Genetic association study of *CYP1A1* polymorphisms identifies risk haplotypes in nonsmall cell lung cancer

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ABSTRACT: Lung cancer remains a leading cause of disease globally, with smoking being the largest single cause. Phase I enzymes, including cytochrome P₄₅₀, family 1, subfamily A, polypeptide 1 (*CYP1A1*), are involved in the activation of carcinogens, such as polycyclic aromatic hydrocarbons, to reactive intermediates that are capable of binding covalently to DNA to form DNA adducts, potentially initiating the carcinogenic process. The aim of the present study was to investigate the association of *CYP1A1* gene polymorphisms and haplotypes with lung cancer risk.

A case–control study was carried out on 1,040 nonsmall cell lung cancer (NSCLC) cases and 784 controls to investigate three *CYP1A1* variants, *CYP1A1*2A* (rs4646903; thymidine to cytosine substitution at nucleotide 3801 (3801T>C)), *CYP1A1*2C* (rs1048943; 2455A>G; substitution of isoleucine 462 with valine (exon 7)) and *CYP1A1*4* (rs1799814; 2453C>A; substitution of threonine 461 with asparagine (exon 7)) using PCR restriction fragment length polymorphism methods.

The *CYP1A1*2A* and *CYP1A1*2C* variants were significantly over-represented in NSCLC cases compared with controls, whereas the *CYP1A1*4* variant was under-represented. *CYP1A1* haplotypes (in allele order *CYP1A1*4*, *CYP1A1*2C*, *CYP1A1*2A*) CGC and CGT were associated with an increased risk of lung cancer, whereas AAT was associated with decreased lung cancer risk in this population.

The present study has identified risk haplotypes for *CYP1A1* in NSCLC and confirmed that *CYP1A1* polymorphisms are a minor risk factor for NSCLC.

KEYWORDS: Cytochrome P₄₅₀, cytochrome P₄₅₀ 1A1 gene, haplotype, lung cancer, nonsmall cell lung cancer

Lung cancer is a leading cause of cancer death internationally, with smoking being the largest single cause. Smoking is responsible for 85–90% of lung cancers [1], yet <20% of lifelong smokers develop lung cancer, suggesting that other factors, including genetics, may play a role [1]. Phase I enzymes (mainly cytochrome P₄₅₀) metabolically activate carcinogens, such as polycyclic aromatic hydrocarbons and *N*-nitrosamines, to reactive intermediates [2]. These intermediates are capable of binding covalently to DNA to form DNA adducts, potentially initiating the carcinogenic process. Two functionally important nonsynonymous polymorphisms have been described for the cytochrome P₄₅₀, family 1, subfamily A, polypeptide 1 (*CYP1A1*) gene, a base substitution at codon 462 in exon 7, resulting in substitution of isoleucine with valine (Ile462Val (exon 7))

(*CYP1A1*2C*; National Center for Biotechnology Information single nucleotide polymorphism (SNP) identifier rs1048943; adenine (A) to guanine (G) substitution at nucleotide 2455 (2455A>G)) and a point mutation (thymine (T) to cytosine (C)) at the *MspI* site in the 3'-untranslated region (*CYP1A1*2A*; rs4646903; 3801T>C) [3, 4]. A third polymorphism, substituting threonine (Thr) for asparagine (Asn) (*CYP1A1*4*; rs1799814; 2453C>A; Thr461Asn (exon 7)), has also been reported two bases upstream of *CYP1A1*2C*; however, its functional effects remain to be fully elucidated.

Ethnic differences in the distribution of the *CYP1A1*2C* and *CYP1A1*2A* genotypes have been reported in lung cancer subjects, with few reports available for *CYP1A1*4*. An over-representation of the Val allele (*CYP1A1*2C*) among lung cancer

