IRX4 at 5p15 suppresses prostate cancer growth through the interaction with vitamin D receptor, conferring prostate cancer susceptibility

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Recent genome-wide association studies (GWAS) identified a number of prostate cancer (PC) susceptibility loci, but most of their functional significances are not elucidated. Through our previous GWAS for PC in a Japanese population and subsequent resequencing and fine mapping, we here identified that IRX4 (Iroquois homeobox 4), coding Iroquois homeobox 4, is a causative gene of the PC susceptibility locus (rs12653946) at chromosome 5p15. IRX4 is expressed specifically in the prostate and heart, and quantitative expression analysis revealed a significant association between the genotype of rs12653946 and IRX4 expression in normal prostate tissues. Knockdown of IRX4 in PC cells enhanced their growth and IRX4 overexpression in PC cells suppressed their growth, indicating the functional association of IRX4 with PC and its tumor suppressive effect. Immunoprecipitation confirmed its protein–protein interaction to vitamin D receptor (VDR), and we found a significant interaction between IRX4 and VDR in their reciprocal transcriptional regulation. These findings indicate that the PC-susceptibility locus represented by rs12653946 at 5p15 is likely to regulate IRX4 expression in prostate which could suppress PC growth by interacting with the VDR pathway, conferring to PC susceptibility.

INTRODUCTION

Prostate cancer (PC) is the fourth most common malignancy in males worldwide (1). Although the precise mechanisms of PC initiation and progression are not fully elucidated, it is evident that genetic factors play some important roles in the etiology of PC (2). A positive family history of PC has been recognized as one of the most important risk factors for PC (2), and twin studies indicated that the contribution of genetic factors to the development of PC is larger than in other types of human common tumors (3). Recent genome-wide association studies (GWAS) have identified more than 40 single nucleotide polymorphisms (SNPs) on various genes or chromosome loci which are associated to PC susceptibility (4–12). However, most of these SNPs are merely the associated markers and only a few variants that functionally affect PC carcinogenesis have been clarified (13–15). Recently, our GWAS in the Japanese population identified rs12653946 at

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chromosome 5p15 as a genetic marker for PC susceptibility (10), which is 600 kb far away from the TERT locus (11). Its linkage disequilibrium (LD) block spanning 20 kb does not include any protein-coding gene and in a 100 kb region surrounding this locus, only one gene, IRX4 (Iroquois homeobox 4), is annotated.

IRX4 is a member of the Iroquois family of homeobox transcription factors that are dominantly expressed in the ventricle of the heart and play an important mediator of ventricular differentiation during cardiac development. Adult Irx4-null mice showed abnormal ventricular gene expression and cardiomyopathy, and Irx4 activates the expression of the ventricle myosin heavy chain 1 (VMHC1) gene and suppresses the expression of the AMHCl/slow MyHC3 gene (16–18). Interestingly, in human, cardiomyopathy is reported to be one of the risk factors of human PC (19). Furthermore, Irx4 was demonstrated to suppress MyHC3 gene expression by forming an inhibitory complex with vitamin D receptor (VDR) and retinoic X receptor (RXR) heterodimer that binds at the VDR response element of the MyHC3 promoter (20). On the other hand, several IRX family members are involved in carcinogenesis. IRX1 was down-regulated and could function as a tumor suppressor gene in gastric cancers (21). IRX3 was down-regulated in LNCaP cells (22), and IRX5 was suggested to be involved in the regulation of both the cell cycle and apoptosis in human PC cells (23). However, the role of IRX4 in carcinogenesis has not yet been elucidated so far.

In this study, we performed re-sequencing of the PC-susceptibility region representing rs12653946 at 5p15 and identified variants in a putative enhancer region that could alter IRX4 expression in the prostate, and we found that the expression of IRX4 in the prostate was associated with haplotype at the 5p15 region. We also demonstrate that IRX4 is likely to behave as a tumor suppressor possibly through interaction with the VDR pathway. These findings should provide new insights into the molecular pathogenesis and genetics of PC and may facilitate in developing new approaches to prevent or diagnose PCs.

