Identification of functionally active, low frequency copy number variants at 15q21.3 and 12q21.31 associated with prostate cancer risk


Copy number variants (CNVs) are a recently recognized class of human germ line polymorphisms and are associated with a variety of human diseases, including cancer. Because of the strong genetic influence on prostate cancer, we sought to identify functionally active CNVs associated with susceptibility of this cancer type. We queried low-frequency biallelic CNVs from 1,903 men of Caucasian origin enrolled in the Tyrol Prostate Specific Antigen Screening Cohort and discovered two CNVs strongly associated with prostate cancer risk. The first risk locus (P = 7.7 × 10⁻⁴, odds ratio = 2.78) maps to 15q21.3 and overlaps a noncoding enhancer element that contains multiple activator protein 1 (AP-1) transcription factor binding sites. Chromosome conformation capture (Hi-C) data suggested direct cis-interactions with distant genes. The second risk locus (P = 2.6 × 10⁻⁵, odds ratio = 4.8) maps to the α-1,3-mannosyl-glycoprotein 4-β-N-acetylglucosaminyltransferase C (MGAT4C) gene on 12q21.31. In vitro cell-line assays found this gene to significantly modulate cell proliferation and migration in both benign and cancer prostate cells. Furthermore, MGAT4C was significantly overexpressed in metastatic versus localized prostate cancer. These two risk associations were replicated in an independent PSA-screened cohort of 800 men (15q21.3, combined P = 0.006; 12q21.31, combined P = 0.026). These findings establish noncoding and coding germ line CNVs as significant risk factors for prostate cancer susceptibility and implicate their role in disease development and progression.

Prostate cancer is a leading cause of cancer death in males throughout the world (1) and demonstrates the largest estimated effect of heritability among the most common tumor types, as determined by a Scandinavian twin-based registration study (2). Emerging insights into the genetics of constitutional disease etiology demonstrate that germ line polymorphisms in the form of copy number variants (CNVs) (3, 4), both de novo and inherited, are associated with diseases, including Alzheimer’s, Parkinson’s, mental retardation, autism, and schizophrenia (5). CNVs also confer risk to developing cancers, such as neuroblastoma, and are enriched in Li-Fraumeni syndrome (6, 7). Therefore, given the strong heritable nature of prostate cancer, we sought to detect clinically-informative prostate cancer risk CNVs in the setting of widespread prostate-specific antigen (PSA) screening, as practiced in the United States (8) and Western Europe (9). To this end, we examined peripheral blood samples from the Tyrol Early Prostate Cancer Detection Program (10, 11). This cohort is a population-based prostate cancer screening program started in 1993 that intended to evaluate the utility of intensive PSA screening in reducing prostate cancer-specific death. We here report the results of a comprehensive large scale, unbiased study using this patient population to study the contribution of germ line CNVs toward prostate cancer risk.

Results

All men enrolled in the program came from the same “at risk” population, characterized by elevated age-adjusted PSA levels (10, 11). Cases were defined as men with biopsy-confirmed prostate cancer, and controls were defined as men with benign prostate biopsy results and no cancer diagnosis on available follow-up data. Demographics are presented in Dataset S1, Table S1. Cases and controls were profiled using the Affymetrix 6.0 Whole Genome SNP Array platform. After applying strict data-quality filters (12), the final study cohort consisted of 1,903 unrelated individuals of Caucasian origin (867 cases and 1,036 controls).

SNPs Associated with Prostate Cancer Risk. Because several past genetic studies have reported on SNPs associated with risk for prostate cancer (13), we first queried 53 such reported risk SNPs in our study cohort (SNP selection criteria in Methods) and replicated associations for 22 of them (Dataset S1, Table S2), including SNPs at 3q21.3, 4q24, 6q25.3, 7p15.2, 7q21.3, 8q24.21, 11p15.5, 11q13.2, 11q13.3, 12q13.13, and 17q24.3. The strongest signal was detected for prostate cancer and for aggressive (14) prostate cancer at 11q13.3 [r²7130881; odds ratio (OR) = 1.52, P = 8 × 10⁻⁵ and OR = 1.624, P = 6 × 10⁻⁵, respectively]. The risk SNP replication...
rate is comparable to other recent studies (15, 16) and could be attributed to cohort and disease heterogeneity (17, 18).

