Gazi Tıp Dergisi / Gazi Medical Journal

ARAŞTIRMA - RESEARCH ARTICLE

2007: Cilt 18: Sayı 3: 121-126

ANGIOPOIETIN-2 GENE POLYMORPHISM IN SPORADIC PROSTATE CANCER

H. İlke ÖNEN[1] M.Sc., Ece KONAÇ[1]* Ph.D., Muzaffer EROĞLU[2] MD., Abdullah EKMEKCİ[1] Ph.D.

Purpose: Angiogenesis is a critical requirement for local proliferation and metastasis of prostate cancer. Single nucleotide polymorphisms in angiogenesis-dependent genes affect the degree of cancer development and progression. Angiopoietin-2 *(ANGPT-2)* is one of the principal regulators of vascular growth and regression; however, its role in normal prostate and prostate tumors is largely unknown. DNA sequence variations in *ANGPT-2* may alter the production outcomes or activities of the genes. In this study, we aimed to determine how the changes in the *ANGPT-2* exon 4 G/A affect sporadic prostate cancer patients in the Turkish population.

Materials and Methods: A case-control study on 52 sporadic prostate cancer patients and 52 healthy control subjects was conducted. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses were performed to identify different *ANGPT-2* alleles.

Results: The distribution of genotype and allele frequencies of the polymorphism did not yield a statistically significant difference between patients and controls (P>0.05). Furthermore, classification of patients by tumor, lymph nodes, metastasis (TNM), Gleason scores (GS), and serum prostate-specific antigen (PSA) levels showed no significant differences among the *ANGPT-2* exon 4 G/A genotypes (P>0.05).

Conclusion: This is the first report on the *ANGPT-2* exon 4 G/A polymorphism in patients with sporadic prostate cancer demonstrating that the investigated polymorphism is not associated with prostate cancer in the Turkish population.

Key Words: Angiopoietin-2, polymorphism, prostate cancer, risk factor.

SPORADİK PROSTAT KANSERİNDE ANJİYOPOİETİN-2 GEN POLİMORFİZMİ

Amaç: Anjiyogenezis, prostat kanserinin lokal ilerleyişi ve metastazında kritik bir role sahiptir. Anjiyogenezise bağımlı genlerde meydana gelen tek nükletit polimorfizmleri, kanserin oluşum ve ilerleme derecesini etkilemektedirler. Anjiyopoietin-2 *(ANGPT-2)*, vasküler büyüme ve regresyonun en önemli düzenleyicilerinden olmakla beraber, prostat ve prostat tümörlerindeki rolü bilinmemektedir. ANGPT-2'de var olan DNA sekans varyasyonları, genlerin ürünlerini veya aktivitelerini değiştirebilirler. Bu çalışmada *ANGPT-2* exon 4 G/A'da meydana gelen değişimlerin Türk popülasyonunda sporadik prostat kanser hastalarını nasıl etkilediğini belirlemeyi amaçladık.

Gereç ve Yöntemler: 52 sporadik prostat kanser hastası ve 52 sağlıklı kontrolü kapsayan hasta kontrol çalışması yapılmıştır. Farklı *ANGPT-2* alellerini tanımlamak amacıyla, Polimeraz Zincir Reaksiyonu (PCR) ve Restriksiyon Parçacık Uzunluk Polimorfizmi (RFLP) yöntemleri uygulanmıştır.

Bulgular: Polimorfizme ait genotip ve alel frekans dağılımları hastalar ve kontrol grubu arasında istatistiksel olarak anlamlı bir farklılığa işaret etmemiştir (P>0.05). Ayrıca, hastaların tümör lenf nodu metastazı (TNM), Gleason skoru (GS) ve serum prostat-spesifik antijeni (PSA) seviyelerine göre sınıflandırılması, *ANGPT-2* exon 4 G/A genotipleri arasında anlamlı farklılıklar göstermemiştir (P>0.05).

Sonuç: Bu çalışma, sporadik prostat kanseri hastalarında *ANGPT-2* exon 4 G/A polimorfizmi üzerine yapılan ve bu polimorfizmin Türk popülasyonunda prostat kanseri ile ilişkili olmadığını gösteren ilk çalışmadır.

