Expression Levels of GABA-A Receptor Subunit Alpha 3, Gabra3 and Lipoprotein Lipase, Lpl Are Associated with the Susceptibility to Acetaminophen-Induced Hepatotoxicity

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Abstract

Drug-induced liver injury (DILI) is the serious and fatal drug-associated adverse effect, but its incidence is very low and individual variation in severity is substantial. Acetaminophen (APAP)-induced liver injury accounts for >50% of reported DILI cases but little is known for the cause of individual variations in the severity. Intrinsic genetic variation is considered a key element but the identity of the genes was not well-established. Here, pre-biopsy method and microarray technique was applied to uncover the key genes for APAP-induced liver injury in mice, and a cause and effect experiment employing quantitative real-time PCR was conducted to confirm the correlation between the uncovered genes and APAP-induced hepatotoxicity. We identified the innately and differentially expressed genes of mice susceptible to APAP-induced hepatotoxicity in the pre-biopsied liver tissue before APAP treatment through microarray analysis of the global gene expression profiles (Affymetrix GeneChip® Mouse Gene 1.0 ST for 28,853 genes). Expression of 16 genes including Gdap10, Lpl, Gabra3 and Ccrn4l were significantly different (t-test: FDR <10%) more than 1.5 fold in the susceptible animals than resistant. To confirm the association with the susceptibility to APAP-induced hepatotoxicity, another set of animals were measured for the expression level of selected 4 genes (higher two and lower two genes) in the liver pre-biopsy and their sensitivity to APAP-induced hepatotoxicity was evaluated by post hoc. Notably, the expressions of Gabra3 and Lpl were significantly correlated with the severity of liver injury (p<0.05) demonstrating that these genes may be linked to the susceptibility to APAP-induced hepatotoxicity.

Key Words: Acetaminophen, Hepatotoxicity, Toxicogenomics, GABA-A receptor subunit alpha 3, Lipoprotein lipase

INTRODUCTION

Acetaminophen (APAP) is one of the most widely used analgesics and antipyretics, and it is considered safe to use within the maximum daily dose of 3,250 mg and the maximum single dose of 650 mg (Krenzelok, 2009). However, its overuse or co-existing risk factors that can exacerbate liver function such as narcotic, alcohol drinking or polypharmacy, often cause drug-induced liver injury (DILI) (Watson et al., 2004; Larson et al., 2005). DILI, namely hepatotoxicity induced by drugs is one of the most frequent and severest forms of adverse drug reaction (Lazarou et al., 1998). The incidence of DILI is low varying from 1/100,000 to 1/10,000 but, its impact is huge since acute liver failure with high mortality can occur (Chang and Schiano, 2007; Stephens et al., 2012). More than 1,000 drugs were known to cause DILI in human (Lewis, 2000; Reuben et al., 2010). Of these APAP-induced DILI accounts for 46% of cases of fulminant liver failure in the United States (Ostapowicz et al., 2002). More than 200 million persons take APAP annually and among them about 200 persons die of ful-
minant hepatic failure from over-dosage (Park, 2006).

APAP induces hepatotoxicity via the generation of reactive and electrophilic metabolites like N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome P450 that can result in glutathione (GSH)-adduct formation, covalent binding to vital endogenous macromolecules, and depletion of cellular antioxidant reserve (Bessem and Vermeulen, 2001). N-acetylcysteine given (NAC) within 12 h seems to be effective in protecting most patients against severe liver damage induced by APAP (Prescott et al., 1977) but frequently APAP-induced DILI could be recognized only after symptoms have developed by when NAC is no longer effective, reflecting that APAP-induced hepatotoxicity progresses in multiple stages and diverse factors may be involved that include inflammation, metabolism and lipid synthesis (Hinson et al., 2010). Moreover, APAP-induced DILI appears with variable degrees of severity ranging from minimal increases in the levels of serum alanine transaminase (ALT)/aspartate transaminase (AST) to severe hepatic necrosis and fatal hepatic failure (Larson et al., 2005; Watkins et al., 2006; Yun et al., 2014), reflecting the existence of “individual factors” in the manifestation of APAP-induced hepatotoxicity.

