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Characteristics of Japanase Thalassemia

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KYE WORDS α-thalassemia; β-thalassemia; dominat-type thalassemia; deletion; PCR

ABSTRACT Thalassemia is relatively rare in Japan. Of 387 β-thalassemia cases of Japanese, homozygotes for β^+- and β^0-thalassemias were twenty-two (5.7%) and one (0.26%), respectively. The rest (94%) were of β-thalassemia trait. Ten different kinds of β-thalassemia mutations comprised about 80% of all cases. Half of these "common" mutations were unique to Japanese and another half were possibly from abroad. Sixty-nine α-thalassemia cases were analyzed, in which HbH and α-thalassemia trait comprised nineteen and fifty, respectively. Most of the α^o- and α^+-thalassemia chromosomes were of Southeast Asian type (--SEA) and $-\alpha^{3.7}$ type, respectively. The frequency of α^+-thalassemia as well as triplication of α-globin gene was high in northern Japan. Recent increase in the number of immigrants from Southeast Asia seems to raise the number of α-thalassemia found in Japan. They comprise at least 20% of the α-thalassemia. Six mutants classified into dominant-type β-thalassemia were found. All of them exhibited moderate anemia and marked anisopoikiocytosis. Heinz body varied in degree from copious to rare or even absent. Any mutation at initiation codon demonstrated marked microcytosis and erythremia. The breakpoint determination for large deletion-type thalassemia became feasible by estimation of gene dosage and PCR. Thus, the precise breakpoints for Filipino-type α^o-thalassemia (--FIL) and Japanese type-2 δβ-thalassemia were disclosed. The genetic diagnosis for these thalassemias are now readily conducted by gap PCR. The characterization of several new large deletions is being carried out.

INTRODUCTION

Thalassemia (thal) syndrome is relatively rare in Japan. It is found in one of 670-1,000 general population (Hattori 2001a). The reason might be due to the absence of malaria infection in Japan that presumably is a selective pressure for thal mutation (Nagel et al. 1990). Most thal mutations of Japanese would be present as neutral in this respect. Recent increase in the number of thal in Japan largely depends on the immigration of people from Southeast Asia where thal is endemic. Thus, the occurrence of thal major is concerned about in a couple at risk. The understanding of thal might become an urgent medical issue because most physicians are unaware of thal in Japan. We have analyzed hundreds of cases of thal in Japan, mostly being heterozygous. Genetic abnormalities and peculiar characteristics of thal in Japan as well as recent progress in the genetic analysis for α thal with a large-deletion are described here.

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METHODS

Samples suspected of thal were referred to our laboratory for gene diagnosis. The blood sample was obtained by venipuncture in EDTA vials, and kept at 4°C until its arrival at our laboratory. More than a thousand of samples suspected of thal were referred to our laboratory in the past 15 years. Among them 386 families including those reported elsewhere (Ohba et al. 1997) had thal mutations. Several families remained yet undetermined with our conventional techniques described below. All samples were subjected to a set of primary screening composed of HbF and HbA2 levels, isoelectrofocusing, glycerol lysis time ½, supravital stains for HbH and Heinz bodies, and isopropanol test. The α- or β-thal surmised in this screening was further subjected to gene analysis using PCR. The mutation was surveyed using single strand conformation polymorphism (SSCP) (Okayama et al. 1995), and confirmed by allele-specific PCR (Newton et al. 1989; Hattori 1998) or direct sequencing. Diagnosis with only a single method often leads to misdiagnosis; hence gene diagnosis was endorsed by two of the three techniques mentioned above. Gap PCR was employed for the deletions of α- and δβ-thal's whose breakpoints are precisely known. Thus, -$\alpha^{3.7}$, -$\alpha^{4.2}$, Southeast Asian-type α^0-thal (--

