

ASSOCIATION OF THE SULT1A1 R213H POLYMORPHISM WITH COLORECTAL CANCER

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Summary

1. Sulphotransferases are a superfamily of enzymes involved in both detoxification and bioactivation of endogenous and exogenous compounds. The arylsulphotransferase SULT1A1 has been implicated in a decreased activity and thermostability when the wild-type arginine at position 213 of the coding sequence is substituted by a histidine. SULT1A1 is the isoform primarily associated with the conversion of dietary *N*-OH arylamines to DNA binding adducts and is therefore of interest to determine whether this polymorphism is linked to colorectal cancer.

2. Genotyping, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, was performed using DNA samples of healthy control subjects ($n = 402$) and patients with histologically proven colorectal cancer ($n = 383$). Both control and test populations possessed similar frequencies for the mutant allele (32.1 and 31%, respectively; $P = 0.935$). Results were not altered when age and gender were considered as potential confounders in a logistic regression analysis.

3. Examination of the sulphonating ability of the two allozymes with respect to the substrates *p*-nitrophenol and paracetamol showed that the affinity and rate of sulphonation was unaffected by substitution of arginine to histidine at position 213 of the amino acid sequence. 4. From this study, we conclude that the SULT1A1 R213H polymorphism is not linked with colorectal cancer in this elderly Australian population.

Key words: colorectal cancer, genetic polymorphism, SULT1A1.

Introduction

Genetic polymorphisms of xenobiotic-metabolizing enzymes have been considered to be important in the determination of an individual's susceptibility to cancer. 1 Sulphotransferases represent a family of xenobiotic-metabolizing enzymes involved in both the detoxification and bioactivation of endogenous and exogenous chemicals. Human aryl sulphotransferase (humSULT1A1) is expressed in various tissues in the body, such as the liver, gastrointestinal tract, kidney and lung. 2 Its primary role is thought to be the metabolism of phenolic compounds. As part of this role, SULT1A1 is also involved in metabolizing food-derived mutagens, resulting in the production of highly reactive electrophiles that are capable of binding to DNA. 3 The cooking of red meat at temperatures between 150 and 250 °C leads to the pyrolysis of creatine and amino acids present in the meat to form heterocyclic amines that are further metabolized by cytochrome P450 1A2 into *N*-hydroxy (*N*-OH) arylamines. 4 Catalysed by *N*-acetyltransferases and sulphotransferases, subsequent esterification of the *N*-OH metabolite produces a DNA-binding adduct. 5 Studies have indicated that the increased amounts of *N*-OH

arylamines can increase the chance of colorectal cancer development. 5 Therefore, *SULT1A1* represents a worthy candidate for study in association with colorectal cancer because it has been shown that this enzyme is able to sulphonate food-derived *N*-OH arylamines into highly reactive electrophiles that are capable of binding to DNA.

To date, three polymorphisms causing an amino acid change within the *SULT1A1* gene have been identified. The R213H polymorphism was first reported in 1997 and results from a transition of a guanine to an adenine at nucleotide 638 of the *SULT1A1* coding region. 6 The R213H allele was present in approximately 30% of the population studied. An important phenotype–genotype correlation was established for this polymorphism in that lower thermostability and enzyme activity were observed in all subjects homozygous for the mutant histidine allele (substrate, *p*-nitrophenol). 6 Ozawa *et al.* 7 also reported that the sulphonation of certain substrates (2-naphthol(+)-isoproterenol and minoxidol) was compromised when histidine was encoded at amino acid 213 of *SULT1A1*.

The aim of the present study was to investigate the potential genetic link between the *SULT1A1* R213H polymorphism and colorectal cancer in a case–control association study and in functional studies investigating potential differences in the sulphonation of compounds that may play a vital role in colorectal cancer by wild-type or mutant *SULT1A1*.

Methods

DNA extraction

Blood samples were obtained from healthy control subjects and patients with histologically proven colorectal cancer from south-east Queensland, all of whom were of Caucasian descent. DNA was extracted from peripheral blood leucocytes using the Clontech Nucleospin Blood Mini Kit (Progen, Madison, WI, USA). Of the 402 control samples analysed in the present study (mean age 71.27 years), 229 were male and 151 were female. The colorectal cancer test population consisted of 383 samples (mean age 68.36 years), 215 males and 166 females. No statistical differences in age and gender between the two populations were detected ($P = 0.99$). Written informed consent was obtained and ethical approval was obtained through the Human Ethics Committee of the University of Queensland.

