are the two most commonly used test. These two methods can identify most known Hb variants. It is not surprising that asymptomatic Hb variants often found during the screening of thalassemia due to their unique electrophoretic mobility or their intervention of HPLC chart. Moreover, they sometimes inappropriately increased or decreased HbA1c measurement. In this last section, we briefly describe the tests that are usually used for the Hb study.

a. Hb Electrophoresis and Chromatography

Protein is negatively charged, therefore, when it is introduced on electrical gel such as acrylamide and cellulose acetate, protein will move towards the cathode. However, an addition of ampholyte or pH agent will cause protein move based on their net charge or its pI. This principle is widely used in Hb Electrophoresis by IEF **[18]**. In many laboratories, quantification of HbA, HbF, and HbA_2 are examined by this method. As patient's sample and control are being run on the gel, they are quantitatively compared. However, the result seems too objective because it is based on the human eye and is not computed automatically. Therefore, this method is less accurate but still powerful as Hb S, Hb D, Hb E, and some abnormal Hbs are migrated in different pI. A mutation that does not alter the charge may be silent and not detectable by electrophoresis, because it may not affect the net charge of the molecule, however interaction with the matrix can also affect the migration **[19]**.

b. Half Glycerol Lysis Time (GLT_{50}**)**

The measurement of red blood cells' lysis time is based on the exposure of glycerol solution. Determination of GLT_{50} is important to distinguish most of the thalassemia cases. Prolonged GLT_{50} in thalassemia and some abnormal Hbs are not completely understood. However, it is likely caused by free globin monomers that attached to the inner membrane of the RBC. Impaired the permeability of the membrane. By an unknown mechanism, it extended the lysis time of the cells. GLT_{50} is a measure of osmotic fragility based on the time necessary for hemolysis of erythrocytes in a mixture of glycerol and buffered saline solution **[20]**.

c. Stability Test

The stability test is more sensitive to Hb variants that have characteristics to cause hemolytic anemia. This test examines the resistance of Hb molecule to denature after chemical compound exposure such as isopropanol. The ordinary time for Hb to denature by isopropanol

is about 20-30 minutes **[21].** Unstable Hb variants tend to denature faster than stable one. However, the isopropanol test may give false negative-positive result when super unstable variants are present, and high level of HbF could alter the lysis time **[22].** In the super unstable variants, the protein has no longer remained in the hemolysate; it might give false negative result since nothing to denature as well. In the other side, HbF is found to be more resistance to alkaline than HbA. Therefore, when the HbF is higher than 3%, it could give false positive result due to its mild instability **[21]**. It is important to note that fresh whole blood is suitable for the isopropanol test. The whole old blood may develop methemoglobin formation. Since metHb could interfere the result. The other stability test is supravital staining of red blood cells, which is used to assess the presence of aggregated globin in the red blood cells, which is usually called inclusion bodies, such as HbH inclusion body and Heinz bodies. The latter can be detected by supravital staining using methylene blue or crystal violet. The presence of Heinz bodies may indicate the precipitation of unstable Hb.

d. Functional Study: Oxygen Equilibrium Curve (OEC), Hill's Plot, and Bohr Effect

In the cell, CO_2 is produced as a product of cell respiration, the accumulation of CO_2 enters the plasma and red cells. The presence of carbonic anhydrase catalyzes the reaction CO_2 and H_2O form carbonic acid. The ionization of carbonic acid is raising an H^+, therefore decreasing intracellular pH. In the physiologic condition, pH range is 6.6 to 7.6. Therefore, $\Delta \log P_{50} / \Delta \log pH$ is about -0.6 for normal human Hb. Each globin subunit binds to 1 mol of O_2, and four subunits of globin work as a team and this result in cooperativity binding. Hill's equation is used to measure the cooperativity binding of four subunits. Unlike the myoglobin which has a hyperbolic curve, oxy-Hb binding has a sigmoid shape because of each subunit cooperate, then enhance the oxygen binding. Besides that, they have allosteric effect from 2,3 DPG as a by-product of erythrocytes metabolism and pH changes which caused by the accumulation of CO_2 change the pH therefore eventually make Hb release oxygen and in turn, bind CO_2 and carry it to the lung.

Newly identified abnormal Hb are usually subjected to functional study. Because the mutation that occurs is unclear whether affect the oxygen affinity or no effect. Particularly, the Hb variants that have an amino acid substitution affect the affinity for oxygen and alter the Bohr effect coefficient or 2,3 DPG effect, is usually subjected to functional study. To determine the Bohr effect coefficient in a test tube, the hemolysate is placed in a buffer with a two different pH, for example, pH 7.2 and 7.4, then drawing the OEC curve. While the 2,3 DPG effect is also done in a similarly, in the addition of 2,3 DPG solution.

Hemox analyzer is the commercial apparatus for conducting the Hb functional study. The equipment itself is widely used in some laboratory. Fortunately, our institution owns this equipment, make it possible to conduct the study (Fig. 6). The principle of this equipment is based on the dual wavelength and the Clark electrode. The optical properties of Hb are measured by dual wavelength spectrophotometry, presented in X-axis, while the oxygen partial pressure is measured by a Clark electrode and presented on Y-axis. Then PC plots the resulting curve **[23]**. The Bohr effect and Hill's coefficient are more likely to represent the Hb affinity in any pH condition and to describe the cooperativity of each subunit, in our research, we included three data, such as $P_{50,}$ Bohr coefficient (d), and Hill's coefficient (n).

Figure 6. TCS Hemox Analyzer

The aparatus is designed to conduct a functional study of Hb. The automated calculation can draw the oxyHb or deoxyHb curve. The sample for the measurement can use the whole blood or hemoglobin solution.

e. Genetic analysis: PCR and Sequencing

PCR and DNA sequencing categorize as confirmatory test yet has remarkable ability to determine the status of Hb variant and thalassemia. Its ability to amplify a small amount of genetic information is widely used as a diagnostic tool. Most of the hemoglobinopathy in the recent day has been described by way of utilizing PCR followed by sequencing. Since most of the abnormal Hbs are silent and no clinical phenotype, only the structurally unstable or the clinically cause an abnormality in phenotypes such as shift oxygen affinity or characteristic like thalassemia, can often be easily detected. Protein can be readily observed by electrophoresis or chromatography, however, thalassemia detection is more reliable when using PCR based protocol such as Gap PCR in most common α thalassemia and β thalassemia. Recently, measurement of glycated Hb (HbA1c) in most hospitals, also contribute to the finding of abnormal Hbs that is accidentally found during the measurement.

f. Mass Spectrometry as an Additional Tool to Detect Electrophoretically Silent Hb variants

Isoeletcric focusing is the most used method for the routine analysis of Hb study. The first attempt of Hb study by using IEF is described by Basset *et al*. in 1978 **[18].** This technique emphasizes the isoelectric point (pI) of the Hb variant. The substitution charged amino acid will, therefore, could separate due to change in pI. Since then, IEF is widely used for Hb study and are still reliable as a tool to quantify Hb's component in hemolysate. It can distinguish the presence of the abnormal Hbs as well. However, the weakness of this technique is mostly unable to separate the neutral to neutral substitution amino acids. Hb variants that caused by a neutral to neutral amino acids substitution and moreover, the location where it occurs determine the structure of new variant. Most of them are electrophoretically silent when detected by using IEF. When IEF failed to identify those silent variants, more highly sensitive methods are required. In those case, mass spectrometry, especially MALDI MS or ESI, provide a more sensitive analytical tool for those Hb variants because of their ability to generate soft ionization of a large molecule such as proteins.

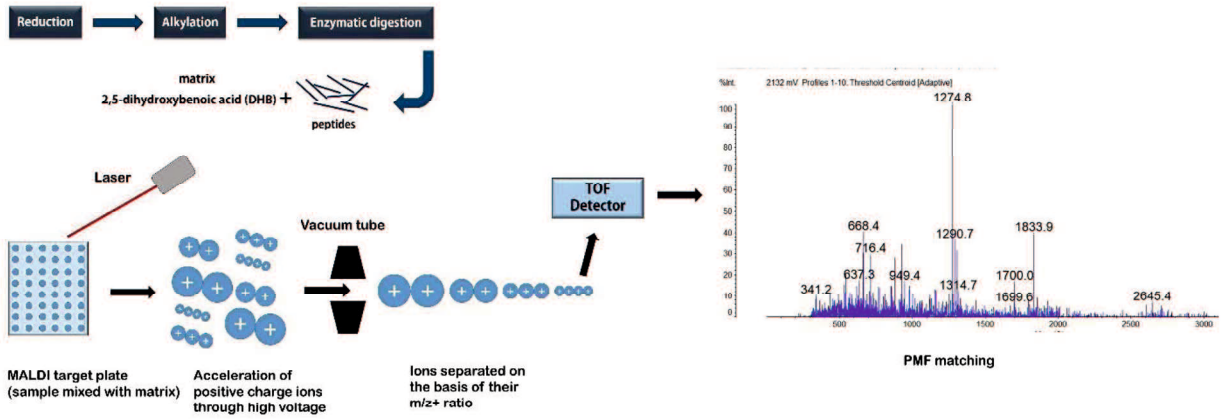


Figure 7. Workflow of MALDI-MS

In MALDI analysis, the sample was first subjected to reduction and alkylation step to minimize the interference of unnecessary component. The sample is digested by a proteolytic enzyme, such as trypsin. Through the high voltage, the sample coated with matrix solution is shot by the laser beam and ions are separated on their basis m/z ratio. The data acquisition is obtained from the spectra which are analyzed by peptide mass fingerprint (PMF).

Mass spectrometry is a method that relies on the principle of the ionization of chemical compounds into a charged molecule, where the ratio of their mass to charge is measured. In MS, two most common methods are ESI and MALDI. In both methods, the addition of one or more H^+, or known as positive mode, or by the loss of one or more H^+, known as negative mode, convert the peptides into ionized phase by the soft ionization process. However, the

interpretation of ESI MS is way more complicated than MALDI TOF MS. In ESI MS, sample mixture resources are better separated by chromatography to simplify the analysis. However, in MALDI, a mixture of sources can be interpreted merely because MALDI system is integrated with organism database that is provided by the manufacturer. For its rapid, sensitive and high throughput, MALDI is an obvious choice for proteomics research. However, MALDI MS is not without limitation. The limitation of the MALDI is not suitable to detect the species larger than 25 kDa. Moreover, the identification of new isolates, such as new protein would be possible if the spectral database contains peptide mass fingerprint (PMF) of the type of specific species. Commercial MALDI platform has built the organism database as a complement to their product. The database is continuously updated because the increasing of discovery new organism **[24]**.

As illustrated in Figure 7, the sample is prepared by mixing with matrix solution, an organic compound that acts as an energy absorbent. Numerous organic compounds are used as matrices such as the α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid). Matrix is mixed with an organic solvent containing ethanol/methanol or acetonitrile and a strong acid like trifluoroacetic acid (TFA) as matrix solution. The sample was placed first on the MALDI plate, and matrix solution was added. Therefore, the matrix solution coated and trapped the sample, similar to the process of co-crystallization **[25]**.

