Identification of novel SNPs associated with risk and prognosis in patients with castration-resistant prostate cancer

Aim: Metabolism and transport play major roles in life-long exposure to endogenous and exogenous carcinogens. We therefore explored associations between polymorphisms in absorption, distribution, metabolism and elimination genes and the risk and prognosis of castration-resistant prostate cancer (CRPC). **Materials & methods:** A total of 634 genotypes were tested in 74 patients using the Affymetrix DMETv1.0 platform. **Results:** No relation to risk was found. Three SNPs were associated with CRPC prognosis in Caucasians: *ABCB11* rs7602171G>A ($p = 0.003$; $n = 30$; hazard ratio [HR]: 0.307), *GSTP1* rs1799811C>T ($p = 0.001$; $n = 38$; HR: 0.254) and *SLC5A6* rs1395 ($p = 0.004$; $n = 35$; HR: 3.15). Two other polymorphisms among Caucasians were associated with interesting trends: *ABCB4* rs2302387C>T ($p = 0.039$) and *ABCC5* rs939339A>G ($p = 0.018$). **Conclusion:** This exploratory study is the first to show that polymorphisms in several absorption, distribution, metabolism and elimination genes may be associated with CRPC prognosis.

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Keywords: castration-resistant prostate cancer • genotype • metabolism • outcome • survival • transport

There is strong evidence that sterol/androgen disposition [1,2], obesity [3,4] and carcinogen activation [5] are related to prostate carcinogenesis and/or disease progression. The liver is central to regulation of each of these processes, and variation in gene-encoding enzymes and polypeptides that metabolize and transport endogenous and environmental carcinogens may explain why some are predisposed to cancer, or a poor prognosis. It is therefore surprising that no study has yet applied a broad genotyping array including genes involved in absorption, distribution, metabolism and elimination (ADME) to explore if such alleles are related to the incidence and prognosis of prostate cancer (PCa).

Previous studies have determined that individual candidate SNPs in the pregnenolone/17- α -hydroxypregnenolone hydroxy-

lase/lyase, encoded by *CYP17*, are associated with the risk of developing PCa [6–13] while others have not found this association [14–16]. An SNP in *UGT2B15*, rs4148269, was associated with PCa risk due to variable expression of a *cis*-regulatory element resulting in differential expression of the steroid hormone glucuronidase [17,18].

Still other studies have investigated genes involved in so-called ‘multiple chemical sensitivity’, which is linked to the idiopathic development of several diseases through various endogenous and environmental exposures [19]. For instance, *CYP1A1* is an oxidative-metabolizing enzyme that both mediates the activation of carcinogens and metabolizes estrogen. Researchers have found correlations between the risk of developing PCa and a single polymorphism (Ile462Val) in the *CYP1A1* gene [20–22], although the find-

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ings have not been confirmed by other studies [23,24]. The Phase II enzyme family of *N*-acetyltransferases (NATs) metabolizes hydralazines and arylamines, including potential carcinogens, and is therefore also considered to play a role in multiple chemical sensitivity. Hamasaki *et al.* found that Japanese men with the *NAT2*5* slow acetylator phenotype had a higher rate of PCa [25], whereas Hein *et al.* reported that individuals with a combined *NAT1*10* and *NAT2*5* genotype had over a sevenfold higher risk of developing the disease [26].

To study how ADME genes affect PCa risk and progression, we genotyped a cohort of patients with castration-resistant prostate cancer (CRPC) using the Drug Metabolizing Enzymes and Transporters Array (DMET; Affymetrix, CA, USA) and compared genotypes to survival from diagnosis. We also genotyped *CYP17*, *CYP1A1*, *NAT2* and *UGT2B15* polymorphisms in a cohort of cancer-free controls to ascertain whether SNPs in these genes conferred risk of CRPC in the present group of subjects.

Materials & methods

Subjects

Patients diagnosed with CRPC were enrolled in a randomized Phase II clinical trial comparing docetaxel to docetaxel with thalidomide. The results of this trial have been previously reported [27]. In brief, 74 patients with CRPC were enrolled with a 2:1 randomization, to receive either docetaxel and thalidomide, or docetaxel alone. Docetaxel was dosed at 30 mg/m² weekly for 3 weeks followed by a 1-week rest. For patients assigned to the combination arm, they received docetaxel on the same schedule, as well as thalidomide at 200 mg orally each day. Forty-nine patients were on the combination arm, and 25 patients were on the control arm. Of these, 35 patients had bone-only disease, 31 had bone and soft-tissue lesions and eight had soft-tissue-only disease. The study was approved by the National Cancer Institute (NCI) Institutional Review Board.

