Multiple novel prostate cancer susceptibility signals identified by fine-mapping of known risk loci among Europeans

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Abstract

Genome-wide association studies (GWAS) have identified numerous common prostate cancer (PrCa) susceptibility loci. We have fine-mapped 64 GWAS regions known at the conclusion of the iCOGS study using large-scale genotyping and imputation in 25 723 PrCa cases and 26 274 controls of European ancestry. We detected evidence for multiple independent signals at 16 regions, 12 of which contained additional newly identified significant associations. A single signal comprising a spectrum of correlated variation was observed at 39 regions; 35 of which are now described by a novel more significantly associated lead SNP, while the originally reported variant remained as the lead SNP only in 4 regions. We also confirmed two association signals in Europeans that had been previously reported only in East-Asian GWAS. Based on statistical evidence and linkage disequilibrium (LD) structure, we have curated and narrowed down the list of the most likely candidate causal variants for each region. Functional annotation using data from ENCODE filtered for PrCa cell lines and eQTL analysis demonstrated significant enrichment for overlap with bio-features within this set. By incorporating the novel risk variants identified here alongside the refined data for existing association signals, we estimate that these loci now explain ~38.9% of the familial relative risk of PrCa, an 8.9% improvement over the previously reported GWAS tag SNPs. This suggests that a significant fraction of the heritability of PrCa may have been hidden during the discovery phase of GWAS, in particular due to the presence of multiple independent signals within the same region.

Introduction

Prostate cancer (PrCa) is one of the most commonly diagnosed cancers and leading causes of cancer-related deaths for men in developed countries. An increased incidence of PrCa among first-degree relatives of patients, together with results from twin studies, provides strong evidence for a heritable component to PrCa (3). In recent years, many studies have sought to identify genetic variants that predispose towards the development of PrCa. Candidate gene studies have demonstrated that rare (minor allele frequency, MAF < 1%) loss-of-function variants in DNA repair genes, in particular BRCA2, as well as a recurrent missense variant in HOXB13 confer moderately increased disease risks; however, these explain only a limited fraction of the overall heritability (2,3). In addition to these rare, higher risk mutations, ~100 common, low-penetration variants have currently been identified through GWAS. These variants confer only modest increases in risk individually, but appear to combine multiplicatively thereby exerting a more substantial effect that is currently estimated to explain 33% of the familial relative risk (FRR) of the disease (4).

The specific low penetrance variants identified in GWAS are generally unlikely themselves to be causative for PrCa, since they are typically correlated with many other variants, one or more of which is functionally related to the disease. Fine-mapping studies are therefore performed to enable a more thorough evaluation of variation in associated regions, in order to narrow down the number of potential causal variants for subsequent evaluation and validation through functional assays. In addition, it has become clear that a small number of regions associated with many traits harbor multiple independent association signals (a classic example of which is the Chr8q24 region centromeric to MYC, which is associated with many forms of cancer including PrCa). However, in most cases, it is unclear whether these independent signals modulate risk through a common or separate functional mechanism, since the causal variants themselves remain unresolved. A key first step towards identifying the precise causal variants and functional mechanisms that confer risk is to comprehensively evaluate the evidence for association for non-genotyped potentially relevant variants within the region, to refine the original GWAS signal. In principle, re-sequencing associated regions in large case-control series would provide the most thorough data, but this approach is currently prohibitively expensive for routine application. However, since GWAS signals are expected to be predominantly driven by relatively common variants, large-scale genotyping together with imputation provides a cost-effective approach to evaluate the majority of likely causal variants. To date, only a small number of PrCa susceptibility regions published by the end of the iCOGS study have been fine-mapped. In analyses conducted by the PRACTICAL Consortium, for which the largest set of PrCa samples and genotype data are available, we previously identified the KLK3 locus at Chr19q13 a more strongly associated missense coding variant that has been demonstrated to alter protein function (5), and at two regions, Chr8q24 and TERT at Chr5p15, fine-mapping demonstrated the presence of multiple independent risk variants (6,7). In this study, we have fine-mapped, functionally annotated and curated a set of the most promising candidate susceptibility variants for all PrCa susceptibility regions published by the end of the iCOGS genotyping project, aside from the three that we had previously analyzed individually.

Results

We have fine-mapped 64 known PrCa regions through a combination of genotyping and imputation. Region boundaries for this analysis were defined as 500 kb either side of any known PrCa associated GWAS SNPs; where such regions overlapped, they were merged to form a single larger region (extended boundaries were employed at regions Chr3p12, Chr4q22, Chr8p21, Chr11q13 and Chr17q12). We used genotype data for 25 723 cases and 26 274 controls of European ancestry from two UK GWAS studies and from the 32 studies in the PRACTICAL Consortium genotyped using the iCOGS array. After imputation to a 1000 Genomes reference panel, data were available for 283 910 SNPs across these 64 regions. For 23 of the 64 regions the iCOGS array contained a dense panel of markers that included almost all variants correlated with the original GWAS hit, thereby facilitating particularly high-resolution interrogation of these loci.

