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REVIEW

Advances in the diagnosis of drug-induced liver injury

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Abstract: Drugs are used to prevent, diagnose, and treat diseases, as well as improve physiological function and health level. With the increasing number of drug types and the lack of formal drug methods, the incidence of adverse reactions to drugs is also rising. Drug-induced liver disease is so common that in addition to poor drug quality, irrational drug use is the main reason, including drug abuse, misuse, low medical quality, and knowledge level of patients and their families; in addition, social problems such as lack of scientific management, medical ethics, and medical staff. Therefore, it is imperative to strengthen drug management, improve drug safety, and reduce the incidence of adverse reactions. Here we it is introduce and elaborate the current situation and progress in the diagnosis of drug-induced liver disease in recent years, so as to build a learning platform for the prevention and treatment of drug-induced liver disease in China.

Key words: drug induced liver injury, diagnostic criteria, gene assay, apoptosis index, glutamate dehydrogenase, HMGB 1, exon, gene assay.

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***Abbreviations*:** DILI, Drug-induced liver injury; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TBIL, total bilirubin; FDA, Food and Drug Administration; CDER, Center for Drug Evaluation and Research; AUC, the area under the curve; AI, apoptotic index; CCK 18, caspase-cleaved keratin 18; K18, keratin 18; AILI, acetaminophen-induced liver injury; KCC, King's College criteria; GLDH, Glutamate dehydrogenase; miRNAs, MicroRNAs; HMGB 1, High mobility group 1.

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Introduction

In China, adverse drug reactions account for approximately 10%–30% of hospitalized patients, and at present, humans are exposed to more than 60000 chemicals. Drug-induced liver injury (DILI) accounts for approximately 2%–5% of inpatients with jaundice, 10% of inpatients with so-called "acute hepatitis," and 20% of elderly liver diseases. Although drug-induced liver disease is common, it has not attracted sufficient attention from patients and clinicians. DILI accounts for approximately 30%–40% of the causes of acute liver failure in European and American countries, while 36% of DILI in the United States is caused by non-steroidal anti-inflammatory drugs. According to statistics, 20%–40% of all adverse drug reactions affect the digestive tract. The incidence of drug-induced liver injury is second only to that of skin reactions, mucosal damage, and drug-induced fever. At the end of 2007, the Chinese hepatology branch of the Chinese Medical Association established the pharmaceutical liver disease group, which provides an organizational and technical platform for the prevention and treatment of pharmaceutical liver disease in China [1].

The phenotype of liver injury is classified by its R value and is defined as ALT / ULN: ALP / ULN ratio. An R value ≥ 5 indicates hepatocyte injury, ≤ 2 indicates cholestatic injury, and 2-5 indicates mixed injury [2].

1. Traditional biochemical markers

For nearly half a century, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in hepatocytes have been commonly used in clinical detection and management of DILI. In the event of acute DILI, the increase in serum ALT and AST levels is closely related to the rate of hepatocyte death and release. An increase in serum ALP usually indicates damage to the bile duct membrane or bile duct epithelial cells. An increase in TBIL level can reflect reduced liver function, bilirubin production (hemolysis), or processing changes. However, these traditional serum markers have limitations. None of these markers are targeted at liver injury alone and do not provide a deep understanding of the mode of injury. In addition, when hepatocyte injury occurs, cell injury biomarkers such as ALT and AST are released into the circulation. Although these biomarkers can detect hepatocyte injury, they cannot be used to identify drug-induced liver injury because an increase in serum ALT and AST levels may occur during drug treatment, even when the drugs do not pose a great risk of progressive liver injury (such as statins, heparin, and choline). Furthermore, the elevation of serum ALT and AST caused by most drugs can be controlled, even if they result in clinically significant drug injury [3].

