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Title page

Genetic Variants of Human UGT1A3: Functional Characterization and Frequency Distribution in a Chinese Han Population

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Running title page

Running title: Function and Distribution of Human *UGT1A3* Variants

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Abbreviations used in the paper are: UGT1A3, UDP-glucuronosyltransferase 1A3; UDPGA, uridine diphosphate glucuronic acid; SNPs, single nucleotide polymorphisms; PCR, polymerase chain reaction; FBS, fetal bovine serum; PVDF, polyvinylidene fluoride; ASA, allele specific amplification.

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Abstract

UDP-glucuronosyltransferase1A3 (UGT1A3) contributes to glucuronidations of many important endogenous compounds and xenobiotics, including some flavonoids. Recently, a total of six single nucleotide polymorphisms (SNPs) have been identified in the human *UGT1A3* gene. Among them, four SNPs (A17G, $Q^{6}R$; T31C, $W^{11}R$; C133T, $R^{45}W$ and T140C, $V^{47}A$) cause amino acid substitutions. Variants caused by these SNPs showed an activity change in estrone metabolism, while their activities towards other substrates were not examined. In the present study, three common flavonoids, quercetin, luteolin and kaempferol, were used as substrates for glucuronidations by wild-type and variant UGT1A3s. Our results demonstrated that the activities of three variants, UGT1A3.2, UGT1A3.3 and UGT1A3.5, were remarkably lower than that of UGT1A3.1. In contrast, UGT1A3.4 exhibited an approximately four times increase in the glucuronidation efficiency and a clear preference to quercetin 7- and 3- hydroxyl groups. The frequency distributions of *UGT1A3* alleles and SNPs in *UGT1A3* in a Chinese Han population were statistically different from the reported value in German-Caucasian ($*p < 0.05$). UGT1A3 variants have an altered glucuronidation activity towards quercetin, luteolin and kaempferol, and may alter human susceptibility to flavonoids exposure.

Introduction

UDP-glucuronosyltransferase 1A3 (UGT1A3) is a phase II drug metabolism enzyme localized in the endoplasmic reticulum and the nuclear envelope (Radominska-Pandya et al., 1999). It catalyzes the conversion of hydrophobic endogenous compounds and xenobiotics to hydrophilic glucuronides and hence plays a major role in metabolism and detoxification (Miners and Mackenzie, 1991; Wells et al., 2004).

The *UGT* genes are highly polymorphic. Recently, Ehmer et al. (2004) and Iwai et al. (2004) have identified six novel single nucleotide polymorphisms (SNPs) in exon 1 of human *UGT1A3* gene (Fig. 1). Of these, four cause amino acid substitutions (A17G, $Q^{6}R$; T31C, $W^{11}R$; C133T, $R^{45}W$ and T140C, $V^{47}A$) and the remaining two are silent (G81A, $E^{27}E$ and A477G, $A^{159}A$). The missense variants showed a significant difference in their activity in the estrone metabolism (Iwai et al., 2004). Considering a wide substrate spectrum of UGT1A3 (Green et al., 1998) and the fact that the activity alteration for a given enzyme could be substrate-specific, it is worth conducting a further functional evaluation of these UGT1A3 variants with other important substrates.

Flavonoids are dietary antioxidants widely existed in fruits, vegetables, cereals, dry legumes, chocolate, and beverages (Scalbert et al., 2005). Experimental studies support an important role of flavonoids in the prevention of cardiovascular diseases and cancers (Galati et al., 2000; Kris-Etherton and Keen, 2002; Vita, 2005). Since glucuronidation is an essential pathway for the elimination and biological activity of flavonoids (da Silva et al., 1998), genetic polymorphisms of UGTs are potentially of toxicological and physiological

importance on the pharmacokinetics, pharmacologic effects and toxicity of flavonoids (Wittig et al., 2001). In the present study, wild-type and variant human UGT1A3s were expressed in a Bac-to-Bac baculovirus expression system. Three common flavonoids, quercetin, luteolin and kaempferol, were used as substrates to characterize their glucuronidating capacity. Regioselectivity in quercetin metabolism by these UGT1A3 variants was also observed. Finally, the frequency distribution of *UGT1A3* polymorphisms in a Chinese Han population was reported in this work.