RESULTS

Re-sequencing and fine mapping of the 5p15 PC-susceptibility locus

The SNP rs12653946 at chromosome 5p15 was the most significantly PC-associated variant among the five loci that our GWAS identified in Japanese population (10). We previously performed further mapping analysis of the candidate region using 36 tag-SNPs, and found that rs12653946 represented an associated region spanning 20 kb (Chr5: 1,945,455–1,965,921 in NCBI36/hg18). In this study, we conducted re-sequencing for this 20 kb region and its surrounding region, spanning ~35 kb (Chr5: 1,934,946–1,969,860), using DNA of 94 PC cases, and identified 101 variants including 17 novel ones. Among them, we genotyped 73 variants with a minor allele frequency (MAF) of 0.05 or higher, and revealed that a newly identified Variant06 (Chr5: 1,942,304 bp) was the strongest association with PC susceptibility in a Japanese population \( P = 2.18 \times 10^{-11}, \text{odds ratio} = 1.34, \text{95\% confidence interval} (CI) = 1.23–1.46, \) (Fig. 1). Variant06 is a multiple-nucleotide length polymorphism (MLNP) which involves a dozen of nucleotides in a diallelic manner (24, Supplementary Material, Fig. S1), and detailed genotyping data are shown in Supplementary Material, Table S1. Variant06 represents a 9.1 kb LD block spanning from Variant06 to rs260410 (Chr5: 1,942,304–1,951,501 bp, Fig. 1). Of these variants within this LD block, we selected 12 strongest associated variants for the candidates to be subjected to further functional analysis (Table 1). Haplotype analysis using these 12 variants indicates that there are two main haplotypes whose frequency is >5%, and the risk haplotype represents more significant association with PC than each of the single variant \( P = 6.0 \times 10^{-13}, \) Supplementary Material, Table S2).

The expression level of IRX4 was associated with haplotype at 5p15

Since no known protein-coding genes were annotated within this LD block, we first examined the expression levels for one expressed sequence tag (EST) (BC039383) which was found to be located within this region, but no expression was observed in prostate and several normal organs in reverse transcription–polymerase chain reaction (RT–PCR) and Northern blot analyses (data not shown). In a 100 kb region surrounding this critical LD block, only one gene, IRX4, is annotated (Fig. 1). Therefore, we next focused on IRX4, which was located at ~6.4 kb upstream of Variant06. RT–PCR of IRX4 showed its specific expression in normal prostate and heart (Fig. 2A), and 2.4 kb transcript was observed in the prostate as well as the heart in Northern blot analysis (Fig. 2B). Moreover, quantitative RT–PCR analysis of 21 normal prostate tissue samples revealed a significant association between the genotypes of rs12653946 and IRX4 expression levels \( P = 0.033 \) by the Kruskal–Wallis test), and an addition of a susceptible allele of rs12653946 significantly decreased the expression of IRX4 in normal prostate tissues (Fig. 2C). These findings implicate that the PC-susceptibility locus at 5p15 can be regulatory elements to control IRX4 expression in the prostate.

Cloning of additional 5′-UTR and the first exon of IRX4

The transcript size of IRX4, shown by Northern blot analysis (2.4 kb), was larger than its RefSeq-registered size (2.2 kb, Accession no. NM_016358), and the presence of one EST (2.4 kb), was larger than its RefSeq-registered size (2.2 kb, Accession no. AB690778). Sequencing analysis of the RACE products showed that the registered first exon of IRX4 (Fig. 2D, Accession numbers AB690778, AB690779, AB690780 and AB690781). Sequencing analysis of the RACE products showed that the registered first exon was shorter than the registered sequence for IRX4 in its 5′-UTR, and there were at least two kinds of the first exon which were located ~4 kb upstream of the registered first exon of IRX4. As a result, the distance between the IRX4 gene (Chr5: 1,930,541–1,940,293 bp in NCBI36/hg18) and the critical LD block associated with PC susceptibility (Chr5: 1,942,304–1,951,501 bp) is shortened to 2.0 kb.
The novel first exons are located in an extremely guanine-cytosine content (GC)-rich region (~70%), indicating a strong potential of the proximal promoter region around this new 5′ exons of IRX4.

