It is well known that HNF4α positively regulates the expression of bile acid-synthesizing enzymes such as CYP7A1 and CYP8B1. Particularly, CYP7A1 catalyzes the first and rate-limiting step in the bile acid synthetic pathway (Pikuleva, 2006). The induction of miR-24 and miR-34a would result in decreased bile acid synthesis *via* mainly CYP7A1. In addition, gluconeogenic enzyme PEPCK (phosphoenolpyruvate carboxykinase) was also down-regulated by the decrease of the HNF4α expression by miRNAs. Thus, miR-24 and miR-34a affect the various hepatic functions through the negative regulation of HNF4α expression (Takagi *et al.*, 2010). The new information was provided on the negative feedback regulation of bile acids synthesis as well as that of various downstream genes and aberrant cell cycles, which contributes to the study of the mechanism of the bile acid-dependent toxicity.

【*Human ERα (Estrogen Receptor α)*】

ERα is an important marker for the prognosis and is predictive of the response to endocrine therapy in breast cancer patients. Up to one-third of patients with breast cancer lack ERα at the time of diagnosis. It was previously reported that ERα regulates the expression of human CYP1B1, which catalyzes the metabolism of estradiol to the toxicologically active endogenous metabolite, 4-hydroxyestradiol (Tsuchiya *et al.*, 2004). It was first demonstrated by Adam *et al.* (2007) that human ERα is regulated by miR-206, whereas the activation of ERα results in the decreased expression of miR-206, showing mutually inhibitory regulation. MiR-221 and miR-222 inhibited human ERα expression at the translational level (Zhao *et al.*, 2008; Xiong *et al.*, 2010). The miR-22 expression level and ERα protein expression were

inversely associated and ERα is the primary target (Pandey and Picard, 2009). MiR-375 was a potential target of RASD1 (dexamethasone-induced Ras-related protein 1), and it was found that RASD1 negatively regulates the ERα expression (Simonini *et al.*, 2010). MiR-27a indirectly regulates human ERα *via* ZBTB10, a specific protein repressor for Sp2, Sp3, and Sp4 (Li *et al.*, 2010). From these lines of data, the authors suggested that these miRNAs may be potential targets for anti-estrogen therapy, however, experimental conditions varied considerably when using cultured hepatoma cell lines, established cells from tumor tissues and/or tissue samples, and there was a lack of quantitative consideration of the data.

【***Other Nuclear Receptors***】

Peroxisome proliferator-activated receptor α (PPARα) is an important transcriptional factor that regulates genes encoding endo/xenobiotic enzymes and lipid metabolizing enzymes. The overexpression and inhibition of miR-21 or miR-27b in HuH7 cells significantly decreased and increased the PPARα protein level, respectively, but not PPARα mRNA level (Kida *et al.*, 2011). These miRNAs negatively regulate the expression of PPARα in human liver, affecting the expression of its downstream gene. Since PPARα is an important regulator of fatty acid catabolism, miR-21 and miR-27b would be one of the factors controlling lipid metabolism.

In spite of the different experimental conditions in each research group, miR-27a and miR-27b were reported to be targets of peroxisome proliferator-activated receptor γ (PPARγ) (Karbiener *et al.*, 2009; Lin *et al.*, 2009; Jennewein *et al.*, 2010; Kim *et al.*, 2010). Interestingly, the inhibition of miR-27b, induced by lipopolysaccharide (LPS), reversed the

PPARγ mRNA degradation, whereas miR-27b overexpression decreased PPARγ mRNA, which affect the LPS-induced expression of the pro-inflammatory cytokines, tumor necrosis factor α (TNFα) and interleukin (IL)-6 (Jennewein *et al.*, 2010). The expression of miR-27a and miR-27b was increased in fat tissue of obese mice and was regulated by hypoxia, (Lin *et al.*, 2009). MiR-130 potently repressed PPARγ expression by targeting both the PPARγ mRNA coding and 3'-UTR regions, thereby controlling adipocyte gene expression programs (Lee *et al.*, 2010). In addition, since the PPARγ agonists pioglitazone and rosiglitazone upregulated the transcription of human CYP26, the enzyme responsible for all-trans-retinoic acid metabolism and elimination, it would be interesting to examine their possible involvement in idiosyncratic drug-induced liver injury.