SEA)(Nicholls et al. 1987, Chang et al. 1992), Filipino-type α^0-thal (--FIL) (Hattori Y et al. 1999) and Hb Lepore (Boston -Washington type) (Yamashiro et al. 1997) were readily identified. Unknown deletion-type α- or δβ-thal was subjected to the measurement of gene dosage using PCR at several portions of DNA (Borreillo et al. 1994), and deleted portion was roughly estimated. Based on these results gap PCR was attempted using several primers prepared around the presumed but not defined breakpoints (Hattori et al. 1999). Another efficient strategy was also taken in which only one of the two breakpoints was approximated by the same methods above. Subsequently genome DNA was digested with eight kinds of endonucleases that were involved in the multi-cloning site of pUC18 vector, and ligated to the pUC vector digested with the relevant endonuclease. The recombinant mixtures were subjected to gap PCR using a M13 primer and primers around the suspected breakpoint. An abnormal-sized PCR product was looked for in an agarose gel electrophoresis, and if present, extracted by SUPREC-01 (TaKaRa, Japan). The base sequence of the extract was determined to disclose precise breakpoints.

RESULTS

I. β-thal Mutation

Mutation Spectrum and Frequencies

The spectrum of β-thal mutation was heterogeneous, being composed of forty-seven different kinds of mutations (Table 1). Its mutation spectrum exhibited in decreasing frequency; –31 A-G (16.3 %), codon 90 GAG-TAG (15.0 %), IVS-II-654-C-T (13.7 %), IVS-II-1 G-A (12.7 %), codons 41/42 TTCTTT-TTC (8.3 %), codons 127/128 CAGGCT-CCT (4.4 %), initiation codon ATG-GTG (2.8 %), codon 85 TTT-TTTT (2.8 %), codon 121 GAA-TAA (2.6 %), codon 17 AAG-TAG (2.3 %) and thirty-seven rare mutations (19.1 %). Thus, ten kinds of mutations were found in more than 80% of all thal families. The remaining 20 % varied in the mutation, and that occurred rarely or sporadically. The –31 A-G demonstrates a β^+ phenotype, and a number of homozygotes for this mutation were found. This mutation reveals a thal intermedia phenotype. This would account for the fact that the –31 A-G is most common among Japanese β-thal mutations. Another homozygous β^+-thal, "silent" mutation at codon 24, was seen in a family. A homozygous β^0-thal patient was found with the nonsense mutation at codon 90 whose clinical information was not obtained.

Origin of Japanese β-thal's

The mutations at –31, codon 90, codons 127/128, initiation GTG and codon 85 are exclusively found in Japanese (Table 1), whereas those at IVS-II-654, IVS-II-1, codons 41/42, codon 121 and codon 17 are found in other ethnic groups, such as Chinese, Southeast Asian and European, too (Huisman 1997). Above all, the mutations at IVS-654 and codon 17 are prevalent among habitants in southern China. The codons 41/42 mutation is seen among all Asians. The splicing mutation at IVS-II-1 is mainly seen in Far East countries. It is

Table 1: Frequency of Japanese β-thalassemia mutations

Mutation	*Mechanism*	*Phenotype*	*Family[homo]*	*Frequency(%)*
-31A-G	transcription	β^+	63[21]	16.3
codon 90 GAG-TAG	nonsense	β^0	58[1]	15.0
IVS-II-654 C-T	splicing	β^0	53	13.7
IVS-II-1 G-A	splicing	β^0	49	12.7
codon 41/42 TTCTTT-TT	frameshift	β^0	32	8.3
codon 127/128 CAGGCT-CCT	super unstable Hb	β^0	17	4.4
initiation ATG-GTG	translation	β^0	11	2.8
codon 85 TTT-TTTT	frameshift	β^0	11	2.8
codon 121 GAA-TAA	nonsense,dominant	β^0	10	2.6
codon 17 AAG-TAG	nonsense	β^0	9	2.3
others (24 species)			74[1]	19.1
Total			387[23]	100%

Bold letter denotes the mutation peculiar to Japanese. The bracket is a number of homozygotes. HbE is not included.

intriguing that IVS-II-1 G-A is also found in Korean that is linked in cis to polymorphism at codon 91 C-T as that found in Far East (Park et al. 2002), while both polymorphisms were found in Japanese IVS-II-1 G-A.

