CLINICAL STUDY

Association between estrogen and androgen receptor genes and prostate cancer risk

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Abstract

Objective: Prostate cancer (PC) is one of the principal causes of death among men. Steroid hormones are involved in normal prostate growth and carcinogenesis. The purpose of our study was to investigate the effects on PC risk of polymorphisms from three steroid hormone receptor genes: the androgen (AR), and the α (ESR1) and β (ESR2) estrogen receptors.

Design and methods: The study was performed on a Caucasian population of 1045 PC patients and 814 controls. Using a logistic regression model, the different alleles and genotypes from those polymorphisms were analyzed according to case/control status, the tumor aggressiveness, and the age at onset.

Results: A significant association between PC risk and the pooled 4/5, 5/6, and 6/6 genotypes of the GGGA repeat located in the first intron of ESR1 (odds ratio (OR) = 3.00, 95% CI = 1.32–6.82, $P = 0.008$) was observed. When we stratified the cases, this association was confined to patients with a Gleason score of 2–4 (OR = 8.34, 95% CI = 2.91–23.91, $P < 0.0001$) or late onset PC (OR = 2.91, 95% CI = 1.22–6.93, $P = 0.016$). An association between a short AR CAG repeat (less than 17 repeats) was also observed among patients with late onset PC (OR = 2.34, 95% CI = 1.15–4.76, $P = 0.019$).

Conclusions: These findings suggest that the GGGA repeat from ESR1 and the CAG repeat from AR may be associated with risk of late onset PC.

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Introduction

Prostate cancer (PC) is one of the major causes of death among men in developed countries (1). The principal risk factors are age, familial history, and ethnicity. Sex steroid hormones like androgens and estrogens are important in PC development. Indeed, evidence supports the hypothesis that exposure to endogenous variations in androgens and estrogens across a man's life span contributes to or may be a causal factor in the development of PC. While androgen deprivation and administration of estrogens are recognized therapies for PC, early exposure to estrogens is suspected to initiate carcinogenesis among different tissues, including the prostate gland. A decrease in the ratio of androgens to estrogens with aging could also be responsible for prostate carcinogenesis.

Growth and differentiation of the prostate are controlled by androgens such as testosterone and 5 α -dihydrotestosterone. Their effects are mediated through their interaction with the androgen receptor (AR) that is expressed in the prostatic epithelial cells (2). Estrogens

could also have an indirect role through repression of the hypothalamic–pituitary–gonadal axis and direct effects on the testis. Estrogens produced by peripheral aromatization of testosterone exert their effects on prostatic tissue by interaction with their receptors, estrogen receptor α (ESR1), and β (ESR2). These receptors are expressed with distinct tissue and cell-specific patterns. In the normal prostate, ESR1 is only found in stromal cells whereas ESR2 is expressed in the majority of epithelial cells and in some stromal cells (3).

In tumoral tissue, AR expression is observed in primary cancers and during progression to hormone refractory carcinoma. Immunohistological studies have shown a heterogeneous expression pattern of AR in PC (4). ESR1 has been reported to be expressed in both stromal and epithelial cells in prostate tumors (5); however, results are inconsistent among studies with other reports showing only stromal expression (3). By contrast, ESR2 is commonly expressed in the epithelial cells of prostate tumors (3).

A reduction in *ESR2* expression has been observed during carcinogenesis, suggesting a role for this receptor in the maintenance of normal prostate epithelium (6, 7). Cancers retaining *ESR2* expression seem to be associated with a highly malignant phenotype (8). Decreased expression of the *ESR1* gene has also been found in PC and particularly, in hormone refractory tumors (7).

Moreover, experimental studies performed in rodents have also demonstrated that estrogens through their receptors are potentially carcinogenic in the prostate. In rodents, neonatal estrogenization inhibits prostate growth and function, and promotes the development of dysplastic lesions (9). Experiments with ER-deficient mice have indicated that the effects of neonatal exposure to high doses of estrogens are largely mediated through stromal *ESR1*, but are not associated with *ESR2* (10). Weihua *et al.* (11) reported that ER β KO mice displayed hyperplastic foci and increased expression of the proliferation marker Ki-67 in prostate. By contrast, in another study, no prostatic abnormality was observed in other ER β KO mice (12). Estrogens have also been shown to induce prostate tumors in rats when associated with androgens (13, 14).