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Methods

Materials. Polymerase chain reaction (PCR) primers and blood genomic DNA isolation kit were from Sangon (Shanghai, China). Restriction endonucleases, DNA molecular marker and T4 ligase were obtained from MBI Fermentas (Amherst, NY). pGEM-T plasmid was from Promega (Madison, WI). Mutanbest kit was obtained from Takara (Tokyo, Japan). Cellfectin® reagent, pFastBac1 vector, DH10Bac competent cells and Grace's medium were from Invitrogen (Calsbad, CA). *Spodoptera frugiperda* Sf9 insect cells were kind gifts from YangShengTang Pharmaceutical (Hangzhou, China). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Mouse anti-his antibody and peroxidase-conjugated goat anti-mouse secondary antibody were supplied by Amersham Biosciences (Uppsala, Sweden) and Zhongshan Biotechnology (Guangdong, China), respectively. The polyvinylidene fluoride (PVDF) membrane was acquired from Amresco (Solon, OH). Uridine diphosphate glucuronic acid (UDPGA), *d*-saccharic acid 1, 4-lactone monohydrate, Brij® 58, alamethicin and *β*-glucuronidase were provided by Sigma (St. Louis, MO). Quercetin, luteolin and kaempferol were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China)，and their purities were 99%.

Construction of UGT1A3 variants. All five reported UGT1A3 enzymes were constructed in the present study: UGT1A3.1, UGT1A3.2, UGT1A3.3, UGT1A3.4 and UGT1A3.5. A *UGT1A3*1* gene had been obtained in our laboratory previously, with an *Afl* II site and a *Xho* I site at two sides of coding sequence and a his-tag ahead of the stop codon. In this research, using pGEM-T- *UGT1A3*1* as the template (Chen et al., 2005a), *UGT1A3*

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alleles were generated by site-directed mutagenesis following the instruction of Mutanbest kit. Oligonucleotide primers for introducing nucleotide transversions are shown in Table 1. To ensure that no mistake happened during the mutagenesis, all PCR products were verified by DNA sequencing.

Expression of UGT1A3s. Expression of UGT1A3s was performed in an insect Bac-to-Bac system as we described previously (Chen et al., 2005a). In brief, the *UGT1A3* gene was excised from the pGEM-T vector, subcloned into the pFastBac1 vector and transformed into *Escherichia coli* DH10Bac. PCR with *UGT1A3*-specific primer and M13 primer was used to confirm the transposition of *UGT1A3* into the Bacmid. The minipreparation of Bacmid-*UGT1A3* was transfected into Sf9 cells. After 72 h, the first passage of baculovirus was harvested from supernatant and titrated by viral plaque assay. Infection conditions were optimized for the subsequent amplification or expression. Infected cells were collected by centrifugation and disrupted by sonication. Cell homogenate was quantified by Genequant (Amersham Biosciences Inc, Piscataway, NJ) at 280 nm and stored at -80°C until further analysis. Transfection with an untransposed bacmid was performed as a negative control. All five UGT1A3 enzymes were expressed in the same condition.

Western blot. To ascertain the relative expression level of each UGT1A3 enzyme, a semiquantitative western blot method was established using the UGT1A3.1 preparation as a standard. The cell homogenate of each UGT1A3 in the same loading amount was separated by a 12% SDS-polyacrylamide gel electrophoresis, transferred onto a PVDF membrane and probed with the mouse anti-his antibody (1:3000). A peroxidase-conjugated goat anti-mouse

antibody (1:5000) was used as the secondary antibody. Immunocomplexes were exposed to 3, 3'-diaminobenzidine detection solution for 10 min. The relative expressions of UGT1A3s were measured with a universal hood image system (Bio-Rad laboratories，Segrate，Italy) using Quantity One 1-D analysis software. To optimize the loading amount, the linear range of western quantification was determined by assaying UGT1A3.1 homogenates at different concentrations.

Glucuronidation assay. Some preliminary experiments were performed to determine the optimal incubation condition. The effects of pH value (7.0, 7.4, 7.8, 8.0, 8.2 and 8.6), *β*-glucuronidase inhibition reagent (*d*-saccharic acid 1, 4-lactone monohydrate) and de-latency reagents (alamethicin and Brij® 58) on the glucuronidation assay were evaluated. A typical incubation mixture (100 µl of total volume) contained 100 mmol/L Tris-HCl buffer, pH 8.0, 10 mmol/L $MgCl_2$, 2 mmol/L UDPGA (The UDPGA apparent K_m is 200 µmol/L ~ 300 µmol/L with the various UGT isoforms as previously determined (Green et al., 1998)), 15 µg/mg pro alamethicin, 5 mmol/L *d*-saccharic acid 1, 4-lactone monohydrate, 0.5 mg/ml cell homogenate and 0.1 mmol/L flavonoids. The reaction was initiated by adding UDPGA, incubated at 37°C and terminated with 290 µl of methanol. After adding an internal standard (morin for quercetin, quercetin for kaempferol, and kaempferol for luteolin), the mixture was centrifuged to remove precipitated protein. Twenty-five microliters of supernatant were injected into a LC-10A HPLC system (Shimadzu, Kyoto, Japan), equipped with two LC-10AD pumps, a UV detector and a Diamonsil™ C18 column (5 µm particle size, 250 mm × 4.6 mm). Data acquisition and integration were performed using a HS2 000