Distribution of β-thal Mutations

Japanese β-thal is not evenly distributed in Japan. The mutations at -31 and codon 90 are mainly found in eastern and western Japan, respectively. Diverse haplotypes are noted for –31 A-G, while a single haplotype for codon 90 GAG-TAG.

II. α-thal

Mutations and Frequency

The α-thal is relatively uncommon, and comprises one-fifth of β-thal. Many of them are heterozygous for α^{o}-thal or α-thal minor, and may go unnoticed. Whereas, HbH disease that is clinically overt as a hemolytic anemia comprises about one-third of α-thal cases (Table 2). In HbH disease, nearly 90% of the α^{o}-thal chromosomes are of--SEA, and coexisting α^{+}-thal chromosomes are mainly of $-\alpha^{3.7}$ type (79%). Small numbers of α^{Pakse} and α^{CS} were found among immigrants from Southeast Asia. Two thirds of the α^{o}-thal chromosomes of Japanese were of -- SEA type, and 3% of -- FIL. The rest of them (non-SEA, 38%) were different from these two, and have undefined gene deletions involving α-globin gene clusters (Fig.1). Their deletions estimated by gene dosage seemed to be large and heterogeneous. Interestingly enough was a case that removed LCR leaving α-globin genes intact. The precise breakpoints remained undetermined due to technical difficulties.

The frequencies of α^{+}-thal chromosome in the

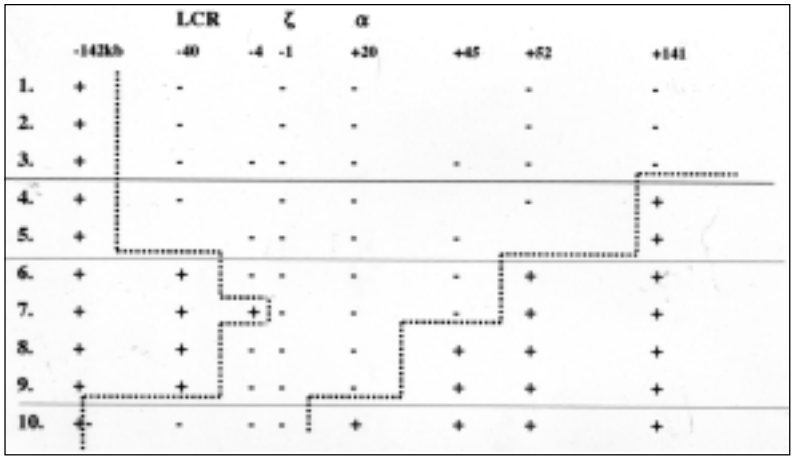

Fig.1. Rough estimation of breakpoints of α-thal chromosomes by gene dosage methods. The letter "-" and "+" denote the absence and presence of the DNA, respectively. Broken lines represent the region of breakpoints. Ten samples were analyzed. Nos. 1-3 had large deletions more than 200kb. Nos. 4-5 had restricted deletion of less than 300kb Nos. 6-9 had deletions less than 100kb No.10 retained two α-globin genes but lost LCR. Subtelomere region was spared in all samples. No.7 was completely analyzed to be found--FIL.