**CNVs.** We systematically identified CNVs along the genome of each individual from the Tyrol cohort (see *Methods*). This process resulted in a map of 2,611 CNVs, where 11.5% of CNVs were smaller than 20 kb in size and 41.7% of CNVs mapped to gene-coding areas. The vast majority of CNVs (94.6%) were present in the population with two or three copy number states and overall 34.1% of the CNVs exhibited minor allele frequencies \( \leq 5\% \). Dataset S1, Table S3 summarizes the CNV characteristics (i.e., size, frequency).

To prioritize association studies of the identified CNVs, we reasoned that new functionally important risk markers for prostate cancer would be deletions with moderate or low frequency. Deletions are often associated with diseases (19), and less-common variants generally have a higher impact in driving human diseases (20). In addition, low-frequency CNVs are not well-tagged by SNPs (21) and, therefore, would carry distinct information for their potential association with prostate cancer (22). We anticipated that functionally active low-frequency deletions might unravel an as yet unexplored portion of the genetic background.

Therefore, priority was given to CNVs that had functional constraints, including potentially altering gene function (e.g., gene-coding variants and variants overlapping regulatory elements). Regulatory elements are characterized by the presence of consistent H3K4Me1 histone marks in the ENCODE ChIP-DNA sequencing (ChIP-seq) data (23). Overall, 238 deletion CNVs fulfilled the selection criteria, including functional constraints with minor allele frequencies below or equal to 10%, and were deemed candidates for the association analysis with the disease state. The genomic characteristics of the 238 CNVs are summarized in Fig. 1.

![Fig. 1. Genomic characteristics of the transcriptionally active, low-frequency, deletion CNVs selected for prostate cancer risk association analysis. (A) Genomic location along the 22 autosomal chromosomes. (B) CNV size distribution.](image)

Table 1 shows the characteristics of the top CNVs identified as susceptibility markers for prostate cancer in the Tyrol cohort upon age and preoperative PSA adjustment (\( P \leq 0.01 \) and false-discovery rate < 0.2). Dataset S1, Table S4 includes extended association results for all of the 238 variants included in the analysis.

The CNV that showed the strongest association with prostate cancer risk maps to a noncoding area on 15q21.3 (\( P = 7.7 \times 10^{-4}\), OR = 2.78); that is, the risk allele (deletion allele) poses 2.8-times higher odds of having prostate cancer compared with the normal allele. Interestingly, this variant also scores as significant for aggressive prostate cancer association (\( P = 0.009\), OR = 2.36). Among the top-ranked gene coding significant risk variants (Table 1), \( \alpha \)-1,3-mannosyl-glycoprotein 4-\( \beta 
\)N-acetylglucosaminyltransferase C (MGAT4C) CNV (\( P = 2.6 \times 10^{-3}\), OR = 4.8), located on 12q21.31, also exhibited significant deregulation of the gene transcript expression with respect to copy number state (where ZFP14 did not), and was therefore prioritized for further investigation together with the 15q21.3 locus.

These two genomic regions, 15q21.3 and 12q21.31, have both been previously recognized as copy number variable by Conrad et al. (24) and by other high-resolution/sequencing studies, including Pang et al. (21) and Ju et al. (25). The estimated minor allele frequencies for the HapMap samples of Utah residents with ancestry from northern and western Europe (CEU) origin were equal to 0.017 [95% confidence interval (CI) 0.004, 0.054] and 0.018 (95% CI: 0.005, 0.056) for the 15q21.3 and 12q21.31 CNV regions, respectively. These data were consistent with the frequency of the exposed allele in the control set of our study, 0.0289 (95% CI: 0.0220, 0.0377) and 0.0131 (95% CI: 0.0087, 0.0196), respectively. As expected (21), the two low-frequency variants are not in linkage disequilibrium with known SNPs and thus have not been previously explored as risk polymorphisms for prostate cancer. Fig. S2 shows the University of California at Santa Cruz (UCSC) browser view of the haplo-block information for these two regions.

