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RESEARCH

Direct Detection of the *AR*-E211 G>A Gene Polymorphism from Blood and Tissue Samples Without DNA Isolation

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Abstract The pathogenesis of prostate cancer (CaP) involves alterations in a gene structure of the androgen receptor (AR). The single nucleotide polymorphism *AR*-E211 G>A localized in exon 1 of the *AR* gene (G1733A) was detected using direct polymerase chain reaction and restriction digestion (PCR-RFLP) method on blood and tissue samples without prior DNA isolation. We used blood samples of patients with a diagnosis of benign prostatic hyperplasia (BPH) or CaP. From monitored group of CaP patients were selected specimen in formalin-fixed paraffin-embedded tissue blocks with morphology of BPH and CaP. The main objective of our study was to develop a method based the direct PCR-RFLP analysis from blood and tissue without prior DNA isolation for faster genotyping analysis of a large number of samples. We found no statistically significant differences in allelic % of the *AR*-E211 G>A polymorphism between BPH and CaP patients ($p \leq 0.8462$). Genotyping of the *AR*-E211 G>A variant in blood was not identical with tumor tissue genotyping analysis. Significant agreement between blood and tissue *AR*-E211 G>A polymorphism only in non-tumor tissue focus was confirmed. Although we analyzed a limited number of the tissue samples, we suppose that a presence of the minor allele A may be associated with cancer transformation-induced changes of the modified *AR* gene.

Keywords Gene polymorphism · Prostate cancer · Androgen receptor · Direct method

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Introduction

The androgen receptor (AR) is a member of the superfamily of nuclear receptors that function as ligand-dependent transcription factors.

A single copy human *AR* gene located in the X chromosome (q11 – 12) is highly polymorphic. There are two polymorphic CAG (polyglutamine) and GGC (polyglycine) tandem repeats, and *AR*-E211 single-nucleotide polymorphism (SNP) localized on exon 1 of the *AR* gene. Variations in the CAG trinucleotide repeats in the *AR* gene have been implicated in various diseases including several cancers (prostate, breast, male breast, liver), hyperandrogenic disorders (male pattern baldness, hirsutism, acne), benign prostatic hyperplasia (BPH), androgen insensitivity syndrome (AIS), progressive neurodegenerative diseases, such as spinal bulbar muscular atrophy (SBMA, also called Kennedy's disease) and/or spinobulbar motor neuronopathy associated with mild AIS (MAIS) [1, 2]. The CAG and GGC repeat lengths have been inversely correlated with the AR transcription activity [3, 4] and their potential impact on prostate cancer formation have been studied with inconsistent results [5–9]. The frequency distribution of the *AR* gene CAG repeats also varies among different racial/ethnic groups and the ethnicity seems to be a significant risk factor for prostate cancer. Shorter alleles are found more frequently in African-American men who have a higher incidence of prostate cancer. On the other hand, Caucasian and Asian populations at lower risk of prostate cancer, exhibit a relatively high number of CAG repeats [10–12].

The G1733A single nucleotide polymorphism of the *AR*-E211 gene (UCSC code: rs6152), designated the G and A alleles is a synonymous change. The presence of the G allele at nucleotide 1733 abolished a *Stu*I restriction enzyme recognition site, which is recognized on the A allele. The allele frequencies in 92 American Caucasian men were 13 % for allele A and 87 % for allele G [13]. Among healthy American Africans, allele G of the *AR*-E211 polymorphism was shown

to be associated with a three-fold higher prostate cancer risk among the men under the age of 65 years [14]. This *AR*-E211 gene polymorphism is in partial linkage disequilibrium with both CAG and GGC repeats [14]. It is established that nucleotide repeat sequences are highly polymorphic, whereas dimorphic polymorphisms are more stable with lower rates of mutation [15].

The main goal of our study was a development of the procedure of direct PCR-RFLP method from blood and/or tissue samples without prior DNA isolation for faster genotyping analysis of a large number of the blood samples.

Methods

Subjects

Blood samples were obtained from 226 patients at the 44–88 ages diagnosed with benign prostatic hyperplasia (BPH) and histopathologically confirmed prostate cancer (CaP). The BPH and CaP patients were recruited from Department of Urology and archived in Department of Clinical and Molecular Pathology of the University Palacky Olomouc. The CaP patients were divided into two groups of patients with Gleason grade < 7 who had histopathologically designed well moderately differentiated CaP (Grade 1 and 2), and in patients with Gleason grade ≤ 7 who had poorly differentiated or undifferentiated CaP (Grade 3 and 4). From monitored group of CaP patients were selected specimen in formalin-fixed paraffin-embedded (FFPE) tissue blocks of 10 patients after radical retropubic prostatectomy (RRP). In the tissue sections were identified foci with morphology of BPH and CaP and from these areas were obtained cells for following analysis.

