Published in final edited form as:
Cancer Epidemiol Biomarkers Prev. 2011 December ; 20(12): 2603–2609. doi: 10.1158/1055-9965.EPI-11-0749.

A case-control study of a sex-specific association between a 15q25 variant and lung cancer risk

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Abstract

Background—Genetic variants located at 15q25, including those in the cholinergic receptor nicotinic cluster (*CHRNA5*) have been implicated in both lung cancer risk and nicotine dependence in recent genome-wide association studies. Among these variants, a 22 base pair insertion/deletion, rs3841324 showed the strongest association with CHRNA5 mRNA expression levels. However the influence of rs3841324 on lung cancer risk has not been studied in depth.

Methods—We have therefore evaluated the association of rs3841324 genotypes with lung cancer risk in a case-control study of 624 Caucasian subjects with lung cancer and 766 age- and sex-matched cancer-free Caucasian controls. We also evaluated the joint effects of rs3841324 with single-nucleotide polymorphisms (SNPs) rs16969968 and rs8034191 in the 15q25 region that have been consistently implicated in lung cancer risk.

Results—We found that the homozygous genotype with both short alleles (SS) of rs3841324 was associated with a decreased lung cancer risk in female ever smokers relative to the homozygous wild-type (LL) and heterozygous (LS) genotypes combined in a recessive model ($OR_{adjusted}$ = 0.55, 95% CI = 0.31–0.89, P = 0.0168). There was no evidence for a sex difference in the association between this variant and cigarettes smoked per day (CPD). Diplotype analysis of rs3841324 with either rs16969968 or rs8034191 showed that these polymorphisms influenced the lung cancer risk independently.

Conclusions and impact—This study has shown a sex difference in the association between the 15q25 variant rs3841324 and lung cancers. Further research is warranted to elucidate the mechanisms underlying these observations.

Keywords

lung cancer; *CHRNA5*; Chromosome 15q25; rs3841324; sex-specific association

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Disclosures: No potential conflicts of interest.

Introduction

Lung cancer is the leading cause of cancer death in the U.S. It accounts for 13% of all cases and 23% of all deaths from cancer worldwide and represents a major public health problem (1;2). Although tobacco smoking is the major etiologic risk factor for lung cancer (3), genetic-epidemiological studies have provided compelling evidence that genetic factors also play a significant role (4–6). Recently, three genome-wide association studies demonstrated the association of a region of chromosome 15q24-25.1 with lung cancer (7–9). This region contains six genes - iron-responsive element-binding protein 2 (IREB2), *AGPHD1*, *PSMA4,* cholinergic receptor nicotinic α5 (*CHRNA5*), cholinergic receptor nicotinic α3 (*CHRNA3*), and cholinergic receptor nicotinic β4 (*CHRNB4*) that are good candidates to harbor variants that influence lung cancer risk.

CHRNA5, CHRNA3, and *CHRNB4* have been well studied and are known to encode nicotinic acetylcholine receptor subunits (nAChRs), a family of pentameric (mostly heteropentameric) ligand-gated ion channels that can mediate fast signal transmission at synapses (10) and modulate the release of several neurotransmitters (11). nAChRs are the initial physiological targets of nicotine in the central and peripheral nervous systems. Within a few seconds of smoking, nicotine is delivered to the synapses where these receptors are expressed to produce physiological and pharmacological responses. In addition, recent studies have also shown that nicotine can stimulate cellular proliferation, tumor invasion, and angiogenesis and inhibits apoptosis through nAChR-mediated processes (12–14). Therefore, it is biologically plausible that genetic variations in these genes may influence lung cancer incidence by affecting either smoking behavior mediated by the nAChRs genes or by directly increasing cancer risk in a genotype dependent manner.

