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Prediction of drug-induced liver injury using keratinocytes

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Abstract

Drug-induced liver injury (DILI) is one of the most common adverse drug reactions. DILI is often accompanied by skin reactions, including rash and pruritus. However, it is still unknown whether DILI-associated genes such as S100 calcium-binding protein A and interleukin (IL)-1 β are involved in drug-induced skin toxicity. In the present study, most of the tested hepatotoxic drugs such as pioglitazone and diclofenac induced DILI-associated genes in human and mouse keratinocytes. Keratinocytes of mice at higher risk for DILI exhibited an increased IL-1 β basal expression. They also showed a higher inducibility of IL-1 β when treated by pioglitazone. Mice at higher risk for DILI showed even higher sums of DILI-associated gene basal expression levels and induction rates in keratinocytes. Our data suggest that DILI-associated genes might be involved in the onset and progression of drug-induced skin toxicity. Furthermore, we might be able to identify individuals at higher risk of developing DILI less invasively by examining gene expression patterns in keratinocytes.

Keywords

keratinocyte; drug-induced liver injury; prediction; IL-1 β ; hepatotoxicity

Introduction

Adverse drug reactions (ADRs) are undesirable effects of drugs beyond their anticipated therapeutic effects (Pirmohamed *et al.*, 1998). A large number of drugs have been withdrawn due to their severe ADRs. Drug-induced liver injury (DILI) accounts for a significant proportion of fatal ADRs (Farmer & Brind, 2011). Skin rash, pruritus, fever, general malaise, nausea and jaundice can be observed in patients with DILI. It was reported that 4.8% of patients with DILI developed severe skin rash such as Stevens–Johnson syndrome or toxic epidermal necrolysis (Devarbhavi *et al.*, 2016), even though the incidence of Stevens–Johnson syndrome and toxic epidermal necrolysis was estimated as 1–2 cases per

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Supporting information

Additional supporting information may be found online in the supporting information tab for this article.

Conflict of interest

The authors did not report any conflict of interest.

one million people (Rzany *et al.*, 1999). Moreover, skin reactions such as rash and pruritus are often observed as initial symptoms of DILI (Shizuma *et al.*, 2002). Takikawa *et al.* (2009) reported that skin rash and pruritus were observed in 12% and 14% of DILI cases at diagnosis, respectively. These indicate that there might be a similarity in responsiveness to drugs in the liver and skin.

Damage-associated molecular patterns such as high-mobility group box 1 (HMGB1) and DNA are molecules that are released from stressed cells. Hepatotoxic drugs can cause release of damage-associated molecular patterns outside the cell, activating toll-like receptors (TLRs) (Piccinini & Midwood, 2010). The activated immune cells can further produce inflammatory mediators that largely contribute to the progression of tissue inflammation (Lotze *et al.*, 2007). In the liver, TLRs activate Kupffer cells and intrahepatic lymphocytes, resulting in the release of inflammatory mediators (Hoek & Pastorino, 2002). In the skin, activation of keratinocytes, the predominant cell type in the epidermis, by TLRs can result in the release of inflammatory mediators (Lebre *et al.*, 2007). These similarities in the liver and skin might be an underlying mechanism of drug-induced liver and skin toxicity.

Hepatic interleukin (IL)-1 β mRNA was increased 30-fold 6 h after a diclofenac administration in mice (Yano *et al.*, 2012). In the study, neutralization of IL-1 β by the antibody attenuated diclofenac-induced hepatotoxicity. It was also demonstrated that S100 calcium-binding protein A (S100A) significantly increased in rat livers by hepatotoxic carbamazepine (Sasaki *et al.*, 2016). These suggest that IL-1 β and S100A might be involved in the diclofenac- and carbamazepine-induced liver injury, respectively. Similarly, it was suggested that immune- or inflammation-related factors such as a receptor for advanced glycation end products (RAGE) (Sasaki *et al.*, 2016), NACHT, LRR and pyrin domain-containing protein 3 (NALP3) and HMGB1 (Yano *et al.*, 2012) were involved in the onset and progression of hepatotoxic DILI. It was further demonstrated that S100A and HMGB1 activated Kupffer cells, natural killer cells, and natural killer cells with T-cell receptors in the liver (Lotze & Tracey, 2005). These activated cells produced cytokines and chemokines such as IL-1 β and IL-17, leading to progression of liver injury (Holt & Ju, 2006). As mentioned earlier, there might be similarities in inflammatory systems between the liver and skin. Therefore, inflammatory-related factors such as S100A, NALP3, IL-1 β and RAGE could be involved in the onset and progression of drug-induced skin toxicity as well.

In the present study, to investigate immune and inflammatory factors involved in the onset and progression of drug-induced skin toxicity, human and mouse keratinocytes were treated with various kinds of hepatotoxic and non-hepatotoxic drugs. We examined mRNA expression levels of S100A8, S100A9, NALP3, IL-1 β and RAGE in the non-treated and drug-treated cells. Furthermore, we investigated whether keratinocytes could be useful for the prediction of DILI in individuals.

Materials and methods

Chemicals

Amoxicillin, atropine, carbamazepine, dicloxacillin, digoxin, erythromycin, estradiol, furosemide, halothane, streptomycin, ticlopidine and lipopolysaccharide (LPS) were

purchased from Sigma-Aldrich (St. Louis, MO, USA). Allopurinol, azathioprine, diclofenac, diphenhydramine, flutamide, ibuprofen, imipramine, indomethacin, isoniazid, kanamycin, ketoconazole, metronidazole, nifedipine, phenobarbital, phenytoin, pioglitazone, sulfamethoxazole, troglitazone and valproic acid were purchased from Wako Pure Chemical (Osaka, Japan). Primidone was purchased from the Tokyo Chemical Industry (Tokyo, Japan). Primers were commercially synthesized at Life Technologies (Carlsbad, CA, USA). HaCaT cells were purchased from CLS Cell Lines Service (Eppelheim, Germany). CnT-Prime (CnT-PR) Epithelial Culture Medium and CnT-Prime 2D Diff (CnT-PR-D) Epithelial Culture Medium were from CELLnTEC Advanced Systems (Bern, Switzerland). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

HaCaT cell cultures and treatment

HaCaT cells, immortalized human keratinocytes, were grown in Dulbecco's modified Eagle's medium supplemented with 1 mM sodium pyruvate, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 0.1 mM non-essential amino acids, 2 mM Gluta-Max™-I and 10% fetal bovine serum with 5% CO₂ at 37°C.

