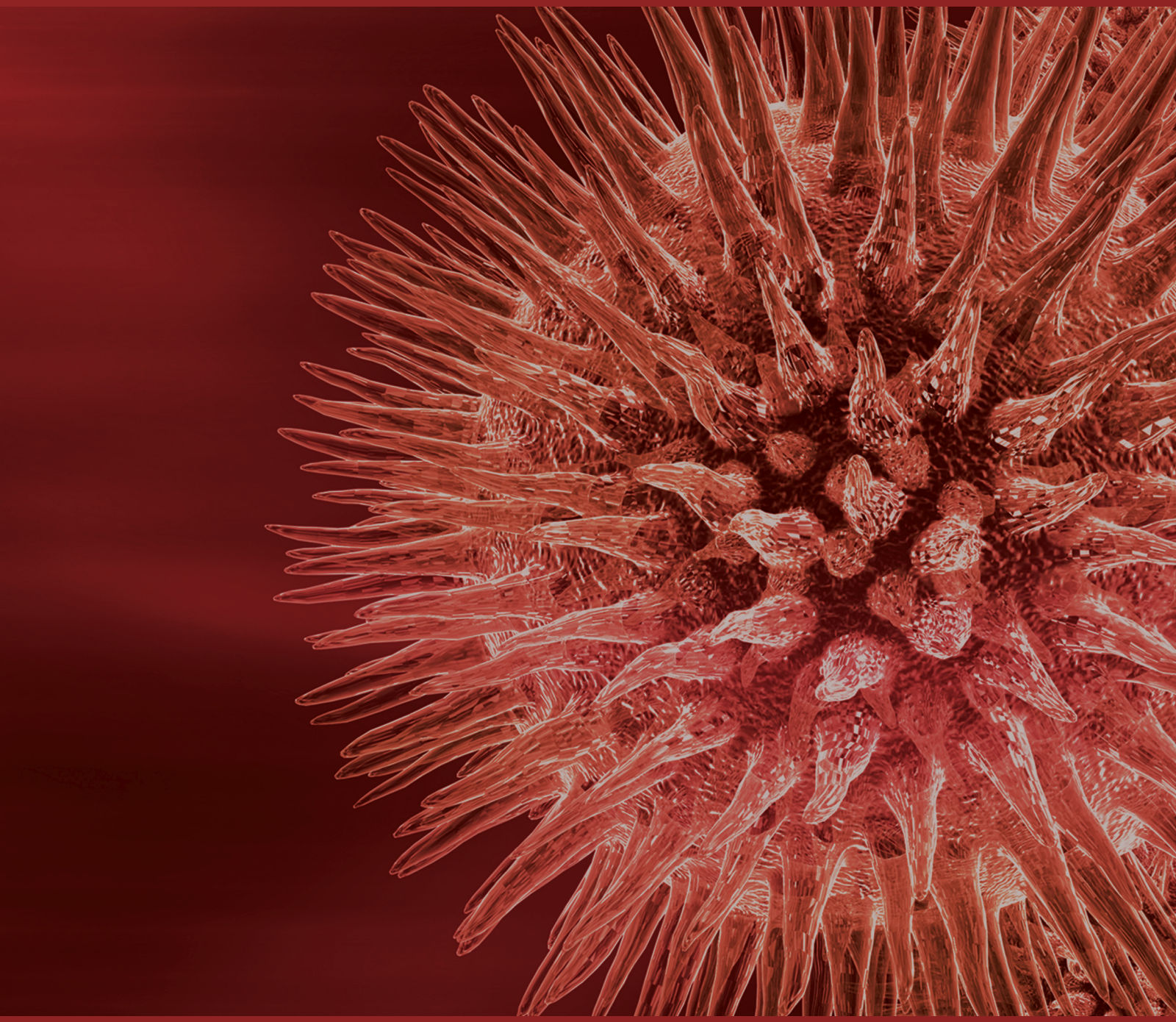
BioMed Research International

Pharmacogenomics in Personalized Medicine and Drug Metabolism

Guest Editors: Wei-Chiao Chang, Shuen-Iu Hung, Koichi Handa, Yan Gong, Jing Li, and Dongquan Shi



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Editorial

Pharmacogenomics in Personalized Medicine and Drug Metabolism

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Enormous progress in human genetic studies has been made in the past decade. With the international research collaboration and advances in genome sequencing techniques, understanding of human genome and disease pathogenesis has dramatically increased. In particular, the information from human genome gives us more ideas to understand disease susceptibility and drug responses. Personalized medicine and pharmacogenomics are to determine unique molecular characteristics between the individuals and to apply this genetic information to diagnose an individual's disease accurately, select better treatments, and reduce possible drug adverse reactions.

This special section contains eleven articles. S.-I. Hung et al. carefully reviewed recent findings in genetic variations and adverse drug reactions. R.-H. Wong et al. described their work that indicated that pesticide exposed individuals with susceptible MDR1-129 genotypes may have increased risk of DNA damage. M.-S. Wu et al. reported a genetic polymorphism in ORAI1 calcium channel that associated with elevated serum calcium levels in chronic kidney disease patients. Q. Jiang et al. showed that ESR1 gene is considerably associated with knee osteoarthritis etiology in the Chinese Han population. Y. Wang et al. identified that polymorphisms in interleukin-4 (IL-4) and interleukin-6 (IL-6) are associated with increased risk of rheumatoid arthritis. Using bioinformatics, E. Y. Chuang's group and C.-H. Yang's group successfully identify more genes linked with breast cancer and Alzheimer's disease. J.-Y. Wang et al. reported that chip including DPYD, TYMS, TYMP, TK1, and TK2 genes is a potential tool to predict responses in locally advanced rectal cancer patients treated with fluoropyrimidine-based chemoradiotherapy. J.-H. Li et al. provided convincing evidence that CYP2B6 785G allele and ABCB1 2677T allele have positive effects on the methadone plasma concentrations. J. Wei et al.'s work indicated that PDZ-binding motif (TAZ) is a potential marker that is overexpressed in signet ring cell carcinoma. In addition, K.-S. Hung et al. reported new clinical applications to improve motor function after traumatic brain injury. Y.-W. Cheng et al. identified a new compound (TW01003) with potent anticancer and antiangiogenesis activities.

In conclusion, the field of genomic medicine has largely advanced in a relatively short time. With the new techniques now available, new findings will be revealed in the future.

Wei-Chiao Chang

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Research Article

In Search of the Active Metabolites of an Anticancer Piperazinedione, TW01003, in Rats

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TW01003, a piperazinedione derivative designed as an antimitotic agent, exhibited potent anticancer and antiangiogenesis activities in mice. However, oral administration of this compound in rats led to poor systemic bioavailability which suggested that *in vivo* efficacy might come from its metabolites. This report describes the identification of TW01003 metabolites in pig and Wistar rats. Following intravenous administration of TW01003, pig urine samples were subjected to sulfatase and glucuronidase treatment to monitor the biotransformation products. Rats were given TW01003 both intravenously and orally, and blood samples were collected and then analyzed by HPLC to quantitatively determine the metabolic transformation of TW01003 to its metabolite. A sulfate conjugate, TW01003 sulfate, was identified as the major metabolite for TW01003 after intravenous injection in both pig and rats. However, in rats, the glucuronide conjugate became major metabolite 30 min after TW01003 oral dosing. Pharmacokinetic analysis after intravenous administration of TW01003 indicated that TW01003 sulfate had a systemic bioavailability 2.5 times higher, volume of distribution three times higher, residence time seven times longer, and clearance rate 2.3 times lower compared to TW01003. Our results indicate that the potent anticancer and antiangiogenesis activities of TW01003 might not come from TW01003 *per se* but from its metabolites TW01003 sulfate.

1. Introduction

Tubulin binding agents, which result in mitotic arrest of tumor cell and then apoptosis, are now standard treatment in cancer chemotherapy [1–4]. (3E,6E)-3-Benzylidene-6-[(5-hydroxypyridin-2-yl)methylene]piperazine-2,5-dione (TW-01003) (Figure 1), a piperazinedione derivative synthesized in this laboratory as an antimitotic agent, exhibited a broad spectrum of antitumor activities in 60 human disease-oriented cancer cell panel screenings [5–7]. A profound antiangiogenesis effect of this compound was demonstrated in mice; after oral treatment of TW01003 (3 mg/kg), the hemoglobin count of the matrigel with vascular endothelial growth factor- (VEGF-) induced angiogenesis was reduced to <1% (unpublished data). However, a preliminary pharmacokinetic study indicated poor bioavailability upon oral administration of this compound to rats, with only 1.72% of the oral fraction absorbed (unpublished data). This led to a suspicion that the potent antiangiogenesis effect of TW01003 might come from its metabolites.

In this report, we describe the identification of the metabolites of TW01003 in pig following intravenous (i.v.) administration. We also investigated the metabolic profiles of TW01003 in rats following i.v. and oral administration. The transformation of TW01003 to its major metabolite was determined by pharmacokinetic studies in rats.

2. Materials and Methods

2.1. Materials. TW01003 potassium salt (TW01003-K) was prepared in this laboratory [5]. Analytical grade chemicals for biological studies were from Sigma-Aldrich (St. Louis, MO, USA), E. Merck KG (Darmstadt, Germany), Fluka

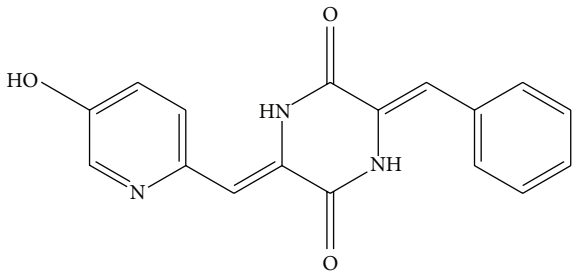

Figure 1: Structure of TW01003 in mice.

Chemika (Buchs, Switzerland), Acros (Morris Plains, NJ, USA), and Wako (Richmond, VA, USA). High-performance liquid chromatography- (HPLC-) grade acetonitrile and methanol were purchased from Alpus Pharmaceutical Industries Co. (Gifu, Japan). Equipment used in the preparation of biological samples consisted of the following: Branson Sonifier 450 sonicator (Danbury, CT, USA), Kubota 2010 (Tokyo, Japan), Eppendorf AG 5415C centrifuge (Hamburg, Germany), and Model 905 incubator (Ballrechten-Dottingen, Germany). β-Glucuronidase (type B-1 from bovine liver, containing 1,240,000 units/g of β-glucuronidase) and sulfatase (type H-1 from *Helix pomatia*, containing 14,000 units/g of sulfatase and 498,800 units/g of β-glucuronidase) were purchased from Sigma-Aldrich.

Crossbred 3-4-month-old pigs weighing 40–50 kg, obtained from the Animal Technology Institute, Taiwan (Miaoli, Taiwan), were used in a preliminary metabolic study for establishing the HPLC analytical method for the identification of TW01003 and its metabolites. The phase II conjugate TW01003 sulfate was first isolated from pig urine as an authentic sample for analysis. Male Wistar rats (200–250 g) for pharmacokinetic studies were purchased from the Laboratory Animal Center of National Taiwan University (Taipei, Taiwan). The animals were pathogen free and allowed to acclimate to the environmentally controlled quarters (24 ± 1°C and 12 : 12 h light-dark cycle) for at least 5 days before the experiments. Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals [8].

2.2. Analytical Sample Preparation. D(+)-Glucose-monohydrate (D5W) solution (5%) was prepared by mixing 25 g of D5W with double deionized water, filtered through a 0.22 μm Millipore filter, and adjusted to 500 mL. Vehicle V was prepared by mixing 5% D5W, Cremophor EL, and ethyl alcohol in the ratio of 90 : 5 : 5 (v/v/v). Standard TW01003 solution (1 mg/mL) was prepared by dissolving TW01003-K (1.16 mg) in 1 mL of vehicle V and 160 μL of aqueous 25% NH_4OH solution to become a stock solution with a concentration of 1 mg/mL. The solution was stored at 4°C until use.

The stock solution was diluted with vehicle V to prepare standard solutions with concentrations ranging from 4.0 μg/mL to 0.00781 μg/mL. The TW01003 standard solutions (100 μL) were mixed with blank plasma (100 μL) and an internal standard solution (400 μL of a solution of HPW044X11 in ethyl acetate 0.025 μg/mL) and centrifuged (5,585 g) for 10 min. The supernatant was blown to dryness with nitrogen gas. The D5W vehicle (200 μL) was then added to the residue as the test solution, and 100 μL was subjected to HPLC analysis.

2.3. Chromatography and Validation of Assay Methods. TW01003 test solutions were analyzed by HPLC. The HPLC system consisted of an autosampler (AS950, Jasco, Tokyo, Japan), a Waters Model 600E solvent delivery pump (Millipore, Milford, MA, USA) coupled with an ultraviolet detector monitored at wavelength 350 nm (Bioanalytical Systems, Inc., West Lafayette, IN, USA), and an integrator (Macintosh LC II computer with Macintegrator I, Rainin; Apple, New York, NY, USA).

The samples were eluted in a C18 reversed-phase microbore column (particle size 5 μm, 150 × 1 mm; Bioanalytical Systems) at a flow rate of 1 mL/min. The eluent was filtered through a Millipore filter (0.22 μm) and degassed prior to analysis. Mobile phases for gradient elution were 0.1% acetic acid aqueous solution ($HOAc_{(aq)}$) : acetonitrile (ACN) = 60 : 40 at 0–10 min and 0.1% $HOAc_{(aq)}$: ACN = 50 : 50 at 10–23 min.

Assay methods were validated by determining the precision and accuracy of intra- and interday analyses of serum standards over a period of 6 days. The lower limit of quantification (LOQ) between intraday assays of TW01003 was 7.46 ± 0.67 ng/mL ($n = 3$, $r^2 = 0.9995$), with coefficients of variation less than 9%. The lower LOQ between interday assays was 7.17 ± 0.54 ng/mL ($n = 3$, $r^2 = 0.9999$) with coefficients of variation less than 15%.

2.4. Animal Experiments. Single-dose TW01003 in a solution (1 mL) containing a 9 : 1 (v/v) ratio of vehicle V : 10% $Na_2CO_{3(aq)}$ was used for animal studies in pig or in male Wistar rats [9]. For metabolic studies, the TW01003 test solution was administered intravenously (7 mg/kg, $n = 1$) to the tail vein or orally (36 mg/kg, $n = 1$) by a feeding tube. For pharmacokinetic studies, the test solution was administered intravenously to the tail vein of Wistar rats (2.0 mg/kg, 1 mL, $n = 6$). The rats were put under a heating lamp to maintain body temperatures at 37°C throughout the experiment. All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

Blood samples (0.5 mL) were withdrawn from the carotid artery of rats at time intervals of 5, 15, 30, 45, 60, 90, 120, 180, and 240 min. Heparin sodium (25 IU/mL in 0.3 mL of saline) was added, and the blood samples were centrifuged (5,585 g) at 4°C for 8 min. The plasma was frozen immediately and kept at −78°C until analysis. The plasma sample (150 μL) was mixed with a sulfatase solution (200 IU/mL, 50 μL) at 37°C for 2 h. The plasma sample (150 μL) mixed with 50 μL of a buffer solution (pH 5.0) was used for the control group. The internal standard solution was then added, and the solution was centrifuged (5,585 g) for 10 min. The supernatant was concentrated to dryness with nitrogen gas. The D5W vehicle

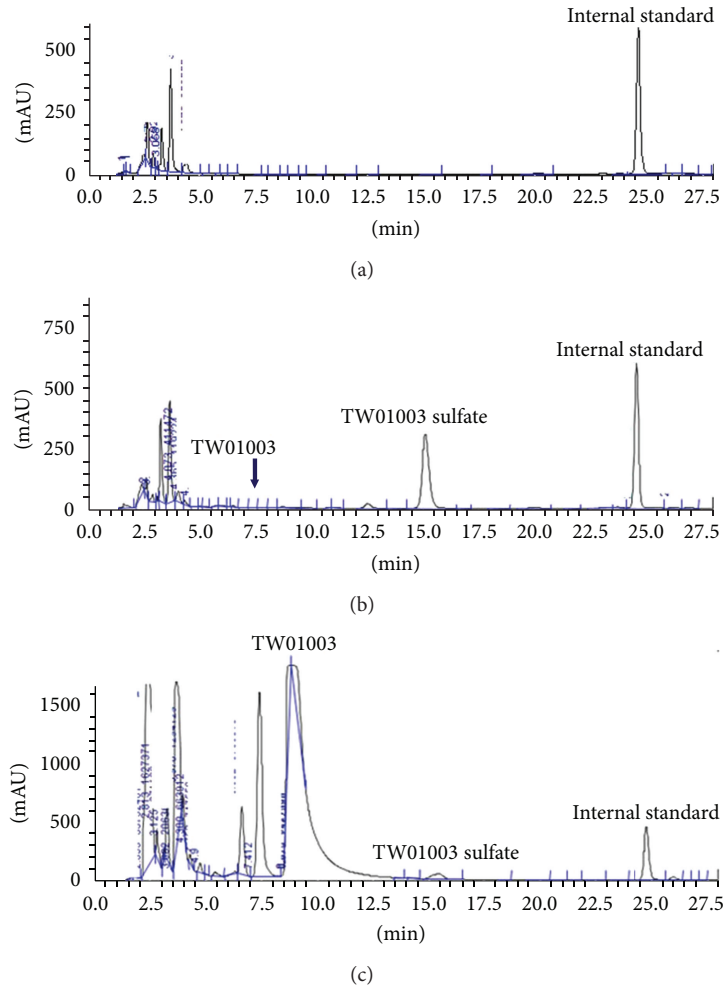

Figure 2: Typical high pressure liquid chromatograph of untreated urine of pig (a); pig urine after TW01003 administration (b); and sulfatase-treated pig urine after TW01003 dosing (c). Parent compound TW01003, TW01003 sulfate, and internal standard HPW044X11 were identified with retention times at 9.17 min, 16.59 min, and 25.85 min, respectively. TW01003 glucuronide with a retention time at 12.5 min was barely visible.

(200 μL) was then added to the residue, and the solution was subjected to HPLC analysis.

2.5. Pharmacokinetic Studies. We established the plasma concentration-time profile [10, 11]. The area under the plasma concentration-time profile (AUC) and other pharmacokinetic parameters (peak concentration: C_{max}; time to reach C_{max}: T_{max}; area under the moment curve: AUMC; volume of distribution: V_D) were calculated using the log-linear trapezoidal rule. Plasma concentrations of TW01003 and the major metabolite were calculated using WINNONLIN 3.1 software by a noncompartment model. Terminal half-life ($t_{1/2}$) was compartment model-independently estimated. Data analysis was performed using Microsoft Excel, and data were represented as mean ± standard deviation (SD) for n experiments. Treatment differences were evaluated by the paired t-test.

3. Results

3.1. Chromatographic Identification of TW01003 and TW-01003 Sulfate. In order to establish a feasible analytical method for the identification of TW01003 metabolites in a biological system, a test solution containing 200 mg of TW01003 was first injected into a 40–50 kg pig. Fresh urine (1 mL) was collected 60 min after dosing, filtered through a 0.22 μm Millipore filter, and subjected to HPLC analysis. Typical HPLC chromatograms of urine sample of untreated pig (Figure 2(a)), urine samples upon i.v. dosing of TW01003 (Figure 2(b)), and urine samples after sulfatase treatment (Figure 2(c)) are depicted.

3.2. Metabolic Study of TW01003 in Rats. Metabolic studies of TW01003 were conducted using male Wistar rats. The preliminary data were studies in one rat. In this experiment,

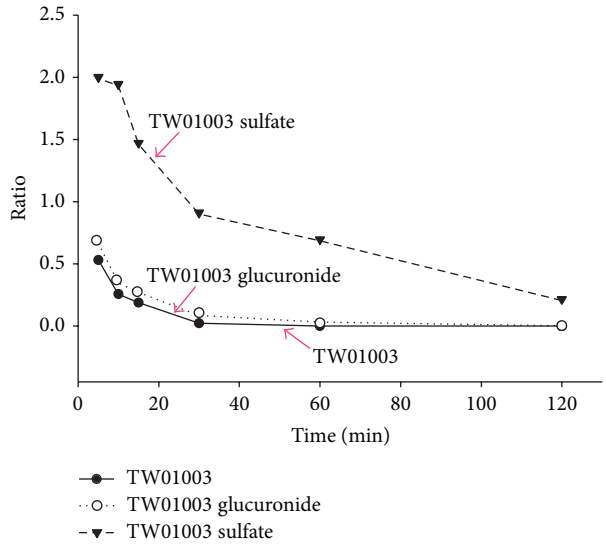

Figure 3: Plasma concentration-time curve after i.v. administration of TW01003 (6.7 mg/kg) to a rat. Plasma samples were collected and subjected to enzymatic treatment by sulfatase or glucuronidase. This method was used for the identification of TW01003 sulfate or glucuronide conjugate after TW01003 dosing.

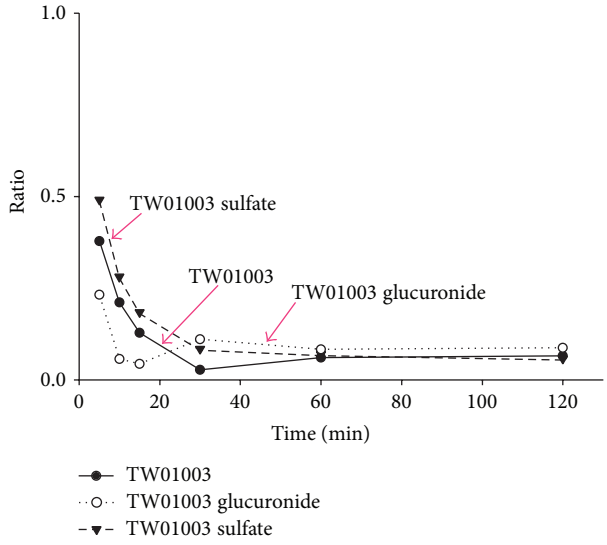

Figure 4: Plasma concentration-time curves of TW01003 and its metabolites after oral administration of TW01003 (36 mg/kg) to a rat ($n = 1$).

we had established plasma concentration-time curves of parent TW01003 and its major metabolites after i.v. (6.7 mg/kg, $n = 1$, Figure 3) or oral administration (36.0 mg/kg, $n = 1$, Figure 4) of TW01003. Plasma samples were collected and subjected to enzymatic treatment by sulfatase or glucuronidase for the identification of TW01003 sulfate and glucuronide as phase II metabolic conjugates.

The results indicated that TW01003 was cleared fairly rapidly, regardless of administration method. As sulfonation and glucuronidation are the most common phase II

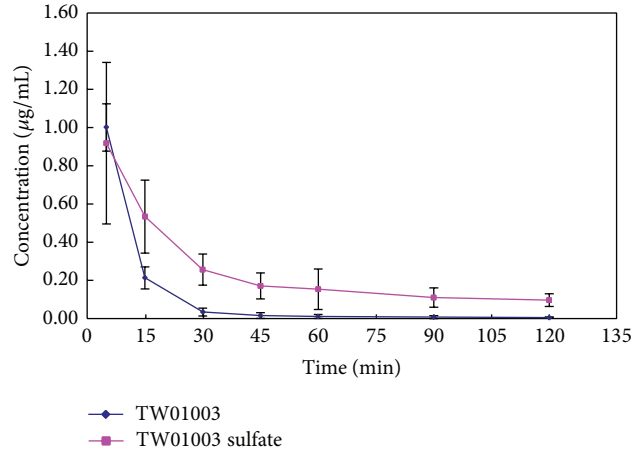

Figure 5: Plasma concentration-time profile of TW01003 and TW01003 sulfate after i.v. administration of TW01003 ($n = 6$).

metabolic pathways for compounds containing an aromatic hydroxyl group, we first identified the metabolites in rat urine. Urine samples were subjected to sulfatase or glucuronidase treatment prior to HPLC analysis. The method used for analyze TW01003 was also used to identify TW01003 sulfate or glucuronidase conjugate.

3.3. Pharmacokinetics upon I.V. Administration of TW01003 in Rats. Plasma samples were subjected to sulfatase treatment for the identification of TW01003 sulfate conjugate as a metabolite. The plasma concentration-time profile of TW01003 and TW01003 sulfate after i.v. administration of TW01003 is depicted in Figure 5 ($n = 6$). Pharmacokinetic (PK) parameters of TW01003 and TW01003 sulfate are summarized in Table 1.

4. Discussion

This study investigated the biotransformation of TW01003 in rats and in pig. As depicted in Figure 2(b), we observed a fast clearance upon administration of TW01003 in pig. After 60 min of TW01003 dosing, TW01003 sulfate (retention time 16.59 min) was identified as the major metabolite. Upon sulfatase treatment, this phase II conjugate was hydrolyzed to TW01003 (Figure 2(c)), confirming that the peak of retention time 16.59 min is TW01003 sulfate conjugate. We had also purified the TW01003 sulfate from pig urine, which showed the same retention time in HPLC (data not shown). This enzymatic method was used to quantify the biotransformation process of TW01003 biotransformation to TW01003 sulfate.

We conducted pharmacokinetic studies to monitor the blood level of TW01003 and TW01003 sulfate. In Wistar rats (Figure 5, $n = 6$), the pharmacokinetic parameters of TW01003 and TW01003 sulfate after i.v. administration of TW01003 were derived from noncompartmental model, based on plasma concentration-time profile. The systemic exposure of TW01003 sulfate was 2.5 times higher than that of

Table 1: Summary of pharmacokinetic parameters of TW01003 and TW01003 sulfate after i.v. administration of TW01003 ($n = 6$).

PK parameters	TW01003 Mean ± SD	TW01003 sulfate Mean ± SD
C_{max} (μg/mL)	2.20 ± 0.25	1.31 ± 0.64
$t_{1/2}$ (h)	0.76 ± 0.14	1.31 ± 0.22
AUC_{all} (h∗μg/mL)	0.28 ± 0.04	0.52 ± 0.20
AUC_{INF} (h∗μg/mL)	0.29 ± 0.04	0.71 ± 0.25
CL (L/h/kg)	7.05 ± 1.12	3.08 ± 0.90
$AUMC_{INF}$ (h∗h∗μg/mL)	0.06 ± 0.02	1.01 ± 0.36
MRT_{INF} (h)	0.20 ± 0.06	1.44 ± 0.33
V_D (L/kg)	1.41 ± 0.41	4.43 ± 1.52

C_{max}: peak concentration; $t_{1/2}$: half-life; AUC_{all}: area under curve from time 0 to the last sampling time; AUC_{INF}: area under curve from time 0 to infinite; CL: clearance; $AUMC_{INF}$: area under the moment-time curve from time 0 to infinite; MRT_{INF}: mean residence time from time 0 to infinite; V_D: volume of distribution.

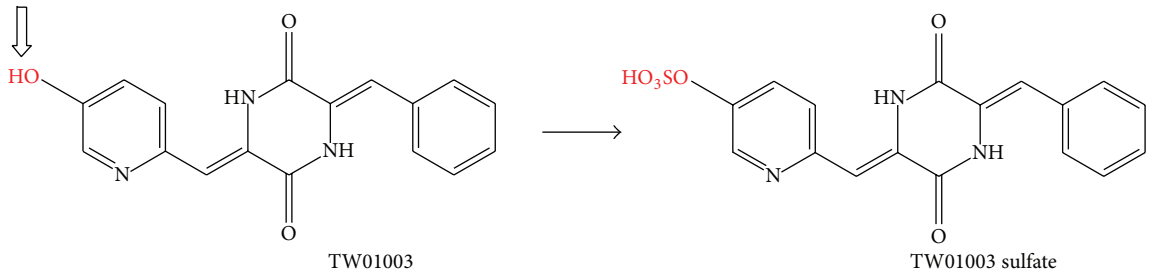

Figure 6: Biotransformation of TW01003 to TW01003 sulfate.

TW01003 (AUC from time 0 to infinite, AUC_{INF} 0.71 ± 0.25 versus 0.29 ± 0.04 h·μg/mL). Interestingly, TW01003 sulfate demonstrated a three times higher volume of distribution (V_D 4.43 ± 1.52 versus 1.41 ± 0.41 L/kg), seven times longer residence time (MRT_{INF} 1.44 ± 0.33 versus 0.20 ± 0.06 h), and 2.3 times lower clearance rate (CL 3.08 ± 0.90 versus 7.05 ± 1.12 L/h/kg) than those of TW01003.

Sulfonation and glucuronidation are the most common metabolic pathways of compounds with aromatic hydroxyl groups [12–14]. This kind of phase II metabolism is also demonstrated in TW01003. The plasma concentration-time profile after i.v. administration of TW01003 to Wistar rats demonstrated a fast metabolism of this compound to its sulfate conjugate as the major metabolite (Figure 3). However, the glucuronide conjugate became the major metabolite 30 min after TW01003 oral dosing (Figure 4). Enterohepatic circulation might explain the increased bioavailability of this metabolite in the systemic circulation. The difference in the sulfonation product level after i.v. or oral dosing of TW01003 may have resulted from tissue distribution of the sulfonation enzyme sulfotransferase [15, 16].

Sulfotransferase, a family of enzymes encoded by sulfotransferase (SULT) genes, possesses important physiological functions in the regulation of bile acid enterohepatic circulation [17]. It also transfers a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate to xenobiotics, primarily to aromatic alcohol, to enhance the hydrophilicity of its substrate and thus improve the clearance of the foreign molecules through bile or renal secretion. From the chemical structure of TW01003 shown in Figure 6, the only viable aromatic hydroxyl group is on the pyridine ring, which is not commonly seen in biotransformation reactions [18].

There were various mutations identified in the SULT enzyme family and they were reported to exhibit different catalytic specificity and capacity in each polymorph form [15]. For new drug development, identification of the metabolic pathway is essential. More studies are needed to explore the enzyme system involved in the sulfonation process.

TW01003 is an antiangiogenesis agent that exhibits strong potency in an *in vivo* model. However, the limited half-life failed to explain its *in vivo* efficacy. Sulfonation in metabolism is mainly regarded as a detoxification mechanism. It may also serve as a bioactivation process [18, 19] that can activate TW01003 with improved exposure, longer half-life, and longer mean residence time. Further investigations are needed to confirm the antiangiogenesis activity of TW01003 sulfate metabolites.

5. Conclusion

Administration of TW01003 exhibited extensive metabolism in both pig and rats. Extensive metabolism leading to the high clearance rate of TW01003 in this study explains the unsatisfactory pharmacokinetics of TW01003. As TW01003 exhibited potent anticancer and antiangiogenesis activities, the low systemic bioavailability of TW01003 failed to explain

the fact that the activity came from TW01003 *per se*. From the chemical structure of TW01003, we suggested an unusual pyridine-OH sulfonation metabolite, and this may serve as an active metabolite for its antiangiogenesis potency. Further studies are needed to identify if the metabolites that demonstrated the anticancer and antiangiogenesis activities are active species.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Multidrug Resistance 1 Gene Variants, Pesticide Exposure, and Increased Risk of DNA Damage

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The P-glycoprotein, encoded by the multidrug resistance (*MDR)1* gene, extrudes fat-soluble compounds to the extracellular environment. However, the DNA damage of pesticides in subjects with genetic variation in*MDR1* has not been investigated. In this study, the comet assay was applied to examine the extent of DNA damage in the peripheral blood of 195 fruit growers who had been exposed to pesticides and 141 unexposed controls. The *MDR1* polymorphisms were identified. Questionnaires were administered to obtain demographic data and occupational history. Results showed subjects experiencing high (2.14 μm/cell, $P < 0.01$) or low pesticide exposure (2.18 μm/cell, $P < 0.01$) had a significantly greater DNA tail moment than controls (1.28 μm/cell). Compared to the *MDR1* T-129C (rs3213619) TC/CC carriers, the TT carriers had increased DNA tail moment in controls (1.30 versus 1.12 μm/cell, $P < 0.01$). Similar results were observed in the high and low pesticide-exposed groups. Combined analysis revealed that pesticide-exposed fruit growers with *MDR1* -129 TT genotype had the greatest DNA damage in the subjects with the combinations of pesticide exposure and *MDR1* -129 genotypes. In conclusion, pesticide exposed individuals with susceptible *MDR1* -129 genotypes may experience increased risk of DNA damage.

1. Introduction

Although pesticide exposure has been linked to an increased risk of many cancers [1–4], epidemiologic data on the cytogenetic effects in pesticide-exposed farmers have been inconsistent [5–7]. Previously, our studies in Taiwan reported that pesticide-exposed fruit growers had a higher risk of DNA damage [8, 9]. In particular, genetic susceptibility has a substantial contribution to DNA damage in pesticide-exposed population.

The P-glycoprotein (P-gp) is encoded by the human multidrug resistance protein 1 (*MDR1* or *ABCB1*) gene. Importantly, this protein can extrude lipophilic compounds to the extracellular space by the ATP-dependent efflux transport mechanism, including chemotherapeutic agents and pesticides [10, 11]. It has also been reported that P-gp acts as an epithelial barrier and performs as excretory functions in various normal human tissues [11]. An animal study found that constructed *mdr1a*-disrupted mice which compared to normal *mdr1a* mice had the increased toxicity

by the pesticide, and there was decreased elimination of this compound [12]. In particular, P-gp is capable of interacting with a large group of structurally diverse pesticides [13]. Therefore, P-gp might play a critical role in the detoxification of pesticide.

Alterations in P-gp expression and function potentially depend on structural variations of the *MDR1* gene. Human *MDR1* is located on chromosome 7q21.1, and many single nucleotide polymorphisms (SNPs) within this gene have been identified. The two common synonymous SNPs are C3435T (rs1045642), located in exon 26 at position 3435, and C1236T (rs1128503), located in exon 12 at position 1236 [14]. It has been found that the mRNA expression and P-gp activity of 3435T and 1236T alleles each were significantly lower than those of the 3435C and 1236C alleles [15–17]. The other frequent nonsynonymous SNP is G2677T/A (rs2032582), which is located in exon 22 at position 2677. This polymorphism could change the amino acid from alanine (*Ala*) to Serine (*Ser*) or threonine (*Thr*) and result in the lower P-gp expression [15]. A previous study conducted in Chinese subjects observed that the *MDR1* C3435T, C1236T, and G2677T/A genetic polymorphisms were significantly associated with a higher risk of developing Parkinson's disease [18]. These three polymorphisms were also indicated to be closely related to linkage disequilibrium. Thus, the haplotypes composed of different *MDR1* SNPs might have a better representation of a change in P-gp function [19, 20]. Another important *MDR1* SNP is T-129C (rs3213619). This polymorphism is located in the promoter region, and it has been established that -129C allele has a decreased P-gp expression [21].

Although the role of *MDR1* polymorphisms, particularly C3435T, C1236T, G2677T/A, and T-129C, has been evaluated in previous studies [22–24], little is known about their potential effect on the genotoxicity of pesticide. In this study, we investigated the association of these four *MDR1* polymorphisms in pesticide-exposed fruit growers with cellular DNA damage, as measured by the comet assay.

2. Materials and Methods

2.1. Study Population and Epidemiological Information. The study design and final report were approved by the institutional review board of the Chung Shan Medical University, Taichung, Taiwan. All participants were provided with a written description of the study. Those who were unable to read the description had it read to them. All subjects gave written informed consent prior to inclusion in the study.

Previously, we conducted a cross-sectional study to explore the association between DNA damage and metabolic and DNA repair traits among 135 pesticide-exposed fruit growers and 106 nonexposed controls in Tungshin Town, which is located in central Taiwan. Criteria for selection of the study population are described in detail elsewhere [9]. In order to acquire greater statistical power to detect a difference in the level of DNA damage, sample size in the current study was increased to 195 pesticide-exposed fruit growers and 141 nonexposed controls. Fruit growers who were exposed to pesticides and unexposed controls were invited to participate in our study. The occupations of control subjects included housewives, teachers, clerks, nonfarm laborers, skilled workers, small-business persons, and professionals. We tried to minimize biases due to variations in ethnicity and lifestyle by selecting unexposed controls who were from the same residential area and of the same ethnicity as the pesticide-exposed subjects. None of the study subjects had received any therapeutic irradiation, and none were taking any medication.

A questionnaire on demographic characteristics, smoking, alcohol drinking, and occupational and medical histories was completed by each subject. The number of cigarettes smoked daily and the duration of the subject's smoking habit were also noted. Most of these farmers have been alerted to the risk of alcohol induced liver damage and understand that drinking alcohol makes the effects of pesticide poisoning worse. In general, alcohol drinking during the period of pesticide application is not allowed. We are concerned that if pesticide-exposed subjects with this condition were included, they would have a lower rate of alcohol drinking than the controls. Therefore, subjects who drank alcohol were not included in this study.

2.2. Assessment of Pesticide Exposure. The assessment of pesticide exposure has been described previously [8, 9, 25]. On the farms of our study area, pesticides are regularly applied all year. Information on past pesticide use by name, amount, area of pesticide application, numbers of treatments per season, years of agrochemical exposure, and use of personal protection equipment was obtained via interviewer-administered questionnaires in this study. Types of work in the orchards were also obtained. The pesticides used by the fruit growers during the 6 months before the medical examination consisted of almost 40 different compounds. On average, each farmer had applied pesticide about 3 times a month, with an average cumulative spraying duration of about 7 h/month (range, 2–28 h/month). Because of the lack of environmental monitoring data and the degree of personal protection used during handing pesticides, it is difficult to reconstruct an individual's previous pesticide exposure history. Thus, fruit growers were categorized as having low or high pesticide exposure by a modification of the criteria developed by Scarpato et al. [26]: (a) for each subject spraying pesticides, the number of hectares treated was determined, and pesticide exposure was calculated by multiplying the average number of treatments by the number of hectares sprayed; (b) the median value of the distribution obtained in (a) was determined, and fruit growers with exposure values less than or greater than the median were assigned to the low or high exposure class, respectively; and (c) subjects who did not directly handle pesticides (e.g., only involved in cutting or harvesting fruit) were considered to have low exposure. There was a good correlation between individuals' long-term exposure as estimated by our exposure model and acetylcholine esterase level. Thus, our estimation for pesticide exposure in this study should be acceptable.

2.3. Comet Capture and Analysis. In the present investigation, blood samples were collected in a single season (March-May), and each fruit grower was sampled at the beginning

of a midweek working day. Blood samples from the study subjects were collected in heparinized tubes. The comet assay was conducted under alkali conditions according to Singh et al. [27]. For each subject, 100 randomly captured comets from slides (25 cells on each of four comet slides) were examined at ×400 magnification using an epifluorescence microscope connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, United Kingdom). Images acquired by the computerized image analysis system were used to compute the integrated intensity profiles for each cell, estimate the comet cell components, and evaluate the range of derived parameters. To quantify DNA damage, the tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. A single reader, who was blind to the status of subjects, scored all slides.

2.4. Genotyping of Polymorphic MDR1 Genes. Genomic DNA was extracted from peripheral blood using the AxyPrepTM Blood Genomic DNA Miniprep Kit (Axygen Scientific, Union City, CA, USA). *MDR1* C3435T (rs1045642) polymorphism was analyzed by polymerase chain reaction (PCR)- based restriction fragment length polymorphisms [16]. Primers used for the amplification of the rs1045642 were 5′-TGC TGG TCC TGA AGT TGA TCT GTG AAC-3′ and 5′-ACA TTA GGC AGT GAC TCG ATG AAG GCA-3′. PCR products were digested with *Dpn*II. *MDR1* C1236T (rs1128503), G2677T/A (rs2032582), and T-129C (rs3213619) polymorphisms were determined by the StepOne Real-Time PCR System (Applied Biosystems) and analyzed by SDS v3.0 software (Applied Biosystems), using the TaqMan assay (assay IDs: C_7586662_10 for rs1128503, C-11711720C_30 for rs2032582 A/C, C-11711720D_40 for rs2032582 C/T, and C_27487486_10 for rs3213619) [28]. Approximately 10% of the randomly selected samples were directly sequenced to examine the initial genotyping results.

2.5. Statistical Analysis. The continuous variables were presented by mean ± standard error (SE) and were compared by Student's t-test and ANOVA among different pesticide exposure groups and control groups. The categorical variables among different pesticide exposure groups and controls were presented by numbers (%) and were compared by χ^2-test or Fisher's exact test. Hardy-Weinberg equilibrium was performed to test *MDR1* genotypes for goodness of fit. Subsequently, the crude DNA tail moment was evaluated using an analysis stratified by pesticide exposure and different factors. ANOVA was used to compare difference in DNA tail moment by different pesticide exposure groups and control groups, and Student's t-test or ANOVA was used to test the association of the DNA tail moment with age, gender, smoking status, and *MDR1* genotypes. Linkage disequilibrium (LD) coefficients, $D' = D/D_{\max}$ (or $D/D_{\min}$ if the D' value is negative), were assessed for pairs of alleles between *MDR1* rs1045642, rs3213619, rs1128503, and rs2032582 polymorphisms by the expectation-maximization algorithm. We estimated the common haplotypes by the expectation-maximization algorithm. Differences in DNA tail moment among different haplotypes were evaluated by ANOVA in the different pesticide exposure groups and controls, respectively. Further, the association of pesticide exposure and *MDR1* genotypes with the DNA tail moment was analyzed using a general linear model (GLM) and adjusting the effects of confounding factors. In addition, least squares means were calculated to predict adjusted DNA tail moment for study subjects stratified by pesticide exposure status and genotypes; and tests for differences in least squares means were also performed. All P values were calculated using two-tailed statistical tests, and statistical significance was defined at $P < 0.05$. All data were analyzed using SAS 9.1 software (SAS Institute, Cary, NC, USA).

Table 1: Basic characteristics of pesticide-exposed fruit growers and controls.

Variables	Controls	Pesticide exposure	
		Low	High
Number of subjects	141	82	113
Age (years)	49.0 ± 0.9*	55.5 ± 1.2	54.7 ± 1.1
Gender: male (%)	68 (48.2%)*	47 (57.3%)	84 (74.3%)
Duration of pesticide exposure (years)	0	29.9 ± 1.7	30.1 ± 1.4
Size of orchard (ha)	0	0.8 ± 0.1	1.7 ± 0.1#
Smoking habit			
Currents smoker (%)	26 (18.4%)*	23 (28.0%)	33 (29.2%)
Pack-years	3.7 ± 0.8*	8.2 ± 1.7	9.4 ± 1.5

Data represent numbers of individuals or mean ± SE for continuous variables.
*$P < 0.01$; control group differed significantly from the high and low pesticide-exposed groups.
#$P < 0.01$ compared with the low pesticide-exposed group.