Anahtar Kelimeler: Anjiyopoietin-2, polimorfizm, prostat kanseri, risk faktörü.

[1]Department of Medical Biology and Genetics, Faculty of Medicine, Gazi University, Beşevler, 06510, Ankara, Turkey.
[2]Department of Urology, Faculty of Medicine, Abant İzzet Baysal University, Gölköy, Bolu, Turkey.

INTRODUCTION

Prostate cancer is the most frequent type of malignant tumor among men aged 50 years and over (1). The causes of prostate cancer are heterogeneous, possibly involving both genetic and environmental factors (1-4).

Many molecular epidemiological studies have shown that gene polymorphisms of the enzymes involved in the metabolism of androgenic and non-androgenic hormones and those involved in phase I (cytochrome P450 variants) and phase II [glutathione *S*-transferase (GST) variants; N-acetyltransferases (NATs)] might be associated with the risk of prostate cancer development and progression (5-10). Three studies, to date, have been conducted on genetic polymorphisms of these enzymes (especially biotransformation and steroid metabolism) in Turkish men with prostate cancer (11-13).

The initiation of new blood vessels through angiogenesis is critical to tumor growth and the process of metastasis (14). Angiogenesis has been suggested to provide important prognostic information in prostate cancer (15). Growth of experimental prostate cancer is inhibited by anti-angiogenic treatment (16, 17). The vasculature also plays an important role in the regulation of growth and regression of normal prostate tissue (17). In the normal and malignant prostate, a variety of factors regulating blood flow and angiogenesis are produced by glandular epithelial and stromal cells. Three human angiopoietins, which are the angiogenesis regulating factors, have been identified: angiopoietin-1 (*ANGPT-1*), angiopoietin-2 (*ANGPT-2*), and angiopoietin-4 (*ANGPT-4*) (18). The Angpt-2 protein acts through inhibition of the endothelial cell-specific receptor tyrosine kinase (Tie-2) signaling and leads to a loosening of cell-matrix and cell-cell contacts, allowing access to angiogenic inducers, such as vascular endothelial growth factor (VEGF). That is, angiopoietins in tumor growth and prognosis are often in concert with VEGF (19). Expression and polymorphism analysis of VEGF shows that their regulation of tumor angiogenesis may be important in the formation and progression of prostate cancer (20-23). Although no polymorphism has been detected in the coding region of *ANGPT-1*, three independent polymorphisms in exons 2, 4, and 5 have been identified for *ANGPT-2* (18). The most common polymorphism of *ANGPT-2* is a G/A polymorphism in exon 4, a silent mutation, and does not result in amino acid changes in the encoded protein (18). However, silent mutations are often associated with expression differences in various genes on the protein level (24, 25). Although *ANGPT-2* is preferentially expressed in the female reproductive tract, hypoxia is one of the key regulating mechanisms for *ANGPT-2* expression, which makes the hypothesis reasonable that hypoxic conditions could alter the expression of *ANGPT-2* (25). Many molecular genetic studies pointed to the association between single nucleotide polymorp-

hisms (SNPs) and increased risk of developing prostate cancer and explained the variations in its incidence among different populations. *ANGPT-1*, *ANGPT-2*, their receptor Tie-2, and interrelationships between them may be important regulators in the angiogenesis of prostate cancer (21, 26-28). Although *ANGPT-2* is a candidate for the angiogenic switch (29), there are only a small number of studies on the *ANGPT-2* polymorphism involving obstetric conditions and/or diseases (25, 30, 31). Indeed, the molecular effects of the angiopoietins have not been fully ascertained in normal prostate and prostate tumors.

The capability of *ANGPT-2* exon 4 G/A polymorphism to affect the growth and invasion rates of the tumor may be a useful molecular indicator of prostate cancer prognosis. We, therefore, investigated whether the mentioned polymorphism is associated with sporadic prostate cancer in the Turkish population, a relationship not studied so far.

