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Review

The Emerging Role of Human Esterases

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Summary: In this review, novel aspects of the role of esterases, which contribute to the metabolism of 10% of therapeutic drugs, are described. Esterases hydrolyze the compounds that contain ester, amide, and thioester bonds, which cause prodrug activation or detoxification. Among esterases, carboxylesterases are well known to be involved in the hydrolysis of a variety of drugs. Additionally, other esterases have recently received attention for their pharmacological and toxicological roles. Arylacetamide deacetylase (AADAC) is involved in the hydrolysis of flutamide, phenacetin, and rifamycins. AADAC is associated with adverse drug reactions because the hydrolytic metabolites of flutamide and phenacetin appear to be associated with hepatotoxicity and nephrotoxicity/hematotoxicity, respectively. Paraoxonase and butyrylcholinesterase hydrolyze pirocarpine/simvastatin and succinylcholine/bambuterol, respectively. Although the esterases that hydrolyze the acyl-glucuronides of drugs have largely been unknown, we recently found that α/β hydrolase domain containing 10 (ABHD10) is responsible for the hydrolysis of mycophenolic acid acyl-glucuronide in human liver. Because acyl-glucuronides are associated with toxicity, ABHD10 might function as a detoxification enzyme. Thus, various esterases, which include enzymes that have not been known to hydrolyze drugs, are involved in drug metabolism with different substrate specificity. Further esterase studies should be conducted to promote our understanding in clinical pharmacotherapy and drug development.

Keywords: esterases; drug hydrolysis; activation of prodrugs; drug detoxification; drug adverse reaction

Introduction

Drug metabolism refers to the biochemical transformation of a compound into its more polar chemical form. Drug-metabolizing enzymes are responsible for the detoxification of many drugs and xenobiotics to facilitate their excretion, which is an important determinant of drug action. Drug metabolism is classified into phase I and phase II reactions. Phase I metabolism usually results in a change in molecular weight or water solubility of the substrate, and its reactions confer the sites at which phase II metabolism occurs. Phase II conjugation causes an appreciable increase in substrate molecular weight and water solubility. Of the enzymes involved in phase I reactions, cytochrome P450 enzymes play a pivotal role in drug metabolism (*i.e.*, approximately 75% of clinically used drugs), followed by esterases, which contribute to the metabolism of 10% of the clinical, therapeutic drugs that contain ester, amide, and thioester bonds.[1)]

Esterases are responsible for prodrug activation and drug detoxification and have been divided into 3 categories (A-, B-, and C-esterases) based on substrate specificity and sensitivity to various inhibitors (*i.e.*, organophosphates and sulfhydryl reagents). A-esterases rapidly hydrolyze aromatic esters, which include organophosphate diisopropyl fluorophosphates (DFP). The representative A-esterase enzyme is paraoxonase (PON). B-esterases are inhibited by organophosphates, carbamate, and organosulfur compounds. In humans, most esterases, such as carboxylesterases (CESs) and cholinesterases, are B-esterases and members of the serine esterase superfamily that possess a serine residue in the conserved -Gly-X-Ser-X-Gly- motif at the active site. C-esterases neither hydrolyze organophosphate esters nor are inhibited by them. To our knowledge, the C-esterases that likely hydrolyze drugs have not been identified. Nowadays, the above classification is not generally applicable because the function of each esterase has been clarified.

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Table 1. Summary of esterases responsible for drug metabolism

Enzyme		EC number	Expressed tissue	Representative substrate drug
CES1	Carboxylesterase 1	EC3.1.1.1 EC3.1.1.56	Liver, lung	Capecitabine, clopidogrel, imidapril, methylphenidate, oseltamivir
CES2	Carboxylesterase 2	EC3.1.1.1 EC3.1.1.56 EC3.1.1.84	Liver, small intestine, kidney	CPT-11, flutamide, heroin, prasugrel
AADAC	Arylacetamide deacetylase	EC3.1.1.3	Liver, small intestine	Flutamide, phenacetin, rifampicin, rifabutin, rifapentine
BCHE	Butyrylcholinesterase	EC3.1.1.8	Liver, plasma	Bambuterol, cocaine, CPT-11, mivacurium, succinylcholine
PON1	Paraoxonase 1	EC3.1.1.2 EC3.1.1.81 EC3.1.8.1	Liver, plasma	Olmesartan medoxomil, pilocarpine, prulifloxacin
PON3	Paraoxonase 3	EC3.1.1.2 EC3.1.1.81 EC3.1.8.1	Liver, plasma	Lovastatin, simvastatin, spironolactone
ABHD10	α/β Hydrolase domain containing 10	EC3.4.-.-	Liver, small intestine	Mycophenolic acid acyl-glucuronide
APEH	Acylpeptide hydrolase	EC3.4.19.1	Liver	Valproic acid acyl-glucuronide
SIAE	Sialic acid 9-O-acetylesterase	EC3.1.1.53	Liver	Alacepril
CMBL	Carboxymethylenebutenolidase homolog	EC3.1.-.- EC3.1.1.45	Liver, kidney, small intestine	Olmesartan medoxomil, faropenem medoxomil, lenampicillin
BPHL	Biphenyl hydrolase-like protein	EC.3.1.-.-	Small intestine, liver, lung	Valacyclovir, valganciclovir
PAFAH	Platelet-activating factor acetylhydrolase	EC3.1.1.47	Erythrocytes	Aspirin
ALDH	Aldehyde dehydrogenase	EC1.2.1.3	Liver	Nitroglycerin
Albumin		EC6.1.1.16	Liver, plasma	Aspirin, ketoprofen acyl-glucuronide

Among these esterases, CES enzymes are well known for their involvement in drug metabolism. Some human esterases other than CES enzymes have also been demonstrated to hydrolyze several drugs. For example, we found that arylacetamide deacetylase (AADAC), which was previously recognized as a lipase, and α/β hydrolase containing (ABHD) 10, whose function was previously unknown, are involved in the hydrolysis of clinical drugs.[2–5] Therefore, recent evidence demonstrates that various esterases are involved in drug metabolism (**Table 1**). A comprehensive understanding of esterases would be useful in clinical pharmacotherapy and drug development. This review describes the novel aspects of the roles of the enzymes that are responsible for drug hydrolysis.

Carboxylesterase

CES enzymes are members of the serine esterase superfamily that possesses the serine residue of the conserved -Gly-X-Ser-X-Gly- motif at the active site and are responsible for the hydrolysis of a wide variety of xenobiotic and endogenous compounds. Human CES enzymes are classified into 5 subfamilies: CES1, CES2, CES3, CES4A, and CES5A.[6] The *CES1* subfamily is composed of 2 genes, *CES1* (a functional gene, previously termed *CES1A1*) and *CES1P1* (a pseudogene, previously termed *CES1A3*). Previously, another functional *CES1* gene (previously termed *CES1A2*) was reported to be located at the locus that corresponds to the *CES1P1* gene, but our later study determined it to be a *CES1P1* variant.[7] The other human CES subfamilies are composed of the corresponding single gene. CES1 and CES2 are reported to be responsible for the biotransformation of a number of clinically used drugs and prodrugs. CES1 is predominantly expressed in the human liver and marginally expressed in the gastrointestinal tract.[8] The monomeric molecular weight of CES1 is 60 kDa, and CES1 is present as a 180-kDa trimer.[9] CES2 is expressed in the liver as well as in extrahepatic tissues, such as the gastrointestinal tract and kidney.[10] In contrast to CES1, CES2 is a 60-kDa monomer.[11] These CES enzymes contain a hydrophobic signal peptide at the N-terminus for trafficking through the endoplasmic reticulum and a retention sequence at the C-terminus for interacting with the Lys-Asp-Glu-Leu (KDEL) receptor. Therefore, it has been hypothesized that CES enzymes are specifically localized in the lumen side of the endoplasmic reticulum.[12] However, CES1 and CES2 enzymes are also present in human liver cytosol at comparable levels; although, it is unclear whether cytosolic CESs initially possess no signal peptide, the signal peptide had been removed before CESs move to the endoplasmic reticulum, or CESs in endoplasmic reticulum move to the cytosol.[10,13]

Although the amino acid homology of CES1 and CES2 around a serine residue in an active site is high, they exhibit significant differences in their substrate specificity. Clinical drugs such as imidapril, methylphenidate, osertamivir, and clopidogrel are specifically hydrolyzed by human CES1.[14–17] Capecitabine and oxybutinin are hydrolyzed by CES1 but are also hydrolyzed by CES2 to a minor extent.[18,19] CPT-11 and prasugrel are preferentially hydrolyzed by CES2.[20,21] A small alcohol group and large acyl group are common features of CES1 substrates, except for oxybutinin, whereas CES2 substrates have a large alcohol group and small acyl group (**Table 1**).

The hydrolysis of drugs that are catalyzed by CES enzymes exhibits large interindividual variability,[17] which may be due to quantitative and qualitative differences in the CES enzymes. Our previous study reported that CES1 protein expression exhibited approximately 22-fold and 58-fold differences in microsomes and cytosol, respectively, among 12 human livers.[7] Xu *et al.* reported that the CES2 protein expression exhibited 3-fold and 15-fold differences in microsomes and cytosol, respectively, among 12 human livers.[10] Two single nucleotide polymorphisms (SNPs) of g.9486G>A and g.12754T>del were found to be associated with an increased area under the blood concentration time curve (AUC) of methylphenidate.[22] The former SNP leads to an amino acid substitution of G143Q, and the latter causes a frameshift at codon 260 that leads to 39 altered residues and a subsequent premature stop codon. These mutations were also reported to be a causal factor of defective trandolapril hydrolase activity.[23] In CES2, amino acid substitutions of R34W and V142M have been reported to lose CPT-11 hydrolase activity.[24] Therefore, a large degree of interindividual variability of CES enzymes is due to the differences in CES expression levels as well as genetic polymorphisms.