To study the role of estrogen receptors in the risk of PC, we screened the *ESR2* gene for polymorphisms and compared their distribution in PC patients and healthy controls. We also evaluated two previously reported polymorphisms in the *ESR1* and *AR* genes in a large population. The first polymorphism is the GGGGA repeat localized in the first intron of the *ESR1* gene, which our group has previously shown to be associated with increased PC risk in a smaller study (15). The second is the highly variable CAG repeat (encoding polyglutamine) which is located in the first exon of the *AR* gene corresponding to the transactivation domain (16).

Subjects and methods

Study population

In this study, 1045 PC cases and 814 controls of Caucasian origin were included. All the participants gave informed consent. The protocol was approved by the Institutional Review Board (IRB) from CCP Ile de France IV (Paris Saint-Louis). Cases were recruited from three French departments of urology (Paris, Nancy, and Brest) and were diagnosed with a histological confirmed adenocarcinoma of the prostate. Aggressiveness of the cancer was based on pathological TNM Classification 2004, Gleason score and PSA level (mean $\pm$ s.d.: 99.36 ng/ml $\pm$ 707.43 ng/ml). Subjects ranged from 40 to 98 years of age (mean = 67 years).

Controls were selected from men invited to a systematic health screening, under the proposition of the national health insurance, performed in the same geographical areas than the hospitals where the cases were collected. They

were checked by digital rectal examination, their prostate specific antigen (PSA) value had to be less than 4 ng/ml, and they could exhibit no signs of prostate carcinoma. Their mean age was 63 years (range 40–92 years).

Genomic DNA was extracted from peripheral blood leukocytes using standard methods.

Detection of polymorphisms in the *ESR2* gene

Specific primers encompassing each coding exon of the human estrogen β receptor were designed. Each fragment of the gene was amplified as follows. In a final volume of 25 μ l, 5 ng genomic DNA, 200 μ M dNTP, 0.02 M MgSO₄, 0.2 μ M of each primer, and 0.375 U of Taq Platinum (Gibco) were mixed. To rapidly screen for the presence or absence of polymorphisms, three PCR products either from three patients or three controls were pooled. The pooled samples were then analyzed by DHPLC as described in patent FR-2793262.

Genotyping

Genotyping of the *ESR2* polymorphisms was done using fluorescence polarization template-direct dye-terminator incorporation technology, as described in (15). The AcycloPrime-FP SNP Detection Kit (Perkin-Elmer Life Sciences Inc., Boston, MA, USA) was used for the extension step. Fluorescence polarization of the two dye terminators was analyzed directly in the final reaction mix with an Analyst fluorescence reader from LJI Biosystems (Boston, MA, USA).

Genotyping was performed as already described in (17) for the CAG repeat from *AR* and in (15) for the GGGGA repeat from *ESR1*. Briefly, primers encompassing the CAG repeat (5'-tccagaatctgttcagagcgtgc-3' and 5'-actgcggctgtgaaggttgctgt-3') or the GGGGA repeat (5'-gcccgctgatgctactgc-3' and 5'-tgggtggagagtacgttttt-3') were selected. One primer was fluorescently labeled. For the *AR* CAG repeat, PCR amplification was carried out in a final volume of 50 μ l, containing 50 ng DNA, 20 pmol of each primer, 100 μ M each of dNTP, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.3, 50 mM KCl, and 1 unit Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA), in the following cycling conditions: 10 min at 94 °C, then 35 cycles of 40 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C. For the *ESR1* GGGGA repeat, PCR was carried out in a final volume of 20 μ l, containing 50 ng genomic DNA, 250 μ M dNTP, 1 μ M each primer, 2.5 U Taq Gold (Applied Biosystems), 2.5 mM MgCl₂, and 5% DMSO, in the following cycling conditions: 5 min at 95 °C, then 35 cycles of 30 s at 94 °C, 30 s at 53 °C, 30 s at 72 °C. The final extension step at 72 °C was extended to 10 min. PCR products were then loaded on 5% denaturing polyacrylamide gel and detected with an ABI Prism 377 DNA automated sequencer. Genotypes were determined with Genescan Analysis 3.1 software (Applied Biosystems).

