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Original Article

Quantified gene expression levels for phase I/II metabolizing enzyme and estrogen receptor levels in benign prostate from cohorts designated as high-risk (UK) versus low-risk (India) for adenocarcinoma at this organ site: a preliminary study

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Abstract

Risk of clinically significant prostate adenocarcinoma (CaP) varies worldwide, although there is a uniform prevalence of latent disease. A hormone-responsive tissue, the prostate possesses the metabolizing capacity to biotransform a variety of environmental procarcinogens or endogenous hormones. Whether such metabolizing capacity or estrogen receptor (ER) status underlies these demographic differences in susceptibility to CaP remains unclear. With appropriate ethical permission, verified-benign tissues were obtained following transurethral resection of the prostate from a high-risk region (n = 12 UK-resident Caucasians) and a typically low-risk region (n = 14 India-resident Asians). Quantitative gene expression analysis was employed for cytochrome P450 (*CYP*)*1B1*, *N*-acetyltransferase (*NAT*)*1*, *NAT2*, catechol-*O*-methyl transferase (*COMT*), sulfotransferase (*SULT*)*1A1*, *ERα*, *ERβ* and aromatase (*CYP19A1*). To quantify the presence or absence of CYP1B1, ERα or ERβ, and to identify their *in situ* localization, immunohistochemistry was carried out. The two cohorts had reasonably well-matched serum levels of prostate-specific antigen or hormones. Expression levels for the candidate genes investigated were similar. However, clear differences in protein levels for CYP1B1 and ERβ were noted. Staining for CYP1B1 tended to be nuclear-associated in the basal glandular epithelial cells, and in UK-resident Caucasian tissues was present at a higher (P = 0.006) level compared with that from India-resident Asians. In contrast, a higher level of positive ERβ staining was noted in prostates from India-resident Asians. These study findings point to differences in metabolizing capacity and ER status in benign prostate tissues that might modulate susceptibility to the emergence of clinically significant CaP in demographically distinct populations.

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1 Introduction

Risk of prostate adenocarcinoma (CaP) varies worldwide and the etiological mechanisms for this remains obscure. Genetic, environmental, dietary and

other lifestyle factors may influence the progression of clinically significant CaP [1]; three lines of evidence implicate non-heritable environmental and/or lifestyle factors. First, occurrence of CaP increases in ethnic groups that migrate from low-risk to high-risk regions [2–5]. Second, autopsy studies on latent CaP show a uniform prevalence of the disease in different populations despite the apparent disparity in occurrence of the clinically significant entity. This could suggest that, although initiating factors for latent CaP may be similar in different populations, the mechanisms through which the disease is promoted and/or progressed so that it becomes invasive might be different [6]. Finally, there is evidence of a dramatic rise in CaP incidence in traditionally low-risk countries that might be associated with the adoption of a more western lifestyle and diet [7, 8].

There is almost an 80-fold difference in CaP occurrence between low-risk (for example, China) and high-risk (for example, USA) regions; the differences in mortality between the same areas is almost 16-fold [8, 9]. CaP incidence in the India-resident Asian population is approximately ninefold lower than that among UK-resident Caucasians. Yet when groups from the same India-resident population migrate to this higher-risk region, they acquire a similar occurrence of clinically significant CaP as in the UK overall; these epidemiological observations point toward an important role for environmental/dietary factors [5, 10]. Various endogenous and exogenous substances including procarcinogens, xeno-estrogens or hormones may be biotransformed by the phase I and/or II metabolizing enzymes [11, 12]. These procarcinogenic substances may be converted into electrophiles that as ultimate carcinogens are capable of causing cellular damage and DNA mutations that in turn give rise to cancer [11]. The presence in the prostate of many such phase I and II enzymes has been shown [13]. For instance, cytochrome P450 (CYP)1B1 will bio-activate a wide range of different procarcinogens [14], and this phase I enzyme is expressed at a higher level in the cancer-susceptible peripheral zone (PZ) compared with the transition zone (TZ) of the gland [15].

CaP occurs in a hormone-responsive tissue that possesses the apparent metabolizing capacity to biotransform a variety of environmental procarcinogens [12, 15] or endogenous hormones [16, 17] to DNA-reactive species. Whether such mechanisms underlie the main initiation, promotion and/or progression events giving rise to CaP, remains to be determined. To the best of our knowledge, there have been no molecular studies designed to investigate whether differences in such fundamental mechanisms exist between individuals from high-risk compared with low-risk regions. To this end, we obtained two separate cohorts of prostate tissues, one from a high-risk region (n = 12, UK-resident Caucasians) and the other from a typically low-risk area (n = 14, India-resident Asians). Collected following transurethral resection of the prostate (TURP), these two cohorts of tissues were examined using quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) to differentially compare the expression of candidate genes for phase I and II metabolizing enzymes (*CYP1B1*, *N*-acetyltransferase [*NAT*]*1*, *NAT2*, catechol-*O*-methyl transferase [*COMT*] and sulfotransferase [*SULT*]*1A1*), estrogen receptor (*ER*) isoforms and aromatase (*CYP19A1*). The aim of this preliminary study was to determine whether in the two reasonably well-matched verified-benign prostate tissue sets, there might be evidence of distinguishing molecular factors that might modulate susceptibility to the emergence of clinically significant CaP in demographically distinct populations.