Since HbA is a tetramer molecule composed of α and β globin chains, the use of LC/MS might be a more sensitive way. Liquid chromatography is the first step where the tetramers were separated by their physical characteristics based on their hydrophobicity. The system is divided into two-phase, stationary phase and mobile phase. The former phase causes the molecule to remain in the column because of its hydrophobicity. Then at the later phase, the addition of organic solvents, such as methanol or acetonitrile (ACN) which is more hydrophobic than the molecule, will be preferable to bind to the column. Therefore, the molecule will be released at different retention time, hence separated from its subunit based on their hydrophobicity. After that, in the second step, they entered ionization phase in mass spectrometry.

4. Hemoglobinopathy in Japan

Hb is the most well-known protein. The amino acids sequence was well studied everywhere. Moreover, the Association of Hb researchers founded the Globin Gene Server. To date, there have been reported 1,307 variants of Hb at Globin Server Repository **[26]** (http://globin.bx.psu.edu/). Many abnormal Hbs are well reported, and some of them are

polymorphic due to the high occurrence in the particular region such as Hb S in Africa and Hb E in Southeast Asia **[27]**. Thalassemia was first described in Mediterranean and was found in the endemic of malaria area in a high prevalence. However, with a huge research and study in thalassemia and abnormal Hb all over the world, this could broaden the definition of the disease, since the diseases are not only found in that area, but also to the northern part area such as Japan. In Japan itself, global migration is considered to play a role in raising risk and number of thalassemia and abnormal Hbs in Japan, however, it is interesting to note that one of the large deletion types of β thalassemia is reported from Japanese subject **[28]**. Abnormal Hbs are found 1/3000 while α thalassemia and β thalassemia were 1/3000 and 1/1000, respectively **[3]**. Since thalassemia always shows clinical manifestation, it is likely to be more frequently reported rather than the abnormal Hbs. However, with the advanced of the technology, abnormal Hbs tend to be found unintentionally during HbA1c measurement.

Here in our study, new abnormal Hbs was initially identified in the person who has chronic hemolytic anemia. Our proband had hemolytic anemia that was first considered to be caused by highly unstable Hb variant because several Heinz bodies or extremely denatured Hb were detected and no abnormal Hb was separated. However, DNA analysis disclosed the presence of Hb Hachioji. The Hb variant was named as Hb Hachioji after the place of the residence of the proband. A follow-up family study later performed 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 could lead to misdiagnosis and even inappropriate treatment. This scenario points to a shortcoming of characterization of a new abnormal Hb.

5. Case Report

The proband, a 32-year-old Japanese male has been suffering from chronic hemolytic anemia since childhood. His attending doctor suspected hereditary spherocytosis (HS). The proband had subsequently undergone splenectomy when he was three years old with no clinical improvement. His laboratory data were summarized in Table 2. The peripheral smears of the proband showed moderate anisocytosis and marked poikilocytosis, or macrocytosis with many target cells and spherocytes (Fig 8a). In addition, some Heinz bodies by supravital staining were observed (Fig 8b). Thus, the presence of hemolytic anemia was considered. The analysis of the proband's parents revealed they were clinically healthy. Their laboratory data are presented in Table 2. Ethical approval including informed consent was obtained.

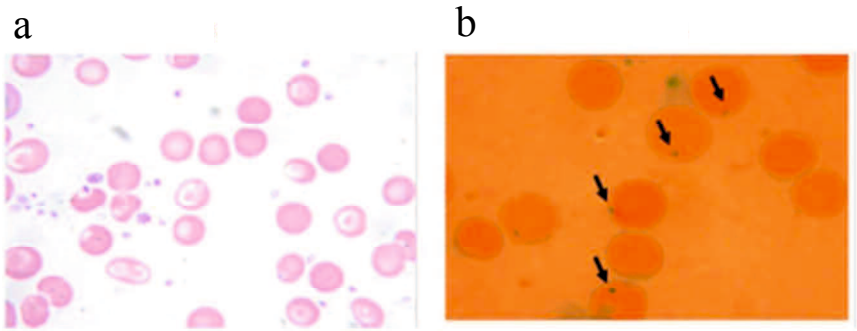


Figure 8. Peripheral blood smear
A number of target cells and spherocytes are seen in the proband's peripheral blood smear (a). In the Heinz body staining, relatively smaller Heinz bodies, it is likely due to the splenectomy, are observed in many red cells (b).

Table 2. Hematological and laboratory data from proband and his parents

Parameter	Reference range	Proband	Father	Mother
RBC (10^{12}/L)	4.30-5.70	1.98	4.61	3.80
Hb (g/dL)	11.0-14.0	8.6	14.7	12.3
MCV (fL)	82.7-101.6	121.7	96.5	95
MCH (pg)	28.0-34.6	43.4	31.9	32.4
MCHC (g/dL)	31.6-36.6	35.7	33.0	34.1
T. Bilirubin (mg/dL)	0.2-1.2	2.4	0.3	0.6
D. Bilirubin (mg/dL)	< 0.4	0.7	0.0	0.6
LD (IU/L)	120-230	488	501	313
Reticulocyte (%)	0.2-2.7	8.1	1.5	1.2
Haptoglobin mg/dL	19.0-170.0	14.7	not tested	not tested

CHAPTER III
METHODOLOGY

To address detail study of this new Hb variant, we divided methodology section into three section: hemoglobinopathy and thalassemia screening (1), genetic analysis (2) and Hb study (3) as illustrated in Figure 9.

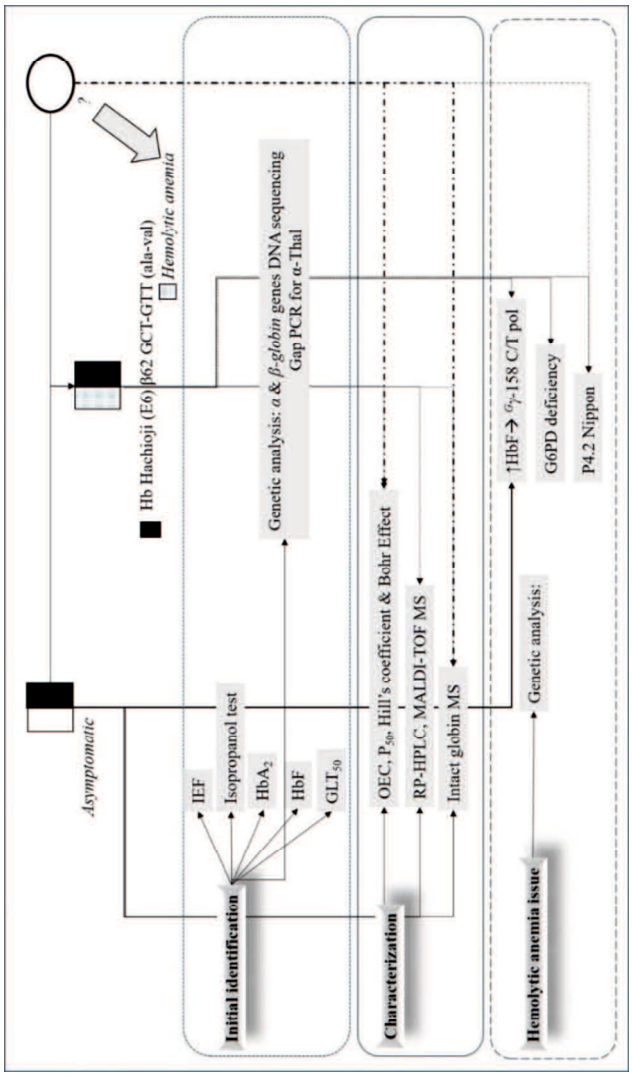


Figure 9. The schematic research workflow of Hb Hachioji study

The study was divided into three workflows; the initial identification based on the laboratory findings and the hemoglobinopathy screening test. At the next stage, the characterization of the new identified abnormal Hb and the determination of the causative factor of hemolytic anemia. Samples are being used in the study including the analysis at the genomic level to the protein level.

1. HEMOGLOBINOPATHY AND THALASSEMIA SCREENING

a) HbA_2 and HbF Measurements

Reagents

Tris-EDTA-borate (TBE) buffer, pH 8.6, Drabkin's solution: 0.05 g of KCN, 0.2 g of potassium ferricyanide and 1 g of sodium bicarbonate dissolved up to 1 L with H_2O, cellulose acetate membrane.

Procedures

The hemolysate was prepared for the HbF and HbA_2 quantification. HbF was quantitated by cation exchange HPLC (HPLC 723 G8®, TOSOH, Tokyo, Japan), while HbA2 was measured by cellulose acetate membrane electrophoresis **[29]**. To quantify HbF, a hemolysate was subjected to the routine analysis as performed for HbA1c measurement. On the other side, cellulose acetate (CA) membrane soaked into TBE buffer pH 8.6. An initial electrophoresis at 150 V for 10 minutes was run. The hemolysate was applied on the original gel, the electrophoresis at 150 V for 150 minutes was carried out. After electrophoresis is completed, each strip of HbA and HbA_2 was excised and transferred into the test tubes. 2 mL and 4 mL of extraction solution (Drabkin's solution) were added into respectively test tubes and waited until the lysate is completely eluted from the membrane. Measurement of each lysate absorbance at 420 nm by using UV-Vis.

b) Stability Test by Isopropanol

Reagents

Isopropanol and 0.1 M Tris-HCl pH 7.4

Procedure

The isopropanol test was first described by Carrel and Kay to study the stability of Hb **[30].** 300 µl of isopropanol buffer was mixed with 10% of hemolysate. The solution is incubated at 30°C and is being monitored at 5, 10, 15, 20, and 60 minutes to qualitatively observe the changing in turbidity. It is observed by eyes under sufficient illumination against the dark background.

c) Glycerol Lysis Time (GLT_{50})

Reagents

Isotonic phosphate buffer or phosphate-buffered saline (PBS), 0.3 M glycerol solution (FW 92.09, density 1.261 g/cm^3 at 20°C).

Procedure

GLT_{50} test was first described by Gottfried **[31,32]**. PBS was prepared by mixing 0.15 M NaCl NaCl and 0.1 M phosphate buffer with ratio 9:1 v/v. Addition 22 mL of 0.3 M

glycerol then the volume is adjusted with deionized H_2O to 100 mL. Instead of hemolysate, ten µL of blood was mixed with 2.5 mL PBS. In the other side, 2 mL of 0.3 M glycerol solution is prepared in a cuvette; the procedure hereafter should be carried out quickly due to time is the most important parameter. Add 1 mL of a mixture of blood and PBS above was added into glycerol solution in the cuvette, then sealed promptly with parafilm®, followed by brief mixing, and placed in the spectrophotometer. Monitoring at 625 nm absorbance was started immediately to observe the temporal reduction of the turbidity by hemolysis. The time required for half hemolysis or GLT_{50} was measured by the absorbance curve. Due to the time elapsed from the sample preparation, the time on chart recorder was corrected by the addition of 7 seconds.

d) Inclusion Body

Reagents

0.5% Brilliant Green, 0.5% Neutral Red.

