

Inherited Variants in the Chemokine *CCL2* Gene and Prostate Cancer Aggressiveness in a Caucasian Cohort

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Abstract

Purpose: Though C–C chemokine ligand 2 (*CCL2*) has been shown to play a pivotal role in prostate cancer tumorigenesis and invasion, the role of inherited variation in the *CCL2* gene in prostate cancer progression and metastases remains unanswered. This study is aimed to determine the influence of *CCL2* germline variants on prostate cancer aggressiveness.

Experimental Design: We performed an association study between six single nucleotide polymorphisms (SNP) in the *CCL2* gene and prostate cancer clinicopathologic variables in a large hospital-based Caucasian patient cohort ($N = 4,073$).

Results: Genetic variation at *CCL2* is associated with markers of disease aggressiveness. Three SNPs, each in strong linkage disequilibrium, are associated with a higher (>7) biopsy Gleason score: *CCL2* –1811 A/G, –2835 A/C, and +3726 T/C ($P = 0.01, 0.03, \text{ and } 0.04$, respectively). The *CCL2* -1811 G allele is additionally associated with advanced pathologic stages in patients who underwent radical prostatectomy ($P = 0.04$). In haplotype analysis, we found that the frequency of a common haplotype, H5, was higher among patients with D'Amico good risk features ($P_{\text{permutation}} = 0.04$).

Conclusions: These results support the influence of *CCL2* variants on prostate cancer development and progression. *Clin Cancer Res*; 17(6); 1546–52. ©2010 AACR.

Introduction

Chemokines and chemokine receptors are major mediators of leukocyte trafficking into the sites of the immune response. They participate in defense against microbial infection, in Th1/Th2 polarization of the immune response, in allograft rejection, and in angiogenesis, as well as in tumorigenesis and metastasis (1, 2). C–C chemokine ligand 2 (*CCL2*), also known as monocyte chemoattractant protein-1 (MCP-1), is a member of the C–C beta chemokine family that is produced by macrophages, fibroblasts, and endothelial cells to stimulate chemotaxis of monocyte/macrophages and other inflammatory cells through its receptor, CCR2 (2, 3). *CCL2* and its receptor CCR2 have recently been shown to play key roles in promoting tumorigenesis and metastasis via distinct

mechanisms (4–7). First, *CCL2* has a direct promotional effect on tumor cell growth and survival. Second, *CCL2* has a modulatory effect on the tumor microenvironment by promoting macrophage mobilization and infiltration into the tumor bed. Third, *CCL2* can promote osteoclast maturation in the bone tumor microenvironment. Fourth, *CCL2* can suppress cytotoxic T lymphocytes. The multiple roles of *CCL2* in the promotion of tumorigenesis make the *CCL2*/*CCR2* axis an attractive therapeutic target for cancer treatment.

Single-nucleotide polymorphism (SNP) analysis of *CCL2* has suggested that this chemokine may play a role in host susceptibility to the development of cancer and/or cancer metastasis (8–10). Seven *CCL2* polymorphisms have been studied in their relationship to disease susceptibility or severity (11): 5 of them are in the promoter regulatory region of the *CCL2* gene, –927 G/C, –1811 A/G, –2136 A/T, –2518 A/G, –2835 C/A; 1 in the first intron, +764 C/G; and 1 in the 3' flanking region: +3726 T/C. Four of the above-mentioned SNPs (–2136 T, –2518 G, –2835 A, +764 G) in the *CCL2* gene were found to be associated with increased circulating levels of *CCL2* protein, but any true association may be due to a single variant in the region, because these SNPs are in strong linkage disequilibrium (LD; ref. 12).

Prostate cancer is the most commonly diagnosed malignancy in men in the United States (13). Although metastatic prostate cancer is initially treatable by castration, advanced prostate cancer remains incurable owing to the

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doi: 10.1158/1078-0432.CCR-10-2015

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Translational Relevance

The chemokine CCL2 may play a pivotal role in prostate cancer tumorigenesis and invasion. Our study analyzes the role of 6 common polymorphisms in the CCL2 gene, as well as the haplotypes they composed, on the risk of developing aggressive prostate cancer. We found that 2 promoter region SNPs, -1811 A/G and -2835 A/C, a 3' untranslated region SNP, +3726 T/C, and a common haplotype are associated with more aggressive prostate cancer. These findings support the relevance of CCL2 in the development of aggressive prostate cancer.

inevitable emergence of androgen-independent cells (14). So far, only a few inherited genetic variants have been associated with aggressiveness or response to treatment of prostate cancer (15–17). CCL2 may play a role in prostate cancer tumorigenesis and invasion and is highly expressed in the tumor microenvironment by human bone marrow endothelial cells. However, the role of CCL2 genetic polymorphisms in prostate cancer progression and metastases remains an open question. To date, only one association study has evaluated the CCL2 -2518 G/A polymorphism and prostate cancer risk and no association was observed in 296 cases and 311 controls (8).

