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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.[2,3] 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.[4] 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.[4] 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,[4] 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

L1-like repeat by Waye but in the "new L1-like" repeat newly discovered by us at more 3' region of the L1-like repeat. It may be ascribed to the fact that the L1-like repeat and new L1-like repeat at the 3' of *β-globin* gene keep substantially high homology. In order to validate our new definition, we further expected the restriction fragments of Southern blot analysis based on our result. In addition, as we cannot get samples diagnosed as FIL β-thal in the past, we analyzed four more cases of deletion-type β-thal from Filipino by gap PCR specific to the FIL β-thal.

Materials and Methods

Among more than 2,800 samples referred to our laboratory from hospitals in Japan for diagnosis of hemoglobinopathies, there were 15 cases of Filipinos. Five patients of these Filipinos had microcytic anemia with no iron deficiency state. Clinical data and Hb screening data were suggestive of β-thal minor (Table 1).

DNA extraction and PCR

DNA was extracted from the peripheral blood leukocytes by phenol-chloroform methods. Whole *β-globin* gene, including promoter region and 3'UTR, was amplified by PCR using primers (forward, 5'-AGTAGCAATTT-GTACTGATGGTATGG-3'; reverse, 5'-TTTC-CCAAGGTTTGAACTAGCTCTT-3').[7]

DNA sequencing

Sequencing reaction was performed using BigDye® Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems). After purification by ethanol/EDTA/sodium acetate, capillary sequencing was performed by Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems).

Quantitative PCR

Gene dosage was measured by quantitative PCR (LightCycler™1.5 Instrument, Roche Diagnostic. Japan) at LCR, ε, $^{G}\gamma$, $^{A}\gamma$, δ, inter δ-β, β, new L1 and 3'HS of *β-globin* gene cluster[6] for the case 1. Each segment was measured

Table 1 Laboratory data

	Case1	Case2	Case3	Case4	Case5	Referance Range
Age	13	9	4	20	37	
Sex	M	M	M	M	F	
RBC	7.01	4.38	5.41	5.57	5.46	M:4.05 ～ 5.50*10^6/μl, F:4.00 ～ 4.50*10^6/μl
Hb	14.2	9.3	10.6	11.4	11.2	M:14 ～ 18 g/dl, F:12 ～ 16 g/dl
PCV	0.457	0.286	0.327	0.353	0.352	M:0.40 ～ 0.55 l/l ,F:0.37 ～ 0.47l/l
MCV	65.2	65.4	60.4	63.4	64.5	87 ～ 103 fl
MCH	20.3	21.2	19.6	20.4	20.5	27 ～ 32 pg
MCHC	31.1	32.4	32.4	32.1	31.8	32 ～ 36 %
Ret	1.6	2.5	1.3	1.7	0.9	0.5 ～ 1.5 %
T,Bil	0.6	0.6	0.3	0.5	0.4	0.3 ～ 1.2 mg/dl
D,Bil	0.3	0.2	0.1	0.1	0.1	0.1 ～ 0.3 mg/dl
LDH	237	300	333	285	135	115 ～ 217 IU/l
Haptoglobin	62.7	30	213	124	131	19 ～ 170 mg/dl
Transferrin	247	nd	216	nd	266	190 ～ 320 mg/dl
Feritin	49.8	76.7	47.6	187	17.6	M:19 ～ 261 ng/ml, F:15 ～ 65 ng/ml
Serum Iron	nd	66	122	74	56	M:50 ～ 200 mg/d, F:40 ～ 180 μg/dl
HbF	1.6	3.0	5.7	2.4	3.1	<1.6 %
HbA2	5.9	7.7	7.3	7.5	6.6	2.5 ～ 3.5 %
Isopropanol test	−	−	−	−	−	−
GLT50	67	nd	105	91	64	22 ～ 55 sec
Inclusion Body	−	nd	−	−	−	−
IEF	−	−	−	−	−	−

GLT_{50}: Glycerol lysis time, IEF: Isoelectrofocusing

in triplicate, and standardized by that of another single gene, or *Glucuronyltransferase* gene.[6] 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[6]

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,[3] 4kb by Motum[2]), *Bgl*II 8.5 (nd, 8.6), *Eco*RI 8.8 (nd,

