REVIEW

Current and emerging biomarkers of hepatotoxicity

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Abstract: Drug-induced liver injury (DILI) is of great concern to human health. Generally, liver function and injury is evaluated based upon clinical signs, a select group of serum clinical biomarkers, and occasionally liver biopsies. While alanine aminotransferase, the most commonly used biomarker of hepatocellular injury, is a sensitive marker of liver injury, it is not necessarily specific for liver injury. Furthermore, alanine aminotransferase levels may not always correlate with the extent of injury. Therefore, new hepatotoxicity biomarkers are needed that are more predictive and specific indicators of liver injury and altered function. In addition, no current biomarker provides prognostic information about ultimate outcome once injury occurs, and any new biomarker filling this need is desperately needed. The omics technologies, including genomics, proteomics, and metabolomics, are being used in preclinical animal studies as well as clinical studies to evaluate markers of hepatotoxicity in easily obtained biofluids, such as urine and serum. Recently, the evaluation of circulating microRNAs in urine and blood has also shown promise for the identification of novel, sensitive markers of liver injury. This review evaluates the current status of proposed biomarkers of hepatotoxicity from the omics platforms, as well as from analysis of microRNAs. A brief description of the qualification of proposed biomarkers is also given.

Keywords: biomarkers, hepatotoxicity, metabolomics, microRNA, proteomics, transcriptomics

Introduction

Drug-induced liver injury (DILI) has been established as a major cause of acute liver failure in the United States. Navarro and Senior[1] reported that up to 10% of DILI patients will develop jaundice and die. DILI further represents a major reason why use of an approved drug is restricted or removed from the market.[1–3] Additionally, many preclinical compounds fail somewhere along the drug development pipeline due to drug-induced toxicity, with half of the compounds removed due to hepatotoxicity, including necrosis, steatosis, cholestasis, proliferation, inflammation, and bile duct hyperplasia.[4] A major limitation of the traditional serum clinical biomarkers used to evaluate hepatotoxicity, including alanine aminotransferase and aspartate aminotransferase, is that they are generally sensitive but not necessarily specific for the target organ.[4] Alanine aminotransferase is highly abundant in the cytosol of hepatocytes and, therefore, more liver-specific than aspartate aminotransferase, which is also abundant in blood, skeletal muscle, and heart.[5] However, increased transaminase levels may reflect processes other than liver injury, including muscle injury, pancreatitis, and others.[6] Other commonly measured serum biomarkers of liver injury including lactate dehydrogenase, alkaline

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phosphatase, and γ-glutamyl transpeptidase, can also be elevated due to processes other than liver injury.[6] Bilirubin is fairly specific for liver injury, but is not very sensitive given that levels increase significantly only after the liver has lost approximately half of its excretory capacity.[6]

There is a strong need for new biomarkers that can identify potential hepatotoxicity prior to the development of clinical signs of DILI, which typically develop only after significant injury has occurred. Furthermore, there is a need to find biomarkers that predict a person's potential sensitivity to liver injury and ones that are prognostic about the course of injury and whether the person will adapt to the insult or exhibit liver failure. Ozer et al[4] described the ideal attributes of a hepatotoxicity biomarker, including: organ specificity; a strong correlation with histopathology; out performance of serum transaminases or ability to provide additional information about liver toxicity when measured with alanine and/or aspartate aminotransferase; adaptability of screening assays to high throughput, commercially available platforms; found in easily collected biofluids such as serum or urine; and translation from preclinical species to humans. The omics technologies, including genomics, proteomics, and metabolomics, have shown promise in the area of biomarker development. Figure 1 shows the progression from altered gene expression to downstream metabolite production, and describes the challenges associated with each omics platform.[3,7–9]