Regulatory functions of the variants within the 5p13 critical region

To screen for functional variants as regulatory elements among the 12 variants that make the critical haplotype in the PC-susceptibility LD block (Fig. 3A), we performed electrophoretic mobility shift assays (EMSAs) for these variants. For each variant, two 31 bp oligo pairs containing non-risk and risk alleles were used as probes to detect nuclear-binding proteins. Of the 12 variants examined, 8 SNPs (rs12655062, rs10866528, rs34695572, rs12656007, rs12653946, rs35010507, rs35326077 and rs4975758) showed differential binding affinities to the nuclear proteins from LNCaP cells between their non-risk and risk alleles (Fig. 3B). Furthermore, to demonstrate their potentials as regulatory elements, we cloned the regions around each allele of the eight candidates and Variant06 into the upstream of the SV40 promoter of pGL3 luciferase expression vector for luciferase assays. After transfections of these vectors into 22Rv1 and HeLa cells, we observed that the risk allele of rs12656007 displayed the most significant and consistent reduction in the luciferase activity when compared with the non-risk allele (P-value = 0.00044 for 22Rv1 cells; P-value = 0.00036 for HeLa cells) (Fig. 3C). In two other variants rs34695572 and rs35010507, the risk alleles also displayed significantly lower luciferase activity than their non-risk alleles (P-value = 0.0094 and 0.00042 for 22Rv1 cells; P-value = 0.00487 and 0.07574 for HeLa cells, respectively), but other variants did not show any difference of the luciferase activity between the risk and non-risk alleles. Hence, these variants, especially rs12656007, within the critical LD block are likely to function as an enhancer element that can regulate the expressions of IRX4, conferring PC susceptibility.

IRX4 suppresses PC cell proliferation

To understand the biological role of IRX4 expression in PC cells, we knocked down endogenous IRX4 expression by transfecting siRNA oligonucleotides into LNCaP cells that highly expressed IRX4. RT–PCR (Fig. 4A) confirmed the knock-down effect on IRX4 expression by two IRX4-specific siRNAs (si1 and si2), and 3-94,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine (MTT) assays revealed that the reduction in IRX4 expression significantly promoted the growth of LNCaP cells with the P-value of 0.00167 for si1 and 0.00046 for si2 compared with the control siEGFP (Fig. 4A). Next, we overexpressed IRX4 in 22Rv1 and PC3 cells that showed low levels of IRX4 expression. MTT
assays observed a significant suppression of cell growth in 22Rv1 cells in accordance with the amount of IRX4 expression (Fig. 4B, $P$-value ¼ 0.0091 and 0.0184 in 22Rv1 cells transfected with 2 and 3 μg plasmid, respectively) and in PC3 cells (Fig. 4C, $P$-value = 0.0045). These findings indicate that IRX4 can function to suppress PC cell proliferation.

Table 1. Selected 12 variants in the critical LD block at the 5p15 region

<table>
<thead>
<tr>
<th>SNP_ID</th>
<th>Location a</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Risk Allele</th>
<th>MAF Case</th>
<th>MAF Control</th>
<th>$P$-value</th>
<th>Odds ratio (95% CI)</th>
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<tr>
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<td>1943877</td>
<td>A</td>
<td>G</td>
<td>A</td>
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<td>0.41</td>
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<td>G</td>
<td>G</td>
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<td>7.42E-10</td>
<td>1.31 (1.20–1.43)</td>
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<td>G</td>
<td>G</td>
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<td>1.21E-10</td>
<td>1.33 (1.22–1.45)</td>
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<td>1947424</td>
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<td>–</td>
<td>TCA</td>
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<td>1.78E-09</td>
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<tr>
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<td>4.29E-10</td>
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<td>0.43</td>
<td>8.61E-10</td>
<td>1.31 (1.20–1.42)</td>
</tr>
</tbody>
</table>

aChromosomal location based on NCBI Human Genome Build 36.
bVariant06 is a MNLP involving a dozen of nucleotides in a diallelic manner (Supplementary Material, Fig. S1).
cAllele 1: GCCGACTGTGATTGTAGCCCTTTCGGGACCAACAGGGACAGT.
cAllele 2: CAGGACTTGCAAGCTCATGTG.