3. Results

Basic characteristics of pesticide-exposed fruit growers and controls are presented in Table 1. The control group was significantly younger ($P < 0.01$, ANOVA) and with a lower proportion of males ($P < 0.01$, χ2-test) compared to the high and low pesticide-exposed groups. The control group also had fewer pack-years of smoking than the pesticide-exposed groups ($P < 0.01$). In addition, the mean size of the orchards differed significantly between the high and low pesticide-exposed groups ($P < 0.01$, t-test). The prevalence of *MDR1* genotypes among the study subjects is shown in Table 2. In all subjects, the *MDR1* C3435T (rs1045642, $P = 0.08$) and C1236T (rs1128503, $P = 0.36$) genotypes conformed to the Hardy-Weinberg equilibrium, whereas the G2677T/A (rs2032582) and T-129C (rs3213619) genetic polymorphism did not (Ps < 0.001). The prevalence of *MDR1* C3435T, C1236T, G2677T/A, and T-129C polymorphisms among the different pesticide exposure and control groups was not significantly different.

The crude associations of DNA tail moment with various factors are presented in Table 3. Subjects in the low (2.18 μm/cell, $P < 0.001$) and high (2.14 μm/cell, $P < 0.001$)

Table 2: Prevalence of *MDR1* genotypes among pesticide-exposed fruit growers and controls.

MDR1 genotypes		Controls	Pesticide exposure		*P* value
			Low	High	
Number of subjects		141	82	113	
C3435T	CC	64 (45.4%)	29 (35.4%)	47 (41.6%)	0.62
(rs1045642)	CT	56 (39.7%)	40 (48.8%)	46 (40.7%)	
	TT	21 (14.9%)	13 (15.8%)	20 (17.7%)	
C1236T	CC	22 (15.6%)	12 (14.6%)	12 (10.6%)	0.71
(rs1128503)	CT	59 (41.8%)	33 (40.3%)	54 (47.8%)	
	TT	60 (42.6%)	37 (45.1%)	47 (41.6%)	
G2677T/A	GG	36 (25.5%)	23 (28.1%)	30 (26.5%)	0.78
(rs2032582)	GT/GA	54 (38.3%)	36 (43.9%)	48 (42.5%)	
	TA/TT/AA	51 (36.2%)	23 (28.0%)	35 (31.0%)	
	Non-GG	105 (74.5%)	59 (71.9%)	83 (73.5%)	0.92
T-129C	TT	120 (85.1%)	72 (87.8%)	102 (90.3%)	0.72*
(rs3213619)	TC	15 (10.6%)	8 (9.8%)	9 (8.0%)	
	CC	6 (4.3%)	2 (2.4%)	2 (1.8%)	
	TC/CC	21 (14.9%)	10 (12.2%)	11 (9.7%)	0.46

*Frequencies of T-129C genotype among the groups of low and high pesticide exposure and controls were compared by Fisher exact test.

pesticide-exposed groups had higher DNA tail moment than controls (1.28 μm/cell), respectively. In the control group, subjects younger than 53 years (mean age of all subjects), males, and those who smoked more than 10 pack-years also showed higher DNA tail moment than those older than 53 years (1.31 versus 1.20 μm/cell, $P < 0.01$), females (1.34 versus 1.22 μm/cell, $P < 0.01$), and those who smoked less than 10 pack-years (1.39 versus 1.26 μm/cell, $P < 0.01$), respectively. Interestingly, the DNA tail moment was found to be significantly higher for control subjects with the *MDR1* -129 TT genotype than that of subjects with TC or CC genotypes (1.30 versus 1.12, 1.11 μm/cell, $P < 0.01$; ANOVA). Since the expression of P-gp is lower in subjects with the *MDR1* -129C allele than subjects with the *MDR1* -129T allele [21], thus those with *MDR1* -129 TC and CC genotypes were further combined for the analysis. Significant difference in the DNA tail moments still remained between the groups of those with *MDR1* -129 TT and TC/CC genotypes (1.30 versus 1.12 μm/cell, $P < 0.01$). However, the DNA tail moment was not associated with the *MDR1* C3435T, C1236T, and G2677T/A genotypes. Similar results were observed in the high and low pesticide-exposed groups.

Furthermore, haplotype analysis using the expectation-maximization algorithm showed that the rs1045642, rs1128503, and rs2032582 are in tight linkage disequilibrium with each other (D' value of >0.7, Figure 1) but the rs3213619 is not in linkage disequilibrium with the former ones. Therefore, the haplotype determination was limited to rs1045642, rs2032582, and rs1128503. The average DNA tail moments per cell stratified by *MDR1* C3435T, C1236T, and G2677T/A haplotypes are presented in Table 4. Among the 12 possible haplotypes, TTC (30.1%), CGC (24.6%), and CGT (19.5%) were predominant in all study subjects. The average DNA tail moments per cell in these haplotypes were not significantly different among high and low pesticide-exposed groups and controls.

Figure 1: Linkage disequilibrium (LD) and haplotype block structure of *MDR1* gene. Numbers in squares represent the pairwise D' value.

A multiple linear regression model for the relationship between DNA tail moment and age, gender, smoking status, pesticide exposure, and genotypes of *MDR1* T-129C is shown in Table 5. The DNA tail moment was significantly and

Table 3: Average tail moment per cell stratified by pesticide exposure status and various factors.

Variables	Controls		Pesticide exposure			
			Low		High	
	n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
All	141	1.28 ± 0.01	82	2.18 ± 0.05*	113	2.14 ± 0.04*
Age (years)						
≥53	44	1.20 ± 0.02*	46	2.15 ± 0.07	60	2.17 ± 0.06
<53	97	1.31 ± 0.02	36	2.22 ± 0.08	53	2.11 ± 0.04
Gender						
Males	68	1.34 ± 0.02*	47	2.27 ± 0.08#	84	2.21 ± 0.04*
Females	73	1.22 ± 0.01	35	2.05 ± 0.06	29	1.95 ± 0.05
Smoking status						
>10 pack-years	19	1.39 ± 0.04*	21	2.30 ± 0.11	33	2.15 ± 0.07
≤10 pack-years	122	1.26 ± 0.01	61	2.14 ± 0.06	80	2.14 ± 0.04
MDR1 C3435T genotype						
CC	64	1.29 ± 0.02	29	2.02 ± 0.07	47	2.13 ± 0.05
CT	56	1.29 ± 0.02	40	2.26 ± 0.08	46	2.16 ± 0.06
TT	21	1.22 ± 0.03	13	2.27 ± 0.14	20	2.13 ± 0.08
MDR1 C1236T genotype						
CC	22	1.31 ± 0.03	12	2.12 ± 0.15	12	2.06 ± 0.11
CT	59	1.29 ± 0.02	33	2.14 ± 0.08	54	2.19 ± 0.05
TT	60	1.25 ± 0.02	37	2.23 ± 0.08	47	2.11 ± 0.06
MDR1 G2677T/A genotype						
GG	36	1.30 ± 0.03	23	2.22 ± 0.10	30	2.15 ± 0.07
GT/GA	54	1.25 ± 0.02	36	2.17 ± 0.08	48	2.12 ± 0.06
TA/TT/AA	51	1.29 ± 0.02	23	2.15 ± 0.09	35	2.16 ± 0.07
Non-GG	105	1.27 ± 0.01	60	2.16 ± 0.06	83	2.14 ± 0.04
MDR1 T-129C genotype						
TT	120	1.30 ± 0.01*	72	2.21 ± 0.06*	102	2.15 ± 0.04*
TC	15	1.12 ± 0.01	8	1.89 ± 0.09	9	2.07 ± 0.13
CC	6	1.11 ± 0.01	2	2.10 ± 0.03	2	1.92 ± 0.18
TC/CC	21	1.12 ± 0.01*	10	1.93 ± 0.08	11	2.04 ± 0.11

Comparisons among different pesticide-exposed status groups or (three) genotype groups conducted with ANOVA; comparisons between age, gender, smoking status, and (two) genotype groups conducted with *t*-test.
*$P < 0.01$.
#$0.01 < P < 0.05$.

Table 4: Average DNA tail moment per cell stratified by *MDR1* haplotypes.

Haplotype			Controls		Pesticide exposure				All	
C3435T	G2677T/A	C1236T			Low		High			
(rs1045642)	(rs2032582)	(rs1128503)	*n**	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
T	T	C	83	1.25 ± 0.02	51	2.24 ± 0.07	68	2.13 ± 0.05	202	1.79 ± 0.04
C	G	C	65	1.26 ± 0.02	43	2.19 ± 0.07	57	2.17 ± 0.05	165	1.81 ± 0.04
C	G	T	56	1.30 ± 0.02	34	2.16 ± 0.08	41	2.08 ± 0.06	131	1.77 ± 0.05
x	A	x#	43	1.31 ± 0.03	26	2.07 ± 0.09	37	2.18 ± 0.06	106	1.80 ± 0.05
	Others$		35	1.30 ± 0.03	10	2.14 ± 0.19	23	2.17 ± 0.09	68	1.72 ± 0.07

*Number of alleles.
#Haplotypes contained the variant A allele at the G2677T/A locus (including CAT, CAC, TAT, and TAC).
$Rare haplotypes with frequencies <5% and not part of the variant A allele at the G2677T/A locus (including CTT, CTC, TGT, TGC, and TTT).

Table 5: Multiple regression model for tail moment per cell.

Variables	Regression coefficient	SE	*P* value
Intercept	1.20	0.10	<0.01
Age: per 1-year increment	−0.003	0.002	0.09
Gender: males versus females	0.19	0.04	<0.01
Smoking status: >10 versus ≤10 pack-years	−0.02	0.05	0.64
Pesticide exposure			
High versus control	0.83	0.04	<0.01
Low versus control	0.90	0.05	<0.01
MDR1 T-129C (rs3213619) genotype			
TT versus TC/CC	0.15	0.05	<0.01

positively associated with males, high pesticide exposure, low pesticide exposure, and *MDR1* -129 TT genotype (*P*s < 0.01). Subsequently, a least squares mean analysis was performed to assess the joint effect of the *MDR1* T-129C polymorphisms and pesticide exposure on DNA tail moment after adjusting for the confounding effects (Figure 2). As statistical power was considered, the low and high pesticide exposure groups were combined. Controls with *MDR1* -129 TC/CC genotypes were selected as the referent group. Compared to the referent group (1.19 ± 0.05 μm/cell, $n = 21$), pesticide-exposed fruit growers with *MDR1* -129 TT had significantly higher tail moment (2.17 ± 0.03 μm/cell, $n = 174$, $P < 0.01$), followed by pesticide-exposed fruit growers with *MDR1* -129 TC/CC genotypes (1.99 ± 0.07 μm/cell, $n = 21$, $P < 0.01$) and controls with *MDR1* -129 TT genotype (1.30 ± 0.03 μm/cell, $n = 120$, $P = 0.02$).

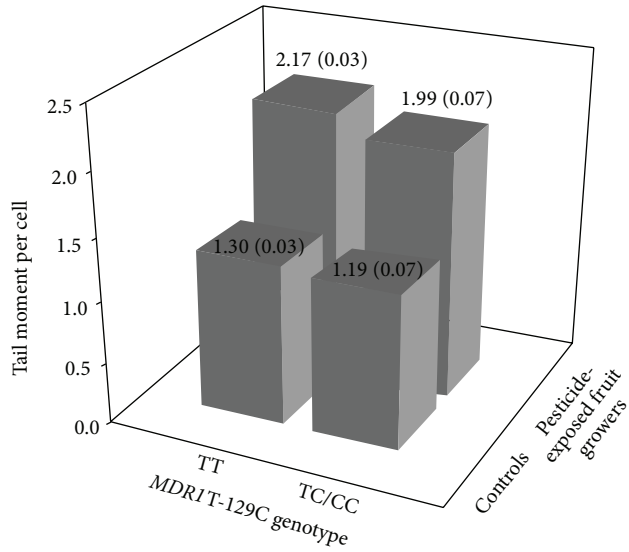

Figure 2: DNA tail moment per cell stratified by the *MDR1* T-129C (rs3213619) genotypes and pesticide exposure (standard errors in parentheses). Least squares mean analysis was performed to adjust for the effects of age and gender.

4. Discussion

It is important to identify the potential susceptibility factors affecting individual genotoxicity in response to pesticide exposure. In the present study, we investigate the association of pesticide exposure and cellular DNA damage, as measured by the comet assay, which is a sensitive method of assessing DNA damage. The comet assay of peripheral blood samples in our study and several previous studies has revealed greater DNA damage in individuals who had been exposed to complex mixtures of pesticides [29, 30]. In our previous studies, genetic variability in the enzymes that metabolize agricultural chemicals or repair DNA damage was also observed to be involved in the genotoxic process in response to pesticide exposure [8, 9]. In the present study, we observed that *MDR1* -129 TT genotype carriers had significantly higher DNA tail moment than TC/CC genotypes carriers. Further, pesticide-exposed fruit growers with *MDR1* -129 TT genotype had the greatest DNA damage in subjects with combinations of pesticide exposure and *MDR1* C-129T genotypes.

DNA damage can be induced by environmental carcinogens like pesticides and/or through metabolic or poor DNA repair processes that increase genomic instability [8, 9]. In addition to metabolic and DNA repair genes, transmembrane transporters on the surface of cells also may have an important role in the protection against gene instability and cancer initiation induced by long-term pesticide exposure. The P-glycoprotein, encoded by *MDR1* gene, is an efflux pump to minimize exposure to chemicals by removing compounds from cells in mammals [10, 11]. It has been found that several *MDR1* genetic polymorphisms are related to a functional variation of the protein [15–17, 21]. Therefore, there is a strong rationale for exploring the role of *MDR1* polymorphisms in genetic susceptibility to DNA damage among pesticide-exposed fruit growers in our current study.

In the current study, it was found that the *MDR1* C3435T and C1236T genotypes conformed to the Hardy-Weinberg equilibrium, while the *MDR1* G2677T/A and T-129C polymorphisms did not conform. However, reports in the NCBI Variation Database indicate that both the G2677T/A and the T-129C polymorphisms from persons of Chinese descent (HAPMAP CHB) also did not conform to the Hardy-Weinberg equilibrium. In addition, the frequency of the *MDR1* 3435T allele (37.2%) in our subjects is consistent with the result of HAPMAP CHB report (38.7%). The prevalence

of the *MDR1* 1236T allele (64.6%) in our study also appears to be quite similar to that previously reported for ethnic Chinese (63.8%) [31]. The frequencies of the *MDR1* 2677 GG genotype (26.5%) and G allele (47.0%) from our subjects were similar to those reported from HAPMAP CHB (GG genotype: 26.8%; G allele: 43.9%). The prevalence of the *MDR1* -129C (7.7%) in our study subjects was close to that reported from HAPMAP CHB (6.1%). These findings, to some extent, validate the practice and results of our genotyping technique. In addition, the current study recruited 195 pesticide-exposed fruit growers and 141 nonexposed controls. Given a type I error (α) level of 0.05, the numbers of our subjects with *MDR1* T-129C TT genotype and those with TC/CC genotypes were 294 and 42, respectively; and the detectable difference of average tail moment between the subjects carrying *MDR1* T-129C TT genotype (mean (SE): 1.82 ± 0.03) and those carrying TC/CC genotypes (1.55 ± 0.08) was 0.27. We acquired a sufficient statistical power of 0.88.

It has been proposed that P-gp is capable of interacting with a large group of structurally diverse pesticides [13]. Interestingly, we observed that *MDR1* -129 TT genotype carriers had the significantly higher DNA tail moment than TC/CC genotypes carriers. The *MDR1* T-129C polymorphism is located in the promoter region, 7 bp downstream from the transcription initiation site. The *MDR1* -129C allele has also been reported to have a lower P-gp expression than the -129T allele [21]. From the combined analysis, we further observed that pesticide-exposure fruit growers with *MDR1* -129 TT genotype had the greatest DNA tail moment, followed by pesticide-exposure fruit growers with *MDR1* -129 TC/CC genotypes and controls with *MDR1* -129 TT genotype. The DNA tail moment of controls with *MDR1* -129 TC/CC genotypes was significantly smaller than those of other groups of combined pesticide exposure and *MDR1* -129 genotypes. Thus, our findings suggest that the *MDR1* T-129C polymorphism may modulate susceptibility to the genotoxicity of pesticides. To the best of our knowledge, this is the first study on the association of *MDR1* genetic polymorphisms in pesticide-exposed fruit growers with cellular DNA damage. However, our results need to be replicated in other populations since it is likely that the *MDR1* T-129C polymorphism may be a susceptibility factor for genotoxicity of pesticides only in certain ethnic groups.

A previous study observed that the *MDR1* C3435T, C1236T, and G2677T/A genetic polymorphisms were significantly associated with a higher risk of developing Parkinson's disease in Chinese subjects [18]. However, these SNPs were not significantly associated with childhood acute lymphoblastic leukemia [32]. In the present study, the DNA tail moment was also not associated with the *MDR1* C3435T, C1236T, and G2677T/A genotypes. Further, the role of *MDR1* C1236T-G2677T/A-C3435T haplotypes has also been examined and provided evidence of a differential effect of indoor insecticide exposure on acute lymphoblastic leukemia risk in children with different haplotypes [32]. As expected, *MDR1* C3435T, C1236T, and G2677T/A were in tight linkage disequilibrium with each other, but the *MDR1* -129 was not in linkage disequilibrium with the former ones in our haplotype analysis. However, differences in the average DNA tail moment per cell stratified by *MDR1* haplotypes did not reach statistical significance among high and low pesticide-exposed groups and controls. Since some genetic polymorphisms may exert population-specific effect, the "at-risk" allele in one person may not be an "at-risk" allele in another. Therefore, the lack of any association of the DNA tail moment with the *MDR1* C3435T, C1236T, and G2677T/A individual genotype or haplotype in our study may partly be due to different environmental exposure and different study populations.

In our study area, most of the younger residents have a low regard for agricultural work. Thus, the agricultural population tends to be older, and our control group was significantly younger than pesticide exposure groups. As expected, the older farmers who smoked also had more pack-years of smoking than younger farmers. Although adjustment was also performed for the confounding factors such as age, gender, and smoking status in our multiple regression model, the effect of selection bias might remain. The present study showed that smoking was not associated with DNA tail moment, which is probably because fewer cigarettes were smoked by subjects in the current study than in other studies [33]. In addition, nondifferential misclassification of pesticide exposure in the current study is likely to occur and, if apparent, can lead to an underestimation of the risk of DNA damage. Furthermore, data pertaining to individual exposure were obtained without the knowledge of health outcome. Lastly, it is not surprising that the P-gp activity phenotypes will provide additional information about the risk of DNA damage in pesticide-exposed subjects that was not provided by genotype alone from our study.

In conclusion, the results reveal that individuals with susceptible *MDR1* -129 genotypes may experience an increased risk of DNA damage due to pesticide exposure.

Conflict of Interests

The authors have no conflict of interests to declare.

Authors' Contribution

Chun-Chieh Chen and Ruey Hong Wong conceived and designed the experiments. Chun-Huang Huang, Chia-Hsuan Chou, Tzu-Yen Tseng, Fang-Yu Chang, and Ying-Ti Li performed the experiments. Chun-Huang Huang, Chia-Chen Huang, Chun-Cheng Tsai, and Ruey Hong Wong analyzed the data. Chun-Chieh Chen, Man-Tzu Marcie Wu, Tsung-Shing Wang, and Ruey Hong Wong contributed with reagents/materials/analysis tools. Chun-Chieh Chen and Ruey Hong Wong wrote the paper.

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Research Article

Genetic Polymorphisms of *ORAI1* and Chronic Kidney Disease in Taiwanese Population

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Taiwan has very high incidence and prevalence of chronic kidney disease (CKD), which easily progresses to end-stage renal disease (ESRD). The association between inflammation and CKD has been explored in several studies. *ORAI1* functions as a pore-forming subunit of the store-operated calcium channels which are involved in the regulation of immune system. Hence, we conducted a case-control study to determine whether the genetic polymorphisms of *ORAI1* gene is a susceptibility factor to CKD and its clinical features in a Taiwanese population. Five hundred seventy-nine CKD patients from a hospital-based CKD care program were included in the study. Five tagging single nucleotide polymorphisms (tSNPs) of *ORAI1* were selected from the genotyping data of the Han Chinese population from the HapMap project. Among these polymorphisms, rs12313273 was found to be significantly associated with elevated serum calcium levels, which has been linked to increased risk of death in CKD patients. To have a better management of serum calcium, we suggest that *ORAI1* polymorphisms might be used as a potential biomarker for initiating non-calcium-based phosphate binder in CKD patients in the future.

1. Introduction

Chronic kidney disease (CKD) is an important global public health concern because of its high incidence, prevalence, morbidity, and mortality [1]. According to the US Renal Data System (USRDS) report, Taiwan has the highest incidence and prevalence of end-stage renal disease (ESRD) [2]. The prevalence of CKD in Taiwan was 9.8–11.9% and owing to the differences in the data sources, study subjects, and definition of CKD, the reasons behind this high incidence and prevalence are multifactorial [3].

CKD has been well known to be associated with low-grade inflammation, endothelial dysfunction, and platelet activation, even among those in the early stage of CKD [4]. Serum levels of the proinflammatory cytokines, such as IL-1, IL-6, CRP, and TNF-α were significantly high in CKD

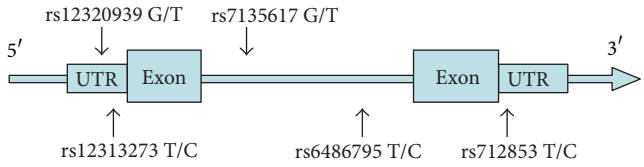

Figure 1: A graphical overview of the genotyped polymorphisms identified in relation to the exon/intron structure of the human *ORAI1* gene.

patients [5–8], and these inflammation markers may replace albumin, which is currently used as the predictive marker for mortality, to predict patient outcomes [9].

Calcium signaling controls diverse cellular functions such as enzyme metabolism, muscle contraction, immune response, and cell cycle regulation [10, 11]. In nonexcitable cells such as T cells and B cells, immunological reactions are regulated via Ca^{2+} entry mainly through store-operated calcium channels [12]. *ORAI1* consists of four transmembrane domains and functions as a pore-forming subunit of the store-operated calcium channels [13]. Functional analysis of *ORAI1*- (also called *CRACM1*-) deficient mice revealed dysfunction of mast cells and attenuation of cytokine (TNF-α and IL-6) release [14].

Recent studies on the genetic susceptibility and the progression of CKD have yielded promising results [15–17]. The results of a genome-wide association study showed that several loci were associated with CKD and estimated glomerular filtration rate (eGFR) [16]. The evolution of ApoL1 variants as survival factors may have contributed to the high prevalence of renal disease among African Americans [17]. To the best of our knowledge, there is no previous research established regarding the association between genetic polymorphism of ORAl1 and the severity of CKD in Taiwanese population. Therefore, in this case-control study, we examined the association of the *ORAI1* genetic polymorphisms with CKD susceptibility, eGFR, and serum phosphorus and calcium levels.

2. Materials and Methods

2.1. Study Subjects and Data Collection. Five hundred seventy-nine unrelated CKD patients (323 (55.8%) men; age range, 18–90 years old; mean age, 61 ± 14 years old) were included in the study at the time of their enrolment for the CKD Care Program at the Kaohsiung Medical University Hospital, Kaohsiung, Taiwan; written informed consent was obtained from all patients. All included patients were >18 years of age, and their detailed clinical history was recorded as part of the CKD Care Program. The study protocol conformed to the Declaration of Helsinki and was approved by the Institutional Review Board of the Kaohsiung Medical University Hospital. Serum creatinine levels were calculated using a modified kinetic Jaffe reaction. eGFR was estimated using the abbreviated equation developed in the Modification of Diet in Renal Disease Study [18], and the cases were categorized according to the staging system described in the Kidney/Dialysis Outcome Quality Initiative Clinical Practice Guidelines for CKD: Evaluation, Classification, and Stratification [19]. The patients were divided into two groups according to their eGFR: patients with eGFR above 45 mL/min/1.73 m^2 were classified as having early-stage CKD [3, 20, 21], whereas those with lower eGFR were classified as having late-stage CKD. In Taiwan, the "nationwide CKD preventive project with multidisciplinary care program" implemented by Health Promotion Administration divided CKD patients into "early" and "pre-ESRD" stages, according to the eGFR ≥45 mL/min/1.73 m^2 or <45 mL/min/1.73 m^2 [22]. Different treatment strategy and management plans are applied in those two groups. In our study, we divided patients into two groups as above to investigate the differences of genetic polymorphism. Their clinical history and biochemical data were recorded.

2.2. DNA Extraction. Venous blood was collected from the patients during medical visit, stored at 4°C, and processed on the same day. The blood was centrifuged to separate serum and cells. DNA extraction from the blood cells involved an initial treatment with 0.5% SDS lysis buffer followed by treatment with protease K (1 mg/mL, for the digestion of nuclear protein) for 4 h at 60°C. Total DNA was harvested using the Gentra extraction kit and was precipitated using 70% alcohol.

2.3. SNP Selection. From the HapMap database (http://www.hapmap.org, HapMap Data Rel 27 PhaseII+III, Freb09, on NCBI B36 assembly, dbSNP b126), five tagging single nucleotide polymorphisms (tSNPs) of *ORAI1* (rs12313273, rs6486795, rs7135617, rs12320939, and rs712853) with minor allele frequency (MAF) >10% and $r^2 > 0.8$ were selected from chromosomal region 120,545,838–120,561,329 of the Han Chinese population in Beijing (CHB). A graphical overview of the physical and chromosomal location of the five tSNPs is shown in Figure 1. Two *ORAI1* polymorphisms (rs12313273 and rs1232093) were located in the promoter region, two (rs6486795 and rs7135617) in the intron region, and one (rs712853) in the 3′-untranslated region (UTR).

2.4. Genotyping. Genotyping was performed using TaqMan PCR. In brief, TaqMan probes were first labeled with different fluorescent markers. PCR primers and TaqMan probes were designed to target the 5 tSNPs. Reactions were performed in 96-well microplates in the ABI 9700 Thermal Cycler (Applied Biosystems, Foster City, USA) and fluorescence was detected and analyzed using the System SDS software version 1.2.3.

2.5. Statistical Analysis. The genotype distribution of the five tSNPs was tested for Hardy-Weinberg equilibrium (HWE). The Chi-square test was used for comparing the genotype distribution or allele frequencies of the early-stage and late-stage CKD patients. One-way ANOVA was used to assess the difference in mean values of the eGFR and the serum levels of calcium and phosphate in the groups created based on genotyping results. All statistical analyses above were performed using the JMP 8.0 statistical software. Linear

Table 1: Basal characteristics of patients with chronic kidney disease.

Characteristics	Patients with CKD
Number of subjects	579
Gender: male, number (%)	323 (55.8%)
Age (years)[a]	61.1 ± 13.7
Range (years)	18–90

[a]Means ± SD.

Table 2: Difference in the value of eGFR among CKD patients stratified by different *ORAI1* genotypes.

SNP	Genotype	Sample number (%)	eGFR[a]
rs12320939	TT	122 (21.2)	31.75 ± 24.28
	TG	295 (51.2)	32.26 ± 24.04
	GG	159 (27.6)	31.34 ± 24.28
	P value		0.9229
	Adjusted *P* value[b]		0.8764
rs12313273	CC	50 (8.7)	34.64 ± 23.93
	CT	245 (42.5)	31.08 ± 22.45
	TT	281 (48.8)	31.90 ± 24.70
	P value		0.6229
	Adjusted *P* value[b]		0.7209
rs7135617	TT	98 (17.0)	29.06 ± 20.71
	TG	285 (49.6)	32.71 ± 24.31
	GG	192 (33.4)	32.29 ± 25.44
	P value		0.4138
	Adjusted *P* value[b]		0.3691
rs6486795	CC	71 (12.3)	33.49 ± 25.23
	CT	271 (47.0)	31.79 ± 22.61
	TT	235 (40.7)	31.93 ± 24.90
	P value		0.8619
	Adjusted *P* value[b]		0.8608
rs712853	CC	56 (9.7)	34.36 ± 26.12
	CT	238 (41.4)	31.95 ± 24.39
	TT	281 (48.9)	31.53 ± 23.09
	P value		0.7220
	Adjusted *P* value[b]		0.7038

[a]Means ± SD. [b]Adjusted age by linear regression.

regression and logistic regression were used to adjust the influence of age in eGFR and CKD staging, which were performed using the SNPassoc 1.9-1 statistical software. A P value < 0.05 was considered significant.

3. Results

3.1. Association between ORAI1 tSNPs and eGFR in the CKD Patients. Patient characteristics are shown in Table 1. We tested whether genetic polymorphisms in *ORAI1* are associated with eGFR in CKD patients. None of the tSNPs were found to be significantly associated with CKD susceptibility. We further adjusted our result by age which showed no significant associations (Table 2).

3.2. Association of ORAI1 tSNPs in Early- and Late-Stage CKD Patients. Next, we evaluated whether the genotype and allele frequency of *ORAI1* were associated with the stage of CKD. After being adjusted by age using logistic regression, no association was observed between tSNPs and the stage of CKD (Table 3).

3.3. Association between the ORAI1 Polymorphisms and Serum Calcium Levels in CKD Patients. Abnormalities in the levels of calcium, phosphorus, and intact parathyroid hormone (PTH) are evident early in CKD patients who are not on dialysis [19]. Since abnormalities in calcium and phosphate levels are associated with increased mortality and CKD progression in non-dialysis-dependent CKD patients [23, 24], we also investigated the associations between *ORAI1* genetic polymorphisms and serum calcium concentration. We found that rs12313273 was significantly associated with serum calcium levels in CKD patients (Table 4). We also observed that patients with the CC genotype of rs12313273 showed significantly higher calcium levels than those with other genotypes did. However, we found no correlation between the genetic polymorphisms and the serum phosphorus levels.

4. Discussion

We systematically investigated five *ORAI1* tSNPs (rs12313273, rs6486795, rs7135617, rs12320939, and rs712853) in CKD patients. None of the tSNPs of *ORAI1* were associated with the risk of CKD. However, rs12313273 was found to be significantly associated with increased serum calcium levels. Patients with CC genotype showed higher serum calcium levels than those with other genotypes. Impaired calcium and phosphate homeostasis have been reported in the early stages of CKD. We frequently used calcium-based or non-calcium-based phosphate binder to manage hyperphosphatemia, yet calcium-based binders often result in hypercalcemia [25]. Recent studies showed that CKD patients with high serum calcium levels (>2.75 mmol/L) have a higher risk of death than patients with low serum calcium levels do [26, 27]. Moreover, high calcium-phosphate product is associated with increased risk of vascular calcification and cardiovascular mortality [28, 29]. Our findings showed that patients with CC genotype of rs12313273 were associated with higher calcium levels. Therefore, we may take *ORAI1* polymorphism into account when prescribing calcium or non-calcium-based phosphate binder to CKD patients with hyperphosphatemia.

ORAI1-mediated calcium signaling plays critical roles in inflammatory diseases. Chang et al. identified several polymorphisms in *ORAI1* from Taiwanese and Japanese atopic dermatitis patients [30]. In addition, the CC genotype of rs12313273 in *ORAI1* was strongly associated with the risk and recurrence of calcium nephrolithiasis [31]. Furthermore, the *ORAI1* haplotypes (rs12313273 and rs7135617) are associated with the risk of HLA-B27-positive ankylosing spondylitis [32]. Consistent with the findings of previous studies, our results confirm the functional role of *ORAI1* polymorphism rs12313273 in modulating the serum calcium concentration.

Table 3: Genotyping and allele frequency of *ORAI1* gene in chronic kidney disease patients.

	Genotype	Late stage (%) (n = 453)	Early stage (%) (n = 126)	Allele	Late stage (%) (n = 453)	Early stage (%) (n = 126)	Genotype P value	Dominant P value	Recessive P value	Allelic P value
rs12320939	TT	94 (20.8)	28 (22.4)	T	424 (47.0)	115 (46.0)	0.6482	0.4482	0.7955	0.7363
	TG	236 (52.3)	59 (47.2)	G	478 (53.0)	135 (54.0)				
	GG	121 (26.8)	38 (30.4)							
rs12313273	CC	36 (8.0)	14 (11.3)	C	271 (30.0)	74 (29.8)	0.3780	0.5522	0.3148	0.9939
	CT	199 (44.0)	46 (37.1)	T	633 (70.0)	174 (70.2)				
	TT	217 (48.0)	64 (51.6)							
rs7135617	TT	80 (17.7)	18 (14.5)	T	378 (41.9)	103 (41.5)	0.4551	0.6285	0.3425	0.8567
	TG	218 (48.3)	67 (54.0)	G	524 (58.1)	145 (58.5)				
	GG	153 (33.9)	39 (31.5)							
rs6486795	CC	53 (11.8)	18 (14.2)	C	323 (35.8)	90 (35.7)	0.5856	0.6638	0.4536	0.9574
	CT	217 (48.1)	54 (42.9)	T	579 (64.2)	162 (64.3)				
	TT	181 (40.1)	54 (42.9)							
rs712853	CC	46 (10.2)	10 (8.0)	C	274 (30.4)	76 (30.4)	0.6072	0.6717	0.4667	0.9998
	CT	182 (40.4)	56 (44.8)	T	626 (69.6)	174 (69.6)				
	TT	222 (49.3)	59 (47.2)							

Late stage: eGFR <45, early stage: eGFR ≥45.
All P values had been adjusted by age using logistic regression.

Table 4: Difference in the value of Ca^{2+} and phosphorous among CKD patients stratified by different *ORAI1* genotype.

SNP	Genotype	Sample number (%)	Calcium (mg/dL)[a]	P value	Phosphorous (mg/dL)[a]	P value
rs12320939	TT	122 (21.2)	9.32 ± 0.53	0.0528	4.26 ± 1.02	0.5243
	TG	295 (51.2)	9.10 ± 0.94		4.25 ± 1.02	
	GG	159 (27.6)	9.17 ± 0.80		4.37 ± 1.02	
rs12313273	CC	50 (8.7)	9.32 ± 0.61	**0.0389***	4.33 ± 0.89	0.0831
	CT	245 (42.5)	9.23 ± 0.57		4.18 ± 1.01	
	TT	281 (48.8)	9.08 ± 1.03		4.38 ± 1.03	
rs7135617	TT	98 (17.0)	9.21 ± 0.87	0.1017	4.42 ± 1.05	0.3290
	TG	285 (49.6)	9.09 ± 0.81		4.24 ± 0.97	
	GG	192 (33.4)	9.25 ± 0.85		4.29 ± 1.07	
rs6486795	CC	71 (12.3)	9.33 ± 0.60	0.1586	4.36 ± 1.08	0.2622
	CT	271 (47.0)	9.17 ± 0.77		4.21 ± 1.03	
	TT	235 (40.7)	9.12 ± 0.96		4.35 ± 0.98	
rs712853	CC	56 (9.7)	8.99 ± 1.27	0.2356	4.47 ± 1.06	0.3133
	CT	238 (41.4)	9.16 ± 0.87		4.30 ± 1.12	
	TT	281 (48.9)	9.20 ± 0.68		4.24 ± 0.92	

*Significant ($P < 0.05$) values are in bold. [a]Means ± SD.

The calcium-dependent pathway is involved in multiple physiological and cellular functions such as modulation of immune responses, activation of inflammation, and enzyme metabolism [33, 34]. Inflammation is an important mediator of CKD progression and is a contributing factor in malnutrition and increased risk of cardiovascular morbidity [35]. A vast body of evidence supports the important role of calcium in kidney disease. Mutations in transient receptor potential canonical 6 (TRPC6) channels and polycystin-2, a prototypical member of a subfamily of the TRPC channel superfamily, have been reported to cause familial focal segmental glomerulosclerosis and autosomal dominant polycystic kidney disease, respectively [36–41].

Recently, Lu et al. demonstrated a significant correlation between *TPRC1*, *ORAI1*, *STIM1*, and parathyroid cells [42]. PTH plays a key role in serum calcium regulation. PTH itself is also regulated by extracellular calcium through stimulating the calcium-sensing receptor (CaSR) expressed on the surface of parathyroid cells [43]. CaSR, a G-protein PLC-linked receptor, has been shown to be involved in the TRPC1-mediated transient calcium oscillation in human embryonic

kidney cells [44]. Our results suggest that the genetic polymorphisms of *ORAI1* may alter *ORAI1* gene expression in store-operated calcium channels, which in turn may affect PTH secretion and thereby serum calcium levels.

This study has several limitations. First, we did not consider several factors that are known to influence calcium levels, such as concomitant drug usage and underlying disease. Second, the underlying comorbidities were not identified in this study, and a possible relationship between the different comorbidities and the tSNPs of *ORAI1* cannot be ruled out. Our results showed that the genotype of *ORAI1* was not associated with CKD susceptibility. However, owing to the moderate size of our cohort, our analyses may not have sufficient power for detecting minor genetic effects. Therefore, we cannot exclude rare causal genetic polymorphisms in *ORAI1*. Direct *ORAI1* sequencing using larger samples may be useful for identifying new SNPs in the *ORAI1* gene and for clarifying the association of *ORAI1* polymorphisms with CKD susceptibility. Further investigation on other variants of the genes of the SOC pathway and of the genes involved in calcium homeostasis are needed to fully understand CKD susceptibility and progression.

In conclusion, our results showed that the *ORAI1* polymorphism rs12313273 is associated with higher serum calcium levels in Taiwanese CKD patients. To have a better management of serum calcium, *ORAI1* polymorphism might be used as a potential biomarker for initiating non-calcium-based phosphate binder in CKD patients in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Author's Contribution

Daw-Yang Hwang and Shu-Chen Chien contribute equally to the paper.

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Research Article

Association of Single Nucleotide Polymorphisms in Estrogen Receptor Alpha Gene with Susceptibility to Knee Osteoarthritis: A Case-Control Study in a Chinese Han Population

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Osteoarthritis (OA) is the most prevalent form of arthritis and its multifactorial nature has been increasingly recognized. Genetic factors play an important role in OA etiology and estrogen receptor alpha (ESR1) gene polymorphisms may be involved. This study tried to explore whether the ESR1 gene single nucleotide polymorphisms (SNPs) were associated with primary knee OA in the Chinese Han population. Two SNPs, rs2234693 and rs9340799, were genotyped in 469 cases and 522 controls. Rs2234693 was associated with knee OA in the dominant genetic model (TT + TC versus CC) ($P = 0.025$) and a higher T allele frequency existed ($P = 0.047$) among females. The combined genotype (TT + TC) ($P = 0.025$) and T allele ($P = 0.016$) were related with mild knee OA only. For rs9340799, A allele was associated with knee OA in all subjects ($P = 0.031$) and females ($P = 0.046$). Statistical differences were detected in the dominant genetic model (AA + AG versus GG) among females ($P = 0.030$). The combined genotype (AA + AG) ($P = 0.036$) and A allele ($P = 0.039$) were merely correlated with mild knee OA. ESR1 gene is considerably associated with knee OA etiology in the Chinese Han population.

1. Introduction

Osteoarthritis (OA, OMIM#165720) is a multifactorial disorder characterized by progressive cartilage loss, osteophyte formation, and subchondral sclerosis, which accounts for a large amount of elderly individuals with pain and disability [1, 2]. Joint arthroplasty surgery, mostly at the knee and the hip, acts as the major therapeutic method for severe OA in current times despite its invasive nature and a large economic burden [3]. In this regard, a better understanding of the etiology of OA is much required for a more effective and targeted prevention.

To date, several large-sample studies and genome-wide association studies (GWAS) have shed further light on potential chromosome regions that may carry OA susceptibility genes which have been subsequently confirmed by replication studies in different populations [4–6]. Asia population have an especially high incidence of knee OA and a recent study has also indicated that the prevalence of symptomatic and radiographic knee OA in Chinese females aged 60 and over was 15.4% and 42.8%, respectively [7]. Combined with the observation that women were more vulnerable to more severe knee OA especially after menopausal age [8], it could be hypothesized that estrogen may be involved in the onset or progression of knee OA.

The human estrogen receptor has two isoforms: ESR1 and ESR2, which are members of the steroid/thyroid hormone superfamily of nuclear receptors and encoded by separate genes [9, 10]. ESR1 is expressed in chondrocytes, stromal cells, and osteoblasts [11], which potentially indicated that both

bone and cartilage can be regulated by ESR1 gene. Genetically, there have been several studies on associations between two common ESR1 gene polymorphisms (rs2234693 and rs9340799) and OA, and the findings were controversial [12]. A considerable number of published reports have indicated a significant association of ESR1 gene polymorphisms and risk for knee, hip, or generalized OA in different populations, although the potential mechanisms await clarifications [13–19]. Still, some investigators failed to demonstrate the possible effects of ESR1 gene polymorphisms on knee and hip OA [20]. For the first time, this study is an attempt to examine the genetic association of the ESR1 gene polymorphism (rs2234693 and rs9340799) with knee OA in the Chinese Han population.

2. Materials and Methods

2.1. Subjects. A total of 991 subjects were enrolled in this study. 469 patients with primary knee OA (357 females and 113 males) were recruited consecutively at the Center of Diagnosis and Treatment for Joint Disease, Drum Tower Hospital, affiliated to the Medical School of Nanjing University, and 522 age-matched healthy controls (124 females and 398 males) were consecutively selected in a plenty of more than 2000 individuals at the Center of Physical Examination. All subjects included in this study were Han Chinese living in and around Nanjing and no one dropped out. The study was approved by the ethics committee of the Medical School of Nanjing University, and informed consent was obtained from patients and control participants.

The inclusion criteria for both OA patients and controls were previously described by Jiang et al. [21]. Informationregardingthe health conditions which mainly include hypertension, hyperglycemia or diabetes, and hyperlipidemia were collected according to their self-report due to the potential confounding effects exerted on knee OA by these disease conditions [1]. Notably, considering the significant associations between ESR1 gene polymorphisms and hypertension in several case-control studies [22–24], patients with hypertension failed to be included. There were 11 (2.35%) and 19 (3.64%) patients who had hyperglycemia or diabetes in case and control groups, respectively. No patients had hyperglycemia. We then calculated the body mass index as weight (kg) divided by height squared (m^2) for all the participants to estimate obesity roughly. All the knee OA patients had not only definite symptoms and signs (pain, tenderness, swelling, or restricted motion) of OA for at least 5-month duration but also radiographic evidences which were diagnosed by standardized anteroposterior radiographs of the knee joint in extension. For each patient, the Kellgren/Lawrence (K/L) grading system was adopted to evaluate radiographic OA [25], and only patients with K/L grades of 2 or higher were included. We then classified radiographic OA findings into mild OA (K/L grade 2) and severe OA (K/L grade 3 or 4) for a clear definition of the severity. Exclusion criteria for this study include other inflammatory arthritis (rheumatoid, autoimmune, or polyarthritic disease), posttraumatic or postseptic arthritis, and developmental dysplasia or skeletal dysplasia.