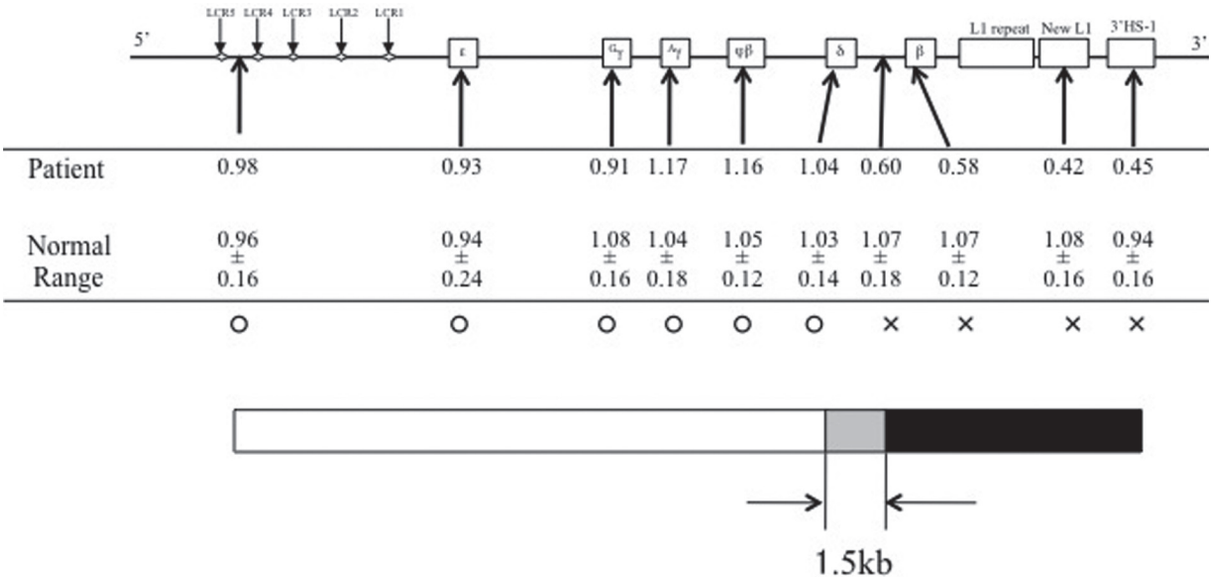

Fig. 1 Estimation of the approximate range of the deletion by gene dosage measurement.

The diploid segments give about "1.0" expressed by open circle (○) (n=10), while hemizygotic one is nearly "0.5" by a christcross (×). DNA upstream of the *δ-globin* gene was present (open column), while it is deleted downstream from the middle portion between *δ*- and *β-globin* genes (δβ region) (solid column). Therefore, the 5' breakpoint is located within 1.5 kb portion between *δ-globin* gene and inter δ-β region (shaded column). New L1: New L1 repeat, 3'HS: 3' Hypersensitive region for DNase I.

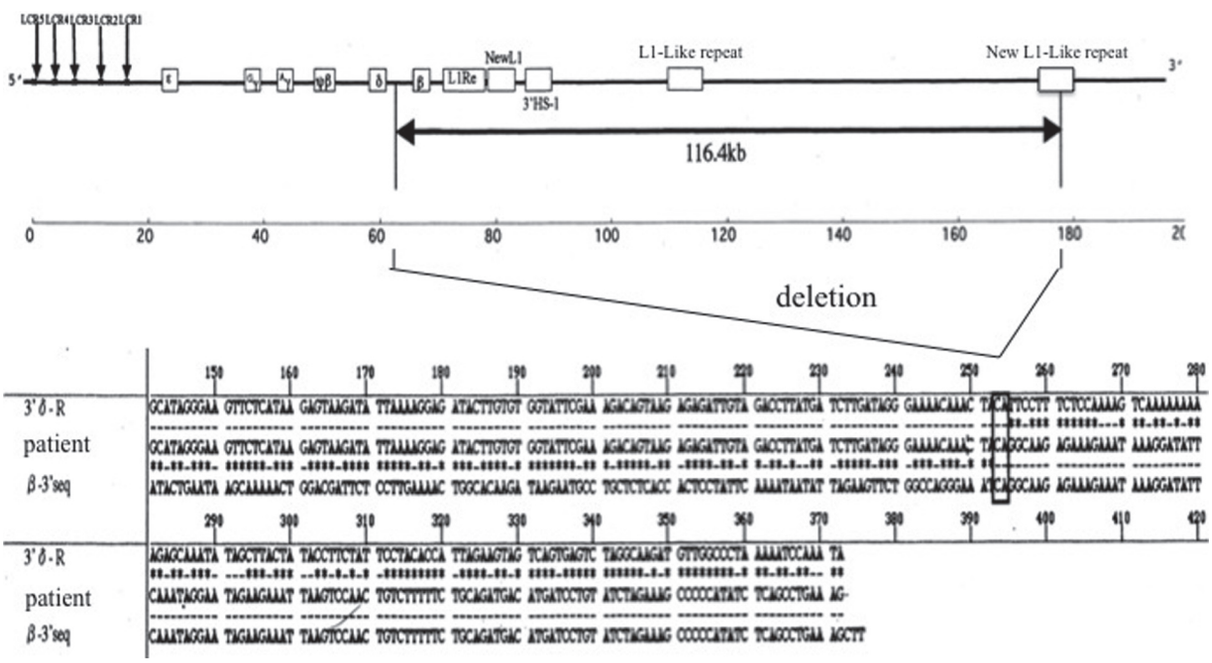

Fig. 2 DNA sequence of the FIL β-thal around the deletion junction.

Its organization and the homology with the normal sequences at 3' portion of *δ-globin* gene (upper, or 3'δ-R) and new L1-like elements far downstream to *β-globin* gene (lower, or β-3'seq) are presented. The asterisk denotes mismatch, and the bar accordance. Note that L1-like sequence and new L1-like sequence reside 39 kb and 114 kb 3' to the *β-globin* gene, respectively, and that complete homology of the FIL allele with β-3'seq, or new L1-like sequence is seen. The recombination occurred between the dinucleotides (CT) at 3'δ-R and at β-3' seq.

