large deletion in ch19 spanning 824 kb (**Figure 3D**). However, there were no deletions detected in ch19 in patient #24 (**Figure 3D**). Genes estimated to reside within a large deletion are listed in **Supplemental Table 1**. Consistent with these s-q-PCR results, 6 of 7 large deletions were detected and confirmed as deleted regions, and these large deletions contained *RPL5, RPL35A, RPS17*, and *RPS19* (**Table 4 and Supplemental Table 1**). Other large deletions in RP genes were not detected by this analysis. From these results, we conclude that the synchronized multiple q-PCR amplification method has a detection sensitivity comparable with that of SNP arrays.

Detailed examination of a patient with intragenic deletion in the RPS19 allele (patient #24)

Interestingly, for patient #24, in whom we could not detect a large deletion by SNP array at s-q-PCR gene copy number analysis, 2 primer sets for *RPS19* showed a 1-cycle delay (RPS19-36 and -40), but 2 other primer pairs (RPS19-58 and -62) did not show this delay (**Figure 4A**). We attempted to determine the deleted region in detail by testing more primer sets on *RPS19*. We tested a total of 9 primer sets for *RPS19* (**Figure 4B**) and examined the gene copy numbers. Surprisingly, 4 primer sets (S19-24, 36, 40, and 44) for intron 3 of *RPS19* indicated a 1-cycle delay, but the other primers for *RPS19* located on the 5'UTR, intron 3, or 3' UTR did not show this delay (S19-57, 58, 28, 62, and 65) (**Figure 4B** and **4C**). These results suggest that the intragenic deletion occurred in the *RPS19* allele. To precisely confirm this deleted region, we performed genomic PCR on *RPS19*, amplifying a region from the 5'UTR to intron 3 (**Figure 4B**). In patient #24, we observed an abnormally-sized PCR product at a low molecular weight by agarose gel electrophoresis (**Figure 4D**). We did not detect a wild type PCR product

from the genomic PCR. This finding is probably because PCR tends to amplify smaller molecules more easily. However, we did detect a PCR fragment at the correct size using primers located in the supposedly deleted region. These bands were thought to be from the products of a wild type allele. Sequencing of the mutant band revealed that intragenic recombination occurred at a homologous region of 27 nucleotides, from –1400 to –1374 in the 5' region, to +5758 and +5784 in intron 3, which resulted in the loss of 7157 base pairs in the *RPS19* gene (**Figure 4E**). The deleted region contains exons 1, 2, and 3, and therefore the correct *RPS19* mRNA could not be transcribed.

Genotype-phenotype analysis and DBA mutations in Japan

Patients with a large deletion in DBA genes had common phenotypes (**Table 4**). Malformation with growth retardation (GR), including short stature or small-for-date were observed in all 7 cases. In patients who had a mutation found by sequencing, approximately half had GR (11/22) (status data of DBA patients with mutations found by sequencing are not shown). GR may be a distinct phenotypic feature of large deletion mutations in Japanese DBA patients. Familial mutations were analyzed for parents for 5 DBA patients with a large deletion (#3, #24, #60, #62, and #72) by s-q-PCR. There are no large deletions in all 5 pairs of parents in DBA responsible genes. Four of the 7 cases responded to steroid therapy. We have not observed significant phenotypic differences between patients with extensive deletions and other patients with regard to blood counts, responsiveness to treatment or other malformations.

Discussion

Many studies have reported RP genes responsible for DBA. However, mutations have

not been determined for approximately half of all patients. There are two possibilities for this finding. One possibility is that patients have other genes responsible for DBA, and the other is that patients have a complicated set of mutations in RP genes that are difficult to detect. In the current study, we focused on the latter possibility, since we have found fewer Japanese DBA cases with RP gene mutations (32.4%) compared with another cohort study with 117 DBA patients and 9 RP genes (~52.9%)[4]. With our newly developed method, we identified 7 new mutations with a large deletion in *RPL5, RPL35A, RPS17,* and *RPS19*.

The frequency of a large deletion was approximately 25.9% (7/27) in a group of patients who were not found to have mutations by genomic sequencing. Therefore, total RP gene mutations were confirmed in 42.6% of Japanese patients (**Table 5**). Interestingly, mutations in *RPS17* have been observed at a high rate (5.9%) in Japan, relative to that in other countries (1%) [5, 15, 16]. Although the percentage of DBA mutations differs among different ethnic groups[8, 17-19], a certain portion of large deletions in DBA responsible genes are likely to be determined in other countries by new strategies.

In this study, we analyzed patient data to determine genotype-phenotype relations. To date, large deletions have been reported with RPS19 and RPL35A in DBA patients [3,6,13]. *RPS19* large deletions/translocations have been reported in 12 cases, and *RPL35A* large deletions have been reported in 2 cases[19]. GR in patients with a large deletion have been observed previously with *RPS19* translocations [3, 19-21], but it was not found in 2 cases with *RPL35A* deletion[6]. Interestingly all of our patients with a large deletion had a phenotype of GR including short stature and small-for-gestational age, which suggests

that this is a characteristic of DBA with a large gene deletion in Japan. Our study results suggest the possibility of GR being associated with extensive deletion in Japanese patients. Although further case studies will be needed to confirm this possibility, screening of DBA samples using our newly developed method will help understanding of the broader implications of mutations and the correlation of the DBA genotype-phenotype.

Copy number variation analysis of DBA has been performed by linkage analysis, where the *RPS19* gene was first identified as a DBA susceptibility gene. Comparative genomic hybridization array technology has also been used to detect DBA mutations in *RPL35A*, and multiplex ligation-dependent probe amplification has been used for *RPS19* gene deletion analysis[3, 6, 13, 22]. However, these analyzing systems have problems in mutation screening. Linkage analysis is not convenient to screen for multiple genetic mutations, such as those in DBA, since it requires a high level of proficiency. Although comparative genomic hybridization technology is a powerful tool to analyze copy number comprehensively, this method requires highly specialized equipment and analyzing software, which limits accessibility for researchers. While q-PCR-based methods for copy number variation analysis are commercially available (TaqMan), they require a standard curve for each primer set, which limits the number of genes that can be loaded on a PCR plate. To address this issue, a new method of analysis is expected. By stringent selection of PCR primers, the s-q-PCR method enables analysis of many DBA genes in 1 PCR plate and the ability to immediately distinguish a large deletion using the q-PCR amplification curve. In our study, 6/7 large deletions in the RP gene detected by s-q-PCR were confirmed by SNP arrays (**Figure 3**). Interestingly, we

detected 1 large intragenic deletion in *RPS19*, which was not detected by the SNP array. This agreement between detection results suggests that the s-q-PCR copy number assay could be useful for detecting large RP gene deletions.