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cases has been reported in Asian and Caucasian populations [5–7]. Although relatively frequent in Asian populations (0.18–0.25) [8, 9], the Val allele is quite rare in Caucasian control populations, occurring in ~7–13% of people [7, 10]. In a previous study, no interactive effects were shown between *CYP1A1**2C and polymorphisms of the phase II enzyme genes encoding glutathione S-transferase pi 1 (*GSTP1*), theta 1 (*GSTT1*) or mu 1 (*GSTM1*). However, risk genotypes of the myeloperoxidase gene (*MPO*) and *CYP1A1**2C interacted to increase the overall risk of nonsmall cell lung cancer (NSCLC) (odds ratio (OR) 2.88; 95% confidence interval (CI) 1.70–5.00; $p=0.001$) [11]. Similarly, the *CYP1A1**2A variant has been strongly associated with increased lung cancer risk in Asian populations, especially in relation to tobacco smoking and in combination with polymorphisms of the phase II enzyme genes *GSTM1* and *GSTT1* [12, 13]. In contrast, studies in Caucasian populations have not clearly established an association between *CYP1A1* polymorphisms and increased lung cancer risk [14–19]. Relatively frequent incidences of *CYP1A1**2A variants have been reported in Asian (39%) [20] and Caucasian control populations (18–23%) [14, 16, 21]. Conversely, few studies have reported population frequencies for *CYP1A1**4 in lung cancer subjects. CASCORBI *et al.* [6] reported variant allele frequencies of 3% in Caucasian lung cancer cases and controls. In contrast, SONG *et al.* [13] detected no polymorphic sites in an Asian population, supporting similar polymorphism studies of Japanese liver subjects [10]. Similar frequencies (2–3%) have been observed in healthy Caucasian populations [22, 23]. A recent pooled analysis of HUNG *et al.* [24] reported an increase in lung cancer susceptibility by more than two-fold for *CYP1A1**2A and *CYP1A1**2C in nonsmokers. The authors suggested these polymorphisms may be implicated in lung carcinogenesis at low levels of tobacco exposure, possibly in combination with phase II enzymes, including *GSTM1* [24], although this has not been replicated by other groups [21].

Many studies investigating the relationships between *CYP1A1* polymorphisms and lung cancer have been limited by small sample numbers, leading to a lack of statistical power. Pooled analyses to increase sample size have tried to address this issue. Conflicting results between groups may be due to population differences (*i.e.* ethnicity) or failure to control for other potential confounders, including age and sex. *CYP1A1* haplotype studies have potential in determining whether combinations of *CYP1A1* (*CYP1A1**2A, *CYP1A1**2C, *CYP1A1**4) together confer a greater risk of lung cancer than single polymorphisms. One recent study of 200 case-matched controls from an Indian population, showed that only one haplotype, CGC (3801C, 2455G, 2453C), was significantly associated with increased lung cancer risk, although the study was limited by its small sample numbers [3]. Few *CYP1A1* haplotype association studies have been conducted in a Caucasian population. A study of HAN *et al.* [25] investigated the genotype frequencies of 13 SNPs found in the promoter region of *CYP1A1* in 21 Caucasian individuals. Subsequent functional studies identified two *CYP1A1* haplotypes (2923C>T, 2875G>A, 3777T>G; and 2923C>T, 3777T>G, 4553G>A) demonstrating moderate increases in basal activity compared with the wild-type *CYP1A1* constructs (1.38 and 1.50, respectively; $p<0.05$). These were considered unlikely to be of functional significance

considering the magnitude of difference in *CYP1A1* expression in response to benzo[a]pyrene and cigarette smoke extract [25].

In order to further investigate the role of *CYP1A1* polymorphism variation (*CYP1A1**2C, *CYP1A1**2A and *CYP1A1**4) in lung cancer risk, *CYP1A1* haplotype analyses were performed in a large sample of Australian lung cancer cases with the aim of identifying risk-modifying *CYP1A1* haplotypes.

MATERIALS AND METHODS

Study population

The present study population has been described previously [11, 26]. Cases were subjects with confirmed primary lung cancer treated at The Prince Charles Hospital (Brisbane, Australia) during 1980–2007 ($n=1,040$). Controls consisted of subjects with chronic obstructive pulmonary disease (COPD) treated at the same hospital ($n=506$) or healthy smokers ($n=278$) who, at the time of recruitment to the study, did not have doctor-diagnosed lung cancer. The ethnicity of the study population was >99% Caucasian. The study was approved by the Ethics Committee at The Prince Charles Hospital. Cases and controls gave informed written consent for use of resected lung tissue or peripheral blood. The demographics of cases and controls were checked by a research nurse or the treating physician against patient medical charts or the hospital lung cancer database (table 1).

