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UCRL-JRNL-207090

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October 7, 2004

Mutation Research

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Delores J. Grant[1,2], Ingrid J. Hall [3], David A. Eastmond [4], Irene M. Jones[5], Douglas A. Bell [1]*

[1] Environmental Genomics Section, Laboratory of Computational Biology and Risk Assessment, National Institute of Environmental Health Sciences.

[2] Cancer Research Program, JLC-Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, NC, USA;

[3]Division of Cancer Prevention and Control, National Center for Chronic Disease Prevention and Health Promotion, Centers for Disease Control and Prevention, Atlanta, Georgia, GA, USA;

[4]Environmental Toxicology Graduate Program UC-Riverside, University of California, Riverside CA.

[5]Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, Livermore, CA

*To whom correspondence should be addressed, NIEHS, C3-03, P.O. Box 12233, Research Triangle Park, NC 27709, USA, 919-541-7686, BELL1@niehs.nih.gov

Abstract

A dinucleotide repeat polymorphism (5-, 6-, 7-, or 8-TA units) has been identified within the promoter region of UDP-glucuronosyltransferase 1A1 gene (*UGT1A1).* The 7-TA repeat allele has been associated with elevated serum bilirubin levels that cause a mild hyperbilirubinemia (Gilbert's syndrome). Studies suggest that promoter transcriptional activity of *UGT1A1* is inversely related to the number of TA repeats and that unconjugated bilirubin concentration increases directly with the number of TA repeat elements. Because bilirubin is a known antioxidant, we hypothesized that *UGT1A1* repeats associated with higher bilirubin may be protective against oxidative damage. We examined the effect of *UGT1A1* genotype on somatic mutant frequency in the hypoxanthine-guanine phosphoribosyl-transferase (HPRT) gene in human lymphocytes and the glycophorin A (GPA) gene of red blood cells (both N0, NN mutants), and the frequency of lymphocyte micronuclei (both kinetochore (K) positive or micronuclei K negative) in 101 healthy smoking and nonsmoking individuals. As hypothesized, genotypes containing 7-TA and 8-TA displayed marginally lower GPA_NN mutant frequency relative to 5/5, 5/6, 6/6 genotypes ($p<0.05$). In contrast, our analysis showed that lower expressing *UGT1A1* alleles (7-TA and 8-TA) were associated with modestly increased *HPRT* mutation frequency ($p<0.05$) while the same low expression genotypes were not significantly associated with micronuclei frequencies (K-positive or K-negative) when compared to high expression genotypes (5-TA and 6-TA). We found weak evidence that *UGT1A1* genotypes containing 7-TA and 8-TA were associated with increased GPA_NØ mutant frequency relative to 5/5, 5/6, 6/6 genotypes ($p<0.05$). These data suggest that *UGT1A1* genotype may modulate somatic mutation of some types, in some cell lineages, by a mechanism not involving bilirubin antioxidant activity. More detailed studies examining *UGT1A1* promoter variation, oxidant/antioxidant balance and genetic damage will be needed.

Keywords: *UGT1A1*, genotypes, repeat polymorphisms, somatic mutation, HPRT, Glycophorin A, micronuclei

I. Introduction

The human UDP-glucuronosyltransferase 1A1 (*UGT1A1*) gene is one of thirteen genes at the *UGT1A* gene locus on chromosome 2 (*UGTA1-UGT1A13p*) that produces an enzyme that catalyzes the glucuronidation of bilirubin as part of the normal catabolism of free heme (1, 2). Serum bilirubin levels are elevated in individuals who have a TA repeat polymorphism in the promoter region (3, 4) or a Gly71Arg polymorphism in the first exon (5, 6, 7) of the gene. Individuals with seven TA (7-TA) repeats in the *UGT1A1* promoter have elevated serum bilirubin levels that cause a mild hyperbilirubinemia (Gilbert's syndrome), but individuals with the more common six TA (6-TA) repeats do not have this condition (3, 4). In some cases, the 7-TA polymorphism as well as other mutations has also been associated with severe (Crigler-Najjar type II disease) hyperbilirubinemia (5, 7, 8, 9).

