The role of vitamin D, estrogen, calcium sensing receptor genotypes and serum calcium in the pathogenesis of prostate cancer

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Introduction: *Prostate cancer is the second leading cause of cancer death among men in developed countries. Estrogen receptor-alpha (ER-α), vitamin D receptor (VDR), and the calcium-sensing receptor (CaSR), partly through their effects on calcium levels are implicated in the proliferation and carcinogenesis in the prostate gland. VDR, ER-α and CaSR genes show polymorphisms in humans that appear to have clinical significance in many pathological conditions, such as prostate cancer. Our aim was to evaluate the role of ER-α (PvuII, XbaI), VDR (BsmI) and CaSR (A986S) gene polymorphisms and serum calcium levels in the pathogenesis of prostate cancer.*

Material and methods: *Two hundred four patients with prostate cancer and 102 healthy controls were recruited into a hospital-based case control study. After genotyping, the relationship between the individual genotypes and prostate cancer was investigated.*

Results: *Both the ER-α XbaI and the VDR BsmI polymorphisms were significantly related to the risk of prostate cancer. An age adjusted logistic regression limited to controls and patients not receiving bisphosphonate therapy showed that higher corrected serum calcium and the VDR Bb/BB genotypes independently increased the risk of prostate cancer.*

Conclusions: *ER-α XbaI and VDR BsmI genetic polymorphisms had a significant association with the risk of prostate cancer. Both VDR BsmI genotypes and serum calcium levels were independently related to the risk of prostate cancer, suggesting an influence of VDR on the development of this malignancy.*

Key Words: prostate cancer, vitamin D receptor, estrogen-alpha receptor, calcium-sensing receptor, serum calcium

Introduction

Prostate cancer is one of the most common malignancies and the second cause of cancer death among men in Europe and North America.[1] Recognized risk factors for prostate cancer include age, positive family history of prostate cancer and ethnicity.[1] Both environmental and genetic factors are thought to underline these ethnic differences.[2]

Estrogen is a crucial hormone participating in the proliferation and carcinogenesis of the prostate gland. The prostate expresses both α and β estrogen receptors. Estrogen receptor (ER)-α, but not ER-β, is essential for prostate development. Genetic alterations of the ER-α gene are known to be associated with an increased risk for prostate cancer.[3,4] The XbaI and PvuII restriction site polymorphisms are also thought to be involved in the development of breast cancer,[5] osteoarthritis,[6] and bone mineral density.[7]

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Estrogens upregulate vitamin D receptor (VDR) expression thus influencing the effects of vitamin D3 (1,25-dihidroxy-colecalciferol).[8] According to some authors, vitamin D3 is protective against several types of cancer.[9-12] The anticancer effects of vitamin D3 in prostate cancer occur via increases in apoptosis, inhibition of cell cycle progression, interaction with the insulin-like growth factor axis, as well as a reduction of metastatic potential of prostate tumors. It is possible that VDR gene polymorphisms could affect the binding of biological active vitamin D3 and modulate the antiproliferative effects of vitamin D3. A number of VDR gene polymorphisms (including BsmI) were studied as prostate cancer risk factors, with inconsistent findings. A meta-analysis of the BsmI polymorphism also showed equivocal results.[12]

Ca^{2+} is essential for a wide variety of biologic functions such as cell division, intercellular adhesion, signal transduction cascades and cell differentiation.[13] Twin studies indicated that more than half of the variability in serum calcium may be influenced by genetic factors.[14] The calcium-sensing receptor (CaSR) is a key element for maintaining Ca^{2+} homeostasis[15] and other diverse and cell type specific functions, including regulation of cell differentiation, proliferation, and apoptosis. Loss and gain of function mutations of CaSR show altered intracellular signaling in response to changes in Ca^{2+}. While several diseases were found to be associated with CaSR mutations,[15] its 986 Ala/Ser polymorphism (A986S) was reported to be related to serum calcium levels in healthy adults.[16]

Our aim was to evaluate the potential role of the above polymorphisms of estrogen, vitamin D and calcium sensing receptor genes in the pathogenesis of prostate cancer.