HaCaT cells proliferate rapidly. To avoid overconfluence of HaCaT cells, the cells were first seeded into six-well plates at 1 × 10⁵ cells well⁻¹ and treated with drugs for 48 and 6 h when the cell confluence reached 50% and 80%, respectively. We followed a method that was previously published (Choi *et al.*, 2010). Drugs were dissolved in dimethyl sulfoxide (DMSO) and the final concentration in the medium of DMSO was less than 0.1%.

The drug concentration was selected according to a previous study by O'Brien *et al.* (2006), in which 80% of drugs with human toxicity potential were identified as hepatotoxicants when they treated HepG2 cells with drugs at 100 µM.

Animals

Acyl glucuronides, which are formed by UDP-glucuronosyltransferases (UGTs), are one of the reactive metabolites associated with drug-induced toxicity (Mitsugi *et al.*, 2016; Usui *et al.*, 2009). To overcome species differences in UGT functions (Kutsuno *et al.*, 2013, 2014), humanized *UGT1* mice were used in the present study. *Tg(UGT1A1*28)Ugt1^{-/-}(hUGT1)* mice were developed previously in a C57BL/6 background (Fujiwara *et al.*, 2010, 2012). The mice were housed under controlled conditions (22 ± 2°C; 55 ± 5% relative humidity; 12/12 h light/dark cycle) and received food and water ad libitum. Mouse handling and experimental procedures were conducted in accordance with the animal care protocol approved by Kitasato University.

Newborn mice (less than 3 days old) were injected subcutaneously with LPS (0.3 or 3.0 mg kg⁻¹).

Primary mouse keratinocyte isolation and treatment

Newborn mice were anesthetized by diethyl ether inhalation. Isolated skin was rinsed with 1× phosphate-buffered saline and incubated overnight (15–18 h) in CnT-PR medium containing 5 mg ml⁻¹ dispase (Wako Pure Chemical), 500 U ml⁻¹ penicillin and 500 µg

ml⁻¹ streptomycin at 4°C. After the epidermis was separated from the dermis, keratinocytes were dissociated from epidermis by incubating them in a trypsin solution for 10 min at room temperature. Mouse primary keratinocytes were collected by centrifugation and were cultured in CnT-PR medium containing 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin with 5% CO₂ at 37°C. The culture medium was changed every 48 h.

Primary pooled mouse keratinocytes ($n = 3$) were seeded into six-well plates at 5×10^5 cells well⁻¹. After 48 or 90 h incubation, the culture medium was changed to CnT-PR-D medium containing drugs and the cells were treated for 48 and 6 h, respectively, with drugs. All of the tested drugs were dissolved in DMSO and their final concentration in the medium was less than 0.1%.

Keratinocytes were also collected from mice at higher risk of developing DILI. The skin was collected 6 h after the LPS administration.

Quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated from control and drug-treated cells using TRIzol (Invitrogen, Carlsbad, CA, USA). The complementary DNA (cDNA) was synthesized from total RNA using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo), and the reactions were run in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primer sequences are shown in Table 1. After an initial denaturation at 95°C for 1 min, amplification was performed by denaturation at 95°C for 5 s, annealing at 60°C for 15 s and extension at 72°C for 30 s for 40 cycles. mRNA expression levels in HaCaT cells and mouse keratinocytes were normalized with human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and mouse cyclophilin (CPH) mRNA level, respectively. C_t method was used to normalize gene expression (Livak & Schmittgen, 2001). GAPDH and CPH were measured separately with each drug concentration.

Statistical analysis

Data are presented as mean ($\pm$ SD). Statistical analyses were performed using one-way analysis of variance, followed by Dunnett's or Games–Howell *post-hoc* test. The Jonckheere–Terpstra trend test was used to test the association between DILI risk and IL-1 β expression levels in keratinocytes. $P < 0.05$ was considered statistically significant.

Results

mRNA expression of S100A8, S100A9, NALP3, interleukin-1 β and receptor for advanced glycation end products in diclofenac-, flutamide- and ketoconazole-treated human keratinocytes

It was reported that S100A8, S100A9, NALP3, IL-1 β and RAGE were commonly induced in the liver with increased serum aspartate aminotransferase and alanine aminotransferase after administration of hepatotoxic drugs to mice (Yano *et al.*, 2014), suggesting that these genes are involved in the onset and progression of DILI. To investigate whether S100A8,

S100A9, NALP3, IL-1 β and RAGE are also involved in the onset and progression of drug-induced skin toxicity, mRNA expression levels of S100A8, S100A9, NALP3, IL-1 β and RAGE were examined in HaCaT cells treated with hepatotoxic diclofenac, ketoconazole and flutamide. As shown in the Supporting information Fig. S1, the expression of IL-1 β was significantly increased by 10 and 100 μ M diclofenac and 100 μ M flutamide in HaCaT cells. S100A8 mRNA was also induced approximately 2-fold by 10 and 100 μ M diclofenac and 100 μ M flutamide in HaCaT cells. NALP3 and RAGE were increased by 100 μ M diclofenac and 100 μ M flutamide. S100A9 expression levels remained unchanged by diclofenac, ketoconazole and flutamide. Increases of DILI-associated genes were observed in a concentration-dependent manner except for S100A9 when HaCaT cells were treated with drugs for 6 h. Figure 1 shows mRNA expression levels of S100A8, S100A9, NALP3, IL-1 β and RAGE in HaCaT cells after 48 h drug treatments. IL-1 β mRNA was increased by diclofenac in a concentration dependent manner (Fig. 1). Ketoconazole and flutamide also induced IL-1 β in a concentration-dependent manner. S100A8 was increased 1.5–2-fold by 10 μ M diclofenac and 100 μ M flutamide. S100A9 was induced by approximately 2-fold using 10 and 100 μ M diclofenac and 100 μ M flutamide. NALP3 expression levels in HaCaT cells were induced 7-fold by 100 μ M ketoconazole. In contrast, the 100 μ M diclofenac and 100 μ M ketoconazole treatment for 48 h reduced S100A8 expression. RAGE expression level did not change by any drug treatment. A concentration- and time-dependent analysis revealed that these hepatotoxic drugs highly induced NALP3 and IL-1 β in human keratinocytes when the cells were treated with 48 h (Fig. 1).

mRNA expression of NALP3 and interleukin-1 β in mouse primary keratinocytes treated with diclofenac, flutamide and ketoconazole

