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Tumor necrosis factor gene polymorphisms are associated with chronic obstructive pulmonary disease

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ABSTRACT

Tumor necrosis factor-α (TNF-α) has been shown to be an important factor in animal models of Chronic Obstructive Pulmonary Disease (COPD); however, human studies of *TNF* polymorphisms in COPD have been equivocal. We investigated six *TNF* single nucleotide polymorphisms (SNPs) (-1031C/T, -863C/A, -857C/T, -237G/A, -308G/A, +487G/A) and their haplotypes in 423 Caucasian smokers (298 with spirometric evidence of COPD patients and 125 without airflow obstruction). The -308 minor allele (A) was associated with a higher odds ratio (OR) of being associated with COPD in multivariate analysis (controlling for age, sex, pack-years; OR 1.9; 95%CI 1.1-3.2, p=0.03) and was also associated with worse FEV_1/FVC (p=0.03). The -237 minor allele (A) had a lower OR of being associated with COPD (OR 0.40; 95%CI 0.19-0.86, p=0.02). In COPD patients, the -857 minor allele (T) had a lower OR of being associated with severe stages of COPD (GOLD stage 3 and 4 versus stage 1 and 2; OR 0.46; 95%CI 0.24-0.88, p=0.02). Other TNF SNPs were not associated with COPD; however the -1031/-863 haplotype CC/TC had a lower OR in COPD patients versus smoking controls (OR 0.22; 95%CI 0.05-.97, p=0.05). This study adds further evidence that *TNF* genotypes play a role in susceptibility to cigarette smoke.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) will develop in only 25-40% of cigarette smokers [1]; however, risk factors for susceptibility to COPD in smokers has not been completely determined. α_1-antitrypsin deficiency, which is the best documented genetic risk factor for COPD, accounts for only an estimated 1% to 2% of cases [2, 3]. Other host factors are suspected to be involved in the remaining 98-99% of cases.

One candidate susceptibility gene for COPD is tumor necrosis factor (*TNF*), the gene coding for the protein that is processed to TNF-α. In mice, TNF-α overproduction has led to pulmonary emphysema and inflammation [4, 5], and is thought to drive approximately 70% of cigarette smoke-induced emphysema and inflammation [6, 7]. TNF-α may exert its effects by stimulating the release of other enzymes such as macrophage metalloelastase [8]. In COPD patients, there is a higher concentration of TNF-α in bronchial biopsies [9], induced sputum [10], and bronchoalveolar lavage fluid (BALF) compared to control subjects [11]. TNF-α levels in sputum are also increased significantly during acute exacerbations of COPD [12, 13]. A recent meta-analysis found an association between COPD and elevated serum TNF-α levels [14]. In contrast, healthy smokers have no increase in TNF-α in BALF [11, 15], and their alveolar macrophages have decreased release of TNF-α [16, 17].

The factors that lead to increased TNF-α in COPD patients but not in smokers with normal lung function are unknown. One possibility is that *TNF* expression is regulated by single nucleotide polymorphisms (SNPs) in the gene. For instance, increased transcriptional activity of the *TNF* gene has been associated with the –308A allele in various disorders [18-23]. The *TNF* –863A allele has been associated with increased gene expression [24] and increased TNF-α expression from peripheral blood mononuclear cells [25]. The –857T and –1031C alleles have been associated with increased transcriptional activity of the *TNF* gene [25]. The –237A allele has shown mixed results in association with TNF-α protein production [26-28]. Despite these promising *in vitro* studies, not all of these SNPs have been studied in COPD populations and those that have been studied often have conflicting results (Table 1). These inconsistencies may be due to study design limitations such as small number of subjects, genotyping with a limited number of informative SNPs, lack of haplotyping with multiple SNPs, failure to adjust for confounding variables such as age, and lack of comparison to healthy smoking controls, the most relevant comparison group. In this study we address some of these limitations by genotyping six *TNF* SNPs with biologic activity in a large number of subjects with well-defined physiologic phenotypes. We also use a multivariate analysis and examine TNF-α genotype/haplotype associations with COPD.

