ELECTRONIC LETTER

Genomic deletion within *GLDC* is a major cause of non-ketotic hyperglycinaemia

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Background: Non-ketotic hyperglycinaemia (NKH) is an inborn error of metabolism characterised by accumulation of glycine in body fluids and various neurological symptoms. NKH is caused by deficiency of the glycine cleavage multienzyme system with three specific components encoded by *GLDC*, *AMT* and *GCSH*. Most patients are deficient of the enzymatic activity of glycine decarboxylase, which is encoded by *GLDC*. Our recent study has suggested that there are a considerable number of *GLDC* mutations which are not identified by the standard exon-sequencing method.

Methods: A screening system for *GLDC* deletions by multiplex ligation-dependent probe amplification (MLPA) has been developed. Two distinct cohorts of patients with typical NKH were screened by this method: the first cohort consisted of 45 families with no identified *AMT* or *GCSH* mutations, and the second cohort was comprised of 20 patients from the UK who were not prescreened for *AMT* mutations.

Results: *GLDC* deletions were identified in 16 of 90 alleles (18%) in the first cohort and in 9 of 40 alleles (22.5%) in the second cohort. 14 different types of deletions of various lengths were identified, including one allele where all 25 exons were missing. Flanking sequences of interstitial deletions in five patients were determined, and *Alu*-mediated recombination was identified in three of five patients.

Conclusions: *GLDC* deletions are a significant cause of NKH, and the MLPA analysis is a valuable first-line screening for NKH genetic testing.

Non-ketotic hyperglycinaemia (NKH), also called glycine encephalopathy, is an inborn error of glycine metabolism caused by deficiency of the glycine cleavage system (GCS).[1–3] Classically, NKH presents in the first few days of life with progressive lethargy, hypotonia, myoclonic jerks, hiccups and apnoea, usually leading to coma and death unless the patient is treated adequately.[4] Patients with atypical glycine encephalopathy often lack neonatal symptoms, but manifest aggressive behaviour, cognitive impairment, and impaired work or school performance.[5 6] Atypical patients manifest only non-specific clinical symptoms with most patients remaining undiagnosed and thus without the benefit of early diagnosis and treatment.[7] The fundamental defect of NKH lies in the mitochondrial GCS (EC2.1.2.10)[8] that consists of four individual proteins:[9] glycine decarboxylase encoded (also called P-protein) by *GLDC*; aminomethyltransferase (T-protein) encoded by *AMT*; hydrogen carrier protein (H-protein) encoded by *GCSH*; and dihydrolipoamide dehydrogenase encoded by *GCSL*. Dihydrolipoamide dehydrogenase is a housekeeping enzyme that serves as an E3 component of other enzyme complexes such as pyruvate dehydrogenase. The three GCS-specific genes are mapped on different chromosomes: *GLDC* on chromosome 9p24,[10] *AMT* on 3p21.1–21.2[11] and *GCSH* on 16q24.[12] Enzymatic analysis has shown that approximately 80% of patients with NKH are deficient of glycine decarboxylase activity.[13]

In Finnish patients we reported a common missense mutation, S564I, that accounts for 70% of mutant alleles.[14] Toone *et al*[15] reported a missense mutation, R515S, in 5% of Caucasian mutant alleles. Most of the reported mutations are, however, private, found in only a single family,[16–20] thus making DNA analysis difficult. Recently, we have undertaken a comprehensive mutation screening of the three genes, *GLDC*, *AMT* and *GCSH*, in patients with neonatal, infantile and late-onset types of NHK.[21] Various *GLDC* and *AMT* mutations were identified in patients with neonatal and infantile types of NHK, but not in those with the late onset type. Among 56 patients with the neonatal type, *GLDC* mutations were found in 36 patients, whereas *AMT* mutations were identified in 11 patients. In 14 of 36 patients, *GLDC* mutations were identified in only one allele, suggesting that some mutations are not detected by the exon-sequencing method. We have reported several patients with deletion of *GLDC* exon 1,[22] and Sellner *et al*[20] have reported a patient with deletion of the *GLDC* exons 2–15. These studies suggest that a considerable number of deletions may remain unidentified in *GLDC*.

The purpose of the present study was to establish a method of screening for deletions within *GLDC* and determine their frequency in patients with NKH. A multiplex ligation-dependent probe amplification (MLPA) method[23] was used to screen 65 patients with NKH. Using this method, 14 different types of exonic deletions were found in 25 of 130 alleles (19%) in patients with NKH. Our results suggest that deletions in the *GLDC* gene are a common cause of NKH, and that MLPA analysis is a useful first-line screening in NKH genetic testing.

METHODS

Patients with NKH

DNA samples were obtained from two cohorts of patients with typical NKH with a neonatal onset. Our original cohort of 56 patients with neonatal-type NKH[21] was found to contain 11 patients with *AMT* mutations. We excluded those 11 patients and defined a new cohort of the remaining 45 patients with NKH (the *AMT*-mutation negative cohort). The second cohort contained 20 patients (14 Caucasian and 6 from the Indian subcontinent) with neonatal-type NKH, who were referred to the Birmingham Children's Hospital, Birmingham, UK, for enzymatic and genetic confirmation of the clinical diagnosis of NKH. In the second cohort, screening for only the R515S and

Abbreviations: GCS, glycine cleavage system; MLPA, multiplex ligation-dependent probe amplification; NKH, non-ketotic hyperglycinaemia; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism

Table 1 Multiplex ligation-dependent probe amplification probes and polymerase chain reaction products in control participants