HaCaT cells are human-derived keratinocytes. To understand whether there is a species difference in the responsiveness to hepatotoxic drugs in keratinocytes, we treated pooled mouse keratinocytes ($n = 3$) with diclofenac, ketoconazole and flutamide for 6 and 48 h and determined NALP3 and IL-1 β mRNA levels, which were increased in a concentration-dependent manner in HaCaT cells (Fig. 1 and Supporting information Fig. S1). NALP3 expression was increased in mouse keratinocytes by treatments with 10 and 100 μ M ketoconazole for 6 h (Fig. 2B). IL-1 β was induced by approximately 2-fold by treatments with 10 μ M diclofenac and 100 μ M ketoconazole for 6 h. NALP3 was increased 2-fold and 3-fold by treatments with 100 μ M ketoconazole and flutamide for 48 h, respectively (Fig. 2B). No IL-1 β expression change was observed by diclofenac, ketoconazole and flutamide. These suggested that NALP3 and IL-1 β were more induced by treatment with hepatotoxic drugs for 6 h in mouse keratinocytes.

mRNA expression of S100A8, S100A9, NALP3, interleukin-1 β and receptor for advanced glycation end products in hepatotoxic drug-treated human keratinocytes

To investigate further whether S100A8, S100A9, NALP3, IL-1 β and RAGE can be involved in the onset and progression of drug-induced skin toxicity, we treated HaCaT cells with 24 hepatotoxic drugs for 48 h and measured mRNA expression levels of DILI-associated genes by qRT-PCR. We selected 24 hepatotoxic drugs according to the Liver Toxicity Knowledge Base Benchmark Dataset (<http://www.fda.gov/ScienceResearch/BioinformaticsTools>; Chen *et al.*, 2011). Based on drug labeling approved by the Food and Drug Administration, the

Liver Toxicity Knowledge Base Benchmark Dataset grouped drugs into three categories, i.e., drugs of most, less and no DILI concern. In this study, allopurinol, amoxicillin, azathioprine, carbamazepine, diclofenac, erythromycin, estradiol, flutamide, furosemide, ibuprofen, imipramine, indomethacin, isoniazid, ketoconazole, nifedipine, phenobarbital, phenytoin, pioglitazone, sulfamethoxazole, ticlopidine, troglitazone and valproic acid were used as hepatotoxic drugs with most and less DILI concern. Dicloxacillin and halothane were also used as hepatotoxic drugs due to their higher risk of DILI (Yano *et al.*, 2014). We calculated the sum of the relative expression levels of S100A8, S100A9, NALP3, IL-1 β and RAGE mRNA in HaCaT cells. Pioglitazone significantly induced S100A8 and S100A9, showing the highest total gene expression level (Fig. 3). Ketoconazole and diclofenac induced NALP3, exhibiting a higher total expression level. Troglitazone highly induced S100A8 and IL-1 β , resulting in a higher total expression level. Halothane and dicloxacillin induced S100A8, showing higher total expressions. Estradiol and phenobarbital slightly induced all DILI-associated genes, resulting in a higher total expression. IL-1 β was commonly induced by many drugs such as ketoconazole, flutamide, diclofenac and nifedipine, although RAGE was not remarkably increased by any drug treatment. Several hepatotoxic drugs including isoniazid, valproic acid and carbamazepine showed poor inducibility of the biomarkers in human keratinocytes, resulting in a low sum expression level. Expression levels of S100A8, S100A9, NALP3, IL-1 β and RAGE mRNA were also investigated in HaCaT cells treated with non-hepatotoxic drugs. Atropine, digoxin, diphenhydramine, kanamycin, metronidazole, primidone and streptomycin were used as non-hepatotoxic drugs with no DILI concern (Chen *et al.*, 2011). No induction of the biomarkers was observed by kanamycin, streptomycin, digoxin or metronidazole (Fig. 3). Primidone induced S100A8 and S100A9. Atropine and diphenhydramine slightly induced NALP3 and IL-1 β , respectively. Of the tested 24 hepatotoxic drugs, 16 showed more than five total expression levels, indicating that S100A8, S100A9, NALP3, IL-1 β and RAGE might be involved in the onset and progression of drug-induced skin toxicity. The data indicate that keratinocytes and hepatocytes might share similar responsiveness to hepatotoxic drugs. Calculated sensitivity and specificity were 70.8% and 57.1%, respectively, in our study (Table 2).

Interindividual variability in responsiveness to pioglitazone in keratinocytes

The fact that there are individuals who are likely to develop DILI suggests that there might be an interindividual variability in responsiveness to hepatotoxic drugs in the liver (Bleibel *et al.*, 2007). To examine interindividual variability in responsiveness to pioglitazone in keratinocytes, IL-1 β mRNA expression levels were determined in individual mouse keratinocytes that were treated with or without 100 μ M pioglitazone for 6 h. Among the thiazolidinediones, pioglitazone has less risk of hepatotoxicity than rosiglitazone and troglitazone (Tolman *et al.*, 2004). However, there are still many case reports of pioglitazone-induced liver injury (Table 3). Floyd *et al.* (2009) reported that the rate of acute liver failure by pioglitazone was higher than the background rate for idiopathic acute liver failure. We used pioglitazone as a test compound also because it showed the highest total expression levels of DILI-associated genes (Fig. 3). In this study, keratinocytes were derived from single mice and we did not pool these cells during culture. The IL-1 β mRNA basal expression in mouse no. 13 was 15 times higher than that in mouse no. 1 (Fig. 4). The IL-1 β mRNA basal expression in mouse no. 2 was seven times higher than that in mouse no. 1.

IL-1 β mRNA in mouse no. 1 was decreased to 50% by pioglitazone. Meanwhile, the IL-1 β expression level in mouse no. 9 was induced 8-fold by pioglitazone (Fig. 4). Thus, it was shown that there was a wide interindividual variability in the expression of IL-1 β ranging from 1- to 7-fold in keratinocytes. In addition, a wide interindividual variability in the inducibility of IL-1 β by pioglitazone was also observed, ranging from 0.5- to 8-fold.

mRNA expression of interleukin-1 β in keratinocytes from the lipopolysaccharide-injected mouse

It has been reported that LPS enhanced the liver damage produced by hepatotoxic drugs such as diclofenac (Deng *et al.*, 2006), ranitidine (Luyendyk *et al.*, 2003) and trovafloxacin (Shaw *et al.*, 2007) in rodents. In this study, low-, medium- and high-risk mice were established by treating mice with different doses of LPS. Mice at medium and high risk for DILI were prepared by injection of 0.3 and 3 mg kg⁻¹ LPS, respectively. Control mice were used as low-risk models. Pooled keratinocytes ($n = 3$) were obtained from low-, medium- and high-risk mice, respectively. We measured IL-1 β basal expression levels in the three different groups. The IL-1 β basal expression level in medium-risk mice was similar to that in low-risk mice (Fig. 5A). The IL-1 β expression level in high-risk mice was seven times higher than that in low-risk mice. The IL-1 β expression level was not changed by pioglitazone in the low-risk mice (Fig. 5A). In contrast, IL-1 β expressions in medium- and high-risk mice were induced 7- and 3.5-fold by pioglitazone, respectively. These results indicated that keratinocytes in mice at higher risk for DILI exhibited higher IL-1 β basal expression levels and inducibility of IL-1 β by pioglitazone. We calculated the sum of IL-1 β basal expression levels and induction rates (Fig. 5B). The sum was increased in accordance with the potential risk for DILI ($P < 0.05$), suggesting that the sum of the IL-1 β expression levels and induction rate in keratinocytes can be used for the DILI prediction.