Several single-nucleotide polymorphisms (SNPs) in the 15q24-25.1 region have been found to be significantly associated with lung cancer risk (5;7–9;15), such as *CHRNA5* rs16969968 ($P = 1\times10^{-20}$) (8) and it's highly correlated SNP rs8034191 ($P = 3\times10^{-18}$) in *AGPHD1*) (7). Although it is unclear that whether this association with lung cancer is a direct effect on lung cancer vulnerability or through the indirect effect of increased risk of smoking, this locus is a risk factor for nicotine dependence and smoking quantity. Therefore, it is likely that the risk allele of SNPs in this locus increase the lung cancer risk through smoking behavior mediated by nicotine dependence susceptibility. rs16969968 is a missense variant that results in an aspartic acid (G allele) change to asparagine (A allele) at codon 398 of *CHRNA5*. A recent study (16) reported that α5 Asn398 lowers Ca^{2+} permeability and increases short-term desensitization in (α4β2)2α5, the most abundantly expressed receptor subtype in the brain. It has also been reported that individuals with one copy of the A "risk allele" for rs16969968 have a 1.3 fold increase in nicotine dependence susceptibility (17). Expression of the A "risk allele" *in vitro* reduces nAChR function through regulation dopamine-mediated reward signaling, thereby facilitating dependence (5). Interestingly, one copy of A "risk allele" for rs16969968 also has a 1.30 fold increase in lung cancer risk (8). These results suggest that the lung cancer risk allele matches the risk allele for nicotine dependence (17). The rs8034191 (T->C) is a non-coding variant located in the third intron of *AGPHD1*. Individuals with one copy of C "risk allele" for rs8034191 have a 1.28 fold increase in lung cancer risk (7) and smoked on average 1.3 more cigarettes per day than individuals who did not carry the risk allele (18). rs8034191 is in nearly complete linkage disequilibrium with rs16969968 and these two SNPs are the most consistently associated with lung cancer (7;8).

Previously, we performed sequencing analysis of *CHNRA5* gene and identified a 22 bp insertion/deletion (indel) at position −71 upstream of the transcription start site (unpublished data), which later was reported as rs3841324 in NCBI. This indel rs3841324 showed the

highest association with CHRNA5 mRNA levels in both brain and lung tissue (19–21). The lower mRNA expression of CHRNA5 along with the G "non-risk allele" of rs16969968 is reported to be protective for nicotine dependence and lung cancer (20). Therefore, the rs3841324 that was previously shown to affect expression level would associate with variation in risk for lung cancer and possibly smoking behavior. However the influence of rs3841324 on lung cancer risk has not been studied further in depth. To address this gap in knowledge, we compared the distribution of the rs3841324 in a case-control study that included 624 Caucasian patients with lung cancer and 766 cancer-free control subjects, evaluated the association between this variant and lung cancer risk and then tested whether this variant work jointly with rs16969968 and rs8034191.

Materials and Methods

Study population

The study participants for this case-control study were a consecutive series of lung cancer cases recruited for an ongoing lung cancer study that has been accruing participants at The University of Texas MD Anderson Cancer Center since 1995. The control subjects were recruited from the Kelsey-Seybold Clinic, Houston's largest multidisciplinary physician practice. The control subjects were frequency matched to the cases on age (±5 years), sex and smoking status (22). Only Caucasian subjects (624 cases and 766 controls) were included in this study and all of them were genotyped for rs3841324 as described below. Among these subject, 441 cases and 520 controls were included in a recently reported Genome wide association study of lung cancer conducted at MD Anderson that was reported recently (7) and 183 case and 246 control of the participants genotyped using an Illumina iSelect Infinium platform (23). All participants provided informed consent and the study was approved by the MD Anderson Institutional Review Board.

DNA extraction and genotyping

Ten milliliters of blood from each study participant was drawn into a Vacutainer tube containing ethylenediamine tetraacetic acid (EDTA) (Becton Dickinson Vacutainer System, Rutherford, NJ). DNA was isolated with Qiagen Kits (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. For rs3841324 genotyping, agarose gel electrophoresis was used following amplification using Polymerase chain reaction (PCR). The primer sequences for PCR were: 5′-GCT AGG AGC AGA CAG GGT TG-3′ (forward) and 5′-GAG ACA AAA CGA GGG CAG AC-3′ (reverse). The PCR amplification was carried out in a final volume of 30-μL mixture, containing 10 ng of DNA, 0.4 mM of each primer, deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) each at 0.25 mM, KCl at 50 mM, $MgCl_2$ at 1.5 mM, Tris-HCl at 10 mM (pH 8.3), and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). The reaction was started at 95°C for 10 minutes, followed by 35 cycles of amplication (95°C for 30 seconds, 61°C for 30 seconds, and 72°C for 40 seconds) and then extension at 72°C for 7 minutes. The PCR product was then subjected to 2% agarose gel electrophoresis, the expected size of PCR product are 269 base pair for L allele (L: long allele) and 247 base pair for S allele (S: short allele-deletion) respectively. The genotype of LL, LS and SS was read independently by two people and there was no discrepancy between the readout by two individuals. Genotypes of rs16969968 and rs8034191 were obtained either from the GWAS (7) that was previously conducted using an Illumina HapMap 300 version 1.1 array or using data derived from analysis of an Illumina iSelect Infinium Array (23).