To identify factors that determine the severity of APAP-induced hepatotoxicity or to develop a biomarker to screen susceptible individuals, various approaches have been attempted. Liu et al. (2010) have analyzed hepatic gene expressions related to APAP toxicity using resistant strain SJL/J and three sensitive strains C57BL/6J, DBA/2J, and SM/J. Stamper et al. (2010) have found several genes with significant differences in the expression between non-toxic APAP regio-isomer 3-hydroxyacetanilide and APAP in TGF-α transgenic mouse hepatocytes. Also, Umbright et al. (2010) have reported that many blood genes associated with inflammation, immune, stress responses and energy metabolism were statistically different in their expression levels following APAP treatment.

While these approaches are effective in the identification of target molecules or markers that can diagnose the extent of APAP-induced hepatotoxicity, their applicability as biomarkers to screen-out susceptible individuals is limited since the exposure to APAP might have extensively altered the genetic landscape due to direct or collateral tissue damages and subsequent inflammatory responses. Recently, to draw an intact genetic picture without the interference from APAP-induced toxicity, we examined through the comparison of pre-dose blood genes and the severity of APAP-induced hepatotoxicity posterior in rats in vivo, which demonstrated that protein kinase A (PKA) inhibitor alpha (Pkiα) expression level in pre-dose blood can be employed to predict susceptible individuals without the interference from the exposure to APAP (Yun et al., 2014). More importantly, this study has provided an insight into the role of PKA in the manifestation of APAP-induced hepatotoxicity. This approach has been further corroborated by Lu et al. (2015) who demonstrated that four genes, including Incenp, Rpgrip1, Sbf1, and Mmp12, which are associated with cell proliferation and tissue repair functions, in the blood collected from individuals prior and posterior to APAP administration can be used for identifying susceptible population to DILI. However, these studies offered surrogate genetic biomarkers in blood rather than that of direct target, liver, therefore, its utility and implication might be limited. In this study, we further investigated the transcriptome in the biopsy of liver pre-dose to uncover the genetic factors for individual susceptibility of APAP-induced hepatotoxicity in mice in vivo in an effort to provide a clue to understand the progression of, and defense mechanisms against APAP-induced DILI.

MATERIALS AND METHODS

Animals

Outbred ICR mice was selected as the experimental species for this study since rats do not manifest a clear profile of blood chemistry to APAP-induced hepatotoxicity when compared with the responses to carbon tetrachloride or D-galactosamine (Shin et al., 2014). Male ICR mice aged 7 weeks were purchased from Jung-Ang Lab Animals (Seoul, Korea) and housed in a specific pathogen-free (SPF) facility of Ewha Womans University. We used ICR mice, one of the outbred strains, which retain a certain level of genetic diversity in the test population in present study. The mice were kept under controlled environmental conditions (23 ± 3°C, 40-60% rela-
tive humidity, 12/12 h dark-light cycle) with *ad libitum* access to laboratory normal diets (Purina Co., Seoul, Korea) and tap water. All protocol involving animals were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Ewha Womans University.