Sample preparation and genotyping

DNA from cases diagnosed with NSCLC was extracted from peripheral blood or resected cryopreserved normal lung tissue as described previously [26]. Cases and controls from a previous study of *CYP1A1**2C were included [11, 26]. DNA from control subjects was extracted from peripheral blood. In 592 cases, DNA was extracted from more than one source (blood lymphocytes and fresh frozen normal lung tissue). In these cases, both sources were genotyped, with identical results in all cases, reinforcing the reproducibility of the methods. PCR-based restriction fragment length polymorphism (RFLP) methods were used to analyse *CYP1A1**2A [27] and *CYP1A1**4 [6] polymorphisms. Approximately 10% of the samples were randomly selected for repeat genotyping by PCR-RFLP in order to test reproducibility.

DNA sequencing and single-base extension genotyping

In order to confirm the accuracy of the PCR-RFLP methods, two representative samples per genotype were confirmed by DNA sequencing for each polymorphism. Samples were purified using the Wizard PCR Cleanup and Gel extraction kit (Promega, Madison, WI, USA) and sequenced at the Australian Genome Research Facility (AGRF, Brisbane, Australia) for DNA sequencing using BigDye Terminator V3.1 chemistry (Applied Biosystems, Foster City, CA, USA). The sequencing primers were identical to those used in PCR amplification. Sequences were visualised using Chromas V1.4 (C. McCarthy, School of Biomolecular and Physical Sciences, Griffith University, Brisbane, Australia).

In addition, an independent genotyping method, iPLEX single-base extension (Sequenom, San Diego, CA, USA), was also used to genotype a subset (89%) of subjects genotyped by PCR-RFLP methods. All experiments were performed by the AGRF. Briefly,

TABLE 1 Demographics of cases and controls using the PCR restriction fragment length polymorphism method

	NSCLC	Controls [#]	p-value [†]
Subjects n	1040	784	
Sex			
Male	710 (68)	531 (68)	0.807
Female	330 (32)	253 (32)	
Age yrs	65.7 ± 10 (32–92)	63.1 ± 12.7 (19–93)	<0.0001
Smoking history	48 ± 35.1 (0–270)	49.1 ± 33.5 (0–246)	0.466
pack-yrs⁺			
Histology			
AC	407 (39)		
SCC	373 (36)		
Other	260 (25)		
Tumour stage			
I	422 (41)		
II	202 (19)		
III	231 (22)		
IV	113 (11)		
Unknown	72 (7)		

Data are presented as n (%) or mean ± sd (range) unless otherwise indicated. NSCLC: nonsmall cell lung cancer; AC: adenocarcinoma; SCC: squamous cell carcinoma. [#]: subjects with chronic obstructive pulmonary disease combined with healthy smokers; [†]: Chi-squared test or t-test, cases versus controls, with level of significance (α) considered to be 0.05 and significant values shown in bold type; ⁺: a measure of cumulative smoking exposure, defined as the mean number of packs (20 cigarettes·pack⁻¹) of cigarettes smoked daily multiplied by the number of years of smoking.

genomic DNA was amplified using primers with 10-mer tags, designed to amplify a 75–150-bp amplicon. Following this, shrimp alkaline phosphatase was added to each reaction in order to remove any unincorporated deoxyribonucleoside triphosphates (dNTPs) by cleavage of phosphates from dNTP groups. An iPLEX reaction master mix consisting of primer, enzyme, buffer and mass-modified nucleotides was then added and the samples placed in a thermocycler in order to permit addition of nucleotides to the polymorphic site, producing allele-specific base extension products of differing sizes. Products were then run on a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer in order to determine product size.

Statistical analysis

Distributions of genotypes and demographic variables were compared between cases and controls using Chi-squared tests for categorical outcome variables and two-sided unpaired t-tests for continuous outcome variables. ORs and 95% CIs were estimated in order to measure the association between lung cancer and genotype/haplotype frequency. Standard Chi-squared statistics were used to determine whether or not the three *CYP1A1* variants were in Hardy–Weinberg equilibrium (HWE). All statistical analyses for genotypes were performed using the SPSS software package (Version 13.0 for Windows; SPSS, Inc., Chicago, IL, USA). Haplotype analyses (haplotype frequency estimates and linkage disequilibrium) were carried

out using Haploview linkage software (Version 4.0) [28]. A p-value of <0.05 (two-tailed) was considered significant. Power calculations to evaluate ability to detect associations between NSCLC and *CYP1A1* variants among the present study population were determined using an α of 0.05 and 80% power. The present study had the power to detect ORs of 1.39, 1.48 and 1.69 for the variant alleles of *CYP1A1*2A*, *CYP1A1*4* and *CYP1A1*2C*, respectively, with 80% confidence at an α of 0.05.