dFigure 2. The expression pattern of IRX4 and its gene structures. (A) RT–PCR of IRX4 in normal organs. IRX4 is expressed specifically in normal heart and prostate tissues. (B) Northern blot analysis of IRX4 in normal organs. The transcript of IRX4 is present as a 2.4 kb band in normal heart and prostate tissues. (C) Quantitative RT–PCR of IRX4 in normal prostate tissues. Box plot shows expression level of IRX4 according to the genotype of rs12653946. The horizontal lines indicate the median values, and the boxes cover 25–75 percentiles. Points outside them show up as outliers. The Kruskal–Wallis test was used for statistical analysis and $P$-values are indicated. (D) The IRX4 gene structure with newly identified first exons and splicing variants (Accession numbers v1: AB690778, v2: AB690779, v3: AB690780 and v4: AB690781). The coding exons of IRX4 were open boxed.
Figure 3. Functional variants at the 5q15 region. (A) The PC-susceptibility LD block at 5q15 including the 12 variants that makes the critical haplotype is located 2 kb upstream of the newly identified first exons of the IRX4 gene, which are located in an extremely GC-rich region. (B) EMSAs to screen for functional SNPs showing differential affinities to nuclear proteins between the risk and non-risk alleles. Differential bands between the risk and non-risk alleles of each SNP (rs12655062, rs10866528, rs34695572, rs12656007, rs12653946, rs35010507, rs35326077 and rs4975758) are indicated as arrows. (C) Luciferase assays in 22Rv1 (upper panel) or HeLa cells (lower panel) evaluated the enhancer/silencer activity of the SNPs selected from EMSAs and Variant06. The averages of three independent experiments are shown. The values of the relative luciferase activity are shown as mean ± SD (*P < 0.01, Student’s t-test).
IRX4 is reported to interact with a heterodimer of VDR and RXR to regulate the expression of their target genes in cardiomyocytes (20). First, we attempted to confirm the protein–protein interaction between IRX4 and VDR/RXR by co-transfecting FLAG-IRX4 expression vector with Myc-VDR or HA-RXR expression vector in PC3 cells. As shown in Figure 5A, the Myc-VDR protein was co-immunoprecipitated with FLAG-IRX4 by anti-FLAG antibody, and FLAG-IRX4 was detected as a co-immunoprecipitated protein with Myc-VDR. In contrast with VDR, the HA-RXR protein was not detected in an immunoprecipitated complex of FLAG-IRX4 (data not shown). These results indicate that VDR, but not RXR, can bind to IRX4 in PC cells. VDR is a nuclear transcriptional receptor to transactivate its downstream genes, and we checked whether IRX4 binding to VDR could affect the transactivation activity of VDR or not. However, we did not observe that IRX4 could affect the transactivation activity of VDR (data not shown).

Next, we investigated whether IRX4 and VDR expressions could affect each other in PC cells. We knocked down IRX4 in LNCaP cells by siRNA and then assessed the mRNA level of both IRX4 and VDR by quantitative RT–PCR. As shown in Figure 5B, IRX4 knockdown resulted in reduction of VDR at the mRNA level with the P-value of 0.0022 for si1 and 0.0003 for si2, indicating that IRX4 is involved in the regulation of VDR expression. Furthermore, we overexpressed VDR in LNCaP cells and checked the IRX4 expression. Quantitative RT–PCR analysis demonstrated that the overexpression of VDR significantly suppressed IRX4 expression at the mRNA level (P-value = 0.00046 and 0.00032 in the cell transfected with 2 and 3 μg plasmids, respectively), when compared with cells transfected with mock vector (Fig. 5C). Furthermore, when we treated LNCaP cells with 50 nm vitamin D3 (VD3), real-time PCR observed that the IRX4 mRNA level was decreased at first but increased maximum at 12 h after VD3 treatment, while the VDR mRNA level was slowly increased significantly and reached to a maximum at 12 h (Supplementary Material, Fig. S2). These findings suggest a complicated feedback transcriptional regulation between IRX4 expression and VDR expression, in which high VDR expression can reduce the IRX4 expression level and consequently can reduce the expression of VDR.

**Functional interaction of IRX4 with VDR**

IRX4 is reported to interact with a heterodimer of VDR and RXR to regulate the expression of their target genes in cardiomyocytes (20). First, we attempted to confirm the protein–protein interaction between IRX4 and VDR/RXR by co-transfecting FLAG-IRX4 expression vector with Myc-VDR or HA-RXR expression vector in PC3 cells. As shown in Figure 5A, the Myc-VDR protein was co-immunoprecipitated with FLAG-IRX4 by anti-FLAG antibody, and FLAG-IRX4 was detected as a co-immunoprecipitated protein with Myc-VDR. In contrast with VDR, the HA-RXR protein was not detected in an immunoprecipitated complex of FLAG-IRX4 (data not shown). These results indicate that VDR, but not RXR, can bind to IRX4 in PC cells. VDR is a nuclear transcriptional receptor to transactivate its downstream genes, and we checked whether IRX4 binding to VDR could affect the transactivation activity of VDR or not. However, we did not observe that IRX4 could affect the transactivation activity of VDR (data not shown).

