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Evaluation of *PPP2R2A* as a prostate cancer susceptibility gene: comprehensive germline and somatic study

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Abstract

PPP2R2A, mapped to 8p21.2, encodes for the α isoform of the regulatory B55 subfamily of the protein phosphatase 2 (PP2A). PP2A is one of the four major Ser/Thr phosphatases and is implicated in the negative control of cell growth and division. Because of its known functions and location within a chromosomal region where evidence for linkage and somatic loss of heterozygosity was found, we hypothesized that either somatic copy number changes or germline sequence variants in *PPP2R2A* may increase prostate cancer (PCa) risk. We examined *PPP2R2A* deletion status in 141 PCa samples using Affymetrix SNP arrays. It was found that *PPP2R2A* was commonly (67.1%) deleted in tumor samples including a homozygous deletion in 3 tumors (2.1%). We performed a mutation screen for *PPP2R2A* in 96 probands of hereditary prostate cancer (HPC) families. No high risk mutations were identified. Additionally, we reanalyzed 10 SNPs of *PPP2R2A* in sporadic PCa cases and controls. No significant differences in the allele and genotype frequencies were observed among either PCa cases and controls or PCa aggressive and non-aggressive cases. Taken together, these results suggest that a somatic deletion rather than germline sequence variants of *PPP2R2A* may play a more important role in PCa susceptibility.

Keywords

PPP2R2A; homozygous deletion; prostate cancer

Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer death among men in the United States. In 2010, it was estimated there will be 217,730 men diagnosed with PCa and 32,050 men died (1). Although the etiology of PCa is unknown, age, race/ethnicity, and family history are three well-established risk factors. Furthermore,

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evidence for a major PCa susceptibility gene has been consistently provided by multiple segregation studies (2). Several chromosomal regions likely harbor PCa susceptibility genes, and several candidate genes in these linkage regions have been reported (2, 3).

Suggestive evidence for PCa linkage at chromosomal region 8p has been reported independently in multiple study populations of familial PCa (4-9). The likelihood of a PCa susceptibility gene in this region is strengthened by the accumulation of evidence that 8p is the site of the most frequent loss of heterozygosity (LOH) in prostate tumors (10). Two important consensus PCa-susceptibility regions 8p21.3 (20.6-23.7 Mb) and 8p23.1 (9.8-11.2 Mb) had been identified by combined somatic deletion and germline linkage analyses (11). Furthermore, a more important recurrent homozygous deletion (HD) region (0.3 Mb) at 8p21.2 was found to be associated with PCa in our previous study (12), in which this HD was found in the PCa cell line VCaP and 1 out of 72 clinical PCa samples. The recurrent HD strongly implies the presence of tumor suppressor genes in the location. There are two genes in this 8p21 recurrent HD region, including *PPP2R2A* and *BNIP3L*. *PPP2R2A* is inferred to be a candidate tumor suppressor gene because of its belonging to the protein phosphatase 2 (PP2A) regulatory subunit B family. PP2A is one of the four major Ser/Thr phosphatases, and is implicated in the negative control of cell growth and division through a variety of cellular processes, including signal transduction, DNA replication, apoptosis, and cell cycle progression (13). A study of the fusion of *CHEK2* and *PPP2R2A* in childhood teratomas found that the deregulation of *CHEK2* and/or *PPP2R2A* is of pathogenic importance in at least a subset of germ cell tumors (14). A more recent study showed that miR-31 acts as an oncogenic miRNA in mouse and human lung cancer by targeting specific tumor suppressors including *PPP2R2A* (15). Additionally, mutations in *PPP2R1A* and *PPP2A1B* genes, both of which belong to the structural subunit of PP2A, have been detected in melanomas and carcinomas of the lung, breast, colon and ovary (16-18). However, to our knowledge, no mutation screening for *PPP2R2A* in prostate tumors or genomic DNA of PCa patients or comprehensive germline and somatic analyses for this gene have been reported.