METHODS

Selection and Description of Participants

All subjects were studied under protocols approved by the Institutional Review Board at National Jewish Medical and Research Center or the Colorado Multiple Institution Institutional Review Board with guidelines recommended by the National Institutes of Health. Signed informed consent was obtained for all subjects. Control subjects were healthy volunteers recruited from local community by word of mouth and advertising, had no report of respiratory symptoms or disease, and had a greater than 20 pack-year history of smoking. Patients with COPD were recruited from an outpatient pulmonary clinic. The diagnosis of COPD was made using GOLD criteria [29] using a post bronchodilator maximum volume of air expired one second (FEV_1) after the onset of full expiration compared to that predicted for one's age, sex, and race (FEV_1%) based on a sample of the general U.S. population [30]. GOLD state I patients ($FEV_1/FVC < 0.7$ and $FEV_1\% \geq 80\%$ of predicted) were included as cases. Patients with emphysema on HRCT and normal pulmonary function test (PFT) (n=11) were placed in GOLD Stage 1 for analysis (Table 2).

Blood Collection

Six ml of blood was withdrawn from the antecubital vein into a sterile 13 × 1000 mm sodium heparin Vacutainer Plus (BD, New Jersey, USA). The sample was immediately spun at 2100 × *g* for 10 minutes at room temperature. The buffy coat was removed and stored at –80°C.

DNA extraction/isolation and genotyping

Buffy coats were used to extract DNA using QIAamp 96 DNA Blood Kit (QIAGEN, Valencia, CA), and the DNA was eluted with nuclease free water. A total of 6 SNPs were tested in all subjects. The polymorphisms -1031T/C, -863C/A, -857C/T were tested using PCR conditions and primers as previously described [31]. We also tested direct haplotypes using two pairs of primers for –857C/T with -237G/A and –308G/A with +488G/A as previously described [31, 32]. Each SNP was analyzed by using a sequence specific primer to detect the presence of that SNP.

Data Analysis

Genotype frequencies of each polymorphism in the case and control populations were evaluated for departures from Hardy-Weinberg equilibrium, using Chi-Square goodness-of-fit tests ($p < 0.001$). Tests for genotypic differences between cases and controls were conducted in the context of logistic regression, univariately and multivariately (adjusting for gender, age and smoking pack-years where appropriate). A dominant model was used for the genetic analysis. Caucasians accounted for the vast majority of subjects and thus were the only group analyzed in this study. Univariate and multivariate linear regression models were used to assess the influence of genotypes on cross-sectional continuous severity outcomes (FEV_1 percent predicted and

FEV_1/FVC). Normalizing transformations were performed on continuous outcome variables when necessary to better approximate model assumptions. For analysis of severity, GOLD stage was dichotomized to a mild-moderate (GOLD stage = 1 or 2) and severe disease (GOLD stage = 3 or 4). Genotypic differences between cases with severe and mild-moderate disease were evaluated in the context of logistic regression univariately and multivariately. All analyses were performed using SAS (SAS Institute, Inc. version 9.1.3). D' and r^2, measures of linkage disequilibrium, were calculated using Haploview (Copyright 2003-2005 Whitehead Institute for Biomedical Research). In addition, Haploview was also used to determine haplotype blocks using the confidence interval method [33].

Haplotype frequencies were estimated using Haplo.Score [34]. This software uses an Expectation Maximization (EM)-based algorithm to calculate the posterior probability of each possible haplotype combination for each individual when haplotype phase is unknown. To adjust for the uncertainty in haplotype assignments, we used a weighted logistic regression model. Each person could appear in the data set more than once, with each entry weighted by the probability of that haplotype combination for that individual, so that the total contribution of each individual was one observation. We tested for both haplotype combination and single haplotype effects: presence of a specific haplotype pair versus no presence, and carrying at least one specified haplotype versus not carrying the specified haplotype.

RESULTS

Demographics

The COPD and control groups were significantly different in percent-predicted FEV_1 (COPD: 45.0 ± 19%, Controls: 98.5 ± 18%, $p < 0.0001$) (Table 2). Compared to COPD patients, controls were more likely to be male, but had a similar pack-year smoking history. Only Caucasians (Whites) were analyzed in this study to minimize the effects of population stratification.

