Severe phenotype of a new mutant of β-thalassemia complicated with $P4.2_{Nippon}$ may have developed based on the oxidative state of the β-thalassemia

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Chris Adhiyanto

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Abstract

A new β-thalassemia frameshift mutation was found at codon 102 (AAC>ATCAC) in a 17-year-old Japanese male and his 14-year-old sister. Both demonstrated a more severe phenotype of mild hemolytic involvement than the usual β-thalassemia minor. No mRNA derived from the thalassemic allele, or β^TmRNA, was detected in the sequencing analysis of the whole mRNA (cDNA). However, a small amount of the β^TmRNA in the whole βmRNA was specifically amplified by amplification refractory mutation system, and furthermore, the quantitative PCR demonstrated a negligible amount of β^TmRNA. Thus, their more severe phenotype was not caused by the "dominant type" β-thalassemia in which a considerable amount of the β^TmRNA is present. In addition, the whole βmRNA of the patient was as half as normal. The cause of the β-thalassemia by the frameshift mutation was ascribed to the reduced amount of βmRNA. We further searched for the cause of their severe phenotype. However, factors that exacerbated the phenotype of β-thalassemia, such as *α-globin* gene triplication, coexisting iron deficiency and infection were not found. Finally, we noticed that the red cell morphology revealed ovalocytosis and small numbers of stomatocytes that were seen in the hereditary spherocytosis (HS), especially by mutations of membrane protein P4.2. The sequence of the *P4.2* gene of our patient disclosed heterozygous $P4.2_{Nippon}$, or missense mutation at codon 142 (GCT>ACT) on exon 3, the most common mutation of Japanese HS. Frequent mutations of other membrane proteins, Band 3 and Ankyrin that were also a common cause of HS in the Japanese population other than P4.2 were not detected. When HS by $P4.2_{Nippon}$ develops it is homozygous, and no P4.2 protein is observed in the red cell ghost by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, while, in our case, the amount of the P4.2 was almost normal. We suspected the oxidation by free excess α-globin chain in β-thalassemia minor might impair the function of altered membrane protein P4.2 by $P4.2_{Nippon}$. However, there are few reports about the oxidative state of β-thalassemia minor measured by malondialdehyde (MDA) and total antioxidant capacity (TCA). Here, our study of thirty-three Japanese patients of β-thalassemia minor disclosed that the β-thalassemia minor was related to oxidation that was evidenced by higher MDA and lower TCA ($p<0.001$). In addition, glycerol lysis time (GLT_{50}) that was invariably prolonged in thalassemia was highly correlated with both MDA and TCA ($p<0.01$), or oxidation. Thus, the GLT_{50} prolongation in thalassemia may reflect the membrane alteration or injury by the oxidation. Such alterations of the membrane in β-thalassemia may precipitate the further derangement of the red cell membrane by heterozygous $P4.2_{Nippon}$ to cause hemolytic involvement.

Keywords

β-Thalassemia, frameshift mutation, membrane protein, $P4.2_{Nippon}$, thalassemia minor, oxidation, malondialdehyde (MDA), total antioxidant capacity (TCA), GLT_{50}

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Abbreviations and Symbols

α	alpha
AQP	aquaporin
ARMS	amplification refractory mutation system
β	beta
bp	base pair
cDNA	copy DNA
C	Celsius
CBB	coomassie brilliant blue
CBC	cell blood count
CRP	C-reactive protein
DNA	deoxiribunucleic acid
EDTA	ethylenediamine tetraacetic acid
GAPDH	glycerolaldehyde-3-phosphate dehydrogenase
GLT_{50}	glycerol lysis time 50
Hb	hemoglobin
HPLC	high performance liquid chormatography
HS	hereditary spherocytosis
IEF	isoelectric focusing
mRNA	messenger RNA
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MDA	malondialdehyde
P4.2	protein 4.2
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PCV	pack cell volume
qPCR	quantitative PCR
RBC	red blood cell
RDW	red cell distribution width
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
T	thalassemia
TAC	total antioxidant capacity
TBAR	thiobarbituric acid regression
TIBC	total iron binding capacity
WBC	white blood cell

CHAPTER I

Introduction

We had a case of hemolytic anemia with β-thalassemia carrier (heterozygote), which is usually asymptomatic in respect to the hemolysis. It was found the patient also had red blood cells membrane defect of P4.2 whose heterozygote is also asymptomatic. Thus, we suspected that there might be some synergic effect for hemolytic involvement on their coexistence. The thesis is composed of two parts. First, it disclosed that the patient was the double heterozygote for β-thalassemia of the new mutation and membrane protein P4.2 abnormality.[1] Subsequently, the association of reactive oxygen species (ROS) on β-thalassemia minor was studied, because the inherent membrane damage by the ROS in β-thalassemia minor may be the cause of the deterioration of the β-thalassemia on the coexistence of P4.2 defect.[2] The membrane injury by ROS is well recognized in the β-thalassemia major, but remains uncertain for β-thalassemia minor. Thus, we suspected that the subtle membrane injury or dysfunction of the β-thalassemia minor by ROS may be a background of exacerbation of the symptom by $P4.2_{Nippon}$. In addition, the association of glycerol lysis time (GLT_{50}), which is prolonged in β-thalassemia with the possible membrane injury by ROS is also studied.

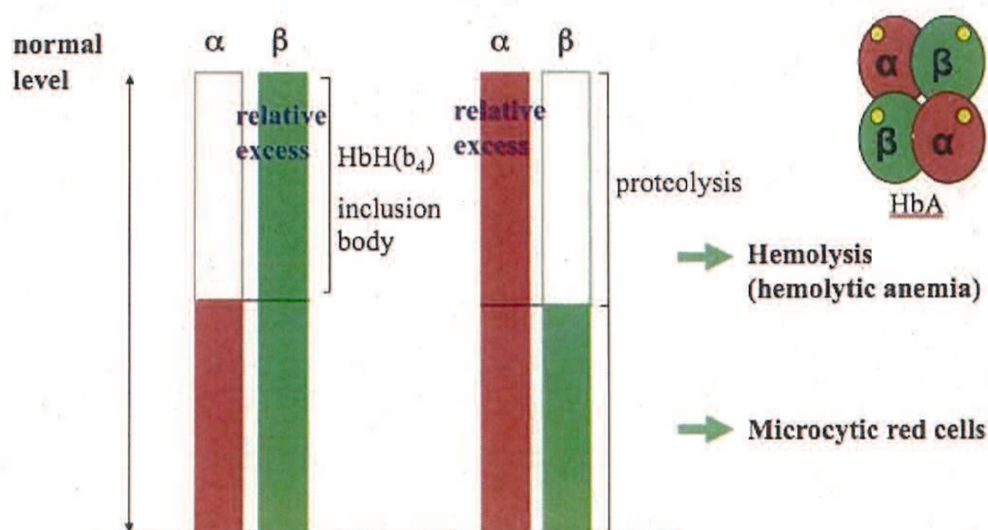

Fig 1. Thalassemia, reduce of globin chains production.

β-Thalassemia is a disease caused by reduced production of β-globin of Hb A ($\alpha_2\beta_2$) by the complete absence of the production of the β-chain (β^0) or the reduced but some production (β^+).[3-5] More than 200 mutations in β-thalassemia have been up to now reported. This disease is basically autosomal recessive except for some dominant-type β-thalassemia.[6,7] Most of the heterozygote display microcytosis but clinically asymptomatic, called thalassemia minor. However, their clinical symptoms may be often affected by additional factors, and may sometimes reveal more severe manifestations than the usual thalassemia minor.[5,8,9-11] One of such factors may be an abnormality of the membrane protein. The β-thalassemia heterozygote that has coexisting elliptocytosis by membrane abnormality showed severe phenotype.[12,13] We present here a new frameshift mutation considered giving rise to β-thalassemia minor.

However, the patient had more severe symptom, or more severe anemia. No abnormal βmRNA, or β^TmRNA was detected. Thus, the possibility of the dominant-type β-thalassemia was excluded. The patient's blood smear revealed moderate poikilocytosis, including ovalocytes that prompted us to search for the abnormality of the membrane protein of red blood cells. And it was found that he also carried P4.2 abnormality, or $P4.2_{Nippon}$ (heterozygote) whose homozygote demonstrates hereditary spherocytosis (HS).

Thalassemia major is reported to be related to ROS and is in enhanced oxidation status, which is reflected by increased malondialdehyde (MDA) by membrane lipid peroxidation and the decreased total antioxidant capacity (TCA). However, there was little evidence for β-thalassemia minor on the association with oxidation state. On the other hand, *in vitro* hemolysis by the exposure of red blood cells to glycerol solution that is measured as GLT_{50} is abnormally prolonged exclusively in thalassemia.[14] The reason for the prolongation of GLT_{50} in thalassemia is not well understood. Thus, the association of GLT_{50} with the membrane alteration by oxidative stress is also studied. The GLT_{50} is likely to be a direct evidence of membrane alteration or injury in β-thalassemia.

CHAPTER II

Materials and Methods

Table 1. Routine laboratory exams and screening data for hemoglobinopathy.

Parameters	Proband (M-17)	Sister (F-14)	Reference ranges
WBC ($\times 10^9$/L)	8.2	5.3	3.9-9.8
RBC ($\times 10^{12}$/L)	5.26	4.24	4.27-5.70
Hb (g/dL)	10.9	9.0	13.5-17.6
PCV (L/L)	0.35	0.28	0.40-0.52
MCV (fl)	66.7	66.0	82.7-101.6
MCH (pg)	20.7	21.2	28.0-34.6
MCHC (g/dL)	31.1	32.1	31.6-36.6
RDW (%)	25.6	21.6	11.0-15.0
Mentzer Index	12.7	15.6	>13
Reticulocyte (%)	1.1	1.0	0.2-2.7
Platelet ($\times 10^9$/ L)	352	291	131-362
Total bilirubin (mg/dL)	1.7	1.0	0.2 – 1.2
Direct bilirubin (mg/dL)	0.7	0.4	< 0.4
CRP (mg/dL)	0.05	0.05	≤0.3
Haptoglobin (mg/dL)	10	<10	19-170
TIBC (μg/dL)	297.0	308.0	250-390
Serum iron (μg/dL)	129.0	158.0	50-200
Serum ferritin (ng/mL)	120.7	80.0	12.9 – 301.3
Hb F (%)	6.1	5.6	<1.0
Hb A_2(%)	4.4	4.5	2.0 – 3.5
Isopropanol test	(+)15 min	(+) 20 min	-
GLT_{50} (seconds)	243.0	>300.0	22.0– 55.0
Inclusion body	-	-	-
IEF profile (abnormal Hb)	-	-	-
Anisocytosis	moderate	moderate	slight
Poikilocytosis	moderate	moderate	slight

Case Report

A17-year-old Japanese male proband was referred to a hospital by a mild hemolytic anemia of Coomb's negative nature. His 14-year old sister also demonstrated the same manifestation. For further examination, five-milliliter of the blood sample was collected from each individual with EDTA as an anticoagulant. Informed consent was obtained prior to the blood sampling. The CBC, tests for clinical chemistry and hemoglobinopathy screening that included HbF and HbA_2 levels, isopropanol test, GLT_{50} and isoelectric focusing (IEF), were performed at Fukuyama Rinshou KK (Fukuyama, Japan). Their data demonstrated mild microcytic anemia, reticulocytosis and reduced level of haptoglobin, suggesting overt hemolytic anemia with microcytosis. Status of serum iron, TIBC and ferritin were not consistent with iron deficiency. The levels of HbF by HPLC and HbA_2 by cellulose acetate membrane were increased. The instability test by isopropanol and tests for HbH inclusions and Heinz bodies were negative. No abnormal hemoglobin was separated in the IEF. The GLT_{50} was longer than normal. The blood smear showed not only hypochromia and moderate aniso-poikilocytosis but some

ovalocytes and occasional stomatocytes. These findings suggested that the patient was afflicted with β-thalassemia minor (Table I). The same data were shared by his 14-year-old sister who also was considered to have β-thalassemia minor.

I. Analysis of β-thalassemia

1. Identification of α- and β-thalassemia

DNA Analysis

The DNA of the proband and his sister were extracted from 2-ml of blood by the conventional phenol/chloroform method.[15] Molecular identification of the thalassemia was carried out by DNA sequencing and by gap PCR for β-thalassemia and α-thalassemia as described below.

Amplification of *β-globin* gene by PCR

The 1.9-kb DNA segment of *β-globin* gene was amplified by PCR using a pair of primers (forward, 5'-AGTAGCAATTTGTACTGATGGTATGG-3'; reverse, 5' - TTTCCCAAGGTTTGAACTAGCTCTT-3'). The PCR condition for amplification of *β-globin* gene are, initial denaturation: 94^0C for 3 minutes; followed by 35 cycles of denaturation at 94^0C for 40 seconds, annealing at 62^0C for 30 seconds and extension: 72^0C for 1 minute. Finally, extension at 72^0C for 2 minutes was added. The PCR product was isolated by agarose gel electrophoresis, excised and purified by Qiagen-QIAquick gel extraction kit.[16] After dideoxy reaction using BigDye ® Terminator v1.1 Cycle Sequencing Kit (Applied BioSystems, Tokyo, Japan), it was subjected to DNA sequencer (Applied Biosystems, 3130 Genetic Analyzer). The BigDye Terminator condition are, initial denaturation at 96^0C for one minute, followed by 25 cycles of denaturation at 96^0C for 10 seconds, annealing at 50^0C for five seconds and extension at 60^0C for 4 minutes, and after completion, kept at 4^0 C until used.

Amplification of *α-globin* gene by PCR

About1.0-kb DNA segments of *α1-globin* gene and *α2-globin* gene were amplified by PCR using a pair of primer (common forward, 5'-GATGCACCCACTGGACTCCT GG-3'; reverse for α2, 5'-AACACCTCCATTGTTGGCACATTCC-3', reverse for α1, 5'-CGCCCATGCTGGCACGTTTGCTGAG-3').

The primers for gap PCR for α-thalassemia ($-\alpha^{3.7}$) and α-triplication ($\alpha\alpha\alpha^{anti3.7}$) are: 5'-GATGCACCCACTGGCATCCTGC-3'and 5'-CCCATGCTGGCACGTTTCTGAG-3' (for $-\alpha^{3.7}$), and 5'-GATGCACCCACTGGCACTCCTGC-3'and 5'-AACACCTCCATTGTTGGCACATTCC-3' (for $\alpha\alpha\alpha^{anti3.7}$). The PCR condition for amplification of *α-globin* gene are, initial denaturation: 94^0C for 3 minutes; followed by 45 cycles of denaturation at 94^0C for 50 seconds, annealing at 63^0C for 1 minute and extension at 72^0C for 1 minute. The last extension at 72^0C for 5 minutes was added.

RNA analysis

The presence of abnormal mRNA derived from thalassemic allele (β^TmRNA) was examined using packed red blood cells washed three times with saline and stored at -80℃ for a couple of months. The RNA was extracted from 100 μl of packed red blood cells using Trizol Reagent (Invitrogen, catalogue No.15596-018). After ethanol purification, it was subjected to RT-PCR using Primescript™ RT reagent Kit (code RR0037A, TaKaRa, Kyoto, Japan) using a primer pair (forward, 5'-

TCTGTCCACTCCTGATGCTGTTAT-3', reverse, 5'-CCAGTTTAGTAGTTGGACTTAGGGA-3'). The product was processed as the same as the aforementioned genomic DNA analysis, and subjected to the sequencing analysis.[17]

2. Possibility of dominant type β-thalassemia
ARMS analysis.

In order to confirm the presence of β^TmRNA, if any, the β^TcDNA produced above was subjected to the amplification refractory mutation system (AMRS)[18] using specific (forward: 5'-AGCTGCACGTGGATCCTGAGATCAC-3', in which the underlined are inserted nucleotides at codon 102 in the thalassemic allele) and common primers (reverse: 5'-CCAGTTTAGTAGTTGGACTTAGGGA-3'). Cycling conditions were as follows: initial denaturation (95^{0}C, 1 min.), followed by 35 cycles of denaturation (95^{0}C, 30 seconds), annealing (60^{0}C, 30 seconds), extension (72^{0}C, 30 second), and final extension (72^{0}C, 10 seconds) followed by cooling.