We present an association study on CCL2 polymorphisms and prostate cancer aggressiveness in a large hospital-based Caucasian patient cohort. Our data suggest the influence of CCL2 germline variants on prostate aggressiveness.

Materials and Methods

Study population

Details of the studied Dana-Farber Harvard Cancer Center SPORC (Gelb Center) Prostate Cancer cohort have been previously described (18, 19). Briefly, the study cohort contains a total of 4,073 prostate cancer patients diagnosed between 1976 and 2007, who had been consented during 1993 to 2007 to provide information, and tissue and blood samples for research purposes. To control the quality of the ethnicity information from the self-reported data, we sampled 3% of self-reported Caucasian ($n = 180$) and performed genotyping by using 26 SNPs which can distinguish Caucasian population from non-Caucasian populations (20); the genotyping data showed that none of the tested samples were in discordance. This confirmed the reliability of self-reported Caucasian ethnicity. For all individuals who ambiguously reported their ethnicity, such as reported as "American," or who do not have the ethnicity information, their Caucasian identity was determined by genotyping, using the same set of 26 SNPs. Only reliably self-reported or SNP confirmed Caucasians were eligible for this study. Age at diagnosis was calculated from the date of the first positive biopsy. Using the D'Amico risk classification criteria, prostate cancer patients were identified as at

low, intermediate, or high risk of clinical recurrence after primary therapy (21). Because the original D'Amico risk classification was set to predict biochemical outcome of localized patients, in this study, patients diagnosed with N1 or M1 diseases were regarded as high D'Amico risk class. Within the entire cohort, 1,716 of 4,073 patients received radical prostatectomy (RP) as the primary treatment. Pathologic Gleason scores and pathologic stages of RP specimen were acquired by reviewing pathology reports.

Selection of SNPs

Six SNPs in the CCL2 gene were selected for our study because of their previous associations seen in other disease types (11, 12). Three SNPs are located in the distal regulatory region of the CCL2 gene, -2835 C/A (rs2857654), -2139 A/T (rs1024610), -1811 A/G (rs3760399); 1 in promoter region, -927 G/C (rs3760396); 1 in the first intron, +764 C/G (rs2857657); and 1 in the 3' flanking region, +3726 T/C (rs2530797). The LD within the CCL2 gene locus is very strong and all r^2 values between selected SNPs except -1811 A/G are greater than 0.80. These 6 SNPs can cover most genetic variants information of studied region (Fig. 1).

DNA, SNPs, and genotyping assays

All DNA samples were extracted from peripheral whole blood by QIAamp DNA Blood mini kit (QIAGEN Inc.). Genotyping was performed with Sequenom iPLEX matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technology. For quality control, approximately 5% of randomly selected duplicates were included. No discrepancy between duplicates was observed in the genotyping data of all 6 SNPs. All SNPs had greater than 99% genotype passing rates.

Statistical methods

We analyzed each SNP as a categorical variable with a common homozygote, a rare homozygote, and a heterozygote. Observed genotype distributions were tested for departure from Hardy-Weinberg equilibrium by Pearson's goodness-of-fit test. No SNP violated Hardy-Weinberg equilibrium ($P > 0.10$).

To investigate the association between genotypes and biopsy Gleason grade at the time of diagnosis, we estimate ORs and their 95% CIs, using unconditional logistic regression. In a subcohort of patients who received RP, we also examined the association between CCL2 SNPs and RP pathologic stages with unconditional logistic regression. These analyses were adjusted for age at diagnosis. Cochran-Armitage test for trend was exploited to assess for the presence of genotype dose effect. Prostate cancer aggressiveness at diagnosis was categorized according to D'Amico risk classes (low, intermediate, or high risk) with criteria described previously. Haplotypes were constructed by an accelerated Expectation-Maximization (EM) algorithm similar to the partition/ligation method (22). Haplotype frequencies in low D'Amico risk group and intermediate or high group were estimated