**Replication of Prostate Cancer Risk Variants in Independent PSA Screening Cohort.** The association of the two risk variants with prostate cancer was then queried in an independent United States PSA-screening cohort from the Early Detection Research Network (26) (EDRN) consisting of 346 cases and 454 controls (Dataset S1, Table S5) from three recruitment sites. The 15q21.3 variant showed significant association with the aggressive phenotype (\( P = 0.05 \)) consistent with the Tyrol cohort data and with cancer risk in the Cornell subcohort (\( P = 0.009 \)). The noncoding variant mapping to MGAT4C showed consistent association with the aggressive phenotype (\( P = 0.067 \)), with a stronger signal observed when the control set was confined to men of greater age (\( P = 0.037 \)). The analysis for prostate cancer risk and aggressive phenotype in the combined Tyrol and EDRN cohorts showed significant association for both the 15q21.3 variant (\( P = 0.006 \) and \( P = 0.052 \), respectively) and the 12q21.31 variant (\( P = 0.026 \) and \( P = 0.059 \), respectively). Overall, the 15q21.3 and 12q21.31 associations detected in the Tyrol cohort were recapitulated in the EDRN series.

**Role of the Noncoding Risk CNV.** To investigate the potential functional role of the noncoding CNV, 15q21.3, in conferring prostate cancer risk, we analyzed the recently published next-generation sequencing data from seven human prostate cancers (27) to identify any potential nonannotated transcribed gene at 15q21.3. This analysis did not show any evidence of transcript expression (Fig. 2A). Hence, we surmised this region was likely a distal regulatory element. Consistent with the presence of a regulatory element (H3K4me1), ENCODE ChIP-seq data also showed that the locus was bound by three members of the activator protein 1 (AP-1) complex of transcription factors (c-Jun, JunD, and FOSL2) in various cell lines. We therefore interrogated this locus in RWPE1 benign prostate cells by ChIP-seq (see SI Methods) where we detected a H3K4me2 double peak and confirmed c-Jun binding signal (Fig. 2B). In addition, this locus

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Table 1. Low-frequency deletion CNVs associated with prostate cancer risk in the Tyrol cohort (P ≤ 0.01, false-discovery rate < 0.2)

<table>
<thead>
<tr>
<th>CNV genomic region (GRCh37/hg19)</th>
<th>Gene symbol/Entrez gene ID</th>
<th>Minor allele frequency</th>
<th>Risk</th>
<th>P</th>
<th>OR (CI 97.5%)</th>
<th>False-discovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr15:54197663–54203357</td>
<td>ZFP1457677</td>
<td>0.030</td>
<td>Deletion</td>
<td>0.0008</td>
<td>2.78 (1.55–5.14)</td>
<td>0.087</td>
</tr>
<tr>
<td>chr19:36828985–36847652</td>
<td>MGAT4C25834</td>
<td>0.014</td>
<td>Deletion</td>
<td>0.0018</td>
<td>0.49 (0.31–0.76)</td>
<td>0.095</td>
</tr>
<tr>
<td>chr12:86428508–86435479</td>
<td>CSMD1164748</td>
<td>0.038</td>
<td>Deletion</td>
<td>0.0026</td>
<td>4.80 (1.86–14.89)</td>
<td>0.097</td>
</tr>
<tr>
<td>chr8:3718722–3720335</td>
<td>CYHR1505626</td>
<td>0.012</td>
<td>Deletion</td>
<td>0.0049</td>
<td>6.24 (1.97–27.63)</td>
<td>0.108</td>
</tr>
<tr>
<td>chr7:4089162–4092270</td>
<td>SDK11730351</td>
<td>0.009</td>
<td>Deletion</td>
<td>0.0106</td>
<td>3.97 (1.39–11.75)</td>
<td>0.111</td>
</tr>
</tbody>
</table>

harbors several putative androgen and estrogen receptor binding sites (28, 29), suggesting that it may be under hormonal regulation.

