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Differential allelic expression of c.1568C>A at *UGT2B15* is due to variation in a novel *cis*-regulatory element in the 3′UTR

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ABSTRACT

Differential allelic expression (DAE) is a powerful tool to identify *cis*-regulatory elements for gene expression. The UDP-glucuronosyltransferase 2 family, polypeptide B15 (UGT2B15), is an important enzyme involved in the metabolism of multiple endobiotics and xenobiotics. In the present study, we measured the relative expression of two alleles at SNP c.1568C>A (rs4148269) in this gene, which causes an amino acid substitution (T523K). An excess of the C over the A allele was consistently observed in both liver ($P=0.0021$) and breast ($P=0.012$) samples, suggesting that SNP(s) in strong linkage disequilibrium (LD) with c.1568C>A can regulate *UGT2B15* expression in both tissues. By resequencing, one such SNP, c.1761T>C (rs3100) in 3′ untranslated region (UTR), was identified. Reporter gene assays showed that the 1761T allele results in a significantly higher gene expression level than the 1761C allele in HepG2, MCF-7, LNCaP, and Caco-2 cell lines (all $P<0.001$), thus indicating that this variation can regulate *UGT2B15* gene expression in liver, breast, colon, and prostate tissues. Considering its location, we postulated that this SNP is within an unknown microRNA binding site and can influence microRNA targeting. Considering the importance of UGT2B15 in metabolism, we proposed that this SNP might contribute to multiple cancer risk and variability in drug response.
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1. Introduction

Glucuronidation is an important clearance pathway for many endogenous and exogenous molecules, including steroid hormones, bile acid, carcinogens and clinical drugs (King et al., 2000; Tukey and Strassburg, 2000; Belanger et al., 2003). This reaction can transfer the glucuronic acid from UDP glucuronic acid to appropriate substrates, which can make them more water soluble and more easily excreted through the biliary and renal systems than their parent compound, and is catalyzed by UDP-glucuronosyltransferase (UGT) family in human body (King et al., 2000; Guillemette, 2003; Mackenzie et al., 2005). In this family, UGT2B15 (Chen et al., 1993; Turgeon et al., 2000) has particular importance due to its relatively high expression level (Ohno and Nakajin, 2009) and activity (Turgeon et al., 2001), especially on steroid hormones and multiple clinical agents (Tukey and Strassburg, 2000; Court et al., 2004). The *UGT2B15* expression is mainly observed in liver, breast, prostate, and colon (Gardner-Stephen and Mackenzie, 2008; Nakamura et al., 2008; Ohno and Nakajin, 2009). Considering that steroid hormones play a central role in multiple cancers and that UGT2B15 is essential in the metabolism of steroid hormones, it has long been proposed that this gene is involved in breast (Sparks et al., 2004) and prostate (MacLeod et al., 2000; Hajdinjak and Zagradisnik, 2004; Park et al., 2004) cancer risk (Nagar and Remmel, 2006).

Differential allelic expression (DAE, or allelic imbalance, AI) has been shown to be a robust and accurate way to identify *cis*-regulatory elements (Pastinen and Hudson, 2004; Stamatoyannopoulos, 2004; Yan and Zhou, 2004; Bray and O'Donovan, 2006). Recently, by studying DAE at the c.253G>T (relative to translation start, rs1902023) site, two tissue-specific *cis*-regulatory elements for *UGT2B15* were identified in the promoter region (Sun et al., 2010). Besides c.253G>T, there are other coding region variants in this gene, such as c.1568C>A (rs4148269) (Iida et al., 2002), which causes a T523K amino acid substitution but is not likely to influence enzyme activity, at least on oxazepam (Court et al., 2004). However, the DAE based on this variation has not been investigated so far.

MicroRNA (miRNA) is a group of endogenous, noncoding, and small (~22 nt in mature type) RNA molecule (Bartel, 2004). It has been established that miRNA is involved in a broad range of physiological processes, including cell proliferation, differentiation, apoptosis, signal transduction, viral infection, and tissue morphogenesis (including myogenesis, cardiogenesis, hematopoiesis, etc.), and in the development of various human diseases, especially cancer (Esquela-Kerscher and Slack, 2006; Kloosterman and Plasterk, 2006; Bushati and Cohen, 2007; Chang and Mendell, 2007). In most cases,

Abbreviations: UGT, UDP-glucuronosyltransferase; DAE, differential allelic expression; AI, allelic imbalance; miRNA, microRNA; 3′UTR, 3′ untranslated region; LD, linkage disequilibrium; CNV, copy number variation.

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miRNA exerts its function through binding to mRNA 3′ untranslated region (UTR) and negatively regulating gene expression by suppressing translation or cleaving mRNA (Bartel, 2004; Filipowicz et al., 2008; Flynt and Lai, 2008; Ghildiyal and Zamore, 2009). Therefore, some polymorphisms in miRNA target sites, i.e., 3′UTR in mRNA, can influence the miRNA–mRNA interactions and have been observed to associate with phenotype variability, such as muscularity in sheep (Clop et al., 2006), asthma (Tan et al., 2007), stroke (Chen et al., 2010), chondrodysplasia (Simon et al., 2010), and Tourette's syndrome (Abelson et al., 2005) risk. However, no *cis*-regulatory element in 3′ UTR and potential miRNA interaction has been reported in the UGT gene family so far.

In the present study, we investigated the allele specific expression marked by the c.1568C>A coding variant. A relative excess of the C allele was observed in both liver and breast samples, thus suggesting the presence of *cis*-regulatory variation in linkage disequilibrium (LD) with c.1568C>A. Re-sequencing of the *UGT2B15* exon 6 identified one such SNP in nearby region. By comparing luciferase activity for different plasmid constructs, we verified that this SNP could affect *UGT2B15* gene expression. Considering the position of this SNP, we proposed that the regulation might result from alteration of the affinity of an unknown miRNA.

