Original paper

Influences of angiotensin I-converting enzyme gene insertion/deletion polymorphism on prostate cancer risk in Romania

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Abstract

The contribution of genetic variants to prostate cancer risk has been documented. Mutations in ACE (angiotensin I-converting enzyme) gene are found to be related with prostate cancer. The purpose of the present study was to characterize ACE genotypes in prostate cancer cases and to investigate any association between I/D polymorphisms of ACE gene and clinical characteristics in a group of Romanian patients. A total of 46 healthy men and 46 male patients diagnosed with prostate cancer were included in the study. ACE I/D polymorphisms were determined by polymerase chain reaction methodology. The highest genotype frequencies found within the prostate cancer group were detected for DD genotype (55.6%) with a significant difference in the allele frequencies (p = 0.006), compared to the control group, where no significant differences were observed (p > 0.05). The DD genotype was statistically significant associated with the advanced tumor stage and Gleason score higher than 8. Our data suggest that the ACE gene polymorphism can be associated with the evolution of prostate cancer disease, more precisely that DD genotype represented a risk factor for prostate cancer.

Keywords
Prostate cancer, ACE gene, genotype, polymorphism, risk factor.

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Introduction

Prostate cancer (PC) is one of the most commonly diagnosed malignancies in the world and continues to be a major cause of morbidity and mortality in men over 50 years. It is acknowledged that PC is a multifactorial disease, and despite the high prevalence, the disease etiology remains largely unknown. In addition to increasing age and ethnicity, genetic variation or polymorphisms existing in the human genome can confer genetic susceptibility to cancer. A series of polymorphisms where described as being involved in the development of different pathologies in the Romanian population (A.C. BULGAR & al [1], M. TOMA & al [2]). The involvement of different polymorphisms in the pathogenesis of PC (R. EELES & al [3], L. TANG & al [4], D. KACHAKOVA & al [5]) and the detection of these modifications may provide useful molecular indicators of diagnosis.

Angiotensin I-converting enzyme (ACE), a key enzyme in the renin-angiotensin system (RAS), plays an important role in regulating blood pressure and circulatory homeostasis. This enzyme is also able to degrade bradykinin, a potent vasodilator (J.H. VAN BERLO & al. [6]). ACE has been also implicated in the pathogenesis of several malignancies such as lung cancer, breast cancer, prostate cancer, gastric cancer and oral cancer (S. DEVić PAVLIĆ & al [7], E. VAIKAṬARIS & al [8], M. NACAK & al [9], M. SUGİMOTO & al [10], B. YİGİT & al [11], C.A. HAIMAN & al [12]). It has been suggested that Ang II is involved in cellular proliferation (A. MUSCELLA & al. [13]) and has a significant role in tumor growth, angiogenesis and metastasis (M. FUJI_TA & al. [14], M. FUJI_TA & al [15]).

The ACE gene maps to human chromosome 17q23 and contains many variations. The most widely studied polymorphism is the Insertion/Deletion (I/D) variant located on intron 16, described by Rigat et al. [16]. The presence or absence of a 287-bp nonsense repetitive DNA domain leads to three genotypes: II, ID and DD. The I/D polymorphism accounts for 20% to 50% of the variance in ACE gene expression or activity in blood and tissues among individuals (C. RÖCKEN & al [17]). It is well known that different genotypes associate diverse ACE levels, and that DD genotype induces the highest ACE levels in blood (B. RIGAT & al [16]).

The implications of ACE polymorphisms in the progression of human PC remain to be established. Thus, the purpose of the present study was to characterize ACE genotypes in PC cases and to investigate any relation between ACE I/D polymorphism, the risk of PC and clinical characteristics in a group of Romanian patients with PC.

Materials and Methods

Patient selection and clinical investigation. Peripheral blood samples were collected in tubes containing EDTA (ethylene diamine tetra acetic acid) from 46 healthy men, age 55 – 75 years (mean age 65.2 ± 6.22) and 46 male patients with PC, age 55 – 76 years (mean age 65.5 ± 3.5). All PC patients were selected between 2014 and 2016 from the Fundeni Hospital, Bucharest. The diagnosis of prostate carcinoma was confirmed by clinical and laboratory examination. Clinical characteristics of PC patients are presented in Error! Reference source not found. PSA (prostate specific antigen) cut-off values and Gleason grading were considered according to Lotan and Epstein (T.L. LOTAN & al. [18]). Control male were selected among healthy volunteers. All samples were obtained with the informed consent of the participants prior to their inclusion in the study.