RESULTS

Participant characteristics

In order to ensure that observed effects between cases and controls were due to genotype frequency and not other potential confounding factors, such as age, smoking history or sex, mean and frequency distributions were compared between cases and controls (table 1). Mean age differed significantly between cases and controls ($p<0.001$), with controls being slightly younger. In contrast, there were no significant differences in sex distribution ($p=0.81$) or smoking history in pack-yrs ($p=0.47$), excluding these factors as potential study confounders. The majority of NSCLC cases were adenocarcinoma or squamous cell carcinoma histological subtypes (39 and 36%, respectively).

In order to confirm the validity of combining COPD subjects and healthy smokers to form one control group, as done previously [26], Chi-squared tests were used to investigate whether or not there were significant differences in genotype frequency between the two groups. No significant difference in variant frequency was observed between COPD and healthy smokers ($p>0.05$; data not shown), validating the decision to combine these two subgroups.

PCR restriction fragment length polymorphism

Genotype frequencies for all three variants, determined by PCR-RFLP methods, are detailed in table 2. Variant allele frequencies were low in the control population for all of the SNPs. The minor allele frequencies for cases and controls combined were 4.6, 4.5 and 11.4% for *CYP1A1*4*, *CYP1A1*2C* and *CYP1A1*2A*, respectively. Since less than five samples were classified as homozygous for the variant allele in either cases or controls for *CYP1A1*2C* or *CYP1A1*4*, homozygous variants were combined with heterozygous genotypes for statistical analysis (table 3). In the present study, genotypes containing the variant allele of *CYP1A1*2A* occurred in 17.6% of the control population, *CYP1A1*4* in 11% and *CYP1A1*2C* in 5.5%. These values were in relative agreement with previous studies of Caucasian populations (*CYP1A1*2A* 18–23%; *CYP1A1*4* 2–3% and *CYP1A1*2C* 9–10%) [14, 16, 21–23]. In the present study population, the genotype frequencies of *CYP1A1*2A* and *CYP1A1*4* were in HWE for cases and controls; however, *CYP1A1*2C* was not (table 4). Sanger DNA sequencing of two samples per genotype was performed for each polymorphism in order to confirm the accuracy of the PCR-RFLP assays.

From the PCR-RFLP data, it was observed that subjects carrying the *CYP1A1*2A* variant were over-represented in NSCLC cases compared with controls (TT versus CT/CC; OR 1.43 (95% CI 1.35–1.51); $p=0.003$). A decreased risk of NSCLC was also observed for those with the *CYP1A1*4* variant (CC

TABLE 2 Distribution of cytochrome P₄₅₀ 1A1 (*CYP1A1*) genotypes amongst nonsmall cell lung cancer subjects and controls[#]

	Cases		Controls [†]		iPLEX		p-value ⁺	
	PCR-RFLP	iPLEX	PCR-RFLP	iPLEX	Samples failed	Samples not re-genotyped	RFLP	iPLEX
<i>CYP1A1*2C</i>								
AA	929 (89.3)	827 (89.9)	741 (94.5)	634 (94.2)	53 (3.2)	162 (9.7)	<0.001	0.008
AG	103 (9.9)	89 (9.7)	40 (5.1)	37 (5.5)	2 (1.4)	11 (7.7)		
GG	8 (0.8)	4 (0.4)	3 (0.4)	2 (0.3)	1 (8.3)	1 (8.3)		
Total n	1040	920	784	673	56	174		
<i>CYP1A1*2A</i>								
TT	797 (76.6)	675 (77.4)	646 (82.4)	541 (82.3)	67 (4.6)	150 (10.4)	0.008	0.008
CT	219 (21.1)	197 (22.6)	128 (16.3)	116 (17.7)	20 (5.8)	21 (6.1)		
CC	24 (2.3)	24 (2.7)	10 (1.3)	8 (1.2)	1 (2.9)	3 (8.8)		
Total n	1040	896	784	665	88	174		
<i>CYP1A1*4</i>								
CC	965 (92.8)	859 (93.4)	698 (89.03)	607 (90.3)	49 (2.9)	159 (9.6)	0.003	0.049
CA	74 (7.1)	60 (6.5)	81 (10.33)	65 (9.7)	9 (5.6)	14 (8.8)		
AA	1 (0.1)	1 (0.1)	5 (0.64)	0 (0)	0 (0)	0 (0)		
Total n	1040	920	784	672	58	173		