As shown in the current study, 7 DBA patients carried a large deletion in RP genes. This type of mutation could be underrepresented by sequencing analysis, although in the future, genome sequencing might provide a universal platform for mutation and deletion detection. We propose that gene copy number analysis for known DBA genes in addition to direct sequencing should be performed for searching for a novel responsible gene for DBA. Although it may be difficult to observe copy numbers on all 80 ribosomal protein genes in one s-q-PCR assay at present, our method allows execution of gene copy number assays for several target genes in 1 plate. Since our method is quick, easy and low cost it could become a conventional tool for detecting DBA mutations.

Acknowledgments

We thank Ms Momoka Tsuruhara, Ms Kumiko Araki, and Ms Keiko Furuhata for their expert assistance. This work was partially supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Health and Labor Sciences Research Grants (Research on intractable diseases) from the Ministry of Health, Labor and Welfare of Japan.

Authorship Contributions

M.K. designed and performed the research, analyzed the data, and wrote the paper;

A.S-O. and S.O. performed the SNP array; T.M. M.T. and M.O designed the study; T.T, K.T, and R.W. analyzed the mutations and status data; H.K., S.O., A.O., S. K., T.K., K. G., K.K., T.M., and N.M. analyzed the status data; A.M., H.M., K.T., T.M., and K.Y., performed the research and analyzed the data; and E.I. and I.H. designed the study, analyzed the data, and wrote the paper together with all co-authors.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

References

1. Hamaguchi I, Flygare J, Nishiura H, et al. Proliferation deficiency of multipotent hematopoietic progenitors in ribosomal protein S19 (RPS19)-deficient diamond-Blackfan anemia improves following RPS19 gene transfer. *Mol Ther.* 2003;7(5 Pt 1):613-622.
2. Vlachos A, Ball S, Dahl N, et al. Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. *Br J Haematol.* 2008;142(6):859-876.
3. Draptchinskaia N, Gustavsson P, Andersson B, et al. The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. *Nat Genet.* 1999;21(2):169-175.
4. Doherty L, Sheen MR, Vlachos A, et al. Ribosomal protein genes RPS10 and RPS26 are commonly mutated in Diamond-Blackfan anemia. *Am J Hum Genet.* 2010;86(2):222-228.
5. Gazda HT, Sheen MR, Vlachos A, et al. Ribosomal protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. *Am J Hum Genet.* 2008;83(6):769-780.
6. Farrar JE, Nater M, Caywood E, et al. Abnormalities of the large ribosomal subunit protein, Rpl35a, in Diamond-Blackfan anemia. *Blood.* 2008;112(5):1582-1592.
7. Gazda HT, Grabowska A, Merida-Long LB, et al. Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia. *Am J Hum Genet.* 2006;79(6):1110-1118.
8. Konno Y, Toki T, Tandai S, et al. Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia. *Haematologica.* 2010;95(8):1293-1299.
9. Robledo S, Idol RA, Crimmins DL, Ladenson JH, Mason PJ, Bessler M. The role of human ribosomal proteins in the maturation of rRNA and ribosome production. *RNA.* 2008;14(9):1918-1929.
10. Leger-Silvestre I, Caffrey JM, Dawaliby R, et al. Specific Role for Yeast Homologs of the Diamond Blackfan Anemia-associated Rps19 Protein in Ribosome Synthesis. *J Biol Chem.* 2005;280(46):38177-38185.
11. Choesmel V, Fribourg S, Aguissa-Toure AH, et al. Mutation of ribosomal protein RPS24 in Diamond-Blackfan anemia results in a ribosome biogenesis disorder. *Hum Mol Genet.* 2008;17(9):1253-1263.
12. Flygare J, Aspesi A, Bailey JC, et al. Human RPS19, the gene mutated in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. *Blood.* 2007;109(3):980-986.

13. Quarello P, Garelli E, Brusco A, et al. Multiplex ligation-dependent probe amplification enhances molecular diagnosis of Diamond-Blackfan anemia due to RPS19 deficiency. *Haematologica.* 2008;93(11):1748-1750.

14. Yamamoto G, Nannya Y, Kato M, et al. Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. *Am J Hum Genet.* 2007;81(1):114-126.

15. Song MJ, Yoo EH, Lee KO, et al. A novel initiation codon mutation in the ribosomal protein S17 gene (RPS17) in a patient with Diamond-Blackfan anemia. *Pediatr Blood Cancer.* 2010;54(4):629-631.

16. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D. Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. *Hum Mutat.* 2007;28(12):1178-1182.

17. Cmejla R, Cmejlova J, Handrkova H, et al. Identification of mutations in the ribosomal protein L5 (RPL5) and ribosomal protein L11 (RPL11) genes in Czech patients with Diamond-Blackfan anemia. *Hum Mutat.* 2009;30(3):321-327.

18. Quarello P, Garelli E, Carando A, et al. Diamond-Blackfan anemia: genotype-phenotype correlations in Italian patients with RPL5 and RPL11 mutations. *Haematologica.* 2010;95(2):206-213.

19. Boria I, Garelli E, Gazda HT, et al. The ribosomal basis of Diamond-Blackfan Anemia: mutation and database update. *Hum Mutat.* 2010;31(12):1269-1279.

20. Campagnoli MF, Garelli E, Quarello P, et al. Molecular basis of Diamond-Blackfan anemia: new findings from the Italian registry and a review of the literature. *Haematologica.* 2004;89(4):480-489.

21. Willig TN, Draptchinskaia N, Dianzani I, et al. Mutations in ribosomal protein S19 gene and diamond blackfan anemia: wide variations in phenotypic expression. *Blood.* 1999;94(12):4294-4306.

