Apolipoprotein E Genotypes in Patients with Prostate Cancer

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Abstract. *Background: Apolipoprotein E (ApoE) is a potential inhibitor of cell proliferation, immune regulation and modulation of cell growth and differentiation; it also has a substantial role in antioxidant activity. ApoE has a potential role in prostate cancer progression. Materials and Methods: ApoE genotyping was performed using real-time polymerase chain reaction (RT-PCR) for blood samples from a group of patients with prostate cancer (n=68) and a control group (n=78). Results: The frequency of the E3/E3 genotype was significantly higher in patients compared to controls (p=0.004). E3/E3 genotype carriers were 3.6-fold more likely to be patients than controls (odds ratio=3.67, 95% confidence interval=1.451-9.155; p=0.004). Additionally, the patients with E3/E3 genotype had significantly higher Gleason score (p=0.017), and more patients with this genotype had a Gleason score higher than 7 (p=0.007). Individuals carrying the E4 allele were significantly more common in the control group (p=0.006). The frequency of the E3/E4 genotype was found to be significantly higher in controls compared to patients (p=0.007), and patients were significantly less likely to have this genotype than controls (odds ratio=0.89, 95% confidence interval=0.833-0.967, p=0.007). Individuals carrying the E2/E3 genotype had a significantly lower Gleason score (p=0.049)–all of the patients with this genotype had a Gleason score lower than 7 (p=0.024). Conclusion: E3/E3 genotype may be a potential risk factor for prostate cancer and high Gleason scoring. The E4 allele maybe a risk-reducing factor for prostate cancer.*

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Prostate cancer has become one of the most common solid tumors diagnosed in men and the second leading cause of death due to malignancy (1). There are important differences in mortality rates associated with ethnic heritage (2). The most commonly used diagnostic parameter is serum prostate-specific antigen (PSA), and surgical decisions are basically made on the basis of the Gleason scoring system. Diagnostic parameters consisting of serum PSA levels, clinical and pathological stages and Gleason scores are considered as powerful prognostic determinants (3). Although the etiology of prostate cancer is largely unknown, both genetic and lifestyle/environmental factors might contribute to development of prostate cancer (4). Genetic identification can be useful for determining the prognosis of patients and risk of tumor progression.

Apolipoprotein E (ApoE) is a plasma protein which serves as a ligand for low-density lipoprotein receptors and participates in the circulatory transport of cholesterol and other lipids. ApoE synthesis occurs in various organs, such as liver, brain, spleen and kidney. It has high concentrations in interstitial fluid, where it appears to participate in cholesterol transport from cells with excess cholesterol to those requiring cholesterol. ApoE is also involved in the repair of tissue injury and regeneration. Studies have shown that ApoE, unrelated to lipid transport, is a potential inhibitor of cell proliferation (1), having roles in immune regulation and modulation of cell growth and differentiation (5), and has a substantial role of antioxidant activity (6). ApoE has three common isoforms, E2, E3 and E4, which are encoded by three variant alleles of 112/158 codons, ε2 (TGC/TGC), ε3 (TGC/CGC), and ε4 (CGC/CGC) (7). Although ethnic heritage affects the variation, the most to the least common

Apo E genotypes are E3/E3, E3/E4, E2/E3, E2/E4, E2/E2 and E4/E4. The *ApoE* gene variants affect tumor growth and proliferation to different extents (3, 6). Miyata *et al*. showed that the antioxidant activity of ApoE protects cells against oxidative damage *in vitro* (8). Previous studies on the effects of ApoE suggest that it is a potent inhibitor of proliferation in several cancer types (9), including prostate cancer (3, 10, 11). Ifere *et al*. showed that non-aggressive prostate cancer cell lines carry the E3/E4 genotype, while aggressive cell lines carry the E2/E4 genotype (10). A multicountry ecological study hypothesized that ApoE4 may be an important risk factor for Alzheimer disease, atherosclerosis and prostate cancer (11, 12). The aim of this study was to determine the ApoE allelic and genotypic variants in patients with prostate cancer in a Turkish population.