Hy's rule is the rule of thumb when evaluating liver-specific laboratory parameters, that is, when serum ALT > 3 occurs in drug-induced liver injury and serum TBIL > 2 when ULN is elevated, it usually indicates a poor prognosis. Hepatocyte drug-induced liver injury with jaundice is a serious reaction that is widely used to determine the risk of acute liver failure (ALF). It has been determined that serum ALT and AST have limitations in evaluating liver safety in clinical trials of new drug candidates, and the 2009 U.S. Food and Drug Administration (FDA) guidelines will. The case of "Hy's rule" is defined as the most accurate prediction that drugs may cause acute liver failure. The large international registry of drug-induced liver injury confirmed that patients with drug-induced hepatocyte jaundice have at least a 10% chance of developing liver failure. According to a report by the FDA / Center for Drug Evaluation and Research (CDER) in 2014 at the DILI Symposium hosted by the president of the conference, a senior member pointed out that: (1) the severity of liver injury cannot be determined only by ALT levels, and the subject operation characteristic curve often fails for very rare events. (2) DILI cannot be diagnosed only by blood biochemical tests or liver biopsy, but it requires master-relevant detailed clinical data. At present, there is no sufficiently specific biomarker for DILI [4].

It is assumed that the increase in serum TBIL is caused by the total loss of liver function due to the massive loss of hepatocytes. It is worth noting that in a real case of Hy's law, the combined increase in serum ALT and TBIL is not a biomarker for predicting the possibility of severe liver injury but is a biomarker for proving that serious and potentially life-threatening liver injury has occurred in this subject.

One challenge in interpreting Hypnos's rule is that drugs may lead to an increase in serum TBIL levels that is not due to the damage of comprehensive liver dysfunction. The causes include hemolysis (increased heme load), the inhibition of drugs on the uptake or outflow of bilirubin, or the inhibition of major hepatocyte enzymes bound to bilirubin. Quantitative systemic pharmacology has been applied to evaluate the estimation of drug liver exposure and the quantitative effect of the drug on the inhibition of transporters and UGT1A1. Furthermore, an in vitro system method has improved the understanding and prediction of a drug-induced increase in serum TBIL levels [5].

Recently, it was proposed that the percentage of dead hepatocytes in the event of acute drug-induced liver injury can be evaluated by continuous measurement of serum ALT. This is based on the view that each hepatocyte contains a certain amount of ALT,

which is passively released during cell death. Therefore, the area under the serum ALT and time curve should correspond to the number of hepatocytes lost. This method could also be used to estimate the amount of hepatocyte loss, which can predict the increase in serum bilirubin according to the implied loss of aggregated liver function. Animal hepatectomy studies have shown that more than 70% of hepatocytes must be lost before the increase in serum TBIL level is sufficient to cause jaundice. However, in the event of acute drug-induced liver injury, cell death may not occur. In some hepatocytes, the function will be impaired. The data that supports using this measurement in the estimation of hepatocyte loss of ALT includes a liver biopsy study conducted in patients with liver injury caused by acetaminophen overdose that occurred decades previously. According to the data provided by this study, more than 40% of hepatocytes must increase serum TBIL levels to greater than 2 times the ULN. Two patients died before ULN. Serum ALT > 3 times the ULN and TBIL > 2 times the area under the plasma concentration time curve (AUC) of serum ALT in patients with ULN was studied, and according to the current definition, they were all cases of Hy's law. According to a series of serum ALT values, the hepatocyte loss rate of each subject was estimated to be less than 40% [6]. The conclusion of the hundred model is that the increase of serum TBIL is not only due to hepatocyte toxicity. This conclusion will be further supported if systematic pharmacological methods show that the inhibition of drugs on transporters and/or enzymes may lead to an increase in serum TBIL levels. These methods may improve the interpretation of potential Hy's law cases. Continuous serum ALT values are used to evaluate hepatocyte loss, and most of the ALT entering the circulation is the result of hepatocyte death with the release of ALT content. This situation is likely to occur in serious drug-induced liver injury, but the sharp increase in serum ALT levels may also be caused by muscle disease or injury [4,5,7].

