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# Variation in NF- B Signaling Pathways and Survival in Invasive Epithelial Ovarian Cancer

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## Variation in NF- $\kappa$ B Signaling Pathways and Survival in Invasive Epithelial Ovarian Cancer

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### Abstract

Survival in epithelial ovarian cancer (EOC) is influenced by the host immune response, yet the key genetic determinants of inflammation and immunity that impact prognosis are not known. The nuclear factor-kappa B (NF- $\kappa$ B) transcription factor family plays an important role in many immune and inflammatory responses, including the response to cancer. We studied common inherited variation in 210 genes in the NF- $\kappa$ B family in 10,084 patients with invasive EOC (5,248 high grade serous, 1,452 endometrioid, 795 clear cell, and 661 mucinous) from the Ovarian Cancer Association Consortium. Associations between genotype and overall survival were assessed using Cox regression for all patients and by major histology, adjusting for known prognostic factors and correcting for multiple testing (threshold for statistical significance— $p < 2.5 \times 10^{-5}$ ). Results were statistically significant when assessed for patients of a single histology. Key associations were with *CARD11* (caspase recruitment domain family, member 11) rs41324349 in patients with mucinous EOC (HR 1.82, 95% CI 1.41–2.35,  $p=4.13 \times 10^{-6}$ ) and *TNFRSF13B* (tumor necrosis factor receptor superfamily, member 13B) rs7501462 in patients with endometrioid EOC (HR 0.68, 95% CI 0.56–0.82,  $p=2.33 \times 10^{-5}$ ). Other associations of note included *TRAF2* (TNF receptor-associated factor 2) rs17250239 in patients with high-grade serous EOC (HR 0.84, 95% CI 0.77–0.92,  $p=6.49 \times 10^{-5}$ ) and *PLCG1* (phospholipase C, gamma 1) rs11696662 in patients with clear cell EOC (HR 0.43, 95% CI 0.26–0.73,  $p=4.56 \times 10^{-4}$ ). These associations highlight the potential importance of genes associated with host inflammation and immunity in modulating clinical outcomes in distinct EOC histologies.

### Keywords

single nucleotide polymorphism; recurrence; survival; ovarian neoplasms

### INTRODUCTION

Epithelial ovarian cancer (EOC) is the sixth leading cause of cancer death among women in developed countries (1), with a five-year survival rate of only 37% in the United States (2). A key cause of poor survival is a lack of specific symptoms and screening methods; as such, the majority of EOC patients present with distant spread of disease. A number of features in addition to stage are known to impact clinical outcome, including age at diagnosis (3),

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extent of residual disease following initial cytoreductive surgery (optimal versus suboptimal) (4), and baseline performance status (5). Genetic polymorphisms may also influence EOC survival (6, 7). Understanding the totality of potential prognostic factors is key to discerning pathogenic mechanisms that underlie carcinogenesis and progression in EOC. Inflammation is known to play a role in tumorigenesis (8); inflammation from multiple causes, including talc use (9) and endometriosis (9, 10), and the presence of non-specific inflammatory markers such as C-reactive protein (CRP) are associated with increased EOC risk (11). Furthermore, the presence of an ongoing inflammatory response, measured by CRP and hypoalbuminemia, has been shown to independently predict poor prognosis in advanced EOC (12).

The nuclear factor-kappa B (NF- $\kappa$ B) family of transcription factors regulates the transcription of multiple proteins, including cytokines, chemokines, acute phase reactants, complement factors, adhesion molecules, and other proteins involved in inflammation, apoptosis, and cell division (13). In canonical NF- $\kappa$ B signaling, binding of NF- $\kappa$ B-associated receptors leads to phosphorylation and activation of the inhibitor of kappa B kinase (IKK) complex, which leads to phosphorylation and proteasomal degradation of the inhibitor of kappa B ( $I\kappa$ B), thus releasing NF- $\kappa$ B transcription factors into the nucleus to regulate gene transcription (14). Alternatively, receptor binding and IKK activation can lead to processing of the p100 protein into active p52, which binds the NF- $\kappa$ B family member Rel-B, translocates to the nucleus, and regulates gene transcription (14). To assess the role of genetic variation in NF- $\kappa$ B signaling on EOC survival, we evaluated common inherited single nucleotide polymorphisms (SNPs) in key genes which mediate NF- $\kappa$ B activation, inhibit NF- $\kappa$ B function, assist degradation, or regulate nuclear function among patients from the Ovarian Cancer Association Consortium (OCAC).

