RAPID COMMUNICATION

Somatic Mutations of the PIG-A Gene Found in Japanese Patients With Paroxysmal Nocturnal Hemoglobinuria

By Norio Yamada, Toshio Miyata, Kenji Maeda, Teruo Kitani, Junji Takeda, and Taroh Kinoshita

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematologic disorder characterized by intravascular hemolysis and hemoglobinuria. Abnormal blood cells are deficient in the surface expression of multiple proteins that are normally expressed on the cell membrane via glycosylphosphatidylinositol (GPI) anchor. Surface deficiencies of complement regulatory proteins, decay-accelerating factor (DAF) and CD59, render red blood cells (RBCs) sensitive to complement and lead to complement-mediated hemolysis. In this disease, granulocytes, monocytes, platelets, and T, B, and natural killer (NK) lymphocytes as well as RBCs are deficient in GPI-anchored proteins, indicating somatic mutation of hematopoietic stem cells.

The biochemical basis of PNH is the deficient biosynthesis of the GPI anchor. The biosynthetic pathway of the GPI anchor consists of multiple reaction steps basically involving sequential additions of sugars and other components to phosphatidylinositol (PI) from respective donor molecules. Studies with affected lymphocyte cell lines and granulocytes from patients with PNH showed deficiency of the first step of GPI-anchor synthesis. Biochemical and cytogenetic analyses of various complementation classes of GPI-anchor deficient mutant cell lines showed that three genes, those for complementation class A, C, and H, are involved in the first step of biosynthesis. Complementation analysis by cell hybridization showed that affected cell lines established from patients with PNH belong to class A, indicating that abnormality of the class A gene must be responsible for PNH.

A cDNA of the class A gene, termed PIG-A gene, has been isolated by expression cloning based on the complementation of surface DAF and CD59 expression on class A mutant cells. The transfection of PIG-A cDNA to the affected cell lines from two Japanese patients with PNH complemented the surface expression of GPI-anchored proteins. A somatic mutation of PIG-A was indeed identified in one of these cell lines. The PIG-A gene has been localized to the short arm of the X chromosome at Xp22.1, indicating that it is haploid in somatic cells. Therefore, one loss-of-function mutation in PIG-A would result in a GPI-anchor–deficient phenotype.

Because multiple genes are involved in GPI-anchor synthesis, genes other than PIG-A might be responsible for PNH in some patients. However, this does not appear to be true, because all 28 patients analyzed at the RNA and/or DNA levels had PIG-A abnormalities and because all of the other 30 patients studied by means of cell hybridization belonged to class A. The most likely explanation for this uniformity of the responsible gene is that only PIG-A is on the X chromosome and all other genes are autosomal. In fact, two other genes, termed GPI-H and PIG-F, for class H and F mutations, respectively, are autosomal. In autosomal genes, two mutations causing loss of function must occur to gain the GPI-anchor–deficient phenotype, but this would be extremely rare. Another possibility is that PIG-A is a hypermutable gene. For example, PIG-A might causing loss of function must a mutation hot spot. To address this question and to understand the nature of somatic mutations of PIG-A, we analyzed PIG-A in 14 Japanese patients with PNH.

MATERIALS AND METHODS

Patients and blood cell samples. Patients no. 1 through 9, 11, 12, 14, and 16 were described elsewhere. Blood samples were obtained from two additional patients (no. 17 and 18) after obtaining their informed consent (Table 1). All of the patients had a positive

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Harn's test. Patients no. 12 and 17 had aplastic anemia, but the remainder had no other hematologic disorders. Granulocytes (PMN) and mononuclear cells were isolated by sedimentation through ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Cells were stained for DAF and CD59 with biotinylated IA10 and 5H8" monoclonal antibodies, respectively, and with phycoerythrin-conjugated streptavidin and CD59 with biotinylated IA1OZ6 and 5H8" monoclonal antibodies. Flow cytometry of DAF and CD59. Cells were stained for DAF and CD59 with biotinylated IA10 and 5H8" monoclonal antibodies, respectively, and with phycoerythrin-conjugated streptavidin (Biomed, Foster City, CA). The cells were then analyzed in a FACSscan (Becton Dickinson, Lincoln Park, NJ).

