The Impacts of MDR1<sup>C3435T</sup> Gene Polymorphism towards Plasma Rifampicin Levels in Javanese Pulmonary Tuberculosis

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Abstract

Rifampicin, one of the primary tuberculosis drugs, is a PGP substrate that is encoded by MDR 1 gene. The objectives of the research was to study the impacts of MDR<sup>C3435T</sup> gene polymorphism toward plasma rifampicin levels in Javanese patients with pulmonary tuberculosis. The subjects of this research are Javanese tuberculosis patients treated by fixed dosage combination (FDC) of anti-tuberculosis drugs containing rifampicin. There were 74 patients. The examination of MDR1<sup>C3435T</sup> was conducted by employing restriction fragment length polymorphism (RFLP-PCR) method. Blood samplings to assays the rifampicin level were done on day 1; 4; 28; and 56. The measurement of rifampicin levels was done by high performance liquid chromatography (HPLC) method. There are significant difference on the level of plasma rifampicin between MDR1 C3435T CC, CT and TT with p <0.05. The polymorphism of MDR1 C3435T TT gene increase plasma rifampicin levels in Javanese patients with pulmonary tuberculosis.

Keywords: Polymorphism, MDR1<sup>C3435T</sup> gene, rifampicin

INTRODUCTION

Therapeutic level of a drug is influenced by many factors. One factor that becomes the focus of attention is gene polymorphisms. Genetic polymorphisms may affect the pharmacokinetic profile of drugs include absorption, distribution, metabolism and elimination. These polymorphisms may cause sub therapeutic levels of drugs or upper therapeutic levels. The sub therapeutic levels of drugs can failure of the treatment. On the other hand, upper therapeutic levels will create excessive side effects. Some group tuberculosis patients were treated by isoniazid (INH) will deference responses. In individuals with rapid acetylates, blood INH levels are only about 30-40% of individuals with slow acetylates (Petri, 2006). These levels may be below therapeutic levels. Therefore, it will contribute to the failure of tuberculosis treatment.

Rifampicin is one of the main anti tuberculosis drugs (ATD). The others are Isoniazid, Ethambutol, Pyrazinamide, and Streptomycin (MOH, 2006). Rifampicin is a substrate of a PGP (P-glycoprotein) (Prakash et al., 2003). PGP is a xenobiotic pump which effluxes its substrate back into the lumen intestinal. PGP is important in the process of absorption, distribution and elimination of its substrate drug (Zang & Benet, 2001; Prakash et al., 2003). PGP is encoded by a multi-drugs resistant-1 gene (MDR-1) (Brinkmann & Eichelbaum, 2001; Hwan et al., 2009). Drug inhibition by PGP will cause drug levels in PGP substrate becomes higher. An example of this case is the treatment with verapamil, a PGP block, will increase rifampicin levels, which is the substrate of the PGP (Prakash et al., 2003). Polymorphisms changes expression of PGP that alter kinetic profile of rifampin (Pechandova et al., 2006). MDR1<sup>C3435T</sup> gene is one of the common polymorphisms found in Asia. A research conducted by Li et al., 2006 shows the distribution of MDR1<sup>C3435T</sup> in Malaysia are as follows: MDR1 C3435T CC genotype (25%), CT (46%) and TT (28%) (10). This research explore the impact of polymorphism of MDR1<sup>C3435T</sup> toward blood rifampicin levels in Javanese tuberculosis patients

MATERIALS AND METHODS

Patients

Seventy four adult pulmonary tuberculosis with inclusion criteria: new cases of positive acid fastness bacteria; being in the intensive phase of treatment with anti-tuberculosis drugs (ATDs) fixed dose combination (FDC); Routinely consuming ATDs FDC every day; willing to participate in the research by signing a letter of approval to join the research and exclusion criteria: body mass index (BMI) > 23; 2); pregnant; suffering HIV; having hepatic malfunction (SGOT serum > 40 U / mL) and SGPT serum > 30 U / mL); Smokers and Alcoholism; consuming drugs which is inductor and inhibitors of P-glycoprotein. This study was approved by ethic committee of Faculty of medicine of Universitas Gadjah Mada with no KE/FK/32/EC.

