ABCB1 polymorphism is associated with atorvastatin-induced liver injury in Japanese population

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Abstract

Background: To investigate the associations between atorvastatin-induced liver injury (AILI) and polymorphisms in eight genes possibly involved in the hepatic metabolism (CYP2C9, CYP2C19, CYP3A4, CYP3A5 and UGT1A1) and membrane transport (ABCB1, ABCG2 and SLCO1B1) of atorvastatin, we genotyped 30 AILI and 414 non-AILI patients recruited at BioBank Japan for 15 single nucleotide polymorphisms (SNPs).

Results: An SNP in ABCB1 (rs2032582: 2677G > T/A) was significantly associated with AILI (P = 0.00068, odds ratio (OR) = 2.59 with 95% confidence interval (CI) of 1.49-4.50, G allele versus T and A alleles), indicating that the G allele might be a risk factor for AILI. The cytotoxicity test demonstrated that IC50 value of atorvastatin to inhibit the growth and/or viability of Flp-In-293/ABCB1 (2677G) cells was 5.44 ± 0.10 mM, which was significantly lower than those in Flp-In-293/ABCB1 (2677 T) (6.02 ± 0.07 mM) and Flp-In-293/ABCB1 (2677A) cells (5.95 ± 0.08 mM).

Conclusions: These results indicate that ABCB1 rs2032582 may predict the risk of AILI in Japanese population.

Keywords: Atorvastatin-induced adverse reaction, Genetic association, Hepatotoxicity, MDR1 Ala893Ser/Thr/

Background

Atorvastatin (atorvastatin calcium; Lipitor®) is widely used in the treatment of dyslipidemia of low- and high-density lipoproteins in patients with or without heart disease [1]. However, atorvastatin-induced liver injury (AILI) can be caused after atorvastatin treatment [1, 2]. In Japanese post-marketing surveillance of atorvastatin, 1.42% of patients who received atorvastatin treatment suffered from liver injury. In general, drug-induced liver injury (DILI) can be divided into 3 types (hepatocellular injury, cholestatic liver injury and mixed liver injury) based on potential liver toxicity symptoms (e.g., anorexia, nausea, vomiting or jaundice), the presence or absence of risk factors (e.g., viral infection and alcohol consumption) and serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) as well as the ALT/ALP ratio [3]. AILI falls within the hepatocellular injury category because ALT level of two patients treated with atorvastatin reportedly raised three-fold higher than that of the upper limit of normal but ALP and bilirubin levels did not change [4].

Atorvastatin is orally administered in the active acid form and undergoes marked first-pass metabolism by uptake into hepatocytes via passive diffusion and SLCO1B1 (encoding OATP1B1 [5–7]). Atorvastatin is metabolized mainly by CYP3A4, with minor contributions from CYP2C9, CYP2C19, CYP3A5, and UGT1A1 [8–12]. Subsequently, atorvastatin and the metabolites are predominantly eliminated by ABCB1 (encoding P-glycoprotein or MDR1) and ABCG2 (encoding BCRP)-mediated transport from liver into bile [7, 13–15]. Single nucleotide polymorphisms (SNPs) identified in ABCB1 rs1128503 (1236C > T), rs2032582 (2677G > T/A), and rs1045642 (3435C > T) markedly affected area under the plasma concentration versus time curve (AUC) of atorvastatin and the lipid-lowering effects of atorvastatin therapy [16–18]. Therefore, we hypothesized that the genetic variability of eight candidate genes associated with the hepatic metabolism and membrane transport of atorvastatin may affect the risk of AILI because higher concentrations of atorvastatin can cause hepatocellular injury, even at appropriate atorvastatin dosages. However, to our knowledge, there...
are no reports on an association of the functional SNPs of the candidate genes with AILI.

In this study, we investigated whether 15 functional SNPs in eight candidate genes that are possibly involved in the pharmacokinetics of atorvastatin were associated with AILI in Japanese population. We found that ABCB1 rs2032582 was significantly associated with AILI. In addition, the cytotoxicity of atorvastatin was investigated using the Flp-In-293 cells stably expressing ABCB1 proteins encoded by ABCB1 rs2032582 [19]. We clarified that the ABCB1 rs2032582 G allele was a significant AILI risk factor in vivo and in vitro.