chromatography workstation. The mobile phase was used at a flow rate of 1 ml/min, with 52%, 52% or 54% methanol (volume percentage) in 0.02 mol/L phosphoric acid (pH 2.0) for quercetin, luteolin and kaempferol, respectively. Peaks were detected at 368 nm for quercetin, 350 nm for luteolin and 365 nm for kaempferol. Glucuronides were identified with an Agilent 1 100 LC-MS system (Agilent Technologies, Palo Alto, CA) equipped with an electrospray ionization source and operated in a negative ion mode. The enzymatic kinetic assay of each UGT1A3 enzyme was conducted under conditions linear with time and protein concentration. Kinetic parameters were calculated from three independent expressions according to the Lineweaver Burk equation. The detailed concentration of each tested flavonoid is listed in Tables 3 ~ 5.

Regioselectivity of UGT1A3s to quercetin. Regioselectivity of UGT1A3 variants to quercetin was measured using enzymetic kinetic parameters according to our previous work (Chen et al., 2005a). In brief, each monoglucuronide was separated and collected from the HPLC effluent and quantified by determination of the amount of quercetin released after hydrolysis. The identities of the monoglucuronides were determined with UV spectra in methanol and some diagnostic reagents (sodium methoxide solution and unmelted sodium acetate solution).

DNA isolation. One hundred and twenty-five unrelated healthy Chinese Han adults were included in this study. All these recruitments were from the clinical laboratory in the Second Affiliated Hospital of Zhejiang University. The research protocol was approved by the Ethics Committee of Zhejiang University. An aliquot of 2.5 ml of venous blood was drawn from

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each volunteer into a tube containing 0.3 ml of sodium citrate and stored at -80°C before DNA isolation. Genomic DNA was extracted with a UNIQ-10 column blood genomic DNA isolation kit following manufacturer's specifications, and quantified by measuring absorbance at 260 nm and 280 nm with a GeneQuant spectrophotometer (Amersham Biosciences Inc, Piscataway, NJ). Typical yields of DNA were 15 ng/µl ~ 25 ng/µl, and A260/A280 ratios were 1.7 ~ 1.8. Samples were stored in the Tris-EDTA buffer (pH 8.0) at -20°C until the further analysis.

Distributions of *UGT1A3* alleles and SNPs in *UGT1A3* in a Chinese Han population. Two-step allele specific amplification (ASA) was developed for mutation detections. At the first step, genomic DNA was amplified for 20 cycles with two *UGT1A3*-specific primers: AAGAAAGCAAATGTAGCAGGC/TACCTTATTTCCCACCCACTTC. Ten PCR products were chosen randomly for sequencing and as positive controls for the second step. The second amplification was performed following an allele specific procedure. For each SNP in *UGT1A3*, two parallel reactions were performed. A pair of allele-specific primers (Table 2) was designed and added into two parallel PCR reactions, respectively. PCR was performed in 20 µl of reaction mixture containing 1.75 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.3 µmol/L each primer (Table 2), 0.1 µl of product of the first step, 0.5 U of *Taq* DNA polymerase. After an initial denaturation at 94°C for 2 min, the DNA was amplified by 25 cycles of 20 s at 94°C and 60 s at an optimized temperature (Table 2). All PCR processes were performed on a gradient mastercycler (Eppendorf, Hamburg, Germany). Special attention was paid to keep the same load and reaction conditions for two parallel PCR reactions. After PCR, amplification products were electrophoresed on a 1.5% agarose gel containing ethidium

bromide. The images were scanned and analyzed with a universal hood gel image system (Bio-Rad laboratories, Segrate, Italy). Predicted lengths of fragments are also shown in Table 2. For the second step, ten positive controls (sequenced samples) and two negative controls (reactions containing no template) were performed. Analysis result of each example was agreed by two individual operations.

Data analysis. SNPs frequencies were calculated by gene counting. Student's *t* test was used for statistical comparisons. A value of $*p < 0.05$ was considered to be statistically significant. All tests in the present study were two-sided.