To examine the possibility of drug-drug interactions that are caused by CES inhibition, we previously investigated the effects of 11 antidiabetic or 12 antihyperlipidemic drugs on the hydrolase activity of imidapril, which is a CES1-specific substrate, using a recombinant enzyme and human liver microsomes (HLM).[25] Angiotensin-converting-enzyme (ACE) inhibitors, such as imidapril and derapril, which are substrates of CES enzymes, are often coadministered with various antidiabetic or antihyperlipidemic drugs in clinical pharmacotherapy. The imidapril hydrolase activity by CES1 was strongly inhibited by lactone ring-containing statins, such as simvastatin and lovastatin, and thiazolidinediones, such as troglitazone and rosiglitazone, but the inhibitory potency would not be significant *in vivo* because the maximum free concentrations of these inhibitors at the inlet to the liver are lower than the *Ki* values. Therefore, drug-drug interactions *via* CES enzymes may be rare in humans.

In drug development, species differences in enzyme activity, substrate specificity, and tissue distribution require attention. Ces enzymes in the mouse and rat are divided into 5 Ces subfamilies that are similar to human CES enzymes. However, different from human, the Ces1 and Ces2 subfamilies include 8 (Ces1a–Ces1h) and 8 (Ces2a–Ces2h) enzymes in mouse and 5 (Ces1a–Ces1f) and 7 (Ces2a–Ces2j) enzymes in rat.[6] It was reported that one of the Ces1 enzymes, Ces1c, does not contain an ER-lumen retention sequence at the C-terminus, which would cause secretion into the plasma.[26] Ces enzyme is present in the plasma of mice, rats, rabbits, horses, cats, and tigers, but not of humans.[27] The pharmacokinetic profile for irinotecan in Ces1c-deficient mice closely reflects that observed in humans.[28] Additionally, the difference in substrate specificity of CES enzymes between human and experimental animals has also been reported. For example, pranlukast, which is a leukotriene receptor antagonist, is efficiently hydrolyzed by rat Ces enzymes that are expressed in the liver, but it is not hydrolyzed by human CES enzymes.[29] Because the substrate specificity of each CES enzyme in various species is not well known, further elucidation will be required.

Arylacetamide Deacetylase

Human arylacetamide deacetylase (AADAC), which has a molecular weight of 45 kDa, is a microsomal serine esterase that is expressed in the liver and gastrointestinal tissues.[2] There is a single AADAC isoform in humans and other mammalians. Unlike CES enzymes, AADAC is a type II membrane protein that has an uncleaved N-terminal signal anchor sequence to retain on the lumen side of the endoplasmic reticulum.[30] AADAC was first identified as the enzyme that catalyzes the deacetylation of the carcinogen 2-acetylaminofluorene.[31] Although 2-acetylaminofluorene is a metabolite of aminofluorene, which is catalyzed by *N*-acetyltransferase (NAT), the acetyl metabolites of other known NAT substrates, such as sulfamethazine, *p*-aminobenzoic acid, procainamide, and *p*-aminosalicyclic acid, are not hydrolyzed by AADAC.[31] In addition to 2-acetylaminofluorene hydrolysis, AADAC has been recognized as a lipase because the active site of AADAC has strong homology with that of hormone-sensitive lipase.[32] In fact, human AADAC was proven capable of hydrolyzing cholesterol ester when expressed in yeast.[33]

Recently, we found that human AADAC is responsible for the hydrolysis of clinical drugs, such as flutamide, phenacetin, and rifamycins (**Table 1**).[2–4] The clinical significance of AADAC in the hydrolysis of these drugs is described below in detail. Flutamide is a nonsteroidal antiandrogen drug that is used for the treatment of prostate cancer. It occasionally causes severe hepatotoxicity.[34] Flutamide itself is not toxic when used at the appropriate clinical dose, but bioactivation of flutamide has been considered the cause of flutamide-induced hepatotoxicity.[35] Flutamide is mainly metabolized to 2-hydroxyflutamide, which is associated with the therapeutic effect, by human CYP1A2[36] and is also hydrolyzed to 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1)[36,37] (**Fig. 1A**) by AADAC and CES2.[2,38] FLU-1 is an arylamine, whose *N*-hydroxylation is generally recognized to be associated with toxicity.[39] In fact, several studies have suggested that flutamide-induced hepatotoxicity was caused by *N*-hydroxyl FLU-1, a metabolite of FLU-1 whose formation is catalyzed by CYP3A or CYP1A.[40–42] Therefore, the hydrolysis of flutamide by AADAC and CES2 is the initial step that leads to hepatotoxicity.

Phenacetin had been widely used as an analgesic antipyretic drug, but it was withdrawn from the market because of the occurrence of renal failure and hematotoxicity in some patients.[43,44] Phenacetin is mainly metabolized to

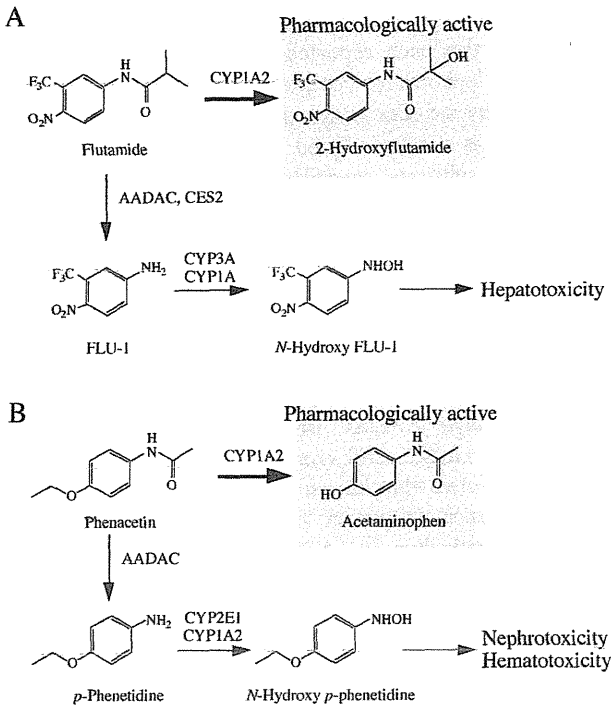

Fig. 1. Metabolic pathways of flutamide and phenacetin in humans

(A) Flutamide and (B) phenacetin are metabolized to pharmacologically active metabolites by CYP1A2. Their hydrolysis by AADAC leads to the formation of toxic metabolites.

acetaminophen, which is a pharmacologically active metabolite, by CYP1A2 and is hydrolyzed to *p*-phenetidine by AADAC (**Fig. 1B**). *p*-Phenetidine is further metabolized to *N*-hydroxyphenetidine, which has been considered to cause renal failure and hematotoxicity, such as methemoglobinemia and hemolytic anemia.[45–47] An *in vitro* analysis demonstrated that phenacetin-induced methemoglobinemia is caused by a hydrolytic metabolite which is formed by AADAC, and subsequent hydroxylation by CYP2E1 or CYP1A2.[48] *p*-Phenetidine is an arylamine, likely FLU-1. Therefore, because AADAC produces arylamines from *N*-acetylarylamine drugs, this would cause toxic effects.

Rifamycins, such as rifampicin, rifabutin, and rifapentine, are a group of structurally similar, complex macrocyclic antibiotics that are produced by *Streptomyces mediterranei* and are important first-line antituberculosis drugs. The main metabolic pathway is 25-deacetylation by AADAC. Rifamycins are known to induce various drug-metabolizing enzymes, such as CYP3A4, but 25-deacetyl rifamycins have no or little induction potency.[4] Rifampicin has been reported to cause toxic injury to hepatocytes.[49] However, we found that 25-desacetyl rifamycins exhibit no or less cytotoxicity compared with the parent rifamycins.[4] Therefore, human AADAC would mediate decreasing the induction rates of drug-metabolizing enzymes and hepatotoxicity by rifamycins.

The common characteristics in the abovementioned AADAC substrates are that they contain a large alcohol group and small acyl group, which is similar to CES2 substrates. In fact, both AADAC and CES2 have been shown to be involved in flutamide hydrolysis in the human liver at low and high concentrations of flutamide, respectively.[38] However, phenacetin and rifamycins are not hydrolyzed by CES2. An understanding of the substrate specificity differences between AADAC and CES2 is of interest.

When we measured flutamide hydrolase activity at 500 μM, at which concentration AADAC is predominantly contributed to flutamide hydrolysis, a 50-fold interindividual variability was observed in microsomes from 24 human livers.[50] Similarly, 22- and 205-fold interindividual variabilities in the hydrolase activities of phenacetin (1 mM) and rifampicin (50 μM), respectively, were observed.[50] Our recent study found a polymorphic mutation in the *AADAC* gene that caused diminished enzyme activity from a human liver sample with remarkably low flutamide hydrolase activity.[50] This allele possesses a SNP of g.14008T>C that causes an amino acid (glutamine) extension at the C-terminus. This allele was found in European-Americans (1.3%) and African-Americans (2.0%), but not in Koreans or Japanese. In the sequence analysis, another SNP, g.13651G>A (V281I), was also found in the four abovementioned populations with relatively high frequencies of 49.5–63.5% and does not appear to cause a marked enzyme activity change. Our recent study found that AADAC protein expression exhibited approximately 200-fold differences in the microsomes among 24 human livers.[50] Because these polymorphic alleles alone could not account for the interindividual variability, further investigation of the mechanisms that affect their expression levels is required.

The species differences in the enzyme activity, substrate specificity, and tissue distribution of AADAC were evaluated using humans and rodents (mouse and rat samples).[51] AADAC mRNA is highly expressed in the liver as well as in the gastrointestinal tract in humans, whereas Aadac mRNA is expressed in the liver at the highest level, followed by the gastrointestinal tract (jejunum) and kidney in rodents. Recombinant mouse and rat Aadac proteins exhibited the hydrolase activities of flutamide and phenacetin but not rifampicin. In support of this finding, rodent liver microsomes did not exhibit rifampicin hydrolase activity. It was reported that 25-deacetyl rifampicin was detected in human plasma as the principal metabolite, whereas it was barely detected in rat and rabbit plasma.[52,53] Additionally, it was reported that dog tissues were also unable to hydrolyze rifampicin.[54] Therefore, 25-deaceyl rifampicin is likely a specific metabolite formed by human AADAC. Species differences in AADAC substrate specificity should receive attention during the preclinical drug development process.

