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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-

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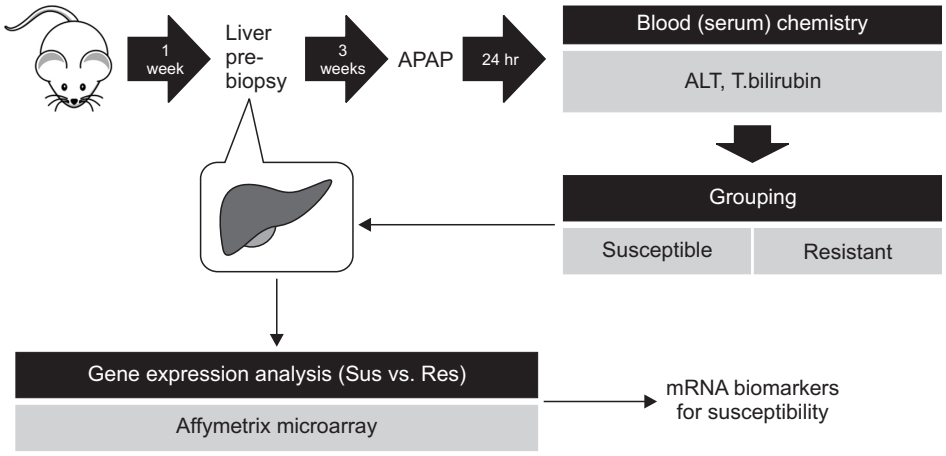

Fig. 1. Experimental scheme for pre-biopsy. To identify the innate genes for individual variation of liver injury, we analyzed the gene expression profiles in the liver pre-biopsied from individual mice prior to APAP administration and compared them with the individual severity of liver injury after APAP administration. ALT: alanine aminotransferase, T.bilirubin: Total Bilirubin, Sus: susceptible, Res: resistant.

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 (Bessems 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 (*Pkia*) 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 tab 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

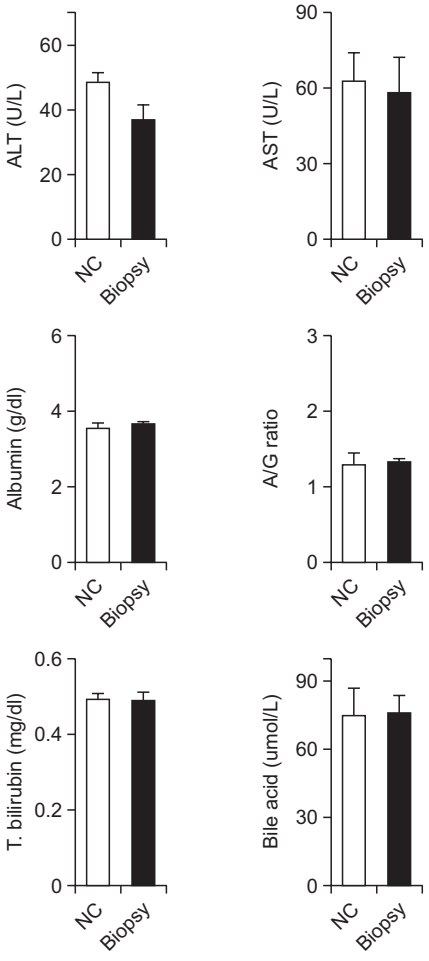

Fig. 2. Effects of the liver biopsy on serum biochemistry after recovery for 3 weeks. Levels of ALT, AST, albumin, A/G ratio, total bilirubin and bile acid in the serum of mice recovered from or not undergone pre-biopsy. The data are presented as means ± SE. NC, Negative control group; Biopsy, Biopsy control group.

(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 GAA GCA GC-3′; reverse *Lpl*, *5′*-ATC TCG AAG GCC TGG TTG TG-3′; forward *Klf10*, *5′*-AGC AAG GGT CAC TCC TCA GA-3′; reverse *Klf10*, *5′*-AGG TTT TTC CCC TGT GTG TG-3′; forward *Malat1*, *5′*-GAC AAA AGG CTA AAG TGG ATG-3′; reverse *Malat1*, *5′*-TTG TGC TGG CTC TAC CAT T-3′; forward *Ccrn4l*, *5′*-CCT GGA