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**DISCUSSION**

Recent GWAS have made successful steps towards deciphering the genetic basis of PC and have been identified more than 40 PC-susceptibility loci, and this number is much larger than those for other types of common cancers. However, many of these PC-susceptibility variants are located in intergenic regions, and there are merely variants of which biological significance is proved to be associated with PC (13,14,15,25). In order to better understand PC genomics and the underlying biology, it is required to analyze the functions of these loci or variants that have been identified by PC GWAS. In this
study, we provide several evidences supporting that the PC-susceptibility locus represented by rs12653946 at 5p15 is a putative regulatory element for the IRX4 gene. First, the IRX4 expression level in normal prostate tissues was significantly associated with the genotype of rs12653946. Secondly, the newly identified first exons of the IRX4 gene are located just 2.0 kb downstream from the critical LD block represented by rs12653946, shortening the physical distance between IRX4 and this locus. Thirdly, several sequence elements, especially sequences involved by rs12656007, within this LD block showed enhancer activity and their PC-risk alleles showed their decreased enhancer activity, when compared with that of the non-risk alleles. When searching for potential transcription factors that could bind the DNA sequences containing these SNPs using TFSEARCH database (26), we found that replacing the non-risk allele with the risk allele of rs12656007 destroys a potential binding site (AACTGTA/CAGTCCAA) for the transcription factors Tst-1/Oct-6. This result corroborates our functional reporter assays that showed that the risk allele had much lower enhancer activity compared with the non-risk allele. Given that IRX4 is possibly regulated by the critical region associated with PC susceptibility, we further examined the causal relationship of IRX4 expression and PC cell proliferation. The down- or up-regulation of IRX4 expression resulted in promotion or suppression of PC proliferation concordantly, indicating that IRX4 is likely to have a tumor suppressive activity in PC. However, it is still not conclusive that this critical PC-susceptibility region can regulate IRX4 expression as an enhancer element directly. In the similar situations, some researchers recently conducted the chromosome conformation capture (3C) technique to detect the physical interaction between distal regulatory elements and target genes, for example, between the 8q24 cancer risk variants and the c-Myc proto-oncogene which is lying ≏300 kb from the cancer-associated region (27). A further study using chromosome conformation analysis or others may provide more direct evidence of the interaction between this critical PC-susceptibility region and IRX4 gene or between other non-coding regions associated with PC susceptibility and functional genes.

In this study, we demonstrated the functional interaction between IRX4 and VDR. VDR is a steroid family receptor of vitamin D, which exerts anti-proliferation and anti-differentiation effect in vitro and in vivo in many types of cancers including PC, and it supports a role for targeting the VDR in either chemoprevention or chemotherapy setting. The molecular mechanism of the tumor suppressive effect of the VDR pathway has been explored, but the whole picture is still difficult to understand and unclear. This functional interaction of a novel PC-susceptible gene IRX4 and VDR could shed light on the mechanism of the anti-proliferation effect of vitamin D and VDR pathway on PC development and progression. The VDR expression has been shown to be regulated by its binding protein named pituitary transcription factor Pit-1 in MCF-7 cells (28,29), and VDR also can repress the transcription of Pit-1 directly (30). Therefore, we
propose that IRX4 may have similar function as Pit-1 in prostate cells and demonstrated that IRX4 and VDR could regulate the expression of each other probably through some feedback systems. However, functional interaction between IRX4 and VDR in prostate cells remains very complex and needs further study.

In summary, we identified some functional variants in putative enhancer elements at the PC susceptibility region at 5p15 that could affect IRX4 expression in the prostate and showed that the IRX4 expression level was significantly associated with the haplotype at the critical region of 5p15. We also identified that IRX4 is a functional gene of this locus. IRX4 could function as a tumor suppressor in prostate via the VDR pathway. These findings indicate that IRX4 may be a promising molecular target for diagnosis and prevention of PC.

