

Original Article**Novel CYP2C19 629c>a mutant gene detection in Japanese subjects and estimation of its effect on conformation**Sayaka Kimura¹, Setsuo Hasegawa¹, Ai Kobayashi¹, Hiroki Yamaguchi¹, Masafumi Yohda³, Takahiro Kubota^{1,2,*}¹ Sekino Clinical Pharmacology Clinic, Tokyo, Japan;² Pharmaceutical Department of Scientific Research, Graduate School of Pharmaceutical Sciences, Chiba Institute of Science, Chiba, Japan;³ Department of Biotechnology and Life Science, Graduate School of Technology, Tokyo University of Agriculture and Technology, Tokyo, Japan.

ABSTRACT: Gene polymorphism is considered to be one of the causes of poor metabolism (PM), and approximately 20 mutants have been reported for CYP2C19 thus far. In our analysis of the CYP2C19*3 mutant gene, we detected new CYP2C19 SNPs by cross checking with different procedures. We confirmed a new c>a mutation at the 629 position. Among the 587 healthy Japanese volunteers studied, two subjects carrying a mutant CYP2C19 allele were found to be heterozygotes (0.17%). Accordingly, we predicted the effect of this novel mutation on CYP2C19 conformation. The 629c>a mutation was located on exon 4 and was an amino acid substitution, in which Thr210 was changed to Asn. The modeled structure of CYP2C19 showed that the hydrogen bond between the main chain oxygen of Ile207 and the side chain O γ of Thr210 would be lost when Thr210 was substituted by Asn; however, no steric constraint was observed, although Asn is larger than Thr in size. Although the CYP2C19 629c>a mutation induces an amino acid substitution, it is predicted to scarcely change its conformation. On the basis of these findings, we speculate that the mutant is not a causative gene for PM in CYP2C19 carriers.

Keywords: CYP2C19, poor metabolism, single nucleotide polymorphisms, allele specific-polymerase chain reaction

1. Introduction

Cytochrome P450 enzymes are the most important

enzymes in phase I metabolism. Cytochrome P450 2C19 (CYP2C19) is a member of the cytochrome P450 enzyme superfamily, and plays an important role in the metabolism of drugs (1,2). For individuals possessing the CYP2C19 gene, drug oxidative metabolic capability varies, and those with poor metabolism (PM) are more frequent among Japanese than among Western populations. Genetic polymorphisms in this enzyme, representing approximately 13-23% of Asians and 3-5% of Caucasians, are responsible for the PM of mephenytoin (3,4). Around twenty mutations in the CYP2C19 gene have been reported (5), and five mutant alleles, namely, CYP2C19*2 (g681a), *3 (g636a), *16 (c1324t), *18 (g986a, a991g), and *19 (a151g, a991g) have already been reported in Japan (6-8).

Genotyping for single nucleotide polymorphisms (SNPs) is of great value to biomedical research and the development of personalized medicine, particularly because it can affect how humans respond to pathogens, chemicals, and drugs. A novel automatic SNP-typing system has been developed in collaboration with ARKRAY, Inc., Kyoto, Japan (9). The system performs SNP typing using analysis of the melting temperature (T_m) of the probe DNA hybridized to the target SNP through a fluorescence quenching probe. In this system, contamination during purification can be avoided because genomic DNA is not purified from a blood sample. We also developed a rapid, low cost and high-throughput genotyping method for detecting polymorphisms of drug-metabolizing enzyme genes using allele specific-polymerase chain reaction (AS-PCR), with detection by SYBR Green I (10). When the CYP2C19*3 (g636a) mutation gene was analyzed with AS-PCR (10) and the above automatic SNP-typing system (9), a difference in the analytical results was recognized.

In the present study, by sequencing the surrounding sequences of CYP2C19*3, we found a new 629c>a variation in exon 4 that creates an amino acid substitution, Thr210Asn, on the probe for the system binding regions. Our study was designed to examine the function of

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Thr210 with regard to its tertiary structure and effect after mutation to Asn on cytochrome P450 2C19. The conformation was constructed using homology modeling.