Polymerase chain reaction

The region of the *SULT1A1* gene containing the mutation was amplified using polymerase chain reaction (PCR). Primers for this procedure were present in intron 6 (5' 2'-GGTTGAGGAGTTGGCTCTGC-3') and exon 6 (5' 2'-AGATGCTGTGGTCCATGAACTC-3') of the *SULT1A1* gene, yielding a 295 bp fragment. These primers were designed to end in three bases specific to the *SULT1A1* gene only such that neither *SULT1A2* nor *SULT1A3* was amplified despite high sequence homology between the isoforms. Reactions were conducted in a final volume of 15 μ L, containing 50 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 3 mmol/L MgCl₂, 200 μ mol/L dNTPs, 1 μ mol/L forward and reverse primer, 0.375 U Failsafe PCR enzyme (Astral Scientific, Caringbah, NSW, Australia) and 30 ng DNA. Reactions were subjected to initial DNA denaturation at 95 °C for 2 min, followed by 94 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min. After five cycles, the primer annealing temperature was increased from 59 to 64 °C to increase annealing specificity; this was repeated for 30 cycles.

Table 1 Genotype and allele frequencies for SULT1A1 R213H polymorphism in control and colorectal cancer populations

	Control	Colorectal cancer	Statistics
Genotype			
R213/R213	178 (44.3)	175 (45.7)	$\chi^2 = 0.28; P = 0.9, 3 \text{ d.f.}$
R213/H213	190 (47.3)	179 (46.7)	
H213/H213	34 (8.4)	29 (7.6)	
Allele			
R213	546 (67.9)	529 (69)	$\chi^2 = 0.24, P = 0.9, 1 \text{ d.f.}$
H213	258 (32.1)	237 (31)	

Data show the number of subjects, with percentages given in parentheses.

Restriction fragment length polymorphism

A RFLP analysis strategy was developed to determine the genotypes of the samples. The R213H polymorphism involves a change in the nucleotide at position 638 of the SULT1A1 coding sequence. The transition of a guanine to an adenine at this nucleotide generates a cleavage site recognized by *Hae* II. *Hae* II (5 U) was added in a volume of 7.5 μ L PCR reaction (supplemented with 50 mmol/L potassium acetate, 20 mmol/L Tris acetate, 10 mmol/L magnesium acetate and 1 mmol/L dithiothreitol, pH 7.9 at 25 °C) and then incubated at 37 °C for 16 h. Following digestion, results were analysed on a 3.75% polyacrylamide 0.6 × Tris–borate–EDTA (TBE) gel, visualized using ethidium bromide and the Corbett Research GS2000 program (Mortlake, NSW, Australia). Subjects homozygous for the wildtype allele yielded fragments of 122 and 173 bp, whereas heterozygotes, in addition, yielded a 295 bp fragment. A single 295 bp fragment was produced by mutant homozygotes, because the restriction enzyme *Hae* II did not recognize the altered restriction site. Samples were genotyped in duplicate, scored by two independent observers and, if conflicting, the samples were genotyped four times.

Statistical analysis

To evaluate the potential association between genotype and having colorectal cancer, the unadjusted odds ratio (OR) was determined. The statistical significance of the OR was determined using Chi-squared (χ^2) analysis in a 2 × 3 contingency table. Logistic regression analysis, with a 95% confidence interval (CI), was undertaken to evaluate whether a particular genotype predicted the presence of colorectal cancer after adjustment for potential confounders (age and gender).