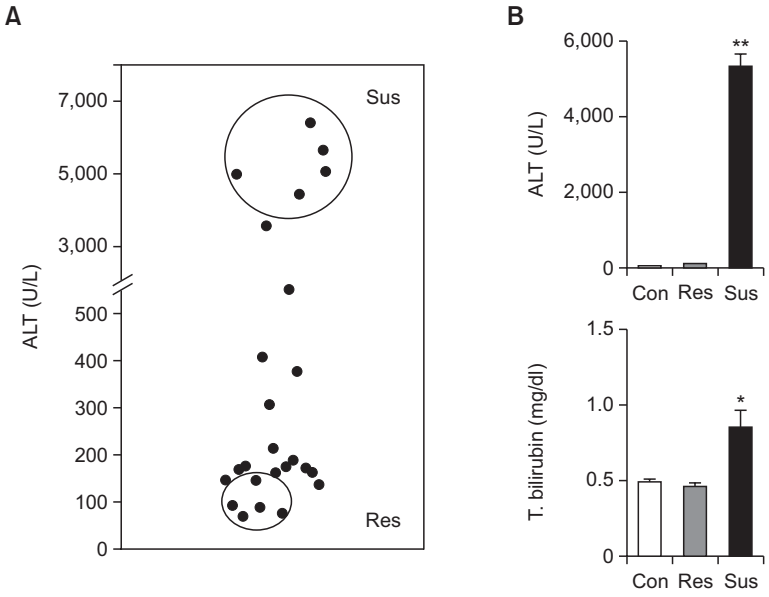

Fig. 3. Selection of the groups based on the serum ALT and total bilirubin after APAP administration. (A) Selection of the samples for microarray analysis (N=5/group). (B) ALT and total bilirubin levels of selected groups. The data are presented as means ± SE (*p<0.05 and **p<0.01). Con, Negative control group; Res, resistant group; Sus, susceptible group.

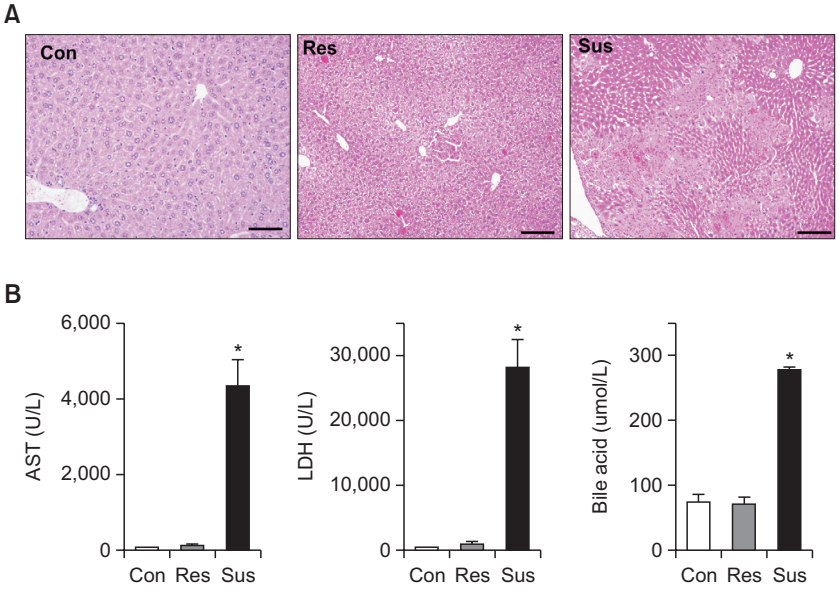

Fig. 4. Biochemical and histological analysis between groups after APAP treatment. The mice were orally administrated with APAP at a dose of 300 mg/kg. At 24 h after APAP administration, histological examination (A) and blood biochemistry for lactate dehydrogenase (LDH, U/L), aspartate aminotransferase (AST, U/L)) and bile acid (μmol/L) (B) were conducted. Representative H&E stained liver tissue (bar = 200 μm). The data are presented as means ± SE. *Significantly different from Con group (p<0.05). Con, Negative control group; Res, resistant group; Sus, susceptible group.