Data are presented as n (%) unless otherwise indicated. RFLP: restriction fragment length polymorphism; A: adenine; G: guanine; T: thymidine; C: cytosine. [#] using PCR-RFLP and Sequenom iPLEX (Sequenom, San Diego, CA, USA) methods; [†]: subjects with chronic obstructive pulmonary disease combined with healthy smokers; ⁺: Chi-squared test or t-test, cases *versus* controls, with level of significance (α) considered to be 0.05 and significant values shown in bold type.

versus CA/AA; OR 0.64 (95% CI 0.62–0.64); $p=0.005$). The wild-type genotype (CC) was slightly over-represented in cases *versus* controls (92.8 *versus* 89%). Data from a previous study on *CYP1A1*2C* were included in the present study. The variant allele was associated with a greater than two-fold increase in the risk of lung cancer.

In order to investigate associations between the three polymorphisms, haplotype analyses were performed to determine whether combinations of polymorphisms conferred a greater lung cancer risk. Five possible haplotypes were identified amongst cases and the larger control group, where the CAT haplotype was considered to be the wild-type (in allele order *CYP1A1*4*, *CYP1A1*2C*, *CYP1A1*2A*). The frequencies of CAT, CAC, AAT, CGC and CGT are presented in table 3. Two haplotypes were significantly over-represented in NSCLC subjects *versus* controls, CGC (4.1 *versus* 2.4%; $p=0.0038$; OR 1.76 (95% CI 1.19–2.60)) and CGT (1.4 *versus* 0.4%; $p=0.0026$; OR 3.26 (95% CI 1.46–7.28)). Conversely, AAT was significantly under-represented in cases *versus* controls (3.4 *versus* 5.4%; $p=0.0014$; OR 0.62 (95% CI 0.45–0.86)). CAC showed a general increase in lung cancer risk, although this did not reach significance (8.5 *versus* 6.9%; $p=0.0747$; OR 1.25 (95% CI 0.98–1.60)). It was found that *CYP1A1*2A* and *CYP1A1*2C* were in linkage disequilibrium (normalised disequilibrium constant (D') 0.749; logarithm of odds (logit; LOD) 66.5; $r^2=0.207$; 95% CI 0.67–0.81) but that *CYP1A1*4* and *CYP1A1*2C* (D' 0.01; LOD 0; $r^2=0$; 95% CI 0.01–0.696) and *CYP1A1*4* and *CYP1A1*2A* were not (D' 0.582; LOD 1.21; $r^2=0.0020$; 95% CI 0.17–0.82).

Single-base extension genotyping

As a secondary method of confirming PCR accuracy and to address the issue of Hardy–Weinberg disequilibrium, single-base extension genotyping methods (iPLEX) were used to

genotype cases and controls. It was possible to successfully re-genotype 89% of cases and controls with iPLEX, with failure rates of 3–5% for the three genotypes. Tables 2 and 3 illustrate the sample numbers and genotype and haplotype frequencies observed using iPLEX. All variants were in HWE for the control population (table 4), with two of three variants in HWE for cases (table 4).

Similar genotype frequencies were observed for *CYP1A1*2C*, *CYP1A1*4* and *CYP1A1*2A*, although relatively fewer variant homozygotes were observed for *CYP1A1*4* (five for PCR-RFLP *versus* none for iPLEX in controls). Observed associations between *CYP1A1* polymorphisms and lung cancer risk remained consistent, with subjects carrying the *CYP1A1*2A* variant over-represented among NSCLC cases compared to controls (TT *versus* CT/CC; OR 1.43 (95% CI 1.35–1.52); $p=0.005$), and carriers of the *CYP1A1*4* variant under-represented in NSCLC cases (CC *versus* CA/AA; OR 0.66 (95% CI 0.66–0.67); $p=0.026$). Carriers of the *CYP1A1*2C* variant were also associated with elevated lung cancer risk (AA *versus* AG/GG; OR 1.83 (95% CI 1.57–2.12); $p=0.002$), consistent with previous findings. Although the findings from PCR-RFLP remained consistent, a decrease in significance was observed, possibly due to the small decrease in sample number genotyped by iPLEX.