Methods

Selection of SNPs and genotyping

A total of 15 functional SNPs in eight candidate genes (ABCB1, ABCG2, CYP2C9, CYP2C19, CYP3A4, CYP3A5, SLCO1B1 and UGT1A1) reportedly altering pharmacokinetics of atorvastatin were genotyped by a multiplex polymerase chain reaction (PCR)-based invader assay as described previously [20] and direct sequencing using ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA) for rs8175347 and rs2032582, according to the manufacturer's protocol of the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems). HLA-A, -B and -C genotyping was carried out using a WAKFlow HLA Typing kit (Wakunaga, Osaka, Japan), which is based on PCR-sequence-specific oligonucleotide probes coupled with multiple analyte profiling (xMAP) technology (Luminex System; Luminex Corporation, Austin, TX). The data analysis was performed using the WAKFlow Typing software (Wakunaga).

Cell culture

HepaRG cells (KAC, Kyoto, Japan) were maintained in HepaRG Thawing and Seeding Medium 670 (KAC) and HepaRG Maintenance and Metabolism medium 620 (KAC) at 37 °C under 5 % CO2 and 95 % air according to the manufacturer’s instructions. Flp-In-293 cells (Life Technologies, Foster City, CA) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10 % heat-inactivated fetal bovine serum (Life Technologies) and Antibiotic-Antimycotic (100×) liquid (Life Technologies) at 37 °C under 5 % CO2 and 95 % air, where 100 μg/ml Zeocin (Life Technologies) and 100 μg/ml hygromycin B (Life Technologies) were also supplemented for the maintenance for parental and ABCB1-expressed Flp-In-293 cells, respectively.

Generation of ABCB1 2677G/T/A variant forms

The pcDNA5/FRT/ABCB1 (2677G) vector was generated by inserting ABCB1 (2677G) in pFastBac1/ABCB1 (2677T) into the pcDNA5/FRT vector (Life Technologies) between the restriction enzyme sites of BamH I and Xho I. ABCB1 2677 T/A variant forms were generated by using the QuikChange Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s protocol, where pcDNA5/FRT/ABCB1 (2677G) vector was used as the template [21]. The PCR reaction consisted of 94 °C for 2 min and then followed by 12 cycles of reactions at 94 °C for 30 sec, 55 °C for 30 sec and at 68 °C for 18 min, where Pfu Turbo DNA polymerase (Agilent Technologies) and the following PCR primers were used: 5′-GAAAAGAATCTAGAGGTTCGCTCTTGGAA GATCGCTAC-3′ and 5′-GTAGGCGATTTTCACAGAAC CTTCTTAGTTCTTCTT-3′ for ABCB1 2677 T, and 5′ GAAAGAATCTAGAGGTTCGCTCTTGGAA GATCGCTAC-3′ and 5′-GTAGGCGATTTTCACAGAAC CTTCTTAGTTCTTCTT-3′ for ABCB1 2677A. After the PCR, the reaction mixture was incubated with DpnI endonuclease at 37 °C for 1 h to digest the original template pcDNA5/FRT/ ABCB1 (2677G) vector. The resulting sequence was examined to confirm the generation of the pcDNA5/FRT/ ABCB1 (2677G/T/A) vectors.