To test the hypothesis that *PPP2R2A* is a PCa susceptibility gene, we performed 3 types of analyses. We continued examining *PPP2R2A* deletion status in another 69 PCa clinical samples. We screened the 5' UTR, all 10 exons, exon-intron junctions, and the 3' UTR of *PPP2R2A* for mutations and sequence variants in 96 HPC probands. Lastly we tested for associations between sporadic prostate cancer and *PPP2R2A* by analysis of 10 SNPs that provide complete coverage of the *PPP2R2A* gene in the JHH study and Illumina IControl.

Materials and Methods

Subjects

The clinical samples used in somatic deletion analysis include total 141 PCa patients. All the primary tumors were from PCa patients undergoing radical prostatectomy (RP) for treatment of clinically localized disease at John Hopkins Hospital (JHH). For the analysis of somatic DNA copy number alterations in these tumors, we selected cases from which genomic DNA of sufficient quantity (>5 µg) and purity (>70% cancer cells for cancer specimens; no detectable cancer cells for normal samples) could be obtained by macrodissection of matched nonmalignant (hereafter referred to as *normal*) and cancer-containing areas of prostate tissue as determined by histologic evaluation of hematoxylin and eosin-stained frozen sections of snap-frozen RP specimens. The detailed clinical characteristics of these samples are described in table 2.

All 96 hereditary prostate cancer (HPC) families, each having at least three individuals affected with PCa and who were first degree relatives, were recruited and studied at the Brady Urology Institute at John Hopkins Hospital (Baltimore, MD). A detailed description

of this series of hereditary prostate cancer families has been previously reported(19). Briefly, PCa diagnosis was verified by medical records for each affected study participant. The mean age at diagnosis was 64.4 years for the cases in these families. Among the participants were 85 European American and 11 African American. Informed consent was obtained from all participants and study protocols were reviewed and approved by the institutional review boards at each institution.

The John Hopkins Hospital (JHH) study population was described in detail previously(20), including 1,402 aggressive cases, 1,445 nonaggressive cases, and 482 controls of European descent (by-self-report). Tumors with a Gleason score of 7 or higher, or stage pT3 or higher, or N+ or M1 (i.e., either high-grade or non-organ-confined disease) were defined as aggressive. We also obtained genotypes from the Illumina iControl database for 3,085 European Americans (EA).

Affymetrix Genome-Wide human single nucleotide polymorphism Array 6.0 analysis

We used Affymetrix SNP array panels to detect DNA copy number alterations, and for this study, we focused entirely on *PPP2R2A* gene. Since this is a part of the ongoing whole-genome copy number change project, we used 500K SNP array (~512,000 SNPs) for the first 72 subjects. Later, with the emergence of the more high-density coverage of whole-genome's 6.0 array (~906,600 SNPs and ~946,000 probes for CNVs), we used 6.0 array for the additional 69 subjects. As to the *PPP2R2A* region coverage, there are a total of 17 SNPs and 20 CNVs mapped to this region on the 6.0 array, and there are only 8 SNPs on the 500K array, with the latter also being included in the 6.0 array. For all subjects, we analyzed both tumor DNA and normal DNA from the same subject using the same Affymetrix SNP array. The procedures and analyses for copy number change using 500K had been described in detail previously (12). For the 6.0 array, 250 ng of genomic DNA were digested with either *Nsp*I or *Sty*I and then ligated to adapters that recognize cohesive four-basepair (bp) overhangs. A generic primer that recognizes the adapter sequence was used to amplify adapter ligated DNA fragments with PCR conditions optimized to preferentially amplify fragments in the 200 to 1,100 bp size range in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). After purification with magnetic beads from Agencourt (Beverly, MA), the PCR product was fragmented using DNase I and a sample of the fragmented product was visualized on a 4% TBE agarose gel to confirm that the average size was smaller than 180 bp. The fragmented DNA was then labeled with biotin and hybridized to the Affymetrix 6.0 chip for 18 hrs.