2.2. Genotyping. The minor allele frequencies of both rs2234693 and rs9340799 were above 0.2 in the Chinese population, respectively. DNA samples were obtained from all the participants from peripheral blood adopting the Chelex-100 method [26] or buccal swabs using the DNA IQ System (Promega, Madison, WI) based on the manufacturer's instructions. The two SNPs were then genotyped using Taqman assay (Applied Biosystems 7500, ABI, Foster City, CA). Genotyping was performed by laboratory personnel who were blinded to case status, and three authors independently reviewed the genotyping results, data entry, and statistical analyses. In addition, we randomly selected 5% samples of case and control subjects for reproducibility tests at least twice in different days and yielded a 100% concordant.

2.3. Statistic Analysis. Mean values were presented with their standard deviation (SD) and assessed by Students' t-test and standard χ^2 tests were employed to determine the significance of differences in allelic and genotypic distributions between cases and controls. Multivariate logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CI) after adjustment for age and BMI for estimating the associations between the SNPs and the risk of knee OA. Stratifications for gender and the severity of OA were subsequently conducted for further analyses. The Hardy-Weinberg Equilibrium (H-WE) was tested by a goodness-of-fit χ^2 test to compare the allele and genotype proportions in case and control subjects. For all the tests, a two-tailed probability value of less than 0.05 was considered as statistically significant. All the data was analysed by SPSS 19.0 (IBM SPSS, Chicago, USA).

3. Results

Baseline characteristics of all the subjects were shown in Table 1. The mean age of knee OA patients (57.3 ± 10.9) and controls (56.4 ± 9.8) are not significantly different ($P = 0.473$). It could be noted that knee OA patients had a higher mean BMI than that of control subjects ($P < 0.001$). Approximately 45.6% of the OA patients had a K/L score of 3 or 4. However, the mismatching in sex distribution between two groups existed in this study ($P < 0.001$). Tables 2 and 3 showed the genotyped and allele distributions for cases and controls based on the stratification of gender and K/L grades. Distributions of genotypes and alleles of two SNPs in healthy control individuals were all confirmed to Hardy-Weinberg Equilibrium (0.997 and 0.736, resp.). The minor allele frequencies of rs2234693 (allele C) and rs9340799 (allele G) in control subjects were 0.379 and 0.185, respectively, which were close to those reported in HapMap for the Chinese Han population (0.402 and 0.211, resp.).

In the association study, for rs2234693, we did not observe any significant difference in any comparison as a whole (Table 4). When stratified by gender after adjustment for age and BMI, we found evident differences in the dominant model (TT + TC versus CC) in female subjects (OR = 2.087; 95% CI, 1.086–4.009; $P = 0.025$). In addition, a higher T allele frequency was also associated with an increased risk

Table 1: Baseline characteristics of subjects.

	Cases	Controls	P value
Subjects, number	469	522	—
Females, number (%)	356 (75.9)	120 (23.0)	$P < 0.001^*$
Mean age, y (SD)	57.3 ± 10.9	56.4 ± 9.8	$P = 0.473$
Mean BMI, kg/m^2 (SD)	26.1 ± 3.9	24.3 ± 2.8	$P < 0.001$
Kellgren-Lawrence grading			
Grade 2, number (%)	255 (54.5)	0 (0.0)	—
Grade 3, number (%)	119 (25.3)	0 (0.0)	—
Grade 4, number (%)	95 (20.2)	0 (0.0)	—

No.: number; y: years; SD: standard deviation.
*Pearson chi-square test.

of knee OA in females (OR = 1.359; 95% CI, 1.003–1.840; $P = 0.047$). For males, no differences were found to be statistically significant. After stratification for the severity of OA, apparent differences were detected in the comparison of CC versus other genotyped combined (TT + TC) after adjustment for age, BMI, and sex in patients with mild knee OA (OR = 1.560; 95% CI, 1.056–2.304; $P = 0.025$) (Table 6). Still, T allele was modestly related with an elevated risk of mild knee OA (OR = 1.320; 95% CI, 1.050–1.615; $P = 0.016$). Unexpectedly, we failed to find any relationship between genotype or allele frequencies of rs2234693 and the risk of severe knee OA.

With respect to rs9340799, A allele was relevant to a higher risk of knee OA in all the patients and controls (OR = 1.272; 95% CI, 1.022–1.583; $P = 0.031$) and this difference remained in female subjects (OR = 1.454; 95% CI, 1.006–2.102; $P = 0.046$) (Table 5). The most significant difference in the dominant model (AA + AG versus GG) was also shown in female subjects (OR = 4.410; 95% CI, 1.027–18.938; $P = 0.030$). Similarly, the combined genotype (AA + AG) (OR = 2.011; 95% CI, 1.036–3.902; $P = 0.036$) and A allele (OR = 1.312; 95% CI, 1.013–1.701; $P = 0.039$) were merely correlated with the elevated risk of mild knee OA (Table 7).

4. Discussion

To our best knowledge, this case-control study firstly described a compelling association of ESR1 SNPs (rs2234693 and rs9340799) and primary knee OA in a Chinese Han population with a complete exclusion of those who had hypertension previously or currently. Significant differences were found between 469 patients and 522 control subjects in the two SNPs. Summarily, we herein suggested the leading roles of T allele of rs2234693 and A allele of rs9340799 in the pathogenesis of primary knee OA, especially for female patients and those subjects with mild disease. For these patients, a potential individualized prevention and treatment of knee OA may be practicable.

Until now, a relative lack of consistency or reproducibility of the association between ESR1 gene (rs2234693 and rs9340799) polymorphisms and OA still exists. Previously, Ushiyama et al. [13] reported an increased risk of generalized OA with genotypes of rs2234693 and rs9340799 polymorphisms in 383 Japanese women. In a large-scale population-based study of Caucasians, Bergink et al. [14] found that ESR1 haplotypes of rs2234693 and rs9340799 polymorphisms were significantly relevant to radiographic knee OA, especially for those who had osteophytes with adjustment for bone mineral density (BMD). Also, one study showed no correlation between ESR1 gene polymorphisms and idiopathic OA of the knee and the hip in a Caucasian population (371 OA patients who underwent total joint replacement and 369 controls) [20]. Recently, ESR1 gene haplotype had been confirmed to be associated with primary knee OA in Korean and Mexican mestizo populations [15, 19]. After accounting for femoral neck BMD, a decreased prevalence of hip OA phenotype which was characterized by moderate to severe joint space narrowing in presence of CC genotype in SNP rs2234693 was also reported in Caucasians [17]. Still, associations of the CC genotype with a reduced risk of knee OA in women and an increased risk of hip OA in men were found in Europeans [18].

Noteworthy, the C allele frequency of rs2234693 and the G allele frequency of rs9340799 in controls were 0.379 and 0.185, respectively, which were similar to those observed in the Japanese and Korean populations [13, 15] whereas different from those in European and American Caucasians to a certain degree [17, 18]. We also proposed the predisposing roles of T allele of rs2234693 in the etiology of knee OA, and this was partially in concordance with the protective effects of C allele in Japanese and Caucasians [17, 18] but against the observation that CC genotype was a risk factor of radiographic knee OA in a Rotterdam study [14]. Still, this difference itself may indicate the heterogeneity in association with specific genetic polymorphisms in different ethnic groups. Environment factors such as anatomical and biomechanical effects and some joint-specific hereditary factors may also be involved in the influencing process on OA susceptibility by certain polymorphic locus [27, 28]. Likewise, potential variations in the nature of OA genetic susceptibility in different joints seem to be a reasonable interpretation [27]. In addition, the different inclusion criteria in the aforementioned studies may also be a possible explanation for the discrepancy. Summarily, further studies on different anatomic OA after unifying the recruitment criteria and OA end points of subjects in various populations will be enormously beneficial to a better understanding of these polymorphisms in OA etiology.

The lack of difference in male participants in this study may be due to the limited sample number, despite a large sex bias of OA incidence in females [8]. With adjustment for age, BMI, and gender, we found that both of the rs2234693 and rs9340799 were in relation with the risk of mild knee OA, whereas the associations disappeared in patients with severe knee OA, which indicated that the SNPs might play a certain role in the progress of OA at different stages. The findings were consistent with that of an Oxford study in patients who accepted joint arthroplasty due to severe OA of the lower limb [20] but against the results reported by Jin et al. in Koreans [15]. The analysis of different OA end points used in different studies may be an explanation.

Table 2: Genotype and allele frequencies of rs2234693 and rs9340799 of the ESR1 gene in the Han Chinese population.

Group	rs2234693							rs9340799						
	Number	Genotype (frequency)			Allele (frequency)		H-WE	Number	Genotype (frequency)			Allele (frequency)		H-WE
		TT	TC	CC	T	C	*P*		AA	AG	GG	A	G	*P*
OA														
All	469	167 (0.356)	217 (0.463)	85 (0.181)	551 (0.587)	387 (0.413)	0.325	469	288 (0.614)	152 (0.324)	29 (0.062)	728 (0.776)	210 (0.224)	0.144
Female	356	119 (0.334)	170 (0.478)	67 (0.188)	408 (0.573)	304 (0.427)	0.649	356	210 (0.590)	122 (0.343)	24 (0.067)	542 (0.761)	170 (0.239)	0.280
Male	113	48 (0.425)	47 (0.416)	18 (0.159)	143 (0.633)	83 (0.367)	0.264	113	77 (0.682)	30 (0.265)	6 (0.053)	184 (0.814)	42 (0.186)	0.192
Control														
All	514	198 (0.385)	242 (0.471)	74 (0.144)	638 (0.621)	390 (0.379)	0.997	522	348 (0.667)	155 (0.297)	19 (0.036)	851 (0.815)	193 (0.185)	0.736
Female	120	47 (0.392)	61 (0.508)	12 (0.100)	155 (0.646)	85 (0.0354)	0.223	124	82 (0.661)	40 (0.323)	2 (0.016)	204 (0.823)	44 (0.177)	0.242
Male	394	151 (0.383)	181 (0.459)	62 (0.158)	483 (0.613)	305 (0.387)	0.528	398	266 (0.668)	115 (0.289)	17 (0.043)	647 (0.813)	149 (0.187)	0.314

No.: number; H-WE: Hardy-Weinberg Equilibrium.

Table 3: Genotype and allele frequencies of rs2234693 and rs9340799 of the ESR1 gene with a stratification by K/L grades.

K/L grading	Number	rs2234693						rs9340799					
		Genotype (frequency)			Allele (frequency)		H-WE	Genotype (frequency)			Allele (frequency)		H-WE
		TT	TC	CC	T	C	*P* value	AA	AG	GG	A	G	*P* value
Grade 2	255	82 (0.321)	120 (0.471)	53 (0.208)	284 (0.557)	226 (0.443)	0.458	156 (0.612)	81 (0.318)	18 (0.070)	393 (0.771)	117 (0.229)	0.105
Grade 3	119	46 (0.387)	54 (0.454)	19 (0.159)	146 (0.613)	92 (0.387)	0.638	74 (0.622)	42 (0.353)	3 (0.025)	190 (0.798)	48 (0.202)	0.295
Grade 4	95	39 (0.410)	43 (0.453)	13 (0.137)	121 (0.637)	69 (0.363)	0.834	57 (0.600)	29 (0.305)	9 (0.095)	143 (0.753)	47 (0.247)	0.079

K/L: Kellgren-Lawrence; No.: number; H-WE: Hardy-Weinberg Equilibrium.

Table 4: Association of the rs2234693 with knee OA in the Chinese Han population with a stratification by gender.

Groups compared	TT versus TC + CC			TT + TC versus CC			T allele versus C allele			All genotyped*
	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value	*P* value
All patients ($n = 469$) versus all controls ($n = 514$)	1.133	0.874–1.469	0.345	1.316	0.937–1.850	0.113	1.149	0.959–1.377	0.133	0.259
Female patients ($n = 356$) and female controls ($n = 120$)	1.282	0.836–1.967	0.254	2.087	1.086–4.009	**0.025**	1.359	1.003–1.840	**0.047**	0.073
Male patients ($n = 113$) and male controls (394)	0.917	0.599–1.405	0.690	0.954	0.538–1.691	0.871	0.943	0.693–1.283	0.709	0.923

OR: Odds Ratio; CI: confidence interval.

*TT, TC, and CC genotypes were grouped together and a 2 × 3 contingency-table analysis was performed.

Table 5: Association of the rs9340799 with knee OA in the Chinese Han population with a stratification by gender.

Groups compared	AA versuss AG + GG			AA + AG versus GG			A allele versus G allele			All genotyped*
	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value	*P* value
All patients (n = 469) versus all controls (n = 522)	1.257	0.969–1.630	0.085	1.745	0.965–3.156	0.063	1.272	1.022–1.583	**0.031**	0.084
Female patients (n = 356) and female controls (n = 124)	1.357	0.885–2.082	0.161	4.410	1.027–18.938	**0.030**	1.454	1.006–2.102	**0.046**	0.069
Male patients (n = 113) and male controls (398)	0.801	0.510–1.256	0.332	1.125	0.433–2.925	0.809	0.869	0.594–1.271	0.468	0.817

OR: Odds Ratio; CI: confidence interval.

*AA, AG, and GG genotypes were grouped together and a 2 × 3 contingency-table analysis was performed.

Table 6: Association of the rs2234693 with knee OA in the Chinese Han population with a stratification by the severity of OA.

Groups compared	TT versus TC + CC			TT + TC versus CC			T allele versus C allele			All genotyped*
	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value	*P* value
Mild OA (255 patients versus 514 controls)	1.322	0.963–1.815	0.084	1.560	1.056–2.304	**0.025**	1.302	1.050–1.615	**0.016**	**0.047**
Severe OA (214 patients versus 514 controls)	0.951	0.686–1.318	0.763	1.045	0.667–1.638	0.846	0.986	0.782–1.245	0.908	0.910

OR: Odds Ratio; CI: confidence interval.

*TT, TC, and CC genotypes were grouped together and a 2 × 3 contingency-table analysis was performed.

Table 7: Association of the rs9340799 with knee OA in the Chinese Han population with stratification by the severity of OA.

Groups compared	AA versus AG + GG			AA + AG versus GG			A allele versus G allele			All genotype*
	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value	*P* value
Mild OA (255 patients versus 522 controls)	1.269	0.930–1.732	0.132	2.011	1.036–3.902	**0.036**	1.312	1.013–1.701	**0.039**	0.073
Severe OA (214 patients versus 522 controls)	1.267	0.911–1.762	0.159	1.573	0.750–3.299	0.227	1.258	0.954–1.658	0.103	0.261

OR: Odds Ratio; CI: confidence interval.

*AA, AG, and GG genotypes were grouped together and a 2 × 3 contingency-table analysis was performed.

Hence a definite conclusion cannot be made and more large-scale population studies will be much required to clarify this finding. Still, we cannot exclude the possibility that the two SNPs may be in linkage disequilibrium with some other more relevant alleles within the ESR1 gene region. Previous studies of candidate genes for OA susceptibility were mainly related to genes encoding collagens, extracellular matrix molecules, bone and cartilage growing factors, and the inflammation pathway [29, 30]. Thus, polygenic effects on the pathogenesis of knee OA should not be ignored. There still exists a necessity to explore other potentially contributing genes involved in the development of knee OA for a better comprehension of genetic regulation on knee OA.

Estrogens act on the skeleton through the binding to ESRs (ESR1 and ESR2), and ESR1 has been increasingly deemed as a mediator of considerable importance in the signal transduction pathway [10]. Given that rs2234693 and rs9340799 are located in intron 1, there is a possibility that TT and AA genotypes of rs2234693 and rs9340799 polymorphisms may exert influence on the expression of ESR1 in bone cells and chondrocytes through transcriptional process. The specific effects may be associated with changes in (juxta articular) bone or articular cartilage, subsequently leading to OA. The truth is that human ESR1 mRNA isoforms are synthesized by splicing of 1 of 6 alternative first exons (1A–1F) to a common acceptor site, which may lead to differential patterns of expression of ESR1 in different tissues and cells [31]. There also exist three isoforms of ESR1 of 36, 46, and 66 kDa, which were generated by alternative splicing of the gene and had certainly transcriptional and functional differences [32]. Previously, C allele of rs2234693 was found to produce a favorable and functional binding site for the *myb* transcription factor that can initiate a great promotion of transcription [33]; however, little has been known in terms of this potential influence on ESR1 expression up to now. On the other hand, Riancho et al. [18] recently pointed out that genotypes of rs2234693 were correlated with the statistically differences in CYP19A1 transcript abundance in a large-sample study, which might propose an underlying transinteraction of the two genes situated in different chromosomes. Accordingly, considering the fact that the expression pattern of ESR1 isoforms in the skeleton especially the cartilage remains unclear, further exploration concerning the impacts of ESR1 gene on OA pathogenesis from the perspective of transcriptional regulation will still be of critical importance.

OA has been seen as a chronic inflammatory disease which could affect the cartilage, the synovium, the subchondral bone, and the other joint tissues [34]. Much attention has been paid to the effects of estrogen on articular cartilage, and estrogen can also affect the synovium, muscles, ligaments, the preiarticular bone, and the joint capsule [35]. Conflicting results have been reported in experimental animal models in terms of the role of estrogen in the development of OA [36–38]. In clinic, several observational studies have shown the protective effects of estrogen replacement therapy (ERT) on OA, especially at the hip joint [39–41]. But, other published reports found short term of ERT may increase the risk of OA in the hip and hand [42, 43]. For all that, the fact that estrogen may have both anti- and proinflammatory properties depending on the involved joint tissues and the situation has been gradually accepted but the specific mechanisms are still unknown [44]. Therefore, more large-sample retrospective and prospective studies in terms of the duration of ERT in different joints will be much needed. By combining with the established association of polymorphisms in the ESR1 gene and OA susceptibility, it will also help to conduct a more resultful and targeted adjuvant treatment on these patients especially for postmenopausal women. Particularly, ERT should target the joint as a whole rather than focusing only on the damage of cartilage. A view that combined estrogen and progesterone therapy could reduce the severity of OA in a murine model of OA is likely to provide us a novel research direction again [45]. Estrogen has been found to decrease the levels of reactive oxygen species, inhibit the synthesis of interlukin-1β-mediated nitric oxide, and reduce the production of some proinflammation factors in articular cartilage [44, 46]. Getting insight into the mechanisms with which estrogen works on OA will also be conducive to a more comprehensive understanding of the genetic influence on OA by ESR1 gene.

Our study still has a few limitations. The relatively limited sample size and unmatched gender in patients and controls may influence the statistical power of any existing association. We failed to systematically obtain X-rays of the knee in control subjects to avoid the interference of asymptomatic OA. Also, we only evaluate the risk of knee OA and ESR1 gene polymorphisms, and the results cannot be well generalized to other joint sites. Nevertheless, there is reason to believe that the findings are of considerable credibility and veracity. Given the fact that genetic factors may vary with disease pattern and severity and according to individuals' characteristics such as gender and age [19], we still have a long way to go in the genetic research of OA.

5. Conclusions

In conclusion, we suggested an association of the polymorphisms of rs2234693 and rs9340799 in the ESR1 gene and susceptibility to primary knee OA in the Chinese Han population. This association remained in female patients and those who had mild OA. Future investigations should focus on sex-specific mechanisms on the etiology of knee OA and determine whether there are genetic factors which can be targeted through prevention and therapy strategies to mitigate the seemingly increased prevalence of knee OA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xiaoyu Dai and Chao Wang were considered to contribute equally to this work.

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Review Article

Digging Up the Human Genome: Current Progress in Deciphering Adverse Drug Reactions

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Adverse drug reactions (ADRs) are a major clinical problem. In addition to their clinical impact on human health, there is an enormous cost associated with ADRs in health care and pharmaceutical industry. Increasing studies revealed that genetic variants can determine the susceptibility of individuals to ADRs. The development of modern genomic technologies has led to a tremendous advancement of improving the drug safety and efficacy and minimizing the ADRs. This review will discuss the pharmacogenomic techniques used to unveil the determinants of ADRs and summarize the current progresses concerning the identification of biomarkers for ADRs, with a focus on genetic variants for genes encoding drug-metabolizing enzymes, drug-transporter proteins, and human leukocyte antigen (HLA). The knowledge gained from these cutting-edge findings will form the basis for better prediction and management for ADRs, ultimately making the medicine personalized.

1. Introduction

Adverse drug reactions (ADRs) are side effects occurring within the approved dosage and labeling recommendations. Severe ADRs, which require hospitalization, are a significant clinical problem in drug therapy because they can be permanently disabling or result in death. The incidence of severe ADRs has been estimated at 6.2–6.7% in hospitalized patients and the incidence of fatal ADRs is estimated to be 0.15–0.3% [1]. From a clinical aspect, ADRs can be broadly divided into two types, type A and type B [2]. Type A reactions are considered as a magnification of a drug's therapeutic effect and represent the majority of ADRs. This type of condition is predictable from the known pharmacology of a drug and typically dose dependent. By contract, type B reactions are less common and do not involve the pharmacological effects of a drug. Moreover, most individuals are not susceptible to type B ADRs, which are, thus, being termed "idiosyncratic." With the advance of the current understanding in their underlying mechanisms, some type B reactions now become potentially avoidable although totally unpredictable in the past.

In addition to the impact on health care, ADR remains a huge cost burden for pharmaceutical industry. It has been reported that 56 out of 548 newly approved drugs in the US either had to be withdrawn from the market or achieved a black box warning due to adverse reactions that were unpredicted by clinical trials from 1975 to 1999 [3]. Although controversial, there is an estimate that the cost of bringing a single new drug to market is US$802 million [4]. Thus, severe ADRs pose tremendous challenges both to patient care and to pharmaceutical development. The current successes in discovering specific genotypes that are highly associated with certain ADRs are encouraging; however, a more comprehensive understanding is essential for dealing with this complex problem. In this review, we discuss the pharmacogenomic techniques used to explore the pathogenesis of ADRs and summarize the current progresses concerning

genetic associations and predictors for the occurrence of ADRs, with a focus on genetic variants for genes encoding drug-metabolizing enzymes, drug-transporter proteins, and human leukocyte antigen (HLA).

2. Pharmacogenomic Strategies for Studying ADR

In the past few decades, many genes which are implicated in simple, monogenic disorders have been discovered by using linkage analysis and positional cloning approaches. However, these methods were less successful in mapping genes that are involved in complex diseases, like ADRs, because such diseases typically are caused by several genes, each with a portion of overall contribution. Researchers, thus, began to conduct the association studies using the candidate-gene approach to search for the statistical correlation between genetic variants and a disease. These genetic association studies, through which the relation of selected genes/genotypes with the etiological role of a disease in a group of population-based samples from affected and unaffected (case versus control) individuals was analyzed, are likely to be more useful than linkage studies for studying complex traits because they can have greater statistical power to find numerous genes of small effect [5]. In spite of its advantage, it has been reported that the association studies of the same disease using such candidate-gene approach are often inconsistent in their findings and that the first study to report an association often presents a stronger effect than that observed in subsequent studies [6].

With the completion of the human genome project [7] and the availability of comprehensive data on variability in human genome from the HapMap [8], huge strides have been made in our understanding of single nucleotide polymorphism (SNP) and the impact of interindividual genetic variants on the risk of complex diseases. These findings together with the development of modern methods and techniques allowing the prosecution of large-scale association studies have evolved the studies of complex disorders from the candidate-gene approach to the genomewide association study (GWAS). Unlike the candidate-gene approach that highlighted the selected genes, GWAS aims to analyze the genotype of SNPs throughout the whole genome, not simply focusing on those that are obvious candidates for effects on the disease of interest. Due to this open nature, GWAS does not require an initial hypothesis for exploring the genetic predisposing factors to a complex disease. However, a limitation of GWAS is that a large sample size is required to discover the SNPs with relatively low odds ratios [9]. This often harnesses the studies on severe, idiosyncratic ADRs which occur at very low frequencies, unless samples are collected via international collaboration [10].

In addition, advances in DNA sequencing technologies that allow substantial increases in sequencing content while dramatically decreasing the cost per base have facilitated the advent of high-throughput sequencing methods, often referred to as next-generation sequencing (NGS). These techniques, including whole-genome sequencing that reads the complete sequence of an individual's genome at a single time and whole-exome sequencing that captures only the parts of the DNA which code for proteins, have been successfully applied to numerous disease-targeted tests in disease diagnostics [11]. The central advantage of NGS over GWAS on exploration of genetic etiology of polygenic diseases is that NGS can directly identify the causal variants whereas GWAS primarily is designed for seeking markers that are intended to represent causal variation indirectly. Furthermore, these sequencing-based methods possess higher explorative power than does GWAS, enabling to discover the causal variations with low allele frequencies (<5%) in complex traits [12], although the development of chip-based genotyping advances greatly. Yet, until recently, studies of uncovering the genetic susceptibility to complex diseases or severe ADRs using NGS techniques are still very limited.

3. Drug-Metabolizing Enzymes

Interindividual differences in drug disposition have been recognized as important and common causes of adverse drug reactions [13]. Drug metabolism is generally classified into two phases, termed phase I and phase II. Phase I reactions encompass oxidation or reduction reactions, usually through the actions of cytochrome p450 oxidative enzymes or reductases. These phase I drug-metabolizing enzymes (DME) process the parent drugs for phase II reactions by creating a conjugation site on the drug. Subsequently, phase II DME acts to conjugate a hydrophilic entity onto the intermediate product, allowing the formation of a more polar metabolite that can be excreted in the urine or bile [14]. Genetic variants of DME genes such as SNP, insertion/deletion, and gene duplication may alter either the expression level or the functional activity of an enzyme, resulting in aberrant pharmacokinetics and ultimately leading to ADR. With the substantial progress in current pharmacogenomic studies, numerous genetic variants of DME have been identified as predisposing factors to ADRs. A comprehensive review that covers the genetic variations in phase I and phase II DMEs to the safety and toxicity of drug therapy has been published recently [15]. Here, we summarize only "known valid" DME biomarkers and their effects on drug safety.

The majority of DMEs belong to the CYP gene superfamily, which encodes a phase I enzyme family, the cytochrome p450 superfamily [16]. Polymorphisms in CYP1A2, CYP2C9, CYP2C19, and CYP2D6 have been evaluated to contribute to clinically significant differences in exposure to several drugs [17]. Among these SNPs, several CYP2C9 variants (predominantly CYP2C9$*$2 and $*$3 alleles) are relevant to adverse effects of numerous antiepileptics, antidepressants, nonsteroidal anti-inflammatory agents, sulfonylurea antidiabetic drugs and, most critically, oral anticoagulants (e.g., acenocoumarol and warfarin) [18]. Myriads of clinical studies have shown that the CYP2C9 polymorphism should be considered in warfarin therapy [19]. Similar information is known for another member of CYP2C subfamily, CYP2C19. The CYP2C19$*$2 allele was associated

with a marked decrease in platelet responsiveness to clopidogrel, an anticoagulant [20] while the pharmacokinetics of citalopram, an antidepressant, were influenced by the CYP2C19∗2 and CYP2C19∗17 alleles [21, 22]. Dose adjustments for these drugs based on CYP2C19 genotypes have been suggested. In addition, CYP2D6, another most extensively studied polymorphic, CYP, is involved in the metabolism of a large number of drugs, such as antiarrhythmics, tricyclic and second-generation antidepressants, antipsychotics, β-blockers, opioid analgesics, and anticancer drugs [23]. Carriers of duplicated variants of CYP2D6 (CYP2D6∗2) have been shown to be susceptible to the ADR of codeine treatment [24, 25]. Cumulative pharmacokinetic data from patients and healthy volunteers have also suggested a reduction in drug dosage for several antidepressants based on CYP2D6 phenotypes [25].

Furthermore, genetic polymorphisms of phase II DMEs are also known to influence the drug metabolism and the development of ADRs. An association of the genetic variation in the promoter region of uridine diphosphate glucuronosyltransferase 1A1 gene (UGT1A1∗28) with irinotecan-associated toxicity has been prescribed [26, 27]. The wild-type allele of UGT1A1 has six TA repeats in the promoter region while the UGT1A1∗28 has seven TA repeats, producing an enzyme with reduced activity [26]. Another notable example is the involvement of thiopurine S-methyltransferase (TPMT) allelic variants (predominantly TPMT∗2, TPMT∗3A, and TPMT∗3C) in mercaptopurine- or azathioprine-related adverse events [28–30]. Other phase II DMEs whose genetic polymorphisms have been correlated with drug toxicity are *N*-acetyltransferase type I (NAT1) and type II (NAT2) [31]. By comparison with the NAT2 genes, only a small number of NAT1 variants result in alteration of phenotypes. An increased incidence of drug toxicities in subjects carrying polymorphic NAT2 alleles has been reported when received hydralazine and sulfasalazine [32–34].

4. Drug-Transporter Proteins

Drug-transporter proteins (DTPs) represent another group of important determinants that govern the pharmacokinetics. These transporters are integral membrane proteins that mediate the influx or efflux transport of drug metabolites across the membrane using active and passive mechanisms [35]. Influx DTPs are mainly composed of the solute carrier (SLC) superfamily, including the organic cation transporters (OCTs), the multidrug and toxin extrusion (MATE) transporters, the organic anion transporters (OATs), and the organic anion transporting polypeptides (OATPs), while efflux transporters consist of members of the ATP-binding cassette (ABC) superfamily, such as P-glycoprotein (P-gp/MDR1), breast cancer resistance protein (BCRP), and transporters of the multidrug resistance-associated protein (MRP) family. For a more detailed description regarding the impact of DTPs on drug efficacy and toxicity, refer to a recent comprehensive review [36]. Here, we highlight those with well-defined pharmacogenomic roles in the development of ADRs.

OATP1B1, encoded by SLCO1B1, remains one of the most extensively studied influx DTPs, owing to the prevalence of clinically relevant polymorphisms [37]. A well-characterized SLCO1B1 variant is the loss-of-function polymorphism c.521T>C (rs4149056). The genetic association of rs4149056 with myopathy induced by simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase inhibitor used for controlling elevated cholesterol, has been identified [38, 39]. It is, thus, recommended that genetic tests of SLCO1B1 genotypes may be clinically useful tools for preventing simvastatin-induced muscle toxicity [40]. Similar finding was also observed in the OCTs, whose expressions and activities are crucial for the delivery of antineoplastics to the target tissues. A SNP of OCT2 gene (SLC22A2), rs316019, was found to be associated with reduced nephrotoxicity from cisplatin in cancer patients [41]. This observation was supported by the pharmacokinetic study of cisplatin in OCT2 knockout mice. In addition, another group of influx DTPs that moves small organic anions against their concentration gradient using a Na+ gradient is the OAT family. Of particular significance in drug disposition are OAT1 and OAT3, encoded by SLC22A6 and SLC22A8, respectively. A SNP in the intergenic region between SLC22A6 and SLC22A8 (rs10792367) was recently identified to be associated with hypertension to hydrochlorothiazide [42], although association studies of genetic variants in genes encoding OATs with changes in drug disposition are very limited.

Polymorphisms in efflux transporters are also known to be involved in the toxicity to drug treatment or predisposition to ADRs. A noteworthy example is the pharmacogenomic finding regarding P-glycoprotein (ABCB1/MDR1), the first human ABC transporter gene formerly characterized through its ability to confer a multidrug resistant (MDR) phenotype to certain chemotherapy drugs in cancer cells [43]. Among numerous variants of ABCB1 identified, a correlation of the ABCB1 3435T>C (rs1045642) was observed with cyclosporine-induced nephrotoxicity [44, 45]. In addition, functional effects of genetic variants in the ABCB1 gene have been considered as haplotypes rather than independent SNPs, as the use of ABCB1 haplotypes has been applied to predict the pharmacokinetics of many drugs [46–48]. Other lines of evidence also indicate that the SNPs of another ABC gene, ABCC4 (encoding MRP4), showed an association with ADRs induced by cyclophosphamide and methotrexate in cancer patients [49, 50]. A brief summary of the association between genetic variations involved in pharmacokinetics and pharmacodynamics and their related ADRs is shown in Table 1.

5. Human Leukocyte Antigen (HLA)

Other than genes involved in pharmacokinetics and pharmacodynamics, an immune etiology has been suggested for

Table 1: Associations between genetic variants involved in pharmacokinetics and pharmacodynamics and their related ADRs.

Genetic variants	ADR	Drug	Reference
ABCB1 (rs1045642)	Nephrotoxicity	Cyclosporine	[44, 45]
ABCC4 (rs9561778)	Leukopenia/toxicity	Cyclophosphamide	[49]
CYP2C19∗2	Decreased platelet responsiveness	Clopidogrel	[20]
CYP2C19∗2, CYP2C19∗17	Altered pharmacokinetics	Citalopram	[21, 22]
CYP2D6∗2	Opioid intoxication	Codeine	[24]
Polymorphic NAT2	Toxicity	Hydralazine, sulfasalazine	[32–34]
SLC22A2 (rs316019)	Reduced nephrotoxicity	Cisplatin	[41]
SLCO1B1 (rs4149056)	Myopathy	Simvastatin	[38, 39]
TPMT∗2, TPMT∗3A, TPMT∗3C	Hematologic toxicity	Mercaptopurine, azathioprine	[30]
UGT1A1∗28	Toxicity	Irinotecan	[26, 27]

ABCB1: ATP-binding cassette subfamily B member 1; ABCC4: ATP-binding cassette subfamily C member 4; CYP: cytochrome p450 superfamily; NAT2: *N*-acetyltransferase type II; SLC22A2: solute carrier family 22 member 2; SLCO1B1: solute carrier organic anion transporter family member 1B1; TPMT: thiopurine S-methyltransferase; UGT1A1: uridine diphosphate glucuronosyltransferase 1A1.

a great number of ADRs, in particular, type B reactions [76]. Many attempts to search for the associations with specific HLAs have been made, and the findings often are drug and ethnicity specific as summarized in Table 2. Such type of ADRs is recognized as drug-induced hypersensitivity reactions that involves major histocompatibility- (MHC-) restricted drug presentation and subsequent activation of specific immune responses. Two types of the classical MHC molecules mediate this process: the MHC class I molecules, expressed by most nucleated cells, and the MHC class II molecules, expressed by specialized antigen-presenting cells (APCs). In humans, the classical MHC class I molecule is encoded by three loci known as HLA-A, HLA-B, and HLA-C; the classical MHC class II molecule is encoded by three loci known as HLA-DR, HLA-DQ, and HLA-DP. MHC class I and class II molecules may regulate the drug hypersensitivity by presenting antigenic drugs to CD8+ (cytotoxic) and CD4+ (helper or regulatory) T cells, respectively. Because drugs are usually too small to likely trigger an immunogenic response, several mechanistic models, including the hapten/prohapten model, the p-i model, and the altered repertoire model, have been proposed to explain how small molecular synthetic compounds are recognized by T cells in an MHC-dependent/independent fashion. The hapten/prohapten concept proposes that the drug or its metabolite (hapten/prohapten) reacts with a self-protein through covalent binding to generate a haptenated, *de novo* product. This product then undergoes antigen processing to create a novel MHC ligand that is loaded onto the MHC and trafficked to the cell surface, where it activates antigen-specific T lymphocytes [77, 78]. In addition, a second concept, the p-i (pharmacological interaction with immune receptors) model, describes that a noncovalent, labile interaction of the drug with the MHC receptor at the cell surface is involved in MHC-dependent/independent T-cell stimulation by various drugs [79]. Neither cellular metabolism nor antigen processing is required in such an interaction. This model, to some extent, explains certain cases where drug hypersensitivity occurs rapidly, since the immunogenic complexes produced by drug presentation are unlikely to depend on antigen processing and cellular metabolism. Another concept, the altered repertoire model, has recently been proposed, according to which the drugs or its metabolites can bind noncovalently within the pocket of the peptide binding groove of certain MHC molecules with extraordinary specificity, allowing a new repertoire of endogenous self-peptides to be bound and presented. This concept is supported by the findings from various studies of abacavir-mediated drug hypersensitivity that the binding of abacavir to the antigen-binding cleft of HLA-B∗5701 sterically hindered the binding of the original repertoire of peptides, thereby prompting the binding of a new repertoire of peptides bearing immunogenic neoepitopes [80–82].

The striking examples of HLA associations with ADRs are HLA-B∗1502 with carbamazepine-induced Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) in many regions of Southeast Asia [59–61], HLA-B∗5801 with allopurinol-induced SJS/TEN/hypersensitivity syndrome (HSS) [53–55], and HLA-B∗5701 with abacavir-induced hypersensitivity syndrome in the Caucasian population [51, 52]. These HLA-linked ADRs typically occur in defined populations owing to the prevalence of the specific alleles. The knowledge gained from such pharmacogenomic studies has led to a further development of genetic tests for identifying individuals at risk of these serious conditions [76]. Moreover, regardless of the diversity of genetic backgrounds and the difference in sample sizes examined, other HLA-drug associations that contribute to the pathogenesis of ADRs have been reported: HLA-A∗3101 and HLA-B∗1511 with carbamazepine-induced HSS [63, 65, 66, 83], HLA-B∗1301 with dapsone-induced hypersensitivity syndrome [68], HLA-B∗1502 with phenytoin-induced SJS/TEN [59, 70], HLA-B∗3505 and HLA-BRB1∗0101 with nevirapine-induced cutaneous ADRs [74, 84], HLA-B∗5701 with

Table 2: Genetic associations of HLA alleles with severe ADRs.

Drug	HLA allele	ADR	Ethnic population	Reference
Abacavir	B∗5701	HSS	Caucasian	[51, 52]
Allopurinol	B∗5801	SJS/TEN/HSS	Han Chinese, Thai, Japanese, European	[53–56]
Aminopenicillins	A∗2, DRw52	DHS	Italian	[57]
Amoxicillin-clavulanate	A∗0201 DQB1∗0602	DILI	Caucasian	[58]
Carbamazepine	B∗1502	SJS/TEN	Han Chinese, Thai, Indian	[59–62]
	B∗1511		Japanese	[63]
	B∗5901		Japanese	[64]
	A∗3101	HSS	Han Chinese, Japanese, European	[65–67]
Dapsone	B∗1301	HSS	Han Chinese	[68]
Flucloxacillin	B∗5701	DILI	Caucasian	[69]
Lamotrigine	B∗1502, B∗38 B∗5801, A∗6801, Cw∗0718, DQB1∗0609, DRB1∗1301	SJS/TEN	Han Chinese European	[55, 70, 71]
Lumiracoxib	DRB1∗1501 DQB1∗0602 DRB5∗0101 DQA1∗0102	DILI	Multiple populations	[72]
Methazolamide	B∗5901, CW∗0102	SJS/TEN	Korean, Japanese	[73]
Nevirapine	B∗3505	DHS	Thai	[74]
	DRB1∗0101	DHS	Australian	[75]
Oxicam	B∗73, A∗2, B∗12	SJS/TEN	European	[55, 71]
Oxcarbazepine	B∗1502	SJS/TEN	Han Chinese	[70]
Phenytoin	B∗1502	SJS/TEN	Han Chinese, Thai	[59, 70]
Sulfamethoxazole	B∗38	SJS/TEN	European	[55]

HLA: human leukocyte antigen; HSS: hypersensitivity syndrome; SJS/TEN: Stevens-Johnson syndrome/toxic epidermal necrolysis; DHS: delayed-type hypersensitivity reaction; DILI: drug-induced liver injury.

flucloxacillin-induced hepatitis [69], HLA-DPB1∗0301 with aspirin-induced asthma [85], and HLA-DQA1∗0201 with lapatinib-induced hepatotoxicity [86].

6. Miscellaneous

In addition to those mentioned above, genetic variations of many genes that are unrelated to pharmacokinetics/pharmacodynamics and HLA-restricted immune responses have been found to be associated with drug toxicity. These include, but not limited to, various cytokine gene promoters [87–89], epidermal growth factor receptor (EGFR) [90, 91], Fc gamma receptor [92], and microRNAs [93].

7. Conclusions

A decade has passed since the completion of the human genome project. During this period, human genetic research has revealed that the genetic backgrounds between the individuals can contribute to differences in the susceptibility to various ADRs. Thousands of genetic variations that are associated with drug safety and toxicity have been identified, many of which have shown high accuracy at predicting drug responses and adverse events. However, the molecular mechanisms through which these biomarkers influence disease risk and/or phenotypic expression still need to be further elucidated. More importantly, to determine which patients will benefit or suffer from a particular drug, the major challenge lies in translating the findings into clinical practice, which perceivably is a key component of the advancement to "personalized medicine."

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Improved Candidate Drug Mining for Alzheimer's Disease

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Alzheimer's disease (AD) is the main cause of dementia for older people. Although several antidementia drugs such as donepezil, rivastigmine, galantamine, and memantine have been developed, the effectiveness of AD drug therapy is still far from satisfactory. Recently, the single nucleotide polymorphisms (SNPs) have been chosen as one of the personalized medicine markers. Many pharmacogenomics databases have been developed to provide comprehensive information by associating SNPs with drug responses, disease incidence, and genes that are critical in choosing personalized therapy. However, we found that some information from different sets of pharmacogenomics databases is not sufficient and this may limit the potential functions for pharmacogenomics. To address this problem, we used approximate string matching method and data mining approach to improve the searching of pharmacogenomics database. After computation, we can successfully identify more genes linked to AD and AD-related drugs than previous online searching. These improvements may help to improve the pharmacogenomics of AD for personalized medicine.

1. Introduction

Alzheimer's disease (AD), the most common form of dementia, was first reported in 1906 [1]. In 2006, there were about 26.6 million AD patients worldwide and it was also common in southern Taiwan [2]. Although AD has been identified for a long time, most research progress was made in the recent 30 years [3]. However, no definitive cure is available for this disease and eventually it leads to death. Therefore, the drug discovery for Alzheimer's disease remains challenging.

Single nucleotide polymorphisms (SNPs) are the most common variation in human genomes [4]. The importance of SNPs has been reviewed in genome-wide association studies for its association with disease susceptibility and drug metabolism [5, 6]. About 60–90% of the individual variation of drug response depends on pharmacogenomic factors. Therefore, SNP genotyping for candidate genes, pharmacological research, and drug discovery may play an increasingly important role in AD treatment. Meanwhile, increasing amounts of related information require the assistance of bioinformatics to construct the suitable databases and web servers.