Table 2: α-thal mutations and family numbers for HbH and α-thal trait found in Japan

	HbH disease		*α-thal trait*	
Japanese	$--^{SEA}/-\alpha^{3.7}$	11	$--^{SEA}$ / N	19
	$--^{FIL}/-\alpha^{3.7}$	2	non-SEA / N	19
	$--^{SEA}/-\alpha^{4.2}$	1	$-\alpha^{3.7}/-\alpha^{3.7}$	3
non-Japansese	$--^{SEA}/-\alpha^{3.7}$	2	$--^{SEA}$ / N	7
	$--^{SEA}/\alpha^{CS}$	2	$-\alpha^{3.7}/-\alpha^{3.7}$	2
	$--^{SEA}/\alpha^{Pakse}$	1		
total		19		50

N: normal

Table 3: Frequency of α-globin gene abnormalities

	α^{+}-thal (-α/)	*triplication (ααα/)*
Northeast (Touhoku)	24 (n=1548, 1.55%) ($-\alpha^{3.7}$ type, 24)	36 (n=1515, 2.3%)
West (Ube)	2 (n=803, 0.25%) ($-\alpha^{3.7}$, 1; $-\alpha^{4.2}$, 1)	6 (n=803, 0.76%)
	p<0.01	p<0.01

western (Ube) and eastern (Tohoku) parts of Japan were 0.25 and 1.55 %, respectively (Table 3). Those of triplicated α-globin genes were 0.76% and 2.3%, respectively. This is the cause that homozygous α^+-thal (-α/-α) is relatively rare in Japan. The frequency of α^+-thal as well as its counterpart or triplicated α-globin gene is higher in the eastern part of Japan than the western part ($p<0.01$).

Origin and Distribution

All of the immigrants with α^o–thal from Southeast Asia had - SEA as α^o-thal chromosome (Table 2).

III. Dominant-type β-thal's

Six kinds of β-thal mutations found in Japanese were classified as dominant-type thal (Fig.2) (Kazazian et al. 1992; Huisman et al. 1997). These were as follows in decreasing order of Hb levels: codon 110 C-A (Hb Showa-Yakushiji), codons 127/128 CAGGCT-CCT (Hb Gunma), codon 121 GAA-TAA, codon 131 CAG-TAG, codon 115 C-A(Hb Hradec Kralove) and codon 125 CCA-CC. All of them had variable degrees of anemia, and resided in the 3rd exon. They seem to fall into three classes by phenotypes, varying from thal minor to thal major: [1] codon 110, [2] codon 127/128, codon 121, and [3] codon 115, codon 125. Heinz bodies that characterize the dominant type as "inclusion body β-thalassemia" were invariably seen in the last two mutations. However, Heinz bodies were not detected at all in the mutations at codons 110 and 131. Mutations at codons 127/128 and 121 were prone to demonstrate Heinz bodies by only one or two in a whole slide examined. The clinical symptoms of categories [1] and [2] were negligible and mild, respectively. However, they were different from usual thal minor. The common feature for all of these dominant types was the presence of moderate to marked anisopoikilocytosis in the blood film. This morphological abnormality was "persistently" observed for these dominant-type mutations but not for others that displayed typical thal minor (eg, codon 90 GAG-TAG and codons 41/42 TTCTTT-TT). The latter, however, sometimes revealed "transient" exacerbation of anemia mostly during pregnancy and infection, and exhibited moderate anisopoikilocytosis.