Retinoid X receptor α (RXRα) is the target of miR-27a and miR-27b in rat primary hepatic stellate cells (Ji et al., 2009). The sequences of MRE on the RXRα mRNA are highly conserved among species, implying that human RXRα may also be regulated by miR-27. RXRα is involved in multiple signaling pathways related to cell proliferation and differentiation, mainly as the heterodimeric partner of several nuclear receptors (Imai *et al.*, 2001). Therefore, miR-27 seems to be involved in the regulation of a wide variety of transcriptional factors affecting inter- and intraindvidual differences in drug response, adverse reactions and toxicity outcomes. In addition, as mentioned above, CYP1B1 is a direct target of human miR-27b (Tsuchiya *et al.*, 2006).

The miRNA regulatory networks are complex because a single miRNA can target numerous mRNAs, often in combination with other miRNAs and nuclear receptors, and a single target can be regulated by different kinds of miRNA. This research field contributes

greatly to understanding the mechanisms of toxicological outcomes and for predicting the risk susceptibility for drugs, chemical toxicants and environmental pollutants.

Potential Modulation of Toxicology-related MiRNA Expression In Vivo and In Vitro

The role of miRNA in the response to xenobiotics, drugs and chemical toxicants, remains to be established, but there can be little doubt that miRNAs are important in the cellular and *in vivo* responses to xenobiotics (Taylor and Gant, 2008). However, the regulatory networks of miRNAs are complex, and the decreased expression of miRNAs will generally lead to high expression of the target proteins and subsequent toxicological phenomena. The increased expression of miRNAs will generally be associated with the low probability of toxicological phenomena. Therefore, changes of miRNA-related gene regulation should affect overall the toxicological processes. From these lines of consideration, comprehensive studies by using miRNA arrays as well as DNA microarrays and proteomics analyses will be used as powerful tools to investigate the mechanisms of individual susceptibility to toxicants and adverse drug reactions. Recently, a large number of studies on the roles of miRNAs in cancer have been investigated, but few studies have reported the altered expression profiles of miRNA in drug-related adverse reactions and in toxicology-related outcomes. In this paragraph, potential modulation of toxicology-related miRNA expression in vivo and in vitro will be discussed.

In plant, it is reported that oxidative stress induces superoxide dismutase (SOD) genes post-transcriptionally accompanied by the downrgulation of plant miR-398 (Sunker *et al.*, 2006). Human miR-222 regulates the matrix metalloproteinase 1 (MMP1) expression level

through both direct cis-regulatory mechanisms (targeting MMP1 mRNA) and direct trans-regulatory mechanisms (indirect controlling of MMP1 gene expression by targeting SOD2) (Liu *et al.*, 2009). SOD2-dependent up-regulation of MMPs may, at least in part, contribute to the increased invasion and metastatic capacity of tumors displaying elevated SOD2 levels. Arsenite, which is known to activate nuclear factor-erythroid 2-related factor 2 (Nrf2) (Aono *et al.*, 2003), affects miRNA expression in human lymphoblastoid TH-6 cells (Marsit *et al.*, 2006). Cigarette smoking causes the down-regulation of many miRNAs in the lungs of both mice and rats (Izzotti *et al.*, 2011) as well as in human airway epithelial cells (Schembri *et al.*, 2009). The mechanistic relationships between the stress and miRNA expression profiles were studied only in vitro conditions, thus experiments to elucidate the mechanism of *in vivo* responses of miRNA are necessary.

