Malays. Appl. Biol. (2016) 45(2): 131–138

PROSTATIC DISEASES: IS IT TOXIC OR GENETIC? STUDY OF TNF ALPHA GENE POLYMORPHISM AND CIGARETTE SMOKING IN CASES OF PROSTATE CANCER AND BENIGN PROSTATIC HYPERPLASIA

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Accepted 28 October 2016, Published online 21 December 2016

Abstract

Inflammation has been implicated as an etiological factor, in several human cancers. Allelic variants of the genes involved in inflammatory pathways are logical candidates as genetic determinants of prostate cancer risk. Besides genetic factors, environmental factors such as smoking are an important risk factor for prostate cancer. This study aimed to investigate whether 308 G/A single nucleotide polymorphism of tumor necrosis factor-α (TNF-α) gene promoter region was associated with outcomes of prostate cancer and to analyze the gene environment interaction between 308 G/A TNF polymorphism and cigarette smoking. A total of 282 patients with prostate cancer (143 smokers, 139 non smoker) and 212 patients with benign prostatic hyperplasia (105 smokers, 107 non smokers) along with 115 healthy control were enrolled in the study. Urinary Cotinine and serum TNF and PSA levels were measured using ELISA technique. TNF genotyping was performed using PCR-RFLP technique. Prostate cancer was significantly associated with TNF G/G genotype and this is accompanied by elevated plasma TNF, PSA and urinary Cotinine. Cancer smokers showed a high frequency of TNF-α 308 G allele compared with other patient groups associated with increased TNF levels. Results of this study support the hypothesis that polymorphism in proinflammatory genes may be important in prostate cancer development and the sequence variants in these inflammatory genes may interact with environmental modifiers such as cigarette smoking to increase prostate cancer risk.

Key words: cigarette smoking, cotinine, gene polymorphism, prostate, cancer

INTRODUCTION

Inflammation has been suggested to have a role in prostate cancer development (De Marzo *et al*., 2007), and epidemiologic research on factors such as non-steroidal anti-inflammatory drug use, obesity, and prostatitis (MacInnis & Englis, 2006; Hsing & Chokkalingam, 2006; Mahmud *et al*., 2004) provides indirect support for the hypothesis. While inflammation might promote carcinogenesis directly, via cellular or DNA damage, it might also promote carcinogenesis indirectly by increasing cell turnover and levels of pro-inflammatory factors, such as tumor necrosis factor-alpha (TNF-α), which themselves affect cancer risk (Smith *et al.*, 2001; Palapattu *et al*., 2005; De Marzo *et al*., 2007). Despite the importance of TNF as a mediator of the inflammatory process (Balkwill, 2002), few studies have examined associations between TNF polymorphisms and prostate cancer risk. Results from existing studies have been mixed, with one observing significant associations with prostate cancer risk (Oh *et al*., 2000) while others did not (Wu *et al*., 2004; McCarron *et al*., 2002; Danforth *et al*., 2008).

Tumor necrosis factor-alpha (TNF-α) is an important inflammatory cytokine that may play a role in controlling the progression of prostate cancer. Two common polymorphisms in the *TNF-α* gene, -308G/A and -238C/T, have been suggested to alter the risk for prostate cancer, but the results have been inconclusive so far (Ma *et al*., 2014).

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Therefore, the present research was done to study if there is association between TNF variants and prostate cancer risk.

METHODS

The current study included adult Egyptian males with a recent diagnosis of prostate cancer (n=282), patients with benign prostatic hyperplasia (n=212) presented at the General surgery department of Mansoura University Hospital and Mansoura Oncology Center. Clinical diagnosis of primary adenocarcinoma of the prostate was histo-pathologically confirmed after abnormal serum PSA findings. Patients were selected randomly with a mean age of 67.4 ± 5.8 years and mean PSA level of 31.6 ± 5.7. Healthy unrelated men (n=115) were selected as controls; these were recruited from men attending to our Hospital for unrelated complaints and with no history of prostate cancer according to Registry records. They were age matched, clinically free as regard the urogenital examination and the mean PSA level was 2.20 ± .07. The mean of age of the controls was 59.4 ± 3.7 years.