Statistical methods

Hardy-Weinberg equilibrium of allele frequencies was tested using a Fisher's exact test. Univariate and multivariate logistic regression analysis was used to estimate odds ratios

(ORs) and 95% confidence intervals (CIs). Chi-square tests were used to compare genotypic frequencies in the cases and controls. The association between rs3841324 genotype and number of cigarettes smoked per day (CPD) and smoking duration was evaluated using one-way analysis of variance, with a test for variability among genotypic group. The recessive effects of rs3841324 were modeled using a dichotomous indicator variable. Age, race, gender, and smoking history were included in the multivariate logistic regression model when appropriate. The level of significance was set to $P < 0.05$ for all statistical analysis. All statistical analyses were performed using SAS/STAT and SAS/Genetics software, version 9 (SAS Institute, Inc, Cary NC).

Results

Patient characteristics

The demographic data for 624 lung cancer patients and 766 unaffected control subjects are described in Table 1. There were no statistically significant differences in the distribution of sex and smoking status between cases and controls because of frequency matching. The patients were, on average, 1.3 years old than the control subjects, but within the study 5 year matching range (P=0.0217).

Genotype frequency

The genotypic frequency of rs3841324 was consistent with the Hardy-Weinberg equilibrium (P=0.7864). The minor allele (S allele) frequency for rs3841324 was 0.44, which is similar to 0.47 reported previously by Wang et al for an European American sample (20). The distribution of genotypes between case and control subjects is shown in Table 2.

Effect of rs3841324 genotype on lung cancer risk

The rs3841324 has been associated with *CHRNA5* mRNA level (19–21). In *Wang* et al (21), subject with rs3841324 SS genotype has statistically significant difference in mRNA expression compared to the ones with either LL or LS genotype and there was no significant difference between LL and LS genotype. Based on this observation, we evaluated the effect of rs3841324 genotype on lung cancer risk in a recessive model. We grouped the patients by gender and smoking status (with ever smokers including both current and former smokers) and then stratified by rs3841324 genotype (Table 3) and found that in ever smokers the recessive genotype of rs3841324 (that is, rs3841324 SS) significantly reduced lung cancer risk in women ($OR_{adjusted}$ = 0.55, 95% CI = 0.31–0.89, P = 0.0168) but had little effect in men ($OR_{adjusted}$ = 1.17, 95% CI = 0.78–1.75, P = 0.444). No significant association between rs3841324 genotype and lung cancer risk was observed in never smokers, but the sample size was small.

Association of rs3841324 genotype and CPD or smoking duration in ever smoker

We examined whether rs3841324 genotype was associated with cigarettes smoked per day (CPD) or smoking duration among ever smokers in cases and controls and among females and males respectively. Both CPD and smoking duration did not differ between groups stratified by rs3841324 genotypes, sex or case-control status, suggesting that no association between rs3841324 and CPD or smoking duration (Supplementary data, Table 1).

Association of rs3841324 and rs16969968, and rs8034191

Genetic variants rs16969968 (Asp398Asn) and rs8034191 (T->C) located at 15q25 have been associated with lung cancer risk (5;7–9;15). Here we observed a protective effect of rs3841324 SS genotype on lung cancer risk for female Caucasians smokers. Therefore, we performed diplotype analysis to test whether the rs3841324 genotype and the presence of

rs16969968 (Asp398Asn) or rs8034191 (T->C) jointly influence lung cancer risk in female Caucasian smokers.

