Influence of Cytochrome P450 2C19 Gene Variations on Pharmacokinetic Parameters of Thalidomide in Japanese Patients

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Received August 4, 2011; accepted November 24, 2011; published online December 15, 2011

Purpose: Cytochrome P450 (CYP)2C19 polymorphisms may partly explain the variability of thalidomide concentration and adverse drug effects by altering its metabolism. To compare the genetic and clinical factors responsible for the adverse effects and efficacy of thalidomide treatment, we investigated CYP2C19 genetic polymorphisms in Japanese subjects. Materials and Methods: Variations in the CYP2C19 gene in 6 patients treated with thalidomide were analyzed. The dosage of thalidomide, concentrations of (R) and (S)-thalidomide in whole blood, and clinical laboratory test results were used as pharmacokinetic and pharmacodynamic indices. Using genomic DNA, CYP2C19*2 and *3 allele frequencies were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays. Results: The frequencies of CYP2C19 PM and hetero EM (hetEM) genotypes in Japanese patients taking thalidomide were 2 (33.3%) and 4 (66.7%), respectively. The areas under the curve (AUC) of (R)-thalidomide were 3.42 and 5.33 μg·h/L, and those of (S)-thalidomide were 1.64 and 2.46 μg·h/L for hetEM and PM, respectively. Conclusions: This study provided new insights regarding the contribution of CYP2C19 gene variations to adverse responses to thalidomide. Genotyping of CYP2C19*2 and *3 can be considerably simplified by using KOD FX as a polymerase for prediction of adverse effects to thalidomide by the PCR-RFLP method. CYP2C19 PM patients tend to have high serum thalidomide concentrations.

Key words thalidomide; cytochrome P450 2C19; polymorphism; multiple myeloma

MATERIALS AND METHODS

Subjects Blood was obtained from 6 unrelated Japanese patients (4 men and 2 women, mean age: 60.3, range: 47–72 years) treated with thalidomide at Shinshu University Hospital. DNA analysis was approved by the Institutional Review Board for Clinical Trials of Shinshu University Hospital and the Ethical Committee for Human Genome Analysis at Shinshu University. Written consent was obtained from each participant after they had been informed of the experimental procedure and the purpose of the study. The patients received thalidomide therapy for treatment of MM and AL amyloidosis or POEMS syndrome at fixed thalidomide dosages for at least 1 week before blood samples were obtained. Biochemical and hematological tests performed before taking blood showed no evidence of hepatic or renal impairment in any of the subjects. Some patients were treated with concurrent medications, such as vincristine, doxorubicin, and dexamethasone (VAD) therapy.

Study Protocol Patients were taking 100 mg thalidomide capsules (Sauramide; Penn Pharmaceuticals Corporation, Gwent, U.K., or Thaled; Fujimoto Pharmaceutical Corporation, Osaka, Japan) once daily at night for about 1 week. Patients stopped taking thalidomide 2d before blood sample collection. A thalidomide capsule was taken orally at 08:00 (before breakfast) the day before the sampling day. At the same time on the measurement day, venous blood samples were obtained before and 1, 2, 3, 4, 6, 8, 12, and 24h after administration of thalidomide. In patients in poor general condition, blood samples were obtained only 6 h or 0, 1, 2, 4, 6, 8, and 24 h after administration of thalidomide. Whole blood samples were collected in tubes with ethylenediaminetetra-
acetic acid (EDTA)-2K. The concentrations of (R)- and (S)-thalidomide in serum were determined and genetic variations of CYP2C19 were identified as follows. Whole blood samples were immediately mixed with the same volume of 25 mM citrate buffer (pH 1.5) and stored at −80°C until analysis. The (R)- and (S)-thalidomide concentrations in plasma were determined in 200 μL aliquots of thawed samples.