22. Gustavsson P, Garelli E, Draptchinskaia N, et al. Identification of microdeletions spanning the Diamond-Blackfan anemia locus on 19q13 and evidence for genetic heterogeneity. *Am J Hum Genet.* 1998;63(5):1388-1395.

Table 1. Primers used for synchronized genomic q-PCR of ribosomal large subunit proteins.

Table 2. Primers used for synchronized genomic q-PCR of ribosomal small subunit proteins.

Table 3. Summary of mutations and the mutation rate observed in Japanese DBA patients.

Table 4. Characteristic data of DBA patients tested.

Table 5. Total mutations in Japanese DBA patients, including large gene deletions.

Table 1. Primers used for synchronized genomic q-PCR of ribosomal large subunit proteins .

Gene	Primer name	Sequence	Primer name	Sequence	Size (bps)
RPL5	L5-02F	CTCCCAAAGTGCTTGAGATTACAG	L5-02R	CACCTTTTCCTAACAAATTCCCAAT	132
	L5-05F	AGCCCTCCAACCTAGGTGACA	L5-05R	GAATTGGGATGGGCAAGAACT	102
	L5-17F	TGAACCCTTGCCCTAAAACATG	L5-17R	TCTTGGTCAGGCCCTGCTTA	105
	L5-19F	ATTGTGCAAACTCGATCACTAGCT	L5-19R	GTGTCTGAGGCTAACACATTTCCAT	103
	L5-21F	GTGCCACTCTCTTGGACAAACTG	L5-21R	CATAGGGCCAAAAGTCAAATAGAAG	102
	L5-28F	TCCACTTTAGGTAGGCGAAACC	L5-28R	TCAGATTTGGCATGTACCTTTCA	102
RPL11	L11-06F	GCACCCACATGGCTTAAAGG	L11-6R	CAACCAACCCATAGGCCAAA	102
	L11-20F	GAGCCCCCTTTCTCAGATGATA	L11-20R	CATGAACTTGGGCTCTGAATCC	109
	L11-22F	TATGTGCAGATAAGAGGGCAGTCT	L11-22R	ATACAGATAAGGAAACTGAGGCAGATT	98
RPL19	L19-02F	TGGCCTCTCATAAAGGAAATCTCT	L19-02R	GGAATGCAGGCAAGTTACTCTGTT	103
	L19-08F	TTTGAAGGCAAGAAATAAGTTCCA	L19-08R	AGCACATCACAGAGTCCAAATAGG	107
	L19-16F	GGTTAGTTGAAGCAGGAGCCTTT	L19-16R	TGCTAGGGAGACAGAAGCACATC	102
	L19-19F	GGACCAGTAGTTGTGACATCAGTTAAG	L19-19R	CCCATTTGTAACCCCCACTTG	106
RPL26	L26-03F	TCCAAAGAGCTGAGACAGAAGTACA	L26-03R	TCCATCAAGACAACGAGAACAAGT	102
	L26-16F	TTTGAGAATGCTTGAGAGAAGGAA	L26-16R	TTCCAGCACATGTAAAATCAAGGA	102
	L26-18F	ATGTTTTAATAAGCCCTCCAGTTGA	L26-18R	GAGAACAGCAAGTTGAAAGGTTCA	102
	L26-20F	GGGCTTTGCTTGATCACTCTAGA	L26-20R	AGGGAGCCCGAAAACATTTAC	104
RPL35A	L35A-01F	TGTGGCTTCTATTTTGCGTCAT	L35A-01R	GGAATTACCTCCTTTATTGCTTACAAG	121
	L35A-07F	TTTCCGTTCTGTCTATTGCTGTGT	L35A-07R	GAACCCTGAGTGGAGGATGTTC	113
	L35A-17F	GCCCACAACCTCCAGAGAATC	L35A-17R	GGATCACTTGAGGCCAGGAAT	104
	L35A-18F	TTAGGTGGGCTTTTCAGTCTCAA	L35A-18R	ATCTCCTGATTCCCCAACTTTGT	102
RPL36	L36-02F	CCGCTCTACAAGTGAAGAAATTCTG	L36-02R	CTCCCTCTGCCTGTGAAATGA	102
	L36-04F	TGCGTCCTGCCAGTGTTG	L36-04R	GGGTAGCTGTGAGAACCAAGGT	105
	L36-17F	CCCCTTGAAAGGACAGCAGTT	L36-17R	TTGGACACCAGGCACAGACTT	114

Table 2. Primers used for synchronized genomic q-PCR of ribosomal small subunit proteins.