Stocker and coworkers (10) demonstrated that bilirubin had *in vitro* antioxidant properties by inhibiting the formation of a hydrogen peroxide derivative. More recently, these properties have also been observed *in vivo* with bilirubin bound to albumin as it is found in the circulation (11). Oxidative damage caused by the action of reactive oxygen species (ROS) may impact DNA and other cellular macromolecules resulting in physiological changes that lead to disease (12). The production of specific DNA lesions has been associated with a wide variety of cancers including prostate, colon, and breast (12, 13, 14). However, ROS are essential mediators of many cellular functions such as antimicrobial phagocytosis, detoxification reactions by liver detoxification enzymes, and apoptosis (15). Therefore an intricate balance must be achieved between beneficial concentrations of an antioxidant and the generation of ROS. Several studies demonstrated that elevated bilirubin levels in human blood contributed to protection from oxidative damage by various agents (16, 17, 18, 19, 20).

Over the past several years, two new *UGT1A1* TA repeat promoter polymorphisms, 5-TA and 8-TA, have been identified primarily in individuals of African descent (14, 21, 22, 23). Recent experimental work demonstrated that the four different promoters (5-TA, 6-TA, 7-TA, 8-TA) have transient transcriptional activity that is inversely related to the number of TA repeats (21, 22). In contrast, concentrations of unconjugated serum bilirubin found in the circulation have been shown to increase

directly with the number of TA repeat elements (24, 25). Hence, the *UGT1A1* repeat polymorphisms are predictive of serum bilirubin concentration and subsequently the *UGT1A1* genotypes may be indicative of susceptibility to or protection against oxidative damage mediated through modulation of bilirubin levels.

Recent studies suggest a link between the *UGT1A1* gene promoter polymorphisms, estrogen hormone metabolism, and cancer (22, 26); however there have been no published reports examining the association between *UGT1A1* promoter polymorphisms and antioxidant activity or damage from oxidants. To explore whether the *UGT1A1* TA repeat polymorphisms could influence levels of somatic mutation, which may, in part, be due to oxidative damage, we examined the effect of *UGT1A1* TA repeat polymorphisms in smoking and nonsmoking individuals on several measures of somatic genetic damage. Genetic damage was assayed by measuring somatic cell mutant frequencies of five genotoxic endpoints: mutants at the hypoxanthine-guanine phosphoribosyl-transferase (HPRT) and glycophorin A genes (both N mutants and NN mutants), and micronuclei (both Mn kinetochore (K) plus or Mn K minus). Because the mutant frequencies of *HPRT* and glycophorin A and induction of micronuclei have been associated with oxidative damage (27, 28, 29) and genotoxic effects by anti-cancer drugs (30), we hypothesized that the higher expressing *UGT1A1* alleles (5-TA and 6-TA) and presumed lower bilirubin levels, would generally be associated with more somatic genetic damage.

II. Materials and Methods

Subjects

A convenience sample from a previously described study population (31, 32) was genotyped for this study. In summary, a community-based sample of healthy, unrelated (19-53 yr old) volunteers from Durham and Chapel Hill, North Carolina were recruited by newspaper advertisement. Blood samples were acquired from African-American and Caucasian subjects with informed consent and the study was carried out under a NIH/NIEHS approved human subject protocol (OH86-E-0037). Race, age, and smoking status of study subjects were determined by self-report.

The investigation of the genotoxic markers and *UGT1A1* genotyping was accomplished using a subset (n=101) of this population for whom sufficient DNA was available. DNA was extracted from peripheral blood using conventional phenol-chloroform methods (33). All 101 samples were genotyped for *UGT1A1* polymorphism and analyzed for *HPRT* mutation frequency. Results from two smaller subsets, previously analyzed for glycophorin A mutant frequency and micronuclei frequency, were also assessed for association with *UGT1A1* genotype.