Table 2 SULT1A1 R213H allele frequency in different ethnic groups

Population ethnicity	Allele frequency		No. samples	Mean age (years)	Female : male ratio	References
	Wildtype	No. Mutant				
Caucasian	0.68	0.32	402	71.27	0.66	Present study
Caucasian with colorectal cancer	0.69	0.31	383	68.36	0.77	
Caucasian with colorectal cancer	0.65	0.35	226	67.9	–	18
Caucasian	0.68	0.32	293	53.6	0.56	12
African	0.73	0.27	52	–	–	
Caucasian	0.66	0.34	150	40–64	Male	13
Caucasian	0.69	0.31	150	–	–	6
Japanese	0.832	0.168	143	61	0.81	7
Caucasian	0.609	0.391	189	–	1.27	14
Caucasian with prostate cancer	0.649	0.351	134	–	–	
Caucasian	0.659	0.341	156	–	Female	15
Caucasian with breast cancer	0.584	0.416	332	–	Female	
Caucasian	0.64	0.36	211	–	0.66	16
African	0.74	0.26	40	–	0.66	
Caucasian	0.656	0.332	242	–	–	17
African-American	0.477	0.294	70	–	–	
Chinese	0.914	0.08	290	–	Female	

–, parameter not provided.

Site-directed mutagenesis

Site-directed mutagenesis was used to construct the wild-type (R213) form of SULT1A1 using the mutant (H213) as a template. The R213 SULT1A1 coding region cloned into pET28a(+) was generated using the QuikChange™ Site-Directed Mutagenesis approach using *Pfu Turbo* polymerase (Stratagene, La Jolla, CA, USA) and the 5' primer, 5'-GGAGTTTGTGGGGCGCTCCTGCCAGAGG-3' and the 3' primer, 5'-CCTCTGGCAGGGAGCGCCCCACAAACTCC-3', according to the manufacturer's instructions. Incorporation of the correct mutation was confirmed by DNA sequencing (ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit; PE Applied Biosystems, Foster City, CA, USA; sequence analysis was performed by the Australian Genome Research Facility, University of Queensland, Brisbane, Qld, Australia).

Bacterial expression and purification of recombinant wild-type and mutant SULT1A1 enzymes

A single *Escherichia coli* BL21 (DE3) pLysS cell colony, transformed with pET28a(+) containing either the mutant or wild-type SULT1A1 insert, was used to inoculate 10 mL LB broth, supplemented with 30 µg/mL kanamycin and 34 µg/mL chloramphenicol and incubated at 37 °C overnight. This overnight culture was then used to inoculate 250 mL LB broth and expression was conducted essentially as described by Elvin *et al.*⁸ Protein purification was effected using TALON Metal Affinity Resin Column (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Purified protein was stored in 20% glycerol at –80°C until required. Protein concentrations were determined using the Pierce BCA reagent (Progen).

Sulphotransferase kinetic assays

Radiometric sulphotransferase assays were performed as established by Foldes and Meek,⁹ with modifications as described by Brix *et al.*¹⁰ Final assay volume was 500 µL, consisting of 10 mmol/L potassium phosphate buffer (pH 7.0), 10–20 µmol/L 32phosphoadenosine-52-phosphosulphate (PAPS) and substrate. The reaction was initiated upon addition of 0.08–0.2 µg purified recombinant protein and reactions were incubated at 37°C for 20 min. For each assay, appropriate substrate blanks were used to correct for background activity.

Results obtained from substrate assays were analysed as described previously by Hempel *et al.*¹¹ Kinetic data reported represent mean values determined from triplicate experiments.

Results

A total of 785 elderly Australians were genotyped for the R213H polymorphism of SULT1A1 using a PCR-RFLP strategy. The genotype and allele frequencies were found to be in Hardy–Weinberg equilibrium. Unadjusted genotype and allele frequencies are provided in Table 1. Table 2 provides a comparison of the genotype and allele frequencies obtained in this elderly Australian population with other published data.

As recorded in Table 1, genotype frequencies were similar for both the control and colorectal cancer populations. No significant differences in genotype were demonstrated between the two populations in the present study ($\chi^2 = 0.28$; $P = 0.9$). Similarly, differences in allele frequency for the wild-type or mutant alleles were not observed to be statistically significant ($\chi^2 = 0.24$; $P = 0.9$).

The risk of a subject who was homozygous for the mutant allele developing colorectal cancer was observed to be lower than for those who were homozygous for the wild-type allele (OR = 0.87), but this value did not reach statistical significance ($\chi^2 = 0.29$; $P = 0.9$; 95% CI = 1.40–0.37, 3 d.f). Conversely, the risk of a mutant homozygote subject falling in the control population compared with a wild-type homozygote was calculated to be 0.21 ($\chi^2 = 2.65$; $P = 0.10$). Logistic regression analysis showed that age, gender and position of colorectal cancer did not represent confounders to this study.