We washed and stained the arrays using an Affymetrix fluidics Station 450 and scanned the arrays using a GeneChip Scanner 3000 7G (Affymetrix, Inc., Santa Clara, CA). The Affymetrix GeneChip® Operating Software (GCOS) was used to collect and extract feature data from Affymetrix GeneChip® Scanners. We used Affymetrix® Genotyping Console™ Software 2.1 for genotype analysis. The average call rate for all samples was >97.7%.

Allele-specific genomic analysis was performed using the Partek Genomic Suite (PGS) version 6.4 allele-specific analysis algorithm, which takes advantage of genotype information and allele-specific intensities from paired samples to estimate DNA copy number for each heterozygous SNP. Allele-specific analysis can also help determine the effect of normal DNA contamination from nonmalignant cells in the tumor samples through comparison of allele ratios inside and outside regions of apparent hemizygous deletion. Please note that the currently released PGS allele-specific copy number algorithm for single sample analysis assigns one allele "Max" status and colors its data red, and assigns the other allele "Min" status and colors its data blue based on the estimated copy number for the different alleles (max=red, min=blue). This labeling is meant to convey the structure of contiguous regions with differing allele prevalence, but by itself does not imply haplotype

phase across regions of similar allele prevalence. Examples of homozygous deletion displayed in Figure 1 were identified by examination of the allele-specific copy number data using a combination of relative and absolute copy number (both alleles generally well below 0.5 copy number) and genomic length of affected segment containing more than approximately 20 probes (each dot in Figure 1 represents data from 10 probes).

Sequencing for mutation

We directly sequenced the PCR products of the ~500 bp promoter, all exons, and exon-intron junctions in 96 HPC probands. Table 1 lists the primers used to amplify the PCR products, the sizes of amplified PCR fragments, and the annealing temperatures for each pair of primers. All PCR reactions were done in a 20 μ l volume consisting of 40 ng genomic DNA, 4 μ l 10 \times buffer, 0.3 μ M of each primer, 0.3 mM dNTP, 0.75 mM MgCl₂, 0.025 U *Pfx* Polymerase. PCR cycling conditions were as follows: 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, specific annealing temperature for 30 seconds, and 68°C for 1 minute, with a final extension of 68°C for 7 minutes. Amplified PCR products were purified using an ExelaPure ultrafiltration membrane (Edgebiosystems, Gaithersburg, MD) to remove deoxynucleotide triphosphates and excess primers. All sequencing reactions were done using dye terminator chemistry (BigDye, ABI, Foster City, CA) and then precipitated using 75 \pm 5% ethanol. Samples were loaded onto an ABI 3730 DNA analyzer after adding 8 μ l formamide. Single nucleotide polymorphisms (SNP) were identified using Sequencer software version 4.8 (gene Codes Corp., Ann Arbor, MI).

Statistical methods

Associations between clinical features and deletion were tested using parametric logistic regression method

A Hardy-Weinberg equilibrium test was performed using the Fisher's exact test. Haplotype blocks were estimated using a computer program Haploview (21), and a default Gabriel method (22) was used to define each haplotype block.

We imputed all of the known SNPs in the 998-kb region of *PPP2R2A* based on the genotyped SNPs and haplotype information in the HapMap phase II data (CEU) using a computer program, IMPUTE (23). A posterior probability of 0.9 was used as a threshold to call genotypes. Imputed SNPs that has a call rate >90% in JHH were included in the following analysis.

Allele frequency differences between case patients and control subjects were tested for each SNP using a χ^2 test with 1 degree of freedom. Allelic OR and 95% confidence interval (95% CI) were estimated based on a multiplicative model.