2 Materials and methods

2.1 Study participants

This study was conducted with appropriate ethical approval at the two centers (LREC No. 06/Q1309/76 for UK-resident participants; institutional ethical approval Workhardt Hospital [Kolkata, India] for India-resident participants). Patients undergoing TURP were identified and prospectively consented on the basis of their having a low risk of harboring CaP (no previous history of CaP, benign-feeling gland on digital rectal examination and prostate-specific antigen (PSA) < 10 ng mL^{-1} serum), except for one patient who had an open prostatectomy for a > 200 g-sized prostate (PSA = 34 ng mL^{-1}, final histology benign) (CAU 3; see Appendix). Morning (08:00–01:00 hours) blood samples were also collected from all participants to measure serum levels of testosterone, 17β-estradiol (E_2) and serum hormone-binding globulin (SHBG).

2.2 Tissue collection and storage

Following TURP, prostate chips were immersed in cold normal saline (0.9%) and transported to the laboratory within 5 min. Using aseptic precautions, the prostate tissue from each patient was processed as

follows:

(i) A few prostate chips were immersed in RNAlater solution (QIAGEN Ltd, Crawley, West Sussex, UK), kept at –4ºC for 24 h and then transferred for storage at –85ºC.

(ii) A few prostate chips were formalin-fixed, after which they were paraffin-blocked for subsequent immunohistochemistry (IHC).

The tissues from India were transported to the UK with adequate precautions taken to maintain the appropriate temperature throughout the journey.

2.3 Quantitative real-time RT-PCR

The method of RNA extraction, reverse transcription and real-time RT-PCR for prostate tissues has been described previously [12, 15]. Briefly, the prostate tissue was ground under liquid nitrogen. Total RNA extraction was performed using the Qiagen RNeasy Kit in combination with the Qiagen RNase-free DNase kit (QIAGEN Ltd.). RNA (0.4 μg) was reverse transcribed in a final volume of 20 μL containing Taqman reverse transcription reagents (Applied Biosystems, Warrington, Cheshire, UK): 1 × Taqman RT buffer, $MgCl_2$ (5.5 mmol L^{-1}); oligo d(T)16 (2.5 μmol L^{-1}); dNTP mix (dGTP, dCTP, dATP and dTTP; each at a concentration of 500 μmol L^{-1}), RNase inhibitor (0.4 U μL^{-1}), reverse transcriptase (MultiScribe) (1.25 U μL^{-1}) and RNase-free water. Reaction mixtures were then incubated at 25ºC (10 min), 48ºC (30 min) and 95ºC (5 min). cDNA samples were stored at –20ºC before use.

Primers (Table 1) for *CYP1B1*, *NAT1*, *NAT2*, *COMT*, *SULT1A1*, *ERα*, *ERβ*, *CYP19A1* and the endogenous control *β-ACTIN* were chosen using Primer Express software 2.0 (Applied Biosystems, Warrington, UK) and designed so that one primer spanned an exon boundary. Specificity was confirmed using the NCBI BLAST search tool. Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Reaction mixtures contained 1 × SYBR Green PCR master mix (Applied Biosystems), forward and reverse primers (Invitrogen, Paisley, UK) at a concentration of 300 nmol L^{-1} for *CYP1B1*, *NAT1*, *NAT2*, *COMT*, *SULT1A1*, *ERα*, *ERβ* and *CYP19A1* amplification 20 ng cDNA template or for *β-ACTIN* amplification 5 ng cDNA template, made to a total volume of 25 μL with sterile H_2O. Thermal cycling parameters included activation at 95ºC (10 min) followed by 40 cycles each of denaturation at 95ºC (15 s) and annealing/extending at 60ºC (1 min). Each reaction was performed in triplicate and 'no-template' controls were included in each experiment. Dissociation curves were run to eliminate non-specific amplification, including

Table 1. Primers used for quantitative real-time RT-PCR analyses.

Gene symbol (GenBank accession No.)	Name	Sequence (5′→3′)
CYP1B1 (NM_000104)	*CYP1B1*-F	GTACCGGCCACTATCACTGACA
	CYP1B1-R	CACATCAGGATACCTGGTGAAGAG
NAT1 (NM_000662)	*NAT1*-F	TGGGTTTGGACGCTCATACC
	NAT1-R	TCCGTCAAACGGAAGACACA
NAT2 (NM_000015)	*NAT2*-F	TGCTGGCCAAAGGGATCA
	NAT2-R	GGGAACAGCCCGGATCTG
COMT (NM_007310)	*COMT*-F	CTGGAGGCCATTGACACCTACT
	COMT-R	TCCACGATCTTGCCTTTCTTG
SULT1A1 (NM_177536.1)	*SULT1A1*-F	TTCTACGCCGGTATGAGCATT
	SULT1A1-R	AGGTGTTCCAGAATTTCTGTTTCAG
ERα (NM_000125.3)	*ERα*-F	TGGACAGGAACCAGGGAAAAT
	ERα-R	GAGATGATGTAGCCAGCAGCAT
ERβ (NM_001040275.1)	*ERβ*-F	TGTAAACAGAGAGACACTGAAAAGGAA
	ERβ-R	CCTCTTTGAACCTGGACCAGTAA
CYP19A1 (Aromatase) (NM_000103.2)	*CYP19A1*-F	ATACCAGGTCCTGGCTACTGCAT
	CYP19A1-R	GATCCCCATCCACAGGAATCT
β-ACTIN (AK222925)	*β-ACTIN*-F	CCTGGCACCCAGCACAAT
	β-ACTIN-R	GCCGATCCACACGGAGTACT

Nucleotide sequences were obtained from GenBank; F, forward primer; R, reverse primer.

primer-dimers.

An unpaired *t*-test was used to determine any significant difference in differential expression of these genes between the two population sets. Pearson's correlation coefficient was employed to investigate any relevant correlations.

