ABSTRACT

This study was aimed to examine the relationship between CYP2Cs genes on chromosome 10, which control the enzyme activities of cytochrome P450, and the occurrence of phenytoin induced severe cutaneous adverse reactions (SCARs) in Thai epilepsy children. DNA samples from SCARs and tolerances. Single Nucleotide polymorphisms (SNPs) composing of rs1592037 (CYP2C8), rs17110192 (CYP2C18), and rs3758581 (CYP2C19) were genotyped by allele-specific PCR. Significant associations were observed between heterozygous genotypes for mutant allele located on rs3758581 and rs17110192 and phenytoin-induced SCARs in Thai epilepsy pediatric patient (OR = 14.52; 95% CI 1.18 – infinity, p value = 0.044).

Key Words: cytochrome P450; pharmacogenomics; aromatic anti-epileptic drugs; severe cutaneous adverse reaction; pediatric

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INTRODUCTION

Severe Cutaneous Adverse Reactions (SCARs) were undesirable skin or mucosal epithelium conditions caused by culprit drugs especially aromatic antiepileptic drugs (AEDs) such as phenytoin. Steven-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug rash with eosinophilia and systemic symptoms (DRESS) were the members of SCARs symptoms. The manifestation of SJS and TEN are detachments of the epidermis and mucosal epithelium. In case of SJS, the separation presences less than 10 % of body surface area and cause 1-5 % death. Turning to consider TEN, more than 30 % of body surface area is destroyed, and the mortality rates from TEN are 20-30 %. These conditions often presented with internal organs involvement (Rzany, 1996) (Schneck, 2008) (Aihara, 2011). DRESS, another life-threatening SCAR, is a group of symptoms including extensive mucocutaneous rash, fever, lymphadenopathy, hepatitis, eosinophilic infiltration, and multiple organ damages. Onset of symptoms is between two weeks to three months. They are rare events such as 1.2 to 6 cases per million person-years approximately in SJS and TENs, and 1 case in 1000 to 10000 drug exposures in DRESS (Criado, 2012) (Roujeau, 1995). However, SCARs cause prolong hospitalization, morbidity, or fatal.

SCARs are bizarre reaction which cannot be predicted from pharmacological properties. Thus, SCARs are difficult to prevent. Nowadays, pharmacogenetic study becomes a promising tool for SCARs prediction. In 2007, the US Food Drug Administration (USFDA) recommended to perform genetic screening on HLA-B*15:02, which has been strongly associated with serious skin reactions, in all Asian ancestry patients prior to starting carbamazepine treatment. Recent predicting genetic markers are investigated focusing on metabolizing enzymes. GWAS studies of Taiwanese detect strong signals on chromosome 10 at CYP2Cs gene positions. In their study, approximately one-third of phenytoin hypersensitivity patients carried the combination of rsl057910 (CYP2C9*3), rs3758581, rsl7110192, rs9332245 or rsl592037 (especially for rsl7110192, rsl057910 and rs3758581) (Chung, 2014).

In addition to the genetic polymorphisms, the variability of hepatic CYP2Cs expression and their catalytic activity in children may cause SCAR easier than adult. For instance, the CYP2C9 activity of children achieves normal adult level within 2 years old and the clearance of phenytoin is twice time longer than adult in children under 6 years old (Caudle 2014) (Suzuki, 1994) (Koukouritaki, 2004). The goals for this study were to investigate the association of interesting SNPs on CYP2Cs gene and phenytoin-induced SCARs in Thai epilepsy children.
MATERIALS AND METHODS

1. DNA samples

DNA samples were provided by Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital (Manuyakorn, 2013). In brief, all samples were epilepsy patient age under 18 years old and they were assigned phenytoin for their treatments. Some of them were experienced serious skin reactions and some of them can tolerate to these drugs (no skin reaction was present least 8 weeks after treatments). In this study, thirty-three DNA samples were included and allocated into phenytoin-induced SCARs (17 subjects) and phenytoin tolerances (16 subjects). The manuscripts were approved under-consideration of the human rights and ethic committee of Faculty of medicine, Ramithibodi Hospital, Mahidol University. Informed consents were written by each participant and their parents.