Target		Upstream probe			Downstream probe				Control values (n=18)		
Gene	Exon	Name	Location	Length (base)	Name	Location	Length (base)	PCR product size (bp)	Peak area (mean (SD)) arbitrary unit	Relative peak area (mean (SD))*	SD/mean (%)
GLDC	Exon 1	M-GLDC-E1U	Exon 1	50	M-GLDC-E1D	Intron 1	50	100	26557 (1944)	11.52 (0.57)	5
	Exon 2	M-GLDC-E2U	Exon 2	54	M-GLDC-E2D	Intron 2	54	108	41534 (1673)	18.04 (0.47)	3
	Exon 3	M-GLDC-E3U	Exon 3	58	M-GLDC-E3D	Intron 3	58	116	44796 (1710)	19.46 (0.31)	2
	Exon 4	M-GLDC-E4U	Exon 4	62	M-GLDC-E4D	Intron 4	62	124	36206 (1716)	15.72 (0.42)	3
	Exon 5	M-GLDC-E5U-2	Intron 4	66	M-GLDC-E5D-2	Exon 5	66	132	43086 (2700)	18.72 (1.07)	6
	Exon 6	M-GLDC-E6U	Exon 6	70	M-GLDC-E6D	Intron 6	70	140	42903 (1261)	18.64 (0.30)	2
	Exon 7	M-GLDC-E7U	Exon 7	74	M-GLDC-E7D	Intron 7	74	148	36397 (1739)	15.80 (0.33)	2
	Exon 8	M-GLDC-E8U	Exon 8	78	M-GLDC-E8D	Intron 8	78	156	26102(1643)	11.34 (0.71)	6
	Exon 9	M-GLDC-E9U	Exon 9	84	M-GLDC-E9D	Intron 9	80	164	30208 (1499)	13.15 (0.37)	3
	Exon 10	M-GLDC-E10U	Exon 10	92	M-GLDC-E10D	Intron 10	80	172	19567 (1174)	8.49 (0.25)	3
	Exon 11	M-GLDC-E11U	Exon 11	100	M-GLDC-E11D	Intron 11	80	180	24892 (1987)	10.80 (0.61)	6
	Exon 12	M-GLDC-E12U	Exon 12	108	M-GLDC-E12D	Intron 12	80	188	16336 (949)	7.09 (0.21)	3
	Exon 13	M-GLDC-E13U-2	Exon 13	112	M-GLDC-E13D	Intron 13	80	192	23856 (1097)	10.36 (0.26)	2
	Exon 15	M-GLDC-E15U	Exon 15	52	M-GLDC-E15D	Intron 15	52	104	45980 (1888)	19.97 (0.54)	3
	Exon 16	M-GLDC-E16U	Exon 16	56	M-GLDC-E16D	Intron 16	56	112	40630 (1275)	17.66 (0.53)	3
	Exon 17	M-GLDC-E17U	Exon 17	60	M-GLDC-E17D	Intron 17	60	120	43310 (1651)	18.82 (0.57)	3
	Exon 18	M-GLDC-E18U-2	Intron 17	64	M-GLDC-E18D-2	Exon 18	64	128	20771 (1002)	9.02 (0.31)	3
	Exon 19	M-GLDC-E19U	Exon 19	68	M-GLDC-E19D	Intron 19	68	136	33266 (1049)	14.45 (0.28)	2
	Exon 20	M-GLDC-E20U	Exon 20	72	M-GLDC-E20D	Intron 20	72	144	27145 (1199)	11.79 (0.32)	3
	Exon 21	M-GLDC-E21U	Exon 21	76	M-GLDC-E21D	Intron 21	76	152	26832 (1246)	11.65 (0.24)	2
	Exon 22	M-GLDC-E22U	Exon 22	80	M-GLDC-E22D	Intron 22	80	160	23008 (1083)	10.00 (0.51)	5
	Exon 23	M-GLDC-E23U	Exon 23	88	M-GLDC-E23D	Intron 23	80	168	20375 (1428)	8.84 (0.38)	4
	Exon 24	M-GLDC-E24U-2	Intron 24	96	M-GLDC-E24D-2	Exon 24	80	176	20051 (609)	8.72 (0.31)	4
	Exon 25	M-GLDC-E25U	Exon 25	104	M-GLDC-E25D	Intron 25	80	184	29957 (1235)	13.10 (0.25)	2
GLDCP	Processed pseudogene	M-GLDC-E1U†	Pseudogene	50	M-GLDCP-1D	Pseudogene	47	97	33495 (1483)	–	–
EXT2	Exon 13	M-EXT2-E13U	Exon 13	41	M-EXT2-E13D	Exon 13	44	85	67822 (4013)	–	–
AMT	Exon 1	M-AMT-E1U	Exon 1	44	M-AMT-E1D	Intron 1	44	88	51455 (2056)	–	–
	Exon 4	M-AMT-E4U	Exon 4	45	M-AMT-E4D	Intron 4	46	91	27010 (1212)	–	–
	Exon 9	M-AMT-E9U-2	Intron 8	47	M-AMT-E9D-2	Exon 9	47	94	50490 (2102)	–	–

PCR, polymerase chain reaction.
*Peak area of each *GLDC* exon/sum of peak areas of *GLDCP, EXT2, AMT* exon 1, *AMT* exon 4 and *AMT* exon 9; †shared with upstream primer for *GLDC* exon 1.

Table 2 Nucleotide sequences of the MPLA probes for detection of *GLDC* deletions