2.4 Immunohistochemistry (IHC) staining

Tissues were fixed in formalin before wax embedding and subsequent IHC staining of tissue sections (4-μm thick) was performed manually. Staining took place following de-waxing and re-hydration, and endogenous peroxidase was blocked by flooding the tissue sections with peroxidase blocking reagent (Dako, Envision FLEX, DM801). High-temperature antigen retrieval was performed by heating the tissue sections in citrate buffer (pH 6.0) for 4 min, under pressure and at full power (800 W) in a microwave oven. The antisera anti-CYP1B1 (ME001; Alpha Diagnostic, San Antonio, TX, USA), anti-ERα (ABCAM; ab9269) or anti-ERβ (ABCAM; ab288) were diluted at 1:200 (anti-CYP1B1), 1:20 (anti-ERα) or 1:50 (anti-ERβ) in 0.2% bovine serum albumin in Tris-buffered saline (BSAT) (pH 7.6). The tissue sections were incubated with primary antibody for 30 min at room temperature (except for anti-ERβ, which was incubated overnight at 4ºC). Following the manufacturer's instructions for the Vectastain universal Elite ABC kit (Vector Laboratories, Peterborough, UK), the tissue sections were washed with Tris-buffered saline (TBS) for 5 min, incubated for 30 min with secondary antisera (goat anti-rabbit) in BSAT and washed with TBS for 5 min. The tissue sections were then incubated with tertiary antisera (avidin–biotin complex) in BSAT for 30 min and washed again with TBS for 5 min. 3,3′-Diaminobenzidine chromogen in 0.05 mol L^{-1} Tris/HCl buffer (pH 7.4) with 0.1% H_2O_2 was applied to preparations for 5 min after which they were washed for 5 min with tap water. Finally, slides were stained (15 s) with Harris' hematoxylin, rinsed with tap water, blued in warm tap water (15 s) and rinsed again. Preparations were dehydrated with graded alcohol solutions through to xylene and mounted with coverslips using Styrolite mounting medium (VWR International, Poole, UK). Parallel control slides, in the absence of primary antibody, were prepared to verify the absence of non-specific staining.

On the basis of the amount of staining, a quantity score was assigned (that is, no staining = 0, 1%–10% positively staining cells = 1, 11%–50% positively staining cells = 2, 51%–80% positively staining cells = 3 and 81%–100% positively staining cells = 4). Furthermore, an intensity score was given depending on whether there was no staining (0), weak staining (1), moderate staining (2) or strong staining (3). For quantification, an IHC score per sample was calculated, which was taken as a multiple of the quantity score and the intensity score for that tissue. The scoring was assigned to be completed by a single senior uro-pathologist (Caroline M. Nicholson) with > 15 years of experience in reporting prostate pathology. Scoring was conducted by the pathologist in a blinded fashion, that being, in a random order without knowledge of the origin of the stained slides or clinical details of the individual participants.

3 Results

For this study, a cohort of prostate tissues was obtained from each of two distinct demographic regions designated as either high-risk (UK-resident Caucasians, $n = 12$) or low-risk (India-resident Asians, $n = 14$) for CaP. These two cohorts were reasonably well matched in terms of age (years), PSA levels (ng mL^{-1}) and serum hormone levels (Table 2). In terms of individual patient demographics (see Table 1S, Appendix), the India-resident cohort tended to be younger ($P < 0.05$) than their UK counterparts and to exhibit a lower PSA level. There were no significant differences in the serum levels of testosterone or E_2. However, a lower ($P < 0.0005$) level of SHBG was observed in the India-resident cohort. All the retrieved tissue sets were verified as benign by a single pathologist.

3.1 Quantitative gene expression (mRNA) analysis

Quantitative analyses of candidate genes were carried out without earlier knowledge of the histopathological findings. The ranges of averaged threshold cycle (C_T) values of amplified cDNA for *CYP1B1* were 28.7–38.8 and 28.2–36.1 (one undetectable, IND 5), for *COMT* 27.7–33.9 and 26.2–34.5, for *NAT1* 30.9–36.9 and 30.2–37.9, for *SULT1A1* 30.8–37.5 and 30.4–37.9, for *ERα* 30.6–37.5 and 29.4–36.4, for *ERβ* 30.7–38.3 (one undetectable, CAU 10) and 29.7–38.9, and for *CYP19A1* 31.0–39.3 and 30.3–38.5, for UK-resident Caucasians and India-resident Asians, respectively (see Table 2S, Appendix). However, *NAT2* mRNA transcripts were quantifiable in only three (CAU 6, CAU 8 and CAU 12) of 12 UK-resident Caucasian tissues

Table 2. Demographics of the two participant groups.

Participant demographics	UK-resident Caucasians (n = 12) (mean; median [range])	India-resident Asians (n = 14) (mean; median [range])	P-value
Age (years)	74.2; 73.0 (62–82)	65.9; 64.5 (47–79)	< 0.05*
PSA (ng mL^{-1})	7.3; 5.0 (2.5–34.0§)	1.5; 1.3 (0.2–3.2)	< 0.05*
S. Testosterone (nmol L^{-1})	18.0; 17.5 (12.1–25.6)	16.1; 16.6 (7.3–29.4)	0.43
S. 17β-Estradiol (pmol L^{-1})	127.2; 125.4 (64.8–183.6)	118.3; 115.6 (36.7–238.6)	0.62
S. SHBG (nmol L^{-1})	56.3; 52.4 (34.4–98.0)	31.4; 28.4 (19.0–50.0)	< 0.0005*
Final histology	Benign; BPH	Benign; BPH	

Abbreviations: BPH, benign prostatic hyperplasia; S, serum; SHBG, serum hormone-binding globulin.
§Except for a single patient who had open prostatectomy for a > 200 g-sized prostate (PSA = 34 ng mL^{-1}, final histology benign), all other samples had PSA < 10 ng mL^{-1} serum.
*Statistically significant.

and five (IND 1, IND 6, IND 8, IND 9 and IND 10) of 14 India-resident Asian tissues. Despite this, expression was generally readily quantifiable in all the tissues examined and results were in agreement with previous findings [12, 15].

