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ORIGINAL ARTICLE

Mutation analysis of the PIG-A gene in Korean patients with paroxysmal nocturnal haemoglobinuria

J H Yoon, H I Cho, S S Park, Y H Chang, B K Kim

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Aim: Paroxysmal nocturnal haemoglobinuria (PNH) is caused by deficient biosynthesis of the glycosylphosphatidylinositol (GPI) anchor in haemopoietic stem cells. Mutation of the phosphatidylinositol glycan class A (PIG-A) gene, an X linked gene that participates in the first step of GPI anchor biosynthesis, is responsible for PNH. The characteristics of somatic mutation of the PIG-A gene in Korean patients with PNH were studied.

Methods: Twenty four patients with PNH were selected. Ham tests and sucrose haemolysis tests were carried out on all patients. The expression of CD59 in erythrocytes and granulocytes was investigated in 14 and five patients, respectively, to confirm the diagnosis. Dideoxy fingerprinting (ddF) was used to screen mutations, and direct sequencing of DNA was performed to characterise the mutations.

Results: Gene mutation was detected in 12 of the 24 patients. The other 12 patients were negative in ddF screening. Ten new mutations and two known mutations were detected. The mutations consisted of five deletions, six substitutions, and one insertion. These mutations resulted in six premature terminations, three abnormal splicings, one missense mutation in exon 2, and two nonsense mutations. Two patients with venous thrombosis showed mutations in exon 3 only. Substitution mutations were seen in six patients and frameshift mutations in the other six.

Conclusions: There were 10 new mutations among the 12 mutations in the Korean patients with PNH and the characteristics of the mutations varied, with no significant hot spots in sites or types.

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Paroxysmal nocturnal haemoglobinuria (PNH) is a clonal stem cell disorder. The pathogenic mechanism of PNH is a deficiency of glycosylphosphatidylinositol (GPI) anchored proteins, which results in an abnormal sensitivity of red cells to complement.[1–3] Anchored protein deficiency is caused by mutation of the phosphatidylinositol glycan class A (PIG-A) gene, an X linked gene that encodes the anchor protein of the first stage of synthesis.[4] A frameshift mutation is the most common mutation found.[5–20] Cluster regions have not been noted.

> "The pathogenic mechanism of PNH is a deficiency of glycosylphosphatidylinositol anchored proteins, which results in an abnormal sensitivity of red cells to complement"

PNH is the second most common haemolytic anaemia in Korea, with autoimmune haemolytic anaemia being the most common form.[21] PNH is diagnosed on the basis of the clinical symptoms, which include intravascular haemolysis with recurrent episodes of emission of dark urine in the morning, and following confirmation by the Ham test. However, it is often not easy to make a definitive diagnosis of PNH because of the complexity of the clinical picture. Molecular testing for mutations in the PIG-A gene can also serve as a confirmation test of PNH. Hence, our study was designed to obtain information on the mutation sites of the PIG-A gene in Korean patients with PNH.

METHODS

Twenty four Korean patients with PNH who had been diagnosed based on the Ham test were selected. Ten of these patients were female. We obtained bone marrow smears from 10 patients and peripheral blood specimens from 14 for our study. CD59 expression was investigated in the red blood cells of 14 patients and the granulocytes of five using flow cytometry (Becton Dickinson, Franklin Lakes, New Jersey, USA). Differences in CD59 expression in red blood cells between mutation positive and mutation negative groups were analysed by means of the Wilcoxon rank sum test, using PC-SAS 6.04 (SAS Institute, Cary, North Carolina, USA) software.

Genomic DNA was isolated from blood using the salting out method and by phenol extraction from bone marrow smears.[22 23] Five polymerase chain reaction (PCR) primers for the PIG-A gene were prepared (Operon Technologies Inc, Alameda, California, USA)[5] (fig 1). Mutations were screened by the dideoxy finger printing (ddF) method, a modified PCR single strand conformation polymorphism method. The PCR product was purified using the BM High Pure PCR purification kit (Roche, Basel, Switzerland) and the ddF reaction was performed using the ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Pharmacia Biotech, Cleveland, Ohio, USA). Electrophoresis was performed on a 0.35 mm, quarter diluted non-denaturing mutation detection enhancement gel (FIMC, Rockland, Maryland, USA).