Determination of *UGT1A1* Promoter Alleles

Fragments, 252-258 bp in length, were amplified by PCR using fam (fluorescent phosphoramidite) labeled primers to analyze with GeneScan (ABI Perkin-Elmer, Foster City, CA). Two sets of primers, famUGTA-F and UGTA-R; or famUGTA R and UGTA-F, were used in this analysis (Table 1) (4). The PCR mix contained 1X NEB (21.95 mg/ml ammonium sulfate, 0.08% BSA, 0.070 mM EDTA, 0.67 M Tris, pH 8.8, 0.35% b-mercaptoethanol), 2.5 mM $MgCl_2$, 0.8 mM dNTP mixture, and 0.833 pmol of each primer (forward and reverse), 0.75 U of *Taq* polymerase (Promega, Madison, WI), and 100 ng of genomic DNA. After 4 min of denaturing at 94^{o}C, the samples were amplified with 32 cycles at 94^{o}C for 30 sec, 60^{o}C for 30 sec, and 72^{o}C for 30 sec followed by a 4 min extension at 72^{o}C. The products were diluted 1:20 with sterile water and electrophoresed on a denaturing polyacrylamide gel (6%) on an ABI 373 Automated Sequencer with XL upgrade. The sequencing of PCR-amplified genomic samples was performed by direct automated DNA sequencing (ABI Perkin-Elmer, Foster City, CA). Primers 641F, 1029F, 1453F, 471R, 895R, 1353R and 1681R2 were used for the automated sequencing (Table 1).

Mutant Frequency Analysis

Genetic damage, evaluated by measuring five genotoxic endpoints (hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) mutant frequency, micronucleus (kinetochore positive and kinetochore negative) frequency, and glycophorin A (N and NN) mutant frequency), was assessed as described below.

HPRT Mutant Frequency

HPRT mutant frequencies were measured in lymphocytes from 101 (table 2) individuals from North Carolina as previously described (32). In summary, phytohemagglutinin (1mg/ml) stimulated mononuclear cells were plated into round-bottomed wells in complete medium (RPMI 1640 with 5% fetal bovine serum, 20% HL-1, antibiotics, supernatant from lymphokine-activated killer cell (10 %v/v), phytohemagglutinin (0.1 g/ml), irradiated lymphoblastoid feeder cells, and -mercaptoethanol (50mM)) with and without thioguanine (1 g/ml). Growth was measured at two time points, 12-15 days, and 1 week later. Cloning efficiency (CE) was determined by quantitation of colonies grown without thioguanine, while mutant efficiency (ME) was determined by quantitation of cells selected with thioguanine. Estimates of mutation frequency (MF) were obtained by dividing ME/CE as previously described (32).

Micronucleus Frequency

Kinetochore (K) positive and negative micronucleus frequencies were measured in a subset (n=63, table 2) of participants. Micronuclei were assayed in lymphocytes using the protocol as described by Yager et al. (34). In summary, phytohemagglutinin (1.5%) stimulated lymphocytes were incubated in complete RPMI medium for 44 hours and harvested to slides after treatment with cytochalasin B (3ug/ml). The kinetochore staining was performed as previously described (35). Micronuclei were scored per 1000 binucleated cells using simultaneous phase contrast and DAPI fluorescence.

Measurement of Glycophorin A Variants in Erythrocytes

Whole blood samples were typed for the M and N antigens and for MN heterozygotes assayed for mutant red blood cells by flow cytometry as described previously (36, 37). There were 35 individuals (Table 2) who were MN heterozygous that could be analyzed for GPA variant frequency from whom DNA was available for UGT genotyping. Approximately 5 million erythrocytes were analyzed for each sample.

Statistical Analysis

We evaluated the effect of UGT1A1 genotype on the genotoxic endpoints of *HPRT* mutant frequency, micronucleus frequency, and glycophorin A mutation frequency by estimating the least-square (LS) mean frequency (*HPRT*, K -positive and K-negative micronuclei, and GPA_N0 and GPA_NN) for each genotype using an analysis of covariance model. This model was adjusted for age and smoking as previously described (32). We also examined the LS mean frequency stratified by smoking status. Analyses were performed in SAS (SAS version 6.12, Cary, NC) using Proc GLM. Statistical significance was assessed at P=0.05.

The mutant frequency of combinations of *UGT1A1* genotypes of interest were compared with the mutant frequency observed for a referent set of genotypes. Genotypes that might have similar phenotypic characteristics (UGT expression) were grouped together in 4 different models (see Table 3). In models 1, 2, and 3, the referent category was comprised of homozygous or heterozygous *UGT1A1* genotypes with few repeats (5-TA, 6-TA). These were compared to combinations of *UGT1A1* genotypes with greater numbers of repeats that were predicted to have lower *UGT1A1* expression. However it is unclear if heterozygous individuals express intermediate phenotypes. In model 4, putative low activity genotypes were contrasted against all other genotypes. These analyses were repeated for each of the individual genotoxic endpoints.

