

Association of decreased mRNA expression of multidrug and toxin extrusion protein 1 in peripheral blood cells with the development of flutamide-induced liver injury

Kazuhiko Nakano¹ · Hitoshi Ando² · Shinsuke Kurokawa¹ · Keiko Hosohata² · Kentarou Ushijima² · Makoto Takada³ · Masato Tateishi³ · Atsushi Yonezawa⁴ · Satoshi Masuda⁴ · Kazuo Matsubara⁴ · Ken-ichi Inui⁵ · Tatsuo Morita¹ · Akio Fujimura²

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Abstract

Purpose The anti-prostate cancer drug flutamide occasionally causes hepatotoxicity, and predictive biomarkers of flutamide-induced liver injury (FILI) are needed to improve safety of this drug. The aim of this prospective study was to identify such a biomarker by analyzing peripheral blood samples from patients before flutamide therapy.

Methods Blood samples were obtained from 52 patients with prostate cancer before flutamide therapy. FILI was defined as treatment-related elevation of the serum concentration of aspartate or alanine aminotransferase to more than twice the upper limit of the reference range. The patients were monitored for at least 6 months regarding FILI. Microarray and quantitative real-time PCR analyses were conducted to compare gene expression profiles between the groups with and without FILI.

Results Seventeen patients developed FILI. Microarray analysis of the training set in 15 patients detected 11 annotated genes showing >twofold expression changes between

the groups ($p < 0.005$). Quantitative PCR analysis of both the training set and validation set confirmed that mRNA levels of multidrug and toxin extrusion protein 1 (MATE1 or SLC47A1, encoded by 1 of the 11 genes) were significantly lower in patients with FILI. A small experiment on mice (three per group) showed that *Mate1* knockout mice had an elevated serum concentration of 4-nitro-3-(trifluoromethyl)phenylamine, a major metabolite of flutamide, 2 h after administration of the drug, suggesting that *Mate1* could affect the pharmacokinetics of flutamide.

Conclusions The *MATE1* mRNA level in peripheral blood is a possible negative predictive biomarker of FILI.

Keywords Flutamide · Liver injury · Multidrug and toxin extrusion protein 1 · Prostate cancer · Solute carrier family 47 · Member 1

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
FILI	Flutamide-induced liver injury
FLU-1	4-Nitro-3-(trifluoromethyl)phenylamine
MATE1	Multidrug and toxin extrusion protein 1
SLC47A1	Solute carrier family 47 (multidrug and toxin extrusion), member 1
SNP	Single nucleotide polymorphism

Introduction

Flutamide is an oral nonsteroidal antiandrogen drug used for the treatment for prostate cancer [1]. Combined androgen blockade with flutamide and castration improves survival compared to castration alone in patients with advanced prostate cancer [1, 2]. In addition, flutamide is

Kazuhiko Nakano and Hitoshi Ando have contributed equally to this study.

✉ Akio Fujimura
akiofuji@jichi.ac.jp

¹ Department of Urology, Jichi Medical University, Shimotsuke, Japan

² Division of Clinical Pharmacology, Department of Pharmacology, Jichi Medical University, Yakushiji 3311-1, Shimotsuke, Tochigi 329-0498, Japan

³ Division of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Nagasaki International University, Nagasaki, Japan

⁴ Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital, Kyoto University, Kyoto, Japan

⁵ Kyoto Pharmaceutical University, Kyoto, Japan

sometimes effective against prostate cancer resistant to bicalutamide (a commonly used drug of the antiandrogen class) [3, 4]. Flutamide is considered an effective medication for the treatment for prostate cancer.

Flutamide causes liver injury at the rate of ~5 % in Western countries [5, 6] and, in rare cases, may lead to death as a result of fulminant hepatitis [7, 8]. Although flutamide is usually given at a dose of 375 mg/d in Japan, which is a half of that in Western countries, flutamide-induced liver injury (FILI) occurs more frequently in Japan, at the rate of ~30 % [9]. The mechanism behind FILI is likely to be idiosyncratic and has yet to be identified [6, 10]. One approach to improving the safety and efficacy of flutamide is to develop a method for prediction of FILI. The caffeine test for the activity of cytochrome P450 1A2, which is involved in the major metabolic pathway of flutamide, is reported to be a useful method [11] but has not been adopted widely in clinical practice probably due to the complexity of the procedure.