Reverse transcription-polymerase chain reaction (RT-PCR). We isolated the total RNA from PMN and reverse transcribed the coding region of PIG-A using RNA from 10⁶ cells and primer B13 (Table 2 and Fig 1) with 200 U of RNAse H-free reverse transcriptase (Superscript; GIBCO-BRL, Grand Island, NY) in a final volume of 20 µL at 37°C for 90 minutes. The coding region was amplified with primers A11 and B12 (Table 2 and Fig 1) by PCR, each cycle of which consisted of 1 minute of denaturation at 95°C, 1 minute of annealing at 55°C, and 2 minutes of extension at 72°C. The reactions were repeated 25 times using [α-³²P]deoxyctydine triphosphate (Amersham, Buckinghamshire, UK) and the products were resolved by electrophoresis in 2% agarose gels, followed by autoradiography. For cloning, the reactions were repeated 36 times and the products were cloned into pBluescript II (Stratagene, La Jolla, CA) for nucleotide sequencing or into Epstein-Barr virus-based mammalian expression vector, pEB, for transfection.

Amplification of the PIG-A gene. DNA was isolated from PMN and MNC. Regions of PIG-A gene were amplified by PCR using the primer sets, AIT1 and B1, A1 and BIT1, AIT2 and BIT2, AIT3 and BIT3, and AIT4 and B12 (Table 2 and Fig 1) for 36 cycles and the products were used for hetero-duplex analysis or cloned into pBluescript II for sequencing. Nucleotide sequences of the cloned PCR products were determined by dideoxy chain termination using either the Taq dye primer cycle sequencing kit or the Taq dye terminator cycle sequencing kit or the Taq dye terminator cycle sequencing kit and a Model 370A DNA sequencer (Applied Biosystems, Foster City, CA).

Table 2. Primers Used for Analyses of PIG-A

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>CD59-Deficient PMN (%)</th>
<th>Site/Codon</th>
<th>Mutation</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>100</td>
<td>3' Splice site</td>
<td>A to G, base change</td>
<td>Exon 4 deletion</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>90</td>
<td>5' Splice site</td>
<td>G to A, base change</td>
<td>Exon 5 deletion</td>
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<tr>
<td>3</td>
<td>F</td>
<td>97</td>
<td>Codon 129</td>
<td>A to G, base change</td>
<td>His to Arg</td>
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<td>4</td>
<td>F</td>
<td>50</td>
<td>Codon 100</td>
<td>C to T, base change</td>
<td>Stop</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>65</td>
<td>Codon 437</td>
<td>C deletion (CTC → TC)</td>
<td>Frame shift</td>
</tr>
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<td>6</td>
<td>M</td>
<td>72</td>
<td>Codon 191</td>
<td>A deletion</td>
<td>Frame shift</td>
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<td>7</td>
<td>F</td>
<td>60</td>
<td>Codon 128</td>
<td>A to G, base change</td>
<td>His to Arg</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>37</td>
<td>Codon 431</td>
<td>T to A, base change</td>
<td>Stop</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>96</td>
<td>Codon 82</td>
<td>A insertion (TAC → TAAC)</td>
<td>Frame shift</td>
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<tr>
<td>11</td>
<td>M</td>
<td>83</td>
<td>Codon 136</td>
<td>T deletion</td>
<td>Frame shift</td>
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<tr>
<td>12</td>
<td>M</td>
<td>94</td>
<td>Codon 312</td>
<td>A deletion</td>
<td>Frame shift</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>43</td>
<td>Codon 112/113</td>
<td>GC to T, base change</td>
<td>Frame shift</td>
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<tr>
<td>16*</td>
<td>F</td>
<td>98</td>
<td>5' Splice site</td>
<td>T deletion</td>
<td>Exon 5 deletion</td>
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<tr>
<td>17</td>
<td>F</td>
<td>40</td>
<td>Codon 90</td>
<td>T insertion (TA → TTA)</td>
<td>Frame shift</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>95</td>
<td>5' Splice site</td>
<td>G to A, base change</td>
<td>Exon 4 deletion</td>
</tr>
</tbody>
</table>