It was demonstrated that miR-17* was able to suppress the primary mitochondrial antioxidant enzymes, such as SOD2, glutathione peroxidase-2 (GPX2) and thioredoxin reductase-2 (RXR2), in prostate cancer PC-3 cells (Xu *et al.*, 2010). The luciferase reporter activities were suppressed by the overexpression of miR-17* and disulfiram, a dithiocarbamate drug, induced the expression level of mature miR-17*. It was previously reported that miR-17 is able to silence hypoxia-inducible factor (HIF)-1α expression (Taguchi *et al.*, 2008). From these reports, it is conceivable that the miR-17 and miR-17* might involve in maintaining the homeostasis against cellular redox stress.

Several studies employing toxicogenomics were attempted to evaluate the responses of miRNAs in rodent liver and were intended to establish biomarker(s) for toxicological risk assessment. It was reported that single administration of acetaminophen or carbon

tetrachloride to rats resulted in different expression profiles of miRNA in the liver (Fukushima *et al.*, 2007). MiRNA-298 and miR-370 presumably target oxidative stress-related enzymes, thioredoxin reductases. The sample size in this study was very small and no statistical analyses could be conducted. However, miRNA suppression occurred as early as 6 hours later, which coincided with the early phase toxicity, prior to cellular necrosis.

It has been demonstrated that let-7C, a miRNA important in cell growth, was inhibited with the potent PPARα agonist WY-14,643 in wild-type mice (Shah et al., 2007). In addition, let-7C was also shown to target c-myc *via* direct interaction with the 3'-untranslated region of c-myc, which subsequently increasing the expression of the oncogenic miR-17-92. Thus, a let-7C signaling cascade will be critical for PPARα agonist-induced liver proliferation.

The effect of TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) on hepatic miRNA expression in TCDD-resistant H/W rats and TCDD-sensitive L-E rats was investigated (Moffat *et al.*, 2007). TCDD treatment did not cause any potent changes in hepatic miRNA expression. The same data were also obtained in mice (Moffat *et al.*, 2007). The authors concluded that dwonregulation of hepatic miRNA by TCDD is unlikely to play a significant role in TCDD toxicity in adult rodent liver. In addition, BaP (3 daily doses of 150 mg/kg) was orally administered to male mice (Yauk *et al.*, 2011). DNA microarray analyses showed widespread changes in gene expression (>400 genes), but almost no changes in miRNA expression. Although miRNA expression would be co-ordinately regulated with the mRNA transcript, it is interesting that hepatic miRNA *in vivo* is not directly responsive to AHR-agonists such as TCDD and BaP *in vivo* in rodent (Yauk *et al.*, 2011).

Concerning *in vitro* studies, cells treated with γ-irradiation resulted in no alteration in the miRNA expression level, but exposure to sodium arsenite resulted in significant increases in the miRNA level (Marsit *et al.*, 2006). Human lymphoblast cell line IM9 treated with γ-irradiation exhibited various changes in miRNAs in a dose-dependent manner (Cha *et al.*, 2009). The controversial results regarding γ-irradiation were possibly due to the cell specificity or experimental conditions. In addition, TCDD in rodent exhibited vastly different responses compared to hepatic cell cultures (Moffat *et al.*, 2007). From these lines of evidence, it is conceivable that there are different responses in miRNA changes between *in vitro* and *in vivo*, and an *in vitro* study alone may not able to predict the *in vivo* responses of miRNA for these kinds of toxic chemicals in vivo. The up- or down-regulated miRNAs could alter the expression of numerous target mRNAs in vivo and lead to changes of toxicological phenotypes.