To identify a predictive biomarker of drug efficacy or toxicity, microarray gene expression analysis of peripheral blood from patients is known to be useful [12–14]. For example, using comparison of gene expression profiles in peripheral blood cells between patients and controls, Devouassoux et al. [12] identified the *GALECTIN-10* mRNA level as a predictive biomarker of aspirin-induced asthma. In this study, we tried to identify a predictive biomarker of FILI by analyzing peripheral blood samples from patients with and without FILI before the initiation of flutamide therapy.

Materials and methods

Patients and study design

This prospective clinical study was conducted between September 2008 and October 2011. In total, 60 adult Japanese male patients who were scheduled to receive androgen

deprivation therapy were recruited from the outpatient clinic at Jichi Medical University Hospital (Shimotsuke, Japan). We excluded patients with liver disease or severe anemia and those who had been treated with androgen deprivation therapy previously. This study was approved by the Ethics Committee of Jichi Medical University (Shimotsuke, Japan) and was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent prior to enrollment in the study.

Before the initiation of treatment with flutamide, a venous blood sample for RNA isolation was collected into a PAXgene Blood RNA tube (Becton–Dickinson and Co. Japan, Tokyo, Japan) and stored at -80°C until RNA extraction. Then, all patients received 375 mg/d flutamide (Odyne tablet, Nippon Kayaku, Tokyo, Japan; one 125-mg tablet, three times a day) in usual clinical settings (i.e., with no study-related restrictions on concomitant medications and concurrent treatments). The patients were followed for at least 6 months in relation to FILI. Blood biochemical liver function tests were performed at least once monthly for the first 3 months in most patients. In this study, we defined FILI as follows: (1) elevation in the serum concentration of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) to more than twice the upper limit of the reference range (10–30 IU/l) during flutamide therapy, (2) amelioration of the elevated concentration of AST or ALT within 3 months after cessation of flutamide therapy, and (3) no obvious cause of liver injury other than flutamide. Patients whose serum concentrations of AST and ALT remained within the reference range during at least the first 6 months served as a control group.

As shown in Fig. 1, we assigned the first 16 patients to the training set for the screening of predictive biomarkers and the remaining 44 patients to the validation set. Five patients (one training case and four validation cases) were excluded from the analysis because they developed liver injury other than FILI. Additionally, three patients in the validation set dropped out for other reasons. Eventually, 17

Fig. 1 Study design and the number of patients. *FILI* flutamide-induced liver injury

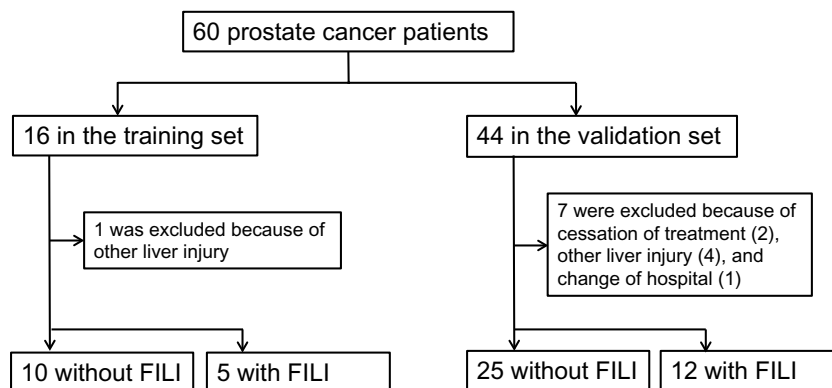


Table 1 Probe sets for which expression levels differed markedly (>2.0 -fold; $p < 0.005$) between the groups of patients with and without flutamide-induced liver injury