The changes from the gene expression level down to the metabolite level occur on different, timescales and do not necessarily have a one-to-one correlation between a particular transcript and metabolite as an example. Genomics encompasses a variety of different assessments, including mRNA transcript changes, DNA methylation patterns, single nucleotide polymorphisms, and microRNA (miRNA) levels. Proteomics evaluates time-dependent and dose-dependent changes in proteins, and metabolomics evaluates changes in endogenous and xenobiotic metabolites. Omics changes can be assessed directly in tissue or in biofluids, such as blood and urine. For biomarker development, it is advantageous to develop biomarkers in biofluids because they can be easily obtained in both preclinical and clinical settings, making it feasible to evaluate a marker in a longitudinal manner. Temporal information on change in a biomarker may provide additional insight into disease severity and outcome. A systems biology approach that employs multiple omics platforms is advantageous because it can identify affected pathways and provide a context for the identified biomarker. However, in a systems approach, a longitudinal study is necessary since the metabolite changes are generally downstream of the transcripts and proteins; the metabolite profile is representative of the phenotype encoding both the genotype and environmental factors.[10] Given that the omics platforms have been reviewed elsewhere,[11–14] this
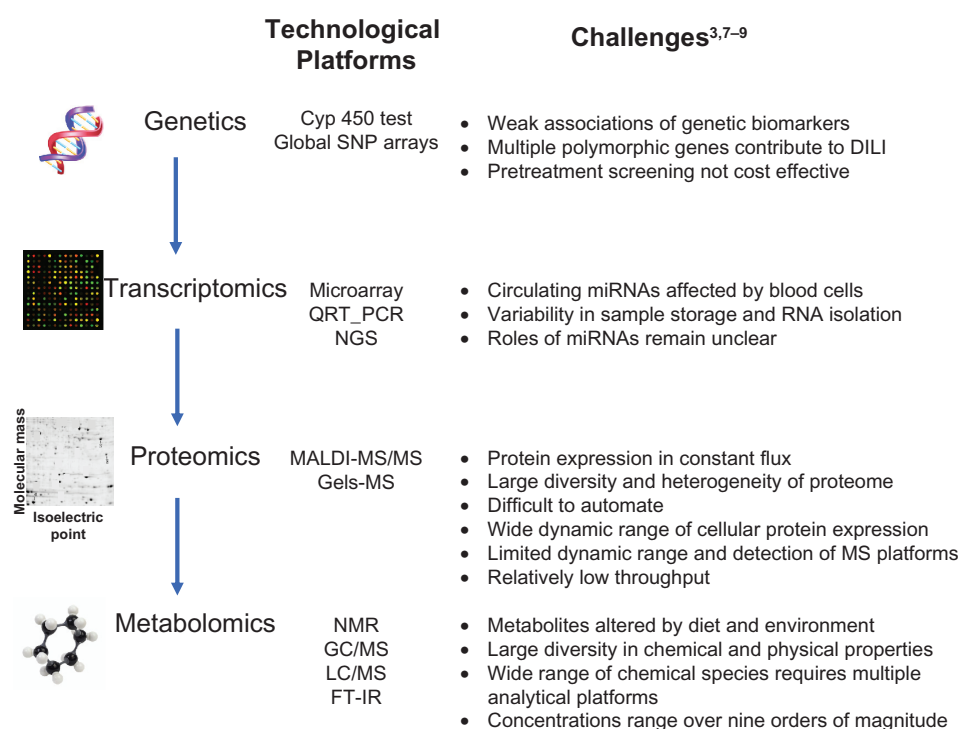

Figure 1 Progression of the omics markers from altered gene expression to downstream metabolite production and challenges with the associated platforms.

review focuses on the status of recently reported potential hepatotoxicity biomarkers.

Serum clinical chemistry biomarkers

Current serum markers

Table 1 lists the commonly measured serum markers in pre-clinical screening for hepatotoxicity. Assays generally evaluate serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and γ-glutamyl transpeptidase activity, as well as total bilirubin and/or bile acids. As noted in Table 1, these markers are not necessarily specific for liver injury and some are more sensitive than others. Alanine aminotransferase is widely considered to be a serum biomarker of hepatocellular injury, but can be elevated due to injury to heart tissue or skeletal muscle, or in response to some medications that do not cause liver injury. Therefore, other serum markers are needed that can be measured alone or in conjunction with alanine aminotransferase to evaluate hepatic injury and liver function better. As noted in Table 1, many of the current markers are potentially elevated by nonhepatic injury. As such, there is a need to identify more liver-specific biomarkers.

Emerging serum markers

A variety of different biomarkers based on serum protein levels has been investigated for assessing liver injury. Most of these biomarkers are based on the concept of leakage of the target protein from damaged hepatocytes into the circulation, with corresponding increases in serum protein levels and activity.[15] However, all of these biomarkers are at early stages of development and none of them has been adequately qualified for preclinical or clinical use at this time. Recent studies have suggested that measuring the alanine aminotransferase isozymes, ie, ALT1 and ALT2, may aid in differentiating the source of injury.[15] ALT1 has been noted to be localized in human hepatocytes, renal tubular epithelial cells, and in salivary gland epithelial cells. ALT2, on the other hand, is localized to the human adrenal gland cortex, neuronal cell bodies, cardiac myocytes, skeletal muscle fibers, and the endocrine pancreas.[15] Other emerging serum biomarkers include sorbitol dehydrogenase, glutamate dehydrogenase, serum F protein, glutathione-*S*-transferase alpha, and arginase I.[4] Sorbitol dehydrogenase is primarily located in the cytoplasm and mitochondria of the liver, kidney, and seminal vesicles, and is a marker of acute hepatocellular injury in rodents. Glutamate dehydrogenase in rats is found primarily in the liver, and serum activity increases with hepatocellular injury. Unlike alanine aminotransferase and aspartate aminotransferase, glutamate dehydrogenase activity is more liver-specific and not affected greatly by muscle damage. Serum F protein has been shown to be a sensitive and specific marker of liver damage, and to have a strong correlation with histopathology in humans.[16] However, it has not been fully investigated in preclinical animal models. Glutathione-*S*-transferase alpha expression is restricted to the liver and kidney, with a high concentration in centrilobular cells within the liver.[4] Therefore, it may serve as

Table 1 Summary of current clinical serum biomarkers of liver function and injury