To evaluate whether the sum of IL-1 β expression levels and induction rates in keratinocytes can identify an individual that can possibly develop DILI, we calculated the sum of IL-1 β basal expression levels and induction rates in individual mice at low ($n = 4$), medium ($n = 4$) and high risk ($n = 4$). Blinding was not performed because gene expression analysis was a quantitative method. High-risk mice exhibited higher sums (Fig. 6). On the other hand, low-risk mice showed lower sums. The Jonckheere-Terpstra test revealed a significant trend toward higher risk for DILI with increasing the sum of IL-1 β basal expression levels and induction rate in keratinocytes. ($P < 0.01$). This suggests that the sum of DILI-associated gene basal expression levels and induction rates in keratinocytes might be a tool to identify individuals at higher risk for DILI.

Identification of genes commonly increased by hepatotoxic drugs in liver

In human keratinocytes, S100A8, S100A9, NALP3, IL-1 β and RAGE were not increased by hepatotoxic sulfamethoxazole, carbamazepine, allopurinol, azathioprine, valproic acid, furosemide and isoniazid (Fig. 3). This finding indicates that it might be difficult to predict liver injury caused by these drugs by determining expression levels of S100A8, S100A9, NALP3, IL-1 β and RAGE in keratinocytes. To identify genes that are commonly increased in hepatocytes and keratinocytes by hepatotoxic drugs, we conducted re-analysis of microarray data from Open TG-GATES (<http://toxico.nibiohn.go.jp>) (Uehara *et al.*, 2010).

We analyzed gene expression changes in the rat livers that were treated with allopurinol, azathioprine, carbamazepine, furosemide, isoniazid and valproic acid for 28 days. We identified six genes that were commonly increased in the liver of rats treated with these drugs (Table 4). Glutathione *S*-transferase alpha (Gsta) in the rat livers was increased by allopurinol, carbamazepine, furosemide and valproic acid. Sulfotransferase family 2A, dehydroepiandrosterone-preferring, member 6 (Sult2a6) and Cd36 were increased in the liver of rats treated with allopurinol, azathioprine, carbamazepine and valproic acid. Serine peptidase inhibitor, Kazal type 3 (Spink3) was induced by azathioprine, furosemide and valproic acid administration. Carbonic anhydrase 2 (Ca2) in the rat livers was slightly increased by azathioprine and furosemide. Administration of allopurinol, azathioprine, carbamazepine, furosemide and valproic acid to rats highly induced aldehyde dehydrogenase family 1, member A1 (Aldh1a1) in the liver.

Azathioprine is one of the hepatotoxic drugs that did not induce S100A8, S100A9, NALP3, IL-1 β and RAGE in human keratinocytes (Fig. 3). To investigate whether GST α , SULT2A1 (rat Sult2a6), CD36, SPINK1 (rat Spink3), CA2 and ALDH1A1 can be induced by azathioprine in human keratinocytes, we measured expression levels of those genes in HaCaT cells that were treated with 100 μ M azathioprine for 48 h. GST α , CD36, CA2 and ALDH1A1 were not significantly increased by azathioprine (Supporting information Fig. S2). SULT2A1 in HaCaT cells was slightly increased by azathioprine. Meanwhile, SPINK1 was increased 13-fold by azathioprine, suggesting that SPINK1 might be useful for identifying individuals at higher risk for azathioprine-induced liver injury.

Discussion

Previously, it was demonstrated that hepatotoxic drugs induced the expressions of S100A8, S100A9, NALP3, IL-1 β and RAGE in the liver (Yano *et al.*, 2014). However, it was unknown whether these genes were also involved in hepatotoxic drug-induced skin toxicity. In this study, we demonstrated that those genes were induced by hepatotoxic drugs even in keratinocytes (Figs 1–3). It has been reported that S100A8 and S100A9 can bind to TLR4 and RAGE, leading to activation of nuclear factor- κ B and mitogen-activated protein kinase signaling pathways in both hepatocytes and keratinocytes (Ehrchen *et al.*, 2009; Liu *et al.*, 2002). Activation of these pathways can result in the release of cytokines such as IL-1 β and IL-18 (Barker *et al.*, 2011), which play an important role in the inflammatory response. Meanwhile, NALP3 has been reported to activate caspase-1, which is involved in the promotion of tumor necrosis factor α - and IL-1 β -associated inflammatory responses (Martinon *et al.*, 2006; Willingham *et al.*, 2009). Therefore, induction of S100A8, S100A9, NALP3, IL-1 β and RAGE can be the underlying mechanism of the same responsiveness to hepatotoxic drugs in hepatocytes and keratinocytes.

HaCaT cells might not be the best models reflecting human physiology. However, as observed in primary human keratinocytes, IL-1 β secretion via NALP3 was demonstrated in HaCaT cells (Cho *et al.*, 2012; Dai *et al.*, 2011). Furthermore, HaCaT cells express functional TLRs and RAGE (Dejean *et al.*, 2012; Pivarcsi *et al.*, 2003), which S100A8 and S100A9 bind to. These suggest that HaCaT cells can be used in evaluating the induction of

S100A8, S100A9, NALP3, IL-1 β and RAGE. Our data were also reproducible in primary mouse keratinocytes.