DNA isolation

A total of 100µL of cell lysis solution was added in 300 µL of buffy coat samples were incubated 10 minutes.
On room temperature, mix solutions were centrifuged 13,000 for 1 minute. The supernatant discarded, 300 uL solutions was added on the residue. The mixture was vortexed for 10-15 seconds. These mixtures were incubated on 37°C. The resulting mixture was stored in room temperature and added by protein presipitation solution 100 µL, then was vortexed for 10-15 seconds and centrifuged on 13,000 for 3 min on 37°C. Three hundred (300) µL supernatant was taken and put in 1.5 mL tube then added 300 µL isopropanol. The solution was mixed by inversion until the white treads-like strands of DNA form a visible mass, then was centrifugated on 13,000 for 1 min on 37°C. The ethanol was removed by inversion. The residue was added by DNA rehydration solution (100 µL for 300 µL sample volume) and incubated at 65°C for 60 min. Periodically, the solution is mixed. The samples were store at 4°C.

Polymerase chain reaction restriction fragment length polymorphism (RFLP-PCR) MDR1\(^{\text{C3435T}}\)

Total 12.5mL master mix and 6.5 mL dH2O, forward: 5'- TTG ATG GCA AAG AAA TAA AGC-3' , 2µL reverse 5'-CTT ACA TTA GGC AGT GAC TCG-3') and 2µL DNA (25µL total) was run by PCR. The conditions of amplification as follows: 94°C for 5 min, the 40cycles by 30 seconds on 94°C, 30 seconds on 55°C, dan 1 min on 72°C with 5 min final extension at 72°C. After PCR amplification, 3 µL of PCR + 5µL buffer (2x buffer) with 1 µLMboI enzyme (10U) and 1µL dH2O (total 10 µL ) was digested by 10 U MboI for 16 h at 37°C. The digested PCR products were analyzed by electrophoresis on 1.5% agarose gel , detected by ethidium bromide can be seen on figure 1.

Determination of rifampicin level:

Determination of rifampicin level was used by employing high performance of chromatography system (HPLC) with reversed phase C-18 column (250mmx0, 4mm, 5µm). The mobile phase are methanol and phosphate buffered PH 7.4 (18.7 mL 0.02M KH2PO4 and 80.3mL 0.02M Na2HPO4.2H2O) in the rasio 75:25. Detection eluent with UV detector on wave length 475nm, flow rate 1.5 mL/min. The retention time is on third minute. From the figure, MDR1\(^{\text{C3435T}}\)CC cut off at 145bp & 62 bp, MDR1\(^{\text{C3435T}}\)CT cut off at 207 bp, 145bp and 62bp. MDR1\(^{\text{C3435T}}\)TT not cut off. Frequency of genotype and allele MDR1\(^{\text{C3435T}}\) shown in the table 2.

| Table 1. The characteristic demographic of research subject (n=74) |
|-----------------|------------------|------------------|
| Subjects        | x±SD             |                   |
| Sex (%)         |                  |                  |
| - Male          | 40(53,33)        |                  |
| - Female        | 35 (46,67)       |                  |
| Age (years)     | 42,1±16,1        |                  |
| Body weight (kg)| 47,1±10,4        |                  |
| Height (cm)     | 160,8±8,3        |                  |
| BMI             | 17,47±2,6        |                  |

The result of DNA isolation was performed by PCR (RFLP- PCR). The products digested of PCR-product that analyzed by electrophoresis with 1.5% agarose gel , detected by ethidium bromide can be seen on figure 1.

RESULTS AND DISCUSSION

There are 74 subjects’ tests who meet inclusion and exclusion criteria. The characteristics of research subject can be seen in table 1.
The mean of rifampicin level from subject test can be seen in Table 4.

Table 4. The mean of rifampicin level on day 1; 14; 28 and 56 and percent of sub therapeutic level of rifampicin.