Cholinesterase

In human cholinesterases, there are 2 principal enzymes: acetylcholinesterase (ACHE) and butyrylcholinesterase (BCHE).[55] ACHE is predominantly expressed in the tissues, such as brain and muscles, and erythrocytes. ACHE in tissues and erythrocytes possesses a large common catalytic domain but has a different C-terminal sequence that is produced by alternative splicing.[56] ACHE exists as polymers of catalytic subunits.[57] The globular forms, G1, G2, and G4, contain 1, 2, or 4 subunits, respectively. The asymmetric form, A12, contains 12 subunits. ACHE is involved in the acceleration of amyloid formation, which is associated with Alzheimer's disease in the brain. Therefore, for the treatment of Alzheimer's disease, several ACHE inhibitors, such as donepezil and rivastigmine, are used.[58] However, knowledge concerning drugs that are hydrolyzed by ACHE is limited.

BCHE is expressed in the liver, lung, brain, and heart tissues.[59] BCHE is predominantly present in plasma because BCHE that is synthesized in the liver is secreted into the plasma. BCHE has similar catalytic properties to ACHE. The G4 tetramer constitutes the majority of the plasma BCHE.[60] In contrast to ACHE, several drugs have been reported to be substrates of BCHE. The neuromuscular blocking agents, such as succinylcholine and mivacurium, are predominantly hydrolyzed by BCHE. Low plasma BCHE enzyme activity, which is caused by organophosphate poisoning and *BCHE* genetic polymorphisms, in patients is associated with prolonged apnea following clinical exposure to succinylcholine and mivacurium.[61–63] Bambuterol, which is a β-adrenoceptor agonist that is used for the treatment of asthma, is hydrolyzed primarily by BCHE to terbutaline, which is a pharmaceutically active metabolite. Bambuterol has the potency to inhibit BCHE enzyme activity, resulting in the slow conversion of bambuterol to terbutaline. This can explain the long acting feature of bambuterol.

Cocaine, which is a stimulant of the central nervous system, an appetite suppressant, and a topical anesthetic, contains 2 ester bonds and is hydrolyzed to ecgonine methyl ester and benzoylecgonine.[64] The ecgonine methyl ester, which is a major metabolite found in urine, is formed by hydrolysis by BCHE. However, in tissues, CES2 can also catalyze this hydrolysis reaction.[11] The relative contribution of these enzymes to ecgonine methyl ester formation is unknown. A second metabolite, benzoylecgonine, which is found in urine, is produced by the cleavage of another ester bond by CES1 in human liver. Therefore, cocaine is hydrolyzed by multiple esterases to produce 2 metabolites.

CPT-11 is known to be hydrolyzed by CES and BCHE enzymes.[65] The *Km* value of CPT-11 hydrolysis by human BCHE is $42.4 \pm 10.6\,\mu M$, which is higher than that by human CES2 ($3.4 \pm 1.4\,\mu M$).[20] BCHE is predominantly expressed in human plasma, whereas CES enzymes are expressed in human tissues, such as the liver and intestine. The contribution of human BCHE to the hydrolysis of CPT-11 is unclear.

Paraoxonase

Different from esterases described above, paraoxonases (PONs) do not belong to the serine esterase family and require calcium to exert their activities and stabilities.[66] The human PON family consists of three isoforms, PON1, PON2, and PON3, in which the gene loci are adjacent on the long arm of chromosome 7q21.3–22.1.[67] These genes are approximately 65% homologous at the amino acid level and have similar molecular weights (43–45 kDa).[67] In mammals, 3 PON isoforms are well conserved; PON orthologs share greater than 80% amino acid homology.[67,68] It has been reported that PON1 and PON3 are synthesized primarily in the liver and partially secreted into the plasma, whereas PON2 is ubiquitously expressed in human tissues except plasma.[69] In the liver, PON enzymes are mainly localized in the endothelial reticulum.[70] PON enzymes are considered to be luminal endoplasmic reticulum proteins, because they have one disulfide bridge between Cys residues, which would favor a luminal protein orientation. In plasma, PON1 resides on the high-density lipoprotein (HDL) cholesterol-carrying particles. Apolipoprotein A-I, which is the major structural protein on HDL, appears to interact with PON1, to make PON1 highly stable and an active conformation.[71]

PON1 hydrolyzes organophosphates, such as paraoxon, sarin, and soman.[72,73] In drug metabolism, PON1 hydrolyzes lactone- or cyclic carbonate-containing drugs, such as prulifloxacin,[74] pilocarpine,[75] and olmesartan medoxomil.[76] Pilocarpine hydrolysis causes pharmacological inactivation, whereas prulifloxacin and olmesartan medoxomil hydrolysis causes pharmacological activation. PON enzymes exhibit a calcium-dependent activity at calcium concentrations of 0–10 μM and a maximum activity at a 20-μM calcium concentration.[66] The plasma calcium concentration is normally 1 mM.[66] In contrast, the calcium concentration in the endoplasmic reticulum in the liver is normally 0.1 to 1 μM.[66] Therefore, PON could exert enzyme activity in human liver, although the activity in plasma might be higher compared with that in human liver.

PON1 has 2 common polymorphisms, L55M and Q192R. The effects of the amino acid substitution of Q192R are substrate dependent. For example, paraoxon is more efficiently hydrolyzed by PON1 192R, but soman and sarin are more efficiently hydrolyzed by 192Q.[77] In drug metabolism, we previously investigated the effect of PON1 Q192R on pilicarpine hydrolysis (**Fig. 2**).[75] **Figure 2B** represents the pilocarpine hydrolase activity in 50 individual human plasma samples with different *PON1* genotypes. The activities in the plasma of 192R homozygote subjects were significantly higher than those in the plasma of subjects with the 192Q polymorphism. This result was not changed by correcting the pilocarpine hydrolase activity with phenyl acetate

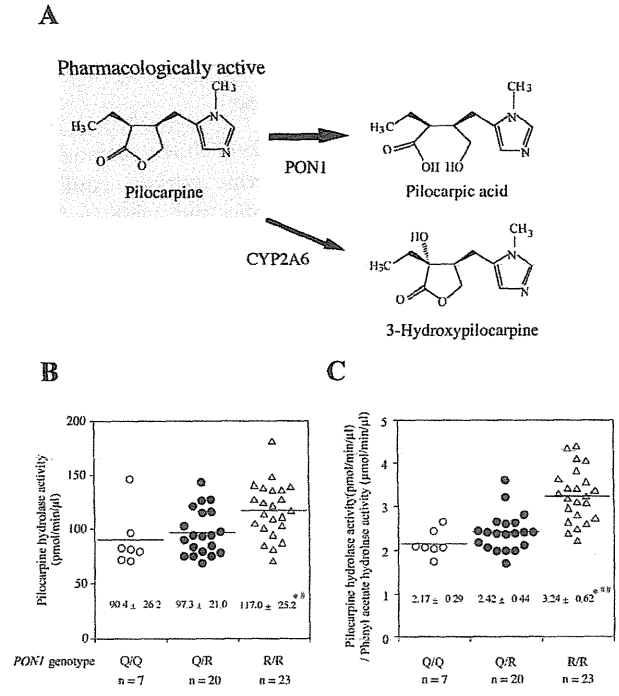

Fig. 2. The effects of PON1 polymorphisms (Q192R) on the pirocarpine hydrolase activity in human plasma

(A) Metabolic pathways of pilocarpine in humans. Pilocarpine is metabolized to pilocarpic acid by PON1 and 3-hydroxypilocarpine by CYP2A6. These metabolites are detected in human blood and excreted into the urine at a level approximately equal to that of the parent drug pilocarpine after oral administration. Pilocarpine hydrolase activities in human plasma with different PON1 genotypes are presented with (B) raw data and (C) corrected data with the phenyl acetate hydrolase activities. Phenyl acetate hydrolase activity was used as a PON1 expression index because it was reported that the PON1 Q192R polymorphism does not affect phenyl acetate hydrolase activity. Human plasma samples from 50 Japanese subjects were used. Seven, 20, and 23 subjects were genotyped as 192Q/Q, 192Q/R, and 192R/R, respectively. Pilocarpine hydrolase activities in plasma were determined at a substrate concentration of 3 mM. *$p < 0.05$ compared with 192Q/Q; #$p < 0.05$; ##$p < 0.005$ compared with 192Q/R.

hydrolase activity, which has been reported to be unaffected by the PON1 Q192R polymorphism (Fig. 2C). Additionally, the catalytic efficiency of recombinant PON1 192R was significantly higher compared with that of recombinant PON1 192Q.[75] Therefore, we found that pilocarpine hydrolysis is more efficiently catalyzed by PON1 192R. The catalytic efficiency of olmesartan medoxomil by PON1 192R has been reported to also be higher compared with that by PON1 192Q.[76] The allele frequency of PON1 Q192R differs by ethnic group as follows: 63%, 60%, and 26% in African-Americans, Asians (Japanese), and Caucasians, respectively.[78–80] The amino acid substitution of L55M unlikely affects catalytic activity, but PON1 55M is associated with decreased PON1 protein levels in plasma.[81,82] This would be because of a strong linkage disequilibrium with g.−108T>C in the promoter region.[83] In the development of PON1-hydrolyzed drugs, taking note of the *PON1* genetic polymorphisms may be required.

The only known substrate of PON2 is *N*-(3-oxododecanoyl)-L-homoserine lactone, which is a key autoinducer that is synthesized by *Pseudomonas aeruginosa*.[84] To date, no PON2-hydrolyzed drugs have been reported.

PON3 is capable of hydrolyzing lovastatin, simvastatin, and spironolactone, which contain a lactone ring.[85] Although pilocarpine also contains a lactone ring, it cannot be hydrolyzed by PON3.[75] Therefore, PON3 and PON1 can catalyze the hydrolysis of lactone-containing drugs, but the differences in substrate specificity between them are unclear. Several polymorphic mutations in the *PON3* gene are known,[86,87] but the effects on drug metabolism have not been determined.

In summary, PON enzymes primarily catalyze lactone ring-containing drugs. PON2 and PON3 are much less studied compared with PON1; therefore, the roles of PON2 and PON3 in drug metabolism require elucidation.