Gene	Primer name	Sequence	Primer name	Sequence	Size (bps)
RPS7	S7-11F	GCGCTGCCAGATAGGAAATC	S7-11R	TTAGGGAGCTGCCTTACATATGG	102
	S7-12F	ACTGGCAGTTCTGTGATGCTAAGT	S7-12R	ACTCTTGCTCATCTCCAAAACCA	102
	S7-16F	GTGTCTGTGCCAGAAAGCTTGA	S7-16R	GAACCATGCAAAAGTGCCAATAT	112
RPS10	S10-03F	CTACGGTTTTGTGTGGGTCACTT	S10-03R	CATCTGCAAGAAGGAGACGATTG	102
	S10-15F	GTTGGCCTGGAGTCGTGATTT	S10-15R	ATTCCAAGTGCACCATTTCCTT	101
	S10-17F	AATGGTGTTTAGGCCAACGTTAC	S10-17R	TTTGAACAGTGGTTTTTGTGCAT	100
RPS14	S14-03F	GAATTCCAAACCCTTCTGCAAA	S14-03R	TTGCTTCATTTACTCCTCAAGACATT	104
	S14-05F	ACAACCAGCCCTCTACCTCTTTT	S14-05R	GGAAGACGCCGGCATTATT	102
	S14-06F	CGCCTCTACCTCGCCAAAC	S14-06R	GGGATCGGTGCTATTGTTATTCC	102
	S14-09F	GCCATCATGCCGAAACATACT	S14-09R	AACGCGCCACAGGAGAGA	102
	S14-13F	ATCAGGTGGAGCACAGGAAAAC	S14-13R	GCGAGGGAGCTGCTTGATT	111
	S14-15F	AGAAGTTTTAGTGAGGCAGAAATGAGA	S14-15R	TCCCCTGGCTATTAAATGAAACC	102
	S14-19F	GATGAATTGTCCTTTCCTCCATTC	S14-19R	TAGGCGGAAACCAAAAATGCT	102
RPS15	S15-11F	CTCAGCTAATAAAGGCGCACATG	S15-11R	CCTCACACCACGAACCTGAAG	108
	S15-15F	GGTTGGAGAACATGGTGAGAACTA	S15-15R	CACATCCCTGGGCCACTCT	108
RPS17	S17-03F	ACTGCTGTCGTGGCTCGATT	S17-03R	GATGACCTGTTCTTCTGGCCTTA	121
	S17-05F	GAAAACAGATACAAATGGCATGGT	S17-05R	TGCCTCCCACTTTTCCAGAGT	114
	S17-12F	CTATGTGTAGGAGGTCCCAGGATAG	S17-12R	CCACCTGGTACTGAGCACATGT	102
	S17-16F	TAGCGGAAGTTGTGTGCATTG	S17-16R	CAAGAACAGAAGCAGCCAAGAG	102
	S17-18F	TGGCTGAATCTGCCTGCTT	S17-18R	GCCTTGTATGTACCTGGAAATGG	103
	S17-20F	GGGCCCTTCACAAATGTTGA	S17-20R	GCAAAACTCTGTCCCTTTGAGAA	101
RPS19	S19-24F	CCATCCCAAGAATGCACACA	S19-24R	CGCCGTAGCTGGTACTCATG	120
	S19-28F	GACACACCTGTTGAGTCCTCAGAGT	S19-28R	GCTTCTATTAACTGGAGCACACATCT	114
	S19-36F	CTCTTGAGGGTGGTCTGGAAAT	S19-36R	GTCTTTGCGGGTTCTTCCTCTAC	102
	S19-40F	GGAACGGTGTCAGGATTCAAG	S19-40R	AGCGGCTGTACACCAGAAATG	101
	S19-44F	CTGAGGTTGAGTGTCCCATTTCT	S19-44R	GCACCGGGCCTCTGTTATC	104
	S19-57F	CAGGGACACAGTGCTGAGAAACT	S19-57R	TGAGATGTCCCATTTTCACTATTGTT	101
	S19-58F	CATGATGTTAGCTCCGTTGCATA	S19-58R	ATTTTGGGAAGAGTGAAGCTTAGGT	102
	S19-62F	GCAACAGAGCGAGACTCCATTT	S19-62R	AGCACTTTTCGGCACTTACTTCA	102
	S19-65F	ACATTTCCCAGAGCTGACATGA	S19-65R	TCGGGACACCTAGACCTTGCT	102
RPS24	S24-17F	CGACCACGTCTGGCTTAGAGT	S24-17R	CCTTCATGCCCAACCAAGTC	101
	S24-20F	ACAAGTAAGCATCATCACCTCGAA	S24-20R	TTTCCCTCACAGCTATCGTATGG	105
	S24-32F	GGGAAATGCTGTGTCCACATACT	S24-32R	CTGGTTTCATGGCTCCAGAGA	105
RPS26	S26-03F	CGCAGCAGTCAGGGACATTT	S26-03R	AAGTTGGGCGAAGGCTTTAAG	104
	S26-05F	ATGGAGGCCGTCTAGTTTGGT	S26-05R	TGCCTACCCTGAACCTTGCT	102
RPS27A	S27A-09F	GCTGGAGTGCATTCGCTTGT	S27A-09R	CACGCCTGTAATCCCCACTAA	102
	S27A-12F	CAGGCTTGGTGTGCTGTGACT	S27A-12R	ACGTCCATCTTCCAGCTGCTT	103
	S27A-18F	GGGTTTTTCCTGTTTGGTATTTGA	S27A-18R	AAAGGCCAGCTTTGCAAGTG	111
	S27A-22F	TTACCATATTGCCAGTCTTTCCATT	S27A-22R	TTCATATGCATTTGCACAAACTGT	106

Table 3. Summary of mutations and the mutation rate observed in Japanese DBA patients.

Genes	Sequencing analysis
RPS19	10
RPL5	6
RPL11	3
RPS17	1
RPS10	1
RPS26	1
RPL35A	0
RPS24	0
RPS14	0
Mutations	22 (32.4%)
Total analyzed	68

Table 4. Characteristic data of DBA patients tested.

Patient No.	Age at diagnosis	Gender	Hb (g/dL)	Large deletion Q-PCR	Large deletion SNP array	Inheritance	Malformations	Response to first steroid therapy
Patients with a large deletion in RP genes								
3*	1Y	M	-	RPL5	RPL5	Sporadic	Short stature, thumb anomalies	Response
14*	5Y	M	5.5	RPS17	RPS17	Sporadic	White spots, short stature	Response
24*	1M	F	5.5	RPS19	N	Sporadic	Short stature, SGA	Response
60*	2M	F	2.4	RPS17	RPS17	Sporadic	SGA	NT
62*	1M	F	6.2	RPS17	RPS17	Sporadic	Small ASD, short stature, SGA	Response
71	0Y	M	5.3	RPL35A	RPL35A	Sporadic	Thumb anomalies, synostosis of radius and ulna , Cohelia Lange-like face, cleft plate, underdescended testis, short stature, cerebellar hypoplasia, fetal hydrops	NT
72	0Y	M	2	RPS19	RPS19	Sporadic	Thumb anomalies, flat thenar, testicular hypoplasia, fetal hydrops, short stature, learning disability	No
Patients without a large deletion in RP genes								
5*	1 Y 0M	F	3.1	N	N	Sporadic	N	Response
15*	1M	F	1.6	N	N	Sporadic	N	Response
21*	1Y	F	2.6	N	N	Sporadic	N	Response
26*	1Y1M	F	8	N	N	Sporadic	Congenital hip dislocation, spastic quadriplegia, hypertelorism, nystagmus, short stature, learning disability	Response
33*	2M	F	1.3	N	N	Sporadic	N	Response
36*	0Y	M	8.2	N	N	Familial	N	Response
37*	4Y	M	6.1	N	N	Sporadic	Hypospadias, underdescended testis, SGA	NT
45*	0M5D	M	5.1	N	N	Sporadic	Short stature, microcephaly, mental retardation, hypogammaglobulinemia	Poor
50*	2M	F	3.4	N	N	Familial	N	Response
61*	9M	M	4	N	N	Sporadic	N	Response
63*	0Y	M	6.8	N	N	Sporadic	Micrognathia, hypertelorism, short stature	Response
68	1Y8M	M	5.9	N	N	Sporadic	N	NT (CR)
69	1Y	M	9.3	N	N	Sporadic	N	Response
76	0Y	M	4	N	N	Sporadic	N	Response
77	0Y	M	7.8	N	N	Familial	Short stature	No
83	9M	F	3	N	N	Sporadic	N	NT
90	10M	M	9	N	N	Sporadic	N	No
91	0Y	F	3.8	N	N	Sporadic	N	Response
92	2M	M	3.7	N	N	Sporadic	ASD, PFO, melanosis, underdescended testis, SGA, short stature	Response
93	11M	M	2.2	N	N	Sporadic	White spots, senile face, corneal opacity, underdescended testis, syndactyly, ectrodactyly, flexion contracture, extension contracture	Response

