

Comprehensive analysis of the cytokine-rich chromosome 5q31.1 region suggests a role for *IL-4* gene variants in prostate cancer risk

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Although inflammation is emerging as a candidate prostate cancer risk factor, the T-helper cytokine-rich [interleukins (*IL*)-5, 13 and 4] chromosomal region at 5q31.1 has been implicated in prostate cancer pathogenesis. In particular, *IL-4* has been associated with prostate cancer progression, whereas the *IL-4* -589C>T (rs2243250) promoter variant has been associated with differential gene expression. We genotyped rs2243250 and 11 tag single-nucleotide polymorphisms (SNPs) spanning 200 kb across the 5q31.1 region on 825 cases and 732 controls from the Risk Factors for Prostate Cancer Study. The minor alleles of rs2243250 and an *IL-4* tagSNP rs2227284 were associated with a small increase in prostate cancer risk. Per allele odds ratios (ORs) are 1.32 [95% confidence interval (CI) 1.08–1.61, *P* = 0.006] and 1.26 (95% CI 1.07–1.48, *P* = 0.005), respectively. Although these associations were not replicated in an analysis of the Melbourne Collaborative Cohort Study, including 810 cases and 1733 controls, no clinicopathological characteristic was implicated for this divergence. Correlating rs2243250 genotypes to *IL-4* gene transcript levels and circulating *IL-4* plasma levels, we observe in contrast to previous reports, a non-significant trend toward the minor T-allele decreasing the likelihood of *IL-4* activity. From our observed association between a low *IL-4* producing promoter T-allele and prostate cancer risk, our study suggests an antitumor role for *IL-4* in prostate cancer. Although we saw no association for *IL-5* or *IL-13* gene variants and prostate cancer risk, our findings call for further evaluation of *IL-4* as a contributor to prostate cancer susceptibility.

Introduction

Prostate cancer is the most common cancer affecting men in the Western world and is among the highest contributor to cancer-related deaths worldwide (1). Despite such vast prevalence, little is known about the etiology of the disease. Genetic and environmental factors have been implicated in prostate cancer risk, although it can be difficult to distinguish between the effects. To date, the most recognized

risk factors include age, ethnicity and a family history of prostate cancer (2). In recent years, inflammation has emerged as a potential contributor to prostate cancer pathogenesis (3).

Several epidemiological studies have reported an increased risk of prostate cancer associated with clinical prostatitis (prostatic inflammation) and prior exposure to sexually transmitted infections, as well as a decreased risk with the use of non-steroidal anti-inflammatory drugs (reviewed in ref. 4). A pathologically defined region of chronic inflammation associated with focal atrophy and epithelial proliferation, termed proliferative inflammatory atrophy, has been described as a potential precursor lesion to prostate cancer development (5). Chronic inflammation may be modulated by the production of cytokines involved in maintaining homeostasis within the host immune response. Thus genetic variability that alters gene expression within key cytokines may be associated with prostate cancer risk as has been reported previously (6–8).

Several reports have implicated regions located within the long arm of chromosome 5 at positions q31–qter as potential prostate cancer risk loci. Specifically, a linkage study identified 5q31.3–q33.1 as a candidate region for inherited susceptibility to aggressive prostate cancer (9). While one study demonstrated frequent loss of the region spanning 5q31–qter in prostate cancer tissue (10), a more recent report demonstrates amplification of chromosomal region 5q31.3–q32 (11). A 200 kb cytokine rich region at chromosomal position 5q31.1 harbors interleukins (*IL*s)-5, 13 and 4 (Figure 1). *IL-5* has pleiotropic effects on the immune system and acts on various immune cell types (most notably eosinophils) to promote cellular proliferation, differentiation and survival (12). *IL-13* and *IL-4* are structurally and functionally analogous. Both cytokines similarly have pleiotropic effects in the immune system acting on a number of cellular components (13). Sharing some of its functions with *IL-13*, *IL-4* induces the activation and differentiation of macrophages, induces B cells to undergo immunoglobulin (Ig) switching (14) and plays a key role in determining a T-cell response against infectious and malignant cells (15). All three cytokines have been linked to allergic inflammation (12). In addition, a gene, which encodes a protein involved in DNA double-strand break repair, *RAD50* encompasses a large region between *IL-5* and *IL-13*. A truncating variant within *RAD50* has been previously implicated in breast cancer pathogenesis (16,17); however, to our knowledge, there is currently no evidence supporting a genetic contribution of *RAD50* to prostate cancer risk.

Naive CD4⁺ T cells differentiate into different effector T-helper (Th) cell subsets depending on the microenvironment and cytokine stimulus. Although *IL-5*, *IL-13* and *IL-4* are all Th2 cytokines, the role of *IL-13* and *IL-5* in prostate cancer pathogenesis has been less well documented. *IL-4* is the central differentiation factor driving Th2 development, responsible for eliminating extracellular pathogens and inhibiting Th1 differentiation, which is involved in surveillance and elimination of tumor cells (18,19). A paradoxical effect of *IL-4* is to act synergistically with interferon- γ to prime maturing antigen presenting dendritic cells to produce high levels of a Th1 cytokine *IL-12* (20). *IL-12* subsequently induces the differentiation of tumor-specific Th1-cells and cytotoxic T lymphocytes. Thus, *IL-4* may also contribute to antitumor immunity. It has been suggested that *IL-4* may be involved in androgen-independent tumor progression (21–25). Prostate cancer cells are generally reliant on hormones, most notably androgen for growth; however, in the absence of androgen, growth factors including cytokines can activate the androgen receptor enabling androgen-independent cellular proliferation.