Quantitative PCR (qPCR)

The cDNAs of βmRNA and β^TmRNA were prepared using the wild type and mutation-specific primers above, and used as the standard in this experiment. However, in order to get pure standard, we cloned from each βcDNA or βmRNA of the proband by TA-cloning method using the pGEM-T Easy vector system I (Promega, Tokyo, Japan) and the PureYield™ Plasmid Miniprep System (Promega, Tokyo, Japan) for purification. The cDNA of glyceroladehyde-3-phospate dehydrogenase (GAPDH) mRNA as internal control was also prepared using primer pairs 5'-GGCCATGCCAGT GAGCTTCCCGTTC-3' (forward) and 5'-CTGCACCACCAACTGCTTAGCACCC-3' (reverse). It was also cloned and used as above. The best clone was selected by the DNA sequencing among several clones. The amount, or copy numbers of βmRNA, β^TmRNA and GAPDH mRNA in red blood cells of the proband and normal control were measured by qPCR using LightCycler®480 SYBR Green I Master (Roche, Tokyo, Japan).

II. Analysis of the membrane abnormalities

1. SDS polyacrylamide gel electrophoresis (SDS-PAGE) analysis of erythrocyte membrane

RBC ghosts were prepared from 2ml of blood by the hypotonic lysis. The ghosts[19] were electrophoresed on SDS-PAGE according to Laemmli and on the 3.0-10.0% gradient gel by the Fairbanks buffer systems.[20,21] The gels were stained with Coomassie Brilliant Blue (CBB), and the relative amounts for P4.1, P4.2, α-spectrin, β-spectrin, Band 3 and Ankyrin against that of actin were measured on the digital picture by Multi Gauge Ver3.X software.

2. DNA analysis of membrane protein gene

As a coexisting abnormality of the membrane protein was suggested by the morphology of the blood smear, the genetic abnormality of the red blood cells membrane protein was surveyed for all 13 exons of the *P4.2* gene. All exons including

exon/intron boundaries of the *P4.1* gene that is often the cause of Japanese elliptocytosis were also analyzed.[22] Other membrane proteins, Ankyrin and Band 3 were also investigated considering their frequency of abnormality in the Japanese population, in which exons 1, 5, 6, 16, 17, 22, 26, 28, 31 and 38, and exons 5 and 6 were analyzed for *Ankyrin* and *Band 3* genes, respectively (Table 2). The presence of Southeast Asian ovalocytosis (SAO) by Band 3 mutation was also analyzed.

The procedure of PCR condition for membrane protein *P4.2* gene (exons 1 to 3) was: Initial denaturation (94^0C, 2 min.), followed by 35 cycles of denaturation (94^0C, 30 seconds), annealing (62^0C, 30 seconds), extension (72^0C, 1 minute), and final extension (72^0C, 2 minutes) followed by cooling. The PCR condition for *P4.2* gene exons 4 to 12 were only different on annealing temperature (60^0C, 1 minute) and extension (70^0C, 30 seconds for exons 4 to 6; and 11, 12; and 70^0C, 1 minute for exons 7 to 10, 13).

The PCR condition for membrane protein *P4.1* gene was the same as that for exon 1 of *P4.2* gene. Meanwhile, the PCR program for *Ankyrin* gene (exons 5,16,17, 22, 28, 38) and *Band 3* gene (exons 5 and 6) were: Initial denaturation (94^0C, 1 min.), followed by 30 cycles of denaturation (94^0C, 30 seconds), annealing (60^0C, 30 seconds), extension (72^0C, 1 minute), and final extension (72^0C, 2 minutes) followed by cooling. The PCR program for exons 1, 6, 26, and 31 of *Ankyrin* gene were: Initial denaturation (94^0C, 3 min.), followed by 35 cycles of denaturation (94^0C, 30 seconds), annealing (62^0C, 30 seconds), extension (72^0C, 30 seconds), and final extension (72^0C, 2 minutes) followed by cooling.

III. Analysis of oxidation status of common β-thalassemia minor

Thirty-three blood samples of β-thalassemia minor and 12 normal controls were collected from individuals living in Japan.

Analysis of malondialdehyde (MDA) and total antioxidant capacity (TAC)

The MDA in plasma that reflects membrane lipid peroxidation was measured using Cayman's TBAR Assay Kit (catalogue number: 10009055). The TAC which indicates the sum of redox capacity was measured using Abcam (catalogue number: ab65329).

Statistical analysis was performed using StatFlex V6 English version (ArtechCo.,Ltd, Osaka, Japan, statflex@statflex.net).

Table 2. The primers of PCR for erythrocyte membrane abnormalities.

	P4.2	
exon	**5' primer**	**3' primer**
1	5'-TAACCTTTCTACCCCACTGCCCAGA-3'	5'-AGTTATACCACCATCTCCCCGCCTA-3'
2	5'-GACAGTTCCACACTCAGCC TCACTC-3'	5'-AGGCACATAATCTCGTGGCA CCCTC-3'
3	5'-GGCCTCATTTCTAGGTGGG TCACTT-3'	5'-ACTGGGTGTTGGTGCTGTCC TTAGA-3'
4	5'-GTAGAGACAGAGTCTTGCT GTGTTG-3'	5'-GGTTATGGGACAGTCACTCT ACACA-3'
5	5'-TAGGACAGTCAGGGTTCAC TAAATG-3'	5'-CTATTGCCTGTCTTTTTGTC CTCCT-3'
6	5'-TTTAACCAGTACCAGGTGG CTCTAA-3'	5'-CTAGGGCAGGGTTAGGGAAG TAAGT-3'
7-8	5'-ACTCCTCTTCTTTCCAAGTT TTCAG-3'	5'-CATGCCTCGTGCTTCTACTG TTTTC-3'
9	5'-TATTCCTGTCATATCCATAG TAAAG-3'	5'-ACTGAATTTTCCTATGTGAA AGAAT-3'
10	5'-GTTTGGAAGAGACACACTG TATCGG-3'	5'-TCCTGCCACTGTACTCTGTT AAACT-3'
11	5'-CCCTGTCTCTAAAACAAAACAAAAA-3'	5'-TAATAGGGCATGTAAGAAAC TATGT-3'
12	5'-CTTGGCAGAGATGAGGAAA CTTACT-3'	5'-AAGCCACTTGGTAGCCCTAA TCCTT-3'
13A	5'-TGGCTCCTGTCTTCTATTCC TTTAT-3'	5'-GTAAGTTCTCGCTCTATATG TCCCT-3'
13B	5'-CTTCCACTTATGCAGGAAG ATGAAG-3'	5'-AGTGTCAAGGCAACAAAATA ACATA-3'
13C	5'-TGGTCCAGTAGAGAAATAT AAAAGT-3'	5'-TTTTTACCAGAAATACAGTG GATTG-3'
	Ankyrin	
exon	**5' primer**	**3' primer**
1	5'-ACCAGGAAAGGCAAATTGTGTAGAT-3'	5'-AAAAATATGAAACTGGCTTCAGCGA-3'
5	5'-TTGTAATAATCCCAGGACGCCAGTC-3'	5'-CTTTGTCATTAGTCCCTTGCAGAAC-3'
6	5'-AAGAGGGCTTTGGTGGAGGT AGGTC-3'	5'-CTGGGCATCTGCCAGGGATTTAGAG-3'
16	5'-CTTCTCCGGGCAAGTGTATTGATCT-3'	5'-GAATAGCGTGATTACAGGGCAGGTA-3'
17	5'-CCCTGCTTGTGTGTTGATATTGTCA-3'	5'-GGGAATAAGCAACCTTTCTGACTCT-3'
22	5'-AAAAATGTGTGCGGCTCTAGTTTGG-3'	5'-TATAGTGCTTGGGCGTGTTGTAAGG-3'
26	5'-GGCATCTAAAGAGTATGATGAGGAC-3'	5'-GACACCTTCGTGTGTCAGGAAAAGT-3'
28	5'-TCCCGCTGTACTTCGTGATCATGTC-3'	5'-CCTCACCCCTTCCCACTTAACTCTC-3'
31	5'-TCTGGATGGAAAGGATGCTCTCTGT-3'	5'-AGGGTCTTGTCCACTTTATCATCTG-3'
38	5'-ACCCCGAATGTGCATGCATCTCTGT-3'	5'-TACCCAGGAGCCCAGAAGACAAAAA-3'
	Band 3	
exon	**5' primer**	**3'primer**
5-6	5'-CCTGGACTATGCCTGATGAACTAAC-3'	5'-AGGACGTTGTAGATGTAAGTGGGTG-3
	P4.1	
exon	**5' primer**	**3'primer**
1	5'-AGCAAGACCCCAGAGGCAGAGGGAG-3'	5'-ATTGCATTGTATCATGTGACCAAAAA-3
2	5'-TTTTAGGGTTTAGGGTTTTGCCAAC-3'	5'-ACCGCGCCCAGCCCAAATACTCTTA-3'
3	5'-GTTCATGCTTTTATTGGTAAGATTA-3'	5'-GTGAGTCTTATCGTGGGCAAAATCA-3'
4	5'-AGCTATGATCAGGCCACTGTACTCC-3'	5'-TCAATGATGAAAGAATACACCAGCA-3'
5	5'-GCACTATTTGAAGATAGTCAGTGAT-3'	5'-TTCCACCTTCTGTTACTCTCAACTC-3'
6	5'-AATGTTTGAATATTTTGAGGTACTT-3'	5'-AATATCCATGATAAGCAAGGCCAGT-3'
7	5'-CTGTGTCCTTATTTTTTCTCTCTCT-3'	5'-TAGGCAAACAATGAACCAATACCAT-3'
8	5'-TAAAATACAGTGAATATATAAATGT-3'	5'-GGGTCTTGCTCTGTCGCCAGGGCTG-3'
9	5'-TTGGTTGTTATCTTCAGATTATCTA-3'	5'-CTCAGATGACTTAATAAGGAAAATG-3'
10	5'-CTCTGGTACTGTATCACTGTAATCT-3'	5'-CCCATTCCTTACTCCTTCTCTTCCTT-3'
11	5'-TATATGGAAACCCTGAGAATTGTGA-3'	5'-TTAATTCGGTTCTCTTATAGATAAT-3'
12	5'-ACACTCTAACATTCGTGTGTATCCT-3'	5'-AATGAAGAATAACTTTGTTGAGTCT-3'
13/14	5'-AGCTTATTTGGAAACAACACTATTC-3'	5'-TGGGTGAAATGGCAATGTAGTTTTG-3'
15	5'-ATGCTGCGCTGTGGACATTTATTTT-3'	5'-GAATCAGAAGGCGAGATGTTTGCAT-3'
16	5'-TCCTGGATGTGGTATGTTTTCTACC-3'	5'-TTTCAGCTCTAAGCTTTCTCTCAGA-3'
17	5'-ATTAAAGCTAACACTTTGATCAGAT-3'	5'-ATTTTTTGCTTTAGATAAAGTAGTC-3'
18	5'-ATGAGATTTTTGCATCAGAATGTGC-3'	5'-CAGAGAGACAGATGTGTAAAGAGAA-3'
19	5'-GATTACAAGTGTGAACCACTAAACC-3'	5'-CCAAAGCTCATGTTCTTTCCATTAC-3'
20	5'-CAGGAGTATTGGATCTGTCAGAACA-3'	5'-GCTGGCACTGCTACAGGCTGCTTTA-3'

CHAPTER III

Results

Analysis of β-thalassemia
DNA and RNA analysis for the thalassemia

The abnormal allele had two nts (TC) inserted at codon 102 (AAC>A<u>TC</u>AC) on exon 2 (Fig. 2). This frameshift is expected to give rise to a β^0-thalassemia, while no such mutation was detected in the sequencing of the whole βmRNA (βcDNA) (Fig. 3).

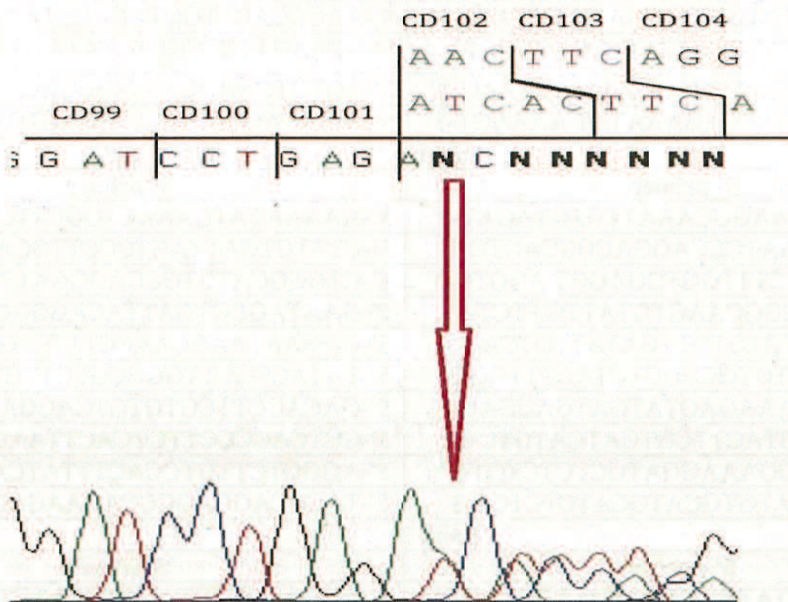

Fig. 2. DNA sequencing of the proband's *β-globin* gene. The nucleotide TC was inserted at codon 102 after the first nt, adenine (AAC>ATCAC). The addition of two nts results in a frameshift of the reading frame. The arrow indicates the site of insertion of the two nts.

The ARMS using a mutant-specific primer barely demonstrated the β^TcDNA (Fig.4), which was not found in the normal control. No cross annealing to the wild type allele was seen in the mutant-specific primer.

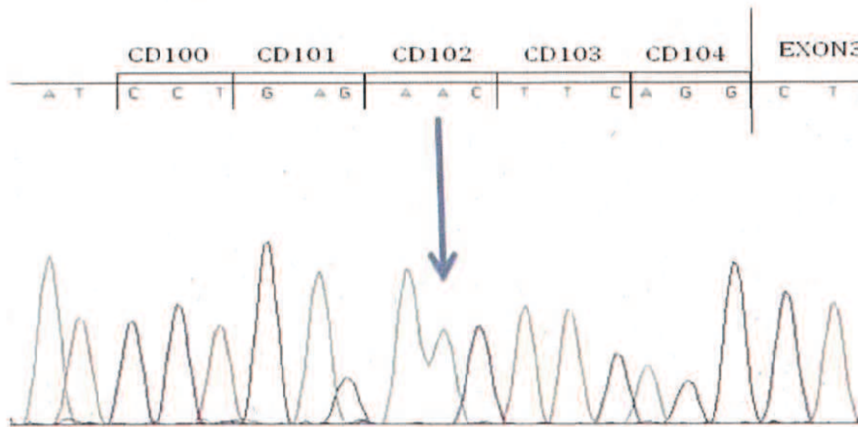

Fig. 3. The sequencing of the whole βmRNA as RT-PCR product revealed only the normal allele. No β^{T}cRNA derived from β^{T}mRNA was observed. It is suggested that β^{T}mRNA, if present, was negligible.