A long time exposure study of drugs or toxic chemicals in rodent would produce different results in miRNA expression profiles compared to acute toxicity studies as mentioned above. It was reported that tamoxifen, a potent hepatocarcinogen in rats, was administered to Fisher 344 rats for 24 weeks leading to statistically significant differential expression of 33 miRNAs (20 genes up-regulated; 13 genes down-regulated) in the liver (Pogribny *et al.*, 2007). A significant up-regulation of oncogenic miRNAs, such as the miR-17-92 cluster, miR-106a, and miR-34, were observed. A number of miRNAs, including miR-152 and miR-195, were down-regulated in the livers of tamoxifen-treated rats. These miRNAs are frequently down-regulated in solid tumors (Murakami et al., 2006). In addition, the differential expression of 55 miRNAs (31 genes up-regulated; 25 genes down-regulated)

in mice fed a diet containing heahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a common environmental contaminant, at 5 mg/kg for 28 days (Zhang and Pan, 2009). A significant up-regulation of oncogenic miRNAs and a significant down-regulation of tumor-suppressing miRNAs, such as let-7, miR-17-92 cluster, miR-10b, miR-15, miR-16, miR-26, and miR-181, were observed. Thus, chronic administration of toxic chemicals will affect the changes of miRNA expression in the liver *in vivo*, which are different compared to those of acute administration.

Role of MiRNA in Immune-mediated Drug-induced Liver Injury

Cytokines have been known as a causal factor in the depression of CYP-associated drug metabolism in humans during inflammation and infection (Abdel-Razzak *et al.*, 1993), which influence the susceptibility to various drugs and toxic chemicals. Statistically significant effects of cytokines on the down-regulation of CYP activity in the human population have also been reported. IL-6 was demonstrated to decrease both the rifampicin- and phenobarbital-mediated induction of CYP2B6, CYP2C8, CYP2C9 and CYP3A4, whereas, the transcriptional activity of PXR and CAR is not affected by IL-6 (Pscussi *et al.*, 2000).

Halothane- and α-naphtylisothiocyanate-induced liver injury is reported to mediate by IL-17 (Kobayashi *et al.*, 2009; Kobayashi *et al.*, 2010). IL-4 mediated dicloxacillin- and flutamide-induced liver injury in mice (Higuchi *et al.*, 2011). IL-6 and IL-4 are essential ILs for the differentiation of Th17 and Th2 cells respectively from naïve T cells. The generation of reactive metabolites by the administered drugs is catalyzed by P450s, which are suggested

to be a major causal factor for the initiation of drug-induced liver injury (DILI), and thereafter the exacerbation of DILI will mediated by ILs.

During the last years, many studies have highlighted the fact that miRNAs play a critical role in the differentiation and function of the adaptive and innate immune system (Carissimi *et al.*, 2009). Indeed, several studies demonstrated the involvement of ILs in relation to miRNA-related diseases and cancer as follows. The expression of miR-148a, miR-152 and miR-301 was decreased in IL-6-overexpressing malignant cholangocytes. IL-6 can increase the exprssion of DNA methyltransferase-1, which is a target of miR-148a and miR-152 (Braconi *et al.*, 2010). The expression of bone morphogenic protein receptor type II (BMPR2) through a miR-17/92 pathway is modulated by IL-6. Because IL-6 signaling is mainly mediated by STAT3, the expression of STAT3 was knocked down by small interfering RNA, which abolished the IL-6-mediated expression of miR-17/92 (Brock *et al.*, 2009). MiR-21 contributes to the oncogenic potential of Stat 3 in multiple myeloma cells. MiR-21 induction by IL-6 was strictly Stat 3 dependent through a highly conserved enhancer (Loffler *et al.*, 2010). Six miRNAs, let-7a, miR-26, miR-146a/b, miR-150, and miR-155 were significantly up regulated in the IL-17 producing T cells. MiR-146a is associated with IL-17 expression in the peripheral blood mononuclear cells (PBMC) in rheumatoid arthritis patients (Niimoto *et al.*, 2010). Interestingly, microRNA expression analysis during the tolerized state of THP-1 cells showed only miR-146a overexpression, suggesting its important role in LPS tolerance. Transfection of miR-146a into THP-1 cells mimicked LPS priming, whereas transfection of miR-146a inhibitor largely abolished LPS tolerance (Nahid *et al.*, 2009).