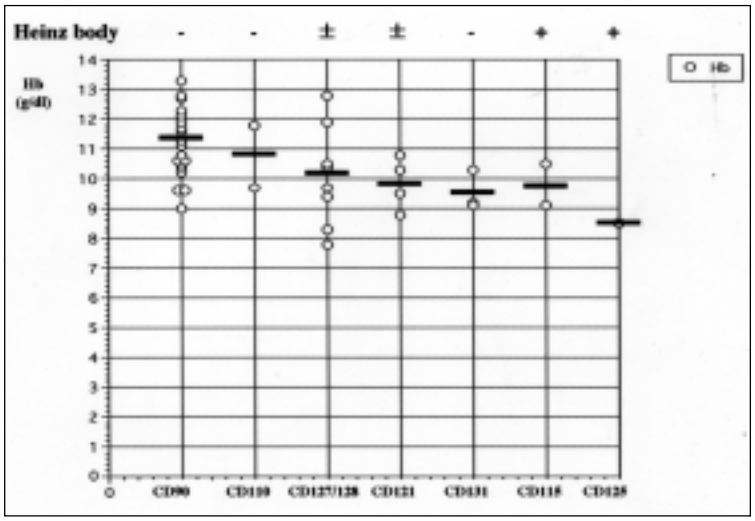

Fig. 2. Hemoglobin level of dominant-type β-thal
The presence of Heinz bodies is presented above the figure for each mutation. The symbol "±" means that Heinz bodies are occasionally found. The Hb levels for codon 90, non-dominant type, were cited as controls that reveal average Hb levels of 11.4 g/dl. Most dominant-types reveal Hb levels lower than 11.4g/dl.

IV. Erythremia

When the average red cell count of each β-thal mutation, mostly being heterozygous, is plotted against its MCH (or MCV), it places itself on a line of Hb=11.4 g/dl (Fig.3). This finding may indicate that thal minor behaves to keep

hemoglobin level at 11.4 g/dl. Dominant type β-thal's and homozygotes for β^+-thal (-31 A-G) are out on the line. Thus, their anemia is not compensated for by the erythrocytosis presumably because of enhanced hemolysis. Why most β-thal traits keep the hemoglobin levels subnormal is unknown. Particularly interesting are initiation codon mutations that reveal extreme microcytosis and give rise to remarkable erythremia (Fig. 3). In addition, more decreased MCHC and more prolonged GLT1/2 than other thal mutants were noted. All initiation codon mutants we experienced exhibited marked erythremia.

V. Thal's with Large Deletions

Once the precise breakpoints of a large deletion-type thal are unraveled, the diagnosis of the same mutation can be easily made by gap PCR (Chan et al. 1991). The --SEA and Hb Lepore (Washington-Boston) were thus analyzed.

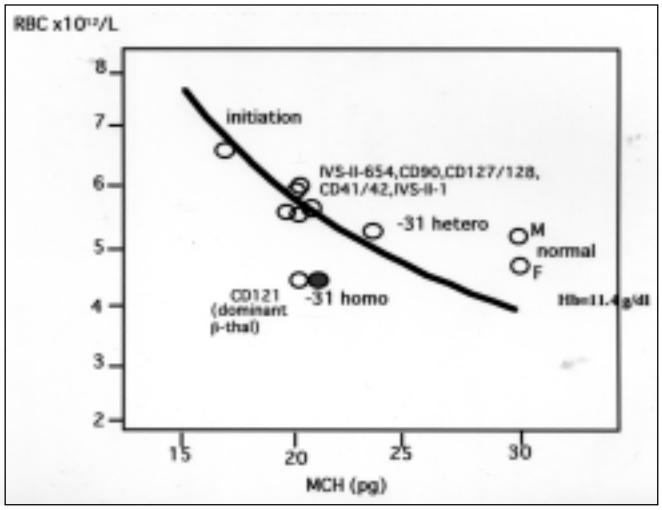

Fig. 3. Red cell counts vs MCH for β-thalassemiacs
The mean value for each mutation is depicted. Note that codon 121 (dominant-type) and homozogote for –31 A-G are not on the line.