CYP1B1 was expressed in readily quantifiable levels in all the samples except one India-resident sample (IND 5). Up to a five-fold difference in inter-individual expression of this enzyme was observed (Table 3) but there was no significant difference between the two population samples (P = 0.6). The results of the quantitative gene expression analysis of the examined phase II metabolizing enzymes (*COMT*, *NAT1*, *NAT2*, and *SULT1A1*) are also shown in Table 3. There was a low but uniformly quantifiable level of *COMT* expression in both cohorts. *NAT1* expression was more variable with up to a 10-fold inter-individual difference, whereas *NAT2* was present in quantifiable amounts in only eight samples. The expression of *SULT1A1* was also more uniform with only up to three-fold inter-individual variation in both cohorts. No significant difference in the quantified gene expression levels for these enzymes was observed between UK-resident and India-resident cohorts.

The expression levels of both *ER* isoforms exhibited wide inter-individual variation (Table 3). *ERα* was quantifiable in all the samples with up to a 15-fold expression difference in levels of mRNA transcripts between the samples in both cohorts. *ERβ* was quantifiable in all but one sample (CAU 10) with up to a 50-fold difference compared with the calibrator control (CAU 1). Similarly, aromatase (*CYP19A1*) expression was up to 70-fold differentially expressed between the prostate tissues derived from study participants (Table 3). However, the overall expression of *ERα*, *ERβ* or *CYP19A1* did not point to either a consistent under- or over-expression between the two populations and no significant inter-ethnic differences were observed. The *ERα*/*ERβ* ratios were also examined and no differences between the two population cohorts were observed (data not shown).

Additionally, no significant correlation was observed between the serum-tested sex steroid hormone levels and the levels of expression of the phase I and/or II metabolizing enzymes or ER isoforms. However, there were positive correlations between *ERα* and *NAT1* (P < 0.0001; Pearson's r = 0.7960) or *CYP19A1* (P < 0.0001; Pearson's r = 0.8628). Similarly strong correlations were seen between *ERβ* and *NAT1*, and between *ERβ* and *CYP19A1*. Correlation was significant though less so between both *ER* isoforms and *SULT1A1*.

3.2 IHC

IHC analysis of de-waxed 4-μm thick tissue sections for CYP1B1 protein showed that it was detected in nine (of 11 examined) UK-resident Caucasian and nine (of 14) India-resident Asian prostate tissue samples (Table 4). In the glandular elements, characteristic nuclear-associated staining was noted especially in the flattened basal epithelial cells; strong staining in the stroma was also noted (Figure 1A and B; Figure 1S A and B, see Appendix). Quantity scores were higher for the UK-resident Caucasian samples, although most of the samples showed weak staining. A higher (P = 0.006) IHC score was observed in the UK-resident Caucasian compared with the India-resident Asian samples. Although not quantified, this difference seemed to be confined to the epithelial cells lining the glandular

Table 3. Relative gene expression in prostate tissues derived from UK-resident Caucasians versus India-resident Asians.

Code	*CYP1B1*	*COMT*	*NAT1*	*NAT2*	*SILT1A1*	*ERα*	*ERβ*	*CYP19A1*
CAU 1	1*	1*	1*	UD	1*	1*	1*	1*
CAU 2	3.138	0.710	10.853	UD	0.735	12.154	40.504	54.317
CAU 3	2.732	0.442	0.310	UD	0.689	0.698	0.546	0.332
CAU 4	2.915	0.749	1.248	UD	0.204	1.866	2.726	3.287
CAU 5	4.019	0.691	1.414	UD	0.727	1.950	5.205	8.112
CAU 6	1.883	0.107	0.133	1*	0.214	0.236	0.060	0.015
CAU 7	0.696	0.179	0.308	UD	0.253	0.345	0.277	0.924
CAU 8	2.308	0.229	0.398	5.762	0.408	0.168	0.126	0.075
CAU 9	3.802	0.159	1.104	UD	0.509	2.738	4.779	10.363
CAU 10	1.584	0.395	0.431	UD	0.529	0.210	UD	1.087
CAU 11	1.655	0.321	1.228	UD	0.323	3.724	15.816	14.554
CAU 12	1.042	0.144	0.065	0.749	0.147	0.290	0.142	0.063
IND 1	4.106	0.483	0.318	0.788	0.247	0.451	0.017	0.012
IND 2	3.234	0.833	3.287	UD	1.786	12.381	15.455	7.362
IND 3	1.569	0.801	1.717	UD	0.554	3.074	8.037	3.991
IND 4	1.471	0.847	0.476	UD	0.334	0.412	0.149	0.090
IND 5	UD	0.540	0.693	UD	2.949	2.316	3.124	1.419
IND 6	2.502	0.208	0.973	0.747	0.511	3.672	18.442	11.713
IND 7	1.950	0.213	3.379	UD	0.297	7.484	50.917	22.943
IND 8	4.970	0.156	0.154	0.456	0.085	0.116	0.036	0.022
IND 9	0.891	0.449	0.306	0.675	0.226	0.091	0.069	0.020
IND 10	0.765	0.452	0.187	1.382	0.218	0.458	0.541	0.122
IND 11	1.146	0.372	0.237	UD	0.287	0.132	0.015	0.046
IND 12	3.900	0.589	3.767	UD	1.079	15.067	16.564	71.506
IND 13	0.515	0.200	0.068	UD	0.196	0.235	0.039	0.224
IND 14	1.240	0.430	0.282	UD	0.358	0.502	0.561	3.146

Abbreviations: CAU, UK-resident Caucasian; IND, India-resident Asian; UD, undetectable or unquantifiable.