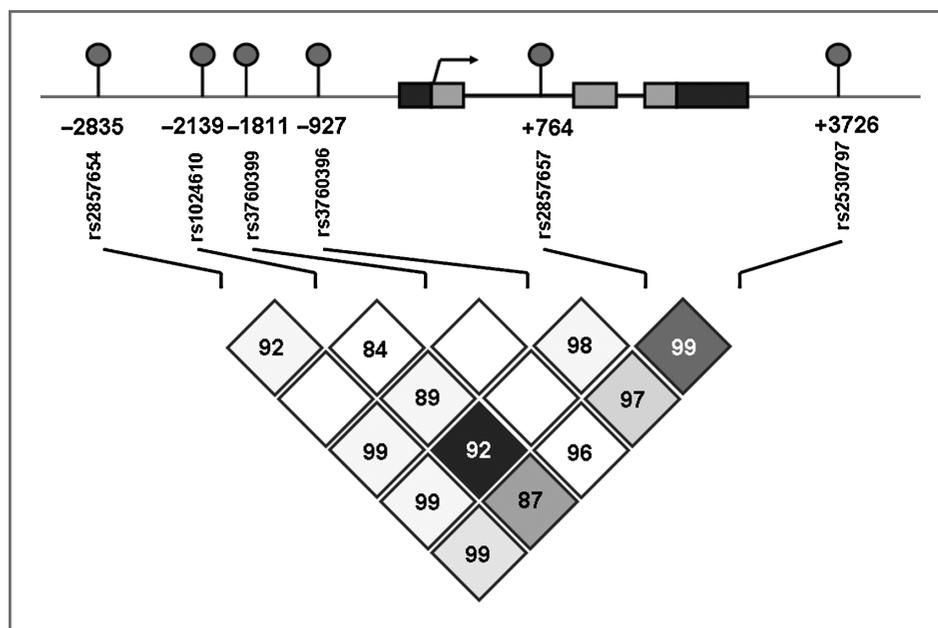


Figure 1. SNPs in the *CCL2* gene locus and their LD status. Top, diagram of *CCL2* gene locus and selected SNPs. Light gray boxes, coding region; black boxes, untranslated regions; arrow, transcription start site; round circles, selected SNPs. Bottom, LD status between selected SNPs in our Caucasian patients cohort generated by using Haploview 4.1. Number in diamonds, r^2 values between 2 indicated SNPs.

separately. Two-sided χ^2 tests were performed to determine the association between each haplotype and risk of having intermediate or high D'Amico risk class prostate cancer at the time of diagnosis with the low-risk group as reference for comparison. A total of 1,000 permutation tests were exploited to correct multiple testing bias in association analysis of haplotypes and D'Amico risk class.

Unconditional logistic regression tests were performed by SAS version 9.1 (SAS Institute Inc.) and $P < 0.05$ (2-sided) was considered statistically significant. The haplotype association analyses were done by using Haploview 4.1 (23). $P < 0.05$ (2-sided) was considered statistically significant.

Results

Subject characteristics

Selected clinical characteristics of study participants are described previously (19). Briefly, the cohort contains 4,073 patients and all participants are Caucasian. The mean age at diagnosis is 61.3 years (range, 42–91 years). The biopsy Gleason score was <7 in 1,771 (47%), 7 in 1,272 (34%) patients, and >7 in 707 (19%) patients. Among the patients who had sufficient information for modified D'Amico risk classification, 1,004 (30%) patients were of low-risk group, 1,357 (40%) patients were of intermediate-risk group, and 986 (30%) patients were of high-risk group. Of the 1,716 patients who underwent RP, 1,161 (68%) men had organ-confined (T1 or T2) disease at the time of surgery, whereas 475 (28%) men had extraprostatic tumor (T3 or T4) and 80 (4%) men had metastatic tumor (N1 or M1).

Correlation of *CCL2* SNPs with biopsy Gleason score

We first estimated associations between *CCL2* SNPs and biopsy Gleason score (Table 1). The -2835 AA genotype

had an OR of 1.42 (95% CI, 1.04–1.94) of having tumor biopsy Gleason score >7 compared with Gleason score <7 . Similarly, the -1811 AG or GG genotype had an OR of 1.47 (95% CI, 1.08–2.01) for having a biopsy Gleason score >7 prostate cancer at the time of the diagnosis compared with the AA genotype. The $+3726$ TT genotype also had an OR of 1.33 (1.01–1.75) of having biopsy Gleason >7 tumor compared with CC genotype. Because these 3 SNPs are strongly correlated, the observed associations could be because of 1 causal SNP rather than 3 independent results. We did not observe any statistically association when comparing genotypes of cases biopsy Gleason score of 7 with Gleason score <7 .