An *IL-4* promoter polymorphism, rs2243250 (–589C>T), was first identified ~15 years ago (26) and suggested to influence *IL-4* activity by creating a transcription factor-binding site, nuclear factor for activated T-cells (27). Despite several reports associating this variant

Abbreviations: BPH, benign prostatic hyperplasia; CI, confidence interval; Ig, immunoglobulin; *IL*, interleukin; LD, linkage disequilibrium; MCCS, Melbourne Collaborative Cohort study; mRNA, messenger RNA; OR, odds ratio; RFPSC, Risk Factors for Prostate Cancer study; SNP, single-nucleotide polymorphism; Th, T-helper.

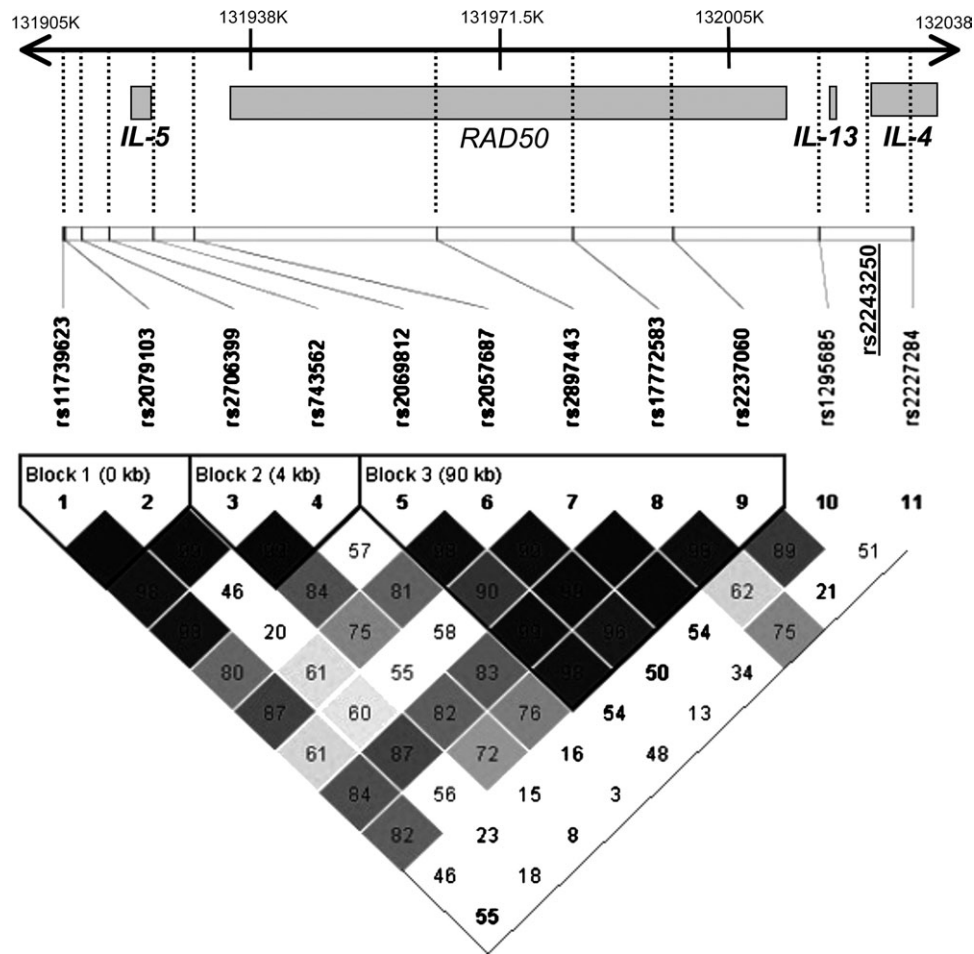


Fig. 1. Gene and variant locations within ~200 kb region (131 889 826–132 089 825) on chromosome 5q31.1. rs2243250 (bold and underline) is a functionally significant promoter variant of *IL-4* and was not included in haplotype analysis. The alternative 11 variants are tagSNPs to capture 100% of all 68 common alleles present within this region. The box color intensity correlates to the degree of LD between variants as a measure of the normalized disequilibrium coefficient (D'). Nine variants display tight linkage, excluding two variants, rs1295685 and rs2227284.

with elevated *IL-4* and subsequent IgE levels or T-cell balance (26,28,29), the original report is unique in that it is the only study to show a direct functional role for this variant *in vitro*. Using luciferase reporter and electromobility shift assays, Rosenwasser *et al.* (26) demonstrated elevated *IL-4* activity associated with the rare T-allele of rs2243250. In response to the suggested functional role, this variant has been investigated for genetic susceptibility in several immune-related diseases. Significant associations have been reported for asthma (26,30), atopic dermatitis (31), pulmonary tuberculosis (32), brucellosis (33) and several cancers including renal cell carcinoma (34), colorectal cancer (35) and oral cancer (36). To date, however, there have been no reports on the rs2243250 variant and prostate cancer risk.