Statistical analysis

Genotypes of *ESR1* and *ESR2* were tested for Hardy–Weinberg equilibrium. The distribution of polymorphisms was tested with a χ^2 test or Fisher's exact test. Odds ratios and a 95% confidence interval (95% CI) were calculated with logistic regression with adjustment for age. Statistical analyses were performed using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA).

Results

The coding sequence of the *ESR2* gene was initially screened for polymorphisms in 96 patients (mean age at diagnosis: 67.9; range 45–86 years) and 96 controls (mean age: 69.2; range 59–86). Eleven polymorphisms were identified (Table 1), four in the coding region (one was already reported: rs1256049), two in introns (one already described: rs944050), and five in the 3'UTR (three already described: rs4986938, rs928554 and rs28440970). Three of the four polymorphisms located in exons modified the protein sequence: S112Stop, Del-N181 and R221G. When we compared the genotype or allele distribution between cases and controls, none of the polymorphisms showed significant association with PC risk (Table 1). Four of them (S112Stop, rs4986938, rs928554, and rs28440970) were further investigated in 290 patients (mean age at diagnosis: 69.2; range 45–98 years) and 290 controls (mean age: 69.8; range 48–92). Their genotype distributions were in agreement with Hardy–Weinberg equilibrium. Again, we noted an absence of association with PC risk (Table 1).

We identified three alleles for the GGGA repeat from the *ESR1* gene corresponding to four, five, or six repeats; the five-repeat allele was the most frequent. The Hardy–Weinberg equilibrium was respected in the control population. Twenty-three PC cases, but only eight controls had a genotype other than 5/5. A significant association was then observed between the pooled genotypes 4/5, 5/6, and 6/6 and PC risk (OR = 3.00, 95% CI = 1.32–6.82, $P = 0.008$; Table 2). When the alleles 4 and 6 were pooled, the association was also statistically significant (OR = 3.65, 95% CI = 1.73–7.72, $P = 0.001$).

The AR CAG repeats from cases ranged from 10 to 34 with a mean length of 21.9 repeats (s.d. 3.04, median = 22). In controls, they ranged from 11 to 34 with a mean length of 22.1 repeats (s.d. 3.03, median = 22). For the statistical analyses, study subjects were considered as having a short CAG repeat allele (less than 17) or a long allele (17 or more) as already performed in a previous study (16). No significant association was observed between this polymorphism and PC risk ($P = 0.085$; Table 2).

To study the impact of these polymorphisms on PC aggressiveness, patients were stratified according to Gleason score and divided in four classes: 2–4, 5–6, 7, and 8–10. We found that the association between the

Table 1 Localization and distribution of *ESR2* polymorphisms in prostate cancer cases and controls.

Region	Position ^b	db. SNP	Amino acid	Alleles ^a	Cases			Controls			P-value
					1/2		2/2	1/2		2/2	
					1/1	1/2		1/1	1/2		
Exon 2	45749114		S112Stop	C/A	287	1	0	287	0	0	1
Exon 4	45735377–379		Del-N181	A/G	95	1	0	95	1	0	1
Exon 5	45727199		R221G	A/G	94	0	0	93	1	0	1
Exon 6	45723804	rs1256049	V328V	G/A	88	8	0	89	7	0	0.79
Intron 8	45701401		A274	G/A	94	1	0	96	0	0	0.50
Intron 8	45699798	rs944050	A35	A/G	88	8	0	89	7	0	0.79
3'UTR	45699569	rs4986938	A264	G/A	138	100	48	122	116	47	0.34
3'UTR	45693948	rs928554	A255	A/G	141	96	51	124	103	58	0.41
3'UTR	45693911	rs28440970	A292	A/G	260	27	1	250	36	2	0.19
3'UTR	45693835		A368	A/G	81	13	2	82	13	1	0.84
3'UTR	45551692		A102	G/A	96	0	0	95	1	0	1

^a1 and 2 are the two alleles of each SNP.

^bPosition is relative to the genomic config NT_026437 from the National Center for Biotechnology Information.

Table 2 Association between *ESR1* and *AR* polymorphisms and prostate carcinoma risk.