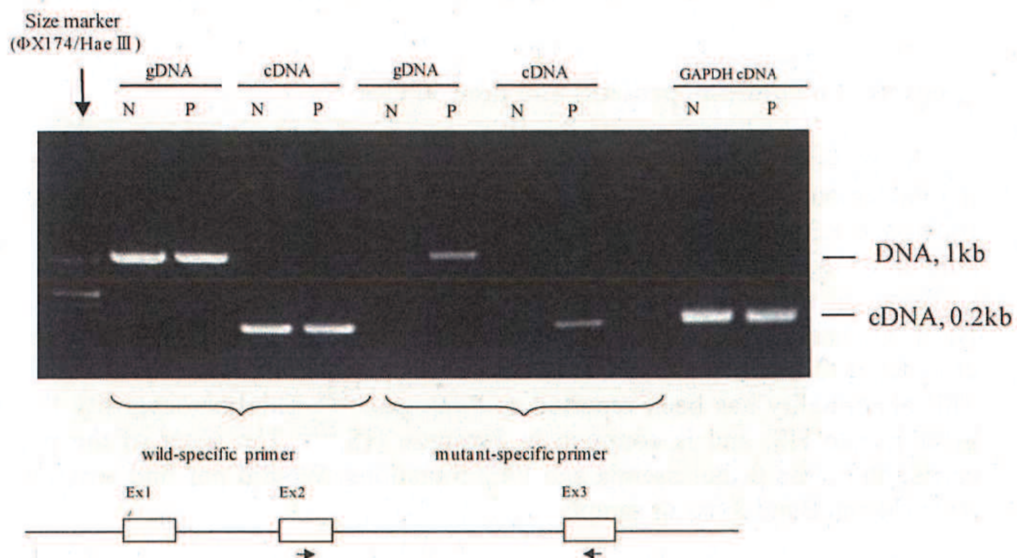

Fig. 4. Amplification refractory mutation system for the β^{T}mRNA (β^{T}cDNA).

The segment from exon 2 to 3 of the *β-globin* gene and βmRNA was amplified by PCR and RT-PCR, respectively, using the forward primer specific to the wild type or β^{T}allele and the common reverse primer. The arrows indicate the place of the forward and reverse primers. When the wild type primer set is used, both the control and proband's *β-globin* genes (1.2 kb long) and βcDNA (0.23 kb long) were amplified by PCR. However, when mutation-specific primer was used, only the proband's *β-globin* gene and β^{T}mRNA (β^{T}cDNA) were amplified. This result indicates that β^{T}mRNA is surely present in the proband, although the level seems very low. gDNA: genomic DNA, N: control, P: proband.

The β^TmRNA in the proband's red blood cells determined by qPCR comprises only 0.01% (0.182/[1438t0.182]) of the whole βmRNA, while it is absent in those of the normal control (Table 3). In addition, the amount of the βmRNA of the patient was 49.5% (1438/2904) of that of normal control (Table 3). The alleles of α-thalassemia (–$\alpha^{3.7}$) and α triplication ($\alpha\alpha\alpha^{anti\ 3.7}$) were not present. The α^0-thalassemia alleles (--SEA and --FIL), frequent in Japanese, were also not found.[23-25]

Table 3. The amount of the total, wild and mutant βmRNA's of the normal control and patient, measured by qPCR.

	sample	normal control		patient	
		copy number		copy number	
		actual	corrected**	actual	corrected**
	GAPDH* cDNA	792		1210	
primer	wild cDNA	2300000	2904	1740000	1438
	mutant cDNA	0.0171	0.00002	220	0.182

**glyceroaldehyde-3-phophate dehydrogenase* (GAPDH) gene

** The wild and mutant βmRNA (βcDNA) were corrected by GAPDH, internal control.

Analysis of membrane proteins and their alleles

The SDS-PAG electrophoretogram of the proband's membrane protein exhibited a normal amount of Band 3, P4.1 and P4.2 that were corrected by actin, i.e., 1.27 (reference 1.31 ± 0.25), 1.33 (1.28 ± 0.22) and 1.12 (1.12 ± 0.15), respectively. The amount of α-, β-spectrins and Ankyrin did not seem to be decreased by the Fairbanks method, but may not have been determined precisely because of sample aging. The DNA sequencing of membrane protein P4.2 showed a heterozygous missense mutation at codon 142 (GCT>ACT) in exon 3, a replacement of alanine by threonine (Fig. 5). This abnormality has been reported as P4.2$_{Nippon}$.[22,26] The homozygosity for P4.2$_{Nippon}$ gives rise to HS, and is common in Japanese HS.[22,26] The sister of the proband also carried the same β-thalassemia and P4.2 mutations. We did not find any mutations for Ankyrin and Band 3 in our sample.

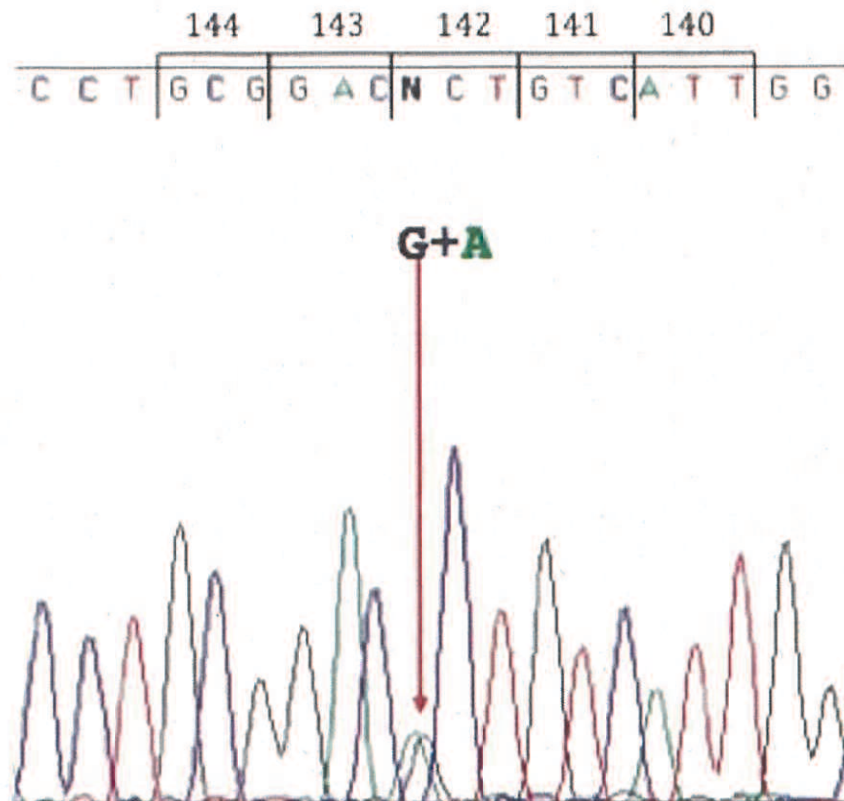

Fig. 5. Sequencing of the red blood cells membrane protein, *P4.2* gene. A missense mutation (GCT>ACT), indicated by an arrow, was found at codon 142 on exon 3 of the *P4.2* gene.

Analysis of oxidative state

To investigate an oxidation state associated with β-thalassemia minor, the level of MDA and TAC in plasma of the affected individuals was examined.

1) The MDA and TAC in β-thalassemia were evidently higher and lower, respectively than the normal control ($p<0.001$). Thus, it is suggested that the ROS affected considerably in the β-thalassemia minor (Table 4).

2) The GLT_{50} of β-thalassemia have demonstrated evident prolongation ($p<0.001$).

Tabel 4. GLT_{50}, MDA and TAC value for β-thalassemia and normal control.

Parameter	β-thalassemia (n=33)	Normal (n=12)	P value β-thalassemia versus normal
Hb (g/dL)	10.9 ± 2.1	n.d	-
GLT_{50} (sec)	146.3 ± 83.9	31.3 ± 1.4	p<0.001*
MDA serum (uM)	9.62 ± 2.8	4.5 ± 0.9	p<0.001*
TAC serum (nM TE)	5.62 ± 0.72	7.2 ± 0.96	p<0.001*

Hb: hemoglobin, MDA: malondialdehyde, GLT_{50}: glycerol lysis time, TAC: total antioxidant capacity, n.d: not determined, *: showed significantly.

When we see the all of the samples together, and see the correlation between the parameters (Table 5),

3) The MDA and TAC displayed a remarkably significant reverse correlation (r: -0.786, $p<0.001$*). Thus, when lipid peroxidation reflected by MDA is enhanced, the TAC is decreased.

4) The GLT_{50} has considerable correlation with TAC and MDA ($p<0.01$), especially with the former. Thus, GLT_{50} seems to be associated with oxidative state.

5) Hb level has no significant correlation with GLT_{50}, MDA and TAC (not included in the table).

Table 5. Correlation of GLT_{50} and oxidation status.

Parameter	GLT_{50}	MDA	TAC
GLT_{50}	-	r: 0.381 $p<0.01$	r: -0.572 $p<0.01$
MDA	-	-	r: -0.786 $p<0.001$

MDA: malondialdehyde, GLT_{50}: glycerol lysis time, TAC: total antioxidant capacity, r: regression.

CHAPTER IV

Discussion

The patient in this study had hemolytic anemia possibly related to thalassemia, while, the examination for the hemoglobinopathy and gene analysis indicated β-thalassemia minor, or heterozygote (β^0/β) for the new β^0-thalassemia mutation at codon 102 (AAC>ATCAC). The patient's symptom is evidently severer than usual β-thalassemia minor (heterozygote). In such situation, there are two possibilities. One is dominant-type β-thalassemia for this new mutation, and another complication of pregnancy, infection, iron deficiency anemia, *α-globin* gene triplication ($\alpha\alpha\alpha^{anti3.7}$) or others such as membrane disorders. Our patients were not related to infection and pregnancy. They are not in iron deficiency state. The $\alpha\alpha\alpha^{anti3.7}$ was not present. Therefore, possibility of the dominant-type β-thalassemia was considered at first. Most β-thalassemia has decreased amount of β^TmRNA, while, in the dominant-type β-thalassemia, β^TmRNA is normally expressed, and subsequently sufficient amount of β^T-globin is formed, which, however, destroyed immediately after it is translated, possibly by the defect of the formation of globin tetramer or even αβ dimer due to its extreme instability. Generally speaking, β-thalassemia has burden of proteolysis of the normally produced α-globin that has lost the β-globin partner (called free excess α-globin). However, the dominant-type β-thalassemia has extra burden to hydrolyze degraded β^Tglobin in addition to the free excess α-globin, which exceeds the capacity of hydrolysis, and results in the formation of Heinz bodies. The Heinz body deranges the membrane, leading to hemolytic involvement. However, our patients demonstrated no Heinz bodies, and had negligible amount of β^TmRNA, which are consistent with common β-thalassemia minor (Fig. 6). Thus, the possibility of the dominant-type β-thalassemia was completely ruled out.

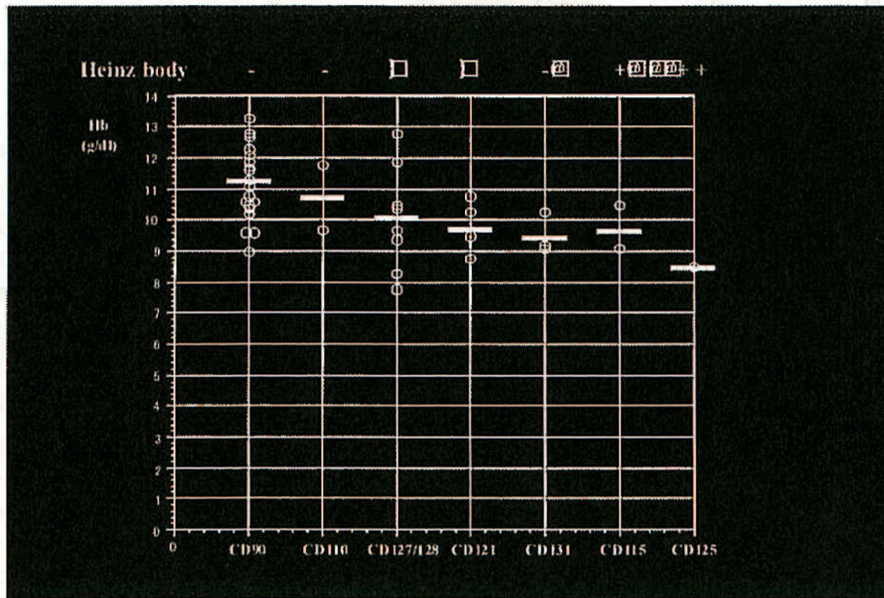

Fig. 6. Dominant-type β-thalassemia (Hb, Heinz Bodies vs Mutation). The left most of codon 90 mutant is not a dominant type but typical β-thalassemia minor, and cited as a reference. Heinz bodies appear with the severity of hemolytic anemia (rightward). (Copywrite permission from Y Hattori, PhD)

The second possibility was red blood cells membrane defect. The presence of the ovalocytes, teardrop cells and occasional stomatocytes implied its possibility (Fig. 7). The SDS-PAGE did not suggest the deficiency of Band 3, P4.1 and P4.2, and that of α-, β-spectrins and Ankyrin looked unlikely to be. The gene analysis for *P4.2* revealed a mutation at codon 142 (GCT>ACT) in exon 3 (heterozygote), or $P4.2_{Nippon}$ whose homozygote gives hemolytic anemia, and most common in the gene abnormalities of Japanese HS. Thus, the double heterozygosity for the β-thalassemia and $P4.2_{Nippon}$ was distinct.

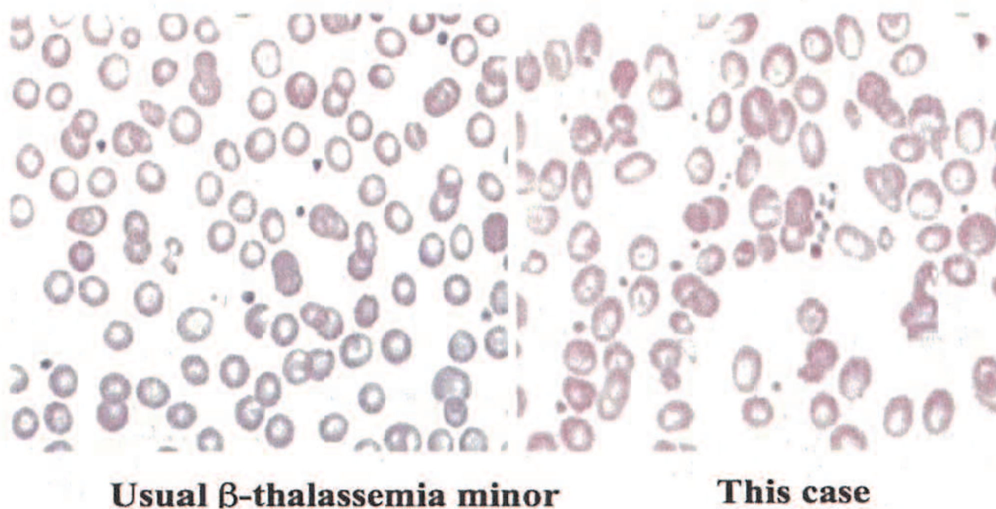

Fig. 7. Aniso-poikilocytosis usual β-thalassemia minor versus our cases.