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 $2^{-\Delta\Delta Ct}$ 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 $2^{-\Delta Ct}$. 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

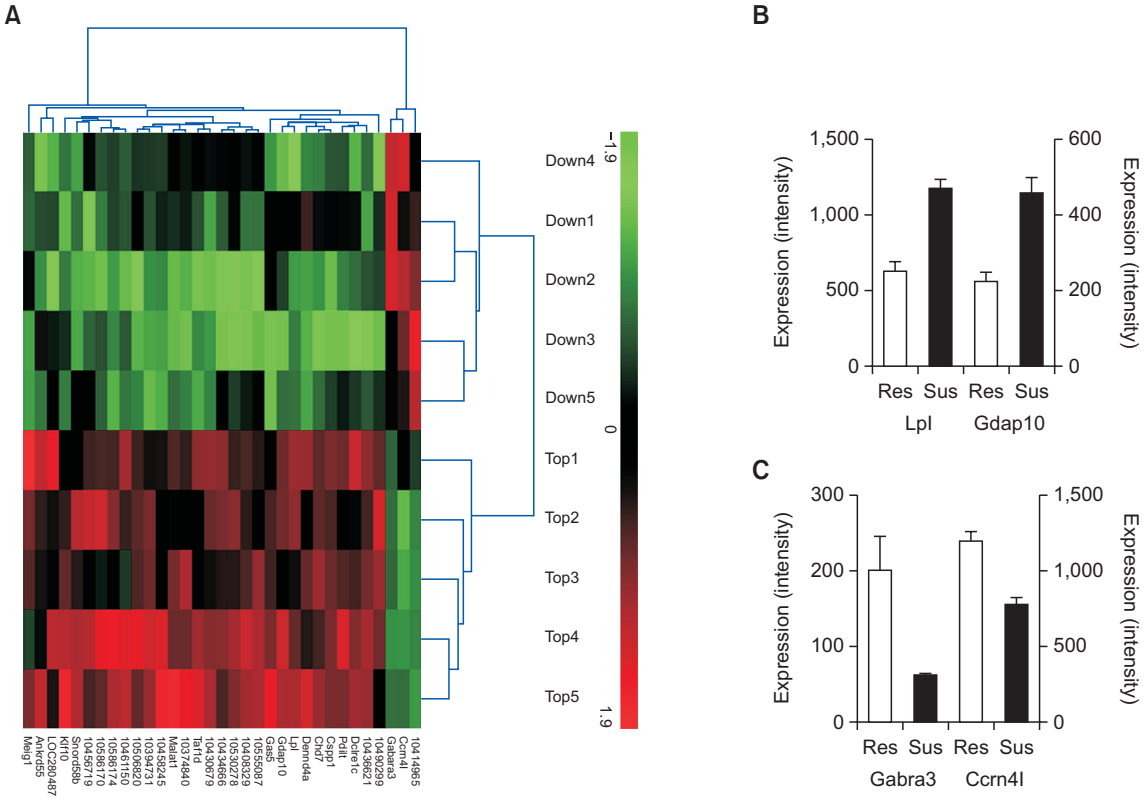

Fig. 5. Hierarchical clustering that exhibited altered expression (p<0.05, fold change >1.5) of the microarray assay. (A) Comparison between the 5 most susceptible mice and the 5 resistant mice. The x-axis of the dendrogram represents the gene symbol. The y-axis represents the severity of liver injury. 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.

target gene in the sample with the lowest level of the normalized gene expression of the target genes, expressed as $2^{-\Delta\Delta Ct}$.

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.

Table 1. Significantly different genes in the liver pre-biopsy of Susceptible group vs. Resistant