Procedure

The inclusion body is to stain the aggregate formed inside the red cell. 2 or 3 drops of blood is placed into a test tube. The staining solution was 2-3 drops added. The solution is mixed and incubated at 37°C for 15-20 minutes. 2 or 3 drops of blood were added into a tube containing 2-3 drops of staining solution. After incubation completed, a blood smear was prepared and after drying, examine by microscope under immersion oil.

e) Isoelectric Focusing (IEF)

Reagents

20% acrylamide solution: acrylamide monomer 19.4 g and bis (N'N'-methylene-bis acrylamide) 0.6 g was dissolved with H_2O to make up to 100 mL. 0.05% KCN solution, tetramethylenediamine (TEMED), carrier ampholytes (Pharmalyte) pH 6.7-7.7, pH 5.0-8.0 and pH 8.0-10.5, 10% ammonium persulfate (APS) which was prepared immediately before use, 1N NaOH, 1M phosphoric acid, TCA solution (230 g TCA, 69 g sulfosalicylic acid, 600 mL methanol, and water up to 2,3 L), washing solution (160 mL glacial acetic acid, 500 mL methanol and water up to 2 L).

Procedure

Gel composed of 3 mL of 20% bis-acrylamide, 7.8 mL of 0.05% KCN solution, 0.47 mL of Pharmalyte (pH 6.7-7.7, pH 5.0-8.0, and pH 8.0-10.5) was degassed under the negative pressure by the vacuum pump for about 3 minutes, and at last added by 7 µl of TEMED and 70 µl of 10% APS and mixed well. The gel mixture was poured into the slit between to the gel plates, and let stand at room temperature for 1-2 hours until the gel is solidified.

The sample was prepared by mixing 6.25 µl of hemolysate and 25 µl of 0.05 KCN solution on micro-tray and followed by agitation for 15 minutes. Pre-electrophoresis was initiated by pouring water on the platform to let the gel plate firmly adhere to it, then followed by placing a piece of filter paper soaked each with 1N NaOH and 1M phosphoric acid at cathode and anode, respectively. Pre-electrophoresis was carried out at 2232 V for 10 minutes before applying the samples on the plate. Small tips of filter paper were soaked into the sample solution, placed on the gel, and the electrophoresis was started. During electrophoresis, the gel was cooling at 4°C. When all bands move no more, the electrophoresis was stopped, and gel piece was transferred into a filter paper then cut into two pieces. One of the protein paper was soaked by TCA solution letting the protein fixed for 30 minutes, while the other was soaked by CBB solution for 1 hour to overnight. The destaining of CBB was done twice with washing solution for 1 hour each.

2. GENETIC ANALYSIS

a) DNA Extraction

Reagents

H_2O, saline (0.85% NaCl), lysing solution (NH_4Cl 14 g and NH_4HCO_3 0.144 g are dissolved with H_2O to make up to 1000 mL), Proteinase K (New England Biolabs, MA, USA), 10% sodium dodecyl sulfate (SDS) solution, saline-Tris-EDTA (STE) buffer, pH 7.4, TE (10 mM Tris-HCl, 1mM EDTA-2Na), pH 7.6, TE-saturated phenol, pH8.0 containing 0.1% 8-hydroxyquinoline as an antioxidant, chloroform, 99.5% ethanol, 70% ethanol.

Procedure

DNA was extracted from the whole blood leukocytes by the conventional phenol-chloroform treatment. Briefly, the whole blood was centrifuged in separate serum, buffy coat, and red cells. Buffy coat was recovered and further processed with the addition of lysing solution to selectively hemolyze the remaining red cells. After the buffy coat was washed several times with the lysing buffer, STE, 10% SDS, and 1 mg/mL of Proteinase K solution were added by 400 µl, 20 µl, four µl, respectively, and incubated overnight at 37°C. The crude DNA was treated with phenol/chloroform (1:1) and gently mixed on the shaking rocker to denature the contaminant proteins in the sample, followed by the same treatment by chloroform twice to remove the phenol in the DNA solution completely. The DNA in the supernatant solution was purified by alcohol precipitation, and ethanol in the DNA pellet was vapored, and finally dissolved in TE.

b) *β-globin G*ene Amplification and Sequencing

Reagents

TaKaRa *Ex Taq* DNA Polymerase (TaKaRa Bio Inc, Shiga, Japan), H_2O, DNA template, a set of primers (Table 3), 50-fold concentrated 40 mM Tris-20 mM acetic acid- 1 mM EDTA buffer (50x TAE), pH 8.0, 0.8% agarose gel (Bio-Rad Laboratories, Hercules, California) containing a drop of 0.5% ethidium bromide solution. The BigDye Terminator Cycle Sequencing Kit ver1.1 (Applied Biosystems, Tokyo, Japan), sequencing primer (order-made at Greiner Japan, Tokyo Japan), DNA template from PCR product, H_2O, 99.5% ethanol, 70% ethanol, 125 mM EDTA, 3M sodium acetate and Hidi-formamide (Sigma-Aldrich, St. Louis, Missouri).

Procedure

The *β-globin* gene was amplified by PCR which was conducted on a total volume of 20 µl. The cycling conditions were as follows: initial denaturation (95°C, 3 min) followed by 35 cycles of denaturation (96°C, 40 sec), annealing (60°C, 60 sec), and extension (60°C, 90 sec), and the final extension (72°C, 3 min) followed by cooling. The 1,946 bp PCR product was subjected to 0.8% agarose gel electrophoresis. Target band was excised and purified using QIAquick gel extraction kit (Qiagen, Tokyo, Japan) for sequencing. Optimal dye terminator cycles with Astec thermal cycler prior to the sequencing analysis was as follows: initial denaturation at 96°C, 1 min, followed by 25 cycles of denaturation at 96°C- 10 sec, annealing at 50°C- 5 sec, extension at 60°C- 4 min (Dye terminator step), and followed by rapid ramp to 4°C and held on until ready for purification by ethanol precipitation before applying to sequencer [Applied Biosystem 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan)].

c) *β-globin* cDNA Sequencing

Reagents

Denaturing solution (4-5 M guanidium thiocyanate, 0.5% sodium N-lauryl sarcosine, 25 mM trisodium citrate, 0.1 M β-mercaptoethanol), chloroform, 99.5% ethanol, 70% isopropanol, diethylpyrocarbonate (DEPC) water, reverse transcriptase PCR kit (Primescript^{TM} RT Reagent Kit, TaKaRa, Shiga, Japan), primer set that amplify exon 2-3 of *β-globin* gene, *Ex Taq* DNA Polymerase (TaKaRa Bio Inc, Shiga, Japan), TAE Buffer, 2% agarose gel containing a drop of 0.5% ethidium bromide, Big Dye Terminator v3.1 cycle sequencing kit (Applied Byosystems, Warrington, UK), primers for sequencing, DNA template from PCR product, H_2O, 70% ethanol, 125 mM EDTA, 3M sodium acetate and hidi-formamide (Sigma-Aldrich, St. Louis, Missouri, USA).

Procedure

The RNA was extracted from 100 µl of packed red cells by using acid guanidinium-phenol-chloroform (AGPC) methods. It was subjected to the reverse transcriptase PCR according to the manufacturer's direction. The cDNA was diluted 100 fold before subjected to PCR. The cDNA was amplified using a set of primer (Table 3) that amplified exons 1 and 2 of the β-globin coding region. The 270 bp product proceeded to the sequencing analysis.

d) *Glucose 6 phosphate dehydrogenase (G6PD)* Gene Amplification and Sequencing

Reagents

TaKaRa *Ex Taq* DNA Polymerase (TaKaRa Bio Inc, Shiga, Japan), H_2O, DNA template, a set of primers to amplify 13 exons as listed in Table 2, TAE Buffer, 2% agarose gel containing a drop of 0.5% ethidium bromide, the BigDye Terminator Cycle Sequencing Kit ver1.1 (Applied Biosystems, Tokyo, Japan), sequencing primer, DNA template from PCR product, H_2O, 99.5% ethanol, 70% ethanol, 125 mM EDTA, 3M sodium acetate and hidi-formamide (Sigma-Aldrich, St. Louis, MO).

Procedure

The *G6PD* gene was amplified by the PCR which was performed with a total volume of 20 µl. Primers are listed in Table 3, as described by Adhiyanto *et al.* **[33]**. Target band was excised and purified using QIAquick gel extraction kit for sequencing. Optimal dye terminator cycles with Astec thermal cycler before the sequencing analysis that aforementioned above.

e) Polymorphism at -158 (promoter area) of $^{G}\gamma$ *globin* Gene

Reagents

TaKaRa *Ex Taq* DNA Polymerase (TaKaRa Bio Inc, Shiga, Japan), H_2O, DNA template, a set of PCR primers: TAE buffer, 2% agarose gel containing a drop of 0.5% ethidium bromide.

Procedure

Since the elevated HbF level was found in the proband, the presence of hereditary persistence of fetal Hb (HPFH) was suspected. The C to T polymorphism at -158 (promoter area) of the $^{G}\gamma$ *globin* gene, the most common HPFH, was studied by hybridization probe methods **[34]**. The $^{G}\gamma$ *globin* gene was amplified by first PCR using a set of primer (forward:, reverse:), and followed by nested PCR using a pair of inner primer. The PCR template thus prepared was hybridized with the probes (Sensor: 5'-AAC GGT TCC TGG CTA AAC T-Fluorescence; Anchor: LCRed640- 5'-ACC CAT

GGG TTG GCC AGC CTT GCC TTG A-Phosphate), and the differentials of the melting curve were drawn by LightCycler^{TM}480 (Roche, Tokyo, Japan).

f) *P4.2* Gene Amplification and Sequencing

Reagents

KOD FX Polymerase (Toyobo Co. Ltd, Osaka, Japan), H_2O, DNA template, a set of PCR primers, TAE buffer, 2% agarose gel containing a drop of 0.5% ethidium bromide.

Procedure

The third exon of *P4.2* gene was amplified by first PCR using a set of primer listed in Table 3, as described by Adhiyanto *et al.* **[33]**. The cycling conditions were as follows: initial denaturation (95℃, 3 min), followed by 35 cycles of denaturation (96℃, 30 sec), annealing (60℃, 60 sec) and extension (60℃, 30 sec). After final additional extension (72℃, 1 min) the sample was kept cool. The PCR product was subjected to 2% agarose gel electrophoresis. Target band was excised and purified using QIAquick gel extraction kit for sequencing. Optimal dye terminator cycles with Astec thermal cycler before the sequencing analysis that aforementioned above.