First, we tested the association of lung cancer and rs3841324-rs16969968 diplotype. We observed nearly complete linkage disequilibrium between these two SNPs (r2=0.389; D′=0.955). As showed in Table 4, the risk genotypes of LL_LS at rs3841324 occurred on both the risk allele (A) and the non-risk allele (G) of rs16969968, while the protective genotype of rs3841324 (SS) almost always occurred on rs16969968 GG genotype. Subject with the SS_GG diplotype had a decreased risk of developing lung cancer ($OR_{adjusted}$ = 0.62, CI = 0.33–1.67) relative to the subject with the LL_GG or LS_GG diplotype. Conversely, subjects with the LL_AA or LS_AA diplotype had the highest risk for lung cancer ($OR_{adjusted}$ = 1.60, CI = 0.85–3.00). This analysis illustrated that the two variants may independently influence the risk for lung cancer, suggesting that they function through different mechanisms. However the sample size is limited and further test will be needed.

Next, we tested the association of lung cancer and the rs3841324-rs8034191 diplotype. The result was similar to what we observed for the rs3841324-rs16969968 diplotype: nearly complete linkage disequilibrium between rs8034191 and rs3841324 was observed (r2 = 0.399; D′ = 0.970). As shown in Table 4, the risk genotype of rs3841324 (LL_LS) occurred on both the risk allele (C) and the non-risk allele (T) of rs8034191, while the protective genotype of rs3841324 (SS) almost always occurred on the rs8034191 TT genotype. Subject with the SS_TT diplotype has a decreased risk of developing lung cancer ($OR_{adjusted}$ = 0.66, CT = 0.35–1.24) relative to subjects with the LL_TT or LS_TT diplotype, while those with the LL_CC or LS_CC diplotype had the highest risk for lung cancer ($OR_{adjusted}$ = 1.78, CI = 0.94–3.36). These observations suggested that the rs8034191 and rs3841324 genotypes may influence the risk of lung cancer independently. This observation deserves further replication due to small sample size.

Discussion

On the basis of our results, we conclude that susceptibility to lung cancer in our cohort of Caucasian women is influenced by the genotype of rs3841324, a functional promoter polymorphism in the *CHRNA5* subunit, which has been clearly identified as a susceptibility gene for lung cancer. We observed that rs3841324 SS genotype is protective against lung cancer in female smokers but not in male. Further analysis showed no significant difference in CPD or smoking duration between groups stratified by rs3841324 genotypes, sex or case-control status. In addition, no joint effect between rs3841324 and rs16969968 or rs8034191 was observed.

Previously, functional characterization of *CHNRA5* using luciferase assays in human cell lines has demonstrated that the −240/+53 region, which contains the rs3841324 indel, is the core promoter (24;25). Using a standardized reporter gene assay system, Buckland et al. found that the rs3841324 minor allele (S allele) decreased promoter activity in HEK293 cells by 1.5-fold (26). Recently, Zheng et al. (27) demonstrated that the rs3841324 S allele was associated with hypoactivity of the promoter, resulting in a 2- to 6-fold decrease in *CHRNA5* transcription compared with that of major allele (L allele) in A549 cells. In addition, the rs3841324 L allele showed higher affinity to nuclear extraction proteins of A549 cells than did the S allele, suggesting a difference in *CHRNA5* transcription by influencing DNA-protein interactions (27). The potential transcription factor prediction with TFSEARCH [(28); accessed on June 9, 2011] show that rs3841324 contains a predicted binding site for the Sp1 transcription factor, which recognizes and specially binds to GC-rich regions such as the GC-box (29;30). Therefore, it is very likely that deletion of

rs3841324 would reduce the number of binding sites for SP1 transcription factor, thus influencing DNA-protein interactions and cause difference in *CHRNA5* transcription.

In the present study, we showed that the SS variant of rs3841324 was significantly associated with reduced risk of lung cancer in female smoker. There is some biological plausibility for this protective association. In the mammalian brain, nAChRs include homopentameric α7 receptors and a variety of heteropentamers, but predominantly α4β2*. CHRNA5 is most commonly found in heteromeric receptors composed α4β2α5 subunits. Inclusion of the α5 subunit in α4β2 receptors significantly increases the rate of desensitization of α4β2* nAChRs (31–33). α4β2 nAChRs play important roles in regulation of anti-inflammatory processes, immune processes, and fundamental pathways involved in cell survival (34–36), therefore, desensitization of α4β2 may be an important force in the development of human cancer. Hypoactivity of the promoter of *CHRNA5* decreases the rate of desensitization of α4β2 nAChRs, thereby potentially reducing the risk of lung cancer. In addition, inclusion of the α5 subunit in α4β2 nAChRs significantly increases Ca^{2+} permeability (37–39). Ca^{2+} signals are pivotal in regulating nAChRs-mediated gene expression and cell signaling which may lead to gene activation (in addiction) (40;41) or to cell proliferation (in cancer) (42). Therefore, further studies investigating the mechanism by which CHRNA5 affects the risk of lung cancer are needed to elucidate this new protective pattern.