The patients were classified according to their smoking history into the following groups: Group 1 include non smoker patients with BPH (n=105), group 2 include smoker patients with BPH (n=107), group 3 include non-smoker patients with cancer (n=139) and group 4 include smoker patients with cancer (n=143). The control group included 115 age matched nonsmoker men. Exclusion criteria were: diabetes mellitus, chronic renal failure, coronary artery disease, end stage liver disease, positive serum antinuclear antibody (ANA). Patients and controls had no history of other malignancy.

All subjects were interviewed by a researcher and signed their informed consent to participation in the study, which was approved by the Ethics Committee of Mansoura University Hospital.

All subjects were instructed to fast for at least 12 hours. A blood sample was withdrawn 3 mls were delivered to centrifuge tubes containing K2EDTA (stored as EDTA anti-coagulated blood sample at -30 C for DNA extraction). Another 5 ml blood sample was allowed to clot for 15 minutes and centrifuged at 7000 rpm for 10 minutes for serum separation to determine: serum PSA, TNF-α. Urine sample was collected for cotinine estimation.

DNA extraction

Genomic DNA was extracted from EDTA–anticoagulated peripheral blood leucocytes using QIA amp DNA Blood Mini Kit supplied by Qiagen GmbH (Cat. No. 51104, Hiden, Germany) (Schur *et al*., 2001). The average DNA concentration (0.127±0.005µg/µl) was determined from absorbance at 260 nm (Jenway, Genova Model, UK). All samples had a 260/280 nm absorbance ratio between 1.6 and 1.79. The integrity of the DNA was checked by electrophoresis on 0.8% agarose gel stained with ethidium bromide.

Genotyping of TNF-α -308 gene

TNF-α -308 gene polymorphism study was carried out by allele-specific polymerase chain reaction (ASPCR) methods described by Tronchon *et al.* (2008) as follow: A TNF-R primer (5'-TCTCGGTTTCTTCTCCATCG) was used with either 308-G (5'-ATAGGTTTTGAG GGGCATGG) or 308-A (5'-ATAGGTTTTGAGGGGCATGA) to amplify a 184 bp fragment of the TNFα gene, which includes the polymorphic site at the nucleotide position -308. The primer pair TNF-F (5'-GAGTCTCCGGGTCAGAATGA)/TNF-R was used to amplify a 531 bp TNF gene fragment that was used as an internal control in the allele-specific polymerase chain reaction (ASPCR). Primer TNF-F was also used as a competitor for the TNF-R/A and TNF-R/G primer pairs to improve the specificity of the ASPCR assay (Zhu & Clark, 1996). Gene specific primers were purchased from Biolegio. BV, PO Box 91, 5600 AB Nijmegen, Netherlands. PCR was carried out in 50 microliters final reaction volume using ReadyMix (RED. Taq-PCR Reaction Mix) (purchased from Sigma Aldrich, Saint Louis, USA). The following mixture was prepared for each sample: 25 µl RED-Taq PCR reaction Mix (1X), 1 µl (20 pmole) of forward primer for each allele, 1 µl (20 pmole) of reverse primer, 2 µl (200 ng) of genomic DNA and 20 µl of double distilled deionized water. This mix was put in a thin wall PCR microcentrifuge tube and gently centrifuged to collect all components to the bottom of the tube. Then 50 µl mineral oil was added to prevent evaporation. Amplification was performed in a Thermal Cycler (Minicycler-PTC-150) using the following program: After initial heating at 95°C for 10 min, 30 PCR cycles were performed and consisted of heat denaturation (95°C for 45 s), annealing (for 150 s at 60°C for the TNF primer pair) and extension (72°C for 45 s). A final extension (72°C for 9 min) was performed. The resulting PCR product was 184 bp in length for each allele and 531 bp TNF gene fragment for the internal control. The products were subjected to agarose gel electrophoresis using 2% agarose stained with ethidium bromide and visualized via Light UV Transilluminator (Model TUV-20, OWI Scientific, Inc. 800 242-5560, France) and photographed.