Immune-mediated DILI might be regulated by miRNAs. The mechanism of idiosyncratic DILI, in which immune-mediated factors will be essential, will be clarified by progress in miRNA research as well as studies of the metabolic activation reactions catalyzed by P450s.

Relevance of MiRNA-related Genetic Polymorphism to Pharmacogenetics/genomics Studies.

The human genome contains about 3 billion base pairs, and single-base variations (called SNPs, single nucleotide polymorphisms) are on the average as 1/1000 bases. Thus, the SNPs may affect either the expression or activities of various enzymes, and are associated with the differences in drug responses and adverse effects of drugs and toxic chemicals. SNPs are present not only in the mRNA but also in mature miRNA sequences. SNPs in primary (pri)-miRNAs, precursor (pre)-miRNAs or mature miRNA could modify various biological processes by influencing the processing or target selection of miRNAs (Iwai and Naraba, 2005; Duan *et al.*, 2007). SNPs in pri- or pre-miRNA are relatively rare. Only ~10% of human pre-miRNAs have documented SNPs and <1% of miRNAs have SNPs in the functional seed sequence region (Saunders *et al.*, 2007). Although seed region variations in miRNA seem to be very rare, they have the potential to influence the expression of hundreds of genes and related pathways. An interesting study regarding the correlation between SNPs in the miRNA sequence and the clinical drug therapy was published by Boni *et al.* (2010). A SNP [rs7372209] in the pri-miR26a gene and a SNP [rs1834306] in the pri-miR-100 gene were significantly associated with the tumor response or time to progression in 61 metastatic

colorectal cancer patients treated with 5-fluorouracil and CPT-11 (Boni *et al.*, 2010). This is the first report to suggest a relationship between the clinical outcome of drug therapy and SNPs in the miRNA-biogenesis machinery, in both primary and precursor miRNAs, but the molecular mechanisms by which these polymorphisms act have not yet been clarified (Shomron, 2010).

Compared to the low level of variation in the functional regions of miRNAs, a considerable level of variation at the target sites is conceivable. Many miRNA target-related polymorphisms are reported to be associated with the phenotypes of diseases, because a gain-of-function or loss-of-function would result in changes in the expression of target mRNAs (Sethupathy and Collins, 2008). However, actually, there are few examples regarding miRNA-related polymorphisms affecting the drug response, adverse reactions and toxicological outcomes, such as human dehydrofolate reductase (DHFR) and sulfotransferase isoform 1A1 (SULT1A1) as mentioned later.

Recent genome-wide analyses of human SNPs have revealed that many polymorphisms exist in the miRNA binding sites. Approximately 400 SNPs were found at verified target sites or predicted target sites, and about 250 SNPs potentially create novel target sites for miRNAs in humans (Saunders *et al.*, 2007). More recently, roughly 20,0000 miRNA target-related polymorphisms were systematically searched using Patrocles (http://www.patrocles.org/Patrocles.htm) and PolymiRTS (http://compbio.uthsc.edu/miRSNP/). Those *in silico*-predicted database should be carefully validated by functional studies in the future.

Concerning a miRNA target polymorphism in relation to drug metabolizing enzymes, it was demonstrated that a C829T SNP, a naturally occurring SNP, at the miR-24 recognition site in the 3'UTR of human dehydrofolate reductase (DHFR) leads to DHFR overexpression and methotrexate resistance (Mishra *et al.*, 2007). Cells with the mutant 3'-UTR had a 2-fold increase in DHFR mRNA half-life, expressed higher levels of DHFR mRNA and DHFR protein, and were 4-fold more resistant to methotrexate as compared with wild type cells. In a case-controlled study of childhood leukemia patients, those possessing C to T SNP occurred with 14.2% allelic frequency in the Japanese population. The T allele of the SNP resulted in the loss of the miR-24-mediated regulation of DHFR, high DHFR protein levels and methotrexate resistance. This finding may be useful in predicting the clinical efficacy of methotrexate treatment (Mishra *et al,* 2007, Mishra and Bertino, 2009).