Esterases Responsible for Deglucuronidation of Acyl-glucuronides

Glucuronidation that is catalyzed by UGT enzymes accounts for the metabolism of more than 35% of all drugs that are metabolized by phase II enzymes.[88] In general, glucuronides are neither active nor reactive and are rapidly excreted from the body.[89] However, it has been reported that acyl-glucuronides, which are formed from compounds that contain carboxylic acid, can bind covalently to proteins and other macromolecules because of their electrophilicity, which suggests that they are associated with immunogenicity and toxicity.[89] The formed glucuronides are primarily hydrolyzed by β-glucuronidase, especially in the gut. However, because acyl-glucuronides contain an ester bond, they may be hydrolyzed by esterases that are expressed in tissues as well as β-glucuronidase. As a drug that forms acyl-glucuronide, we studied mycophenolic acid (MPA). MPA is primarily metabolized to the inactive phenolic glucuronide (MPAG),[90] and partially to MPA acyl-glucuronide (AcMPAG) (Fig. 3A).[91] Although the AcMPAG deglucuronidation was detected in human liver homogenates, the deglucuronidation was not inhibited by D-saccharic acid 1,4-lactone, a β-glucuronidase inhibitor.[5] Our recent study found by purifying the responsible enzyme from human liver cytosol (HLC) that α/β hydrolase domain containing (ABHD) 10 catalyzes the deglucuronidation of AcMPAG.[5] The theoretical molecular weight of human ABHD10 is 34 kDa, but the purified ABHD10 shows a 28-kDa band by Western blotting. According to the National Center for Biotechnology Information database (accession number Q9NUJ1), 52 amino acids on the N-terminal end and the residual 254 amino acids of the human ABHD10 precursor are regarded as the transit peptide and mature chain, respectively. The purified ABHD10 would correspond to the mature form.

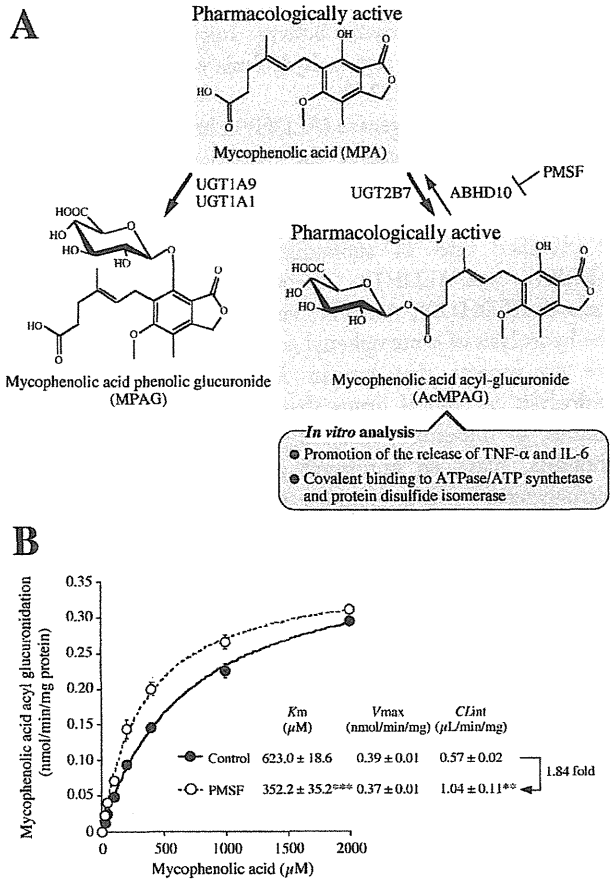

Fig. 3. The role of ABHD10 in the formation of mycophenolic acid acyl-glucuronide in human liver

(A) Major metabolic pathways of MPA in humans. MPA is metabolized to its phenolic glucuronide (MPAG) and acyl-glucuronide (AcMPAG). AcMPAG deglucuronidation is catalyzed by ABHD10. (B) Kinetic analyses of AcMPAG formation from MPA in human liver homogenates in the presence or absence of 1 mM PMSF, which is an ABHD10 inhibitor. The *CLint* value in the presence of PMSF is 1.84-fold higher compared with that in the absence of PMSF. Each data point represents the mean ± SD of triplicate determinations. **p < 0.01 and ***p < 0.001 compared with the control sample (Student's *t*-test).

Recombinant human ABHD10 could not hydrolyze phenolic glucuronide. ABHD10 is expressed in both of HLM and HLC. The catalytic efficiency of AcMPAG formation in human liver homogenates was increased by phenylmethylsulfonyl fluoride (PMSF), which is an ABHD10 inhibitor (**Fig. 3B**). It has been reported that AcMPAG promotes the release of TNF-α and IL-6, which are proinflammatory cytokines, from human mononuclear leukocytes.[92] Additionally, AcMPAG can covalently bind to ATPase/ATP synthetase and protein disulfide isomerase, which are essential for the control of energy and the redox state of cells.[93] Therefore, because AcMPAG appears to be associated with immunogenicity and toxicity, ABHD10 may function to protect against the adverse effects of MPA. The substrate specificity of human ABHD10 is under investigation in our laboratory. The human ABHD family consists of 19 isoforms (ABHD1–ABHD11, ABHD12(A), ABHD12B, ABHD13, ABHD14A, ABHD14B, ABHD15, ABHD16A, and ABHD16B), but other than ABHD10, the involvement of ABHD enzymes in drug metabolism remains unknown.

Recently, it was reported that the deglucuronidation of valproic acid acyl-glucuronide in HLC is catalyzed by acylpeptide hydrolase (APEH).[94] The monomeric molecular weight of APEH is 75 kDa, and APEH is present as a homotetrameric protein (300 kDa).[95] APEH is originally recognized as the peptidase that catalyzes the hydrolysis of the N-terminus of proteins to release the N-terminal-acetylated amino acid. Information is limited for the involvement of this enzyme in drug metabolism. However, plasma valproic acid concentrations are decreased by concomitant use with carbapenem antibiotics in epileptic patients.[96] The drug-drug interaction can be explained by the inhibition of APEH by panipenem.

Human serum albumin (HSA) has an esterase-like activity for the deglucuronidation of acyl-glucuronide of fenoprofen, etodolac, ketoprofen, and gemfibrozil.[97–100] HSA is a major protein in plasma to which various compounds bind.[101] The reversible binding of acyl-glucuronides to HSA would promote acyl-glucuronide hydrolysis. Additionally, HSA might be a target for forming a covalent adduct of acyl-glucuronide.[102] To date, the effect of HSA on the formation of acyl-glucuronide in the body is unknown.

Various esterases and proteins catalyze acyl-glucuronide deglucuronidation. Studies of clofibric acid in rabbits and zomepirac in guinea pigs have demonstrated that esterases, rather than β-glucuronidase, are primarily involved in acyl-glucuronide deglucuronidation.[103,104] Although information for rabbit and guinea pig ABHD10 was not reported, ABHD10 between human and rodents (mouse and rat) share approximately 75% amino acid homology. APEH between human and other animals, such as guinea pigs and rabbits share more than 90% amino acid homology. However, the contribution of these enzymes to the hydrolysis of acyl-glucuronide in animals remains to be clarified. In mice that lack hepatic β-glucuronidase, it was reported that more bilirubin acyl-glucuronides are excreted in bile compared with control mice.[105] Further study will be required to evaluate the extent to which acyl-glucuronide deglucuronidation of drugs by the abovementioned esterases and proteins have an *in vivo* effect.

Other Enzymes Responsible for Drug Hydrolysis

This section describes the other esterases for which evidence of involvement in drug hydrolysis is accumulating.

It was reported that sialic acid 9-*O*-acetylesterase (SIAE) enzymes that are expressed in the lysosome and cytosol were encoded by a single gene by different usage of exons at the N-terminus.[106] Usui *et al.* found that SIAE is an enzyme that

catalyzes the hydrolysis of alacepril by the purification of the responsible enzyme from rat liver cytosol.[107] However, they suggested that lysosomal SIAE was detected in the cytosol because frozen rat livers were used as the sample material for purification.[107] It has not been examined whether cytosolic SIAE can hydrolyze alacepril. SIAE cannot hydrolyze other ACE inhibitors, which are classified as dicarboxylate-containing agents, such as imidapril, delapril, and temocapril.[14] In humans, there are 2 types of SIAEs, lysosomal and cytosolic enzymes, which are similar to rat SIAEs. Human and rat SIAE showed 75% amino acid homology. To date, it is unknown whether human SIAEs can catalyze alacepril.

Olmesartan medoxomil is hydrolyzed by carboxymethylenebutenolidase homolog (CMBL) in human tissues, mainly in the liver and intestine cytosol.[108] As described above, PON1 also efficiently hydrolyzes olmesartan medoxomil to olmesartan in human plasma. Because it was reported that ormesartan alone was detected in plasma after the oral administration of olmesartan medoxomil,[109] PON1 and CMBL in the small intestine, portal blood, and liver would be highly involved in the hydrolysis of olmesartan medoxomil. CMBL can hydrolyze faropenem medoxomil and lenampicillin, but not prulifloxacin despite their structural similarity. In contrast, PON1 hydrolyzes prulifloxacin, which demonstrates a substrate specificity difference toward CMBL.

Valacyclovir and valganciclovir are amino acid ester prodrugs of acyclovir, which is pharmacologically active. Their oral bioavailabilities are several-fold higher than that of acyclovir.[110] After absorption from the gut, they are efficiently hydrolyzed to acyclovir. Kim *et al.* demonstrated that one enzyme responsible for the hydrolysis of valacyclovir and valganciclovir is biphenyl hydrolase-like protein (BPHL), which is highly expressed in human intestine, liver, and kidney.[111,112] The substrate specificity of BPHL resides mainly in the acyl moiety and to a lesser extent in the alcohol moiety.[113] Considering the chemical structures of valacyclovir and valganciclovir, it is proposed that BPHL should be termed an α-amino acid ester prodrug-activating enzyme rather than a nucleoside prodrug-activating enzyme. Considering the substrate specificity of BPHL based on the amino acid acyl group,[114] new prodrugs, especially antiviral and anticancer nucleoside prodrugs, with increased absorption could be developed.