Results

Expression of UGT1A3s. In the present study, five *UGT1A3* sequences were obtained by mutagenesis and confirmed by sequencing. Their amino acid sequences correspond to those of all known UGT1A3s in population: UGT1A3.1, UGT1A3.2, UGT1A3.3, UGT1A3.4 and UGT1A3.5. To investigate the catalytic activity of the UGT1A3 variants *in vitro*, we established a Bac-to-Bac insect expression system, which has been used for functional characterizations of many *UGT* alleles (Jinno et al., 2003) and applied in our laboratory for expressions of *UGT1A3*, *UGT1A4*, *UGT1A6*, *UGT1A9* and *UGT2B7* (Chen et al., 2005a; Qian et al., 2004; Zheng et al., 2004). The optimized expression conditions were 5 of multiplicity of infection value and 72 h of infection time. Typical concentrations of cell homogenates were 15 mg/ml ~ 18 mg/ml. Expressions were finally confirmed by western blot. We used anti-his as the primary antibody, so the immunoreactivity of UGT1A3s will not alter with mutations. The linear range of western quantification was 0.2 mg ~ 0.5 mg of cell homogenate. We blotted one sample of each UGT1A3 on the same membrane. Three individual membranes were measured and confirmed. According to DNAstar software (DNASTAR Inc. Madison, MI), molecular weight of UGT1A3 was 60 kD. Protein bands in approximate 60 kD were detected in all expression models except the negative control transfected with blank bacmid. As shown in Fig. 2, expression levels of UGT1A3 variants were 0.782 to 1.186 times as many as that of UGT1A3.1. These data were used for the normalization of glucuronidation activity.

Glucuronidation of UGT1A3s. At the pH 8.0, the glucuronidation catalyzed by recombinant enzymes reached its maximal velocity, and then showed a plateau. Alamethicin

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showed a positive effect on the glucuronidation at the concentration of 12 μg/mg pro ~ 30 μg/mg pro, while Brij® 58 had no detectable effect on it. In addition, *d*-saccharic acid 1, 4-lactone monohydrate was used to inhibit the *β*-glucuronidase activity in the incubation, although few *β*-glucuronidase activities were detected in sf9 preparation, which is different from the liver microsome (O'Leary et al., 2001). The glucuronidations catalyzed by UGT1A3.1 produced more than one monoglucuronide for each tested flavonoid. All products were confirmed by LC-MS. Glucuronidations were proportional to the concentration of cell homogenate up to 1.2 mg/ml. At 0.5 mg/ml, the reaction was linear with time up to 90 min. The apparent K_m, V_{max} and catalytic efficiency (V_{max}/K_m) showed in Table 3 ~ Table 5 represent a composite of all glucuronides formed using quercetin, luteolin or kaempferol as substrate. Glucuronidation activities of 100, 11, 23, 464 and 77% to quercetin were observed for UGT1A3.1, UGT1A3.2, UGT1A3.3, UGT1A3.4 and UGT1A3.5, respectively. It suggests statistically significant differences among five UGT1A3s ($*p < 0.05$). The UGT1A3.4 also improved the function in glucuronidation of luteolin and kaempferol, with a relative efficiency (V_{max}/K_m) approximately four times of UGT1A3.1. While UGT1A3.2 and UGT1A3.3 led to a considerable decrease of glucuronidation to less than 20%. It explained the reason why only one glucuronide was identified in our previous activity assay of quercetin with UGT1A3.2 (Chen et al., 2005b). Compared with UGT1A3.2 and UGT1A3.3, UGT1A3.5 showed a relative mild decrease in glucuronidation efficiency for all tested flavonoids.

It is interesting that regioselectivities of UGT1A3s to hydroxyl groups in quercetin were also significantly different (Table 6). UGT1A3.3 showed an obvious preference to

3'-glucuronide. It catalyzed 3'- glucuronidation with a more than five times efficiency of 7-glucuronidation. UGT1A3.2, UGT1A3.4 and UGT1A3.5, produced much less 4'-glucuronide and 3'- glucuronide than 7- glucuronide and 3- glucuronide. Especially for the latter two, almost no 4'- glucuronide and 3'- glucuronide were detected after incubation.

Distributions of *UGT1A3* alleles and SNPs in *UGT1A3* in a Chinese Han population. The 125 genomic DNA samples from whole blood were screened by ASA to examine the distribution frequency of *UGT1A3* alleles and SNPs in *UGT1A3* in a Chinese Han population (Table 7 and Table 8). Sequences of ten positive controls were identical with the results of sequencing. The prevalences of T31C, G81A and T140C mutants were detected in 26.8%, 26.8%, and 10.4% of donors, respectively, which are significantly different from those reported for German-Caucasian ($*p < 0.05$) (Ehmer et al., 2004). German-Caucasian has much higher distribution frequencies (65% to T31C, 65% to G81A and 58% to T140C) than those in a Chinese Han population. While other three SNPs (A17G, C133T and A477G) reported in Japanese (Iwai et al., 2004) but not in German-Caucasian were also detected in the Chinese Han population. Significant distribution differences of *UGT1A3* alleles were observed between German-Caucasian and Chinese Han population except *UGT1A3*3a*. It reveals the types and incidences of *UGT1A3* alleles and SNPs in *UGT1A3* have significant race-differences. Such difference is relatively weak between Japanese and the Chinese Han population, with C133T has a significant difference ($*p < 0.05$) in the SNPs distribution and *UGT1A3*4* shows a statistically significant frequency difference between Chinese Han population and Japanese. In the previous report (Iwai et al., 2004), the 477 substitution is always found to be together with other two SNPs (T31C and G81A) in the Japanese

population, while some subjects without 477 substitution also show 31 and 81 mutations in a Chinese Han population. It leads to the significant distribution difference of *UGT1A3*2a* and *UGT1A3*3a* between Chinese Han population and Japanese.