Table 1. Clinical characteristics of patients with prostate carcinoma

| Cases (n = 46) | Age | Mean 65.5±3.5 |
|---------------|-----|---------------|
| < 70          | 40  |               |
| ≥ 70          | 6   |               |
| PSA values (ng/ml) | Mean 15±5 |
| < 10          | 14  |               |
| ≥ 10          | 32  |               |
| Tumor stage   |     |               |
| Early         | 4   |               |
| Advanced      | 42  |               |
| Gleason score |     |               |
| < 8           | 25  |               |
| ≥ 8           | 21  |               |

DNA analysis. Genomic DNA from PC patients and control groups was isolated from whole blood samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

The ACE genotype was determined by PCR (polymerase chain reaction) methodology as described previously (V. SHANMUGAM & al. [19]) with some modifications. Briefly, genomic DNA samples were subjected to PCR amplification using the following primer pair sequence that target the ACE I/D polymorphism: 5’-CTG GAG AGC TAC TCC CAT CTT TCC T-3’ (sense) and 5’-GAC GTC GCC ATC ACA TTC GTC AGA T-3’ (antisense) synthetized by ThermoFisher Scientific (Carlsbad, CA). Amplification was carried out in a 25 µl volume containing template DNA (0.5–1.0 µg), primers (1 µM of each), dNTPs (0.2 mM each), 1X PCR buffer and 0.04 U/µl of Taq polymerase Recombinant (Invitrogen by ThermoFisher Scientific, Carlsbad, CA). After initial denaturation at 95°C for 5 min, the DNA was amplified by 30 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 45 s and primer extension at 72°C for 45 s. Final extension was performed at 72°C for 10 min. The PCR products were visualized under UV trans-illuminator following electrophoresis on 2% agarose gel and staining with ethidium bromide.

Statistical analysis. Statistical analysis was performed using the SPSS software package, version 22.0 for Windows.
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Clinical laboratory data were presented as mean ± standard deviation. Hardy–Weinberg equilibrium (HWE) test was conducted for alleles and genotypes distribution. Chi-square test ($\chi^2$) and chi-square goodness-of-fit test were used to compare the distribution of genotypes and alleles in cases and control groups. To analyze the association of I/D ACE genotypes and clinicopathological features (tumor stage, PSA serum levels and Gleason score) Fisher’s exact test was used. A p value < 0.05 was considered the threshold for statistical significance.

**Results and Discussion**

**Frequency of ACE polymorphisms.** The I/D ACE genotype was determined by the length of the PCR product: 190-bp in case of the deletion and 490-bp in the presence of the insertion. Each DNA sample revealed one of three patterns after electrophoresis: a 490-bp band (genotype II), a 190-bp band (genotype DD), or both 490-bp and 190-bp bands (genotype ID) (Figure 1).

A total of 46 pathological samples and 46 controls were genotyped. I/D ACE polymorphism could not be amplified in one of the PC samples due to poor DNA quality. Allele frequencies and I/D ACE genotype distribution for each study group are presented in Error! Reference source not found.. The observed genotype distributions are consistent with Hardy–Weinberg equilibrium among the control group ($p = 0.926$) and PC cases ($p = 0.256$).

| ACE genotype | Study groups |
|--------------|--------------|
|               | Control ($n=46$) | Prostate cancer ($n=45$) |
| II           | 8 (17.4%)      | 5 (11.1%) |
| ID           | 22 (47.8%)     | 15 (33.3%) |
| DD           | 16 (34.8%)     | 25 (55.6%) |
| Allele       |               |             |
| I            | 38 (41.3%)     | 25 (27.8%) |
| D            | 54 (58.7%)     | 65 (72.2%) |

The distribution of ACE genotypes and alleles for the PC group did not differ significantly from the control group (genotype: $\chi^2 = 3.981$, $p = 0.136$; allele; $\chi^2 = 3.677$, $p = 0.055$), although the patients group had a more frequent DD genotype (55.6%) compared to the control group (34.8%). The highest genotype frequencies found within the control group were detected for the ID heterozygous genotype for ACE (47.8%). For the PC group there were significant differences in the allele distribution ($p = 0.006$). No significant differences were observed between I and D alleles ($p = 0.095$) in the control group.

**Correlation between clinicopathological features of PC and ACE polymorphism.** The majority of PC patients included in this study were diagnosed with advanced tumor stages. Fisher’s exact test was conducted and did not detect a statistically significant association between the tumor stage and I/D ACE genotype of the patients ($p = 0.184$). Because of the small number of individuals with early tumor stages (only 8.7%), there were used only the data
obtained from the patients diagnosed with advanced tumor stages (91.3%), and the chi-square goodness-of-fit test showed a significant difference between the three genotypes in the PC group with advanced tumor stage ($\chi^2 = 21.732$, $df = 2$, $p < 0.001$). The DD genotype is most frequent associated with the advanced tumor stage, followed by ID genotype and II genotype with the lowest frequency (Figure 2).

Subsequent analysis evaluated the association of the ACE I/D genotypes and the PSA serum levels (Figure 3) and the Fisher’s exact test revealed a two-tailed $p$ value of 0.1821 that is not statistically significant.

**Figure 2.** Frequency of ACE I/D polymorphism according to PC tumor stage

![Graph showing frequency of ACE I/D polymorphism according to PC tumor stage](image)

**Figure 3.** Distribution of PSA serum levels in PC group according to ACE genotypes

![Graph showing distribution of PSA serum levels in PC group according to ACE genotypes](image)

Data regarding the association of ACE I/D genotypes and the Gleason score (Figure 4), Gleason score $< 8$ (low-grade tumor) and Gleason score $\geq 8$ (high-grade tumor), showed that there is a statistically significant association between the two subgroups ($p = 0.035$); otherwise, Gleason score $\geq 8$ is more frequently associated with the DD genotype (64%) compared to ID and II genotypes (27% and 20% respectively).