Materials and Methods

Patient selection. Our study included 68 patients with prostate cancer (mean age=67.61±7.34 years) who were treated at the Department of Urology, Yeditepe University Hospital and Göztepe Research and Education Hospital. The diagnosis of prostate cancer was confirmed by clinical, laboratory and pathological examinations. The tumor differentiation status was evaluated using Gleason scoring criteria. The clinical T-stage classified as early-stage (T1 and T2) and late-stage (T3 and T4) by clinical examinations. Pathological T-stage was classified as T2a, T2b, T2c, T3a and T3b. A total of 78 age-matched controls (mean age=67.53 ±8.77 years) were selected from the healthy cases with unimportant complaints at Urology Clinics from the same hospitals. Clinical parameters (body mass index, serum PSA level, Gleason score and smoking habit) for each participant were collected. Serum samples were taken after obtaining informed consent and the study was conducted prospectively.

DNA extraction. Blood samples from all participants were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Genomic DNA was extracted from 350 μl peripheral whole blood using Invitrogen iPrep PureLink gDNA blood isolation kit with a iPrep Purification Instrument (Invitrogen, Life Technologies, Carlsbad, CA, USA). The isolation procedure was performed in a closed system and it took 45 min; 100 μl of DNA was obtained at the end of the procedure. Consequently, sample DNA concentrations (mean=80±9.62 ng/μl) and optical density ratios (at 260/280 nm) (mean=1.9±0.2) were measured by Nanodrop 2000 (Thermoscientific, Waltham, MA, USA). Isolated DNA samples were preserved at 4˚C until genotyping assessments were conducted.

Genotyping. Analysis of ApoE genotype variants were performed in A LightCycler 4800 RT PCR (Roche Diagnostics, Mannheim, Germany) with an ApoE mutation detection kit (Roche Diagnostics, TIB MOLBIOL GmbH, Berlin, Germany). A 228 bp fragment of the human ApoE gene was amplified with specific primers. The resulting PCR fragments were analyzed with a probe (ApoE C112R, detected in channel 530 465-510 nm) and with probes labeled with LightCycler Red 640 (ApoE R158C, detected in channel 640 498-640 nm). The simultaneous analysis of the two polymorphic codons (codons 112 and 158) in a single reaction was conducted using two reporter dyes with the different excitation and emission spectrum LightCycler-color compensation software (Roche Diagnostics, Mannheim, Germany) to correct the temperature-dependent crossover among the emission spectra of the dyes. The ApoE codon 112 exhibits a melting temperature (Tm) of 49.0˚C in channel 530 for allelic variant 112C and a Tm of 59.0˚C in channel 530 for allelic variant 112R. The ApoE codon 158 exhibits a Tm of 63.0˚C in channel 640 for allelic variant 158R and a Tm of 53.0˚C in channel 640 for allelic variant 158C.The different genotypes were then determined by performing the melting curve analysis using the two different channels. In this study, we used LightMix Kit-Color Compensation 530/640 (Roche Diagnostics, TIB MOLBIOL, Berlin, Germany) for color compensation file generated with a prerequisite to run the duplex reaction. The resulting melting peaks in the different fluorescence channels allowed us to discriminate among the homozygous as well as the heterozygous genotypes. At these two codon sites (112/158), E2, E3, and E4 alleles contain TGC/TGC (Cys/Cys), TGC/CGC (Cys/Arg), and CGC/CGC (Arg/Arg), respectively (7).

Statistical analyses. Statistical analyses were performed using SPSS version 23 software (SPSS Inc, Chicago, IL, USA). Values are given as the mean±standard deviation (SD). Student's *t*-test was used to examine the significance of differences between the two groups and χ^2 and Fisher's exact tests were used to compare demographic information with expression. Individuals homozygous for the common genotypic variants were used as the reference to test for any association of genotype with prostate cancer by calculating the odds ratio (OR) with 95% confidence interval (CI). *p*-Values lower than 0.05 denote statistical significance.