* This patient has been previously described.†

Table 2. Primers Used for Analyses of PIG-A

| A1          | 5'-ACAGGCAACGACCTTCTTACAG   |
| A2          | 5'-AATTTTACAGCTCCTAGTGC     |
| A3          | 5'-GCTGAGTCAGCTGCGCTTAC     |
| A4          | 5'-GGTTGACGACCATGTTTACAG    |
| A5          | 5'-GATAGGCACTGCTAGAGGCTG    |
| A6          | 5'-GAGGAGAGGAGCAGAAGAAGTC   |
| A7          | 5'-ACTGAAAGACCTTGCGATGGC    |
| A11         | 5'-GCTTGGCTTAAGAAGGTGTC     |
| A14         | 5'-AAGGCACTGCAGCTGACGAGAA   |
| A16         | 5'-AGACGAGCTGTTGGTTTAC      |
| AIT1        | 5'-AGAGGAGAATGCTGCTTACTCT   |
| AIT2        | 5'-TGATTGCTGACGGCTTGAGCA    |
| AIT3        | 5'-TCATCCCTCTCTCCCTCT       |
| AIT4        | 5'-GCTCTCTCTCTGCTCGATAC     |
| B1          | 5'-GAAAGAAGAACTAGTGAAAGAT   |
| B2          | 5'-AGGATGAAAGAGGCTGCGCTG    |
| B3          | 5'-TTCAGGGCTCTAGGATACAG     |
| B4          | 5'-CCTCCTCTTGGCTCCCTCTCT    |
| B7          | 5'-CCTCCTAGAAGAAGGATTTTAC   |
| B10         | 5'-GACCTCTCAACTCCCGACTAC    |
| B11         | 5'-CAACAAADTAGAAAGAAGAGAA   |
| B12         | 5'-TCTACAATCTGAGGCTCCTCT    |
| B13         | 5'-AATGATAGAGAGTAGATACAG    |
| BIT1        | 5'-GCGCAAAACATGATATAAAC     |
| BIT2        | 5'-CTTCCCTCTCGAAGAAGAAGAA   |
| BIT3        | 5'-GTACGTAACATCGAAGAAGAAGAG |

Fig 1. Schematic representation of PIG-A cDNA (A) and the genomic gene (B), as well as the primers used for PCR and reverse transcription.
**Hetero-duplex analysis.** The coding region of PIG-A was amplified by PCR in seven overlapping fragments using the total RT-PCR product from each patient as a template and the primer sets A11 and B2, A2 and B3, A3 and B4, A4 and B7, A5 and B10, A16 and B11, and A14 and B12 (Table 2 and Fig 1). For hetero-duplex formation, the PCR products from a patient with 80% or more affected PMN were mixed with the corresponding PCR products from normal cDNA to obtain an approximate 1:1 ratio of the mutant to the normal products. The PCR products from a patient with less affected PMN were analyzed without any additions. Samples were denatured for 3 minutes at 95°C and then were kept at room temperature for 1 hour to allow duplex formation. These samples were resolved by electrophoresis in MDE gel (Hydrolink; AT Biochem, Malvern, PA) for 4.5 hours at 800 V and bands were visualized by staining with ethidium bromide. If a region containing a mutation was detected with RT-PCR products, cDNA clones containing that region were prepared. For this, a bigger segment containing the region of interest was amplified by PCR from total cDNA and the products were cloned. These cDNA clones were then used as templates for PCR and the products were analyzed by MDE gel electrophoresis after mixing with equal amounts of normal cDNA fragments. cDNA clones that showed hetero-duplex were sequenced to determine the mutation. Regions of PIG-A cDNA were also analyzed in a similar way.

**Transfection.** We examined the functional activity of PIG-A cDNA by transfection into a PIG-A-deficient mutant human B-lymphoblastoid cell line, JY-5. The cells (5 X 10⁶) were mixed with 10 μg of pEB bearing PIG-A cDNA in 0.8 mL of HeBS transfection buffer. The mixtures were electroporated at 250 V and 960 μF with a Gene Pulser (Bio-Rad, Hercules, CA). After culture and selection with 400 μg/mL hygromycin B, the transfected cells were stained for CD59 and DAF to assess the complementation of the PIG-A-deficient phenotype of the mutant.

**Construction of chimeric PIG-A cDNAs from normal cDNA and that of patients with missense mutations.** A segment of PIG-A cDNA containing a missense mutation and the remaining segment were prepared by restriction enzyme digestion of a mutant cDNA that contained the coding region but lacked most of the 3’ untranslated region. The fragments were isolated by means of agarose gel electrophoresis. The corresponding segments of normal PIG-A cDNA were also prepared, but the full-length PIG-A cDNA was used to help determine the origins of the fragments. Chimeric cDNAs were constructed by ligation of these fragments and pEB vector.