2. New biomarkers of drug-induced liver injury

2.1 Apoptosis index

Recently, an apoptotic index (AI) based on serum biomarkers was proposed to estimate the relative contribution of apoptosis and necrosis to liver injury. AI is caspase-cleaved keratin 18 (CCK 18), and total keratin 18 (K18) includes full-length K18 and caspase cleavage fragments. K18 is a type I intermediate filament that exists in epithelial cells and provides structural support for cells. In the early stage of apoptosis, K18 is cleaved by caspase to form a stable protein fragment (CCK 18) and released into the circulation. When necrotic, full-length K18 is passively released into the circulation, a small amount of CCK 18 should be released. In acetaminophen-induced liver injury (AILI), mass spectrometry (mouse) or immunoassay analysis (for humans) have been used to detect the concentration of K18 or CCK 18 in the blood to determine if they are related to the degree of necrosis and apoptosis in the liver. It has been found that, in mice, the increase in CCK 18 levels is closely related to the expression of caspase 3, the treatment of caspase 3, DNA breakage, and the appearance of apoptotic hepatocytes. In addition, it has been found that the ratio of CCK 18 to K18 reflects the transformation of the liver from mixed apoptosis / necrosis to main necrosis. It was also noted that in AILI, the levels of K18 and CCK 18 increased significantly before the ALT level. These data show that quantifying K18 and CCK 18 can not only detect hepatocyte death early but also quantitatively estimate the relative number of hepatocytes that underwent apoptosis and necrosis. Since K18 is not limited to epithelial cells of liver origin, at least in preclinical models, using K18 as a biomarker of drug-induced liver injury may be problematic. Although the ratio of miR-122 or GLDH / K18 may be more useful, this has not been investigated [8].

The quantification of serum K18 and CCK 18 was also carried out in the clinical context of drug-induced liver injury. Compared with ALT, these two methods are more sensitive in detecting liver injury. Serum K18 and CCK 18 may also be used as biomarkers to predict liver failure and death. In these investigations, they meet the national King's College criteria (KCC). The levels of K18 and CCK 18 were significantly higher in patients who did not meet these criteria, which are used for predicting AILI liver failure. AI estimates showed that KCC patients had lower values (more necrosis) than patients without KCC. These results are consistent with the idea that necrotic liver injury is more serious and dangerous than apoptotic injury [9-11].

However, it should be noted that computing artificial intelligence may be a challenge when using commercially available ELISAs. Experimental evidence in the serum of cancer patients shows that this ratio may not be useful when the levels of K18 and CCK 18 are low. Therefore, it is recommended that AI be calculated only when the values of K18 and CCK 18 exceed a background threshold. When the level of K18 is low, the measured CCK 18 level may exceed the measured total K18 level due to the increase in non-specific background binding level. Similarly, this supports the view that AI can be calculated only when the value is higher than the specific background level and when the K18 level exceeds the CCK 18 level. When AI needs to be calculated, the ELISA manufacturer recommends the use of an earlier version of the K18 ELISA (M65)®. Finally, these ELISAs are currently not suitable for rodents [12].

2.2 Glutamate dehydrogenase

Glutamate dehydrogenase (GLDH), an enzyme located in the mitochondrial matrix, is involved in the oxidation of amino acids and the production of urea. This protein is primarily expressed in the central peripheral region of the liver, but there is also a low level of GLDH expression in the kidney and brain. In a recent AILI study in rodents, GLDH was found to be slightly better than ALT in identifying hepatocyte necrosis and showed a correlation with the severity of injury. Similarly, in a dog study, the serum GLDH level could more accurately reflect the liver injury observed by histology at the end of the study than the ALT level. A clinical study showed that there was a strong correlation between serum GLDH and ALT levels, indicating that GLDH has a high diagnostic ability for liver injury in patients with liver injury caused by multiple causes. This suggests that the half-life of GLDH is shorter than the half-life of ALT (16 h and 47 h, respectively); thus, the level of serum GLDH can more accurately reflect the progress of liver injury [13,14].