To understand the function of this enhancer region, we carried out an association analysis between the CNV deletion allele (risk allele) and transcript expression levels of the AP-1 trans-target genes (30) by querying data generated from human benign and cancer prostate tissues (Dataset S1, Table S6). This analysis demonstrated that the presence of the risk allele in both the prostate tissue datasets was significantly correlated with transcript expression of the proto-oncogene B-cell lymphoma 2 (BCL2), an AP-1 target gene located on 18q21.33 (Fig. S3). Individuals carrying the risk allele showed higher transcript expression.

Next, we used a nonbiased approach of chromosome conformation capture, referred to as Hi-C (31) (see SI Methods) to identify potential long-range cis–gene interactions. We used the benign prostate epithelial cell line, RWPE1, for performing the chromatin–interaction experiment. This study revealed significant interactions between the locus on 15q21.3 and multiple gene promoters and microRNA promoter sites within a 50-Mb window (Dataset S1, Table S7). Gene-set enrichment analysis showed transcriptional regulation activity as being significantly overrepresented in our gene set (P = 0.017). Association analysis was performed between the CNV copy number states and transcript expression of the cis–interacting genes. Among the genes with consistent transcript level association, pygopus homolog 1 (PYGO1) showed the strongest signal (P = 0.0002) (Dataset S1, Table S7).

Taken together, these data suggest that the noncoding locus identified as protective against prostate cancer development is a regulatory region, potentially involved in the active regulation of both cis- and trans-gene expression (Fig. 2C).

Functional Characterization of the Coding Risk CNV. Next, we carried out functional characterization of the gene coding CNV associated with prostate cancer risk involving the MGAT4C gene. Association analysis demonstrated an intronic deletion in MGAT4C to be the prostate cancer risk allele. MGAT4C transcript abundance analysis suggested an inverse association between copy number states and transcript levels in human prostate tissues (both benign and tumor) and in lymphoblastoid cells (P = 0.049, P = 0.041, and P = 0.032, respectively) (Fig. S4). This risk CNV in the intronic region of MGAT4C likely overlaps a repressor/inhibitor element, such that a deletion in this region leads to transcript overexpression, a scenario seen in several other instances (32–34). Two recent studies (34, 35) identified several deletion CNVs that were associated with increased transcript expression, similar to our observations with MGAT4C. To evaluate the effect of changes in transcript expression, we carried out in vitro experiments using prostate cell lines (SI Methods). Overexpression of MGAT4C in benign (RWPE1) and cancer (VCaP) prostate cell lines (Fig. S5), resulted in significant increase in cell proliferation (P < 0.0001) (Fig. 3A) and migration (P = 0.0011 and P = 0.0017, respectively) (Fig. 3C and D). Knock down of MGAT4C expression with siRNA in one benign (RWPE1) and two prostate cancer cell lines (LNCaP and VCaP) resulted in significant decrease in proliferation (P ≤ 1 × 10–3, P < 1 × 10–5, and P < 1 × 10–5, respectively) (Fig. 3B). We next asked if MGAT4C levels vary with human prostate cancer progression and found that transcript levels were significantly higher in metastatic versus localized prostate cancers (P = 0.00004) (36) (Fig. 3E). Taken together, these data show that transcript overexpression associated with the deletion CNV results in changes associated with tumor progression.

Discussion In this unique large-scale study to investigate the association of functional CNVs and prostate cancer, we identified and characterized two low-frequency risk CNVs located on 15q21.3 and 12q21.31. The first risk CNV overlaps a noncoding gene desert area. Such noncoding CNVs might indirectly modulate gene transcription through distal regulatory elements and long-range interactions. Targeted disruption of enhancers has been shown to exert tumorigenic effects through remote transcriptional dysregulation in acute myeloid leukemia (37). We demonstrated that this risk CNV might be a regulatory site, as evidenced by the presence of experimentally determined AP-1 binding sites. AP-1 is a dimeric transcription factor that includes the JUN, FOS, activation transcription factor, and musculo-aponeurotic-fibrosarcoma protein families, with FOS and JUN being the most prevalent proteins in mammalian cells. AP-1 exerts both oncogenic and tumor-suppressive roles and does so by regulating genes involved in cell proliferation, differentiation, apoptosis, angiogenesis, and tumor invasion (30). FOS and JUN expression has been linked to prostate cancer progression, with JUN also being associated with relapse-free survival (38).