Some epidemiologic evidence suggests a sex difference in the association between the 15q25 variants and lung cancer(43–46). Although speculative, some data have linked nAChRs signaling to sex hormones. For example, studies have shown that steroid hormones, including progesterone, are noncompetitive antagonists of nAChRs (43;47). There are reports that sex hormones regulate nAChR expression or activity in the rat hippocampus (48). In addition, a putative progesterone responsive element was found in the promoter of α5 nAChR subunit, and progesterone has been shown to have an effect on the α5 expression level both in vitro and in vivo (49). It is biologically plausible that the interplay between sex hormones and α5-containing nAChRs may play a direct or indirect role in the mediation of sex differences in susceptibility to lung cancer. Our present results revealed a sex-specific association of rs3841324 on lung cancer risk. In order to exclude the possibility that the observed sex-specific association could potentially be caused by a difference in variant allele frequency in male and female controls, we calculated the frequency of rs3841324 variant allele for male controls (MAF=0.45) and female controls (MAF=0.44) in the study and found there was little difference (P=0.5949), suggesting that this hospital-based study is unlikely to have yielded any significant bias in estimating the genotype-specific ORs.

Previous studies have consistently found that the genetic variants rs16969968 and rs8034191 in 15q25 are associated with lung cancer risk and nicotine dependence (5;7–9;15). The diplotype analysis showed high linkage disequilibrium between rs3841324 and both rs16969968 and rs8034191. Because rs16969968 (D398->N398) is believed to alter receptor activity while rs3841324 is assumed to correspond to mRNA expression level, the influences of these variants on lung cancer risk are independent. rs3841324 SS_rs16969968 GG diplotype was associated with decreased risk for lung cancer, and the rs3841324 LL_rs16969968 AA diplotype exhibited the highest risk. These findings suggest that the risk associated with the amino acid change might counteract the protective effect of the change in gene expression to some degree. The underlying mechanism by which rs8034191 (T->C) influences lung cancer is unclear. However, diplotype analysis of rs3841324_rs8034191 showed a trend similar to that of rs3841324 SS_rs16969968; the rs3841324 SS_rs8034191 TT diplotype was associated with decreased risk and the rs3841324 LL_rs8034191 CC diplotype with the highest risk. Together, these results suggest that although variation in 15q25 influences the risk for lung cancer and nicotine dependence, different polymorphisms

and different mechanisms of action are responsible for their effects. However, these observations deserve further test due to small sample size.

In summary, the current study indicates that the rs3841324 SS genotype is protective against lung cancer in Caucasian female smokers. In contrast, there is little such effect in Caucasian male, implying that the effect is sex-specific. Our results also indicate a new association between *CHNRA5* promoter activity and susceptibility to lung cancer, implying that *CHRNA5* plays a complex role in lung cancer. The underlying mechanism of sex differences in susceptibility to lung cancer remains unclear and will require in-depth molecular analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Kathryn Carnes and Sarah Bronson for manuscript editing. This research is supported in part by the National Institutes of Health through MD Anderson's Cancer Center Support Grant CA016672, research grants CA55769, CA127219, R01 CA121197, 1P50 CA70907, and U19 CA148127. This research was also supported by Cancer Prevention Research Institute of Texas grant RP100443.