Methods

Patients

A total of 204 (age range: 52-88 years) consecutive Caucasian prostate cancer patients treated at the Department of Urology, Semmelweis University, between 2003-2005 were enrolled in this case-control study. All patients were diagnosed histologically from specimens obtained from transrectal needle biopsy or transurethral resection of the prostate. Patients were excluded, if they had other malignant diseases except for non-melanoma skin cancer, or if they had indolent, low volume, insignificant, symptomless, incidental pT1a prostate cancer, or if Gleason grades were lower than 5. Since prostate cancer is a heterogenous malignancy, we tried to select from all clinical stages. One hundred three patients had organ confined disease, and were treated naïve at the time of inclusion, while the remaining 101 patients had advanced metastatic disease, and received hormone deprivation and/or bisphosphonate therapy at the time of the study procedures. The patients under hormone deprivation and bisphosphonate therapy were excluded from serum calcium, PSA and testosterone level analyses. Clinical data were collected at the time of diagnosis.

One hundred two controls were recruited from our outpatient clinic. Prostate cancer was excluded in controls based on serum PSA levels (< 2ng/mL) and negative rectal digital findings. Controls were also free from any other medical conditions or treatments with known effect on calcium metabolism, or history of malignancies other than basal cell carcinoma of the skin. Fasting blood samples were collected from all participants between 7 am and 9 am. Written informed consent was obtained from each participant, and the project has received ongoing approval from the Regional and Institutional Committee of Science and Research Ethics, Semmelweis University.

Genotyping

Genomic DNA was isolated from blood samples of the patients using the Magnesil KF Genomic System (Promega, Madison, WI, USA). For the ER-alpha gene, the following primers were used: primer S: 5' CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC 3' 34-mer, primer A: 5' TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA 3' 33-mer (10 µM final concentration). The PCR reaction was carried out using the following materials (Promega, Madison, USA): 5 µL 10x Mg free reaction buffer, 1 µL 10mM dNTP, 5 µL 25 mM $MgCl_2$, 10 µL DNA, 1-1 µL 10 µM primers A and S, 0.4 µL (2 U/µL) Taq and 26.6 µL 2D PCR water. The PCR program was the following: 95°C for 2 min, 85°C for 3 min, 5x (70°C for 210 sec, 95°C 30 sec), 30x (70°C for 105 sec, 90°C for 30 sec), 10x (70°C for 210 sec, 95°C for 30 sec) and 70°C for 15 min. The PCR product was digested by using PvuII and XbaI restriction endonucleases at 37°C overnight. The lack of PvuII/XbaI restriction sites corresponds to the P/X alleles and they are present in the p/x alleles.

For the VDR gene, the following primers were used: primer A: 5' AAC CAG CGG GAA GAG GTC AAG GG 3' 23-mer, primer B: 5' CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA 3' 30-mer (2.5 µM final concentration). PCR reaction was carried out using the following materials (Dynazyme, Espoo, Finland): 2 µL 10 x PCR reaction buffer, 0.5 µL dNTP (10 mM, 200 µM final concentration), 0.5 µL (2 U/µL) Taq polymerase, 1-1 µL primers A, B and 15 µL (1 µg) purified DNA. The following reactions were applied: 95°C for 3 min, 35 x (94°C for 45 sec, 72°C for 90 sec), 72°C for 10 min.

The amplified PCR product was digested by using BsmI restriction enzyme (BioLabs, Beverly, USA) for 90 min at 65°C. The BsmI restriction site is missing in the B allele and is present in the b allele.

The polymorphic region of the CaSR gene was amplified by allele-specific PCR technique. The following primers were used: primer M: 5′ ACG GTC ACC TTC TCA CTG ACG TTT GAT GAG CCT CAG AAG TAC T 3′ 43-mer, primer W: 5′ GCT TTG ATG AGC CTC AGA AGA TCG ′ 24-mer and primer R: 5′ CTC TTC AGG GTC CTC CAC CTC T 3′ 22-mer (10 µM final concentration). The PCR reaction was carried out using the following materials (Promega, Madison, USA): 2 µL 10x Mg free reaction buffer, 4 µL dNTP (1mM), 1.2 µL 25 mM $MgCl_2$, 1 µL DNA (25 ng/ml), 3-2-1 µL (primer R, -W, -M), 0.1 µL (0.5 U/µL) Taq and 5.7 µL 2D PCR water. The PCR program was the following: 94°C for 12 min, 35x (94°C for 20 sec, 55°C 20 sec, 72°C 30 sec) and 72°C for 5 min. There are two types of CaSR allele, the allele A and allele S, thus, the genotypes are the following: AA, AS, SS.