The release of GLDH into the serum may be a signal of mitochondrial toxicity and a mechanism of liver injury. This idea stems from the fact that if necrosis does not lead to mitochondrial toxicity, it can be centrifuged and create "post mitochondrial supernatant." The released intact mitochondria are then removed from fresh serum. Using this technique, mouse studies have demonstrated evidence of GLDH as a biomarker of mitochondrial damage. In this study, the content of GLDH in the post-mitochondrial supernatant obtained from liver toxicity induced by AILI or furosemide. However, the patterns of peripheral necrosis and elevated serum alanine aminotransferase peak caused by these two poisons were similar, but the level of GLDH in serum supernatant increased significantly only after AILI and not in furosemide-treated mice. This is consistent with the known mitochondrial toxicity of acetaminophen and the lack of mitochondrial toxicity caused by furosemide, suggesting that the analysis of GLDH in mitochondrial supernatant during drug-induced liver injury may be caused by a variety of drugs [3,15].

2.3 miR-122

MicroRNAs (miRNAs) are small compared to coding RNAs and contribute to the regulation of post-transcriptional genes. An attractive feature of miRNAs is their high stability in biological fluids. MicroRNA-122 is specifically expressed by hepatocytes and accounts for 70% of the total miRNAs in the liver. Therefore, it may be an ideal candidate as a biomarker for drug-induced liver injury because traditional biomarkers, such as ALT, are not entirely liver-specific [16].

In AILI mice, the level of miR-122 in plasma was high and decreased in the liver tissue. In addition, it was related to ALT, while circulating miR-122 levels increased in both early and low-dose APAP. Clinically, multiple studies of drug-induced liver injury have shown that circulating miR-122 levels increase before ALT levels increase. In another study, serum miR-122 levels increased in patients with acute liver injury, while they did not increase in healthy controls and patients taking excessive acetaminophen without liver injury. Studies have shown that the serum half-life of miR-122 is shorter than that of ALT; therefore, it can more accurately reflect the degree of sustained liver injury [9,17,18].

A large number of miRNA analysis studies have also been carried out on clinical AILI datasets obtained from adults and children. In all studies, miR-122 was one of the most detected miRNAs in the circulation. Interestingly, in a study involving the evaluation of AILI and ischemic liver injury, there were significant differences in the distribution of miRNAs between the two injury types [19,20].

Although there is great interest in miR-122 as a sensitive and specific biomarker of liver injury, some recent observations suggest that the hepatocyte release of miR-122 may be regulated, not just passively released during hepatocyte death. For example, a recent study in rats showed that two hours after acetaminophen treatment compared with the control group. Furthermore, it has been recently reported that miR-122 is released during acute liver reactions and may move to the kidney due to anemia, which is often observed in a chronic inflammatory state. MiR-122 has also been found to have a large degree of internal and internal variation in healthy volunteers, which can counteract the advantages of miR-122 as a biomarker of drug-induced liver injury, while GLDH has the advantage of low inter-disciplinary and internal variability [20,21].

3. Biochemical marker prediction of specific drug-induced liver injury

Many DILI reactions are caused by the adaptive immune attack of the liver. This forces the human immune system to regard the liver as foreign, which may be the result of the new antigen produced by class I HLA molecules on the surface of hepatocytes induced by drugs. However, introducing a new antigen is not enough to cause an adaptive immune attack in the liver. Therefore, innate immune cells, especially Kupffer cells, in the liver must also be activated. This requires the drug-induced release of the molecular pattern related to injury (Fig. 1). In this case, serum ALT increases or decreases in the absence of DAMP release, and the lack of activation of innate immune cells should prevent adaptive immune attack in the liver. Therefore, researchers have been looking for appropriate DAMPs released by hepatocytes and biomarkers that can detect the activation of innate

immune cells in the liver. Thus far, high migration rate group 1 (HMGB 1) and its various post-translational modifications have been the most extensively studied [22].

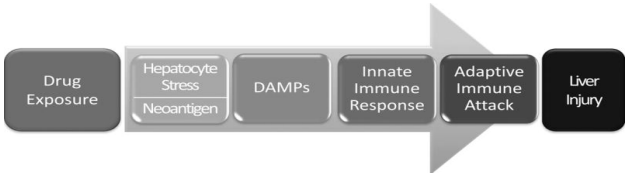

Figure 1. Proposed sequence of events involving adaptive immunity in special DILI.