Establishment of ABCB1 2677G/T/A variant forms-expressing cells

Flp-In-293 cells having the Flp Recombination Target (FRT) site at the telomeric region of only one of the pair of chromosomes 12 were transfected with the pcDNA5/FRT/ABCB1 (2677G/T/A) and the Flp recombinase expression plasmid pOG44 vectors as previously reported [19]. Single colonies resistant to hygromycin B (Life Technologies) were picked and sub-cultured as Flp-In-293/ABCB1 (2677G), Flp-In-293/ABCB1 (2677T) and Flp-In-293/ABCB1 (2677A) cells. Protein expression levels of ABCB1 in these cells (2 × 10⁷ cells) were determined using Membrane Protein Extraction Kit (BioVision, Milpitas, CA) and Human permeability glycoprotein (P-gp/ABCB1) ELISA kit (Cusabio Biotech, Wuhan, China) by a microplate reader (ARVOmx, PerkinElmer, Waltham, MA).
Cytotoxicity studies
In atorvastatin (Sigma-Aldrich, St Louis, Mo) cytotoxicity experiment, HepaRG (5 × 10⁵ cells/well), Flp-In-293/ABCB1 (2677G), Flp-In-293/ABCB1 (2677 T) and Flp-In-293/ABCB1 (2677A) cells were cultured in monolayers at 37 °C for 24 h in 24-well collagen type I-coated plates (Iwaki Glass, Chiba, Japan). After the preculture, cells were cultured in the presence of different concentrations of atorvastatin (0, 0.3, 1, 3, 10, 30, and 100 μM) for HepaRG cells, 0, 0.6, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM for Flp-In-293/ABCB1 (2677G/T/A) cells for 24 h. After the culture, 50 μl of WST-8 working solution (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) was added to each well and the plates were incubated at 37 °C under 5 % CO₂ and 95 % air. Optical density at 450 nm was measured by a microplate reader (ARVOmx, PerkinElmer, Waltham, MA). Lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and ALT releases from HepaRG cells into the medium were determined according to the manufacturers’ protocol of LDH, AST and ALT activity assay kits (Biovision, Milpitas, CA).

Statistical analysis
Association studies were conducted by using Fisher’s exact test under an allelic model. P values were corrected according to Bonferroni correction. A significance level was set at 0.0029 (0.05/17) in Table 1. In case of ABCB1 rs2032582, the patients were divided into two groups (T/A versus G, G/A versus T or G/T versus A) to evaluate the association of the three alleles by using the Fisher’s exact test (Table 1). The haplotype analysis was performed using SNPAlzye software (version 8.0.1, Dynacom, Chiba, Japan). Statistical analysis of cytotoxicity test of HepaRG and ABCB1 protein expression levels in Flp-In-293 cells was performed by using one-way analysis of variance with Dunnett’s and Tukey’s post-hoc test using GraphPad Prism software.

Results
No significant association of disease background was observed between AILI and non-AILI patients (Additional file 1: Table S1). The median age values were 61 years (range 27–82) and 66 years (32–89) in AILI and non-AILI groups, respectively. The 60.0 and 53.9 % were male in AILI and non-AILI groups, respectively. All

Table 1: Association of 15 functional SNPs in eight candidate genes with atorvastatin-induced liver injury