TNF SNP Genotypes

TNF genotype frequencies are listed in Table 3. A genotype with the –308 minor allele (GA or AA) was significantly more frequent in COPD subjects in both univariate and multivariate analyses (OR 1.9; 95%CI 1.1-3.2, p=0.03 and OR 1.9; 95%CI 1.1-3.4, p=0.03, respectively) when comparing COPD and smoking control subjects (Table 4). There was significantly less chance of having the genotype with the –237 minor allele (GA or AA) in COPD subjects than control subjects by multivariate analysis (OR 0.40; 95%CI 0.19-0.86, p=0.02) (Table 4).

A genotype with a minor allele for the -857 SNP (CT or TT) had a statistically significant lower OR in COPD subjects with severe COPD (GOLD stage 3 and 4) compared to those with mild-moderate COPD (stage 1 and 2) in both univariate and multivariate analyses (OR 0.5; 95%CI 0.26-0.95, p=0.03 and OR 0.46; 95%CI 0.24-0.88, p=0.02, respectively) (Table S3). There was no association found with any of the genotypes and FEV_1% (data not shown), although the -308 genotype (GG) was associated with a higher mean FEV_1/FVC (Table S4). There were no statistically significant associations with COPD for SNPs –1031, -863, and +488.

Haplotype Analysis

Haplotype blocks were constructed using the confidence interval method of Gabriel [33] in cases and controls separately using Haploview. This method uses both an estimate of D' and a measure of its precision (confidence bounds) to construct haplotype blocks (Figure1). Blocks with pairwise D' less than 1 have actual D' values in the squares. Although the estimated pairwise linkage disequilibrium (LD) between many of the SNPs was high, the precision of the estimates was not high enough to fulfill the criteria for construction of haplotype blocks utilizing all of the SNPs. The estimate of LD between the -1031 SNP and the -863 SNP was the only estimate that had sufficient precision to warrant construction of a haplotype block. The estimated frequencies can be found in Table S5. There were no haplotypes associated with COPD (data not shown).

Meta-analysis of the -308 and -237 SNPs in COPD

To put our findings in perspective, we searched PubMed for previously reported frequencies of *TNF* SNPs. For the -308 SNP, we were able to extract data from all the studies listed in Table 1, except for one which did not report frequencies for case and control separately [35], two that only reported rapid decliners with no cases [36, 37], and one that only reported emphysema in COPD subjects [38]. Combining our data with the remaining 16 studies (N=610 cases, 1612 controls), we found that a genotype with the -308 minor allele has an OR of 1.28 (standard error (SE) 0.03) for COPD. Four of these studies either did not use healthy smokers as their control group or did not state whether their control group was smokers [39-42]. Therefore, we pooled data from only studies that compared COPD subjects to healthy smokers (Figure 2) and found an OR of 1.29 (SE 0.04) for COPD with the -308 SNP.

We also compared SNP frequencies for the -237 *TNF* SNP to other populations. Combining our results with the pooled data from three other studies listed in Table 1, the odds ratios were not statistically significant either for all subjects (OR 1.25, SE 0.12) or studies just comparing COPD subjects to smokers (OR 1.22, SE 0.14) (Figure 3).

DISCUSSION

TNF-α plays an integral role in the pathogenesis of COPD. In this study, we performed the most comprehensive haplotype evaluation to-date using six single nucleotide polymorphisms in *TNF*. These SNPs were chosen based on their association with changes in biologic activity and results from previous studies in COPD. Some of these SNPs have been previously reported to be independently associated with COPD [38, 41-45]. For instance, we confirmed that a genotype (GA or AA) with the minor allele of the –308 SNP was associated with a higher odds ratio of having COPD compared to smoking controls. Other SNPs (e.g. -237) and haplotypes were associated with a lower odds ratio of having COPD or less severe disease.

The strongest association we observed was for the most studied SNP, -308 (Table 1). This SNP is in the promoter region of the *TNF* gene and is associated with increased gene transcription [18-23]. Although several earlier studies have shown an association of the –308 minor allele with COPD [38, 41, 43-45], four of five of these studies have been in Asian populations [38, 41, 44, 45], in which the minor allele frequency is much lower. This is only the second study to show this association in Caucasians, the first being a large family study from the Boston Early-Onset COPD Study [43]. Despite multiple studies showing positive associations, there are also 13 studies reporting no association between the -308 genotype (GA and AA) and COPD, including 10 in Caucasian populations [35-37, 39, 42, 46-49]. Previously it was felt that the difference in studies were due small sample size (under powering) or to low minor allele frequency [42]; however, a Forest plot (Figure 2) suggests that there is an association despite variability among populations.