In this fine-mapping study, 15 previously reported PrCa susceptibility variants did not replicate at genome-wide significance (P < 5×10^{-8}). For four of these variants, the association with PrCa had previously been reported only in East Asian populations (rs1938781, rs2252004 (8) and rs9600079 (9) in Japanese and rs103294 (10) in Chinese individuals). We found no evidence
suggestive of association with PrCa at any of these regions in individuals of European ancestry ($P > 0.4$), which may indicate that these variants modulate risk through a mechanism predominate among individuals with specific genetic backgrounds, or alternatively require further confirmation in Europeans through additional larger studies. For another variant that did not replicate at genome-wide significance in this study, rs1571801, the previously reported association with PrCa achieved statistical significance only in relation to aggressive disease (11). In our data, the most strongly associated correlated variant within this region showed some tendency towards association with PrCa although remained non-significant (rs200543781, OR = 1.36, $P = 5.1 \times 10^{-4}$); this could reflect the fact that our sample panel was not enriched for aggressive disease. A further four regions had previously been identified in studies by the PRACTICAL Consortium; however, in each case, a larger sample size was available in the original study or subsequent replication set than for this fine-mapping analysis. For these four SNPs (rs6869841, rs2427345 and rs11902236 published in Eeles et al. (12) and rs6769391 in Kote-Jarai et al. (13)), $P$-values in this analysis were close to genome-wide significance (Supplementary Material, Table S1), and the failure to replicate at genome-wide significance most likely reflects the smaller sample size. For the remaining six regions where the original index SNP did not reach genome-wide significance, our recent meta-analysis that included additional datasets (35,093 cases and 34,599 controls, Amin Al Olama et al. (4)) observed associations at genome-wide significance, and therefore these regions were included in this analysis. Of the 55 regions in our final analysis (after excluding the four non-European and five European regions which did not reach genome-wide significance in this study or the meta-analysis), after stepwise logistic regression, 39 could be categorized as ‘simple regions’ defined by a single association signal (Table 1) and 16 as ‘complex regions’, each of which contained more than one independent association signal (Table 2).

For each of the 75 independent association signals identified across the 55 regions analyzed, we have selected a set of the most promising correlated candidate causal variants. Since a greater density of variants are interrogated during fine-mapping than through the ‘tag SNP’ approach used in the discovery phase of GWAS, the causal variants responsible for PrCa risk at each region would generally be expected to associate with PrCa at a similar level to the refined lead SNP, as well as exhibiting relatively high levels of linkage disequilibrium (LD). Consequently, we selected our ‘best candidate’ variants that most warrant further interrogation for functionality and PrCa causality for each association signal using an overlap between two criteria: likelihood ratio of $\geq 1/1000$ relative to the refined lead SNP (‘1000 worse’ list = 6537 SNPs) and correlation with the lead SNP at LD $r^2 > 0.7$ (LD list = 2202 SNPs). This best candidate list comprised 1623 SNPs across the 55 regions studied, with between 1 and 93 SNPs per association signal and a median of 13 candidate variants (Supplementary Material, Table S2). These best candidate SNPs were annotated for overlap with functional elements in PrCa cell lines. For this analysis, bio-features were annotated according to the methodology used in Hazelet et al. (14). Of our 1623 best candidate SNPs, 413 (25%) were either coding or within an annotated bio-feature, with enhancer elements accounting for the largest single class of element represented (Supplementary Material, Table S2). We also analyzed the best candidate SNP list for potential eQTLs using TCGA data available for prostate tissue and a much larger set of EuroBATS data for three different tissue types (skin, LCL and adipose). To aid in the interpretation of data from this study, we developed Locus Explorer, a Shiny R application which allows the interactive graphical illustration of all the regions we have fine-mapped and which can be accessed at https://github.com/oncogenetics/LocusExplorer (manuscript in preparation). In the Locus Explorer plots for this study, we have included LD structure, statistical association data, functional annotation, gene transcripts and eQTL data; however, this application is customizable as required.

For 4 of the 39 simple regions, the originally reported SNP remained the most strongly associated variant after fine-mapping, whereas in the remaining 35 regions a new lead SNP was identified (Table 1). The novel lead variants were generally in strong ($r^2 > 0.7$) or moderate LD ($r^2 = 0.3–0.7$) with the original GWAS tag SNP; however, for five regions, the new lead SNP was only in weak LD with the original signal ($r^2 < 0.3$). In 10 regions, the originally reported variant was excluded from our set of the best candidate SNPs to further investigate for possible causal functional effects. A good illustration of the refinement of the association signal within simple regions is at ChrXq12 where the original signal, rs5919432, was situated 71 kb 3′ of the androgen receptor (AR) gene. This has now been replaced by rs4466888 which is intronic to AR and in strong LD with a number of variants within the coding sequence, while the original tag SNP did not remain among our list of the best candidate functional variants (Table 1, Fig. 1A; Supplementary Material, Table S2). It may also be possible to further prioritize the 46 selected candidate SNPs at this region based on statistical and functional evidence; a cluster of five variants were more strongly associated than the remainder and these flank a known AR-binding site within intron 2 that has been reported to function as an enhancer in LNCaP (15).

Two of the simple regions had been reported previously as PrCa susceptibility variants only in East Asian populations (rs1983891 at Chr6p21 and rs817826 at Chr9q31); in this study, we have demonstrated for the first time that these locus are also associated with PrCa risk in individuals with European ethnicity. The lead SNP describing the association signal for Europeans at Chr6p21, rs6458228, is strongly correlated with the Japanese hit ($r^2 = 0.92$); both are intronic in FOX4 and in LD with variants overlapping a number of bio-features. Our top European hit on Chr9q31 near RAD23B, rs1771718, is not correlated ($r^2 = 0.03$) with the index SNP reported in the Chinese population and itself overlaps a DNAS1 hypersensitivity site in the LNCaP PrCa cell line. Based on TCGA data, this signal is also an eQTL for RAD23B in normal prostate tissue but not tumor (Table 1 and Fig. 1B).