2. Primer design and validation

Primers for allele-specific PCR were newly designed. These following primers were exploited for SNPs detection at rs1592037 (CYP2C8): forward primer 1 (5’ CTT ATA GGT AAG AGC TGA AGA 3’), reverse primer 1 (5’ GAC CTG CTT TCA AAG CTG ATA C 3’), forward 2 (5’ TGT GTC ATC AGA TTT TCT ATT GTG 3’), and reverse 2 (5’ AAG TAT ATA CAC ATA TGC ATT AAC C 3’); for SNPs detection at rs17110192 (CYP2C18): forward primer 1 (5’ CAA CCC TGT TAT TTT GTG AAC AC 3’), reverse primer 1 (5’ TCC AAA TGG GGA AAG GGA GAC 3’), forward 2 (5’ CAG CTC TTC AGA TCT ATC ACC ACA AC 3’), and reverse 2 (5’ TCA CTC TGA ATC TAC ACC ACC ACA AC 3’); for SNPs detection at rs3758581 (CYP2C19): forward primer 1 (5’ CAG GAA GAG ATT GAA CGT GTC A 3’), reverse primer 1 (5’ CAC TTC TCT CAC CCA GTG ATG 3’), forward 2 (5’ ATG TAC CCC TGA ATT GCT AGA AC 3’), and reverse 2 (5’ GGG CTC CGG TTT CTG CCA AC 3’). Both pairs of primers for each SNP amplify the specific PCR products which were different in length (Table 1). Length of PCR products from forward 1 and reverse 1 were used to determine particular SNPs.

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1/R1</td>
</tr>
<tr>
<td>rs1592037 (CYP2C8)</td>
<td>230*</td>
</tr>
<tr>
<td>rs17110192 (CYP2C18)</td>
<td>226*</td>
</tr>
<tr>
<td>rs3758581 (CYP2C19)</td>
<td>277*</td>
</tr>
</tbody>
</table>

In order to prevent the formation of primer-dimers, hairpin, and inappropriate melting temperature, the novel primers were analyzed on electronic database such as the sequence manipulation suite database (Stothard, 2000) (examine the general properties of primers) and the
nucleotide blast database (Altschul, 1997) (blast novel primers with human genome sequence to determine DNA specificity). The known genotype samples were used as positive and negative controls to validate designed primers.

3. Allele specific PCR and genotype interpretation

Genotyping assay including allele-specific PCR (AS-PCR) method and size determination by gel electrophoresis were selected. PCR amplification was performed on T100 Thermal Cycler (Biorad, USA). The compositions of PCR reaction were 20 ng of DNA templates and 1X of KAPA2G™ Fast Multiple PCR Kit, 0.2 μM of each primer. KAPA2G™ contains Hot start DNA polymerase (1 U per 25 μl reaction), KAPA2G Buffer A (1.5X at 1X), dNTPs (0.2 mM each dNTP at 1X), MgCl₂ (3.0 mM at 1X) and stabilizers. Gradient temperature method in accordance with primer profiles was used to optimize conditions for each PCR reaction. Appropriate PCR conditions were listed as following: an initial denaturation 95 °C for 3 minutes, 30 cycles of amplification [95 °C for 15 Sec, 62-68 °C for 30 Sec (as appropriate), 72 °C for 30 Sec], a final extension 72 °C for 1 min. Size of PCR product was determined in gel electrophoresis by running 5 μl of PCR products in 2 % agarose gel with 0.5X TBE buffer followed by ethidium bromide staining. The presence of PCR band was observed under UV light with gel documentation (Unidoc, UK). The genotype interpretation was indicated from the length of PCR product compared with 100 bps ladder, (Invitrogen, USA).

STATISTICAL ANALYSIS

The protocol was designed with 80 % power to detect a significant difference (p value = 0.05, two-sided). Statistical analysis was performed by SPSS software, version 16. Kolmogorov Smirnov Test was used to test the normality of continuous data. Demographic data (continuous variable) was calculated and demonstrated as mean ± standard deviation, median, or frequency. Student t test (normal distribution) or Mann-Whitney U test (if the data is not normal distribution) was analyzed to compare the differences of continuous data. The results were presented as frequencies, p-value, odd ratio, and 95 % confidence interval. When there was an absence in genotype frequency, Haldane’s modification, adds 0.5 to all cells, is applied for all variables in order to calculate odd ratio.

For categorical variables such as gender, chi-square test was used. The association test between genotypes frequencies and the incident of SCARs was performed by Fisher Exact Test. The Fisher’s exact significant level, 95 % CI, and odd ratios, were computed in R program using “Exact 2X2” package (Fay, 2014). P-value which is less than 0.05 reveals statistical significances. The exact test for Hardy–Weinberg disequilibrium was performed to exclude the genotype error that might distort the proportion of heterozygotes and homozygotes with significant departure from Hardy–Weinberg equilibrium (HWE).
RESULTS

1. Patient characteristics

Seventeen patients of phenytoin-induced SCARs, 16 of phenytoin tolerances were included in this study. Medians of age were 9.5, 11.59 year in phenytoin cases, phenytoin tolerances, respectively (Table 2).