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean (µg/mL±SD) sub therapeutic level (&lt;4 µg/mL) (%)</th>
<th>Number of subject (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.11±1.43</td>
<td>52.7%</td>
</tr>
<tr>
<td>14</td>
<td>3.92±1.64</td>
<td>50.7%</td>
</tr>
<tr>
<td>28</td>
<td>3.68±1.72</td>
<td>51.5%</td>
</tr>
<tr>
<td>56</td>
<td>3.59±1.82</td>
<td>58.2%</td>
</tr>
</tbody>
</table>

The mean of rifampicin level from subject test each MDR1CT3435T gene can be seen in Table 5.

Table 5. The mean of rifampicin level each MDR1CT3435T gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean on day 1 (µg/mL±SD)</th>
<th>Mean on day 14 (µg/mL±SD)</th>
<th>Mean on day 28 (µg/mL±SD)</th>
<th>Mean on day 56 (µg/mL±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1CT3435T CC</td>
<td>3.61±1.68</td>
<td>3.44±1.60</td>
<td>3.20±1.08</td>
<td>3.26±1.07</td>
</tr>
<tr>
<td>MDR1CT3435T CT</td>
<td>4.31±1.26</td>
<td>4.31±1.25</td>
<td>4.29±1.36</td>
<td>4.21±1.57</td>
</tr>
<tr>
<td>MDR1CT3435T TT</td>
<td>4.72±0.93</td>
<td>5.11±1.16</td>
<td>5.16±1.08</td>
<td>4.95±1.14</td>
</tr>
</tbody>
</table>

The statistic test with anova, LSD and regression analysis were used to analyze difference of rifampicin level from each MDR1CT3435T gene.

Rifampicin level on 2 hours after taking drug can be classified into: toxic level (>20µg/mL); therapeutic level (8-20 µg/mL); low therapeutic level (4-8µg/mL); and sub-therapeutic level (<4µg/mL) (<4µg/mL). On Febrinasari research in 2010, it was revealed that the average of rifampicin level of tuberculosis patients in 2 hours after taking the drug is 4.12±0.46 µg/mL in 56th day (Febrinasari, 2010). Other research conducted by Van Crevel et al. stated that about 70% tuberculosis patients in Indonesia carried out sub-therapeutic rifampicin level (<4µg/mL), whereas toxic level was not found (Van Crevel et al., 2002). In the research, in 56th day after ATDs are given about 60% tuberculosis patients have sub-therapeutic rifampicin level. Narita et al. in her research in 2001 in a hospital in Florida found that 60% patients treated by ATDs have rifampicin and isoniazid level less than the standard (Narita et al., 2001). The factors that may cause this includes the low drugs manufacturing quality (Van Crevel et al., 2002) and low dosage of rifampicin given to the patient (it is only 10mg/kg bb) (Alisjahbana, 2006). The research was done by Nijland et al. found C_max of blood rifampicin of tuberculosis patients in Indonesia was 6.74 mg/L (Nijland, 2006).

The rifampicin level in MDR1CT3435TT gene group is higher than MDR1CT3435T or heterozygote MDR1CT3435T CT group. This happens because MDR1CT3435TT causes little PGP formation in villi intestinally (Brinkmann & Eichelbaum, 2001; Hwan et al., 2009). The PGP was encoded by MDR1 gene. An individual with MDR1CT3435TT has lower PGP expression than an individual with MDR1CT3435TCC (Larsen et al., 2007; Miladpoor et al., 2006). Thus, an individual with low PGP expression (MDR1CT3435TT) will have higher plasma rifampicin level. The relation between polymorphism of MDR1CT3435T gene and rifampicin level in the 1st, 14th, 28th, and 56th day is tested by analysis of variant (ANOVA). On ANOVA test, it is found that among the MDR1CT3435T gene variants have significant differences on the rifampicin level in the 1st, 14th, 28th, and 56th day. There are some researchers conducted to study the impact of MDR1CT3435T gene polymorphism toward PGP expression and kinetic profile. Hoffmeyer et al., in their research found that MDR1 gene polymorphism influence PGP expression and its function (Hoffmeyer et al., 2003). Other research found MDR1CT3435TT causes little PGP formation in villi intestinal (Brinkmann & Eichelbaum , 2001; Hwan et al, 2009).