Serum biomarker	Tissue localization	Injury	Specific damage marker	Comments
ALT	Primarily localized to liver	Elevated due to liver necrosis and with heart and skeletal muscle injury (necrosis)	Hepatocellular Necrosis	Commonly used to assess hepatocellular injury
AST	Localized in heart, brain, skeletal muscle and liver tissue	Elevated due to liver or extrahepatic tissue injury	Hepatocellular Necrosis	Less specific than ALT
Total Bilirubin	Taken up, conjugated in liver and secreted into bile	Marker of hepatobiliary injury and liver function; also increased due to hemolysis	Cholestasis, biliary; Liver function	Conventional biliary; in conjunction with ALT, better indicator of disease severity in humans
ALP	Broad tissue localization	Marker of hepatobiliary injury	Cholestasis	Conventional biliary; associated with drug-induced cholestasis in humans
GGT	Activity localized to kidney > liver, pancreas	Marker of hepatobiliary injury	Cholestasis, biliary	Conventional biliary; high sensitivity in humans
Bile Acids	Bile duct	Elevated with liver injury and functional change	Liver function	Levels influenced by diet and fasting
Clotting Time		Increased with severe liver injury	Liver function	Liver fails to produce clotting factor increasing time; international normalized ratio equivalent to prothrombin time
Protein Levels		Decreased with severe liver injury	Liver function	Liver fails to synthesize enough protein especially albumin

a region-specific marker of liver injury. However, it should be noted that synthesis of glutathione-S-transferase alpha can be induced by a range of compounds. Glutathione-S-transferase alpha and other proposed biomarkers of liver injury must be qualified for use in order to be clinically useful and may need to be measured in conjunction with another biomarker to obtain the desired specificity. Finally, serum arginase I is highly liver-specific, and has a strong correlation with aspartate and alanine aminotransferase activity.[17] In a preclinical animal study, arginase I was shown to have the earliest and largest increase following thioacetamide-induced liver injury.[18] There is a clear need to find additional biomarkers in serum and/or urine that can be measured in conjunction with alanine aminotransferase or outperform alanine aminotransferase with respect to specificity for liver injury. The omics methods are well suited to identify novel biomarkers of hepatotoxicity that have the desired specificity and potentially indicate injury earlier than the current serum markers.

Genomic evaluation of hepatotoxicity

Over the past decade, the use of a genomics approach to identify patterns of changes in mRNA transcripts, referred to as toxicogenomics, has gained popularity for identification of DILI biomarkers.[19,20] Most studies have used microarray analysis of the rodent liver to identify unique gene expression profiles as biomarkers and to elucidate the molecular mechanisms of DILI drugs. There is considerable interest in using genomic biomarkers from rodents or primary hepatocytes to predict drugs that cause idiosyncratic liver toxicity in humans.[21] Currently, toxicogenomic data sets, generated from animals receiving DILI drugs, have been constructed and are available in the public domain.[29] These datasets can be used to generate gene expression signatures associated with different drugs or chemicals that cause liver injury. These genomic biomarkers can be used in the preclinical stage of drug development, where the hepatic gene expression signature of a new compound is compared and contrasted with the hepatic signatures of drugs that cause DILI along with appropriate negative controls.

However, the use of liver tissue-based genomic biomarkers is not optimal because biomarkers requiring liver biopsy have limited value in a clinical setting, especially for monitoring DILI progress in patients. Thus, genomic biomarkers from a minimally invasive source, such as the blood, have been explored in rodents. In rats treated with acetaminophen, it was demonstrated that whole blood transcriptome profile changes, particularly in immune and inflammatory pathways, were more sensitive and specific predictors of the extent of liver injury than traditional histological or clinical chem-

istry markers.[23] More interestingly, applying the rat blood transcriptomic signatures to human blood data was able to differentiate patients suffering from acetaminophen overdose from healthy controls.[23] This study indicated that alterations in genes involved in the inflammatory response were the best discriminators between subtoxic/nontoxic and toxic exposure to acetaminophen. In another study, downregulation of mitochondrial genes involved in complex I of the oxidative phosphorylation pathway was noted in blood from humans after exposure to acetaminophen consistent with earlier rat studies.[24] A concurrent metabolomics analysis showed increased serum lactate, supporting the gene expression findings.[24] Lobenhofer et al[25] showed that gene expression profiles from rat blood samples could be used as biomarkers to indicate the severity of liver injury induced by known hepatotoxicants. In a recent study, a more comprehensive approach was used to demonstrate that transcriptomic signatures extracted from blood can predict liver injury caused by a wide variety of hepatotoxicants.[26]

Next-generation sequencing to evaluate DILI in future studies

Recently, next-generation sequencing technologies have become available for fast, inexpensive sequencing of whole static genomes and dynamic transcriptomes.[27] While the microarray platform monitors the expression levels of most annotated genes within the cell, powerful and rapidly evolving next-generation sequencing technology allows for precise quantification of gene expression, including transcripts that have not been sequenced previously. Initially, there was concern regarding the comparability of next-generation sequencing with microarray platforms, in terms of gene expression and biological variability in a real-life toxicological study design. The MicroArray Quality Control group evaluated the robustness of next-generation sequencing for detecting differentially expressed genes, and reported that consistent biological interpretation was generated from both the next-generation sequencing platform and microarrays.[28] The next-generation sequencing data are fundamentally different from microarray data, and translating these short sequences to genomic biomarkers must overcome several obstacles. One of the major challenges lies in the handling of immense volumes of sequence data generated by next-generation sequencing.[27,29] Powerful bioinformatics tools are needed to assure sequence quality, conduct sequence alignment to the relevant genome and/or transcriptome, and provide biological interpretation from the complex datasets. Over the past five years, next-generation sequencing has provided a more comprehensive understanding of complex transcriptomes,

with a turnaround time and cost that is comparable with microarrays.[30] Next-generation sequencing technologies are anticipated to accelerate toxicogenomics research and play a pivotal role in identifying new DILI biomarkers.