*calibrator control;

Patients who donated prostate tissue were chronologically numbered. Following resection, mRNA transcripts were compared in tissue sets taking the first measurable sample as the calibrator control. Quantitative gene expression was carried out exactly as previously described [15].

elements and there seemed to be relatively equal levels of positive staining for CYP1B1 in the surrounding stroma of both cohorts.

ERα-positive staining was only noted in five prostate tissue samples: one UK-resident Caucasian (CAU 3) and four India-resident Asians (IND 1, IND 3, IND 8 and IND 12); in general, the staining for this protein was negative (Figure 1C and D). However, positive staining for ERβ protein was observed in all the tissues examined; although no significance ($P = 0.086$) between the UK-resident Caucasian and India-resident Asian cohorts was noted, an elevated level of staining (nuclear-associated and cytosolic) was noted in the latter low-risk group (Figure 1E and F; Figure 1S C and D, [see Appendix]).

4 Discussion

Differences in the expression of various metabolizing enzymes and ER isoforms in the prostate among high-risk (UK-resident Caucasians) compared with low-risk (India-resident Asians) populations might point to factors or mechanisms responsible for demographic differences in susceptibility to the emergence of clinically significant CaP. In the main, hormone serum levels (testosterone or E_2) are similar between the two population cohorts (Table 2) but this might not reflect tissue micro-environmental production or metabolism [12]. Although there was a significant difference in SHBG levels (Table 2), the relevance of this significance in such a small study might be

Table 4. Immunohistochemical scores for prostate tissues derived from UK-resident Caucasians versus India-resident Asians.

Code	CYP1B1			ERα			ERβ		
	Quantity score*	Staining intensity score+	IHC score (quantity score × intensity score)	Quantity score	Staining intensity score	IHC score (quantity score × intensity score)	Quantity score	Staining intensity score	IHC score (quantity score × intensity score)
CAU 1	2	1	2	0	0	0	2	3	6
CAU 2	2	1	2	0	0	0	3	3	9
CAU 3	3	1	3	1	2	2	4	3	12
CAU 4	3	1	3	0	0	0	3	2	6
CAU 5	3	1	3	0	0	0	3	2	6
CAU 6	ND	ND	ND	ND	ND	ND	ND	ND	ND
CAU 7	0	0	0	0	0	0	2	1	2
CAU 8	0	0	0	0	0	0	2	1	2
CAU 9	3	2	6	0	0	0	3	3	9
CAU 10	3	1	3	0	0	0	3	3	9
CAU 11	2	1	2	0	0	0	2	2	4
CAU 12	3	1	3	0	0	0	3	2	6
IND 1	0	0	0	1	1	1	4	3	12
IND 2	0	0	0	0	0	0	4	3	12
IND 3	2	1	2	1	1	1	4	3	12
IND 4	0	0	0	0	0	0	4	2	8
IND 5	1	1	1	0	0	0	3	2	6
IND 6	0	0	0	0	0	0	3	2	6
IND 7	2	1	2	0	0	0	3	2	6
IND 8	1	1	1	2	2	4	3	2	6
IND 9	0	0	0	0	0	0	3	2	6
IND 10	1	1	1	0	0	0	3	2	6
IND 11	2	1	2	0	0	0	4	3	12
IND 12	1	1	1	1	1	1	3	3	9
IND 13	1	1	1	0	0	0	3	3	9
IND 14	2	1	2	0	0	0	3	3	9

Abbreviations: CAU, UK-resident Caucasian; IND, India-resident Asian; ND, immunohistochemistry not performed; IHC, immunohistochemistry.

*Quantity score: no staining = 0, 1%–10%; positive staining = 1; 11%–50% positive staining = 2; 51%–80% positive staining = 3 and 81%–100% positive staining = 4.

+Staining intensity score: none = 0; weakly-staining = 1, moderate staining = 2 and strong staining = 3.

questionable, as previous larger population studies examining the role of serum steroid hormones have been inconclusive [16]. Examining the tissues themselves, the findings of this study point to differences at the protein level in the expression of CYP1B1 and ERβ in the glandular epithelial cells from which CaP might arise.

Several studies have looked into the presence and role of phase I and/or II enzymes in the prostate. Primary cultures of prostate epithelial cells are capable of bio-activating various procarcinogens [18] and the gland *per se* harbors many of the enzymes that are capable of activating these agents [19]. Amongst the cytochrome P450 (CYP) enzymes, we chose to examine CYP1B1 because previous studies show it to be consistently expressed in this tissue, whereas other CYP1A enzymes are inconsistently detected [13, 15, 16]. CYP1B1 is capable in transforming polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines (HAAs) into reactive electrophile metabolites [13], forming carcinogenic catechol estrogens [20, 21] and deactivating anticancer drugs [22]. COMT metabolizes and deactivates catechol estrogens that mediate estrogen-induced tumorigenesis [23]. SULT and NAT can form