Of particular interest, within the 55 regions included in our final analysis, we identified 16 ‘complex regions’ that harbor more than one independent association signal after conditional analysis and contained a total of 36 separate risk signals (Table 2). Only five of these regions had previously been reported to contain independently associated SNPs (CHMP2B at Chr3p12 (8,16), PDLIM5 at Chr4q22 (17), SLC25A37/NKX3.1 at Chr8p21 (17), Chr11q13 (16,18) and HNF1B at Chr17q12 (19,20)). At both Chr3p12 and Chr8p21, the two originally reported association signals are within 50 kb of one another however situated adjacent to different genes. As this proximity felt comfortably within the flanking distance we defined in this study for each region they were nonetheless merged into a single region for this analysis. Multiple independent risk variants within the same gene had previously been identified within PDLIM5 and HNF1B, while Chr11q13 is a gene desert previously reported to contain three independent hits. For all of these five regions that were known to be complex prior to our fine-mapping, in this study, we confirmed each of the previously reported independent associations, have refined the spectrum of variation that best describes these
association signals, and we have identified a novel, third independent signal at HNF1B/Chr17q12. Furthermore, we have discovered additional novel signals within 11 regions previously known to contain only a single risk variant.

To illustrate the utility of fine-mapping in combination with functional annotation for the refinement of GWAS signals towards promising candidate causal variants, we present detailed findings for four representative complex regions in this manuscript. In the Chr3q31 region, we identified two independent signals (Table 2 and Fig. 1C). The most strongly associated SNP, rs13410475, is intronic in ITGA6 and in complete LD with the original index SNP rs12621278 ($r^2 = 1$). This signal represents a tight cluster of four potentially causative variants. The novel additional lead SNP, rs12151618, is...
Table 2. Complex regions: multiple independent associations were identified in 16 regions following stepwise logistic regression.

| Chr (region) | Previous hit(s) | Region boundaries (Mb) | Best signal in meta-analysis (P-value) | Independent lead SNPs | Alleles (ref/salt) | Imputation quality r² | LD (r²) with original index SNP | Number of best candidate SNPs (number overlapping bio-features) [eQTLs] |
|-------------|-----------------|------------------------|----------------------------------------|-----------------------|------------------|---------------------|-----------------------------|--------------------------------|
| 2p15* (2.4) | rs721048        | 62631731-63631731      | rs58235267 (3.9 × 10⁻²⁶)               | rs8235267             | C/G              | 0.88 (0.85-0.90)   | 0.88: 0.12: n/a             | 1 (1)                          |
| 2q31* (2.6) | rs12621278      | 172811553-173811553    | rs1340475 (8.3 × 10⁻²⁶)               | rs1340475             | A/C              | 0.90 (0.74-0.77)   | 0.97: 0.0: n/a              | 3 (1) EHBP1                    |
| 2q37 (2.8)  | rs3771570       | 24188286-24288286      | rs111770284 (1.6 × 10⁻¹⁵)             | rs111770284            | T/C              | 0.92 (0.88-0.95)   | 0.93: 0.09: 0.09            | 4 (0)                          |
| 3p12* (3.1) | rs2660753       | 86610674-87967332      | rs2088396 (6.5 × 10⁻²⁵)               | rs2088396             | C/G              | 0.90 (0.88-0.93)   | 0.99: (0.03/0.01): n/a      | 17 (0)                         |
| 3q21* (3.3) | rs10934853      | 127710474-128284711    | rs2811485 (1.1 × 10⁻¹⁵)              | rs2811485             | A/G              | 0.93 (0.90-0.96)   | 0.97: (0.36/0.33): 0.06     | 42 (1)                         |
| 3q26* (3.5) | rs10936632      | 169689793-170395852    | rs7841326 (6.4 × 10⁻²⁵)               | rs7841326             | C/G              | 0.83 (0.79-0.86)   | 0.80: 0.13: n/a             | 2 (0)                          |
| 4q22* (4.2) | rs12500426      | 95018784-95600782      | rs7682375 (4.0 × 10⁻¹⁵)              | rs7682375             | A/G              | 0.93 (0.90-0.96)   | 0.95: (0.76/0.30): 0.30     | 7 (0) BMFRP1                   |
| 6p25* (6.7) | rs9364554       | 160374745-16132288     | rs4662824 (3.2 × 10⁻⁴)               | rs4662824             | T/G              | 0.98 (0.90-0.99)   | 0.97: 0.002: 0.002          | 2 (0)                          |
| 7p15* (7.2) | rs10486567      | 27550633-28012614      | rs10486567 (7.3 × 10⁻²²)             | rs10486567            | A/G              | 0.88 (0.85-0.91)   | 0.99: (0): 0.008            | 8 (2)                          |
| 8p21* (8.1) | rs2928679       | 23100674-23548146      | rs13272392 (1.3 × 10⁻⁶)              | rs13272392            | A/G              | 0.83 (0.83-0.89)   | 0.98: (0.37/0.018): 0.019   | 49 (31)                        |
| 11q13* (11.3)| rs7931942      | 68811777-69494148      | rs12275055 (4.7 × 10⁻⁵)              | rs12275055            | A/G              | 0.83 (0.79-0.87)   | 1: 0.19: n/a                 | 5 (1)                          |
| 12q24 (12.3)| rs1270884       | 114632506-115103229    | rs1270884 (6.8 × 10⁻⁸)               | rs1270884             | A/G              | 0.80 (0.73-0.88)   | 0.75: 0.002: 0.002          | 16 (13)                        |
| 14q24 (14.2)| rs7141529       | 68974508-69135457      | rs7141529 (6.5 × 10⁻¹⁰)              | rs7141529             | T/C              | 0.92 (0.89-0.94)   | 0.97: 0.001: 0.001          | 1 (0)                          |
| 17q12* (17.2)| rs11649743     | 35740855-36249855      | rs11263763 (2.1 × 10⁻⁶)              | rs11263763            | A/G              | 0.80 (0.79-0.94)   | 0.97: (0.08/0.94): n/a      | 5 (3)                          |
| 17q21 (17.3)| rs11650494      | 46845186-47936749      | rs138263737 (7.0 × 10⁻¹³)            | rs138263737           | T/C              | 0.93 (0.92-0.99)   | 0.98: (0.76/0.005): 0.005   | 4 (4)                          |
| 22q13 (22.2)| rs5759167       | 43000212-44000212      | rs5759167 (1.8 × 10⁻⁸)               | rs5759167             | T/C              | 0.87 (0.85-0.90)   | 0.97: 0.002: 0.002          | 15 (6) TTIL2/MCAT              |