Proteomic evaluations of hepatotoxicity
Cytokines as markers of hepatotoxicity

Proteomic analyses have identified several classes of markers that may serve as markers of hepatotoxicity. One class includes the cytokines, which are associated with inflammation, immune reactivity, tissue injury or repair, and organ dysfunction. They can be generally grouped based upon structure and function, and include interleukins, growth factors, interferons, tumor necrosis factors, and chemokines.[31–33] The major source of cytokine production in the liver is thought to be the Kupffer cells, although all liver cells are capable of producing cytokines.[34–36] The cytokine signals that arise following tissue damage may serve as biomarkers of cellular response. Generally, elevations of cytokine levels in plasma occur rapidly and, therefore, may precede the formation of lesions and increases in transaminases. Cytokine levels decline rapidly, so in order for them to serve as useful plasma biomarkers, not only does the correlation with histopathology need to be established, but also a timeline over which they should be evaluated.[37] Interleukin-1 has been proposed to be a biomarker for liver toxicity.[37,38]

Cellular stress markers

Additional proteomics studies have evaluated biomarkers associated with the cellular stress response. The cellular stress response pathways represent a set of pathways through which cells respond to a toxicant by either mounting a homeostatic response or making cell fate/death decisions.[39] In a recent review, Amacher[11] summarizes proteomic biomarkers associated with cellular stress response or toxicity pathways. Liver tissue protein markers associated with chemically-induced hepatocarcinogenesis in rats were evaluated by two-dimensional gel electrophoresis-mass spectrometry proteomics[40] and iTRAQ™ reagent technology.[41] An initial two-dimensional gel electrophoresis-mass spectrometry proteomics approach identified proteins in liver tissue potentially associated with hepatotoxicity.[40] A follow-up study using iTRAQ reagent technology was used to prevalidate the proteins identified.[41] In both studies, potential protein biomarkers in liver tissue of the annexin family and those involved in anabolic and catabolic functions as well as drug metabolism were identified. Furthermore, carbonic anhydrase III, aflatoxin B1 aldehyde reductase, and gluta-

thione *S*-transferase P-form, which play important roles in hepatocarcinogenicity, were identified by both technological platforms. The ability to validate potential biomarkers via repetitive measurements on multiple platforms is critical in the acceptance of new markers, whether they are transcripts, proteins, metabolites, or miRNAs. Other proteins that have been evaluated as markers of hepatocellular stress include the keratins and high mobility group box protein 1. Keratins are expressed by epithelial cells and are responsible for cell structure and integrity. Cytokeratin-18 has been used to monitor apoptosis and necrosis in blood.[42] High mobility group box protein 1 has also been proposed as a marker of hepatotoxicity, because it alerts the immune system to dying cells. In addition to being a marker of inflammation, high mobility group box protein 1 also has been shown to be a marker of necrosis but not apoptosis.[43]

Other proposed protein biomarkers

Amacher et al[44] treated rats with four different compounds toxic to the liver and then used proteomics methods to evaluate serum protein biomarkers of hepatotoxicity. The candidate proteins that correlated with histopathology included malate dehydrogenase, purine nucleoside phosphorylase, and paraoxanase 1. Malate dehydrogenase has been correlated with both liver and heart injury[45] and shown to be increased following acetaminophen-induced and thioacetamide-induced hepatotoxicity.[45,46] Purine nucleoside phosphorylase is primarily located in liver tissue and has been shown to increase following dosing with galactosamine[47] and at an earlier time point than alanine aminotransferase after dosing with endotoxin.[48] Paraoxanase 1 is primarily produced in the liver and released into the circulation bound to high-density lipoprotein. Unlike other serum markers that indicate leakage, a decrease in paraoxanase 1 is noted in serum after damage. Therefore, it is likely that liver damage reduces paraoxanase 1 synthesis and secretion. Paraoxanase 1 has been shown to be decreased after dosing with phenobarbital and acetaminophen[44] and in humans with chronic liver disease.[49,50] In a separate study evaluating the mechanism(s) of hydrazine-induced hepatotoxicity, paraoxanase 1 was also noted to decrease following treatment.[51] This may represent a causative response to oxidative stress as an initial response to a toxin. Bell et al[52] recently used a mass spectrometry-based quantitative proteomic approach to evaluate serum samples from patients with DILI and controls. Ninety-two serum priority 1 proteins were identified as having significant differential expression between DILI patients and controls. Among these, apolipoprotein E expression was able to differentiate the two groups, with 89% correct classification.

Apolipoprotein E was also noted to be differentially expressed in the serum of patients who developed DILI after treatment with ximelagatran.[53] Therefore, apolipoprotein E may also serve as a candidate biomarker of DILI.

Metabolomic applications in hepatotoxicity

Metabolic profiles are affected by both genetic and environmental factors. As such, a baseline reflective of the phenotype can be established and evaluated in a longitudinal manner following a stressor. Generally, easily obtained biofluids, such as urine and serum, are evaluated in a metabolomics analysis. The potential to identify new biomarkers of toxicity in such biofluids makes it feasible to translate preclinical markers to a clinical setting. Many nuclear magnetic resonance (NMR) spectroscopy and ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS) studies have been used to evaluate hepatotoxicity in preclinical animal models and identified metabolites from several pathways in multiple reports.