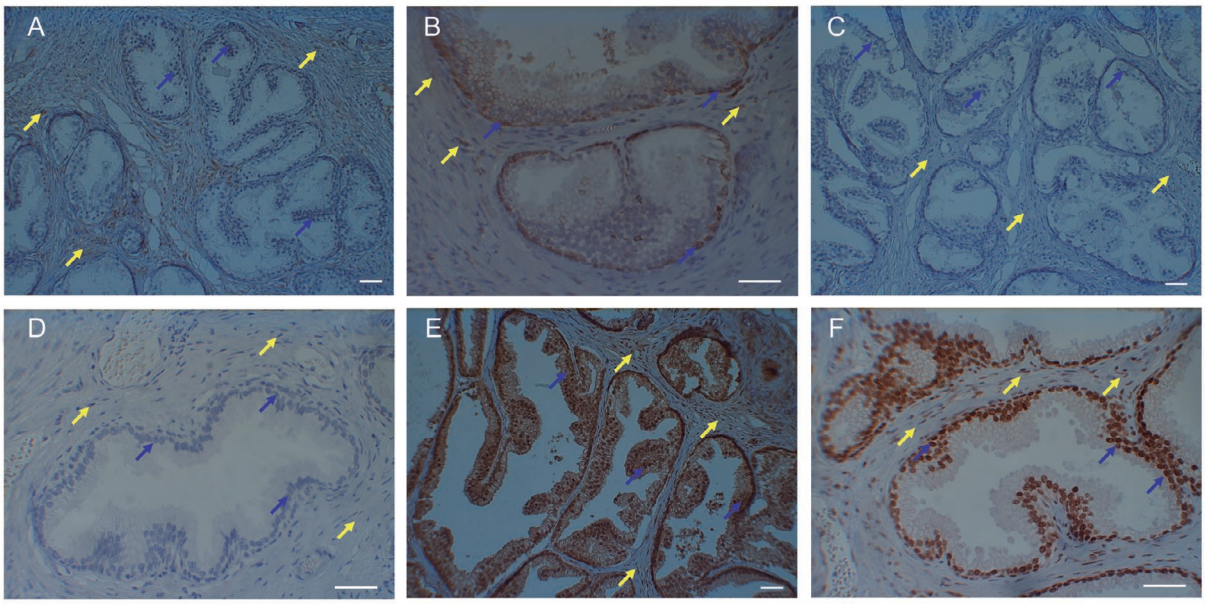

Figure 1. Immunohistochemical staining of prostate tissues. Anti-CYP1B1 staining in (A) tissue derived from an India-resident Asian prostate showing the absence of nuclear-associated staining in cancer-free glandular epithelial cells (blue arrows) but which is present in stroma (yellow arrows); and (B) tissue derived from a UK-resident Caucasian prostate showing the presence of nuclear-associated staining in cancer-free glandular epithelial cells (blue arrows) that is also present in stroma (yellow arrows). Anti-ERα staining in (C) tissue derived from an India-resident Asian prostate showing the absence of nuclear-associated staining in cancer-free glandular epithelial cells (blue arrows) and stroma (yellow arrows); and (D) tissue derived from a UK-resident Caucasian prostate showing the absence of nuclear-associated staining in cancer-free glandular epithelial cells (blue arrows) and stroma (yellow arrows). Anti-ERβ staining in (E) tissue derived from an India-resident Asian prostate showing the presence of intense nuclear-associated and cytosolic staining in cancer-free glandular epithelial cells (blue arrows); and (F) tissue derived from a UK-resident Caucasian prostate showing the presence of nuclear-associated staining in cancer-free glandular epithelial cells (blue arrows). Scale bars = 50 μm.

unstable conjugates of PAHs and HAAs that are DNA-reactive [24, 25].

No significant differences in the expression of the examined phase I and/or II metabolizing enzymes were detected between the two population cohorts (UK-resident Caucasians versus India-resident Asians) (Table 3). However, such mRNA quantification gives no indication of the protein activities of these enzymes. In addition, polymorphic variants of these enzymes, which we have not attempted to determine, might also contribute to inter-ethnic differences in susceptibility to CaP [26]. Certain variants of CYP1B1 have been seen to modify CaP risk in different ethnicities [27] and predict response to chemotherapy for CaP [28]. Similarly, for phase II enzymes the polymorphism or enzyme activity (that is, slow versus fast acetylation status) may dictate varying risk for a particular disease between different populations [29, 30]. Despite inter-individual variation, protein expression of CYP1B1 detected using IHC showed a significant difference in the IHC score between the population samples. This enzyme is known to be over-expressed in CaP tissues when compared with benign or pre-malignant prostatic lesions [31].

Similar to studies on serum levels of other sex hormones, serum estrogen levels may not accurately reflect the intra-prostatic estrogenic milieu. Prostate tissue itself has the necessary enzyme aromatase to produce estrogen locally from testosterone [32, 33]. Estrogens at low concentrations can be genotoxic [34], and such effects might have a role in the promotion of CaP [16, 35]. In rodent models, estrogen is capable of inducing a variety of tumors and CaP is seen to develop at a shorter interval and with increased frequency when this hormone is administered in addition to testosterone [36, 37]. Apart from the direct genotoxicity of catechol estrogens, estrogen might also exert its action through the ER isoforms. ERα is expressed in the cells of the

stromal compartment, whereas ERβ is predominantly found in the basal epithelial cells and to a lesser extent in the stromal cell nuclei. There seems to be a progressive loss of ERβ expression in CaP, suggesting a regulatory role for this receptor in suppressing the abnormal growth of the gland [38, 39]. Paradoxically, ERβ re-emerges as the predominant ER isoform in advanced metastatic CaP [39], suggesting that a simple regulatory role might be an oversimplification of its function. In our study, *ER* expression was uniform across both population cohorts. Immunostaining pointed to sparse ERα positivity, whereas staining for ERβ was much more pronounced, especially in the tissues obtained from the India-resident group. This finding is actually counter-intuitive, as E_2-mediated transcription activation of *CYP1B1* is thought to be mediated through ERα [40], although this latter observation was based on cell lines.