**Discussions**

Despite years of cancer genes research, the mechanisms of PC progression and the molecular base of tumor cells remain largely unknown. As a multifactorial disorder, cancer is influenced by genetic, as well as environmental factors, life style and age. Among genetic factors, beside oncogenes and tumor suppressor genes, polymorphisms in more than 100 genes, which confer moderate or high risk of cancer, have been identified (N. RAHMAN [20]). Such an example is the RAS, which regulates cell proliferation, angiogenesis, blood pressure and cardiovascular homeostasis and has also been involved in various malignancies (P.R. GARD [21], A.J. GEORGE & al [22], X. WANG & al [23]). The role of ACE in the development of human cancers and in the progression of PC mostly, is still under debate. Studies in various populations (Chinese, Turkish, Mexican and Netherlands) have produced conflicting results.
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Figure 4. Graphical display of Gleason score associated with DD, ID and II genotypes in PC group

(Y. XIÉ & al [24]). Ruiter et al. [25], in a meta-analysis of 24 studies, found an increased risk of PC for the carriers of ACE DD genotype, while noting a large variation in the analyzed results. Another meta-analysis study conducted by Xie et al [24], suggested that ACE I/D polymorphism could be a potential genetic biomarker for risk of PC.

In this study, we analyzed the relation between ACE I/D polymorphism and clinical characteristics in a group of Romanian patients diagnosed with PC.

The highest levels of circulating and tissue ACE activity are known to be found in carriers of DD genotype (B. RIGAT & al [16]). It is reported by Medeiros et al [26] that the DD genotype was significantly associated with advanced disease and experimental studies demonstrated that Ang II can promote angiogenesis, an important determinant in the growth and progression of many human cancers. Another study showed that different genotypes of ACE I/D polymorphism found in patients with PC did not differ significantly from the control group, but when the risk ratio was examined, having a DD genotype increases the risk factor for PC 1.35 times (B. YİGİT & al [27]).

In the present study, we observed a high frequency of DD genotype compared to the other genotypes (55.6%) in patients with PC; these patients had a high probability to be diagnosed with an advanced tumor stage compared with ID and II genotype carriers. When comparing allele frequencies in PC and control groups, the D allele showed the highest frequency in the PC group. We observed a higher frequency of DD genotype among the PC patients with Gleason score ≥ 8. Consequently, compared with the DD genotype and D allele, the ID and II genotype can be associated with a decreased risk of PC. Similar results were found in the previous studies which indicated the I allele could have a protective effect against carcinogenesis (B. YİGİT & al [11], X. WANG & al [23], R. MEDEİROS & al [26], E. SIERRA DÍAZ & al [28], R. VAN DER KNAAP & al [29]).

Yigit et al [11] have shown that DD genotype is significantly present in PC and demonstrated that the DD genotype is statistically over-represented in PC. Sierra Díaz et al [28] suggested that D allele, particularly the presence of DD homozygous genotype, is a risk factor for PC. Contrariwise, the results of Hasanzad et al [30] suggested that the D allele has a protective effect on PC development, although not significantly (p = 0.4), and the presence of II genotype increased the risk of cancer more than 38% compared with DD genotype.

Previous studies suggested that the DD genotype can be associated with high ACE levels, leading to angiogenesis (B. YİGİT & al [11], B. RİGAT & al [16], R. MEDEİROS & al [26], E. SIERRA DÍAZ & al [28]), which could cause detrimental effects leading to lymph node metastasis. The association between ACE I/D polymorphism and clinicopathological parameters in PC patients revealed that a statistically significant difference was present between the advanced tumor stage and distribution of the DD genotype, which shows a potential relationship between the two variables.

This investigation is the first addressing the I/D polymorphism of ACE gene in Romanian population and complements previous studies targeting the association of D allele with the PC progress, in various ethnic backgrounds, as this polymorphism is viewed as a potential risk factor in carcinogenesis.
Conclusion

In this study, we have provided further evidence that polymorphism of ACE gene is associated with the progression of disease in PC patients, more precisely that DD genotype represented a risk factor for PC. The statistical analysis revealed that there is an association between the ACE polymorphism and clinicopathological parameters of PC patients.

Some limitations of our study should be considered: the relatively small number of recruited PC patients and the lack of data regarding family history, that could lead to a better understanding of the relation between genetic variants and prostate malignancy.

Therefore, future studies with larger groups of subjects are needed to establish if ACE I/D polymorphism can be used in developing a genetic profile that may be useful in the prediction of the outcome of PC.

Furthermore, additional studies concerning the association of different genetic factors involved in angiogenesis and inflammatory processes related to oncogenesis are of great importance. This type of findings could lead to the development of preventive measures concerning individuals that are at risk for PC.

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