GeneChip Probe Set ID	Probe p value*	Fold change (\log_2)	Gene symbol	Gene name	NCBI RefSeq ID	TaqMan gene expression assay ID
211753_s_at	0.0002	2.13	RUN1	Relaxin 1	NM_006911	Hs00762019_sl
204550_x_at	0.0003	-2.12	GSTM1	Glutathione S-transferase μ 1	NM_146421	Hs02341468_ml
208159_x_at	0.0006	2.00	DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, <i>Saccharomyces cerevisiae</i>)	NM_004399	Hs01035967_ml
224685_at	0.0009	2.10	MLLT4	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, <i>Drosophila</i>); translocated to, 4	NM_001040001	Hs00984486_ml
239385_at	0.0019	2.05	TFG	TRK-fused gene	NM_001007565	Hs00607175_ml
243578_at	0.0023	-2.20	-	-	-	-
213913_s_at	0.0025	-2.02	TBC1D30	TBC1 domain family, member 30	NM_015279	Hs00392437_ml
236989_at	0.0028	2.40	-	-	-	-
227099_s_at	0.0030	-2.03	LOC387763	Hypothetical LOC387763	-	-
219525_at	0.0032	-3.80	SLC47A1	Solute carrier family 47, member 1	NM_018242	Hs00217320_ml
213846_at	0.0032	2.35	COX7C	Cytochrome c oxidase subunit VIIc	NM_001867	Hs01595220_g1
239206_at	0.0040	3.50	CRIL	Complement component (3b/4b) receptor 1-like	NM_175710	Hs01369566_ml
215333_x_at	0.0044	-2.00	GSTM1	Glutathione S-transferase μ 1	NM_000561	Hs02341468_ml
204146_at	0.0047	2.23	RAD51AP1	RAD51-associated protein 1	NM_001130862	Hs00272413_ml
221297_at	0.0048	-2.01	GPRC5D	G-protein-coupled receptor, family C, group 5, member D	NM_018654	Hs03045339_ml

* Student's t test

patients developed FILI, whereas 35 did not develop liver injury during the observation period.

RNA isolation and microarray analysis

Total RNA was extracted from blood samples using the PAXgene Blood RNA Kit (Qiagen Japan, Tokyo, Japan) according to the manufacturer's instructions. RNA quality was evaluated using an Agilent 2001 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and only samples with RNA integrity number >7 were used for microarray and real-time PCR analyses.

Fragmented, biotin-labeled amplified cDNA was prepared from 100 ng of total RNA using the Ovation Biotin System (NuGEN Technologies, San Carlos, CA) and was then hybridized to the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). The expression data were analyzed using the GeneSpring GX10 software (Agilent Technologies). After global normalization, the expression level of each gene was compared between the groups with and without FILI using Student's *t* test.

Quantitative real-time PCR analysis

Total RNA was reverse-transcribed using the High-Capacity cDNA Archive Kit (Life Technologies, Carlsbad, CA), and quantitative real-time PCR analysis was performed using the Applied Biosystems StepOnePlus Real-time PCR system (Life Technologies). The specific sets of primers and TaqMan probes are shown in Table 1 (reagents for the TaqMan gene expression assays were from Life Technologies). To adjust the data for variation in the amount of cDNA available for PCR in different samples, expression of target sequences was normalized to that of an endogenous control, glyceraldehyde 3-phosphate dehydrogenase. The data were analyzed using the comparative threshold cycle method.

The animal experiment

Wild-type and *Mate1* knockout mice [15] (three per group) were maintained in a temperature-controlled room with a 12-h light/dark cycle and given a standard diet and water ad libitum. At 17–18 weeks of age, the mice were given a single intraperitoneal dose of 30 mg/kg flutamide (Sigma-Aldrich, St. Louis, MO) dissolved in Solutol HS-15 (9 % in saline). We selected this dose because 100 mg/kg flutamide was lethal in a preliminary experiment. Two hours after the administration of flutamide, the mice were anesthetized with pentobarbital and killed to obtain blood and liver samples. All animal procedures were performed in accordance with the Guidelines for Animal Research with approval of

the Institutional Animal Experiment Committee of Jichi Medical University.