Correlation of SNPs with pathologic stages

In the patients who underwent RP, we classified men as either having evidence of extraprostatic (T3/T4) or metastatic (N1 or M1) disease or localized disease (T1/T2) at prostatectomy. When analyzing the association of *CCL2* genotypes and pathologic stages in RP patients (Table 2), we only found that the -1811 AG or GG genotype was significantly correlated with the development of extraprostatic or metastatic prostate cancer (OR, 1.50; 95% CI, 1.03–2.18; $P = 0.04$), compared with AA genotype. All other 5 investigated genotypes were not found to be associated with pathologic stages in RP patients.

Correlation of haplotypes with D'Amico risk classification

D'Amico risk classifications system uses integrated clinical information to estimate the prostate cancer aggressiveness. Because the analysis on the association of individual *CCL2* SNP with D'Amico risk classifications was null (data not shown), to more fully understand the extent of *CCL2* genetic variation effect on prostate cancer

Table 1. Genotype frequencies, odds ratios and 95% CI comparing biopsy Gleason grade at diagnosis < 7, 7, and >7

SNPs	n (%)			OR (95% CI) ^a			
	< 7	7	> 7	7 vs. < 7	P ^b	> 7 vs. < 7	P ^b
-2835 C/A (rs2857654)							
CC	945 (53.4)	683 (53.7)	345 (48.9)	Reference	0.37	Reference	0.02
AC	687 (38.8)	511 (40.2)	288 (40.9)	1.01 (0.87–1.18)		1.12 (0.93–1.36)	
AA	139 (7.8)	78 (6.1)	72 (10.2)	0.77 (0.57–1.04)		1.42 (1.04–1.94)	
Total	1,771 (100.0)	1,272 (100.0)	705 (100.0)				
-2139 A/T (rs1024610)							
AA	1,096 (62.8)	769 (61.0)	461 (65.7)	Reference	0.41	Reference	0.15
AT	573 (32.8)	438 (34.7)	216 (30.8)	1.10 (0.94–1.28)		0.91 (0.75–1.11)	
TT	76 (4.4)	54 (4.3)	25 (3.5)	1.03 (0.72–1.48)		0.80 (0.50–1.28)	
Total	1,745 (100.0)	1,261 (100.0)	702 (100.0)				
-1811 A/G (rs3760399)							
AA	1,645 (93.2)	1,172 (92.6)	636 (90.2)	Reference	0.51	Reference	0.01
AG+GG	120 (6.8)	94 (7.4)	69 (9.8)	1.10 (0.83–1.46)		1.47 (1.08–2.01)	
Total	1,765 (100.0)	1,266 (100.0)	705 (100.0)				
-927 G/C (rs3760396)							
GG	1,158 (65.6)	791 (62.3)	452 (64.1)	Reference	0.09	Reference	0.46
CG	538 (30.5)	426 (33.5)	223 (31.6)	1.16 (0.99–1.36)		1.06 (0.88–1.29)	
CC	69 (3.9)	53 (4.2)	30 (4.3)	1.12 (0.77–1.61)		1.10 (0.70–1.71)	
Total	1,765 (100.0)	1,270 (100.0)	705 (100.0)				
+764 C/G (rs2857657)							
CC	1,063 (63.5)	745 (62.0)	453 (66.3)	Reference	0.47	Reference	0.20
CG	547 (32.7)	411 (34.2)	207 (30.3)	1.08 (0.92–1.27)		0.89 (0.74–1.09)	
GG	64 (3.8)	46 (3.8)	23 (3.4)	1.05 (0.71–1.55)		0.84 (0.51–1.37)	
Total	1,674 (100.0)	1,202 (100.0)	683 (100.0)				
+3726 T/C (rs2530797)							
CC	274 (15.8)	199 (16.0)	94 (13.5)	Reference	0.93	Reference	0.01
CT	833 (48.1)	595 (47.9)	310 (44.4)	0.97 (0.78–1.20)		1.06 (0.81–1.39)	
TT	625 (36.1)	448 (36.1)	294 (42.1)	0.96 (0.77–1.20)		1.33 (1.01–1.75)	
Total	1,732 (100.0)	1,242 (100.0)	698 (100.0)				

^aAdjusted by age at diagnosis.^bCochran–Armitage test for trend.