2. Materials and Methods

2.1. CYP2C19 genotyping

The novel automatic SNP-typing system performs SNP typing by analysis of the melting temperature (T_m) of the probe DNA hybridized to the target SNP site, using a fluorescence quenching probe (ARKRAY, Inc., Kyoto, Japan). The system enables fully automated SNP genotyping from sample pretreatment to gene amplification and signal detection. We need only to set up two cartridges and apply the samples to the cartridges (9). We performed genotyping of CYP2C19 polymorphisms using blood samples obtained from 587 healthy volunteers.

Five hundred and eighty-five typing sample results were consistent with the results obtained from the SNP typing kit for allele-specific PCR, obtained from Toyobo (Toyobo Co., Ltd., Fukui, Japan). AS-PCR for CYP2C19*2, and *3 has been previously described (10).

All subjects provided written consent for participation in the study, after having been informed both verbally and in writing of the experimental procedures and purpose of the study. The study protocol was approved by the Ethics Committee of Sekino Clinical Pharmacology Clinic (Tokyo, Japan).

2.2. PCR conditions and DNA sequencing

PCR amplification of *2 was performed simultaneously in a buffer containing genomic DNA, 1.25 U Gene Taq FP (Nippon Gene Co., Ltd., Toyama, Japan), 10× Gene Taq Universal Buffer and 0.2 mM dNTPs with 0.5 μM of each primer (5'-GAATTTTCTTCTCAAATCTTGT-3' and 5'-CTCCTTGACCTGTAAACATCC-3'). PCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension at 72°C for 7 min. PCR amplification of *3 was performed simultaneously in a buffer containing genomic DNA, 1.25 U TaKaRa Ex Taq (Takara Bio Inc., Shiga, Japan), 10× EX Taq Buffer and 0.2 mM dNTPs with 0.5 μM of each primer (5'-CAT AGGTAAGATATTACTTAAA-3' and 5'-CCAAAGTAC TTTATAGAAAC-3'). PCR conditions consisted of initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C 30 sec and extension at 72°C for 30 sec, followed by a final extension at 72°C for 7 min.

CYP2C19*16, *18 and *19 were amplified by PCR under previously reported reaction conditions (7,8). Sequencing was performed on PCR products using an ABI PRISM 3130 DNA sequencer with a PRISM Dye

Terminator Cycle Sequencing kit, and sequence analysis software (Life Technologies Japan, Inc., Tokyo, Japan).

2.3. Novel SNP evaluation

The 3D structure of CYP2C19 is not yet registered in the Protein Data Bank (PDB). Among the PDB structures of the CYP2 family, human 2C9 (PDB code: 1OG5) was selected as a template structure for homology modeling, which was performed using the Molecular Operating Environment (MOE) (Chemical Computing Group, Inc., Canada). CYP2C19 and 2C9 share 90% amino acid identity, which is sufficiently high to construct a good model. There was also no insertion-deletion region on the sequence alignment, and this also indicated that the model structure would be reliable. AMBER99 was used for energy optimization. Of the 490 amino acids of CYP2C19, the modeled structure covered the region from amino acid No. 30 to No. 490. Thr210 was mutated to Asn using MOE. No structural optimization calculation was made for the mutated structure obtained here.

3. Results

When the CYP2C19*3 (g636a) mutation was analyzed with AS-PCR (10) and a novel automatic SNP-typing system (9), a difference in the analytical results was recognized. Therefore, when we sequenced the surrounding sequences of this mismatched allele in the present study, we found a new variation 629c>a on the probe for the system binding regions. Five mutant alleles, namely, CYP2C19*2 (g681a), *3 (g636a), *16 (c1324t), *18 (g986a, a991g), and *19 (a151g, a991g) have already been reported in the Japanese population (6-8). These five mutant alleles were not found in subjects with the 629c>a variant and thus the 629c>a variation was clearly identified as a novel variant. The new variation was found in two subjects as heterozygotes at a frequency of 0.17% (95% confidence level, 0.0-0.6%). The variation was 629c>a in exon 4 resulting in an amino acid change of Thr210 to Asn (Figure 1).