Kinetic constants for R213 SULT1A1 and H213 SULT1A1 proteins towards PAPS, using a saturating concentration of *p*-nitrophenol, are given in Table 3. Neither K_m or V_{max} values were observed to be statistically different between the wild-type and mutant enzyme ($P = 0.98$, *t*-test). Sulphonation of *p*-nitrophenol and paracetamol by the wild-type and mutant SULT1A1 enzymes is given in Table 4. No difference in sulphonating capacity of either substrate was apparent between the R213 and H213 allozymes ($P = 0.91$ and 0.84 , respectively, *t*-test).

Table 3 Kinetic analysis of wildtype (R213) and mutant (H213) humSULT1A1 towards 3'-phosphoadenosine-5'-phosphosulphate*

	K_m ($\mu\text{mol/L}$)	V_{max} (nmol/min per mg)
HumSULT1A1*1	0.18 ± 0.02	20.43 ± 0.13
HumSULT1A1*2	0.19 ± 0.10	19.62 ± 0.70

Data are the mean \pm SD ($P = 0.98$, *t*-test).

*Concentration of *p*-nitrophenol saturating ($1 \mu\text{mol/L}$) with varying concentrations of 3'-phosphoadenosine-5'-phosphosulphate

Table 4 Kinetic analysis of wildtype (R213) and mutant (H213) humSULT1A1

	R213 SULT1A1		H213 SULT1A1	
	K_m ($\mu\text{mol/L}$)	V_{max} (nmol/min per mg)	K_m ($\mu\text{mol/L}$)	V_{max} (nmol/min per mg)
<i>p</i> -Nitrophenol*	0.095 ± 0.018	21.96 ± 1.08	0.13 ± 0.02	26.10 ± 1.06
Paracetamol†	296.7 ± 52.66	27.87 ± 1.21	404.1 ± 105.1	24.99 ± 1.82

Data are the mean \pm SD. * $P = 0.91$ (*t*-test); † $P = 0.84$ (*t*-test).

Discussion

The present study investigated the relationship between the SULT1A1 R213H polymorphism and colorectal cancer. The frequency of this polymorphism has been determined in several ethnic populations to date.^{6,7,12–18} In the population examined in the present study, the frequency of the mutant allele was similar to that observed in the African, UK Caucasian and Japanese populations.^{7,12} Thus, it appears from these studies that no ethnic bias exists in the distribution of this polymorphism. In contrast, Carlini *et al.*¹⁷ have found striking ethnic variations in their study examining Caucasian, African-American and Chinese subjects. Limitations to the study, such as the exclusion of subjects over 70 years of age, the use of only Chinese women and the small sample size of the African-American subjects, may have possibly accentuated distribution of this polymorphism between ethnic groups in the study of

Carlini *et al.*¹⁷ Because of conflicting data, further studies are required in order to determine whether the frequency of the SULT1A1 R213H polymorphism is subject to ethnic or age bias.

Our study used a group of 785 Caucasians with an average age of 69.81 years and the overall frequency of wild-type homozygotes was approximately 45%. Coughtrie *et al.*¹² observed in their study, consisting of a population of 293 UK Caucasians with an average age of 53.6 years, that the proportion of wild-type homozygotes rose from 39% in the under 40 age group to 59% in the age group over 70 years. The number of subjects in the > 70 years group in the Coughtrie *et al.* study¹² was not supplied. This difference observed between the two studies can be accounted for by differences in population mean age. It may be that the > 70-year-old subset in the study of Coughtrie *et al.*¹² had a mean age significantly higher than the 69.8 years in our population. In the study conducted by Carlini *et al.*,¹⁷ no significant age-related differences in allele frequencies in Caucasian subjects were observed, although there was a significant increase in the mutant enzyme as age increased in the Chinese women investigated in their study.