III. Results

UGT1A1 Genotyping

The genotypes were determined using GeneScan analysis of fluorescent-labeled PCR products from genomic DNA and verified with automated DNA sequence analysis using primers listed in Table 1. The results from the GeneScan and sequence analysis are summarized in table 2 (UGT genotype). Counts for each possible allele combination are presented for the smoker and non-smoker categories.

UGT Polymorphism Genotype and Somatic Mutant Frequency

We calculated the LS mean frequency for various combinations of *UGT* genotypes that could putatively translate to UGT phenotypes using models that adjusted

for age and smoking. *HPRT* mutant frequency was observed to be marginally higher for individuals with the lower activity *UGT1A1* 7/7 and 7/8 genotypes (Table 3, models 2 and 4) relative to genotype combinations with higher activity. Stratification by smoking status did not modify the association although the difference in the LS mean HPRT mutant frequency was no longer significant.

We used the same models as above to evaluate the effect of *UGT* genotype on K-positive and K-negative micronucleus frequency (Table 4 and Table 5). Among all subjects, there was a trend for individuals with the lower activity 7/7 and 7/8 UGT genotypes to have higher LS mean K-positive micronucleus frequencies relative to 5/5, 5/6, and 6/6 genotypes (Table 4, models 2, and 4,), although these differences were not significant. This pattern persisted among smokers but no differences were observed among nonsmokers (nonsmokers were 15 of the 63 subjects). The trend was reversed for K-negative micronucleus frequency (Table 5) where no differences were detected between *UGT* genotypes among all participants or smokers. However, among nonsmokers, *UGT* genotypes with lower activity (7/7, 7/8; models 2, and 4) had lower levels of damage, although LS mean differences were not statistically significant.

Examining GPA_N0 mutant frequency, we observed that the grouped heterozygous UGT 5/7, 6/7 and 6/8 genotypes displayed higher mutation frequencies than either of the putative high (5/5, 5/6, 6/6) or low (7/7, 7/8) expression genotypes (Table 6, models 1, 3, and 4). Only the differences for models 1 and 3 were significant. This pattern persisted after stratification by smoking ($p \leq 0.01$). Data were very sparse for nonsmokers.

Considering the GPA_NN mutant frequency (Table 7), the pattern of results was somewhat different and varied by smoking status. Among all participants and in smokers the putative high *UGT1A1* expression (5/5, 5/6, 6/6) genotypes had higher mutant frequency values than each of the other combinations (models 1, 2 and 3). The magnitude of the effect was greatest in smokers and achieved statistical significance in model 2, which contrasts the high (5/5, 5/6, 6/6) vs low (7/7, 7/8) expression genotypes. Conversely, among the very small number of nonsmokers with data (n=6), high *UGT1A1* expression (5/5, 5/6, 6/6) genotypes had lower mutant frequencies (models 1 and 3).

IV. Discussion

We explored whether *UGT1A1* TA repeat polymorphisms were associated with differences in genotoxic damage in peripheral blood cells in an effort to determine the utility of UGT1A1 promoter polymorphisms as biomarkers of susceptibility to cellular oxidative damage. We hypothesized that higher mutant frequencies would be observed among those with higher expressing *UGT1A1* alleles (5-TA and 6-TA, associated with low bilirubin levels) and that lower mutant frequencies would be observed among those with lower expressing *UGT1A1* alleles (7-TA and 8-TA). Only the higher GPA_NN frequency among smokers with 5/5, 5/6, 6/6 genotypes was consistent with the bilirubin as antioxidant hypothesis. Contrary to expectation, some combinations of low expression *UGT1A1* genotypes and alleles (7-TA and 8-TA) were associated with marginally significant, slightly increased frequency of *HPRT* mutants, K -positive micronuclei and GPA_N0 mutants.

In general, the results do not support a strong role for either the antioxidant properties of bilirubin in protecting against genotoxic damage or for a role for UGT1A1 expression in modulating genotoxicity. Bilirubin may not be functioning as an antioxidant at the physiological levels present in lymphocytes or red blood cell progenitor cells. Perhaps oxidative damage is only a minor contributor to the genotoxic damage measured here and much higher levels of oxidative stress would be needed in order to detect a modulating effect for bilirubin levels (and UGT genotypes). Studies by Mireles et al (18) demonstrated that bilirubin's antioxidant activity was within a narrow physiologic range. Specifically, at high exposure to oxidative damage caused by H_2O_2 and $CuSO_4$, low concentrations of bilirubin bound to albumin (<60 mg/dL) decreased the rate of oxidative damage in fetal erythrocytes, but higher concentrations of bilirubin did not. Indeed, studies attempting to demonstrate an effect of antioxidants in protecting against DNA damage and mutation have had mixed results (38, 39, 40).