Following multiple stepwise logistic regression analyses, multiple independent association signals were identified in 16 regions. The TERT at Chr15p15 and Chr8q24 regions which are also known to harbor multiple independent PCa susceptibility loci were not included in this analysis as they had previously been fine-mapped individually [6,7]. Imputation quality and correlation (LD) between the novel lead SNPs and the original GWAS signal(s) are indicated, as are the correlations between the most strongly associated variant in this analysis and the additional independent hits within the region. Best Candidate SNPs are variants correlated at r² > 0.7 with the lead variant describing an association, and with odds of association ≥1/1000 relative to the lead variant for the region. eQTL data indicate statistically significant correlation between the new index SNP and gene expression in 145 prostate tumor samples from the TCGA dataset.

*These regions were densely genotyped on the iCOGS chip to fine-map PCa associations known at the time of design.
weakly correlated with this signal ($r^2 < 0.07$) and situated 5 kb upstream of PDK1 (pyruvate dehydrogenase kinase isozyme 1) in the promoter region. PDK1 is believed to be upregulated by Myc and HIF-1 to facilitate cell survival and proliferation under hypoxia, and is reported to be commonly overexpressed in cancer cells (21,22).

Two independent signals were also identified at Chr2q37, where the previously reported hit rs3771570 was intronic in FARP2 (Table 2 and Fig. 1D). In our analysis, the most strongly associated novel lead SNP, rs111770284, is not in LD with the original GWAS hit ($r^2 = 0.03$). rs111770284 is intronic in ANO7 (also known as NGEP, New Gene Expressed in Prostate), overlaps a
DNase1 site in LNCaP and implicates a set of four correlated variants as putative functional candidates. ANO7 is an androgen regulated gene and its expression appears to be prostate specific (23,24), with reduced expression associated with increased PrCa malignancy (25). The second independent signal, rs183997311, is intronic in FARP2. This variant is not in LD with either the original GWAS tag SNP or rs111770284. rs183997311 is relatively rare (MAF = 1%) and is correlated to two variants that overlap regulatory elements in LNCaP. It is also notable that the original hit rs3771570 was excluded from our list of best candidate SNPs after fine-mapping of this region.

At Chr14q24, the original signal rs7141529 remained the most significantly associated variant after stepwise logistic regression; however, two further additional independent signals described by rs2189517 and rs17105852 were identified (Table 2 and Fig. 1E). These novel variants are both situated within the same long intron of RAD51B but are not correlated with each other or with rs7141529. This region also harbors two independent risk signals for breast cancer, one of which is additionally associated with breast cancer in males; however, there is no correlation between these variants and any of the three PrCa risk SNPs (26,27) (Supplementary Material, Fig. S3).