Results

Detection of somatic *PPP2R2A* deletion status in prostate cancer (PCa)

The deletion status of *PPP2R2A* in 141 pairs of samples were analyzed using our Affymetrix 500k/6.0 SNP array data. With the application of allele-specific analysis algorithm, which takes advantage of genotype information and allele-specific intensities from paired samples to estimate DNA copy number, we found total 87 tumors (61.7%) showed *PPP2R2A* gene deletion, including 3 (2.1%) homozygous deletion and 84 (59.6%) hemizygous deletion, among 141 prostate tumor samples. The homozygous deletions were shown in Figure 1.

To clarify the relationship between clinical features and *PPP2R2A* deletion, we categorized PCa-specific death (dead or live), Gleason Scores (GS ≤ 7 or >7), \log_2 transformed preoperative serum prostate specific antigen (PSA, ≤ 10 ng/ml or >10 ng/ml), and tumor stage (TNM, T3a or $>T3a$) into two groups. Overall, the deletion of *PPP2R2A* was not correlated with PCa-specific death, GS, PSA or stage shown in Table 2.

We also reanalyzed germline *PPP2R2A* deletion status in a JHH study population consisting of 500 nonaggressive and 448 aggressive PCa cases. A subset of these DNA samples were used as normal controls in our somatic copy number analyses since no matching normal prostate tissues were available. No germline hemizygous or homozygous deletions were found in any PCa cases, including the three subjects in which we observed homozygous deletion in tumor tissues.

Identification of mutations and sequence variants in *PPP2R2A*

The *PPP2R2A* gene consists of 10 exons and spans ~ 79.6 -kb genomic DNA. The full length of mRNA transcript of this gene is 2,385 bp (NM_002712.2), with a 5'-untranslated region (UTR) of 302 bp and a long 3'-UTR of 720 bp.

A total of 25 sequence variants were identified in the sequenced region of *PPP2R2A* among 96 HPC probands. The location and frequency of each sequence variant is listed in Table 3. Among 25 germ-line variants identified in this analysis, there were 15 known SNPs and 10 new sequence variants. Most new sequence variants were rare, with the exception of 2 new sequence variants with minor allele frequency (MAF) ≥ 0.05 (-482T/C MAF=0.072 and -78C/G MAF=0.058) and in high LD ($r^2 = 0.82$). SNPs 70985A/G (L253L) and 74521C/T (L333L) are in coding regions, however, neither of these SNPs result in amino acid change, nor were located in exonic splicing enhancer and may not have significant effect on pre-mRNA splicing. No consensus-binding sites of transcription factors were found for the two promoter variants. For the two SNPs located in the 3'-UTR, neither of them are located in the pentamer AUUUA or nonamer UUAUUUAUU of AU-rich regulatory sequence motifs. The remaining 18 SNPs are in intronic regions.

Association between prostate cancer (PCa) susceptibility and the SNPs in *PPP2R2A*

All the SNPs and sequence variants identified were in HWE in 96 probands. Pair-wise LD tests for all SNPs were also performed and results are as follow: new SNP -482T/C, -78C/G and rs3808574 are in strong LD with each other, with estimated r^2 of 0.82 (-482T/C and -78C/G), 0.82 (-482T/C and rs3808574) and 0.75 (-78C/G and rs3808574); similarly, SNPs rs17309515, rs17309529 and rs17400920 also showed strong LD, with estimated r^2 of 1 (rs17309515 and rs17309529), 0.89 (rs17400920 and rs17309529) and 0.89 (rs17400920 and rs17309515); and another pair of SNPs rs12682345 and rs13264882 is in strong LD with r^2 of 0.92.

Since 15 known SNPs were also found in sequencing our 96 HPC probands, we want to see whether there is any association between these known SNPs and sporadic PCa. Among these 15 SNPs, there were 10 genotyped or imputed SNPs spanning the entire *PPP2R2A* included in the JHH population study and the Illumina IControl database. Re-analysis showed that there were not any statistical differences in the allele frequencies between 1,909 cases and 3,085 Illumina Icontrols for sporadic PCa, similarly to the lack of statistical difference between 1,402 aggressive and 1,445 non-aggressive PCa cases in JHH study (Table 4).