There are several mechanisms by which this noncoding risk CNV may lead to prostate carcinogenesis. The first evidence is based on the modulation of gene transcription in the presence of the noncoding risk CNV. To test the hypothesis that the risk locus bound by AP-1 family members exerts an effect in a tissue-specific manner in prostate (39), we queried mRNA expression of AP-1 target genes with respect to the risk-allele status in tumor and benign prostate tissues. Strikingly, we observed a statistically significant association between the expression of the BCL2 oncogene, an AP-1 target gene (30), and the noncoding CNV status in both the prostate tissue sets. Although not definitive, this result indicates that the noncoding CNV may play a role in tumor initiation and maintenance through control of BCL2.

The second evidence that suggests a potential functional role of this CNV comes from analysis of chromatin cis–interactions deduced from Hi-C data. We found cis-genes, the mRNA expression of which correlates with CNV status, and that are predicted to physically interact with the noncoding CNV according to Hi-C. These genes include PYGO1, one of the Pygopus proteins that are involved in Wnt signaling, where they act as coactivators by binding to the β-catenin complex. PYGO2, a member of the Pygopus family, has been shown to be overexpressed in breast cancer (40). Interestingly, an additional gene that was shown to interact with the risk CNV by Hi-C, Ras-related
Fig. 2. Genomic region for the noncoding CNV on chromosome region 15q21.3 associated with aggressive prostate cancer risk. (A) DNA and RNA views of seven individuals at chr15q21.3 locus. Paired DNA (Left) and RNA (Right) data are visualized for seven individuals with different numbers of DNA copies (two copies, one-copy loss, two-copy loss) at the locus of interest (27). No transcripts are present. (B) The tracks include the following information: Prostate cancer-associated CNV on 15q21.3 (dark red; coordinates include midpoint distance to neighboring marker on the genotyping platform; black vertical bars on platform indicate probe locations); the locus corresponding to Variation_66804 in Conrad et al. (24) (black); RefSeq track for validated transcripts; ENCODE DNaseI hypersensitivity; ENCODE mono and trimethylation tracks, H3K4Me1 and H3K4Me3, consistent with enhancer activity (48); ENCODE transcription factor-binding signal (intensities are proportional to binding support); H3K4Me2 and c-Jun ChIP-seq tracks in RWPE cells; predicted androgen receptor (AR) (black) and estrogen receptor (ER) (blue) binding sites (28, 29); and sequence conservation tracks. The positions are to scale and adapted from UCSC genome browser, Feb2009 (GRCh37/hg19) assembly (http://genome.ucsc.edu/). (C) Summary of functional effects of the intergenic 15q21.3 prostate cancer risk locus. This CNV overlaps a potential regulatory element with AP-1 transcription factor binding sites. Our data support a trans- and cis-regulatory role for this region. Presence of deletion in this CNV region is associated with expression of target genes both in cis and trans, potentially leading to prostate cancer predisposition.

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protein Rab-27A (RAB27A), a small GTPase, was seen to be differentially expressed in aggressive prostate cancer (41) and also, more recently, identified as a “driver” oncogene in aggressive melanoma (42). Overall, our finding suggests that the risk locus, which has all of the hallmarks of an AP-1–bound enhancer, often localizes with a transcription factory that also contains multiple genes involved in transcriptional regulation activity. Under some conditions, such as when AP-1 and its factors are active, this functionally coherent gene set may then be activated or repressed by our risk locus.