The second most significant association we observed was with the -237 genotype (GA and AA). This SNP has been associated with decreased transcriptional activity and reduced TNF-α production from peripheral blood mononuclear cells [50]. Our multivariate analysis of Caucasian subjects showed that this genotype was less likely to be found in COPD subjects (7% in controls versus 4% in COPD subjects). Previous studies have reported no association of this SNP with COPD [42, 46]; these studies had minor allele frequencies in controls (4% and 6% respectively), slightly lower than ours (7%). In another study [43] the sample size was larger (N=718), yet minor allele frequencies were also lower in control subjects (4% or controls and 6% in cases; personal communication Craig Hersh) suggesting that this association may have been due to a false-positive association (Figure 3).

Although other *TNF* SNPs appear to have biologic activity *in vitro*, only two of these other SNPs (-376 and+488) have been previously reported in COPD studies [42, 43, 46, 51]. The +488 SNP has been associated with renal cell carcinoma [52] and prostate carcinoma [53]. This SNP is in the first intron of the *TNF* transcript, but its significance is unclear [31, 54, 55]. In most studies there was no association between this SNP and COPD [43, 51] except for one study that compared COPD patients to healthy donors [42]. Surprisingly this study found that the association between the +488 SNP and COPD was even stronger for COPD subjects that had no or limited radiographic evidence of disease [42]. Although we did not study the -376 SNP, it is

associated with an increase in *TNF* transcriptional activity and very strongly associated with an increased risk of cerebral malaria [56].

This is the first report of genotype frequencies of the -857 SNP in COPD, although it has been studied in other pulmonary diseases. This SNP is thought to be associated with decreased transcription by affecting binding of OCT1 and NF-κB transcription factors in the *TNF* promoter region, although reports have been conflicting [25, 57, 58]. Although in this investigation the OR for this SNP was not statistically significant different, it was associated with mild-moderate COPD (GOLD stages 1 and 2). We speculate that the -857T allele affects TNF-α production negatively in certain inflammation pathways using different transcription factors, thus leading to less inflammation of airways and destruction of lung parenchyma – a model similar to that postulated for the gut in inflammatory bowel disease [57].

One strength of this study is that we investigated multiple *TNF* SNPs and conducted a haplotype analysis. Although only two SNPs were in significant LD, we found that the haplotype block -1031/-863 was associated with COPD. This region of *TNF* is also thought to play a role in binding of OCT1 and NF-κB transcription factors to the promoter region of *TNF* and the minor alleles are felt to cause increased *TNF* transcription [24, 25, 59]. The minor allele for the -1031 and -863 SNPs have also been reported to be associated with gastric ulcers and gastric cancer [60]. In the haplotype in which COPD was found to be less likely, CC/TC -1031/-863, we hypothesize that several minor alleles at these sites makes one less likely to have increased TNF-α production and therefore less likely to develop COPD.

In summary, this is one of the larger and more comprehensive studies of *TNF* polymorphisms in COPD. We confirmed that the well studied -308 SNP is associated with COPD and also report other associations with the *TNF* gene, suggesting that *TNF* polymorphisms may play a role in the susceptibility to tobacco smoke. Additional large independent studies in both Caucasian and non-Caucasian populations are needed to replicate these findings.

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Table 1 – Previous TNF-α SNP Association Studies in COPD