To cause an adaptive immune attack in the liver, drugs cause hepatocyte stress or death, which leads to the release of molecular patterns related to hepatocyte injury, and then activate natural immune cells, especially Kupffer cells, in the liver. Activated natural immune cells release cytokines and chemokines as well as introduce inflammatory cells into the liver. Biomarkers may help detect the initial step before the occurrence of severe drug-induced liver injury events.

3.1 High mobility group 1 (HMGB 1)

High mobility group box 1 (HMGB 1) is a ubiquitous nuclear protein that plays a role in DNA binding and transcriptional regulation; however, it can also be actively secreted into the extracellular environment. There is evidence that HMGB 1 is passively released into the circulation by damaged or dead hepatocytes and can dampen or triggering an immune response. This function seems to be caused by the redox of specific residues in HMGB 1State mediated. HMGB 1 subtypes that are completely or partially reduced at these residues are released upon necrosis and promote the release of innate immune cell chemotaxis and cytokines. In contrast, HMGB 1 is completely oxidized at these key sites and is released by apoptotic cells and does not cause an innate immune response [23].

Importantly, HMGB 1 can also be actively secreted during a process that requires high acetylation of key lysine residues. When detected in serum in drug-induced liver injury events, most highly acetylated HMGB 1 is considered to be released by innate immune cells, meaning that it was activated by DAMPs; however, hepatocytes have also been shown to secrete this atypia under certain conditions.

Both non-clinical and clinical studies have shown that HMGB 1 is a sensitive biomarker of DILI. The increase in serum HMGB 1 in mice was significantly earlier than that of ALT and returned to the baseline at a faster speed, which is similar to the histopathological observation of liver necrosis. Consistent with the view that HMGB 1 exuded by necrotic cells promotes immune cell inflammation and HMGB 1 activity release, the increase of highly acetylated HMGB 1 in serum is related to inflammatory cells in the liver. Furthermore, in a recent mouse study, a chimeric humanized anti-HMGB 1 antibody alleviated acetaminophen-induced liver injury, supporting the role of the protein in the progression of drug-induced liver injury [8,24].

In a clinical AILI study, the sensitivity of HMGB 1 to hepatotoxicity was higher than that of ALT [20]; in one study, measurements were collected from patients whose ALT levels were within the normal range at the time of admission. In these samples, the total level of HMGB 1 increased significantly in patients with subsequent liver injury [25]. In a second study, highly acetylated HMGB 1 was observed only in AILI patients who died or needed liver transplantation, indicating that, compared to those who survived spontaneously, the form of HMGB 1 may be an indicator of poor prognosis after AILI. Although total HMGB 1 can be quantified by immunoassay, there is no antibody that can measure the different isomers; therefore, mass spectrometry is needed to determine the post-translational state of HMGB 1. However, in the above AILI study, the determination of total HMGB 1 has also been proven to be a sensitive biomarker of drug-induced liver injury [25].

3.2 Exons

As mentioned earlier, DAMPs can be released through hepatocyte necrosis and activate innate immune cells in the liver, which is considered necessary, but not sufficient, to trigger an adaptive immune attack of the liver. However, there is increasing evidence that hepatocyte necrosis is not necessarily a prerequisite for the release of DAMPs and liver adaptive immune attacks. For example, in clinical trials of melagatran, lumiracoxib, and lapatinib, a strong HLA correlation was observed, and the increase of serum ALT was relatively small. In addition, in the case of DILI caused by the antibiotic isoniazid, drug reactive T cells have been found in the patient's blood before the increase in serum ALT. These observations show that the slight increase of serum ALT does not necessarily reflect hepatocyte necrosis caused by drugs but will trigger adaptive immune attack, and the initial hepatocyte death is mediated by adaptive immune attack. In the absence of hepatocyte death, an adaptive immune attack on the liver can be triggered, which is consistent with hepatitis B infection. Hepatitis B virus does not cause cytolysis but produces liver specific adaptive immune attacks, leading to hepatocyte necrosis and clinical diseases. Recent data suggest that in the absence of cell death, DAMPs may spread in hepatocyte extracellular bodies through drug-induced hepatocyte stress. The observation also showed that, compared with the exons released by control hepatocytes, the exons released by hepatocytes treated with acetaminophen in a sub toxic dose of acetaminophen had greater activation of monocytes. It is reasonable to emphasize that, but not dead hepatocytes release damps (possibly new antigens) in