**RESULTS**

To identify somatic mutations of PIG-A in 14 patients with PNH, we first analyzed PIG-A mRNA in PMN by RT-PCR. When the mRNA had a size abnormality, we analyzed DNA from PMN to determine the mutation responsible for that abnormal mRNA. When the mRNA showed an apparently normal profile on RT-PCR analysis, we analyzed normal-sized PIG-A cDNA because it is a mixture of mutant and normal types. We cloned the RT-PCR products and distinguished mutant clones from the normal clones by transfection. To find a mutation, we sequenced either the entire mutant cDNA or only a relevant region that had been localized by hetero-duplex analysis. The mutation found at the mRNA level was confirmed by analysis of DNA.

**Patients no. 1 and 2: Abnormal splicing.** Among the PMN of patients no. 1 and 2, 100% and 90%, respectively, were CD59- (Table 1). Previous analysis of the RNA from PMN showed that they had abnormal PIG-A mRNA, exons 4 and 5, respectively, being missing. To identify the somatic mutations that caused these abnormal mRNA, we analyzed the DNA from PMN. Because a likely defect was abnormal splicing, we amplified a segment containing junctions of exons 4 and 5 by PCR with primers AIT3 and BIT3 (Fig 1B), cloned the products, and sequenced several clones. In samples from patient no. 1 (female), we found an A to G base change within the consensus 3’ splice site sequence AG located immediately 5’ to exon 4. This mutation was found in 4 of 10 clones obtained in two independent PCR (Table 1). There was no mutation in the 5’ splice site flanking exon 4.

The analysis of patient no. 2 (male) showed that five of six clones from two independent PCR had a G to A base change within the consensus GT sequence of the 5’ splice site that flanks exon 5 (Table 1). There was no mutation in the 3’ splice site flanking exon 5. These results indicated that the abnormal mRNA of these patients were formed by abnormal splicing caused by somatic mutations within splice sites.

**Patients no. 4 and 8: Nonsense mutations.** In patient no. 4, 50% of the PMN were CD59- (Table 1). From PMN of this patient, we previously cloned the cDNA of nonfunctional PIG-A mRNA that should have a mutation within the coding region. We sequenced three nonfunctional clones and found a base change, C to T, in codon 100 that generates a stop codon, TAG (Table 1). To confirm presence of this mutation in DNA, we amplified the corresponding region of DNA from PMN using primers AIT1 and B1 (Fig 1B) and sequenced the cloned products. Two of six clones had the same mutation as that found in the cDNA (data not shown). Because this patient is female and 50% of her PMN were affected, these results are in agreement with an idea that the mutant PIG-A allele is on the active X chromosome of her affected PMN.

We sequenced three nonfunctional clones of PIG-A cDNA prepared from PMN of patient no. 8 and found a T to A base change in codon 431, which generates the stop codon, TAG (Table 1). To confirm the presence of this mutation in DNA, we sequenced PCR products from the DNA of PMN amplified using primers AIT4 and B12 (Fig 1B). We found the same mutation as that in the cDNA in two of five clones (data not shown). This finding is reasonable because patient no. 8 is male and 37% of his PMN were affected.

**Patients no. 3 and 7: Missense mutations.** Among the PMN of patients no. 3 and 7, 97% and 60%, respectively, were CD59- (Table 1). Mutations in these patients were identified in a similar way as described above at the cDNA level by sequencing three nonfunctional clones. Both patients had the same missense mutation, namely an A to G change within codon 128 that causes a change from His to Arg (Table 1). This mutation was confirmed at the DNA level. Two of three and two of five clones of DNA amplified by PCR from patients no. 3 and 7, respectively, had the mutation. To confirm that this missense mutation accounted for the loss of function of PIG-A, portions of cDNA that contain codon 128 were exchanged between the nonfunctional clone from patient no. 7 and the wild-type cDNA. Introduction of the mutant sequence from the nonfunctional clone into wild-type cDNA resulted in a complete loss of...
ability to restore the surface expression of CD59 on PIG-A−deficient mutant cells (Fig 2A). The portion of cDNA introduced was sequenced before the exchange to ensure that this mutation was the only nucleotide change. Conversely, introduction of the wild-type sequence into the nonfunctional clone resulted in the expression of functional activity (Fig 2A), indicating that this missense mutation was responsible for the loss of function.