In light of previous associations within and surrounding the 5q31.1 region and prostate cancer development (9–11) and the proposed link between inflammation and prostate cancer (3), we aimed to investigate genetic variability within the cytokine rich 5q31.1 region for association with prostate cancer risk using a large well-defined Australian-based epidemiological study. To encompass the entire region surrounding the three Th2-cytokines, *IL-5*, *IL-13* and *IL-4*, we adopted a tagged single-nucleotide polymorphism (SNP) approach, genotyping a total of 11 haplotype tagSNPs. In addition, we sought to determine if the proposed functional *IL-4* rs2243250 promoter variant may be associated with prostate cancer risk. For further assessment of functional impact, we associated rs2243250 genotype with levels of prostate-specific gene transcript and circulating protein.

Materials and methods

Epidemiological study samples

The Risk Factors for Prostate Cancer study (RFPCS) is a population-based case-control study of 825 cases and 732 controls from the Melbourne and Perth metropolitan areas of Australia. The men of predominantly European descent were recruited between 1994 and 1998 and described in detail elsewhere (37). Eligible cases were diagnosed with histopathologically confirmed prostate cancer via the State Cancer Registries. Cases were diagnosed before age 70 years with a Gleason score >4 . Tumor stage (tumor, node and metastasis stages I–IV (38)) and grade (moderate: Gleason score 5–7; high grade: Gleason score 8–10) were obtained from histopathological reports. Controls were randomly selected from the State Electoral Rolls and frequency matched to the expected age distribution of cases. Demographic information including country of birth, family history of prostate cancer and lifestyle were recorded during a face-to-face interview.

Samples from participants in the Melbourne Collaborative Cohort study (MCCS) were genotyped for the variants associated with prostate cancer risk in the RFPCS in order to replicate these associations. The MCCS is a prospective cohort study of 41 514 people (including 17 045 men), aged between 27 and 80 years at baseline ($>99\%$ aged between 40–69 years), recruited in Melbourne between 1990 and 1994. Individuals diagnosed with cancer were ascertained during follow-up through regular correspondence with the Victorian Cancer Registry and the National Clearing House Statistics. Case samples consisted of a total of 810 MCCS participants who were diagnosed with prostate cancer by the end of 2006. A control group consisting of 1733 men was randomly selected from the remaining MCCS samples.

Prostate tissue samples

Total RNA and DNA were isolated from matched cancerous and non-malignant prostate tissue of 36 individuals with prostate cancer (72 samples) and 8 individuals with benign prostatic hyperplasia (BPH) using QIAGEN RNeasy and DNA mini kits (QIAGEN Pty Ltd, Doncaster, VIC, Australia). The cancerous and non-malignant samples were sections of prostate cores (5 mm) collected during pathology work-up of retropubic radical prostatectomy specimens, snap frozen and stored at -80°C in optimal cutting temperature. The cancer tissue was extracted from individuals with predominantly Gleason score 7 tumors and varying percentage of cancerous cells. BPH samples were collected from transurethral resection of the prostate. All tissue samples were supplied by the Australian Prostate Cancer BioResource, an initiative of the Australian Prostate Cancer Collaboration (www.apccbioresource.org.au). RNA was also isolated from four prostate cancer cell lines obtained from ATCC, the Global Bioresource Centre (<http://www.atcc.org/>) including two androgen sensitive (LNCaP and PCa2b) and two androgen insensitive cell lines (DU145 and PC3).

Plasma samples

A total of 53 whole blood samples were collected in ethylenediaminetetraacetic acid tubes from Southern African men ($n = 53$) diagnosed with various stages of prostate cancer at the Urology clinic, Tygerberg Hospital, South Africa (Tygerberg samples). Cell-free plasma was isolated via centrifugation and stored at -80°C for measurement of antibodies against IL-4. Buffy coat was isolated for DNA extraction using a QIAGEN Allprep kit.

SNP selection

The IL-4 promoter variant rs2243250 was selected for genotyping as a result of previous reports of functional significance. Using the HapMap genome browser (http://www.hapmap.org/cgi-perl/gbrowse/hapmap3r2_B36/) and based on the CEU population (Utah residents with Northern and Western European Ancestry), an additional 11 haplotype tagSNPs (r^2 co-efficient cutoff of 0.8, mean $r^2 = 0.96$) with a minor allele frequency between 0.2 and 0.5 were selected to capture 100% of all 68 common alleles reported within a 200 kb region (nucleotide range 131 889 826–132 089 825) surrounding IL-4, IL-13 and IL-5 at chromosomal position 5q31.1 (Figure 1).

High-throughput genotyping

All twelve selected SNPs were genotyped on one or both the RFPCS and MCCS using pre-designed TaqMan allelic discrimination assays according to manufacturer's guidelines (Applied Biosystems, Scoresby, VIC, Australia) for high-throughput analysis. Random study repetition ($>10\%$) was performed for each variant for determination of assay reliability.