Many new *in vitro* models have been developed based on DILI mechanisms to screen compounds that have a higher risk of developing DILI. However, a number of DILI cases including poor outcome have still been reported (Björnsson *et al.*, 2013; Hayashi *et al.*, 2015; Robles-Diaz *et al.*, 2014). Bleibel *et al.* (2007) reported that certain people are extremely susceptible to developing DILI. Previous studies reported that gene expression analysis in the liver was useful for identification of such individuals who have a higher risk for DILI (Yun *et al.*, 2009, 2010). However, obtaining liver tissue samples in patients is an invasive technique and involves risks including bleeding, pain, infection and even death (Firpi *et al.*, 2005; Forssell *et al.*, 1981). Therefore, an alternative less-invasive method is needed to predict individuals who can possibly develop DILI less invasively. Skin tests have been extensively used for the prediction and diagnosis of ADRs and allergy. Its utility has been widely accepted in terms of safety and convenience (Barbaud *et al.*, 2013; Fatteh *et al.*, 2014). It was demonstrated in the present study that the basal expression and induction rates of IL-1 β in keratinocytes could be a tool that is useful for the identification of individuals at higher risk for DILI (Fig. 6). Application of drugs to the skin followed by isolation of keratinocytes from skin surface by tape stripping could be a method for the skin-based prediction of DILI.

Although S100A8, S100A9, NALP3, IL-1 β and RAGE were induced by most hepatotoxic drugs, these were not increased by certain hepatotoxic drugs including azathioprine (Fig. 3). This indicates that it might be difficult to predict such DILI by just determining expression levels of S100A8, S100A9, NALP3, IL-1 β and RAGE in keratinocytes. In the present study, the re-analysis of microarray data showed that hepatic expressions of Gsta, Sult2a6, Cd36, Ca2, Spink3 and Aldh1a1 were commonly induced by hepatotoxic drugs that did not induce S100A8, S100A9, NALP3, IL-1 β and RAGE in keratinocytes (Table 4). Interestingly, SPINK1 was significantly induced by azathioprine in human keratinocytes (Supporting information Fig. S2), suggesting that SPINK1 could be a marker gene that can identify individuals at higher risk for azathioprine-induced liver injury. Although each DILI could be predictable by determining the expression of each marker gene in keratinocytes, there might be a useful marker gene(s) that can predict any types of DILI.

The present study demonstrated that S100A8, S100A9, NALP3, IL-1 β and RAGE were induced by most hepatotoxic drugs in keratinocytes. Furthermore, we showed that mice at higher risk of DILI exhibited a higher basal expression and induction of the DILI-associated genes in keratinocytes. Therefore, we might be able to identify individuals less invasively at higher risk of developing DILI by examining gene expression patterns in keratinocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Asakura M, Karaki F, Fujii H, Atsuda K, Itoh T, Fujiwara R. Vildagliptin and its metabolite M20.7 induce the expression of S100A8 and S100A9 in human hepatoma HepG2 and leukemia HL-60 cells. *Sci Rep*. 2016; 6:35633. [PubMed: 27759084]
- Barbaud A, Collet E, Milpied B, Assier H, Staumont D, Avenel-Audran M, Grange A, Amarger S, Girardin P, Guinnee MT, Truchetet F, Lasek A, Waton J. A multicentre study to determine the value and safety of drug patch tests for the three main classes of severe cutaneous adverse drug reactions. *Br J Dermatol*. 2013; 168:555–562. [PubMed: 23136927]
- Barker BR, Taxman DJ, Ting JP. Cross-regulation between the IL-1 β /IL-18 processing inflammasome and other inflammatory cytokines. *Curr Opin Immunol*. 2011; 23:591–597. [PubMed: 21839623]
- Björnsson ES, Bergmann OM, Björnsson HK, Kvaran RB, Olafsson S. Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. *Gastroenterology*. 2013; 144:1419–1425. [PubMed: 23419359]
- Bleibel W, Kim S, D'Silva K, Lemmer ER. Drug-induced liver injury. *Dig Dis Sci*. 2007; 52:2463–2471. [PubMed: 17805971]
- Chase MP, Yarze JC. Pioglitazone-associated fulminant hepatic failure. *Am J Gastroenterol*. 2002; 97:502–503. [PubMed: 11866308]
- Chen M, Vijay V, Shi Q, Liu Z, Fang H, Tong W. FDA-approved drug labeling for the study of drug-induced liver injury. *Drug Discov Today*. 2011; 16:697–703. [PubMed: 21624500]
- Cho KA, Suh JW, Lee KH, Kang JL, Woo SY. IL-17 and IL-22 enhance skin inflammation by stimulating the secretion of IL-1 β by keratinocytes via the ROS-NLRP3-caspase-1 pathway. *Int Immunol*. 2012; 24:147–158. [PubMed: 22207130]
- Choi BM, Oh GS, Lee JW, Mok JY, Kim DK, Jeong SI, Jang SI. Prenylated chalcone from *Sophora flavescens* suppresses Th2 chemokine expression induced by cytokines via heme oxygenase-1 in human keratinocytes. *Arch Pharm Res*. 2010; 33:753–760. [PubMed: 20512474]
- Dai X, Sayama K, Tohyama M, Shirakata Y, Hanakawa Y, Tokumaru S, Yang L, Hirakawa S, Hashimoto K. Mite allergen is a danger signal for the skin via activation of inflammasome in keratinocytes. *J Allergy Clin Immunol*. 2011; 127:806–814. [PubMed: 21272927]
- Dejean E, Foisseau M, Lagarrigue F, Lamant L, Prade N, Marfak A, Delsol G, Giuriato S, Gaits-Iacovoni F, Meggetto F. ALK + ALCLs induce cutaneous, HMGB-1-dependent IL-8/CXCL8 production by keratinocytes through NF- κ B activation. *Blood*. 2012; 119:4698–4707. [PubMed: 22394598]
- Deng X, Stachlewitz RF, Liguori MJ, Blomme EA, Waring JF, Luyendyk JP, Maddox JF, Ganey PE, Roth RA. Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. *J Pharmacol Exp Ther*. 2006; 319:1191–1199. [PubMed: 16990511]
- Devarbhavi H, Devarbhavi H, Raj S, Aradya VH, Rangegowda VT, Veeranna GP, Singh R, Reddy V, Patil M. Drug-induced liver injury associated with Stevens-Johnson syndrome/toxic epidermal necrolysis: Patient characteristics, causes, and outcome in 36 cases. *Hepatology*. 2016; 63:993–999. [PubMed: 26439084]
- Ehrchen JM, Sunderkötter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J Leukoc Biol*. 2009; 86:557–566. [PubMed: 19451397]
- Farley-Hills E, Sivasankar R, Martin M. Fatal liver failure associated with pioglitazone. *B M J*. 2004; 329:429.
- Farmer AD, Brind A. Drug-induced liver injury. *Medicine*. 2011; 39:536–540.
- Fatfeh S, Rekkerth DJ, Hadley JA. Skin prick/puncture testing in North America: a call for standards and consistency. *Allergy Asthma Clin Immunol*. 2014; 10:44. [PubMed: 25221603]