Recently, PharmGKB (the Pharmacogenetics and Pharmacogenomics Knowledge Base) has been constructed to provide a comprehensive database for pharmacogenomic studies [7]. PharmGKB provides the pharmacogenetics research network in terms of SNP discovery and drug responses [8] with the fully curated knowledge for drug pathways, drug-related genes, and relationships among genes, drugs, and diseases. However, some information of different functions of PharmGKB is insufficient to allow convenient crosstalking between each other.

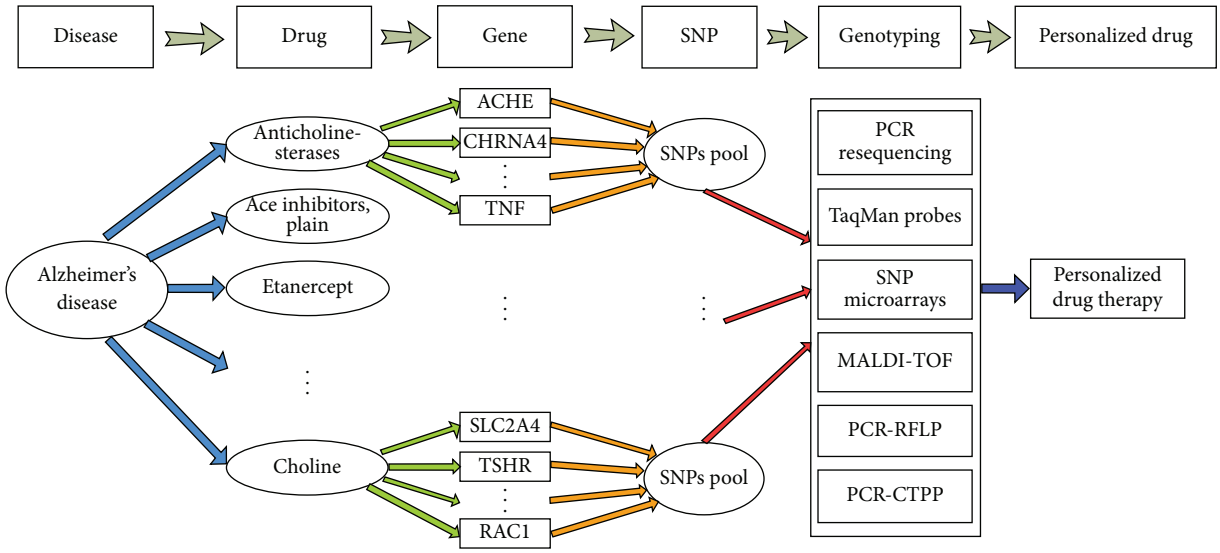

Figure 1: The flowchart for PharmGKB-based pharmacogenomics of AD in this study.

To solve this problem, we propose data mining method to improve the searching of pharmacogenomics of AD based on the download dataset of the PharmGKB resource.

2. Materials and Methods

The flowchart for pharmacogenomics in AD for personalized drug studies is shown in Figure 1. First of all, the AD-related drugs and genes are retrieved from PharmGKB download data using approximate string matching method and data mining approach. The genes associated with AD and the genes associated with a single Alzheimer's drug are identified and compared with the online searching of PharmGKB. Then, numerous SNPs of genes associated with AD are identified. Through some SNP genotyping tools or assays, the association studies to AD-related drugs may be evaluated. Finally, the relevant information may be helpful for the personalized drug research.

2.1. AD-Related Drugs Using Approximate String Matching Based on PharmGKB Download Data. In order to study the pharmacogenomics of AD, we downloaded the known PharmGKB (the Pharmacogenetics and Pharmacogenomics Knowledge Base) (http://www.pharmgkb.org/downloads/) [9, 10] as source by the approximate string matching method [11] to find out all AD-related drug classes. The meaningful keywords associated with "Alzheimer's disease" are shown in Table 1. Then, these found drug classes are used to find out associated genes by data mining approach. The description of the approximate string matching method for all AD-related drug classes gives a pattern string $P = p_1 p_2 p_3 \cdots p_m$, that is, the meaningful keywords associated with "Alzheimer's disease" and a text string $T = t_1 t_2 t_3 \cdots t_n$, that is, the description for drug and disease retrieved from PharmGKB. Find a substring $T_{i,j} = t_i t_{i+1} t_{i+2} \cdots t_j$ in T that has the smallest edit distance [12] to the pattern P. The pseudocode for the edit distance is shown in Algorithm 1.

2.2. Data Mining Method for PharmGKB Download Data. In this study, we used a priori algorithm [13] for frequent item set mining and association rule learning over PharmGKB. The pseudocode for the a priori algorithm for data mining in PharmGKB is shown in Algorithm 2. At first, a priori algorithm has to find out the frequent gene in drug class for "Alzheimer's disease." A set of genes can be mined from each drug class. A priori algorithm is a "bottom up" approach, where frequent gene subsets are extended one item at a time (i.e., candidate generation) and groups of candidates are tested against the data. This algorithm is terminated when no further successful extensions are found.

2.3. SNP Searching for Genes Using the NCBI dbSNP. Every gene contains numerous SNPs. In order to find out SNPs of single gene for Alzheimer's pharmacogenomics, NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp) is used to search in the study.

3. Results and Discussion

3.1. AD Information Based on PharmGKB Search. In PharmGKB online searching, the SNP variants, related genes, and drugs for AD are able to be retrieved. For example, the SNP information such as rs2066853 and rs6313 is provided (Figure 2). As shown in Figure 3, the AD-related genes such as ADRB1, AHR, HTR2A, MTHFR, and PTGS2 are identified and the related drugs such as olanzapine and risperidone are searched. This information may assist the researchers to study the pharmacogenomics of AD. Unfortunately, this

Table 1: The meaningful keywords associated with "Alzheimer's disease" are retrieved from PharmGKB and they are applied to discover the drug classes*.

ID	Keywords
1	AD
2	Alzheimer's disease
3	AD—Alzheimer's disease
4	Acute Confusional Senile Dementia
5	Alzheimer Dementia, Presenile
6	Alzheimer Disease, Early Onset
7	Alzheimer Disease, Late Onset
8	Alzheimer Type Dementia
9	Alzheimer Type Senile Dementia
10	Alzheimer's Disease, Focal Onset
11	Alzheimer's disease, NOS
12	Dementia, Alzheimer Type
13	Dementia, Presenile
14	Dementia, Presenile Alzheimer
15	Dementia, Primary Senile Degenerative
16	Dementia, Senile
17	Dementias, Presenile
18	Dementias, Senile
19	Disease, Alzheimer
20	Disease, Alzheimer's
21	Early Onset Alzheimer Disease
22	Focal Onset Alzheimer's Disease
23	Late Onset Alzheimer Disease
24	Presenile Alzheimer Dementia
25	Presenile Dementia
26	Presenile Dementias
27	Primary Senile Degerative Dementia
28	Senile Dementia
29	Senile Dementia, Acute Confusional
30	Senile Dementia, Alzheimer Type
31	Senile Dementias
32	MeSH: D000544 (Alzheimer Disease)
33	MedDRA: 10001896 (Alzheimer's disease)
34	NDFRT: N0000000363 (Alzheimer Disease [Disease/Finding])
35	SnoMedCT: 26929004 (Alzheimer's disease)
36	UMLS: C0002395 (C0002395)

*Drug class is one of the functions listed in the ParamGKB download data.

PharmGKB online searching just provides limited information and it insufficiently copes with the complexity of the drug researches for Alzheimer's personalized medicine.

3.2. PharmGKB-Based Data Mining of AD Information of Drug Classes or Gene Symbols. In current study, our proposed method is used to perform data mining for PharmGKB download data in terms of the keyword "Alzheimer's disease." As shown in Table 2, 22 kinds of AD-related drug classes are identified from "drug classes" of PharmGKB. Their corresponding PharmGKB accession ID, PubMed PMID, and the number of genes that are associated with AD-related drug

```
(1)  // initialization
(2)  for i ← 0 to m do
(3)      E(i, 0) ← i
(4)  end for
(5)  for j ← 0 to n do
(6)      E(0, j) ← 0
(7)  end for
(8)  // edit distance E(i, j)
(9)  for i ← 0 to m do
(10)     for j ← 0 to n do
(11)         if(T(j) = P(i)) then
(12)             E(i, j) ← (i − 1, j − 1)
(13)         else
(14)             min ← MIN[E(i − 1, j), E(i, j − 1)]
(15)             E(i, j) ← min + 1
(16)         end if
(17)     end for
(18) end for
(19) return E
```

Algorithm 1: Pseudocode for the edit distance used for approximate string matching.

```
(1)  Apriori(PharmGKB, ε)
(2)  L_1 ← (frequent genes in drug class for Alzheimer's disease)
(3)  k ← 2
(4)  while L_{k−1} ≠ ϕ
(5)      C_k ← {a ∪ {b} | a ∈ L_{k−1} ∧ b ∈ ⋃L_{k−1} ∧ b ∉ a}
(6)      for each drug class ∈ PharmGKB
(7)          C_t ← {gene | gene ∈ C_k ∧ gene ⊆ drug class}
(8)          for each candidate gene ∈ C_t
(9)              count[gene] ← count[gene] + 1
(10)         end for
(11)     end for
(12)     L_k ← {gene | gene ∈ C_k ∧ count[gene] > ε}
(13)     k ← k + 1
(14) end while
(15) return ⋃_k L_k
```

Algorithm 2: Pseudocode for a priori algorithm for the data mining in PharmGKB, where ε is a support threshold, L is the frequent gene subsets that satisfy the support threshold, k is the number of current iterations, and C is the candidate set, and count[gene] accesses a field of the data structure that represents gene candidate set.

classes are also presented. In total, 495 genes are identified for AD information of drug classes (see Supplementary file 1: gene information includes PharmGKB Accession Id, gene symbol, and publications are providing in different classes; it is available online at http://dx.doi.org/10.1155/2014/897653). Alternatively, 99 genes associated with AD are identified from "gene symbols" of PharmGKB in terms of the keyword "Alzheimer's disease." These results suggest that the same keyword, for example, Alzheimer's disease, may identify different numbers of AD-associated genes between "drug classes" or "gene symbols" of PharmGKB.

After detailed examination, 67 genes in the gene symbols searching (bold fonts of gene names as shown in Table 3) are absent from the genes in the drug class searching (Table 2). Furthermore, genes corresponding to the drug "memantine" listed in Table 2 (drug classes) are not found in Table 3 (gene symbols). Therefore, some current drugs have identified a small number of AD-related genes in the drug class searching; however, the remaining AD-related genes that may affect AD-related drugs may be partly discovered in the gene symbols searching. These novelly identified AD-related genes may be the potential candidates for further drug development of AD. These results demonstrated that our proposed data mining method may be an improved AD pharmacogenomics study.

3.3. SNP Information of AD-Related Genes. The SNP statuses for 99 AD-related genes are also provided in Table 3. This SNP status for each gene is calculated from the online NCBI

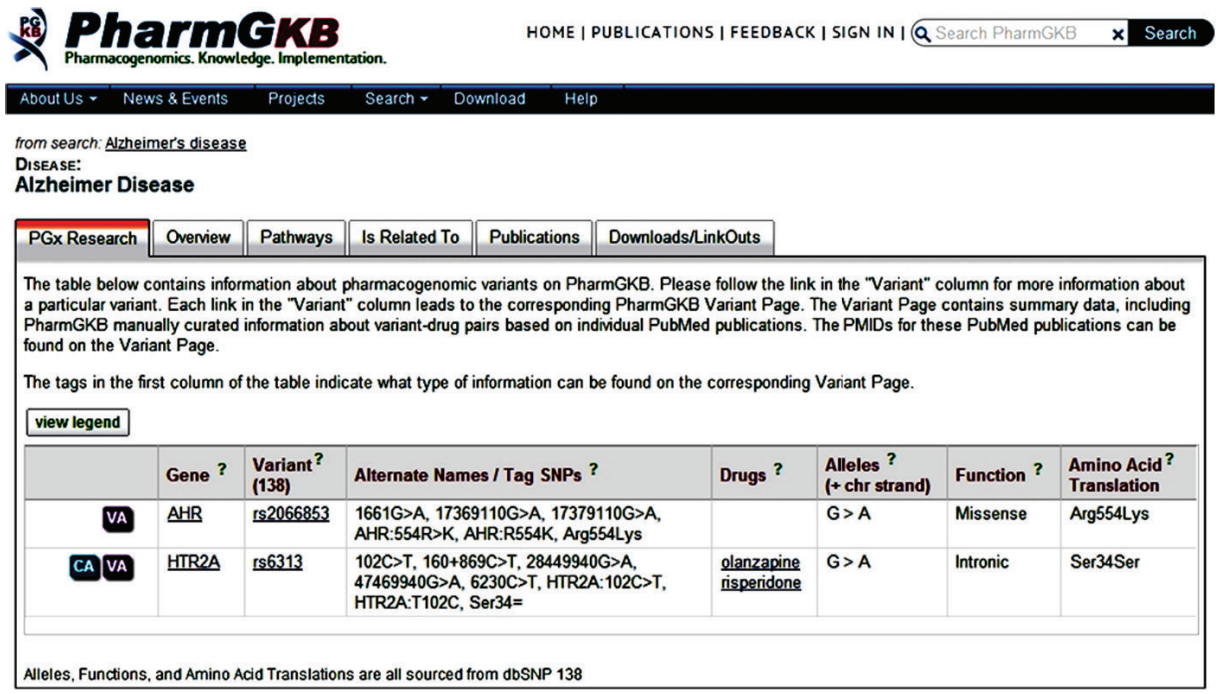

FIGURE 2: PharmGKB-pharmacogenomics online query for the variant information (SNP rs#ID) of "Alzheimer's disease." Retrieval source: http://www.pharmgkb.org/disease/PA443319?previousQuery=Alzheimer's%20disease.

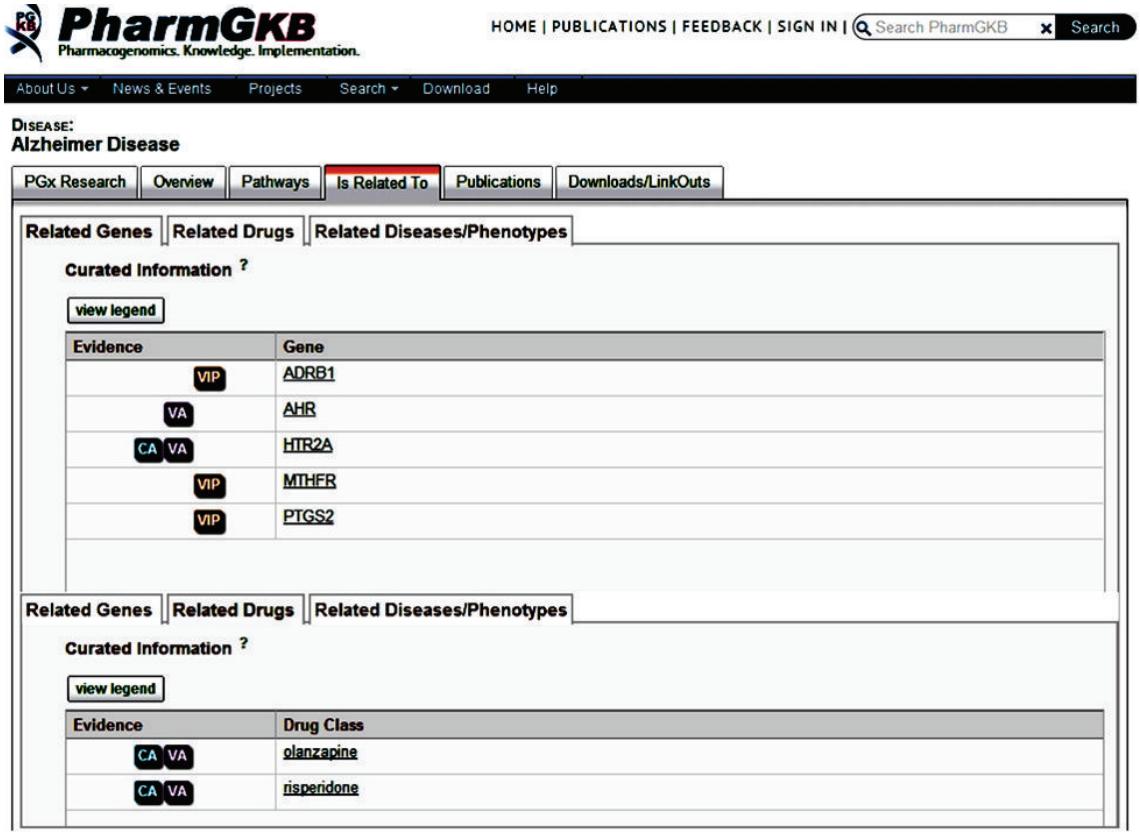

FIGURE 3: Gene and drug related information of "Alzheimer's disease" online query from PharmGKB. Retrieval source: http://www.pharmgkb.org/disease/PA443319?previousQuery=Alzheimer's%20disease#tabview=table 3&subtab=33.

Table 2: PharmGKB-based data mining results in terms of the PharmGKB accession ID, drug class, publications, and the number of gene information of Alzheimer's disease.

No.	PharmGKB accession ID	Drug classes	Publications*1	Gene no.*2
1	PA164712423	Anticholinesterases	PMID: 20644562 20644562 14674789	6
2	PA164712308	Ace inhibitors, plain	PMID: 17362841	24
3	PA449515	Etanercept	PMID: 19027875	12
4	PA451262	Rivastigmine	PMID: 20644562 16323253 17082448 20644562 15289797 17522596	2
5	PA450243	Lithium	PMID: 17082448	13
6	PA10384	Anti-inflammatory and antirheumatic products, nonsteroids	PMID: 17082448 17082448	11
7	PA449760	Glatiramer acetate	PMID: 17082448	4
8	PA133950441	Hmg coa reductase inhibitors	PMID: 17082448	39
9	PA151958596	Curcumin	PMID: 17082448	2
10	PA451898	Vitamin c	PMID: 17082448	16
11	PA451900	Vitamin e	PMID: 17082448	1
12	PA452229	Antidepressants	PMID: 17082448	43
13	PA452233	Antipsychotics	PMID: 17082448	46
14	PA449726	Galantamine	PMID: 20644562 16323253 17082448 15853556 20644562 14674789 12177686	7
15	PA10364	Memantine	PMID: 17082448	0
16	PA451283	Rosiglitazone	PMID: 16770341	34
17	PA448031	Acetylcholine	PMID: 15695160	8
18	PA450626	Nicotine	PMID: 15695160	88
19	PA137179528	Nimesulide	PMID: 16331303 11810182	3
20	PA449394	Donepezil	PMID: 20859244 20644562 16323253 16424819 17082448 20644562 1973817012142731	9
21	PA451576	Tacrine	PMID: 9521254 17082448 10801254 9777427 18004213	6
22	PA448976	Choline	PMID: 8618881	122

*1PMID: PubMed article ID number.
*2The full gene names for each of the "drug classes" have been provided in the Supplementary file 1.

dbSNP queries. In general, many SNPs are found in these AD-related genes. Some SNPs of these genes have been reported to be associated with AD. For example, the APOE gene is found in Table 3 and one of its SNPs, such as ApoE epsilon 4 allele, has been reported to be associated with AD [14]. With suitable tools for SNP genotyping, these SNP candidates are warranted for the pharmacogenomics research of AD.

Currently, there are many high throughput SNP genotyping methods developed (as shown in Figure 1), including PCR resequencing [15], TaqMan probes [16], SNP microarrays [17], Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) [18], and others [19, 20]. Furthermore, some SNP genotyping tools or databases are also developed, such as SNP-RFLPing2 for comprehensive PCR-RFLP information based on SNPs [21–24], algorithmic PCR-RFLP primer design and restriction enzymes for SNP genotyping [25, 26], and primer design for PCR-confronting two-pair primers (PCR-CTPP) [27, 28]. These tools and methods can provide useful and convenient information for SNP genotyping in the AD pharmacogenomics studies.

4. Conclusions

AD is the most common form of dementia for older people. The pharmacogenomics of AD still remains a challenge. In this study, we propose the pharmGKB-based data mining method to improve the gene discoveries for the potential AD-related drug candidates. With the assistance of bioinformatics, this improvement can help researchers to develop personal therapeutic drugs of AD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 3: PharmGKB-based data mining results of gene symbols of Alzheimer's disease and NCBI dbSNP-based query results for SNP number for the genes of Alzheimer's disease.

No.	PharmGKB accession ID	Gene symbols*	SNP no.	No.	PharmGKB accession ID	Gene symbols*	SNP no.	No.	PharmGKB accession ID	Gene symbols*	SNP no.
1	PA20	ACHE	899	34	PA37597	**ZNF225**	813	67	PA125	CYP2C8	993
2	PA26490	CHRNA4	1518	35	PA38499	**DEFB123**	330	68	PA126	CYP2C9	1605
3	PA128	CYP2D6	482	36	PA134902026	**SORCS2**	19073	69	PA30864	MME	3323
4	PA130	CYP3A4	899	37	PA134949387	**SORCS3**	13969	70	PA142671271	**NCSTN**	741
5	PA26620	**CLU**	644	38	PA38274	**TOMM40**	462	71	PA36153	**SST**	120
6	PA26855	**CR1**	19859	39	PA162397694	**NLRC5**	2297	72	PA36457	**TF**	1501
7	PA33287	**PICALM**	3169	40	PA24641	**AHR**	991	73	PA31930	OPCML	28437
8	PA46	**ALOX5**	1992	41	PA134950706	**DNMBP**	3312	74	PA29561	**HTR7**	2623
9	PA293	PTGS2	579	42	PA24910	APP	9411	75	PA162393285	**KIF20B**	2109
10	PA108	CETP	1246	43	PA238	MAPT	4399	76	PA26971	**CSRP3**	907
11	PA32996	**PCDH11X**	15199	44	PA128394579	TMED10	1079	77	PA231	**LMNA**	1486
12	PA24507	**ADAM12**	10827	45	PA162397475	**NGF**	1286	78	PA27029	**CTSD**	460
13	PA25165	**ATP8A1**	5983	46	PA25232	**BACE1**	794	79	PA29629	**IDE**	2755
14	PA26243	**CD86**	1385	47	PA36022	**SORL1**	4394	80	PA31374	**MYH7**	1157
15	PA26935	**CSF1**	569	48	PA33796	**PRNP**	452	81	PA272	**PLN**	343
16	PA27342	**DISC1**	11813	49	PA37302	**VEGFA**	561	82	PA33855	**PSEN1**	2343
17	PA28597	**GBP2**	625	50	PA114	CHRNA7	3714	83	PA33856	**PSEN2**	959
18	PA220	**KCNMA1**	19081	51	PA37155	**UBQLN1**	1400	84	PA304	SCN5A	3380
19	PA25512	**KCTD12**	235	52	PA26123	**CBS**	924	85	PA36638	TNNT2	739
20	PA164724093	NOS2	1820	53	PA26976	**CST3**	233	86	PA139	ACE	1108
21	PA33614	**PPP1R11**	215	54	PA25623	C1QB	356	87	PA37935	**SIRT1**	1145
22	PA143485670	**WWC1**	5070	55	PA162380954	**CALHM1**	247	88	PA55	APOE	184
23	PA37596	**ZNF224**	490	56	PA30748	**MEOX2**	2140	89	PA24357	**A2M**	1385
24	PA162380963	**CALHM2**	192	57	PA26448	CHAT	2572	90	PA192	HTR1A	186
25	PA51	APOC1	243	58	PA38239	**CLSTN2**	15608	91	PA182	GSTM1	264
26	PA34958	**ATXN1**	11910	59	PA134952303	**NMNAT3**	39	92	PA183	GSTT1	200
27	PA26210	**CD33**	465	60	PA134904440	**C1orf49**	348	93	PA268	ABCB4	1915
28	PA28478	**GAB2**	5119	61	PA134864387	**RALGPS2**	3980	94	PA115	CHRNB2	698
29	PA34052	**PVRL2**	1344	62	PA134870196	**RGSL1**	3300	95	PA156	**ESR1**	10108
30	PA37754	**ZNRD1**	316	63	PA25294	BCHE	1796	96	PA134934259	**GAPDHS**	361
31	PA38114	**TRIM15**	466	64	PA120	CRP	977	97	PA245	MTHFR	790
32	PA134927803	**MTHFD1L**	7229	65	PA127	**CYP2C18**	1353	98	PA36458	**TFAM**	376
33	PA144596420	**INTS1**	1820	66	PA124	CYP2C19	2692	99	PA435	TNF	268

*Gene names in bold fonts are not identified in Table 2.

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Research Article

TAZ Is Highly Expressed in Gastric Signet Ring Cell Carcinoma

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Transcriptional coactivator with PDZ-binding motif (*TAZ*) is known to bind to a variety of transcription factors to control cell differentiation and organ development. We examined *TAZ* protein levels in 146 stage II–IV gastric cancer using immunohistochemistry (IHC), while *TAZ* mRNA was confirmed by quantitative reverse-transcription polymerase chain reaction (QRT-PCR) in 84 samples with enough tissue. *TAZ* protein expression was positive in 113 out of 146 (77.4%) gastric cancer samples. In parallel, *TAZ* mRNA expression was successfully detected in 81 of the 84 (96.4%) samples. Protein levels of *TAZ* were positively correlated with its mRNA levels ($P = 0.018$). High expression of *TAZ* protein was observed with higher percentage in gastric cancer samples with histology of signet ring cell carcinoma (SRCC) than adenocarcinoma (85.7% versus 60.2%, $P = 0.001$). Similarly, *TAZ* mRNA level was higher in SRCC than in adenocarcinoma ($P = 0.003$). When correlated with survival, the median overall survival (OS) is 14 months (95% CI: 12.2–15.8 months) in all patients. There was no significant association between survival and other clinical characteristics or *TAZ* expression levels. Our results show that *TAZ* is highly expressed in SRCC. *TAZ* might be considered as a target for the treatment of gastric SRCC in future.

1. Introduction

Gastric cancer is the fourth most common cancer with the leading causes of cancer death in East Asian countries and some western countries [1, 2]. Signet ring cell carcinoma (SRCC) is characterized by cells with abundant mucin in the cytoplasm and nuclei located at the cell periphery. This type of carcinoma appears to be relatively frequent in women and young patients [3, 4]. It has long been thought to have a worse prognosis than other forms of gastric cancer. Recently, studies have begun to question this idea. Several studies find that the survival of patients with early SRCC was not significantly different from other types of gastric carcinoma [5]. This was because SRCC of the stomach is less likely to lymph node metastasis and it had a higher proportion in the early stage of gastric carcinoma than other carcinomas [6, 7]. The genetic background of SRCC has rarely been investigated, and the molecular basis of their growth, differentiation, and metastasis still remains unclear. Therefore, studies of the molecular profile of gastric SRCC and identification of new molecular markers are both relevant to improve the diagnosis and the prognosis of the tumor.

Transcriptional coactivator with PDZ-binding motif (*TAZ*), also called WW-domain containing transcription regulator 1 (*WWTR1*), has been defined for its role in the nucleus [8–10]. It functions directly as a transcriptional regulator by interacting with several nuclear factors and plays a central role in the Hippo pathway, which regulates the size and shape of organ development [8–12]. *TAZ* was described as controlling gene important for muscle differentiation, lung and respiratory epithelia differentiation, cardiac and limb development, adipogenesis and osteogenesis, and tumorigenesis. Most human tissues, except thymus and peripheral blood leucocytes, express *TAZ* mRNA, with the highest levels in kidney, heart, placenta, and lung [8–12]. *TAZ* has been identified as an oncogene and has an important role in tumorigenicity of many cancers, such as non-small cell lung cancer

Table 1: Primers and probes of *TAZ* and β-actin.

Primer	*TAZ*	β-actin
Forward primer	5′ CCAGTGCCTCAGAGGTCCA 3′	5′ TGAGCGCGGCTACAGCTT 3′
Reverse primer	5′ ATCTGCTGCTGGTGTTGGTG 3′	5′ TCCTTAATGTCACGCACGATTT 3′
Probe	**6FAM** 5′ CCAAATCTCGTGATGAAT 3′ **MGB**	**6FAM** 5′ ACCACCACGGCCGAGCGG 3′ **TAMRA**

[13, 14], papillary thyroid carcinoma [15], and colon cancer [16]. They found that *TAZ* gene expression signature was over-represented in poorly differentiated tumors compared with well-differentiated low-grade tumors. Importantly, *TAZ* confers cancer stem cell-related traits in breast cancer cells [17–19], further highlighting its importance in tumor initiation and progression. According to present studies, *TAZ* is significantly associated with poor survival of cancer, so *TAZ* may be a novel prognostic indicator for cancer progression. But so far, no report has been published concerning the relationships between *TAZ* expression and clinicopathological features and prognosis of gastric cancer patients. Therefore, the objectives of this study were to evaluate the relationships between *TAZ* expression and the clinicopathological parameters of gastric cancer and to evaluate its potential role as a prognostic biomarker and an anticancer target.

2. Materials and Methods

146 gastric samples were collected from patients of the Comprehensive Cancer Center, Drum Tower Hospital Affiliated to Medical School of Nanjing University, from November 2007 to August 2011. All samples have been pathologically proven to be cancer. *TAZ* protein levels were examined by IHC in 146 samples. Meanwhile, *TAZ* mRNA levels were confirmed by quantitative reverse-transcription polymerase chain reaction (QRT-PCR) in 84 samples with enough tissue. This project has been approved by Institutional Review Board of Drum Tower Hospital.

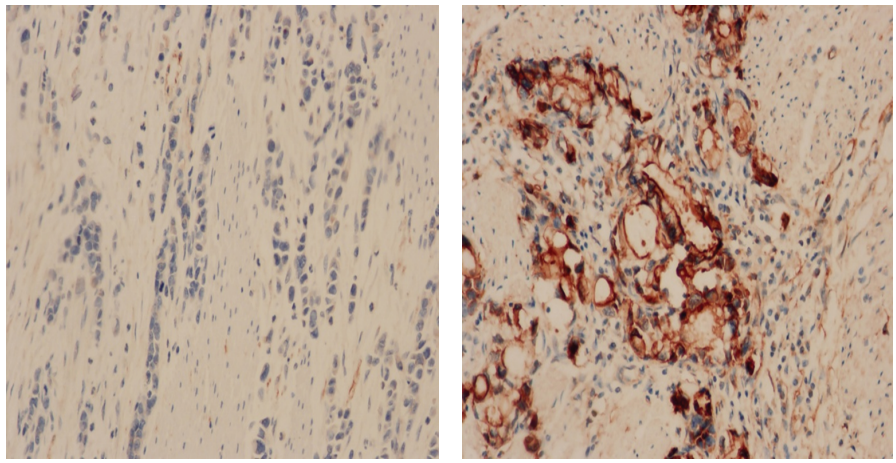
2.1. Immunohistochemical Staining for TAZ . After dewaxing in xylene and rehydrating stepwise in ethanol, sections were subjected to heat-induced antigen retrieval. The endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxide (H_2O_2) in methanol. In the negative control, the primary antibody was omitted. Skeletal muscle was used as positive control. Pretreated sections were incubated with rabbit polyclonal *TAZ* antibody (T3467, 1 : 50, Epitomics) at 4°C overnight, followed by secondary antibody. Immunohistochemical staining was evaluated independently by two pathologists without knowledge of patient characteristics, and discrepancy was resolved by consensus review. Tissue was scored (H score) based on the total percentage of positive cells ((≤5%) = 0, (6%~25%) = 1, (26%~50%) = 2, (51%~75%) = 3, and (>75%) = 4) and the intensity of the staining (0, 1, 2, or 3), where H is the percentage of positive score multiply intensity score. The sample was considered negative if $H = 0$ and positive if H was more than 0. Positive samples were also categorized as weak (1+) if $H = 1$ to 4, middle (2+) if $H = 5$ to 8, and strong (3+) if H was more than 8 [20]. A minimum of 100 cells were evaluated in calculating the H score. Patients with negative or weak staining were considered as lower group, while patients with middle and strong staining were considered as higher group.

2.2. Quantitative Reverse-Transcription Polymerase Chain Reaction (QRT-PCR) Assessment of TAZ Expression. Three 5 μm sections were prepared from FFPE tumor blocks that contained at least 80% tumor cells. After hematoxylin-eosin staining, RNA was isolated in accordance with a proprietary procedure as we published before [21]. Briefly, paraffin was removed by xylene, and macrodissected tissues were lysed in a proteinase K-containing buffer at 60°C for 16 h. RNA was purified by phenol and chloroform extractions followed by precipitation with isopropanol in the presence of sodium acetate at −20°C. The RNA pellet was washed in 70% ethanol and resuspended in RNase-free water followed by DNase. M-MLV Reverse Transcriptase Kit (Ambion, Carlsbad, CA) was used to generate cDNA for quantitative reverse-transcription polymerase chain reaction (QRT-PCR) to detect the expression of β-actin (used as endogenous control) and *TAZ*. Commercial human total RNA was used for each RT reaction as calibrator. Template cDNA was amplified with specific primers and probes (Table 1) for β-actin and *TAZ* using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA). The QRT-PCR was performed to quantify gene expression using ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR conditions were 50°C for 2 min and 95°C for 15 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Relative gene expression quantifications were calculated according to the comparative Ct method [21] and analyzed with the Applied Biosystems analysis software. *TAZ* mRNA levels were further divided into three groups according to tercile levels.

2.3. Statistical Analysis. Correlations between *TAZ* protein expression and clinicopathological parameters were analyzed by χ^2 test. Correlations between TAZ protein expression and mRNA were also analyzed by χ^2 test. The Mann-Whitney U test and the Kruskal-Wallis test were used to test the associations between *TAZ* mRNA levels and clinical characteristics. Survival curves were assessed by the Kaplan-Meier method. Two-sided $P < 0.05$ was considered statistically significant. All analyses were performed with the SPSS 17.0 software package (SPSS Inc., Chicago, USA).

3. Results

A total of 111 males and 35 females were included with ages ranging from 24 to 92 years (median, 61 years). Eighty-three patients (56.8%) with the histology of adenocarcinoma and 63 patients (43.2%) were confirmed as signet ring cell



(a) (b)

Figure 1: TAZ protein expression in gastric cancer. (a) Negative case at high magnification (×400). (b) Positive case (H score = 12) with signet ring cell phenotype at high magnification (×400).

Table 2: *TAZ* protein levels and mRNA levels.

TAZ mRNA	Protein low expression (IHC: 0-1+)	Protein high expression (IHC: 2+-3+)	P
Low expression	13 (44.8%)	16 (55.2%)	
Intermediate expression	6 (23.1%)	20 (76.9%)	0.018
High expression	3 (11.5%)	23 (88.5%)	

carcinoma. There were 6 patients (4.1%) with stage II (4 stage IIA and 2 stage IIB), 136 patients (93.2%) with stage III (22 stage IIIA, 36 stage IIIB, and 80 stage IIIC), and 4 patients (2.7%) with stage IV disease. 40 patients received 5-FU and/or oxaliplatin-based chemotherapy. The median follow-up time was 14.3 months (95% CI = 2.53 to 27.5 months).

3.1. Relationship between TAZ Protein Expression and mRNA Expression. *TAZ* protein expressions were positive in 113 of 146 (77.4%) samples. *TAZ* had nuclear and cytoplasmic expression (Figure 1). In parallel, 84 samples had enough tissue to detect *TAZ* mRNA. *TAZ* mRNA expression was found in 81 of the 84 (96.4%) samples. In *TAZ* mRNA low expression group, 44.8% of patients had low level of *TAZ* protein. Protein levels of *TAZ* were correlated with its mRNA levels (P = 0.018). There were 88.5% of patients with high *TAZ* protein levels in *TAZ* mRNA high group, 76.9% of patients with high *TAZ* protein levels in mRNA intermediate group, and 55.2% of patients with high *TAZ* protein levels in mRNA low group (Table 2).

3.2. Relationship between TAZ Protein Expression and Clinicopathological Characteristics. *TAZ* protein levels were higher in SRCC than in adenocarcinoma (P = 0.001) and higher in Grade 3 cancer than in Grade 2 cancer (P = 0.004). However, there was no difference between *TAZ* protein levels and age (P = 0.294), gender (P = 0.376), tumor site (P = 0.159), lymph node metastasis (P = 0.232), or stage (P = 0.785) (Table 3).

3.3. Relationship between mRNA Expression and Clinicopathological Characteristics. *TAZ* mRNA level in signet ring cell carcinoma was higher than adenocarcinoma (median levels: 4.64 versus 2.02, P = 0.003). However there was no difference between *TAZ* mRNA levels and patients' age (P = 0.374), gender (P = 0.696), tumor site (P = 0.069), lymph node metastasis (P = 0.899), p-TNM stage (P = 0.492), or histological grade (P = 0.375) (Table 3).

3.4. Survival for Gastric Cancer Patients According to TAZ Protein and mRNA Levels. The median overall survival (OS) is 14 months (95% CI = 12.2 to 15.8 months) in all patients. The median OS is longer in younger patients (16.9 months, 95% CI = 11.9–22.6 months) than in elder patients (12.4 months, 95% CI = 9.3–14.9). Patients with stage II had a longer OS (23.25 months, 95% CI = 10.4–36.1 months) than stage III (14 months, 95% CI = 12.4–15.7 months) and stage IV (5.1 months, 95% CI = 4.6-5.6 months). There was no significant association between OS and gender (P = 0.652), tumor site (P = 0.312), differentiation (P = 0.477), lymph node metastasis (P = 0.294), *TAZ* protein levels (P = 0.481), or *TAZ* mRNA levels (P = 0.132) (Table 4).

4. Discussion

The Hippo pathway plays an important role in cell proliferation, organ size control, and cancer development and progression. *TAZ* is a transcriptional coactivator that is inhibited by Hippo pathway [22, 23]. Aberrant inactivation of the Hippo pathway and/or overexpression of *TAZ* results

Table 3: The relationship between *TAZ* and clinicopathological characteristics.

Characteristics	*TAZ* protein levels		*P*	*TAZ* mRNA levels	*P*
	low IHC (0~1+)	High IHC (2+~3+)			
Age			0.294		0.374
<60	15 (24.2%)	47 (75.8%)		4.45 ± 4.84	
≥60	27 (32.1%)	57 (67.9%)		3.92 ± 4.93	
Sex			0.376		0.696
Female	8 (22.9%)	27 (77.1%)		4.71 ± 6.44	
Male	34 (30.6%)	77 (69.4%)		3.99 ± 4.32	
Histology			0.001		0.003
Adenocarcinoma	33 (39.8%)	50 (60.2%)		3.15 ± 3.25	
SRCC	9 (14.3%)	54 (85.7%)		6.71 ± 7.02	
Tumor site			0.159		0.069
Distal stomach	13 (26.5%)	36 (73.5%)		5.43 ± 6.02	
Proximal stomach	17 (41.5%)	24 (58.5%)		2.2 ± 2.31	
Whole stomach	11 (22.9%)	37 (77.1%)		4.37 ± 4.82	
Unknown	1 (12.5%)	7 (87.5%)		2.94 ± 1.51	
Lymph node			0.232		0.899
N0-1	8 (40%)	12 (60%)		4.8 ± 6.99	
N2-3	34 (27%)	92 (73%)		4.04 ± 4.45	
Stage			0.785		0.492
II	1 (16.7%)	5 (83.3%)		3.60 ± 2.30	
III	40 (29.4%)	96 (70.6%)		4.24 ± 5.05	
IV	1 (25%)	3 (75%)		2.80 ± 2.59	
Histological grade			0.004		0.375
G2	16 (48.5%)	17 (51.5%)		4.05 ± 5.86	
G3	26 (23%)	87 (77%)		4.19 ± 4.54	

in transcriptional activation of their downstream targets. *TAZ* overexpression induces cell proliferation and epithelial-mesenchymal transition (EMT) and inhibits apoptosis and contact inhibition [24, 25]. EMT is a process in which cells lose epithelial-like characteristics, such as cell-cell adhesion and polarity, and acquire mesenchymal properties that include increased motility. Most carcinomas exhibit a partial EMT, which is thought to promote the formation of cell populations that are enriched in cancer stem cells (CSCs). Cordenonsi et al. [19] found that *TAZ* was required to sustain self-renewal of breast CSCs and to induce their tumorigenic potential. And most interestingly, *TAZ* was overrepresented in poorly differentiated breast tumors compared with well-differentiated ones. *TAZ* protein levels increase during EMT and that this is required for mammosphere formation, which is also promoted by EMT. Bhat et al. [26] found that *TAZ* expression was lower in proneural glioblastomas (GBMs) and lower grade gliomas compared with GBMs that had a mesenchymal phenotype. *TAZ* expression in GBMs is positively correlated with the expression for mesenchymal genes and is also predictive of poor overall survival. Moreover, *TAZ* is significantly associated with poor survival of colon cancer patients in two independent colon cancer datasets, comprising 522 patients [16]. In present study, we successfully detected and compared *TAZ* protein and mRNA expressions in gastric tumor tissues (Table 2) and correlated *TAZ* levels with clinicopathological parameters and survival (Table 3).

We also found that *TAZ* was higher expressed in SRCC than adenocarcinoma in either protein or mRNA levels (Table 3). SRCC has long been thought to have a worse prognosis than other forms of gastric cancer. Recently, SRCC has been known to have different biologic characteristics between early stage and advanced stage gastric cancer. In early gastric cancer, SRCC has been reported to have better prognosis than others because of less lymph node metastasis and a more grossly depressed type, which is helpful for diagnosis. However, in advanced gastric cancer, SRCC has been characterized to be a more grossly infiltrative type, although the reason is still unclear. Few molecular markers had been proven to have relationship with SRCC, such as the M2 isoform of pyruvate kinase (*PKM2*), bone morphogenetic proteins (*BMP-7*), and transcriptional factor forkhead box P3 (*FoxP3*). *PKM2* was identified as a driver of aerobic glycolysis and has been shown to be the isoform preferentially overexpressed in tumor cells. Well and moderately differentiated adenocarcinoma showed significantly higher expression of *PKM2* than SRCC. *PKM2* protein expression was found to negatively correlate with survival in SRCC patients [27]. *BMP-7* is signaling molecule belonging to the transforming growth factor (TGF) superfamily. Recent

Table 4: The median overall survival for patients according to *TAZ* levels.