Recently, it was reported that human erythrocytes exhibit more efficient hydrolysis activity of aspirin, a classic nonsteroidal anti-inflammatory agent, rather than human plasma, and identified erythrocyte type I platelet-activating factor acetylhydrolase (PAFAH) as the responsible enzyme in human erythrocytes.[115] This enzyme exists as a trimer that is composed of homo- or heterodimers of 2 catalytic subunits, either PAFAH1B3 or PAFAH1B2 and a noncatalytic PAFAH1B1 protein subunit. Aspirin ineffectiveness is observed in some individuals. The interindividual variability of PAFAH enzyme activity might cause this ineffectiveness, although aspirin hydrolysis is catalyzed by other esterases, such as CES2.[14]

Aldehyde dehydrogenase (ALDH) in human liver, which is widely known to catalyze the oxidation of both aliphatic and aromatic aldehydes to their corresponding carboxylic acids, appears to participate in hydrolysis.[116] Human ALDH is classified into 11 subfamilies (*i.e.*, ALDH1–ALDH9, ALDH16, and ALDH18). Among them, several enzymes, such as ALDH1A1, ALDH2, and ALDH3A1, can catalyze the hydrolysis of *p*-nitrophenyl acetate.[117,118] Although there are no reports that human ALDH is involved in the hydrolysis of clinical drugs that contain carboxylic esters, amides, or thioesters, ALDH2, which is localized in mitochondria, plays an important role for the hydrolysis of nitroglycerin, which contains nitroester.[119] Nitroglycerin hydrolysis generates 1,2-glyceryl dinitrate and nitrite (NO^{2-}) that is further converted to nitric oxide, which is associated with the treatment of angina pectoris. A polymorphic *ALDH2* mutation is associated with a lack of nitroglycerin efficacy in Chinese patients.[120]

Conclusions and Further Prospects

Over time, evidence has accumulated concerning the roles of esterases in drug metabolism. Prodrugs are hydrolytically activated after absorption from the gastrointestinal tract. Conversely, some drugs are hydrolytically inactivated. Additionally, drug hydrolysis is sometimes associated with toxicity. Therefore, esterases play important roles in the regulation of drug efficacy and toxicity.

The differences in the substrate specificities of esterases have not been fully elucidated. In CES enzymes, CES1 prefers substrates with a small alcohol group and large acyl group, whereas CES2 prefers substrates with a large alcohol group and small acyl group. AADAC also prefers substrates with the same characteristics as CES2. BCHE also hydrolyzes the same substrates as CES2. It will be a challenge to clarify the substrate specificity of each esterase. Furthermore, in some cases, the hydrolysis of a drug is catalyzed by more than 2 esterases in various tissues and blood. Predicting the contribution of each esterase to the hydrolysis of drugs in the body is also important.

There are several clinical drugs for which the esterase(s) that are involved in their hydrolysis are unknown. Additionally, there are uncharacterized esterases involved in the hydrolysis of clinical drugs. Unidentified esterase(s) involved in drug metabolism may exist in humans. Further studies of esterases that catalyze the hydrolysis of clinical drugs will facilitate our understanding of the pharmacological and toxicological importance of esterases.

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—Review—

シトクロム P450 と転写因子の microRNA による発現制御

中島美紀

Role of MicroRNAs in the Regulation of Cytochrome P450s and Transcriptional Factors

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MicroRNAs (miRNAs) are endogenous ~22-nucleotide non-coding RNAs that regulate gene expression through the translational repression or degradation of target mRNAs. The human genome contains over 1400 miRNAs and over 60% of human mRNAs are predicted to be targets of miRNAs. The miRNAs have roles in fine-tuning the expression of their target genes forming intricate networks. Research on miRNA is growing exponentially, and it is now clear that miRNAs can potentially regulate every aspect of cellular processes such as differentiation, proliferation and apoptosis as well as a large range of physiological processes such as development, immune response, metabolism, tumor formation, and disease development. The roles of miRNAs in the metabolism of xenobiotics and endobiotics have only recently been revealed. This review describes the current knowledge on the regulation of cytochrome P450s and transcriptional factors by miRNAs, and its physiological and clinical significance, which were disclosed in our studies. The miRNA expression is readily altered by chemicals, carcinogens, drugs, hormones, stress, or diseases, and the dysregulation of specific miRNAs might lead to changes in the drug metabolism potency or pharmacokinetics as well as pathophysiological changes. Utilizing miRNAs opens a new era in the fields of drug metabolism and pharmacokinetics as well as toxicology.

Key words——microRNA; cytochrome P450; nuclear receptor; post-transcriptional regulation

1. はじめに

薬の効果や副作用に認められる個人差は，薬の体内動態並びに薬物代謝酵素活性の個人差に起因することが多い．その個人差の理解のため，薬物代謝酵素の遺伝子多型や転写調節の研究が行われてきた．しかし，それでもなお薬物代謝能の個人差を説明できない事象が存在する．薬物代謝能を制御する可能性のある新たな因子として microRNA（miRNA）が考えられた．miRNA はタンパク質をコードしない 22 塩基程度の小さな RNA で，標的となる mRNA に結合して翻訳を抑制あるいは mRNA を分解することにより，タンパク質の発現を負に制御する機能を有している．1993 年線虫で最初に発見されたのち，2001 年にヒトにも存在することが明らかになり，以降その存在意義が解明され，発生，分化，増殖，アポトーシスなど，重要な生命現象に係わっていることがわかってきた．これまでにヒトでは 1400 種以上の miRNA が同定され，ヒト遺伝子産物の 60%以上が，また二次的な影響を含めるとほぼすべての遺伝子産物が miRNA によって調節されていると考えられている．本稿では，miRNA の生合成と発現抑制機構について簡単に解説し，ある遺伝子の発現制御に係わる miRNA を同定するために用いられる一般的な方法について述べた後，筆者らの研究によって明らかになったヒトシトクロム P450 と転写調節因子の miRNA による発現制御とその意義について概説する．

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本総説は，平成 23 年度日本薬学会学術振興賞の受賞を記念して記述したものである．

2. miRNA の生合成と発現抑制機構

miRNA は，通常 RNA ポリメラーゼ II によってヘアピン構造を有する 200–5000 塩基ほどの転写産物 primary microRNA（pri-miRNA）として転写され，核内で Drosha などによりプロセッシングを受けて，ステムループ構造を持つ約 70 塩基の precur-

sor microRNA (pre-miRNA) となる．その後，pre-miRNA は Exportin-5 を介して細胞質に輸送され，Dicer によってプロセッシングを受けて約 22 塩基の二本鎖 miRNA:miRNA*となり，Dicer, Agonaute 2 (Ago2), tar-RNA-binding proteins (TRBP) などで構成される RNA-induced silencing complex (RISC) と複合体を形成する．そして，一本鎖化された mature miRNA がガイド鎖となり，標的 mRNA の主に 3′-非翻訳領域 (untranslated region, UTR) に存在する認識配列(miRNA recognition element, MRE) に結合する．miRNA の標的 mRNA への結合は部分相補的であり，miRNA の 5′末端 2–7 塩基である seed 配列が相補的であることが重要と言われている．RISC はリボソーム・サブユニット会合の阻害，リボソームの脱落，キャップ構造の脱離，脱アデニル化など，種々のメカニズムを介して翻訳を抑制又は mRNA を分解し，遺伝子サイレンシングを起こす．

3. 発現制御に係わる miRNA の同定

1 つの miRNA は数百種類の mRNA を標的とする可能性があり，1 つの mRNA は複数の miRNA によって認識されることもあるため，遺伝子の発現調節に係わっている miRNA を予測することは容易ではない．いくつかの予測プログラムが利用可能であるが，それぞれのプログラムで用いるアルゴリズムが異なっており，予測されてくる miRNA が異なることも多い．偽陽性の確率も高く，実際に標的となるかどうかは実験により確かめなければわからない．また，予測プログラムでは miRNA の発現量は考慮されていないため，当該遺伝子が発現している組織に予測された miRNA がどの程度発現しているかは別途考慮する必要がある．

遺伝子の発現制御に miRNA が係わっているか調べる一般的な方法は以下の通りである．まず，細胞に miRNA を過剰発現させ，当該タンパク質又は mRNA の発現量が低下するか調べる．miRNA の過剰発現による人為的な影響の結果である可能性を否定するためには，miRNA に対するアンチセンスオリゴヌクレオチド (AsO) を導入し，内因性の miRNA を抑制した際に，当該タンパク質又は mRNA の発現量が増加するか調べることが肝要である．しかし，このような実験では，miRNA の作用が直接的なものか，別の標的遺伝子に作用した二次的な結果なのか判断できない．そこで有用なのが 3′-UTR や MRE をルシフェラーゼ遺伝子の下流に組み込んだプラスミドを用いたルシフェラーゼアッセイである．miRNA の過剰発現又は AsO の導入によってルシフェラーゼ活性に変動が認められれば，直接的な発現制御を証明することができる．MRE への変異の導入や欠失，複数連結などによるルシフェラーゼ活性の変動を調べることで，MRE の機能性を確認することも重要である．細胞内の miRNA の発現量を人為的に変動させることなく，常在的な状態で miRNA の関与を提唱するには，当該タンパク質発現量と miRNA の発現量に負の相関関係が認められるか調べることも有用である．正常細胞とがん細胞の比較，複数の細胞株間での比較，複数の個人サンプル間の比較など，様々なパターンで適用できる．

4. miR-27b によるヒト CYP1B1 の発現制御[1)]

CYP1B1 は多環芳香族炭化水素や芳香族アミンの代謝的活性化を触媒し，またエストロゲンを DNA 損傷性の代謝物に変換することから，発がんに関与している分子種である．CYP1B1 は卵巣，子宮，乳腺などの組織において mRNA レベルでは高く発現しているものの，タンパク質レベルではほとんど検出できないことから，転写後調節の寄与が示唆され，miRNA による発現制御の可能性を検討した．CYP1B1 mRNA の長さは約 5.2 kb であり，そのうち 3′-UTR は約 3.1 kb と半分以上を占める．CYP1B1 mRNA の配列をヒト，マウス，ラットで比較すると翻訳領域の相同性は 80%以上と高いのに対し，3′-UTR 全体の相同性は 30%ほどしかない (Fig. 1)．ところが，ポリ A に近い領域に 86%と高い相同性を示す領域が 44 bp ほど存在し，その中に miR-27b との結合が予想される配列が存在していた (Fig. 1)．miRNA の配列は種を超えて保存されていることが多く，MRE の配列も種で保存されているほど，その miRNA によって制御されることに意義があるものと推定される．ルシフェラーゼ遺伝子の下流に MRE や 3′-UTR を組み込んだプラスミドを Jurkat 細胞に pre-miR-27b とともに導入するとルシフェラーゼ活性の低下が認められた (Fig. 2)．一方，miR-27b の発現量の高い MCF-7 細胞に導入した際，MRE や 3′-UTR を組み込んだプラスミドでコントロールプラスミドと比べてルシフェ