PCR-RFLP Analysis from Blood Without DNA Extraction

The PCR-RFLP (Restriction Fragment Length Polymorphism) assay was performed from blood samples without DNA purification (Fig. 1a, b). In the first step, genomic DNA was amplified directly from 2 μl whole blood using Finnzymes's Phusion® Blood Direct PCR Kit (Finnzymes, Espoo, Finland). The *AR*-E211 G>A polymorphism was detected by amplification of a 416 bp fragment using forward primer 416/F 5′- CAC AGG CTA CCT GGT CCT GG −3′ and reverse primer 416/R 5′- CTG CCT TAC ACA ACT CCT TGG C – 3′ [16, 17] on high-speed Piko® Thermal Cycler (Finnzymes, Espoo, Finland) by initial denaturation at 98 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 5 s, annealing at 58 °C for 4 s, elongation at 72 °C for 30 s, and the final extension at 72 °C for an additional 3 min after the last cycle. After PCR, the reactions were

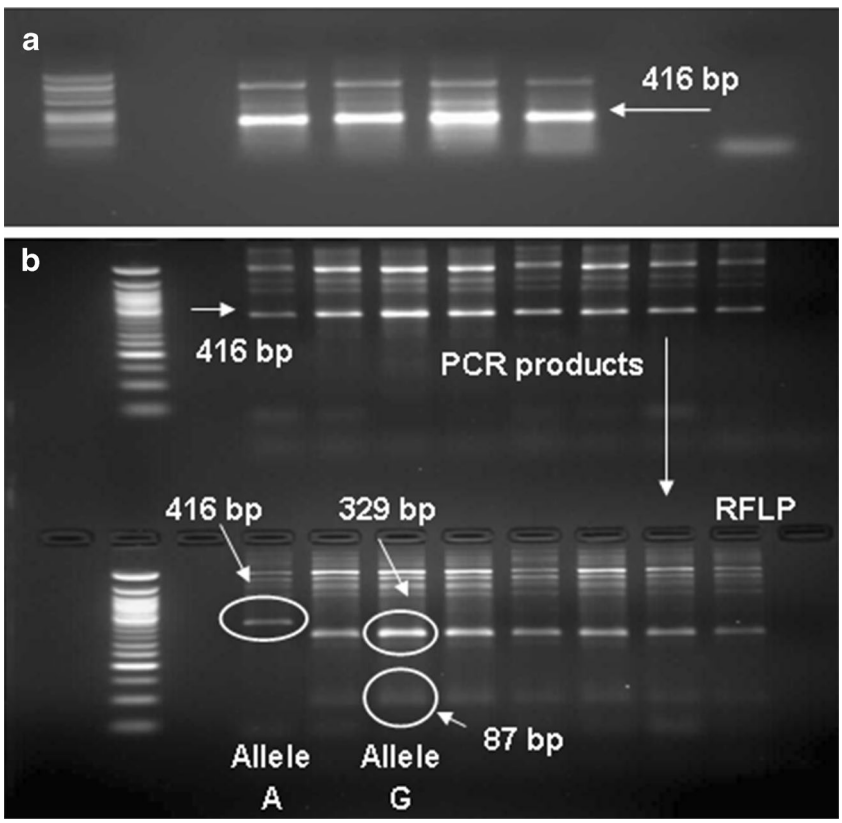

Fig. 1 PCR-RFLP directly from blood. Agarose gel electrophoresis showing 416 bp PCR products detected after PCR where blood was used as a template (**a**). A 416 bp DNA fragment indicates the presence of the allele A. The allele G is determined a presence of the 329 bp and 87 bp DNA fragments (**b**)

centrifuged at 2,500 rpm for 3 min and the supernatants were collected for restriction digestion. The digestions were prepared from 5 μl of supernatants by addition of 10 units of *Stu*I (New England Biolabs, Ipswich, MA) and incubated at 37 °C for 1 h. Digested products were electrophoresed through a 3 % TAE agarose gel and visualized by ethidium bromide staining (Fig. 1).