SNP rs#	Study	Ethnicity/Race	Cases/controls	Association	Haplotype Analysis
-376 rs1800750	Kucukaycan et al 2002 [42]	Caucasian	169/358	No	No
	Brogger et al 2006 [46]	Caucasian	244/248	No	No
-308 rs1800629	Brogger et al 2006 [46]	Caucasian	244/248	No	No
	Ruse et al 2006 [35]	Caucasian	220/141	No	No
	Patuzzo et al. 2000 [39]	Caucasian	66+23(DB)/98(HC)+45(NOPD)	No	Yes
	Hersh et al 2005 [43]	Caucasian	304/441 Case-Control 127/503(FDR)/273(SDR)/46(Sp) BEOCS	Positive for -308 in BEOCS	Yes
	Kucukaycan et al 2002 [42]	Caucasian	169/358(RBD)	No	No
	Seifart et al 2005 [47]	Caucasian/German	113(CB)/113(MC)/243(HC)	No	No
	Keatings et al 2000 [61]	Caucasian	106/99(from Cardiology clinic)	No. AA had less reversibilty and higher mort	No
	Higham et al 2000 [48]	Caucasian	86/63(SC)/199(HC)	No	No
	Sanford et al 2001 [36]	Caucasian	283 (RD)/308 (NRD)	No	Yes
	Tanaka et al 2007 [37]	Caucasian	279 (RD)/304 (NRD)	No	Yes
	Ferrarotti et al 2003 [49]	Caucasian/Italian	63/86 Male	No	No
	Hegab et al, 2005 [51]	Asian/Japanese	88/61	No	Yes
	Sakao et al 2001 [45]	Asian/Japanese	106/110/129	Yes, OR 2.58	No
	Sakao et al 2002 [38]	Asian/Japanese	44 severe VSE/11 mild VSE	Yes, OR 2.15	No
	Ishii et al 2000 [62]	Asian/Japanese	53/65	No	No
	Huang et al 1997 [44]	Asian/Taiwanese	42(CB)/42(MC)/99kids	Yes, OR 11.1	No
	Chierakul et al 2005 [63]	Asian/Taiwanese	57/67(SC)/116(Anon)	No	No
	Jiang L et al 2005 [41]	Asian/Chinese	111/97(smokers and non-smokers	Yes, OR 5	No
	Danilko et al 2007 [40]	Russian	319 COPD vs 403 healthy	Yes	No
	Hegab et al, 2005 [64]	Egyptians	106/72	No	Yes
-237 rs361525	Hersh et al 2005 [43]	Caucasian	304/441 Case-Control 127/503(FDR)/273(SDR)/46(Sp) Family Study	No	Yes
	Kucukaycan et al 2002 [42]	Caucasian	169/358(RBD)	No	No
	Brogger et al 2006 [46]	Caucasian	244/248	No	No
+488 rs1800610	Hegab et al, 2005 [51]	Asian/Japanese	88/61	No	Yes
	Hegab et al, 2005 [51]	Egyptians	106/72	No	Yes
	Hersh et al 2005 [43]	Caucasian	304/441 Case-Control 127/503(FDR)/273(SDR)/46(Sp) Family Study	No	Yes
	Kucukaycan et al 2002 [42]	Caucasian	169/358(RBD)	Yes, OR 1.9 (Subgroup w/o Emphysema; OR 3.6)	No
rs769178	Hersh et al 2005 [43]	Caucasian	304/441 Case-Control 127/503(FDR)/273(SDR)/46(Sp) Family Study	No	Yes

DB=Disseminated Bronchiectasis, HC=Healthy Control, NOPD=Non-obstructive pulmonary disease, FDR=1st degree relative, SDR=2nd degree relative, Sp=Spouse, MC=Matched controls, RBD=Random blood donors, RD=Rapid Decline in FEV_1; NRD=non-Rapid Decline in FEV_1; Anon=Anonymous donors, VSE = visual score for emphysema.

Table 2 – Study Subject Characteristics

	COPD (n=298)	Control (n=125)	p-Value
Age, mean years(SD)	65.6(9.8)	58.7(10.4)	<0.0001
Male (%)	52.0	70.3	0.0007
Pack Year,mean(SD)	55.2(30)	52.3(26)	.34
FEV_1%, mean(SD)	45.0%(19%)	98.5%(18%)	<0.0001
FEV_1/FVC, mean(SD)	0.48(.13)	0.72(.09)	<0.0001