The P4.2 binds Band 3 and Ankyrin. These three membrane proteins are associated with each other, and when P4.2 or Ankyrin is reduced, Band 3 is also decreased in amount, which is seen in the homozygous state for the $P4.2_{Nippon}$ allele.[26] Tachavanich *et al*[27] and Kedar *et al*[28] using EMA method have reported that patients with HS showed decreased amount of Band 3 other than P4.2, but not for those with only β-thalassemia. Our analysis of the Band 3, P4.1 and P4.2 by SDS-PAGE by Fairbanks method demonstrated no evident difference between patient and controls, meanwhile the gene analysis demonstrated no mutations other than $P4.2_{Nippon}$. Thus, the evident quantitative reduction of P4.2, which was seen in the $P4.2_{Nippon}$ homozygote was not observed in our double heterozygote for β-thalassemia and $P4.2_{Nippon}$.

Several studies have reported that increased oxidative stress in β-thalassemia major is related to an excess of denatured α- or β-globin chain, iron overload and decreased Hb level.[29] The oxidation that occurs mainly in the erythroblasts is expected to cause changes in membrane structure of the erythroblasts and induces premature apoptosis[30,31], leading to the intramedullary hemolysis, or ineffective erythropoiesis. Although thalassemia minor may share the same pathophysiology, the amount of the denatured α- or β-globin chain would be much less than that of thalassemia major, and the clinical pictures often become asymptomatic. However, it was found in our experiments that even thalassemia minor is under enhanced oxidation state that is reflected by higher MDA and lower TAC levels (Table 4). Thus, the red blood cells of β-thalassemia minor are basically in a milieu of decreased redox state. The dominant β-thalassemia that is characterized by hemolytic involvement, demonstrated extraordinary elevated MDA level in our past study. Excess burden of elimination of denatured, highly unstable β-globin in addition to unpaired free α-globin would give rise to increased ROS generation. When slight α-globin excess is complicated as $\alpha\alpha\alpha^{anti3.7}$ on β-thalassemia, the β-thalassemia phenotype is often exacerbated.[32] Thus, the capacity against oxidation of the β-thalassemia minor is limited, and would be vulnerable to the further oxidative stress.

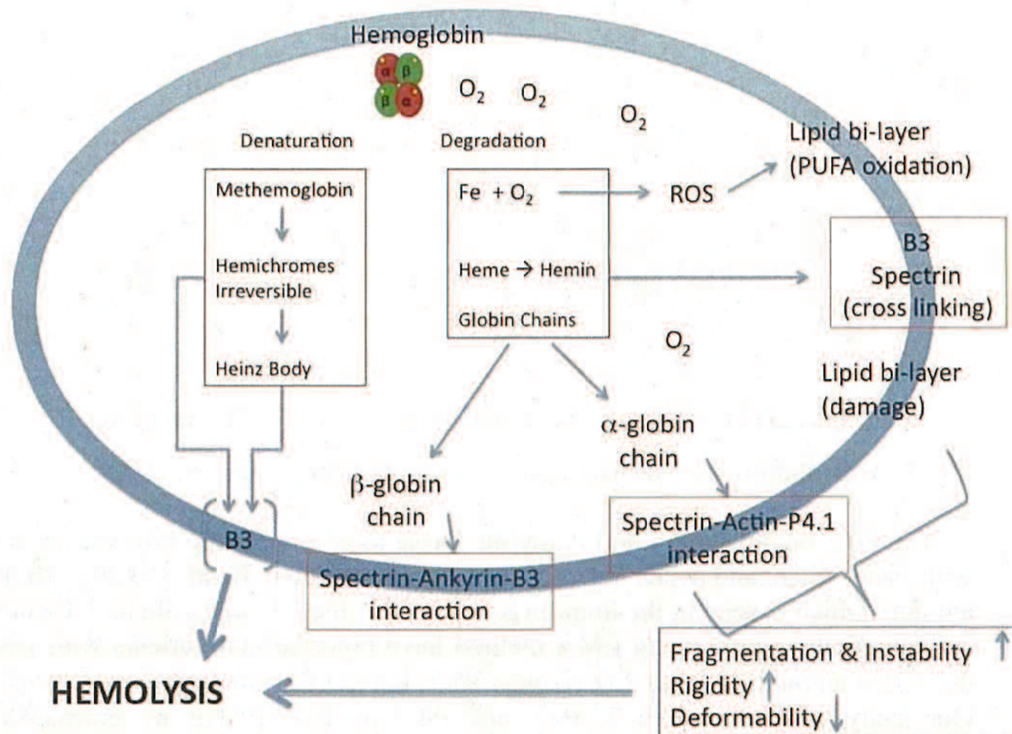

Fig. 8. Mechanism leading to red blood cells membrane dysfunction in hemoglobinopathies. ROS: Reactive Oxygen Radicals, PUFA: Polyunsaturated Fatty Acid, P4.1: Protein 4.1, B3: Band 3. O_2: Oxygen. (Modification from Hemoglobinopathies and red blood cells membrane function. Shinar E, Rachmilewitz AE. Bailiere's Clin. Haematol. 1993;6:p357-369)[42]

The free α-globin in the β^0-thalassemia is extremely susceptible to oxidation, and form hemoglobin thioyl radicals.[33,34] This radical would easily attack the red blood cells cytoskeleton Band 3, oxidize and impair its function. The Band 3 is known for the denatured hemoglobin to attach it and to form Heinz bodies.[35-37] Although, our patients do not have Heinz body nor evident decrease in the amount of Band 3, its function as Band 3 network may be sub-clinically deteriorated by the $P4.2_{Nippon}$, on which oxidative stress by coexisting β-thalassemia was further imposed, and Band 3 network may be functionally broken. Our case represents the first report in Japan of the double heterozygote for the β^0-thalassemia mutation and P4.2 mutation of $P4.2_{Nippon}$ type.

The thalassemia minor demonstrates poikilocytosis, mainly of target cells and decreased osmotic fragility. Therefore, the membrane of thalassemic red blood cells is evidently abnormal, or altered. GLT_{50} is known to have close association with osmotic fragility test where the thalassemic red blood cells are resistant to osmotic lysis.[38] Thus, it is suspected that the membranes of the thalassemic red blood cells are osmotically "rigid". The GLT_{50} is invariably prolonged in thalassemia minor, or heterozygous thalassemia[39,40], and has been in effect used for the screening of thalassemia. We examined here the correlation of GLT_{50} to oxidation markers, or MDA and TAC in β-thalassemia minor. It was highly correlated with both MDA and TAC (Table 5). Therefore, GLT_{50} is very likely to be associated with oxidation. Kahane and Rachmilewitz described that osmotic fragility of thalassemia was normalized by

administration of vitamin E or thiol groups. The resistance to osmotic fragility seems to be caused by oxidative stress leading to cross-linked and rigid membranes.[39] On the other hand, it is reported that both glycerol and water flow across the red blood cells membrane via Aquaporin 3 (AQP3), one of membrane proteins.[41] The excess free α-globin in β-thalassemia, very susceptible to oxidation generates globin radicals, which later reacts more easily with cytoskeletal and membrane proteins, such as AQP3. AQP3 may cause impairment of the function, resulting in delayed entrance of glycerol and delayed hemolysis. This explains the reason for GLT_{50} prolongation in β-thalassemia. Although the cause of the prolonged GLT_{50} in thalassemia still remains uncertain, it may be related to AQP3 denaturation. Further studies on permeability of glycerol as well as water in thalassemic red blood cells are necessary.

CHAPTER V

Conclusion

1. A new frameshift mutation of β^0-thalassemia at codon 102 (AAC>ATCAC) was found.
2. No exacerbating factors of the β-thalassemia including dominant-type was found.
3. Morphological abnormality of red blood cells led the discovery of the co-existence of abnormal membrane protein, or $P4.2_{Nippon}$ (heterozygote) in our patients whose homozygote gives overt hemolysis as HS.
4. The increased membrane oxidation and reduced TAC in β-thalassemia minor (n=33) was shown by elevated MDA and decreased TAC, respectively. Prolonged GLT_{50} is an example of membrane protein damage by the oxidation.
5. Although there is no firm evidence, the aggravation of β-thalassemia in our patients (hemolytic involvement) may be induced by impaired $P4.2_{Nippon}$ (heterozygote) based on the oxidative background of β-thalassemia.

CHAPTER VI

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ORIGINAL ARTICLE

A NEW β^0-THALASSEMIA MUTATION (CODON 102, AAC>A*TC*AC) IN COEXISTENCE WITH A HETEROZYGOUS *P4.2 NIPPON* GENE

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A new β-thalassemia (β-thal) frameshift mutation was found at codon 102 (AAC>ATCAC) in a 17-year-old Japanese male and his 14-year-old sister. Both demonstrated a more severe phenotype than the usual β-thal minor with mild hemolytic involvement. No mRNA derived from the thalassemic allele, or β^T *mRNA, was detected in the sequencing analysis of the whole mRNA (cDNA). However, the* β^T *mRNA from the whole βmRNA was specifically amplified by amplification refractory mutation system (ARMS), and was actually found to be present. Furthermore, quantitative polymerase chain reaction (q-PCR) demonstrated a negligible amount of* β^T *mRNA. Thus, their more severe phenotype was not caused by the "dominant type" β-thal in which a considerable amount of the* β^T *mRNA would be expected. In fact, our proband had a total βmRNA level that was mostly normal. Thus, the cause of a β-thal phenotype by the frameshift mutation was ascribed to the reduced amount of mRNA. We further searched for the cause of their severe phenotype. However, factors that exacerbated the phenotype of β-thal, such as α-globin gene triplication, coexisting iron deficiency and infection were not found. Finally, we noticed that the red cell morphology revealed ovalocytosis and small numbers of stomatocytes that were seen in the hereditary spherocytosis (HS), especially by* P4.2 *mutations. The sequence of the* P4.2 *gene disclosed heterozygous* P4.2 Nippon, *or missense mutation at codon 142 (GCT>ACT) on exon 3, the most common mutation of Japanese HS. Frequent mutations of other membrane proteins, Band 3 and ankyrin that are common cause of HS in the Japanese population, other than* P4.2, *were not detected. When HS by* P4.2 Nippon *develops it is homozygous, and no* P4.2 *protein is observed in*

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hepatosplenomegaly and no jaundice. His 14-year-old sister also had mild anemia, and was studied as well. Written, informed consent was obtained prior to blood sampling.

His CBC data revealed a moderate microcytic anemia with mild hemolytic involvement (Table 1). No iron deficiency was noted. The blood picture exhibited hypochromia and moderate anisopoikilocytosis including ovalocytes. The levels of Hb F (9) and Hb A_2 (10,11) were mildly elevated (5.6 and 4.5%, respectively). Glycerol lysis time (GLT_{50}) was markedly prolonged (12). The isopropanol test (instability test) became positive after only 15 min. incubation, probably due to the increased Hb F level (13). No abnormal hemoglobin (Hb) was separated by isoelectrofocusing (IEF) (14). No Heinz bodies or Hb H inclusion bodies were found. These data suggested the presence of a β-thal abnormality. However, the phenotype was more severe than that of a β-thal minor. His sister also presented similar data, and was suspected of carrying a severe β-thal minor.

TABLE 1 Data of Routine Hematological Examinations, Hemoglobin Screening and Some Genetic Analyses

Parameters	Proband (M-17)	Sister (F-14)	Reference Ranges
WBC (10^9/L)	8.2	5.3	3.9–9.8
RBC (10^{12}/L)	5.26	4.24	4.30–5.70
Hb (g/dL)	10.9	9.0	11.0–14.0
PCV (L/L)	0.35	0.28	0.42–0.52
MCV (fL)	66.7	66.0	82.7–101.6
MCH (pg)	20.7	21.2	28.0–34.6
MCHC (g/dL)	31.1	32.1	31.6–36.6
RDW (%)	25.6	21.6	9.0–15.5
Meltzer index	12.7	15.6	15.0–25.0
Reticulocytes (%)	1.1	1.0	0.2–2.7
Platelet (10^9/L)	35.2	29.1	15.0–45.0
Total bilirubin (mg/dL)	1.7	1.0	0.2–1.2
Direct bilirubin (mg/dL)	0.7	0.4	<0.4
C-reactive protein (mg/dL)	0.05	0.05	<3.0
Haptoglobin (mg/dL)	10.0	<10.0	19.0–170.0
Total iron binding capacity (μg/dL)	297.0	308.0	243.0–430.0
Serum iron (μg/dL)	129.0	158.0	43.0–184.0
Serum ferritin (ng/mL)	120.7	80.0	12.9–301.0
Anisocytosis	moderate	moderate	slight
Poikilocytosis	moderate	moderate	slight
Hb F (%)	6.1	5.6	<1.0
Hb A_2 (%)	4.4	4.5	2.0–3.5
Isopropanol test (seconds)	[+] 15 min.	[+] 20 min.	–
GLT_{50}	243.0	300.0	22.0–55.0
$^G\gamma$ genes (–158, C>T)	[–] (C>C)	[–] (C>C)	C>C
HBS1L (exon 1a: codon 32, C>G)	[+] (G>G)	[+] (G>G)	C>C

NB: No inclusion bodies were found and the IEF test was negative. Neither the proband or his sister carried the $-\alpha^{3.7}$, $\alpha\alpha\alpha^{anti\,3.7}$, $--^{SEA}$ or $--^{FIL}$ mutations.

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Table 2 Primers Used For Polymerase Chain Reaction of *P4.2*, *Ankyrin*, *Band 3* and *P4.1* Genes

Exon	5′ Primer (5′>3′)	3′ Primer (5′>3′)
P4.1 Gene		
1	TAA CCT TTC TAC CCC ACT GCC CAG A	AGT TAT ACC ACC ATC TCC CCG CCT A
2	GAC AGT TCC ACA CTC AGC CTC ACT C	AGG CAC ATA ATC TCG TGG CAC CCT C
3	GGC CTC ATT TCT AGG TGG GTC ACT T	ACT GGG TGT TGG TGC TGT CCT TAG A
4	GTA GAG ACA GAG TCT TGC TGT GTT G	GGT TAT GGG ACA GTC ACT CTA CAC A
5	TAG GAC AGT CAG GGT TCA CTA AAT G	CTA TTG CCT GTC TTT TTG TCC TCC T
6	TTT AAC CAG TAC CAG GTG GCT CTA A	CTA GGG CAG GGT TAG GGA AGT AAG T
7–8	ACT CCT CTT CTT TCC AAG TTT TCA G	CAT GCC TCG TGC TTG TAC TGT TTT C
9	TAT TCC TGT CAT ATC CAT AGT AAA G	ACT GAA TTT TGC TAT GTG AAA GAA T
10	GTT TGG AAG AGA CAC ACT GTA TCG G	TCC TGC CAC TGT ACT CTG TTA AAC T
11	CCC TGT CTCT AAA ACA AAA CAA AAA	TAA TAG GGC ATG TAA GAA ACT ATG T
12	CTT GGC AGA GAT GAG GAA ACT TAC T	AAG CCA CTT GGT AGC CCT AAT CCT T
13A	TGG CTC CTG TCT TCT ATT CCT TTA T	CTA AGT TCT CGC TCT ATA TGT CCC T
13B	CTT CCA CTT ATG CAG GAA GAT GAA G	AGT GTC AAG GCA ACA AAA TAA CAT A
13C	TGG TCC AGT AGA GAA ATA TAA AAG T	TTT TTA CCA GAA ATA CAG TGG ATT G
Ankyrin Gene		
1	ACC AGG AAA GGC AAA TTG TGT AGA T	AAA AAT ATG AAA CTG GCT TCA GCG A
5	TTG TAA TAA TCC CAG GAC GCC AGT C	CTT TGT CAT TAG TCC CTT GCA GAA C
6	AAG AGG GCT TTG GTG GAG GTA GGT C	CTG GGC ATC TGC CAG GGA TTT AGA G
16	CTT CTC CGG GCA AGT GTA TTG ATC T	GAA TAG CGT GAT TAC AGG GCA GGT A
17	CCC TGC TTG TGT GTT GAT ATT GTC A	GGG AAT AAG CAA CCT TTC TGA CTC T
22	AAA AAT GTG TGC GGC TCT AGT TTG G	TAT AGT GCT TGG GCG TGT TGT AAG G
26	GGC ATC TAA AGA GTA TGA TGA GGA C	GAC ACC TTC GTG TGT CAG GAA AAG T
28	TCC CGC TGT ACT TCG TGA TCA TGT C	CCT CAC CCC TTC CCA CTT AAC TCT C
31	TCT GGA TGG AAA GGA TGC TCT CTG T	AGG GTC TTG TCC ACT TTA TCA TCT G
38	ACC CCG AAT GTG CAT GCA TCT CTG T	TAC CCA GGA GCC CAG AAG ACA AAA A
Band 3 Gene		
5–6	CCT GGA CTA TGC CTG ATG AAC TAA C	AGG ACG TTG TAG ATG TAA GTG GGT G
P4.1 Gene		

(72°C, 30 seconds) and final extension (72°C, 10 seconds) followed by cooling.