*: Status data of Japanese probands #3 to #63 is from a report by Konno et al (Reference 8). N, not detected; NT, not tested; SGA, small for gestational age; ASD, atrial septal defect; PFO, persistent foramen ovale. Large deletions of the parents of 5 DBA patients (#3, #24, #60, #62, and #72) were analyzed by s-q-PCR, but there were no deletions in DBA genes in all 5 pairs of the parents.

Table 5. Total mutations in Japanese DBA patients, including large gene deletions.

Genes	Mutation rate
RPS19	12 (17.6%)
RPL5	7 (10.3%)
RPL11	3 (4.4%)
RPS17	4 (5.9%)
RPS10	1 (1.5%)
RPS26	1 (1.5%)
RPL35A	1 (1.5%)
RPS24	0
RPS14	0
Mutations	29 (42.6%)
Total analyzed	68

Figure legends

Figure 1. Synchronized q-PCR can determine a large gene deletion in DBA. (A) Concept of the DBA s-q-PCR assay. The difference in gene copy number between a healthy sample and that with a large deletion is 2-fold (i). When all genomic q-PCR for genes of interest synchronously amplify DNA fragments, a 2-fold difference in the gene copy number is detected by a 1-cycle difference of the Ct scores of the q-PCR amplification curves (ii). Dot plot of the Ct scores (iii). **(B)** Results of the amplification curves of s-q-PCR performed with a healthy person (i) and a DBA patient (#03) (ii). Upper panel: result of PCR cycles. Lower panel: extended graph of the PCR cycles at logarithmic amplification. **(C)** The graph shows Ct scores of s-q-PCR. If all specific primer sets for DBA genes show a 1-cycle delay relative to each other, this indicates a large deletion in the gene. Gene primer sets with a large deletion are underlined in the graph. **: P<0.00.1

Figure 2. Detection of 7 mutations with a large deletion in DBA patients. Genomic DNA of 27 Japanese DBA patients with unknown mutations were subjected to the DBA gene copy number assay. **(A)** Amplification curve of s-q-PCR of a mutation with a large deletion. The deleted gene can be easily distinguished. **(B)** Ct score (cycles) of representative q-PCR with DBA genomic q-PCR primers. Results of the 2 gene specific primer pairs indicated in the graph are representative of at least 2 sets for each gene specific primer (carried out in the same run). **: P<0.001, *: P<0.01

Figure 3. Results of single nucleotide polymorphism genomic microarray (SNP-chip) analysis. Genomic DNA of 27 Japanese DBA patients with unknown

mutations was examined using a SNP array. Six patients had large deletions in their chromosome (ch), which included one DBA responsible gene. **(A)** Patient #03 has a large deletion in ch1, **(B)** Patient #71 has a deletion in ch3, **(C)** Patients #14, #60, and #62 have deletions in ch15, and **(D)** Patient #72 has a deletion in ch19.

Figure 4. Result of s-q-PCR gene copy number assay for patient #24. (A) Result of s-q-PCR gene copy number assay for *RPS19* with 4 primer sets. **(B)** (i) The *RPS19* gene copy number was analyzed with 9 specific primer sets for *RPS19* that span from the 5' UTR to the 3'UTR. (ii) Primer positions of genomic PCR for *RPS19*. (iii) Region determined to be an intragenic deletion in *RPS19*. **(C)** Results of gene copy number assay for *RPS19* show a healthy person (i, iii) and a patient (ii, iv), and Ct results are shown (iii, iv). Patient #24 showed a "1-cycle delay" with primers located in the intron 3 region, but other primer sets were normal. **(D)** Results of genomic PCR amplification visualized by agarose gel electrophoresis to determine the region of deletion. N1, N2: healthy sample. *: nonspecific band. **(E)** Results from the genomic sequence of the 3-kb DNA band from patient #24 genomic PCR show an intragenic recombination from –1400 to 5784 (7157 nt) in *RPS19*. **: $P<0.001$