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exons, and then move and activate innate immune cells. This is a critical topic of study because, due to the porous barriers of liver endothelial cells, liver derived exons can enter the blood circulation and could be used as blood biomarkers. Therefore, the isolation of exons from peripheral blood and HMGB 1 may be an important biomarker for early prediction of clinical drug-induced liver injury [3,17,26].

4. Gene assay

Scholars who study hepatotoxicity have found risk alleles that have a much higher risk of susceptibility to DILI. Furthermore, HLA allele detection had a high negative predictive value. Therefore, because of the particularity of drugs, it has been used to exclude DILI: when patients take more than one hepatotoxic drug, the high negative predictive value of genetic testing has been used to identify the correct drug for DILI, and the inclusion of genetic testing can improve the performance characteristics of the DILI causality assessment tool. The variation among individuals in the human genome has been considered to be one of the reasons for the difference in sensitivity to drug reactions and adverse drug reactions [27].

4.1 Application of gene detection in clinical diagnosis

The performance of the "gene test" for the diagnosis of DILI is usually described as the sensitivity and specificity calculated from case-control studies aimed at identifying risk alleles, which have very high negative predictive values (> 0.95).Therefore, when the importance of diagnosis is clear, they can be used to exclude adverse liver reactions caused by specific drugs, allowing continued effective drug treatment and drawing attention to the manifestations of an atypical, treatable situation, such as autoimmune hepatitis, which is characterized by remission and recurrence in the absence of a classical autoantibody pattern. The high negative predictive value of the gene test can be used to identify the drug that cause liver injury when the patient has been exposed to multiple drugs. In a recent report, a patient with cholestatic hepatitis was exposed to fluxicillin and androgen synthetic steroids. The lack of alleles was used to exclude fluxicillin as an offending agent, and the cause of the liver injury considered to be the synthetic steroids. Another clinical example of effective use of gene detection is in the evaluation of jaundice. In a study on acute liver injury, clinicians used gene detection as a diagnostic tool, among which DILI is a differential diagnosis, and causality assessment of suspected DILI is a challenging process. The inclusion of genetic testing in specific cases may improve the consistency and accuracy of causality assessment tools such as the Roussel Uclaf causality assessment method (rucam).*Hla-B*5701* [28,29].

4.2 future of pharmacogenetics

Current research has confirmed that some common gene variants are closely related to DILI; however, the basic knowledge of genetic factors of hepatotoxicity is not sufficient to make it widely used in clinical settings. Additionally, rare gene variants may have a much greater impact on DILI because of its inherent methodological limitations, and GWAS cannot detect these variants. Whole genome sequencing technology involves mining the genetic contribution of the whole human genome to adverse drug reactions, which is expected to greatly increase the proportion of adverse reactions that can be associated with genotyping or diagnosis.

In the past decade, the understanding of non-genetic factors affecting DILI susceptibility has improved significantly. For example, if one in every 10000 people exposed to flucloxacillin has DILI, the risk of DILI is 1 / 500 for people carrying flucloxacillin, and the estimated risk of people over 65 years of age carrying dangerous alleles increases to 1 / 100. Therefore, the combination of drug-related, genetic, and non-genetic host factors of lumiracoxib, which affects the development risk of DILI, has a greatly improved performance compared with the genetic test used alone; therefore, the clinical applicability of an integrated algorithm is increased. *Hla-B*5701* [30].

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