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Discussion

Genetic polymorphisms have been reported for several functional human *UGT* genes (Maruo et al., 2005), including *UGT1A1* (Bosma, 1995; Maruo et al., 1999), *UGT1A3* (Ehmer et al., 2004; Iwai et al., 2004), *UGT1A4* (Ehmer et al., 2004), *UGT1A6* (Ciotti et al., 1997), *UGT1A7* (Guillemette et al., 2000), *UGT1A8* (Huang et al., 2002), *UGT1A10* (Jinno et al., 2003), *UGT2B4* (Levesque et al., 1999), *UGT2B7* (Jin et al., 1993) and *UGT2B15* (Levesque et al., 1997). These genetic variants may cause different phenotypes and play an important role in drug efficacy and xenobiotic toxicity, as well as in hormonal regulation and pathogenesis. For example, mutant *UGT1A1* alleles lead to inherited diseases of bilirubin metabolism (Ritter et al., 1992; Bosma et al., 1994; Labrune et al., 2002). *UGT1A6*, *UGT1A7*, *UGT1A9*, UG*T2B7* and *UGT2B15* polymorphisms have been proposed for toxicological and clinical significance (Bhasker et al., 2000; Vogel et al., 2001; Miners et al., 2002; Carlini et al., 2005; Chung et al., 2005). Novel *UGT1A10* polymorphisms are associated with orolaryngeal carcinoma risk (Elahi et al., 2003). Recently, in German-Caucasian and in a Japanese population, six *UGT1A3* SNPs have been identified (Ehmer et al., 2004; Iwai et al., 2004). Some UGT1A3 variants show significantly different activities in estrone metabolism. However, their activities towards other substrates have not been determined. Since flavonoids are common constituents of foods of plant origin and display positive effects on many important diseases, such as cardiovascular diseases and cancers (Galati et al., 2000; Kris-Etherton and Keen, 2002; Vita, 2005), three wide existing flavonoids were used in this study as the substrates for glucuronidations to increase understanding on *UGT1A3* variants.

Although statistically significant differences in catalytic activities of UGT1A3s to

flavonoids were observed in the present study, results are not the same as reported observations for estrone glucuronidation (Iwai et al., 2004). The UGT1A3 variants clearly showed different activity alteration depending on substrates. UGT1A3.5 exhibited a mild decrease in both flavonoids and estrone. UGT1A3.3 had a mild increase in estrone glucuronidation but a considerable decrease in flavonoids glucuronidation. A distinct activity difference was observed in UGT1A3.2 and UGT1A3.4. The former, having a 369% activity in estrone metabolism, showed a decreased activity in the metabolism of flavonoids to approximate 10%. The latter showed an approximate four times glucuronidation efficiency to flavonoids of UGT1A3.1, with a decreased activity to 70% to estrone. These results indicate that amino acid residues at positions of 45 and 47 are in a key region of UGT1A3 protein. Polymorphism researches on UGT1A4, which shares more than 93% similarity in DNA sequence with UGT1A3 (Green et al., 1998), also indicated such an assumption. A $L^{48}V$ UGT1A4 mutant lost completely dihydrotestosterone glucuronidation activity ((Ehmer et al., 2004) and gave twice the efficiency for clozapine as the wild-type UGT1A4. Its efficiencies for some important clinical drugs, trans-androsterone, imipramine, cyproheptadine and tigogenin, also changed (Mori et al., 2005). Attention should be paid to mutations in this region in the structural and functional research of *UGT1A3*.

It is of interest to observe a regioselectivity variation in quercetin metabolism by the UGT1A3 variants. It has been reported that different quercetin glucuronides showed different free radical scavenging, antioxidant, antithrombotic and anti-carcinogenic activities (Moon et al., 2001; Silva et al., 2002). The quercetin 7-glucuronide and 3-glucuronide were the main active forms exerting the physiological activity *in vivo* (Morand et al., 1998). In the

previous study, we have described that UGT1A3.1 played its minor role in quercetin physiological activities for preferring low-activity 3'- and 4'- glucuronides to high-activity 7-glucuronide and 3- glucuronide (Chen et al., 2005a). In the present work, our data showed that three variants (UGT1A3.2, UGT1A3.4 and UGT1A3.5) had much higher glucuronidation efficiency to 7- and 3- hydroxyl groups of quercetin than the other hydroxyls. Among these variants, the UGT1A3.4 may alter susceptibility to quercetin exposure, because UGT1A3.4 was a 464% total glucuronidation efficiency of UGT1A3.1 and preferably produced high-activity 7-glucuronide with the efficiencies more than ten times of that to 3'- and 4'-hydroxyls.