aggressiveness, we performed a haplotype-based association analysis of D'Amico risk classifications. We found that the *CCL2* gene was encompassed in one haplotype block in our participants (Fig. 1). Six *CCL2* SNPs delineate 7 common haplotypes (H1 through H7) that accounted for 98% of all haplotypes in our studied Caucasian patients (Table 3). Haplotypes were then constructed in patients having low D'Amico risk and intermediate or high D'Amico risk class tumors, respectively. One major *CCL2* haplotype (H5, CAAGCT), which can only be defined by all 6 SNPs, was significantly associated with a reduced risk of having more aggressive prostate cancer at the time of diagnosis when compared all other haplotypes ($P = 0.01$). This result indicated that interaction between *CCL2* genetic variants may exist. The frequency of H5 was higher in low D'Amico risk group

(10.9%) than in intermediate or high-risk group (8.3%). The H5 carriers had a reduced risk of having intermediate- or high-risk prostate cancer when compared with noncarriers (OR = 0.80, 95% CI 0.66–0.96). This result remains significant after 1,000 permutation tests (adjusted $P = 0.04$). No other haplotype was significantly associated with D'Amico risk classification.

Discussion

Chemokines and their receptors have been detected in most tumors (1, 2). *CCL2* remains one of the best studied chemokines. It has been shown that *CCL2* may play a role in prostate cancer tumorigenesis and metastasis (4, 5). *CCL2* is not only involved in inflammatory responses, but also may stimulate prostate cancer cell chemoattraction,

Table 2. Genotype frequencies, odds ratios and 95% CI comparing pathologic stage in RP patients

SNPs	n (%)		OR (95%CI) ^a	P ^b
	T1 or T2	T3 or T4, or N1 or M1		
–2835 C/A (rs2857654)				
CC	622 (53.6)	291 (52.4)	Reference	0.59
AC	458 (39.4)	222 (40.0)	1.02 (0.82–1.26)	
AA	81 (7.0)	42 (7.6)	1.12 (0.75–1.67)	
Total	1,161 (100.0)	555 (100.0)		
–2139 A/T (rs1024610)				
AA	705 (61.5)	320 (58.3)	Reference	0.28
AT	389 (33.9)	203 (37.0)	1.14 (0.91–1.42)	
TT	53 (4.6)	26 (4.7)	1.06 (0.65–1.75)	
Total	1,147 (100.0)	549 (100.0)		
–1811 A/G (rs3760399)				
AA	1,080 (93.5)	503 (90.8)	Reference	0.04
AG+GG	75 (6.5)	51 (9.2)	1.50 (1.03–2.18)	
Total	1,155 (100.0)	554 (100.0)		
–927 G/C (rs3760396)				
GG	729 (63.1)	369 (66.6)	Reference	0.26
CG	380 (32.9)	161 (29.1)	0.82 (0.65–1.03)	
CC	47 (4.0)	24 (4.3)	1.05 (0.62–1.79)	
Total	1,156 (100.0)	554 (100.0)		
+764 C/G (rs2857657)				
CC	696 (63.1)	321 (60.7)	Reference	0.49
CG	358 (32.5)	187 (35.3)	1.12 (0.89–1.40)	
GG	48 (4.4)	21 (4.0)	0.92 (0.54–1.58)	
Total	1,102 (100.0)	529 (100.0)		
+3726 T/C (rs2530797)				
CC	184 (16.1)	78 (14.2)	Reference	0.61
CT	541 (47.5)	271 (49.5)	1.14 (0.84–1.55)	
TT	415 (36.4)	199 (36.3)	1.07 (0.78–1.48)	
Total	1,140 (100.0)	548 (100.0)		

^aAdjusted by the age at diagnosis.^bCochran–Armitage test for trend.

proliferation, and survival (6, 7). Overexpression of CCL2 in human prostate cancer cells significantly increased local tumor burden *in vivo* (24, 25). Other *in vivo* data further showed that CCL2-neutralizing antibodies effectively inhibit prostate cancer growth (26). SNPs located in the regulatory region of the gene may increase or decrease transcriptional activity and polymorphisms affecting the gene encoding proteins may affect the function of encoded protein, potentially leading to the association with disease susceptibility or severity (15, 27). Therefore, it is biologically plausible that genetic polymorphisms that increase CCL2 expression may be associated with prostate cancer aggressiveness, reflected by clinicopathologic traits such as biopsy Gleason score, tumor grade, or an integrated risk estimator such as D'Amico classification. In this study, we correlated genetic variations of the CCL2 gene to 3 clinical traits in a large hospital-based prostate cancer patient cohort.