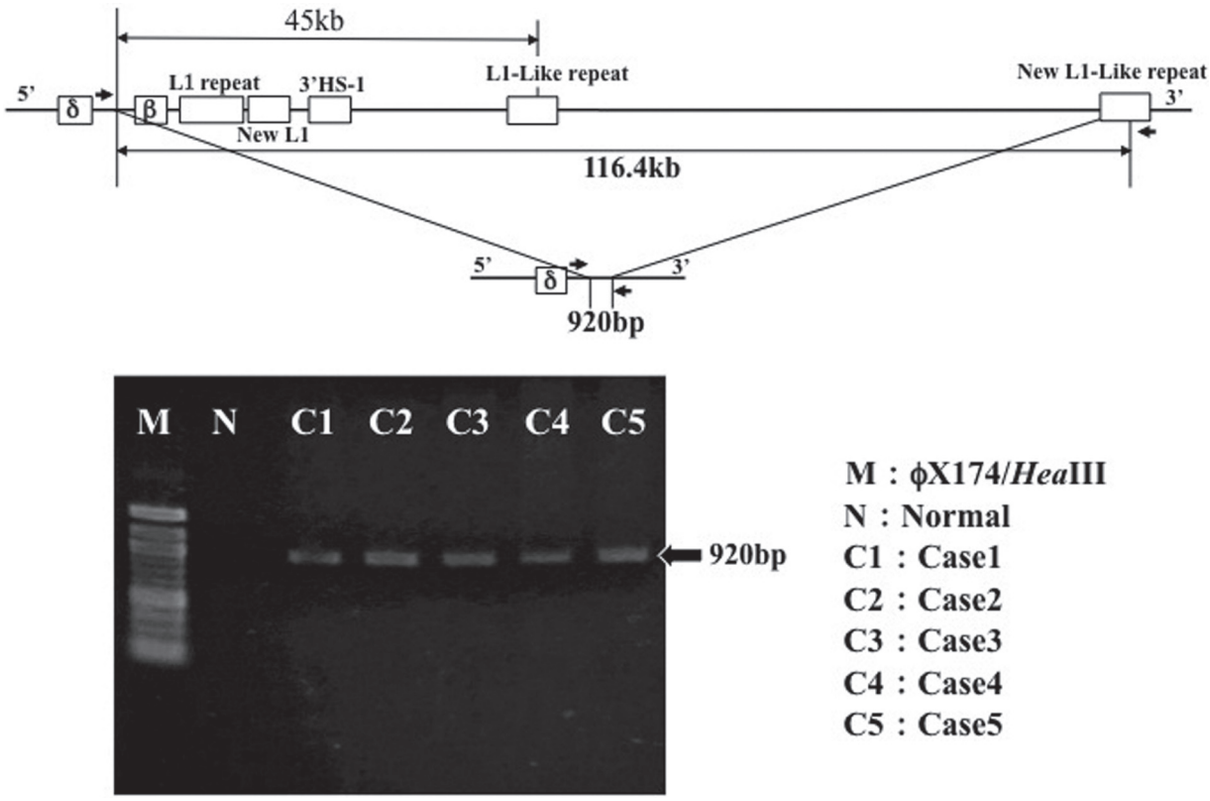

Fig. 3 Gap PCR for FIL deletion.

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.[2,3] The sequencing analysis was once performed by Waye using inverse PCR for the *Ava*II fragment from the FIL β-thal allele.[4] They were successful, but the lack of the genomic database in those

days may have prevented them from resolving the correct 3' breakpoint of the deletion. The FIL-type deletion, however, has widely been successfully determined by the specific gap PCR based on their result.[8,9] Our analysis was conducted by a different method, or Junction PCR, and confirmed the correct breakpoints.

A number of large deletions are associated with Alu and L1 repetitive sequence.[10] The 3' breakpoint of the FIL β-thal is indeed within a kind of L1 repeat that has approximately 80% homology with the 3' half of the L1.[11] However, 5' breakpoint is not related to L1 or L1-like sequences at all. Thus, the FIL-type deletion has occurred by illegitimate recombination.

In the past 10 years we had 43 cases with large deletions of β globin gene cluster out of 2800 samples referred to our laboratory. Twenty cases of them were εγδβ-thal and fifteen cases were δβ-thal. Screening for the FIL β-thal, common in Filipino people,[8] by gap PCR established above disclosed five positive samples of the deletion, and they are all Filipinos. Thus, our new definition for the breakpoint applies to FIL β-thal in general.

All of the five cases of FIL β-thal above were related to marriages with Filipino females who immigrated to Japan. With internationalization, such immigration is increasing in Japan. Thus, the FIL β-thal may distribute in time throughout Japan, where it has not been found before. The same situation is seen in HbE, which is prevalent in Southeast Asia.[12]

Conflict of Interest

The authors state no conflict of interest.

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