Discussion

This is the first report in which comprehensive somatic and germ-line analysis for *PPP2R2A*, a gene that is a potential tumor suppressor gene, were systemically evaluated in

141 clinical PCa samples, 96 HPC probands, as well as 983 aggressive cases and 572 controls from the JHH study. Interestingly, *PPP2R2A* was commonly deleted (61.7%) among prostate tumors with 2.1% homozygous deletion. Again, multiple common and rare germ-line variants in *PPP2R2A* were identified, however the variants we found were not associated with sporadic PCa in the JHH population and Illumina IControls.

PPP2R2A is a candidate gene for PCa susceptibility because of its chromosomal location and potential tumor suppressor function. Furthermore, the results from our study suggest that *PPP2R2A* may play a more important role in PCa tumorigenesis on somatic levels. This conclusion is based on the following three observations. *PPP2R2A* was commonly deleted in clinical 141 PCa tumors (61.7%), with 3 recurrent homozygous deletion (2.1%). Second, no potentially important mutation, such as missense mutation or synonymous mutation in exonic splicing enhancer, was found after re-sequencing of *PPP2R2A* in germ line DNA samples of 96 HPC probands. And lastly, no significant association between common SNPs of *PPP2R2A* and sporadic PCa was observed after analysis of the 10 SNPs, spanning ~78.8 kb and providing good coverage of the *PPP2R2A* gene (~79.6 kb), in JHH PCa case and Illumina IControl studies. It has been postulated that protein phosphatases are involved in the suppression of cellular growth and cancer development by antagonizing protein kinases. PP2A is a multimeric serine/threonine protein phosphatase consisting of a 36 kDa catalytic subunit (PP2Ac) and a 65 kDa scaffolding subunit (PR65 or A subunit) (13). An additional regulatory subunit is associated with this dimer, including four classes (B, B', B'' and B'''). Many studies had showed that PR65/A (*PPP2R1A* and *PPP2R1B*) is a tumor suppressor (16, 17, 24-27). The *PPP2R2A* gene encodes α isoform (aka B55 α or PR55 α) of the regulatory B55 subfamily of PP2A. A role for the PR55/B subunit in the regulation of mitosis is suggested by the finding that deletion of this gene is associated with cytokinesis defects in both yeast and *Drosophila*(28). In mammalian cells, PR55/B subunit seems to be essential for the intermediate filaments dephosphorylation (29). Furthermore, PP2A has been thought to regulate cellular transformation (30) and function as a tumor suppressor (13). PP2A-B55 α had been found to contribute to this tumor suppressor activity by negatively regulating pathways such as the MAPK and Akt signaling to ensure normal growth of cells (31).

High frequencies of a deletion of *PPP2R2A* combined with no germline sequence variations associated with PCa from our results suggest that to fully understand the contribution of *PPP2R2A* in the PCa pathogenesis, somatic mutation and methylation should be explored in further studies. Other important candidate genes in the 8p regions should be analyzed by applying multiple genetic and epigenetic approaches, such as cytogenetics, sequence variants, germline CNV, somatic mutation, copy number change and promoter methylation.

Conclusions

In summary, *PPP2R2A* may play a more important role in the PCa pathogenesis on a somatic level. To fully explore the potential candidate tumor susceptibility role of *PPP2R2A*, further studies will be necessary to understand the contribution of other somatic changes in the prostate carcinogenesis.