Table I. *Demographic characteristics of the study population*.

Parameter	Prostate cancer (n=68)	Control (n=78)	*p*-Value
Age (years), mean±SD	67.61±7.34	67.53±8.77	0.967
Body mass index (kg/m^2), mean±SD	27.01±3.71	27.28±3.55	0.773
Smoking (pack years), mean±SD	30.56±18.68	27.75±17.04	0.594
PSA (ng/ml),mean±SD	32.43±44.99	3.03±2.66	0.006*
Family history of cancer, n (%)			
Yes	31 (44.9%)	-	-
No	38 (55.1%)	-	-
Gleason score, mean±SD	7.74±0.88	-	-
Pathological T-stage, n (%)			
T2a	9 (13.2%)	-	-
T2b	10 (14.7%)	-	-
T2c	28 (41.2%)	-	-
T3a	11 (16.2%)	-	-
T3b	10 (14.7%)	-	-
Clinical T-stage n (%)			
cT1c	26 (38.2%)	-	-
cT2	34 (50%)	-	-
cT3	8 (11.8%)	-	-

PSA: Prostate-specific antigen, n: number of individuals; SD: standard deviation; *statistically significant difference.

Table II. *Apolipoprotein E (Apo E) genotypic and allelic frequencies in patients with prostate cancer and the control group.*

	Prostate cancer (n=68)	Control (n=78)	*p*-Value	Odds ratio	95% Confidence interval
Genotype	n (%)	n (%)			
E3/E3	61 (89.6%)	55 (70.5%)	0.004*	3.67	1.451-9.155
E3/E4	0 (0%)	8 (10.3%)	0.007*	0.89	0.833-0.967
E2/E3	5 (7.4%)	12 (15.4%)	0.131	0.42	0.141-1.26
E2/E4	0 (0%)	3 (3.8%)	0.380	0.36	0.037-3.56
E2/E2	1 (1.5%)	0 (0%)	0.283	1.01	0.986-1.044
E4/E4	1 (1.5%)	0 (0%)	0.127	1.03	0.989-1.072
Allelle	Allelic count (%)	Allelic count (%)			
E2	7 (28.6%)	15 (71.4%)	0.069	0.38	0.141-1.063
E3	127 (46.8%)	130 (53.2%)	0.764	1.36	0.221-8.386
E4	2 (8.3%)	11 (91.7%)	0.006*	0.91	0.131-0.973

n: Number of individuals; *statistically significant difference.

Results

The demographic and clinical characteristic of control and patient groups are given in Table I. The patient group had significantly higher levels of PSA (p=0.006) when compared to the control group, as expected. No significant differences were found between patients and controls in terms of median age, body mass index and smoking habit (p>0.05).

ApoE genotypic and allelic frequencies in patients with prostate cancer and controls are given Table II. There was a significant difference in ApoE genotype between patient and control groups (χ^2=15.581; p=0.008). The frequency of the E3/E3 genotype was found to be significantly higher in patients compared to controls (χ^2=8.197; p=0.004). Those with E3/E3 genotype were ~3.6-fold more likely to be patients than controls (OR=3.67, 95% CI=1.451-9.155; p=0.004). Patients were significantly less likely to have the E3/E4 genotype than were the controls (χ^2=7.379; OR=0.89, 95% Cl=0.833-0.967; p=0.007). Despite these results, there were no significant differences between the groups in the frequency of ApoE3 allele (χ^2=0.111; p=0.764). We also found the E4 allelic frequency to be significantly higher in the control group compared to that for patients (χ^2=7.694' OR=0.91, 95% CI=0.131-0.973; p=0.006).