**Experimental protocol**

To identify the innate genes for individual variation of liver injury, we analyzed the gene expression profiles in the liver pre-biopsied from individual mice prior to the oral administration of APAP (Sigma, St. Louis, MO, USA) and compared them with the individual severity of liver injury after APAP administration according to the method described previously (Yun *et al*., 2009, 2010) (Fig. 1). In brief, the mice were randomly assigned into three groups consisting of negative control group (no biopsy group) (N=5), biopsy control group (N=5), and APAP group (N=32). The operation was performed for minimum liver biopsies (about 10 mg) from left lobe of the liver of anesthetized mice in biopsy groups (biopsy control group and APAP group) using an isoflurane vaporizer (Midmark, Orchard Park, OH, USA) with an isoflurane of 1.5% to 3% and an oxygen flow of 0.5 L/min. After conducting the sutures to close the subcutis tissue and the skin using Surgifit 6-0 (AILEE, Busan, Korea) and Black silk 6-0 (AILEE). For the assessment of postoperative recovery, ALT, AST, albumin, albumin/globulin ratio (A/G ratio), total bilirubin and bile acid were measured using serum samples from blood collected via retro-orbital plexus of anesthetized animals into a gel serum separator plain blood tubes (MiniCollect 0.8 ml Z Serum Sep, Greiner Bio-One, Frickenhausen, Germany). After recovery for 3 weeks, the mice were administered with APAP through oral gavage at a dose of 300 mg/kg (dissolved in deionized water) according to a previous method (Saha and Nandi, 2009) with minor modifications. At 24 h after administrations of APAP, we performed the biochemical analysis using the blood collected via the postcaval vein from anesthetized animals, and then conducted microarray analysis using the pre-collected liver samples of the 10 animals (5 susceptible and 5 resistant) selected based on the results of the biochemical analysis and compared the gene expressions with the individual severity of liver injury after APAP administration to identify the innate genes for individual variation of liver injury. Additionally, to identify whether the innate genes selected by microarray experiment can predict the susceptibility of liver injury, we conducted the real-time PCR analysis for selected genes using liver samples biopsied from another set of animals (N=32) and then compared the gene expression with individual severity of liver injury after APAP administration (300 mg/kg, p.o.). The severity of liver injury were analyzed with the biochemical indicators, including ALT, total bilirubin, AST, lactate dehydrogenase (LDH) and bile acid at 24 h after administrations of APAP.

**RNA isolation and microarray analysis**

The liver tissue biopsied before APAP administration was processed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolation of total RNA. RNA precipitates were dissolved in RNase free DEPC treated water (USB, Cleveland, OH, USA). The concentration of RNA was determined Nano drop 1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Affymetrix (Santa Clara, CA, USA) GeneChip1 Mouse Gene 1.0 ST arrays were used to analyze the differential gene expressions, as described previously (Yun *et al*., 2009). The normalized scanned probe array data were compared between the groups to generate p-value and signal log ratio (fold change). Unpaired *t*-test was applied to determine statistically reliable probe sets.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Relative expression levels of mRNAs were measured by quantitative real-time PCR. Total RNA, extracted from liver prior to APAP treatment, was used to synthesize cDNA using pre-master mix with oligo dT (Bioepis, Seoul, Korea). Each reaction was performed using power SYBR Green PCR master mix in a 7300 real-time PCR machine (Applied Biosystems, Warrington, UK). The sequence of primers of mice liver were as follows: forward Gdap10, 5′-GCC TGG CAC GTA GAA CAA AC-3′; reverse Gdap10, 5′-ACT AGT TTG CTG CCC TCG TT-3′; forward Lpl, 5′-TCA GAG CCA AGA GGA GGA GC-3′; reverse Lpl, 5′-ATC TCG AAG GCC TGG TTG TG-3′; forward Klf10, 5′-AGG AAG GGT CCC TGC TCA GA-3′; reverse Klf10, 5′-AGG TTT TTC CCC TGG GTG TG-3′; forward Malat1, 5′-GAC AAAAGG CTAAG TGG AGT-3′; reverse Malat1, 5′-TTG TGC TGG CTC TAC CAT T-3′; forward Ccm4l, 5′-CCT GGA
GTG CAA GGA GTC TG-3'; reverse Ccrn4l, 5'-CTG GGT GAT GTT CTG CAG GT-3'; forward Gabra3, 5'-CCA ACA GCG ATT GCT TCA CC-3'; reverse Gabra3, 5'-GGC AAA GAG CAC AGG GAA GA-3'; forward GAPDH, 5'-CTA CCC CCA ATG TGT CCG TC-3'; reverse GAPDH, 5'-AAG TCG CAG GAG ACA ACC TG-3'. Annealing temperature were 51°C. For real-time PCR data, fold change results were calculated using 2^{-ΔΔCt} relative to the internal reference gene (GAPDH) and the mean of all samples. The change in the Ct (ΔCt) of the target genes was calculated as ΔCt=(Ct of target genes)-(Ct of GAPDH). The ratio of the target gene to the housekeeping gene was calculated and expressed as 2^{-ΔCt}. This ratio was then used to evaluate the expression level of the target gene within each animal. To determine the fold changes in gene expression among animals, the normalized gene expression of the target genes was divided by the normalized expression of the same
target gene in the sample with the lowest level of the normalized gene expression of the target genes, expressed as \(2^{-\Delta\Delta C_{\text{t}}}.\)