The other prostate cancer risk CNV we identified and followed-up on maps to an intron of the gene MGAT4C. This gene belongs to the family of glycosyltransferases, two of whose members, MGAT4A and MGAT4B, have been shown to be differentially regulated in pancreatic cancer (43). Glycosyltransferases are involved in the transfer of N-acetylgalactosamine to the core mannose residues of N-linked glycans. By regulating N-glycan branching of cell adhesion molecules, such as E-cadherin, these enzymes play an important role in maintaining cell-cell adhesion in normal epithelia where the extent of branching is inversely correlated with epithelial cell adhesion (44). MGAT4C promotes branching, which results in reduced cell-cell adhesion (44). From our results, it appears that the presence of a germ line deletion CNV at this locus results in overexpression of the gene and, hence, demonstrates a mechanistic basis for the observed increase in invasion of prostate cells in the in vitro cell line assays. Hence, our results strongly support a role whereby MGAT4C could promote disease initiation and progression by reducing cell-cell adhesion and resulting in increased cell motility and migration.

Taken together, our findings demonstrate association of two low-frequency functionally active CNVs with prostate cancer risk. Functional characterization of these risk CNVs show that gene coding and noncoding “gene desert” germ line CNVs may directly or indirectly modulate the transcriptome machinery of known oncogenic pathways in prostate cancer enabling carcinogenesis. The genes and loci identified in this study are candidates for further functional investigation and for replication in independent cohorts and provide alternative information in the assessment of prostate cancer risk, so far limited to SNP variants. In vivo studies in transgenic mice or zebrafish model systems will potentially help elucidate the role of CNVs in disease development, as recently shown for type 2 diabetes and obesity (45).

Methods

The Tyrol PSA Screening Cohort represents a population-based PSA testing cohort in asymptomatic men initiated in 1993 and intended to evaluate the utility of intensive PSA screening in the reduction of prostate cancer-specific death. Cases were defined as men with biopsy-confirmed prostate cancer. Controls were defined as men with a benign prostate biopsy result and no cancer diagnosis in available follow-up data. The demographics and full description of this cohort and of sample collection and preparation for this study are available in Dataset S1, Table S1 and the SI Methods, respectively.

EDRN PSA-Screening Cohorts consists of three Prostate Cancer Clinical Validation Center institutions of Beth Israel Deaconess Medical Center (“Harvard”), Harvard University, Cambridge, MA), the University of Michigan (“Michigan,” Ann Arbor, MI), and Weill Cornell Medical College (“Cornell,” New York, NY). Using a common research protocol at the three institutions, men are prospectively enrolled who are at risk for prostate cancer in three catchment areas in the United States: Boston, MA, Southeast Michigan, and New York, NY, respectively. For this cohort, cases are defined as men diagnosed with prostate cancer and controls are men who have undergone prostate needle biopsy without any detectable prostate cancer and no prior history of prostate cancer. The demographics, cohort collection protocol of men enrolled in this trial and sample preparation are presented in Dataset S1, Table S5 and SI Methods, respectively. A total of 800 DNA samples from Caucasian individuals (based on self-declaration) (346 cases and 454 controls) that passed all quality controls were included in the validation study.

CNV Detection and Selection of CNVs for Prostate Cancer Risk Association Analysis. For CNV characterization, the across-sample detection approach from Banerjee et al. (35) was applied. This approach takes advantage of the polymorphic signal across the entire sample set and helps improve on CNV detection. To benchmark the CNV detection algorithm and genotyping approach in the current study, we quantitatively compared its performance with the data from Conrad et al. (24), verified copy number states using high-coverage DNA sequencing data (27, 46), and performed qPCR on a selected set of CNVs (SI Methods, and Figs. S6 and S7). With the intent to ultimately query a comprehensive and well-characterized set of CNVs, we...
combined the variants detected by the across-sample approach (35) with variants from Conrad et al. (24), for a total number of 2,611 CNVs. All CNVs were genotyped as in Banerjee et al. (35).

The inclusion criteria for biallelic deletion, low-frequency (≤ 10%), gene coding, or functionally active (histone mark analysis) CNVs led to the selection of 238 variants (Dataset S1, Table S38).

Quantitative PCR Validation for CNVs. As additional validation step, a subset of CNVs was selected for genotype verification using TaqMan copy number assays (Applied Biosystems) as in Setlur et al. (47) (Fig. S6). The quantitative PCR assays developed for the prostate cancer risk CNVs at 15q21.3 and risk allele identifi

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