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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.

Keywords

UGT2B15; Differential allelic expression; *cis*-regulation; gene expression; 3′untranslated region

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

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Conflict of interest statement

The authors declare no conflicts of interest.

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, 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 were 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.

Materials and methods

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.

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 $A_{quantity}/C_{quantity}$. The robustness and sensitivity of this method has been fully described in our recent study (Sun et al., 2010).

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′-TGGCTAAAGTAAAACAAAAAT-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′-TTTTCAAAGACCATCCATAG-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) were calculated by Slider (http://genapps.uchicago.edu/labweb/index.html).

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.

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.

Transient transfection

Cells (10^5) were seeded into a 24-well plate 24 hours 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.

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 $P< 0.05$.

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 breast, most individuals (76.7%) also showed the same pattern (minimum, 0.33; median, 0.85; maximum, 1.56; mean ±SD, 0.88 ± 0.25; 95% CI, 0.66–0.91, *t*-test, P=0.012, Fig 1b). The observed allele intensity ratio did not violate normal distribution in either liver (P=0.325) or breast (P=0.611). On average, the proportion of C allele was 17.6–212.5% and 9.9–51.5% higher than that of A allele in liver and breast samples, respectively. These results indicated that c.1568C>A variant, or other SNPs in LD with it, influence *UGT2B15* expression levels in both tissues.

A recent study found that one or more promoter SNPs in LD with c.253G>T can modify *UGT2B15* expression (Sun et al., 2010). To investigate whether these SNPs can influence DAE at c.1568C>A, we classified the individuals in our sample into heterozygotes (n=8 in liver and 16 in breast) and homozygotes of either allele for c.253G>T (n=5 in liver and 14 in breast) (Sun et al., 2010) and compared the mean of the ratio of the two alleles at c.1568C>A by an independent *t*-test. No significant difference was observed between these two groups in both liver (P=0.67) and breast (P=0.46), thus suggesting that DAE at c.1568C>A is likely to be independent on c.253G>T status or that our test does not have sufficient power.

It has been proposed that *UGT2B17* CNV can influence *UGT2B15* expression (Jakobsson et al., 2008). To test whether *UGT2B17* CNV can influence *UGT2B15* DAE at c.1568C>A, we retrieved the *UGT2B17* CNV genotype data for the current samples from our recent study (Sun et al., 2010; Sun et al., 2011). We found 0, 7, and 6 liver and 2, 16, and 12 breast

samples that are homozygous of deletion, heterozygous, and homozygous of non-deletion, respectively. When linear regression was performed, no correlation was observed in either liver (r=0.103, P=0.737) or breast (r=0.126, P=0.506), thus indicating that *UGT2B17* CNV does not influence *UGT2B15* c.1568C>A DAE. However, this conclusion should be interpreted with caution since multiple groups have small sample size.

A recent study has also showed that *UGT2B15* is differentially expressed in female and male in liver (Sun et al., 2011). To test whether gender contributes to c.1568C>A DAE, we compared the DAE between gender groups. The mean±SD for DAE was 0.81±0.27 in male while 0.64±0.21 in female. Consequently, no significant difference was observed in liver (t-test, P=0.24).

It has been observed that *UGT2B15* expression in liver increases with age (Sun et al., 2011). In our liver sample group, the age varies from 32–79 years, with mean±SD 58.4±13.4 and median 57. For our breast cohort, the minimum, median, maximum age, and mean±SD was 15, 42, 62, and 38.3±13.1 years, respectively. To test whether age influences c.1568C>A DAE, we performed linear regression and no significant result was obtained in liver (r=0.215, P=0.481) and breast (r=0.098, P=0.613).

Although our sample size was modest, which might limit the power of our test, all above evidence indicated that c.1568C>A DAE is likely to be independent of c.253G>T genotype, *UGT2B17* CNV, gender, and age. To investigate whether DAE is affected by the combination of all these factors, we further performed multiple regression and failed to observed any significant correlation (P>0.39 and >0.18 for all variables in liver and breast, respectively). These analysis suggests that c.1568C>A or SNP(s) in LD with it can alter *UGT2B15* expression liver and breast. Because c.1568C>A lies in the coding region, it could influence *UGT2B15* expression by altering the mRNA secondary structure and stability, as previously reported for other genes (Nackley et al., 2006). To explore this possibility, we used the software RNA Mfold (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=mfold#) (Mathews et al., 1999; Zuker, 2003) to predict and compare the secondary structure induced by these two alleles. For all predictions for each haplotype, the Gibbs free energy (dG) fluctuated around 570kcal/mol, thus indicating there was not much difference between their predicted mRNA structures. Therefore, it was more likely that the observed DAE should be attributed to some other nearby SNP(s).