The patients with E3/E3 genotype had significantly higher Gleason score (χ^2=19.650, p=0.017). When we divided all genotype groups by Gleason score ≤7 and >7 (not shown in the table), 54.1% of the patients (n=33) with E3/E3 genotype had a Gleason score higher than 7 (p=0.007). Individuals carrying the E2/E3 genotype had a significantly lower Gleason score (χ^2=14.815; p=0.049), in fact all of the patients with this genotype had a Gleason score lower than 7 (p=0.024). Interestingly, all the patients with E2 allele had a Gleason score lower than 7 (χ^2=6.205, p=0.013). ApoE genotypes and allelic frequencies in relation to Gleason scoring and PSA levels are shown in Table III.

Table III. *Apolipoprotein E (ApoE) genotypic and allelic frequency and Gleason score and prostate-specific antigen (PSA) levels in patients with prostate cancer.*

	Gleason score (mean)	*p*-Value	PSA (ng/ml)	*p*-Value
Genotype (n=68)				
E3/E3 (n=61)	7.84±0.898	0.017*	34.51±47.08	0.397
Not E3/E3 (n=7)	7±00		19.07±21.15	
E3/E4 (n=0)	0	-	-	-
Not E3/E4 (n=68)	7.75±0.887		32.87±45.19	
E2/E3 (n=5)	7±00	0.049*	24.02±23.70	0.652
Not E2/E3 (n=63)	7.81±0.895		33.59±46.56	
E2/E4 (n=0)	0	-	4.5±0	0.531
Not E2/E4 (n=68)	7.84±0.898		33.30±45.40	
E2/E2 (n=1)	7±00	0.399	4.5±0	0.670
Not E2/E2 (n=67)	7.76±0.889		33.30±45.40	
E4/E4 (n=1)	7±00	0.229	6.7±3.11	0.410
Not E4/E4 (n=67)	7.77±0.894		33.69±46.61	
Alleles (n=68)				
E2 (n=6)	7±00	0.029*	20.76±22.64	0.496
Not E2 (n=62)	7.82±0.897		34.08±46.80	
E3 (n=66)	7.77±0.891	0.228	33.69±45.66	0.410
Not E3 (n=2)	7±00		6.7±3.11	
E4 (n=1)	7±00	0.399	8.9±00	0.397
Not E4 (n=67)	7.76±0.889		33.24±45.44	

n: Number of individuals; *statistically significant difference.

Analysis of clinical stages showed that 23 of those with E3/E3 genotype (total n=61) had cT1c stage (37.7%), 30 had

cT2 stage (49.2%) and eight had cT3 (13.1%). E3 allele carriers (total n=66) had a similar profile to that for E3/E3 genotype (cT1c: n=24, 36.4%; cT2: n=34, 51.5%; cT3: n=8, 12.1%). Four patients who had E2/E3 genotype (total n=5) had cT2 stage (80%) and one of the patients in this group had cT1c stage (20%). One patient from each E2/E2 and E4/E4 group had cT1c clinical stage.

For the pathological stage analysis, most patients had T2c disease by genotype (E3/E3: 41%; E2/E3: n=2, 40%; E4/E4: n=1, 100%) and also by allele (E2: n=2, 33%, E3: n=27, 40.9%; E4: n=1, 100%).

Discussion

In this study, we explored the association between ApoE genotypic and allelic frequencies in patients with prostate cancer. We also investigated effects of ApoE genotypes and allelles on the prognosis of prostate cancer cases by Gleason score. Venanzoni *et al*. showed that ApoE expression correlated directly with Gleason score in prostate cell lines (3). In the present study, we focused on the relationship between ApoE genotypes and their effects on prostate cancer *in vivo*.

Currently, there exist no clearly defined mechanisms to explain associations between ApoE isoforms and cell proliferation. Understanding the molecular mechanisms underlying the development and progression of prostate cancer may help answer clinical questions about its diagnosis and treatment. It is remarkable that there is a lack of reliable markers predictive of malignancy. Current surgical strategies are still based exclusively on Gleason score, which is considered highly predictive of prostate cancer progression. Venanzoni *et al*. also found that ApoE expression correlated directly with the Gleason score in tissue sections (3). In our study, we show that patients with E3/E3 genotype had a potential risk for prostate cancer and higher Gleason score.