Measurement of flutamide concentrations

An internal standard (IS, Nilutamide) and acetonitrile were added to mice plasma. The extract was cleaned up on a C18 column. After vortexing and centrifugation, the supernatant was used for further analysis. A homogenate of the mice liver spiked with IS was subjected to extraction with acetonitrile. The sample was centrifuged, and the supernatant was evaporated completely under a nitrogen stream. The residue was dissolved in 50 % acetonitrile, and the resulting solution was cleaned up on a C18 column (reversed-phase Kinetex C18, 50 × 2.1 mm I.D., 2.6 μm). Ammonium acetate (100 mM, solution A) and acetonitrile (solution B) served as mobile phases (gradient curve: 0–10 min, linear change from 10 to 95 % B; 10–15 min, 95 % B; after 15 min, a return to 10 % B; the run time 20 min). The flow rate was set to 0.5 ml/min. We used a time-of-flight mass spectrometer with an electrospray ionization interface (JEOL Ltd., Tokyo, Japan). The detection was based on negative ions. Theoretical *m/z* values of the $[M-H]^-$ ion were 275 for flutamide, 291 for hydroxyflutamide (an active metabolite of flutamide), 205 for 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1, a major intermediate metabolite of flutamide), and 316 for nilutamide.

Statistical analysis

The data were expressed as mean ± SE or % (n/N) and analyzed using either Student's *t* test or the χ^2 test. A *p* value <0.05 was assumed to denote statistical significance. All calculations were performed using the SPSS Statistics software (version 17.0 for Windows; Japan IBM, Tokyo, Japan).

Results

As shown in Table 2, baseline characteristics, including age, body mass index, and smoking status, did not differ between the groups with and without FILI in both the training and validation sets. Microarray analysis of the training set detected 15 probe sets, including 11 annotated genes, that showed *p* < 0.005 and >twofold expression changes between the groups with and without FILI (Table 1). Moreover, quantitative real-time PCR analysis validated the microarray data for two genes (Fig. 2) but not for the remaining nine annotated genes (data not shown). Therefore, we next compared mRNA expression levels of the

Table 2 Characteristics of the patients

	Training set			Validation set		
	Patients without FILI	Patients with FILI	<i>p</i> value*	Patients without FILI	Patients with FILI	<i>p</i> value*
<i>n</i>	10	5		25	12	
Age (years)	72 ± 2	73 ± 2	0.77	70 ± 2	68 ± 2	0.43
Body mass index (kg/m ²)	23.6 ± 0.9	24.2 ± 0.9	0.68	23.5 ± 0.6	24.6 ± 0.9	0.28
Body surface area (m ²)	1.60 ± 0.05	1.59 ± 0.03	0.79	1.70 ± 0.04	1.74 ± 0.04	0.60
Time since initiation to discontinuation of flutamide therapy (days)	–	55 ± 42	–	–	77 ± 52	–
Current smoker	40 (4/10)	20 (1/5)	0.44	16 (4/25)	8 (1/12)	0.52
Current drinker	30 (3/10)	40 (2/5)	0.70	44 (11/25)	50 (6/12)	0.73
Prostate-specific antigen (ng/ml)	34.8 ± 12.2	1529.8 ± 1500.3	0.38	31.1 ± 19.5	11.8 ± 3.0	0.50
Testosterone (ng/ml)	4.6 ± 0.6	5.7 ± 0.6	0.24	3.8 ± 0.3	4.1 ± 0.5	0.58
Creatinine (mg/dl)	0.82 ± 0.05	0.93 ± 0.11	0.45	0.78 ± 0.04	0.86 ± 0.04	0.25
AST (IU/l)						
Basal	23 ± 1	28 ± 7	0.57	21 ± 2	22 ± 2	0.78
Maximum, during flutamide therapy	28 ± 3	108 ± 12	<0.01	30 ± 2	83 ± 10	<0.01
ALT (IU/l)						
Basal	17 ± 2	21 ± 4	0.41	20 ± 2	20 ± 2	0.92
Maximum, during flutamide therapy	23 ± 3	158 ± 45	0.04	31 ± 3	146 ± 25	<0.01
Lactate dehydrogenase (IU/l)	188 ± 11	177 ± 15	0.56	183 ± 5	179 ± 12	0.66
Alkaline phosphatase (IU/l)	224 ± 12	290 ± 82	0.47	344 ± 67	255 ± 21	0.21
White blood cells (×10 ³ /μl)	6.8 ± 0.6	6.2 ± 1.0	0.60	6.3 ± 0.4	5.8 ± 0.4	0.51
Hemoglobin (g/dl)	14.0 ± 0.3	12.8 ± 1.1	0.15	13.5 ± 0.3	14.4 ± 0.7	0.21
Platelet (×10 ⁴ /μl)	23.4 ± 2.0	23.7 ± 2.4	0.93	22.6 ± 1.3	25.7 ± 4.0	0.37