**Determination of (R)- and (S)-Thalidomide Concentrations in Plasma** (R)- and (S)-thalidomide concentrations in plasma were determined by high-performance liquid chromatography (HPLC). Briefly, 20 μL of 1.56 mM phencacetin as an internal standard, 100 μL of 25 mM citrate buffer (pH 1.5), and 1 mL of ethyl acetate were added to 100 μL of whole blood. The mixture was shaken for 1 min followed by centrifugation at 1500 × g for 5 min. The upper layer (0.84 mL) was evaporated to dryness under reduced pressure at 40°C. The residue was reconstituted with 100 μL of 5 mM citrate buffer (pH 3.0)/acetonitrile (75/25, v/v), and 2 μL of the solution was injected into the HPLC system (Hitachi ELITE LaChrom; Hitachi, Tokyo, Japan), composed of a Hitachi L-2100 pump, Hitachi L-2200 auto sampler, Hitachi L-2300 column oven, and Hitachi L-2400 UV detector. The column was a CHIRALPAK AD-RH column (150 mm × 4.6 mm i.d., Daicel Chemical Industries, Osaka, Japan) with a guard column of the same type (10 mm × 4.6 mm i.d.). The mobile phase consisted of 75% water and 25% acetonitrile. Column temperature was 40°C, and flow rate was 0.1 mL/min. The wavelength of the detector was 220 nm. The areas under the curve (AUC) were approximated by the trapezoidal method.

**Polymerase Chain Reaction (PCR) Amplification of CYP2C19*2 and *3 Regions** Genomic DNA in whole blood was amplified using KOD FX (40 μL KOD FX polymerase; 12.5 μL of 2×PCR buffer for KOD FX; 5 μL of 2 mM deoxyribonucleotide triphosphates (dNTPs); Toyobo, Osaka, Japan) containing nuclease-free water in a total volume of 25 μL, with PCR primers at a concentration of 0.32 μM. Genotyping procedures for identifying CYP2C19*2 and *3 were performed by PCR-restriction fragment length polymorphism (RFLP) using allele-specific primers with minor modifications. Primers were as follows: CYP2C19*2, forward primer (5′-ATTACAACCAGCTTGGC-3′) and reverse primer (5′-TATCATTCCATATAAAGCAAG-3′); CYP2C19*3, forward primer (5′-TTATTTATCTGTTAACAATAGTA-3′) and reverse primer (5′-ACTTCAACGGGCTTGGTCAATATAG-3′). Amplification of these two regions was performed in separate tubes using a MyCycler thermal cycler (Bio-Rad, Hercules, CA, U.S.A.). PCR conditions were as follows: CYP2C19*2, an initial denaturation step of 94°C for 2 min followed by 35 cycles of 98°C for 10 s, 53°C for 30 s, and 68°C for 15 s; CYP2C19*3, an initial denaturation step of 94°C for 2 min followed by 30 cycles of 98°C for 10 s, 53°C for 30 s, and 68°C for 20 s. Restriction enzyme cleavage was performed at 37°C for 3 h after addition of 18 units of SmaI for CYP2C19*2 and 20 units of BamHI for CYP2C19*3. The digested PCR products were analyzed by electrophoresis on 3% agarose gels and stained with ethidium bromide.

**Statistical Analysis** The correlations among CYP2C19 genotype, age, sex, concurrent interacting medications were evaluated by χ² test to compare the allele frequency of each variant, and p-values were corrected according to Bonferroni’s method. Results are presented as means±S.D. In all analyses, p<0.05 was taken to indicate statistical significance.

### RESULTS

**Factors Contributing to Adverse Effects of Thalidomide** To evaluate the relative strengths of the factors important for the adverse effects of thalidomide, genotype, age, sex, diagnosis, and concurrent CYP2C19 substrate medications were analyzed. The results of simple analysis of sex, diagnosis, and concurrent medications are shown in Table 1. Age and sex did not show clear effects on thalidomide dose in the Japanese population. In patients taking concurrent drugs, thalidomide dosages were not reduced in patients taking CYP2C19 substrates.

**Incidence of Adverse Drug Reactions According to Thalidomide Treatment** All patients developed adverse reactions to thalidomide. Major adverse drug effects were constipation, somnolence, peripheral neuropathy of grade 1—2 (based on CTCAE v3.0). Only one patient (CYP2C19*2/*2; patient No. 6 in Tables 1, 2) taking thalidomide developed dyspea as a side effect but it improved following the termination of thalidomide.