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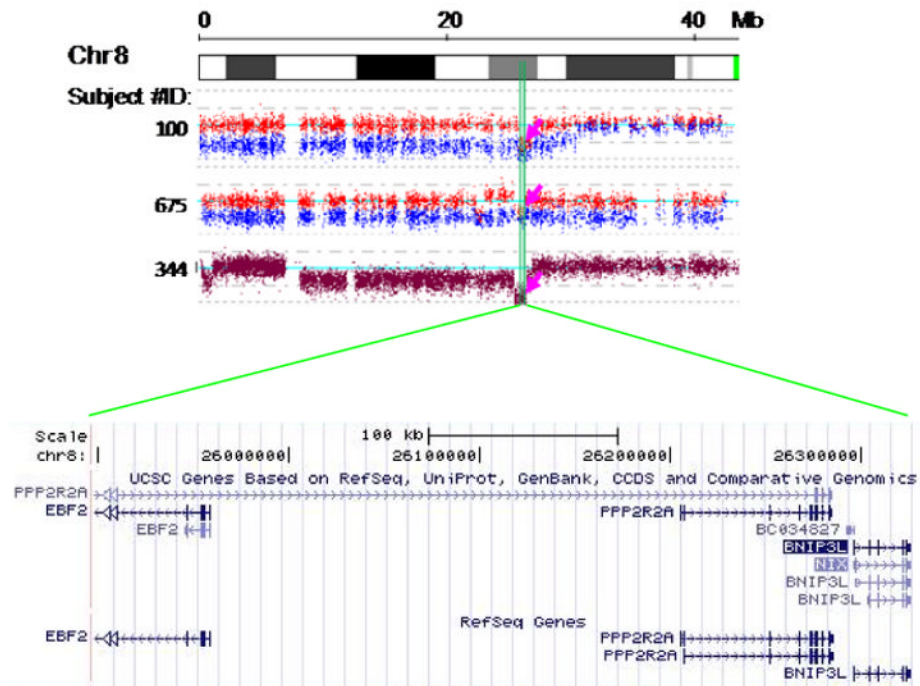


Figure 1. Mapping of concurrent homozygous deletions on 8p21.2 in three PCa tumor samples
 The consensus homozygous deletion regions (chr8: 25,897,499-26,348,514) marked by green lines. Detailed gene physical distributions within this region were extracted from UCSC Genome Browser (NCBI36/hg18) website. Paired samples 100 and 675 were analyzed with allele-specific analysis, with one allele marked with red color and another one labeled with blue color. Sample 344 was analyzed using non-allele-specific analysis, since no normal sample was available at the time of analysis.

Table 1
Primers used for mutational analysis and SNPs identification

PCR ID	Forward primer	Reverse Primer	PCR product (bp)	Annealing temperature (°C)
1	TCCTTGCAGGAGAGCACGTA AACT	AATAAGGAAGCGGTGCCTTGTCAG	671	60
2	ACTGGTGCTCTTGGTGGTAGTTGT	CTGAGCCAAATGTTGATGGCACCT	608	60
3	TGAAGGCACATAGCACGAAG	ATCACTTCCACCCCTTCT	906	68
4	TGAGAATGTGCAGGGTCCTTTGGA	ACCTGCCCAAGATTACGAGACTGA	563	58
5	GGGATAGAGACCTGTCTGTGACTT	ACCAGAGATGAAGTCTGATGGCGT	616	60
6	TTCACGCCATCAGACTTCATC	ATTATGCCCTTTATGTTTAGCA	839	54
7	AGTTGTAGTGGTGTGCGCCTGTAA	GCCCAACCGCAAGTTACCAAAGAA	749	62
8	ACCTGGTGCACCTGAAGTCAGAAGT	TTTCTCCAATCCTCTTCCTGCCCA	786	68
9	CCTGAAGGGCTTGTGTGTTT	GTTATCTGCCGCTGAATGT	619	58
10	GCCTGGTTGATCACATAGCCTGTAGT	TCACTGTGCCCAAAGGTATGACAC	1386	60

All primers are 5'→3' oriented.