Samples & genotyping

For 47 of the 74 originally enrolled patients, stored buffy coat samples were archived and available for DNA extraction. This included 33 patients on the combination arm of the trial and 14 patients on the docetaxel-alone arm. Whole blood was collected from patients, and the buffy coat layers were resuspended and frozen in Trizol (Invitrogen). Genomic DNA was extracted from serum or white blood cell buffy coat layers using the QIAamp DNA Blood Kit as described by the manufacturer (Qiagen, CA, USA).

The Affymetrix DMETv1.0 (Drug Metabolizing Enzymes and Transporters) platform was used to

detect 1243 genetic variations in 169 genes involved in substrate metabolism. These include 49 *CYP450* genes, 73 non-*CYP* genes and 47 transporters that metabolize endogenous substrates, exogenous carcinogens and chemotherapy agents as described in a previously published article using the same patient cohort for a different purpose [28].

For direct-sequencing assays, primer pairs were designed on the basis of the gene sequence available at Genbank, and the sequences of each primer pair are listed as follows: *CYP17* rs743572 F1 5'-TTGCACTCTGGAGTCATTCA-3' R1 5'-AGTCTTG-GTGCCCATACGAAC-3'; *UGT2B15* rs4148269 F1 5'-AACCAATGAAGCCCCTGGATC-3' R1 5'-TCTCCAAATGCTATCCTTCC-3'; *NAT2* F1 5'-ACTGACATTCTTGAGCACCA-3' R1 5'-TG-AATCACTCTGCTTCCCAA-3'. PCR reactions were carried out in a 50-μl reaction mixture containing 200 ng of genomic DNA, 1× PCR buffer (Invitrogen), 1.5 mmol/l MgCl₂, 0.2 mM deoxynucleotide triphosphates, 800 nM of each primer (i.e., F1 and R1) and 1.25U Platinum Taq DNA polymerase (Invitrogen, CA, USA) using a GeneAmp PCR system 9700 (Applied Biosystems, CA, USA) as a thermocycler with the following thermal profile (primary PCR): 40 cycles of denaturation at 94°C for 30 s, annealing at 66°C for 30 s and extension at 72°C for 30 s. After amplification, the quality of the amplified PCR products was verified by agarose gel electrophoresis. Sequencing PCR was carried out with Big Dye (v3.1; Applied Biosystems) using the following PCR primers: *CYP17* rs743572 F3 5'-CCAGGAGATCTTTCCACAA-3' R3 5'-CTTGGGGTACTTGGCACCAG-3'; *UGT2B15* rs4148269 F2 5'-TCTTTGGATGTGATAGCATTTC-3' R2 5'-TGAA-ATAAAGGAGGAGTCCC-3'; *NAT2*CORE F4 5'-CCACAATGTTAGGAGGGTATTTT-3' CORE R4 5'-ACTGCTCTCTCCTGATTTGGT-3'. The PCR products were then sequenced on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) per the manufacturer's instructions. *CYP1A1*2C* rs1048943 and *PPARγ* rs1801282 were genotyped with TAQman genotyping assays (Applied Biosystems) and run on an Applied Biosystems StepPlus RealTime PCR machine as per the manufacturer's instructions.

PCa risk analysis

The frequency of SNPs in genes previously reported to correlate with PCa risk were determined and compared with a de-identified PCa-free Caucasian population, as determined by digital rectal exam (purchased from Valley Biomedical, VA, USA). The mean age of the controls ± standard deviation was

40.4 years (13.3 years). These data were compared with Caucasian patients with CRPC (n = 43), of which 30 patients were on the combination arm of the trial and 13 patients were on the docetaxel-alone arm. Data analysis was limited to Caucasian patients to control for interethnic variation in SNP frequencies. SNP variants in all genes included on the DME-T 1.0 panel were also tested for an association with survival. Clinical data included in the analysis were overall survival (OS), measured from date of diagnosis to date of death or last contact. Results were censored if the patient was alive at the last contact.

Statistical analysis

Divergence from Hardy–Weinberg equilibrium (HWE) was determined using an exact Chi-square test with 2 degrees of freedom. To compare the genotype frequencies between these CRPC patients with those found in the general population, Fisher's exact tests were conducted comparing variant alleles to the reference genotype. Odds ratios (ORs) were calculated comparing heterozygote and homozygote variant to homozygote wild-type.

To test for an association between genetic variants and OS, a Cox regression model was used. Two dichotomous (dummy) variables were created to represent either the presence of a wild-type or variant allele. In cases where $p < 0.01$ and there were at least eight patients in each group, Kaplan–Meier plots were assembled and a log–rank test was performed to compare the groups; median OS (95% CI) is reported for select groups. Exact log–rank p-values were used as appropriate. Hazard ratios (HRs) and their 95% CIs are reported as well.