To further interrogate the variants in our best candidate list for potential functional effects, we examined TCGA data from 145 prostate tumor and 45 normal prostate tissue samples for differential expression of nearby genes associated with these SNPs. Figure 2. Circos plot overview of functional annotation and eQTL data for fine-mapped PrCa risk loci generated using Circos (http://circos.ca, 62). The outer ring is a circular ideogram of the human genome annotated with chromosome number. The positions of the novel index SNPs for PrCa susceptibility identified through fine-mapping are indicated adjacent to this and are color coded for overlap with enhancer elements in LNCaP in orange, promoter regions in green, coding SNPs in red, variants within UTR regions in purple and variants with no annotated functionality in black. The inner ring denotes potential candidate genes for the refined PrCa regions. Genes for which an SNP in the best candidate list is a significant eQTL in prostate tissue in TCGA data are indicated in red, eQTLs in skin tissue from EuroBATS data are marked in brown, eQTLs for both prostate and skin in green and for regions with no significant eQTL in either tissue the closest flanking gene is indicated in black. Gene interaction networks between potential candidate genes are shown as links in the central portion of the plot. The genes annotated on the inner ring were used to construct a network using the BioGRID interaction database filtered to exclude ubiquitin and interactions with more than a single intervening gene between the candidate genes. Red links indicate an interaction network with the AR gene, other examples of interaction highlighted in color: blue—RAD23B, green—BMPR1B, orange—PDK1 and all other interactions are marked in gray.
We observed significant associations with gene expression for 16 of our association signals (Fig. 2, Tables 1 and 2; Supplementary Material, Table S2). Several of these eQTLs have been reported previously (28,29) and most recently Li et al. described significant eQTLs at 31 of 69 PrCa GWAS regions they tested (45%), implementing a mapping strategy for 19 of these regions which selected candidate causal variants ranging from 1 to 33 SNPs (30,31). Here, we report prostate tissue eQTLs at ~20% of the loci we analyzed; however, this is likely to be an underestimate due to the relatively small set of prostate samples currently available in TCGA (30). We subsequently examined EuroBATS (32,33) eQTL data for a much larger set of samples from lymphoblastoid cell lines (814 samples), skin (716 samples) and adipose (766 samples) tissues in order to investigate whether any of the eQTLs identified in the TCGA prostate tumor tissue dataset act ubiquitously and also whether additional signals are present within these tissues that might also be detectable in a larger set of prostate samples. Only one region (Chr10q24 for C10orf32) showed evidence for ubiquitous eQTL association in all four tissue types interrogated. There were nine signals across seven regions which had at least one concordant eQTL between the TCGA FRAD tumor and any tissue type in EuroBATS. Of the eQTL signals which are confirmed in both TCGA and at least one EuroBATS tissue, five have been described before using the TCGA dataset (A53MT, VPS53, MLPH, IRX4 and ROS17) (30). We therefore consider the four novel signals identified here (C10orf32, TMEM180, MMP7 and TTHL12) as robust candidates for functional follow-up, especially due to the large sample set used in the EuroBATS project (Supplementary Material, Table S2). TTHL12 is a particularly interesting candidate susceptibility gene as its expression has been shown to increase during cancer progression and metastasis (34). In addition to these eQTLs replicated between the two separate datasets, we also report a strong eQTL for ZNF652 within the TCGA prostate tissue data solely. We have also detected eQTLs for RAD51B within the EuroBATS data only and for RAD23B in normal prostate TCGA data, but not in prostate tumor. These potentially interesting associations may therefore warrant further follow-up within a larger prostate tissue set. In the central portion of our circcos plot (Fig. 2), we have illustrated potentially interesting interaction networks between the candidate genes identified through this fine-mapping study. This highlights in particular that a large number of these plausible candidate genes are regulated by the AR, as shown in red.

To evaluate whether the refinement of previously identified PrCa loci that we have achieved in this study reflects a greater enrichment towards variants with annotated functionality, we assessed our list of 1623 best candidate SNPs for overlap with bio-features and compared this against the full set of imputed SNPs within the 55 regions that were significant in this analysis (243,627 SNPs). Using the hypergeometric test, we observed a significant enrichment in overlap with bio-features for the variants in our best candidate SNP list (P = 2.01 × 10-20, Supplementary Material, Fig. S4). This enrichment was even stronger when only enhancer elements were included in the analysis (P = 1.67 × 10-22).

In order to estimate the extent to which fine-mapping of these known PrCa susceptibility regions could improve understanding of the genetic factors that influence PrCa risk, we compared the FRR of PrCa explained by the original GWAS tag SNPs with the FRR explained by the novel lead SNPs that we have identified in this study. We used estimated variant effect sizes and allele frequencies from the samples in the iCOGS study to calculate both estimates to avoid the potential for inflation from the UK GWAS study. We accounted for the LD between variants in the complex regions containing more than one independent SNP to avoid overestimating FRR. The estimated FRR explained when substituting for our refined lead SNPs and introducing the newly identified independently associated variants from this study was 38.9%, compared with 30% for the originally reported GWAS tag SNPs; an improvement of 8.9% overall, and nearly a third greater than had been previously attributed to these known PrCa susceptibility signals.

**Discussion**

In this study, we used imputation of existing genotype data to fine-map 64 PrCa GWAS regions in European ancestry populations comprising 25,723 PrCa cases and 26,274 controls from three studies (iCOGS and UK GWAS Stages 1 and 2). Twenty-three of these regions were fine-mapped at very high resolution on the iCOGS chip. Nine previously reported GWAS signals were not replicated at genome-wide significance in this study due to either decreased power in comparison with the original studies or having only previously been associated with PrCa susceptibility in a non-European population.