Figure 1

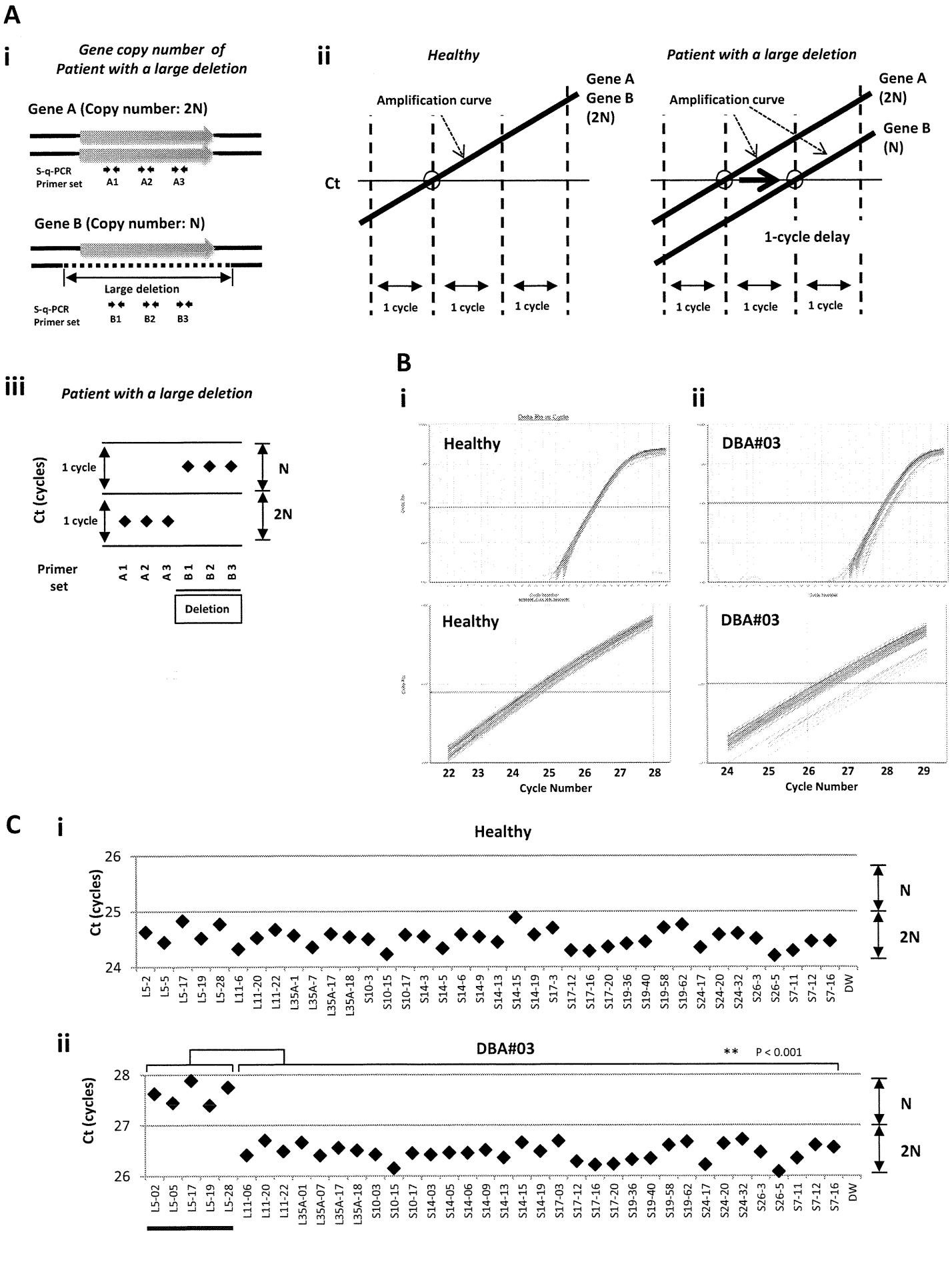

A
i
Gene copy number of Patient with a large deletion
Gene A (Copy number: 2N)
S-q-PCR Primer set
A1
A2
A3
Gene B (Copy number: N)
Large deletion
S-q-PCR Primer set
B1
B2
B3
ii
Healthy
Amplification curve
Gene A
Gene B
(2N)
Ct
1 cycle
1 cycle
1 cycle
Patient with a large deletion
Amplification curve
Gene A
(2N)
Gene B
(N)
1-cycle delay
1 cycle
1 cycle
1 cycle
iii
Patient with a large deletion
Ct (cycles)
1 cycle
1 cycle
N
2N
Primer set
A 1
A 2
A 3
B 1
B 2
B 3
Deletion
B
i
ii
Healthy
DBA#03
Healthy
DBA#03
22 23 24 25 26 27 28
Cycle Number
24 25 26 27 28 29
Cycle Number
C
i
Healthy
Ct (cycles)
26
25
24
N
2N
L5-2 L5-5 L5-17 L5-19 L5-28 L11-6 L11-20 L11-22 L35A-1 L35A-7 L35A-17 L35A-18 S10-3 S10-15 S10-17 S14-3 S14-5 S14-6 S14-9 S14-13 S14-15 S14-19 S17-3 S17-12 S17-16 S17-20 S19-36 S19-40 S19-58 S19-62 S24-17 S24-20 S24-32 S26-3 S26-5 S7-11 S7-12 S7-16 DW
ii
DBA#03
** P < 0.001
Ct (cycles)
28
27
26
N
2N
L5-02 L5-05 L5-17 L5-19 L5-28 L11-06 L11-20 L11-22 L35A-01 L35A-07 L35A-17 L35A-18 S10-03 S10-15 S10-17 S14-03 S14-05 S14-06 S14-09 S14-13 S14-15 S14-19 S17-03 S17-12 S17-16 S17-20 S19-36 S19-40 S19-58 S19-62 S24-17 S24-20 S24-32 S26-3 S26-5 S7-11 S7-12 S7-16 DW

Figure 2.