Target gene	Probe name	Nucleotide sequences (5′ to 3′)
GLDC	M-GLDC-E1U	GGGTTCCCTAAGGGTTGGAGAGAGAGATGCTGCAGACCTTGGGGCTGGCG
	M-GLDC-E1D	*GTAAGGACCTCCACCCGGCCCTCCGCGTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E2U	GGGTTCCCTAAGGGTTGGATTTGAAAAGACCCTTGAAAATGGAAGACCCTGTTT
	M-GLDC-E2D	*GTAAGTGGCCGGGAGGGCTCCCTTGGACTTATCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E3U	GGGTTCCCTAAGGGTTGGAACAGACGATTTTGCGGAACTTACTGGAGAACTCAGGATG
	M-GLDC-E3D	*GTAATGTATTTCTCAGTTCAGGAACAGGATGACTGTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E4U	GGGTTCCCTAAGGGTTGGAATGAGGGGACTGCAGCCGCAGAGGCACTGCAGCTGTGCTACAG
	M-GLDC-E4D	*GTGAGAGGCCCTCTCAAAGTGCTGGAATTCCAGTTGTGGGTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E5U-2	GGGTTCCCTAAGGGTTGGACTATTTATTTAATGTTCACGTTGGAATGTGCTTTTTCTTTTCAACAG
	M-GLDC-E5D-2	*ACACAACAAGAGGAGGAAATTTCTCGTTGATCCCCGTTGCCACTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E6U	GGGTTCCCTAAGGGTTGGAGGGAAGGTGGAAGACTTTACGGAACTCGTGGAGAGAGCTCATCAGAGTGGG
	M-GLDC-E6D	*GTAGGTATACCTTTCTTGTGGGGGGTCCGTGGAGGCGTATCCCAACTTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E7U	GGGTTCCCTAAGGGTTGGACTGTCCGAGAAAGCTTGGTGAGAATGATGCCTGGAAGAATGGTGGGGGTAACAAG
	M-GLDC-E7D	*GTAAAGGGGCTCATGTTTCTCTACTTTTATTGTGATTATGATTTCCCTGATTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E8U-2	GGGTTCCCTAAGGGTTGGACCATTTTCTCAGTGGGAACTAAGGGCGGGCCTCTTCAGTTCCCAC
	M-GLDC-E8D-2	*CTGAGCATTCATATTTGCCCCGTCTAGGTAGACCTGTCCTCTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E9U	GGGTTCCCTAAGGGTTGGAATGTTCCCATGGGCTGGAGCATATTGCTAGGAGGGTACATAATGCCACTTTGATTTTGTCAGAAG
	M-GLDC-E9D	*GTGAGTTGGTAATCTGTCTAAAACATTTGGGCATAATAAAATTGATAAATTTGAGTATCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E10U	GGGTTCCCTAAGGGTTGGAGCTGCTAGTGAAGGAGGTCTTGGGCAGGGCCGCTCAGCGGCAGATCAATTTTCGGCTTTTT GAGGATGGCACA
	M-GLDC-E10D	*GTAAGTCAAATTTTCAGTATTTTTACCAGTTTTTCAAATTTTCACATTGTTTCTCATTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E11U	GGGTTCCCTAAGGGTTGGACTTGGTATTTCTCTTGATGAaACAGTCAATGAAAAAGATCTGGACGATTTGTTGTGGATCTTTGG TTGTGAGTCATCTGCA
	M-GLDC-E11D	*GTAAGTAAAATAAAAACATGCGTTCCTCAtCATAACTATTGGAGGTGGTAGCAAAAGTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E12U	GGGTTCCCTAAGGGTTGGAGCTGAAAGCATGGGAGAGGAGTGCAGAGGTATTCCAGGGTCTGTGTTCAAGAGGACC AGCCCGTTCCTCACCCATCAAGTGTTCAACAG
	M-GLDC-E12D	*GTTTGTGTGTCTTGTGTGACTTCTGCGTTTTGTGCTTTGGTAATCAGCTAGGGAGTTTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E13U-2	GGGTTCCCTAAGGGTTGGATGTTCACAGCTACCACTCTGAAACAAACATTGTCCGGTACATGAAGAAACTGGAAAATAAAGA CATTCCCTTGTTCACAGCATGATTCCACT
	M-GLDC-E13D	*GGTAGTTATTTGTGGCCTTTTTTCTCATTTCCAAGCTACCCCAATCCCACGTCTCTTTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E15U	GGGTTCCCTAAGGGTTGGAAGGTTATGACCAGGTCTGTTTCCAGCCAAACAG
	M-GLDC-E15D	*GTAAGGGCATTTCTTTTCTTATTGTTTCATCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E16U	GGGTTCCCTAAGGGTTGGAAGCCTACTTAAACCAGAAAGGAGAGGGGCACAGAACG
	M-GLDC-E16D	*GTGAGTATGGCAGGAGGTGGCGCTTGCTCACCATCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E17U	GGGTTCCCTAAGGGTTGGAATAAATATGGGAATATCGATGCAGTTCACCTCAAGGCCATG
	M-GLDC-E17D	*GTACTTGTCTTCTCCTTAGCAGATGGGAGAGGCCGGATCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E18U-2	GGGTTCCCTAAGGGTTGGACCATTTTCTCAGTGGGAACTAAGGGCGGGCCTCTTCAGTTCCCAC
	M-GLDC-E18D-2	*CTGAGCATTCATATTTGCCCCGTCTAGGTAGACCTGTCCTCTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E19U	GGGTTCCCTAAGGGTTGGATCTGCATTCCCCACGGAGGAGGTGGTCCTGGCATGGGGCCCCATCGGAGT
	M-GLDC-E19D	*GTAAGTTCTGGGCTGCTGGTTTCAGGATGGCTTTGGAGACAGAATTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E20U	GGGTTCCCTAAGGGTTGGACGGCCCCATGGGGCTCCAGTTCCATCTTGCCCATTTCCTGGGCTTATATCAAG
	M-GLDC-E20D	*GTGAGGCCTGGGAGTATGTGCAGGTGTGCAGGTGGGTGGGGGCGTCAGGTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E21U	GGGTTCCCTAAGGGTTGGAACTACATGGCCAAGCGATTAGAAACACACTACAGAATTCTTTTCAGGGGTGCAAGAG
	M-GLDC-E21D	*GCAAGTATCAACTTTAATCTATCATTACTTGGTTTTTTTCTTGGCCAAACTAATCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E22U	GGGTTCCCTAAGGGTTGGACCCTTCAAAAAGTCTGCAAATATTGAGGCTGTGGATGTGGCCAAGAGACTCCAGGATTATG
	M-GLDC-E22D	*GTAAGTGGCTTTTGACATTCATGCCGCCGCCCATGCTGGCTGTGGACCACTTCCTAATCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E23U	GGGTTCCCTAAGGGTTGGAATCAGCATTCGGCAGGAAATTGCTGACATTGAGGAGGGCCGCATCGACCCCAGGGTCAAT CCGCTGAAG
	M-GLDC-E23D	*GTGCGTAGGCCCTGGAACATTGCTTGAAATGTTCCTTAAACTAGAAAATGATGTCTGTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E24U-2	GGGTTCCCTAAGGGTTGGAGCTAAGAGCGTACACCCGTCAGGATAGGAGCTGGCCCATGCCTTCCCAGCTGGCACATTC AGATTCAGAGAACTTAC
	M-GLDC-E24D-2	*GAGTGGGAATGCTGCCACCTCTCTGGAATAAGGCCGGTCCCAGTGGGAAGATGTAACTCTAGATTGGATCTTGCTGGCAC
	M-GLDC-E25U	GGGTTCCCTAAGGGTTGGATGTGGGACTAGCATTGCCACCTCCTTTGCCCTAAGAGAAACCTCCCAGAACATCTCACAGCA TTTCCATCTTTTGTCCTTTGCAG
	M-GLDC-E25D	*CCCTTCGTGAAACCAGAGAACAAATTCTGGCCAACGATTGCCCGGATTGATGACATATCTAGATTGGATCTTGCTGGCAC
GLDCP	M-GLDCP-1D	*AGCATTGATGAATTGATCGAGAAGTCTAGATTGGATCTTGCTGGCAC
EXT2	M-EXT2-E13U	GGGTTCCCTAAGGGTTGGACAGCCATAGATGGGCTTTCACT
	M-EXT2-E13D	*AGACCAAACACACATGGTGGATCTAGATTGGATCTTGCTGGCAC
AMT	M-AMT-E1U	GGGTTCCCTAAGGGTTGGAGATGCAGAGGGCTGTAAGTGTGGTG
	M-AMT-E1D	*GCCCGTCTGGGCTTTCGCCTGTCTAGATTGGATCTTGCTGGCAC
	M-AMT-E4U	GGGTTCCCTAAGGGTTGGAAGGGCCACCTGTATGTGGTGTCCAAC
	M-AMT-E4D	*GCTGGCTGCTGGGAGAAAGATTTCTAGATTGGATCTTGCTGGCAC
	M-AMT-E9U-2	GGGTTCCCTAAGGGTTGGATGATGCGTGGCTTATGCTTGCTTGACAG
	M-AMT-E9D-2	*GTACTGTGACTAGTGGCTGCCCCTTCTAGATTGGATCTTGCTGGCAC