We found that SNP –1811 A/G, which occurs in the promoter region of CCL2 gene, was associated with higher biopsy Gleason grade (>7) at diagnosis in all patients, and also associated with advanced pathologic stage in RP patients. Another promoter region polymorphism, –2835 C/A, and a downstream SNP, +3726 T/C, are associated with higher biopsy Gleason grade. Because of the tight LD between these 3 SNPs, it is very difficult to separate their influence in our genetic association study.

Variation at the CCL2 promoter has been reported to affect transcriptional binding sites, consequently, influencing expression (12, 28–30). Binding of IFN regulatory factor-1 and/or Prep1/Pbx2 transcription factor complex is influenced by the CCL2 –2518 A/G polymorphism (28, 31). Another functional study showed that the –927 C SNP is associated with PARP-1 and aryl hydrocarbon receptor nuclear translocator (ARNT) binding, and the –362 G is associated with a STAT binding site. CCL2 –2518, –927,

Table 3. Frequency distributions of constructed haplotypes with D'Amico risk classifications

	Haplotypes ^a	All subjects	Low risk	Intermediate or high risk	P ^b	P ^c
H1	AAAGCT	0.271	0.275	0.271	0.68	0.99
H2	CAACCT	0.196	0.185	0.200	0.18	0.67
H3	CTAGGC	0.189	0.195	0.190	0.64	0.98
H4	CAAGCC	0.186	0.188	0.181	0.52	0.96
H5	CAAGCT	0.089	0.102	0.083	0.01 ^d	0.04
H6	CAGGCT	0.039	0.032	0.042	0.05	0.35
H7	CAAGGC	0.012	0.008	0.015	0.03	0.18
	Overall					0.04

^aThe SNPs order is the same as given in Figure 1.

^bTwo-sided χ^2 test, each haplotype compared with all other haplotypes.

^cAfter 1,000 permutation test.

^dOR for carriers versus noncarriers, 0.80 (95% CI, 0.66–0.96).

and –362 polymorphisms are associated with altered transcriptional activity *in vitro* (32, 33). Intriguingly, both of the promoter SNPs which are associated with prostate cancer aggressiveness in this study, *CCL2* –1811 A/G and *CCL2* –2835 C/A, could disrupt a potential transcription factor Sp1 binding site, predicted by the web software TRANSFAC 4.0 (34), though the hypothesis remains to be validated.

Another possibility is that the observed association between *CCL2* polymorphisms and *CCL2* aggressiveness is due to linkage with another as-yet unknown polymorphisms. For example, the SNP rs4430796, which is located in the same chromosome region as the *CCL2* gene, 17q12, was correlated to prostate cancer risk and aggressiveness recently (35–38). However, there is more than 3.5 Mbp distance between rs4430796 and *CCL2* gene locus. More comprehensive resequencing and functional studies are needed to explore functional sites.

To better evaluate the influence of *CCL2* genetic variants on prostate cancer aggressiveness, we augmented our analyses with haplotypes and D'Amico risk classification to examine more comprehensively. The haplotype frequency distributions in our participants are in consistent with previous heart study in a population of European ancestry (12). It showed that one common haplotype H5 was associated with D'Amico risk classification. Because H5 is defined by all the 6 studied SNPs, only interaction of all 6 SNPs together identifies H5 and its correlation with

D'Amico risk. The association of H5 remained significant after 1,000 times permutation tests.

In conclusion, our results indicate that inherited variants in the *CCL2* gene are associated with prostate cancer aggressiveness in Caucasian patients. This finding also provides evidence that *CCL2* may be involved in prostate cancer tumorigenesis and metastasis. Additional follow-up of our sample, further validation in other larger set of prostate cancer samples and functional study will help to clarify the role of *CCL2* genotype in prostate cancer aggressiveness.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by a SPORE in Prostate Cancer 2 P50 CA090381-06, SPORE in Prostate Cancer 2 P50 CA69568-06, the Prostate Cancer Foundation, and Department of Defense (DoD) Prostate Cancer Training Award W81XWH-09-1-0372.

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Received July 27, 2010; revised October 27, 2010; accepted November 24, 2010; published OnlineFirst December 6, 2010.

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Clin Cancer Res 2011;17:1546-1552. Published OnlineFirst December 6, 2010.

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