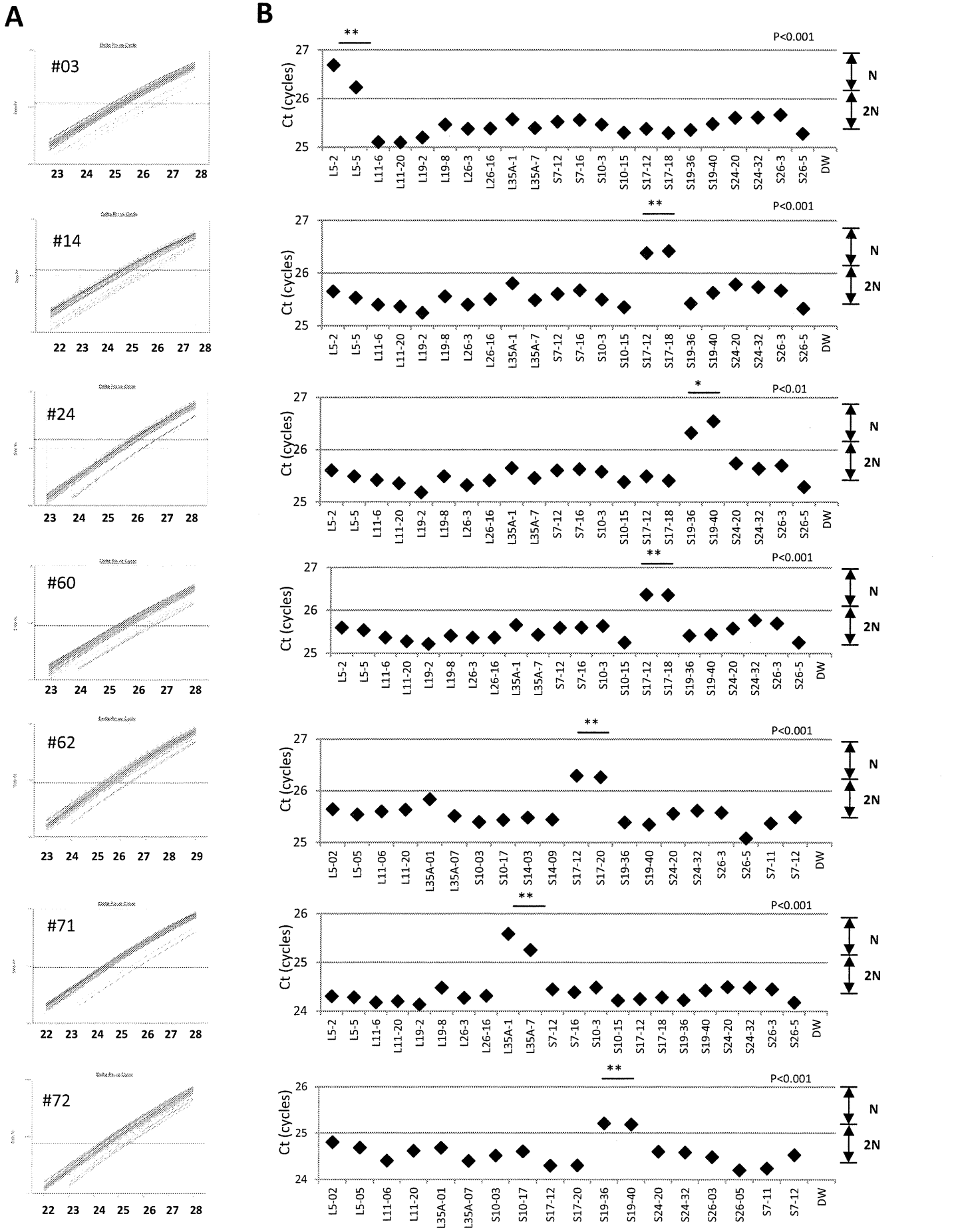

A
#03
#14
#24
#60
#62
#71
#72
B
Ct (cycles)
P<0.001
P<0.01
N
2N
L5-2
L5-5
L11-6
L11-20
L19-2
L19-8
L26-3
L26-16
L35A-1
L35A-7
S7-12
S7-16
S10-3
S10-15
S17-12
S17-18
S19-36
S19-40
S24-20
S24-32
S26-3
S26-5
DW
L5-02
L5-05
L11-06
L35A-01
L35A-07
S10-03
S10-17
S14-03
S14-09
S17-20
S19-36
S19-40
S24-20
S24-32
S26-3
S26-5
S7-11
S7-12
S26-03
S26-05