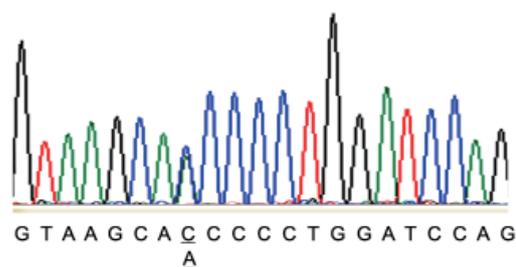


Figure 1. Nucleotide sequences of the CYP2C19 gene in exon 4 containing the 629c>a polymorphism. Both strands were sequenced, although only the sequences for sense strands are shown. The variation at 629c>a in exon 4 resulted in an amino acid change from Thr210 to Asn. Underlining indicates the position of the variant nucleotide.

There were four reasons for selecting the human 2C9 structure (PDB code: 1OG5) as a template for homology modeling. First, the amino acid identity was the highest among the CYP2C family. There were three human 2C9 structures that shared 90% identity with human 2C19. Second, there was no insertion-deletion region near the mutation site, Thr210. Third, there were Pro residues between each helix of HelixF, HelixF', HelixG', and HelixG, which probably acted to bend the helices. The position of Pro matched perfectly with that of 1OG5 (Alignment: Figure 2A, Structure: Figure 2B). Fourth, 1OG5 was a co-crystal structure. In many cases, information regarding ligand interaction is useful. As mentioned above, 1OG5 was selected as a template for homology modeling of human 2C19.

The model structure of CYP2C19 obtained is shown in Figure 3A. Thr210 was located between the F and F' helices. The side chain O γ of Thr210 had a hydrogen bond with the main chain carbonyl oxygen of Ile207 of helix F (Figures 3B and 3C). On the basis of these findings, we could speculate that Thr210 functions to maintain the bent configuration of the F-F' helices. When Thr210 was mutated to Asn, observation of the modeled structure of Thr210Asn revealed that Asn had no specific steric constraints with the other residues (Figure 4). On the basis of this finding, Thr210Asn would not cause any steric hindrance; however, the energy contribution due to hydrogen bonding between Thr210 and Ile207 might be lost.