Table 3. List of primers used for genetic analysis in this research

Gene	Primer pairs
β-globin	**Gene** **F:** 5'- AGT AGC AAT TTG TAC TGA TGG TAT GG -3' **R:** 5'-TTT CCC AAG GTT TGA ACT AGC TCT T -3' **cDNA** **F:** 5'-ACA TTT GCT TCT GAC ACA ACT GTG TT-3' **R:** 5'-CTT GAG GTT GTC CAG GTG AGC CA-3'
G6PD	**Exon 1-2** F: 5'-TCGCCTGAGGCGGGTCCGCTCAGCC-3' R: 5'-CCCTGCAACAATTAGTTGGAAAAGC-3' **Exon 3-4** F: 5'-GCCCAGTAGTGATCCTGAGTAGTGC-3' R:5'-AATGGGGGTCTCAAGGAAGTACGAG-3' **Exon 5** F: 5'-ACACACGGACTCAAAGAGAGGGGCT-3' R:5'-GGAAAGGCGGTGTTTCGTGGAGCAA-3' **Exon 6-7** F: 5'-CAGCTCTGATCCTCACTCCCCGAAG-3' R:5'-CTCTGCCACCCTGTGCCAGCCTCCC-3' **Exon 8** F: 5'-AGTCTTGCAGCTTGTCACTAGGAAG-3' F: 5'-CAAGCTGAGGCCCAGAGAGGCAATG-3 **Exon 9-10** F: 5'-ATCCCTGCACCCCAACTCAACACCC-3' R:5'- GCCACTGCCTGCCACCATGTGGAGT-3' **Exon 11-13**

	F: 5'-ACTCCACATGGTGGCAGGCAGTGGC-3' R:5'- GGGGTCGGGCGGCGGGAAGGAGGGT-3'
$^{G}\gamma$-globin	F-1: 5'-GCA CTG AAA CTG TTG CTT TAT AGG A-3' F-2: 5'- TGG TAT CTT CTA TGG TGG GAG AAG A-3' R-1: 5'- TCA GAC GTT CCA GAA GCG AGT GT-3'
P4.2	F: 5'GTT CAT GCT TTT ATT GGT AAG ATT A-3' R: 5'GTG AGT CTT ATC GTG GGC AAA ATC A-3'

3. HEMOGLOBIN STUDY

a) Reverse Phase HPLC

Reagents

Degassed H_2O, 0.1% trifluoroacetic acid (TFA) (solvent A), 0.1% TFA-80% acetonitrile (ACN) (Solvent B).

Procedure

Before injecting hemolysate into the HPLC instrument, the sample was filtered by Millipore filter syringe (Millex GV, Millipore, Ireland) to remove cell debris in the hemolysate. An aliquot (1µl) of the filtered sample was applied to the Symmetry300TM C18 5µm column (3.9×150mm: Waters, Massachusetts, USA), and the globin chains were eluted by a linear gradient of 40 to 50% as acetonitrile of the solvent B in 75 minutes at a flow rate 0.5 mL/min. Each sample was collected into glass tube by fraction collector LKB 2211 Superrac (Pharmacia, Sweden). The protein solution was purified and concentrated by using Pierce concentrator 7mL/9K (Thermo Scientific, USA) before tryptic digest MALDI analysis.

b) MALDI Mass Spectrometry

Reagents

50 mM ammonium bicarbonate-50% acetonitrile (ACN), 100 mM ammonium bicarbonate, 10 mM dithiothreitol (DTT), 55 mM iodoacetamide, trypsin in 1% octyl β-D-glucopyranoside, ACN/Milli-Q/Trifluoroacetic acid (TFA) (66:33:1) solution, 50% ACN-0.1% TFA, 0.1% TFA, 2,5-dihydroxybenzoic (DHB) acid matrix (5 mg/mL in 50% ACN, 0.1% TFA) and sinapinic acid matrix solution (SA, MW = 224.22] in TA30 (saturated solution, final concentration, 0.067 g/dL).

Procedure

The HbA bands of the proband and his father separated in IEF were in-gel digested by trypsin and subjected to MS. Matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) MS was performed using Utraflextreme® (Bruker-Daltonics, Billerica, MA, USA). The measurement was performed by positive ion detection reflector mode of

MALDI-TOF MS. The excised gel was dehydrated in acetonitrile (CAN), and hydrolyzed by trypsin solution (Promega, USA) at 37°C for 24 hours. The peptides were extracted by solution a containing 5% trifluoroacetic acid (TFA) and 50% ACN from the gel, evaporated by centrifuge evaporator and concentrated up to 10 µL. The concentrated sample was desalted by ZipTip C18 (Millipore, USA). One µL of the eluate was applied to AnchorChip™ 600 TF plate, on which 1 µL of matrix solution that is composed of 0.4 mg/mL of a-cyano-4-hydroxycinnamic acid (HCCA) in 85% ACN, 0.1% TFA and 1mM ammonium dihydrogen phosphate was added. It was dried again in a clean bench and used for mass analysis. The spectra were acquired over a mass range of 100 to 6000 m/z. Mass data collected during MALDI TOF were processed and searched by using Mascot (Matrix Science, London, UK) server.

While in doing whole lysate analysis, the step was initially by desalting as a pretreatment was followed by MS measurement by the double layer method. The hemolysate was diluted with 0.1% of TFA, and ten µL of it (final concentration, 0.167 g/dl) was bound to ZipTip C18 (Millipore) and eluted by five µL of a mixture of 0.1% TFA and 75% ACN. The matrix thin-layer was prepared on a Target Ground Steel TF plate. The desalted sample was diluted five-fold with a matrix solution (sinapinic acid). One microliter of the sample (0.67 µg) was applied to the thin layer, dried, and subjected to the MS measurement using Ultraflextreme® (MALDI-TOF/TOF; Bruker-Daltonics, Billerica, MA, USA). Spectra were acquired in a linear positive ion mode and at a scan range of 840–20137 Da.

c) Functional Analysis of Hemoglobin by Hemox Analyzer : OEC, P_{50}, Hill's Plot and Bohr Effect

Reagents

HS-500 Hemox Buffer, Additive A (22% BSA) and anti-foaming were supplied by the manufacturer (TCS Scientific Corp., New Hope, Pennsylvania, USA), 0.05M Bis-Tris buffer, pH 7.2 and pH 7.4 containing 0.1 M NaCl

Procedure

Functional properties were studied using the Hemox Analyzer™ (TCS Scientific Corporation, New Hope, PA, USA). Unfortunately, the functional analysis of the proband's father was unfeasible. The proband's erythrocytes were washed with saline and centrifuged at 1,600 g for 5 minutes, 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 minutes. A 30-50 µl of the hemolysate was added into 4 mL HS-500 hemox buffer with the addition of 20 µl additive A (22% BSA) and ten µl anti-foaming agent, mixed gently and applied to the cuvette of Hemox Analyzer for OEC measurement.The OEC for whole blood was measured at pH 7.4 at 37°C using Hemox buffer (TCS Scientific Corporation), while Bohr effect was measured for hemolysate using 0.05 M bis-tris buffer (containing 0.1 M NaCl), pH 7.2 and pH 7.4.

CHAPTER IV
RESULTS

1. Hemoglobinopathy and Thalassemia Screening

As summarized in Table 4, the proband's HbA_2 was normal in quantity, and its electrophoretic profile was also normal. An elevated HbF level was noted. Instability by isopropanol precipitation test was normal. However, GLT_{50} was extended. The IEF showed no abnormal band (Fig. 10). The result from his parents were also found no abnormalities. Except for the elevated in HbA_2 of his father.

Table 4. The summary data of hemoglobinopathy and thalassemia screening of proband and his parents

Parameter	Result			Reference range	Unit
	Proband	Father	Mother		
HbF	↑ 4.4	0.9	0.5	1.0 (adult)	%
HbA_2	3.1	↑ 4.2	3.0	2～3.5	%
Isopropanol Test	(－)	(－)	(－)	(－)	(－)
GLT_{50} (sec)	↑ 157	34	33	22～55	Sec
Inclusion Body	Heinz bodies	(－)	(－)	(－)	
Isoelectric Focusing (IEF)	(－)	(－)	(－)	(－)	
Band3	ND	ND	ND	47.4～60.4	

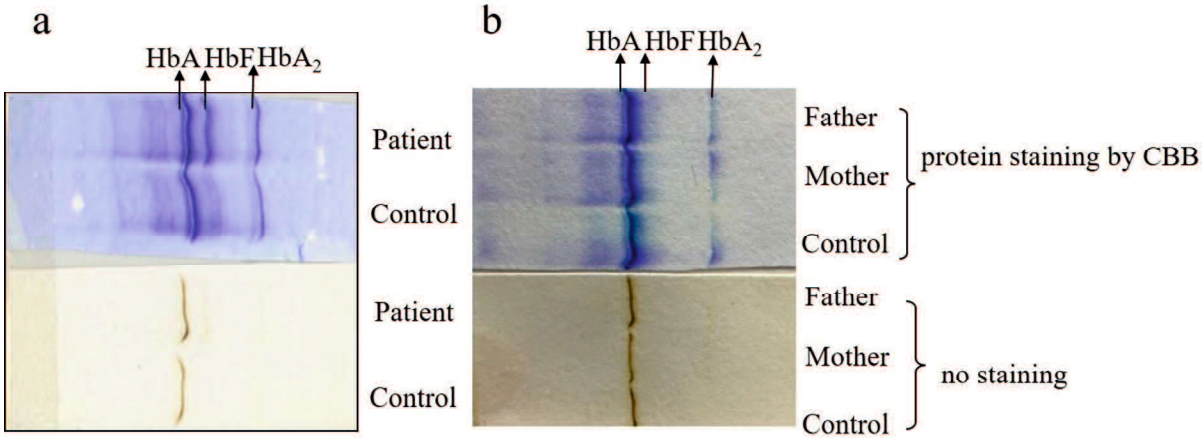


Figure 10. Isoelectric focusing result from proband and his parents.
The IEF of proband (a) and his parents (b) were done separately. No abnormal band was observed on the IEF, however, the elevated of HbF was noticed in the proband's. The upper figure stained by Coomassie brilliant blue (CBB) while the lower figure was not stained.

2. Genetic Analysis

Table 5 summarized the result from the genetic analysis performed in this study. The sequencing analysis demonstrated GCT to GTT mutation at codon 62 of the *β-globin* gene that

codes for alanine to valine, respectively (Fig. 11a). Common α thalassemia mutations, including the most common $-\alpha^{3.7}$ deletion were absent. The presence of the $\beta^{Hachioji}$ mRNA, as well as β^{nomal} mRNA, was confirmed by cDNA sequencing (Fig. 11b). The peaks for $\beta^{Hachioji}$ (T) and β^{normal} (C) mRNA's were almost the same height, and it was surmised that a considerable amount of the $\beta^{Hachioji}$ mRNA was present. Thus, a sufficient amount of the transcript of the mutant allele was present, to provide satisfactory $\beta^{Hachioji}$ production.

Table 5. Summary of genetic analysis for hemoglobinopathy and thalassemia

Method	Genetic testing performed	Result		
		Proband	Father	Mother
Gap-PCR	-3.7	(—)	(—)	(—)
	anti3.7	(—)	(—)	(—)
Sequencing	*β-globin*	Cd 62 G**C**T-G**T**T Ala-Val	Cd 62 G**C**T-G**T**T Ala-Val	(—)
	α2-globin	(—)	(—)	(—)
	α1-globin	(—)	(—)	(—)
	G6PD	(—)	ND	(—)
	P4.2	(—)	ND	(—)
Hybri-Probe	$^{G}\gamma$-158 Pol C/T	C/T	C/T	C/T

Apart from the attempt in Hb Hachioji characterization, we did detection of $^{G}\gamma$-158 promoter area C/T polymorphism both proband and his parents. The melting curve from the hybri-probe analysis showed proband and his parents are having C and T alleles. This result may explain the elevated HbF level in the proband, since proband experiences hemolytic anemia, his HbF level raised in response to hematopoietic stress. However, it has nothing to do with proband's parent are not having hemolytic anemia. On the other side, the analysis of *G6PD* gene also found no abnormalities. Further, the coexistence of P4.2 Nippon type red cell membrane abnormality was negative as well.