Bile acid metabolism

A targeted UPLC-MS method was employed in a study of galactosamine-induced hepatotoxicity to profile bile acids rapidly.[54] Galactosamine is known to induce bile duct proliferation, and leakage of bile acids due to necrosis. In the above study, taurine-conjugated and glycine-conjugated bile acids in serum were increased after dosing. Bile acid profiles have been evaluated with other hepatotoxicants and show distinct patterns based upon the mechanism of toxicity.[54] Want et al[55] exposed rats to galactosamine and then evaluated bile acids in serum extracts. Glycine and taurine conjugates were shown to be elevated markedly, and to have a strong correlation with the severity of liver damage as measured by histopathology scoring. A targeted metabolic profiling study based upon gas chromatography-mass spectrometry and capillary electrophoresis-mass spectrometry identified urinary cholic acid and lithocholic acid as potential liver toxicity biomarkers following dosing with acetaminophen or carbon tetrachloride.[56] Acetaminophen were evaluated in rat serum after carbon tetrachloride-induced and alpha-naphthylisothiocyanate-induced liver failure.[57] Carbon tetrachloride and alpha-naphthylisothiocyanate cause distinct types of liver injury, and the two could be distinguished based upon the pattern of altered bile acids. In a recent study of acetaminophen-induced toxicity, decreases in the expression of bile acid synthesis-related genes (*Cyp7a1* and *Cyp8b1*) and cholesterol transporter gene (*Abcg8*) and increases in the expression of bile acid transporter genes (*Mrp3* and *Mrp4*) combined with increases in serum bile acids suggested a mild form of intrahepatic cholestasis (unpublished data). The aforementioned studies indicate that bile acids may be a sensitive marker of DILI and may also be able to differentiate specific types of liver injury.

Biomarkers of hepatic glutathione depletion
Ophthalmic acid

Many hepatotoxicants that form reactive metabolites induce oxidative stress contributing to cell death. During induction of oxidative stress, the protective molecule glutathione scavenges reactive oxygen species but is consumed in the process. Glutathione production proceeds via the folate-dependent transmethylation and trans-sulfuration pathways. The ophthalmic acid biosynthesis pathway proceeds via a biosynthetic route similar to that of glutathione. Ophthalmic acid is an analog of glutathione in which the cysteine residue is replaced by 2-aminobutyrate. Synthesis of ophthalmic acid is catalyzed by the same enzymes (γ-glutamylcysteine synthetase and glutathione synthetase) involved in the synthesis of glutathione. Gamma-glutamylcysteine synthetase may be activated during glutathione depletion, increasing synthesis of ophthalmic acid.[58] Serum ophthalmic acid has been reported as a potential biomarker of oxidative stress and glutathione depletion following a hepatotoxic dose of acetaminophen in mice.[58] The results of that study indicated that elevations in ophthalmic acid were noted with concomitant decreases in glutathione. Following the report of ophthalmic acid as a potential marker of glutathione depletion, other methods have been developed to assess this marker further.[59–61] Geenen et al[59] developed a liquid chromatography-mass spectrometry method to evaluate ophthalmic acid in vitro and in vivo. In order to validate the method, rat serum samples were evaluated following repeat dose administration of methapyrilene. In contrast with the acetaminophen study in mice, ophthalmic acid was shown to be decreased despite large rises in aspartate aminotransferase levels, indicating hepatotoxic effects of the drug. While there is interest in ophthalmic acid as a potential oxidative stress biomarker in the early stages of liver toxicity, more studies are needed to understand fully the relationship between this marker and liver damage. As a complication, there are multiple biosynthetic pathways involved in the biosynthesis of glutathione and ophthalmic acid, making it difficult to correlate fully the changes noted between the two metabolites.

5-Oxoproline

5-Oxoproline (pyroglutamate) is an intermediate in the glutathione biosynthesis pathway and, therefore, may be more reflective of glutathione content and cell status. 5-Oxoproline has been noted to be elevated in biofluids and tissues in animal studies following administration of hepatotoxicants, such as acetaminophen,[62] bromobenzene,[63] and ethionine.[64] The aforementioned compounds are known to deplete glutathione. Increased 5-oxoproline has also been noted in humans with inborn errors of metabolism that affect glutathione synthesis,[65–69] and as a result of chronic use of acetaminophen.[70] It has been reported that 5-oxoproline was increased in human liver epithelial cells following exposure to acetaminophen[71] using a liquid chromatography-mass spectrometry method. A concurrent decrease was noted in cellular glutathione content. In the same study, levels of 5-oxoproline were measured in serum from rats 24 hours after dosing with methapyrilene, a known hepatotoxicant. No significant changes were noted, which may be related to the rapid clearance of 5-oxoproline in urine.[71] Glutathione consumption has also been implicated in hydrazine-induced hepatotoxicity where 5-oxoproline was increased in a dose-dependent manner in urine, plasma, and liver tissue.[72] Based upon the available literature, 5-oxoproline may be more directly coupled with glutathione depletion and, therefore, may be more reflective of glutathione status than ophthalmic acid.