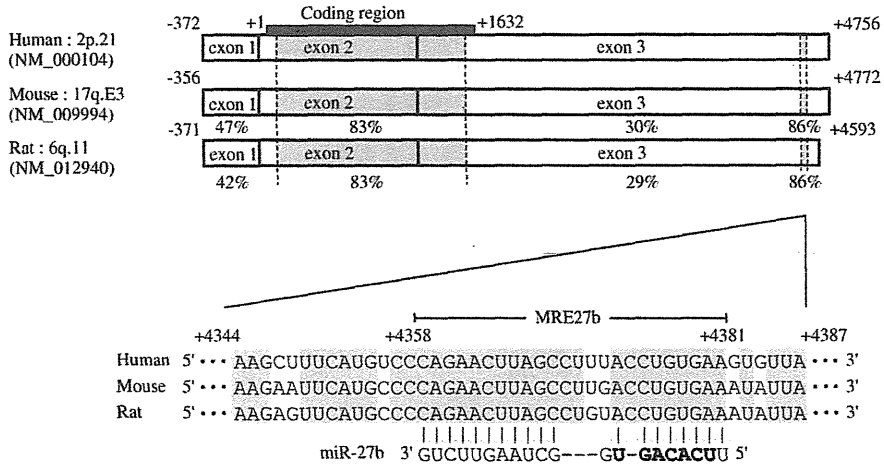

Fig. 1. Homology between Human, Mouse, and Rat CYP1B1 mRNAs and the Predicted Target Sequences of miR-27b

CYP1B1 mRNAs in human, mouse, and rat are ~5 kb in length and consist of three exons. The numbering refers to the translation start site as 1. The sequence of MRE27b is located on +4358 to +4381 in the 3′-UTR of human CYP1B1. Highly conserved regions are shown in gray color. Bold letters: seed sequence.

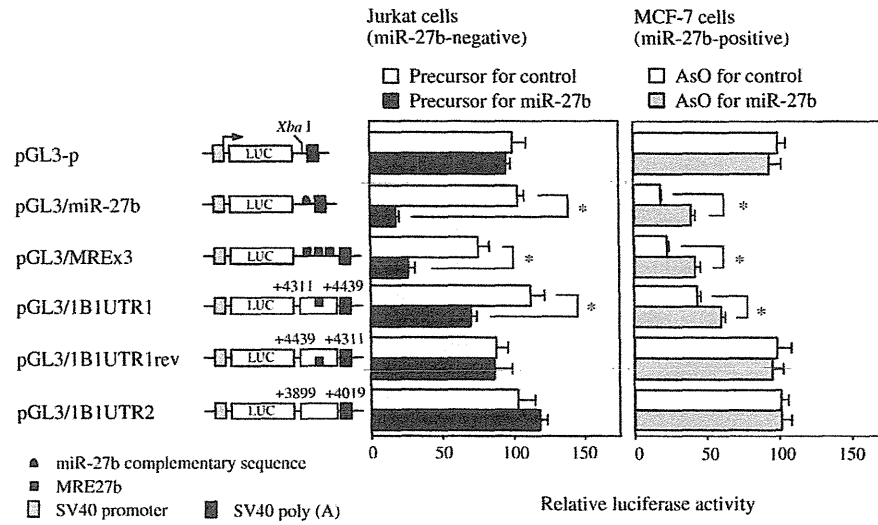

Fig. 2. Luciferase Assay with Reporter Constructs Containing MRE27b or 3′-UTR of Human CYP1B1 in Jurkat or MCF-7 Cells

A series of reporter constructs was transfected into Jurkat cells with precursor for miR-27b or control, or into MCF-7 cells with AsO for miR-27b or control. Values are expressed as percentages of the relative luciferase activity of pGL3 promoter plasmid. Each column represents the mean±S.D. of three independent experiments. $^{*}p<0.05$.

ラーゼ活性は低値を示したが，miR-27b に対する AsO の導入により活性の回復が認められたことから，予測された MRE に miR-27b が結合し，発現を抑制していることが示された．また，miR-27b に対する AsO の導入により，MCF-7 細胞における内因性 CYP1B1 の発現量の増大が認められ，CYP1B1 が miR-27b によって制御されていることが明らかになった．この発現制御機構の生体内における意義を解明するにあたり，CYP1B1 タンパク質発現量は正常組織よりもがん組織で多いことに注目し，その現象に miR-27b が係わっている可能性を考慮した．乳がん組織とその周辺の非がん部における miR-27b の発現量を調べたところ，がん部では発現量が少ないことが明らかになり [Fig. 3(A)]，がん部における miR-27b の発現量と CYP1B1 タンパク質発現量との間に負の相関関係が認められた [Fig. 3(B)]．したがって，正常組織中では miR-27b が CYP1B1 の発現を抑制的に制御しており，がんで CYP1B1 が高発現している理由の 1 つとして miR-27b の低下が挙げられることを明らかにした．これ

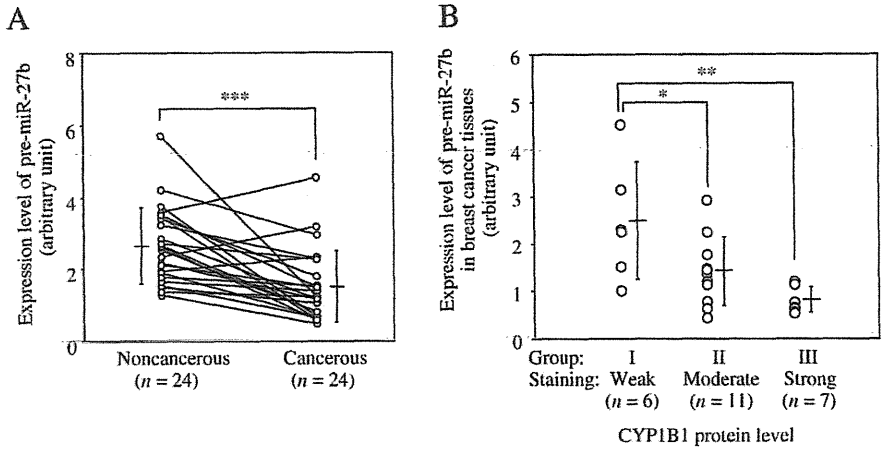

Fig. 3. Expression of miR-27b in Human Breast Cancerous and Adjacent Noncancerous Tissues (A) and the Relationship between the Expression Levels of miR-27b and CYP1B1 Protein Level in Human Breast Cancer (B)

The expression levels of pre-miR-27b and CYP1B1 protein were determined by real-time RT-PCR and immunostaining, respectively. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

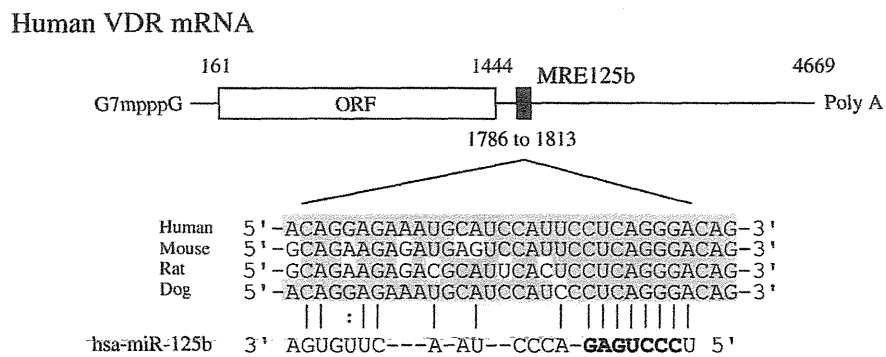

Fig. 4. Schematic Representation of Human VDR mRNA and the Predicted Target Sequence of miR-125b

The numbering refers to the 5′-end of mRNA as 1. The sequence of MRE125b is located on +1786 to +1813 in the 3′-UTR of human VDR. Highly conserved sequence is shown in gray color. Bold letters: seed sequence.

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はmiRNAが薬物代謝酵素の発現制御に係わっていることを示した世界初の研究成果である.[1)]

5. miR-125bによるビタミンD受容体（VDR）[2)]とCYP24[3)]の発現制御

ビタミンD_3は血中カルシウム濃度の恒常性維持や骨代謝に重要な役割を担う一方，細胞増殖抑制作用や分化誘導及びアポトーシス誘導作用を有しており，抗がん薬としての可能性が期待されている．ビタミンD_3の作用はVDRを介して発揮される．VDRは，mRNA発現量としてはがん部位と正常部位で差が認められないものの，タンパク質発現量としては正常部位に比べがん部位で高いことが報告されており，転写後調節の関与が示唆された．VDR mRNAに結合する可能性のあるmiRNAを探索したところ，いくつかのmiRNAが予測されたが，中でもmiR-125bの認識部位MRE125bの配列は種を超えて高く保存されていた（Fig. 4）ことから，miR-125bがVDRの発現を制御している可能性を検討した．ルシフェラーゼアッセイによりMRE125bが機能的に働いていることが示された．miR-125bがVDRのタンパク質発現量を抑制しているか，ヒトがん由来細胞株におけるVDR発現量をウェスタンブロットで解析したが，市販の抗体では非特異的なバンドが多く，検出が困難であった．そこで，ゲルシフトアッセイを利用した検出を試みた．VDRは活性化されるとレチノイドX受容体α（RXRα）とヘテロダイマーを形成して，標的遺伝子の応答配列に結合して転写を活性化する．MCF-7細胞に