Figure 3

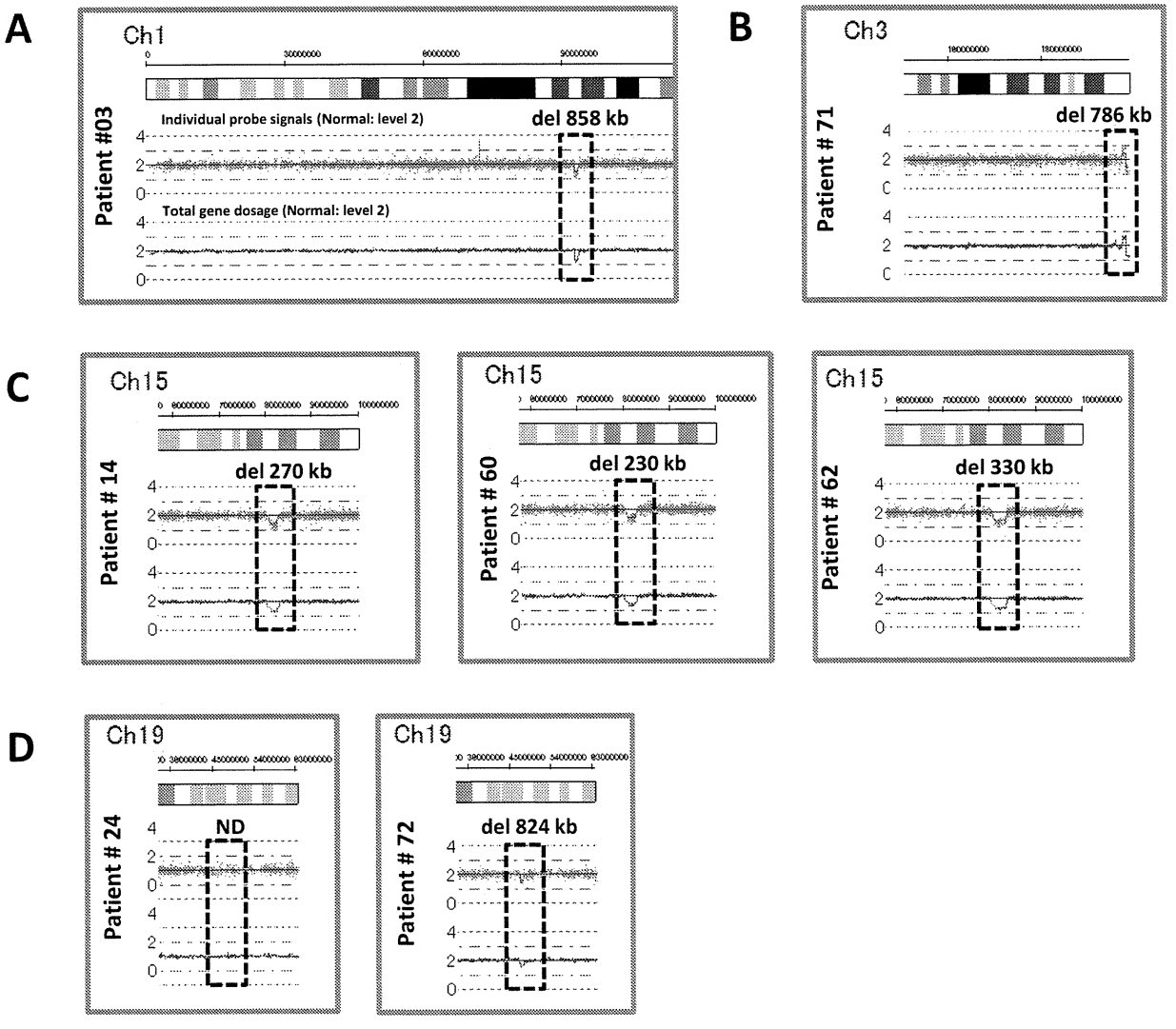
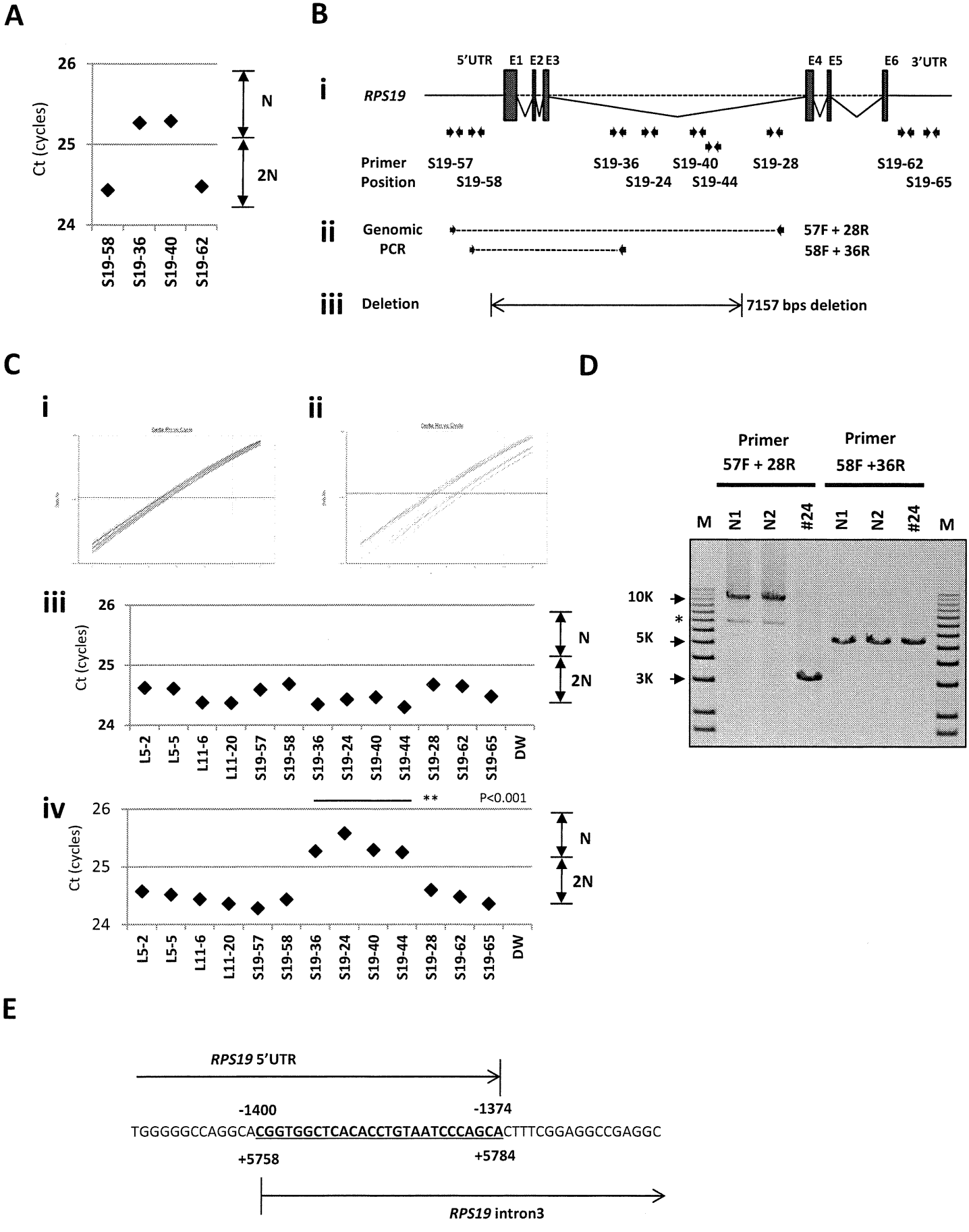

A
Ch1
Patient #03
Individual probe signals (Normal: level 2)
del 858 kb
Total gene dosage (Normal: level 2)
B
Ch3
Patient # 71
del 786 kb
C
Ch15
Patient # 14
del 270 kb
Ch15
Patient # 60
del 230 kb
Ch15
Patient # 62
del 330 kb
D
Ch19
Patient # 24
ND
Ch19
Patient # 72
del 824 kb

Figure 4


A
B
C
D
E
Ct (cycles)
N
2N
S19-58
S19-36
S19-40
S19-62
5'UTR
E1 E2 E3
E4 E5
E6
3'UTR
RPS19
Primer Position
S19-57
S19-58
S19-36
S19-24
S19-40
S19-44
S19-28
S19-62
S19-65
Genomic PCR
57F + 28R
58F + 36R
Deletion
7157 bps deletion
Primer 57F + 28R
Primer 58F +36R
M
N1
N2
#24
10K
5K
3K
L5-2
L5-5
L11-6
L11-20
S19-57
S19-58
S19-36
S19-24
S19-40
S19-44
S19-28
S19-62
S19-65
DW
**
P<0.001
RPS19 5'UTR
-1400
-1374
TGGGGGCCAGGCACGGTGGCTCACACCTGTAATCCCAGCACTTTCGGAGGCCGAGGC
+5758
+5784
RPS19 intron3