DNA sequencing was carried out on the samples obtained from the patients screened by ddF to confirm the mutation sites. Direct sequencing was achieved using the ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Pharmacia Biotech) and an 8% polyacrylamide gel.

RESULTS

We detected somatic mutations in 12 of the 24 patients with PNH who had been diagnosed by means of Ham tests. The other 12 patients were negative in ddF screening. Mutations

Abbreviations: ddF, dideoxy fingerprinting; GPI, glycosylphosphatidylinositol anchor; PCR, polymerase chain reaction; PIG-A, phosphatidylinositol glycan class A; PNH, paroxysmal nocturnal haemoglobinuria

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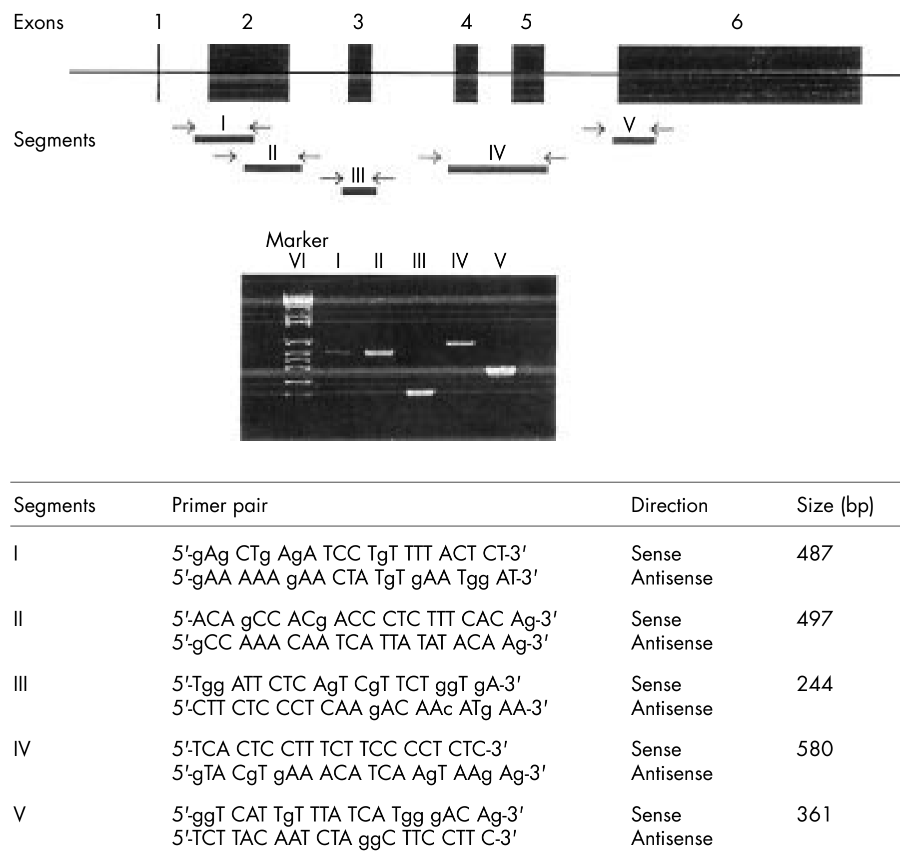

Segments	Primer pair	Direction	Size (bp)
I	5′-gAg CTg AgA TCC TgT TTT ACT CT-3′ 5′-gAA AAA gAA CTA TgT gAA Tgg AT-3′	Sense Antisense	487
II	5′-ACA gCC ACg ACC CTC TTT CAC Ag-3′ 5′-gCC AAA CAA TCA TTA TAT ACA Ag-3′	Sense Antisense	497
III	5′-Tgg ATT CTC AgT CgT TCT ggT gA-3′ 5′-CTT CTC CCT CAA gAC AAc ATg AA-3′	Sense Antisense	244
IV	5′-TCA CTC CTT TCT TCC CCT CTC-3′ 5′-gTA CgT gAA ACA TCA AgT AAg Ag-3′	Sense Antisense	580
V	5′-ggT CAT TgT TTA TCA Tgg gAC Ag-3′ 5′-TCT TAC AAT CTA ggC TTC CTT C-3′	Sense Antisense	361

Figure 1 Map and sequence of the primers used for mutational analysis of the PIG-A gene.

were detected in only two of the 10 female patients with PNH. The remaining mutations were detected in 10 of the 14 male patients.