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Table 1

Demographic characteristics of study subjects

	Combined			Female			Male		
	cases n=624	**controls n=766**	***P***	**cases n=307**	**controls n=386**	***P***	**cases n=317**	**controls n=380**	***P***
Sex, No. (%)			0.6582						
Male	317(50.80)	380(49.61)							
Female	307(49.20)	386(50.39)							
Smoking Status, No. (%)			0.0576			0.0247			0.4502
Never	142(22.76)	136(17.75)		100(32.57)	90(23.32)		42(13.25)	46(12.11)	
Former	264(42.31)	356(46.48)		104(33.88)	146(37.82)		160(50.47)	210(55.26)	
Current	218(34.94)	274(35.77)		103(33.55)	150(38.86)		115(36.28)	124(32.63)	
Mean age (SD)	61.8(11.6)	60.5(10.3)	0.0217	61.4(11.6)	59.6(10.8)	0.0248	62.2(11.6)	61.6(9.8)	0.2901
No. of cigarettes per day (SD)	21.3(16.6)	20.0(15.3)	0.1552	15.9(14.3)	16.6(14.0)	04538	26.6(17.0)	23.4(15.9)	0.0106
Years smoked (SD)	27.4(18.5)	25.4(16.8)	0.0269	23.5(19.5)	23.2(17.0)	0.9228	31.2(16.7)	27.5(16.4)	0.0040

P values were calculated with a X2 test for categorical variables and with a Wilcoxon test for continuous variables. All statistical tests were two-sided.

Never smokers were defined as those who had smoked fewer than 100 cigarettes in their lifetime.

Table 2

Distribution of select variables by rs3841324 genotype

	Case (N=624)				Control(N=766)			
	LL No. (%)	LS No. (%)	SS No. (%)	*P*	LL No. (%)	LS No. (%)	SS No. (%)	*P*
				0.0316				0.8147
Male	100(50.00)	150(47.47)	67(62.04)		114(47.90)	188(50.27)	78(50.65)	
Female	100(50.00)	166(52.53)	41(37.96)		124(52.10)	186(49.73)	76(49.35)	
				0.7726				0.7626
Ever	151(75.50)	247(78.16)	84(77.78)		195(81.93)	311(83.16)	124(80.52)	
Never	49(24.50)	69(21.84)	24(22.22)		43(18.07)	63(16.84)	30(19.48)	
				0.4602				0.8372
Current	66(43.71)	118(47.77)	30(40.48)		88(45.13)	134(43.09)	52(41.94)	
Former	85(56.29)	129(52.23)	50(59.52)		107(54.87)	177(56.91)	72(58.06)	
				0.4819				0.5852
<65	111(55.50)	182(57.59)	55(50.93)		148(62.18)	247(66.04)	97(62.99)	
≥65	89(44.50)	134(42.41)	53(49.07)		90(37.82)	127(33.96)	57(37.01)	

S: short allele-deletion; L: long allele

Table 3

Association of rs3841324 genotype and lung cancer risk in group stratified by sex and smoking status

	Ever smoker*						Never smoker^					
Genotype	**Male**			**Female**			**Male**			**Female**		
	N	**OR(95%CI)**	***P***	**N**	**OR(95%CI)**	***P***	**N**	**OR(95%CI)**	***P***	**N**	**OR(95%CI)**	***P***
LL+LS	481	1.00 (Ref)		423	1.00 (Ref)		71	1.00 (Ref)		153	1.00 (Ref)	
SS	128	1.17(0.78, 1.75)	0.4440	80	0.55(0.31, 0.89)	0.0168	17	0.50(0.16, 1.51)	0.2181	37	0.84(0.41, 1.73)	0.6357

*adjusted for age, CPD and years of smoking

^adjusted for age

Table 4

Association of rs3841324 genotype plus rs16969968 or rs8034191 genotype in female ever smoker

	rs3841324	
rs16969968	**LL_LS (cases /control)**	**SS (cases /control)**
GG	45/73	22/55
OR (95% CI)	1.00 (reference)	0.62(0.33, 1.67)
GA	99/133	0/2
OR (95% CI)	1.12(0.71, 1.79)	NC
AA	32/32	1/0
OR (95% CI)	1.60(0.85, 3.00)	NC
rs8034191		
TT	42/72	22/55
OR (95% CI)	1.00 (reference)	0.66(0.35, 1.24)
TC	100/135	0/2
OR (95% CI)	1.19(0.74, 1.91)	NC
CC	33/31	1/0
OR (95% CI)	1.78(0.94, 3.36)	NC

NC: Not calculated due to small sample size of subjects with rs3841324 SS genotype

OR: adjusted for age, CPD, years of smoking