In order to ensure that the haplotype findings from PCR-RFLP remained consistent, new haplotype analyses were also performed on the iPLEX data. Only samples with iPLEX genotype data for all three polymorphisms were included in these analyses. Four possible haplotypes (CAT, CAC, CGC and AAT) were observed amongst cases and controls (in allele order *CYP1A1*4*, *CYP1A1*2C*, *CYP1A1*2A*; table 3), where CAT was considered to be the wild-type. No CGT haplotypes

TABLE 3 Distribution of cytochrome P₄₅₀ 1A1 (*CYP1A1*) genotypes and haplotypes amongst nonsmall cell lung cancer (NSCLC) subjects and controls[#]

	PCR-RFLP				iPLEX			
	NSCLC	Controls [†]	p-value ⁺	OR (95% CI)	NSCLC	Controls [†]	p-value ⁺	OR (95% CI)
Subjects n	1040	784						
Genotype								
<i>CYP1A1</i> *2C								
AA	929 (89.3)	741 (94.5)	≤0.001	2.057 (1.77–2.39)	827 (89.9)	663 (94.2)	0.002	1.829 (1.57–2.12)
AG or GG	111 (10.7)	43 (5.5)			93 (10.1)	39 (5.8)		
<i>CYP1A1</i> *2A								
TT	797 (76.6)	646 (82.4)	0.003	1.427 (1.35–1.51)	675 (75.3)	541 (81.4)	0.005	1.428 (1.35–1.52)
CT or CC	243 (23.4)	138 (17.6)			221 (24.7)	124 (18.6)		
<i>CYP1A1</i> *4								
CC	965 (92.8)	698 (89.0)	0.005	0.637 (0.62–0.64)	859 (93.4)	607 (90.3)	0.026	0.664 (0.66–0.67)
CA or AA [‡]	75 (7.2)	86 (11.0)			61 (6.6)	65 (9.7)		
Haplotype[‡]								
CAT	1715 (82.3)	1329 (84.5)	0.0511	1.0 (reference)	1526 (82.8)	1151 (85.3)	0.0536	
CAC	178 (8.5)	109 (6.9)	0.0747	1.251 (0.98–1.60)	157 (8.5)	91 (6.7)	0.0629	
AAT	72 (3.5)	84 (5.6)	0.0014	0.624 (0.45–0.86)	62 (3.4)	65 (4.8)	0.0365	
CGC	85 (4.1)	37 (2.3)	0.0038	1.761 (1.19–2.60)	97 (5.3)	41 (3.0)	0.0023	
CGT	30 (1.4)	7 (0.4)	0.0026	3.259 (1.46–7.28)	NA	NA		

Data are presented as n (%) unless otherwise indicated. RFLP: restriction fragment length polymorphism; OR: odds ratio; CI: confidence interval; A: adenine; G: guanine; T: thymidine; C: cytosine; NA: not applicable. [#]: using PCR-RFLP and Sequenom iPLEX (Sequenom, San Diego, CA, USA) methods; [†]: subjects with chronic obstructive pulmonary disease combined with healthy smokers; ⁺: significant associations shown in bold type; [‡]: no AA genotypes observed in this cohort; [§]: in allele order *CYP1A1**4 (C to A substitution at nucleotide 2453 (2453C>A)), *CYP1A1**2C (2455A>G), *CYP1A1**2A (3801T>C).

were observed in this group. Two of these haplotypes were significantly associated with either increased (CGC; 5.3 *versus* 3.0%; p=0.0023) or decreased lung cancer risk (AAT; 3.4 *versus* 4.8%; p=0.037), with CAC showing a trend towards increased lung cancer risk (8.5 *versus* 6.7%; p=0.063). These risk haplotypes were also identified in the present PCR-RFLP haplotype analyses. *CYP1A1**2A and *CYP1A1**2C remained in linkage disequilibrium (D' 1.0; LOD 98.17; r²=0.324; 95% CI 0.97–1.00), with *CYP1A1**4 and *CYP1A1**2C (D' 0.991; LOD 0.67; r²=0.002; 95% CI 0.11–0.98) and *CYP1A1**4 and *CYP1A1**2A not exhibiting disequilibrium (D' 1.0; LOD 2.76;

r²=0.006; 95% CI 0.48–1.00), as observed in the PCR-RFLP findings.