Extracted DNA concentrations were between 3.0 and 930.9 ng; however, 2000 ng is recommended for optimal DMET analysis. Therefore, since we used lower than the recommended DNA concentration for several samples, we obtained call rates of between 88 and 98% for all samples containing at least 92 ng of DNA, and lower call rates for other samples (n = 15; 21–91% call rates). In the few cases in which there was evidence for an association with survival, we also evaluated the missing data as a group to ascertain whether there was potential for bias. We recognize that if the missing data group was similar to only one of the other groups, there was potential bias in the results.

In general, given the very large number of evaluations performed with data only for 47 patients, strong associations are assigned to those cases in which $p < 0.005$, and those cases in which $0.005 < p < 0.05$ are considered notable trends. p-values should only be used as a relative measure of potential importance and used in conjunction with confidence intervals. Sta-

tistical analyses were performed using SAS/STAT(R) software (version 9.3). Results are to be interpreted as hypothesis generating and in the context of a large number of exploratory evaluations performed.

Results

Genotyping

Patient characteristics at baseline are reported in Table 1. Of the 1243 successful genotyping assays conducted on the DMET array, 609 assays demonstrated no variation in Caucasians with PCa (n = 43). Of the remaining 634 genotypes, 22 assays were out of HWE ($p_{\text{[exact]}} < 0.01$). As the presence of advanced PCa could influence random inheritance in the population, we did not exclude SNPs that were out of HWE in patients.

SNPs versus OS after diagnosis

Genotypes were evaluated for association with OS from diagnosis in Caucasian men with CRPC. The combination of the Cox model screening process and careful examination of Kaplan–Meier plots revealed that only three variables were strongly associated with OS. The rs7602171 G>A SNP in *ABCB11*, encoding the bile salt export pump, was associated with a 5.1-year shorter OS (median OS [95% CI]: 5.47 years [3.67–8.62]) than those carrying only wild-type alleles (10.6 years [7.71–12.8]; $p = 0.003$; adjusted $p = 0.006$; Figure 1A). The rs1799811 C>T SNP in *GSTP1*, encoding the glutathione S-transferase pi, was associated with prolonged OS (12.4 years [6.2–15.1]) when compared with those carrying only wild-type alleles (7.40 years [5.30–8.92]; $p = 0.001$; Figure 1B).

Table 1. Patient characteristics at diagnosis.

Patient characteristics	Summary statistics
Patients (n)	47
Race:	
– Caucasian	43
– African–American	3
– Asian	1
Age (years)	61.8 (40.3–72.8)
Gleason	7.9 (6–10)
PSA	57.9 (0.20–1777.0)
Hemoglobin	13.5 (8.4–15.4)
Lactate dehydrogenase	183 (116–777)
Alkaline phosphatase	116 (50–591)
ECOG	ECOG 0: n = 12 ECOG 1: n = 45
Summary statistics are expressed as median and minimum to maximum. On-study patient characteristics are reported in Deeken <i>et al.</i> [28]. ECOG: Eastern Cooperative Oncology Group; PSA: Performance status.

Figure 1. Overall Survival by genotype. (A) *ABCB4* rs2302387 (median OS = 9.7 vs 4.7 years); (B) *ABCB11* rs7602171 (median OS = 10.6 vs 5.5 years); (C) *SLC10A1* rs4646285 (median OS = 4.7 vs 8.9 years); (D) *PPARδ* rs1883322 (median OS = 8.3 vs 9.8 vs 2.7 months); (E) *ABCC5* rs939339 (median OS = 9.8 vs 5.0 years). DDx: Date of diagnosis.

Carriers of the rs1395 SNP in *SLC5A6*, encoding the sodium-dependent multivitamin transporter, experienced poor OS (6.75 years [4.07–8.92]) when compared with those carrying wild-type alleles (9.78 years [4.68–12.8]; $p = 0.004$; adjusted $p = 0.008$; Figure 1C).

Whereas findings for *ABCB4* and *ABCC5* did not meet the *a priori* criteria for Kaplan–Meier analyses, these findings are considered notable trends. For *ABCB4*, encoding the hepatic phosphatidylcholine floppase, carriers of the rs2302387 C>T SNP had poor OS (4.68 years [3.67–6.23]) compared with those carrying only wild-type alleles (9.65 years [6.20–10.6]; $p = 0.039$; Figure 1D). Carriers of the rs939339 A>G SNP in *ABCC5*, encoding the multidrug resistance-associated protein 5, also had poor OS (5.00 years [2.52–7.76])

compared with those carrying only wild-type alleles (9.75 years [6.72–11.2]; $p = 0.018$; adjusted $p = 0.036$; Figure 1E). See Table 2 for the HRs and their 95% CIs.