Ten novel mutations and two known mutations were detected (table 1). The mutations consisted of five deletions (three multiple base deletions and two single base deletions), six substitutions, and one insertion. These mutations resulted in six premature terminations, three abnormal splicings, two nonsense mutations, and one missense mutation. The missense mutation was in exon 2 (fig 2). Two patients with venous thrombosis showed mutations in exon 3 only. Substitution mutations were seen in six patients and frameshift mutations in the other six. No hot mutation spots were encountered.

PNH III red blood cells comprised 60–93.7% (mean, 80.2%) of the total red blood cells in the mutation positive group and 9.0–98.7% (mean, 46.7%) in the mutation negative group. PNH I red blood cells comprised 2.8–20.3% (mean, 9.0%) of the total red blood cells in the mutation positive group and 1.3–61.0% (mean, 28.1%) of the mutation negative group (table 2). There was a significant difference between the mean values in the mutation positive and mutation negative groups for the PNH III red blood cells ($p = 0.0298$), but not for the PNH I red blood cells ($p = 0.0967$).

DISCUSSION

We detected somatic mutations in only 12 of the 24 patients with PNH who had been diagnosed by Ham tests. Such a low

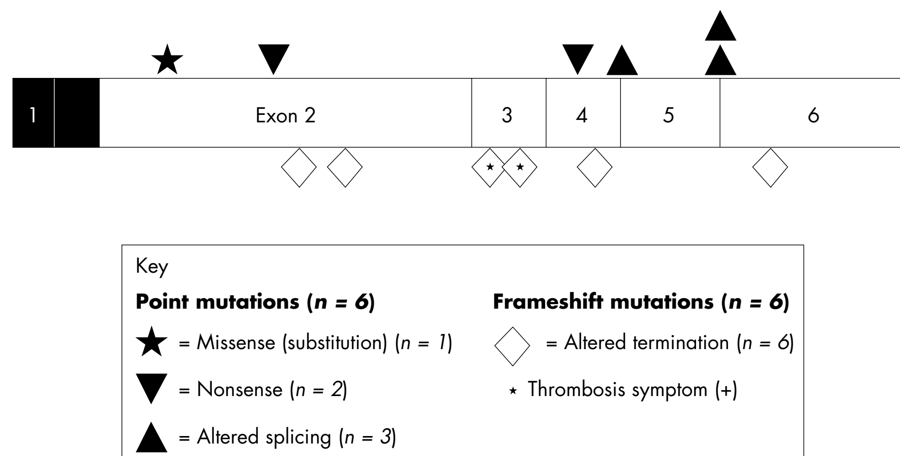

Figure 2 Summary of the 12 mutations in the PIG-A gene seen in paroxysmal nocturnal haemoglobinuria.

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Table 1 Mutations of the PIG-A gene of patients with paroxysmal nocturnal haemoglobinuria

Case	Sex/Age	Site/Codon	Mutation	Consequence	Suggested site of termination
1	M/25	Codon 165–172	22 base deletion (TGCTTACAAACAAGCTTCTAAC)	Premature termination	Codon 194
2	M/20	Intron 4 (5′ splice site)	G to A	Altered splicing	
4	M/36	Intron 5 (3′ splice site)	G to A	Altered splicing	
5	M/23	Codon 409–417	25 base deletion (GGACAAACGACTGGACAGACTTATT)	Premature termination	Codon 423
7	F/36	Codon 316	ATG→AG (T deletion)	Premature termination	Codon 329
12	M/39	Codon 302	GGA→TGA (G to T)	Nonsense	Codon 302
15	M/23	Codon 282	GAC→AC (G deletion)	Premature termination	Codon 290
20	M/52	Codon 127	TCA→TGA (C to G)	Nonsense	Codon 127
22	M/61	Codon 48	GGC→GTC (G to T)	Missense (Gly→Val)	
23	M/47	Codon 139	CTC→C (CT deletion)	Premature termination	Codon 171
24	M/37	Intron 5 (5′ splice site)	G to A	Altered splicing	
25	F/23	Codon 248	CCT→ACCT (A insertion)	Premature termination	Codon 248