Low-throughput genotyping

DNA extracted from prostate tissue (Australian Prostate Cancer BioResource samples) and matched plasma and buffy coat prostate cancer patient samples (Tygerberg samples) were amplified using Roche FastStart Taq DNA polymerase (Roche Diagnostics Corp., Indianapolis, IN) with an annealing temperature of 57°C for a 91 bp fragment encompassing the IL-4 promoter variant, rs2243250. The forward primer for amplification contained a 40 bp GC-rich tail on the 5' end to prevent complete strand dissociation and improve genotype resolution using denaturing gradient gel electrophoresis. Primer information available upon request. A 40% gradient (30–70%) of denaturants, urea and formamide was used to resolve the different genotypes. Denaturing gradient gel electrophoresis banding patterns were validated via direct Sanger sequencing.

IL-4 real-time polymerase chain reaction

One microgram of each total RNA extracted from prostate tissue was converted to complementary DNA using the Promega reverse transcription system (Promega Corporation, Madison, WI). The entire reverse transcription reaction was loaded onto a custom-designed 48-plexed Applied Biosystems low-density array card for analysis of IL-4 transcript levels by real-time polymerase chain reaction. Together with IL-4, the low-density array also contained 3 housekeeping genes *18S*, *HPRT1* and *ALAS1* and 44 genes not forming part of this study. Total messenger RNA (mRNA) was detected by monitoring fluorescent signals over 45 cycles on the AB 7900 real-time polymerase chain reaction machine according to manufacturer's guidelines. A total of four samples across four cards were run in duplicate to assess intra-array variability.

IL-4 enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay was used to measure IL-4 cytokine levels in the plasma of 53 prostate cancer patients (Tygerberg samples). Ninety-six-well plates were coated with capture antibody (mouse IgG1 anti-human IL-4 unconjugated antibody at $1\text{ }\mu\text{g/ml}$, Pharmingen, San Diego, CA, Clone 8D4-8) by overnight incubation at 4°C . The wells were blocked with 2%

fetal calf serum in phosphate-buffered saline and incubated at 37°C for 1 h. Plates were washed in phosphate-buffered saline–Tween solution before adding plasma samples (three wells per sample, neat, 1/3 and 1/9) to incubate for a further 1 h at 37°C . Plates were then incubated with the biotinylated antibody (rat IgG1 anti-human IL-4 biotinylated antibody, Pharmingen, Clone MP4-25D2) at $1\text{ }\mu\text{g/ml}$ for 1 h at 37°C . Streptavidin–horseradish peroxidase conjugate was added and allowed to bind at 37°C for a further hour before adding tetramethylbenzidine substrate solution (BD kit) until color developed. Sulphuric acid (1 M) was added to stop the reaction before analysis on a microplate reader at a wavelength of 450 nm and a reference of 620 nm. IL-4 concentrations were calculated from standard curves obtained by incubating serial dilutions (lower limit = 0.6 pg/ml) of recombinant IL-4.

Statistical analysis

For the case–control and case–cohort studies, allele and haplotype frequency estimates and tests of deviation from Hardy–Weinberg Equilibrium were carried out using standard procedures based on asymptotic likelihood theory (39). Unconditional logistic regression analysis was used to test for independence between the variants and age (<55 , $55\text{--}59$ and >60), country of birth (Australia and others) and family history of prostate cancer (affected first-degree relative and two or more first-degree relatives affected). For both RFPCS and MCCS, analysis was conducted using unconditional logistic regression and odds ratio (OR) estimates and their 95% confidence intervals (CIs) were derived under likelihood theory. Tests for association between genotypes/haplotypes and case–control status were performed using dominant, recessive, co-dominant and log-additive models. Heterogeneity in the ORs by tumor stage [stages I–II (0) and stages III–IV (1)] and grade [moderate grade (0) and high grade (1)] were tested using polytomous logistic regression models. Heterogeneity in the ORs by age and family history of prostate cancer in first-degree relatives (0 or at least 1) was tested by including an interaction term in the logistic model. For the SNPs tested in both the RFPCS and MCCS, a pooled analysis was performed fitting an unconditional logistic model to the combined data. Heterogeneity between the ORs for the two studies was tested by including an interaction term between genotype and study. Haplotype analyses were performed with methods based on the score statistics (40). The linkage disequilibrium (LD) and haplotype analyses were performed using the R statistical language (www.r-project.org). A chi-square test for trend was used to determine statistical significance between tissue phenotype (cancerous, non-malignant and BPH) as well as rs2243250 genotypes and IL-4 activity (detectable/non-detectable levels of IL-4) in prostate tissue (Australian Prostate Cancer BioResource samples) and/or plasma samples (Tygerberg samples). All statistical analyses were performed using Stata 10 (Stata Corporation, College Station, TX). All tests were two sided and a 5% level was used as the threshold for statistical significance.

Results

Genetic association with prostate cancer risk

Genotype calls were achieved for $>97\%$ and 99% of the RFPCS and MCCS samples, respectively. We obtained 100% genotyping concordance using the TaqMan allelic discrimination method for an average 12 and 15% random repetition of RFPCS and MCCS, respectively. Genotype distributions were consistent with Hardy–Weinberg Equilibrium for both cases and controls ($P > 0.09$) with the exception of rs2227284, which displayed Hardy–Weinberg disequilibrium for RFPCS cases ($P = 0.003$), but not for controls ($P > 0.93$). In the MCCS, genotype distribution for this SNP was consistent with Hardy–Weinberg Equilibrium for both cases and controls ($P > 0.4$).