In 39 of the remaining 55 regions, we found evidence for a single PrCa association signal only. The original GWAS tag SNP remained the most significant association for just 4 of these, while at 35 regions, we identified a more significantly associated replacement lead SNP. Importantly, we also identified 16 complex regions containing multiple variants independently associated with PrCa. Only five of these had previously been identified as containing multiple independent variants (Chr3p12, Chr4q22, Chr8p21, Chr11q13 and Chr17q12); however, our analysis helped to further refine these five regions and identified one additional previously unknown hit at Chr17q12 within the promoter region of HNF1B. For the remaining 13 complex regions, this study provides the first evidence for the presence of multiple independent PrCa susceptibility variants in close genomic proximity to one another. An alternative explanation for the observation of multiple apparently independent association signals would be that both could in fact be moderately correlated with a single untyped causal variant, despite exhibiting limited LD between themselves (35,36). We believe that this hypothesis is unlikely to underpin a substantial proportion of the multiple signals we have observed in this study, since 11 of the 16 identified complex regions had been densely genotyped on the iCOGS array, enabling very thorough imputation of additional untyped variants within the region. However, this phenomenon cannot be completely excluded without deep re-sequencing of each of these loci in a large sample panel, which would facilitate the evaluation of all correlated variation within the region and subsequently the identification of the precise causal variants. Our functional annotation of the statistically most promising correlated candidate causal variants also provides provisional evidence to implicate a contribution by several new potential candidate genes in PrCa risk.

We have annotated our set of statistically significant SNPs in order to prioritize the most likely candidate causal variants within each region. We firstly excluded all SNPs that were associated with PrCa risk at a conservative threshold of ≤1/1000 compared with the association likelihood of the novel lead SNP for each signal. This generated a list of 6537 variants across the 55 significantly associated regions in our final analysis. To further prioritize within this list, we trimmed based on LD structure and selected only those variants that were strongly correlated (LD r2 > 0.7) with the lead SNP based on 1 KB EU data. The intersection of these two selection criteria generates a list of 1623 variants, which we would expect to retain and be enriched for the causal functional variants. However, we cannot exclude the
possibility that in some instances the causal variant(s) may be in lower LD with the novel index variants, particularly in the regions that were less densely genotyped. In addition, our imputed association data may be underpowered to detect any instances where rare causal variants give rise to the association signal through a ‘synthetic association’ (37). The incorporation of functional annotation in addition to statistical data and LD criteria may also help to further prioritize our list of the best candidate SNPs for PrCa risk causality towards those with the strongest evidence for biological effect, to facilitate potential laboratory follow-up. For 62 of the 75 independent association signals we detected, one or more of the best candidate variants in our list overlaps with bio-features in PrCa cell lines (413 of the 1623 variants were annotated for functionality in total, median of 4 per signal). We furthermore observed eQTLs for differential gene expression in TCGA prostate tumor or normal tissue for one or more variants in our best candidate list for 16 of these 75 independent association signals, with additional eQTLs at other regions also observed within a larger set of EuroBATS data for three additional tissues.

An important aspect of this mapping study is the improvement of the estimated FRR of PrCa explained by the refined and newly identified independently associated variants. This is now substantially higher, at ∼39% compared with 30% estimated for the original GWAS tag SNPs (12). Fine-mapping of these 55 known regions has therefore improved our understanding of the genetic basis of PrCa and incorporating these novel variants into future risk models should enhance the capability to predict individuals at greater risk. It is also interesting to note that of the 23 regions analyzed in this study that were known at the time of design of the iCOGS array and therefore fine-mapped through a more dense set of directly genotyped markers; these represent 11 of the 16 complex regions and only 12 of the 39 simple regions. This might suggest that the presence of multiple independent PrCa susceptibility variants within previously identified GWAS regions could be even more widespread than we have been able to identify in this fine-mapping study. Consequently, additional susceptibility signals could yet reside within the regions that remain to be interrogated through very dense marker resolution in a sufficient sample size; an experiment which is currently being undertaken by the OncoArray Consortium (38).

A concurrent PrCa fine-mapping study by Han et al. (Hum. Mol. Genet., submitted) has examined 69 risk regions among a multi-ethnic sample panel comprising European (8600 cases and 6946 controls), African (5327 cases and 5136 controls), Asian (2563 cases and 4391 controls) and Latino (1034 cases and 1046 controls) ethnicities. After performing a meta-analysis for marginal significance in our study, the original GWAS tag SNP was replaced significantly associated SNP at 47 (85.4%). For the 46 significant regions that overlapped between these two studies’ final datasets, 32 of the refined SNPs identified by Han et al. were among our list of best candidate SNPs (69.5%). Within these overlapping regions, we identified 12 novel independent signals, 9 of which were nominally significant in the multi-ethnic fine-mapping but none were included in their top order or putatively functional SNPs. Comparing final putative functional candidates for the 46 overlapping regions, 29 (63.0%) of these regions have at least one overlapping functional candidate SNP. More notably, 11 of these 46 regions (23.9%) have a single overlapping functional candidate SNP, which should therefore be assigned high priority as potential causal candidates for future experimental follow-up. Overall, the comparisons between these two approaches highlight the increased power provided by the larger sample set available within our study; in particular in respect to the identification of multiple independent association signals within already known regions, but also demonstrate that the ability to incorporate multiple ethnic populations may further improve the efficiency of fine-mapping. This suggests that large meta-analysis based fine-mapping studies comprising individuals of diverse ancestries may represent the most robust strategy for imputation-based fine-mapping where such data are available.