MATERIALS AND METHODS

Study population and clinical classification. The present case-control study included 104 subjects, consisting of 52 healthy volunteers as controls and 52 sporadic prostate cancer patients selected from patients admitted to the Department of Urology, Faculty of Medicine, Abant İzzet Baysal University. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association and the study was approved by the Ethical Committee of Gazi University. The patients and controls submitted their written consent before donating blood samples. In all cases, age at first diagnosis, clinical staging, which was carried out according to the 1997 TNM (Tumor, Lymph, Metastasis) system of the American Joint Committee on Cancer (32), pathological grades (33), and prostate-specific antigen (PSA) levels were obtained from the patients' files. The TNM staging system describes the extent of the primary tumor (T), the absence or presence of metastasis to nearby lymph nodes or glands (N), and the absence or presence of distant metastasis (M). The categories of clinical staging were organ-confined prostate cancer (pT1a to pT2c) and extra-capsular invasion prostate cancer (pT3a to pT4b). The categories of pathological grade were Gleason score 2-6, Gleason score 7, and Gleason score 8-10. Serum PSA levels were classified as ≤ 4, 5-9 and 10 ≤. None of the patients had a family history of prostate cancer or benign prostatic hyperplasia (BPH). The control group consisted of 52 randomly selected healthy men who had no family history of prostate cancer. These men underwent a clinical urologic examination, which included a digital rectal examination (DRE), transrectal ultrasound of the prostate (TRUS), residual urine volume, measurement of serum PSA, and physical check-up. Any samples with abnormal DRE, suspicious lesions detected by TRUS, or elevated serum levels of PSA 4 ng/mL ≤ were excluded from the control group. Clinical characteristics of the patients and controls are shown in Table 1.

Genotyping. For extraction of genomic DNA and amplification of the gene *ANGPT-2*, genomic DNA was isolated from peripheral blood using a DNA extraction kit (Heliosis®, Metis Biotechnology, Turkey) according to the manufacturer's instructions. Amplifications of the gene encoding for *ANGPT-2* were carried out by placing in a Mastercycler gradient (Eppendorf, Germany) thermal cycler a total volume of 50 µL of PCR mixture containing 50 ng of genomic DNA, 2.5 mM $MgCl_2$, 100 µM dNTP, 50 pmol/µl of each primer, and 1.0 U/µl of Taq DNA polymerase. Oligonucleotide primers flanking exon 4 of *ANGPT-2* were used. The sequence of the forward primer was 5' CATTAGAATAGCCTTCAC 3'. The reverse primer sequence was 5' GAGTGTTTACTGACTAAAGG 3' (Gene bank accession No. AC018398; nucleotides 83277-83611). For genotyping, a fragment of 335 bp (base pairs) was amplified. We set the PCR cycling conditions for the *ANGPT-2* gene as explained by Pietrowski et al. (25). The *ANGPT-2* exon 4 G/A polymorphism was analyzed by digestion of the PCR product with restriction endonuclease *Eco57I* (Fermantas, USA). After digestion, the G allele generated two fragments of 193 bp and 142 bp, whereas the A allele remained uncut (335 bp). The PCR products and restriction fragments of *ANGPT-2* exon 4 were loaded directly onto 2% agarose gel (containing 0.5% ethidium bromide) (Fig. 1). The products were separated by electrophoresis and visualized by Gel Logic 100 gel image system (Kodak, USA).

Statistical analysis. We used Pearson's two-way chi-square (χ^2) test to evaluate whether the distribution of genotype frequency varied among the cases and controls. The relationships between genotypes and/or alleles and prostate cancer risks were determined by obtaining the odds ratios (ORs) through a logistic regression method [95% confidence interval (CI)]. Adjusted ORs for the other epidemiological covariant factors such as TNM, Gleason score, and PSA levels were determined by using a multivariable logistic regression method. χ^2 values with a probability (p) value greater than 0.05 were not statistically significant. Statistical analysis was performed using SPSS V.11.5.