Single-underlined and double-underlined sequences are binding sites for forward and reverse polymerase chain reaction (PCR) primers, respectively.
*5′ phosphorylation.

A389V mutations in the *GLDC* gene was conducted. The study was approved by the Ethics Committee of Tohoku University School of Medicine, Sendai, Japan, and all patients or their legal representatives gave informed consent for DNA analysis.

Synthetic MLPA probes

In all, 29 pairs of MLPA probes were designed for analysis (table 1). As there is a processed pseudogene (*GLDCP)* which is 98% homologous with *GLDC* exons,[22] probes for the *GLDC* gene

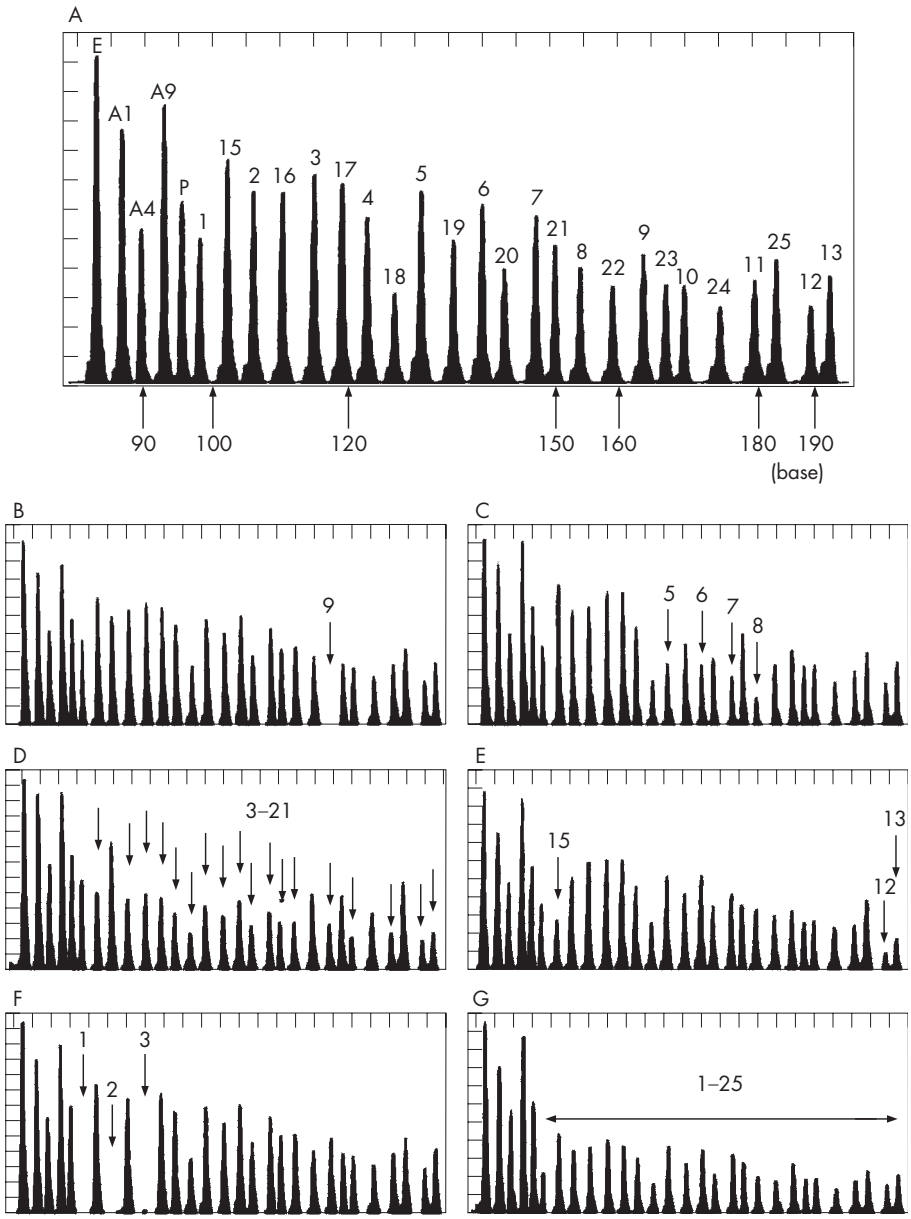

Figure 1 Multiplex ligation-dependent probe amplification (MLPA) analysis of a control subject and patients with non-ketotic hyperglycinaemia (NKH) with neonatal onset. A representative MLPA chromatogram of a control participant (A). The five control peaks include *EXT2* exon 13 (E), *AMT* exons 1, 4 and 9 (A1, A4, A9), and *GLDCP* (P). The number on each peak indicates the exon number of the *GLDC* gene. MLPA probe for *GLDC* exon 14 was not used in this assay. MLPA analysis of patients with NKH: homozygotic deletion of exon 9 (B), heterozygotic deletion of exons 5–8 (C), heterozygotic deletion of exons 3–21 (D), heterozygotic deletion of exons 12–15 (E), homozygotic deletion of exons 1–3 (F) and heterozygotic deletion of all 25 *GLDC* exons (G).

were placed at the 5′ or 3′ junction of each exon. No probe for exon 14 was used, as it lies only 175 bp from exon 13. Probes for *AMT* exons 1, 4 and 9, *EXT2* exon 13, and *GLDCP* were used as gene dose controls for estimation of *GLDC* copy number. The length of the synthetic MLPA probes ranged from 41 to 112 bp in size. Table 2 shows their nucleotide sequences. The probe for *EXT2* exon 13 was synthesised as reported previously.[24] We first tested the 3′ end of each exon as the target site. However, this did not work for *GLDC* exon 5, 18, 24 and *AMT* exon 9, so probes were designed at these 5′ regions of the exons. All downstream MLPA probes were 5′ phosphorylated for ligation with the upstream probes. The MLPA probe mixture was prepared by mixing 2 nmol/l of each MLPA probe, and used as described below.

MLPA procedures

An MLPA P0 FAM detection kit (MRC Holland, Amsterdam, The Netherlands) was used in this study. This kit contains all the necessary reagents except the MLPA probe mixture. MLPA was performed essentially according to the manufacturer's instructions (www.mrc-holland.com). Briefly, 50–250 ng of genomic DNA was used as the starting material, and after hybridisation, ligation and amplification, the PCR products were size-separated by an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). For normalisation, relative peak areas were calculated by dividing each measured peak area by the sum of the five control peak areas (table 1). Mean and SD were obtained by testing 18 control DNA samples.