**Patients no. 5, 6, 9, 11, 12, 14, and 17: Frame shift mutations.** Among the PMN of patients no. 5, 6, 12, and 17, 65%, 72%, 94%, and 40%, respectively, were CD59− (Table 1). Previous RT-PCR analysis and that shown in Fig 3 (lane 3) demonstrated that they have apparently normal-sized PIG-A transcripts. Transfection showed that fractions of these transcripts were nonfunctional. To identify the mutations in these patients, we subjected the RT-PCR products to hetero-duplex analysis and then sequenced the relevant regions. Patients no. 5, 12, and 17 showed a hetero-duplex in one of seven segments on MDE gel electrophoresis (Fig 4A, B, and C). For example, hetero-duplex formation was found when exon 6 was analyzed in patient no. 5 (lane 7 of Fig 4A). We sequenced the cloned RT-PCR products that showed the same hetero-duplex profile (lanes 1 through 3 and 5 of Fig 4A) and found a mutation. In this way, we identified one mutation in the sample from each patient: a deletion of C in exon 437 in patient no. 5, a deletion of A in exon 312 in patient no. 12, and an insertion of T in exon 90 in patient no. 17 (Table 1). We identified the deletion of A in exon 191 in patient no. 6 by sequencing the whole coding region of three nonfunctional clones of RT-PCR products. These deletion and insertion mutations caused a frame shift and the appearance of stop codons 16, 9, 50, and 116 nt downstream in patients no. 5, 6, 12, and 17, respectively. The presence of these mutations in DNA was confirmed by sequencing the PCR products amplified from the DNA of PMN (data not shown).

Hetero-duplex and sequencing analyses of the cDNA of patient no. 12 identified a silent base change of T to C in exon 175 in addition to the above base deletion. This base change must be genetic polymorphism rather than a somatic mutation, because all PCR clones amplified from DNA of MNC, only 10% of which were CD59−, had this change (data not shown).

Mutations in patients no. 9 and 11, insertion of A in codon 82 and deletion of T in exon 136, respectively, were previously identified at the cDNA level. These mutations in the DNA were shown by restriction mapping PCR-amplified DNA fragments, because the former mutation generates a new Hpa I site and the latter eliminates a Nla III site (data not shown).

As reported previously, patient no. 14 had skewed profile of PIG-A transcripts on RT-PCR, the band of 1,500 bp, which corresponds to the normal size coding region, being greatly decreased, and, instead, a band of 850 bp, which is a product of the alternative splicing, being increased. Because the 850-bp band lacked a 3′-portion of exon 2, we sequenced exon 2 after PCR amplification of DNA using primers A11 and BIT1. We found a change from GC to T in exon 112/113 (nt 336/337) in 3 of 10 clones obtained in two independent PCR (data not shown). Because 43% of PMN of this male patient were CD59− (Table 1), this ratio of mutants to normal clones is reasonable. This mutation caused a frame shift and a stop codon 34 nt downstream.

**Patient no. 18: Double PNH clones.** As shown in Fig 3, the RT-PCR of PMN from patient no. 18 showed double bands of 1,500 and 1,400 bp as well as two shorter, alternatively spliced bands of 850 and 750 bp. Because 95% of her PMN were DAF− and CD59− (Fig 5A), the normal sized 1,500-bp band might also be abnormal.

We analyzed the abnormally shorter 1,400-bp band first. Restriction mapping of the cloned 1,400-bp band showed that exon 4 was missing. To identify the mutation responsible for this defect, we amplified, cloned, and sequenced the junctions of exon 4 from DNA of PMN. Two of six clones had a G to A change within the consensus GT sequence of the 5′ splice site (Table 1). Because this patient is female and because the ratio of 1,500- to 1,400-bp bands was about 1 to 2 (lane 4 in Fig 3), this result supported a notion that two thirds of her PMN had this mutation on the active X chromosome that causes abnormal splicing of exon 4.

We then analyzed the 1,500-bp cDNA. Hetero-duplex analysis of cDNA segments amplified by PCR from the total RT product showed a mutation in a segment corresponding to the 5′ half of exon 2 (lane 5 in Fig 4D). Three of four clones of the PCR product showed a similar hetero-duplex when mixed with the corresponding PCR product from normal cDNA (lanes 2 through 4 in Fig 4D). Sequence analysis of these clones identified a missense mutation, a T to A change in codon 35 that changes Ile to Lys (Table 1). To confirm the presence of this mutation in DNA, we sequenced...
Fig 3. RT-PCR analysis of PIG-A mRNA in PMN from patients no. 17 and 18. Samples of RNA were reverse-transcribed and the coding region of PIG-A was amplified by PCR. The products were resolved by agarose gel electrophoresis and visualized by autoradiography. Lane 1, amplified coding region of PIG-A cDNA; lane 2, a normal individual; lane 3, patient no. 17; lane 4, patient no. 18. Sizes are indicated on the right.