**MATERIALS AND METHODS**

**Genotyping samples**

All PC cases and controls were obtained from the BioBank Japan at Institute of Medical Science, The University of Tokyo. For fine mapping of the 5p15 PC-susceptibility region, we utilized the same set of the cases and controls that we used in our GWAS (10). From the registered samples in the BioBank Japan, we selected 1583 individuals that were clinically diagnosed as having PC based on the pathological evaluation of prostatic biopsy. The controls consisted of 2480 individuals that were registered in the BioBank Japan as subjects with 13 diseases other than PC and 906 healthy volunteers collected at the Osaka-Midosuji Rotary Club. All participants provided written informed consent. This research project was approved by the ethical committees at Institute of Medical Science, The University of Tokyo and Center of Genomic Medicine, RIKEN.

**Genotyping and re-sequencing**

Genotyping was performed using the multiplex PCR-based Invader assay (31). We assessed case–control association analysis using a one-degree-of-freedom Cochrane–Armitage trend test. LD and haplotype analysis were performed by using the Haploview (Broad Institute, MA, USA). Direct sequencing of the critical 20 kb region and its surrounding region, spanning ~35 kb (Chr: 1,934,946–1,969,860), were performed in 94 PC cases selected randomly from our GWAS samples, regardless of the genotype of rs12653946, described above using ABI3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

**Cell lines and clinical prostate samples**

PC cell lines LNCaP, 22Rv1 and PC3 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). LNCaP was grown in RPMI (Invitrogen, Carlsbad, CA, USA) supplemented with 10^{-8} M dihydrotestosterone (Teikoku Hormone MFG, Kawasaki, Japan), 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA) and 1% antibiotic/antimycotic solution (Sigma-Aldrich, St Louis, MO, USA). 22Rv1, PC3 and HeLa cells were grown in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution. Cells were incubated at 37°C in atmosphere of humidified air with 5% CO2. Normal prostate tissues were obtained from patients who underwent surgical resections for bladder cancer or localized PC in Iwate Medical University under appropriate informed consents.

**Semi-quantitative and quantitative RT–PCR**

DNAs and RNAs were extracted from 21 normal prostate tissue samples by using QIAamp DNA Mini Kit (QIAGEN, Chatsworth, CA, USA) and RNeasy Mini Kit (QIAGEN), respectively. We also purchased polyadenylated RNAs isolated from 13 normal organs from Takara Clontech (Kyoto, Japan). cDNA was prepared using SuperScript III First-Strand Synthesis System (Invitrogen). We apply appropriate dilutions of each cDNA for subsequent PCR by monitoring amount of ACTB (β-actin) as a quantitative control. We also carried out real-time PCR on Light Cycler 480 using LightCycler Fast Start DNA Master SYBR Green I (Roche, Basel, Switzerland). Primer sequences for IRX4 were 5′-CCGC CTTCCTACTGCTGA-3′ and 5′-GAGCTGCTGCTAAGGG TTAG-3′. The results were normalized to ACTB and analyzed by the standard curve method.

**Northern blot analysis**

Human multiple-tissue blots (Takara Clontech) and PC cell line blots were hybridized with [α-32P]dCTP-labeled PCR products (912 bp) of IRX4. The probe for IRX4 was prepared by RT–PCR using the following primer set: 5′-CCAGA CTGAGTTTCCGTAGTA-3′ and 5′-TAGCCTCTGAACGAG GCAAT-3′. Pre-hybridization, hybridization and washing were done according to the supplier’s recommendations. The blots were auto-radiographed with intensifying screens at −80°C for 14 days.

**Electrophoretic mobility shift assays (EMSAs)**

Nuclear cell lysates were prepared from LNCaP cells using TransFactor Extraction Kit (Clontech) according to the manufacturer’s instruction. EMSAs were performed using DIG Gel Shift Kit, 2nd Generation (Roche) according to the manufacturer’s instructions. Thirty-one base sense and anti-sense strand for each SNP allele were synthesized and annealed to generate the double-stranded oligonucleotides (Supplementary Material, Table S1). After pre-incubation with poly(dI)C and poly-l-lysine, double-stranded DIG-labeled probes were incubated with 10 μg of nuclear extract prepared from LNCaP cells for 20 min at room temperature. The mixtures were then separated by electrophoresis on a 6% non-denaturing polyacrylamide gel with 0.5 x tris-borate-EDTA buffer. The protein–oligonucleotide complexes were visualized by autoradiography. All EMSAs were repeated to check for reproducibility.