Since not all samples were genotyped by DMET, we compared the OS of patients' missing genotype data to the other genotype groupings. The 'missing' data group overlapped with one of the genotype groupings in some cases; therefore, results pertaining to three SNPs may be potentially biased: *ABCB4*, *ABCC5* and *SLC5A6*.

Finally, when patients of other races ($n = 3$) were included with Caucasians ($n = 43$), the HR for *ABCB11* rs7602171 G>A became only slightly larger (0.307 vs 0.326; both with the same 95% CI of 0.149–0.714). Likewise, for both *GSTP1* rs1799811 and *SLC5A6* rs1395, the HRs changed very little with the addition of three non-

Table 2. Hazard rates.

Gene	Variant	Alleles compared (n) [†]	HR	HR 95% CI
<i>ABCB11</i>	rs7602171	GG (15) vs GA/AA (15)	0.307	0.149–0.714
<i>GSTP1</i>	rs1799811	CC (30) vs CT (8)	0.254	0.094–0.690
<i>SLC5A6</i>	rs1395	AA (15) vs AG/GG (20)	3.15	1.39–7.09
<i>ABCB4</i>	rs2302387	CT (7) vs CC (27)	0.278	0.110–0.699
<i>ABCC5</i>	rs939336	AA (27) vs AG (8)	4.50	1.78–11.4

[†]Reference allele given first.
HR: Hazard ratio.

Caucasian patients (OR [n = 43–46]: 0.254–0.256 and 3.15–2.96, respectively; CIs were nearly identical).

Variation in genes implicated in PCa risk

Next, we genotyped *NAT2**5 (341T>C; rs1801280), *CYP1A1**2C (I462V; rs1048943), *PPAR* γ (intronic variant; rs1801282), *UGT2B15* (K523T; rs4148269), *COMT* (V158M; rs4680) and *CYP17* (-34T>C; rs743572) in Caucasian controls and compared the genotype frequencies to the CRPC population. All candidate SNPs data and HWE p-values are reported in Table 3. We had previously published that the *CYP17* -34C allele was not related to risk of CRPC in

a cohort consisting of these and several other patients with CRPC [29]. Consistent with the findings of that study, the frequency of the *CYP17* -34C allele was not considerably different in cases than in controls (Table 3). However, the *NAT2**5 allele was associated with risk but with a wide confidence interval (OR [95% CI] = 3.97 [1.12–17.6]; see Table 2) indicating low precision of the estimated OR.

Discussion

To our knowledge, this is the first time that a broad genotyping array, which represents numerous polymorphisms in genes involved in ADME of drugs and

Table 3. Comparison of genotypic frequencies of candidate SNPs previously linked to prostate cancer risk in Caucasian patients diagnosed with castration-resistant prostate cancer versus healthy Caucasian controls.

Gene	Type	Cases	Controls	p-value [†]	OR	OR 95% CI
<i>CYP17</i> [‡]	TT	15	21			
	TC	14	36	0.248	0.544	0.220–1.35
	CC	4	13	0.235	0.431	0.117–1.58
<i>UGT2B15</i>	GG	5	14			
	TG	24	33	0.28	2.04	0.583–8.16
	TT	10	23	1.0	1.22	0.298–5.50
<i>NAT2</i>	TT	4	20			
	TC	27	34	0.024	3.97	1.12–17.6
	CC	11	14	0.062	3.93	0.89–19.9
<i>CYP1A1</i>	II	0	2 [§]			
	IV	6	4	0.45	NA	NA
	VV	27	61	1.0	NA	NA
<i>PPAR</i> γ	GG	24	53			
	GC	11	15	0.34	1.62	0.577–4.42
	CC	1	1	0.54	2.21	0.027–177

[†]Fisher's exact test.

[‡]*CYP17* -34T>C genotype was previously compared versus prostate cancer risk in a larger population [13]. However, in the present study, we identified genotyping errors in the previous data set, which were a function of old genotyping technology (restriction fragment length polymorphism detection). Therefore, corrected data obtained from drug metabolizing enzyme and transporter analysis and direct sequencing are reported for this cohort.

[§]Inheritance was not consistent with Hardy–Weinberg equilibrium (*CYP1A1**2C; p = 0.010).