| Gene  | SNP          | Allele (1/2) | Amino acid change | Other name | AILI⁵  | Non-AILI⁵ | RAFA  | P value⁶  | HWE |
|-------|--------------|--------------|------------------|------------|-------|-----------|-------|-----------|-----|
|       |              |              |                  |            |      | 11 12 22  | 11 12 22 | 11 12 22  |     |          |
| ABCB1 | rs1045642    | C/T          | I1145I           | C3435T     | 12 15 3 | 151 196 67 | 0.65 0.60 | 0.50 0.70 0.60 | 0.59 0.80 |
|       | rs2032582    | T, A/G       | S7893A           | G2677 T/A  | 2 16 12 | 138 191 85 | 0.67 0.44 | 0.00006 0.0017 0.020 | 0.27 0.21 |
|       |              | G, A/T       | A7893S           |            | 15 14 1 | 158 193 63 | 0.73 0.61 | 0.073 0.24 0.10 | 0.29 0.75 |
|       |              | G, T/A       | A5893T           |            | 26 4 0  | 283 114 17 | 0.93 0.82 | 0.031 0.039 0.62 | 0.70 0.21 |
|       | rs1128503    | T/C          | G412G            | C1236T     | 15 12 3 | 146 197 71 | 0.70 0.59 | 0.10 0.12 0.45 | 0.79 0.74 |
| ABCG2 | rs2231142    | C/A          | Q141K            | C421A      | 12 15 3 | 195 180 39 | 0.35 0.31 | 0.57 0.57 0.76 | 0.59 0.78 |
| CYP2C9| rs1798583    | C/T          | R144C            | CYP2C9*2   | 30 0 0  | 412 0 0  | 1.00 1.00 | 1.00 1.00 1.00 | 1.00 1.00 |
|       | rs1057910    | A/C          | I359L            | CYP2C9*3   | 28 2 0  | 393 20 0  | 0.03 0.02 | 0.66 0.65 1.00 | 0.85 0.61 |
| CYP2C9| rs424285     | G/A          | P272P            | CYP2C19*2  | 16 9 5  | 197 180 36 | 0.32 0.31 | 0.88 0.58 0.18 | 0.09 0.57 |
|       | rs4986893    | G/A          | W212X            | CYP2C19*3  | 27 3 0  | 321 86 6  | 0.95 0.84 | 0.16 0.14 0.77 | 0.93 0.93 |
| CYP3A4| rs12721627   | C/G          | T185S            | CYP3A4*16  | 29 1 0  | 405 8 0  | 0.02 0.01 | 0.47 0.47 1.00 | 0.93 0.84 |
|       | rs28371759   | T/C          | L292P            | CYP3A4*18  | 29 1 0  | 399 14 0  | 0.98 0.98 | 1.00 1.00 1.00 | 0.93 0.73 |
| CYP3A5| rs776746     | G/A          | –                | CYP3A5*3   | 18 11 1 | 223 165 26 | 0.78 0.74 | 0.54 0.57 0.66 | 0.54 0.54 |
| SLC01B1| rs2306283   | G/A          | N130D            | SLC01B1*18 | 14 12 4 | 185 179 50 | 0.67 0.66 | 1.00 0.85 0.77 | 0.58 0.51 |
|       | rs4149056    | T/C          | V174A            | SLC01B1*5  | 18 11 1 | 306 95 13 | 0.22 0.15 | 0.14 0.13 1.00 | 0.66 0.10 |
| UGT1A1| rs4183223    | G/A          | G71R             | UGT1A1*6   | 25 5 0  | 281 118 15 | 0.92 0.82 | 0.075 0.10 0.61 | 0.62 0.35 |
|       | rs8175347    | (TA)6/(TA)7  | –                | UGT1A1*8   | 23 7 0  | 332 76 6  | 0.12 0.11 | 0.83 0.64 1.00 | 0.47 0.49 |

Abbreviation: AILI: atorvastatin-induced liver injury, RAF: risk allele frequency, ALLELIC: Allelic model, Dom: Dominant model, REC: Recessive model, HWE: Hardy–Weinberg equilibrium

⁵AILI, N = 30; Non-AILI, N = 414

⁶The lowest significant P value after Bonferroni correction among three models is shown in bold (P < 0.0011)
SNPs met quality control criteria (call rate > 95 %, Hardy–Weinberg equilibrium P value > 10⁻³ and minor allele frequency > 1 %). *ABCB1* rs2032582 was found to be associated with an increased risk of AILI (P = 0.00068, odds ratio (OR) = 2.59 with 95 % confidence interval (CI) of 1.49–4.50, G allele versus T and A alleles) by genotyping 444 Japanese subjects for 15 functional SNPs in eight candidate genes that reportedly affect the pharmacokinetics of atorvastatin (Table 1). No other polymorphisms showed a significant association with AILI. The frequency for *ABCB1* rs2032582 G allele in AILI patients was significantly higher than that in non-AILI patients whereas the frequencies of *ABCB1* rs2032582 T and A alleles were not significantly different between AILI and non-AILI groups, indicating that the G allele might be a risk factor for AILI (Table 1 and Additional file 1: Table S2). Although we performed haplotype analysis using three SNPs of *ABCB1* (rs1128503, rs2032582 and rs1045642), no haplotype constructed from the SNPs showed an extremely smaller P value than a single marker association of the *ABCB1* rs2032582 (Table 2). No association of HLA-A, -B and -C genotypes with AILI was shown (Additional file 1: Table S3, Additional file 1: Table S4 and Additional file 1: Table S5).