A. Patient 5

B. Patient 12

C. Patient 17

D. Patient 18

Fig 4. Hetero-duplex analysis for patients no. 5, 12, 17, and 18. (A) Patient no. 5. Samples of cDNA amplified with primers A14 and B12 are shown. Lanes 1 through 5, mixtures of patient’s cloned cDNA and normal cDNA; lane 6, normal cDNA; lane 7, patient’s total cDNA. Clones shown in lanes 1 through 3 and 5 had a mutation. (B) Patient no. 12. Samples of cDNA amplified with primers A5 and B10 are shown. Lanes 1 through 4, mixtures of patient’s cloned cDNA and normal cDNA; lane 5, normal cDNA; lane 6, a mixture of patient’s total cDNA and normal cDNA. All four clones had a mutation. (C) Patient no. 17. Samples of cDNA amplified with primers A11 and B2 are shown. Lane 1, normal cDNA; lane 2, patient’s total cDNA; lanes 3 and 4, mixtures of patient’s cloned cDNA and normal cDNA; lanes 5 and 6, patient’s cloned cDNA shown in lanes 3 and 4, respectively, alone. Both clones had a mutation. (D) Patient no. 18. Samples of cDNA amplified with primers A11 and B2. Lanes 1 through 4, mixtures of patient’s cloned cDNA and normal cDNA; lane 5, patient’s total cDNA; lane 6, normal cDNA. Clones shown in lanes 2 through 4 had a mutation.
A. PMN

![Graph showing PMN cell number and fluorescence intensity for patient and normal samples.]

B. Lymphocytes

![Graph showing lymphocyte cell number and fluorescence intensity for patient and normal samples.]

**Fig 5.** FACS analysis of PMN (A) and lymphocytes (B) from patient no. 18. Lines 1 and 2, cells from patient no. 18 stained for CD59 and its negative control, respectively; lines 3 and 4, cells from a normal individual stained for CD59 and its negative control, respectively.

the PCR products from the DNA of MNC. In the MNC, 10% of lymphocytes (Fig 5B) and 90% of monocytes (data not shown) were CD59⁺. Two of 20 clones from the MNC had the same missense mutation (data not shown).

We then tested, by means of transfection, whether the missense mutation causes a functional loss. The cloned 1,500-bp cDNA that bears the missense mutation did not complement the surface expression of CD59 on the PIG-A-deficient mutant cells (Fig 2B). Replacement of a portion of the mutant cDNA containing codon 35 with that of the normal cDNA restored the activity to complement the mutant cells. Conversely, replacement of that portion of the normal cDNA with that of the mutant cDNA resulted in the absence of activity to complement surface expression of CD59 (Fig 2B), indicating that the missense mutation is responsible for the loss of function.

To determine whether this missense mutation is present in a clone of PMN different from that bearing the splice abnormality, we sequenced the 1,400-bp, abnormally spliced cDNA and found no mutation in codon 35 (data not shown). This indicated that the patient’s PMN consist of two PNH clones, with about 60% bearing a mutation in a splice site, about 30% bearing a mutation within the coding region, and 5% being normal cells.

**DISCUSSION**

We analyzed here the PMN from 14 Japanese patients with PNH and identified 15 somatic mutations of PIG-A (Table 1). Mutations were first found in mRNA and then confirmed in DNA. The mutations included eight single-base changes and seven frame shift mutations. The single-base changes were two nonsense and three missense mutations, as well as three examples of abnormal splicing caused by single-base changes. The frame shift mutations were four single-base deletions, two single-base insertions, and a replacement of two bases with one base. These mutations were all different except for one missense mutation found in both patients no. 3 and 7. The mutations were distributed in various regions of the gene, suggesting that they occurred at random sites and that there is no mutation hot spot in the PIG-A gene. Thus, we now have no evidence that mutation rate of the PIG-A gene is abnormally high. This finding further supports the notion that X-chromosomal location of the PIG-A gene accounts for the uniformity of the responsible gene for PNH.