Gene	Genotype or allele	Cases/controls	OR ^a	95% CI ^b	P-value
<i>ESR1</i>	5/5 Genotype	550/541	1		
	4/5, 5/6, 6/6 Genotype ^c	23/8	3.00	1.32–6.82	0.008
	5 Allele ^c	1114/1089	1		
	4, 6 Allele	32/9	3.65	1.73–7.72	0.001
<i>AR</i>	≥ 17 repeats ^d	966/757	1		
	< 17 repeats	29/12	1.83	0.92–3.66	0.085

^aOR, odds ratio adjusted for age.^bCI, confidence interval.^c4, 5, and 6 correspond to the number of GGGA repeat.^d17 corresponds to the number of CAG repeat.

pooled genotypes 4/5, 5/6, and 6/6 of *ESR1* and PC was confined to patients with Gleason score of 2–4 (OR = 8.34, 95% CI = 2.91–23.91, $P < 0.0001$; Table 3).

The age at onset of the PC cases was also divided in two classes: early onset if their cancer was diagnosed ≤ 64 years and late onset if it was diagnosed > 64 years. The association between the *ESR1* pooled genotypes (4/5, 5/6, and 6/6) or *AR* CAG repeat < 17 alleles and PC risk was confined to cases with late onset: OR = 2.91, 95% CI = 1.22–6.93, $P = 0.016$ and OR = 2.34, 95% CI = 1.15–4.76, $P = 0.019$ respectively (Table 3).

Discussion

By screening the coding sequence of the *ESR2* gene, we identified eleven polymorphisms but did not find any association between those polymorphisms and PC risk.

Table 3 Association between *ESR1* and *AR* polymorphisms and risk of prostate cancer, stratified by Gleason score or by age at onset.

Gene	Genotype	OR ^{a,b}	95% CI ^c	P-value
<i>ESR1</i>				
Gleason score				
2–4	4/5, 5/6, 6/6 ^d	8.34	2.91–23.91	<0.0001
5–6	4/5, 5/6, 6/6	2.49	0.92–6.74	0.07
7	4/5, 5/6, 6/6	1.81	0.53–6.13	0.34
8–10	4/5, 5/6, 6/6	2.52	0.65–9.77	0.18
Onset				
≤ 64 years	4/5, 5/6, 6/6	2.69	1.00–7.27	0.051
> 64 years	4/5, 5/6, 6/6	2.91	1.22–6.93	0.016
<i>AR</i>				
Gleason score				
2–6 ^e	< 17 repeats ^f	1.95	0.90–4.21	0.09
7	< 17 repeats	1.43	0.55–3.71	0.46
8–10	< 17 repeats	1.57	0.49–4.99	0.45
Onset				
≤ 64 years	< 17 repeats	1.19	0.47–3.05	0.72
> 64 years	< 17 repeats	2.34	1.15–4.76	0.019

^aOR, odds ratio adjusted for age only for Gleason score.^bFor Gleason score or onset, the category of reference is the *ESR1* genotype 5/5 (OR = 1) or the *AR* allele ≥ 17 (OR = 1).^cCI, confidence interval.^d4, 5, and 6 correspond to the number of GGGA repeat.^ePatients with Gleason score of 2–4 and 5–6 were pooled because the class Gleason 2–4 included too few patients.^f17 corresponds to the number of CAG repeat.

Our results agree with a previous study performed by Thellenberg–Karlsson *et al.* (18), which analyzed 28 SNPs spanning the entire *ESR2* gene in 1415 PC cases and 801 controls. In this study, only one polymorphism (rs29877983) located in the promoter region was significantly associated with PC risk (OR = 1.22, 95% CI = 1.02–1.46) and with localized carcinomas (OR = 1.33, 95% CI = 1.08–1.64). Because our study focused on the *ESR2* coding region, this part of the promoter was not analyzed. Together, these results are not in favor of a major role of the *ESR2* gene in PC susceptibility. Among the identified exonic polymorphisms, the S112Stop, which leads to a truncated protein, was observed in one of the 288 PC cases and none of the 288 controls. This variant should thus be considered as an infrequent mutation.