Table 3 –Genotype frequencies of TNF-α SNPs

TNF - α SNP		COPD N=298	Control N=125
-1031 rs1799964	TT	195(65.4%)	83(66.4%)
	TC	93(31.2%)	36(28.8%)
	CC	10(3.4%)	6(4.8%)
-863 rs1800630	CC	219(73.5%)	94(75.2%)
	CA	72(24.2%)	29(23.2%)
	AA	7(2.3%)	2(1.6%)
-857 rs1799724	CC	253(84.9%)	103(82.4%)
	CT	44(14.8%)	21(16.8%)
	TT	1(.3%)	1(.8%)
-308 rs1800629	GG	220(73.8%)	105(84.0%)
	GA	67(22.5%)	18(14.4%)
	AA	11(3.7%)	2(1.6%)
-237 rs361525	GG	277(93.0%)	110(88.0%)
	GA	20(6.7%)	13(10.4%)
	AA	1(.3%)	2(1.6%)
+488 rs1800610	GG	264(90.1%)	116(94.3%)
	GA	28(9.6%)	7(5.7%)
	AA	1(.3%)	0(0.0%)

Table 4 – Univariate and Multivariate Analyses (Dominant Model)

Genotype Comparison	Univariate Analysis		Multivariate Analysis*	
COPD vs Control	Odds Ratio (95%CI)	p-value	Odds Ratio (95%CI)	p-value
-1031 (CC or TC vs TT)	1.0 (.67-1.6)	.85	1.1 (.71-1.8)	.59
-863 (AA or CA vs CC)	1.1 (.68-1.8)	.71	1.3 (.74-2.1)	.40
-857 (TT or CT vs CC)	0.83 (.48-1.5)	.52	0.70 (.39-1.3)	.24
-237 (AA or GA vs GG)	0.56 (.28-1.1)	.10	0.40 (.19-.86)	**.02**
-308 (AA or GA vs GG)	1.9 (1.1-3.2)	**.03**	1.9 (1.1-3.4)	**.03**
+488 (AA or GA vs GG)	1.8 (.78-4.3)	.17	1.7 (.71-4.2)	.23

*Adjusted for gender, age and pack years

Figure Legends

Figure 1: Linkage disequilibrium (LD) plots of TNF haplotypes. Haplotypes were constructed from genotyping data from Caucasians using the Gabriel block method. Significant D' values are shown. There was only one block of SNPs (-1031 and -863).

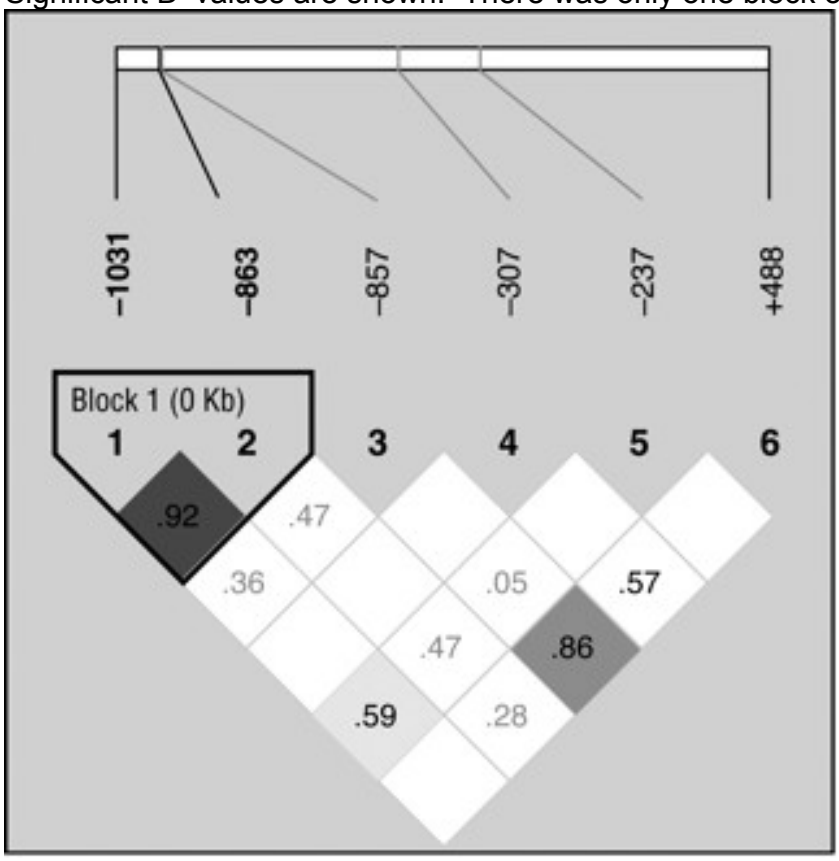


Figure 2: Forest plot of the odds ratio (OR) of having COPD with genotype of GA or AA of -307 SNP in this study and previously published studies. The square indicates the point estimate and the whiskers are 95% confidence intervals (CI). The size of the square is proportional to the number of subjects in the study. The diamond at the bottom of the plot represents the combined OR and CI for all studies (Total) and studies comparing COPD patients to smoking controls (Smokers only).