Other metabolic pathways reflective of hepatotoxicity

Pathways in addition to bile acid metabolism and glutathione biosynthesis have been noted to be altered following a hepatotoxic insult. Kumar et al[56] used a global profiling method for the initial discovery of potential biomarkers of hepatotoxicity in urine. These markers were then quantified using gas chromatography-mass spectrometry and capillary electrophoresis-mass spectrometry. In addition to identifying two significantly altered bile acids after dosing with acetaminophen or carbon tetrachloride, four steroids and six amino acids were selected as liver toxicity biomarkers that could be evaluated in future drug toxicity studies. In the hydrazine-induced hepatotoxicity study noted above, not only was 5-oxoproline elevated but several amino acids were altered in urine and plasma.[72] Amino acid precursors of glutathione, including cysteine, glutamine, and glycine, were elevated along with other amino acids. The amino acid changes were associated with fatty degeneration and glycogen depletion. Amino acid metabolism was also altered in response to bromobenzene-induced hepatic necrosis.[73] A metabolic profiling study evaluated

safety biomarkers for atorvastatin in rat urine and identified estrone, cortisone, proline, cystine, 3-ureidopropionic acid, and histidine as markers related to liver toxicity following dosing.[74] Nontargeted and targeted metabolic profiling identified 3-hydroxy-2′-deoxyguanosine and octanoylcarnitine as urinary markers of hepatotoxicity after exposure of rats to valproic acid.[75] Chen et al[76] evaluated the inhibition of fatty acid beta-oxidation in mice after dosing with acetaminophen. Liquid chromatography-mass spectroscopy analysis of serum samples showed accumulation of long-chain acylcarnitines and free fatty acids in serum, indicating a disruption of fatty acid beta-oxidation. The pattern of accumulation of acylcarnitines in the above study indicated that they might be useful as complementary biomarkers for monitoring acetaminophen-induced hepatotoxicity. Multiple biological matrices were evaluated after exposure of rats to aflatoxin-B1.[77] Analysis of NMR spectral data showed significant elevations in amino acids in plasma and liver tissue, indicating that aflatoxin-B1 altered protein biosynthesis. While bile acids, 5-oxoproline, and ophthalmic acid, are promising potential markers of hepatotoxicity, fatty acids, amino acids, and steroids may also be indicators of liver damage that warrant further investigation.

Metabolomic flux analysis utilizing a ^{13}C-labeled precursor to evaluate metabolite flux and turnover rates in specific pathways has also been used for evaluation of biomarkers of hepatotoxicity. Use of noninvasive, nonradiating ^{13}C tracers permits real-time quantification of synthesis and turnover of select metabolites. Stable isotope ^{13}C-labeled glucose was used to investigate the toxic effects of valproic acid[78] and usnic acid (a dietary supplement promoted for weight loss).[79] The fate of the labeled glucose was analyzed in plasma, urine, liver, brain, and kidneys from control and valproic acid-treated mice. The results indicated that liver toxicity may be due to a disruption of the flux of acetate and its disposal via plasma cholesterol. Usnic acid was shown to be cytotoxic to rat primary hepatocytes in a time-dependent and concentration-dependent manner, and isotopomer distributions indicated a reduction in oxidative phosphorylation and gluconeogenesis at the high dose, consistent with cytotoxicity and ATP depletion in the cells. Stable isotope methods have the potential to develop translational isotopomer profiles in biofluids and tissues that can be used as toxicity markers in humans.

MicroRNAs as new markers for DILI assessment

miRNAs are approximately 22 nucleotides long, single-stranded, noncoding RNA that have recently been recognized

as novel agents exercising post-transcriptional control over most eukaryotic genomes.[80,81] miRNAs are highly conserved among the species, ranging from worms to humans, revealing their very ancient ancestry. To date, over 1500 mature human miRNAs have been identified and recorded in the miRBase registry,[82] and they are predicted to regulate the activity of about 50% of human genes.[83] miRNAs have been implicated in a wide diversity of basic cellular functions, including development, cellular differentiation, proliferation, apoptosis, cell-cycle control, metabolism, and cancer.[84,85] Similar to mRNA, some miRNAs are produced in cell-specific or tissue-specific manners.[86,87] Recently, miRNA-altered expression patterns have been observed in many human pathologies, including cancer, suggesting that miRNAs may serve as new biomarkers of organ injury.[7,88,89] For example, miRNA expression profiles have been used as biomarkers to distinguish patients with different types of cancer and even provide prognostic information about disease outcome.[90]

Similar to mRNA, most miRNAs are transcribed as a part of a long transcript through RNA polymerase II.[91] Within the nucleus, these primary miRNA transcripts are processed by the ribonuclease III enzyme Drosha in cooperation with the double-strand RNA binding protein, DGCR8, releasing small, approximately 70 nucleotide-long, hairpin miRNA molecules called precursor miRNAs. These precursor miRNAs are then translocated to the cytoplasm with the assistance of protein Exportin 5 in a GTP-dependent process. Once in the cytoplasm, the precursor miRNAs are processed into approximately 22 nucleotide-long mature miRNAs by a protein complex. In mammals, miRNAs typically negatively regulate the expression level of the target mRNAs. A single miRNA often regulates multiple mRNAs, and multiple miRNAs often target a single mRNA. Therefore, miRNA regulation of gene expression appears to act like a rheostat to fine-tune gene expression of many genes as opposed to gross regulation of a single gene. Generally, miRNAs affect target genes through a perfect or near-perfect match between miRNAs and their binding sequences, referred to as a "seed" sequence, within the 3′ untranslated regions of the target mRNAs. The targeted sequences are then degraded or repressed at the translational level.