PCR-RFLP Analysis from FFPE Tissue Without DNA Extraction

Phusion® High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was performed in direct PCR without DNA extraction from disrupted FFPE tissue (Fig. 2). Single 15 μm^2 sections of FFPE tissues were treated with 0,2 mg/ml of proteinase K in 100 μl 1 x Phusion HF Reaction Buffer (Finnzymes, Espoo, Finland). The samples were incubated overnight at 60 °C in thermoblock, after which proteinase K was inactivated by increasing the temperature to 98 °C for 10 min. After centrifugation at 14,500 rpm for 2 min, 1 μl of tissue suspension from the supernatant was used directly for PCR. Amplification of a 100 bp PCR product with rs6152S/forward primer 5′- AAC AGC AGC AGG AAG CAG TAT C – 3′ and rs6152S/reverse primer 5′- GTC CCC CCT AAG TAA TTG TCC T – 3′ was performed on Piko® Thermal Cycler (Finnzymes, Espoo, Finland) by initial denaturation at 98 °C for 5 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 20 s, elongation at 72 °C for 30 s and the final extension at 72 °C for an additional 10 min after the last cycle. After centrifugation of the PCR products, the supernatants were collected for restriction digestion with 10 units of *Stu*I (New England Biolabs, Ipswich, MA) and incubated at 37 °C for 1 h. Digested products were electrophoresed in 3 % TAE agarose gels and visualized by ethidium bromide staining (Fig. 2).

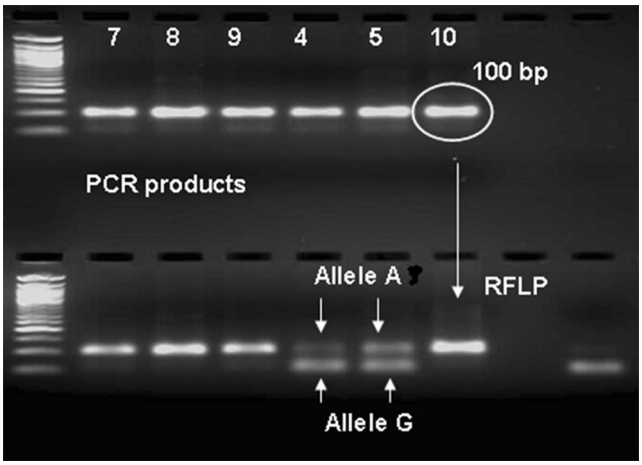

Fig. 2 PCR-RFLP from FFPE tissue without DNA extraction. The Agarose gel showing DNA fragments detected with *Stu*I restriction enzyme after digestion of 100 bp PCR product, where tissue suspension was used as a template. An undigested 100 bp PCR product indicates the presence of allele A. An occurrence of the allele G suggests the presence of 54 and 46 bp DNA fragments

Table 1 Absolute and relative frequencies of the both alleles in BPH and CaP patients (Blood samples)

	A n[a] (%)[b] SD	G n[a] (%)[b] SD	Column Total n[a] (%)[b] SD
BPH	14 (14.4)	83 (85.6)	97 (100)
	0.400	0.435	0.429
CaP	21 (16.3)	108 (83.7)	129 (100)
	0.600	0.565	0.571
Row total	35	191	226
	0.155	0.845	

Pearson's χ^2 - test with Yates'continuity correction

χ^2 - test=0.376; df=1; p-value=0.8462

SD Standard of deviation

[a] Absolute frequency

[b] Relative frequency

Statistical Analysis

Comparison of allele frequencies between the groups of BPH and CaP patients, and between the groups of CaP patients with low Gleason grade (< 7) and CaP patients with high Gleason grade (≤ 7) were analyzed by χ^2 – tests. We assumed that the null hypothesis was valid, when between the relative frequencies of alleles in the groups of BPH and CaP patients, and the groups of patients with<7 and≤7 Gleason grades were not

Table 2 Absolute and relative frequencies of the both alleles in BPH and CaP patients according Gleason grade (Blood samples)

	A n[a] (%)[b] SD	G n[a] (%)[b] SD	Column Total n[a] (%)[b] SD
BPH patients	14 (14.4)	83 (85.6)	97 (100)
	0.400	0.435	0.429
CaP patients with Gleason Grade<7	8 (16)	42 (84)	50 (100)
	0.229	0.220	0.221
CaP patients	13 (16.5)	66 (83.5)	79 (100)
Gleason grade≥7	0.371	0.346	0.350
Row total	35	191	226
	0.155	0.845	