## MATERIALS AND METHODS

### Study Participants

A total of 10,084 women with invasive EOC (37,171 person-years follow-up) and greater than 90% estimated European ancestry were analyzed as described previously (15, 16). Participants were from 28 OCAC studies (Supplemental Table 1) based in Europe, North America, and Australia which conducted follow-up for vital status, including 12 studies (AUS, BAV, HAW, HSK, LAX, MAL, MAY, NCO, NEC, ORE, PVD, and SRO) followed for disease recurrence or progression.

### SNP Selection

We identified 210 key genes (Supplemental Table 2) known to encode NF- $\kappa$ B subunits or molecules key to NF- $\kappa$ B activation (in signaling cascade), inhibition (inhibitory role), degradation (involved in proteasomal degradation), and nuclear function (nuclear proteins involved in transcription) (6). TagSNPs within 5 kb based on  $r^2 \geq 0.8$ , minor allele frequency (MAF)  $\geq 0.05$  in Europeans were identified using the most informative source for each gene from among HapMap Phase II Release 24 (<http://www.hapmap.org>), the 1000 Genomes Project Low-Coverage Pilot (<http://www.1000genomes.org/>), SeattleSNPs (<http://pga.mbt.washington.edu/>), Innate Immunity PGA (<http://innateimmunity.net/>), and NIEHS

SNPs (<http://egp.gs.washington.edu>) (17). Additional putative-functional SNPs were also included, regardless of linkage disequilibrium (LD), with European MAF  $\geq 0.05$  which were 1 kb upstream, non-synonymous, or resided in a 3' untranslated region (UTR), 5' UTR, splice site, or miRNA binding site (<http://www.microrna.org/microrna/home.do>, <http://www.targetscan.org/>). Finally, SNPs with an Illumina design score  $< 0.4$  or in LD ( $r^2 > 0.80$ ) with a SNP found to be null ( $p > 0.05$ ) in a small prior analysis (16) were excluded. With this approach, 76% of significant SNPs with MAF  $\geq 0.05$  were adequately tagged if we used HapMap as our reference.

### Genotyping and Quality Control

Germline genotyping was conducted using an Illumina Infinium iSelect BeadChip as part of the Collaborative Oncological Gene-environment Study (COGS) (16). Centralized genotyping used raw intensity data files and a cluster file generated with HapMap2 European, African, and Asian samples. Samples were excluded with 1) conversion rate  $< 95\%$ , 2) heterozygosity  $>$  five standard deviations from the European mean heterozygosity, 3) ambiguous sex, 4) lowest call rate from an observed first-degree relative pair, or 5) duplicate samples that were non-concordant for genotype or genotypic duplicates that were not concordant for phenotype. SNPs were excluded with 1) no genotype call, 2) monomorphism, 3) call rate  $< 95\%$  with MAF  $> 0.05$  or call rate  $< 99\%$  with MAF  $< 0.05$ , 4) deviation from Hardy-Weinberg equilibrium ( $p < 10^{-7}$ ), or 5)  $> 2\%$  duplicate discordance.

### SNP Imputation

Imputation to the 1000 Genomes (1000G) Phase I Integrated Release Version 3 haplotypes was carried out in MaCH (18) using all 1,092 1000G samples and excluding monomorphic and singleton sites.