**Statistical analysis**

All data were analyzed by student \(t\)-test or one-way analysis of variance (ANOVA) followed by post hoc Tukey’s multiple range tests to determine treatment effect and to compare differences between group means. Differences were considered to be significant at \(p<0.05.\)

**RESULTS**

**Assessment of the influences of liver biopsy on liver function of ICR mice**

Pre-biopsy scheme needs the liver biopsy at the naïve condition prior to APAP administration to identify the innate genetic factors associated with the individual variation of APAP-induced liver injury afterwards (Fig. 1). Firstly, to check if pre-biopsy procedure does not affect normal liver function of mice, small amount of liver tissue (about 10 mg) was pre-biopsied from the identical region of left lobe and liver function was evaluated after sufficient postoperative recovery (3 weeks) by measuring ALT, AST, albumin, A/G ratio, total bilirubin, and bile acid in the serum. As a result, liver function of biopsy control group was not statistically different from those in Negative control group (Fig. 2), suggesting that the effects of pre-biopsy scheme on the liver function are minimal for mice as shown in rats previously (Yun et al., 2010).

**Hepatotoxicity induced by APAP administration after liver biopsy**

After liver pre-biopsy and postoperative recovery for 3 weeks, APAP was orally administered to the ICR mice (N=32), an outbred strain at a dose of 300 mg/kg. The APAP-treated mice exhibited clear signs of liver injury, as indicated by increased serum ALT and total bilirubin (Fig. 3). Importantly, there was a substantial inter-animal variation in the level of these biochemical indicators, suggesting that mice can display different susceptibility to APAP. On the basis of ALT and total bilirubin levels, animals could be grouped into 2 groups, that is, susceptible and resistant animals (top 5, susceptible and bottom 5, resistant, Fig. 3A). Serum ALT and total bilirubin in susceptible group were substantially higher when compared with both Negative control group and resistant group (Fig. 3B, 3C). Further histological examination (Fig. 4A) and blood biochemistry (Fig. 4B) also revealed that the animals grouped as susceptible manifested higher toxic response to APAP than resistant which could be readily determined by significantly higher liver toxicity markers and massive hepatic injury including numerous apoptotic cells and inflammatory cell infiltration in the liver tissue.

![Fig. 5. Hierarchical clustering that exhibited altered expression (p<0.05, fold change >1.5) of the microarray assay. (A) Comparison between the 5 most susceptible mice and the 5 resistant mice. The x-axis of the dendrogram represents the gene symbol. The y-axis represents the severity of liver injury. Colors range from bright green (log2 value -1) to bright red (log2 value +1). (B, C) The difference of gene expressions (intensity) in microarray analysis between susceptible and resistance group.](https://doi.org/10.4062/biomolther.2016.076)
<table>
<thead>
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<th>Gene</th>
<th>NCBI RefSeq No.</th>
<th>Description</th>
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<th>Absolute fold change</th>
<th>p-value</th>
<th>GO Process</th>
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<td>Chromodomain helicase DNA binding protein 7</td>
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Gene expression analysis with microarray to identify genes associated with susceptibility to APAP-induced hepatotoxicity

To determine the genetic factors associated with these inter-individual variations to APAP-induced hepatotoxicity, microarray analysis was performed with the liver samples of 5 susceptible and 5 resistant animals pre-obtained before APAP treatment as described in scheme (Fig. 1). The reliability of the transformed and normalized data was statistically analyzed using one-way ANOVA. This was visualized by hierarchical clustering of the calculated data from the experiment (Fig. 5A). From this analysis, 16 genes (excluding unknown sequences and noncoding genes) were found to be different with statistical reliability at \( p < 0.05 \) with more than 1.5 fold difference between two groups in their expression levels (Table 1). Among them, uppermost two genes expressed higher innately in susceptible group were found to be Gdap10 and Lpl, and lowermost two genes expressed lower innately in susceptible group were Gabra3 and Ccrn4l (Fig. 5B, 5C).