Estimation of serum TNF-α level

Quantitative determination of serum TNF-α level were performed by RayBio Human TNF alpha ELISA Kit (RayBiotech, Inc. Cat#: ELH-TNFalpha-

001). This assay employs the quantitative sandwich ELISA technique which measures TNF-α in 5 hours. It was performed according to the manufacturer's instructions. The absorbance of each sample was read on plate ELISA reader (Tecan, Sunrise Absorbance reader) at 450 nm wavelength (Bonavida, 1991).

Estimation of serum PSA level

Quantitative determination of serum PSA level were performed by DRG® Total PSA ELISA (DRG International, Inc., USA. Cat#: EIA-3719). This assay employs the quantitative sandwich ELISA technique. It was performed according to the manufacturer's instructions, the absorbance of each sample was read on plate ELISA reader (Tecan, Sunrise Absorbance reader) at 450 nm wavelength with blanking at 630nm wavelength (Price *et al*., 2001).

Estimation of urinary cotinine level

Quantitative determination of urinary cotinine level was performed by Cotinine ELISA kit (Catalog No. 40-101-325056, GenWay Biotech, Inc. 6777 Nancy Ridge Drive San Diego, CA 92121). This assay employs the quantitative solid phase competitive ELISA technique. It was performed according to the manufacturers instructions, the absorbance of each sample was read on plate ELISA reader (Tecan, Sunrise Absorbance reader) at 450 nm wavelength (Yeh *et al*., 2008).

RESULTS

Results showed significant difference in PSA level among the studied groups. PSA was significantly higher in cancer patients (32.05 ± 11.51 ng/ml) than BPH patients (11.76 ± 3.84 ng/ml). It showed also that smoking has significant effect in BPH (10.70 ± 2.12 ng/ml) and cancer (56.04 ± 4.39 ng/ml) patients in comparison to the non smokers (11.76 ± 3.84 ng/ml and 32.05 ± 11.51 ng/ml respectively) (Table 2). Table 3 showed significant difference in TNF level among the studied groups. TNF was significantly higher in cancer patients (35.72 ± 6.33 ng/ml) than BPH patients (17.76 ± 4.29 ng/ml). It showed also that smoking has significant effect in BPH (19.46 ± 5.28 ng/ml) and cancer (41.56 ± 8.63ng/ml) patients in comparison to the non smokers (35.72 ± 6.33 ng/ml and 17.76 ± 4.29 ng/ml respectively). Table 4 showed significant difference in cotinine level among the studied groups. Cotinine was significantly higher in cancer patients (425.97 ± 125.34 ng/ml) than BPH patients (376.69 ± 130.97 ng/ml). It showed also that smoking has significant effect in BPH (1379.6 ± 252.64 ng/ml) and cancer (1755.4 ± 230.62 ng/ml) patients in comparison to the non smokers (376.69 ± 130.97 ng/ml and 425.97 ± 125.34 ng/ml respectively). Correlation studies (Table 5 and Graph 1) showed significant correlation between the level of PSA and TNF (0.832), PSA and cotinine (0.581),

Table 1. Clinical character of patients

	Control	Group 1 BPH	Group 2 BPH smokers	Group 3 Cancer	Group 4 Cancer smokers
No.	(n=115)	(n=105)	(n=107)	(n=139)	(n=143)
Age	59.4±3.7	60.1±3.7	64.74±3.5	65.33±3.72	67.02±4.88

Table 2. Plasma PSA (ng/ml) in the studied groups

Group 4 Cancer smokers (n=143)	Group 3 Cancer (n=139)	Group 2 BPH smokers (n=107)	Group 1 BPH (n=105)	Control (n=115)	
56.04 ± 4.39	32.05 ± 11.51	10.70 ± 2.12	11.76 ± 3.84	2.20 ± .07	Mean ± SD
t=-128.5 p=0.000	t=-27.68 p=0.000	t=-38.01 p=0.000	t=-25.58 p=0.000		t test Comparison with the control group
t=-23.24 p=0.000		t=2.49 p=0.014			Comparison between group 1 & 2 and 3 & 4 (Effect of smoking)
	t=-17.34 p=0.000				Comparison between group 1 & 3
t=-98.59 p=0.000					Comparison between group 2 & 4