### Statistical Methods

HapMap2 genotypes were used to define intercontinental ancestry; among Europeans ( $> 90\%$  European ancestry), we used 37,000 unlinked non-NF- $\kappa$ B markers in population stratification principal components (PC) analysis (16). Cox regression accounting for left truncation and right censoring at 10 years estimated hazard ratios (HRs) and 95% confidence intervals (CIs) for association with overall survival, defined as time to death from any cause. Censoring at 10 years was performed to minimize competing causes of mortality, which become more common after 10 years from EOC diagnosis. HRs were calculated based on the ordinal number of copies of the minor allele for all genotyped SNPs and allele dosage variables for all imputed SNPs. Analyses were conducted overall and within the four most common histologic subtypes (high grade serous, mucinous, endometrioid, and clear cell). Analyses adjusted for study site and the first five population substructure PCs, as well as the following covariates which associated with survival in these data ( $p < 0.05$ , Supplemental Table 3): age (continuous), tumor stage summarized from FIGO or SEER stage (localized, regional, distant), tumor grade (well, moderately, poorly, or undifferentiated), oral contraceptive use (ever, never), and, for analysis of all cases only, histology (serous, mucinous, endometrioid, clear cell, mixed cell, undifferentiated, unknown). Sensitivity analyses included covariates only for age, five population substructure PCs, and study site. Analyses were also conducted with a recurrence endpoint defined as time to disease

recurrence or death (377 additional events), among cases which were optimally debulked in cytoreductive surgery (2,078 cases having no residual deposits of cancer that were >1 cm) and among cases where surgical debulking was suboptimal (1,215 cases with >1 cm residual disease).

To address multiple testing concerns, we used spectral decomposition of the observed genotype matrix (19) to account for observed LD and estimated that the effective number of independent tests for each analysis was 2,000. As a result, only SNPs with p-values  $<2.50 \times 10^{-5}$  ( $0.05/2,000$ ) were considered statistically significant. We used SAS (SAS Institute, Inc., Cary, NC) and R (R Foundation for Statistical Computing, Vienna, Austria), and, in regions of interest, LocusZoom (Standalone Version) (20) and Haploreg v2 (21) for plotting and annotation respectively.

## RESULTS

We analyzed 2,254 SNPs in 210 genes for clinical outcome among 10,084 EOC cases. The strongest survival association in any of the histology subgroups was seen in 661 mucinous EOC with the *CARD11* intronic SNP rs41324349 (HR =1.82,  $p =4.13 \times 10^{-6}$ , Table 1). In addition, five of the fifty-six genotyped *CARD11* SNPs were associated at  $p <0.005$ , including two independent SNPs ( $r^2 <0.20$ ) with  $p <0.001$  (Table 1). The distribution of p-values and correlation with rs41324349 across *CARD11* are shown in Figure 1 for both directly genotyped and imputed SNPs. Imputation revealed that the *CARD11* SNP rs2527513, which was in strong LD with rs41324349, was highly correlated with survival. For 1,452 patients with endometrioid EOC, the *TNFRSF13B* 3' UTR SNP rs7501462 showed the strongest association (HR =0.68,  $p =2.33 \times 10^{-5}$ ). Out of eighteen additional *TNFRSF13B* SNPs, two others (rs7212800 and rs11078362) showed association ( $p <0.005$ ) in endometrioid EOC patients; these additional SNPs were in moderate LD with rs7501462 ( $r^2 =0.26$  and  $r^2 =0.76$ , respectively).

For 5,248 high grade serous EOC patients, the *TRAF2* SNP rs17250239 showed the most significant association (HR =0.84,  $p =6.49 \times 10^{-5}$ ), although this was just beyond our pathway-wide threshold for statistical significance ( $p <2.50 \times 10^{-5}$ ). The rs17250239 SNP is located in an intronic sequence within the *TRAF2* gene. In 795 clear cell EOC patients, *PLCG1* rs11696662 showed the most significant association (HR = 0.43,  $p =4.56 \times 10^{-4}$ ), but this was not within our pathway-wide threshold for statistical significance. Finally, among all cases, the SNPs rs61764220 and rs518162 (within the genes *MAPK3* and *PGR*, respectively) had the strongest survival associations (HR =0.81,  $p =6.50 \times 10^{-4}$ , and HR =0.87,  $p =8.11 \times 10^{-4}$ , respectively, Table 1). However, these results did not meet our threshold for statistical significance taking into account multiple comparisons ( $p <2.50 \times 10^{-5}$ ), and so there were not clear associations between polymorphisms in *MAPK3* and *PGR* and survival in EOC.