One must use caution in interpreting these results as there are a number of possible confounding effects. While we have hypothesized that genetic determinants of UGT expression may affect bilirubin levels and risk, Schwertner (41) observed that higher levels of cigarette smoking were associated with decreased serum bilirubin concentrations. Thus smoking may induce UGT1A1 expression (lowering bilirubin) and

this induction effect may counteract any genetic regulation of basal UGT expression levels. Others have suggested that UGT1A1 expression levels could affect hormone metabolism (42). There is suggestive evidence that the lower expressing alleles (7-TA and 8-TA) are associated with increased risk of breast cancer in premenopausal African American women (22). The relationship between the endpoints studied here and breast cancer risk has not been determined.

Recently Malfatti and Felton (43) have shown that UGT1A1 is particularly effective in conjugating the mutagen N^2-hydroxy-PhIP that is found in cooked foods and Peters et al observed that *UGT1A1* genotype modulated urinary mutagenicity following consumption of food containing mutagenic heterocyclic amines (44). Thus, low expression level of this enzyme could reduce one's ability to conjugate and excrete heterocyclic amines. In the event these compounds contribute to mutagenicity, the low UGT expression genotypes (7-TA, 8-TA) could be a risk factor independent of associated bilirubin levels. In the case of GPA, perhaps NØ (gene loss or inactivation, (45)) mutations are initiated by bulky aromatic DNA adducts increased by 7-TA, 8-TA *UGT1A1* genotypes. In contrast, NN (recombination generated) mutations among smokers with the high risk 5/5, 5/6, 6/6 genotypes may be associated with oxidative damage (which would be consistent with the bilirubin as antioxidant hypothesis).

In this study, we found that *UGT1A1* alleles were associated with various differences in mutation frequency; however, the mechanism for the effects is unclear. The small number of participants limited statistical power to detect differences among groups, particularly the very small group of nonsmokers. It is unclear if any of the observed significant differences have a magnitude great enough to impact human risk from mutagens in cigarette smoke. While *UGT1A1* promoter polymorphisms appear to be predictive of serum bilirubin levels and Gilbert's syndrome (46), we are just beginning to explore the impact that variation in *UGT1A1* has on the development of cancer. Larger and more detailed studies will be needed to elucidate the impact that the *UGT1A1* promoter polymorphisms have on genetic damage and susceptibility to cancer.

Acknowledgments

We thank Dr. Claudia Thompson, NIEHS, for help in organizing this study and in coordinating the sample distribution. We also thank Dr. Richard Langlois, Lawrence Livermore National Laboratory for Glycophorin A analysis and Dr. Xuguang Guo, Analytic Sciences Inc, Durham NC for data analysis. This work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract No. W-7405-Eng-48 with support from Interagency Agreement Y01-ES-80171 from the National Institute of Environmental Health Science.

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Table 1. Primers used in genotyping and sequence Analysis

Description	Primers
Primers to amplify 252-258 fragment for GeneScan Analysis (ABI)	
famUGTA-F	5'fam-aag tga act ccc tgc tac ctt
famUGTA-R	5'fam-cca ctg gga tca aca gta tct
UGTA-F	5'aag tga act ccc tgc tac ctt
UGTA-R	5'cca ctg gga tca aca gta tct
Primers for Automated Sequence Analysis	
Hind2F	5'ccc ggg aag ctt ggt gtg ggg agg agc ttc agt g
641F	5'tga agg atg gaa act tgc tta g
1029F	5'acc cac ctg ttc att tcc ttc t
1453F	5'tta cca gag agg aag aag gac g
471R	5 'tct tcc aaa ttc ctt ttc aga g
895R	5'tga acc tga aag agc cag tc
1353R	5'cta tgt agt gaa caa gtt agg c
1681R2	5'ccc ggg tct aga gcc ttt gct cct gcc aga ggt t

Table 2. Characteristics of 101 participants genotyped for UGT1A1 by smoking status.