The cytotoxicity study using HepaRG cells demonstrated concentration-dependent effects of atorvastatin on cell viability as well as on LDH, AST and ALT release from the cells (Additional file 1: Figure S1). To estimate the effects of *ABCB1* rs2032582 on cytotoxicity induced by atorvastatin, we conducted cytotoxicity study using Flp-In-293 cells stably expressing ABCB1 proteins encoded by 2677G wild-type [Flp-In-293/ABCB1 (2677G) cells], 2677A [Flp-In-293/ABCB1 (2677A) cells] and 2677 T [Flp-In-293/ABCB1 (2677 T) cells] alleles.

### Table 2 Association of haplotypes consisting of three SNPs of *ABCB1* with atorvastatin-induced liver injury

| rs1128503 | rs2032582 | rs1045642 | Number of carriers | P value* |
|-----------|-----------|-----------|--------------------|---------|
| 2677 T    | 2677 G    | 2677 A    | 0 (0.0) 2 (0.5) 1.00 |
| 2677 G    | 2677 T    | 2677 A    | 0 (0.0) 2 (0.5) 1.00 |

**Abbreviation:** AILI atorvastatin-induced liver injury

*AILI, N = 30; Non-AILI, N = 414

*The significant P value after Bonferroni correction is less than 0.005*

The significant P value for *rs1128503*, *rs2032582*, and *rs1045642* was observed in Flp-In-293/ABCB1 (2677T/A) cells. The IC₅₀ value in Flp-In-293/Mock cells was about two-fold lower than those in Flp-In-293/ABCB1 (2677G/T/A) cells, indicating higher accumulation of atorvastatin in the Flp-In-293/Mock cells compared to those of 2677 G/T/A cells. The IC₅₀ value in Flp-In-293/ABCB1 (2677G) cells was significantly lower than those in Flp-In-293/ABCB1 (2677T) and Flp-In-293/ABCB1 (2677A) cells (Table 3, Additional file 1: Figure S3).

### Discussion

To identify the genetic markers associated with AILI, we genotyped 15 functional SNPs in eight genes that are possibly involved in the hepatic metabolism (CYP2C9, CYP2C19, CYP3A4, CYP3A5 and UGT1A1) and membrane transport (ABCB1, ABCG2 and SLCO1B1) of atorvastatin. *ABCB1* rs2032582 was significantly associated with AILI. *ABCB1* rs2032582 changes ABCB1 amino acid 893 from alanine to serine or threonine, respectively. These variants did not appear to affect ABCB1 protein expression levels in Flp-In-293/ABCB1 (2677G/T/A) cells, but gave a lower IC₅₀ in Flp-In-293/ABCB1 (2677G) cells than those in Flp-In-293/ABCB1 (2677 T/A) cells. ATP-dependent uptake of [³H]-vincreistine into membrane vesicles is also reportedly slower in cells expressing the ABCB1 rs2032582 G allele than those expressing the ABCB1 rs2032582 T/A alleles [22]. Therefore, we speculate that patients carrying the *ABCB1* rs2032582 G allele experience lower atorvastatin efflux activity from the hepatocytes into bile and higher hepatocellular concentrations of atorvastatin than carriers of the *ABCB1* rs2032582 T/A alleles. The higher hepatocellular concentration of atorvastatin can increase the risk of hepatotoxicity because atorvastatin induced concentration-dependent cytotoxicity in HepaRG cells (Additional file 1: Figure S1).