Finally, we determined the frequency distributions of *UGT1A3* alleles and SNPs in *UGT1A3* in a Chinese Han population. A significant race-difference of distributions was noted. *UGT1A3*2*, leading to decreased flavonoids glucuronidations, has a lower frequency in Chinese Han than in German-Caucasian, while *UGT1A3*4*, a key mutant in producing more high-activity quercetin glucuronides, distributes more widely in Chinese Han population than in German-Caucasian, but significantly lower than in Japanese. Chinese Han may have a different susceptibility to flavonoids exposure from the Caucasian and Japanese, and hence affect human susceptibility to cancer and cardiovascular diseases according to beneficial effects of flavonoids to the prevention of these diseases (Galati et al., 2000; Kris-Etherton and Keen, 2002; Vita, 2005).

The present study demonstrated a significant difference in catalytic activities of the UGT1A3s to quercetin, luteolin and kaempferol, and was the first time to determine the

frequencies of *UGT1A3* alleles and several *UGT1A3* SNPs in Chinese Han subjects. The UGT1A3.4 showed a 464% increase in total glucuronidation efficiency and a clear preference to producing quercetin glucuronides with high biological activity. The statistically different distribution frequency of this variant allele in a Chinese Han population indicates that Chinese Han may have a different susceptibility to flavonoids exposure from the Caucasian and Japanese. The clinical relevance of these genetic variants of UGT1A3 remains to be assessed.

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Footnotes

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Legends to figures:

Fig. 1. Seven *UGT1A3* alleles caused by SNPs.

Fig. 2. Western blot of UGT1A3 recombinant enzymes expressed in Sf9 cells. 0.3 mg of cell homogenate was obtained from transfected Sf9 cells, separated on a 12% SDS-polyacrylamide gel electrophoresis, transferred onto a PVDF membrane filter and probed with the mouse anti-his antibody (1:3000). A peroxidase-conjugated goat anti-mouse antibody (1:5000) was used as the secondary antibody. Immunocomplexes were exposed to 3, 3'-diaminobenzidine detection solution for 10 min. The relative expressions of UGT1A3s were measured with a universal hood image system (Bio-Rad laboratories，Segrate，Italy) using Quantity One 1-D analysis software. The results are expressed as a relative percentage compared with UGT1A3.1. Each bar represents the scanning analysis result of expression level of above UGT1A3 enzyme. The results are indicated as mean ± S.D. of three independent experiments.

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Tables

TABLE 1 *Oligonucleotide primers for site-directed mutagenesis using UGT1A3*1 - pGEM-T as template*.

Mutant nucleotides are indicated in italic and bold.

SNPs	Forward primer	Reverse primer
A17G	C***G***GGTTCCCCTGCCG	GAGTCCTGTGGCCATCTTAAG
T31C	TCCCCTGCCG***C***GGC*C*GGCCA	ACCTGGAGTCCTGTGGCCAT
C133T	GCATG***T***GGGAGGTCTTGCGGGA	TGAGCCAGTGGCTGCCATCAAT
T140C	ATGCGGGAGG***C***CTTGCGGGAG	GCTGAGCCAGTGGCTGCCAT

TABLE 2 *ASA conditions for detection of each SNP in UGT1A3.*

SNPs	Allele specific primer	Allele nonspecific primer	Annealing and extension temperature (°C)	Length of product (bp)
17	AGATGGCCACAGGACTCCA AGATGGCCACAGGACTCCG	AGCTCCACACAAGACCTATGATA	68.5	391
31	CAGTCCTGAGGCCAGCCA CAGTCCTGAGGCCAGCCG	TGAGCACAGGGTCAGACGTG	69.5	289
81	CCACCAACACCTTTCCACTC CCACCAACACCTTTCCACTT	TGAGCACAGGGTCAGACGTG	68.0	339
133	CCCGCAAGACCTCCCG CCCGCAAGACCTCCCA	TGAGCACAGGGTCAGACGTG	68.3	388
140	CTCAGCATGCGGGAGGC CTCAGCATGCGGGAGGT	AGCTCCACACAAGACCTATGATA	69.5	268
470	CGTTAACCTCTGCGCGGCA CGTTAACCTCTGCGCGGCG	GAAGACCATGTTGGGCATGATT	72.8	387

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TABLE 3 *Kinetic parameters of UGT1A3 enzymes to quercetin (n=3).*

Assay was performed at 37°C for 10 min using 0.5 mg/ml protein. The concentrations of quercetin were 3.9 µmol/L ~ 100 µmol/L. The results are indicated as the mean ± S.D. from three independent expression experiments.