**Dual luciferase assays**

BamHI sites were added to the 31 bp fragment used as probes in the EMSAs. The annealed double-stranded oligonucleotides
were digested with BamHI, and inserted in reverse orientation into the pGL3 luciferase reporter vector (Promega). Twenty-four hours after plating 3 × 10^5 cells on six-well plates, LNCaP cells were transfected with 1 μg of the luciferase reporter plasmid and 0.2 μg of pRL-TK (Renilla Luciferase) using Fugene 6 reagent (Roche). At 48 h after transfection, the cells were solubilized, and the luciferase activity was measured using Pikkagene Dual Luciferase Assay System (Toyolink, Tokyo, Japan). The luciferase activities were normalized by renilla luciferase activity. Each experiment was carried out more than two times with triplicate replicates.

**siRNA experiments and cell viability assays**

Two siRNA duplexes were designed to IRX4 sequence (GenBank Accession no: NM_016358). The target sequences of these siRNAs are: si1, 5'-GCCUUCUCACUCGUGAA CATT-3'; si2, 5'-CGCUGAACAAGCUCUUGAUATT-3' and siEGFP, 5'-GCAGACACUCUUCUAAAGTT-3' as a negative control. LNCaP cells were grown on 24-well plates, and transfected with each of the siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instruction. The knockdown effects were assessed by RT–PCR using primers: 5'-AGGGCTATGGAACACTACG TG-3' and 5'-CTTCCAGCAGGTCGAAGT-3'. In MTT assay, cell growth was measured in triplicates using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Absorbance was measured at 490 nm, and at 630 nm as a reference, with ARVO MX 1420 multi-label counter (PerkinElmer, Walltham, MA, USA). Both RT–PCR and MTT assay were carried out at 72 h after siRNA transfection.

**Overexpression of IRX4 and cell viability assays**
The full-length cDNA encoding human IRX4 was amplified by forward primer, 5'-GCTGGAAATCTATGCCACTACG TTTG-3' and reverse primer, 5'-CCCTCGAGGGCGCA GAAGGTTTGGC-3', and was inserted into the EcoRI and XhoI sites of the pCAGGS vector. Recombinant plasmids were confirmed by DNA sequencing. FLAG-tagged IRX4 expression vector (pCAGGSn-IRX4) or mock vector was transfected into LNCaP cells by using Lipofectamine 2000 (Invitrogen). Exogenous IRX4 expression was confirmed by western blotting using anti-FLAG antibody (Sigma) at 72 h after transfection. Cell viability was evaluated by MTT assay at 96 h after transfection.

**Vitamin D treatment and VDR/IRX4 expression**
LNCaP cells were sub-cultured 24 h before vitamin D treatment. In the next day, all the cells were changed to medium containing 50 nM vitamin D3 (VD3, Sigma-Aldrich). After treatment, the cells were collected at time points: 0, 1, 2, 6, 12 and 24 h. Total RNAs were isolated and cDNAs were synthesized by SuperScript III (Invitrogen). Real-time quantitative PCR was performed to measure the mRNA level of IRX4 and VDR, described above. ACTB (β-actin) was used as the reference. Primer sequences for VDR were 5'-GCACCACATAAGACCTACGA-3' and 5'-GATGAGGAG AAGCTGGACAAG-3'.

**Immunoprecipitation**
PC3 cells were co-transfected with various combinations of plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Transfected cells were harvested 48 h after transfection and lysed with RIPA lysis buffer supplemented with 10 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 1× protease cocktail mix (Roche). The obtained lysates were pre-cleared with 20 μl of 50% Protein G– or A–Sepharose beads (GE Healthcare) at 4°C for 1 h. Anti-Myc antibody (Sigma) was pre-incubated with Protein A–Sepharose beads at 4°C for 1 h. Then the pre-cleared lysates were incubated with 30 μl of anti-FLAG M2 mAb-conjugated protein G– or A–Sepharose beads (Sigma-Aldrich) or anti-Myc antibodies bound to Protein A–Sepharose at 4°C for 2 h. After incubation, immunoprecipitants were extensively washed with the lysis buffer. The proteins in the immunoprecipitants were eluted by boiling the beads in sodium dodecyl sulphate reagent (SDS) sample buffer (60 mM Tris–HCl, pH 6.7, 3% SDS, 2% 2-mercaptoethanol and 5% glycerol) for 5 min and subjected to western blot analysis with anti-FLAG and anti-Myc antibodies.

**SUPPLEMENTARY MATERIAL**
Supplementary Material is available at HMG online.

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