Table 3 *GLDC* deletions identified in patients with NKH

Deletion	Missing exons	Number of alleles	Family	Ethnicity	Other allele	Comment
First cohort (*AMT*-mutation negative, 45 families)						
1	Exons 1–2	2	P14	Caucasian	c.2714T→G (p.V905G)	
			P36	Caucasian	Deletion (exons 1–17)	
2	Exons 1–3	3	P5	Oriental	Deletion (exons 1–3)	Homzygote, consanguinity (−)
			P70	Oriental	Unidentified	
3	Exons 1–17	2	P36	Caucasian	Deletion (exons 1–2)	
			P40	Caucasian	Unidentified	
4	Exons 1–25	1	P32	Caucasian	c.1786C→T (p.R596X)	
5	Exons 3–4	1	P69	Oriental	c.2311G.A (p.G771R)	
6	Exons 3–8	1	P120	Oriental	c.2574T→G (p.Y858X)	
7	Exons 3–9	1	P47	Oriental	c.2519T→A (p.M840K)	
8	Exons 3–22	1	P48	Caucasian	c.2665+1G→C	
9	Exons 12–15	4	P7	Oriental	c.2266_2268del TTC	
			P8	Oriental	c.2080G→C (p.A694P)	
			P22	Oriental	Unidentified	
			P74	Oriental	c.2311G→A (p.G771R)	
Second cohort (not prescreened for *AMT* mutation, 20 families)						
1	Exon 1	1	B3	Caucasian	Unidentified	
2	Exons 1–2	2	B8	Caucasian	c.1545G→C (p.R515S)	
			B13	Caucasian	c.1545G→C (p.R515S)	
3	Exons 1–16	2	B10	Pakistani	Deletion (exons 1–16)	Homozygote, consanguinity (+)
4	Exons 3–21	1	B6	Caucasian	Unidentified	
5	Exon 9	2	B7	Caucasian	Deletion (exon 9)	Homozygote, consanguinity (+)
6	Exons 5–8	1	B18	Caucasian	c.1545G→C (p.R515S)	

Long-range PCR

To clarify the boundary sequences of the deleted fragments, we used nested and long-range PCR with the LA PCR kit (TaKaRa Co Ltd, Tokyo, Japan) for PCR across the breakpoints. PCR fragments containing the boundary sequences of the deletions were size-separated on 1% agarose gel and bands with the expected sizes were cut out for purification by the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). Purified PCR fragments were subjected to the dye-terminator-sequencing analysis with the BigDye Terminator Sequencing Kit (Applied Biosystems).

RESULTS

MPLA analysis in control DNA

Eighteen control DNA samples were tested to estimate the deviation of each peak area. Figure 1A shows a representative chromatogram with all 29 peaks. Intervals between the peaks correspond to a difference of three or four bases in size of DNA fragments. Each peak area was measured and the mean (SD) was calculated (table 1). The sum of the five control peak areas (*EXT2* exon 13, *AMT* exons 1, 4 and 9, and *GLDCP)* was used to normalise the relative peak area of each *GLDC* exon. As a result, the mean (SD) ranged from 2% to 6%. We therefore set a screening threshold for deletion as <80%, <–3 SD from the mean value.