Quantification of the Wild Type βmRNA and β^TmRNA by Quantitative Polymerase Chain Reaction (q-PCR)

The cDNAs of βmRNA and β^TmRNA were prepared using the wild type- and mutation-specific primers above, and used as the standard in this experiment. However, in order to get pure standard they were cloned from each βcDNA of the proband by a TA-cloning method using the pGEM-T Easy Vector Kit (Tamara Bio Inc.) and the Pure Plasmid Yield™ Syst Promega Miniprep Kit (Promega, Tokyo, Japan) for purification. The cDNA of glyceroaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as internal control was also prepared using primer pairs 5′-GGC CAT GCC AGT GAG CTT CCC GTT C-3′ (forward) and 5′-CTG CAC CAC CAA CTG CTT AGC ACC C-3′ (reverse). It was also cloned and used as above. The best clone was selected by DNA sequencing among several clones. The amount, or copy numbers of βmRNA, β^TmRNA and GAPDH mRNA in red cells of the proband and normal control were measured by q-PCR using the LightCycler® 480 SYBR Green I Master (Roche, Tokyo, Japan).

Red Blood Cell Membrane Protein Analysis by Sodium Dodecilsulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Red blood cell (RBC) ghosts were prepared from the blood by the hypotonic lysis procedure of Dogde *et al.* (19). The ghosts were electrophoresed on SDS-PAGE (12.5%) according to Laemmli (20) and on the 3.0-10.0% gradient gel by the Fairbanks *et al.* (21) buffer systems. The gels were stained with Coomassie Brilliant Blue, and the amount of the separated proteins or Band 3, *P4.1*, *P4.2* and actin as an internal control was measured on the digital picture by Multi Gauge Ver3.X software (Fuji Film Science Lab, Tokyo, Japan).

RESULTS

The DNA sequencing of the β-globin gene showed heterozygosity for the abnormal allele that had two nts (TC) inserted at codon 102 (AAC>A*TC*AC) on exon 2 (Figure 1a). This frameshift is expected to give rise to a β^0-thal trait, while no such mutation was detected in the sequencing of the whole βmRNA (βcDNA) (Figure 1b). The amplification refractory mutation system (ARMS) using a mutant-specific primer barely demonstrated the β^TcDNA (Figure 1c), which was not found in the normal control. Thus, no cross annealing to the wild type allele was seen in the mutant-specific primer. The β^TmRNA in the proband's red cells determined by q-PCR comprises only 0.01% (0.182/

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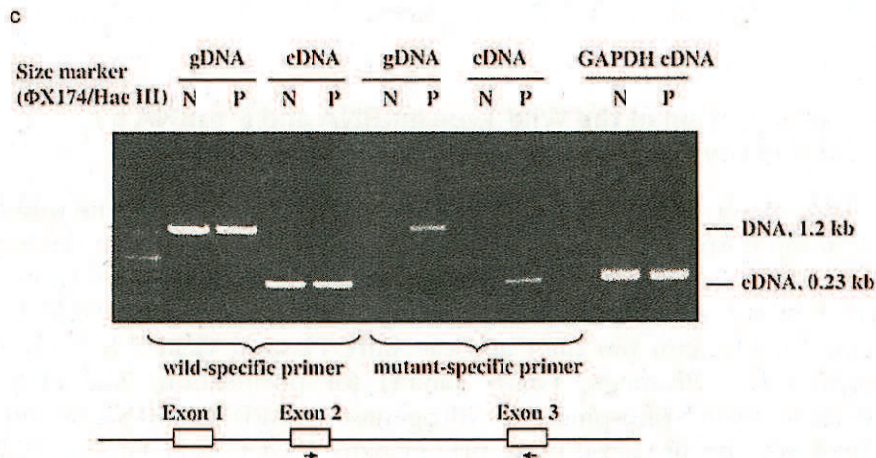

FIGURE 1 (Continued).

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[1438+0.182]) of the whole βmRNA, while it is absent in those of the normal control (Table 3). In addition, the amount of the βmRNA of the patient was 49.5% (1438/2904) of that of normal control (Table 3). The alleles of α^+-thal ($-\alpha^{3.7}$) and α triplication ($\alpha\alpha\alpha^{anti\ 3.7}$) were not present. The α^0-thal alleles ($--^{SEA}$ and $--^{FIL}$), frequent in Japanese, were also not found.

The DNA sequencing of membrane protein P4.2 showed a heterozygous missense mutation at codon 142 (GCT>ACT) in exon 3, a replacement of alanine by threonine (Figure 2b). This abnormality has been reported as *P4.2 Nippon* (16,22). The homozygosity for *P4.2 Nippon* gives rise to HS, and is common in Japanese HS (16,22,23). The sister of the proband also carried the same β-thal and P4.2 mutations. We did not find any mutations for ankyrin and Band 3 in our sample (data not shown). The SDS-PAG electrophoretogram of the proband's membrane protein exhibited a normal amount of Band 3, P4.1 and P4.2 that were corrected

TABLE 3 The Level of βmRNA of the Normal Control and Proband, Measured by Quantitative Polymerase Chain Reaction

Sample	Normal Control		Proband	
	Copy Number		Copy Number	
Primer	Actual	Corrected[a]	Actual	Corrected[a]
GAPDH cDNA	792	–	1210	–
Wild type cDNA	2300000	2904	1740000	1438
Mutant cDNA	0.0171	0.00002	220	0.182

[a] The wild type and mutant βmRNA (βcDNA) were corrected by GAPDH (internal control).

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The routine hematological examination revealed marked aniso-poikilocytosis, extremely reduced haptoglobin level and slight indirect hyper-bilirubinemia, suggesting mild hemolytic involvement, or a severe form of β-thal minor. His sister is exactly the same. Here, the possibility of a dominant type of β-thal arises. The β^TmRNA in dominant-type β-thal should substantially remain. For example, in case of a 6 bp deletion (–TGGCTA) at codons 137, 138 and 139 that reveals dominant-type, the β^TmRNA comprises 40.0% of the total βmRNA (5). Since the amount of β^TmRNA in our proband was negligible, the possibility of the dominant-type β-thal is very unlikely. Although most Japanese thalassemias are of the minor type (28), their clinical severity sometimes varies depending on the presence of other factors (2,3,29). Our proband revealed moderate anemia with hemolytic involvement, and his blood smear demonstrated moderate aniso-poikilocytosis compatible with severe type of β-thal minor (Figure 2a). These findings suggested that there might be other factors that deteriorated the clinical manifestation of the proband. Many factors can affect the severity of the β-thal minor other than dominant-type β-thal, such as a coexisting α-triplication, iron deficiency, abnormal Hb or others (1–3). The gap-PCR assay for α-triplication was negative (30). Our proband also did not have iron deficiency or abnormal Hb. Thus, we searched for another factor that could aggravate the phenotype.

Peripheral blood smears showed frequent ovalocyte and occasional stomatocytes. These morphological pictures are reminiscent of HS and the abnormality of P4.2, one of the membrane proteins. This abnormal morphology is rarely seen in other membrane abnormalities, such as α- and β-spectrins, ankyrin and Band 3. In addition, considerable numbers of Japanese HS cases are due to P4.2 mutations, in particular, *P4.2 Nippon* (22,31). In effect, the *P4.2* gene sequencing result of our proband revealed the heterozygous *P4.2*

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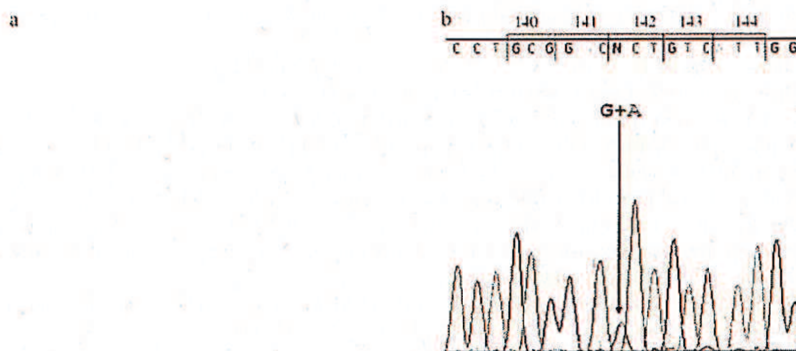

FIGURE 2 (a) Peripheral bloods smear. The hypochromic cells and codocytes (target cells) are predominant, and most of them tend to be oval. Tear-drop cells and schistocytes intermingle with them. (b) Sequencing of the protein *P4.2* gene. A missense mutation (GCT>ACT), indicated by an arrow, was found at codon 142 on exon 3 of the *P4.2* gene.

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ORIGINAL ARTICLE

Oxidation Status of β-Thalassemia Minor and Hb H Disease, and Its Association with Glycerol Lysis Time (GLT_{50})

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Abstract

β-Thalassemia (β-thal), especially β-thalassemia major (β-TM), is reported to be related to reactive oxygen species (ROS) and enhanced oxidation status. It is reflected by increased malondialdehyde (MDA), by membrane lipid peroxidation and decreased by the newly developed total antioxidant capacity (TAC). However, there is less evidence for β-thal minor and Hb H (β4) disease on its association with oxidation status. On the other hand, hemolysis by glycerol lysis time (GLT_{50}) is invariably prolonged in thalassemia. The reason for the prolongation of GLT_{50} is not well understood. The aim of this study was to investigate the oxidation state in β-thal minor and Hb H disease and to find out the association of the oxidation with the prolongation of GLT_{50}. Blood samples from 39 subjects (33 with β-thal minor, six with Hb H disease) were collected from individuals living in Japan. The clinical screening tests and molecular identification of the thalassemias were performed. Malondialdehyde and TAC were measured using spectrophotometric analyses. In β-thal minor and Hb H disease, the plasma MDA level was significantly elevated and the TAC reduced. A highly reversed correlation between MDA and TAC was noted. Their GLT_{50} levels were evidently prolonged, and the GLT_{50} has significant correlations with MDA and TAC. β-Thalassemia minor and mild Hb H disease are evidently in a milieu of reduced redox state, and GLT_{50} prolongation in β-thal minor and Hb H disease has a close correlation with the oxidation state, possibly by oxidative impairment of the membrane protein of the red cell.

Keywords

β-Thalassemia minor, glycerol lysis time, malondialdehyde, oxidation, total antioxidant capacity

History

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Introduction

β-Thalassemia (β-thal) is a hereditary hemoglobinopathy, and common in tropical and subtropical areas. In Japan it is less common and mostly β-thal minor, a clinically milder form. Most thalassemias are caused by defective production of either α- or β-globin chains that form Hb tetramers (α2β2). Another globin produced from intact genes is normally produced in half the amount of a normal person. The net reduction of hemoglobin (Hb) tetramer brings about microcytic red cells (microcytosis) that are universally seen in all types of thalassemias. When the imbalance is extreme, such as in β-thal major (β-TM), it is clinically of a more severe type, the normally produced α-globin denatures in the erythroblasts and eventually lets the red blood cells (RBCs) undergo hemolysis. These hemolytic involvements are not seen in β-thal minor, presumably because the excess globin normally produced in any amount would completely undergo hydrolysis by the proteolytic enzymes in the erythroblast.

On the other hand, the RBCs cells in patients with β-TM are exposed to a high oxidation state in the blood. The oxidation of the denatured globin in erythroblasts or RBCs causes release of free iron. In turn, free iron, by non enzymatic reaction, reacts with oxygen and releases reactive oxygen species (ROS) that initiate the process of oxidation of lipids and proteins the of the red cell membrane, and render the membrane rigid. Membrane rigidity may cause the RBCs to be susceptible to lysis in the circulatory system (1). However, there is still very little known about the association of the oxidation states for β-thal minor, which actually has no hemolytic involvement. Oxidation of RBCs is often measured by the malondialdehyde (MDA) method. Malondialdehyde is a natural organic compound and one marker for oxidative stress. In RBCs, ROS degrade polyunsaturated lipid membrane to form MDA (2,3) whose levels are elevated in β-TM

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Discussion

Several studies have reported that increased oxidative stress in β-TM is related to an excess of denatured α- or β-globin chain, iron overload and decreased Hb level (10). The oxidation that occurs in the erythroblasts is expected to cause changes in membrane structure of the erythroblasts and induces premature apoptosis (4,11), leading to intramedullary hemolysis, or ineffective erythropoiesis. Although β-thal minor may share the same pathophysiology, the amount of the denatured α- or β-globin chain would be much less than in β-TM, and the clinical picture often becomes asymptomatic. However, in our experiments, it was found that even the oxidation status of β-thal minor was under enhanced, which was reflected by higher MDA and lower TAC levels (Table 1). Our Hb H disease cases were all compound heterozygotes for $-\alpha^{3.7}$ and the Southeast Asian α^0-thal ($--^{SEA}$) type that tends to manifest a milder clinical severity (12,13). The RBCs of β-thal minor and mild Hb H disease are thus not only in a milieu of decreased redox state. In addition, even slight oxidative stress may lead to precipitation of Hb that is seen in the *in vitro* addition of brilliant cresyl blue (BCB), mild oxidant in Hb H disease. In our study, the dominant β-thal that is characterized by hemolytic involvement demonstrated extraordinary elevated MDA levels. Excess burden of elimination of denatured, highly unstable β-globin, in addition to unpaired α-globin, may give rise to increased ROS generation.

β-Thalassemia minor demonstrates poikilocytosis, mainly of target cells and decreased osmotic fragility. Therefore, the membrane of thalassemic RBCs is suggested to be altered. The GLT_{50} is known to have close association with the osmotic fragility test where the thalassemic red cells are resistant to osmotic lysis (14). Thus, it is suspected that the membranes of the thalassemic RBCs are osmotically 'rigid.' The GLT_{50} is invariably prolonged in β-thal minor or heterozygous β-thal (8,9), and has been used for the screening of thalassemia. We examined the correlation of GLT_{50} to oxidation markers, or MDA and TAC in β-thal minor and Hb H disease. It was highly correlated with both MDA and TAC (Table 2). Therefore, GLT_{50} is very likely to be associated with oxidation. Kahane and Rachmilewitz (14) described only 50.0% of thiol groups in thalassemia compared to normal membranes and osmotic fragility was normalized by administration of vitamin E. The authors concluded that resistance to osmotic fragility was caused by oxidative stress leading to cross-linked and rigid membranes.