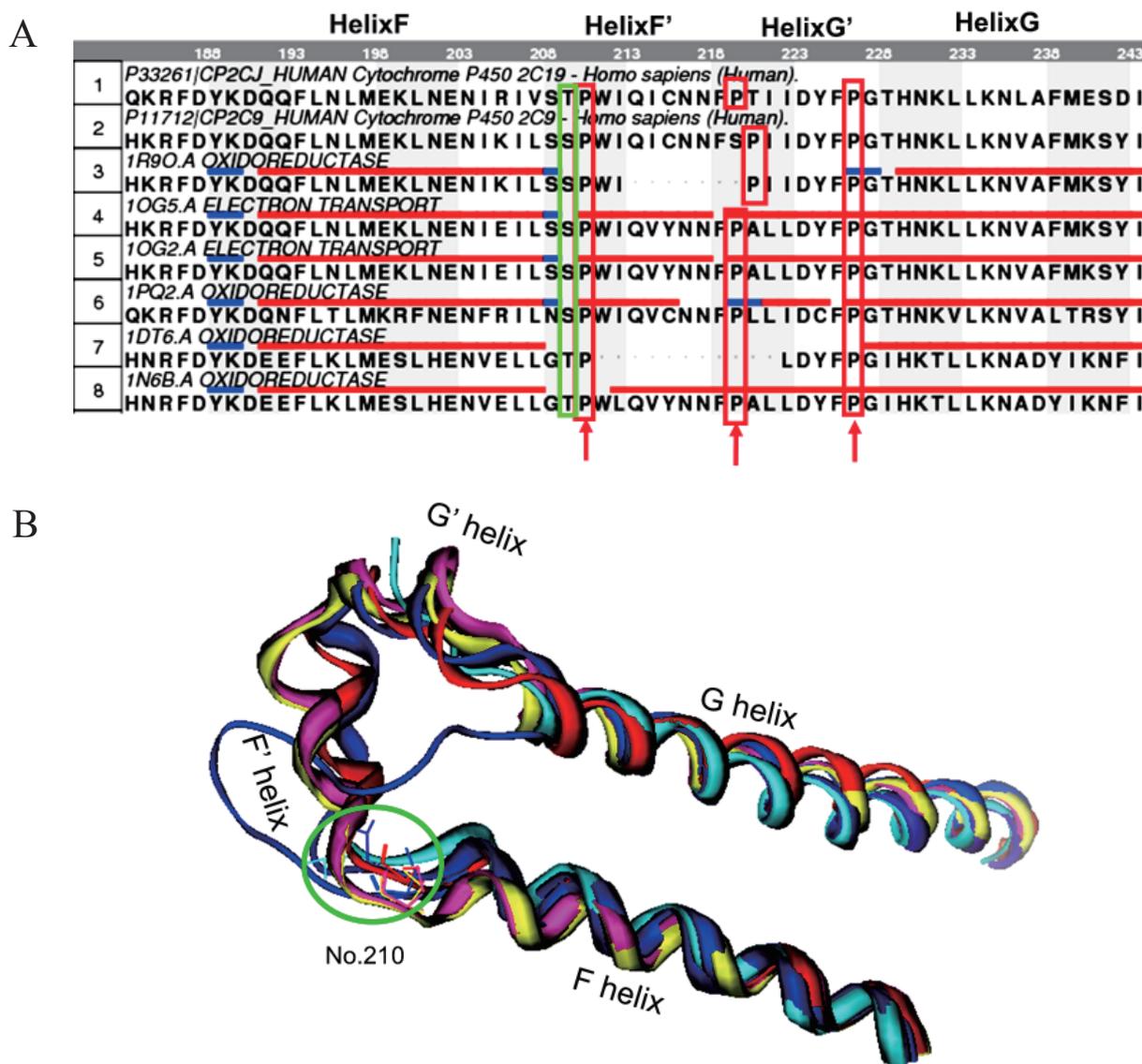


Figure 2. Alignment of CYP2C families and superimposition of F-G helix regions. (A) From top to bottom: 1, human 2C19 sequence; 2, human 2C9 sequence; 3, 1R90 (sequence of human 2C9 in PDB); 4, 1OG5 (sequence of human 2C9 in PDB); 5, 1OG2 (sequence of human 2C9 in PDB); 6, 1PQ2 (sequence of human 2C8 in PDB); 7, 1DT6 (sequence of rabbit 2C5 in PDB); 8, 1N6B (sequence of rabbit 2C5 in PDB). The amino acid corresponding to No. 210 of 2C19 is enclosed in green. The Pro between the F-G helices is colored pink. The horizontal red lines on the sequences indicate α helix regions. Blank spaces indicate an absence of coordinates. (B) Sequence numbers of the alignment shown in Figure 2A: 3, blue; 4, pink; 5, yellow; 6, orange; 7, cyan; 8, purple. The amino acid in Figure 2A is shown as a stick figure and is circled in green.