Recently, a significant numbers of miRNAs have been observed in the body fluids of both healthy and diseased patients and animals. Some studies indicate that miRNAs are actively released from cells via several different processes; however, the function of circulating miRNAs remains unclear.[92,93] Owing to its minimal invasive nature and unique stability, miRNA in body fluids holds unique promise for use as a preclinical and clinical DILI biomarker. Unlike mRNA, miRNA has been shown to be remarkably stable in many body fluids, including blood and urine.[94–96] This stability greatly facilitates its use as a clinical biomarker of disease and injury because sample handling and processing are much less problematic when compared with mRNA.

Circulating microRNAs as DILI biomarkers

Using an acetaminophen-induced mouse model of DILI, Wang et al[86] reported that the level of many plasma miRNAs inversely correlated with the level of hepatic miRNAs, indicating that for these miRNAs, hepatic injury caused the release of miRNAs into the circulation. Specifically, miRNA-122 and miRNA-192, which are predominantly expressed in the liver, increased in plasma with concurrent decreases in the liver. The increases in both miRNAs were detected earlier than an increase in alanine aminotransferase. The increase of serum miRNA-122 and miRNA-192 was confirmed recently in patients with acetaminophen poisoning.[97] Ding et al[98] also showed a marked elevation in serum miRNA-122 levels related to paraquat-induced hepatotoxicity in humans. The results indicated a strong correlation between serum miRNA-122 and liver injury based upon elevations in alanine aminotransferase in drug-induced hepatotoxicity. Furthermore, miRNA-122 was also evaluated in serum from patients with liver injury due to hepatitis or hepatocarcinoma, and the change was much smaller than after drug-induced injury.[98] Therefore, the differential increases in miRNA-122 may allow for discrimination of different types of liver injury. miRNA-122 is enriched in the liver and may prove to be a more specific indicator of liver injury than alanine aminotransferase.[99]

Recently, Yang et al[100] reported that urinary miRNA profiles were altered in rats after administration of hepatotoxic doses of acetaminophen or carbon tetrachloride. Levels of the same 10 urinary miRNAs were increased in acetaminophen-treated and carbon tetrachloride-treated rats. The miRNA expression profiles were able to distinguish patterns of responses to the two hepatotoxicants versus a nonhepatotoxicant (penicillin) and vehicle controls. In addition, at a high dose of acetaminophen, only two of seven animals exhibited dramatically increased alanine/aspartate aminotransferase levels and four of seven animals exhibited histopathological centrilobular necrosis, whereas every animal had elevated urinary miRNA concentrations for at least several of the miRNAs that were screened.[101] For urinary miR-291a-5p, which was increased in the urine in response to the hepatotoxicants, a corresponding decrease

in the liver was observed.[100] Although the functions of the 10 common miRNAs remain unknown, the possible target genes of these miRNAs are related to cell death, cell-to-cell signaling, and major metabolic pathways. Furthermore, the predicted target genes of the urinary miRNAs have some overlap with significantly changed genes measured in the livers of acetaminophen-treated and carbon tetrachloride-treated rats. Although a larger number of chemicals will need to be tested, this study clearly demonstrates the possibility of using noninvasive urinary miRNAs as a biomarker of DILI.

Challenges associated with measurement of miRNAs

Although miRNA detection is theoretically simpler and subject to fewer confounders than protein-based assays, there are some technical issues associated with miRNA measurement (Table 2).

A standardized protocol for measuring miRNA in body fluids and the choice of an appropriate control for data normalization are needed for reproducible quantification of circulating miRNA biomarkers.[7] The available published data show that the level of various miRNAs in serum, plasma, and urine may serve as new biomarkers of DILI. However, use of cell-free miRNAs as biomarkers of DILI is in its infancy, and further investigations are needed to translate animal-based DILI biomarkers into clinical applications. Future studies will help determine the role cell-free miRNAs play in monitoring DILI and whether they will simply be adjuncts to existing biomarkers or will show superior performance and supplant existing biomarkers.

Biomarker qualification and quantification

Applying omics-based technologies in rodents treated with a variety of hepatotoxic drugs, together with a better understanding of the mechanisms associated with hepatotoxicity, may facilitate the identification of novel DILI biomarkers. The primary biomarkers discussed in this review are summarized in Table 3.

However, it is worthwhile to point out that none of the new DILI biomarkers has been qualified for preclinical or clinical use from a regulatory perspective. Furthermore, many of the potential biomarkers discussed in this review are not necessarily specific to the liver and may be the result of other types of tissue injury. True qualification of new biomarkers will ultimately require large numbers of samples obtained from animals and patients treated with many different drugs. A stringent qualification process is required to validate their specificity and sensitivity for DILI before they can supplement and/or replace existing biomarkers. Qualification has been described as "the process of linking a biomarker to a preclinical or clinical end point or to a biological process in a specific context".[102,103] The US Food and Drug Administration and International Conference on Harmonisation have issued guidance on biomarker qualification and the content of data submissions.[104–106] These guidance documents provide a foundation for qualifying a biomarker for a given context of use, such as preclinical versus clinical for example. Although specific testing and qualification plans are not provided in the guidance documents, they do highlight the fact that robust data are required to qualify a new biomarker. A biomarker will require a clearly defined context of use as well as sufficient data to support a full review of its performance characteristics within that context. Furthermore, it is required that the biomarker be measured reliably on multiple analytical platforms. Finally, in order to translate a biomarker successfully from the preclinical to the clinical setting, the marker must be qualified for its intended use and should correlate with lesions observed by histopathology. As part of the qualification process, it will be necessary to quantify a marker and provide a range of normal values in a control state. Metabolomics biomarkers can be quantified by mass spectra based upon a calibration curve or comparison of intensities after spiking a sample with an isotope labeled standard for the compound of interest. NMR metabolite data are quantified based upon the concentration of an internal chemical shift standard. Protein biomarkers can also be quantified by mass spectral methods that include a labeling procedure and tandem mass spectroscopic analysis. A Bradford assay can be used for quantification of a protein of interest. Gene or miRNA biomarkers can be quantified using real-time polymerase chain reaction, microarray, and next-generation sequencing platforms based on the fluorescence signals.