It was reported that both glycerol and water flow across the red cell membrane *via* Aquaporin 3 (AQP3) that lies on the red cell membrane as an oligomeric integral water channel protein (WCP) (15). The excess of free α-globin in β-thal is very susceptible to oxidation and the generation of globin radicals is well established. The latter react more easily with cytoskeletal and membrane proteins rather than penetrate into the lipid bilayer. McMillan et al. (16) reported that the Hb radical is less lipophilic and difficult to penetrate the lipid bilayer. However, interaction between globin radicals and membrane proteins such as AQP3 may cause impaired function from the membrane surface. It might be assumed that AQP3 in thalassemic RBCs denatured by oxidation of its thiol groups, resulting in delayed entrance of glycerol and delayed of hemolysis. The presence of up to six thiols in APQ (16) make, besides intramolecular adducts, intramolecular reaction possible between thiols forming cystine from two cysteins with essential impact on protein conformation and function (16–18).

Similar to Kahane and Rachmilewitz (14), Freisleben et al. (19) reported changes in thiol status in RBC membranes indicating oxidation processes and rigidified environment in membrane proteins. Concomitantly, changes in lipid domains of the RBC membranes of thalassemia patients were detected (20). In β-thal minor patients, isolated RBC membranes exhibited increased order parameters, *i.e.*, increased rigidity (20) correlated with oxidative stress (21). Thus, it may be assumed that the membrane of thalassemic RBCs undergo not only the oxidation by membrane lipid peroxidation suggested by increased MDA level, but also impairment of membrane protein that gives rise to delayed water influx through the membrane, delayed hemolysis, and prolonged GLT_{50}.

However, other WCPs such as Aquaporin 1 (AQP1) that is specific to water channels may still be intact. Hence, GLT_{50} might be more sensitive to thalassemia than to osmotic fragility tests (22). Although the cause of the prolonged GLT_{50} in thalassemia is not certain, it may be related to AQP3 denaturation. Further studies on permeability of glycerol as well as water in thalassemic RBCs are necessary.

The correlation of Hb level as a marker of phenotypic severity with MDA and TAC was not significant. The reason is unknown, but there may be various factors that influence the levels of MDA as discussed in (23) and TAC which was reassessed in (24).

Declaration of interest

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

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Filipino-type β^0-thalassemia has 116 kb Deletion: Its Correct Breakpoints and Five Cases Found in Japan

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Abstract Filipino-type β-thalassemia has been reported to have about 45 kb deletion involving *β-globin* gene and L1 element 3' to it by Southern blot and inverse PCR analyses. However, accurate determination by Junction PCR assigned the 3' breakpoint in a New L1-like sequence far downstream from the L1, or 113 kb apart from the *β-globin* gene. Thus, the correct deletion is 116.4 kb instead of 45 kb. The 3' flanking region from the junction of the Filipino deletion has complete homology with the New L1-like sequence that has 80% homology with the latter half of the L1 element. The 5' brbreakpoint is exactly the same as reported in the past, and it is not related to the L1 element at all. Thus, Filipino deletion arose as an illegitimate recombination. In addition, the corrected deletion elegantly conform to the results of the past Southern blot analysis which has not been completely explained by the 45 kb deletion. We have found five unrelated cases with Filipino-type β-thalassemia in Japan where it has not been previously discovered. All cases were associated with recent immigration from Philippine, and childbearing between Filipinos and Japanese. Thus internationalization is altering the mutation spectrum of β-thalassemia in Japan. The same is noted in HbE that is endemic in the Southeast Asia.

Key words: β-thalassemia, Filipino-type, PCR, L1 element, breakpoint

Introduction

Filipino-type (FIL) β-thalassemia (β-thal), the most common type of β-thal mutation among Filipino was first reported in 1993[1] and characterized by Southern blot analysis its deletion to be about 45 kb beginning approximately 1.5 kb 3' to the *δ-globin* gene.[2-4] However, the 3' breakpoint remained unresolved by the Southern blot analysis. Waye et al. challenged to solve this issue by inverse PCR, and disclosed the sequence around the junction of the recombination.[1] There are a number of L1 and L1-like elements at the 3' of *β-globin* gene.[5] The FIL β-thal sequence by Waye et al.[1] exhibited relatively high homology with the latter, and they assigned the 3' breakpoint in the L1-like repeat. The deletion, thus, was determined as 45 kb. However, there were several mismatches, and they casted some doubt of this determination. Meanwhile, Dimovski, A.J. et al.[3] suspected by Southern blot analysis that the deletion may have extended more than 110 kb. Thus, the exact site of the 3' breakpoint remained undetermined.

Recently, we have analyzed a deletion-type β-thal sample using junction PCR.[6] The 5' and 3' flanking sequences from the junction of our sample were actually the same as those of Waye,[1] and it was likely to be FIL β-thal. However, our sequencing analysis was performed longer than that of Waye, and the 3' breakpoint we assigned was not in the

in triplicate, and standardized by that of another single gene, or *Glucuronyltransferase* gene.[7] Thus, the normal or non-deleted segment gave the ratio of approximately "1", while the hemizygous segment, "0.5". The other four cases were measured at only the *β-globin* gene to see whether gene deletion was present or not.

Junction PCR[8]

We applied for the first case junction PCR which was developed in our laboratory to determine the correct region of the deletion. Briefly, the patient's DNA and pUC 18 DNA were digested by eight types of restriction enzyme (*Hind* III, *Sph*I, *Pst*I, *Xba*I, *Bam*H I, *Kpn*I, *Sac* I, and *Eco*R I). Each of them was ligated to pUC18 vector digested with the same enzyme. After ligation, DNA was subjected to gap PCR using M13 common primer and site-specific primers, prepared at the possible junction that was estimated in the gene dosage analysis above. An abnormal band of the PCR product, if detected in agarose gel electrophoresis, was excised, recovered, and subsequently subjected to DNA sequence analysis by autosequencer (3130 Genetic Analyzer, Applied BioSystems Japan, Tokyo). The 5' and 3' breakpoints were searched for in the Genome-Net database (http://www.genome.jp/).

Gap PCR

Gap PCR by PCR primer pair newly prepared across the deletion and subsequently more specific to this deletion, further confirmed the result. The primers for the Gap PCR are as follows: forward: 5'-GTAAATGAGTAAATGAAGGAATGAT-3'and reverse 5'-TGTGATTTGGCTCTCTTCTTGTCTA-3'. The PCR was conducted initially with 4 min of denaturation at 94 degrees, and followed by 38 cycles of 30 sec of denaturation at 94 degrees, 30 sec of annealing at 60 degrees, and 1 min of extension at 72 degrees. This gave 920bp fragment for the FIL β-thal. The gap PCR is infeasible for normal allele, because it is too long for PCR analysis. Thus, four other Filipino cases as well as the 1st case were subjected to the gap PCR.

Results

Sequencing analysis

Whole *β-globin* gene was analyzed by direct sequencing. However, no mutations were found.

Gene dosage analysis

DNA of the β-thal allele was present from LCR to *δ-globin* gene, but deleted at the *β globin* gene and its 3'flanking region (Fig. 1). Therefore, the 5' breakpoint of this deletion was expected to be lie within 1.5kb between *δ-globin* gene and inter δ-β region.

Junction PCR

Five specific primers were designed within the possible area of the 5' breakpoint, or about 1.5 kb between the *δ-globin* gene and the inter δ-β region to perform Junction PCR. The PCR was carried out between the five primers and M13 common primer outside of the multi-cloning site (MCS) of pUC18 vector. Thus, all 40 kinds of enzymes (8 kinds)-primers (5 sets) combinations, were employed for the PCR. An abnormal band was detected in one of the PCRs on the *Hind* III digest, and its DNA sequence was analyzed. Comparison across the deletion of the *β-globin* gene cluster is presented (Fig. 2). The upstream flanking sequence from the junction (5' breakpoint) of our FIL β-thal allele has complete homology with the sequence between *δ*- and *β-globin* genes and the downstream sequence from the junction (3' breakpoint) has complete homology with the new L1-like sequence except for only one mismatch which may be polymorphism at 17th nucleotide downstream from the junction. The 3' flanking sequence from the junction has high, but relatively less homology with the L1-like sequence where the 3' breakpoint was once believed to be present. Thus, the 5' and 3' breakpoints were assigned at 4.3 kb upstream and at 112.1 kb downstream from the cap site of the *β-globin* gene, respectively (116.4 kb deletion).

The expected abnormal restriction fragments derived from our allele of FIL β-thal were calculated, and compared with those reported in the past (δ IVS-II as a probe): *Ava*II 3.6 kb by ours (3.7kb by Dimovski,[1] 4kb by Motum[2]), *Bgl*II 8.5 (nd, 8.6), *Eco*RI 8.8 (nd,

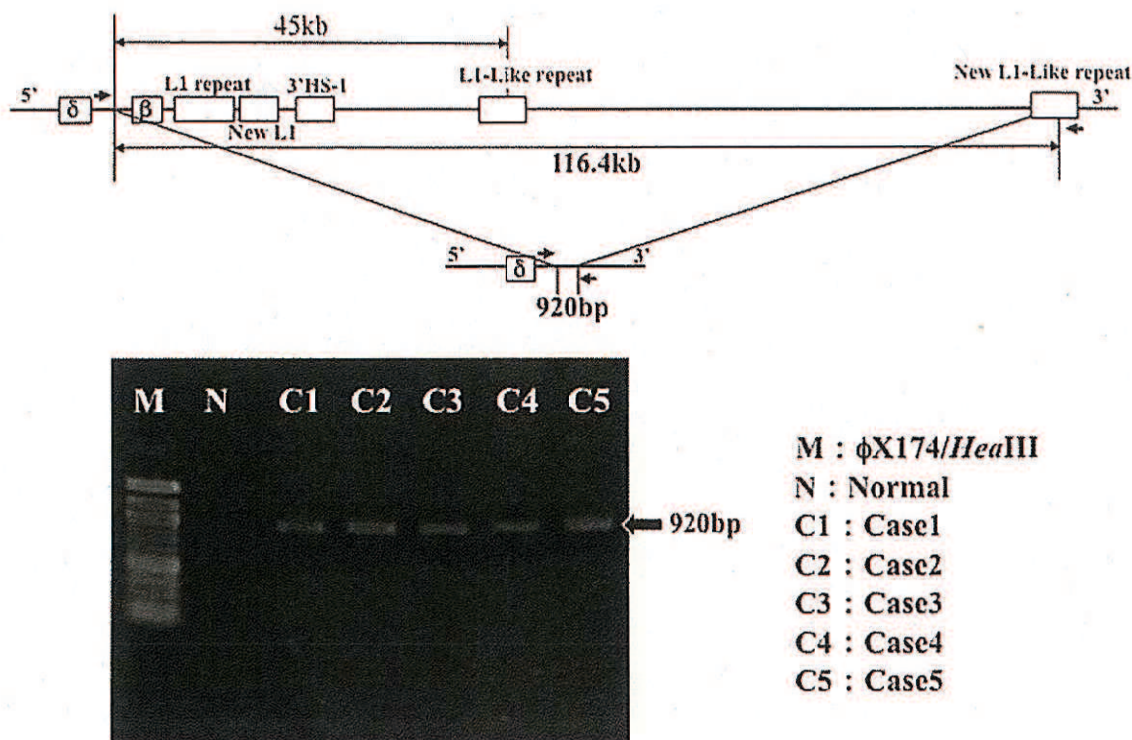

Fig. 3 Gap PCR for FIL deletion.
The FIL β-thal was confirmed by FIL-specific gap PCR that gives 920 bp product. It was identified not only in case 1 but also in cases 2-4. The gap PCR for normal allele (116 kb) is too long for PCR to be successful.

8.6), *Eco*RV 8.6 (8.5, 8.7), *Hind*III 16.0 (16.5, 16), *Nco*I 7.4 (nd, 7.4), *Xba*I 3.6 (3.7, 4.3). Only *Bam*HI fragment is different by 20.8 (11, 9.8).

Gap PCR

The Gap PCR specific to FIL deletion gave the specific band of 920 bp in all the samples (cases 1-5) (Fig. 3). They were, thus, all carriers of FIL β-thal chromosome.

Discussion

We started the determination of the deletion, without taking account of FIL β-thal. The deleted portion of the chromosome, or hemizygous portion, has one copy less than the normal diploid, and gives decreased gene dosage. It was determined with reasonable accuracy by quantitative PCR, and a rough estimation of the large deletion was obtained. The subsequent junction PCR is based on the probability to catch suitable abnormal fragments by gap PCR. In order to increase the possibility, several primers as well as eight kinds of endonuclease were employed, because one of these recognition sites appears every 500-1,000 bp on average in a genome. Usually, one or a few abnormal bands may be obtained in the junction PCR, and their direct sequencing discloses the both 5' and 3' breakpoints at a time.

The 5' and 3' sequences at the junction of the FIL β-thal allele have complete homology except for a single polymorphism with 3'*δ*- and 3'*β-globin* genes throughout the stretch of more than 200 bp and 120 bp, respectively. This high accordance indicates that both breakpoints are true. The results of the past Southern blot analysis are elegantly explained by our breakpoints.[4] The sequencing analysis was once performed by Waye using inverse PCR for the *Ava*II fragment from the FIL β-thal allele.[3] They were successful, but the lack of the genomic database in those

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SHORT COMMUNICATION

IDENTIFICATION OF A NOVEL MUTATION IN THE β-GLOBIN GENE 3′ UNTRANSLATED REGION [+1,506 (A>C)] IN A JAPANESE MALE WITH A HETEROZYGOUS β-THALASSEMIA PHENOTYPE

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β-Thalassemia (β-thal) is characterized by the absent or reduced production of β-globin chains. The precise molecular lesion that causes decreased β-globin synthesis in β^+-thal is difficult to predict when mutations occur in the locus control region (LCR), the promoter, the introns or 3′ untranslated regions (3′ UTRs). Among them, the role of the 3′ UTR of β-globin gene in mRNA stability is poorly understood, mainly due to very few cases that have mutations in this region. So far, only three mutations have been reported in the 3′ UTR of β-globin gene. Although, it is speculated that some of these reported mutations could be associated with mRNA stability, the precise molecular basis still remains unclear. We report here a novel mutation in the β-globin gene 3′ UTR [+1,506 (A>C)] in a 31-year-old Japanese male with hematological parameters suggestive of heterozygous β-thal. Further functional studies on this novel mutation reported here, may help in understanding of the regulation and expression of the β-globin gene and its products.

Keywords β-Thalassemia (β-thal), Novel mutation, 3′ Untranslated region (3′UTR)

β-Thalassemia (β-thal) is a genetically heterogeneous group of disorders characterized by the absent or reduced production of β-globin chains. The free α-globin chains accumulate and form inclusions that damage the cell membrane and cause ineffective erythropoiesis. Reduced production

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TABLE 1 Primers Used for DNA and cDNA Direct Sequencing and Semi-Quantification of mRNA

βF	5′-AGT AGC AAT TTG TAC TGA TGG TAT GG-3′
βD	5′-TTT CCC AAG GTT TGA ACT AGC TCT T-3′
β57	5′-TCT GTC CAC TCC TGA TGC TGT TAT GG-3′
β53-2	5′-CCA GTT TAG TAG TTG GAC TTA GGG A-3′
Q1506F	5′-CCC TGG CCC ACA AGT ATC ACT A-3′
Q1506r	5′-CCC TTC ATA ATA TCC CCC AGT TT-3′
N1506A probe	5′-VIC-AAT TTC TAT TAA AGG TTC CTT TG-MGB-3′
N1506C probe	5′-FAM-TTC TAT TAA CGG TTC CTT TG-MGB-3′

DNA Sequencing

The PCR fragments were purified then directly sequenced by using the BigDye® Terminator cycle sequencing kit version 1.1 on ABI PRISM™ 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan). The same primers as mentioned above (Table 1) were used for the sequencing analysis.