In conclusion, we have demonstrated the importance of genotyping and imputation-based fine-mapping through the discovery of 12 additional independent PrCa associations within known GWAS regions and by refining the vast majority of the previously reported signals. Before fine-mapping efforts were employed, potential causal SNPs at each susceptibility locus were selected primarily on the basis of high correlation to the reported GWAS tag SNP (14) or overlap with functional elements; this strategy can however either introduce substantial noise or a greater likelihood of excluding true causal variants, due to the lack of statistical evidence available when retaining or excluding non-genotyped variants. Through coupling functional annotation and LD information to our imputed association dataset, we believe we have been able to enrich for the most likely candidate causal PrCa susceptibility variants at each association signal while substantially reducing noise within the list. Indeed, this approach selected only a modest number of SNPs per locus, yet these showed evidence for modulating differential gene expression and greater overlap with bio-features in prostate datasets. While complete re-sequencing of GWAS regions in large sample sets would ultimately be desirable, our approach undoubtedly represents the most time and cost-effective method available at the present time to interrogate and prioritize the most plausible causal variants underpinning GWAS studies. We have demonstrated that many of the GWAS regions identified to date for PrCa harbored additional, previously hidden independent association signals, with perhaps more yet to be discovered. This observation may have important implications towards the deconvolution of the functional mechanisms underlying these signals, and in particular could help to facilitate improved risk prediction, since these additional independent association signals could account for a significant proportion of the missing heritability of PrCa and other complex diseases (39).

Materials and Methods

Samples

Analyses were based on data from the iCOGS array, a custom array comprising ∼200 000 SNPs designed to study susceptibility to prostate (PRACTICAL Consortium), breast and ovarian cancers (12), together with data from a UK GWAS study (Stage 1) and subsequent custom array designed as a replication stage of the Stage 1 GWAS that was genotyped in studies from the UK and Australia (Stage 2). The analyses presented here were restricted to 51 997 men (25 723 cases and 26 274 controls) of European ancestry, samples with other ethnicity in the iCOGS study were small in number and were included in a separate study addressing mapping using multi-ethnic sample sets (Han et al., Hum. Mol. Genet., submitted). See Eeles et al. (12) for details of the quality control (QC) procedures. After QC, data were available for 11 338 samples (5504 cases and 5834 controls) from the GWAS, and 40 659 samples (20 219 cases and 20 440 controls) from 32 studies in PRACTICAL genotyped using the iCOGS array. The majority of studies were population- or hospital-based case-control studies, or nested case-control
studies, but some studies selected samples by age or oversampled for cases with a family history; in the latter instance only one case per family was genotyped (Supplementary Material, Table S5). Only the 40 659 iCOGS samples were used for the FRR estimation to avoid inflation from the Stage 1 GWAS.

Genotyping
UK GWAS Stages 1 and 2 were genotyped using the Illumina Infinium HumanHap550 and a custom Illumina iSELECT array, respectively (17,40).

iCOGS genotyping was conducted using a custom Illumina Infinium array (iCOGS) in seven centers, of which five were utilized for PRACTICAL. Genotypes were called using Illumina’s proprietary GenCall algorithm. In addition to SNPs selected for replication of GWAS, the iCOGS array included dense sets of SNPs surrounding susceptibility variants known at the time of design. For PrCa, we included 23 such regions (ccge.medschl.cam.ac.uk/research/consortia/icogs/). To select markers for comprehensive interrogation of these densely genotyped regions, we identified all known SNPs from the March 2010 release of the 1000 Genomes Project with MAF > 0.02 in Europeans and selected all SNPs that were correlated with the published GWAS SNPs (at $r^2 > 0.1$); together with a set of SNPs designed to tag all remaining variants at $r^2 > 0.9$. Approximately 14 000 SNPs were successfully designed across these regions.

Statistical methods
The primary genotype data were used to impute genotypes for ~17 M SNPs/indels using the 1000 Genomes Project (March 2012 release) as a reference panel and IMPUTE V2 (41). Imputation was carried out using pre-phasing with 50 iterations and all known PrCa regions were imputed in an ~5 Mb block size. Per-allele odds ratios and standard errors were estimated for each SNP by logistic regression. Analysis was stratified by study and, for both the GWAS and iCOGS, included eight principal components as covariates. We included imputed data for SNPs with quality information scores >0.3. Analyses were performed using SNPTEST (42). Results from the iCOGS and GWAS were then combined in a fixed effect meta-analysis using METAL (43).

Comparison of number of associated loci among cancerspecific regions
To establish a suitable significance threshold, we examined 59 known breast cancer regions with no known association with PrCa prior to this analysis. Regions were defined as a ±500 kb boundary around the published breast cancer GWAS SNPs. We interrogated our meta-analysis results (iCOGS and Stages 1 and 2 UK GWAS) in order to establish the likelihood that SNPs within these breast cancer regions would be associated with PrCa by chance at $P \leq 10^{-5}$ level. Performing this comparison revealed that an association $P = 10^{-5}$ was sufficiently strict for the avoidance of false-positive results in the discovery of secondary signals within a region after adjusting for the top signal variant.