### Prediction of the susceptibility of animals to APAP-induced liver injury by real-time PCR analysis of the selected genes

To further confirm whether the individual expression levels of Gdap10, Lpl, Ccrn4l and Gabra3 are related to the inter-individual variation in the susceptibility of APAP-induced liver injury indeed, the expression of these genes was analyzed with liver biopsy samples pre-collected from 32 animals before APAP-treatment using quantitative real-time PCR analysis. While a meaningful relationship could not be found with the expression levels of Gdap10 and Ccrn4l with the severity of APAP-induced hepatotoxicity, the innate expression level of Gabra3 matched well with the severity of APAP-induced liver injury as could be determined by the strong correlation with APAP-induced AST (Spearman Correlation Analysis, \( p < 0.05 \); Correlation coefficients, -0.4209) (Fig. 6A) and ALT (Spearman Correlation Analysis, \( p < 0.05 \); Correlation coefficient, -0.4012) decrease (Fig. 6B). Moreover, the innate expression level of Lpl matched well with the severity of APAP-induced liver injury as could be determined by the strong correlation with APAP-induced total bilirubin (Spearman Correlation Analysis, \( p < 0.05 \); Correlation coefficient, 0.4052) increases (Fig. 6C), of which relationships were further corroborated by the comparison of AST, ALT and total bilirubin of grouping based on the genes (Fig. 6D-6F).

### DISCUSSION

Here, we explored the genetic markers in the liver that are associated with the individual susceptibility to APAP-induced hepatotoxicity in ICR mice employing pre-biopsy scheme. Through this approach, we could identify that low Gabra3 and high Lpl expression may be related to the susceptibility to APAP-induced hepatotoxicity. More importantly, we could confirm that susceptible animals could roughly be predicted through determining the expression levels of Gabra3 and Lpl in liver. In addition, this study suggests that Gabra3 may play a protective role against APAP-induced hepatotoxicity in the liver while Lpl may contribute to the development or aggravation of liver damages, which warrant further studies.

In the present study, a substantial inter-individual variation...
on APAP-induced hepatotoxicity was found among individual mouse, a well-known species susceptible to APAP, as evidenced by the histopathological examination and biochemistry analysis. Many previous transcriptomic approaches have examined the genetic landscape after APAP treatment and hence, can have limited applicability for the prediction of the individual susceptibility to APAP-induced hepatotoxicity. We previously demonstrated (Yun et al., 2009, 2010) that the pre-dose scheme using pre-biopsied liver, which has a unique ability to fully regenerate after injury for the maintenance of metabolism and detoxification (Fausto et al., 2006; Michalopoulos, 2007) may provide a useful tool for prec- }

**Fig. 6.** Verification test with a new set of animals (Correlation between expression of candidate genes and serum chemistry, N=32). The expression levels of Gabra3 and Lpl in the pre-collected liver biopsy were measured with quantitative real-time PCR analysis and compared with the blood chemistry data after APAP treatment. Correlation of expression level of Gabra3 with serum ALT (A) or AST (B) and that between Lpl and total bilirubin (C). *Represents statistical significance analyzed by Spearman correlation test and r notes Spearman’s rank correlation coefficient (p<0.05). Comparison of serum ALT (D), AST (E) or total bilirubin (F) levels of animals in 1st quartile of Gabra3 and 4th of Lpl expression levels (Predicted Sus, N=8) with those of 4th of Gabra3 or 1st Lpl (Predicted Sus, N=8). *p<0.05 and **p<0.01.

Although DNA microarray can simultaneously quantitate the expression of thousands of genes, a second methodology including quantitative real-time RT-PCR is required to assess the accuracy of the candidate genes discovered by microarray measurements (Draghici et al., 2006). Furthermore, the margin of difference in the expression is largely small, which necessitates a verification step. To confirm if the gene expression profiles detected with the microarray is reproducible and can be used to predict the animal to be susceptible or resistant to APAP-induced hepatotoxicity indeed, the liver samples pre-
biopsied from another set of animals were undergone real-time PCR analysis for the expression levels of 4 genes including higher two and lower two genes selected from microarray analysis.

