Somatic Mutations of PIG-A in Thai Patients With Paroxysmal Nocturnal Hemoglobinuria

By Patcharin Pramoonjago, Wanchai Wanachiwanwini, Sripapra Chipprasertsak, Kovit Pattanapanayasat, Junji Takeda, and Taroh Kinoshita

Paroxysmal nocturnal hemoglobinuria (PNH) is a hematopoietic stem cell disorder characterized by clonal blood cells that are deficient in the surface expression of glycosylphosphatidylinositol (GPI)-anchored proteins. In the affected cells, the X-chromosomal gene PIG-A, which participates in biosynthesis of the GPI anchor, is somatically mutated. Analyses of Japanese, British, and American patients with PNH have shown somatic mutations of PIG-A in all of them, indicating that PIG-A is responsible for PNH in most, if not all, patients in those countries. Twenty-nine of the reported somatic mutations are small, mostly involving 1 or 2 bases, except for one with a 4-kb deletion. Here we describe an

analysis of PIG-A in neutrophils from 14 patients from Thailand where PNH is thought to be more common. We found small somatic PIG-A mutations in all patients. These consisted of six single base deletions, one each of 2-, 3-, 5- and 10-base deletions, two single base insertions and two base substitutions. Thus, the small somatic mutation in the PIG-A gene is also responsible for PNH in Thailand. However, base substitutions were rarer (2 of 14) than in Japan (8 of 16), and deletions of multiple bases were more common, suggesting various causes of mutation.

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Paroxysmal nocturnal hemoglobinuria (PNH) is a hematopoietic stem cell disorder characterized by the appearance of clonal cells of multiple hematopoietic lineages on which surface expression of glycosylphosphatidylinositol (GPI)-anchored proteins is deficient. Deficiencies of GPI-anchored complement regulatory proteins, such as CD59 and decay-accelerating factor, render red blood cells very sensitive to complement and cause complement-mediated hemolysis and hemoglobinuria.

In the affected cells from patients with PNH, the first step in biosynthesis of the GPI-anchor is defective. Three genes are involved in this reaction step and one of them, an X-linked gene PIG-A, is mutated in affected cells. Granulocytes and B lymphocytes have the same mutation, indicating that a somatic mutation occurs in hematopoietic stem cells. Because PIG-A is on the X-chromosome, one somatic mutation in PIG-A that causes a loss of function would result in deficient biosynthesis of the GPI anchor and the defective surface expression of multiple GPI-anchored proteins.

Approximately 10 genes are involved in biosynthesis of the GPI-anchor. Some of them have been cloned from human cells and characterized. Mutations in any one of them would cause GPI-anchor deficiency. However, studies of 30 Japanese, British, and American patients with PNH have shown abnormalities of PIG-A in all of them. Cyto genetic studies of affected blood cells from about 20 other patients with PNH from the United States and Germany showed that they belong to complementation class A of GPI-anchor-deficient mutants and hence are PIG-A mutants. Therefore, in those countries, PIG-A is responsible for PNH in all patients characterized to date. The most likely explanation as to why PIG-A is always responsible, is that all genes except PIG-A are autosomal. For autosomal genes, mutations on both alleles must occur to cause GPI-anchor deficiency, but this would be very rare. There is evidence that at least two other genes are autosomal, supporting this rationale.

Twenty-nine somatic mutations in PIG-A have been reported. Except for a 4-kb deletion found in one British patient, they are small mutations, mostly involving 1 or 2 bases. The mutations are widely distributed in the coding region and splice sites, indicating that they occurred at random sites. Whether they are spontaneous or induced mutations is not known. Because the frequency of PNH seems variable in different countries, the causes and nature of somatic mutation could also be different. In this investigation, we analyzed PIG-A in 14 patients from Thailand where PNH is thought to be more common.

MATERIALS AND METHODS

Patients and blood cell samples. Blood samples from 14 Thai patients with PNH (T1 through T14) who were being treated at Mahidol University Hospital were analyzed. All patients had DAF- and CD59-deficient erythrocytes and polymorphonuclear leukocytes (PMN). Patients T4, T6, T7, and T9 through T11 had aplastic anemia.