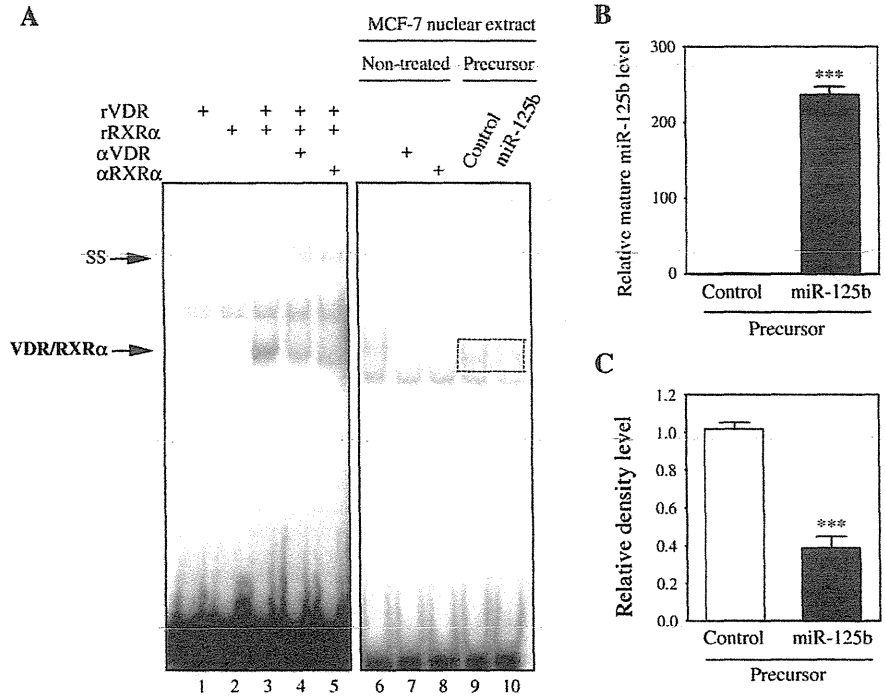

Fig. 5. Electrophretic Mobility Shift Assay to Evaluate the Endogenous VDR Protein Level

The ^{32}P labeled probe containing the VDRE in human CYP24 promoter was incubated with *in vitro*-synthesized VDR (rVDR) and RXRα (rRXRα) or the nuclear extract prepared from the precursors for miR-125b or control-transfected MCF-7 cells (A). The mature miR-125b level was determined by real-time RT-PCR analysis (B). The relative density of the shifted band including VDR/RXRα complex was shown as the mean±S.D. of three independent experiments (C). $^{***}p<0.001$.

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pre-miR-125b を導入して核抽出液を調製し，VDR の標的遺伝子の 1 つである CYP24 の応答配列をプローブとしてゲルシフトアッセイを行ったところ，VDR/RXRα ヘテロダイマーの結合量が低下し，VDR 発現量の低下が示された（Fig. 5）．また，VDR のリガンドである 1α,25-ジヒドロキシビタミン D_3 の処置により，標的遺伝子である CYP24 mRNA は顕著に誘導されるが，その誘導能は miR-125b により有意に抑制された (Fig. 6)．以上より，ヒト VDR が miR-125b によって発現制御されていることが明らかになった．[2)]

また興味深いことに，CYP24 の発現も miR-125b で制御されていることを，過剰発現又は阻害実験及びルシフェラーゼアッセイなどの手法を用いて明らかにした（Fig. 7）．[3)] つまり，CYP24 は miR-125b によって直接的に，及び VDR の発現抑制を介して間接的に発現抑制されていることになる．CYP24 も正常組織に比べてがん組織で高発現しており，がん組織における miR-125b の発現低下が原因であることも示された．様々ながん組織において多くの

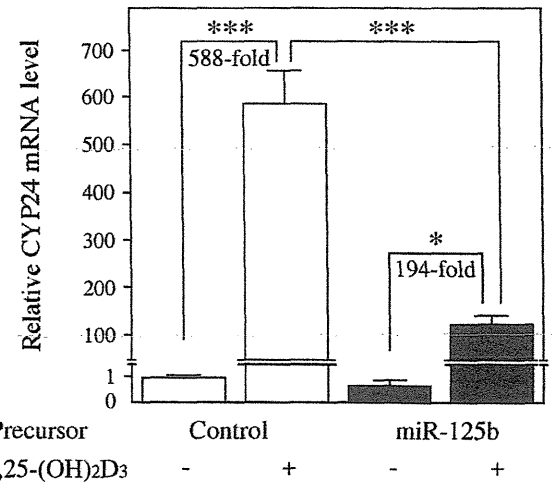

Fig. 6. Induction of CYP24 mRNA in MCF-7 cells by 1α,25-dihydroxyvitamin D_3

The precursors for miR-125b or control (50 nM) were transfected into MCF-7 cells. After 72 h, the cells were treated with 100 nM 1α,25-dihydroxyvitamin D_3 or 0.1% ethanol (vehicle) for 24 h and then CYP24 mRNA levels were determined by real-time RT-PCR and normalized with the GAPDH mRNA level. Each column represents the mean±S.D. of three independent experiments. $^{*}p<0.05$, $^{***}p<0.001$.

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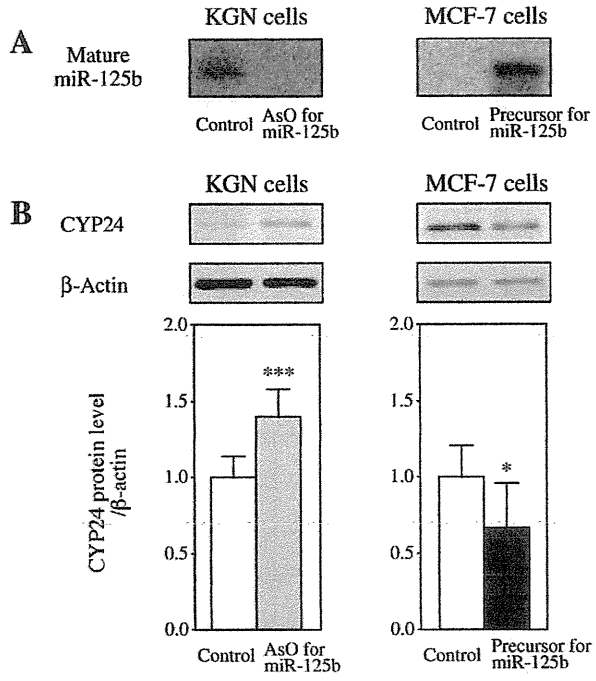

Fig. 7. Effects of miR-125b on the Endogenous CYP24 Protein Level in KGN or MCF-7 Cells

AsO for miR-125b or control (2.5 pmol/4×10^5 cells) were transfected into KGN cells and precursors for miR-125b or control (84 pmol/1.68×10^5 cells) were transfected into MCF-7 cells. After 72 h, total RNA and whole cell lysate were prepared. The expression levels of mature miR-125b were determined by Northern blot analysis (A). The expression levels of CYP24 protein were determined by Western blot analysis and normalized with β-actin protein level (B). Each column represents the mean±S.D. of three independent experiments. *p<0.05, ***p<0.005

This figure is from our previous paper[3] with permission of The American Society for Pharmacology and Experimental Therapeutics.

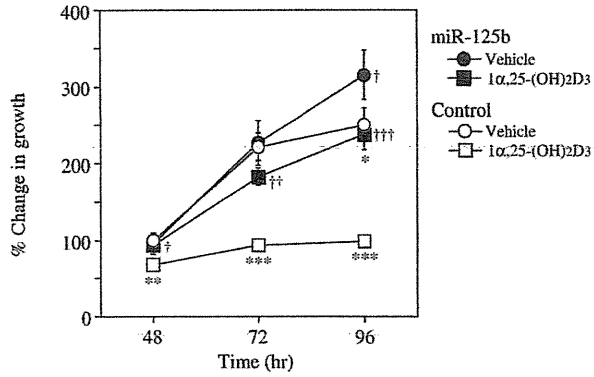

Fig. 8. Antiproliferative Effects of 1α,25-dihydroxyvitamin D_3 in MCF-7 Cells

The precursors for miR-125b or control (20 nM) were transfected into MCF-7 cells. After 24 h, the cells were treated with 1 μM 1α,25-dihydroxyvitamin D_3 or 0.1% ethanol (vehicle) for 48–96 h and then crystal violet assays were performed. Values are expressed as percentages change in growth relative to the cell viability in the precursor for control-transfected cells in the absence of 1α,25-dihydroxyvitamin D_3 after 48 h incubation. Each point represents the mean±S.D. of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, compared with the vehicle. †p<0.05, ††p<0.01, †††p<0.001, compared with the precursor for control.

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miRNA の発現が大きく変動していることが示されている.[4] その原因として，転写活性の変化，エピジェネティック発現調節の変動，遺伝子変異，DNA コピー数の異常など，いくつかの理由が挙げられるが，miRNA をコードする遺伝子の半数以上はがんに関連する fragile site 上に存在することから，DNA コピー数の異常が主な原因と考えられている.[5] miR-125b は pre-miR-125b-1 と pre-miR-125b-2 の 2 つの前駆体から生成されるが，それらをコードする遺伝子はそれぞれ 11q24.1 と 21q11.2 にある. 11q23-24 は乳がん，卵巣がん，肺がんなどで欠失し易く,[6,7] 21q11-21 は乳がん，食道がん，胃がん，卵巣がん，肺がんなどで欠失し易い領域である.[8] そのために miR-125b の発現量ががん組織で低下しているものと考えられる.

CYP24 は活性型ビタミン D_3 の 24 位水酸化反応を触媒し，不活性化する酵素である．活性型ビタミン D_3 の効果発揮に必要な VDR と不活性化に係わる CYP24 がともに miR-125b により制御されていることが明らかになり，それがビタミン D_3 による細胞増殖抑制作用にどのような結果をもたらすかが疑問に残った．そこで，MCF-7 細胞の増殖能を評価することで検討したところ，細胞増殖能は 1α,25-ジヒドロキシビタミン D_3 により有意に抑制され，その抑制効果は pre-miR-125b の導入により低下した（Fig. 8）．したがって，この実験条件下では miR-125b は VDR に対する抑制効果を優先的に示すことが明らかになった．すなわちがん組織では miR-125b の発現が低下しており，VDR を介した抗腫瘍作用を増大させる生体防御機構が働いている可能性が考えられた.