**Chromatography** Chromatograms of 20 μg/mL racemic thalidomide in 80% citrate buffer (25 mM, pH 1.5) and 20% methanol, and internal standard phencacetin in methanol (1.56 mM) are shown in Fig. 1. The retention times for (R)-thalidomide, (S)-thalidomide, and phencacetin were 34.4, 39.1, and 16.9 min, respectively, with a mobile phase consisting of 75% water and 25% acetonitrile at a flow rate of 0.1 mL/min.

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**Table 1. Baseline Patient Characteristics, Diagnosis, and Other Major Drugs**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Height (cm)</th>
<th>Body weight (kg)</th>
<th>Another CYP substrate drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>POEMS</td>
<td>58</td>
<td>Male</td>
<td>162</td>
<td>57</td>
<td>Omeprazole, Nifedipine, prednisolone</td>
</tr>
<tr>
<td>2</td>
<td>AL amyloidosis</td>
<td>47</td>
<td>Female</td>
<td>168</td>
<td>66</td>
<td>Sarpogrelate, Sarpogrelate</td>
</tr>
<tr>
<td>3</td>
<td>AL amyloidosis</td>
<td>55</td>
<td>Female</td>
<td>161</td>
<td>51</td>
<td>Omeprazole, Atorvastatin</td>
</tr>
<tr>
<td>4</td>
<td>MM</td>
<td>55</td>
<td>Female</td>
<td>145</td>
<td>55</td>
<td>Omeprazole, Dexamethasone</td>
</tr>
<tr>
<td>5</td>
<td>POEMS</td>
<td>72</td>
<td>Male</td>
<td>165</td>
<td>62</td>
<td>Lansoprazole, Warfarin, mosapride, Atorvastatin, prednisolone</td>
</tr>
<tr>
<td>6</td>
<td>AL amyloidosis</td>
<td>61</td>
<td>Female</td>
<td>157</td>
<td>61</td>
<td>Lansoprazole, Warfarin, mosapride, Atorvastatin, prednisolone</td>
</tr>
</tbody>
</table>

All patients were taking CYP2C19 substrates.
The baseline was extremely stable. The calibration standard curves for thalidomide enantiomers had a coefficient of determination ($r^2$) > 0.999. Linearity ranged from 0.5 to 20 μg/mL thalidomide samples, i.e., 0.25—10 μg/mL of each enantiomer. The limit of quantification for both thalidomide enantiomers was 25 μg/mL (data not shown).

The relations between CYP2C19 Gene Variations and (S)- and (R)-Thalidomide Concentrations in Plasma

Among 6 patients, 4 (66.7%) were heterozygous carriers with the CYP2C19*1 and CYP2C19*2 or 3 allele and 2 (33.3%) were homozygous carriers for CYP2C19*2/*2, as shown in Table 2. The mean (R)-thalidomide AUC was 35.9% lower in the CYP2C19*1/*3 and *1/*2 groups than in the CYP2C9*2/*2 group, and the mean (S)-thalidomide AUC was 33.5% lower in the CYP2C19*1/*3 and *1/*2 groups than the CYP2C9*2/*2 group, as shown in Fig. 2.

Table 2. Thalidomide Trade Name, Number of Blood Samples, CYP2C19 Genotyping, and Adverse Events

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Trade name</th>
<th>Number of blood samples</th>
<th>CYP2C19 genotype</th>
<th>Adverse effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sauramide®</td>
<td>7</td>
<td>*1/*3</td>
<td>Constipation, somnolence, peripheral neuropathy</td>
</tr>
<tr>
<td>2</td>
<td>Sauramide®</td>
<td>9</td>
<td>*1/*2</td>
<td>Constipation, somnolence, peripheral neuropathy</td>
</tr>
<tr>
<td>3</td>
<td>Sauramide®</td>
<td>1</td>
<td>*1/*2</td>
<td>Constipation, somnolence, peripheral neuropathy</td>
</tr>
<tr>
<td>4</td>
<td>Thaled®</td>
<td>1</td>
<td>*1/*2</td>
<td>Constipation, somnolence, peripheral neuropathy</td>
</tr>
<tr>
<td>5</td>
<td>Thaled®</td>
<td>9</td>
<td>*2/*2</td>
<td>Constipation, somnolence, peripheral neuropathy</td>
</tr>
<tr>
<td>6</td>
<td>Thaled®</td>
<td>9</td>
<td>*2/*2</td>
<td>Constipation, somnolence, peripheral neuropathy, dyspnea</td>
</tr>
</tbody>
</table>