A single proposed functional IL-4 promoter variant, rs2243250 and 11 haplotype tagSNPs that spanned a 200 kb region at chromosomal position 5q31.1 were assessed for association with prostate cancer risk using the RFPCS. We compared genotype distribution between cases and controls and observe a significant association between prostate cancer risk and two IL-4 variants, rs2243250 and intron 2 located rs2227284 (Table I). For each variant, the per allele ORs were 1.32 (95% CI 1.08–1.61) and 1.26 (95% CI 1.07–1.48), respectively. The promoter variant rs2243250 was significantly associated with risk using the recessive (OR = 2.16, 95% CI 1.06–4.40, $P = 0.03$) and dominant (OR = 1.31, 95% CI 1.05–1.65, $P = 0.02$) models of inheritance, whereas rs2227284 was significant only for the recessive model (OR = 1.98, 95% CI 1.30–3.00, $P = 0.001$).

A measure of pairwise LD between the eleven 5q31.1 haplotype tagSNPs, revealed strong LD between nine variants in three LD

Table I. Allele and genotype distributions for 12 variants across the chromosome 5q31.1 locus on the RFPCS, the two *IL-4* variants are repeated for the MCCS and depicted as a pooled analysis of the RFPCS and MCCS

Study	Variant ID	Nucleotide substitution	Gene	Minor allele frequencies (total N)		Per allele OR ^a (95% CI)	P ^b	Heterozygous OR (95% CI)	Homozygous OR (95% CI)	P-value (recessive)	P-value (dominant)
RFPCS	rs1295685	G>A	<i>IL-13</i>	0.20 (799)	0.18 (727)	1.15 (0.96–1.39)	0.1	1.12 (0.90–1.39)	1.48 (0.83–2.63)	0.2	0.2
	rs17772583	A>G	<i>RAD50</i>	0.25 (803)	0.23 (725)	1.14 (0.96–1.34)	0.1	1.12 (0.91–1.39)	1.34 (0.86–2.07)	0.3	0.2
	rs2237060	T>G	<i>RAD50</i>	0.41 (802)	0.43 (726)	0.90 (0.78–1.04)	0.2	0.89 (0.71–1.12)	0.82 (0.61–1.10)	0.3	0.2
	rs2897443	G>T	<i>RAD50</i>	0.19 (798)	0.20 (726)	0.91 (0.76–1.10)	0.3	0.87 (0.70–1.08)	0.99 (0.55–1.78)	0.9	0.3
	rs2057687	G>A	<i>RAD50 / IL-5</i>	0.48 (797)	0.50 (722)	0.93 (0.81–1.078)	0.3	0.96 (0.75–1.23)	0.87 (0.66–1.15)	0.4	0.6
	rs2069812	G>A	<i>IL-5</i>	0.32 (798)	0.29 (726)	1.15 (0.98–1.34)	0.08	1.17 (0.95–1.45)	1.28 (0.89–1.84)	0.3	0.09
	rs743562	C>T	<i>IL-5</i>	0.41 (802)	0.42 (722)	0.98 (0.85–1.13)	0.8	0.87 (0.70–1.09)	1.00 (0.74–1.35)	0.5	0.4
	rs2706399	G>A	<i>IL-5</i>	0.50 (801)	0.50 (727)	1.02 (0.89–1.18)	0.8	1.12 (0.87–1.43)	1.04 (0.79–1.38)	0.8	0.5
	rs2079103	G>T	—	0.23 (800)	0.25 (722)	0.93 (0.78–1.10)	0.4	0.94 (0.76–1.16)	0.83 (0.52–1.32)	0.5	0.5
	rs11739623	G>A	—	0.26 (809)	0.24 (730)	1.08 (0.92–1.27)	0.3	1.15 (0.93–1.42)	1.05 (0.70–1.58)	>0.9	0.2
	rs2243250	C>T	<i>IL-4</i>	0.16 (818)	0.13 (733)	1.32 (1.08–1.61)	0.007	1.25 (0.98–1.58)	2.27 (1.11–4.65)	0.03	0.02
	rs2227284	G>T	<i>IL-4</i>	0.27 (803)	0.22 (727)	1.26 (1.07–1.48)	0.005	1.10 (0.89–1.36)	2.05 (1.34–3.13)	0.001	0.06
	rs2243250	C>T	<i>IL-4</i>	0.13 (810)	0.14 (1731)	0.90 (0.77–1.04)	0.2	0.85 (0.71–1.02)	0.98 (0.61–1.57)	0.9	0.1
	rs2227284	G>T	<i>IL-4</i>	0.24 (810)	0.25 (1733)	0.98 (0.87–1.11)	0.8	0.93 (0.79–1.08)	1.08 (0.80–1.48)	0.5	0.5
MCCS	rs2243250	C>T	<i>IL-4</i>	0.15 (1628)	0.14 (2461)	1.09 (0.96–1.23)	0.2	1.03 (0.89–1.19)	1.47 (0.98–2.21)	0.4	0.07
	rs2227284	G>T	<i>IL-4</i>	0.26 (1613)	0.24 (2457)	1.08 (0.97–1.19)	0.2	0.96 (0.84–1.10)	1.43 (1.11–1.85)	0.8	0.004
Pooled ^c											

^aORs and 95% CIs from unconditional logistic regression analysis. Adjustment for family history of prostate cancer, age and country of birth did not materially change the OR estimates.