The protective effect of the E2 allele has been studied in many diseases. Corder *et al*. demonstrated a protective effect of E2 allele in late-onset Alzheimer disease (13). Miyata *et al*. showed that the E2 allele had antioxidant activity that was suggested to protect tissues from oxidative damage. This had been shown in cultured B12 cells protected from beta-amyloid peptides by E2; E2 is the most protective isoform against the effects of hydrogen peroxide cytotoxicity (8). Furthermore, Lehrer claimed that increased antioxidant activity of ApoE2 allele could be protective against prostate cancer (12). Another study showed that the presence of ApoE2 allele and ApoE4 allele were positively associated with breast cancer cases (14). In our study, we found that the ApoE4 allele was significantly more frequent in the control group. Therefore we suggest that E4 may be a factor reducing prostate cancer risk. But we did not find any relationship for the E2 allele. On the contrary, Lehrer *et al*. published a letter in the British Journal of Cancer in 1998 reporting that the E4 allele was a risk factor for prostate cancer (12). There were many studies which claimed association between the E4 allele and particular diseases. The E4 allele has been reported to be responsible for atherosclerosis (15) and is a strong risk factor for Alzheimer disease (16). Although many studies have shown the negative results of carrying an E4 allele, recent cancer studies suggested there were no associations between the E4 allele and various cancer types (17, 18). A case study showed that the E4 allele was not a risk factor for patients with prostate cancer (17). A meta-analysis of breast cancer suggested that among Caucasians, neither the E4 allele nor the E2 allele showed an association with susceptibility to breast cancer (18). In our study, E4 allele carriers had a lower risk for prostate cancer and the E2 allele might have positive effects on Gleason score. Our hypothesis is supported by another case–control study by Cibeira *et al*. showing that the presence of E4 and E2 alleles was associated with the absence of breast cancer (14). Other studies suggested a protective antioxidant effect of the E2 allele in Alzheimer disease (8, 13). De Feo *et al*. supported the protective effect of E2 allele on gastric cancer risk and progression in a case–control study (19). These studies reported that the protective effect of the E2 allele was associated with its antioxidant properties. In our study, although we did not find any relationship for the E2 allele, all patients with E2 allele head a Gleason score ≤7. Thus this could be the result of The antioxidant effect of E2.

An *in vitro* study showed that ApoE is a potent inhibitor of cell proliferation and has effects on modulating angiogenesis, tumor cell growth and metastasis of endothelial and tumor cells (5). The antiproliferative effect of ApoE might result from binding to cell surface or matrix heparan sulfate proteoglycans and thus have an effect on cellular interactions (9). Scott *et al*. suggested that ApoE may enhance microtubule formation and enhance cell polarity (20). ApoE expressions are controlled by variations of ApoE genotype, so we could hypothesize that E3/E3 genotype carriers have a more aggressive tumor type.

In conclusion, the E3/E3 genotype may be a potential risk factor for prostate cancer and higher Gleason score. The E4 allele appears to be a factor reducing prostate cancer risk and the E2 allele might have a positive impact on Gleason score.

References

1 Brawer MK, Crawford ED, Fowler J, Lucia MS and Schröeder FH: Prostate cancer: epidemiology and screening. Rev Urol *2(Suppl 4)*: S5-9, 2000.

2 Park SY, Murphy SP, Wilkens LR, Henderson BE and Kolonel LN: Fat and meat intake and prostate cancer risk: the multiethnic cohort study. Int J Cancer *121(6)*: 1339-1345, 2007.