**Chromatography**

Selectivity was done by compare blank with rifampicin standard. The result of the assay are: the retention time of rifampicin 3.169 minute with regression linear Y=13691.33+3351.524 and coefficient of correlation linear (R²) is 0.993. Limit of detection (LOD) is 0.078 µg/mL. Limit of quantitation (LOQ) is 0.26 µg/mL. Coefficient of variation (CV) rifampicin standard on 10 and 20 µg/mL are 1,39% and 0.12%.

The mean of rifampicin level on the 1st day is in sub-therapeutic level (<4µg/mL), whereas toxic level was not found (Van Crevel et al., 2002). In the research, in 56th day after ATDs are given about 60% tuberculosis patients have sub-therapeutic rifampicin level. Narita et al. in her research in 2001 in a hospital in Florida found that 60% patients treated by ATDs have rifampicin and isoniazid level less than the standard (Narita et al., 2001). The factors that may cause this includes the low drugs manufacturing quality (Van Crevel et al., 2002) and low dosage of rifampicin given to the patient (it is only 10mg/kg bb) (Alisjahbana, 2006). The research was done by Nijland et al. found C_max of blood rifampicin of tuberculosis patients in Indonesia was 6.74 mg/L (Nijland, 2006).

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Researches on the influence of MDR1 C3435T gene variant toward pharmacokinetic and pharmacodynamics generate different results. Research by Hoffmeyer was done in 2003, concluded that PGP expression in intestinal on individual with MDR1 C3435T TT type two times lower than CC type. This condition cause level of digoxin in TT type higher than in CC type (Hoffmeyer et al., 2003). The AUC 0-4 jam (P, 0.42) and C max (P,0.43) digoxin is greater in MDR1 C3435T TT than in MDR1 C3435T CC (Johne et al., 2002).

A meta-analysis shows that there is no influence of MDR1 C3435T polymorphism to AUC 0-4 h and AUC 0-24h (Johne et al., 2002). The polymorphism of MDR1 C3435T TT gene increase plasma rifampicin levels in Javanese patients with pulmonary tuberculosis.

In the research, we find differences of rifampicin level between individuals with MDR1 C3435T CC and CT. This happened in the 14th, 28th and 56th day. Transport of Rifampicin is influenced by Pgp that encoded by MDR1. In MDR1 C3435T TT type, there is cytosine substitution by thymine. In fact, the substitution does not change amino acid (Ile145Ile), but in this research, there is significant influence of MDR1 C3435T gene toward rifampicin levels. The Statistic analyses were done by analysis of variate (ANOVA). There are significant difference on plasma rifampicin level between individuals with MDR1 C3435T CC (3.44±1.60 µg/mL ) and MDR1 C3435T CT (4.31±1.25 µg/mL) (p,0.016) ; MDR1 C3435T CC (3.44±1.60 µg/mL) and MDR1 C3435T TT (5.11±1.16 µg/mL ) (p,0.006) on day 14; between MDR1 C3435T CC (3.20±1.08 µg/mL) and MDR1 C3435T CT (4.29±1.36 µg/mL) (p,0.001); MDR1 C3435T CC (3.20±1.00 µg/mL) and MDR1 C3435T TT (5.16±1.08 µg/mL) (p,0.006) on day 28 and on day 56 between MDR1 C3435T CC (3.26±1.07 µg/mL) and MDR1 C3435T CT (4.21±1.57 µg/mL) (p, 0.013), between MDR1 C3435T CC (3.26±1.07 µg/mL) and MDR1 C3435T TT (4.95±1.14 µg/mL) (p,0.010). The relation between polymorphism in MDR1 C3435T and PGP expression is not clear. Some assumptions emerge, i.e. 1). The Cytosine or Thymine which encodes isoeluesin located at wobble position (Morita et al., 2003; Huo et al., 2010); 2). Silent mutation in MDR1 C3435T might decrease the effectviness of translation (Schwab et al., 2003); 3). There is a changes translation process in mRNA (Allain et al., 1996; Wang et al., 2005); 4). There is modification post transcription or it might relate to important sequence in mRNA process (Jamrozik et al., 2002). These changes affect the formation/ expression of PGP that influence blood rifampicin levels.

CONCLUSION

The polymorphism of MDR1 C3435T TT gene increase plasma rifampicin levels in Javanese patients with pulmonary tuberculosis.

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