Identification of lead SNPs within each region
To identify independent association signals within a region, we selected all significant SNPs with $P \leq 10^{-5}$ from the fine-mapping dataset (iCOGS, Stages 1 and 2 GWAS) and performed a stepwise logistic regression on this set of SNPs for each region. For regions in which the initial analysis indicated more than one independent SNP, a second round stepwise logistic regression was performed to confirm the presence of additional independent SNPs, after adjusting for the best signal in the region. SNPs with $P \leq 10^{-8}$ from the adjusted results were included in this analysis, alongside the top hit. For regions found to contain multiple independently associated SNPs, haplotype-specific odds ratios were estimated based on the independent SNPs identified through the stepwise logistic regression analysis, using the Haplo.Stats package (http://cran.r-project.org/web/packages/haplo.stats/index.html) (44). SNPs with a likelihood ratio of $>1/1000$ relative to the most significant SNP describing each signal were selected as potential candidate causal variants (45). This list was narrowed down further by filtering variants at $LD^2 < 0.7$ with the lead SNPs to curate a list of best candidate causal SNPs for each signal. For regions with multiple independent SNPs, the lists for the subsequent signals were generated by determining the likelihood ratio relative to the top SNP in that region, adjusted for other signals. We excluded duplicates and used a cut-off $P = 6.7 \times 10^{-5}$ ($P$-value for odds of $>1/1000$ relative to the genome-wide significance level of $5 \times 10^{-8}$) to trim the list where there was a second signal that did not reach the genome-wide significance level ($5 \times 10^{-8}$).

Contribution to familial risk
The contribution of SNPs to the familial risk of PrCa, under a multiplicative model, was computed using the formula

$$\lambda = \frac{\sum \log \lambda_k}{\log \lambda_0},$$

where $\lambda_0$ is the observed familial risk to first-degree relatives of PrCa cases, assumed to be 2 (46,47), and $\lambda_k$ is the FRR due to locus $k$. For a single SNP, $\lambda_k$ is given by

$$\lambda_k = \frac{p_k T_k^2 + q_k}{(p_k T_k + q_k)^2},$$

where $p_k$ is the frequency of the risk allele for locus $k$, $q_k = 1 - p_k$ and $t_k$ is the estimated per-allele odds ratio, estimated from the logistic regression model for that SNP. For regions with more than one SNP in LD, we used the extended formula:

$$\lambda_k = \frac{\sum_j p_j t_j}{(\sum_j p_j t_j)^2}$$

where $p_j$ is the frequency of haplotype $j$ for the multi-locus region $k$ and $t_j$ is the corresponding risk estimate. The haplotype frequencies were estimated using Haplo.Stats, while the haplotype-specific risk estimates were based on the regression analyses, thus preserving the assumption of a log-additive effect of all SNPs.

Locus explorer
We developed Locus Explorer, a Shiny web application for R, to generate the locus plots shown in this manuscript. Locus Explorer can be run locally using R studio with the necessary packages installed. The code is available on GitHub. The application is interactive and enables selecting and zooming on the locus, selecting annotation tracks and downloading the resulting plots and the data used for plotting. Data for plotting are stored in SQLite database which is accessed using R scripts. Plots are displayed mainly using RStudio, sqldf, ggplot and ggbio (48–51). At the time of submission, the application is currently in Beta stage for external users, with most functionality in place and performance issues resolved. We are continuously working on
additional features and for future versions the main focus will be speed of the plotting process. More information is available at GitHub (https://github.com/oncogenetics/LocusExplorer).

Functional annotation

We used a number of publicly available prostate epithelia and PrCa ENCODE datasets of chromatin features to identify putative regulatory regions at each risk locus (14,52). The integration of chromatin bio-feature annotations with SNP positions was performed using FuncSNP (53). These datasets included LNCaP and RWPE1 DNase I HS sites (GSE32970) ENCODE, PrEC DNase I HS sites (GSE29692) ENCODE, LNCaP CTCF ChIP-seq peaks (GSE35213) ENCODE, LNCaP H3K27ac and TCF7L2 (GSE51621) (14), H3K4me3 and H3K4me1 histone modification ChIP-seq peaks (GSE27823) (54), FoxA1 ChIP-seq peaks (GSE28264) (55); AR ChIP-seq peaks (56) and AR binding sites (GSE28219) (57); NKX3-1 ChIP-seq peaks (GSE28264) (55). We also used the highly conserved set of predicted targets of microRNA targeting (miRcode) (58). To determine whether any of the putative functional SNPs potentially affect the binding of known transcription factors, position-specific frequency matrices were employed from Factorbook (14,59).

cis-eQTL analysis

A/TCGA PRAD samples

The genotypes of the variants in the best candidate SNP list in 145 prostate tumor samples and 45 normal tissue samples were downloaded from TCGA database (Feb 2013). All samples were verified as Caucasian descendants. If a variant was not represented in the TCGA data, the genotypes were imputed using M-PUTE2. A cis-eQTL analysis was performed for these variants and any transcript within a 1 Mb interval (500 kb on either side). The abundance of the transcripts is adjusted for somatic copy number changes and CpG methylation changes using methods described previously. The nominal P-values obtained for each risk variant were corrected for the number of transcripts in the interval using Benjamini–Hochberg method. Significant associations were defined as a false discovery rate <0.05.

B/EuroBATS samples

The sample set includes LCLs (N = 814), skin (N = 716) and adipose tissue (N = 766) derived simultaneously from a subset of well-phenotyped healthy female twins (32,33). eQTL discovery is described in detail elsewhere (32). In short, (i) we kept the residuals of a mixed model that removed the effects of the family structure using the implementation in GenAbel R package. (ii) We performed a linear regression of those residuals on the SNPs in a 1 Mb window around the transcription start site for each gene, using MatrixeQTL R package (60). We assessed statistical significance through 2000 permutations. Prior to the analysis, we removed the effects of technical covariates using the factor analysis strategy implemented in PEER (61) and transformed the data using a rank normal transformation.

Supplementary Material

Supplementary Material is available at HMG online.

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