Among four candidate genes, it is notable that the innate expression of Lpl in the liver was significantly correlated well with total bilirubin which was well-correlated with the severity of APAP-induced hepatotoxicity (Spearman correlation coefficients 0.4052, p<0.05). The expression of Lpl in liver was expressed higher innately in susceptible group to APAP-induced hepatotoxicity when compared to resistant group. Lpl, which is a member of the lipase superfamily that includes pancreatic, hepatic and endothelial lipase, is widely expressed in many tissues such as liver, brain, heart and adipose tissues (Wang and Eckel, 2009). Kim et al. (2001) have demonstrated that the overexpression of Lpl, which has been known to be the rate-limiting enzyme involved with triglyceride hydrolysis (Goldberg, 1996), causes profound insulin resistance in liver (Baron et al., 1988). Insulin resistance can lead to selective accumulation of fatty acid-derived metabolites (i.e., fatty acyl CoA, ceramide, diacylglycerol) in liver and may be implicated in the development of acute liver failure (ALF) (Clark et al., 2001) through impaired peripheral glucose utilization and a failure to fully suppress endogenous glucose production, contributing to the catabolic state that occurs in ALF. These suggest potential involvement of Lpl in the susceptibility to hepatotoxicity although little is known currently for the exact mechanism on link with the APAP-induced hepatotoxicity.

We also observed that Gabra3 expression level was found to be correlated well with the APAP-induced hepatotoxicity as shown by significant Spearman correlation coefficient of -0.4209 (p<0.05) and -0.4012 (p<0.05) for AST and ALT increase, respectively. Gabra3 has been originally found in the CNS but its expression in the liver tissue has been reported (Moe et al., 2008; Oh et al., 2009). Moreover, Biju et al. (2001) have showed that hepatic GABA-A receptor can give inhibitory signal for hepatic cell proliferation. Bozogluer et al. (2012) also showed that flumazenil, a GABA-A receptor antagonist, attenuated the APAP-induced hepatotoxicity. Conversely, the expression of Gabra3 was down-regulated following the exposure to a hepatocarcinant, 4,4′-methyleneedianiline in mice (Oh et al., 2009). Despite many contradicting results on the role of GABA-A receptor in the liver, we could speculate that the susceptibility to APAP-induced hepatotoxicity in the animals with innately lower expression of Gabra3 can be attributable to the altered signaling for hepatocyte proliferation which is important for the recuperation processes.

Previously, we demonstrated that high expression level of Pkia in pre-dose blood may be related to the susceptibility to APAP-induced hepatotoxicity (Yun et al., 2014). In our microarray analysis, however, the difference of Pkia expression between susceptible and resistant was only marginal (1.12 fold higher in susceptible with p-value of 0.055, t-test, data not shown) which may be considered confirmatory but unapparent. This discrepancy may be from different species employed for the studies (SD rats for blood genes, ICR mice for liver genes). Here, we employed ICR mice since this species manifests clear blood chemistry profiles for APAP-induced hepatotoxicity in contrast to the rat which needs histology to monitor APAP-induced hepatotoxicity additional to blood chemistry. These results suggest that other experimental animal species or strains may produce different genetic markers owing to distinct physiology and translational clinical research in human is ultimately necessary to draw the solid answers.

In conclusion, we demonstrated that the two gene biomarkers in the liver pre-biopsied prior to administration were related to the inter-individual variation in the severity of APAP-induced hepatotoxicity although other important factors like different gastrointestinal absorption of APAP (Sanaka et al., 1998) or alteration of metabolic capacity like CYP2E1 (Lee et al., 1996) could not be examined due to the limitation of study design and animal species employed. However, the pre-dose expressions of Lpl and Gabra3 in the liver were all correlated well with the post-dose changes of ALT, AST, and total bilirubin. Accordingly, the data presented in this study suggest a novel role of Lpl and Gabra3 in the liver in the manifestation of APAP-induced hepatotoxicity. In addition, we could speculate that these genes can be employed to screen out individuals susceptible to APAP-induced hepatotoxicity although further studies should be conducted.

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