Eighty-eight percentages of phenytoin cases had DRESS symptoms and the rest of them were SJS-TEN patients. Liver enzymes from SCARs patients were slightly higher than normal range in both drug-induced SCARs groups because the majority of cases in this study belong to DRESS, which hepatitis is common characteristic. These SCARs symptoms from this agent prolonged hospitalization for at least one week. The examined alleles did not differ from HWE in both SCARs group and control group (P-value > 0.05).

Table 2: Demographic data of phenytoin-treated patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Case (n = 17)</th>
<th>Tolerance (n = 16)</th>
<th>Total (n = 33)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age(year), median</td>
<td>9.5</td>
<td>11.59</td>
<td>10.83</td>
<td>0.01*</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>8 (47.1)</td>
<td>9 (56.3)</td>
<td>17</td>
<td>0.598</td>
</tr>
<tr>
<td>Female (%)</td>
<td>9 (52.9)</td>
<td>7 (43.8)</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Dose (mg/kg/day)</td>
<td>6.5 (7.2)</td>
<td>6.0 (5.03)</td>
<td>6.5 (5.29)</td>
<td>0.79</td>
</tr>
</tbody>
</table>

2. Genotyping

All of SNPs carriers who were presented in this study were heterozygous. Genotype frequencies of rs1592037, rs17110192, and rs3758581 carriers were 29.4 % in phenytoin-induced SCARs. Only one patient (6.7 %) can be detected rs1592037 in phenytoin tolerances. Zero of phenytoin tolerances carried rs17110192 and rs3758581. Association analysis demonstrates significant relationship between rs17110192, rs3758581 and phenytoin-induced SCARs (Odd ratio = 14.52; 95 % CI = 1.18, P-value = 0.044) (Table 3).
Table 3 Association testing among phenytoin-induced SCARs patients, tolerant patients in Thais

<table>
<thead>
<tr>
<th>SNPs</th>
<th>SCARs (%)</th>
<th>Tolerance (%)</th>
<th>Odds ratio (95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1592037</td>
<td>carrier</td>
<td>5 (29.4)</td>
<td>1 (6.7)</td>
<td>6.25</td>
</tr>
<tr>
<td></td>
<td>non-carrier</td>
<td>12 (70.6)</td>
<td>15 (93.8)</td>
<td></td>
</tr>
<tr>
<td>rs17110192</td>
<td>carrier</td>
<td>5 (29.4)</td>
<td>0 (0)</td>
<td>14.52*</td>
</tr>
<tr>
<td></td>
<td>non-carrier</td>
<td>12 (70.6)</td>
<td>16(100)</td>
<td></td>
</tr>
<tr>
<td>rs3758581</td>
<td>carrier</td>
<td>5 (29.4)</td>
<td>0 (0)</td>
<td>14.52*</td>
</tr>
<tr>
<td></td>
<td>non-carrier</td>
<td>12 (70.6)</td>
<td>16(100)</td>
<td></td>
</tr>
</tbody>
</table>

*Odd ratio with Haldane’s modification

*P<0.05 fisher exact test

DISCUSSIONS

Phenytoin is anticonvulsant drugs for treatment of epilepsy in Thai patients. CYP2Cs enzyme plays major role in phenytoin metabolism especially CYP2C8, CYP2C9, CYP2C18, and CYP2C19. These genetic variants, including rs159037, rs17110192, rs3758581, encode decrease activity enzyme CYP2C8, CYP2C18, and CYP2C19. Both rs17110192 and rs3758581 generate high risk to phenytoin-induced SCARs approximate 14.52 times comparing with pediatric tolerances. These associations demonstrated the same trend as data from Taiwanese (Chung, 2014).

This study used pediatric population to investigate the influence of age along with genetic factor over SCARs from AEDs. However, the impact of young age and risk for developing SCARs could not be proven in the study.

Reduced function of CYP2Cs polymorphism and phenytoin-induced SCARs could not be explained exactly at this time of study. Possible mechanism is that intermediate toxic metabolites might be accumulated more due to slower clearance in combination with nonlinear pharmacokinetic properties of phenytoin. These metabolites bind to cellular macromolecules resulted in hapten formation and stimulated immunological reactions (Shear, 1988) (Leeder, 1998).

There are some limitations in this study. First, sample sizes were small because SCARs were rare events. Another thing is that it is not feasible to measure phenytoin concentration in their plasma due to it is retrospective study and re-challenging is impossible.
CONCLUSIONS
This study is the first report demonstrated the association of rs17110192, rs3758581 to phenytoin-induced severe cutaneous drug reaction in Thai epileptic young patients. Both SNPs may be potential genetic markers to predict SCARs.

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REFERENCES