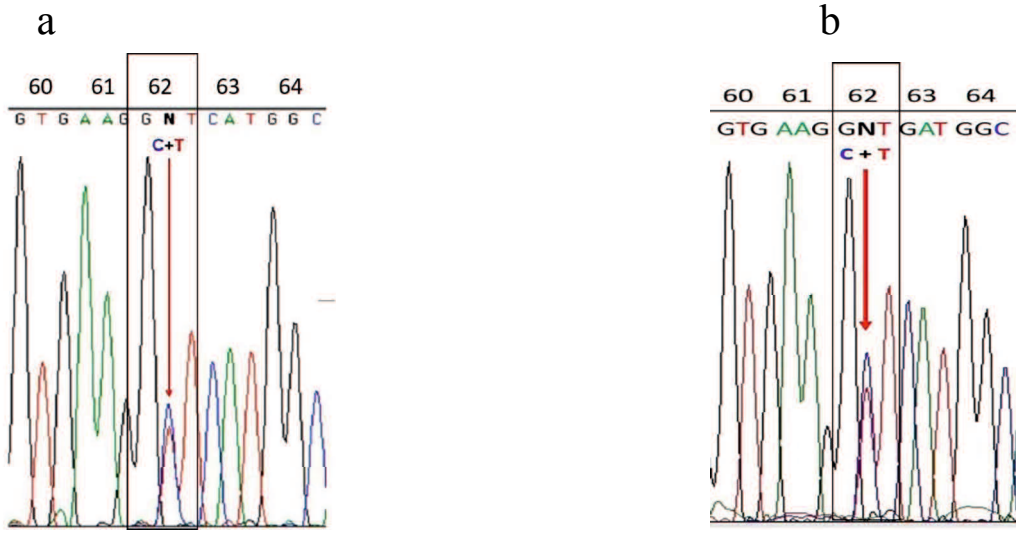


Figure 11. Sequencing result of genomic DNA and cDNA of the *β-globin*.
A heterozygous mutation of GCT (alanine) to GTT (valine) at codon 62(E6) in a sense strand is detected in the proband's DNA (a). A similar result in the cDNA analysis, the peak levels of C and T at codon 62 are almost the same (b). In addition, both peaks are lower than other single C or T peaks immediately after G, suggesting $\beta^{Hachioji}$ mRNA is stable and sufficient amount is present in the hemolysate.

3. Hemoglobin Study

a) Reverse Phase HPLC

In our initial structural study, we conducted chromatography study by RP-HPLC because of its fast, economic, and reliable result. However, the $\beta^{Hachioji}$ was not separated on RP-HPLC (Fig. 12). There would be two possibilities: Abnormal Hb no more remains in the hemolysate or it is overlapped with normal β globin subunit.

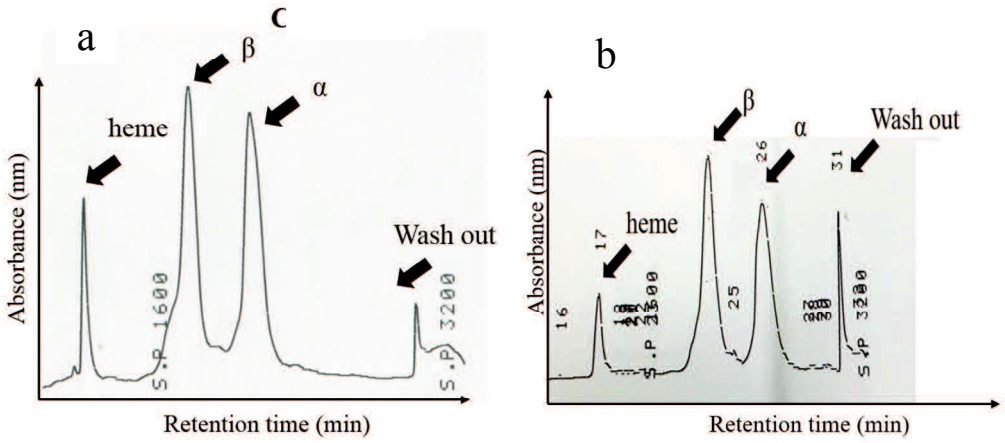


Figure 12. RP HPLC chromatogram obtained from proband and control.
The Hb is separated into its subunits where heme eluted faster than two globin chains. The β globin's retention time is faster than the α globin chain, as seen in control (a). However, no abnormal peak, in this case, is the $\beta^{Hachioji}$ globin, was observed in the proband (b). It was difficult to conclude whether the $\beta^{Hachioji}$ globin was overlapped with β^{A} globin or it was completely denatured.

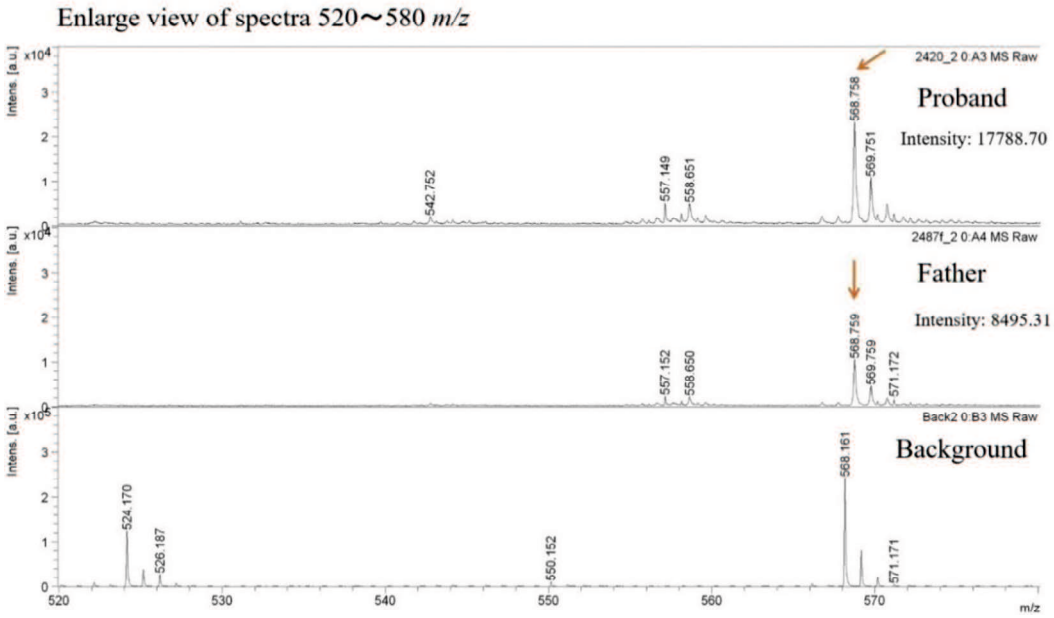


Figure 13. The result of Maldi MS.

The enlarged view (mass range 520~580 *m/z*) is shown. The peak VHGKK (568.35 *m/z*) pointed by red arrows which are likely derived from $\beta^{Hachioji}$ is visible in both proband and his father. It is not found in the background. However, weakness of the result was failed to demonstrate the wildtype peak of the abnormal peak for β^{A}, therefore, MALDI MS was not satisfactorily clarified the stability of Hb Hachioji.

b) Trypsin Digest with MALDI MS

An enlarged spectrum of the MS measurement is presented (Fig. 13). The $\beta^{Hachioji}$ peak (VHGKK) derived from Hb Hachioji with "actual" mass of 568.76 *m/z* (the theoretical value of positive mode, 568.35 *m/z*) was seen both in proband and father's spectra. Thus, it was found that Hb Hachioji was present. However, the peak corresponding to β^{Normal} (AHGKK, theoretical value of positive mode, 540.32 *m/z*) was missing, probably because of an extremely low signal. The content of $\beta^{Hachioji}$ in both carriers may be almost equal when compared with other peak levels, but it is not sure. The MS/MS of peptide fragment was not performed due to a too low level of the peak.

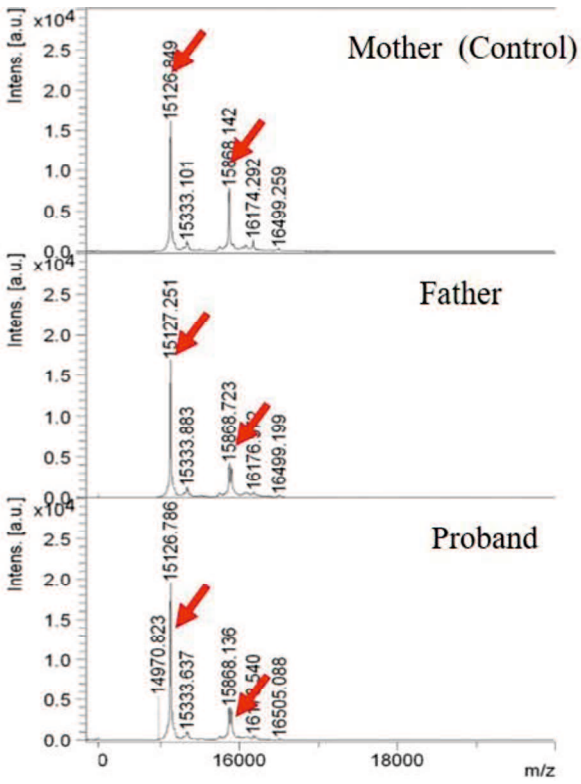


Figure 14. MS spectra obtained from whole lysate MS analysis.
An m/z 15,127.251 and 15868.723 Da from α and β globin (possibly also βHachioji) are pointed by the arrow. It is likely that the proband and his father has split peaks of the β globin, therefore continued to figure 15 for the expanded area.

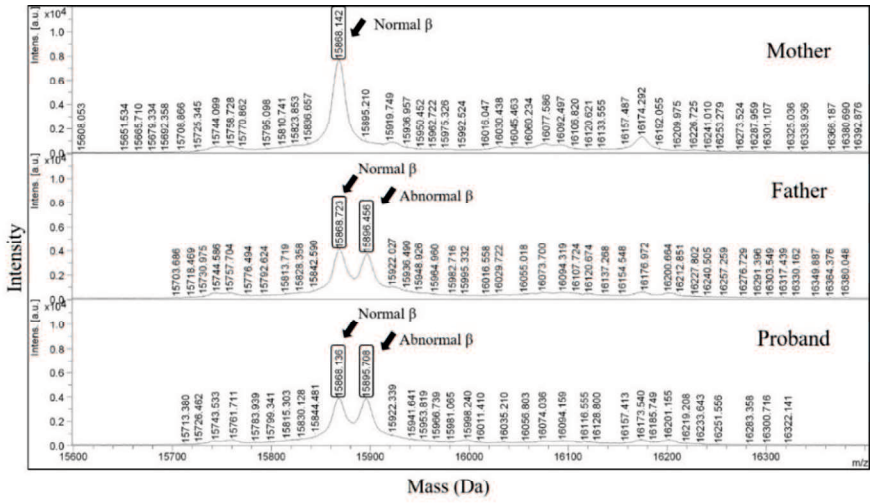


Figure 15. Analysis of the hemolysate demonstrates an abnormal β chain.
The peaks of the β^{nomal} and $\beta^{Hachioji}$ are shown by the arrows. The abnormal β chain with a mass difference from the normal counterpart of 27.46 Da and 27.67 respectively (theoretical value; 28.06 Da) was seen in the proband and his father and absent from the mother/control.