- Firpi RJ, Soldevila-Pico C, Abdelmalek MF, Morelli G, Judah J, Nelson DR. Short recovery time after percutaneous liver biopsy: should we change our current practices? *Clin Gastroenterol Hepatol*. 2005; 3:926–929. [PubMed: 16234032]
- Floyd JS, Barbehenn E, Lurie P, Wolfe SM. Case series of liver failure associated with rosiglitazone and pioglitazone. *Pharmacoepidemiol Drug Saf*. 2009; 18:1238–1243. [PubMed: 19623674]
- Forssell PL, Bonkowsky HL, Anderson PB, Howell DA. Intrahepatic hematoma after aspiration liver biopsy. A prospective randomized trial using two different needles. *Dig Dis Sci*. 1981; 26:631–635. [PubMed: 7249899]
- Fujiwara R, Nguyen N, Chen S, Tukey RH. Developmental hyperbilirubinemia and CNS toxicity in mice humanized with the UDP glucuronosyltransferase 1 (UGT1) locus. *Proc Natl Acad Sci U S A*. 2010; 107:5024–5029. [PubMed: 20194756]
- Fujiwara R, Chen S, Karin M, Tukey RH. Reduced expression of UGT1A1 in intestines of humanized UGT1 mice via inactivation of NF- κ B leads to hyperbilirubinemia. *Gastroenterology*. 2012; 142:109–118. [PubMed: 21983082]
- Hayashi PH, Fontana RJ, Chalasani NP, Stolz AA, Talwalkar JA, Navarro VJ, Lee WM, Davern TJ, Kleiner DE, Gu J, Hoofnagle JH, for the US Drug-Induced Liver Injury Network Investigators. Under-reporting and poor adherence to monitoring guidelines for severe cases of isoniazid hepatotoxicity. *Clin Gastroenterol Hepatol*. 2015; 13:1676–1682. [PubMed: 25724701]
- Hoek JB, Pastorino JG. Ethanol, oxidative stress, and cytokine-induced liver cell injury. *Alcohol*. 2002; 27:63–68. [PubMed: 12062639]
- Holt MP, Ju C. Mechanisms of drug-induced liver injury. *AAPS J*. 2006; 8:48–54.
- Kutsuno Y, Sumida K, Itoh T, Tukey RH, Fujiwara R. Glucuronidation of drugs in humanized UDP-glucuronosyltransferase 1 mice: Similarity with glucuronidation in human liver microsomes. *Pharmacol Res Perspect*. 2013; 1:1–11.e00002.
- Kutsuno Y, Itoh T, Tukey RH, Fujiwara R. Glucuronidation of drugs and drug-induced toxicity in humanized UDP-glucuronosyltransferase 1 mice. *Drug Metab Dispos*. 2014; 42:1146–1152. [PubMed: 24764149]
- Lebre MC, van der Aar AM, van Baarsen L, van Capel TM, Schuitemaker JH, Kapsenberg ML, de Jong EC. Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. *J Invest Dermatol*. 2007; 127:331–341. [PubMed: 17068485]
- Liu S, Gallo DJ, Green AM, Williams DL, Gong X, Shapiro RA, Gambotto AA, Humphris EL, Vodovotz Y, Billiar TR. Role of Toll-like receptors in changes in gene expression and NF- κ B activation in mouse hepatocytes stimulated with lipopolysaccharide. *Infect Immun*. 2002; 70:3433–3442. [PubMed: 12065483]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ Ct method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol*. 2005; 5:331–342. [PubMed: 15803152]
- Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, Devera ME, Liang X, Tör M, Billiar T. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunol Rev*. 2007; 220:60–81. [PubMed: 17979840]
- Luyendyk JP, Maddox JF, Cosma GN, Ganey PE, Cockerell GL, Roth RA. Ranitidine treatment during a modest inflammatory response precipitates idiosyncrasy-like liver injury in rats. *J Pharmacol Exp Ther*. 2003; 307:9–16. [PubMed: 12893837]
- Maeda K. Hepatocellular injury in a patient receiving pioglitazone. *Ann Intern Med*. 2001; 135:306.
- Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*. 2006; 440:237–241. [PubMed: 16407889]
- May LD, Lefkowitz JH, Kram MT, Rubin DE. Mixed hepatocellular-cholestatic liver injury after pioglitazone therapy. *Ann Intern Med*. 2002; 136:449–452. [PubMed: 11900497]
- Mitsugi R, Sumida K, Fujie Y, Tukey RH, Itoh T, Fujiwara R. Acyl-glucuronide as a possible cause of trovafloxacin-induced liver toxicity: Induction of chemokine (C-X-C Motif) ligand 2 by trovafloxacin acyl-glucuronide. *Biol Pharm Bull*. 2016; 39:1604–1610. [PubMed: 27725437]