Though studies examined in the Oncomine gene expression analysis database [41] seem to downplay the role of genes like the *CYP1B1* and *ERβ* in CaP, one should be wary of making such conclusions based on the limited number of transcriptomic studies that such databases contain. Furthermore, such transcriptomic studies give no indication of protein or gene expression relative to susceptibility towards developing disease, something this study attempts to do. Of course, our pilot study findings will require verification in a larger population-based study. Additionally, the interplay between the estrogen-signaling mechanisms and androgen receptor pathways remains unknown and, as studies into green tea consumption suggest, might have important roles in chemoprevention of CaP [42, 43].

In addition to the small sample size, there are other limitations to the current study. The affluent (private hospital-treated), cosmopolitan, non-vegetarian cohort of India-resident Asian patients designated as the low-risk cohort may not be completely ideal to reflect the demographic differences in risk factors, as such participants may be influenced by or practicing a more westernized life style compared currently with the general India population. Also, the tissues examined in this study would be expected to consist mostly of TZ of prostate, as this is the region predominantly resected during TURP. Though the TZ accounts for some 25%–30% CaP, the ideal prostate tissue region to conduct such comparative analyses would be the PZ, which accounts for nearly 75% of CaP occurrence. Finally, though we have tried to employ uniform tissue collection, retrieval and storage methods in the two study centers, other unexpected influences may have impacted on our study findings. However, our observations point to differences in benign tissue between a high-risk and low-risk population for CaP that may have important implications for the etiology of this disease.

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Appendix

Table 1S. Individual patient age (years), PSA and serum hormone levels.

Code	Age (years)	PSA (ng mL^{-1})	S. Testosterone (nmol L^{-1})	S. 17β-Estradiol (pmol L^{-1})	S. SHBG (nmol L^{-1})
CAU 1	81	5.0	22.1	119.7	58.2
CAU 2	62	5.7	17.5	104.4	64.4
CAU 3*	82	34.0	NK	NK	NK
CAU 4	71	3.75	12.2	151.0	41.0
CAU 5	81	4.99	12.1	88.0	34.4
CAU 6	72	NK	17.2	125.4	48.6
CAU 7	72	4.96	12.7	64.8	51.5
CAU 8	73	2.49	25.6	162.0	63.8
CAU 9	74	6.25	16.5	183.6	65.7
CAU 10	79	3.75	20.4	107.0	98.0
CAU 11	71	4.19	21.1	130.0	52.4
CAU 12	73	5.0	21.3	163.3	41.1
IND 1	58	1.2	24.6	124.8	35.1
IND 2	60	2.4	16.8	77.1	44.4
IND 3	63	0.3	16.3	113.8	37.9
IND 4	76	3.2	13.8	80.8	24.0
IND 5	75	0.3	7.7	36.7	50.1
IND 6	65	0.2	8.1	80.8	21.0
IND 7	79	2.5	18.8	238.6	32.8
IND 8	74	0.3	7.8	124.8	19.0
IND 9	65	0.6	7.3	99.1	24.0
IND 10	62	3.3	29.4	157.9	50.0
IND 11	47	2.4	16.8	165.2	20.7
IND 12	64	2.4	13.8	88.1	21.8
IND 13	60	0.3	24.6	117.5	35.2
IND 14	74	1.4	19.6	150.5	24.0

Abbreviations: CAU, UK-resident Caucasian; IND, India-resident Asian; NK, not known; PSA, prostate-specific antigen; S, Serum; SHBG, serum hormone-binding globulin; *open prostatectomy.

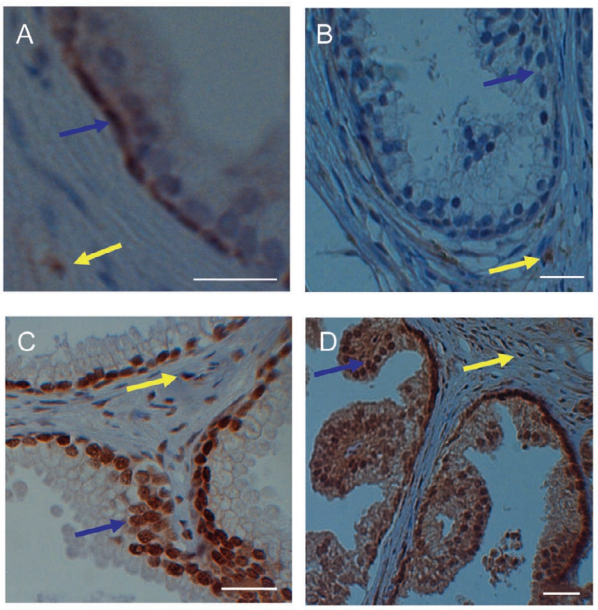

Figure 1S. High magnification of immunohistochemical staining of prostate tissues. Anti-CYP1B1staining in (A) tissue derived from a UK-resident Caucasian prostate showing the presence of nuclear-associated staining in cancer-free glandular basal epithelial cells (blue arrow) that is also present in stroma (yellow arrow); and (B) tissue derived from an India-resident Asian prostate showing the absence of nuclear-associated staining in cancer-free glandular epithelial cells (blue arrow) but which is present in stroma (yellow arrow). Anti-ERβ staining in (C) tissue derived from a UK-resident Caucasian prostate showing the presence of nuclear-associated staining in cancer-free glandular epithelial cells (blue arrow) that is also present in stroma (yellow arrow); and (D) tissue derived from an India-resident Asian prostate showing the presence of intense nuclear-associated and cytosolic staining in cancer-free glandular epithelial cells (blue arrow) that is also present in stroma (yellow arrow). Scale bars = 50 μm.