Characteristics	Number of patients	Median overall survival (months) (95% CI)	*P*
Age			0.029
<60	61 (41.8%)	16.9 (11.9–22.6)	
≥60	85 (58.2%)	12.4 (9.3–14.9)	
Sex			
Female	35 (24.0%)	14.8 (8.3–21.4)	
Male	111 (76.0%)	14.0 (12.6–15.5)	
Tumor site			0.312
Distal stomach	49 (33.6%)	14.1 (10.4–17.9)	
Proximal stomach	40 (27.3%)	13.7 (12.8–14.6)	
Whole stomach	49 (33.6%)	14.1 (9.8–18.3)	
Unknown	8 (5.5%)	6.7 (4.8–8.5)	
Lymph node			0.294
N0~1	20 (13.7%)	18.1 (8.7–27.5)	
N2~3	126 (86.3%)	14.0 (12.2–15.8)	
Stage			0.029
II	6 (4.1%)	23.25 (10.4–36.1)	
III	136 (93.2%)	14.0 (12.4–15.7)	
IV	4 (2.7%)	5.1 (4.6–5.6)	
Histological grade			0.477
G2	33 (22.6%)	12.9 (10.1–15.7)	
G3	113 (77.4%)	14.2 (11.9–16.5)	
TAZ protein expression			0.481
Low expression	42 (28.8%)	13.7 (10.9–16.5)	
High expression	104 (71.2%)	14.1 (11.4–16.7)	
TAZ mRNA expression			0.132
Low expression	29 (35.8%)	8.1 (5.2–10.9)	
Intermediate expression	26 (32.1%)	14.0 (12.6–15.5)	
High expression	26 (32.1%)	9.6 (6.5–12.6)	

studies demonstrated that *BMP-7* expression is found in various human cancers and regulates cell differentiation, proliferation, migration, invasion, and apoptosis [28]. *BMP-7* expression was significantly higher in the differentiated histology group than in the undifferentiated group. And the *BMP-7* positive group had significantly poorer survival than the *BMP-7* negative group in the undifferentiated group. The key role of *FoxP3* is induction of immunesuppressive function to maintain self-tolerance. It is widely accepted that *FoxP3* is expressed not only in mice and humans but also in tumor cells such as melanoma stomach and might have relationship with immunosuppressive effect. Yoshii, et al [29] demonstrated that *FoxP3* was expressed in SRCC. *FoxP3* would allow them to escape from immune surveillance, thereby resulting in cancer progression such as lymph node metastasis. But the molecular pathogenesis of SRCC remains largely unknown. In present study, we find that *TAZ* expression was higher in SRCC than in adenocarcinoma for the first time. We hypothesize that *TAZ* might participate in tumorigenesis and development of signet ring cells. However, future studies were more needed. Our results show the way for future studies aiming to reveal additional insights into the molecular mechanisms of signet ring cell. Since *TAZ* was reported to bind to a variety of transcription factors to control cell differentiation and organ development, such as *p73* (p53 family member), *Runx2* (runt family member 2), *PPAR* γ (peroxisome prolif-erator-activated receptor γ), *TTF-1* (thyroid transcription factor-1), *Pax3* (paired box 3), *Tbx5* (T-box 5), *Smad2/3/4* (SMAD family member 2/3/4), and *TEAD* [8–10]. In present study, the *TAZ* protein is mainly accumulated in the nucleus with a less cytoplasmic presence. *TAZ* might be considered as a novel target for the treatment of gastric cancer, especially in SRCC. However, in the present study, the sample size is rather limited and the distribution between different stages is also scattered, which might be the reason that we did not find any correlations between *TAZ* and stage or prognosis. Further studies with larger number of patients were warranted to valid utility of *TAZ* in gastric cancer patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Author's Contribution

Guofeng Yue and Xia Sun contributed equally to this work.

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Research Article

The Effects of Gene Polymorphisms in Interleukin-4 and Interleukin-6 on the Susceptibility of Rheumatoid Arthritis in a Chinese Population

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Background. Interleukin-4 (IL-4) and interleukin-6 (IL-6) have been reported to associate with pathogenesis of rheumatoid arthritis (RA); however, the role of IL-4 and IL-6 genetic polymorphisms in RA remains unknown. *Method*. A total of 752 unrelated Chinese patients with RA and 798 healthy Chinese volunteers with no family histories of any autoimmune diseases were recruited. The promoter IL-4-590 C/T and IL-6-174 G/C polymorphisms were genotyped. *Result*. The genotype distributions and allele frequencies of IL-4-590 C/T and IL-6-174 G/C polymorphisms in RA patients were significantly different from healthy volunteers. Statistically significant differences were observed in genotypes for IL-4-590 and IL-6-174. The frequencies of both the T allele on the IL-4-590 and the C on the IL-6-174 were significantly increased in RA patients. *Conclusion*. The IL-4-590 and IL-6-174 promoter polymorphisms may be associated with increased risk of RA and could be used as genetic marker for assessing the susceptibility and severity of RA in Chinese.

1. Introduction

Rheumatoid arthritis (RA) is a complex, chronic inflammatory disease that predominantly involves synovial joints, leading to cartilage and bone destruction [1, 2]. Although the etiology of RA remains unknown, numerous genetic factors have been established to contribute as much as 60% to RA susceptibility [1, 3, 4]. Furthermore, the HLA-DR loci were estimated to account for only about one-third of the genetic predisposition to RA [5]. A single-nucleotide polymorphism of ccr6 (rs3093024) was found to be associated with susceptibility to rheumatoid arthritis in Japanese and Taiwanese population [6, 7]. Many cytokine genes were also playing an important role in its pathogenesis [8–12]. Interleukin-4 (IL-4) and interleukin-6 (IL-6) are the two most important cytokine genes associated with RA [4, 13–18]. IL-4 is the first discovered B-cell pleiotropic cytokine that promotes proliferation of T cells and antibodies production of B cells and plays an important role in the immune system [3, 9, 19–21]. IL-6 is a multifunctional B-cell differentiation cytokine which is overexpressed in the affected tissues of RA patients and induces the final maturation of activated B cells into immunoglobulin-secreting plasma cells [8, 11, 22–24]. Therefore, polymorphisms affecting genes of IL-4 and IL-6 can be linked with RA risk and become of great interest to researchers [14, 18, 19]. IL-4-590 promoter polymorphism, a C-to-T base substitution, has been suggested to be associated with RA, especially with early pauciarticular juvenile rheumatoid arthritis [25–28]. Many previous studies examined the association of IL-4 gene polymorphisms with RA [9, 12, 14, 15, 20, 21], but their data are conflicting, so the association of IL-4 gene polymorphisms with RA in Chinese

Table 1: The clinical and demographic characteristics of all subjects.

Variables	Cases (n = 752)	Control (n = 798)
Sex (female/male)	354/398	367/431
Age (years)	52.3 ± 16.3	52.1 ± 17.1
Disease duration (range)	8.2 years (0.2–20.1)	None

could not be deduced and needs further studies. Several polymorphisms have been revealed in the IL-6 gene, including one of the most important single-nucleotide polymorphisms (SNPs) in the promoter, the -174G to C substitution, which affect IL-6 levels and are associated with RA, especially with systemic juvenile chronic arthritis. The association of IL-6-174G/C with RA was studied in many populations, such as Europeans, Turkish, Koreans, and Egyptians; however, besides a very preliminary study in a few Han population in Guangdong, there are not any systematic studies about the association of IL-6-174G/C with RA in Chinese population.

Although the association of IL-4-590 and IL-6-174 gene polymorphisms with RA has been studied by many researchers, its relation with RA in Chinese population remains unknown and could not be deduced. In this study, we enrolled 752 Chinese patients and 798 healthy Chinese volunteers to explore the role of IL-4-590 and IL-6-174 gene polymorphisms in RA.

2. Methods

2.1. Clinical Material. A total of 752 unrelated patients with RA, diagnosed according to the American Criteria of Rheumatology (ACR-2011) classification criteria for rheumatoid arthritis, were recruited from the follow-up and inpatient units. The control group included 798 healthy Chinese subjects with no family histories of any autoimmune diseases. Both RA and control groups were interviewed to obtain demographic data and all of the established risk factors. The clinical and demographic data are presented in Table 1. In the cases group, 354 patients were females and 398 males; the mean age was 52.3 ± 16.3 with a range of 18–76 years; the mean disease duration time was 8.2 with a range of 0.2–20.1 years.

The control group consisted of 798 anonymous healthy Chinese volunteers who did not show any clinical or laboratory signs of autoimmune diseases. They were randomly selected as to match the patients in age, gender, and ethnicity.

2.2. Genetic Analysis. The scientific investigation presented in this paper has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Reaction conditions for genotyping the two polymorphic loci (IL-4-590 and IL-6-174) were performed as follows: genomic DNA was extracted from peripheral venous blood by using the Axygen DNA isolation kit (Axygen, CA) as recommended by the supplier and then stored at −80°C until analyzed; all polymerase chain reaction (PCR) primers were synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China) as references listed in Table 2, and Table 2 shows the primers sequences, annealing temperature, fragment region, and size. All PCRs were carried out in 20 μL of reaction mixture containing 50 ng template DNA, 1 × buffer (Tris-HCl 100 mmol/L, pH 8.3; KCl 500 mmol/L), 0.25 μmol/L primers, 2.0 mmol/L MgCl2, 0.25 mmol/L dNTPs, and 0.5 U Taq polymerase (Invitrogen Corporation, Carlsbad, CA, USA). The PCRs were performed on 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, annealing at 57°C for 30 s and 72°C for 35 s, and a final extension at 72°C for 10 min. All amplified PCR products were preliminarily checked by electrophoresis on 2.0% agarose gel and then observed under UV light. All SNPs of IL-4 and IL-6 promoters were genotyped by PCR-RFLP. Aliquots of 5 μL amplified PCR products were digested with 2 U selected restriction enzymes (MBI Fermentas, St. Leon-Rot, Germany, Table 2) at 37°C for 2 h following the supplier's manual. Digested products were separated by 2.0% agarose gel electrophoresis and observed under UV light. 10% of random samples were reanalyzed by DNA sequencing method (ABI3730xl DNA Analyzer, Applied Biosystems, Foster City, CA, USA) to make sure concordance with the genotyping results from PCR-RFLP. Allele and genotype frequencies were compared by χ^2 analysis.

2.3. Statistical Analyses. The chi-squared (χ^2) test was utilized to evaluate the Hardy-Weinberg equilibrium in genotypic distributions and clinical characteristics between cases and controls. All statistical analyses to evaluate if each SNP was independently associated with RA when adjusted for the potential confounding effects of important clinical variables were performed by using the Statistical Package for Social Sciences software (SPSS, Windows version release 16.0; SPSS Inc.; Chicago, IL, USA). A level of $P < 0.05$ was considered statistically significant.

3. Results

As the demographic and clinical characteristics of all subjects in the study were shown in Table 1, there were no significant differences in sex ratio and age, between RA cases and controls.

The single-nucleotide polymorphism (SNP) was found to be in Hardy-Weinberg equilibrium and the genotype distributions and allele frequencies of IL-4 and IL-6 promoter polymorphisms in RA and control subjects are summarized in Table 3. The genotype frequencies and allele frequencies for both IL-4 and IL-6 promoter polymorphisms are quite significantly different in RA subjects and controls under Hardy-Weinberg equilibrium ($P < 0.001$).

As for the IL-4-590C/T, the frequency of the TT genotype was significantly higher among RA patients (7.05%) compared to controls (2.01%), and the frequency of the CT genotype was also higher among RA patients (29.00% versus 25.94%), but the frequency of the CC genotype was significantly lower among RA patients (63.96%) than controls (72.06%). Accordingly, the T allele frequency was significantly higher in RA patients than controls (21.54% versus 14.97%, $\chi^2 = 22.4713$, $P = 2.1330 \times 10^{-6} < 0.001$). These results showed a significantly increased risk for RA for

Table 2: Primer pairs, PCR-RFLP analysis for IL-4 and IL-6 promoter polymorphisms.

SNP	Primer sequences	Annealing temperature (°C)	Amplification fragment (bp)	Restriction enzyme	Genotype bp	References
IL-4-590 C/T	5′-ACTAGGCCTCACCTGATACG-3′ 5′-GTTGTAATGCAGTCCTCCTG-3′	57	252	*Bsm*FI	CC: 192, 60 CT: 252, 192, 60 TT: 252	[9]
IL-6-174 G/C	5′-GGAGTCACACACTCCACCT-3′ 5′-CTGATTGGAAACCTTATTAAG-3′	57	525	*Hsp*92II	GG: 327, 169 GC: 327, 169, 122 CC: 327, 122	[23]

Table 3: The genotype and allele frequencies of IL-4 and IL-6 promoter polymorphisms in cases and controls.

	Genotype frequencies (%)			Allele frequencies (%)	
C > T, IL-4-590	CC	CT	TT	C	T
Cases (n = 752)	481 (63.96)	218 (29.00)	53 (7.05)	1180 (78.46)	324 (21.54)
Controls (n = 798)	575 (72.06)	207 (25.94)	16 (2.01)	1357 (85.03)	239 (14.97)
	$\chi^2 = 27.1515$, $P = 1.2610 \times 10^{-6}$			$\chi^2 = 22.4713$, $P = 2.1330 \times 10^{-6}$, OR = 0.6414	
G > C, IL-6-174	GG	GC	CC	G	C
Cases (n = 752)	613 (81.52)	124 (16.49)	15 (2.00)	1350 (89.76)	154 (10.24)
Controls (n = 798)	786 (98.50)	10 (1.25)	2 (0.25)	1582 (99.12)	14 (0.88)
	$\chi^2 = 127.0661$, $P = 2.5582 \times 10^{-28}$			$\chi^2 = 132.4104$, $P = 1.2168 \times 10^{-30}$, OR = 0.0776	

the TT genotype and the T allele after adjustment with sex, age, BMI, smoke status, and history of heavy labor work.

As for the IL-6-174G/C, the frequencies of the GG, GC, and CC genotypes were 81.52%, 16.49%, and 2.00% in RA patients, significantly different from those observed in controls, which were determined to be 98.50%, 1.25%, and 0.25%, respectively ($\chi^2 = 127.0661$, $P = 2.5582 \times 10^{-28} < 0.001$). Accordingly, the allelic frequencies in the patients and controls were also significantly different for G allele (89.76% versus 99.12%) and C allele (10.24% versus 0.88%), respectively ($\chi^2 = 132.4104$, $P = 1.2168 \times 10^{-30} < 0.001$). These results also showed a significantly increased risk for RA for the CC genotype and the C allele after adjustment with sex, age, BMI, smoke status, and history of heavy labor work.

4. Discussion

Rheumatoid arthritis (RA) is a common chronic autoimmune disorder characterized by the destruction of articular cartilage and bone, which affects millions of patients worldwide. In this study, we investigated whether IL-4 and IL-6 promoter polymorphisms influence the susceptibility of RA in a Chinese population. Our results showed that the TT genotype carriers had markedly higher risk for RA compared with CC genotype carriers for IL-4 promoter polymorphisms, and the CC genotype carriers had markedly higher risk for RA compared with GG genotype carriers for IL-6 promoter polymorphisms; besides, the T allele of IL-4 promoter polymorphisms and the C allele of IL-6 promoter polymorphisms had shown an association with susceptibility of RA in a Chinese population.

IL-4 is a potent anti-inflammatory cytokine, produced by activated CD4+ lymphocytes, mast cells, and basophils and exerts an important role in the immune system on different cell types [27, 29–33]. In humans the IL-4 gene has been mapped to chromosome 14q32 [34]. The IL-4 gene promoter contains a number of polymorphic loci, which were reported to influence the susceptibility of many diseases, including the IL-4-33C/T [35], IL-4-589C/T [36], and IL-4-590C/T [20, 37, 38]; especially, the genotype and allele frequencies of IL-4-590C/T were well studied and reported to be associated with many diseases, such as rheumatoid arthritis [3, 20], liver disease [37], and gastric cancer [38]. To our surprise, although the role of the genotype and allele frequencies of IL-4-590C/T in association with rheumatoid arthritis has been documented, we did not find any reports with regard to the genetic polymorphisms of IL-4-590C/T with rheumatoid arthritis in Chinese population. In this study, we firstly reported the role of genetic polymorphisms of IL-4 promoter in RA in Chinese population. We found that IL-4-590C/T polymorphisms are associated with the RA risk, and the T allele of IL-4 promoter polymorphisms has significantly increased the susceptibility of RA in Chinese population. This finding suggests that the IL-4-590C/T polymorphisms may be used as a genetic marker for the onset and development of RA in Chinese population.

IL-6 is another multifunctional B-cell differentiation cytokine, which also plays important role in inducing the final maturation of activated B cells into immunoglobulin-secreting plasma cells and influencing the susceptibility of RA [7, 8, 11, 22–24], dermatomyositis and systemic lupus erythematosus [39], liver cirrhosis and hepatocellular carcinoma [40], diabetic microvascular complications [41], coronary heart disease [42], acute appendicitis [43], and so on. Although the association of IL-6-174 G/C with RA was well studied in many populations, such as Europeans, Turkish,

Koreans, and Egyptians, besides a very preliminary study in a few Han population in Guangdong, studies about the association of IL-6-174 G/C with RA in Chinese population do not be reported. In this study, we firstly systematically studied the role of genetic polymorphisms of IL-6 promoter in RA in Chinese population. We found that IL-6-174G/C polymorphisms are also associated with the RA risk, and the C allele of IL-6 promoter polymorphisms has dramatically increased the susceptibility of RA in Chinese population. This finding suggests that, besides the IL-4-590C/T, the IL-6-174C/T polymorphisms may also be used as another genetic marker for the onset and development of RA in Chinese population.

Although our study suggests that the genotype and allele frequencies of IL-4-590C/T and the IL-6-174C/T polymorphisms are associated with the susceptibility of RA in a Chinese population, to be honest, it is also a preliminary study, and the results need to be further confirmed in an ideally larger-scale study.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xiang Li and Wei Chai contributed equally to this paper and should be considered as cofirst authors.

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Research Article

Low Dose of Valproate Improves Motor Function after Traumatic Brain Injury

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Background. Traumatic brain injuries (TBIs) are a major health care problem worldwide. Approximately 1.5 million new TBI cases occur annually in the United States, with mortality rates ranging between 35% and 40% in severe patients. Despite the incidence of these injuries and their substantial socioeconomic implications, no specific pharmacological intervention is available for clinical use. Several studies have indicated that 300 mg/kg or 400 mg/kg of valproate (VPA) exhibits neuroprotective effects in animal models. However, humans cannot tolerate high doses of VPA. This study aims to investigate whether 30 mg/kg of VPA administered to rats affects TBIs. *Methods.* We used a rat model to test the effects of 30 mg/kg of VPA on TBIs. Molecular identifications for histone acetylation and phosphorylation of cAMP response element-binding protein (CREB) and phosphorylated extracellular signal regulated kinase (ERK) were performed. *Results.* The results indicated that treating adult rats with VPA after TBIs significantly decreased the contusion volume and recovery of contusion-related skilled forelimb reaching deficits. Applying VPA also increased histone acetylation, p-ERK, and p-CREB expression in the brain. Furthermore, applying VPA reduced inflammation, glial fibrillary acidic protein activation, and apoptosis. *Conclusion.* This study found that 30 mg/kg of VPA assists in treating TBIs in rat models.

1. Introduction

Traumatic brain injury (TBI) is a major clinical problem that causes substantial mortality rates and a broad spectrum of mental disorders. These complex pathological conditions are characterized by blood brain barrier (BBB) leakage, excessive release of excitatory neurotransmitters, axonal and dendritic disruptions, neuroinflammation, and cell death [1–4]. Given the complexity of brain responses to trauma and the lack of an ideal drug for treating TBIs, the National Institutes of Health TBI working group recommended either a combination of therapies or evaluating agents that act on multiple mechanisms as TBI treatment options [5].

Valproate [2-propylpentanoic acid] (VPA) is one of the most commonly used antiepileptic medications. It has been shown to reduce the neuronal damage associated with epileptic activity. After a TBI, VPA has been shown to be effective in treating posttraumatic seizures [6]. The antiepileptic activity of VPA results from a combination of its influences on several targets in the central nervous system, including inhibiting gamma amino butyric acid transamination, reducing N-methyl D-aspartate excitotoxic amino acid (NMDA)-mediated neuronal excitation, inhibiting histone deacetylases (HDACs) and glycogen synthase kinase (GSK)-3, and blocking voltage-gated sodium and T-type calcium channels [7]. Studies have found that 300 mg/kg or 400 mg/kg

of VPA exhibits neuroprotective effects in animals [8–10]. However, humans cannot tolerate such large doses, which might be toxic [11]. In this study, we administered 30 mg/kg of VPA to rats to test whether this dose is effective in treating TBIs, given that 30 mg/kg of VPA is acceptable for clinical practice.

2. Methods

2.1. Materials. The experimental procedures used in this study conformed to the guidelines approved by the institutional animal care committee at Wan Fang Hospital. Adult male Sprague-Dawley rats (250 to 300 g) were obtained from BioLASCO Taiwan Co., Ltd. The rats were randomly divided into 3 groups: (1) the TBI + VPA (VPA-treated TBI) group, (2) the TBI (vehicle-treated TBI) group, and (3) the sham (sham-operated control) group. They were housed in a temperature-controlled animal room (24°C to 25°C) and exposed to a 12 h light-dark cycle. Standard laboratory rat chow and tap water were available ad libitum. Acetylated histone H2A, acetylated histone H2B, and acetylated histone H3 antibodies were obtained from Millipore (Billerica, MA, USA). Phospho-ERK42/44 and phospho-CREB antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-beta actin and glial fibrillary acidic protein (GFAP) antibodies were obtained from Sigma-Aldrich Biotechnology (Saint Louis, MO, USA). Apoptosis detection kits, including a terminal deoxynucleotidyl transferase-mediated-UTP-biotin nick end labeling (TUNEL) assay and 4′,6-diamidino-2-phenylindole (DAPI) staining kits, were purchased from Oncogene Research Products (Boston, MA, USA).

2.2. Controlled Cortical Impact TBI Model. Surgical anesthesia was induced by intraperitoneally (IP) administering ketamine (90 mg/kg) and xylazine (10 mg/kg). After anesthesia, the animals were secured in a stereotaxic frame and mechanically ventilated. A cortical contusion was produced on the exposed cortex by using a controlled impactor device, the TBI-0200 TBI Model system (Precision Systems and Instrumentation). The scalp and epicranial aponeuroses were retracted, and a 3 mm diameter circular craniotomy was performed with a burr drill, lateral to the midsagittal suture (contralateral to the preferred limb), with its center at the following coordinates: AP = 1 mm and ML = ±2.5 mm from the bregma. The impacting shaft was extended, and the impact tip was centered and lowered over the craniotomy site until it touched the dura mater. The rod was then retracted and the impact tip was advanced to produce a brain injury of moderate severity to a rat (tip diameter, 3 mm; cortical contusion depth, 2 mm; impact velocity, 4 m/s).

2.3. Drug Preparation, Administration, and Grouping. VPA (Depakine lyophilized injection, Sanofi-Aventis) was dissolved in 0.9% sterile saline at a concentration of 100 mg/mL (based on the salt weight). Animals received either an IP VPA injection of 30 mg/kg/d or a vehicle (0.9% sterile saline) 30 min after the TBI from day 0 to day 6. Skilled forelimb reaching tests (n = 8 per group), contusion volume measurements (n = 5 per group), and immunohistochemistry studies (n = 3 per group) were then conducted. Some animals received either a single IP VPA injection of 100 mg/kg, 200 mg/kg, or 400 mg/kg or a vehicle (0.9% sterile saline) 30 min after TBI to perform western blot analyses (n = 3 per group).

2.4. Training or Skilled Forelimb Reaching Test. Animals were trained to criterion in the skilled forelimb reaching task and their performances were assessed. The rats were randomly allocated to 3 groups (n = 8 per group): (1) the TBI + VPA (VPA-treated TBI) group, (2) the TBI (vehicle-treated TBI) group, and (3) the sham (sham-operated control) group. Each animal was tested to assess their skilled forelimb reaching task performance on the first postoperative day and then daily (Monday to Friday) for 6 weeks. On days when VPA or the vehicle was administered (i.e., days 0 to 6), behavior testing was performed before drug injection. Skilled forelimb reaching was tested as described by [12]. Before surgery (contralateral to the preferred limb craniotomy), the baseline performance (defined as the average of the last 3 preoperative testing sessions) of each rat was established. Success was defined as an animal grasping the pellet on its first attempt and placing it into its mouth (this is termed "first reach success"). Each testing session consisted of 20 reaching opportunities, using the preferred forelimb. Attempts using the nonpreferred forelimb were not included in the analyses. The preoperative criterion was at least 16 successes in 20 attempts for 3 consecutive days. A maximal time limit of 5 min per testing session was set.

2.5. Triphenyltetrazolium Chloride Staining. 2,3,5-triphenyltetrazolium chloride (TCC) staining was used to assess the lesion size by comparing various neuronal tissue viabilities. The excised brain was sliced into 2 mm thick sections and incubated in a 1% TTC solution for 30 min at 37°C. In viable neuronal tissue, dehydrogenase enzymes converted TTC into a red pigment that stained the tissue dark red.

2.6. Contusion Volume Measurement. To describe the entire contusion volume for further analysis, the contusion volume areas were specifically analyzed. Images of all sections were captured using a digital camera (Nikon Coolpix 990) and analyzed using ImageJ software (public domain program developed at the National Institutes of Health, Bethesda, MD, USA).

2.7. Western Blotting. Rats received either a single IP VPA injection of 30 mg/kg, 100 mg/kg, 200 mg/kg, or 400 mg/kg or a vehicle (0.9% sterile saline) 30 min after the TBI (n = 3 per group). Ipsilateral frontal cortical segments were obtained 24 h after the VPA or vehicle injection and homogenized. Brain tissue extracts were sonicated (five pulses per second) by using a Sonics Vibra-Cell sonicator (Sonics & Materials, Inc., Newtown, CT, USA) and a 0.4 mm diameter probe. The amount of protein in each sample was determined using a

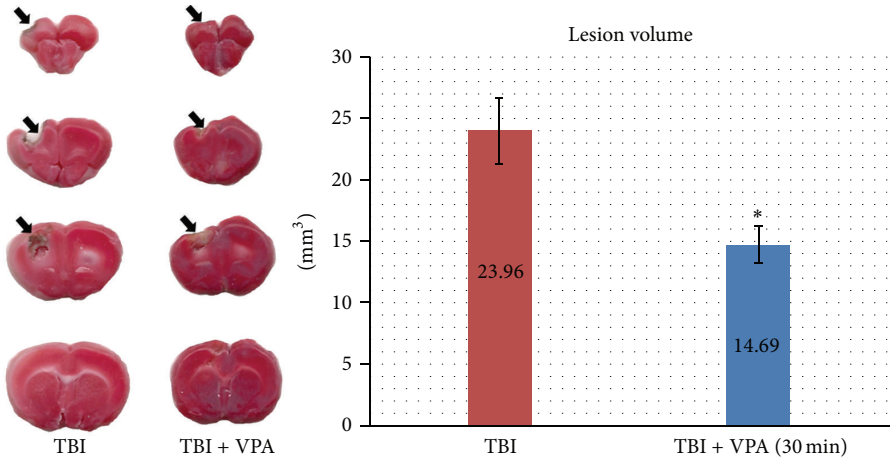

Figure 1: Postinjury administration of 30 mg/kg/day of VPA for 7 days reduces contusion volume. Representative photographs of the brains of a vehicle- and VPA-treated animal. Animals were killed 7 days after injury. Quantification of the volume of cortical contusion revealed that VPA significantly reduced brain contusion volume (mean: 23.96 versus 14.19 mm^3). Rostral-caudal extent of the damage detected in the injured cortex from the vehicle- and VPA-treated animals. (Data are presented as the mean ± SEM, significant difference by 1-way ANOVA. $^*P = .010$, $n = 5$ per group.)

Bradford assay with bovine serum albumin as the standard. Equal amounts of protein were loaded, electrophoresed, and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA) by using the NOVEX X-Cell II system (Invitrogen, Burlingame, CA, USA). Membranes were then washed and incubated with antibodies at room temperature. A chemiluminescence system was used to detect immunoreactivity.

2.8. Immunohistochemistry. To examine the effects of VPA after a TBI, animals were operated on and their brains were sectioned for post-TBI histological evaluation ($n = 3$ per group). Three or 7 days after the TBI, rats were sacrificed for H&E staining, immunocytochemical GFAP analyses, or TUNEL staining. Animals were deeply anesthetized with an IP injection of 150 mg/kg of pentobarbital and perfused through the left ventricle with phosphate-buffered saline, followed by cold 4% paraformaldehyde in 0.15 M sodium phosphate buffer, pH 7.4. The brains were immediately removed, postfixed for 8 h in the same fixative at 4°C, and cryoprotected for 2 to 3 days in 15% and 30% sucrose. The brains were frozen in powdered dry ice and stored at −80°C until required and 30 μm coronal sections were then cut using a freezing, sliding microtome. The sections were prepared for either immunostaining or apoptosis staining.

2.9. Statistics. A one-way ANOVA was used to analyze brain contusion lesion size. Skilled forelimb reaching was independently analyzed using a repeated-measures one-way ANOVA with Tukey post hoc analysis for the treatment effect. Data were considered significant at $P < .05$ and are presented as the mean ± the standard error of the mean (SEM).

3. Results

3.1. VPA Administered after a TBI Reduces Brain Contusion Volume. We investigated continuous IP treatment with 30 mg/kg/d of VPA that began 30 min after the TBI and lasted for 7 days. Seven days after the TBI, VPA and vehicle-injected animals were euthanized and their brains were removed to determine the contusion volume. As shown in Figure 1, the animals treated with 30 mg/kg/d of VPA for 7 days exhibited less cortical tissue loss than the vehicle-treated animals did. Quantification of this tissue loss revealed that 30 mg/kg/d of VPA produced significantly less tissue loss than vehicle injections did ($P = .01$; Figure 1).

3.2. VPA Improves Skilled Motor Function after a TBI. Skilled forelimb reaching, which requires fine digit movement and intact motor and sensory neural pathways, was analyzed using the single pellet retrieval task [13]. Before TBI surgery, animals in all groups showed good skilled reaching without significant difference in performance. Three days after the TBI, all animals exhibited significant deficits in obtaining pellets with the TBI-impaired limb (Figure 2). However, animals that received 30 mg/kg/d of VPA for 7 days began to increase their pellet reaching success rate 7 days after the TBI and showed significant differences to the TBI with vehicle-only animals 14 to 28 days after treatment (14 d, $P = .013$; 21 d, $P = .005$; 28 d, $P = .005$; Figure 2). A repeated-measures one-way ANOVA revealed that a significant treatment effect occurred ($P = .038$) after 6 weeks of testing. The results demonstrated that postinjury VPA treatment improves skilled motor function after TBIs.

3.3. Systemic VPA Administration Increases H3 Histone Acetylation, p-ERK, and p-CREB in the Brain. VPA is an HDAC

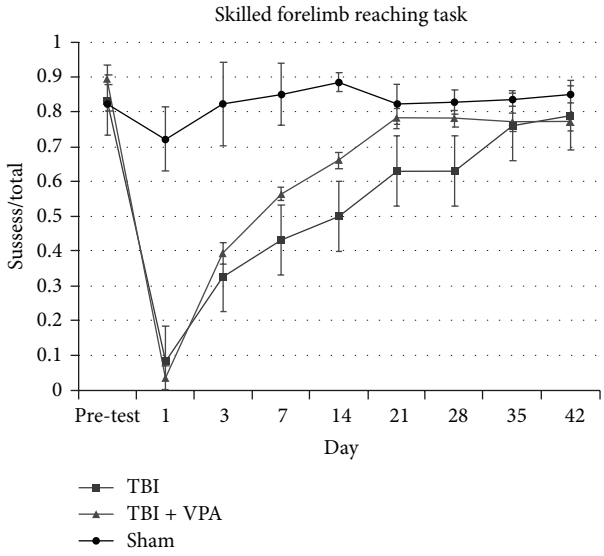

Figure 2: Postinjury treatment of VPA improves skilled forelimb reaching task. All groups had a baseline performance without a significant difference. After TBI, the vehicle- and VPA-treated groups showed marked deficits in successfully obtaining pellets with the TBI-impaired limb, with no significant difference between groups until 7 days after injury. Animals that received VPA treatment, 30 mg/kg/day for 7 days began to exhibit improvements in the pellet reaching success rate at 7 days after TBI and showed a significant difference starting from 14 to 28 days after treatment, compared to TBI with vehicle-only animals (14 days, $P = .013$; 21 days, $P = .005$; 28 days, $P = .005$). Data are presented as the mean ± SEM, significant difference by 1-way ANOVA with Tukey post hoc analysis.

inhibitor and therefore preserves the acetylation of histones [10]. To examine the relationship between systemic VPA administration and histone acetylation in the brain, western blots were performed with acetyl-H2A, acetyl-H2B, and acetyl-H3 histone antibodies. Brain protein extracts were prepared for the western blots. The representative western blots (Figure 3(c)) indicated that 200 mg/kg ($P = .0087$) and 400 mg/kg ($P = .006$) of VPA significantly increased histone H3 acetylation 1 day after VPA treatment, compared with the vehicle-treated controls. No significant changes were detected when the levels of acetyl-H2A and acetyl-H2B histone antibodies were evaluated (Figures 3(a) and 3(b)).

As well as inhibiting HDAC, VPA activates the ERK and CREB pathways [7, 14]. To examine this effect of VPA administration, the p-ERK and p-CREB levels were evaluated for 4 VPA doses (30 mg/kg, 100 mg/kg, 200 mg/kg, and 400 mg/kg). A significant increase in phospho-ERK expression occurred 1 day after the 100 mg/kg VPA treatment, compared with the vehicle-treated controls ($P = .0488$; Figure 3(d)). The 200 mg/kg ($P < .0001$) and 400 mg/kg ($P < .0001$) doses resulted in increased p-CREB expression 1 day after VPA treatment, compared with the vehicle-treated controls (Figure 3(e)). These results demonstrated that systemic VPA administration increases H3 histone acetylation and p-ERK and p-CREB expression in the brain.

3.4. VPA Administered after a TBI Reduces Inflammation, GFAP Expression, and Apoptosis. Figure 4 shows the H&E stain of the pathological change that occurred 7 days after a TBI. The figure shows that 30 mg/kg/d of VPA treatment for 7 days reduced inflammatory cells to a greater extent than the vehicle did. Astrocyte activation contributes to the inflammatory response after a TBI. We investigated the expression of GFAP—a marker of activated astrocytes and potential TBI severity grading. Strong GFAP immunostaining occurred 7 days after the TBI, but the VPA-treated group exhibited significantly weaker activations (Figure 5, $P = .005$, $n = 3$ per group). These results indicated that postinjury treatment with VPA (30 mg/kg/d for 7 d) attenuated the activation of astrocytes and inflammation after the TBI. Additionally, after 7 days of 30 mg/kg/d of VPA treatment, the number of apoptotic cells significantly decreased (Figure 6, $P = .0072$, $n = 3$ per group).

4. Discussion

TBI is a major health care problem worldwide. Approximately 1.5 million new cases occur annually in the United States, with mortality rates ranging from 35% to 40% in severe patients [15]. Therefore, identifying new therapeutic methods that can be used to treat TBI is essential. VPA is a simple branched-chain fatty acid with well-established efficacy for seizures [16]. It is also commonly prescribed for bipolar disorder, acute mania, and migraines [7]. The therapeutic concentration of VPA is 40 to 100 mg/mL. This therapeutic concentration is achieved using a loading dose (as low as 10 mg/kg) followed by maintenance doses (as high as 60 mg/kg). Studies have reported the HDAC inhibitory effects of 300 mg/kg or 400 mg/kg of VPA in animals [8–10]. Postinjury administration of VPA can decrease BBB permeability, reduce neural damage, and improve neurobehavioral outcomes [9]. A 400 mg/kg IP dose of VPA increased histone acetylation and reduced the activity of GSK-3 in the hippocampus [9]. When 400 mg/kg of VPA was administered 30 min after injury, it improved BBB permeability. The same dose also reduced cortical contusion and hippocampal dendritic damage and improved motor function and spatial memory. Consistent with this, HDAC inhibitors can augment memory and synaptic plasticity and promote neuronal outgrowth [17].

Although 400 mg/kg of VPA is useful in a TBI rodent model, this dose is too high for humans. The teratogenicity of VPA also limits its use in women of childbearing age. In this study, we reduced the dose to 30 mg/kg of VPA and tested its effects in rats. The results indicated that treatment with 30 mg/kg of VPA in adult rats with TBIs significantly reduced the contusion volume and the skilled forelimb reaching contusion-related deficit. Histone H3 acetylation and p-ERK and p-CREB expression were also induced in the brain with single injection of VPA at different dosages. The results reflect

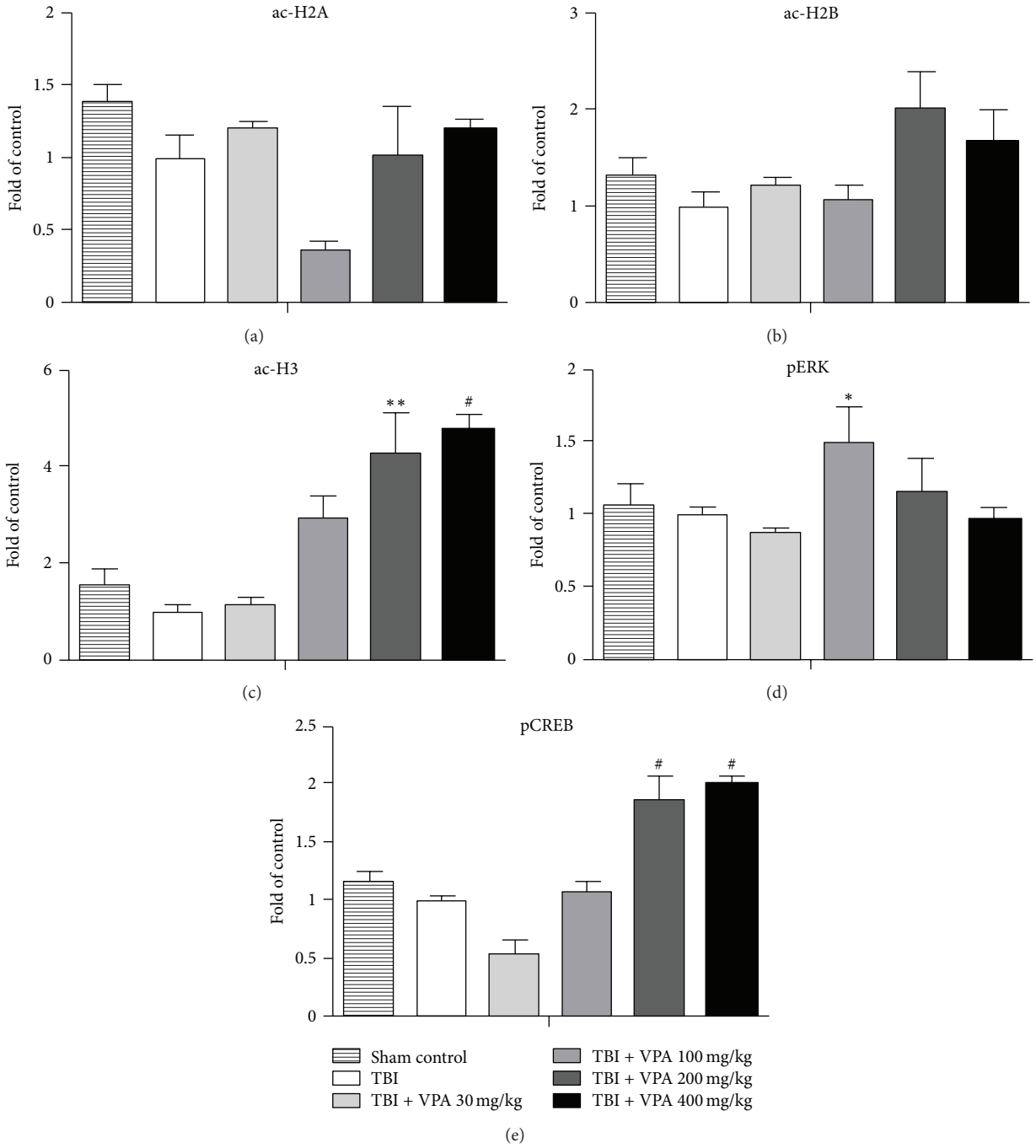

Figure 3: Systemic VPA administration increases H3 histone acetylation, p-ERK, and p-CREB in brain. The statistical analysis of the quantification of western blots (c) showed that systemic VPA administration ($n = 3$ per group) significantly increases histone H3 acetylation with the dosage of 200 mg/Kg ($P = .0087$) and 400 mg/Kg ($P = .006$) 1 day after treatment compared to the vehicle-treated controls. No significant changes were detected when the levels of acetyl-H2A and acetyl-H2B histone antibodies were evaluated (a and b). Significant increase of p-ERK expression was noted 1 day after 100 mg/kg VPA treatment compared to vehicle-treated controls (d), ($P = .0488$). Also increased expression of p-CREB was noted with the dosage of 200 mg/Kg ($P < .0001$) and 400 mg/Kg ($P < .0001$) 1 day after VPA treatment compared to the vehicle-treated controls (e). These results demonstrate that systemic VPA administration increases H3 histone acetylation, p-ERK, and p-CREB expressions in the brain.

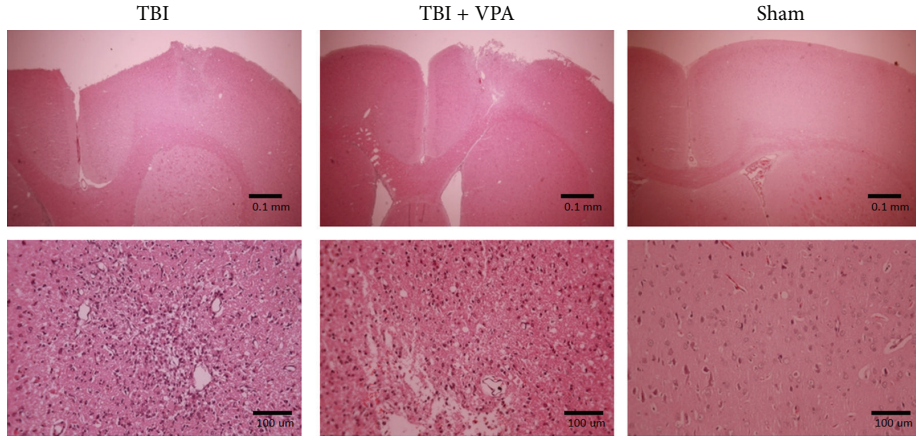

Figure 4: VPA administered after TBI reduces inflammation. H&E staining shows pathological change 7 days after TBI; VPA treatment (30 mg/kg/day for 7 days) was able to reduce the number of inflammatory cells compared to the vehicle alone.