The K_m and V_{max} presented in this table represent a composite of all glucuronides formed using quercetin as substrate. V_{max} and V_{max}/K_m are normalized by expression levels of recombinant UGT1A3 enzymes.

UGT1A3 enzymes	K_m (µmol/L)	V_{max} (pmol/min/mg)	V_{max}/K_m (µl/mg/min)	% of UGT1A3.1 (%)
UGT1A3.1	39.40 ± 2.10	1974 ± 65	50.19 ± 1.42	100
UGT1A3.2	16.34 ± 1.35	90.30 ± 1.19	5.55 ± 0.38	11
UGT1A3.3	39.56 ± 1.64	450.9 ± 7.0	11.41 ± 0.30	23
UGT1A3.4	21.00 ± 1.82	4877 ± 237	233.0 ± 15.9	464
UGT1A3.5	11.05 ± 1.00	427.6 ± 31.6	38.76 ± 1.23	77

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TABLE 4 *Kinetic parameters of UGT1A3 enzymes to luteolin (n=3).*

Assay was performed at 37°C for 10 min using 0.5 mg/ml protein. The concentrations of luteolin were 5.8 µmol/L ~ 120 µmol/L. The results are indicated as the mean ± S.D. from three independent expression experiments.

The K_m and V_{max} values presented in this table represent a composite of all glucuronides formed using luteolin as substrate. V_{max} and V_{max}/K_m are normalized by expression levels of recombinant UGT1A3 enzymes.

UGT1A3 enzymes	K_m (µmol/L)	V_{max} (pmol/min/mg)	V_{max}/K_m (µl/mg/min)	% of UGT1A3.1 (%)
UGT1A3.1	35.60 ± 0.94	1595 ± 42	44.69 ± 0.40	100
UGT1A3.2	27.80 ± 1.85	142.8 ± 10.3	5.13 ± 0.04	12
UGT1A3.3	29.30 ± 2.11	150.2 ± 4.6	5.24 ± 0.27	12
UGT1A3.4	33.27 ± 1.79	6825 ± 533	206.1 ± 27.2	461
UGT1A3.5	33.00 ± 1.84	446.8 ± 48.1	13.50 ± 0.64	30

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TABLE 5 *Kinetic parameters of UGT1A3 enzymes to kaempferol (n=3).*

Assay was performed at 37°C for 10 min using 0.5 mg/ml protein. The concentrations of kaempferol were 5.8 µmol/L ~ 120 µmol/L. The results are indicated as the mean ± S.D. from three independent expression experiments.

The K_m and V_{max} values presented in this table represent a composite of all glucuronides formed using kaempferol as substrate. V_{max} and V_{max}/K_m are normalized by expression levels of recombinant UGT1A3 enzymes.

UGT1A3 enzymes	K_m (µmol/L)	V_{max} (pmol/min/mg)	V_{max}/K_m (µl/mg/min)	% of UGT1A3.1 (%)
UGT1A3.1	37.06 ± 1.60	999.0 ± 48.1	27.04 ± 3.66	100
UGT1A3.2	39.90 ± 1.30	93.80 ± 3.41	2.35 ± 0.03	9
UGT1A3.3	37.97 ± 0.51	102.6 ± 9.1	2.70 ± 0.21	10
UGT1A3.4	23.90 ± 1.90	2532 ± 235	105.8 ± 4.5	391
UGT1A3.5	38.37 ±1.14	644.2 ± 46.9	16.80 ± 0.90	62

TABLE 6 *Regioselectivity of UGT1A3 enzymes to quercetin (n=3).*

V_{max} and V_{max}/K_m are normalized by expression levels of recombinant UGT1A3s. Relative catalytic efficiency of each UGT1A3 enzyme is indicated as % of 7-glucuronide.

	UGT1A3.1(Chen et al., 2005a) [a]		UGT1A3.2		UGT1A3.3		UGT1A3.4		UGT1A3.5	
Monogluc uronide	V_{max}/K_m (µl/mg/min)	% of 7-gluc uronide	V_{max}/K_m(µl/mg/min)	% of 7-gluc uronide	V_{max}/K_m(µl/mg/min)	% of 7-glucuronide	V_{max}/K_m(µl/mg/min)	% of 7-glucuronide	V_{max}/K_m(µl/mg/min)	% of 7-gluc uronide
7-glucuro nide	8.310 ± 0.87	100	2.574 ± 0.17	100	1.214 ± 0.13	100	66.60 ± 5.87	100	20.32 ± 0.42	100
3-glucuro nide	10.51 ± 0.61	126	2.073 ± 0.17	84	1.327 ± 0.05	110	158.7 ± 10.8	238	16.26 ± 0.90	80
4′-glucuro nide	9.216 ± 0.14	111	0.423 ± 0.04	16	1.908 ± 0.10	158	5.866 ± 0.27	9	1.330 ± 0.02	7

3′-glucuronide	22.01 ± 0.90	265	0.461 ± 0.02	18	6.929 ± 0.34	573	1.067 ± 0.10	2	0.787 ± 0.07	4

[a] The data of UGT1A3.1 have been previously reported (Chen et al., 2005a).