Hydrolysis Probe Method (Semi-Quantification of mRNA)

The purified cDNA is semi-quantitated by a hydrolysis probe method using the TaqMan MGB SNP Kit (Applied Biosystems). The PCR amplifications were carried out on a Light Cycler™ (Roche Diagnostics K.K., Tokyo, Japan) using LightCycler® 480 Probes Master reagent (Roche Diagnostics). Q1506F and Q1506r were used as primers and N1506A and N1506C were used as probes (Table 1). Cycling conditions were as follows: initial denaturation (95°C, 10 min.), followed by 55 cycles of denaturation (95°C, 10 seconds), annealing (60°C, 30 seconds), extension (72°C, 1 second) and cooling (40°C, 10 seconds).

The patient, a 31-year-old Japanese male, was referred to us from a regional hospital for further evaluation of microcytosis. An automated hematology analyzer (XE-2100; Sysmex, Hyogo, Japan) was used for complete blood count. This patient had mild anemia (Hb 11.8 g/dL) with a normal RBC count (5.54×10^{12}/L) and microcytosis (MCV 66.9 fL). His serum ferritin level was normal (147 μg/L). These results prompted further hemoglobinopathy screening tests that showed elevated Hb A_2 (5.2%) (5), normal Hb F level (0.7%) and prolonged glycerol lysis time (GLT_{50}, 79 seconds; normal reference range: 35–55 seconds) (6).

His genomic DNA was sequenced using specific primers to amplify the β-globin gene (–500 to +2000 bp). This analysis revealed a rare point mutation in the 3′UTR that has never before been published in the literature (Figure 1). This novel point mutation was identified in the 3′UTR [+1,506 (A>C) or 32 bases downstream of the termination codon of the β-globin gene]. There was no sequence abnormality in other parts of the β-globin gene. We confirmed that the mutation was not a polymorphism by the

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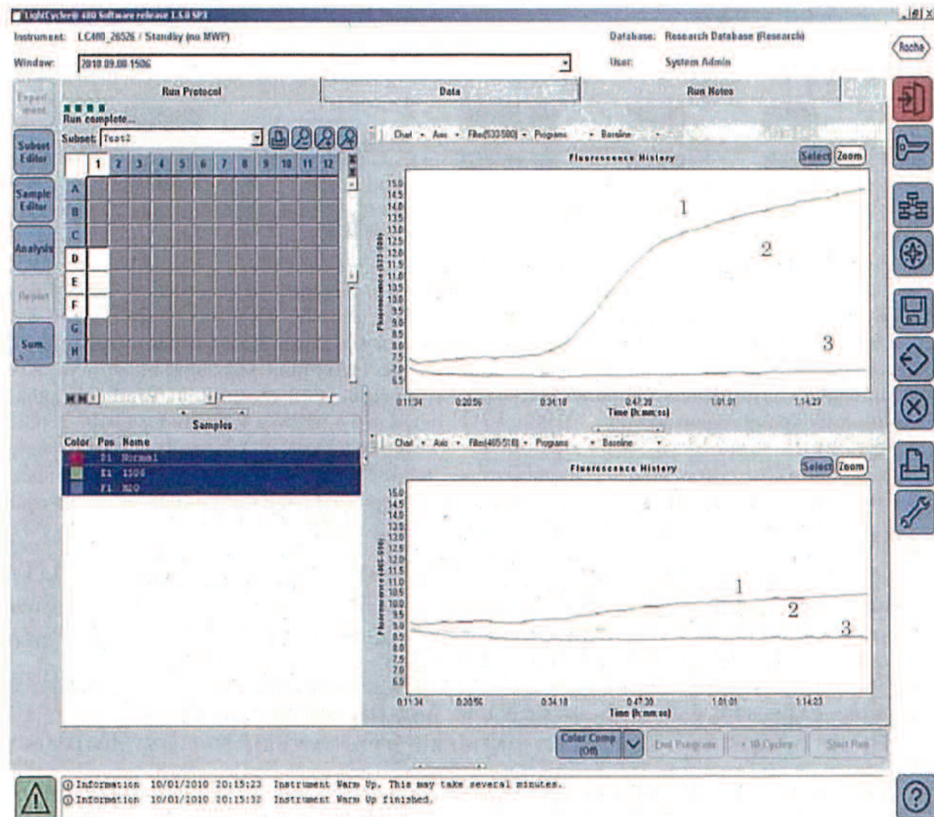

FIGURE 3 The result for the semi-quantification analysis of patient's mRNA. The normal (1), patient (2) and negative control (water) (3), were monitored. When the wild allele-specific probe was used (upper light), fluorescence was obtained in normal (1) and patient (2). However, when the mutant-specific probe was employed (lower right), no fluorescence appeared in the both normal (1) and patient (2). This result demonstrated the absence of mutant mRNA in the patient.

phenotype. Reduced production of β-globin chains is predictable when mutations occur in β-globin gene exons, splice site consensus sequences or promoter regions.

With few exceptions (7–9), stability elements are positioned in the 3′UTR where they are protected from disruption. In this context, it was widely anticipated that 3′UTR mutations were likely to impact β-globin mRNA levels by adversely affecting its stability. This stability is determined by many proteins that are known to affect mRNA stability and protein expression (10–13) through mechanisms that involve mRNA capping, polyadenylation and 3′ end processing.

Three earlier mutations have been reported in the 3′UTR. One such mutation is +1,480 (C>G) in PTB (polypyrimidine tract-binding protein)

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SHORT COMMUNICATION

THE +1,506 (A>C) MUTATION IN THE 3′ UNTRANSLATED REGION AFFECTS β-GLOBIN EXPRESSION

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*The 3′ untranslated region (3′UTR) is known to be important to mRNA stability but the stabilization mechanism on the β-globin gene is not fully elucidated. We speculated in our previous report that +1,506 (A>C) mutation (HGVS nomenclature: *32A>C) on the β-globin 3′UTR causes β-thalassemia (β-thal) in order to destabilize the mRNA. To investigate further, we studied the expression efficiency for the mutation with a luciferase assay. We made recombinant pGL4.74 vectors in which the luciferase 3′UTR was replaced with the wild-type and mutant 3′UTR of the β-globin gene. For a comparison experiment, recombinant vectors were made not only for this mutation but also six other mutations in the β-globin 3′UTR which bring about β-thal or affect mRNA stability.*

The +1,506 mutation led to a 30.0% lower protein expression than normal in this assay. We concluded that this mutation destabilizes mRNA and consequently decreases the β-globin amount to finally cause β-thal. Our study highlights the crucial area of β-globin 3′UTR for protein expression.

Keywords β-Thalassemia (β-thal), β-Globin gene 3′ untranslated region (3′UTR), β-Globin expression

Expression of human globin genes is a highly regulated process. Transcriptional and post transcriptional controls provide a stable expression of globin gene mRNA. These controls dictate the high level, erythroid

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(72°C, 2 min.) and final extension (72°C, 5 min.). These amplicons and the pGL4.74 vector were excised by double digestion using *Xba*I and *BamH*I-HF (New England Biolabs Japan, Tokyo, Japan) restriction enzymes. Then the vector was subjected to ligation with wild type and mutant 3′UTRs, respectively.

Mutagenesis

The excess nts in recombinant pGL4.74 were excised by Mutagenesis Kit (KOD-PLUS Mutagenesis Kit TOYOBO, Osaka, Japan) using B3′UTR-iPCR-U and B3′UTR-D primers (Table 2) under conditions recommended by the manufacturer. In addition, six other mutations in the β-globin 3′UTR were also introduced by mutagenesis into the pGL4.74 construct with the wild-type β-globin 3′UTR (Figure 1). These mutations were known to cause β-thalassemia (β-thal) or β-mRNA instability. They are +1,480 (C>G) (*6C>G) (7), term codon +47 (C>G) (*47C>G) (8), 13 nt deletion at +1,565>+1,577 [*91-*103(del)] (9,10), +1,570 (T>C) (*96T>C) (11), H122 (*67-*72, GGGGGA>AAGCTT) and H124 (*73-*80, TATTAT>AAGCTT) (2). The primers for each mutagenesis are shown in Table 2. Subsequently, recombinant pGL4.74 vectors were transformed and cloned. They were further subjected to a large-scale culture, and purified by ultracentrifugation to get the plasmid with various mutations (12).

TABLE 2 The Mutations and Primers Used for the Preparation of the Mutagenesis

Mutation	HGVS Nomenclature	Primers	Primer Sequences (5′>3′)
		B3′UTR-iPCR-U	TTA CTG CTC GTT CTT CAG CAG GCG C
		B3′UTR-D	GCT CGC TTT CTT GCT GTC CAA TTT CT
+1,480 (C>G)	*6C>G	B3′UTR-iPCR-U	TTA CTG CTC GTT CTT CAG CAC GCG C
		β C1480G-D	GCT CGG TTT CTT GCT GTC CAA TTT CT
+1,570 (T>C)	*96T>C	β T1570C, -1565 to 1577-U	TCA ACGG CCC TTC ATA ATA TCC CCC A
		β T1570C-D	GCA TCC GGA TTC TGC CTA ATA AAA AAC
13 nt del	*91-*103(del)	β T1570C, -1565 to 1577-U	TCA AGG CCC TTC ATA ATA TCC CCC A
		β −1565 to 1577	GCC TAA TAA AAA ACA TTT ATT TTC A
term codon +47 (C>G)	*47C>G	+47C>C-D	GTA AGT CCA ACT ACT AAA CTG GGG G
		β53	GGA ACA AAG GAA CCT TTA ATA G
H122	*67-*72	β 3′UTR H122-U	AGT TTA GTA GTT GGA CTT AGG GAA C
		β 3′UTR H122-D	AAG CTT TAT TAT GAA GGG CCT TGA GC
H124	*73-*80	β 3′UTR H124-D	GGG AAA GCT TGA AGG GCC TTG AGC ATC TGG
		β53-2	CCA GTT TAG TAG TTG GAC TTA GGG A

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Dual-Luciferase Assay

Dual-luciferase assay was processed using Dual-Luciferase® Reporter Assay System (PromegaKK) under the conditions recommended by the manufacturer. *Photinus pyralis* and *Renilla reniformis* luminescences were monitored. The internal control (pGL4.10 vector) emits *Photinus pyralis* fluorescence, and the wild-type and mutant constructs (recombinant pGL4.74) emit *Renilla reniformis* fluorescence. Since *Renilla reniformis* fluorescence is short-lived, immediate measurement is necessary after addition and mixture of the Stop & Glo Reagent.

In order to know the expression efficiency of the mutants, we carried out luciferase assay using HEL and K562 cell lines. A relative luciferase assay activity of mutant constructs against that of the wild-type is shown in Figure 2.

Expression efficiency of the +1,506 mutation (*32A>C) was 0.7 compared with wild-type and was decreased significantly. These findings implied that the mutation surely reduces the expression of protein. The mutation causes β-thal probably due to decreased protein expression.

The +1,480 (*6C>G) is a mutation in PTB (pyrimidine tract-binding protein) binding sites, and reduces the 3′ cleavage activity (3,4). It has been reported to be associated with silent β-thal (6). However, our experiment showed a little higher activity than normal.

The term codon +47 mutation (*47C>G) (8) is an αCP binding site, and the positions of the H122 and H124 anomalies (2) attach nucleolin. Although

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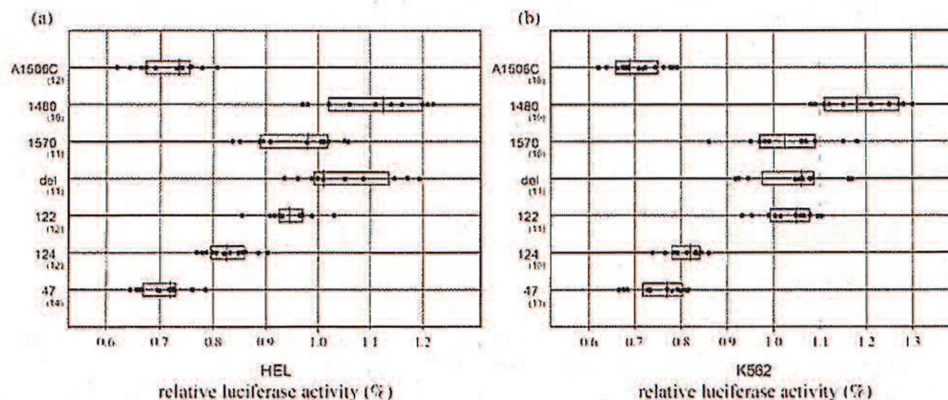

FIGURE 2 The results of the luciferase assay. The assay was conducted using HEL (a) and K562 (b) cells. A1506C = *32A>C, 1480 = *6C>G, 1570 = *96T>C, del = *91-*103(del), 122 = *67-*72, 124 = *73-*80 and 47 = *47C>G, respectively. The number of samples are given in parentheses. When the expression of the wild-type is 1.0, the mutation constructs of *32A>C, *73-*80, *47C>G were decreased significantly to 0.7, 0.8 and 0.75, respectively. The constructs of *96T>C, *91-*103(del) and *67-*72 had almost the same activity as the wild type. The activity of the *6C>G construct was a little higher than normal. The one-factor ANOVA was used and the calculation as well as the graph preparation was performed by Stat Flex (Artec KK, Osaka, Japan), a data analysis tool developed in Japan.

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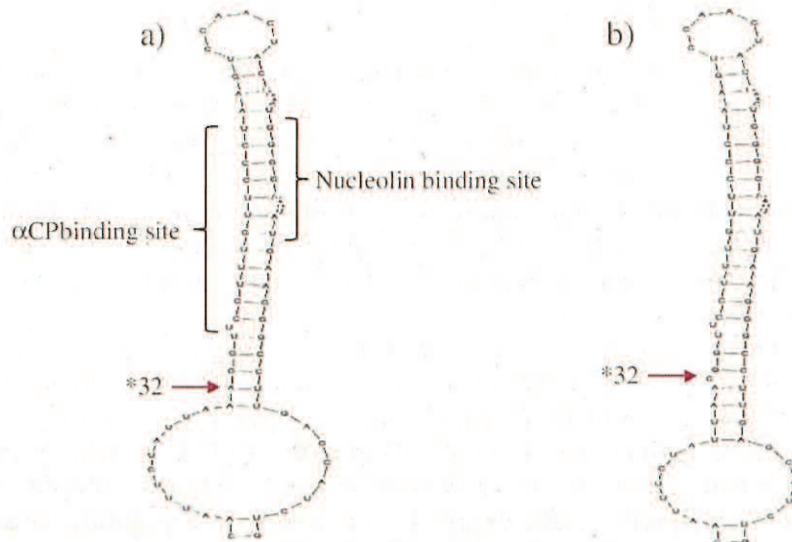

FIGURE 3 The stem-loop structure in the β-globin 3′UTR. a) The +1,506 (A>C) (*32A>C) mutation may alter the stem-loop structure. The access of αCP to the binding site is facilitated by the relaxation of the β-globin stem-loop motif that is attained by the nucleolin binding. The +1,506 mutation may interfere with the relaxation of the stem-loop structure, as the stem becomes longer in the presence of the mutation. The stem-loop structure was designed by RNAdraw (Mazura Multimedia, Stockholm, Sweden).