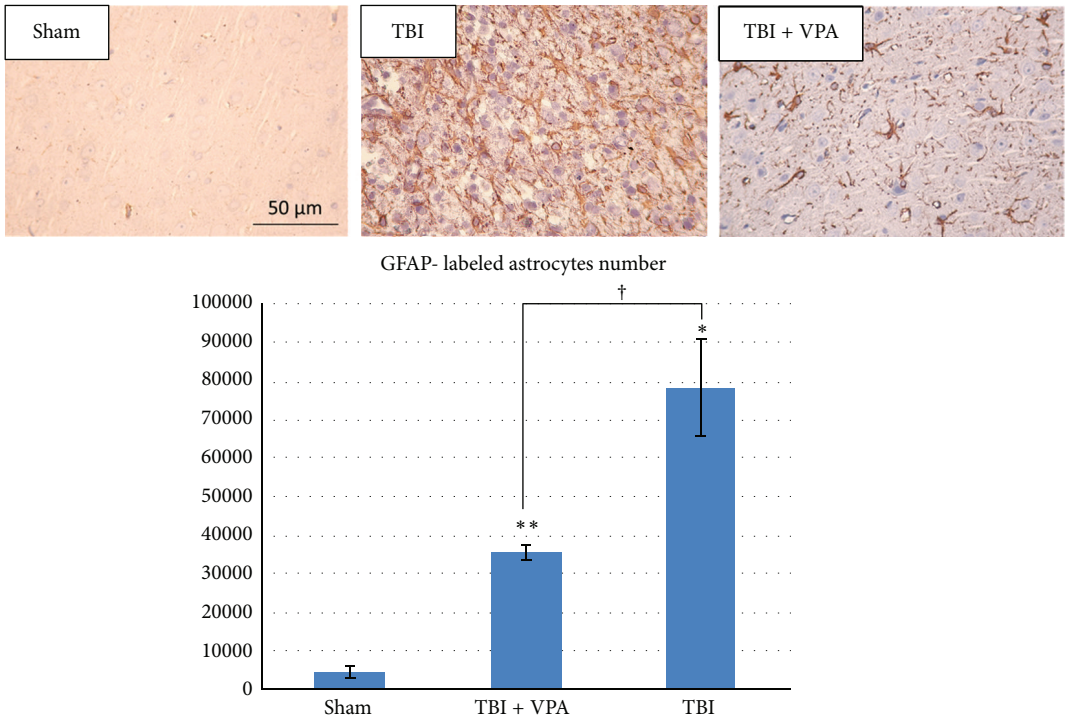

Figure 5: Postinjury administration of 30 mg/kg/day VPA for 7 days reduces GFAP expression. Strong immunostaining of GFAP was demonstrated 7 days after TBI, but these activations were significantly attenuated in the VPA-treated group. (Data are presented as the mean ± SEM, significant difference by 1-way ANOVA followed by Dunnett's post hoc test. $^{\dagger}P = .005$, $n = 3$ per group.)

some of the potential effects of using 30 mg/kg/d of VPA for 7 days in the treatment of TBI.

A recent behavioral study showed that the release, collection, and manipulation hand-shaping movements involved in skilled reaching are similar in rats and humans [18]. Because hand movement plays a critical role in the quality of life of TBI patients, we conducted fine motion evaluations of skilled forelimb reaching before and after TBIs. Once the preferred reaching limb was determined, a CCI injury was performed on the contralateral motor cortex. In our study, VPA treatment reduced the contusion volume of the injured brain and improved skilled reaching motions from 14 to 28 days after a TBI. VPA might offer protection from TBIs by increasing histone acetylation and enhancing the expression of genes involved in neuronal plasticity and survival. Consistent with this, Shein et al. showed that acute treatment of TBI mice with HDAC inhibitor ITF2357 reduced contusion volume and improved motor function [19]. Zhang et al. indicated that DMA-PB (a novel HDAC inhibitor) attenuated the TBI-associated decrease in histone acetylation and reduced microglia-mediated inflammation [20]. VPA can also elicit neuronal growth by activating p-ERK [21]. Dash et al.

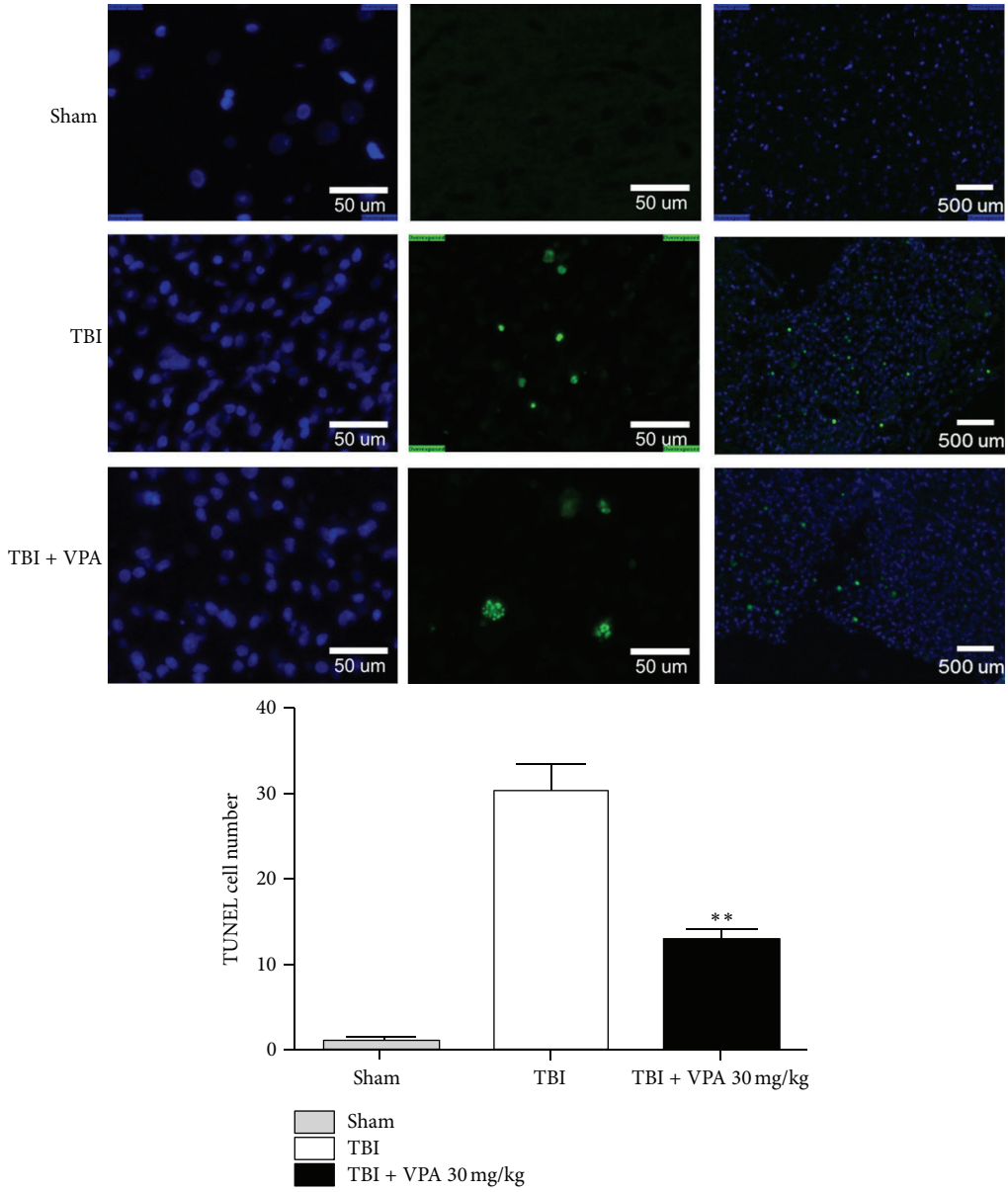

Figure 6: VPA attenuates apoptosis after TBI. TUNEL-positive cells with green fluorescence were easily demonstrated in the TBI group. After VPA treatment (30 mg/kg/day for 7 days), the apoptotic cells significantly decreased. (Data are presented as the mean ± SEM, significant difference by 1-way ANOVA followed by Dunnet's post hoc test. $^{**}P = .0072$, $n = 3$ per group.)

proved that activating p-ERK after a TBI is neuroprotective. Inhibition of p-ERK exacerbates TBI-associated motor and cognitive deficits [22]. Our findings are consistent with the results of other studies [20–22] and we hypothesize that VPA plays a critical role in the neuroprotective mechanism.

The generalizability of this study is limited, because extrapolating conclusions from experiments on animal models to humans requires safety and efficacy validation. Although VPA exhibits a potential neuroprotective effect, its molecular mechanisms remain unclear. Our data indicated that 30 mg/kg of VPA treatments in rats can reduce TBI-mediated inflammation and apoptosis. However, we did not measure the kinetics of VPA in rats. Furthermore, the animal dose used (30 mg/kg) cannot be extrapolated to a human-equivalent dose. Therefore, more pharmacokinetic studies of VPA should be conducted.

In conclusion, this study identified VPA as a rational therapeutic choice for drugs aimed at treating TBI.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yu-Ting Tai and Wen-Yuan Lee contributed equally to this work.

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Research Article

Multiclass Prediction with Partial Least Square Regression for Gene Expression Data: Applications in Breast Cancer Intrinsic Taxonomy

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Multiclass prediction remains an obstacle for high-throughput data analysis such as microarray gene expression profiles. Despite recent advancements in machine learning and bioinformatics, most classification tools were limited to the applications of binary responses. Our aim was to apply partial least square (PLS) regression for breast cancer intrinsic taxonomy, of which five distinct molecular subtypes were identified. The PAM50 signature genes were used as predictive variables in PLS analysis, and the latent gene component scores were used in binary logistic regression for each molecular subtype. The 139 prototypical arrays for PAM50 development were used as training dataset, and three independent microarray studies with Han Chinese origin were used for independent validation ($n = 535$). The agreement between PAM50 centroid-based single sample prediction (SSP) and PLS-regression was excellent (weighted Kappa: 0.988) within the training samples, but deteriorated substantially in independent samples, which could attribute to much more unclassified samples by PLS-regression. If these unclassified samples were removed, the agreement between PAM50 SSP and PLS-regression improved enormously (weighted Kappa: 0.829 as opposed to 0.541 when unclassified samples were analyzed). Our study ascertained the feasibility of PLS-regression in multi-class prediction, and distinct clinical presentations and prognostic discrepancies were observed across breast cancer molecular subtypes.

1. Introduction

Multi-class prediction remains a challenge for high-throughput bioinformatics such as analysis of microarray gene expression data. Numerous machine learning algorithms are readily available for high-throughput data analysis, most of which, however, are limited to scenarios of the classification or prediction with only two classes. This difficulty arises not only from the vast data amount produced by high-throughput microarray or sequencing experiments but from the highly-correlated and nonstochastic nature of genetic/gene expression data. For real-world applications, dichotomous classifications between cancer/normal, alive/dead, and responsive/resistant status are mostly encountered, and many machine learning algorithms and bioinformatics tools perform quite well with sufficient discriminative power [1–3].

One way to tackle the n (experimental samples) $< p$ (genomic/gene expression features) problem inherited in high-throughput microarray or sequencing techniques is to

reduce the high-dimensional data using gene component analysis [4–7]. Gene components, which are synthesized latent factors, and orthogonal transformations of original high-throughput data are interpreted as the projection of high dimensional vector space into a few gene component axes, and the number of gene component (p') is no longer larger than sample numbers (n), facilitating the usage of classical statistical tools.

In previous work we demonstrated that gene component analysis could discriminate estrogen receptor (ER) positive and negative breast cancers and gene component classifiers could be projected into independent samples with high predictive accuracy, as well as an integrated step of automatic gene selection [8]. We also concluded that principle component (PC) regression was more suitable for unsupervised class discovery while partial least square (PLS) was more efficient in supervised class prediction.

The aim of the study was to apply PLS-regression for breast cancer intrinsic taxonomy, of which five distinct molecular subtypes were identified from microarray experiments. Here we extended the applications of PLS-regression from two-class (ER positive versus ER negative and Luminal-A versus Luminal-B subtype) into multiclass prediction of the full spectrum of breast cancer intrinsic taxonomy [9]. We hypothesized that PLS-regression could be an alternative and efficient classification algorithm for breast cancer microarray experiments pertaining intrinsic signature genes.

2. Materials and Methods

2.1. Breast Cancer Intrinsic Taxonomy. In the past decade, microarray experiments have redefined breast cancers as heterogeneous diseases in terms of molecular aberrations, and a number of taxonomic classifications based on gene expression profiles that have been reported have shown some prognostic significance. One such molecular taxonomy is the "intrinsic subtype" proposed by the Stanford/UNC group. Perou identified 476 intrinsic genes from 65 patients with breast cancers and normal individuals; four subclasses: basal-like, Erb-B2+, normal breast-like, and luminal epithelial/ER+ were revealed by class discovery through clustering analysis [10, 11]. The luminal subtype was further divided into luminal-A and luminal-B, and distant metastases were strongly associated with the expression patterns of intrinsic genes [12]. Independent studies supporting the existence of breast cancer intrinsic subtypes followed [13, 14]. By definition, intrinsic genes were those genes that show the highest variation across different subjects and show the least variation within each individual (i.e., pre-/post-chemotherapy changes) [12]. The latest version of intrinsic signature, prediction analysis of microarray 50 gene set (PAM50), was supposed to provide prognostic and predictive values independent of traditional prognostic factors such as hormone receptor, human epidermal growth factor receptor 2 (HER2) status, or proliferation markers [15].

The PAM50 intrinsic signature genes that defined 5 molecular subtypes (luminal-A, luminal-B, normal breast-like, HER2-enriched, and basal-like) were retrieved. The expression values of training samples deriving intrinsic signatures were downloaded from UNC Microarray Database (https://genome.unc.edu/). Centroids were the mean expression values of intrinsic genes corresponding to each molecular subtype. The prototypes included 12 normal breast-like, 57 basal-like, 35 HER2-enriched, 23 luminal-A, and 12 luminal-B tumors.

2.2. PLS-Regression Classifier. Following identification of intrinsic genes, PLS was used for dimension reduction and latent X-factors (gene components) construction. The troublesome n (sample size) $< p$ (gene expression predictors) problem became tractable since a much smaller p' (gene component) was used instead of original microarray gene expression features. At the same time model over-fitting and collinearity of original p genes was avoided due to the limited number of gene components (p') used in classification algorithm and the uncorrelated nature between successive latent factors.

All gene component regressions were essentially the linear transformations of original gene expression values and could be viewed as the projection of high dimensional predictor space into a few orthogonal latent factor axes. PLS maximized the covariance between the predictor and response variables. In matrix algebra, let X_0 and Y_0 be centered and scaled matrix of predictive and responsive variables; one dummy variable Y indicating clinical phenotype was needed for binary classifications in PLS. PLS maximized $X_0'Y_0$ for latent factor construction. PLS predicted X_0 (and Y_0) with the following formula:

$$\begin{aligned} X_0 &= tp', \quad \text{where } p' = (t't)^{-1}t'X_0, \\ Y_0 &= uc', \quad \text{where } c' = (u'u)^{-1}u'Y_0. \end{aligned} \tag{1}$$

The x-scores ($t = X_0w$) and y-scores ($u = Y_0q$) were derived to meet the criteria of maximal covariance of $t'u$ where w and q were associated weighted vectors. The vectors p and c were x- and y-loadings, respectively. It should be noticed that all latent factor extractions were under orthogonal constraints; successive latent factors (gene components) were linearly independent to each other, and usually the corresponding eigen-vectors were normalized to unity (*standardized* linear combinations of original variables or *orthonormal* transformations).

The number of latent factors used for PLS-regression was determined by cross-validation. We used split-sample cross validation to determine the number of latent factors that delivered the minimal predicted residual sum of squares (PRESS) followed by van der Voet's test; the fewest number of gene components that was insignificantly different from the factor number corresponding to the minimal PRESS should be used in regression [16]. In short, it was a randomization-based model comparison test performed on each cross-validation model [17]. Missing values in gene expression values were handled by imputing the missing ones with the non-missing values for the corresponding variable first, then followed by filling in missing values with their predicted

values based on that fitted model and computed the model again (expectation-maximization algorithm).

After the number of gene component was determined and each gene component score was calculated for all samples, binary logistic regression (LR) was applied for classification/prediction. For binary LR, the predicted probability was estimated by

$$\left(1 + \exp^{-(\beta 0+\beta 1\times 1+\cdots+\beta k\times k)}\right)^{-1}, \quad (2)$$

where k was the number of gene component used, and x_k was the kth gene component score. To evaluate classifier performance, leave-one-out cross validation was used to prevent model over-fitting. The threshold of a positive prediction was defined to have a more than 0.5 of cross-validated predicted probability. The process of PLS scores construction and LR prediction was repeated for each of the 5 molecular subtypes. Bonferroni corrections with a reduced α level of 0.01 were applied for all PLS-regression classifiers for multiple comparisons. All samples were categorized into one of the 5 molecular subtypes with the highest predicted probability, assuming that probability exceeding the threshold of 0.5. An ambiguous classification was claimed when more than one predicted probability was higher than 0.5 among all subtypes. A sample was designated as unclassifiable if none of the predicted probabilities of 5 molecular subtypes exceeded the threshold of 0.5.

In each classifier, a binary PLS-regression was fit, with the most relevant genes associated with the subtype enrolled as predictive variables. Each classifier compromised 10 out of the 50 PAM50 signature genes, and this class-specific gene selection avoided using all 50 genes into the PLS regression at the same time.

2.3. Validation Dataset. Our microarray experiments and two publicly available microarray studies fulfilled the purpose of external validation [18–20]. Our study material included 83 breast cancers from Taiwan (GSE48391); sporadic breast cancer samples were collected consecutively during surgery, snapped frozen in liquid nitrogen, and then stored at −80°C. The frozen samples were dissected into slices of 1-2 mm thickness, and more than 90% of cancerous content was a pre-requisite for microarray experiments. Written consent was obtained for all subjects before sample collection with the protocol approved by Institute Review Board of Cathay General Hospital. The criteria of enrolment included incident/invasive breast cancers without neo-adjuvant therapy, no systemic spread (clinical stage I to III), no concurrent secondary malignancy, and less than 70 years of age. Enrolled patients were managed according to standard guidelines with regular follow-up.

For relevant pathological features, ER positivity was defined as the presence of at least 10% of nuclei with positive results by immunohistochemical (IHC) analysis, and breast samples displaying low ER positivity (<10% of nuclei with positive stains) were not assayed in the current study. For HER2 status, the ASCO and CAP guidelines were followed: IHC3+ and IHC2+ with fluorescence in situ (FISH) hybridization amplification were considered to indicate HER2 overexpression.

Total RNA from cancerous breast tissues was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA) and RNA was purified using RNeasy mini kits (Qiagen, Germantown, MD). RNA integration was tested by gel electrophoresis. Affymetrix (Affymetrix, Santa Clara, CA) GeneChip Human Genome U133 plus 2.0 was used for the microarray experiment. Hybridization and scanning were performed according to the Affymetrix standard protocol. Images were scanned using GeneChip Scanner 3000, and the scanned images were processed with GeneChip Operating Software (GCOS). Robust multi-array average (RMA) algorithm was used to normalize 83 array chips [21].

Two publicly available breast cancer microarray depositories, one from Lu et al. and another from Kao et al., were merged with our microarrays to form the validation dataset [18–20]. Both datasets used the same Affymetrix U133 plus 2.0 microarrays as used in our experiments, and all assayed subjects were Han Chinese ethnically. RMA was used for normalization within each dataset [21]. Details of microarray experiments and the demography of the study populations had been described elsewhere [19, 20]. The Lu et al. dataset comprised 125 Chinese breast cancers with known clinical ER and HER2 status, and original Affymetrix CEL files were downloaded from NCBI Gene Expression Omnibus (GSE5460); clinical ER and HER2 status was provided. For the Kao et al. dataset, 327 Taiwanese breast cancers were assayed, and corresponding disease-free survival and overall survival data were available (GSE 20685). The median follow-up time of our 83 breast cancer patients was 3.7 years (range: 0.1 to 5.8 years) with 13 events of recurrence, metastasis, or breast cancer-specific mortality (16%) and 11 deaths (all-cause mortality). For 327 breast cancers from Kao et al. (GSE20685), the median follow-up was 7.7 years with 94 events of recurrence, metastasis, or breast cancer-specific mortality (29%), and 83 deaths (all-cause mortality).

All intrinsic genes were mapped to the Affymetrix gene annotation file, and only the most variable probeset measured by inter-quartile range (IQR) across all arrays was used when multiple probesets per gene were encountered. The 535 breast cancer specimens of the Han Chinese patients were assigned to 1 of the 5 molecular subtypes with the nearest centroid method (single sample prediction, SSP). Spearman's rank correlation coefficients were used, and samples were designated as unclassified if correlation coefficients to all 5 centroids were less than 0.1. To enhance the comparability between the original studies deriving intrinsic genes and independent samples in current study, mean-centering of genes was applied to the expression data of Han Chinese breast cancers, as suggested by the investigators of the Stanford group [22]. All arrays within each study were scaled and centered (mean = 0 and standard deviation = 1) on a gene-by-gene basis before PLS-regression was performed in order to overcome the discrepancies and enhance comparability across microarray studies.

3. Results

3.1. PLS-Regression in Prototypical Arrays. PLS-regression classifiers based on latent gene component scores were built for each molecular subtype from training dataset of 139 prototypical arrays. Table 1 showed the performance of individual classifiers. The number of gene component chosen for PLS regression ranged from 1 to 2. Table 2 tabulated PAM50 prototypes with class labels predicted by PLS- regression. The agreement between PAM50 prototypes and predicted subtype by PLS-regression was excellent (weighted Kappa: 0.988, 95% CI: 0.965–1) after excluding 16 unclassified cases. It should be noted that six cases were ambiguously predicted into luminal-A ($n = 4$), luminal-B, and normal breast-like subtype since these cases were positively predicted by two classifiers.

3.2. PLS-Regression in Validation Arrays. PLS-regression was performed for independent Han Chinese breast cancers including our series and two publicly available microarray depositories. To derive the "gold standard" for intrinsic subtype, centroid-based method (SSP) was used to designate each individual of the three studies into 1 of the 5 molecular subtypes.

Since no missing value was found in Affymetrix microarrays used for validation, there was no need of missing value imputations. Table 3 showed the results of PLS-regression classifiers with centroid-based SSP as the gold standard. At most two gene components were adopted by PLS-regression. Table 4 compared the results of PLS-regression and subtypes designated by SSP. A much compromised agreement between SSP and PLS-regression was observed, with only a fair weighted Kappa statistic of 0.541 (95% CI: 0.486–0.597) reported. The number of ambiguous cases raised to 55. Around one-fourth ($n = 125$) of tested samples were categorized as unclassified by PLS-regression.

3.3. Clinical Presentations and Prognostic Discrepancies among Intrinsic Taxonomy. Clinical and follow up data were available for 208 of Han Chinese breast cancers and we compared ER and HER2 phenotypes between distinct intrinsic subtypes designated by PAM50 SSP and PLS-regression (Table 5). Despite fewer cases analyzed by PLS-regression due to more unclassified samples, characteristics of molecular subtypes were similar between predictive results of PAM50 SSP and PLS-regression.

Figures 1(a) and 1(b) showed disease-free survival of 410 Han Chinese breast cancers with follow up data, classified by PAM50 SSP and PLS-regression, respectively. As expected, luminal-A subtype reported more optimistic results of breast cancer therapy. The prognoses of molecular subtypes other than luminal-A were much more intertwined and compromised.

4. Discussion

In the current study, PLS-regression was used for microarray multiclass predictions. Latent gene component scores were used in binary LR, each time with one molecular subtype tested. For breast cancer intrinsic taxonomy, PLS-regression classifiers were built for five mutually exclusive molecular subtypes. Bonferroni corrections were applied for multiple comparisons (5 times of classifications per each case). If the cross-validated predicted probability was higher than 0.5, a positive prediction was recognized. For most instances, there was only one classifier reported a positive prediction and the sample was categorized into the corresponding subtype. If two classifiers reported a higher than 0.5 predicted probability, the case was classified into the subtype with the highest probability but an ambiguous prediction was identified. If all classifiers failed to deliver a prediction higher than the threshold of 0.5 cross-validated probability, an unclassified sample was claimed.

Applications of gene component methodology for microarray studies had been reported in literature. West et al. demonstrated the "metagene" model, which used principle component (PC) scores from the top 100 genes showing the highest absolute correlations with clinical ER status of breast cancers and used these PC scores as predictive variables in binary regression [23]. Following studies adopting "metagene" concept, which was PC approach in nature, utilized the gene component scores in Bayesian classification tree [24]. On the other hand, Nguyen and Rocke performed binary and polychotomous LR and linear/quadratic discriminative analysis from PLS scores for two-class and multi-class microarray tumor classification problems [5, 6]. The main difference between PC and PLS is that PC extracts latent factors accounting for most of gene expression variations regardless of outcome variables and is unsupervised while PLS maximizes the covariance between latent explanatory and latent dependent variables and is supervised in nature. For this reason, it was postulated that PLS might perform better than PC in microarray classification problem and indeed, successful results of microarray gene component classification with PLS had been reported for several human cancers in past few years [25, 26]. Our previous studies compared predictive performance of gene component approaches, and concluded that PC regression was more suitable for unsupervised class discovery while PLS was more efficient in supervised class prediction [8].

Since PLS automatically produced (predicted) response variable (tumor class label), one-step PLS regression, which predicted tumor class directly from latent y-scores was reported by Pérez-Enciso and Tenenhaus [7]. However, for breast cancer intrinsic taxonomy comprising five molecular subtypes, at least four dummy variables were required, and the mutual exclusive relationships between these responsive variables were not constrained. For these reasons, direct PLS modeling of five molecular subtypes was not practical.

Multi-class prediction of PLS-regression was the extension of the regression for binary responses. The strategy of latent score construction remained the same. It was quite intuitive that polychotomous (ordinal or nominal) LR could fill the task of prediction with multiple responsive levels. However, neither ordinal (with one baseline class) nor nominal LR resulted in a converged model in the training

Table 1: Performance of PLS-regression classifiers for prototypical arrays.

Intrinsic subtype	Basal-like	HER2-enriched	Luminal-A	Luminal-B	Normal breast-like
Number of samples	57	35	23	12	12
PLS-regression					
Number of gene component	1	1	2	1	2
X-variance explained	57.0%	37.1%	74.5%	25.8%	60.2%
Y-variance explained	86.7%	56.2%	64.6%	24.6%	66.5%
Binary LR					
Adjusted *R*-square	0.99	0.73	0.9	0.63	0.99
AUC	1	0.96	0.99	0.96	1
Accuracy	98.6%	89.9%	97.1%	95.0%	100.0%
Sensitivity	98.2%	74.3%	91.3%	50.0%	100.0%
Specificity	98.8%	95.2%	98.3%	99.2%	100.0%

PLS: partial least square, LR: logistic regression, AUC: area under the curve.

Table 2: PAM50 prototypes and predicted subtypes by PLS-regression for prototypical arrays.

PAM50 prototype (sample number)	Predicted subtype					
	Basal-like	HER2-enriched	Luminal-A	Luminal-B	Normal breast-like	Unclassified
Basal-like (57)	57	0	0	0	0	0
HER2-enriched (35)	0	26	0	0	0	9
Luminal-A (23)	0	0	21	0	0	2
Luminal-B (12)	0	1	0	6	0	5
Normal breast-like (12)	0	0	0	0	12	0

Table 3: Performance of PLS-regression classifiers for independent validation dataset.

Intrinsic subtype	Basal-like	HER2-enriched	Luminal-A	Luminal-B	Normal breast-like
Number of samples	97	94	165	121	56
PLS-regression					
Number of gene component	2	1	2	2	1
X-variance explained	71.1%	25.5%	79.9%	61.7%	38.1%
Y-variance explained	56.9%	41.6%	34.5%	30.9%	18.1%
Binary LR					
Adjusted *R*-square	0.86	0.66	0.73	0.61	0.39
AUC	0.98	0.95	0.95	0.93	0.89
Accuracy	96.6%	90.7%	88.2%	86.2%	90.5%
Sensitivity	85.6%	68.1%	81.8%	63.6%	23.2%
Specificity	99.1%	95.5%	91.1%	92.8%	98.3%

PLS: partial least square, LR: logistic regression, AUC: area under the curve.

Table 4: Single sample prediction by PAM50 centroids and predicted subtypes by PLS-regression for independent validation dataset.

PAM50 SSP (sample number)	Predicted subtype					
	Basal-like	HER2-enriched	Luminal-A	Luminal-B	Normal breast-like	Unclassified
Basal-like (97)	83	1	0	1	0	12
HER2-enriched (94)	0	63	0	3	0	28
Luminal-A (165)	0	3	130	8	0	24
Luminal-B (121)	0	5	10	73	0	33
Normal breast-like (56)	1	3	17	1	8	26
Unclassified (2)	0	0	0	0	0	2

Table 5: Association of clinical ER and HER2 status with intrinsic taxonomy, classified by either PAM50 single sample prediction or PLS-regression.

	Basal	HER2	LumA	LumB	Norm
ER			PAM50 SSP		
Negative	40	28	0	3	9
Positive	0	7	67	46	7
HER2					
Normal	37	5	63	30	8
Over-expression	3	30	4	19	8
ER			PLS-regression		
Negative	38	19	1	1	3
Positive	0	5	59	33	2
HER2					
Normal	35	0	56	21	4
Over-expression	3	24	4	13	1

SSP: single sample prediction, Basal: basal-like, HER2: Her2-enriched, LumA: luminal-A, LumB: luminal-B, Norm: normal breast-like subtype.

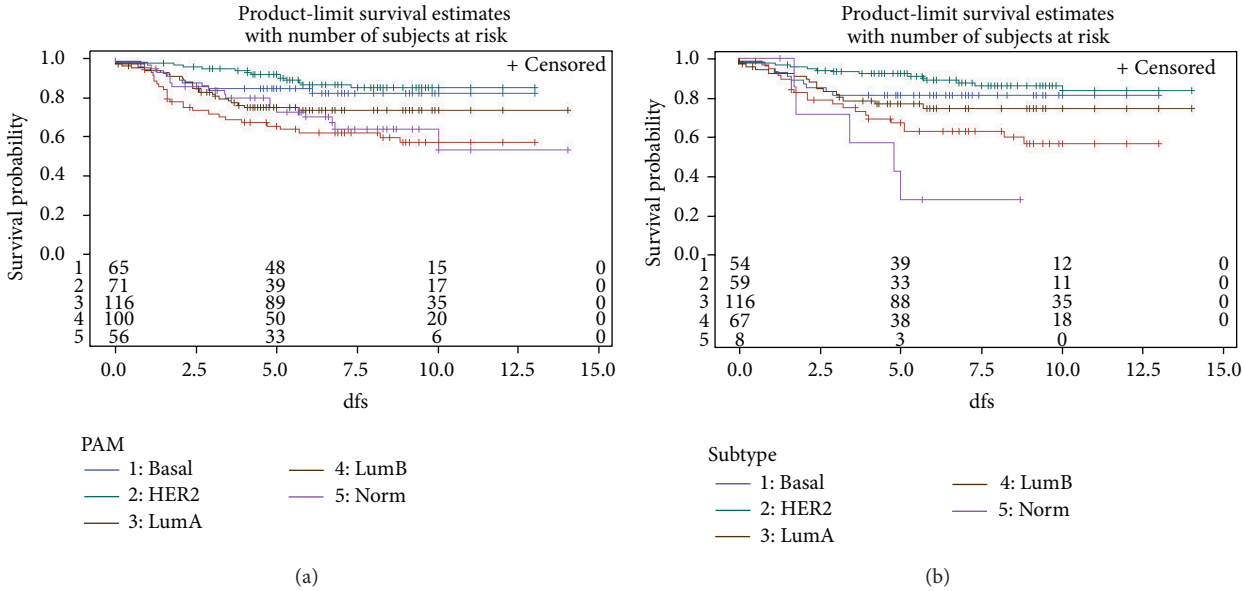

Figure 1: Breast cancer disease-free survival stratified by intrinsic subtypes, classified by either PAM50 single sample prediction (a) or PLS-regression (b). dfs, disease-free survival; Basal, basal-like; HER2, Her2-enriched; LumA, luminal-A; LumB, luminal-B; Norm, normal breast-like subtype.

or validation dataset. Multi-class prediction remained a challenge for high-throughput gene expression data analysis with classical statistical tools.

To overcome aforementioned difficulties, our strategy started with the development of PLS-regression for each of the molecular subtypes individually. In each classifier a binary PLS-regression was fit, with the most relevant genes associated with the subtype enrolled as predictive variables. Table 6 showed the compositions and weight vectors of PLS regressions for each intrinsic subtype. It deserved notice that each classifier compromised 10 out of the 50 PAM50 signature genes, and this class-specific gene selection avoided using all 50 genes into the PLS regression at the same time. These class-specific predictors for PLS-regression were not a coincidence but were revealed in tFhe intermediate step when PAM50 signature genes were selected. It was the ClaNC (classification to nearest centroids) algorithm which determined the composition of these class-specific genes [27]. More details could be disclosed from Figure 2(a) from the original publication of PAM50 [15].

In 139 prototypical arrays, the agreement between PAM50 SSP and PLS-regression was excellent (weighted Kappa: 0.988), indicating the robustness and feasibility of PLS-regression as an alternative classification method to PAM50 SSP. In validation dataset of 535 Han Chinese breast cancer microarrays, the agreement between PAM50 SSP and PLS-regression deteriorated substantially. If we took a close look at Table 4, the compromised performance of PLS-regression

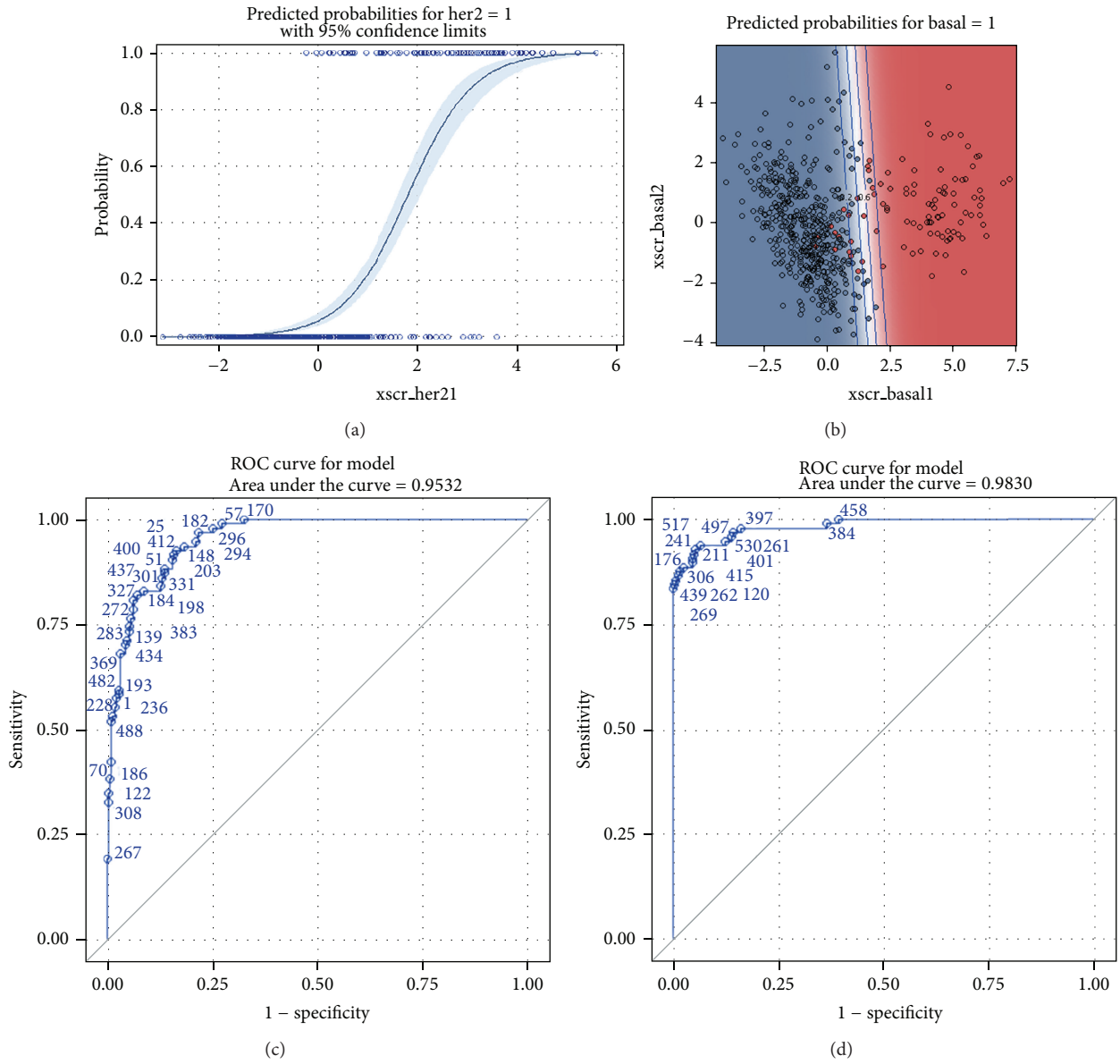

Figure 2: Predicted probabilities and 95% confidence interval as a function of the 1st PLS score or 1st/2nd PLS scores for HER2-enriched and basal-like subtype (a and b) and corresponding ROC curves (c and d). (*x*scr_her21: the 1st *x*-score for HER2-enriched subtype, *x*scr_basal1: the 1st *x*-score for basal-like subtype, *x*scr_basal2: the 2nd *x*-score for basal-like subtype).

Table 6: Compositions and weight vectors of five PLS-regressions for each molecular subtype.

Basal-like		HER2-enriched		Luminal-A		Luminal-B		Normal breast-like	
ANLN	0.271	ACTR3B	−0.316	BIRC5	−0.299	BCL2	−0.325	CCNB1	−0.272
CEP55	0.271	BAG1	−0.083	CDCA1	−0.294	CDH3	−0.667	CDC6	−0.241
ESR1	−0.319	BLVRA	0.317	CENPF	−0.288	CXXC5	0.484	KRT14	0.350
FOXA1	−0.417	CCNE1	−0.067	EXO1	−0.293	EGFR	−0.316	KRT17	0.241
FOXC1	0.370	CDC20	−0.069	MAPT	0.352	KIF2C	−0.050	KRT5	0.276
GPR160	−0.297	ERBB2	0.452	MYBL2	−0.328	MDM2	0.027	MLPH	0.376
KNTC2	0.303	FGFR4	0.365	NAT1	0.421	MKI67	−0.136	MMP11	−0.404
MELK	0.270	GRB7	0.470	PTTG1	−0.299	ORC6L	−0.049	RRM2	−0.359
MIA	0.296	MYC	−0.390	SLC39A6	0.339	PR	−0.143	TYMS	−0.286
TMEM45B	−0.323	SFRP1	−0.343	UBE2C	−0.296	PHGDH	−0.529	UBE2T	−0.374

in independent samples largely resulted from increased number of unclassified cases. If these unclassified samples were removed, the agreement between PAM50 SSP and PLS-regression improved enormously (weighted Kappa: 0.829 as opposed to 0.541 when unclassified samples were analyzed). Another clue came from the fact that if we forced all samples to be categorized into the subtype with the highest predicted probability (given that the highest probability was more than 0.1 as was in PAM50 SSP), the agreement between PAM50 SSP and PLS-regression was ameliorated with a weighted Kappa of 0.704 (95% CI: 0.649–0.758). The unclassified samples reduced to 9 (2% of 535 assayed samples).

An apparent benefit of PLS-regression rather than centroid-based SSP proposed by PAM50 investigators was that the predictive probability was reported. In our study we used the 0.5 of (cross-validated) predicted probability as the threshold of a positive prediction. If the threshold was relaxed to a lower level, the number of unclassified cases decreased but was with the expense of increased ambiguous classifications (two or more than two classifiers reported a positive prediction). Although we could designate samples into the class with the highest predictive probability, there remained a doubt about the validity of molecular taxonomy when more than one classifier passed the predefined threshold and reported a positive prediction. With current threshold of 0.5, there were fewer than 10% of cases with ambiguous classifications.

In centroid-based SSP, since samples were categorized into the subtype with the highest correlation coefficient, and the unclassified threshold was set to a much lower level (less than 0.1 of correlation coefficients to all five centroids), the higher proportion of unclassified cases of PLS-regression in independent dataset was not a drawback of purposed algorithm but indicated a more precise and sophisticated statistical rationale. In our opinion, the threshold of 0.1 correlation coefficient in PAM50 SSP was too loose as the proportion of unclassified cases was erroneously reduced with the expense of compromised reproducibility and robustness. The threshold of positive predicted probability could be viewed as a tuning parameter of PLS-regression, as suggested by high area under the curve (AUC) values of most classifiers (Tables 1 and 3). In the current study, the threshold of 0.5 implied an uninformative prior and an unprejudiced belief in individual classifier of each molecular subtype. Figures 2(a) and 2(b) showed the predicted probability as a function of the latent PLS scores, with one and two gene components incorporated into regression for HER2-enriched and basal-like subtype classifier, respectively. Figures 2(c) and 2(d) showed the corresponding receiver operating characteristic (ROC) curves.

The clinical ER and HER2 status for 208 Han Chinese breast cancers were presented in Table 5; no basal-like breast tumors were ER positive, and most HER2-enriched breast tumors (around four-fifths) were clinically ER negative, whereas most of luminal-A and luminal-B subtypes were ER positive. For clinical HER2 status, most basal-like breast tumors were HER2-normal, most HER2-enriched subtype cases were with HER2 over-expression, and the luminal-B tended to report a higher propensity of HER2 over-expression than the luminal-A subtype, regardless of predictive methods (PLS-regression or PAM50 SSP). Thesse findings, in general, were in agreement of what we learnt from previous studies about intrinsic taxonomy and further evidenced the validity of current study [20]. Disease-free survival from 410 cases of validation dataset was displayed in Figures 1(a) and 1(b). The luminal-A subtype was associated with the best prognosis during the follow-up for Han Chinese breast cancers.