2. Materials and methods

2.1. Tissue samples, RNA and DNA extraction, and genotyping

Thirty-one normal liver (3 European American [CA] and 1 African American [AA], 27 unknown) and 81 normal breast (4 CA and 8 AA, 69 unknown) tissue samples were retrieved from the University of Chicago Tissue Core Facility. RNA and DNA were extracted by RNeasy Lipid Tissue and QIAamp DNA Mini Kit (Qiagen, Valencia, CA), respectively. cDNA was synthesized by High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Genotype of c.1568C>A polymorphism was determined by a Taqman genotyping assay C_9440184_20 (Applied Biosystems) according to the manufacturer's protocol. Among all tissues, 13 liver (6 female and 7 male) and 30 breast (28 female, 1 male and 1 unknown) samples were heterozygous for the c.1568C>A variant.

2.2. DAE

Allele specific real time PCR was performed with the same abovementioned Taqman assay in cDNA sample from c.1568C>A heterozygous individuals. Each Taqman probe, which is specific for one allele, was labeled by a different dye and fluorescence was detected on a StepOne Plus Realtime PCR System (Applied Biosystems). For normalization, a heterozygous genomic DNA sample was serially diluted as standard. The real time PCR was performed in triplicate for each sample and the AI ratio was expressed as $A_{\text{quantity}}/C_{\text{quantity}}$. The robustness and sensitivity of this method has been fully described in our recent study (Sun et al., 2010).

2.3. Resequencing

Fifty-six unrelated HapMap samples (24 Yoruba in Ibadan [YRI], 22 CEPH Collection [CEU] and 10 Han Chinese in Beijing or Japanese in Tokyo [ASN]) were chosen for resequencing. Amplification of *UGT2B15* exon 6 was performed by using the primer pair 5′-TGGCTAAAGTAAAA-CAAAAAT-3′ and 5′-CTTACTTATAGCACTTAGAA-3′. After exonuclease I and Shrimp Alkaline Phosphatase (United States Biochemicals, Cleveland, OH) treatment, sequencing was performed by using internal primers 5′-TGCATCCAGTAACTCGTCATT-3′ and 5′-TTTTCAAAGACCATC-CATAG-3′ and BigDye Terminator v3.1 (Applied Biosystems). Polymorphisms were scored by PolyPhred (Stephens et al., 2006) and confirmed visually. Visual genotype and LD were determined by using the Genome Variation Server (http://gvs.gs.washington.edu/GVS/). F_{ST} (Wright, 1950; Weir and Cockerham, 1984) was calculated by Slider (http://genapps.uchicago.edu/labweb/index.html).

2.4. Plasmid construction

The full 3′UTR region of *UGT2B15* (from positions 1594 to 2065 relative to the translation start site) was amplified by nested PCR from individuals with specific haplotypes. The first round of PCR was performed by using the above PCR primers and the second round by using primers 5′-CAGTC-TCTAGA-TTATATCAAAAGCCTGAAGTG-3′ and 5′-CAGTC-GGATCC-TTTTTATGGCTTGGATGACA-3′, which introduced restriction sites for *Xba*I and *BamH*I (New England Biolabs, Ipswich, MA), respectively. PCR was performed by iProof High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA) to avoid artificial mutations. After digestion, the segment was fused with pGL3-Promoter vector (the original 3′UTR of the vector was removed by the same enzymes, Promega, Madison, WI). Both plasmids were sequenced to rule out PCR errors and to verify the haplotype orientation before transfection.

2.5. Tissue culture

Human hepatocellular carcinoma cell line HepG2 was cultured in minimum essential medium (MEM, ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). Human breast adenocarcinoma cell line MCF-7 was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) with 10% FBS and 0.1% insulin (Sigma, St. Louis, MO). Human prostate carcinoma cell line LNCaP and colon adenocarcinoma cell line Caco-2 were maintained in DMEM with 10% FBS.

2.6. Transient transfection

Cells (10^5) were seeded into a 24-well plate 24 h before transfection. Plasmid constructs (1.9 μg DNA) were transfected by using FuGene HD (Roche, Indianapolis, IN) according to the manufacturer's recommendations. Plasmid pRL-TK (0.1 μg DNA; Promega) was co-transfected as an internal control. Thirty-six hours after transfection, cells were harvested and luciferase activity was read by Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The result was expressed as the ratio between firefly and *Renilla* luciferase. Six replicates were performed for each experiment.

2.7. Statistical analyses

To investigate whether DAE deviated from the null expectation, one-sample *t*-test was performed. Shapiro–Wilk test was utilized to determine whether the allele intensity ratio follows normal distribution. The DAE difference in c.253G>T genotype and gender groups, and luciferase result for different alleles was compared by independent two-tailed *t*-test. The correlation between DAE and *UGT2B17* copy number variation (CNV) or age was determined by linear regression, in which age of donors (coded as a continuous integer) or CNV (coded as 0, 1, and 2 for homozygous of deletion, heterozygous, and homozygous of non-deletion, respectively) was set as an independent variable and the DAE value as dependent. All analyses were performed by SPSS 15.0 (SPSS Inc., Chicago, IL) and the null hypothesis was rejected when $P<0.05$.

3. Results and Discussion

As shown in Fig. 1a, except for one individual (7.7%), all liver samples showed an excess of C allele over the A allele and consequently a significant deviation from 1:1 ratio was observed (minimum, 0.25; median, 0.75; maximum, 1.15; mean ± standard deviation [SD], 0.73 ± 0.25; 95% confidence interval [CI], 0.32–0.85, *t*-test, $P=0.0021$). In

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