To uncover additional SNPs in LD with c.1568C>A, we resequenced the *UGT2B15* exon 6 (Fig 2). The derived allele, 1568A, occurs at lower frequency in CEU (27%) compared to ASN and YRI (90%, Table S1). Besides c.1568C>A, our resequencing survey identified additional 10 SNPs (see Fig 2 and Table S1). Only one SNP, c.1761T>C (rs3100) in 3′UTR region, showed nearly complete LD with c.1568C>A (r^2=0.814, 1, and 1 in YRI, CEU, and ASN, respectively; result not shown). Considering our DAE result, it could be postulated that c.1761T>C can affect *UGT2B15* expression and that the T allele was correlated with a higher expression level than C allele. The remaining 9 SNPs showed a relative low minor allele frequency (<15%; see Fig 2 and Table S1) and low LD (all r^2<0.27; result not shown) with c.1568C>A.

To investigate the *cis*-regulatory potential of c.1761T>C, we constructed a luciferase plasmid containing *UGT2B15* 3′UTR with two different alleles and transfected them into HepG2 and MCF-7 cell lines, which have been frequently used to examine the function of 3′UTR variants (Tan et al., 2007; Huang and Li, 2009; Chen et al., 2010; Simon et al., 2010). The relative luciferase activity of the plasmid with *UGT2B15* 3′UTR was only ~4.5% and ~12.3% (result not shown) of that of pGL3-promoter (empty) vector in HepG2 and

MCF-7 cells, respectively, thus confirming that *UGT2B15* 3′UTR can dramatically reduce gene expression. On the other hand, the luciferase activity of the plasmid with *UGT2B15* 3′UTR was 3–150 thousand times higher than background (empty tube, result not shown) and 3–16 times higher than pGL3-basic plasmid (result not shown), thus indicating that our assay could be used to distinguish the effect of two alleles. When the two alleles were compared, the T allele showed ~14.3% (*t*-test, $P<10^{-4}$, Fig 3a) and ~21.5% (*t*-test, $P<0.001$, Fig 3b) higher activity than C allele in HepG2 and MCF-7, respectively, consistent with the hypothesis that 1761T allele up-regulates *UGT2B15* expression in both tissues.

We also transfected the plasmid into LNCaP and Caco-2 cell lines in order to examine whether this SNP can modify *UGT2B15* expression in prostate and colon, respectively. Similar to the results in HepG2 and MCF-7, the relative luciferase activity of the plasmid with *UGT2B15* 3′UTR accounted for only ~11.1% and ~18.4% of that of pGL3-promoter vector in LNCaP and Caco-2 cell lines, respectively. When the two alleles were compared, the T allele showed ~61.5% (*t*-test, $P<10^{-4}$, Fig 3c) and ~31.6% (*t*-test, $P<10^{-5}$, Fig 3d) higher luciferase activity than C allele in LNCaP and Caco-2 cell lines, respectively. These results strongly suggest that c.1761T>C can alter *UGT2B15* expression in all four tissues.

Since this SNP is located within the 3′UTR, the most plausible mechanism is that it alters miRNA binding affinity. To identify the putative miRNA, we used TargetScan (http://www.targetscan.org/) (Lewis et al., 2005) and MicroInspector (http://bioinfo.uni-plovdiv.bg/microinspector/) (Rusinov et al., 2005) to predict the interaction between *UGT2B15* mRNA and known miRNAs. However, no miRNA target site was obtained in vicinity of c.1761T>C. Considering the fact that the full miRNA profile is unlikely to be known, we hypothesized that c.1761T>C exerts its function through an unknown miRNA. Since c.1761T>C is functional in all four tissues, this miRNA is hypothesized to be ubiquitously expressed. Moreover, the luciferase activity difference between T and C allele varied markedly, from 14% to 61%, among four tissues. This phenomenon, if it is not due to noise in the experimental measurement, could be attributed to differences in abundance of this putative miRNA among tissues.