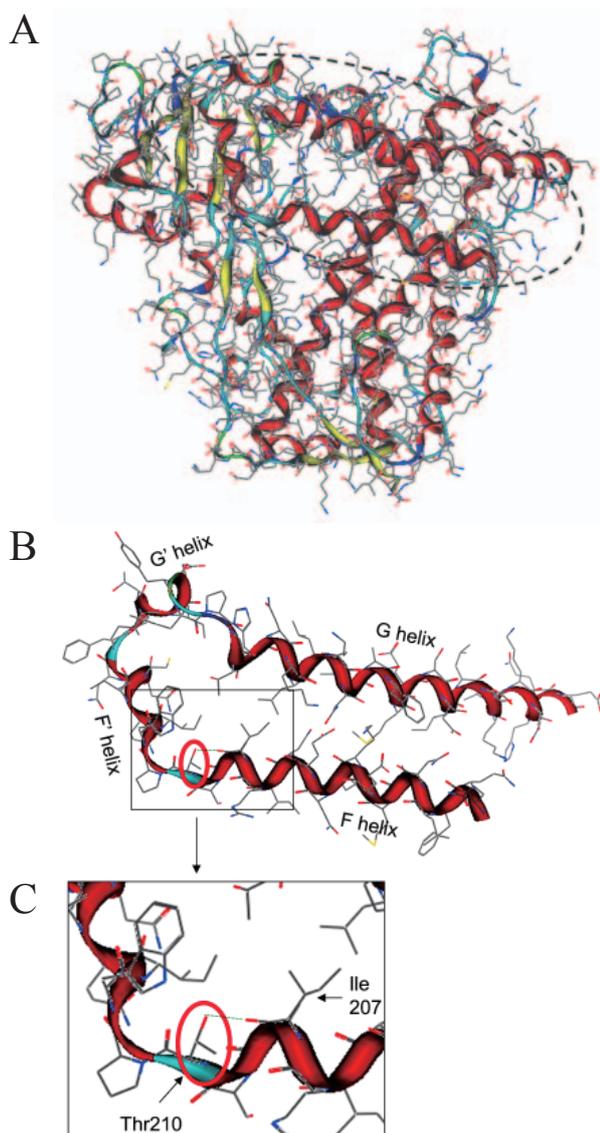


Figure 3. Human CYP2C19 model structure and magnified view of the surrounding area of Thr210 in the CYP2C19 model structure. The entire structure is shown as a stick figure. The main chain is shown as a ribbon shape. The red region in the main chain shows the α helix and the yellow region shows the β strand.

4. Discussion

Operability is improved with the SNP analysis system, which uses various methods. However, there is a limit to each technique, and there is a danger of obtaining inaccurate results. In this study, we were able to detect a novel mutation by comparing the results of two methods (9,10).

It was speculated that Thr210 functions to maintain the bent configuration of the F-F' helices *via* a hydrogen bond with Ile207 (Figure 3C). Observation of the mutated Thr210Asn structure revealed that there were no specific steric constraints with other residues. Thus, it is very likely that metabolic activity is maintained. It is essential to predict the phenotype of a subject on the basis of a genotype that confirms the effect of a

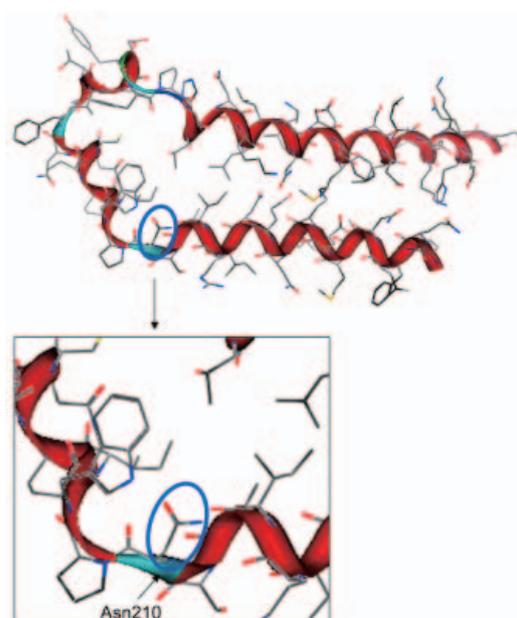


Figure 4. F-G helix region in the Thr210Asn model structure of CYP2C19 and magnified view of the area surrounding Thr210Asn.

novel mutation on function. Accordingly, we examined the function of Thr210 with regard to its tertiary structure and effect after mutation to Asn on the basis of cytochrome P450 2C19, with the conformation constructed using homology modeling. Metabolic activity comparisons are generally performed by protein expression within a different kind of cell. However, we can infer function, as in this report, if the protein crystallization structure is clear.