RESULTS

The mean age of first diagnosis of the prostate cancer patients and that of the controls were not significantly diffe-

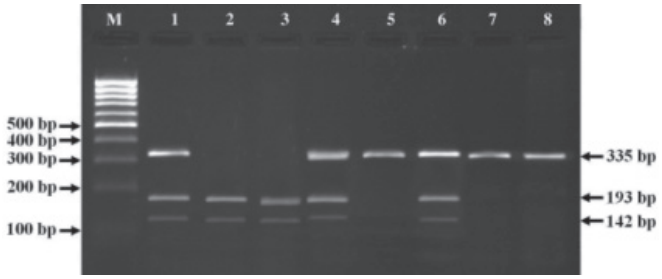


Figure 1: PCR-based restriction analysis of *ANGPT-2* exon 4 gene polymorphism shown on 2% agarose gel electrophoresis. Lanes 1, 4, 6: heterozygous G/A alleles. Lanes 2, 3: homozygous G alleles. Lanes 5, 7: homozygous A alleles. The left lane (M: marker) contains a 100 bp ladder and lane 8 contains a 335 bp PCR product (uncut) of the gene.

Table 1: Clinical characteristics of prostate cancer patients and controls.

Characteristics	Patients (N=52)	Controls (N=52)
Age (mean ± SD)	65 ± 0.1	62 ± 0.1
Age range (years)	45 – 89	53 - 74
Clinical Staging (TNM)[a]		
Stages		
pT1a-pT2c	33	-
pT3a-pT4b	19	-
Lymph Nodes		
N0	52	-
Metastasis		
M0	52	-
Pathological grade (GS)[b]		
2-6	37	-
7	11	-
8-10	4	-
PSA[c] level (ng/mL)		
≤ 4	10	-
5-9	14	-
10 ≤	28	-

[a] Tumor, lymph nodes, metastasis. According to bone scan at the time of diagnosis. N0= no regional lymph node metastasis. M0= no distant metastasis.
[b] Gleason score.
[c] Total serum prostate-specific antigen (PSA).

rent (P>0.05). We observed 23 (44.2%) GG, 22 (42.3%) GA, and 7 (13.5%) AA genotypes in the control subjects and 21 (40.4%) GG, 25 (48.1%) GA, and 6 (11.5%) AA genotypes in the prostate cancer patients (Table 2). The frequency of carriers of an A allele (AA + GA genotypes) in the prostate cancer patients (60%) was approximately equivalent to that in the controls (56%) (Table 2). The frequencies of G alleles were 64.4% in the patients and 65.4% in the controls. The frequencies of A alleles were 35.6% in the patients and 34.6% in the controls (Table 2). The ORs for prostate cancer risk for men with the GA and AA genotypes were 1.25 (P= 0.602, CI 0.55-2.84; Table 2) and 0.94 (P= 0.920, CI 0.27-3.25; Table 2), respectively, in comparison to the GG genotype. The OR for carriers of an A allele for prostate cancer was 1.17 (P= 0.691, CI 0.54-2.55; Table 2). The OR per copy of the "A" allele was 1.04 (P= 0.884, CI 0.59-1.84; Table 2). In other words, the genotype and allele frequencies of the gene were not significantly associated with prostate cancer patients (P> 0.05). Thirty-three out of the 52 prostate cancer patients were at the T1a-T2c stage whereas 19 were at the T3a-T4b stage (Table 1 and Table 3). None of the patients had regional lymph node metastasis or distant metastasis (Table 1). There was no significant correlation between tumor stages and genotypes (Table 3). Thirty-seven patients with Gleason score 2-6, 11 patients with Gleason score 7, 4 patients with Gleason score 8-10, 10 patients with PSA level ≤4 ng/mL, 14 patients with PSA level 5-9 ng/mL, and 28 patients with PSA level 10 ng/mL ≤ were included in the study (Table 4). Multivariable logistic regression analysis demonstrated that the occurrence of prostate cancer was not associated with Gleason score or PSA levels (Table 4). Adjusted ORs for each tumor stage and those for Gleason score and PSA levels in relation to prostate cancer risk are summarized in Table 3 and Table 4, respectively.

DISCUSSION

Prostate cancer growth is angiogenesis dependent, and thus progression and prognosis of prostate cancer are related

Table 2: Distribution of ANGPT-2 exon 4 G/A genotype and allele frequencies in men with prostate cancer and healthy controls.