In addition to OS, we performed sensitivity analyses for time to recurrence, examined results from minimally adjusted analyses, and assessed optimally debulked and suboptimally debulked patients separately. The HRs for recurrence were similar to HRs for survival with and without full covariate adjustment for each of the SNPs that we had considered to have

the most significant associations with survival ( $p < 0.0001$ ) and among optimally debulked compared to suboptimally debulked patients (available on one-third of participants; data not shown).

## DISCUSSION

In this pooled analysis of over 10,000 EOC patients enrolled in 28 different studies within OCAC, we evaluated associations between NF- $\kappa$ B-related SNPs with survival. We did not identify SNPs associating with overall survival among all EOC patients that met our corrected threshold for statistical significance. However, we identified three SNPs, rs41324349, rs2527513, and rs7501462, which associated with overall survival and time to recurrence for EOC subtypes accounting for known prognostic factors. The *CARD11* intronic SNPs rs41324349 and rs2527513 were in high LD with each other and were associated with shortened survival in patients with mucinous EOC, whereas *TNFRSF13B* 3' UTR rs7501462 associated with improved outcome among patients with endometrioid EOC. Sensitivity analyses showed concordance between HRs for overall survival and time to recurrence, and among optimally debulked patients.

*CARD11*, also known as Carma 1, is an adapter protein that functions as a molecular scaffold in leukocytes (22). *CARD11* interacts with the pro-apoptotic protein BCL10, and overexpression of *CARD11* leads to increased NF- $\kappa$ B activation (23). Oncogenic mutations in *CARD11* have been reported in association with several types of lymphoma (24). The expression of *CARD11* in leukocytes suggests that it may influence immune/inflammatory responses to EOC. rs41324349 lies within seven regulatory motifs that would be altered by the base change, which could potentially alter transcription; however, this SNP is not in a conserved domain. Six additional intronic and one synonymous SNPs located in regulatory motifs were correlated with this SNP ( $r^2 = 0.6$ ). Primary mucinous EOC is relatively uncommon, and mechanisms responsible for tumorigenesis, invasion, and metastasis that are specific for mucinous subtype have not yet been clearly demonstrated. Thus, it is not clear how a change in expression or function of *CARD11* would impact survival specifically in this subgroup.

*TNFRSF13B*, more commonly known as TACI (transmembrane activator and calcium-modulating cyclophilin ligand interactor), is a member of the tumor necrosis factor (TNF) receptor superfamily and is found on B lymphocytes (25). TACI interacts with the TNF family members BAFF (B cell activating factor) and APRIL (a proliferation-inducing ligand) to activate NF- $\kappa$ B and other transcription factors in B cells. It is not known whether rs7501462 affects *TNFRSF13B* expression, and it is not located in an evolutionarily conserved domain; however, it falls in a strong enhancer region and *POL2* binding site in B-lymphoblastoid cell lines. As the primary pathologic process associated with endometrioid ovarian carcinomas is endometriosis, alterations in *TNFRSF13B* that affect inflammatory responses to endometriosis may modulate the aggressiveness of endometriosis-associated carcinomas.

Interestingly, while SNPs associated with survival were identified for relatively rare histologies (mucinous and endometrioid histologies), there were no SNP associations



identified for the most common EOC histology (high grade serous). This may simply reflect underdetection of SNPs due to a relatively stringent statistical threshold for significance, as there were several SNPs, most notably rs17250239 (HR =0.84,  $p = 6.49 \times 10^{-5}$ ) which had survival associations not quite meeting our pre-specified threshold for significance ( $p < 2.5 \times 10^{-5}$ ). However, this may also reflect that survival high grade serous EOC, which is characterized by dramatic alterations in DNA macrostructure, may be more closely associated with certain amplified or deleted regions of DNA rather than alterations at the single nucleotide level.

The search for inherited variants associated with EOC outcome has proven challenging, with no published variants reaching genome-wide significance to date (15, 26). Here, by testing a candidate pathway within a consortium, we identified two SNPs from NF- $\kappa$ B-related genes that associated with survival in patients with distinct histologic subtypes of EOC using a pathway-wide statistical significance threshold. Strengths of this report include large sample size and use of centralized genotyping; limitations include missing data on surgical debulking status. For example, analysis by debulking status classified patients based on whether  $<1$  cm or  $\geq 1$  cm residual disease was present, as opposed to complete debulking (no visible residual disease); thus, association in certain patient subsets may have been overlooked. In addition, for some population-based studies, there was possible over-enrollment of women with longer survival; this could also bias results to the null if NF- $\kappa$ B SNPs associate only with very poor survival time.