Together with a previously demonstrated single-base deletion in patient no. 16 (Table 1), all 16 somatic mutations found to date in Japanese patients with PNH are small mutations. Nine mutations in British patients have been reported: two missense and one nonsense mutations, two single-base deletions, the insertion of five bases, two examples of replacements of two bases by one base, and a 4-kb deletion. Four mutations in American patients have been reported: one missense mutation, one single-base deletion, one two-base deletion, and one two-base insertion. Fourteen mutations have recently been identified in Thai patients. They include six single-base deletions; one each of two-, three-, five- and 10-base deletions; two single-base insertions; and two base changes at splice sites (P. Pramoonjago et al, manuscript in preparation). Except for the 4-kb deletion, the largest of the 42 mutations was a 10-base deletion. Thus, a big deletion or a gross rearrangement involving PIG-A gene has not been found. These events may be very rare in the hematopoietic stem cells of patients with PNH or a big deletion might have critical effects on the cells because an essential gene is thought to be located closely to the PIG-A gene.

In patients with PNH, mutant clonal cells occupy significant fractions of the blood cells. During the early stage of the disease, expansion of the mutant clone must occur. It seems unlikely that a mutant clone has intrinsic ability to grow advantageously seems unlikely, because the expansion does not seem to continue but rather to become stabilized at a particular level of occupancy. The level of occupancy by a mutant clone varies in different patients. The patients studied in this investigation had 37% to 100% affected PMN. All mutations in these patients resulted in loss of function, causing complete deficiency of the surface expression of GPI-anchored proteins. This finding implies that the extent to which a mutant clone expands is not determined solely by the mutation of PIG-A but is influenced by some other factor(s). This is consistent with the notion that there is some mechanism(s) that selectively suppresses normal hematopoietic cells during expansion of a mutant clone(s). Stronger suppression would result in a higher extent of occupancy by a mutant clone, and different suppressions would cause various extents of expansion of the mutant cells.
In patient no. 18, we found two abnormal clones of PMN bearing different somatic mutations. Two British patients with two PNH clones have been found.20 The frequency of patients with double clones is, therefore, at least 3 in 39. About one fourth of patients with PNH have two phenotypically different abnormal RBCs, one with a complete deficiency of DAF and CD59 (type II PNH cells) and the other with partial deficiencies of DAF and/or CD59 (type II PNH cells).34,35 These RBCs have different sensitivities to complement and may be different clones bearing different mutations. Thus, the frequency of cases with double clones may be even higher.

So far, we found 4 patients with abnormal splicing (Table 1). In three of them, mutations in the 5' splice site were associated with the splicing out of the immediately upstream exons. In one, a mutation in the 3' splice site was associated with the splicing out of the exon immediately downstream. These are the typical consequences of mutations within the splice sites found in several genetic diseases.36

We found missense mutations in 3 patients. Patients no. 3 and 7 had a mutation in codon 128 that changes His to Arg. His 128 is conserved in mouse Pig-a37 and yeast SPT14,38 a yeast homologue of PIG-A,37,39 consistent with its essential role in the function. Patient no. 18 had an Ile to Lys mutation in codon 35. Ile 35 is conserved in mouse Pig-a but is within the N-terminal 36 amino acids that are truncated in SPT14. Because these N-terminal portions have only 50% amino acid identity, whereas the rest of the molecules have 88% identity,35 so it was uncertain whether these N-terminal portions are functionally important was not clear. Identifying this mutant demonstrated their functional importance.

The mutation in patient no. 14 was the same as that found in a British patient HHH.18 In the former, we analyzed PMN and, in the latter, we analyzed a B-lymphoblastoid cell line. In both, the amounts of normal sized transcript greatly decreased. In the former, the 850-bp product increased, whereas, in the latter, the 1,200-bp product increased.18,19 This difference may be caused by the cell type. Possible explanations for these abnormal transcripts in cells bearing a frame-shift mutation in the coding region were previously discussed.18 One explanation is that the generation of a premature stop codon due to this frame shift resulted in instability of the normal-sized transcript. Another explanation is that the close proximity of the mutation to an alternative splice site resulted in skewed splicing. Further analysis is necessary to clarify this.

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REFERENCES


Somatic mutations of the PIG-A gene found in Japanese patients with paroxysmal nocturnal hemoglobinuria

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