	Patients		Controls		OR*	p values**
Genotypes	N	%	N	%		
GG	21	40.4	23	44.2	1.00	
GA	25	48.1	22	42.3	1.25 (0.55-2.84)	0.602
AA	6	11.5	7	13.5	0.94 (0.27-3.25)	0.920
AA + GA	31	60	29	56	1.17 (0.54-2.55)	0.691
Alleles						
G	67	64.4	68	65.4	1.00	
A	37	35.6	36	34.6	1.04 (0.59-1.84)	0.884

[*] Odds Ratio (OR) at 95% confidence interval (95% CI).
[**] p values were calculated GG vs. GA, AA and AA + GA by χ^2 test.

Table 3: Odds ratios of ANGPT-2 exon 4 G/A polymorphism in patients according to tumor stages.

Genotypes	Controls (N=52)	Patients (N=33)	T1a-T2c OR*	p**
GG	23	13	1.00	
GA	22	14	1.23 (0.43-2.92)	0.808
AA	7	6	1.52 (0.42-5.48)	0.524
AA + GA	29	30	1.83 (0.78-4.28)	0.162
Genotypes	**Controls (N=52)**	**Patients (N=19)**	**T3a-T4b OR***	**p****
GG	23	8	1.00	
GA	22	11	1.44 (0.49-4.24)	0.510
AA	7	0	NC***	-
AA + GA	29	11	1.09 (0.38-3.15)	0.873

* Odds Ratio (OR) at 95% confidence interval (95% CI).
** p values were calculated GG vs. GA, AA and AA + GA by χ^2 test.
*** NC= Not Calculated. There are no AA-genotype patients at T3a-T4b stages.

to angiogenesis and vasculature is under androgenic control in prostate tumors (34, 35). In this study, we aimed to investigate whether the *ANGPT-2* gene polymorphism is responsible for the development of sporadic prostate cancer.

Polymorphisms in the promoter regions of cytokine genes may influence prostate cancer development via regulation of the antitumor immune response and/or pathways of tumor angiogenesis. SNPs associated with differential production of IL-8, IL-10, and VEGF are risk factors for prostate cancer with their influence on angiogenesis (20). However, Yang et al. (36) could not confirm these relationships and examined the most representative SNPs for the IL-8 and its receptor genes (CXCR1 and CXCR2). None of the SNPs studied had major effects on prostate cancer susceptibility (36). On the other hand, variation in the promoter region of cyclooxygenase-2 (COX-2), which plays an important role in stimulating angiogenesis and promoting tumor cell metastasis and invasion, may influence the risk and development of prostate cancer (37). Furthermore, VEGF-460 C/T polymorphism represents a suitable genetic marker for prostate cancer but not necessarily with cancer progression (22).

Genetics as well as social and environmental factors, particularly diet and lifestyle, are determinants of why one man might be at a higher risk of prostate cancer than another. While the association between high penetrance genes (20) and prostate cancer susceptibility highlights the complex and multigenic mode of inheritance of prostate cancer, lower penetrance susceptibility polymorphisms in genes may be implicated in a higher portion of the sporadic prostate cancer disease burden and therefore have more relevance to public health. In our

Table 4: Odds ratios of ANGPT-2 exon 4 G/A polymorphism in prostate cancer patients according to Gleason score (GS) and prostate-specific antigen (PSA) levels.

Genotypes	Controls (N=52)	GS 2-6 Patients (N=37)	OR*	p**	GS 7 Patients (N=11)	OR*	p**	GS 8-10 Patients (N=4)	OR*	p**
GG	23	14	1.00		5	1.00		2	1.00	
GA	22	17	1.27 (0.51-3.18)	0.610	6	1.26 (0.33-4.71)	0.737	2	1.05 (0.14-8.08)	1.00
AA	7	6	1.41 (0.39-5.05)	0.599	0	NC***	-	0	NC***	-
Genotypes	**Controls (N=52)**	**PSA ≤ 4 ng/mL Patients (N=10)**	**OR***	**p****	**PSA 5-9 ng/mL Patients (N=14)**	**OR***	**p****	**PSA 10 ng/mL ≤ Patients (N=28)**	**OR***	**p****
GG	23	2	1.00		8	1.00		11	1.00	
GA	22	6	3.14 (0.57-17.2)	0.256	5	1.65 (0.19-2.31)	0.507	14	1.33 (0.50-3.55)	0.568
AA	7	2	3.29 (0.39-27.8)	0.281	1	0.41 (0.04-3.88)	0.653	3	0.90 (0.19-4.15)	1.00