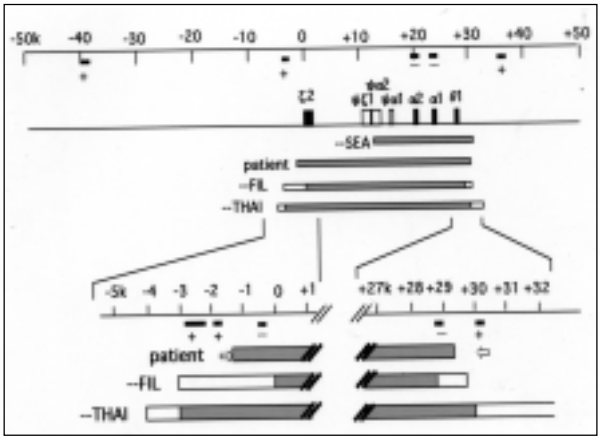

Fig. 4. Stepwise approximation of the unknown deletions by gene dosage.
The symbols "-" and "+" denote the absence and presence of the DNA segment amplified by PCR, respectively. The possible range that the breakpoint resides becomes narrower step by step. If it is followed by successful gap PCR, precise breakpoint is disclosed by sequencing.

However, a novel large deletion, mostly seen as heterozygotes is often encountered during gene analysis of thal. We estimated gene dosage by PCR at randomly-selected several portions within the globin gene cluster. Complete base sequence of β- or α-globin gene cluster was obtained from GenBank. The dosage was measured at three points during PCR before and after it reached a plateau. The possible 5' and 3' breakpoints were gained access stepwise by PCR (Fig.4). The product of the gap PCR was sequenced to disclose the precise breakpoints. This procedure enabled us to determine the breakpoints of --FIL (Fig. 5). The--FIL lost 30.656 kb that extended from 5'ζ to 3'θ1 of α-globin gene clusters. Both 5' and 3' breakpoints reside in Alu repeats at -1,450 /-1,146 and +29,226 / +29,518 bp, respectively, where the "A" of initiation codon ATG of ζ-globin gene is as coordinate +1. This deletion surely occurred within the area predicted by Southern blot analysis for --FIL (Fischel-Ghodsian et al. 1988). One more α^0-thal family was diagnosed as --FIL by gap PCR. Both the families were actually related to Filipino in their ancestors. Since this procedure to determine a novel deletion needs substantial numbers of primers, other methods aforementioned were attempted using a pUC18 vector. A new db-thal mutation found in Japanese was thus disclosed (Fig.6). This removed 26,926 bp that spanned from 5'φβ (2,775bp downstream from the termination codon of $^{A}\gamma$ globin gene) to 3'β (6,955 bp downstream from termination codon of β globin gene, or within L1 repeat), and designated as Jpn

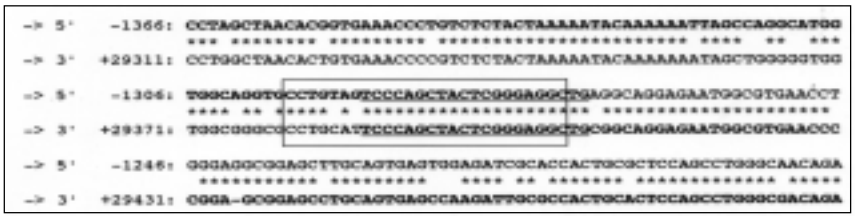

Fig. 5. The crossover region between two distant Alu repeats in -- FIL
The nucleotide sequence of the gap PCR product from the patient is presented by bold letters. The homologous crossover occurred within the twenty-one completely homologous nucleotides underlined. The box indicates 26-bp core sequence, within or around which gene recombination often occurs (Rudiger M et al. 1995).