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

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Practice Article

Internet-based approach to population screening for common hemoglobinopathies in United Arab Emirates

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Abstract This article reports on efforts to overcome common hurdles that were faced during population-based screening for common hemoglobinopathies in the United Arab Emirates. An Internet-based approach was designed and implemented to increase the acceptance of the screening program. The process involved: an awareness campaign, a simple bilingual (Arabic/English) online consent form and registration process, the use of a barcode for sample labeling, an equipment upgrade, electronic communication of a successful registration process, test results, and a counseling process. Before the implementation of the Internet-based system, great concern was noted among the clients in terms of the availability of accurate and timely test results, the need for pretest and post-test counseling, and the way that their personal health information was handled. Lapses in information exchange between the clients who participated in the screening program for the carrier state of inherited disorders and the screening laboratory posed significant challenges. The emphasis on confidentiality and the ease of access to services was instrumental in increasing the level of acceptance of these services in our community. Based on an analysis of > 10,000 samples, we conclude that Internet–based reporting holds much promise for improving the quality of care that clients receive.

Key words hemoglobinopathies, Internet-based, population screening.

INTRODUCTION

The Internet has emerged as a potential vehicle for distributing health communication to millions of individuals because it is interactive, user-controlled, and offers breadth and depth of information (Gawande & Bates, 2000). In recent times, health information technology has emerged as a tool that holds much promise for improving the quality of care that clients receive by preventing medical errors, providing clinicians with better clinical decision-making tools, allowing the sharing of information about patients with other clinicians, tracking health outcomes, and coordinating public health activities. Information technology is now used extensively as a connectivity and reference tool for commercial, personal, and educational purposes (Glowniak, 1995). Many government organizations have been using the Internet to publish vast amounts of health-related information (Hubbard *et al.*, 1995; Kim *et al.*, 2005; Dorfman *et al.*, 2010). To the best of our knowledge, we have not come across a population-based genetic screening program, where an Internet-based approach has been used in its entirety to cover all phases of the screening process.

Genetic blood disorders represent a major public health problem in the Arab world, including in the United Arab Emirates (UAE). Several factors contribute to this situation, including the lack of public measures that are directed towards the early recognition and prevention of inherited diseases (Al-Gazali *et al.*, 2006).

Background

UAE Genetic Diseases Association (UAEGDA) is a non-profit organization that was established under the patronage of H. H. Sheikh Nahyan Mubarak Al Nahyan, the Minister of Higher Education and Scientific Research, UAE, with the main aim being to control and prevent population-specific genetic disorders that are prevalent in the UAE. This organization first initiated a massive public awareness campaign under its first project, "Emirates free from new births of children with thalassemia disease by the year 2012." This effort resulted in the inclusion of sickle cell and beta-thalassemia screening as mandatory under the UAE Federal Pre-marital Legislation Program that has been active since 2005. In 2007, following generous support from its chief patron and strategic partners, UAEGDA started offering free

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included regulations and policies concerning the storage, transmission, retention period of electronic health information, handling of database servers, and database back-up, as well as defining the administrator and user security matrix.

RESULTS

Over 8000 individual samples were processed on this novel Internet-based reporting system. The various sections that were addressed on the LIMS and the work flow at the National Screening Laboratory are illustrated in Table 1 and Figure 1. Specific descriptions of some of the salient areas are described here.

Client registration

A client can register for the screening process through UAE-GDA's website (http://www.uaegda.ae) and this can be done from anywhere and from any computer with Internet access. This registration process can be done in English or Arabic. Before accessing the registration page, the client has to agree to the terms and conditions that represent an online consent form (Fig. 2). After a successful registration process, a unique number and profile is created for each client automatically. The client receives an email communication on the successful completion of the registration process (Fig. 3). This also informs the client about the laboratory's acceptance of the collected blood sample for testing.

Blood sample collection

The process of blood sample collection is the first step in the management and operations process in the laboratory, where the sample collection is planned at a remote laboratory, a collection center, or main laboratory. The sample then is transported in an appropriate state and time. In all cases, the registration process is online and a barcode that is generated from the system is used on the sample collection tubes. The phlebotomists are trained in the client identification process, generation of a unique barcode, sample labelling, and collection process. Once the sample arrives in the laboratory, the receiving desk will check the sample according to a specific checklist (see Table 2) that also needs online documentation. This sample checklist is an important quality assurance step to determine the fitness of the collected sample for the next process in the testing area. If the sample does not meet the checklist criteria, it is rejected with automated feedback to the client about a repeat collection. Once the sample is accepted and registered online, the client gets an email confirming this event. This email also gives instructions to the client on checking the result online through a unique username and password that are generated soon after the successful blood-collection process.

Blood sample analysis

The blood sample is processed through various test process automated equipment (for CBC, HPLC, ferritin, and G6PD assay) and also through other manual tests, depending on the initial screening results. All the automated equipment that is used in our laboratory has both an auto-sampler and a barcode-reading facility. The remaining sample is stored for further needed investigations, including DNA extraction and DNA analysis.

Exporting of the test results to the laboratory information management system and reporting

The result for each client and each test is verified and exported to the database area, to which only the laboratory director has access. Here, the laboratory director can approve or disapprove the generated results or advise the laboratory technologist on a further testing process. Once approved, the results are generated as a test report (bilingual) and can be viewed in the client profile section.

Release of the test results

A certificate of analysis is made available in the client profile as a PDF file after the approval process. Here, the client checks the result online through the unique username and password that were generated soon after the successful blood-collection process (Fig. 4). If the results are negative, there is no need for further action. All the reports are released to the clients within 48–72 h of a working day (Fig. 5). There is an online feedback form on customer satisfaction that all clients can complete at the time of accessing their result. We also conduct independent audits on these services.

Counseling process

If the results are positive at the time of reporting, the laboratory director electronically communicates a scheduled counseling session to the client. Again, the entire process is controlled online. Automated bidirectional email communication between the LIMS and the client confirms the counseling session (Fig. 6). Every counseling session that is conducted is documented. The clinic nurse coordinates this counseling process and the counseling is provided by the clinical geneticist or genetic counselor. In most cases, only the client who tested positive attends the counselling session. On some occasions, when the client is a minor and/or when family studies are required, other members of the family attend the counseling session. All clinic visits and counseling sessions are free. The clinic visit for counseling also concentrates on extended family screening. Most of the time, one counseling session is sufficient as all those who participate in the counseling program have prior knowledge of what to expect through the pretest awareness programs and lectures that have been conducted in the schools and colleges.

DISCUSSION

During the earlier phase of the program's implementation, individual and group surveys were undertaken. Lapses in information exchange between the clients who participated in the screening program for a carrier state of inherited

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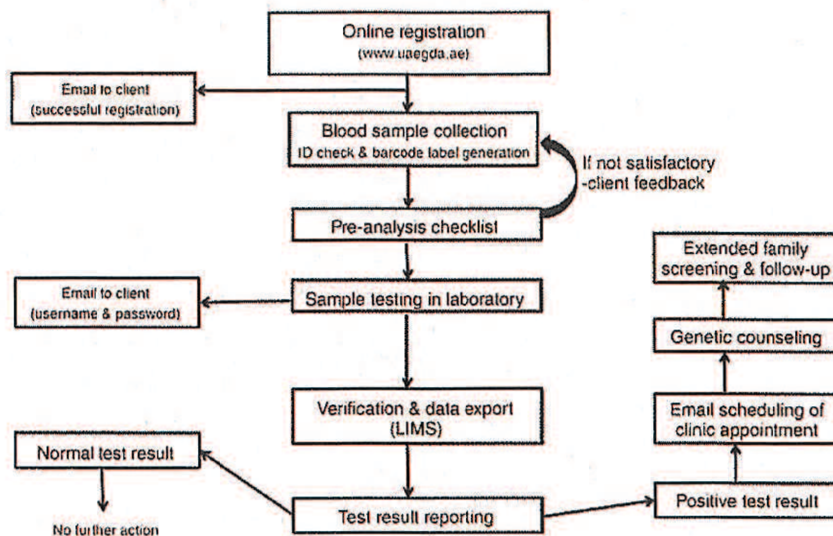

Figure 1. Schematic representation of Internet-based screening for hemoglobinopathies. LIMS, laboratory information management system.

Client registration	
1	I agree to submit a blood sample for testing and analysis.
2	I understand that as per the local health guidelines, if I am a minor, I have my parents guardian's permission for this consent.
3	I understand that both pretest and post-test counseling services are available.
4	I understand that testing involves a blood sample collection (5-10 mL) and it will be professionally handled.
5	I agree to the proposed test(s) requested by the laboratory.
6	I understand that all the listed tests are free.
7	I will receive an ID card/information to access the results.
8	The laboratory will provide me a username and password to access my results online or to my specified email address.
9	I appreciate that the sample might be sent to another laboratory, if required, for any confirmatory testing, and while doing so, my name or other personal details will be kept confidential.
10	I understand that my blood sample will be stored and that, in developing and upgrading the health information data of the country, it might be necessary to use part of the sample anonymously.
[ ] Agree	[ ] Disagree

Figure 2. Online consent form that is seen on the National Screening Laboratory website before the client registers for the screening program.

disorders and the screening laboratory posed significant challenges. Great concern was expressed by the clients in terms of the availability of accurate and timely test results, the need for pretest and post-test counseling, and the way in which their personal health information was handled. The students who participated in the conventional testing process expressed resentment regarding the communication mode of the test results.

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Screening for sickle cell and beta-thalassemia

Client name:	Anna Joseph	**Personal ID:**	102057
Sampling date:	27-Jul-2010	**Laboratory ID:**	101852
Report release date:	01-Aug-2010	**Certificate no.:**	1468

Results of investigations:

- Screening result for beta-thalassemia carrier (trait): **Negative**
- Screening result for sickle cell carrier (trait): **Negative**

Special remarks (if any):

- No evidence of sickle hemoglobin or other common hemoglobin variant and no evidence of thalassemia
- No action needed

Please note:

- Method of analysis:
  a. CBC: Abbott Cell-Dyne 3700 SL (fully-automated); b. HPLC: BioRad Variant II
- This data and the interpretation of the data might be misleading if the person tested has had a recent transfusion. If so, the tests should be repeated at least 4 months after the last transfusion.
- Any genetic predictions following these results assume that family relationships are as stated and sample identification is correct.
- Please contact the laboratory or your institute's health center for further information and counseling.

Why this screening for sickle cell and beta-thalassemia?

- Thalassemia is a major public health problem in our population and also in many parts of the world.
- Thalassemia and sickle cell anemia are a possibility only when both parents are carriers.
- Carriers of these conditions are normal, healthy individuals. They are detected only when such screening tests are done.
- Screening for sickle cell and beta-thalassemia is substantially more cost-effective than treatment.
- Prevention is possible, practical, and successfully implemented in many countries.

This electronic computer-generated report does not require a signature

Figure 5. Example of a screening test result. CBC, complete blood count; HPLC, high performance liquid chromatography.

the benefit of an easy communication process from receiving their results early, the quality of care, and the efficiency of the genetic counseling process. This also resulted in the efficient use of the staff members' time, a fewer number of staff members, less technical errors, less recurring costs, and maximized efficiency. The technical staff and clients both were satisfied and benefited from this electronic communication. At the end of the communication exchange, the client, the laboratory support staff, and the clinician completed information about the communication event and documented the event, as required.

With the implementation of the above processes, the laboratory is able to handle a large number of samples with a minimal staff, comprising one phlebotomist, one assistant technician, one senior laboratory technician, one event coordinator, and one laboratory director. With the provision of Web-based services, the laboratory director not only is able to monitor activities remotely, but also is able to report test results from anywhere with Internet access. The other modules that are included in the LIMS help to effortlessly handle various other sections, including human resources management and purchasing and stores, without the need to recruit skilled staff for these areas. The system has been adopted and customized to meet local needs. Therefore, our clients are able to send secure emails and gain access to appointments with the laboratory and clinician in a timely and efficient manner, instead of a conventional office visit. The laboratory staff and clinician also benefit from this secure communication process and online document control, thereby promoting evidence-based, personalized, better health care. Additionally, the system helps to securely send reminders to clients regarding their follow-up visit and to encourage family members to participate in the testing process, thereby promoting preventive health care more effectively (Matar *et al.*, 2010).

One of the limitations of the application of such Internet-based screening programs is the literacy level of the population that is tested. Our screening program mainly targets the literate student community in various colleges and universities. All of these colleges and universities have good information technology support and every student has an email ID through which the student and faculty communicate. With this level of computer literacy, the implementation of our screening program was relatively easy. However, it will be

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Answers to the comments and questions given before and during the presentation.

By Prof. Ishikawa:
Q1: What is Thalassemia?

Thalassemia is an inherited blood disorder caused by defective production of globin chain (α- or non-α, such as β). Thalassemia can be classified by phenotype into major and minor types. The patients with thalassemia major have severe anemia and need regular blood transfusion. Those with thalassemia minor may only suffer from slight anemia, and usually no blood transfusion is necessary. Most of the thalassemia cases found in Japan are minor type.

Q2: I know that number of Japanese patients are increasing recently. Why the incidence of thalassemia has arisen in the Japan as well as United State?

What is a factor which led to the increasing number of cases of Thalassemia in Japan?

1) Immigration from abroad or population movement, particularly from countries such as Southeast Asia where the thalassemia is common, is increasing.
2) International marriage between Japanese and foreigners coming from countries where the thalassemia is common, is increasing.
3) There are several β-thalassemia mutants unique to Japanese such as a nonsense mutant at CD90 which has spread with the increase in the past population.

Q3: Explain about clinical significances of your cases:

I am not a physician or clinician, I am just considering if the Japanese physician has a patient with a severe anemia, he or she would assume possibility of not only iron deficiency, but thalassemia combined with another abnormality such as certain membrane defect which is common in Japan.

Q4: How to treat this disease with minor symptoms?

For We do not need to treat Thalassemia minor, because it just exhibits slight anemia. The patients with thalassemia minor may have severe anemia under pregnancy or any other complications. In those cases, blood transfusion may be required exceptionally as a supportive care.

By Prof. Ichihara (the question after presentation)

Q: This is a new type of β-thalassemia with combination of HS, Nippon type. What is the prevalence of combination of β-thalassemia and HS recessive allele?

I got no information about the cases of combination of β-thalassemia and recessive HS in Japanese. However, considering the frequency of thalassemia in Japan is 0.1% (1 of 1000) and the recessive HS (P4.2) comprises 45% of 60 Japanese kindreds of HS which has been reported by Prof Yawata, the combination of β-thalassemia and recessive HS would be possible, but rare.

By Prof. Tokuda (the question on presentation)
Q: Is there any reference about Aquaporin?

Some researcher has reported about Aquaporin, and now, there are more than 10 Aquaporins have been reported. However, there is only a few informations about Aquaporin 3 function in red blood. Nathalie Roudier et al (1998 and 2002) has reported about the evidence of Aquaporin 3 in red blood cell. However, the role as a glycerol transporter in erythrocytes is not elucidated.

4. Prof. Tanaka (the question on presentation and interview)
Q: Would you like to explain about GLT_{50}?

GLT_{50} is one of parameter for screening thalassemia trait or minor. The principle of this method is how long the time (second) for 50% hemolysis of red bloods is required when the red cells are exposed to saline-glycerol solution. The basic principle is like osmotic fragility test, but glycerol is used in GLT_{50}. The glycerol will enter across the glycerol transporter (AQP3) from outside of the red blood cell membrane. In thalassemia cases, this protein AQP3 function may be impaired by the oxidation, and the glycerol needs more time to enter the red cell.