MPLA analysis in patients with NKH

Two independent cohorts of patients with neonatal-onset NKH were screened by our MLPA system. Nine different types of *GLDC* deletions were detected in the first *AMT*-mutation negative cohort of patients, whereas six different types of deletions were found in the second cohort of patients, in which no prescreening of *AMT* mutation had been performed (table 3). Figure 1 shows six representative results of *GLDC* deletions: homozygotic deletion of exon 9 (fig 1B), heterozygotic deletion of exons 5–8 (fig 1C), heterozygotic deletion of exons 3–21 (fig 1D), heterozygotic deletion of exons 12–15 (fig 1E), homozygotic deletion of exons 1–3 (fig 1F) and heterozygotic deletion involving all 25 *GLDC* exons (fig 1G). In the first cohort, a total of 16 deletion alleles were identified in 90 mutant alleles (18%). In the second cohort, 9 of 40 (22.5%) alleles were positive for deletion screening. No deletions of *AMT* exons 1, 4 and 9 were detected in this study. MLPA analysis of family P41 suggested that the patient was homozygotic for a deletion of exon 7 (data not shown). Subsequent sequencing analysis of the probe binding sites disclosed a one-bp deletion, c.1054delA, in the M-GLDC-E7U binding site. Similarly, in the MLPA analysis of family B5, both parents appeared to be heterozygotic for a deletion of exon 5 (data not shown). Sequencing of the probe binding sites showed that this was due to a single base substitution in the M-GLDC-E5U-2 binding site on one allele. Unfortunately, no DNA was available from the index case, but the patient from family B5 was presumed to be homozygotic for this c.636-1G→C mutation, which was deduced to abolish the conserved consensus AG at the splicing acceptor sites.

Identification of boundary sequences of the deletions

To confirm the deletions identified by the MPLA study and elucidate the mechanisms of the deletions, we examined the boundary sequences of four interstitial deletions within *GLDC*. We examined the patient homozygotic for a deletion of exon 9 (family B7 in the second cohort), the patient with heterozygotic deletion of exons 5–8 (family B18 in the second cohort), the patient with heterozygotic deletion of exons 3–21 (family B6 in the second cohort) and the patients with heterozygotic deletions of exons 12–15 (families P74 and P8 in the first cohort; fig 2). Nested long-range PCR was employed in this analysis, which was followed by direct sequencing analysis. In the patient of family B7, a 7906-bp deletion was identified, extending from the 3′ end of intron 8 (~6 kb) to the 5′ end of intron 9 (~2 kb) as shown in fig 2A. In the patient in family B18, we found a 10 422-bp deletion beginning at the 3′ end of intron 4 (~3 kb) and including exons 5–8, up to the 5′ end of intron 8 with ~3 kb (fig 2B). The patient in family B6 had the longest deletion, 99 395 bp, among the four patients. Both 5′