TABLE 7 *Distribution of SNPs in UGT1A3 in a Chinese Han population.*

SNPs	Wild-type	Mutant	Amino acid change	Frequency in a Chinese Han population (n = 250)	Frequency in Japanese (n = 200; Iwai et al., 2004) [a]	Frequency in German-Caucasian (n = 162; Ehmer et al., 2004) [a]
17	A	G	$Q^{6}R$	0.044	0.055	-
31	T	C	$W^{11}R$	0.268	0.280	0.650 [b]
81	G	A	$E^{27}E$	0.268	0.280	0.650 [b]
133	C	T	$R^{45}W$	0.056	0.110 [b]	-
140	T	C	$V^{47}A$	0.104	0.125	0.580 [b]
477	A	G	$A^{159}A$	0.208	0.280	-

[a] Frequencies in Japanese and German-Caucasian were published by Iwai et al. (Iwai et al., 2004) and Ehmer et al. (Ehmer et al., 2004), respectively.

[b] Statistically different ($p < 0.05$) versus frequency in a Chinese Han population, two-side student's t test.

TABLE 8 *Distribution of UGT1A3 alleles in a Chinese Han population.*

UGT1A3 alleles	Nucleotide change	Amino acid change	Frequency in a Chinese Han population (n = 250)	Frequency in Japanese (n = 200; Iwai et al., 2004) [a]	Frequency in German-Caucasian (n = 162; Ehmer et al., 2004) [a]
*UGT1A3*1*	-	-	0.676	0.610	0.350 [b]
*UGT1A3*2a*	$T^{31}C/G^{81}A/T^{140}C$	$W^{11}R/E^{27}E/V^{47}A$	0.012	-	0.580 [b]
*UGT1A3*2b*	$T^{31}C/G^{81}A/T^{140}C/A^{477}G$	$W^{11}R/E^{27}E/V^{47}A/A^{159}A$	0.092	0.125	-
*UGT1A3*3a*	$T^{31}C/G^{81}A$	$W^{11}R/E^{27}E$	0.048	-	0.070
*UGT1A3*3b*	$T^{31}C/G^{81}A/A^{477}G$	$W^{11}R/E^{27}E/A^{159}A$	0.072	0.100	-
*UGT1A3*4*	$C^{133}T$	$R^{45}W$	0.056	0.110 [b]	-
*UGT1A3*5*	$A^{17}G/T^{31}C/G^{81}A/A^{477}G$	$Q^{6}R/W^{11}R/E^{27}E/A^{159}A$	0.044	0.055	-

[a] Frequencies in Japanese and German-Caucasian were published by Iwai et al. (Iwai et al., 2004) and Ehmer et al. (Ehmer et al., 2004), respectively.

[b] Statistically different ($p < 0.05$) versus frequency in a Chinese Han population, two-side student's t test.

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Fig.1

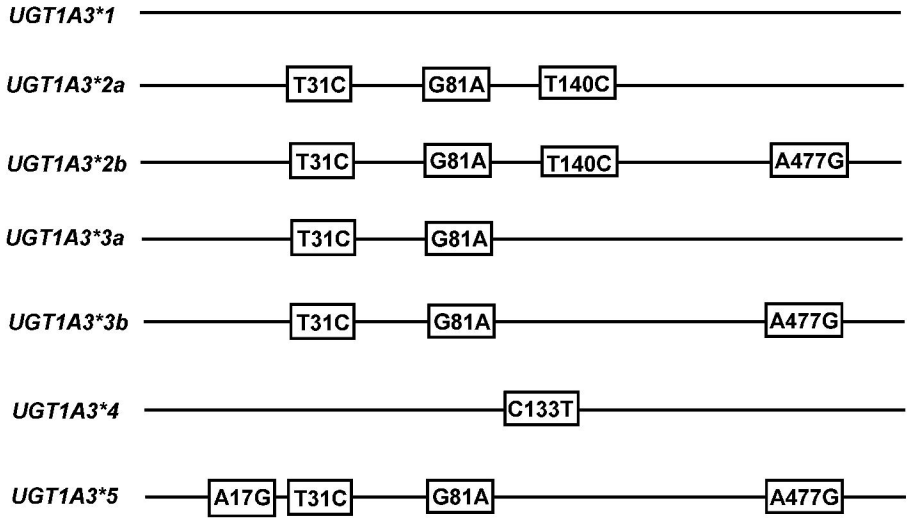

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Fig.2