Table 2
Clinical characteristics of prostate cancer samples with *PPP2R2A* deletion

<i>PPP2R2A</i> deletion (%)	Gleason Score		<i>P</i>
	7	>7	
+	57 (64.8)	25 (58.1)	0.46
-	31 (35.2)	18 (41.9)	
<i>PPP2R2A</i> deletion (%)	PSA (ng/ul)		<i>P</i>
	<10	10	
+	40 (62.5)	47 (61.0)	0.86
-	24 (37.5)	30 (39.0)	
<i>PPP2R2A</i> deletion (%)	PCa-specific death		<i>P</i>
	Dead	Alive	
+	13 (59.1)	69 (61.6)	0.82
-	9 (40.9)	43 (38.4)	
<i>PPP2R2A</i> deletion (%)	T stage		<i>P</i>
	T3a	>T3a	
+	39 (54.9)	38 (67.9)	0.14
-	32 (45.1)	18 (32.1)	

Table 3
Identified sequence variants in the *PPP2R2A* gene

Variant ID	Position	SNP ID	Amino acid change	Minor allele	MAF
-588C/T	Promoter	rs7840855		T	0.396
-482T/C	Promoter			C	0.072
-78C/G	5'UTR			G	0.058
1953A/G	Intron2			G	0.005
1977A/G	Intron2			G	0.005
2102C/T	Intron2			T	0.011
2268T/G	Intron2	rs2046224		G	0.435
47287T/C	Intron3	rs3808574		C	0.068
47297A/T	Intron3	rs3808573		T	0.145
62916G/C	Intron4	rs3779915		C	0.011
68520A/C	Intron5	rs17309515		C	0.051
68939A/T	Intron5	rs17309529		T	0.048
68996T/C	Intron5	rs12682345		C	0.314
70985A/G	Exon7		Leu253Leu	G	0.005
71106A/G	Intron7	rs17309592		G	0.052
74521C/T	Exon9	rs34109536	Leu333Leu	T	0.011
74601C/A	Intron9			A	0.011
74904T/G	Intron9	rs13264882		G	0.304
78050C/G	Intron9	rs17400920		G	0.062
78148C/T	Intron9	rs3200031		T	0.051
78177T/C	Intron9			C	0.006
78263A/C	Intron9	rs3824232		C	0.117
78304A/G	Intron9	rs3808565		G	0.056
79115G/C	3'UTR			G	0.006
79192G/C	3'UTR			C	0.006

Table 4
Association of 10 SNPs at *PPP2R2A* with prostate cancer risk in JHH and Illumina IControl

SNP	Allele	JHH-Illumina IControl				JHH(Aggressive-Non-aggressive)					
		Cases (n=1909)	Illumina IControls (n=3085)	P	OR	% missing data	Aggressive cases (n=1,402)	Non-aggressive Cases (n=1,445)	P	OR	% missing data
rs2046224	G	0.4185	0.4225	0.6908	0.9835	0.0006	0.4165	0.4245	0.5454	0.9680	0.0007
rs3808574	C	0.0393	0.0400	0.8620	0.9812	0.0679	0.0349	0.0326	0.6386	1.0730	0.0527
rs3808573	T	0.1568	0.1452	0.1331	1.094	0.0923	0.1492	0.1473	0.8470	1.0150	0.0889
rs17309515	C	0.0522	0.0548	0.5879	0.9502	0.0509	0.0583	0.0531	0.4015	1.1050	0.0534
rs17309529	T	0.0496	0.0530	0.4457	0.9310	0.0008	0.0558	0.0513	0.4462	1.0940	0.0018
rs12682345	C	0.2904	0.3056	0.1078	0.9300	0.0014	0.2946	0.3057	0.3610	0.9485	0.0011
rs17309592	G	0.0495	0.0532	0.4239	0.9278	0.0004	0.0558	0.0513	0.4500	1.0930	0.0014
rs17400920	G	0.0496	0.0534	0.4045	0.9249	0.0014	0.0558	0.0513	0.4424	1.0950	0.0021
rs3824232	C	0.1462	0.1359	0.1483	1.0890	0.0056	0.1375	0.1398	0.8023	0.9809	0.0035
rs3808565	G	0.0485	0.0438	0.2758	1.1130	0.0138	0.0405	0.0399	0.9018	1.0170	0.0169