Pearson's χ^2 - test with Yates'continuity correction

χ^2 - test=0.149; df=2; p-value=0.9282

SD Standard of deviation

[a] Absolute frequency

[b] Relative frequency

Table 3 Comparison of the *AR*-E211 G>A polymorphism detected from blood, non-tumor and tumor tissues of the CaP patients

Sample	030	014	026	036	159	060	006	003	088	215
Blood	G	G	G	G	G	A	A	A	A	A
Non-tumor	G/A	G/A	G/A	G/A	G/A	G/A	A	A	A	A
Tumor tissue	A	A	G/A	G/A	G/A	G/A	A	A	A	A

statistically significant differences. The null hypothesis was rejected if p value was less than or equal to a predetermined level of significance ($p \leq 0.05$). The data evaluation was used statistical software R (http://r-project.org/).

Results

Frequency of Alleles of the *AR*-E211 G>A Polymorphism in Blood Samples

Since the *AR* gene is located on the X chromosome, males have only one allele on their one X chromosome and females have two alleles on their two X chromosomes [18]. Of the 226 patients who were assessed, 97 males were diagnosed with BPH and 129 with CaP. The frequency of the minority allele A in a group of BPH patients was 14,4 % and in CaP patients 16,3 % (Table 1). We did not find any statistically significant difference ($\chi^2 \leq 0.8462$) in the allele frequencies between BPH and CaP patients at the significance level ($p \leq 0.05$). The analysis showed no association between prostate cancer and the A allele (Table 2). When the division of CaP patients to patients with low (< 7) and with high (≥ 7) Gleason grades was compared with BPH patients, we did not found any statistically significant differences at the significance level ($p \leq 0.05$).

The *AR*-E211 G>A Polymorphism in Tissue

FFPE tissue blocks of the CaP patients after RRP were selected on the basis of the *AR*-E211 G>A polymorphism from blood samples i.e., slices of tumor tissue carrying allele A and ones with allele G. Genotyping of the *AR*-E211 G>A variant in blood was not identical with tissue genotyping analysis. However, the *AR*-E211 G>A polymorphism determined from blood and tissue of non-tumor focus were confirmed in samples with the allele A. Further, in tissue samples either the allele A or both alleles were detected, the slices only with the allele G were not detected (Table 3, Fig. 2).

Discussion and Conclusion

Recent studies have suggested that polymorphisms of the *AR* gene could influence the risk of prostate cancer development and progression [5–9]. However, the CAG and GGC polymorphic repeats in the AR have been studied extensively as markers of prostate cancer susceptibility, with inconclusive results [5–9]. The *AR*-E211 G>A polymorphism is in partial linkage disequilibrium with both CAG and GGC repeats [14] and although in our study, no evidence for an association between the A allele and risk of CaP was described (Table 1), its utilization as a genetic marker should be re-considered. This dimorphic polymorphism can be more stable with smaller mutation probability, and thus could be offered as ideal markers for association studies [19].

Result of the genetic analysis of the *AR*-E211 G>A polymorphisms of patients with histologically confirmed prostate cancer was association of the A genotypic variant with high Gleason grade (≥ 7) tumor in 70.0 % patients and in 30.0 % of patients with low Gleason grade (< 7) [20]. Nevertheless, we found no overall association between the presence of the A allele and CaP patients with low (< 7) and/or with high (≥ 7) Gleason grades, as compared with BPH patients (Table 2).

So far studies have been carried out searching for a relationship between the *AR*-E211 G>A polymorphism detected in blood and various degrees changes in tumor prostatic tissue [19, 20, 21]. The *AR*-E211 G>A polymorphism compared to blood and tissue has not yet been performed. Although, we analyzed only a small sample of patients, in several cases we have repeatedly identified in non-tumor and tumor tissues, both alleles of the *AR*-E211 G>A polymorphism (Fig. 2). It is still studied by the assumption that the minor allele A is associated with higher risk of prostate cancer [19, 20, 21]. Our finding indicates that the minor allele A could be associated with transformation-induced changes of the modified androgen receptor gene or induced another changes caused by tumor transformation. However, the main benefit of our communication is the development and using of a new methodological approach of the direct PCR analysis directly from blood or tissue samples without prior DNA isolation. This methodology allows processing a large number of samples in less time than diagnostic procedures based on the DNA isolation.

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