For the PCR reactions, Hybaid Express thermocycler (Teddington, Middlesex, UK) was used. Electrophoretic separation was carried out in a 7% Spreadex/acrilamide-bis (29:1) gel (Elchrom, Cham, Switzerland).

Laboratory parameters

Serum PSA, total calcium, albumin, 17-beta-estradiol, and testosterone levels were determined by routine laboratory methods. Serum calcium levels were corrected for serum albumin using the standard formula: corrected calcium (mmol/L) = calcium (mmol/L) – 0.02 x (albumin (g/L)).

Statistical methods

For descriptives, continuous variables are presented as mean ± standard deviation (SD) for normally distributed and median [interquartile range – IQR) for non-normally distributed data; categorical variables are shown as percentages. To compare characteristics of the prostate cancer and control subjects for continuous variables 2-sample t-tests or Mann-Whitney U-tests, for categorical variables (genotypes) x^2-tests were performed. Continuous variables were log-transformed as necessary.

Two sets of analyses were performed. First, the association between each genotype and the risk of prostate cancer was investigated using logistic regression on the whole sample of cases and controls (n = 306).

Second, in an analysis restricted cases not receiving bisphosphonate, or anti-androgen therapy and all controls (n = 205), we further investigated the association of the VDR BsmI polymorphism with the risk of prostate cancer using multiple logistic regression adjusting for age and serum calcium, phosphate, albumin, corrected calcium with a stepwise backward selection. Due to the limited power related to the smaller sample we repeated this analysis after collapsing VDR genotypes associated with similar prostate cancer risk. We used the Nagelkerke R^2 as a measure of performance of the overall models.[17]

All genes satisfied the criteria of a Hardy-Weinberg equilibrium ($p > 0.05$ for all 4 SNPs). A two-tailed p value < 0.05 was considered as significant. All data analyses were carried out using SPSS 13.0 for Windows.

TABLE 1. **Baseline characteristics of participants**

	Prostate cancer patients n = 204	**Controls n = 102**	**Reference values**	**p values**
Age (yrs)	70.0 ± 8.2	64.1 ± 10.9	-	< 0.0001*
Serum PSA (ng/mL)	22.5 [54.1]	1.5 [2.2]	< 2.0	< 0.0001*
Serum total calcium (mmol/L)	2.34 ± 0.17	2.41 ± 0.14	2.25-2.61	< 0.0001*
Serum phosphate (mmol/L)	1.10 ± 0.21	1.06 ± 0.17	0.74-1.52	0.15
Serum albumin (g/L)	44 [7]	44 [5]	35-50	0.58
Corrected calcium (mmol/L)‡	2.27 ± 0.11	2.34 ± 0.12	2.25-2.61	< 0.0001*
Serum estradiol (pg/mL)	20.0 [20.3]	28.5 [9.4]	13.0-60.0	< 0.0001*
Serum testosterone (ng/mL)	1.56 [4.13]	4.02 [1.85]	2.80-8.00	< 0.0001*

Comparisons were made using 2-sample t-tests or Mann-Whitney U-tests as appropriate. Data are shown as mean ± SD for normally distributed data and median [interquartile range] for non-normally distributed data.
PSA = prostate-specific antigen
‡corrected calcium (mmol/L) = serum total calcium (mmol/L) – 0.02 x (albumin (g/L) – 40).

Results

Association of genotypes with the risk of prostate cancer in the whole sample

Prostate cancer patients were almost 6 years older compared to controls. Serum calcium, and corrected calcium levels were significantly lower in prostate cancer patients compared to controls. As expected, serum PSA levels were significantly elevated, while estradiol levels were decreased in controls, Table 1.

The distributions of and the risk associated with the investigated genotype can be seen in Table 2. Both the BB and Bb genotypes had a significantly increased odds ratio for prostate cancer, however these two groups had wide and overlapping confidence intervals. The ER-alpha gene also showed a significant relationship with prostate cancer. Participants with the XX genotype had a 2.7 times higher odds ratio for prostate cancer compared to the xx genotype, while the risk for the Xx genotype was intermediate. Neither the PvuII genotypes of the ER-alpha gene, nor the CaSR genotypes showed any significant association with the risk of prostate cancer.