DISCUSSION

It has previously been shown that carriers with the *CYP1A1**2C Val allele (Ile/Val or Val/Val genotype) are significantly over-represented in NSCLC subjects compared to controls (OR 1.9 (95% CI 1.20–2.90); p=0.005), especially in females, those aged <64 yrs and those with <46 pack-yrs of tobacco exposure [26]. It has also previously been shown that the *CYP1A1**2C variant, in combination with the *MPO* risk allele, confers a significantly increased risk of NSCLC (OR 2.88 (95% CI 1.70–5.00); p<0.0001) [11]. In the present report, we present the genotype and haplotype frequencies of two additional *CYP1A1* polymorphisms in relation to lung cancer risk. These data indicate an association between *CYP1A1**2A and lung cancer risk, and support a recent pooled analysis of 2,451 lung cancer subjects and 3,358 controls that showed a clear association between the homozygous *CYP1A1**2A CC allele and lung cancer risk in Caucasians (age- and sex-adjusted OR 2.36 (95% CI 1.16–4.81)) [15]. Although a meta-analysis of HOULSTON [29] provided little support for involvement in lung cancer risk of variations in *CYP1A1*, a recent review investigating the role of polymorphisms in candidate genes for 18 different cancer sites reported an increased risk of lung cancer for carriers of the *CYP1A1**2A variant in Caucasian populations (OR 2.36 (95% 1.16–4.81); p=0.018) in addition to variants of *CYP1A1**2C in Asian populations (OR 1.61 (95% CI 1.24–2.08); p=0.0003) [30]. Associations between *CYP1A1* variations and lung cancer risk have also been observed in never-smokers with lung cancer [24, 31].

TABLE 4 Hardy–Weinberg Chi-squared tests for nonsmall cell lung cancer (NSCLC) subjects and controls

Genotype	PCR-RFLP		iPLEX [#]	
	NSCLC	Controls	NSCLC	Controls
<i>CYP1A1</i> *2A	0.16	0.45	0.04	0.51
<i>CYP1A1</i> *2C	0.03	0.01	0.34	0.07
<i>CYP1A1</i> *4	0.48	0.27	0.96	0.18

p-values of >0.05 were considered to be consistent with Hardy–Weinberg equilibrium. RFLP: restriction fragment length polymorphism; *CYP1A1*: cytochrome P₄₅₀, family 1, subfamily A, polypeptide 1 gene. [#]: Sequenom, San Diego, CA, USA.

*CYP1A1*2A* and *CYP1A1*2C* have been reported to be in linkage disequilibrium in Caucasian [18] and Asian populations [27], although not in people of African descent [32]. Linkage disequilibrium can be influenced by a variety of factors, including genetic linkage, recombination, mutation rates, random drift, nonrandom mating and population structure. In the present study, *CYP1A1*2A* and *CYP1A1*2C* were in linkage disequilibrium, confirming reports from previous studies [18]. Conversely, as in *CASCORBI et al.* [6], no evidence of linkage disequilibrium between *CYP1A1*2C* and *CYP1A1*4* was observed. This may be due to the close proximity of the two variants (only one base pair separates the two polymorphic sites) decreasing the rate of recombination.

The function of *CYP1A1*4* has not been clearly established, although it has been suggested that it shows the greatest enzymatic efficiency of all of the *CYP1A1* polymorphisms [33]. In the present study, very few homozygous variants of *CYP1A1*4* were identified among cases or controls, limiting any ability to draw strong conclusions for a Caucasian population. However, a general decrease in the frequency of heterozygotes was observed in this study population compared to controls. Others have reported no clear association between lung cancer risk and *CYP1A1*4* [3].