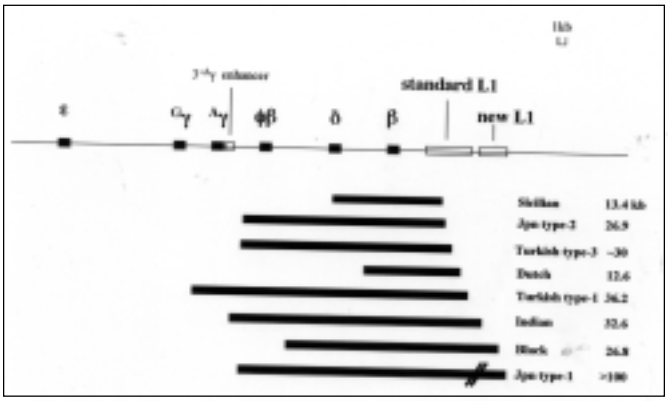

Fig. 6. Schematic representation of the deletion of novel Jpn type-2 δβ-thal
The breakpoints of eight kinds of δβ-thal's are presented. The 5'/3' breakpoints of Sicilian, Jpn type-2 (our case), Turkish type-3, Dutch, Turkish type-1, Indian, Black and Jpn type-1 are assigned in the sequence of GenBank HUMHBB at 55943/69339, 43673/70565, 43220-43565/70840-71185, 59698/72298, 37075/73286, 42151/74772, 49701/76508 and 43120/ ?(unknown), respectively. Jpn type-2 has 5' breakpoint at 3' to those of Jpn type-1, Indian and Turkish type-3 δβ-thal's by 2.26kb, 1.52 kb and several hundreds bp apart, respectively. It's 3' breakpoint is 1.2kb downstream to that of Sicilian type and only several hundreds bp upstream of Turkish type-3, both residing within L1 repeat. The deletion length is shown at the right side of each. Note that all deletion sizes are multiples of 12-13 kb.

type-2 δβ-thal. Our Jpn type-2 δβ-thal was subsequently identified by gap PCR in four other unrelated Japanese families. All carriers of Jpn type-2 were from the same Shimane-Tottori district. They were unrelated, but might share a common ancestor who had the same mutation.

DISCUSSION

Each ethnic group reveals its own mutation spectrum of β-thal (Huisman et al. 1997). In endemic areas of β-thal, a few mutations are found in over 90% of the patients. It is a good contrast with nations like Japan where thal is not endemic and demonstrates relatively diverse mutations (Huisman et al. 1997). These mutations underwent no selective pressure and have probably increased in number with population burst. Both β- and α-thal's in Japan have large influence from abroad. Thus, mutation spectrums of Japanese thal reveal dual origins, Japanese proper and foreign. It is interesting, that the -- SEA and -- FIL tend to be found in southwest part of Japan where ocean current comes up from south. These α^{o}-thal chromosomes seem to have flowed historically into this island from Southeast Asia. Three Japanese--SEA chromosomes were once analyzed (Hattori et al. 1990). Two of them shared the same microsatellites at 5'HVR and 3'HVR as those of Thai --SEA (Winichagoon et al. 1984), whereas, one of the three had different 3'HVR. An evolutional history might be involved.

The mutations unique to Japanese provide some information. The β-thal mutations at codon 90 and –31 are peculiar to Japanese, and they must have arisen after Japan islands were separated from Asian continent about 10,000 years ago or "Jomon-period" when our ancestor Jomon people lived (Omoto K. 1996). Their exact origin is obscure. After BC300 new people called "Yayoi", migrated over to Japan archipelago from the continent, presumably via Korea peninsula. Their technologies of agriculture remarkably increased the population of Japanese. When frequency of –31 A-G was extrapolated against the increase in the population, it was found that it has arisen in the Jomon era. The presence of diverse haplotypes associated with–31 A-G may support this hypothesis (Hattori Y 2001b). On the other hand, codon 90 GAG-TAG has only a single haplotype. Thus, codon 90 GAG-TAG is relatively new. Yayoi people pervaded from the western part of Japan, and indigenous Johmon was driven away to the northeast. Although they soon mixed peacefully and modern Japanese emerged, their historical distribution is reminiscent of the localization of –31 and codon 90 mutations, northeast and west, respectively.