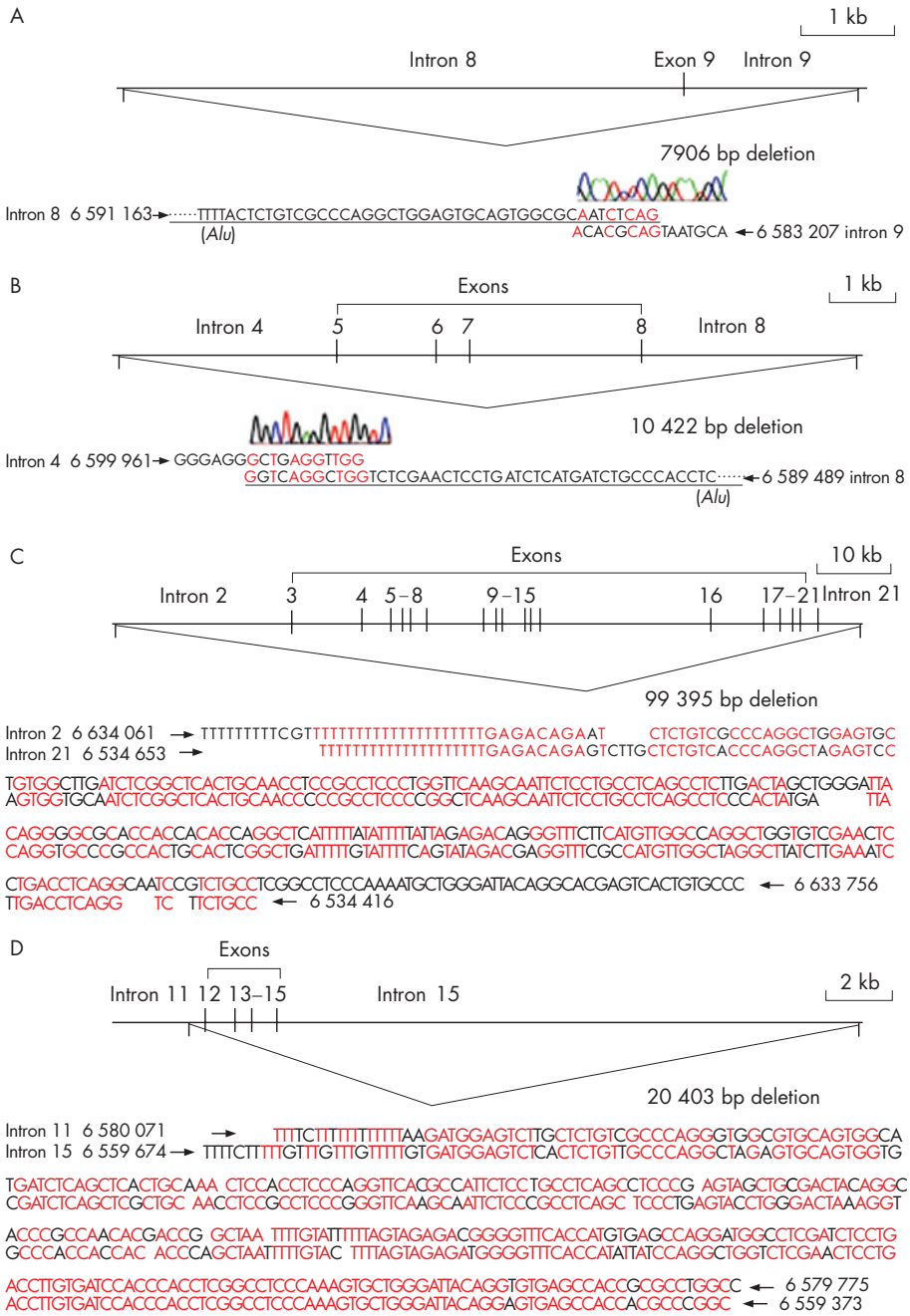

Figure 2 Boundary sequences of the deleted fragments. The boundary sequences of four different types of *GLDC* deletions were identified: the deletion of exon 9 in family B7 (A), the deletion of exons 5–8 in family B18 (B), the deletion of exons 3–21 in family B6 (C) and the deletion of exons 12–15 in family P74 (D). The boundary sequence found in family P74 was the same as that in family P8. Nucleotide sequences in red indicate the identical bases in the 5′ and 3′ flanks of the deletions.

and 3′ fragments shared near identical sequences as shown in fig 2C. In a Japanese patient (P74) with heterozygotic deletion of exons 12–15, a 20 403-bp deletion was identified (fig 2D). The deleted fragment consisted of a short 3′ end of intron 11 (~0.6 kb), exons 12–15 and the 5′ end of intron 15 (~18 kb). The identical breakpoint was also found in the patient from family P8 (data not shown). Thus far, the deleted fragments flanked with *Alu* motifs in B6, P74, and P8 patients, but not in patients from families B7 or B18. Four Caucasian patients (from P14, P36, B13 and B8) had a deletion of exons 1 and 2. However, as the two patients, B13 and B8, from the UK had different haplotypes (data not shown), this deletion has occurred more than once. This observation agrees with our previous finding that the deletions of the *GLDC* exon 1 had multiple origins.[21]

Distribution of deletions in the *GLDC* gene

Figure 3 shows the distribution of the missing exons by *GLDC* deletions. The lengths of the *GLDC* deletions were heterogeneous, ranging from a single exon to all 25. Of the 50 breakpoints of the deletions, 26 (52%) were found 5′ upstream of the *GLDC* gene or in introns 1–3, suggesting that deletions

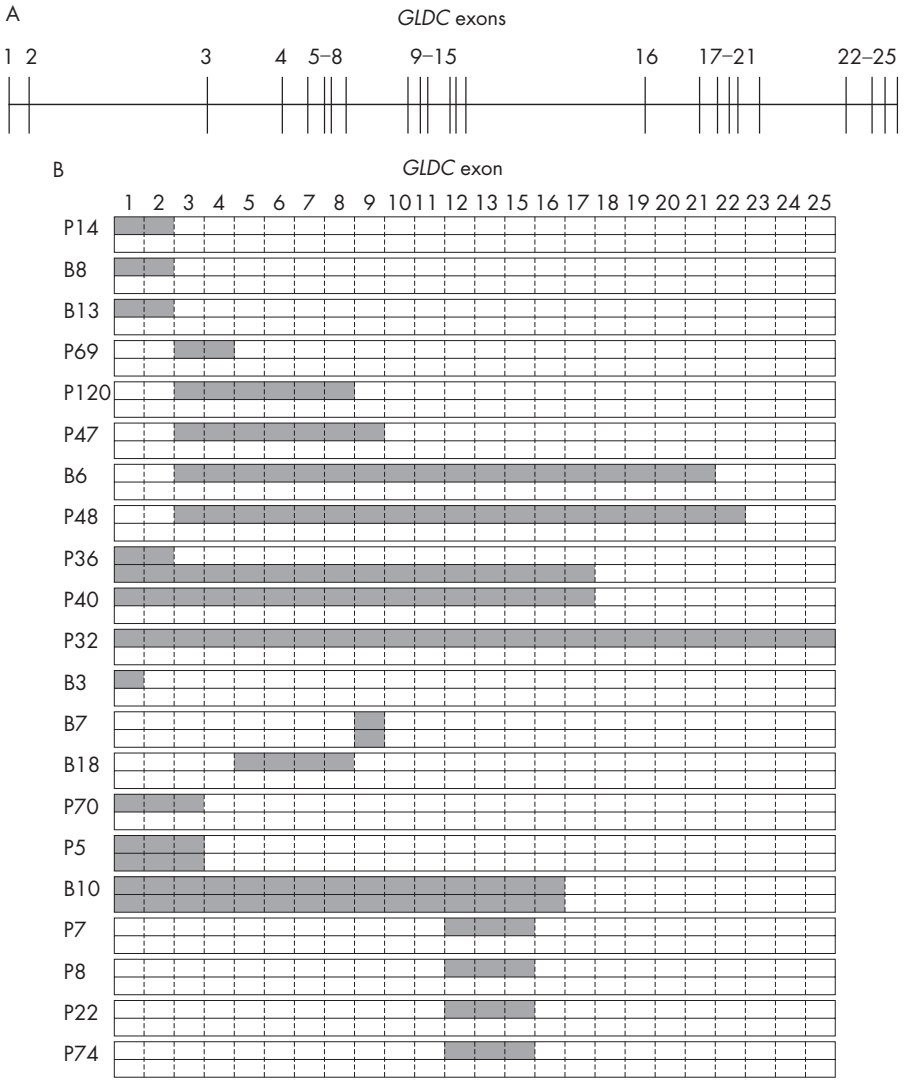

Figure 3 Distribution of missing exons by the *GLDC* deletions. The exon–intron organisation of the *GLDC* gene is illustrated (A). Patients with non-ketotic hyperglycinaemia with *GLDC* deletions were classified by their missing exons (B). Hatched boxes indicate *GLDC* exons involved in the deletions. Note the clustering of the deletion breakpoints in the 5′ end of *GLDC*.

tend to occur in the 5′ part of the *GLDC* gene, a region relatively rich in *Alu* repeats.