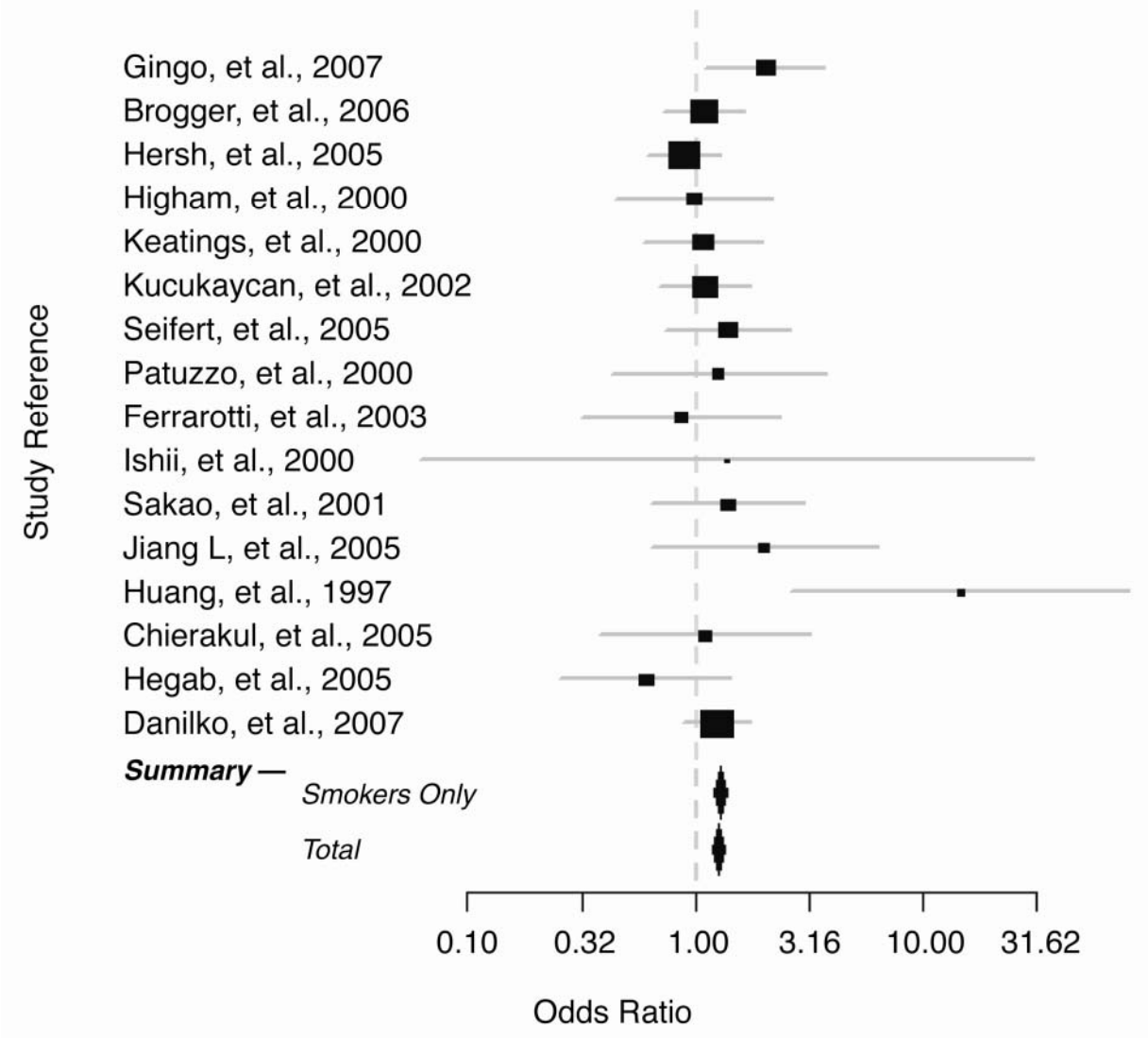
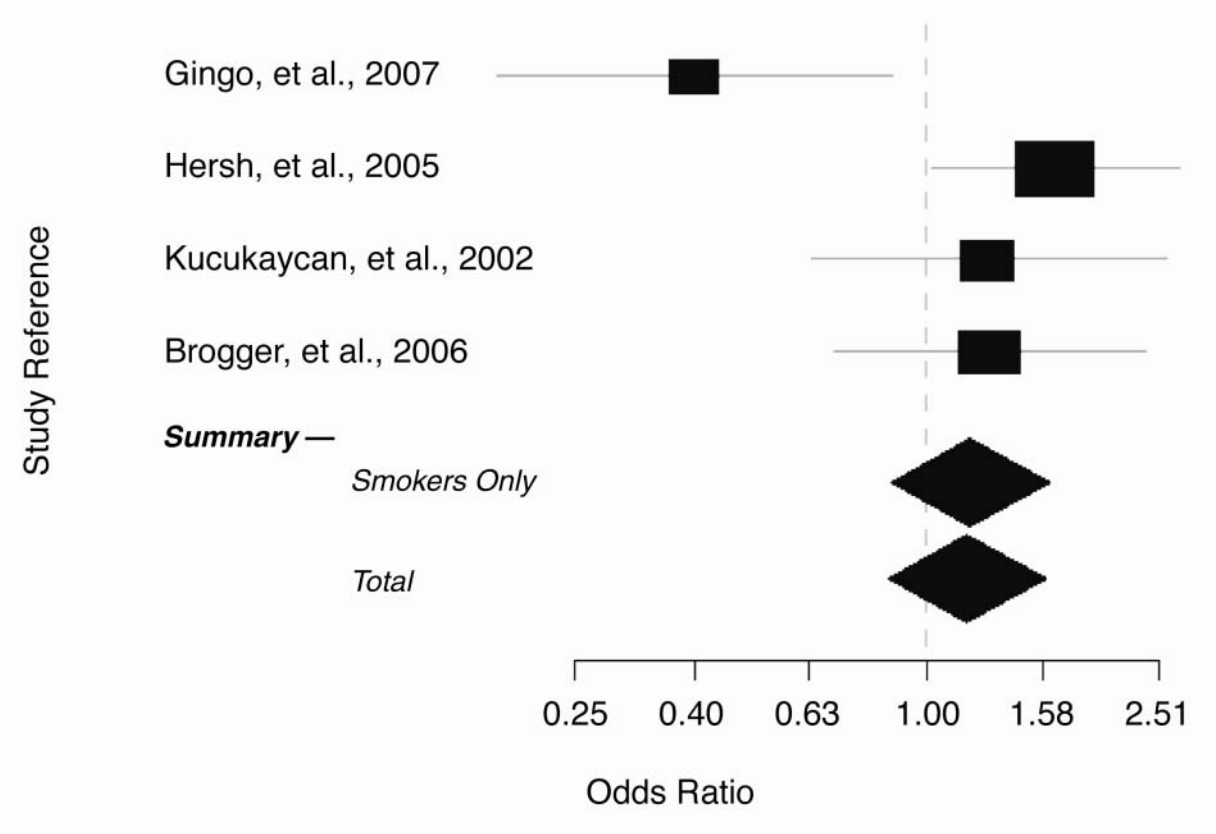


Figure 3: Forest plot of the odds ratio (OR) of having COPD with genotype GA or GA of -237 SNP in this study and previously published studies. The square indicates the point estimate and the whiskers are 95% confidence intervals (CI). The size of the square is proportional to the number of subjects in the study. The diamond at the bottom of the plot represents the combined OR and CI for all studies (Total) and studies comparing COPD patients to smoking controls (Smokers only).


Gingo, et al., 2007
Hersh, et al., 2005
Kucukaycan, et al., 2002
Brogger, et al., 2006
Summary —
Smokers Only
Total
Study Reference
0.25
0.40
0.63
1.00
1.58
2.51
Odds Ratio