^bTest for association between genotype and prostate cancer risk (likelihood ratio test).

^cThe *P*-value for testing the heterogeneity are 0.01 for both rs2243250 and rs2227284.

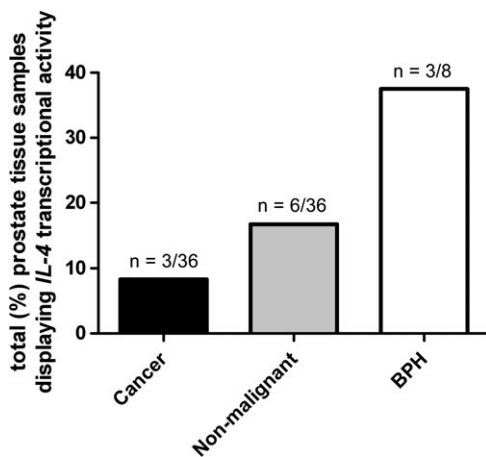


Fig. 2. Percentage of cancerous ($n = 36$), matched non-malignant ($n = 36$) and BPH prostate tissue samples with detectable *IL-4* transcriptional activity by real-time polymerase chain reaction. *IL-4* mRNA was detected least frequently in cancerous prostate tissue (8%, 3/36), followed by the matched non-malignant (17%, 6/36) and BPH (38%, 3/8) samples. The distribution of detectable *IL-4* mRNA was not significantly different between the different tissue types ($P = 0.06$ between BPH and cancerous tissue).

blocks, excluding *IL-13* variant rs1295685 and *IL-4* variant rs2227284 (Figure 1). Strong LD was also observed between the two *IL-4* variants, rs2243250 and rs2227284 ($D' = 0.97$, $r^2 = 0.49$; data not shown). A total of 32 haplotype combinations were predicted using rs1295685, rs2227284 and rs2243250 and one SNP for each block (rs2079103 for block 1, rs743562 for block 2 and rs2057687 for block 3). Haplotype-specific scores were marginally significant for the three rare haplotypes ATGCG, GTCGTG and ATTGCA (haplotype frequency = 0.01, 0.004 and 0.01, respectively) for the additive and dominant models (P -values = 0.02, 0.01 and 0.01, respectively); overall, there was little evidence of association between haplotype and prostate cancer risk (P -values from the global score statistics >0.2 for the additive, dominant and recessive models).

The associations with rs2243250 and rs2227284 observed in the RFPCS were not confirmed in the MCCS. For rs2243250 and

rs2227284 per allele ORs were 0.90 (95% CI 0.77–1.04) and 0.98 (95% CI 0.87–1.11), respectively. Pooled ORs were close to unity and the CIs were not consistent with association with prostate cancer risk. For both variants, there was evidence of heterogeneity in the per allele ORs between the case-control (RFPCS) and case-cohort study (MCCS) ($P_{\text{heterogeneity}} = 0.01$ for both variants).

IL-4 transcriptional activity in prostate tissue

The *IL-4* transcript was detected at only low levels in the minority of prostate tissue samples assessed in this study. We detected *IL-4* complementary DNA in nearly 38% (3/8) of BPH samples compared with ~17% (6/36) and 8% (3/36) of non-malignant and cancerous prostate tissue, respectively (Figure 2). The difference between *IL-4* presenting cancerous and BPH prostate tissue was, however, not statistically significant ($P = 0.06$). For two of the three cancerous samples displaying *IL-4* transcriptional activity, *IL-4* complementary DNA was similarly detected in the matched non-malignant sample. Positive transcript levels were determined at raw cycle threshold (Ct) 38–45, and when normalized to 18S, >26. Thus, low levels of *IL-4* transcriptional activity was observed in these early stage cancerous, matched non-malignant and BPH prostate tissue samples.

Circulating IL-4 levels in prostate cancer patients

Similarly to the prostate tissue samples assessed in this study, plasma levels of *IL-4* in individuals diagnosed with prostate cancer were in most cases below the sensitivity range of the enzyme-linked immunosorbent assay (0.6 pg/ml). Approximately 68% (36/53) of all serum samples contained *IL-4* at levels too low to detect (<0.6 pg/ml). Of the remaining 32% (17/53) of samples, *IL-4* was detected at levels ranging between 3 and 91 pg/ml.