Gene	NCBI RefSeq No.	Description	log2 ratio	Absolute fold change	*p*-value	GO Process
Gdap10	BC052902	Ganglioside-induced differentiation-associated-protein 10	1.01	2.01	0.0018	-
Lpl	NM_008509	Lipoprotein lipase	0.91	1.88	0.0004	Lipid metabolic process // positive regulation of macrophage derived foam cell differentiation // lipid catabolic process // triglyceride biosynthetic process // triglyceride catabolic process
Klf10	NM_013692	Kruppel-like factor 10	0.82	1.77	0.0011	Transcription // induction of apoptosis // bone mineralization // regulation of cell proliferation // regulation of transcription // positive regulation of osteoclast differentiation // response to protein stimulus
Malat1	NR_002847	Metastasis associated lung adenocarcinoma transcript 1	0.81	1.75	0.0040	Biological_process
Gas5	NR_002840	Growth arrest specific 5	0.80	1.75	0.0054	-
Ankrd55	NM_029898	Ankyrin repeat domain 55	0.79	1.73	0.0066	Molecular_function // cellular_component // biological_process
Cspp1	NM_026493	Centrosome and spindle pole associated protein 1	0.73	1.66	0.0024	Biological_process // cellular_component // molecular_function
Dennd4a	NM_001162917	DENN/MADD domain containing 4A	0.70	1.62	0.0048	Biological_process // cellular_component // molecular_function
Chd7	NM_001081417	chromodomain helicase DNA binding protein 7	0.64	1.55	0.0014	Blood vessel development // in utero embryonic development // heart morphogenesis // chromatin assembly or disassembly // transcription // adult heart development // sensory perception of sound // locomotory behavior // adult walking behavior // blood circulation // chromatin modification // female genitalia development // embryonic hindlimb morphogenesis // positive regulation of multicellular organism growth // ear morphogenesis // inner ear morphogenesis // camera-type eye development // nose development // regulation of transcription // palate development chromatin // cellular_component // nucleus nucleotide binding // molecular_function // nucleic acid binding // DNA binding // chromatin binding // helicase activity // ATP binding // hydrolase activity
Snord58b	NR_028552	Small nucleolar RNA, C/D box 58B	0.62	1.54	0.0011	Biological_process //cellular_component // molecular_function //
Dclre1c	NM_146114	DNA cross-link repair 1C, PSO2 homolog (S. cerevisiae)	0.62	1.53	0.0008	Telomere maintenance // DNA repair // double-strand break repair // DNA recombination // response to DNA damage stimulus // response to ionizing radiation // B cell differentiation // chromosome organization nucleus single-stranded DNA specific endodeoxyribonuclease activity // nuclease activity // endonuclease activity // exonuclease activity // 5'-3' exonuclease activity // hydrolase activity
Taf1d	BC056964	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D	0.60	1.52	0.0036	Transcription // biological_process // regulation of transcription cellular_component // nucleus molecular_function // DNA binding

Table 1. Continued

Gene	NCBI RefSeq No.	Description	log2 ratio	Absolute fold change	*p*-value	GO Process
Pdilt	NM_027943	Protein disulfide isomerase-like, testis expressed	0.60	1.51	0.0044	Multicellular organismal development // spermatogenesis // biological_process // metabolic process // cell differentiation // cell redox homeostasis // cellular_component // endoplasmic reticulum molecular_function // isomerase activity
Meig1	NM_008579	Meiosis expressed gene 1	0.60	1.51	0.0066	Meiosis nucleus protein binding
Ccrn4l	NM_009834	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	-0.63	1.55	0.0008	Rhythmic process
Gabra3	NM_008067	Gamma-aminobutyric acid (Saeed et al.) A receptor, subunit alpha 3	-1.56	2.94	0.0034	Transport // ion transport // chloride transport // signal transduction // signal transduction // gamma-aminobutyric acid signaling pathway // synaptic transmission // ion transmembrane transport