- Nagasaka S, Abe T, Kawakami A, Kusaka I, Nakamura T, Ishikawa S, Saito T, Ishibashi S. Pioglitazone-induced hepatic injury in a patient previously receiving troglitazone with success. *Diabet Med.* 2002; 19:347–348. [PubMed: 11943013]
- Nakamura A, Nakajima M, Yamanaka H, Fujiwara R, Yokoi T. Expression of UGT1A and UGT2B mRNA in human normal tissues and various cell lines. *Drug Metab Dispos.* 2008; 36:1461–1464. [PubMed: 18480185]
- O'Brien PJ, Irwin W, Diaz D, Howard-Cofield E, Krejsa CM, Slaughter MR, Gao B, Kaludercic N, Angeline A, Bernardi P, Brain P, Hougham C. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. *Arch Toxicol.* 2006; 80:580–604. [PubMed: 16598496]
- Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. *Mediat Inflamm.* 2010; 2010 pii: 672395.
- Pinto AG, Cummings OW, Chalasani N. Severe but reversible cholestatic liver injury after pioglitazone therapy. *Ann Intern Med.* 2002; 137:857. [PubMed: 12435231]
- Pirmohamed M, Breckenridge AM, Kitteringham NR, Park BK. Adverse drug reactions. *B M J.* 1998; 316:1295–1298.
- Pivarcsi A, Bodai L, Réthi B, Kenderessy-Szabó A, Koreck A, Széll M, Beer Z, Bata-Csörgö Z, Magócsi M, Rajnavölgyi E, Dobozy A, Kemény L. Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. *Int Immunol.* 2003; 15:721–730. [PubMed: 12750356]
- Robles-Diaz M, Lucena MI, Kaplowitz N, Stephens C, Medina-Cáliz I, González-Jimenez A, Ulzurrun E, Gonzalez AF, Fernandez MC, Romero-Gómez M, Jimenez-Perez M, Bruguera M, Prieto M, Bessone F, Hernandez N, Arrese M, Andrade RJ. Use of Hy's law and a new composite algorithm to predict acute liver failure in patients with drug-induced liver injury. *Gastroenterology.* 2014; 147:109–118. [PubMed: 24704526]
- Rzany B, Correia O, Kelly JP, Naldi L, Auquier A, Stern R. Risk of Stevens-Johnson syndrome and toxic epidermal necrolysis during first weeks of antiepileptic therapy: a case-control study. *Lancet.* 1999; 353:2190–2194. [PubMed: 10392983]
- Sasaki E, Iida A, Oda S, Tsuneyama K, Fukami T, Nakajima M, Yokoi T. Pathogenetic analyses of carbamazepine-induced liver injury in F344 rats focused on immune- and inflammation-related factors. *Exp Toxicol Pathol.* 2016; 68:27–38. [PubMed: 26391595]
- Shaw PJ, Hopfensperger MJ, Ganey PE, Roth RA. Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor- α . *Toxicol Sci.* 2007; 100:259–266. [PubMed: 17709330]
- Shizuma T, Obata H, Hashimoto E, Hasegawa K, Hayashi N. Clinical analysis of drug-induced liver injury. *Kanzo.* 2002; 43:309–315.
- Takikawa H, Murata Y, Horiike N, Fukui H, Onji M. Drug-induced liver injury in Japan: An analysis of 1676 cases between 1997 and 2006. *Hepatol Res.* 2009; 39:427–431. [PubMed: 19207579]
- Tolman KG, Fonseca V, Tan MH, Dalpiaz A. Narrative review: hepatobiliary disease in type 2 diabetes mellitus. *Ann Intern Med.* 2004; 141:946–956. [PubMed: 15611492]
- Uehara T, Ono A, Maruyama T, Kato I, Yamada H, Ohno Y, Urushidani T. The Japanese toxicogenomics project: application of toxicogenomics. *Mol Nutr Food Res.* 2010; 54:218–227. [PubMed: 20041446]
- Usui T, Mise M, Hashizume T, Yabuki M, Komuro S. Evaluation of the potential for drug-induced liver injury based on in vitro covalent binding to human liver proteins. *Drug Metab Dispos.* 2009; 37:2383–2392. [PubMed: 19720731]
- Willingham SB, Allen IC, Bergstralh DT, Brickey WJ, Huang MT, Taxman DJ, Duncan JA, Ting JP. NLRP3 (NALP3, cryopyrin) facilitates in vivo caspase-1, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways. *J Immunol.* 2009; 183:2008–2015. [PubMed: 19587006]
- Yano A, Higuchi S, Tsuneyama K, Fukami T, Nakajima M, Yokoi T. Involvement of immune-related factors in diclofenac-induced acute liver injury in mice. *Toxicology.* 2012; 293:107–114. [PubMed: 22285467]

- Yano A, Oda S, Fukami T, Nakajima M, Yokoi T. Development of a cell-based assay system considering drug metabolism and immune- and inflammatory-related factors for the risk assessment of drug-induced liver injury. *Toxicol Lett.* 2014; 228:13–24. [PubMed: 24747151]
- Yun JW, Kim CW, Bae IH, Park YH, Chung JH, Lim KM, Kang KS. Determination of the key innate genes related to individual variation in carbon tetrachloride-induced hepatotoxicity using a pre-biopsy procedure. *Toxicol Appl Pharmacol.* 2009; 239:55–63. [PubMed: 19481104]
- Yun JW, Kim CW, Bae IH, Park YH, Chung JH, Lim KM, Kang KS. Expression levels of pituitary tumor transforming 1 and glutathione-S-transferase theta 3 are associated with the individual susceptibility to D-galactosamine-induced hepatotoxicity. *Toxicol Appl Pharmacol.* 2010; 242:91–99. [PubMed: 19800905]

Figure 1.

Concentration-dependent induction of S100A8, S100A9, NALP3, IL-1 β and RAGE in hepatotoxic drug-treated HaCaT cells. HaCaT cells were treated with diclofenac, ketoconazole and flutamide (10 and 100 μ M) for 48 h. mRNA expression levels of S100A8, S100A9, NALP3, IL-1 β and RAGE were measured by quantitative reverse transcription–polymerase chain reaction. Gene expressions were normalized with the GAPDH expression. Data are the means $\pm$ SD of three independent determinations. * P < 0.05 and ** P < 0.01 compared to the expression level in the vehicle-treated cells.

Figure 2. Concentration- and time-dependent induction of S100A8, S100A9, NALP3, IL-1 β and RAGE in hepatotoxic drug-treated mouse keratinocytes. Primary mouse keratinocytes were pooled ($n = 3$) and cultured for 48 h (A). Isolated keratinocytes were treated with 10 and 100 μ M diclofenac, ketoconazole and flutamide for 6 and 48 h (B). mRNA levels of NALP3 and IL-1 β were measured by quantitative reverse transcription–polymerase chain reaction. Expression was normalized with the cyclophilin mRNA expression. Data are the means $\pm$ SD of three independent determinations. * $P < 0.05$ and ** $P < 0.01$ compared to the expression level in the vehicle-treated cells.

Figure 3.

Induction of DILI-associated gene mRNA expressions in hepatotoxic drug-treated human keratinocytes. HaCaT cells were treated with hepatotoxic drugs (100 μ M) for 48 h. mRNA levels of S100A8, S100A9, NALP3, IL-1 β and RAGE were measured by quantitative reverse transcription–polymerase chain reaction. Sum of the relative expression levels of S100A8, S100A9, NALP3, IL-1 β and RAGE mRNA was calculated. mRNA expression levels were normalized to the level of GAPDH. Drugs shown with a red color exhibited more than 5 of the sum score. DILI, drug-induced liver injury.

Figure 4.

IL-1 β mRNA expression in pioglitazone-treated mouse keratinocytes. Mouse keratinocytes were isolated from 19 mice. Individual mouse keratinocytes were treated with 100 μ M PIO for 6 h and the IL-1 β mRNA expression levels were determined by quantitative reverse transcription–polymerase chain reaction. Expression was normalized with the cyclophilin mRNA expression. Data are the means $\pm$ SD. PIO, pioglitazone.

Figure 5.