Data are presented as mean ± SE or % (n/N)

AST aspartate aminotransferase, ALT alanine aminotransferase

* Student's *t* test or the χ^2 test

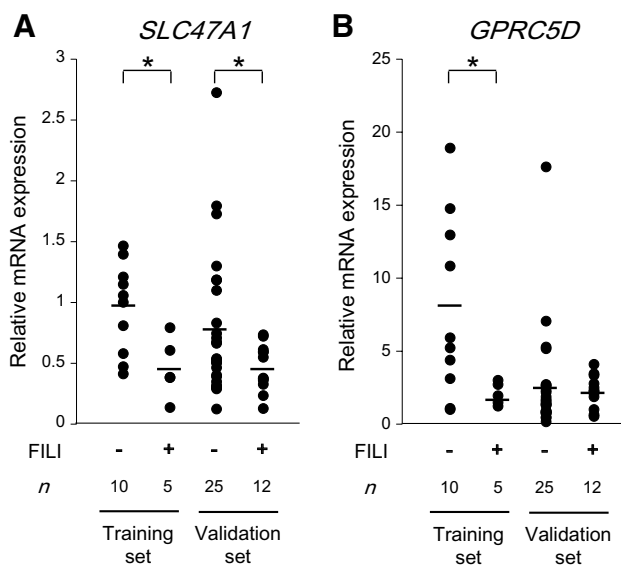


Fig. 2 Relative mRNA expression levels of *SLC47A1* (a) and *GPRC5D* (b). The bars indicate the means. **p* < 0.05

two candidate genes (*SLC47A1* and *GPRC5D*) between the groups in the validation set. As shown in Fig. 2, we confirmed that *SLC47A1* mRNA levels were significantly lower in the patients with FILI.

Because *SLC47A1* encodes a drug transporter protein, multidrug and toxin extrusion protein 1 (MATE1) [16, 17], these clinical data led us to speculate that the concentration of flutamide or its metabolite(s) in blood or liver is influenced by this protein's activity. To test this hypothesis, we conducted a small animal experiment on *Mate1* knockout mice (three per group). Two hours after intraperitoneal administration of flutamide, serum concentrations of flutamide and hydroxyflutamide did not differ between *Mate1* knockout and control mice (Fig. 3). Nevertheless, the serum concentration of FLU-1 was higher in *Mate1* knockout mice than in the control mice. Concentrations of flutamide, hydroxyflutamide, and FLU-1 in the liver were below the detection limit of our methods. Thus, these preliminary results indicated that *Mate1* could affect the pharmacokinetics of flutamide.