Table 6. Summary of *m/z* MS results from proband and his parents

Sample	MW (n=5)	α-globin	β-globin	abn β-globin	difference of abn/nor β	intensity ratio of abn/nor β
theoretical		15126.20	15867.05	15895.11	28.05	
Proband	average	15127.04	15868.70	15896.17	27.46	0.99
	sd	0.89	1.23	1.14	0.11	
father	average	15127.16	15868.69	15896.36	27.67	0.91
	sd	0.61	0.91	0.70	0.25	
mother	average	15127.12	15868.34	-	-	-
	sd	0.90	1.00			

abn: abnormal, nor: normal

c) Whole lysate Mass Spectrometry (MS)

The whole lysate MS revealed α and β globins peak, 15126.65 and 15896.17 Da, respectively, as seen in Figure 14. Two major split peaks were observed for the β globin mass, when the spectra were expanded, the masses are15868.70 ± 1.2 Da and 15896.17 ± 1.1 Da, corresponding to normal β and abnormal β globins, respectively (Fig. 15). As summarized in Table 6, 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 (A>V), or $\beta^{Hachioji}$ was evidenced. The same result was obtained for the proband's father. As expected, his mother did not share the $\beta^{Hachioji}$. The intensity ratio of the abnormal/normal β globin peak for the proband and his father were 0.99 and 0.91, respectively. Thus, $\beta^{Hachioji}$ comprises nearly half of the total β globin, which also suggests that Hb Hachioji is a stable variant.

d) OEC, Bohr effect, and Hill's Coefficient

The first attempt is to describe the functional properties of Hb Hachioji. We draw OEC curve as showed in Figure 16. The P_{50} value, Hill's coefficient (n) and Bohr effect (d) 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 Hb was normal and implies that Hb Hachioji has a normal function. Unfortunately, the fresh whole blood was unavailable for proband's parents, therefore, we could analyze by using their hemolysate. His mother has used as a control. The result of proband's father's sample showed similar P_{50} value againts the control, indicate that his father's hemolysate has normal P_{50}.

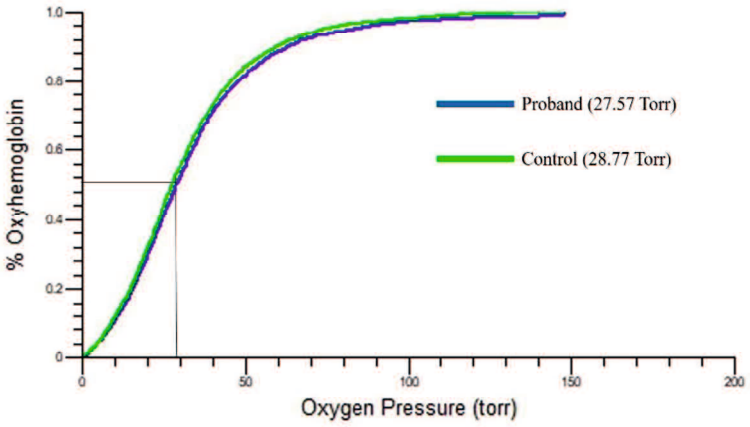


Figure 16. Deoxygenation curve drawn by Hemox Analyzer

The normal pattern of the deoxyHb curve obtained from proband was shown in this future. The P_{50} was in a normal range, suggesting that Hb Hachioji does not have an alteration in the oxygen affinity.

CHAPTER V
DISCUSSION

Our study identified a new stable abnormal Hb, or Hb Hachioji [β62 (E6) c.187C>T, A62V] in Japanese proband and his father. Since the Proband resides in Hachioji city, Tokyo, we named this new Hb variant by using proband residing place. It was based on the international convention in abnormal Hbs nomenclature **[14].** On the initial analysis, Proband came first to our attention since He experienced chronic hemolytic anemia since childhood. At first, his doctor suspected that He has hereditary spherocytosis (HS), the most common cause hemolytic anemia in Japan **[35].** Therefore proband underwent splenectomy when he was three years old. Even after he underwent splenectomy, the condition was not ameliorated. Since then, the doctor sent his sample to undergo further hemoglobinopathy and thalassemia screening test to our institution.

Hereafter, we conducted an initial investigation research in hemoglobinopathy by using proband's sample. Later on, due to the finding of abnormality in his *β-globin* gene, informed consent is obtained. Since this abnormality had never been reported anywhere, no evidence of its presence in the hemolysate and the relation to the clinical aspect, we considered a detailed study is necessary. Therefore, we also invited his parents to involve in the study by obtaining their sample and subjected to the same research. The subsequent result that the *β-globin* gene's abnormality of the proband was inherited from his father. Unexpectedly, his father was clinically healthy and asymptomatic.

By far, Hb Hachioji was only carried by the proband's father. If the proband does not exhibit clinical phenotype, it is unlikely that Hb Hachioji would have been discovered. If the analysis were carried out only in the proband without a family study, Hb Hachioji would have been recognized as a highly unstable variant, which is a misdiagnosis. Therefore, this report highlights the importance of family study to correctly define a new Hb variant. From this result, it is very unlikely that the abnormal Hb itself was responsible for the anemic condition in the proband. There should be coexistence factor that causes that worse condition. At this stage, we further examine for the causative factor of hemolytic anemia that could probably pass from his mother.

The two main purposes of the research were to characterize this new abnormal Hb and to determine what was caused hemolytic anemia in the proband. The result of the research has been clarified, except for the causative factor of hemolytic anemia syndrome of the proband. The discussion will be divided into two section, where we reported the finding of the new

abnormal Hb, and later we discuss the result of the examination of the causative factor of hemolytic anemia.

1. Hb Hachioji as a new stable Hb variant

Hb Hachioji is new abnormal Hb which has a mutation in the *β-globin* gene, at codon 62 GCT to GTT in a heterozygous form, from alanine to valine. The mutation is neutral to neutral amino acid, with mass change approximately 28 Da. Therefore, if the gene were translated into protein, there would be two variant, the normal HbA, and Hbx variant, which we named Hb Hachioji. The position 62 is in the interior of the molecule yet next to distal histidine. The introduction of valine was first though to disrupt the stability of Hb molecule. However, on the screening for hemoglobinopathy, the result of instability test by isopropanol test was negative, could not strongly describe the stability of Hb Hachioji. A negative result might indicate it was a stable variant or it was a highly unstable variant. Another finding was from the IEF. The electrophoresis of total Hb by IEF did not reveal any abnormal patterns. The negative result from IEF could be caused by the neutral amino acid replacement does not sufficiently change the net charge of the molecule **[19]**, that might cause Hb Hachioji was not separated from HbA. Neither IEF nor Instability test could confirm the presence or absence of Hb Hachioji. However, the lysis time of Hb Hachioji sample was extended by 157 seconds. This extension usually occurs in thalassemia and unstable hemoglobinopathies due to oxidation stress **[32]**. Here, our proband showed a phenotype looked like a thalassemia, but the correlation between other parameters did not fit with thalassemia description, except if this Hb variant has a thalassemia phenotype.

A contrasting phenotype showed by his father. He is also a carrier of the same Hb variant, but he is asymptomatic and healthy. On his hemoglobinopathy study, the GLT_{50} was not extended, the isopropanol gave a negative result, and further, IEF did not reveal any abnormal pattern as well as proband. When we went further to DNA analysis, he has the same mutation, thus neglected the assumption of Hb variant with thalassemia phenotype. Looking back to the laboratory data, the proband had macrocytic anemia, probably due to reticulocytosis (Table 2). Elevated HbF was probably related to the presence of hematopoietic stress by the hemolysis, and the proband had a polymorphism at -158 of $^{G}\gamma$ gene (C/T) where T polymorphism raises HbF level on the hematopoietic stress **[36,37]**. The reason that Hb Hachioji was thought as a highly unstable variant was that of the hemolytic anemia presentation, followed by prolonged GLT_{50}. The erythrocyte prematurely denatured and precipitated resulting in Heinz body formation with no more Hb Hachioji remained. The proband's Heinz body injuries the erythrocyte membrane by oxidative stress that has prolonged the GLT_{50}. Fortunately, we had

the opportunity to conduct the family study, and the proband's father was also a carrier of Hb Hachioji but was asymptomatic without hemolytic anemia (Table 2). This unexpected finding changed our initial assumption.

Since we had no clue about the stability of Hb Hachioji, we further investigate to the mRNA level, both proband and his father. Here we attempted to qualitatively measure the β^{normal} mRNA and $\beta^{Hachioji}$ mRNA by cDNA sequencing method. Our findings (Fig.11) confirmed that the peak's ratio between β^{normal} mRNA/$\beta^{Hachioji}$ mRNA is almost same, suggested that the mRNAs are equal in amount. Therefore, we can conclude that Hb Hachioji is likely to be translated into protein.

Thus, we further go to RP-HPLC test. The methods were chosen because of its high throughput and widely used in globin study in many laboratories **[19].** RP-HPLC can readily separate the globin chain monomer, therefore, we could observe the β^{normal} chain and $\beta^{Hachioji}$ chain, as well as quantitatively calculate the proportion. However, as seen in Figure 11, RP-HPLC was unable to separate the $\beta^{Hachioji}$ chain. In RP-HPLC, heme peak has the fastest retention time, followed by α and β globin chain. The α and β globin's peak tends to have the same height since they are quantitatively in the same amount. However, when the unstable suspected, it is easy to see the reduction of the peak's height by 10-30% and up to 50% for α and β globin's peak, respectively. Our result showed no alteration in α/β ratio, gave an unclear result, whether, the $\beta^{Hachioji}$ globin is super-unstable variant or stable and does not separated from its normal counterpart. However, since our target has neutral to neutral amino acids substitution, RP-HPLC failed to separate this globin variant, made us modify the RP-HPLC buffer condition to optimally get the proper separation condition. At last, no proper buffer condition could satisfactorily solve the challenge. This result again failed to explain the absence or presence of Hb Hachioji in the hemolysate.

The failure in our attempt by RP-HPLC could be caused by several factors. First, Our instrument has been used for many similar works for almost 25 years. Even the column was newly purchased prior this study, but the instrument may have deterioration in performance. However, during the study, we also did column qualification control to study other Hb variants; Hb M Saskatoon (β63 (E7) His>Tyr) and Hb Hikari (β61 (E5) Lys>Asn). However, Hb M Saskatoon has cyanosis phenotype, while Hb Hikari was silent. Hb Hachioji has a mutation at codon 62, in the interior region of the β-globin peptide, while Hb M Saskatoon is at codon 63 with amino acid substitution histidine to tyrosine, which is at the exterior because responsible for the binding site. Hb Hikari is at codon 61, with amino acid substitution lysine to asparagine. In the attempt study, we were trying to validate the ability of the column to separate the

abnormal chain. Hb M Saskatoon was successfully separated, and the $\beta^{Saskatoon}$ was accounted for about 20% of total β globin (figure not shown). While Hb Hikari failed to be separated in HPLC, the reference said that Hb Hikari is a stable variant. This result was quite similar to Hb Hachioji. Here we considered that the type of the column is likely to affect the result of chromatographic work. As stated by Reigner **[38]** the substitution occurs in the peptide or protein will influence the chromatographic behavior only when it occurs in the are that has a direct contact to the chromatographic sorbent or conformationally change the peptide or protein structure. The peptide or protein has their contact surface to the chromatographic column. Therefore, the location where the mutation occurs will influence the retention of the eluate. This means when the substitution occurs in the location that is not in the contact region between the peptide and chromatographic column, and there is a tendency will not undetected/unseparated, unless the substitution that alters the structure of the chromatographic contact region. Since the presence or absence of the Hb variant is very significant to the study, we finally decided to try Mass Spectrometry (MS) analysis.