Tables 3 and 4 showed that the agreement between PAM50 SSP and PLS-regression for normal breast-like subtype was extremely low, also indicated by the unsatisfactory adjust R-square as well as compromised sensitivity. Notably, normal breast-like centroid in PAM50 was derived from 29 normal breast samples; in 2009 Parker et al. clained that normal breast-like category of PAM50 should be treated as an internal quality control rather than a breast cancer intrinsic subtype such as normal breast-like subtype in Hu 306 and Sørlie 500 intrinsic signatures [15]. In this sense, none of our samples should be predicted as normal breast-like subtype with PAM50. In our study, the number of samples categorized as the normal breast-like subtype by PLS-regression ($n = 8$) was less than the number designated as normal breast-like by PAM50 SSP ($n = 56$), indicating a more precious and valid prediction of the purposed gene component algorithm. The dubious clinical meaning and doubtful existence of normal breast-like subtype, also reflected in its heterogeneous clinical presentations of ER and HER2 phenotypes, remained unsolved and demanded further evaluations. Perhaps directly assaying true normal breast tissues might shed light on this issue.

5. Conclusion

Our study extended the applications of PLS-regression for gene expression data to multi-class taxonomy such as PAM50 intrinsic subtypes purposed by the Stanford/UNC group. With gene component classifiers and class-specific genes for each molecular subtype, the purposed algorithm was validated in original cohort deriving the PAM50 signature as well as in independent Han Chinese breast cancers with modest sample size. PLS-regression was evidenced to be a feasible and efficient alternative to centroid-based SSP when more than two classes were discerned. The increased proportion of unclassifiable cases in independent samples deserved meticulous evaluation. Whether inconsistency in classification threshold or unrecognized patterns in full spectrum of intrinsic taxonomy resulted in these undetermined cases was speculated; further gene expression studies might be directed to answer these questions in an effort to derive a more sophisticated signature for human breasts cancer.

Authors' Contribution

Chi-Cheng Huang and Shih-Hsin Tu contributed equally to the paper.

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Research Article

DPYD, *TYMS*, *TYMP*, *TK1*, and *TK2* Genetic Expressions as Response Markers in Locally Advanced Rectal Cancer Patients Treated with Fluoropyrimidine-Based Chemoradiotherapy

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This study is to investigate multiple chemotherapeutic agent- and radiation-related genetic biomarkers in locally advanced rectal cancer (LARC) patients following fluoropyrimidine-based concurrent chemoradiotherapy (CCRT) for response prediction. We initially selected 6 fluoropyrimidine metabolism-related genes (*DPYD, ORPT, TYMS, TYMP, TK1*, and *TK2*) and 3 radiotherapy response-related genes (*GLUT1*, *HIF-1α*, and *HIF-2α*) as targets for gene expression identification in 60 LARC cancer specimens. Subsequently, a high-sensitivity weighted enzymatic chip array was designed and constructed to predict responses following CCRT. After CCRT, 39 of 60 (65%) LARC patients were classified as responders (pathological tumor regression grade 2 ~ 4). Using a panel of multiple genetic biomarkers (chip), including *DPYD*, *TYMS*, *TYMP*, *TK1*, and *TK2*, at a cutoff value for 3 positive genes, a sensitivity of 89.7% and a specificity of 81% were obtained (AUC: 0.915; 95% CI: 0.840–0.991). Negative chip results were significantly correlated to poor CCRT responses (TRG 0-1) ($P = 0.014$, hazard ratio: 22.704, 95% CI: 3.055–235.448 in multivariate analysis). Disease-free survival analysis showed significantly better survival rate in patients with positive chip results ($P = 0.0001$). We suggest that a chip including *DPYD*, *TYMS*, *TYMP*, *TK1*, and *TK2* genes is a potential tool to predict response in LARC following fluoropyrimidine-based CCRT.

1. Introduction

Colorectal cancer (CRC) is the third most common malignancy, and morbidity and mortality due to CRC are increasing worldwide [1]. Despite substantial progress in both diagnosis and therapy in recent decades, the prognosis for CRC remains poor. Approximately 35–40% of patients with locally advanced rectal cancer (LARC) will eventually develop distant metastases and die from this disease [2]. One of the leading causes of rectal cancer-related death is

therapy resistance [3]. In locally advanced stages of rectal cancer, clinical outcomes can be improved by preoperative neoadjuvant radiation or concurrent chemoradiotherapy (CCRT). Preoperative CCRT, introduced in the past decade, can achieve better sphincter preservation rates and lower local recurrence rates and can downstage the disease. It has therefore become a consensus treatment modality for LARC [4–8]. Although complete pathological response rates of 10–25% can be achieved, more than one-third of patients either do not respond or show only modest response to treatment [6]. The rate of local recurrence or distant metastasis remains as high as 15–20% for LARC treated with preoperative CCRT [8, 9]. The disease-free survival (DFS) of rectal cancer patients receiving preoperative CCRT with tumor response is better than that of patients with progressive or stable disease [7, 10].

The response of individual tumors to adjuvant therapies is not uniform. This poses a considerable clinical dilemma because patients with *a priori* resistant tumors could be spared exposure to radiation or DNA-damaging drugs, treatments that are associated with substantial adverse effects, and surgery could be scheduled without delay. Alternatively, different adjuvant treatment modalities, including additional chemotherapeutics, could be pursued. Therefore, it would be of significant clinical relevance to identify predictive biomarkers of response in LARC following CCRT.

Accordingly, several studies have investigated the correlation of various gene expression levels and tumor responses to different chemotherapeutic drugs, radiotherapy, and CCRT; however, the predictive value of at least some of these markers remains controversial [11–17]. For instance, thymidylate synthetase (*TYMS*) and other fluorouracil-associated enzymes (such as thymidine phosphorylase (*TYMP*) and dihydropyrimidine dehydrogenase (*DPYD*)) have been analyzed with respect to the local recurrence and development of metastasis of CRC after postoperative 5-fluorouracil (5-FU) chemotherapy [18]. Overexpression of *TYMS* is associated with resistance to 5-FU chemotherapy and can lead to poorer CRC survival rates, both DFS and overall survival (OS) [19]. Traditionally, the methodology used to identify predictive factors for response to fluoropyrimidine-based treatments has been to analyze the expression of enzymes implicated in its metabolism, either directly by immunohistochemistry (IHC) or by an enzyme-linked immune-sorbent assay (ELISA) or indirectly by individual mRNA expression [14, 20, 21]. More recently, the development of high-throughput methods of multiple genetic expression analysis has enabled a broader approach, analyzing multiple genes profiles simultaneously and providing genomic response signatures.

Conventional regimens for treating cancer patients with chemotherapy and radiotherapy do not account for interpatient variability in the expression of particular target genes. Such variability results in unpredictable tumor responses and host toxicity. Hence, our study investigated the role of the genetic expression levels of 6 fluoropyrimidine-based chemotherapy-related genes (*DPYD*, *TYMS*, *TYMP*, thymidine kinase 1, soluble (*TK1*), thymidine kinase 2, mitochondrial (*TK2*), and orotate phosphoribosyl transferase (*ORPT*)) and 3 genes related to radiotherapy (RT) response (glucose transporter member 1 (*GLUT1*), hypoxia-inducible factor 1 (*HIF1*), and hypoxia-inducible factor 2 (*HIF2*)) in the literature, genomic databases, and the Medline database [22–28].

Previously, our laboratory has successfully established a weighted enzymatic chip array (WEnCA) platform that could identify candidate genes as predictive biomarkers for potential clinical implications [29]. In the current study, we collected preoperative CCRT tumor tissues and paired normal tissues from 60 LARC patients. The correlations between the gene expression levels of the 9 candidate genes and the clinicopathological features of LARC patients, in addition to the relationship between gene expression levels and the CCRT tumor response, were analyzed to elucidate the role of a panel of multiple genetic biomarkers as a predictor of tumor response in LARC patients following preoperative CCRT.

This is the first investigation regarding predicting the clinical outcome of CCRT using a panel of multiple genetic biomarkers for LARC patients. The results would have potential clinical implications for predicting which patients would be likely to respond to preoperative CCRT and those who would be unlikely to respond, for whom therapeutic strategies would probably be altered.

2. Materials and Methods

2.1. Patients and Samples Collection. Between November 2006 and June 2011, 60 patients with LARC (T3/T4 disease or any clinical positive N-stage) located within 10 cm of the anal verge and receiving fluoropyrimidine-based preoperative CCRT were enrolled in this study. The study was approved by the ethics committee of our hospital. Baseline assessment before initiation of CCRT included a complete medical history and physical examination, colonoscopy, tumor biopsy, pelvic and abdominal computed tomography (CT), endorectal ultrasonography (if clinically feasible), and/or pelvic magnetic resonance imaging. Complete laboratory tests included a complete blood cell count, liver function tests, electrolytes, creatinine, albumin, and carcinoembryonic antigen (CEA). All patients had Eastern Cooperative Oncology Group (ECOG) performance status <2, were between 18 and 85 years of age, and had adequate hematological, liver, and renal function. Each tissue sample was snap-frozen in liquid nitrogen immediately after surgery or biopsy and stored at −80°C. Samples were further used in experiments for membrane array analysis. Clinical stage and pathological features of primary tumors were defined according to the criteria of the American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC) [30].

2.2. Treatments. Patients were treated with fluoropyrimidine-based chemotherapy. Of the 60 patients, 24 were treated with 5-fluorouracil (5-FU) (350 mg/m^2 IV bolus) and leucovorin (20 mg/m^2 IV bolus) with the fractions of the radiotherapy being administered on days 1 through 5 and days 21 through 25. Thirty-six patients were treated with capecitabine (850 mg/m^2, twice daily, 5 days a week, during the days when radiotherapy was administered). The first daily

dose of capecitabine was given 2 hours before radiotherapy; the second dose was administered 8–10 hours later. Radiotherapy (RT) was planned via computerized dosimetry, and a dose of 1.8 Gy per fraction was prescribed to cover the planned target volume. Pelvic RT consisted of 45 Gy in 25 fractions over a period of 5 weeks. The clinical target volume contained the primary tumor, the mesorectum, the presacral space, and the lymph nodes, which included the perirectal, presacral, internal iliac, and/or external iliac nodes. Patients were evaluated weekly during the course of CCRT to assess acute toxicity and their own compliance with the study. Blood tests were performed each time and consisted of complete blood cell and differential counts. The toxicity was monitored by use of the National Cancer Institute Common Toxicity Criteria, version 3.0 (http://ctep.cancer.gov/reporting/ctc.html; accessed in December 2012). Chemotherapy was withheld if any chemotherapy-related grade 3 or 4 toxicity was noted, in which case appropriate dose adjustment was undertaken. Chemotherapy was restarted at an 80% dose if toxicity levels resolved and was terminated if grade 3 or 4 toxicity was noted again after adjustment of the dosage. If grade 3 or 4 toxicity was clearly related to RT (e.g., with radiation dermatitis), local therapy was administered and chemotherapy was not terminated. After completion of the CCRT, all patients underwent surgery with a total mesorectal excision (TME), and extended visceral resection was performed in the clinical T4 patients. All operations were carried out by a single colorectal surgery specialist (J.-Y. Wang), who had performed more than 300 TMEs in the past 5 years. Anal sphincter-sparing surgery was performed whenever possible, with primary anastomosis and/or temporarily diverting colostomies.

2.3. Tumor Response. The characteristics of each LARC patient, any adverse events, and their responses after the CCRT were recorded. Assessment of pathological tumor response to preoperative CCRT was based on a standardized tumor regression grading (TRG) as described by Dworak et al. [31]. Two pathologists were involved in this study. They were blinded to the results of the array and scored each specimen independently. Any specimen where a difference in scores existed was then scored by consensus using a double-headed microscope. TRG was determined by the amount of viable tumor versus fibrosis, ranging from TRG 4 (no viable tumor cells detected) to TRG 0 (fibrosis completely absent). TRG 3 was defined as a regression of more than 50% with fibrosis outgrowing the tumor mass; TRG 2 was defined as a regression of less than 50%, and TRG 1 was basically defined as a morphologically unaltered tumor mass. In this study, pathological tumor response was defined as ranging between TRG 2 and TRG 4. The determination for downstaging was based on the comparison between the clinical TNM stage before the initiation of CCRT and the postoperative histopathological TNM stage.

2.4. Total RNA Extraction and First-Strand cDNA Synthesis. Total RNA was isolated from each LARC patient's tissue with the GeneCling Enzymatic Gene Chip Detection Kit (Medico-Gene Biotechnology Co., Ltd., LA, USA). RNA purified was quantified by measuring absorption at OD 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and quantitated by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was synthesized from total RNA, using the GeneCling Enzymatic Gene Chip Detection Kit. Reverse transcription was carried out in a reaction mixture consisting of 3 μg/mL oligo (dT) 18-mer primer, 1 μg/mL random 6-mer primer, 100 mmol/L deoxyribonucleotide triphosphate, 200 units of MMLV reverse transcriptase, and 25 units of ribonuclease inhibitor. The reaction mixtures with RNA were incubated at 42°C for a minimum of 2 hours, heated to 95°C for 5 minutes, and then stored at −80°C until analysis.

2.5. Preparation of Biotin-Labeled cDNA Targets and Hybridization. First-strand cDNA targets for hybridization were generated by reverse transcription of the mRNA from the tumor and corresponding normal tissues of LARC patients in the presence of biotin-labeled UTP using the GeneCling Enzymatic Gene Chip Detection Kit. The hybridized arrays were then scanned with an Epson Perfection 1670 flatbed scanner (SEIKO EPSON Corp., Nagano-ken, Japan). Subsequent quantification analysis of intensity of each spot was carried out using AlphaEase FC software (Alpha Innotech Corp., San Leandro, CA, USA). Spots consistently carrying a factor of 2 or more were considered as differentially expressed. A deformable template extracted the gene spots and quantified their expression levels by determining the integrated intensity of each spot after background subtraction. The fold ratio for each gene was calculated as follows: spot intensity ratio = mean intensity of target gene/mean intensity of β-actin. Figure 1 provides the schematic representation of the membrane array with 5 candidate genes, 1 housekeeping gene (β-actin), 1 bacterial gene (Mycobacterium tuberculosis; TB), and the blank control (dimethyl sulfoxide; DMSO).

2.6. Weighted Enzymatic Chip Array (WEnCA) Analysis. The procedure of the membrane array method for gene detection was performed based on our previous work [32]. Visual OMP3 (Oligonucleotide Modeling Platform, DNA Software, Ann Arbor, MI, USA) was used to design probes for target genes and β-actin, and the latter served as an internal control (Table 1). The newly synthesized oligonucleotide fragments were dissolved in distilled water to a concentration of 100 mM and applied to a BioJet Plus 3000 nL dispensing system (BioDot Inc., Irvine, CA, USA), which blotted the target oligonucleotide; the β-actin control was used sequentially (0.05 μL per spot and 1.5 mm between spots) on a SuPerCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) in triplicate. DMSO was also dispensed onto the membrane as a blank control. After rapid drying and cross-linking procedures, the preparation of the membrane array was accomplished. The expression levels of each gene spot measured by the WEnCA method were quantified and then normalized based on reference gene (β-actin) density. When the normalized spot density was 2 or greater, it was defined as an overexpressed gene spot.

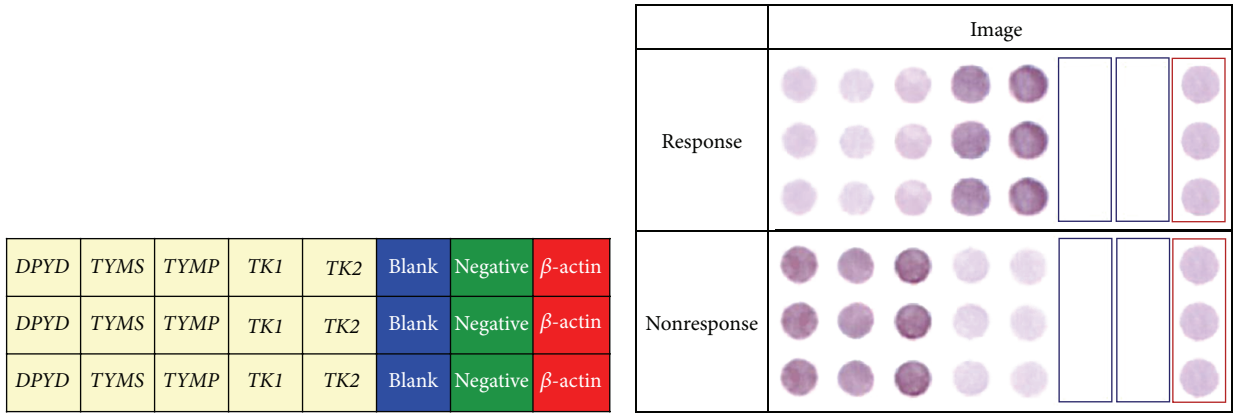

Figure 1: Schematic representations of weighted enzymatic chip array and gene expression patterns of responders and nonresponders. (a) Schematic representation of weighted enzymatic chip array including 5 target genes, one housekeeping gene (β-actin), one negative control gene (Negative), and one blank control (Blank). Five target genes (*DPYD*, *TYMS*, *TYMP*, *TK1,* and *TK2*). (b) A triplicate set of 5 genetic biomarkers for locally advanced rectal cancer patients with response and nonresponse is shown on the nylon membrane.

Table 1: Oligonucleotide sequences of target genes and the *β-actin* gene.

Gene name	Oligonucleotide sequence
DPYD	CAGTCAGAGCCCGTATGTGCACAGCAAAAGAGTGGTAACCAGGATCTATC
ORPT	GCTGCTGAGATTATGCCACGACCTACAATGATGATATCGGAACCTCGTTT
TYMS	GGATCCCTTGATAAACCACAGCAACTCCTCCAAAACACCCTTCCAGAACA
TYMP	CATCTGCTCTGGGCTCTGGATGACATTGAATCCAGGAATAGACTCCAGCT
TK1	AAGGTTGGTGCCACCCATCTTGGTGAAAGATGCTGTTGTTCCTGTGGAAA
TK2	CACTGAACACCGGGCTCCAGCCAAATGCAGCATAATTTTGTGGAAGTCTA
GLUT1	CAACCCCACTTACTTCTGTCTCACTCCCATCCAAACCTCCTACCCTCAAT
HIF-1 α	GTTCTATGACTCCTTTTCCTGCTCTGTTTGGTGAGGCTGTCCGACTTTGA
HIF-2 α	TCAGTGCTTCCTACCTACATGTCACTGACCGACCCAGAGACCTCAGCCAG
β-actin	TCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTC

2.7. Receiver-Operating Characteristic Curves. Receiver-operating characteristic (ROC) curves were constructed by plotting all possible sensitivity/specificity pairs for the WEnCA analysis, resulting from continuously varying the cutoff values over the entire range of results obtained. According to the analysis of ROC curves, the optimal cutoff point for the number of CCRT response-related genes was obtained. At this cutoff point, the sensitivity and specificity of a panel of multiple genetic biomarkers would also achieve optimal levels. Based on the calculated cutoff values, genetic biomarker panel results were interpreted as either positive or negative chip results.

2.8. Statistical Analysis. All statistical analyses were performed using the Statistical Package for the Social Sciences software, Version 14.0 (SPSS Inc., Chicago, IL, USA). ROC curve analyses were performed to analyze the membrane array data of the expression levels of the 9 candidate genes in the tissues of the subjects. The area under the ROC curve (AUC) and the corresponding 95% confidence intervals (CI) were calculated for each gene. The cutoff value at the highest accuracy (with minimal false-negative and false-positive results) was determined. On the basis of the calculated cutoff values, test results were classified as either positive or negative. The sensitivity and specificity of these dichotomous test results and the corresponding 95% CI were determined. A two-sided Pearson Chi-square test and the Fisher exact test were used to analyze the potential correlation between the CCRT response and the clinicopathological features of the study subjects. The multivariate analysis of independent prognostic factors for CCRT response was determined using logistic regression analysis. DFS rates were calculated using the Kaplan-Meier method, and the differences in survival rates were analyzed using the log-rank test. A probability of less than 0.05 was considered statistically significant.

3. Results

3.1. Constructing a Panel of Multiple Genetic Biomarkers. The study used the predictive biomarker panel, including 6 genes related to fluoropyrimidine-based chemotherapy (*DPYD*, *TYMS*, *TYMP*, *TK1*, *TK2*, and *ORPT*) and 3 genes related to the radiotherapy response (*GLUT1*, *HIF1*, and *HIF2*). According to ROC curve analysis between all 9 genes

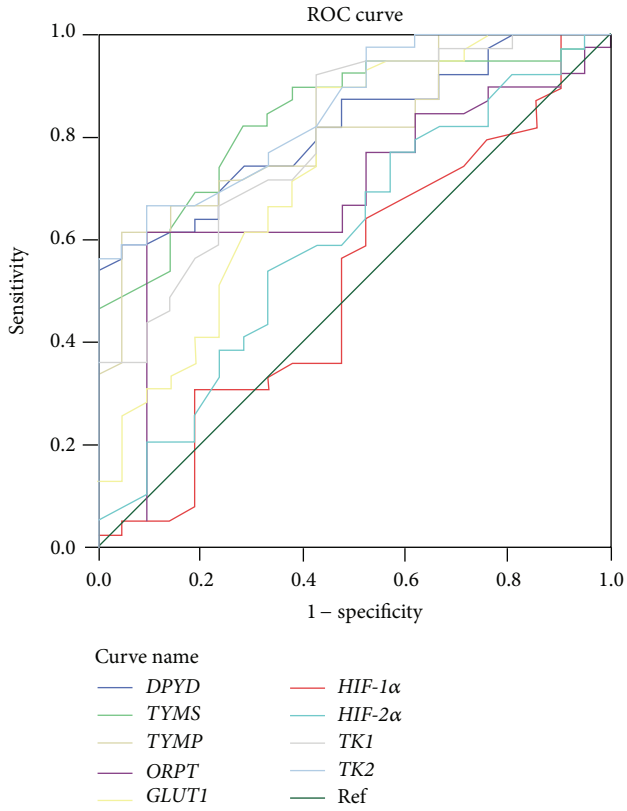

Figure 2: Receiver-operating characteristic (ROC) curves analysis of nine genes and tumor regression grade in 60 rectal cancerous tissues.

and TRG (Figure 2 and Table 2), the cutoff values of the 9 genes (*DPYD*, *TYMS*, *TYMP*, *ORPT*, *GLUT1*, *HIF-1α*, *HIF-2α*, *TK1*, and *TK2*) were 2.885, 2.155, 3.215, 2.330, 2.940, 2.455, 3.910, 1.955, and 3.065, respectively, while the sensitivity of each individual gene was above 70% in predicting the CCRT response. We further selected 5 genes with corresponding specificities above 70%, including *DPYD*, *TYMS*, *TYMP*, *TK1*, and *TK2*, to construct a panel of genetic biomarkers for predicting CCRT response (Figure 1). The definition of positive interpretation for each gene was as follows: *DPYD* gene expression less than 2.885, *TYMS* less than 2.155, *TYMP* less than 3.215, *TK1* gene expression more than 1.955, and *TK2* more than 3.065.

3.2. ROC Curve Analysis of the Multiple Genetic Biomarker Panel. From the results of ROC curve analysis of the multiple genetic biomarker panel and TRG, we found that the best cutoff value was 3 genes. In other words, a multiple genetic biomarker panel, on which no less than 3 genes were interpreted as positive, was considered to be positive. The multiple genetic biomarker panel, can predict CCRT response with a sensitivity of 89.7% and a specificity of 81% (AUC: 0.915; 95% CI: 0.840–0.991; Figure 3).

3.3. Correlation between Clinicopathological Features/Chip and CCRT Response. Sixty LARC patients (34 men and 26 women; mean age: 63.08 ± 12.71 years) were analyzed, and these patients' characteristics and clinicopathological findings are listed in Table 3. After preoperative CCRT, 39 patients (65%) achieved a pathological tumor response (TRG 2–4). The T classification was downstaged in 29 patients (48.3%), and the N classification was downstaged in 34 patients (56.7%). Univariate analysis indicated that negative perineural invasion ($P = 0.022$) was significantly associated with higher tumor response (Table 3) but that perineural invasion was insignificant in multivariate analysis ($P = 0.056$) (Table 4). Univariate or multivariate analysis indicated that the pre-CCRT CEA level (>2.5 ng/mL versus ≤2.5 ng/mL; >5 ng/mL versus ≤5 ng/mL) was not significantly associated with the CCRT tumor response rate (Tables 3 and 4). Other variables, including age, gender, tumor size, stage, clinical

Table 2: The area under the ROC curve (AUC) and the corresponding 95% confidence intervals (CI) of 9 genes.

Gene	AUC	95% CI
DPYD	0.815	0.711–0.919
TYMS	0.836	0.734–0.939
TYMP	0.81	0.701–0.918
ORPT	0.683	0.54–0.827
GLUT1	0.743	0.605–0.881
HIF-1 α	0.514	0.355–0.673
HIF-2 α	0.601	0.449–0.754
TK1	0.798	0.682–0.914
TK2	0.852	0.759–0.946

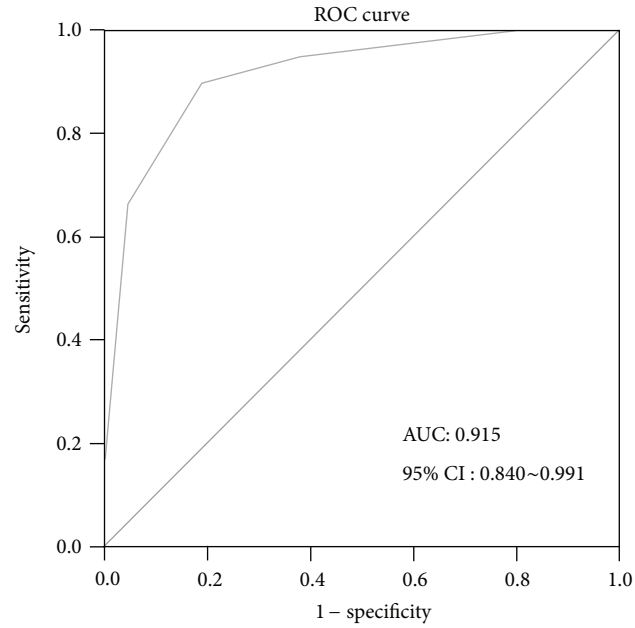

Figure 3: Receiver-operating characteristic (ROC) curve analysis of weighted enzymatic chip array in 60 rectal cancer tissues. In ROC curve analysis of 5 target genes, at a cutoff value of 3 positive genes, a sensitivity of 89.7% and specificity of 81% were obtained (area under ROC curve (AUC): 0.915; and the corresponding 95% confidence intervals (CI): 0.840–0.991) were considered positive results.

Table 3: Correlations between clinicopathological features and response status in 60 locally advanced rectal cancer patients.

Characteristics	Total cases *n* (%)	Nonresponse *n* (%)	Response *n* (%)	*P* value
Gender				
Female	26 (43.3%)	7 (33.3%)	19 (48.7%)	0.251
Male	34 (56.7%)	14 (66.7%)	20 (51.3%)	
Age (years)				
<60	22 (36.7%)	8 (38.1%)	14 (35.9%)	0.886
≥60	38 (63.3%)	13 (61.9%)	25 (64.1%)	
Tumor size				
<5 cm	46 (76.7%)	14 (66.7%)	32 (82.1%)	0.179
≥5 cm	14 (23.3%)	7 (33.3%)	7 (17.9%)	
Stage (UICC)[a]				
II	12 (20.0%)	5 (23.8%)	7 (17.9%)	0.737
III	48 (80.0%)	16 (76.2%)	32 (82.1%)	
Clinical T-stage				
T3	54 (90.0%)	19 (90.5%)	35 (89.7%)	1.000
T4	6 (10.0%)	2 (9.5%)	4 (10.3%)	
Clinical N-stage				
N0	12 (20.0%)	5 (23.8%)	7 (17.9%)	0.846
N1	23 (38.3%)	8 (38.1%)	15 (38.5%)	
N2	25 (41.7%)	8 (38.1%)	17 (43.6%)	
Chemotherapy				
Capecitabine	36 (60.0%)	10 (47.6%)	26 (66.7%)	0.151
5-FU	24 (40.0%)	11 (52.4%)	13 (33.3%)	
Time intervals of CCRT to operation				
<6 weeks (5.35 ± 0.49 weeks)	20 (33.3%)	8 (38.1%)	12 (30.8%)	0.566
≥6 weeks (7.15 ± 1.20 weeks)	40 (66.7%)	13 (61.9%)	27 (69.2%)	
Differentiation[b]				
WD	2 (3.3%)	0 (0.0%)	2 (5.1%)	0.653
MD	47 (78.3%)	17 (81.0%)	30 (76.9%)	
PD	4 (6.7%)	2 (9.5%)	2 (5.1%)	
Unclassified	7 (11.7%)	2 (9.5%)	5 (12.8%)	
Distance to anus				
<5 cm	38 (63.3%)	11 (52.4%)	27 (69.2%)	0.196
≥ 5 cm	22 (36.7%)	10 (47.6%)	12 (30.8%)	
Vascular invasion				
Yes	52 (86.7%)	18 (85.7%)	34 (87.2%)	1.000
No	8 (13.3%)	3 (14.3%)	5 (12.8%)	
Perineural invasion				
Yes	40 (66.7%)	10 (47.6%)	30 (76.9%)	0.022
No	20 (33.3%)	11 (52.4%)	9 (23.1%)	
Pre-CCRT CEA				
>2.5 ng/mL	44 (73.3%)	15 (71.4%)	29 (74.4%)	0.807
≤2.5 ng/ml	16 (26.7%)	6 (28.6%)	10 (25.6%)	
Pre-CCRT CEA[c]				
>5 ng/mL	27 (45.0%)	10 (47.6%)	17 (43.6%)	0.765
≤5 ng/mL	33 (55.0%)	11 (52.4%)	22 (56.4%)	

Table 3: Continued.

Characteristics	Total cases n (%)	Nonresponse n (%)	Response n (%)	P value
Chip[d] result				
Negative	21 (35.0%)	17 (81%)	4 (10.3%)	<0.001
Positive	39 (65.0%)	4 (19%)	35 (89.7%)	

[a]UICC: The American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC, 2002).
[b]WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated.
[c]CEA: carcinoembryonic antigen.
[d]Chip: panel of multiple genetic biomarkers.

Table 4: Univariate and multivariate regression analysis of prognostic indicators and nonresponse status for 60 locally advanced rectal cancer patients.

Parameters	Number	Univariate analysis Hazard ratio (95% CI)	P value	Multivariate analysis Hazard ratio (95% CI)	P value
Sex (female/male)	26/34	0.654 (0.309–1.384)	0.251	0.088 (0.005–1.613)	0.102
Age (≥60/<60)	38/22	0.941 (0.464–1.908)	1.000	0.093 (0.003–2.979)	0.179
Tumor size (≥5 cm/<5 cm)	14/46	1.643 (0.831–3.250)	0.179	4.658 (0.145–50.100)	0.385
Stage (UICC)[a] (II/III)	12/48	1.250 (0.573–2.727)	0.737	7.244 (0.114–458.695)	0.349
Clinical T-stage (T4/T3)	6/54	0.947 (0.289–3.108)	1.000	0.752 (0.023–24.539)	0.873
Clinical N-stage (N2/N1 + N0)	25/35	0.862 (0.421–1.762)	0.681	0.396 (0.011–14.706)	0.616
Chemotherapy (capecitabine/5-FU)	36/24	0.606 (0.306–1.200)	0.151	0.061 (0.002–1.745)	0.102
Differentiation[b] (PD + MD/WD)	51/2	0.627 (0.508–0.775)	0.531	0.000 (0.000–0.000)	0.999
Distance to anus (<5 cm/≥5 cm)	38/22	0.637 (0.324–1.253)	0.196	0.107 (0.002–5.908)	0.275
Vascular invasion (Yes/No)	52/8	0.923 (0.350–2.434)	1.000	2.022 (0.084–48.564)	0.664
Perineural invasion (Yes/No)	40/20	0.455 (0.233–0.886)	0.022	0.043 (0.002–1.0780)	0.056
Pre-CCRT CEA[c] (ng/mL) (>2.5/≤2.5)	44/16	0.909 (0.428–1.933)	0.807	0.314 (0.004–23.578)	0.599
Pre-CCRT CEA[c] (ng/mL) (>5/≤5)	27/33	1.111 (0.558–2.213)	0.765	0.407 (0.018–8.967)	0.569
Chip[d] result (negative/positive)	21/39	7.893 (3.049–20.434)	<0.001	22.704 (3.055–235.448)	0.014

[a]UICC: The American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC, 2002).
[b]WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated.
[c]CEA: carcinoembryonic antigen.
[d]Chip: panel of multiple genetic biomarkers.

T classification, clinical N classification, differentiation, distance to anus, vascular invasion, and type of chemotherapy, were also not significantly associated with the rate of tumor response. For the correlation between multiple genetic biomarker panel (chip) results and CCRT response, negative chip results were more significantly correlated than positive chip results to poor CCRT responses (TRG 0-1; $P < 0.001$ in univariate analysis and $P = 0.014$ in multivariate analysis; Tables 3 and 4).

3.4. Correlation between Multiple Genetic Biomarker Panel (Chip) Results and Disease-Free Survival. The median DFS was 47.01 months in patients with positive chip results; on the other hand, the median DFS was 22.16 months in patients with negative chip results ($P < 0.001$; Figure 4).

4. Discussion

Preoperative infusional 5-FU and concurrent RT, followed by total mesorectal excision, are the current standard of care for LARC [2]. As compared to postoperative 5-FU based CCRT, this preoperative strategy is associated with significantly lower toxicity and better compliance [2]. A large randomized phase II clinical trial has also provided convincing evidence that preoperative CCRT of rectal cancer reduces local recurrence (6% after 5 years) as compared to postoperative (13% after 5 years) multimodality treatment [2]. However, not all tumors respond uniformly, and despite promising results, *a priori* resistance to CCRT poses a thorny problem, since patients with nonresponsive tumors might either be spared the possible side effects of cytotoxic treatment and radiation or be subjected to alternative treatment modalities [33, 34]. Despite the well-known benefits of neoadjuvant CCRT for LARC, approximately 40% of patients have a poor response to this treatment, due to being exposed to unnecessary toxicities and delays in surgical intervention [7].

The factors predicting response to preoperative CCRT in rectal cancer have not been well characterized. Knowledge of such factors may be useful to clinicians and patients for predicting outcomes and thereby making treatment decisions. A better understanding of predictive factors may eventually lead to the development of such risk-adapted

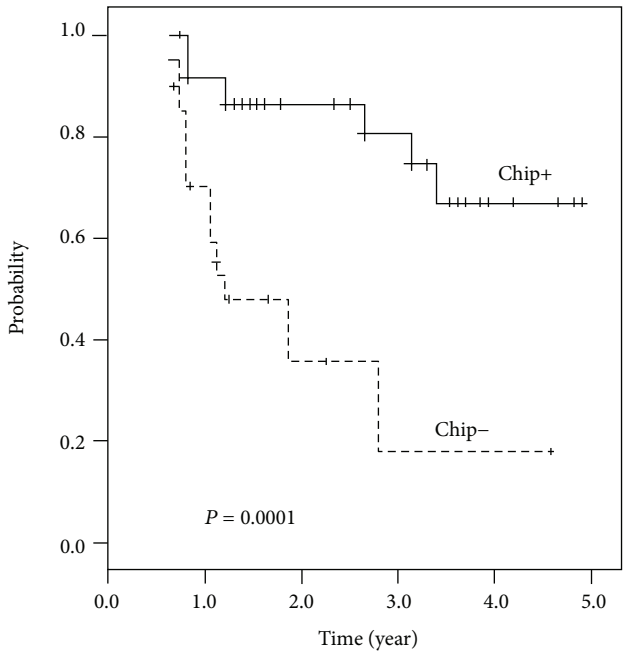

Figure 4: Disease-free survival analysis of 60 locally advanced rectal cancer patients according to results of a multiple genetic biomarkers panel. The median survival rate was 47.01 months in 60 locally advanced rectal cancer patients with positive results, while the median survival rate was only 22.16 months in patients with negative results ($P = 0.0001$).

treatment strategies as more aggressive preoperative regimens in patients less likely to respond to standard therapy. Better knowledge of these predictive factors may also help in the design of clinical trials for newer preoperative regimens.

A retrospective study of 141 patients has demonstrated that pretreatment CEA levels greater than 5 ng/mL are associated with poor response to preoperative CCRT [35]. Das et al. reported that pretreatment serum CEA levels greater than 2.5 ng/mL ($P = 0.015$) were associated significantly with lower pathologic complete response rates [36]. Moreno García et al. have reported that pretreatment CEA levels below or equal to 2.5 ng/mL correlate with higher complete pathologic response (21 versus 9%; $P = 0.05$) [37]. However, the study results indicated that pretreatment CEA levels cannot predict CCRT response with either univariate or multivariate analysis, whether the cutoff value of CEA levels was 2.5 ng/mL or 5 ng/mL.

This study has attempted to move beyond single gene expression to a more comprehensive investigation of multiple gene expression levels in predicting tumor response following fluoropyrimidine-based CCRT. The initial investigation involved the expression levels of 9 functional genes; subsequently, a panel of multiple genetic biomarkers was constructed, including the following 5 genes: *DPYD*, *TYMS*, *TYMP*, *TK1*, and *TK2*. In the present study, the RT response-related genes could not well predict response in LARC following fluoropyrimidine-based CCRT. We hypothesis it result from the RT dose (45 Gy) in preoperative CCRT was lower than the definite RT dose (more than 60 Gy). Therefore, the response predictive value of these RT response-related genes (*GLUT1*, *HIF1*, and *HIF2*) could not be highlighted in the adjuvant role.

TRG was reported to have prognostic value in LARC patients after preoperative CCRT and has also been previously reported as an independent prognostic factor for either local recurrence or DFS [7, 38, 39]. Following preoperative CCRT, TRG may reflect the characteristics of proliferation and resistance to hypoxia of residual cancer cells [40]. In our study, 65% of the 60 LARC patients achieved TRG grades 2~4. On comparing Taiwanese patients with other races with regard to tumor response, Berho et al. reported that, of 86 LARC patients receiving preoperative infusional 5-FU and RT, 73.3% of the Caucasians among them achieved a TRG grade between 2 and 4 [41]. These differences in tumor response may explain the variety of CCRT-related responses that occur worldwide. By analyzing multiple gene expression results and TRG, the prediction efficacy of this multiple genetic biomarker panel was demonstrated.

Fluoropyrimidines are antimetabolite drugs widely used in the treatment of solid tumors including rectal cancer [42]. The principal mechanism of action of fluoropyrimidines has been considered to be the inhibition of *TYMS*, but recent evidence has also shown alternative pharmacodynamic pathways acting through the incorporation of fluoropyrimidine's metabolites into the DNA and RNA of tumors [43, 44]. The fluoropyrimidines are broken down into three metabolites that have pharmacodynamic effects, including fluorodeoxyuridine monophosphate (FdUMP), fluoro-deoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The main mechanism of 5-FU activation is the conversion to FdUMP, which inhibits the enzyme *TYMS*, an important part of the folate-homocysteine cycle and purine and pyrimidine synthesis [43]. The conversion of 5-FU to FdUMP can occur via *TYMP* to fluorodeoxyuridine and then by the action of thymidine kinase to FdUMP or indirectly by fluorouridine monophosphate (FUMP) or fluorouridine (FUR) to fluorouridine diphosphate (FUDP) and then ribonucleotide reductase action to fluorodeoxyuridine diphosphate and FdUMP [43]. The incorporation of dUTP or FdUTP into DNA is the cause of DNA damage of tumor cells of fluoropyrimidines [44]. The rate-limiting step of 5-FU catabolism is *DPYD* conversion of 5-FU to dihydrofluorouracil [45]. To modulate the activity of fluoropyrimidines, inhibitors of *DPYD*, such as uracil and eniluracil, can be coadministered. This slows the degradation of 5-FU and improves the response rate [43]. Meanwhile, metabolites of fluoropyrimidine are crucial for LARC therapy.

The main enzymes implicated in fluoropyrimidine metabolism have been widely studied for response prediction. It has been established that higher *TYMP* (as well as lower *DPYD*) expression in tumors resulted in higher intratumoral concentrations of 5-FU, as well as a more potent antitumor effect of capecitabine [42–44]. In line with this idea, positive immunostaining for *TYMP* has predicted a significantly higher response rate to a capecitabine regimen in advanced stages of CRC [45]. Likewise, Boskos et al. have found that patients with a higher *TYMP*/*DPYD* ratio by ELISA were

more likely to respond to neoadjuvant capecitabine/RT [21]. Thymidylate synthase (*TYMS*) is considered the indirect target of 5-FU. High *TYMS* expression in pretreatment biopsies, measured either by IHC [46] or by mRNA [12], has been linked to a lack of response to neoadjuvant 5-FU/RT. The number of tandem repeats in the *TYMS* promoter region affects the translation efficiency of the protein, leading to increased expression [42]. Patients with triple repeats of this sequence (*TYMS* 3/3) had poorer tumor responses than those with shorter sequences (2/2 or 2/3) [43], suggesting that germline analysis for genetic variants may assist in predicting response. However, these studies are hypothesis-generating and, to date, there are neither studies confirming them nor contradictory findings [14]. In the current study, simultaneous positive interpretations of 3 genes (out of the 5) can predict fluoropyrimidine-based CCRT response with a sensitivity of 89.7% and specificity of 81%. The identification of predictive indicators of CCRT would be extremely useful in selecting feasible patients for fluoropyrimidine-based preoperative CCRT, thereby avoiding unnecessary preoperative treatment. In the present study, the median DFS was 47.01 months in LARC patients with positive chip results; on the other hand, the rate was only 22.16 months in patients with negative results. There were prominent associations between chip results and DFS, which could be used as a pre-CCRT predictor for clinical outcomes of LARC. The study data suggest that positive chip results might be predictors not only of tumor response but also of DFS. This finding could be useful in the future to identify individual risk and to develop more aggressive or alternative therapeutic strategies.