Association of VDR genotypes, age, and laboratory measures with the risk of prostate cancer in participants not receiving bisphosphonate therapy

The subset of patients not receiving bisphosphonates (n = 103) were similar (all $p > 0.05$) to the rest of the prostate cancer cases except that they had 0.05 (95% confidence interval [CI] 0.01-0.10) mmol/L higher serum calcium ($p = 0.03$). The prostate cancer cases not receiving bisphosphonate therapy also had a significantly lower estrogen level compared to the rest of the group (median [IQR] 18.3 [18.7] versus 21.4 [22.9] pg/mL, $p = 0.04$).

Based on an unadjusted logistic regression analysis, participants with the Bb genotype of the VDR BsmI polymorphism had a 1.96 times increased risk of prostate cancer (95% CI 1.01 to 3.83) compared to the bb genotype. The BB genotype had a similarly increased (although not significant) point estimate of 2.16 (95% CI 0.88 to 5.33). These odds ratios did not change substantially after the adjustment for age and corrected calcium levels, however they lost significance, Table 3.

Since the groups with Bb and BB genotypes had similar odds ratios we created a new grouping based on the presence of the B allele and repeated the

TABLE 2. **Frequency distribution of genotypes and risk of prostate cancer**

	Prostate cancer patients (n = 204)	Controls (n = 102)	Odds ratio (95% CI)
VDR BsmI			
bb	52 (25.5%)	53 (52.0%)	1 (ref)
Bb	101 (49.5%)	35 (34.3%)	2.96 (1.67-5.25)
BB	51 (25.0%)	14 (13.7%)	3.76 (1.79-7.90)
		P < 0.0001*	
ERα PvuII			
pp	43 (21.1%)	31 (30.4%)	1 (ref)
Pp	122 (59.8%)	47 (46.1%)	1.84 (0.99-3.43)
PP	39 (19.1%)	25 (24.5 %)	1.01 (0.52-2.28)
		p = 0.092	
ERα XbaI			
xx	35 (17.2%)	29 (28.4%)	1 (ref)
Xx	111 (54.4%)	54 (52.9%)	1.72 (0.91-3.26)
XX	59 (28.9%)	18 (17.6%)	2.73 (1.25-5.94)
		p = 0.037*	
CaSR A986S			
AA	159 (77.9%)	73 (71.6%)	1 (ref)
AS	42 (20.6%)	28 (27.5%)	0.68 (0.38-1.20)
SS	3 (1.5%)	1 (1.0 %)	1.48 (0.15-14.48)
		p = 0.37	

Comparisons between prostate cancer cases and controls were done using x^2-tests and logistic regression.
CI = confidence interval; VDR = vitamin D receptor; ERα = estrogen receptorα; CaSR = calcium sensing receptor

TABLE 3. **The association between vitamin D receptor BsmI polymorphism and prostate cancer before and after adjustment according to logistic regression analysis on prostate cancer cases without bisphosphonate therapy (n = 103) and controls (n = 102)**

	Odds ratio	95% CI	p
Model 1			
VDR BsmI			
bb	1 (ref)		
Bb	1.96	1.01-3.83	0.047*
BB	2.16	0.88-5.33	0.094
Model 2			
Age (yrs)	1.06	1.03-1.10	< 0.0001*
Corrected calcium (mmol/L)‡	0.027	0.001-0.54	0.018*
VDR BsmI			
bb	1 (ref)		
Bb	1.95	0.96-3.98	0.066
BB	2.29	0.87-5.99	0.092
Model 3			
VDR BsmI			
bb	1 (ref)		
Bb or BB	2.02	1.01-3.74	0.026*
Model 4			
Age (yrs)	1.06	1.03-1.10	< 0.0001*
Corrected calcium (mmol/L)‡	0.027	0.001-0.54	0.018*
VDR BsmI			
bb	1 (ref)		
Bb or BB	2.04	1.05-3.95	0.035*

Stepwise backward method (using $p < 0.05$ for entry and $p < 0.10$ for removal of variables). Other variables available for the model: total calcium, phosphate, albumin. Nagelkerke R^2 for the final model (Model 4): 0.188.
‡corrected calcium (mmol/L) = serum total calcium (mmol/L) – 0.02 x (albumin (g/L) - 40).

previous logistic regressions with this new variable that confirmed that the risk of prostate cancer is doubled in participants with the B allele compared to those without (odds ratio 2.04, 95% CI 1.05 to 3.95), Table 3.