In the MS analysis, we performed MALDI MS for its ability of rapid detection and simplicity workflow. The spectra obtained by MALDI analysis can be directly subjected to Peptide Mass Fingerprint (PMF) by using Mascot. The spectra that correspond to a specific mass charge can be determined directly by matching the peptide on the Mascot's database. Therefore we preferred to use MALDI analysis rather than ESI MS. The HbA ($\alpha_2\beta_2$) was isolated from IEF gel. The excised gel was subjected to in-gel trypsin digestion. Trypsin was chosen because it is widely used in the globin study and the tryptic digest peptide has been well described elsewhere. We have been worked with MALDI several times until finally we conclude that the tryptic peptide from the $\beta^{Hachioji}$ β62-66 (VHGKK) and βnormal (AHGKK) was difficult be ionized. Our instrument (Shimadzu Corp.) was unable to find both VHGKK and AHGKK. Thus we decided to use another instrument (Bruker Daltonics Corp.) from other institution and use the same method. As seen in the Figure 12, the expected peptide from $\beta^{Hachioji}$ with the 568.76 *m/z* was detected and confirmed by the Mascot software could a $\beta^{Hachioji}$. With the replacement of alanine by valine, the β62-66 of $\beta^{Hachioji}$ is larger than β^{normal} by addition 28 Da. However, the limitation of the spectra obtained this time is it is failed to demonstrate the β^{normal}'s peak. Neither 540.76 *m/z* was observed on the proband nor his father. Therefore, the MALDI result remained unclear. Thus, we decided to change the method by conducting the whole lysate MS analysis.

The analysis was still conducted in Bruker Daltonic MALDI TOF MS instrument, but this time, instead of using tryptic digest peptide analysis, we just did MS analysis to the whole peptide. The challenge of using whole MS analysis is hard to determine the molecular mass of the unknown protein. Fortunately, Hb molecular weight is precisely determine. HbA consists of two α-chains and two β-chains. The α and β globin's molecular weight are 15127.65 and 15896.17 Da, respectively. The molecular weight of these globin subunits is suitable for whole lysate analysis by MS. The mass that differs 28 Da is no doubt can be easily observed in the MS analysis. As seen in the Figure 15, we could finally distinguish the β^{normal} and $\beta^{Hachioji}$ which is seen in the proband and his father's spectral, but absent from his mother's spectra. It is likely that the desalting process affects the ionization of the whole mass, therefore, the ratio α/β was unequal (Fig. 14). Theoretically, it should be balance. However, looking to the result of mother's spectra could probably prove that the α/β ratio may not represent the imbalance between the two peptides. However, in Figure 15, the proportion of β^{normal} and $\beta^{Hachioji}$ can be calculated directly, it was almost equal in amount, as summarized in Table 6. The identified mass which differs by 28 Da from normal β chain suggested that $\beta^{Hachioji}$ was undoubtly present in the blood. Thus, this result could strongly prove that Hb Hachioji is a stable variant. The amount is approximately equal to normal HbA.

To the best of our knowledge, Hb variant database from Globin Gene Server (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) **[26]**. Hb Duarte is an unstable variant probably due to the break of α-helix by the substituted proline and has increased oxygen affinity **[39]**. In contrast, Hb J-Europa is a stable variant and has a normal oxygen affinity like Hb Hachioji **[40]**. Hb Hachioji is the third variant at this position. The substitution of valine for alanine at β62 of the E6 helix is located at the α1β2 contact and next to the distal histidine β63 (E7) in the heme binding pocket. Thus, the amino acid replacement at the E6 was at first expected to render the instability to the variant Hb molecule**.** The result of MS suggested that Hb Hachioji is an electrophoretically stable variant that overlaps with HbA on IEF, and it may have caused no HbA1c reduction on cation exchange HPLC. Also, 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 were carried out only in the proband without a family study, Hb Hachioji would have been recognized as a highly unstable variant, which is a misdiagnosis. Therefore, this report highlights the importance of family study to correctly define a new Hb variant. Furthermore, this result also confirmed that the Hb variant itself has

nothing to do with the hemolytic syndrome in the proband. This is suggesting that another coexisting factor play a role in causing hemolytic anemia.

2. The Cause of Hemolytic Anemia in the Proband is Not Related to Hb Hachioji

Another important issue in this research is the phenotype's discrepancy between the proband and his father. The two subject may be a carrier of an abnormal Hb variant, but only proband exhibit worse condition. If the hemolytic anemia of the proband is not associated with solely Hb Hachioji, there must be another cause for his hemolytic involvement. Long before the Hb Hachioji was identified, the proband had already undergone a splenectomy. Since then, the microspherocytes were no longer seen in his proband's blood film. This unclear condition made the investigation of HS coexistence was genetically examined. As reported by Yawata *et al.* **[41],** many mutations are found in RBC protein membrane and are related to hereditary red cell membrane disorders. The most frequent of them are HS and the underlying cause of many Japanese HS cases is homozygous P4.2Nippon. Therefore, we examined the presence of the P4.2Nippon in the proband's parents. However, the P4.2Nippon mutation was not detected. Yawata *et al* **[41]** have reported at least 29 mutations in three red cell membrane protein's genes are related to HS and most of them are specific to Japanese population. Recently, we have considered another relevant factor that may clarify the unknown factor, the dehydrated hereditary stomatocytosis (DHS). Since the clinical findings of most DHS cases are the slight macrocytosis and target cells are observed. However, the stomatocytes itself was not seen on the blood film.

Another clinical finding is the presence of Heinz bodies by the supravital staining. Heinz bodies are the final product of denatured Hb and give rise to hemolytic involvement as well. Thus, we considered that hemolytic anemia of our proband might be related to Heinz bodies. Also, when Heinz bodies are removed from the blood in the spleen, it tends to appear after splenectomy. However, usually, after the patient had done splenectomy, Heinz bodies rarely appear in the blood without denaturation of Hb. The cause may be more related to something that oxidizes and denatures normal Hb. Here we considered another possibility, as the proband is male, we further suspected that he might carry an X-linked genetic disorder that was passed onto him from his mother. Hemolysis and Heinz bodies may also be seen in the case of G6PD deficiency on exposure to an oxidant or after fava beans ingestion **[1]**. G6PD deficiency is the most common red cell enzyme abnormality that affects world's population. In Japan, G6PD deficiency is relatively rare (1/1000) **[36]**, nevertheless some cases have been reported **[42,43]**. The mutation that could possibly give rise G6PD deficiency is spreading in many sites of *G6PD* gene. Therefore we examined whole exons of *G6PD* gene. However, we did not find any

abnormalities. The enzymatic assay for G6PD activity was unable. The *G6PD* gene abnormality, therefore, seems to be unlikely as the cause of hemolytic involvement in our proband. Another enzyme deficiency of erythrocytes such as pyruvate kinase (PK) deficiency that occurs next to G6PD in the Japanese population, indeed causes hemolytic anemia but does not give rise to Heinz bodies, and therefore we did not test for this condition. The phenotype may become worse with coexisting red cell membrane abnormality, as is seen in Hb Gunma with congenital elliptocytosis **[44]**. However, elliptocytosis was not present in our patient. A P4.2nippon mutation that is asymptomatic in heterozygote but symptomatic with β-thalassemia minor was absent as stated above **[32]**. Hemolytic anemia is one of the indicators of the diagnosis of super unstable Hb variant.

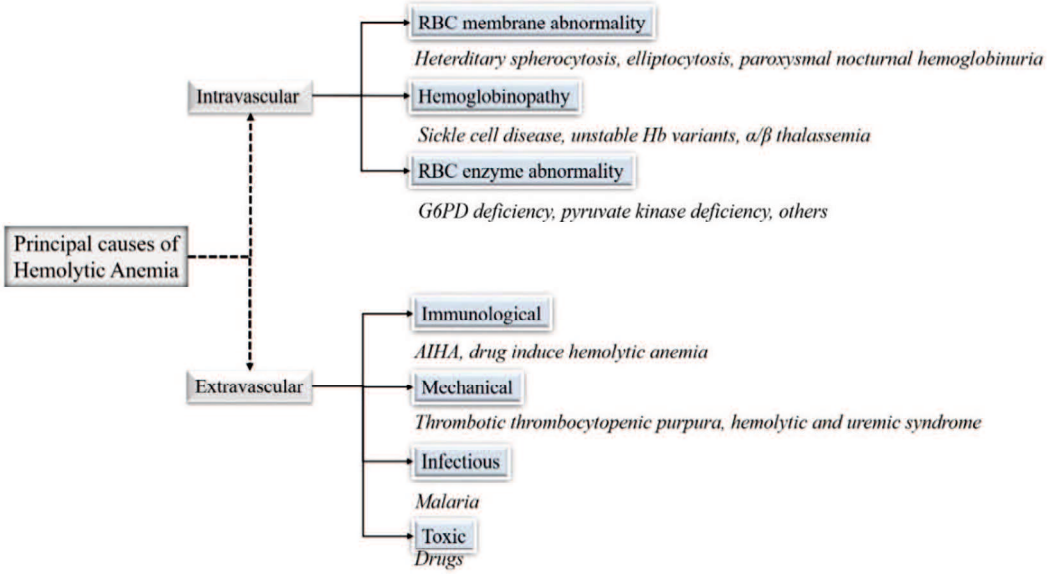


Figure 17. Summary of the cause of hemolytic anemia in adulthood.
The hemolytic anemia syndrome caused by a limitless factor. It can be intravascular or extravascular factors. However, the laboratory findings of our proband indicate that the causative factor is likely to be caused by an intravascular factor, such as the RBC's membrane protein or the enzyme abnormality.

Due to the wide spectrum factors that cause HS and hemolytic anemia, it is not easy and challenging to determine which factor relevant to this. We may spend a lot of DNA sample if we want to examine genetic abnormalities for each the predicted genes. Therefore, to solve the question of the hemolytic anemia in the proband, a genome-wide association study was considered. This may provide a valuable approach to elucidate other mutations relevant to this hemolytic anemia, and be a powerful diagnostic tool **[45, 46]**. However, this time, our research is limited to the sample amount. With this situation, we were unable to further investigate the possibilities at the moment.

In Japan, red cell membrane abnormality accounts for the most prevalent factor to cause hemolytic anemia, followed by hemoglobinopathy. Thus, red cell enzyme abnormality is the least frequent, but still reported quite frequent occurs. Figure 17 outlined some causes for hemolytic anemia to occur, especially in adulthood. Based on the initial laboratory data, it is likely the hemolytic anemia is caused by hereditary factor rather than acquired, because the proband has been continuously experiencing hemolytic anemia since childhood. The intravascular factor plays a more significant role in this case rather than extravascular. The immunological cause, such as autoimmune hemolytic anemia (AIHA) was also ruled out. Since the Coomb's test was examined and gave a negative result. Further, the history of drug intake was also essential to be evaluated, because some drugs are hemolytic anemia inducers. Such as hemolytic anemia drug inducer might also relevant as an extravascular factor. The diagnosis of hemolytic anemia should also include the patient and family medical history taking. Currently, we are unable to determine the last situation to define the hemolytic anemia promoting factor. Whether the hereditary by X-chromosome disease or by the mechanism of a homozygous recessive disorder, is still unclear. Hemolytic anemia itself is a syndrome that caused by many factors.