Table 2 Technical challenges associated with circulating microRNA biomarker quantification

Sample preparation
- Lack of standard protocol for isolating miRNA from body fluids
- Assessment of miRNA quantity and quality is less than ideal

miRNA measurement
- Limited correlation between different platforms for miRNA detection
- miRNA sequence heterogeneity makes it difficult for specific miRNA detection
- Difficult to distinguish between precursor miRNAs and mature forms

Data analysis
- Lack of standard normalization methods

Abbreviation: miRNA, microRNA.

Table 3 Summary of potential omics biomarkers of liver function

Proposed biomarker	Reference(s)	Biofluid evaluated	Origin	Proposed indication
Cytokines	31–36	Plasma	Produced by all liver cells but primarily Kupffer cells	Marker of cellular stress in the liver
Interleukin-1	37–38	Liver tissue Plasma	Produced by a variety of cells	Marker of cellular response to tissue damage
Annexin family of proteins	40,41	Liver tissue	Highly abundant in smooth muscle, cardiomyocytes, and endothelial cells	Marker of hepatocarcinogenicity
Carbonic anhydrase III	40,41	Liver tissue	Present in normal hepatocytes	Marker of hepatocarcinogenicity
Aflatoxin B1 aldehyde reductase	40,41	Liver tissue Serum	Found in a wide range of tissues including heart, lung, liver, placenta, brain, colon, and breast	Marker of hepatocarcinogenicity
Glutathione S-transferase P-form	40,41	Serum	Present in hepatocytes	Marker of hepatocellular injury
Cytokeratin-18	42	Serum	Expressed by epithelial cells	Marker of apoptosis or necrosis
High mobility group box protein 1	43	Serum	Found in multiple tissue types	Marker of necrosis and inflammation
Malate dehydrogenase	4,44–46	Serum	Localized in mitochondria and extramitochondrial compartment; found primarily in liver, but also in skeletal muscle, heart, and brain	Marker of necrosis in liver
Purine nucleoside phosphorylase	4,44,47,48	Serum	Primarily in liver but also present in heart muscle and brain; mainly in cytoplasm of endothelial cells, Kupffer cells, and hepatocytes	Marker of necrosis
Paraoxanase 1	4,44,49,50	Serum	Produced primarily in liver but also found in kidney, brain, and lung	Marker of tissue damage in liver and necrosis
Apolipoprotein E	52,53	Serum	Produced in the liver and many other tissues, including brain and kidney	Marker of drug-induced liver injury
Bile acids	54–58	Urine Serum	Synthesized primarily in the liver	Marker of liver damage including intrahepatic cholestasis
Ophthalmic acid	59,60	Serum	Analog of glutathione produced along a similar biosynthetic route as glutathione	Marker of oxidative stress and glutathione depletion following hepatotoxic insult
5-Oxoproline	65–73	Liver tissue Urine Serum	Intermediate in the synthesis of glutathione	Marker of oxidative stress and glutathione status
Amino acids	56,73,74,78	Liver tissue Urine Plasma	Nontissue specific	Marker of altered protein biosynthesis
Steroids	56,75	Urine	Metabolites of cholesterol	Marker of stress and liver damage
Acylcarnitines	76,77	Urine Serum	Located in multiple tissue types, including heart, muscle, brain, liver, and kidney	Marker of defects in fatty acid oxidation
Fatty acids	77	Serum	Nontissue specific	Marker of disruption of fatty acid β-oxidation
miRNA-122	94,98	Plasma	Liver specific expression	Marker of viral-, alcohol- and chemical-induced liver injury
miRNA-192	94	Plasma	Liver specific expression	Marker of chemical-induced liver injury
miR-291a-5p	101,102	Urine	Unknown	Marker of chemical-induced liver injury

Conclusion

DILI is a major reason for limiting the use of a drug or removal of drugs from the market. At times, the current serum markers of liver toxicity fail to identify a toxic compound in the preclinical development stages and in even clinical trials. Therefore, novel biomarkers are needed that are more sensitive to liver injury. The omics technologies are

well suited to identify novel biomarkers of DILI that can be measured in easily obtained biofluids. Potential markers have been identified by genomics, proteomics, and metabolomics methodologies. In order for any of the proposed markers to move forward, they must be qualified for their intended use. In many cases, the same markers have been noted in multiple studies with overlapping pathways of interest, including

glutathione depletion as an initial response to a cellular stress. Recently, measurement of circulating miRNAs has shown promise in identification of new biomarkers of liver injury. Further studies are needed to evaluate the sensitivity and specificity as well as validate the omics biomarkers of hepatotoxicity discussed in this review before they can be routinely used in preclinical and clinical testing.

Acknowledgment

Disclosure

The opinions expressed in this manuscript do not necessarily represent those of the US Food and Drug Administration.

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