Amplification and analysis of the PIG-A mRNA and the PIG-A gene. We isolated the total RNA from PMN and amplified the coding region of PIG-A mRNA by reverse transcription-polymerase chain reaction (RT-PCR). DNA was isolated from PMN and regions of the PIG-A gene were amplified by PCR. The amplified cDNA and DNA were subjected to heteroduplex analysis to locate regions containing mutations. Mutations were then determined by nucleotide sequencing.

RESULTS

RT-PCR analysis of PIG-A mRNA. Among the PMN of all patients, 6% to 96% were CD59-deficient (Table 1). The PIG-A transcripts in PMN were analyzed by RT-PCR. Patients T1 through T3, T5 through T9, and T13 showed appar-
SOMATIC MUTATION OF PIG-A IN PNH

Table 1. Mutations of PIG-A in Thai Patients With PNH

<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>T1</td>
<td>M</td>
<td>70</td>
<td>Codon 407</td>
<td>T deletion</td>
<td>Frame shift</td>
</tr>
<tr>
<td>T2</td>
<td>M</td>
<td>50</td>
<td>Codon 412</td>
<td>C deletion</td>
<td>Frame shift</td>
</tr>
<tr>
<td>T3</td>
<td>M</td>
<td>96</td>
<td>Codon 427</td>
<td>T deletion</td>
<td>Frame shift</td>
</tr>
<tr>
<td>T4</td>
<td>M</td>
<td>9</td>
<td>Codons 284-287</td>
<td>10-base deletion (TGCGTCTTTT)</td>
<td>Exon 4 deletion</td>
</tr>
<tr>
<td>T5</td>
<td>F</td>
<td>86</td>
<td>Codon 209</td>
<td>T deletion</td>
<td>Frame shift</td>
</tr>
<tr>
<td>T6</td>
<td>M</td>
<td>80</td>
<td>Codon 272</td>
<td>A deletion</td>
<td>Frame shift</td>
</tr>
<tr>
<td>T7</td>
<td>F</td>
<td>21</td>
<td>Codon 203</td>
<td>2-base deletion (GTG to G)</td>
<td>Frame shift</td>
</tr>
<tr>
<td>T8</td>
<td>M</td>
<td>89</td>
<td>Codon 248</td>
<td>C deletion</td>
<td>Frame shift</td>
</tr>
<tr>
<td>T9</td>
<td>M</td>
<td>82</td>
<td>Codon 151</td>
<td>3-base deletion (TTT)</td>
<td>Phe deletion</td>
</tr>
<tr>
<td>T10</td>
<td>M</td>
<td>55</td>
<td>5' Splice site</td>
<td>G to A, base change</td>
<td>Exon 5 deletion</td>
</tr>
<tr>
<td>T11</td>
<td>F</td>
<td>61</td>
<td>5' Splice site</td>
<td>G to A, base change</td>
<td>Exon 4 deletion</td>
</tr>
<tr>
<td>T12</td>
<td>M</td>
<td>95</td>
<td>Codon 193/194</td>
<td>5-base deletion (GTACT)</td>
<td>Frame shift</td>
</tr>
<tr>
<td>T13</td>
<td>M</td>
<td>70</td>
<td>Codon 135</td>
<td>T insertion (GCC to GTCC)</td>
<td>Frame shift</td>
</tr>
<tr>
<td>T14</td>
<td>M</td>
<td>6</td>
<td>Codon 299</td>
<td>T insertion (TTA to TTA)</td>
<td>Frame shift</td>
</tr>
</tbody>
</table>

Somatic mutations in patients T1 through T3, T5 through T9, and T13. Because it was very likely that the normalized transcripts of these patients were a mixture of normal and mutants, the total RT-PCR products were subjected to heteroduplex analysis. As expected, all patient samples included one fragment that formed heteroduplexes. To identify the mutations, we prepared clones from the total RT-PCR products, defined the clones bearing mutations by heteroduplex analysis, and sequenced the relevant regions in those clones. The mutations were identified in all patients (Table 1), including six single-base deletions (T1 through T3, T5, T6, and T8), one each of 1- and 3-base deletions (T7 and T9) and a single-base insertion (T13). These mutations were confirmed in DNA by either restriction enzyme digestion or heteroduplex analysis (data not shown).