Table 3. Plasma TNF (ng/ml) in the studied groups

Group 4 Cancer smokers (n=143)	Group 3 Cancer (n=139)	Group 2 BPH smokers (n=107)	Group 1 BPH (n=105)	Control (n=115)	
41.56 ± 8.63	35.72 ± 6.33	19.46 ± 5.28	17.76 ± 4.29	12.50 ± 5.15	Mean± SD
t=-31.83 p=0.000	t=-31.60 p=0.000	t=-9.94 p=0.000	t=-8.18 p=0.000		t test Comparison with the control group
t=-6.46 p=0.000		t=-2.58 p=0.011			Comparison between group 1 & 2 and 3 & 4 (Effect of smoking)
	t=-25.05 p=0.000				Comparison between group 1 & 3
t=-23.4 p=0.000					Comparison between group 2 & 4

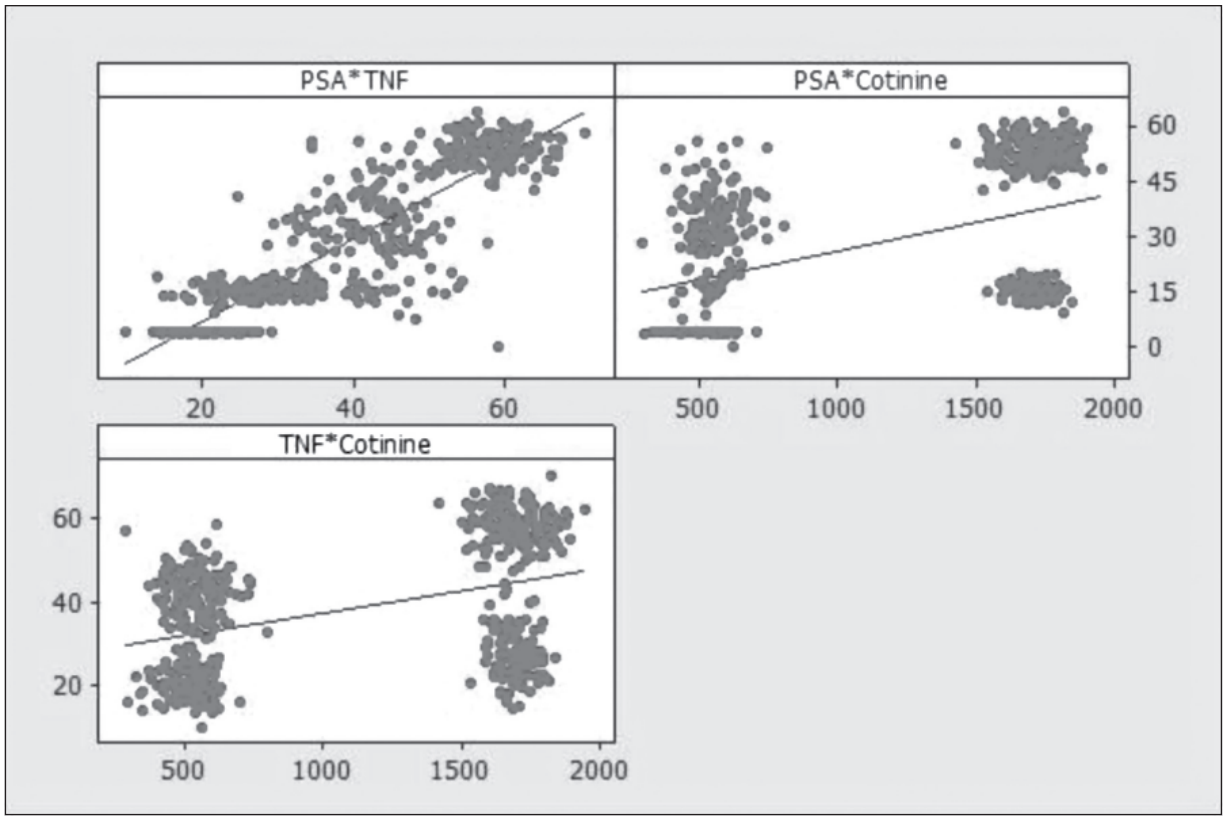
Table 4. Plasma urinary cotinine (ng/ml) in the studied groups