Characteristic	Smokers	Non-smokers
Mean age (yrs)	34.2	30.5
Range	21-53	19-46
	n (%)	n (%)
Race		
Caucasian	35 (49)	23 (79)
African-American	37 (51)	5 (17)
Not reported	0 1 (3)	
Gender		
Male	51 (71)	19 (66)
Female	21 (29)	10 (34)
UGT Genotype		
5/5	1 (1)	0
5/6	2 (3)	1 (3)
5/7	1 (1)	0
5/8	0	0
6/6	25 (35)	12 (41)
6/7	27 (38)	12 (41)
6/8	2 (3)	1 (3)
7/7	11 (15)	2 (9)
7/8	3 (4)	1 (3)
8/8	0	0
Total	72 (100)	29 (100)
Endpoints Analyzed		
HPRT	72	29
GPA_NN	29	6
GPA_NØ	29	6
MnK+	48	15
MnK-	48	15

Table 3. UGT polymorphism genotype and HPRT mutant frequency

Model	Genotype	Total		Smokers		Non-Smokers	
		N	LS Mean (SEM)[a]	N	LS Mean (SEM)	N	LS Mean (SEM)
1	5/7, 6/7, 6/8	43	0.91 (0.09)	30	0.97 (0.10)	13	0.74 (0.18)
	5/5, 5/6, 6/6[b]	39	1.00 (0.09)	27	1.09 (0.11)	12	0.83 (0.19)
2	7/7, 7/8	17	1.36 (0.19)	14	1.30 (0.20)	3	1.67 (0.59)
	5/5, 5/6, 6/6[b]	39	1.01 (0.13)	27	1.08 (0.14)	12	0.84 (0.28)
3	5/7, 6/7, 6/8, 7/7, 7/8	60	1.03 (0.09)	44	1.08 (0.10)	16	0.86 (0.22)
	5/5, 5/6, 6/6[b]	39	1.00 (0.12)	27	1.09 (0.13)	12	0.86 (0.26)
4	7/7, 7/8	17	1.34[c] (0.17)	14	1.31 (0.18)	3	1.54 (0.48)
	5/5, 5/6, 5/7, 6/6, 6/7, 6/8[b]	82	0.95[c] (0.08)	57	1.03 (0.09)	25	0.78 (0.16)

[a]Mutant frequency (per 100,000 cells). Model adjusted for age and smoking status.

[b]Referent group

[c]Least square means (LSMean) are significantly different at the p=0.05 level.

Table 4. UGT polymorphism genotype and MnK+ frequency

Model	Genotype	Total		Smokers		Non-Smokers	
		N	LS Mean (SEM)[a]	N	LS Mean (SEM)	N	LS Mean (SEM)
1	5/7, 6/7, 6/8	26	0.43 (0.05)	22	0.44 (0.06)	4	0.46 (0.13)
	5/5, 5/6, 6/6[b]	27	0.55 (0.05)	17	0.57 (0.06)	10	0.49 (0.08)
2	7/7, 7/8	10	0.70 (0.13)	9	0.76 (0.14)	1	0.55 (0.30)
	5/5, 5/6, 6/6[b]	27	0.56 (0.08)	17	0.57 (0.10)	10	0.50 (0.09)
3	5/7, 6/7, 6/8, 7/7, 7/8	36	0.50 (0.06)	31	0.53 (0.07)	5	0.47 (0.11)
	5/5, 5/6, 6/6[b]	27	0.56 (0.07)	17	0.57 (0.09)	10	0.48 (0.08)
4	7/7, 7/8	10	0.72 (0.11)	9	0.76 (0.12)	1	0.54 (0.24)
	5/5, 5/6, 5/7, 6/6, 6/7, 6/8[b]	53	0.49 (0.05)	39	0.46 (0.06)	14	0.47 (0.06)

[a]Mn frequency (per 100 cells). Model adjusted for age and smoking status.
[b]Referent group