Owing to the importance of UGT2B15, the associations between the genetic polymorphisms in this gene and drug response or cancer risk have been repeatedly investigated (as reviewed by (Desai et al., 2003; Guillemette, 2003; Maruo et al., 2005; Nagar and Remmel, 2006)). However, the role of T1761C in these phenotypes has never been tested. Our results point to the c.1761T>C variant as a strong candidate risk factor for hormone-dependent diseases such as breast and prostate cancer and for inter-individual variability in drug metabolism and response, especially in populations of European ancestry due to the high minor allele frequency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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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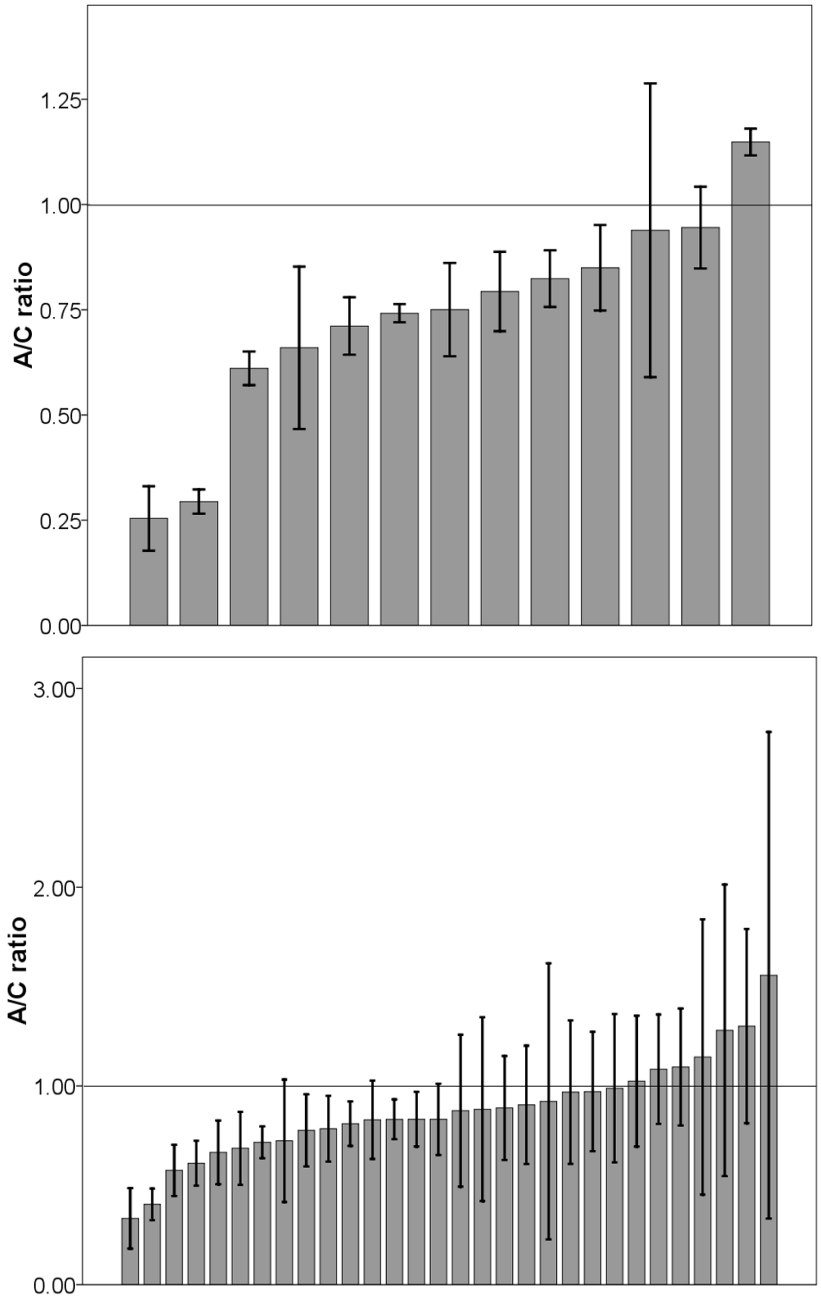

Fig 1.

The ratio of A/C alleles at C1568A in liver (a) and breast (b) samples. Each bar represents one individual and data is expressed as mean ± standard error (SE).