Table 2 CD59 expression in patients with paroxysmal nocturnal haemaglobinuria (PNH)

	CD59 (RBCs) (%)			CD59 (granulocytes) (%)			
Case	PNH I	PNH II	PNH III	PNH I	PNH II	PNH III	Mutation
10	36.8	5.8	57.4	NT	NT	NT	Negative
11	9.0	82.0	9.0	NT	NT	NT	Negative
12	13.0	27.0	60.0	NT	NT	NT	Positive
14	26.0	21.2	52.8	NT	NT	NT	Negative
15	2.8	16.5	80.7	NT	NT	NT	Positive
16	61.0	12.0	27.0	NT	NT	NT	Negative
18	1.3	0.0	98.7	NT	NT	NT	Negative
19	12.0	29.2	58.8	NT	NT	NT	Negative
20	4.6	16.9	78.5	NT	NT	NT	Positive
21	50.8	25.8	23.4	58.5	41.5	0.0	Negative
22	6.3	0.0	93.7	6.4	90.3	3.3	Positive
23	20.3	0.0	79.7	47.0	41.3	11.7	Positive
24	8.3	4.0	87.7	16.2	11.9	71.9	Positive
25	8.0	10.7	81.3	8.0	59.0	33.0	Positive

NT, not tested; RBC, red blood cell.

detection rate might be because we used leucocytes; we were only able to separate granulocytes from the five most recent patients. Four of these five patients showed mutations, but eight of the other 19 patients did not. The male dominant mutation detection rate—10 of 14 in men and only two of 10 in women—might be another factor. A normal PIG-A allele may be present even in PNH cells in women, which might inhibit the detection of mutation; thus, granulocytes may provide a higher mutation detection rate. Another interesting result was that the mean expression of CD59 in red blood cells was significantly different between the mutation positive and mutation negative groups. Although the small number of patients tested is an inevitable limitation, it appeared that the detection rate for mutations increased with the number of PNH III red blood cells that were present.

Because ddF is a convenient method for screening mutations, it may be practical in the clinical laboratory.[24] It is possible that the detection of mutations is lower in 200 bp or larger fragments of DNA,[25] but we cannot say that the ddF method used is responsible for the low rate of detection of mutation in our study until the other factors mentioned above are ruled out.

The somatic mutations of the PIG-A gene in Korean patients with PNH varied in their site distribution and their type. There was no mutation hot spot. Base substitution and deletion were equal in frequency, a frequency nearly equivalent to that reported in a Japanese study,[5] but different from that reported in a Thai study, in which base substitutions were found in 10 of the 37 mutations present in 33 patients.[26] Differences in mutations between ethnic groups can be considered only when a large enough number of mutations have been reported.

The mutations consisted of six base substitutions, two single base deletions, three multiple base deletions, and one single base insertion. The single base substitutions caused a missense mutation in one, a nonsense mutation in two, and a splice site mutation in three cases. Three multiple base deletions and three single base deletions resulted in premature termination.

> "The mean expression of CD59 in red blood cells was significantly different between the mutation positive and mutation negative groups"

Two patients with PNH and venous thrombosis both showed mutations in exon 3 only. Although a lack of correlation between the site of mutation and the clinical expression is well known, this may be an interesting finding. The fact that the only missense mutation was found in exon 2 was another interesting finding; there were also more missense mutations in exon 2 than in the other exons in another report.[27] However, a larger study investigating a greater number of patients with PNH is required to confirm these findings.

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Take home messages

- Gene mutation was detected in only 12 of the 24 Korean patients with paroxysmal nocturnal haemoglobulinuria (PNH)
- There were 10 new mutations among the 12 mutations found in the Korean patients with PNH
- The characteristics of the mutations varied, with no significant hot spots in sites or types
- The mean expression of CD59 in red blood cells was significantly higher in the mutation positive group than was seen in the mutation negative group

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