There was no association between genotype distribution or serum calcium and clinical data including Gleason score, metastatic tumors and survival.

Discussion

In our study, we have demonstrated that the VDR BsmI bb genotype was significantly less frequent in prostate cancer patients. Upon exposure to 1,25-dihydroxivitamin D_3 in vitro, prostate cancer cells possessing VDR are induced to differentiate and inhibited from proliferation.[18] Mikhak et al have found no association between the VDR BsmI genotype and the risk of prostate cancer.[11] However, as mentioned above, there is a considerable variation in the distribution frequencies in different countries.[4] This fact combined with differences in sun exposure suggests that the results may not be extrapolated to the general case.[19,20] Nevertheless, VDR BB has been associated with lower serum calcium due to impaired calcium absorption from the gut.[21] Thus, VDR – at least partly – may act through circulating calcium since lower corrected serum calcium levels are associated with increased risk for prostate cancer.

In addition we have also shown that the association between VDR genotypes and prostate cancer was independent of serum corrected calcium levels and age: the presence of the B allele approximately doubled the risk of prostate cancer. The vitamin D/VDR system may influence the development of malignant processes by directly interfering with a number of biological substances including insulin-like growth factor binding proteins, p21, cyclin D1 and prostaglandins.[22,23] This is also observed in prostate cancer.[24]

We have also demonstrated an association between the ER-alpha gene XbaI polymorphism and the risk of prostate cancer. Growth of prostate cancer cells in vitro can be regulated by estrogen.[25,26] Decreased ER-alpha expression has been found in prostate cancer patients, particularly in hormone-refractory tumors.[27] The role of ER in prostate cancer is further corroborated by the fact that normal prostate development is estrogen-dependent.[28]

In our study, the lower frequency of ER-alpha xx genotype among patients with prostate cancer compared to controls could indicate that the other receptor genotypes may not be able to mediate the anti-proliferative effects of estrogen in the prostate with the same efficiency. The role of ER-alpha in the carcinogenesis of prostate cancer is still controversial.[4,29] Hernandez et al have found ethnical/racial differences between the allelic distribution of both ER-alpha polymorphism (XbaI and PvuII) and they have found an association between the XbaI polymorphism and prostate cancer only in black men.[4] However, Modugno et al, using a multigenic model of prostate cancer susceptibility, supported the role of the ER-alpha polymorphism independently of ethnic origin.[29]

CaSR regulates the secretion of parathyroid hormone and the reabsorption of calcium by renal tubules in response to alterations in serum calcium concentrations. Cole et al observed an association between the A986S polymorphism and serum ionized and total calcium levels in healthy adults.[16] A protective effect of calcium against prostate cancer has been suggested by numerous epidemiological and experimental studies.[11-13,19,20] Decreased (although within normal range) extracellular calcium throughout life determined by genetic factors might contribute to the increased risk for prostate cancer. We observed no difference in CaSR A986S genotype between the prostate cancer patients and the control group. Despite this fact, the significantly lower serum calcium found in prostate cancer patients underlines the putative importance of calcium in the development of prostate cancer.

Based on the results of a meta-analysis of Ming et al, the roles are not clear of the investigated genes in the pathogenesis of prostate cancer.[30] In the single nucleotide polymorphisms association studies one of the key point is the homogeneity of the investigated population. The results of surveys on heterogenic populations are not comparable. In our study we investigated a pure population of Caucasian men, which could be one of the strength of our publication.

In our study, multiple genetic factors affecting calcium homeostasis in relation to prostate cancer have been investigated simultaneously. ER-α XbaI and VDR BsmI genetic polymorphisms as well as serum calcium had a strong association with the risk of prostate cancer. In addition, the effect of the VDR genotypes was independent of that of serum calcium suggesting a role of vitamin D system in the prostate carcinogenesis. □

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