6. miR-148a によるプレグナン X 受容体（PXR）の発現制御と CYP3A4 発現量への影響[9]

ヒト CYP3A4 は肝臓及び小腸に高く発現し，医薬品代謝の約 50％に関与する最も重要な薬物代謝酵素である．CYP3A4 の発現量や酵素活性には 100 倍ほどの大きな個人差が認められるが，遺伝子多型でも個人差を説明できない．筆者らは CYP3A4 とその発現に重要な役割を果たすプレグナン X 受容体（pregnane X receptor, PXR）の 3′-UTR に共通して miR-148a 認識配列が存在することを見い出し

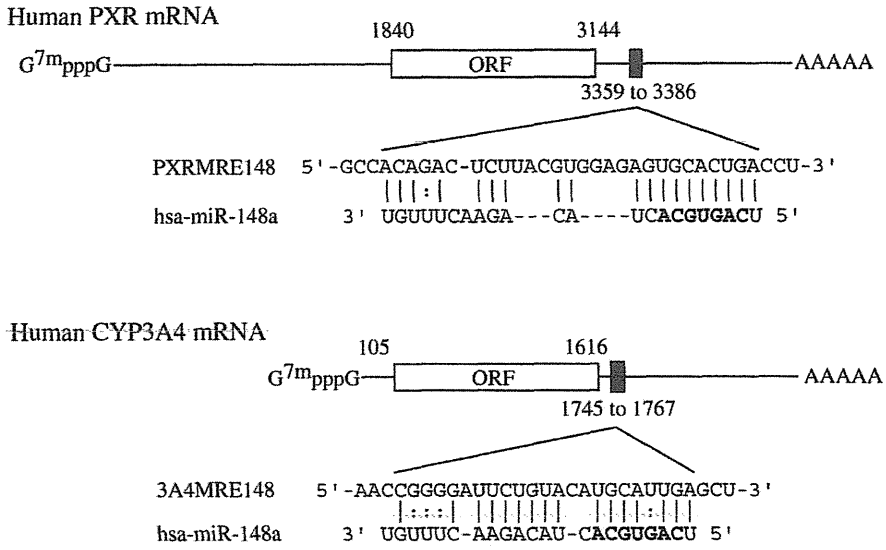

Fig. 9. Schematic Representation of Human PXR and CYP3A4 mRNAs and the Predicted Target Sequence of miR-148a

The numbering refers to the 5′-end of mRNA as 1. The sequence of MRE148a is located on +3359 to +3386 in the 3′-UTR of human PXR mRNA and +1745 to +1767 in the 3′-UTR of human CYP3A4 mRNA. Bold letters: seed sequence.

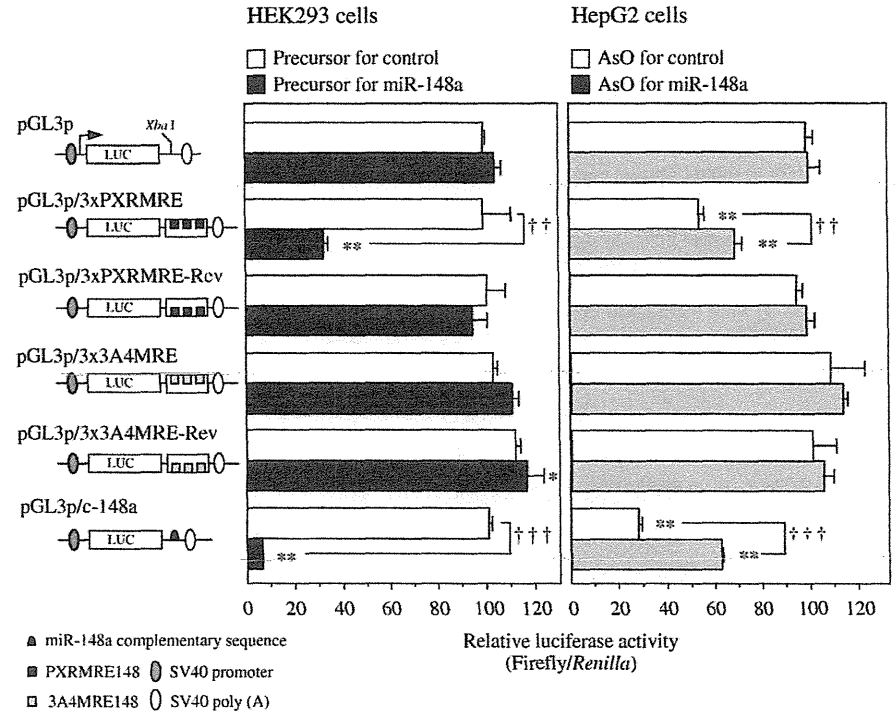

Fig. 10. Luciferase Assay with Reporter Constructs Containing MRE148a in the 3′-UTR of PXR or CYP3A4 in HEK293 or HepG2 Cells

A series of reporter constructs was transfected into HEK293 cells with precursor for miR-148a or control, or into HepG2 cells with AsO for miR-148a or control. Values are expressed as percentages of the relative luciferase activity of pGL-3 promoter plasmid. Each column represents the mean±S.D. of thee independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with pGL3, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared with precursor or AsO for control.

(Fig. 9), 解析したところ, miR-148a は CYP3A4 には直接作用しないが, PXR の発現を抑制的に制御し (Fig. 10), CYP3A4 の発現量に影響を与えていることを明らかにした (Fig. 11). タンパク質は DNA から転写された mRNA に基づいて合成されるため, かならず転写レベルでの調節を受けている.

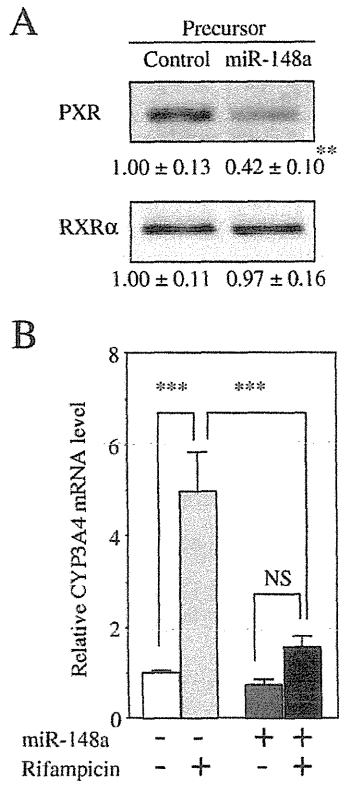

Fig. 11. Effects of Overexpression of miR-148a on the Endogenous PXR Level and the Induction of CYP3A4 mRNA in LS180 Cells

The precursors for miR-148a or control (50 nM) were transfected into LS180 cells. After 72 h, the cells were harvested and nuclear extracts were isolated. The PXR and RXRα protein levels were determined by Western blot analysis (A). The precursor-transfected LS180 cells were treated with 50 μM rifampicin or 0.1% DMSO for 24 h and the CYP3A4 mRNA levels were determined by real-time RT-PCR and normalized with the GAPDH mRNA level. Data are the mean±S.D. of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$; NS: Not significant.

25検体のヒト肝試料を用いた検討において，PXRではmRNA発現量とタンパク質発現量との間に正の相関関係が認められず（r=0.1），転写後調節が大きく寄与していることが示唆された（Fig. 12）．一方，CYP3A4ではmRNA発現量とタンパク質発現量との間に有意な正の相関関係が認められた（$r=0.67, p<0.001$）ことから，転写調節が主要であり，miRNAによる転写後調節の寄与は大きくないことが示された．

7. miR-24とmiR-34aによるhepatocyte nuclear factor 4α（HNF4α）の発現制御と胆汁酸合成への影響[10]

肝臓や腎臓，腸管などに発現しており，非常に多くの遺伝子発現を制御することからマスターレギュレーターとよばれる肝細胞核因子4α（hepatocyte nuclear factor 4α, HNF4α）がmiRNAで制御されている可能性を検討した．興味深いことに，miR-34aは3′-UTRに結合して翻訳を抑制し，miR-24は翻訳領域に結合してmRNAの分解を介して発現を抑制することを明らかにした［Fig. 13(A)］．この発現制御は肝臓中においてHNF4αの下流遺伝子であるCYP7AやCYP8Bなどの胆汁酸合成酵素の発現低下を招くことが示された［Fig. 13(B)］．胆汁酸はHNF4αの発現を低下させ，胆汁酸合成を抑制する，というネガティブフィードバック機構が存在することが報告されていたが，そのメカニズムは不明であった．本研究では，胆汁酸によるプロテインキナーゼCの活性化や活性酸素種の産生を介したシグナル伝達経路の活性化がmiR-24及びmiR-34aの発現を増加させ，それがHNF4αの発現低下をもたらしていることを示し，メカニズムの一因にmiRNAが係わっていることを明らかにした(Fig. 14)．

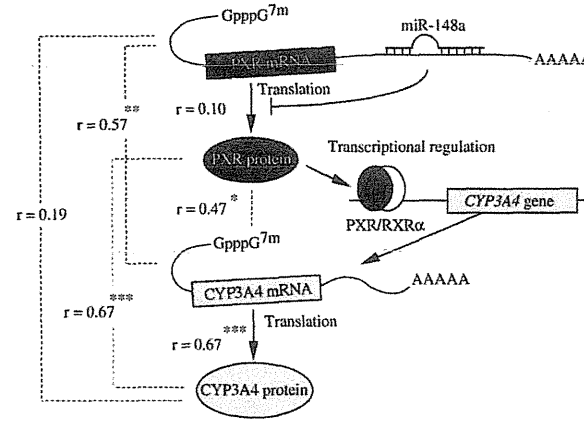

Fig. 12. Schematic Representation of miR-148a-dependent Post-transcriptional Regulation of Human PXR Affecting the Expression Level of CYP3A4 in Human Livers

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

8. おわりに

上述の研究に加え筆者らは，ヒトCYP2E1がmiR-378で制御されていること，[11] ヒトPPARαがmiR-21及びmiR-27bで制御されていること[12]も最近明らかにしており，薬物・異物代謝におけるmicroRNAの役割についてかなり情報が蓄積されてきた．[13] 興味深いことに，ジヒドロ葉酸還元酵素(dihydrofolate reductase, DHFR)[14]や硫酸転移酵素(sulfotransferase, SULT) 1A1[15]の3′-UTRに存在する一塩基多型（single nucleotide polymorphism, SNP）が，miRNAによる結合・制御能に影響を及