In conclusion, the present study indicates that a panel of multiple genetic biomarkers, consisting of the *DPYD*, *TYMS*, *TYMP*, *TK1*, and *TK2* genes, could be a potential aid in clinical predictions to obtain better CCRT response prediction models. It suggests that such a panel could be used to distinguish between LARC patients responding to CCRT and those who do not. Moreover, further studies in larger sample sizes and even multiple centers are mandatory to verify these results.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

Moving toward Personalized Medicine in the Methadone Maintenance Treatment Program: A Pilot Study on the Evaluation of Treatment Responses in Taiwan

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This pilot study simultaneously evaluated the effects of various factors, including genetic variations of *CYP2B6*, *CYP2C19*, and *ABCB1*, demographic characteristics, disease states, methadone-drug interactions (MDIs), and poly-substance use, on the treatment responses among non-HIV patients in the methadone maintenance treatment program (MMTP) in Taiwan. A total of 178 patients were recruited from two major hospitals that provided MMTP services in southern Taiwan, and information regarding concomitant medications and diseases was acquired from the National Health Insurance (NHI) program. The results demonstrated that the methadone maintenance dose, *CYP2B6* 785G allele, and *ABCB1* 2677T allele have positive effects on the methadone plasma concentration. In contrast, patients with HCV coinfection, alcohol problems, and psychiatric diseases may have a negative response to treatment. Thus, a comprehensive evaluation of treatment responses in the MMTP should include not only genetic polymorphisms in methadone metabolism and transporter proteins, but also concomitant diseases, MDIs, and poly-substance use. The results also suggest that personalized medicine may be indispensable for a better outcome of the MMTP.

1. Introduction

Methadone maintenance treatment programs (MMTPs) have been shown to be effective in reducing illicit drug use and risks of human immunodeficiency virus (HIV) and hepatitis C (HCV) infection [1–3]. Achievement of an optimal methadone plasma concentration is crucial for a successful MMTP [4]. However, due to wide interindividual variations in methadone pharmacokinetics [5–7], some patients' methadone plasma concentrations may be too difficult to reach within a therapeutic range even though they receive higher dosages. Between 30% and 80% of patients are considered poor responders to MMTPs [8], and 98.6% of injecting drug users (IDUs) on a MMTP still continue to inject drugs [9].

Some factors are thought to influence the methadone plasma concentration and treatment responses. IDUs may have severe medical complications of substance use disorders,

including psychiatric disease (e.g., depression, anxiety) [10–12], infectious diseases (HIV, HCV) [13], and pain disorders [14].

In addition, many treatment conditions can result in complications. For example, methadone-drug interactions (MDIs) could occur because MMTP clients have a high tendency towards taking concomitant medications (72%) [15]. Approximately 48% of patients have at least one MDI, and the most common MDI is benzodiazepines (38.1%), such as alprazolam and estazolam. Moreover, methadone interactions with some antiretroviral agents are also ubiquitous in HIV-MMTP clients [16, 17]. Some antiretroviral agents are well-documented as cytochrome P450 (CYP450) 3A4, 2B6, or 2D6 strong inducers (e.g., efavirenz) [18] or inhibitors. In a case report [19], interruption in the use of lopinavir-ritonavir triggered Torsade de Pointes (TdP) by increasing the methadone plasma concentration, because lopinavir-ritonavir may induce metabolic clearance of methadone involving CYP3A4, 2B6, and 2D6 enzymes.

Observational and *in vitro* studies have suggested that *CYP2B6* [20–23], *2C19* [21, 22], and *ABCB1* [22, 24] genetic polymorphisms have important roles in gene codes for methadone-metabolizing enzymes and transporter proteins (p-glycoprotein, P-gp). CYP2B6 has been demonstrated to be an important contributor to S-methadone metabolism, and CYP2C19 preferentially metabolizes R-methadone [21, 25]. S-methadone has been found to contribute to higher levels of dissatisfaction and the risk of QT interval prolongation [26, 27]. R-methadone has been reported to be associated with clinical effects as a result of its stronger activation of μ-opioid receptors [28]. Methadone is a substrate of P-gp, encoded by the multidrug resistance1 (*ABCB1*) gene, which has the ability to influence the bioavailability of orally administered methadone in the gastrointestinal tract and hepatocytes and has an effect on mediating methadone transport through the blood-brain barrier (BBB) [29, 30].

Even though joint genetic effects of CYP and P-gp on a MMTP have been demonstrated in some pharmacogenetic studies [20, 31–34], few studies have simultaneously considered other important factors, such as disease states, MDIs, and poly-substance use, in assessing the methadone treatment responses, particularly in the ethnic Chinese populations. It is especially important that medical professionals understand the efficacy of and crucial factors related to MMTPs.

In Taiwan, to encounter the escalating IDUs and HIV infections through needle sharing, the first MMTPs were implemented in July 2006 [35, 36]. However, a thorough evaluation on the treatment responses of a MMTP has not yet been conducted. Therefore, the main purpose of this pilot study was to simultaneously evaluate the influence of genetic variations of *CYP2B6*, *CYP2C19*, and *ABCB1,* disease states, MDIs, and poly-substance use on the methadone steady-state trough plasma level and treatment responses in MMTP patients. In addition, as the methadone plasma dose and plasma concentration may be severely interfered by antiretroviral drugs, we recruited non-HIV patients to avoid the impact of antiretroviral agents on the MMTP and to evaluate the potential factors related to the methadone plasma concentration and treatment responses more precisely.

2. Materials and Methods

2.1. Study Patients. This study was conducted from February 2010 to December 2011 at Jianan Mental Hospital of the Department of Health, the first mental hospital to implement a MMTP, and Chung-Ho Memorial Hospital of Kaohsiung Medical University, a major medical center in southern Taiwan. One hundred and seventy-eight patients with no HIV infection (confirmed by medical records) aged ≥20 years and who were not pregnant were recruited. To ensure that patients' methadone plasma concentrations were at the steady-state condition and have stable methadone doses, patients who had participated in the MMTP ≥ 1 month were recruited.

2.2. Ethics Statement. All information was kept strictly confidential and used for research proposes only. The study was approved by the Institutional Review Boards of Jianan Mental Hospital (Approval number 10-002) and Chung-Ho Memorial Hospital (Approval number KMUH-IRB-980429). Written informed consent was obtained from all participants who were given a detailed description of the study and had the chance to clarify any questions. Before agreeing to join the study, all participants were informed of the purpose of the study and the role and functions of their participation. Participants had the right to decline to take part in this research. They could also stop participating in the research at any time. Treatments of participants and nonparticipants at the hospital were not discriminated in any way.

2.3. Clinical Assessments. The participants were interviewed by a trained research staff to collect information on their sociodemographic characteristics, histories of substance use, and adverse drug reactions. Sociodemographic characteristics included age, sex, weight, educational level (years of education completed), and current marital status. Substance use referred to the use of cigarettes, alcohol, or betel quid, while drug use included illicit use of heroin and amphetamines.

Data on the methadone maintenance dose, admission date to the MMTP, and data regarding hepatitis, including hepatitis B (HBV) or HCV, as defined by a serological blood test with the presence of hepatitis B surface antigen (HBsAg) or detected antibodies to HCV, respectively, were obtained from medical records.

Information regarding concomitant medications and diseases during the period of the MMTP was acquired from the National Health Insurance (NHI) program, a national universal health insurance program with a coverage rate of over 99% for all citizens in Taiwan. General medical and pharmacy records were obtained for all patients. MDIs in the Taiwan MMTP [15] were categorized as follows:

(1) decreased methadone metabolism: by agents that could inhibit CYP3A4, 2B6, and 2D6 enzymes, such as cimetidine, ketoconazole, erythromycin, clarithromycin, and metronidazole;

(2) increased methadone metabolism: by agents that could induce CYP3A4, 2B6, and 2D6 enzymes, such as dexamethasone, carbamazepine, spironolactone, and rifampine;

(3) antianxiety drugs: benzodiazepines (BZDs) and non-BZDs, such as zolpidem and zopiclone;

(4) antipsychotic drugs: chlorpromazine, prochlorperazine, thioridazine, and haloperidol;

(5) opioid-related drugs: morphine, tramadol, nalbuphine, propoxyphene, and bupernorphine;

(6) antidepressive drugs: imipramine, fluoxetine, sertraline, amitriptyline, paroxetine, fluroxamine, and risperidone;

(7) QT prolongation drugs: droperidol, levofloxacin, moxifloxacin, and ciprofloxacin.

2.4. Treatment Responses. Response to treatment is defined by nonconsumption of heroin. Determination of nonconsumption of heroin (responders) was first identified by the patients' self-reporting, followed by confirmation of the negative urine results for illicit opiates use, whereas consumption of drugs (nonresponders) was based on the self-reporting of patients and confirmed by the positive urine results for opiates. Urinalysis results were retrieved from medical records. In Taiwan, poppy seeds are not a traditional food and possession or consumption of poppy seeds is an illegal act. Therefore, false positive results for morphine due to consumption of poppy seeds can be excluded. The urine was thus defined as positive for illicit opiates use when the amount of morphine in the urine sample was equal to or greater than 300 ng/mL, as determined by gas chromatography-mass spectrometry (GC-MS).

2.5. Plasma Sample Analysis. Blood samples were withdrawn prior to next methadone administration (trough methadone plasma concentration). After blood samples (5 mL) were drawn, they were centrifuged and the plasma was stored at −70°C until quantification of the enantiomers of methadone (R-methadone and S-methadone). The concentrations of enantiomers of methadone in the plasma were determined by capillary electrophoresis (CE-UV) [37] after liquid-liquid extraction (LLE) of samples with ethyl acetate/heptane (4 : 1, v/v). A Beckman P/ACE MDQ system with a UV detector (214 nm) was used for the enantioselective determination of methadone and atenolol as an internal standard (I.S.). After LLE, CE was performed in an uncoated fused-silica capillary of 31.2 cm (effective length 20 cm) with a 50-μm internal diameter. A constant voltage of 20 kV was applied and the cartridge temperature was maintained at 25°C. The running buffer was 80 mM phosphate buffer and 3.3 mM carboxymethyl-β-cyclodextrin (CM-β-CD) (pH 3). The relative standard deviation (RSD) and relative error (RE) were less than 5.3% and 7.7%, respectively, and the limit of quantification (LOQ) was found to be 10 ng/mL.

2.6. Genetic Polymorphism Analysis. Genomic DNA was extracted from venous blood samples using a FlexiGene DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For detection of *CYP2B6* A785G, G516T, and C1459T, *CYP2C19* G681A, G636A, and C3402T, and *ABCB1* C1236T, G2677A, and C3435T single nucleotide polymorphisms (SNPs), the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was used.

PCR reactions were performed in a reaction volume of 50 μL with 100 ng genomic DNA, SapphireAmp Fast PCR Master Mix (Takara, Otsu, Shiga, Japan), and 25 pmole of each primer. The protocol was as follows: 35 cycles, with an initial denaturation step at 95°C for 10 seconds, followed by annealing at primer-specific temperatures (56.4°C–62.4°C) for 10 seconds, 20 seconds of extension at 72°C, and cooling to 4°C for 5 minutes. PCR fragments were amplified using a PCR Thermal Cycler Dice TP600 (Takara, Otsu, Shiga, Japan). For the *CYP2B6* A785G, G516T, and C1459T, *CYP2C19* G681A, G636A and C3402T, and *ABCB1* C1236T, G2677A, and C3435T SNPs, the restriction enzymes, *Bsr* I, *Sty* I, *Bgl* II, *Msp* I, *BamH* I, *MnI* I, *Hae* III, *BseY* I, and *Mbo* I, were applied, respectively. DNA fragments were separated by electrophoresis in 2% agarose gel.

The SNPs were also confirmed using direct sequencing. Amplicons were purified using PCR Clean Up-M (Viogene, Taiwan). The PCR products were then directly sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed on an ABI 3730 DNA sequencer (Applied Biosystems, CA, USA).

2.7. Statistical Analysis. Hardy-Weinberg equilibrium was tested for each SNP. R,S-methadone, R-methadone, and S-methadone plasma concentrations were divided by the methadone dose (in milligrams per day) and by the patient weight (in kilograms). Then, the values were natural-log transformed before analysis. The Levene test was applied to verify homogeneity of variance.

For the purpose of assessing the statistical differences between responders and nonresponders and between *CYP2B6*, *CYP2C19*, and *ABCB1* genotypes, analysis of variance (ANOVA), or the t-test was used for data presenting a homogenous distribution, or the Kruskal-Wallis nonparametric test or the Mann-Whitney U test was used for those that did not attain the estimated homogeneity and normality for continuous variables. The chi-square test or Fisher's exact test was used for categorical variables to account for the small sample size.

Stepwise multiple linear regression analysis was performed in order to explore the variables independently related to methadone plasma concentrations. However, because methadone plasma concentrations were skewed positively, the methadone plasma concentrations were natural-logarithmically transformed to achieve a normal distribution. The variables included were age, weight, sex, sociodemographics, methadone dose, current amphetamine use,

methadone treatment duration, drugs related to methadone-drug interactions, and *CYP2B6*, *CYP2C19*, and *ABCB1* genetic variability.

Moreover, stepwise multiple logistic regression analysis was conducted to identify associations between potentially predictive variables (sociodemographics, tobacco use, betel nut use, alcohol use, current amphetamines use, methadone treatment duration, HBV, HCV, pain disorders, depression, anxiety, psychiatric disorders, methadone maintenance dose, *CYP2B6*, *CYP2C19*, and *ABCB1* genetic variability) and treatment responses. A variable was selected using mixed stepwise regression and included if its P value was ≤0.05 and excluded if its P-value was ≥0.05.

All analyses were completed using JMP (version 9.0, SAS Institute, Cary, USA). The statistical tests performed were two-tailed and a P value <0.05 denoted a statistically significant difference.

3. Results

The results were based on the data of 178 patients, including 156 males and 22 females. The mean age (±SD) of the patients was 39.5 ± 7.1 years (range, 25–59 years), and the mean weight was 68.8 ± 12.8 kg (range, 40–118 kg). Of the patients, 160 (89.9%) and 33 (18.5%) had HCV and HBV coinfection, respectively, and 128 (72.7%) patients had coadministration of other medications with methadone. The mean methadone daily maintenance dose was 50.8 ± 30.5 mg (range 5–250 mg/d).

A lack of available genotype data in two male subjects resulted from methodological problems. The mean R,S-methadone, R-methadone, and S-methadone trough levels were 172.9 ± 150.6 ng/mL (range 6.9–1368.0 ng/mL), 92.9 ± 79.8 ng/mL (range 6.9–800.7 ng/mL), and 81.9 ± 73.2 ng/mL (range 7.3–567.3 ng/mL), respectively.

3.1. Demographic Characteristics, Disease State, CYP2B6, CYP2C19, and ABCB1 Genotypes. The demographic characteristics, concomitant diseases, substance use history, and MDIs of patients (split into responders and nonresponders) are shown in Table 1. Responders and nonresponders differed significantly with respect to gender, alcohol use, HCV coinfection, psychiatric disorders, and benzodiazepine use. Although the responders and nonresponders had similar methadone doses, R,S-methadone, R-methadone, and S-methadone concentrations (Table 1), there were statistically significant differences in the distributions of *ABCB1* G2677T and C3435T between the responders and nonresponders (Table 2). No differences were found in the distribution of *CYP2B6* and *CYP2C19* genotypes between responders and nonresponders. *CYP2B6*, *CYP2C19,* and *ABCB1* allele frequencies observed in MMTP Taiwanese were in Hardy-Weinberg equilibrium ($P > 0.05$).

3.2. Methadone Maintenance Dose, Plasma Concentrations, and CYP2B6, CYP2C19, and ABCB1 Genotypes. We investigated the trough R,S-methadone, R-methadone, S-methadone concentrations and methadone dose by the genotypes of the *CYP2B6*, *CYP2C19,* and *ABCB1* genes (Table 3). For *CYP2B6* A785G, though the methadone dose did not differ significantly ($P = 0.42$), the R,S-methadone plasma concentration was significantly different among the allele groups ($P = 0.03$); in particular, that of G/G carriers (5.84 ± 0.77 ng·kg/mL·mg) was higher than that of A/A carriers (5.35 ± 0.81 ng·kg/mL·mg).

For *ABCB1* C1236T, the T homozygous carriers (47.2 ± 24.7 mg) showed a trend towards a lower maintenance methadone dose than the T heterozygous carriers (50.4 ± 28.3 mg) and noncarriers (67.9 ± 50.4 mg) ($P = 0.02$). For *ABCB1* G2677T, the R,S-methadone (G/G group 5.23 ± 0.86 ng·kg/mL·mg versus G/T group 5.24 ± 0.73 ng·kg/mL·mg versus T/T group 5.67 ± 0.86 ng·kg/mL·mg, $P = 0.008$), R-methadone (G/G group 4.12 ± 0.76 ng·kg/mL·mg versus G/T group 4.26 ± 0.62 ng·kg/mL·mg versus T/T group 4.58 ± 0.65 ng·kg/mL·mg, $P = 0.004$), and S-methadone (G/G group 4.00 ± 0.68 ng·kg/mL·mg versus G/T group 4.08 ± 0.67 ng·kg/mL·mg versus T/T group 4.41 ± 0.77 ng·kg/mL·mg, $P = 0.01$) plasma concentrations differed significantly between the different genotypes.

Moreover, we studied the association between the methadone dose and the methadone plasma levels with different genotypes in the responders and nonresponders. The results did not differ significantly in terms of *CYP2B6* and *CYP2C19* genetic variability, with the exception of the *ABCB1* G2677T genotype (Table 4). In the responders, the R,S-methadone (G/G group 5.20 ± 0.98 ng·kg/mL·mg versus G/T group 5.00 ± 0.72 ng·kg/mL·mg versus T/T group 5.97 ± 0.71 ng·kg/mL·mg, $P = 0.001$), R-methadone (G/G group 4.09 ± 0.83 ng·kg/mL·mg versus G/T group 4.15 ± 0.51 ng·kg/mL·mg versus T/T group 4.72 ± 0.72 ng·kg/mL·mg, $P = 0.01$), and S-methadone (G/G group 3.95 ± 0.76 ng·kg/mL·mg versus G/T group 3.93 ± 0.61 ng·kg/mL·mg versus T/T group 4.55 ± 0.75 ng·kg/mL·mg, $P = 0.01$) plasma concentrations were significantly different among the different genotypes, although the methadone dose did not differ significantly among the allele groups. This result suggested that *ABCB1* gene polymorphism may play an important role in the methadone plasma concentrations.

3.3. Factors Associated with Methadone Plasma Concentrations and Treatment Responses. Stepwise multiple linear regression analysis revealed that the methadone maintenance dose ($\beta = 0.0006$, $P = 0.001$), *CYP2B6* A785G ($\beta = 0.44$, $P = 0.03$), and *ABCB1* G2677T ($\beta = 0.37$, $P = 0.003$) were independent predictors determining the R,S-methadone plasma concentration (Table 5). These results showed that the methadone maintenance dose, *CYP2B6* A785G, and *ABCB1* G2677T were positively associated with the R,S-methadone plasma concentration.

Stepwise logistic regression analysis was performed to identify associations of variables with treatment responses. Adjusted analyses showed that significant correlates for nonresponders were HCV coinfection (adjusted odds ratio, AOR = 6.42, $P = 0.03$), psychiatric diseases (AOR = 2.71, $P = 0.02$), alcohol problems (AOR = 2.25, $P = 0.02$), and *CYP2B6* A785G (AOR = 1.74, $P = 0.10$). However, *ABCB1* G2677T (AOR = 0.48, $P = 0.05$) and the female gender (AOR = 0.44,

Table 1: Patient characteristics of responders and nonresponders to the Taiwan methadone maintenance treatment program (MMTP) based on urine morphine screen tests ($n = 178$).

Variables	Responders ($n = 62$)	Nonresponders ($n = 116$)	P value
Age, yr (SD)	39.1 (7.3)	39.6 (7.0)	0.67
Weight, kg (SD)	71.0 (14.5)	68.0 (12.1)	0.14
Gender, *n* (%)			
Male	50 (32.1)	106 (67.9)	0.03*
Education, *n* (%)			
Below high school	39 (36.4)	68 (63.6)	0.57
High school or above	23 (32.4)	48 (67.6)	
Marital status, *n* (%)			
Married or living with partner	14 (31.1)	31 (68.9)	0.65
Never married	36 (37.5)	60 (62.5)	
Divorced/widowed	11 (30.6)	25 (69.4)	
Dose, mg (SD)	51.6 (30.8)	50.4 (30.5)	0.78
R,S-Methadone, ng/mL (SD)	172.9 (134.9)	172.9 (158.8)	0.99
R-Methadone, ng/mL (SD)	92.9 (66.5)	92.9 (86.3)	0.99
S-Methadone, ng/mL (SD)	81.2 (70.6)	82.3 (74.8)	0.92
Substance use history, *n* (%)			
Tobacco	55 (36.7)	95 (63.3)	0.23
Alcohol	26 (44.8)	32 (55.2)	0.03*
Betel nut	21 (43.8)	27 (56.3)	0.15
Current amphetamine use, *n* (%)	10 (27.1)	27 (72.9)	0.52
Treatment duration, mo (SD)	19.4 (13.9)	17.4 (13.9)	0.37
Heroin use history, yr (SD)	7.8 (5.7)	8.5 (5.6)	0.48
HBV, *n* (%)	13 (39.4)	20 (60.6)	0.55
HCV, *n* (%)	60 (37.5)	100 (62.5)	0.03*
Pain disorders, *n* (%)	25 (39.1)	39 (60.9)	0.37
Depression, *n* (%)	11 (44.0)	14 (56.0)	0.29
Anxiety, *n* (%)	15 (46.9)	17 (53.1)	0.11
Psychiatric disorders[a], *n* (%)	15 (57.6)	11 (42.3)	0.01*
Methadone-drug interaction			
(1) Increase methadone metabolism, *n* (%)	17 (46.0)	20 (54.0)	0.11
(2) Decrease methadone metabolism, *n* (%)	9 (36.0)	16 (64.0)	0.89
(3) Benzodiazepines (BZD) or non-BZD, *n* (%)	18 (56.2)	14 (43.8)	0.007*
(4) Antipsychotic drugs, *n* (%)	2 (28.6)	5 (71.4)	0.72
(5) Opioid-related drugs, *n* (%)	12 (40.0)	18 (60.0)	0.53
(6) Antidepressive drugs, *n* (%)	5 (50.0)	5 (50.0)	0.32
(7) QT prolongation, *n* (%)	7 (35.0)	13 (65.0)	0.98

[a]Psychiatric disorders included schizophrenic disorders, hallucinosis, paranoia, panic disorders, and neurotic disorders.
*Statistical significance set at $P < 0.05$; comparisons were performed by the Mann-Whitney U test, t-test, Chi-square test, or Fisher's exact test as appropriate.

$P = 0.09$) were associated with a reduced odds of positive urine test with morphine (Table 6).

4. Discussion

A successful methadone maintenance treatment program (MMTP) for opioid-dependent users is associated with the optimal methadone dosage and methadone plasma concentrations. A number of factors related to the optimal methadone dosage and methadone plasma concentrations, including poly-substance use, concomitant diseases, MDIs, genetic polymorphisms in metabolism enzymes, *CYP2B6* and *CYP2C19*, and transporter proteins, *ABCB1*, were investigated comprehensively in our study. We observed that

Table 2: Frequencies of *CYP2B6*, *CYP2C19,* and *ABCB1* polymorphisms in responders and nonresponders to treatment ($n = 176$)[a].

Genotype	Responders ($n = 61$)	Nonresponders ($n = 115$)	P value
CYP2B6			
A785G (*4)			
A/A	29 (47.5)	72 (62.6)	
A/G	26 (42.6)	35 (30.4)	0.15
G/G	6 (9.9)	8 (7.0)	
G516T (*9)			
G/G	38 (62.3)	86 (74.8)	
G/T	19 (31.1)	27 (23.5)	0.10
T/T	4 (6.6)	2 (1.7)	
CYP2C19			
G651A (*2)			
G/G	27 (44.3)	63 (54.8)	
G/A	32 (52.5)	45 (39.1)	0.21
A/A	2 (3.2)	7 (6.1)	
G636A (*3)			
G/G	53 (86.9)	105 (91.3)	
G/A	6 (9.8)	10 (8.7)	0.14
A/A	2 (3.3)	0 (0)	
C3402T (*17)			
C/C	60 (98.4)	114 (99.1)	
C/T	1 (1.6)	1 (0.9)	0.64
T/T	0 (0)	0 (0)	
ABCB1 Genotype			
C1236T			
C/C	5 (8.2)	15 (13.0)	
C/T	30 (49.2)	49 (42.6)	0.53
T/T	26 (42.6)	51 (44.4)	
G2677T			
G/G	22 (36.1)	26 (22.6)	
G/T	21 (34.4)	63 (54.8)	0.03*
T/T	18 (29.5)	26 (22.6)	
C3435T			
C/C	30 (49.2)	39 (33.9)	
C/T	20 (32.8)	63 (54.8)	0.02*
T/T	11 (18.0)	13 (11.3)	

[a] No available data on genotype for two subjects (1 responder and 1 nonresponder) due to methodological problems.
* Statistical significance set at $P < 0.05$; comparisons were performed by the Chi-square test or Fisher's exact test as appropriate.

the methadone maintenance dose, the *CYP2B6* 785G allele, and the *ABCB1* 2677T allele have positive effects on the methadone plasma concentrations. Furthermore, a protective factor associated with treatment response was the ABCB1 2677T allele and the CYP2B6 785G allele, and the risk factors were HCV infection, alcohol problems, and diagnosis with a psychiatric disease.

Many Taiwan IDUs had an experience of sharing needles or dilution water, and the HIV prevalence among IDUs reached a peak in 2005 [35, 36]. However, many antiretroviral agents [18, 19] have been investigated as CYP3A4, 2B6, or 2D6 strong inducers or inhibitors. In order to avoid an influence of antiretroviral agents on the pharmacokinetics of methadone, we recruited non-HIV patients in this study. Therefore, the data derived from this study can provide more precise evidence in terms of predicting the methadone plasma concentrations and treatment responses.

Previous studies regarding *ABCB1* pharmacogenetics indicated that individuals with the 3-locus genotype pattern TT-TT-TT (C1236T, G2677T, and C3435T) have an approximately 5-fold chance of requiring a higher methadone dose [38]. Patients with C3435T alleles were more likely to require a higher methadone dose than noncarriers [31]. However, the genetic effects of P-gp on the methadone plasma concentration and treatment responses remain unclear.

In this study, we found that the *ABCB1* 2677TT allele has positive effects on methadone plasma concentrations and treatment responses. Subjects with mutations in C1236T, G2677T, or C3435T may have a lower P-gp expression or function at the BBB, such that the CNS exposure to methadone is increased, and a lower dose is required to prevent overdoses [24]. It has been shown that a synonymous SNP in C1236T is linked to G2677T and C3435T SNPs [39]. Thus, we observed that the effects of *ABCB1* G2677T on methadone plasma concentrations were similar to those of C1236T in this study. 1236T homozygous carriers needed lower maintenance doses (47.2 ± 24.7 mg) than heterozygous carriers (50.4 ± 28.3 mg) and noncarriers (67.9 ± 50.4 mg). The effects of *ABCB1* genetic polymorphism on the methadone dose in this study were also consistent with the results of Coller et al. [24], who showed that TT-TT (G2677T, C3435T) carriers (38.0 ± 16.8 mg) required a lower methadone dose than noncarriers (61.3 ± 24.6 mg). However, conflicting studies on the effect of *ABCB1* genetic variability on methadone dose and plasma concentrations have been published [20, 31]. Crettol et al. [20] showed that 2677TT carriers (2.75 ng/mL·mg) had a lower R,S-methadone plasma level than 2677GT carriers (3.23 ng/mL·mg) and 2677GG carriers (3.46 ng/mL·mg). Their study was conducted in 5 methadone dispensing centers in Geneva, Bern, Montreux, Lausanne, and Switzerland. Hung et al. [31] revealed that 3435T carriers have 2.58-fold to require higher methadone dose than noncarriers.

The *CYP2B6* 785GG allele also has positive effects on methadone plasma concentrations. The effects of *CYP2B6* genetic polymorphism on the methadone dose and methadone plasma concentrations in this study were consistent with the results of Levran et al. [38], who showed that Israeli Jewish subjects with 785GG (596.7 ng/mL) had a higher R,S-methadone plasma level than those with 785AA (514.9 ng/mL), while 785GG carriers (88.3 mg) had a lower methadone dose than 785AA (151.4 mg). Our study revealed that patients with 785GG (5.84 ng·kg/mL·mg) had a higher R,S-methadone plasma level than those with 785AA (5.35 ng·kg/mL·mg), while 785GG carriers (40.9 mg) had a lower methadone dose than 785AA (51.4 mg).

Table 3: Influence of *CYP2B6*, *CYP2C19,* and *ABCB1* polymorphism on methadone plasma concentrations ($n = 176$)[a].

Gene	*n* (%)	Dose (SD)	R,S-Methadone[b] (SD)	R-Methadone[b] (SD)	S-Methadone[b] (SD)
CYP2B6 genotype					
A785G (*4)					
A/A	101 (57.4)	51.4 (26.5)	5.35 (0.81)	4.32 (0.69)	4.15 (0.67)
A/G	61 (34.6)	52.6 (38.2)	5.22 (0.81)	4.21 (0.66)	4.05 (0.72)
G/G	14 (8.0)	40.9 (18.4)	5.84 (0.77)	4.60 (0.79)	4.52 (0.89)
P value	—	0.42	0.03*	0.15	0.08
G516T (*9)					
G/G	124 (70.5)	50.4 (24.9)	5.28 (0.82)	4.29 (0.67)	4.10 (0.67)
G/T	46 (26.1)	54.4 (43.1)	5.42 (0.76)	4.29 (0.70)	4.21 (0.78)
T/T	6 (3.4)	36.7 (19.7)	5.99 (0.99)	4.57 (0.99)	4.57 (1.02)
P value	—	0.38	0.09	0.63	0.22
CYP2C19 genotype					
G651A (*2)					
G/G	90 (51.1)	52.6 (35.2)	5.40 (0.79)	4.33 (0.74)	4.18 (0.78)
G/A	77 (43.8)	49.8 (24.9)	5.28 (0.86)	4.29 (0.62)	4.12 (0.64)
A/A	9 (5.1)	45.2 (26.8)	5.38 (0.68)	4.24 (0.72)	3.95 (0.62)
P value	—	0.71	0.66	0.90	0.60
G636A (*3)					
G/G	158 (89.8)	52.1 (31.3)	5.33 (0.83)	4.30 (0.69)	4.15 (0.70)
G/A	16 (9.1)	39.1 (20.9)	5.48 (0.70)	4.28 (0.64)	4.08 (0.87)
A/A	2 (1.1)	62.5 (31.8)	5.81 (0.13)	4.85 (0.16)	4.40 (0.66)
P value	—	0.24	0.55	0.52	0.81
C3402T (*17)					
C/C	174 (98.9)	49.9 (26.7)	5.35 (0.82)	4.30 (0.69)	4.15 (0.72)
C/T	2 (1.1)	140 (155.6)	4.55 (0.32)	4.18 (0.53)	3.79 (0.91)
T/T	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
P value	—	<0.0001*	0.08	0.81	0.47
ABCB1 genotype					
C1236T					
C/C	19 (10.8)	67.9 (50.4)	5.23 (0.66)	4.36 (0.52)	4.21 (0.48)
C/T	80 (45.5)	50.4 (28.3)	5.38 (0.82)	4.29 (0.73)	4.17 (0.72)
T/T	77 (43.7)	47.2 (24.7)	5.37 (0.85)	4.29 (0.68)	4.10 (0.76)
P value	—	0.02*	0.75	0.91	0.79
G2677T					
G/G	48 (27.3)	49.6 (39.4)	5.23 (0.86)	4.12 (0.76)	4.00 (0.68)
G/T	84 (47.7)	53.1 (27.3)	5.24 (0.73)	4.26 (0.62)	4.08 (0.67)
T/T	44 (25.0)	48.5 (25.5)	5.67 (0.86)	4.58 (0.65)	4.41 (0.77)
P value	—	0.68	0.008*	0.004*	0.01*
C3435T					
C/C	69 (39.2)	56.8 (39.3)	5.26 (0.87)	4.31 (0.69)	4.13 (0.63)
C/T	83 (47.2)	47.1 (21.6)	5.36 (0.74)	4.26 (0.71)	4.12 (0.76)
T/T	24 (13.6)	47.7 (26.9)	5.52 (0.91)	4.49 (0.58)	4.27 (0.78)
P value	—	0.13	0.41	0.36	0.63

[a]No available data on genotype for two subjects due to methodological problems.

[b]R,S-Methadone, R-methadone, and S-methadone plasma concentrations were divided by the methadone dose (in milligrams per day) and by the patient weight (in kilograms). The unit of concentration is ng·kg/mL·mg. Then, the values were natural log transformed before analysis. All values are expressed as the mean and standard deviation (SD).

*Statistical significance set at $P < 0.05$; comparisons were performed by the *t*-test, ANOVA, Mann-Whitney *U* test, or Kruskal-Wallis test as appropriate.

Table 4: Influence of *ABCB1* polymorphism on methadone plasma concentrations in responders and nonresponders ($n = 176$)[a].

ABCB1 genotype	n (%)	Dose (SD)	R,S-Methadone[b] (SD)	R-Methadone[b] (SD)	S-Methadone[b] (SD)
Responders ($n = 61$)					
G2677T					
G/G	22 (36.1)	47.6 (31.4)	5.20 (0.98)	4.09 (0.83)	3.95 (0.76)
G/T	21 (34.4)	61.3 (29.8)	5.00 (0.72)	4.15 (0.51)	3.93 (0.61)
T/T	18 (29.5)	47.1 (30.1)	5.97 (0.71)	4.72 (0.72)	4.55 (0.75)
P value	—	0.24	0.001*	0.01*	0.01*

[a]No available data on genotype for two subjects due to methodological problems.
[b]R,S-Methadone, R-methadone, and S-methadone plasma concentrations were divided by the methadone dose (in milligrams per day) and by the patient weight (in kilograms). The unit of concentration is ng·kg/mL·mg. Then, the values were natural log transformed before analysis. All values are expressed as the mean and standard deviation (SD).
*Statistical significance set at $P < 0.05$; comparisons were performed by the ANOVA or Kruskal-Wallis test as appropriate.

Table 5: Methadone maintenance dose and *CYP2B6* and *ABCB1* gene mutation relationships with R,S-methadone plasma concentration[a, b].

Variable	Regression coefficient (β)	95% CI	P value
Dose (mg)	0.0006	0.002–0.009	0.001*
CYP2B6 A785G			
A/G, G/G (versus A/A)	0.44	0.049–0.839	0.03*
ABCB1 G2677T			
G/T, T/T (versus G/G)	0.37	0.124–0.616	0.003*

[a]R,S-Methadone concentration was natural-logarithmically transformed to achieve a normal distribution.
[b]Results are from stepwise multiple linear regression analysis. Age, marital status, weight, current amphetamine use, treatment duration, drugs related to methadone-drug interactions, *CYP2B6* G516T (*9), *CYP2C19* G681A/C (*2), G636A (*3), C3402T (*17), *ABCB1* C1236T, and C3435T were not significantly associated. Only variables significantly contributing to the models are displayed (variables selected using mixed stepwise regression); P value for model: <0.0001.

Table 6: Variables significantly associated with nonresponders in the Taiwan methadone maintenance treatment program (MMTP)[a].

Variable	AOR[c]	95 % CI[d]	P value
Sex			
Female (versus male)	0.44	0.86–6.24	0.09
CYP2B6 A785G (*4)			
A/G, G/G (versus A/A)	1.74	0.89–3.46	0.10
ABCB1 G2677T/A			
G/T, T/T (versus G/G)	0.48	0.23–1.01	0.05
Psychiatric disorders[b]	2.71	1.10–6.53	0.02*
Alcohol use history	2.25	1.12–4.59	0.02*
HCV	6.42	1.14–121.3	0.03*

[a]Age, marital status, tobacco use, betel nut use, current amphetamine use, treatment duration, HBV, pain disorders, depression, anxiety, methadone maintenance dose, *CYP2B6* G516T, *CYP2C19* G681A/C, G636A, C3402T, *ABCB1* C1236T, and C3435T were not significantly associated. In the multiple logistic regression model, variables were selected using mixed stepwise regression; P value for model: 0.0002.
[b]Psychiatric disorders included schizophrenic disorders, hallucinosis, paranoia, panic disorders, and neurotic disorders.
[c]AOR, adjusted odds ratio.
[d]95% CI, 95% confidence interval.

Fonseca et al. [34] reported that the contributions to clinical treatment responses from *ABCB1*, *CYP2B6*, and *CYP2D6* genetic polymorphism are marginal. Nevertheless, these controversial results may be explained by different ethnicities or characteristics of participants.

We observed that 89.9% (160/178) of the patients were infected with HCV, but only 3.4% (6/178) of the patients received HCV therapy (ribavirin plus peginterferon alpha 2A or 2B). A very low proportion of MMTP patients receive HCV treatment [40]. HCV treatment with pegylated interferon-alfa plus ribavirin is often complicated by psychiatric side effects in patients with drug addiction because depression, anxiety, fatigue, flu-like syndromes, and irritability are typical interferon-alfa-associated adverse events. Patients can have an increased risk of discontinuing HCV treatment early in the first three months when most psychiatric adverse events appear and flu-like syndromes may be misunderstood as withdrawal syndromes [41]. However, MMTP clients who do not accept HCV treatment may also have similar uncomfortable feelings, such as fatigue, nausea, loss of appetite, muscle ache, flu-like symptoms, and depression, which may be mistaken as withdrawal syndromes as well. Relapse of illicit drug abuse may then follow. Thus, patients with HCV coinfection may have an increased risk to positive urine tests for morphine.

Poly-substance use, such as consumption of amphetamines, betel nut, cigarettes, and alcohol, has been found to be common among Taiwan MMTP patients [15], especially alcohol drinking [42]. A high proportion of Taiwanese MMTP patients have alcohol problems (31.4%) [42]; this is also true in other countries (41–52%) [43]. Alcohol problems have a negative effect on illicit opioid use. Coconsumption of methadone with alcohol is not only associated with road traffic crashes [44] but also related to an increased risk of relapsing into illicit drug use and discharge from the MMTP, particularly in females [45]. Therefore, alcohol problems among MMTP patients should be monitored closely.

Psychiatric comorbidity, such as schizophrenia, low mood, anxiety, hallucinosis, and panic disorders, often coexists in MMTP patients (78%) [46, 47]. Patients with psychiatric comorbidity may require a higher maintenance dose

(154 ± 84 mg) than patients (99 ± 49 mg) without psychiatric disorders [48]. About 35% of MMTP patients with concurrent psychiatric diseases were found to be regular or problem users of BZDs, and they were more likely to have opioid-positive urine screens during the MMTP [49]. In addition, concurrent psychiatric diseases may reduce quality of life in MMTP clients [47].

There was a tendency that the *CYP2B6* 785GG allele has negative effects on treatment responses (AOR = 1.74, $P = 0.10$). Previous studies [21, 32, 33] found that *CYP2B6* enzyme genetic polymorphisms were related to S-methadone metabolism, which often contributed to uncomfortable feelings and dissatisfaction with the MMTP [27]. Additionally, CYP2B6 slow metabolizers exhibit a reduced ability to metabolize S-methadone and were associated with an increased risk of a prolonged QT interval (OR = 4.5) [26], which may increase the risk of cardiac arrhythmias and sudden death.

The mean methadone maintenance dose in this study was 50.8 ± 30.5 mg, and in other studies Taiwan or Chinese MMTP patients ranged from 35 mg to 54.7 mg [50, 51]. However, the mean methadone maintenance dose was found to vary from 59.2 mg to 134 mg in Caucasians [20, 24, 32, 34], which is much higher than that in ethnic Chinese. The interethnicity or interindividual differences may result from genetic polymorphisms in metabolism enzymes and transporter proteins. We also detected *CYP2B6* C1459T genetic variability in our study and found that all MMTP clients were heterozygous carriers. The frequency of the *CYP2B6* 1459T allele in these Taiwan MMTP patients was about 50%, while it was 11.2% in Caucasians [20, 33]. This may be explained by patients with C1459T mutations having a significantly reduced *CYP2B6* protein expression, which decreases the enzymatic activity [52]. Hence, patients in Taiwan MMTPs may have a lower CYP2B6 metabolism enzyme activity and consequently require a lower methadone maintenance dose than Caucasians. The relationship between *CYP2B6* C1459T genetic polymorphisms and methadone maintenance dose among different ethnicities may be worthy of investigation in the future.

These results should be interpreted within the context of the following limitations. This study was conducted in southern Taiwan, and the results may not be generalized to other regions in Taiwan. In this study, we only recruited non-HIV patients, but some patients may use psychoactive drugs, which may interfere with methadone metabolic disposition. We also observed that psychoactive drugs or other drugs may not be key factors affecting on methadone plasma concentrations (Table 5). Finally, because MMTP patients may have different attitudes towards or habits related to the treatment of diseases, some may use over-the-counter (OTC) medications or Chinese herbal medicines to alleviate withdrawal symptoms. Therefore, the interactions of methadone with OTC drugs or Chinese herbal medicines may be underestimated among MMTP patients who used additional OTC medications or Chinese herbal medicines, and we did not consider these effects in assessing the methadone plasma concentrations and treatment responses.

In summary, we controlled some important factors, such as antiretroviral agents, which may affect the pharmacokinetics of methadone severely and confounded (or obscured) related variables that may have an impact on the methadone plasma concentrations and treatment responses. Therefore, the variables explored in this study can provide more precise evidence in predicting the methadone plasma concentrations and treatment responses.

The results of our study demonstrated that the methadone maintenance dose, *CYP2B6* 785G allele, and *ABCB1* 2677T allele have positive effects on the methadone plasma concentrations. Furthermore, a positive indicator of treatment responses is the *ABCB1* 2677T allele. In contrast, patients with HCV coinfection, alcohol problems, and psychiatric diseases may have negative treatment responses. Thus, the results suggest that a comprehensive evaluation of treatment responses in the MMTP should include not only genetic polymorphisms in methadone metabolism and transporter proteins, but also concomitant diseases, MDIs, and polysubstance use. This pilot study also provides clues that personalized medicine may play an important role in determining a better outcome of the MMTP.

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