3D structures in the PDB show that CYP has both open and closed structures. It is considered that the F-G helices have a role of opening and shutting the gate of the pocket for substrates entering and exiting. This was speculated on the basis of a comparison between the open structure of rabbit CYP2B4 (PDB code: 1PO5) and the closed structure of rabbit CYP2B4 (PDB code: 1SUO) (11-13). The human CYP2C9 (PDB code: 1OG5) used as a template in the present study is a closed structure. Accordingly, our SNP evaluation was used to assess the effect on the closed structure, but not on the open structure, or on movement from open to closed or *vice versa*. Information about the structural movement of a CYP family member when it binds to a substrate would be useful for predicting the effect of SNPs more precisely. To predict the position of the amino acid corresponding to Thr210 of 2C19, the structures of rabbit CYP2B4 (open: PDB code: 1PO5, closed: PDB code: 1SUO) and human 2C9 (PDB code: 1OG5) were superimposed (Figure 5). In the closed structure, the amino acid was located in the position colored green in Figure 5, whereas in the open structure, it shifted to the position colored pink. In CYP2B4, the amino acid colored pink was Ser. Even when Ser was mutated to Asn, no effect appeared to be exerted on the structure as

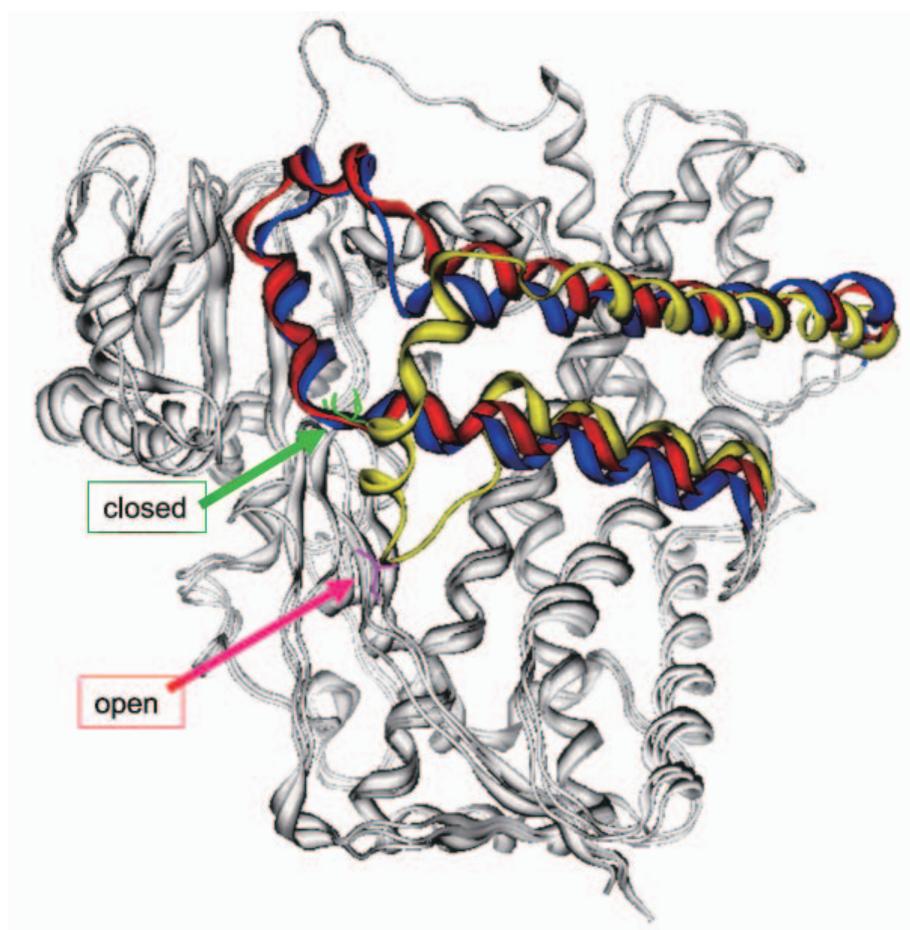


Figure 5. Superimposition of open and closed structures of CYP2B4 and 1OG5. The main chain is shown as a ribbon shape. Only the F-G helices are colored: yellow, CYP2B4 open structure (PDB code: 1PO5); blue, CYP2B4 closed structure (PDB code: 1SU0); red, CYP2C9 closed structure of template (PDB code: 1OG5).

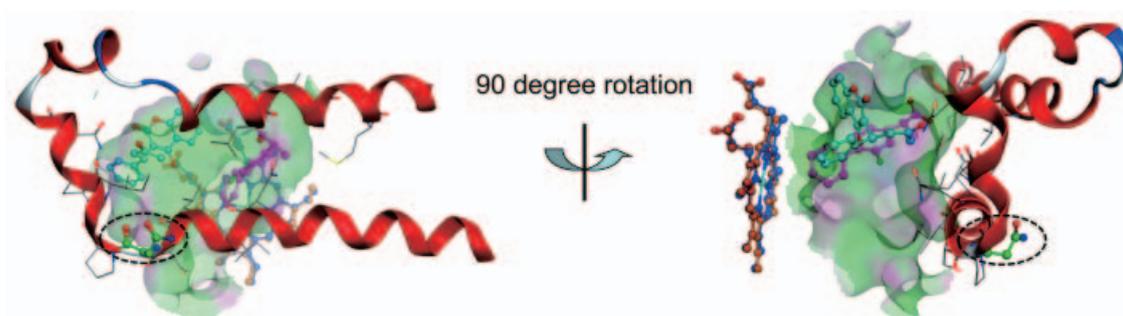