* Odds Ratio (OR) at 95% confidence interval (95% CI).
** p values were calculated GG vs. GA and AA by χ^2 test.
*** NC= Not Calculated. There are no AA-genotype patients having GS ≥7

study, a limited number of samples were analyzed due to the strict criteria set for controls and patients. These criteria, on the other hand, were necessary to display the direct effects of the *ANGPT-2* exon 4 genotypes in the sporadic prostate cancer.

"AA" homozygote genotype was the least common genotype found in our patient and control groups. As a result, "A" allele frequencies of prostate cancer patients and controls were 35.6% and 34.6%, respectively. The distribution frequency of the G/A polymorphism of the *ANGPT-2* did not differ between the patients and the controls. There are few studies on *ANGPT-2* exon 4 G/A polymorphism. The *ANGPT-2* polymorphism is not associated with idiopathic recurrent miscarriage, unexplained intrauterine fetal death, or uterine leiomyoma (25, 30, 31). The allelic and genotypic frequencies of *ANGPT-2* exon 4 gene polymorphism found in our study were in close agreement with those previously published for a German population by Pietrowski et al. (25) and Denschlag et al. (31). However, Huber et al. (30) found that the least common genotype was GA heterozygote in *ANGPT-2* exon 4 G/A polymorphism (17% of the patients and 15% of the healthy controls). The discrepancy in the results concerning the same SNP might be attributed to several factors, including studying different populations, characteristics of controls and subjects, difference in the size of the samples, and functional changes in the *ANGPT-2* protein, which would affect the properties of the protein, giving it an increased or decreased ability to transactivate and cause transcription of its target genes during the progression of sporadic prostate cancer and other cancers.

A significant correlation has been demonstrated between the angiogenesis in prostate cancer and Gleason score (15, 38). In prostate tumors, basal epithelial cells are lost and accordingly the Angpt-1 expression is low in the tumor epithelial cells. Thus, normal homeostasis of the angiopoietins is disrupted in tumors in favor of Angpt-2 expression (28). In addition, Angpt-2 protein is up-regulated in high-grade prostate cancer and becomes able to act in synergy with VEGF to drive angiogenesis in prostate cancer (28). It was also reported in the same study that Angpt-2 protein was significantly correlated to Gleason score, density of endoglin-stained blood vessels, metastases, and cancer specific survival (28). However, in our population, no statistically significant differences were found between prostate cancer patients and genotypes when they were further classified according to clinical stage (TNM), pathologic grade (GS: Gleason score), and total serum prostate-specific antigen (PSA) levels. Due to the lack of published studies with respect to the *ANGPT-2* gene polymorphism in any type of prostate cancer, we were unable to compare our results with those of similar studies covering other populations. Although our criteria for the selection of the cases and controls resulted in a limited sample size, it is important to conduct further studies on polymorphisms of angiogenic genes, including *ANGPT-2*, and their susceptibility to sporadic prostate cancer in different populations in order to better understand the pathways regulating angiogenesis in the normal prostate and how these pathways change during malignant transformation. In addition, as genes tend to act in concert with other genes and environments, it may be worth investigating the possible association of the *ANGPT-2* gene and receptor polymorphisms with prostate cancer and measuring protein levels in relation to genotyping.

Acknowledgments

This study has been particilly supported by the Gazi University Research Fund, with the project code number 11/2003-05.

Correspondence Address
Ece KONAÇ, Ph.D.
Department of Medical Biology and Genetics
Faculty of Medicine Gazi University Beşevler, 06510, Ankara, TURKEY
Phone: (+90-312) 202 46 34
E-mail: ecemercanoglu@yahoo.com
ecem@gazi.edu.tr

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