IL-1 β mRNA expression in keratinocytes isolated from LPS-treated mice. LPS was administered to newborn mice (0.3 or 3 mg kg⁻¹, s.c.). Six hours after the treatment, skins were collected and the pooled keratinocytes ($n = 3$) were cultured. Keratinocytes were treated with 100 μ M PIO for 6 h and IL-1 β expression levels were measured by quantitative reverse transcription–polymerase chain reaction (A). Sums of IL-1 β basal expression levels and induction rates were calculated (B). Expression was normalized with the cyclophilin mRNA expression. Data are the means $\pm$ SD of three independent determinations. * $P < 0.05$ and ** $P < 0.01$ between the groups. LPS, lipopolysaccharide; PIO, pioglitazone.

Figure 6.

Sum of IL-1 β basal expression levels and induction rates in individual mouse keratinocytes. LPS was administered to newborn mice (0.3 or 3 mg kg⁻¹ in saline, s.c., $n = 4$). Six hours after the treatment, skins were collected and the individual keratinocytes were cultured. Keratinocytes were treated with 100 μ M pioglitazone for 6 h and IL-1 β expression levels were measured by quantitative reverse transcription–polymerase chain reaction. The sum of IL-1 β basal expression levels and induction rates in keratinocytes were calculated. Expression was normalized with that of cyclophilin. Data are the means. LPS, lipopolysaccharide.

Table 1

Primer sequences used for real-time reverse transcription–polymerase chain reaction analyses

Species	Gene	F5'-3'/R5'-3'	Sequence	Reference
Human	S100A8	F	GCC GTC TAC AGG GAT GAC CT	Asakura <i>et al.</i> , 2016
		R	CCA CGC CCA TCT TTA TCA CCA	Asakura <i>et al.</i> , 2016
	S100A9	F	GCA GCT GGA ACG CAA CAT AG	Asakura <i>et al.</i> , 2016
		R	TGT GTC CAG GTC CTC CAT GA	Asakura <i>et al.</i> , 2016
	NALP3	F	CCA CGC TAA TGA TCG ACT TC	Yano <i>et al.</i> , 2014
		R	GTA AAC CCA TCC ACT CCT CT	Yano <i>et al.</i> , 2014
	IL-1 β	F	GCT GAT GGC CCT AAA CAG ATG	Yano <i>et al.</i> , 2014
		R	TTC TCC TGG AAG GTC TGT GG	Yano <i>et al.</i> , 2014
	RAGE	F	GGC TGG AAT GGA AAC TGA AC	Yano <i>et al.</i> , 2014
		R	TGG TCT CCT TTC CAT TCC TG	Yano <i>et al.</i> , 2014
	GAPDH	F	CCA GGG CTG CTT TTA ACT C	Nakamura <i>et al.</i> , 2008
		R	GCT CCC CCC TGC AAA TGA	Nakamura <i>et al.</i> , 2008
	GST α	F	GAA GCC CAA GCT CCA CTA CT	–
		R	TGG CAC TTG CTG GAA CAT CA	–
	SULT2A1	F	TCA CTT CAC AGT GGC CCA AG	–
		R	TGG GAA CAG CTC TCG AGG AA	–
	CD36	F	ACG CTG AGG ACA ACA CAG TC	–
		R	GCC ACA GCC AGA TTG AGA AC	–
	SPINK1	F	CCT TGG CCC TGT TGA GTC TA	–
		R	TCA GTC CCA CAG ACA GGG T	–
Mouse	CA2	F	CTG GGG TTC ACT TGA TGG ACA	–
		R	TTT AGC GCT GCC AAC CTT C	–
	ALDH1A1	F	CCT TGG CCC TGT TGA GTC TA	–
		R	TCA GTC CCA CAG ACA GGG T	–
	NALP3	F	AGC CTT CCA GGA TCC TCT TC	Yano <i>et al.</i> , 2014
		R	CTT GGG CAG CAG TTT CTT TC	Yano <i>et al.</i> , 2014
	IL-1 β	F	GTT GAC GGA CCC CAA AAG AT	Yano <i>et al.</i> , 2014
		R	CAC ACA CCA GCA GGT TAT CA	Yano <i>et al.</i> , 2014
	CPH	F	CAG ACG CCA CTG TCG CTT T	Fujiwara <i>et al.</i> , 2012
		R	TGT CTT TGG AAC TTT GTC TGC AA	Fujiwara <i>et al.</i> , 2012

Table 2

Sensitivity and specificity of total drug-induced liver injury-associated gene expression levels in keratinocytes treated with drugs

	Hepatotoxic drugs	Non-hepatotoxic drugs
Total expression levels >5	17	3
Total expression levels ≤ 5	7	4
Accuracy	Sensitivity 70.8%	Specificity 57.1%

Table 3

Case reports of pioglitazone-induced liver injury

Age	Gender	Observations	Reference
67	Male	Elevated AST, ALT, ALP and γ -GTP	Maeda, 2001
78	Male	Elevated bilirubin, AST and ALT/Granular eosinophilic cytoplasm and marked canalicular cholestasis with intact bile ducts	Chase & Yarze, 2002
49	Male	Elevated total bilirubin, AST, ALT and ALP/Mild centrilobular cholestasis in hepatocytes and bile canaliculi	May <i>et al.</i> , 2002
43	Female	Elevated AST, ALT and LDH	Nagasaka <i>et al.</i> , 2002
49	Female	Elevated bilirubin levels/Inflammatory cell infiltrate and marked bile ductular proliferation	Pinto <i>et al.</i> , 2002
63	Male	Elevated bilirubin, AST, ALT and ALP/Liver parenchymal damage	Farley-Hills <i>et al.</i> , 2004

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; LDH, lactate dehydrogenase; γ -GTP, γ -glutamyltransferase.

Table 4

Genes upregulated (2-fold) in liver of rats administered with hepatotoxic drugs

Genes	Fold change					
	Alopurinol	Azathioprine	Carbamazepine	Furosemide	Isoniazid	Valproic acid
Gsta	12.3	NU	11.0	5.2	NU	3.4
Sult2a6	16.3	9.1	4.9	NU	NU	NU
Cd36	10.3	2.3	2.7	NU	NU	2.6
Spink3	NU	3.7	NU	6.6	NU	2.4
Ca2	NU	3.6	NU	2.3	NU	NU
Aldh1a1	9.3	14.1	9.5	4.5	NU	9.9

Aldh1a1, aldehyde dehydrogenase family 1, member A1; Ca2, carbonic anhydrase 2; Gsta, glutathione *S*-transferase alpha; NU, not upregulated; Spink3, serine peptidase inhibitor; Kazal type3; Sult2a6, sulfotransferase family 2A, dehydroepiandrosterone-preferring, member 6.