3 Venanzoni MC, Giunta S, Muraro GB, Storari L, Crescini C, Mazzucchelli R, Montironi R and Seth A: Apolipoprotein E expression in localized prostate cancers. Int J Oncol *22(4)*: 779-786, 2003.
4 Kalay E, Ergen A, Narter F, Agaçhan B, Görmüs U, Yigit N and Isbir T: ARE-I polymorphism on PSA gene in prostate cancer patients of a Turkish population. Anticancer Res *29(4)*: 1395-1398, 2009.
5 Vogel T, Guo NH, Guy R, Drezlich N, Krutzsch HC, Blake DA, Panet A and Roberts DD: Apolipoprotein E: a potent inhibitor of endothelial and tumor cell proliferation. J Cell Biochem *54(3)*: 299-308, 1994.
6 Mahley RW: Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science *240(4852)*: 622-630, 1998.
7 Nauck M, Hoffmann MM, Wieland H and März W: Evaluation of the apo E genotyping kit on the LightCycler. Clin Chem *46(5)*: 722-724, 2000.
8 Miyata M and Smith JD: Apolipoprotein E allele specific antioxidant activity and effects on cytotoxicity by oxidative insalts and betaamyloid peptides. Nat Genet *14*: 55-61, 1996.
9 Niemi M, Hakkinen T, Karttunen TJ, Eskelinen S, Kervinen K, Savollainen MJ, Lehtola J, Makela J, Yla-Herttuala S and Kesanimi YA: Apolipoprotein E and colon cancer Expression in normal and malignant human intestine and effect on cultured human colonic adenocarcinoma cells. Eur J Intern Med *13(1)*: 37–43, 2002.
10 Ifere GO, Desmond R, Demark-Wahnefried W and Nagy TR: Apolipoprotein E gene polymorphism influences aggressive behavior in prostate cancer cells by deregulating cholesterol homeostasis. Int J Oncol *43(4)*: 1002-1010, 2013.
11 Grant WB: A multicountry ecological study of risk-modifying factors for prostate cancer: apolipoprotein E epsilon4 as a risk factor and cereals as a risk reduction factor. Anticancer Res *30(1)*: 189-199, 2010.
12 Lehrer S: Possible relationship of the apolipoprotein E (ApoE) epsilon4 allele to prostate cancer. Br J Cancer *78(10)*: 1398, 1998.
13 Corder EH, Saunders AM, Risch NJ, Strittmatter WJ, Schmechel DE, Gaskell PC Jr, Rimmler JB, Locke PA, Conneally PM, Schmader KE, Small GW, Roses AD, Haines JL and Pericak-Vance MA: Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat Genet *7(2)*: 180-184, 1994.
14 Cibeira GH, Giacomazzi J, Aguiar E, Schneider S, Ettrich B, DE Souza CI, Camey S, Caleffi M, Weber B, Ashton-Prolla P and Moriguchi EH: Apolipoprotein E genetic polymorphism, serum lipoprotein levels and breast cancer risk: A case–control study. Mol Clin Oncol *2(6)*: 1009-1015, 2014.
15 Isbir T, Yilmaz H, Agachan B and Karaali ZE: Cholesterol ester transfer protein, apolipoprotein E and lipoprotein lipase genotypes in patients with coronary artery disease in the Turkish population. Clin Genet *64(3)*: 228-234, 2003.
16 Weisgraber KH and Mahley RW: Human apolipoprotein E: the Alzheimer's disease connection. FASEB J *10(13)*: 1485-1494, 1996.
17 Wessel N, Liestøl K, Maehlen J and Brorson SH: The apolipoprotein E epsilon4 allele is no risk factor for prostate cancer in the Norwegian population. Br J Cancer *85(9)*: 1418, 2001.
18 Saadat M: Apolipoprotein E (APOE) polymorphisms and susceptibility to breast cancer: a meta-analysis. Cancer Res Treat *44(2)*: 121-126, 2012.
19 De Feo E, Simone B, Persiani R, Cananzi F, Biondi A, Arzani D, Amore R, D'Ugo D, Ricciardi G and Boccia S: A case-control study on the effect of apolipoprotein E genotypes on gastric cancer risk and progression. BMC Cancer *12*: 494, 2012.
20 Scott BL, Welch K, deSerrano V, Moss NC, Roses AD and Strittmatter WJ: Human apolipoprotein E accelerates microtubule polymerization *in vitro*. Neurosci Lett *245(2)*: 105-108, 1998.

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