Very few haplotype analyses studying the interactions between *CYP1A1*2A*, *CYP1A1*2C* and *CYP1A1*4* have been performed in populations of Caucasian lung cancer subjects. A recent pooled analysis studying interactions between *CYP1A1* polymorphisms and *GSTM1/GSTT1* in an Asian population identified the GT and AC haplotypes as being associated with lung cancer risk compared to AT (OR 3.41 (95% CI 1.78–6.53) and 1.39 (1.12–1.71), respectively) [34]. Among the present population, the haplotypes CGC and CGT (in allele order *CYP1A1*4*, *CYP1A1*2C*, *CYP1A1*2A*) were associated with an increased risk of lung cancer, whereas AAT was associated with a decreased risk. In an Indian population, a four-fold increased lung cancer risk (hazard ratio 3.90 (95% CI 1.00–5.10); $p=0.025$) was shown for the CGC haplotype [3], suggesting that this haplotype may be an important risk factor for NSCLC. Although the CAC haplotype has been associated with higher enzymatic activity and decreased cancer risk in prostate cancer [35], *SHAH et al.* [3] showed a trend for increased lung cancer risk, although this did not reach significance. The differences observed may be due to tumour type, gene expression or functional role of *CYP1A1*. For example, in addition to detoxification of carcinogens, *CYP1A1* also has a role in the oxidative metabolism of oestrogens [36], which have been implicated in the aetiology of prostate cancer [37, 38]. *CHANG et al.* [35] suggested that, in prostate cancer, *CYP1A1*'s role of oestrogen metabolism may be more important than that of carcinogen detoxification. Therefore, it is possible that the role of *CYP1A1* differs between cancer types, resulting in haplotype associations that vary according to tumour origin.

Interpretation of the data from the present study are curtailed by the low frequency of some variant genotypes (for instance very few *CYP1A1*4* AA genotypes were observed) limiting any ability to draw statistically valid conclusions. Subjects in the control group also have the potential to develop lung cancer in the future, a source of misclassification bias. One of the

polymorphisms, *CYP1A1*2C* was not in HWE in either controls or cases using PCR-RFLP, but was in HWE using iPLEX. In addition, although *CYP1A1*2A* was in HWE using PCR-RFLP, it was not in HWE using iPLEX in NSCLC subjects. This may be due to chance (from the selection of a large subset of the original samples), decreased sensitivity of iPLEX due to differences in sample concentration ($10 \text{ ng} \cdot \mu\text{L}^{-1}$ for iPLEX versus $10\text{--}40 \text{ ng} \cdot \mu\text{L}^{-1}$ for PCR-RFLP, as determined by gel estimation techniques), a nonrandom population (highly unlikely) or a possible association with NSCLC risk. In addition, *CYP1A1*4* and *CYP1A1*2C* are separated by only one nucleotide on exon 7, raising the possibility that misclassification could occur. In the present study, an attempt was made to overcome this by employing published assays using enzymes that selectively digest each polymorphism irrespective of the neighbouring polymorphism, thereby decreasing the risk of misclassification bias. Finally, the present study evaluated three commonly studied *CYP1A1* variants; however, it is possible that other SNPs may be in linkage disequilibrium with these variants, and may also be instrumental in determining lung cancer risk.

Technical validation of the present PCR-RFLP method by iPLEX confirmed the observed associations for all of the *CYP1A1* polymorphisms. Similar genotype frequencies were observed for *CYP1A1*2C*, *CYP1A1*4* and *CYP1A1*2A* in both case and control populations. Haplotype analyses also confirmed association of haplotypes AAT and CGC with decreased and increased lung cancer risk, respectively. No CGT haplotypes were observed in these analyses, and association with lung cancer risk could not be confirmed. Despite the significance of these results decreasing in the iPLEX cohort, it remained possible to confirm the observations obtained using PCR-RFLP, increasing our confidence in the reproducibility of genotyping techniques. Owing to the high reproducibility rate, we are confident that these associations are valid and support a role for *CYP1A1* polymorphisms in altering lung cancer risk.

In conclusion, the results of the present study confirm that *CYP1A1* polymorphisms are a minor risk factor for NSCLC. Although several studies have confirmed associations between these polymorphisms and lung cancer risk in the past, few have explored linkage effects between the three different *CYP1A1* polymorphisms in a Caucasian population. To the present authors' knowledge, this is the first study to show that haplotypes CGC, CGT and AAT are strongly associated with lung cancer risk in Caucasians. Identification of these haplotypes may assist in risk stratification, early detection and improvement of current treatment options for subjects with lung cancer. Larger studies are required in order to explore these risk *CYP1A1* haplotypes further.

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STATEMENT OF INTEREST

None declared.

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