Influence of rs2243250 on IL-4 activity

Correlating the percentage of prostate tissue (Figure 3A) and plasma (Figure 3B) samples presenting with *IL-4* activity, with the rs2243250 genotype combinations (wild-type CC, heterozygous CT and homozygous TT), we concurred an observed trend that wild-type samples are more probably to present with detectable levels of *IL-4* compared with the alternative genotypes. The difference between rs2243250 genotypes presenting with *IL-4* activity was, however, not statistically significant in the tissue samples ($P = 0.3$) and only marginally

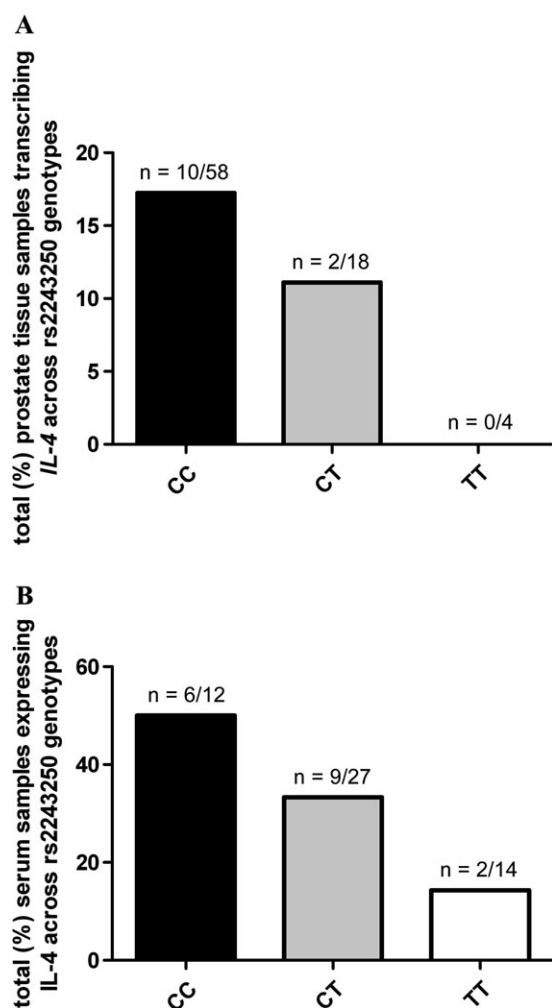


Fig. 3. Percentage of prostate tissue samples with detectable *IL-4* transcription (A) and serum cytokine levels (B) among the rs2243250 genotypes. For both real-time polymerase chain reaction and enzyme-linked immunosorbent assay methods, the C allele of rs2243250 most frequently exhibited detectable *IL-4* activity. (A) In the prostate tissue samples, ~17% (10/58) of CC and 11% (2/18) of CT genotypes presented with a small level of *IL-4* transcriptional activity. No tissue samples homozygous for the mutant T-allele ($n = 4$) revealed any *IL-4* transcription. There was no significant difference between *IL-4* transcription and allelic distribution ($P = 0.3$). (B) Circulating *IL-4* in patients with prostate cancer was detected in 50% (6/12), 33% (9/27) and 14% (2/14) of rs2243250 CC, CT and TT genotypes, respectively. The frequency of detectable *IL-4* was marginally significant between the rs2243250 genotypes ($P = 0.05$).

significant in the plasma samples ($P = 0.05$). In prostate tissue, ~17% (10/58) and 11% (2/18) of samples with the wild-type (CC) and heterozygous genotypes, respectively, presented with a small level of *IL-4* transcriptional activity. In contrast, no tissue samples homozygous for the mutant T-allele ($n = 4$) revealed *IL-4* transcription. A similar, though marginally significant trend ($P = 0.05$) was observed in the plasma samples of patients with prostate cancer. Antibodies against *IL-4* were detected in 50% (6/12), 33% (9/27) and 14% (2/14) of rs2243250 CC, CT and TT genotypes, respectively. In samples with a positive value for either *IL-4* transcription in prostate tissue or *IL-4* plasma levels, there was no significant difference in the average quantity between the various rs2243250 genotypes (data not shown).

Discussion

Recent studies have suggested a role for chronic inflammation in prostate cancer pathogenesis (41). It has been further hypothesized

that an imbalance in Th-cell response may determine whether a tumor-mediated immune response is beneficial (antitumor immunity) or inhibitory (pro-tumor immunity) to the host (42). *IL-4*, *IL-5* and *IL-13* secreting Th2-cells induce a cascade of events that suppress the differentiation and cytokine production of Th1-cells involved in tumor surveillance and elimination (43). Furthermore, elevated levels of Th2-cytokines, *IL-4* and *IL-5* have been associated with a poorer prognosis of prostate cancer (44). Sufficient amounts of *IL-4* secreted upon initial activation of Th-cells trigger a positive feedback loop for further Th2 differentiation (45). However, despite the prominent role of *IL-4* in mediating a Th2 response, the pre-exposure of dendritic cells to *IL-4*, prior to an interaction with Th-cells has been associated with dendritic cell-mediated differentiation of a tumor-specific Th1 and cytotoxic T lymphocyte response, emphasizing the contradictory roles of this cytokine (20). Thus, genetic variation responsible for differential *IL-4* expression and subsequent Th-cell differentiation may be critical in determining a pro-tumor or antitumor immune response.