As additional outcome-associated variants come to light, further work will address the potential prognostic utility of a broad panel of outcome-associated SNPs. For now, we provide evidence that the genetics of the immune/inflammatory response to EOC may impact clinical outcome and suggest that characterization of functional mechanisms will be a key next step to understanding this deadly disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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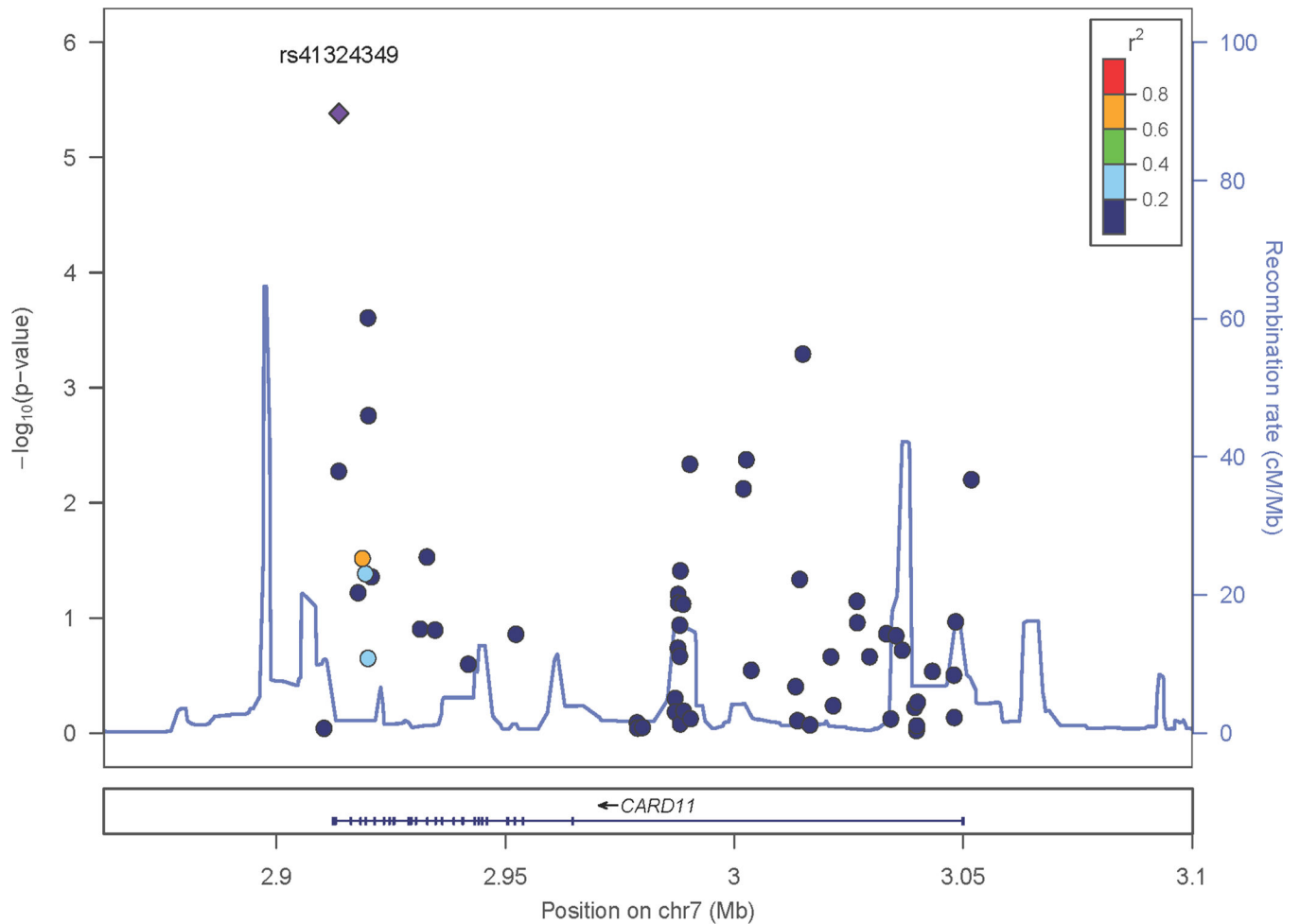
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**Figure 1. Strength of association between *CARD11* genotypes and survival of women with mucinous EOC (N=661)**