Table 5. UGT polymorphism genotype and MnK- frequency

Model	Genotype	Total		Smokers		Non-Smokers	
		N	LS Mean (SEM)[a]	N	LS Mean (SEM)	N	LS Mean (SEM)
1	5/7, 6/7, 6/8	26	0.39 (0.05)	22	0.43 (0.05)	4	0.22 (0.14)
	5/5, 5/6, 6/6[b]	27	0.44 (0.05)	17	0.45 (0.06)	10	0.39 (0.09)
2	7/7, 7/8	10	0.41 (0.09)	9	0.46 (0.10)	1	0.08 (0.32)
	5/5, 5/6, 6/6[b]	27	0.43 (0.06)	17	0.45 (0.07)	10	0.39 (0.10)
3	5/7, 6/7, 6/8, 7/7, 7/8	36	0.39 (0.04)	31	0.44 (0.04)	5	0.20 (0.12)
	5/5, 5/6, 6/6[b]	27	0.44 (0.05)	17	0.45 (0.06)	10	0.39 (0.09)
4	7/7, 7/8	10	0.41 (0.08)	9	0.46 (0.08)	1	0.11 (0.28)
	5/5, 5/6, 5/7, 6/6, 6/7, 6/8[b]	53	0.42 (0.03)	39	0.44 (0.04)	14	0.34 (0.07)

[a]Mn frequency (per 100 cells). Model adjusted for age and smoking status.

[b]Referent group

Table 6. UGT polymorphism genotype and GPA_NØ mutant frequency

Model	Genotype	Total		Smokers		Non-Smokers	
		N	LS Mean (SEM)[a]	N	LS Mean (SEM)	N	LS Mean (SEM)
1	5/7, 6/7, 6/8	15	18.39[c] (1.69)	13	17.05[d] (1.46)	2	22.95 (8.14)
	5/5, 5/6, 6/6[b]	15	11.98[c] (1.69)	11	9.90[d] (1.58)	4	19.78 (5.76)
2	7/7, 7/8	5	14.16 (3.22)	5	11.62 (2.49)	0	-
	5/5, 5/6, 6/6[b]	15	11.83 (1.78)	11	10.06 (1.64)	4	19.85 (5.85)
3	5/7, 6/7, 6/8, 7/7, 7/8	20	17.04[c] (1.53)	18	15.65[c] (1.42)	2	22.95 (8.14)
	5/5, 5/6, 6/6[b]	15	11.69[c] (1.77)	11	9.95[c] (1.81)	4	19.78 (5.76)
4	7/7, 7/8	5	12.93 (3.37)	5	11.36 (3.03)	0	-
	5/5, 5/6, 5/7, 6/6, 6/7, 6/8[b]	30	15.05 (1.33)	24	13.93 (1.35)	6	20.83 (4.14)

[a]Mutant frequency (per 1,000,000 cells). Model adjusted for age and smoking status.
[b]Referent group
[c]Least square means (LSMean) are significantly different at the p=0.05 level.
[d]Least square means (LSMean) are significantly different at the p=0.01 level.

Table 7. UGT polymorphism genotype and GPA_NN mutant frequency

Model	Genotype	Total		Smokers		Non-Smokers	
		N	LS Mean (SEM)[a]	N	LS Mean (SEM)	N	LS Mean (SEM)
1	5/7, 6/7, 6/8	15	9.94 (2.38)	13	9.34 (2.27)	2	14.67[c] (0.91)
	5/5, 5/6, 6/6[b]	15	13.33 (2.38)	11	14.64 (2.47)	4	9.31[c] (0.64)
2	7/7, 7/8	5	5.76 (5.05)	5	5.18[c] (3.84)	0	-
	5/5, 5/6, 6/6[b]	15	14.23 (2.79)	11	16.21[c] (2.54)	4	9.50 (0.79)
3	5/7, 6/7, 6/8, 7/7, 7/8	20	9.54 (1.95)	18	8.97 (1.84)	2	14.67[c] (0.91)
	5/5, 5/6, 6/6[b]	15	13.54 (2.26)	11	15.08 (2.35)	4	9.31[c] (0.64)
4	7/7, 7/8	5	7.45 (4.05)	5	6.63 (3.72)	0	-
	5/5, 5/6, 5/7, 6/6, 6/7, 6/8[b]	30	11.89 (1.60)	24	12.26 (1.66)	6	11.10 (1.34)

[a]Mutant frequency (per 1,000,000 cells). Model adjusted for age and smoking status.

[b]Referent group

[c]Least square means (LSMean) are significantly different at the p=0.05 level.