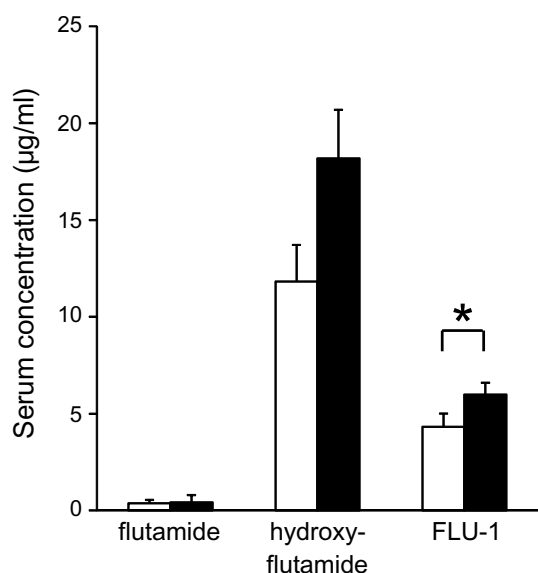


Fig. 3 Serum concentrations of flutamide and its metabolites. Blood samples were obtained from wild-type (white bars) and *Mate1* knockout mice (black bars) 2 h after intraperitoneal injection of flutamide (30 mg/kg). The data are presented as mean + SE (three mice per group). * $p < 0.05$. FLU-1, 4-nitro-3-(trifluoromethyl)phenylamine

Discussion

According to our results, baseline characteristics of patients, e.g., age, body mass index, and liver enzyme levels, cannot predict the development of FILI; these data are in line with another study [6]. On the other hand, we found that the lower mRNA levels of *SLC47A1* (*MATE1*) in peripheral blood can predict FILI with high sensitivity as follows: the sensitivity is 100 % (17/17) when the cutoff level of *SLC47A1* relative expression is set to 0.9. Specificity, however, is low (37 %; 13/35) because many patients without FILI also exhibit lower *SLC47A1* levels. Therefore, this biomarker seems to be useful for the identification of patients who will not develop FILI but not those who will develop FILI.

MATE1 is an H^+ /organic cation exporter and is primarily expressed in the brush border membrane of renal proximal tubular cells and the canalicular membrane of hepatocytes [16, 17]. *MATE1* has been shown to transport various cationic and anionic drugs, such as cimetidine, cephalixin, and metformin, in *MATE1*-transfected HEK293 cells [18]. Moreover, it has been reported that a single nucleotide polymorphism (SNP) of *SLC47A1* affects the therapeutic efficacy of metformin [19]. The hepatic concentration of metformin 60 min after administration is markedly higher in mice lacking *Mate1* (a homolog of human *MATE1*) than in control mice [20]. Thus, *Mate1* appears to affect the pharmacokinetics of several medications. In our study, serum concentrations of FLU-1 differ between *Mate1* knockout and control mice; this finding is consistent with the

hypothesis that *Mate1* is involved in the pharmacokinetics of flutamide, although the mode of action of this transporter remains to be determined.

Aizawa et al. [21] found that the steady-state plasma concentration of FLU-1 but not flutamide and hydroxy-flutamide may be linked to the development of FILI. Further studies are needed to determine whether the plasma concentration of FLU-1 is higher in patients with the low *SLC47A1* mRNA expression in peripheral blood than in patients with strong expression of this gene. Because the *SLC47A1* mRNA level seems to work only as a *negative* predictor of FILI, accurate positive prediction of FILI may require combining the data on *SLC47A1* mRNA with some other biomarker.

The gene encoding *MATE1* has several SNPs that attenuate the transport activity at least in vitro [22]. Some of these SNPs, such as *SLC47A1* -66T>C (rs2252281), 191G>A (rs77630697), and 983A>C (rs111060527), are reported to downregulate *MATE1* expression [22, 23]. Therefore, it is possible that the patients with low *SLC47A1* (*MATE1*) mRNA expression in the present study have such a SNP. To use the *SLC47A1* mRNA level as a negative predictor of FILI in clinical practice, we first need to resolve several technical issues concerning the sampling and measurement methods, including the optimal sampling time, an absolute quantitative procedure, and an optimal cutoff level. On the other hand, the genotyping is relatively easy and readily available for clinical use if the genotype–phenotype relationship becomes clear. We are planning to test whether genotypes of *SLC47A1* are associated with the development of FILI in an upcoming project.

In our prospective study, we found that *SLC47A1* mRNA levels in peripheral blood before the initiation of flutamide therapy are significantly lower in patients who will develop FILI than in those who will not. The *SLC47A1* mRNA level is a possible negative predictive biomarker of FILI. Our results may lead to the development of a safer flutamide-based treatment strategy for patients with prostate cancer.

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Conflict of interest The authors declare that they have no conflict of interest.

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