Figure 6. Position of the mutation site and substrate binding pocket. Left: F-G helices are shown in red ribbon. The surfaces of the substrate binding pocket are colored green (hydrophobic regions) and pink (hydrogen bonding regions), with a slightly transparent view. Two ligands from PDB, S-Warfarin (cyan, PDB code: 1OG5) and Flurbiprofen (pink, PDB code: 1R9O), are shown in the substrate binding pocket. Heme is situated at the most rear site, the structure of which was obtained from 1OG5 and 1R9O. The mutated residue is colored green and enclosed by a dotted black circle. Right: The same structure as that on the left, but rotated -90 degrees around the Y axis.

there was space in the surrounding area. Accordingly, assuming 2C19 has a similar open structure as 2B4, it was speculated that Thr210Asn does not exert an effect on the open structure, and this speculation is consistent with that for the closed structure. On the mutated 2C19 structure, the side chain of Asn210 is directed to the external side of the molecule, which is on the opposite side of the substrate binding pocket (Figure 6). From

this observation, it is speculated that T210N does not affect substrate binding.

On the basis of these findings, the Thr210Asn variant of 2C19 likely exerted an effect that reduced the configuration stability of helices F-F', because the hydrogen bonding of Thr210 with Ile207 on helix F was lost. However, no steric constraints were found. Thus, it is speculated that the variant might not exert an

effect sufficient to transform the structure. This novel mutation CYP2C19 629c>a that we identified results in a single amino acid substitution, Thr210>Asn, and occurs with very low frequency. Because metabolic activity is likely maintained, it does not seem reasonable that the mutation generates the gene responsible for CYP2C19 poor metabolizers.

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