DISCUSSION

We established a detection system for *GLDC* deletions by using the MLPA method, and showed that deletions within this gene are a common cause of NKH. Fourteen different types of *GLDC* deletions were identified in screening 65 patients with neonatal-onset NKH. *GLDC* deletions were identified in 21 of 65 patients with NKH (32.3%), and in 25 of 130 NKH alleles (19.2%) by MLPA analysis. The MLPA method provides a good first-line screen in a condition where there are no common mutations and full sequencing of 25 exons of the *GLDC* gene is a lengthy process. The deletion detection rates by MLPA analysis were 18% and 22.5% in the first and second cohorts, respectively. In our previous study, the exon-sequencing analysis has shown *GLDC* mutations in 41 of 90 alleles (45%).[21] Thus, this MLPA test improved the sensitivity of mutation detection from 45% to 63%. Mutations for NKH are highly heterogeneous: the prevalent mutations previously reported are Finnish S564I mutation (70%)[14] and Caucasian R515S mutation (5%),[15] hampering the genetic testing in diagnosis of NKH. In contrast, *GLDC* deletions seem to be prevalent in different ethnic groups. In a previous study, we analysed the relative allele number of the *GLDC* exon 1 by using *GLDCP* as a copy number control.[22] As MLPA analysis covers the whole gene in one simple assay, it is highly recommended for the first screening in the genetic testing of NKH.

Point mutations in MLPA-probe binding sites may cause false positives in MLPA analysis, notably where a single exon is deleted. A mismatching in the binding site of the MLPA probes is known to reduce the ligation efficiency. In our study, we encountered four single-exon deletions in the analysis of families P41, B3, B5 and B7. Subsequent sequencing analysis of the probe binding sites showed that the patient in family P41 had a 1-bp deletion and that the patient from B5 carried a 1-base substitution at the splicing accepter site of intron 4. Both mutations are predicted to be disease causing. No base change was found in the patient from B3. In the patient from B7, exon 9 of the patient failed to be amplified by PCR and a single-exon deletion was confirmed by subsequent sequencing across the breakpoint (fig 2A). As the MLPA probes for *GLDC* were

Key points

- A screening system for genomic deletions within *GLDC* has been developed by the multiplex-ligation-dependent probe amplification (MLPA) method.
- *GLDC* deletions were identified in approximately 20% of non-ketotic hyperglycinaemia (NKH) mutant alleles.
- The MLPA analysis is useful for first-line screening in the genetic testing of NKH.

designed to bind an exon–intron boundary to avoid detection of the pseudogene of *GLDC*, *GLDCP,* the MLPA method can also detect some mutations that cause aberrant splicing. Sequencing the probe-binding regions of the *GLDC* gene where MLPA analysis suggests a single-exon deletion is therefore necessary before making a diagnosis of *GLDC* deletion.

In a previous study, we diagnosed the patient of family P32 as a homozygote of a nonsense mutation, c.1786C→T (p.R596X), although there was no history of consanguinity.[21] A familial study was not possible because no parental DNA was available. The present study showed that he was heterozygotic for a deletion containing all 25 *GLDC* exons (table 3, fig 1G), indicating that he was a compound heterozygote of the nonsense mutation c.1786C→T and the deletion of exons 1–25. As this deletion was the biggest one so far identified, we looked to see whether it involved any adjacent genes. We performed a microarray analysis to determine the genotypes of many single-nucleotide polymorphisms (SNPs) by using the GeneChip Human Mapping 100 k Set (Affymetrix, Santa Clara, California, USA). *GLDC* is located between base positions 6635650 and 6522467 bp in chromosome 9 (GenBank, NT_008413). The JMJD2C gene (6748083–7165647 bp) is located 5′ upstream of *GLDC* whereas the UHRF2 gene (6403151–6497051 bp) lies 3′ downstream of *GLDC*. The SNP at the base position 6606648 bp, which is located within the *GLDC* gene, was indeed homozygotic in this patient (data not shown). In contrast, two SNPs at the base positions of 6513056 and 6759229 bp were heterozygotic, suggesting that the deletion is <246 kb, and thus that the two adjacent genes are unlikely to be involved in the deletion.

We determined flanking sequences of interstitial deletions in five patients, and *Alu*-mediated recombination was identified in three of five patients. The *Alu* elements, approximately 300 bp in length, compose about 10% of the whole human genome.[25] There are several inherited disorders in which *Alu*-mediated recombination/deletion is a common cause: hereditary angioedema, *C1-INH*;[26] α-thalassemia, α-globin gene;[27] and Ehlers–Danlos syndrome, *PLOD*.[28] Recently, *Alu*-mediated genomic recombination has also been reported in non-inherited human cancer, hepatoma.[29] A total of 120 copies of *Alu* repeats are present in the *GLDC* gene, which has a length of 113 kb, resulting in one *Alu* of 1.1 kb on average. This is much higher than the average density of one *Alu* every 3–4 kb over the whole human genome.[30] The *GLDC* deletions tend to be located in the 5′ end of the *GLDC* gene, which may be explained by the fact that the region contains a high number of *Alu* repeats.

The diagnosis of NKH is difficult to establish on clinical and biochemical grounds alone, and typically requires a liver biopsy for enzyme analysis or DNA studies to confirm a diagnosis. However, the complex nature of the genetics of NKH (three genes and no common mutations) makes DNA analysis a lengthy and difficult process. Our finding that deletions within the *GLDC* gene are one of the most common causes of NKH and the development of a simple assay for such mutations will make genetic analysis for this disorder much more straightforward. Such analysis will reduce the need for a liver biopsy in a sick child, make diagnosis easier, and improve the ease and reliability of antenatal diagnosis.

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