We also did not find a significant association between the *AR* CAG repeat and the risk of PC, even when the cases were stratified according to Gleason score. This is in agreement with other studies including a multiethnic cohort study of 1014 cases (19) and another study of 460 cases (20), which did not observe any association between *AR* CAG repeats and PC risk. Indeed, results regarding the *AR* CAG repeat polymorphism and association with PC risk have been very contradictory as reviewed by Simard *et al.* (21). Several studies have found an association between short CAG repeats (≤ 20 or 22) and the risk of PC in Caucasian populations (22, 23) whereas others have reported a reduced risk of PC associated with short alleles (≤ 22) after an analysis of 1461 and 288 cases respectively (24, 25). Finally, a meta-analysis of 19 case-control studies comprising a total of 4274 cases and 5275 controls with 79% of Caucasians concluded that a modest association existed between the shorter repeats (≤ 21) and PC risk (OR = 1.19, 95% CI = 1.07–1.31) (26).

Nevertheless, when the cases were stratified according to age at onset, we observed a significant association between CAG repeats less than 17 and PC risk among patients with an age at diagnosis above 64 years (OR = 2.34, 95% CI = 1.15–4.76, $P < 0.019$). By contrast, Hardy *et al.* (27) found a positive correlation between CAG repeats ≥ 23 with increased age at diagnosis. However, this divergent study only included 109 cases and the statistical analysis was based on

correlations between length of CAG repeats and age at diagnosis. Finally, the association between the AR CAG repeat and PC risk seems to be limited. These contradictory results could be due to the heterogeneity of many factors such as the number of cases, the various ethnicities, the different gravity and age at diagnosis of cases, and the cut-point of CAG repeats.

In this study, we showed that rare alleles consisting of four or six GGGA repeats from the *ESR1* gene were significantly associated with PC risk (OR = 3.65, 95% CI = 1.73–7.72, $P < 0.001$). In a previous study including 300 cases and 300 controls, we reported that this *ESR1* GGGA repeat polymorphism was significantly associated with PC risk ($P = 0.036$) (15). Here, in a larger population, we confirmed this result and showed a stronger association among patients with Gleason score of 2–4 (OR = 8.34, 95% CI = 2.91–23.91, $P < 0.0001$) or with an age at onset of the cancer above 64 years (OR = 2.91, 95% CI = 1.22–6.93, $P = 0.016$). These results indicate that this *ESR1* polymorphism could be a useful indicator of PC low gravity.

No other study has been performed on the *ESR1* GGGA repeat polymorphism and until now, only a few studies of other *ESR1* polymorphisms and PC risk have been undertaken. Two other polymorphisms: *PvuII* (rs2234693) and *XbaI* restriction sites (rs9340799), located in the first intron of the *ESR1* gene were studied in different ethnic populations. Modugno *et al.* (28) analyzed these polymorphisms in a small population of 88 Caucasian patients and 241 controls and found no significant association with PC risk. However, when the *ESR1 XbaI* polymorphism was combined with the AR CAG repeat, they observed that individuals with a short AR repeat (< 23) and *ESR1 XbaI* –/– or *ESR1 XbaI* –/+ had an increased PC risk. When we performed the same kind of analysis in our population, we also found a significant increased PC risk for individuals with a short AR (< 17) and an *ESR1* genotype 4/5, 5/6, or 6/6 (OR = 2.57, 95% CI = 1.11–5.95, $P = 0.028$). No significant association was found between the *PvuII* polymorphism and PC risk in another study on non-Hispanic Caucasian and African-American men including 488 PC cases and 617 controls (29). These two *ESR1* polymorphisms were also analyzed in three populations of 260 black, 1013 non-Hispanic white and 423 Hispanic white men (30). Only in black men, *XbaI* polymorphism was associated with PC risk (OR = 2.25, 95% CI = 1.07–4.70, $P = 0.031$). The limited number of studies, the small size, and the different origins of the population do not allow firm conclusions to be made regarding the real implication of these two polymorphisms. The association between the GGGA repeat from *ESR1* and PC risk seems to be the most significant. Further studies in other ethnic populations will be worthwhile to confirm our results.

Our results suggested that the role of the *ESR2* gene in PC susceptibility is limited in Caucasian populations. We confirmed an association between the *ESR1* GGGA repeat and PC risk. This polymorphism seemed to be

associated with a favorable Gleason score (2–4) and a late onset PC, whereas the AR CAG repeat (< 17) was only associated with a late onset PC.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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