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

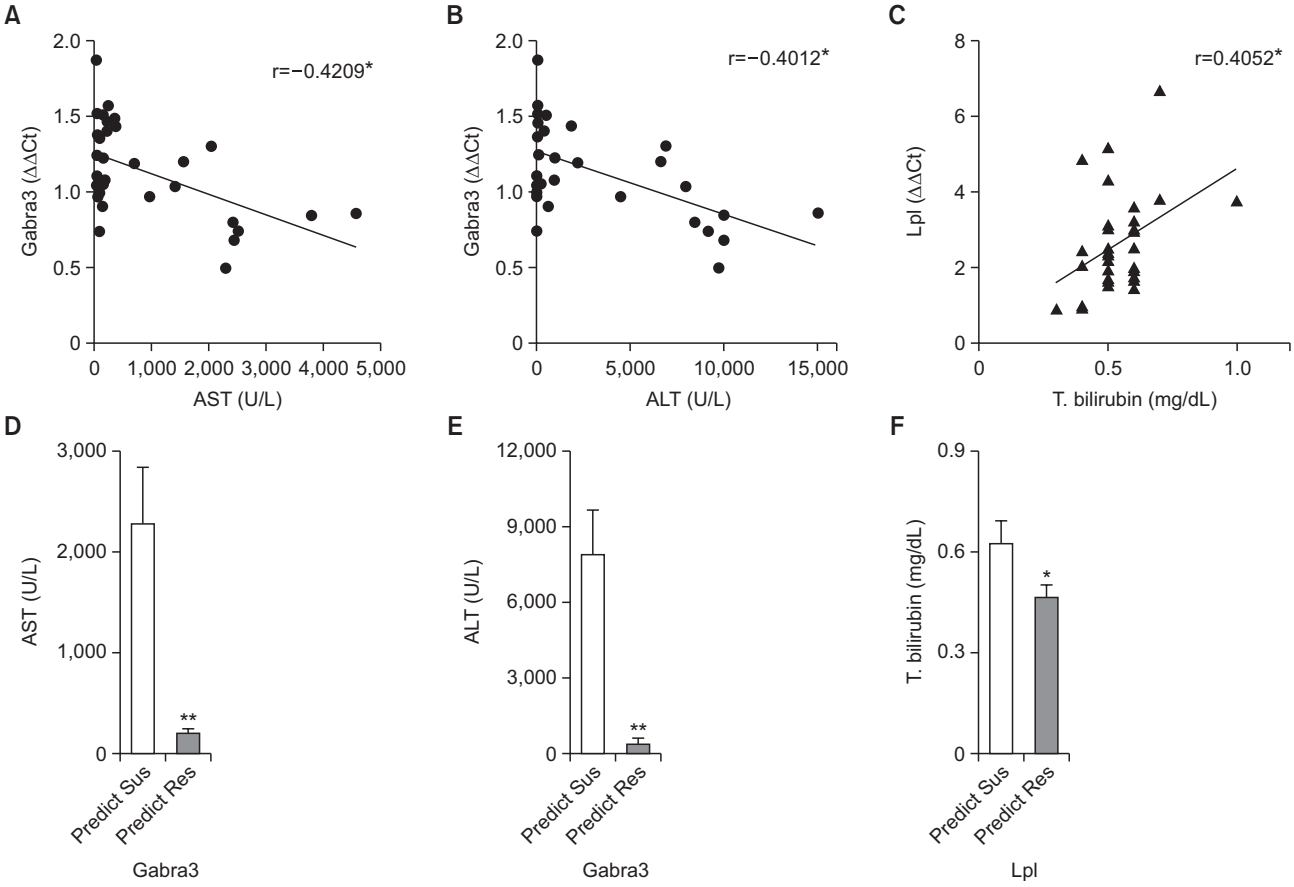

Fig. 6. Verification test with a new set of animals (Correlation between expression of candidate genes and serum chemistry, N=32). The expression levels of *Gabra3* and *Lpl* in the pre-collected liver biopsy were measured with quantitative real-time PCR analysis and compared with the blood chemistry 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 Res, N=8) with those of 4th of *Gabra3* or 1st *Lpl* (Predicted Sus, N=8). *$p<0.05$ and **$p<0.01$.

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 predose scheme using pre-biopsied liver, which has a unique ability to fully regenerate after injury for the maintenance of its functions on metabolism and detoxification (Fausto *et al*., 2006; Michalopoulos, 2007) may provide a useful tool for prescreening of the susceptible individual and for the discovery of key molecules in the manifestation of toxicity. In the current microarray experiment using the pre-biopsy method, we identified 16 genes including *Gdap10*, *Klf10*, *Malat1*, and *Ccrn41* (unpaired *t*-test: FDR <10% and fold change >1.5) as candidate genes predictive of severity on APAP-induced liver injury.

Malat1, which has been known to be associated with a prognostic marker for metastasis in early stages of lung adenocarcinoma (Ji *et al*., 2003; Gutschner *et al*., 2013) and ganglioside-induced differentiation-associated-protein 10 (*Gdap10*), which is one of *Gdap* genes involved in different signal transduction pathway (Liu *et al*., 1999). *Klf10* has been known to regulate TGF-β signaling by blocking expression of the negative regulator, Smad7 (Johnsen *et al*., 2002), and activating expression of the positive effector, Smad2 (Johnsen *et al*., 2002). *Ccrn4l* is a gene that encodes a circadian deadenylase, and its disruption in mice showed lower body weight and reduced visceral fat, reflecting resistance to fatty liver and diet-induced obesity (Green *et al*., 2007). Genes encoding cellular components like *Ankrd55*, *Cspp1*, *Dennd4a*, *Chd7*, *Snord58b*, *Taf1d* and *Pdilt*, are also shown to be associated. Other genes, *Gas5*, *Dclre1c*, and *Meig1* also need further studies to examine their roles in the manifestation of APAP-induced hepatotoxicity.

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 hepatotoxicant, 4,4'-methylenedianiline 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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