Sulfotransferase isoform 1A1 (SULT1A1) is one of the essential enzymes in the metabolism of endo- and exobiotics, and dietary and environmental procarcinogen/promutagen activation and/or detoxification. SULT1A1 activity shows high interindividual variability in the expression of the protein (Jones *et al.*, 1993) and genetic polymorphism was suggested, although not fully accounted for by the variation of SULT1A1 activity. A SNP in the 3'-UTR (972C>T [rs1042157]) is significantly associated with the SULT1A1 mRNA level ($P = 0.029$) and enzymatic activity ($P = 0.012$) (Yu *et al.*, 2010). From subsequent functional analyses, it was found that miR-631 directly regulates SULT1A1 expression in an allele-specific manner of the 3'-UTR (SNP of 972C>T), which provides a new insight into the mechanism of SULT1A1 regulation and also new information for

molecular epidemiology and risk assessment studies of heterocyclic amines such as *N*-hydroxyarylamines, *N*-hydroxy-heterocyclic amines and arylhydroxamic acids.

Drug responses or susceptibility to xenobiotic toxicity will be predicted by miRNA expression profiles. Interindividual variability in adverse drug responses and toxicity will be partly predicted by using miRNA-related polymorphisms. This new class of miRNA-related polymorphisms may contribute to the interindividual differences in drug responses, and toxicant-induced adverse events.

MiRNAs in Plasma as Potential Toxicological Biomarkers.

Circulating miRNA in plasma was first demonstrated as the diagnosis biomarkers of lung (Chen *et al.*, 2008), colorectal (Chen *et al.*, 2008), and prostate cancers (Mitchell *et al.*, 2008). The dynamic changes of circulating miRNAs in the plasma resulting from drug exposure was first demonstrated by Wang *et al.* (2009). Mice dosed with acetaminophen changed miR-122 and miR-192 in liver and plasma in a dose- and exposure-duration manner that paralleled ALT and histopathological changes in the liver. The changes of miRNAs were earlier than those of ALT. This discovery of plasma miRNA opens up the great possibility of using miRNAs as a sensitive, informative and non-invasive potential biomarker for drug induced liver injury and toxicological outcomes. Many studies have already been conducted regarding circulating miRNA and the clinical diagnosis and prognosis of cancer, but few papers have been published in relation to toxicological studies. It was reported that increased plasma concentrations of miR-122, miR-133a, and miR-124 corresponded to injuries in liver, muscle and brain, respectively, while each of these is the abundant and specific miRNA in each organ

(Laterza *et al.*, 2009). The miR-122 concentration in plasma increased earlier than the increase of aminotransferase activities in the blood (Zhang *et al.*, 2010). This change was more specific for viral-, alcohol-, and chemical-related liver injury than other organ damage and was a more stable and reliable biomarker (Zhang *et al.*, 2010). Although several challenges remain to be addressed, circulating miRNAs have great potential in toxicological studies, *e.g.*, as a novel, noninvasive method for the extrapolation of the toxicity data from animal to human.

Conclusions

This review has described several examples of miRNAs and their relationship to drug metabolizing enzymes, their interactions with nuclear receptors as well as implications for toxicological studies. Although the field of miRNA-related drug metabolism and toxicology studies is still in its infancy, we are now entering an interesting period in which the contribution of miRNAs in controlling various pharmacological and toxicological outcome will become more clear. In the near future, miRNA profiling may lead to the discovery of novel miRNA biomarkers that might improve the prediction of metabolic activation and detoxification of drugs *in vivo* in human. As the methodologies for miRNA studies are now becoming more widespread, comprehensive understanding of miRNAs will lead to progress in toxicology research fields.

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