The dominant-type β-thal's are important in Japan where most thal's are asymptomatic. Dominant-type and homozygous β^{+}-thal's, both being relatively uncommon, are symptomatic. Clinical severity of the dominant thal is manifested by the presence of anemia and Heinz bodies. The importance of Heinz body is implied by the synonymous term "inclusion body β-thalassemia"(Kazazian 1992). However, some mutations such as codon 121 and codons 127/128 that are classified into the dominant type, often have less severe phenotypes, and do not necessarily demonstrate Heinz bodies. Their phenotypes fluctuate from thal minor to thal intermedia. This phenotypic variability without presence of triplicated α-globin gene is also observed in the other β-thal of nonsense β127 CAA-TAA (Hall GW et al. 1991). Furthermore, the one of three groups of our dominant-type mutations at codons 110 and 131 was almost asymptomatic, while they tended to exhibit more severe anemia and persistent anisopoikilocytosis. Considering our cases, the dominant thal is delineated from usual thal minor by the mutation at the 3rd exon, persistent anisopoikilocytosis, and presence of anemia and Heinz bodies. Presence of Heinz body would not be a prerequisite though it is most important. What makes these stories ambiguous is that Heinz body is exceptionally encountered during gestation and infections in the mutations that otherwise give rise to thal minor. Such cases experienced in our laboratory usually demonstrate only a few Heinz bodies in a piece of smear.

Some β-thal's are discovered by erythremia. Poor hemoglobinization in a red cell reduces the volume of a red cell (microcytosis), and this is compensated for by increasing the number of erythrocytes or erythremia. The degree of the erythremia depends on the size of red cells or MCV (Fig. 3). The MCH level lower than 17 pg/μl is almost exclusively found in the initiation codon mutations (Ohba et al. 1997). Decreased production of hemoglobin tetramer in each erythrocyte seems to be pronounced in the initiation codon mutations. An association of the extreme microcytosis with defective mRNA that would be completely refractory to translation remains unknown.

The measurement of gene dosage not only approximates a large DNA deletion yet unknown

but also avoids the necessity of meticulous Southern blot. The disadvantage is that the estimation of gene dosage in heterozygote is prone to be inaccurate. Thus, more effective methods using Light Cycler (PE Japan) are being developed. The more the breakpoint is gained access, the more primers have to be prepared because long PCR is not necessarily successful for this purpose. In order to reduce the number of primers, recombinant of DNA segments digested by eight kinds of endonuclease with pUC vector was devised. The recognition sites are presents on average every 500 nucleotideo of genomic DNA. Among a number of PCR products thus prepared, a 1-2 kb DNA segment that involves a breakpoint is expected. This procedure, if successful, discloses the both 5' and 3' breakpoints at a time, and it surely did for Jpn type-2 δβ-thal.

The crossover in --FIL occurred between the completely homologous 21 nucleotides, 5' TCCCAGCTACTCGGGAGGCTG in the Alu repeats. Interestingly, nineteen of the 21 nucleotides are located within the core 26-bp region where a number of homologous crossover events occurred (Fig. 5) (Rüdiger et al. 1995). This concept of core 26-bp facilitated our analysis. The breakpoints of our Jpn type-2 δβ-thal reside very close to Indian (Gilman et al. 1992), Jpn type-1 (Shiokawa et al. 1988) and especially to those of Turkish type-3 whose precise breakpoints are not determined (Öner et al. 1996). However, Jpn type-2 evidently removed DNA inside the deletion range of the Turkish type-3. The 3' breakpoint of Jpn type-2 is close to Sicilian, Turkish type-3, Dutch (Gilman 1987) and Turkish type-1 δβ-thal's. A number of δβ- and β-thal's have the 3' breakpoint within L1 repeat (Henthorn et al. 1990; Huisman et al. 1997). Thus, the 5' and 3' breakpoints of Jpn type-2 seem to have occurred in "hot spots" of gene recombination. However, homologous recombination such as seen between Alu sequences in --FIL is unlikely to be for their occurrence. Many of them tend to remove DNA by multiples of 12-13kb, which casts the speculation that organization of chromatin within the nucleus might be related to the deletion (Nicholls et al. 1987; Gilman et al. 1992). A detailed mechanism for it remains unsolved.

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