The *ABCB1* rs2032582 allele frequencies in our 444 patients (45.2 %, 37.7 % and 17.1 % for G, T and A alleles, respectively) are consistent with the previous

### Table 3 Atorvastatin-dependent cytotoxicity in Flp-In-293 cells stably expressing different ABCB1 proteins

| Cell name | ABCB1 Allele | Amino acid | IC₅₀ (mM) | P value |
|-----------|--------------|------------|----------|---------|
| Mock      | –            | –          | 2.74 ± 0.04 | –       |
| 2677G wild-type | G          | Alanine      | 5.44 ± 0.10 | –       |
| 2677 T    | T            | Serine     | 6.02 ± 0.07 | 0.009   |
| 2677A     | A            | Threonine  | 5.95 ± 0.08 | 0.026   |

**Abbreviation:** SE standard error, CI confidence interval

Experiments were performed in duplicate wells and repeated four times. The significant P value is shown in bold (P < 0.05, versus 2677G wild-type, one-way analysis of variance with Dunnett’s post-hoc test).
report of 154 Japanese subjects (42.9 %, 40.6 % and 16.6 % for G, T and A alleles, respectively) [23]. The above report revealed that the ABCB1 rs2032582 G and T/A allele frequencies in a Japanese population were comparable with those in a Caucasian population (42.9 % vs. 50.0 % and 57.2 % vs. 50.0 % for G and T/A alleles, respectively) [23]. Taking into account that no differences were reported in the systemic exposure to atorvastatin between Asian and Caucasian subjects [24], the ABCB1 rs2032582 allele might be also associated with the risk of AILI in the Caucasian population.

Of the atorvastatin-induced adverse reactions, myopathy is one of the most fatal adverse reactions [25, 26]. No statistically significant difference in AUC and the maximum plasma concentrations was observed between 14 patients with atorvastatin-induced myopathy and 15 healthy controls [27]. However, patients with atorvastatin-induced myopathy showed 2.4- and 3.1-fold higher AUC to atorvastatin lactone and p-hydroxy atorvastatin, respectively, compared to controls [27]. Atorvastatin is converted to its corresponding lactone form spontaneously or via glucuronidation mediated by UGT1A1, 1A3 and 1A4 and is metabolized to p-hydroxy atorvastatin by CYP3A4/5 [11, 28]. The present association studies showed that known functional SNPs of UGT1A1 and CYP3A4/5 were not associated with AILI. The higher accumulation of atorvastatin in the liver of patients carrying the ABCB1 rs2032582 G allele may cause hepatotoxicity, rather than those of atorvastatin lactone and p-hydroxy atorvastatin, the atorvastatin metabolites generated by UGT1A1 and CYP3A4/5. Therefore, the genetic markers might differ between liver injury and myopathy induced by atorvastatin.

In general, DILI can be divided into dose-dependent and idiosyncratic types [29]. The former is related to the pharmacokinetics and/or pharmacological actions of the drug and the latter is related to immune systems, such as human leukocyte antigen (HLA) in a dose-independent manner. In fact, several HLA alleles showed drug-specific associations with DILI, such as HLA-A*33:03 for ticlopidine and HLA-B*57:01 for fluvoxacinillin [30]. Therefore, we examined association of HLA alleles with AILI. However, no significant association was observed for HLA-A, -B and -C alleles with AILI (Additional file 1: Table S3, Additional file 1: Table S4 and Additional file 1: Table S5).

Conclusions
Our results showed that ABCB1 rs2032582 was associated with an increased risk of AILI in the Japanese population. A genetic test of ABCB1 rs2032582 may provide useful information for predicting individuals at higher risk of AILI. However, additional studies with larger sample size are needed before applying this genetic marker in clinical practice.

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Availability of data and materials
The data sets supporting the results of this article are included within the article and its additional files.

Authors’ contributions
Conceived and designed the experiments: KF, MK, TM. Performed the experiments: KF, HN. Analyzed the data: KF, TM. Contributed reagents/materials/analysis tools: HN, TI. Wrote the paper: KF, HN, TI, MK, TM. All individuals who participated in this study provided written informed consent. This project was approved by the ethical committees at the RIKEN University of Tokyo and RIKEN Center for Integrative Medical Sciences, Yokohama Branch, Japan and The Institute of Medical Science, The University of Tokyo and RIKEN Center for Integrative Medical Sciences, Yokohama, Japan.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethical approval and consent to participate
All individuals who participated in this study provided written informed consent. This project was approved by the ethical committees at the RIKEN Yokohama Branch, Japan and The Institute of Medical Science, The University of Tokyo, Tokyo, Japan.

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