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Table 2S. C_T values for mRNA transcripts in human prostate tissues.

Code	Mean C_T values (mean β-ACTIN C_T value)							
	CYP1B1	*COMT*	*NAT1*	*NAT2*	*SULT1A1*	*ERα*	*ERβ*	*CYP19A1*
CAU 1	34.5 (23.4)	30.3 (26.9)	34.6 (27.6)	nd	34.2 (26.8)	33.7 (24.8)	35.9 (25.1)	36.9 (26.4)
CAU 2	32.8 (23.4)	30.6 (26.7)	30.9 (27.4)	nd	34.4 (26.5)	29.2 (23.9)	30.7 (25.3)	31.0 (26.3)
CAU 3	29.3 (19.7)	28.4 (23.8)	33.0 (24.3)	nd	31.5 (23.6)	30.6 (21.1)	33.7 (22.0)	35.3 (23.2)
CAU 4	33.2 (23.7)	31.4 (27.6)	34.4 (27.7)	nd	34.6 (24.9)	33.2 (25.2)	34.7 (25.3)	35.7 (26.9)
CAU 5	32.6 (23.6)	30.8 (26.9)	33.7 (27.2)	nd	35.3 (27.5)	31.9 (24.0)	33.7 (25.3)	34.2 (26.8)
CAU 6	28.7 (18.6)	27.7 (21.1)	32.5 (22.6)	35.6 (23.0)	30.8 (21.1)	30.9 (19.9)	35.6 (20.7)	38.7 (22.2)
CAU 7	37.6 (26.1)	31.5 (25.7)	34.7 (26.0)	nd	35.3 (26.0)	35.8 (25.3)	38.3 (25.6)	36.6 (26.0)
CAU 8	34.9 (25.0)	30.5 (25.0)	34.3 (26.0)	35.7 (25.7)	32.1 (23.4)	34.1 (22.6)	37.8 (24.0)	39.3 (25.0)
CAU 9	37.0 (27.8)	33.9 (27.8)	34.4 (27.6)	nd	35.9 (27.6)	35.3 (27.8)	36.3 (27.8)	34.7 (27.6)
CAU 10	38.8 (28.4)	33.1 (28.4)	36.9 (28.7)	nd	34.9 (26.6)	37.5 (26.4)	nd	38.8 (28.4)
CAU 11	38.3 (28.0)	33.1 (28.1)	35.2 (28.5)	nd	32.9 (23.8)	33.3 (26.3)	33.9 (27.1)	35.0 (28.4)
CAU 12	33.9 (22.9)	29.2 (23.0)	34.4 (23.4)	37.0 (24.0)	32.0 (21.8)	32.0 (21.3)	34.9 (21.3)	37.8 (23.3)
IND 1	28.2 (19.1)	26.2 (21.8)	30.4 (21.7)	34.9 (21.9)	32.3 (22.9)	30.4 (20.3)	37.2 (20.6)	37.5 (20.7)
IND 2	34.0 (24.6)	31.2 (27.5)	32.7 (27.4)	nd	34.8 (28.2)	31.1 (25.8)	33.0 (26.2)	33.9 (26.3)
IND 3	31.8 (21.3)	27.5 (23.8)	30.2 (24.0)	nd	32.8 (24.6)	29.8 (22.5)	30.9 (23.1)	31.0 (22.5)
IND 4	34.1 (23.6)	30.0 (26.3)	34.7 (26.7)	nd	35.2 (26.2)	35.3 (25.1)	38.9 (25.3)	38.5 (24.6)
IND 5	UD (27.2)	34.5 (30.2)	37.7 (30.2)	nd	35.9 (30.1)	36.2 (28.6)	38.2 (29.1)	38.6 (28.6)
IND 6	31.1 (21.3)	29.1 (23.5)	31.5 (24.5)	37.3 (24.3)	32.5 (24.1)	29.4 (22.4)	30.3 (23.7)	30.4 (23.5)
IND 7	34.2 (24.1)	31.2 (25.6)	30.9 (25.7)	nd	34.4 (25.3)	30.1 (24.1)	29.7 (24.6)	30.3 (24.3)
IND 8	28.6 (19.8)	28.4 (22.3)	32.6 (22.9)	34.8 (21.1)	31.5 (20.6)	32.3 (20.3)	36.7 (21.1)	36.1 (20.1)
IND 9	33.2 (21.9)	28.3 (23.7)	33.1 (24.5)	nd	32.8 (23.2)	34.5 (22.2)	37.3 (22.6)	38.5 (22.3)
IND 10	34.4 (22.9)	29.6 (25.1)	35.3 (25.8)	36.7 (23.6)	33.3 (23.7)	34.3 (24.2)	36.1 (24.5)	36.6 (23.1)
IND 11	31.4 (20.5)	25.3 (20.4)	30.3 (21.2)	36.5 (24.3)	30.4 (21.2)	33.2 (21.4)	37.3 (20.4)	36.1 (21.2)
IND 12	35.8 (26.7)	30.9 (26.7)	31.8 (26.8)	nd	34.0 (26.8)	31.6 (26.6)	33.1 (26.7)	31.1 (26.8)
IND 13	32.8 (20.8)	27.0 (21.2)	33.7 (22.9)	nd	32.6 (22.9)	33.7 (22.7)	33.4 (26.7)	35.5 (22.9)
IND 14	36.1 (25.3)	30.5 (25.8)	37.9 (29.1)	nd	37.9 (29.1)	36.4 (26.5)	36.7 (21.2)	37.9 (29.1)

Abbreviation: C_T, threshold cycle; nd, not detectable.

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