A previous report has suggested a 70 bp variable number of tandem repeat polymorphism within intron 3 of *IL-4* may influence *IL-4* production (28). A single study has investigated this variant for involvement with prostate cancer risk and found no significant association (46). The *IL-4* variable number of tandem repeat polymorphism displays tight linkage with the reported functional promoter variant rs2243250 and it was further hypothesized that the *IL-4* variable number of tandem repeat may be a marker for rs2243250 (28). To the best of our knowledge, our study is the first to directly investigate an association between rs2243250 and prostate cancer risk. We found a significant association with the minor T-allele of this variant as well as a second *IL-4* intron 2 variant, rs2227284, with overall prostate cancer risk in the European-centric RFPC case-control study. We found no other tagSNPs within the chromosome 5q31.1 locus to be associated with disease risk. These results are in concordance with current genome-wide association studies that have thus far failed to implicate the 5q31.1 region in prostate cancer risk. However, the growing number of prostate cancer risk loci already identified on genome-wide association studies, demonstrates the complex and heterogeneous nature of the disease, allowing for the possibility that multiple low to modest risk alleles are yet to be identified. Our results are therefore suggestive that *IL-4* may be a candidate gene for prostate cancer susceptibility; however, null results for previous genome-wide association studies and discrepant results between the studies used in this report raise the possibility of false-positive associations.

We failed to replicate the associations observed with prostate cancer risk in the RFPCS on a second prostate cancer epidemiological resource, namely, the MCCS. However, heterogeneity of the per allele ORs would suggest that statistical differences observed between the RFPCS and MCCS are potentially due to diverse clinical and/or methodological factors between the two studies and not a consequence of chance alone. We saw no evidence that *IL-4* variant, rs2243250 or rs2227284 is associated with tumor stage, grade, age or family history of prostate cancer. We are thus unable to explicate differences between association results to intra study discrepancies of these attributes. Limited numbers and reduced power to assess each of these variables, however, indicates they cannot be excluded. Study heterogeneity raises important issues regarding the suitability of these studies for pooled analysis or for comparison of results.

The observed genetic association in the RFPCS imply that *IL-4* may be important in prostate cancer risk. We endeavored to determine whether this cytokine was elevated in patients with prostate cancer as well as investigating the possibility that the promoter variant rs2243250 contributes to differential *IL-4* activity. Despite reports that *IL-4* is involved in androgen-independent growth of prostate cancer, we observed no *IL-4* mRNA in any of the prostate cancer cell lines investigated in this study, including two androgen-independent cell lines. Furthermore, we observed only low levels of *IL-4* transcriptional activity in a minimal number of prostate tissue samples assessed. We found a non-significant 2-fold and 4-fold increase in the number of BPH tissue samples with detectable *IL-4* transcription compared with non-malignant and matched cancerous prostate tissue, respectively.

One explanation for this observation may be that the prostate tissue samples in this section of the study present with early stage, intermediate grade disease (average Gleason score of 7). A previous study has identified a Th17 phenotype to be dominant in the early stages of prostate cancer, whereas Th2 cells (measured by IL-4 expression) were reportedly absent (47). Thus alternative immune cell phenotypes could be suppressing the IL-4 secreting Th2-cells predominating in the BPH tissue samples. In support of a role for IL-4 in BPH, a previous report has similarly revealed elevated expression of IL-4 in BPH tissue compared with normal and cancerous prostate cell lines (48).

Circulating biomarkers are a useful tool for early detection of disease. Although circulating plasma IL-4 were detected in only 32% of prostate cancer patients (17/53), this is more frequent than was observed in a previous report of healthy individuals (10%, 3/30) (49). Patients used in this section of the study, however, are clinicopathologically diverse and it would thus be difficult to determine a precise mechanism for IL-4 involvement in prostate cancer pathogenesis.

To date, a vast majority of IL-4-mediated disease association studies have focused on the promoter variant rs2243250 as a consequence of suggested influences on transcriptional activity (26). We sought to provide further clarification to this assumption by correlating IL-4 mRNA levels in prostate tissue and circulating IL-4 plasma levels to the rs2243250 genotypes. We were unable to concur with previous reports associating the T-allele of rs2243250 with elevated IL-4 activity (26,29,50). A more recent report, however, in support of our findings suggests that the rs2243250 TT genotype correlates with a low genetic ability to produce IL-4 compared with the intermediate CT and high producing CC genotypes (51). In concordance with this recent report, our results suggest a trend toward the common C-allele of rs2243250 being more probably to produce detectable levels of IL-4 compared with samples containing the rare T-allele. Detectable levels of IL-4 mRNA in prostate tissue samples did not significantly differ between the various genotypes. However, the trend was in a similar direction to the marginally significant results observed in plasma samples of patients with prostate cancer. In support of an antitumor role for IL-4, the low producing T-allele of rs2243250 was associated with prostate cancer risk in the RFPCS.

In conclusion, we found no association between variants in IL-5 and IL-13 and prostate cancer risk. Two variants in IL-4 (including promoter variant rs2243250) were associated with a modest increase in prostate cancer risk in the RFPCS. Although lack of replication of association using a case-cohort study design cautions interpretation, functional analysis of rs2243250 suggested association with down-regulation of IL-4 activity. The observed increased risk coupled with decreased IL-4 activity is suggestive of an antitumor role of IL-4 in prostate cancer pathogenesis.

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