Adjusted for study site, first five European ancestry population substructure PCs, age at diagnosis, tumor stage, tumor grade, and oral contraceptive use. Circles represent imputed SNPs, while triangles represent genotyped SNPs.

**Table 1**SNP association with EOC overall survival ( $p < 0.001$ ,  $r^2 < 0.20$ )

Histologic Subtype	Gene	SNP	Alleles	MAF	HR (95% CI)	p-value
Mucinous (N=661)	<i>CARD11</i>	<b>rs41324349</b>	C>A	<b>0.44</b>	<b>1.82 (1.41–2.35)</b>	<b><math>4.13 \times 10^{-6}</math></b>
		rs6944821	A>G	0.31	1.64 (1.26–2.13)	$2.47 \times 10^{-4}$
		rs34251392	A>G	0.34	0.63 (0.48–0.82)	$5.08 \times 10^{-4}$
	<i>TRAF5</i>	rs79776636	G>C	0.04	2.89 (1.70–4.92)	$4.01 \times 10^{-4}$
	<i>IKBKE</i>	rs10836	G>C	0.47	0.62 (0.47–0.82)	$6.04 \times 10^{-4}$
	<i>PIK3R1</i>	rs10940158	G>A	0.52	1.47 (1.17–1.85)	$8.47 \times 10^{-4}$
Endometrioid (N=1,452)	<i>TNFRSF13B</i>	<b>rs7501462</b>	A>G	<b>0.26</b>	<b>0.68 (0.56–0.82)</b>	<b><math>2.33 \times 10^{-5}</math></b>
	<i>PELI2</i>	rs1152468	G>C	0.40	0.75 (0.64–0.87)	$1.86 \times 10^{-4}$
	<i>MAP2K6</i>	rs72847071	G>A	0.09	1.61 (1.26–2.05)	$2.66 \times 10^{-4}$
	<i>IL3</i>	rs40401	G>A	0.22	0.72 (0.59–0.87)	$5.65 \times 10^{-4}$
	<i>TLR5</i>	rs5744157	G>C	0.12	0.66 (0.52–0.85)	$8.28 \times 10^{-4}$
High grade serous (N=5,248)	<i>TRAF2</i>	rs17250239	G>A	0.11	0.84 (0.77–0.92)	$6.49 \times 10^{-5}$
	<i>PRKCA</i>	rs9894564	A>G	0.24	0.90 (0.84–0.95)	$5.83 \times 10^{-4}$
Clear cell (N=795)	<i>PLCG1</i>	rs11696662	G>A	0.07	0.43 (0.26–0.73)	$4.56 \times 10^{-4}$
	<i>MAPK1</i>	rs72847071	T>A	0.43	0.70 (0.57–0.86)	$6.10 \times 10^{-4}$
All (N=10,084)	<i>MAPK3</i>	rs61764220	A>G	0.03	0.81 (0.71–0.92)	$6.50 \times 10^{-4}$
	<i>PGR</i>	rs518162	G>A	0.08	0.87 (0.81–0.95)	$8.11 \times 10^{-4}$

Bold indicates  $p < 2.5 \times 10^{-5}$ ; adjusted for study site, first five European ancestry population substructure PCs, age at diagnosis, tumor stage, tumor grade, oral contraceptive use, and histology (for analyses of all cases only); SNPs with  $p < 0.001$ , but correlated at  $r^2 > 0.20$  SNPs above are not shown; SNP id is dbSNP 137 rsid; MAF, minor allele frequency; HR, hazard ratio; CI, confidence interval; minor allele designation based on allele frequencies in all cases.