CONCLUSION

Our study detected a new stable Hb in the Japanese population, Hb Hachioji, which was identified 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. In this research, since limited to the sample amount, further investigation of the origin of proband's hemolytic anemia could not be done. However, the characterization of the coincidentally new abnormal Hb found in this proband was accomplished. Therefore we were focusing our research on the characterization of this new Hb variant. Moreover, our findings highlight the importance of conducting a comprehensive family study.

FUTURE STUDY

The investigation of the relevant factor that causes the hemolytic anemia of the proband, for example by using genome wide study (GWS), should be put on the next further study. Since obtaining more sample is likely not possible, the establishment of next-generation sequencing method with less sample consuming might be helpful to complete the study.

REFERENCE

1. Steinberg MH and Nagel RL. Unstable hemoglobins, hemoglobins with altered oxygen affinity, hemoglobin M, and other variants of clinical and biological interest. In Steinberg MH, Forget BG, Higgs DR, Weatherall DJ, editors. Disorders of Hemoglobin; Genetics, Pathophysiology, and Clinical Management. 2nd ed. New York (NY): Cambridge University Press; 2009. p589-606
2. Lehmann, H. Human Hemoglobin Variants. In: Bunn, HF, Forget, BG. Hemoglobin: Molecular, Genetic, and Clinical Aspects. Philadelphia: WB Saunders Company 1986. 381-451
3. Hattori Y, Harano T. Abnormal hemoglobin and thalassemia (in Japanese). Nihon Rinsho. 2001; 59(7): 437-451
4. De la Fuente-Gonzalo F, Nieto J, Velasco D, *et al*. HB Puerta del Sol [HBA1: c.148A>C], HB Valdecilla [HBA_2:c.3G>T], HB Gran Vía [HBA_2:c.98T>G], HB Macarena [HBA_2:c.358C>T] and HB El Retiro [HBA_2:c.364_366dupGTG]: description of five new hemoglobinopathies. Clinical Chemistry and Laboratory Medicine (CCLM). 2015;54(4):553-560
5. Boursier G, Trouillier T, Blaizot MG, *et al*. A New high affinity variant Hb Aurillac (β141Leu→Val). Hemoglobin. 2013;37(60):584-588
6. Giambona A, Vinciguerra M, Cassarà F, *et al*. Hb Marineo [β70(E14) ala>val]: a silent hemoglobin variant with a mutation within the heme pocket. Hemoglobin. 2006;30(2):139–148
7. Kumar MK, Judd C, Hoyer JD, *et al*. Hb Manukau [β67(E11) Val → Gly; HBB: c.203T>G]: The role of genetic testing in the diagnosis of idiopathic hemolytic anemia. Hemoglobin. 2014;38(3):211-212
8. Vinciguerra M, Passarello C, Leto F, *et al*. Co-inheritance of the rare β hemoglobin variants Hb Yaounde, Hb Görwihl and Hb City of Hope with other alterations in globin genes: impact in genetic counseling. Eur J Haematol. 2015; 94:322–329
9. Colah RB, Surve R, Sawant P, *et al.* HPLC studies in hemoglobinopathies. Indian J Pediatr. 2007 Jul;74(7):657-662
10. Hardison RC. Evolution of hemoglobin and its genes. *Cold Spring Harbor perspectives in medicine*. 2012;2(12)

11. Orkin SH. The duplicated human alpha globin genes lie close together in cellular DNA. Proceedings of the National Academy of Sciences of the United States of America. 1978;75(12):5950-5954.
12. Sankaran VG, Xu J, Orkin SH. Advances in the understanding of haemoglobin switching. *British journal of haematology*. 2010;149(2):181-194. doi:10.1111/j.1365-2141.2010.08105.x.
13. Bunn HF and Forget BG. Hemoglobin: Molecular, Genetic and Clinical Aspects. Philadelphia: WB Saunders Company 1986
14. Thom CS, Dickson CF, Gell DA, *et al*. Hemoglobin Variants: Biochemical Properties and Clinical Correlates. Cold Spring Harbor Perspectives in Medicine. 2013;3(3)
15. Safo MK, Ahmed MH, Ghatge MS, *et al*. Hemoglobin–ligand binding: Understanding Hb function and allostery on atomic level. Biochimica et Biophysica Acta 1814 (2011) 797–809
16. Giambona A, Vinciguerra M, Passarello C, *et al*. (2010), Co-inheritance of Hb Hershey [β70(E14) Ala→Gly] and Hb La Pommeraie [β133(H11)Val→Met] in a Sicilian subject. European Journal of Haematology, 84: 453–457.
17. Lehmann, H. Human Hemoglobin Variants. In: Bunn, HF, Forget, BG. Hemoglobin: Molecular, Genetic and Clinical Aspects. Philadelphia: WB Saunders Company 1986. 381-451
18. Basset, P, Beuzard, Y, Garel, M, *et al*. Isoelectric focusing of human hemoglobin: its application to screening, to the characterization of 70 variants, and to the study of modified fractions of normal hemoglobins. Blood 1978;51(5):971-982
19. Fabry M and Old JM. Laboratory Methods for Diagnosis and Evaluation of Hemoglobin Disorders. In Steinberg MH, Forget BG, Higgs DR, Weatherall DJ, editors. Disorders of Hemoglobin; Genetics, Pathophysiology, and Clinical Management. 2nd ed. New York (NY): Cambridge University Press; 2009. P658-685
20. Posterano A, Gottfried EL. The diagnostic significance of a prolonged erythrocytic glycerol lysis time (GLT_{50}). Am J Clin Pathol. 1978;70(4):637–641
21. Old J, Harteveld CL, Traeger-Synodinos J, *et al*. Prevention of Thalassaemias and Other Haemoglobin Disorders: Volume 2: Laboratory Protocols [Internet]. 2nd edition. Nicosia, Cyprus: Thalassaemia International Federation; 2012. Chapter 2, haematological methods. Available from: https://www.ncbi.nlm.nih.gov/books/NBK190583/

22. Brosious EM, Morrison BY, Schmidt RM. Effects of hemoglobin F levels, KCN, and storage on the isopropanol precipitation test for unstable hemoglobins. Am J Clin Pathol. 1976 Nov;66(5):878-882.
23. Guarnone R, Centenara E, Barosi G. Performance characteristics of Hemox-Analyzer for assessment of the hemoglobin dissociation curve. Haematologica. 1995 Sep-Oct;80(5):426-430.
24. Singhal N, Kumar M, Kanaujia PK, *et al.* MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front. Microbiol. 2015 6:791. doi: 10.3389/fmicb.2015.00791
25. Horneffer V, Forsmann A, Strupat K, *et al.* Localization of analyte molecules in MALDI preparations by confocal laser scanning microscopy. *Anal. Chem.* 2001;73, 1016–1022. doi: 10.1021/ac000499f
26. HbVar: A database of human hemoglobin variants and thalassemias [Internet]. (Cited on May 12). Available from http://globin.bx.psu.edu/hbvar/menu.html
27. Kwiatkowski DP. How malaria has affected the human genome and what human genetics can teach us about malaria. American Journal of Human Genetics. 2005;77(2):171-192
28. Furuya, C., Yamashiro, Y., Hattori, *et al*. A novel $\epsilon\gamma\delta\beta$ thalassemia of 1.4 Mb deletion found in a Japanese patient. Am. J. Hematology. 2008;83: 84–86. doi:10.1002/ajh.21040
29. Giambona A, Passarello C, Renda D, *et al*. The significance of the hemoglobin A_2 value in screening for hemoglobinopathies. Clin Biochem. 2009; 42:1786–1796
30. Carrel RW, Kay R. A simple method for the detection of unstable haemoglobins. Br J Haematol. 1972;23(5):615–619
31. Posterano A, Gottfried EL. The diagnostic significance of a prolonged erythrocytic glycerol lysis time (GLT_{50}). Am J Clin Pathol. 1978;70(4):637–641
32. Adhiyanto C, Hattori Y, Yamashiro Y, *et al*. Oxidation status of β-thalassemia trait and HbH disease, and its association with glycerol lysis time (GLT_{50}). Hemoglobin. 2013;38(3):169-172
33. Adhiyanto C, Yamashiro Y, Hattori Y, *et al*. A new β^0-thalassemia mutation (codon 102, AAC>A*TC*AC) in coexistence with a heterozygous *P4.2 Nippon* gene. Hemoglobin. 2013;37(3):227-240
34. Yamashiro Y, Hattori Y, Hino M, *et al*. Establishment of gene analysis by improved hybridaization probe method for β-thassemia. Rinsho Byori. 2011; 59(9): 858-863
35. Fujii J, Hemogloenzymopathies, *in* Erythrocyte, ed by Fujii J and Takasou Y (in Japanese). Igakushoin, Tokyo, 1998. pp195-212
36. Alter BP. Fetal erythrophoiesis in stress hematophoiesis. Experimental Hematology. 1979;7(5):200-209

37. Akinsheye I, Alsultan A, Solovieff N, *et al*. Fetal hemoglobin in sickle cell anemia. Blood. 2011;118(1):19-27
38. Reigner FE. The role of protein structure in chromatographic behavior. *Science* 238 (4825), 319-323. DOI: 10.1126/science.3310233
39. Beutler E, Lang A, Lehmann H. Hemoglobin Duarte: (α2β2 62(E6) Ala leads to Pro): a new unstable hemoglobin with increased oxygen affinity. Blood. 1974;43(4):527-535
40. Kiger L, Kister J, Groff P, *et al*. Hb J-Europa [β 62(E6) Ala-->Asp]: normal oxygen binding properties in a new variant involving a residue located distal to the heme. Hemoglobin. 1996;20(2):135-140
41. Yawata Y, Kanzaki A, Yawata A, *et al*. Hereditary red cell membrane disorders inJapan: Their genotypic and phenotypic features in 1014 cases studied. Hematology 2001;6 (6):399-422
42. Nakashima K, Ono J, Abe S, *et al*. G6PD Ube, a glucose-6-phosphate dehydrogenase variant found in four unrelated Japanese families. Am J Hum Genet. 1977;29:24-30
43. Beutler E. G6PD Deficiency. Blood. 1994;84(11):3613-3636
44. Maehara T, Tsukamoto N, Nojima Y, *et al*. Enhanced haemolysis with β-thalassemia trait due to the unstable β chain variant, Hb Gunma, accompanied by hereditary elliptocytosis due to protein 4.1 deficiency in a Japanese family. Brit J Haematol. 2002; 117:193-197
45. Ku CS, Cooper DN, Polychronakos C, *et al*. Exome sequencing: dual role as a discovery and diagnostic tool. Ann Neurol. 2012;7:5-14
46. Nitta T, Kawano F, Yamashiro Y, *et al*. A new kruppel-like factor 1 mutation (c.947G>A or p.C316Y) in humans causes β-thalassemia minor. Hemoglobin. 2015;39(2):121-126