Three patients were treated with Sauramide® capsules, and the other three were given Thaled® capsules.

Fig. 1. Chromatogram Showing the Separation of Phenacetin (Internal Standard), (R)-Thalidomide, and (S)-Thalidomide in Whole Blood Extracts

The retention times for (R)-thalidomide, (S)-thalidomide, and phenacetin (internal standard) were 34.4, 39.1, and 16.9 min, respectively.

DISCUSSION

Thalidomide is frequently used in the management of MM. However, there is as yet no fixed dosage of thalidomide for all MM patients as a standard regimen because of the variable responses among patients to the drug. In addition, the factors associated with the adverse effects of thalidomide and responses in MM patients remain unclear. To predict the adverse effects of thalidomide, it is necessary to clarify the factors that affect the blood concentration of the drug. These factors may also be important for explaining the efficacy of thalidomide. Genetics, age, diagnosis, concurrent interacting medications, and/or environmental factors may contribute to individual differences in thalidomide blood concentration. All patients in this study were treated concomitantly with drugs that were metabolized by CYP2C19. Patients No. 1, 4, and 6 were taking proton pump inhibitors (lansoprazole, omepra-
zole) to prevent problems with the mucous membrane of the stomach produced as a side effect of steroid use. Patients No. 3 and 5 were continuously treated with proton pump inhibitor after they were enforced the VAD therapy and the dexmethasone pulse therapy in the past. We showed here that CYP2C19 gene polymorphism is one of the most important determinants of thalidomide blood concentration in Japanese patients. Thalidomide therapy was discontinued in patient No. 6 due to the development of dyspnea as a side effect. In the case of patient No. 6, the AUCs of (R)- and (S)-thalidomide were about 50% higher than the mean value for heterozygous CYP2C19 mutation patients. This high AUC caused by the CYP2C19*2/*2 genotype may result in the occurrence of severe side effects. Moreover, this study population included no patients with the CYP2C19*1/*1 genotype (wild-type). It is possible that the patients with wild-type CYP2C19 do not develop side effects as they have a low AUC of thalidomide. Further investigations are required to clarify the role of the CYP2C19 genotype in determining the occurrence of side effects associated with thalidomide therapy.

The tendency for $T_{\text{max}}$ to become fast was reported previously in a comparison of Sauramide® and Thaled®. Fujita et al. reported that the dissolution rate of Thaled® capsules was faster than that of Sauramide® capsules. Although it was reported previously that there were no correlations between genetic polymorphisms and curative effects of thalidomide, these polymorphisms may be related to the incidence of side effects.

In conclusion, this study provided new insights regarding the contribution of CYP2C19 gene variations to adverse responses to thalidomide. Genotyping of CYP2C19*2 and *3 can be considerably simplified by using KOD FX as a polymerase for prediction of the adverse effects of thalidomide by PCR-RFLP. CYP2C19 PM patients tend to have high serum thalidomide concentrations and high risks of adverse drug effects, such as constipation, somnolence, and peripheral neuropathy. Further studies to clarify the mechanisms underlying the adverse drug effects of thalidomide are currently in progress in our laboratory.

Acknowledgments We thank N. Kato, MD, PhD, K. Fukushima, MD, PhD, K. Nakamura, MD, T. Yasude, MD, N. Senoo, MD, and A. Hineno MD for collection of blood samples. We also thank Y. Katsuyama, PhD, for advice regarding analysis of thalidomide.

REFERENCES