Except for the 3-base deletion found in patient T9 that caused a deletion of Phe at codon 151, all mutations caused frame shifts and premature stop codons. To show that the single amino acid deletions in T9 resulted in a loss of function, we transfected the mutant cDNA into PIG-A-deficient cells. The mutant cDNA did not restore the surface expression of CD59 on these cells, whereas wild-type cDNA prepared similarly from the same patient restored it (data not shown). Therefore, the deletion of Phe151 is responsible for the loss of function.

Ninety-six percent of the PMN from patient T3 were CD59-deficient. Nevertheless, heteroduplexes were formed without mixing with normal cDNA. This indicated that a substantial fraction of the cDNA from this patient does not have this mutation, but would have another that was not detected by the heteroduplex analysis. Therefore, a second PNH clone was suggested.

Somatic mutations of patients T12 and T14. Although the entire coding region was not amplified well from RNA of these patients, shorter fragments were amplified from patient T12. Using these cDNA fragments as templates for heteroduplex analysis, we identified one segment that contained a mutation and then determined it by sequencing. This patient had a 5-base (GTACT) deletion in codons 193 and 194 that eliminated a Rsa I site. This mutation was confirmed by Rsa I digestion of the PCR-amplified cDNA fragment (data not shown). About a half of the cDNA amplified from the patient's sample was resistant to the enzyme, whereas the product from a normal individual was completely sensitive. Most of this patient's PMN were affected, and the patient is male, suggesting that a half of the PMN had another mutation that was not detected by the heteroduplex analysis.

We did not obtain a complete set of amplified fragments from the RNA of patient T14, so we directly analyzed the DNA. A DNA segment containing exon 4 showed heteroduplexes. Sequence analysis showed a T insertion in codon 299 (Table 1) that caused a frameshift and a premature stop codon 37 nt downstream.

Somatic mutations of patients T4, T10, and T11. RT-PCR analysis showed an abnormally shorter 1,460-bp band in patients T4 and T11, and a 1,300-bp band in patient T10. In these 1,400- and 1,300-bp bands, exon 4 and 5 were missing, respectively (data not shown). To determine mutations that cause these abnormalities, we amplified exon-intron junctions of exons 4 and 5 from DNA of PMN from patients T10 and T11, then cloned and sequenced the products. Two of four clones from patient T10 had a G to A change within the consensus sequence GT of the 5' splice site immediately downstream of exon 5 (Table 1). Four of five clones from patient T11 had a G to A change within the 5' splice site that is immediately downstream of exon 4 (Table 1). These base changes within the consensus splice donor sites should account for the abnormal splicing found in these patients. We also analyzed exon 4 junctions in the cloned DNA of PMN from patient T4 and found a 10-base (TGCGTCTTTT) deletion within the exon very close to its 5' end. This deletion was confirmed by separating the PCR products in an acrylamide gel (data not shown).

DISCUSSION

We analyzed the PIG-A gene in PMN from 14 Thai patients with PNH to determine whether PIG-A is also responsible for PNH in all patients in an area where PNH is more...
common and to understand the nature of the somatic mutations in Thailand. We identified one small somatic mutation of PIG-A in all patients, indicating that this is also responsible for PNH in Thailand. Therefore, this is a common characteristic of patients with PNH from various countries.

The nature of the somatic mutations in the PIG-A gene is relevant to the etiology of PNH. Of the 14 somatic mutations found in Thai patients, only two were base substitutions (Table 1). This proportion is much lower than that in Japanese patients in whom 8 of 16 mutations were base substitutions. This difference is statistically significant (P = .0445 as assessed by Fisher's exact probability test), indicating that base substitutions in the PIG-A gene are more common in Japan than in Thailand. All base substitutions in Japanese patients were null mutations, so this difference is not caused by a higher frequency of mutations that cause partial deficiency in Japanese patients. Moreover, deletions of multiple bases were more common in Thai patients than in Japanese and British patients. There were four such mutations among 14 mutations in Thailand, whereas there was none among 16 mutations in Japan and one in nine in Britain. These results suggest that the nature of the mutations differs geographically and that they are induced by various mechanisms.

To date, 43 somatic mutations have been reported. Distribution of 42 small mutations is shown in Fig 1. These include four cases of mutations found twice in different patients. Other mutations are all different and widely distributed in the coding region and splice sites. Therefore, there is no mutation hot spot in the PIG-A gene and somatic mutations occurred at random sites.

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