OR: Odds ratio; NA: Not applicable.

endogenous molecules, has been applied to study risk or survival from diagnosis of PCa. Most cancers arise from a complex interaction between genetic and environmental factors that are often poorly understood [30], and the liver is integral to the lifelong exposure to many of these factors. Since genetic variability in liver enzymes is often linked to interindividual variation in liver metabolism, the present study suggests that certain important pathways and variants may be behind the risk and prognosis of CRPC.

Of considerable interest are several associations between PCa prognosis and transporters that regulate bodily sterol and fatty-acid disposition. The present study suggests that variants in the major bile-flow transporters are linked to variation in PCa cancer prognosis: *ABCB11* (bile salt export pump) and *ABCB4* (phosphatidyl-choline floppase, MDR3). Notably, a rather nontrivial 5-year OS difference was attributed to the polymorphism in *ABCB11* and *ABCB4*. These findings should be verified by independent studies.

Evidence suggests that decreasing serum cholesterol slows prostate tumor development, at least in part, due to reductions in the uptake of androgens and androgen precursors into prostate tumor tissues [31]. The human bile acid pool undergoes enterohepatic recirculation, which occurs multiple times per meal. As bile acids are eliminated through the fecal route, new bile acids are formed from hepatic cholesterol. Bioconversion of cholesterol into bile acids, and subsequent efflux of sterols through the canalicular membrane represent the main elimination pathway for cholesterol and steroids [32] and are the major mechanism of statin-induced serum cholesterol reductions [33]. Therefore, the present data suggest that interindividual variation in cholesterol efflux from such transporters is potentially responsible for tumor progression.

The DMET array analysis also suggests that SNPs in *MOATC* (*ABCC5*), the sodium dependent-vitamin transporter (*SLC5A6*) and the glutathione *S*-transferase pi (*GSTP1*) were associated with OS from diagnosis. These survival differences are also approximately 5-year differences (3 years for *SLC5A6*). No study has yet posited any role for the SNPs identified *ABCC5* and *SLC5A6*, which are involved in resistance to purine analogs used in cancer and multivitamin uptake, respectively [34,35]. Therefore, future studies should clarify what role these SNPs play in PCa. Another SNP in *GSTP1* has been previously linked to susceptibility of PCa and other genitourinary malignancies, which the authors attribute to the ability of *GSTP1* to detoxify carcinogens, such as benzo(*a*)pyrene diol epoxide [36]. This may also be the case in the present study; however, we could not test that hypothesis in the present group of patients.

The present study is consistent with our previous findings that the -34T>C SNP in *CYP17* does not confer PCa risk [29], although this has been a subject of considerable debate [6–16]. Other SNPs were not associated, although *NAT2*5* was possibly related to risk. This finding is also controversial [25,26].

In this pilot study, we have found evidence that SNPs in bile acid and fat catabolism genes may be related to PCa OS. We did not find evidence that any of the aforementioned SNPs were related to PCa risk, and our previous study showed no evidence that these SNPs are related to docetaxel or thalidomide outcomes [28]. While it is acknowledged that the present study is small and consists of multiple comparisons, these data suggest further studies may be warranted.

Conclusion

In summary, this is the first study to examine hundreds of genotypes in genes encoding DMETs in CRPC. Data suggest that bile acid and fat catabolism genes are related to the overall survival from diagnosis to death in men who are diagnosed with CRPC.

Future Perspective

This exploratory study suggests that future efforts should be devoted to understanding inter-individual variation in cholesterol disposition in men with prostate cancer. While it is known that decreasing serum cholesterol is related to slower prostate cancer progression, the specific pathways that regulate hepatic cholesterol disposition are not well-studied in this disease. Other pathways, such as transport of vitamin D and redox status, are implicated as well, and the present findings require validation.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles

outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Several genes that mediate absorption, distribution, metabolism, elimination and activation are putatively associated with lifetime exposure to endogenous and exogenous carcinogens.
- No study has yet applied a broad genotyping array to study absorption, distribution, metabolism, elimination and activation in prostate cancer (PCa).
- In this exploratory study, 634 genotypes were compared with overall survival from diagnosis in 47 patients with castration-resistant prostate cancer. PCa risk was also compared with genotypes of five commonly associated PCa risk genes in these patients versus cancer-free controls.
- Associations with overall survival differences were detected between: an SNP in each of two transporters involved in bile flow, *ABCB4* (rs2302387) and *ABCB11* (rs7602171), an SNP in a detoxification enzyme, *GSTP1* (rs1799811) and a SNP in each of two genes with pleiotropic function, *ABCC5* (rs939336) and *SLC5A6* (rs1395).
- No associations were detected with risk alleles.

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