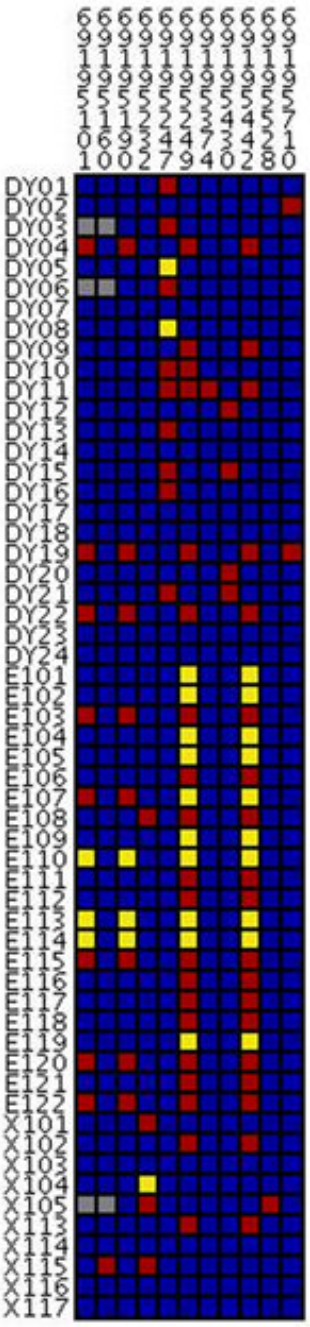

Fig 2.

Visual genotype of the *UGT2B15* exon 6 region. Each column indicates one SNP while each row denotes one individual. Blue, red, yellow, and grey represent homozygous of common allele, heterozygous, homozygous of rare allele, and missing data, respectively. DY, E, and X indicate YRI, CEU, and ASN HapMap populations, respectively. All positions refer to the genome sequence (build 36) for chromosome 4.

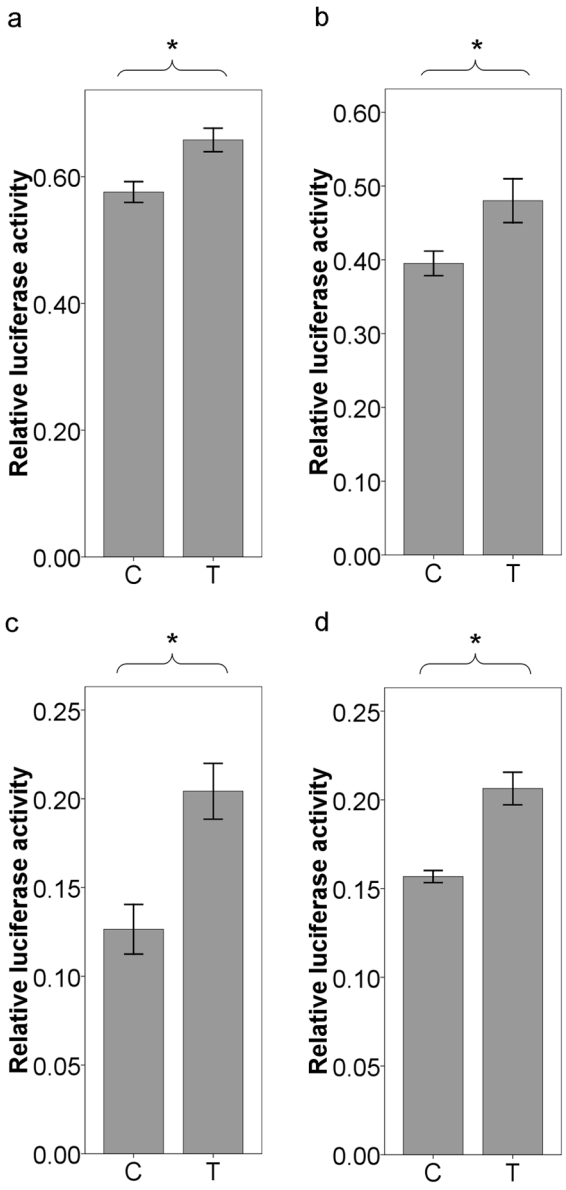

Fig 3.

Transient transfection of plasmid constructs with different rs3100 alleles in HepG2 (a), MCF-7 (b), LNCaP (c), and Caco-2 (d). Data is expressed as mean ± SE. *P<0.001 (t-test).