Our analysis represents the largest population used for determining the frequency of this polymorphism. The size of the sample population used in the present study allowed an 85% probability of detecting a 10% change in allele frequency between the control and test populations (using a two-tailed test).¹⁹ No relationship between the R213H polymorphism of SULT1A1 and colorectal cancer was indicated. Genotype frequencies between control and test populations were not found to be statistically different either before or after adjustments for potential confounders such as age and sex ($\chi^2 = 0.28$; $P = 0.9$). Our study demonstrates that there is no association between the SULT1A1 R213H polymorphism and colorectal cancer development. A small but statistically non-significant trend towards a higher number of mutant homozygotes in the control population compared with the colorectal cancer population was observed ($\chi^2 = 2.65$; $P = 0.10$). Interestingly, this trend was also observed in an association study by Steiner *et al.*,¹⁴ in which the association of the SULT1A1 R213H polymorphism with prostate cancer was examined. It is tempting to speculate that this increase in the frequency of the homozygous mutant genotype in the control group may indicate a weak, protective effect of the homozygous mutant genotype against colorectal cancer. Conversely, it may be possible that the inheritance of the homozygous mutant genotype is detrimental; thus, those subjects with the mutant genotype and colorectal cancer are more likely to die younger than subjects homozygous for the wild-type allele with colorectal cancer. During the preparation of this manuscript, a similar study was reported by Bamber *et al.*¹⁸ In agreement with the findings of our study, there was no significant difference in allele frequency between the control and cancer population when considering the entire population. Furthermore, they also found a trend towards allele bias when the population was segregated according to age, although, in this case, the trend was significant. They found a reduced risk of colorectal cancer in individuals under the age of 80 years and homozygous for the wild-type allele.

The functional significance of the R213H polymorphism was investigated through enzyme kinetic analysis. Preliminary modelling studies, using the SULT1A3 crystal structure²⁰ indicate that a change from an arginine to a histidine at position 213 of the coding region of SULT1A1 does not appear to directly affect the binding site of the substrate or of the universal sulphonate donor PAPS. However, because of the change at physiological pH from a positive to a neutral amino acid at position 213, the conformation of the protein and, thereby, its stability may be altered. Assays using saturating substrate concentrations and varying concentrations of PAPS produced similar kinetic constants for both wildtype and mutant enzymes of SULT1A1, suggesting that the R213H polymorphism does not disrupt the binding of PAPS. Results from substrate assays indicate that there are no differences in the sulphonation of the model substrate *p*-nitrophenol or the drug substrate paracetamol. In contrast with the observations of Raftogianis *et al.*,⁶ this result seems to suggest that the R213H polymorphism of SULT1A1 is not functionally significant. In the study initiated by Raftogianis *et al.*,⁶ enzyme activity was measured using platelet preparations. While important, the use of platelets to determine the activity of a specific enzyme can be misinterpreted due to the inability of the method to distinguish which enzyme is responsible for the activity observed. Moreover, with the recent finding that the R213H polymorphism of SULT1A1 is in linkage disequilibrium with SULT1A2 N235T,^{13,16} it is possible that the activity measured in their study is attributable to SULT1A2 and SULT1A1. Although there are overlapping substrate preferences and expression patterns between these two enzymes, SULT1A2 has not been found to be expressed in the colon. Furthermore, SULT1A2 is much less efficient at metabolizing *N*-OH arylamines compared with SULT1A1 (CF Wong *et al.*, unpubl. obs., 1999). This tends to indicate that SULT1A1 is the most likely of the two to play a role in colorectal cancer development.

The differences in activity between R213 SULT1A1 and H213 SULT1A1 were also highlighted in studies performed by Ozawa *et al.*⁷ Although a recombinant enzyme was used, differences in sulphonating abilities were only slight, which may be explained by the compromise in thermostability the amino acid change causes. Due to conflicting results, further investigation into the sulphonation of more biologically relevant compounds, such as *N*-OH arylamines, may provide a better understanding of the functional implications of this polymorphism.

In conclusion, the present study indicates that, in the elderly Australian population investigated in the present study, there is no significant association between the SULT1A1 R213H polymorphism and colorectal cancer development. Furthermore, results from substrate assays demonstrate that there is no functional difference between R213 SULT1A1 and H213 SULT1A1 in sulphonating the model substrate *p*-nitrophenol, the drug substrate paracetamol or the sulphonate donor PAPS.

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