Group 4 Cancer smokers (n=143)	Group 3 Cancer (n=139)	Group 2 BPH smokers (n=107)	Group 1 BPH (n=105)	Control (n=115)	
1755.4 ± 230.62	425.97 ± 125.34	1379.6 ± 252.64	376.69 ± 130.97	316.77 ± 100.97	Mean± SD
t=-62.25 p=0.000	t=-7.54 p=0.000	t=-41.68 p=0.000	t=-3.82 p=0.000		t test Comparison with the control group
t=-59.92 p=0.000		t=-36.18 p=0.000			Comparison between group 1 & 2 and 3 & 4 (Effect of smoking)
	t=-2.98 p=0.003				Comparison between group 1 & 3
t=-12.24 p=0.000					Comparison between group 2 & 4

Table 5. Correlation study between the PSA, TNF and cotinine in studied groups

Cotinine	TNF	PSA		
			PSA	
0.581**	0.832**	1	Pearson	correlation
0.000	0.000			Sig. (2-tailed)
609	609	609		N
			TNF	
0.463**	1	0.832**	Pearson	correlation
0.000		0.000		Sig. (2-tailed)
609	609	609		N
			Cotinine	
1	0.463**	0.581**	Pearson	correlation
	0.000	0.000		Sig. (2-tailed)
609	609	609		N

** Correlation is significant at the 0.01 level (2-tailed).



Graph 1: Correlation study between the PSA, TNF and cotinine in studied groups.

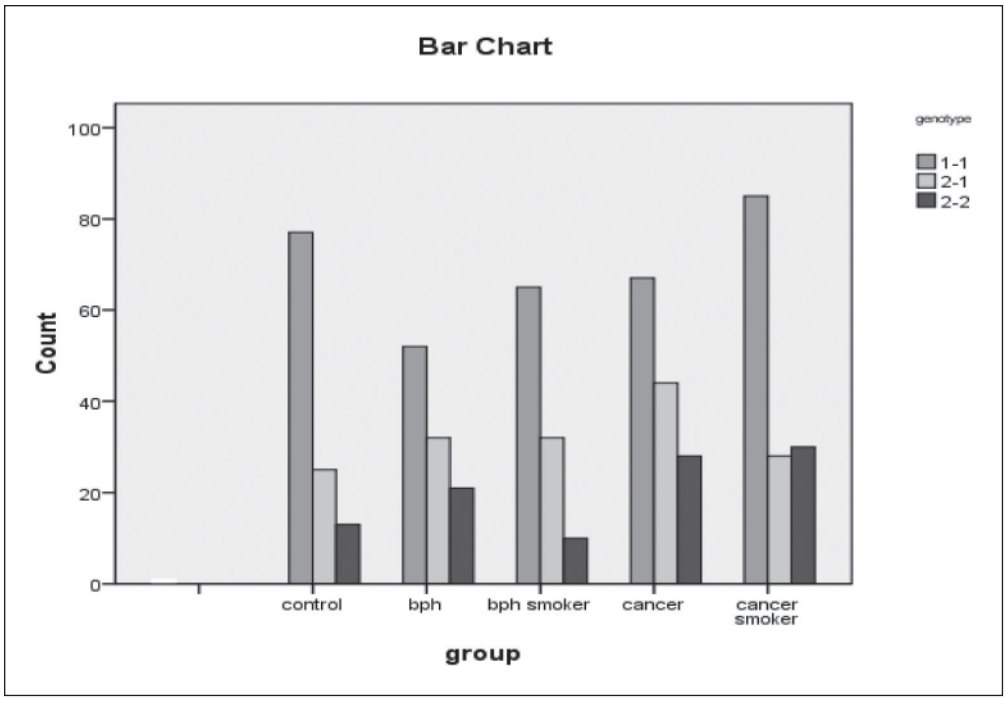
TNF and cotinine (0.463). Studying of genotype distribution of TNF polymorphism showed that genotype (G / G) is mostly associated with smoker patients either in BPH (60.7%) and cancer (59.4%) then non smoker BPH (49.5%) and cancer (48.2%) patients, which confirm the combined effect of smoking and genetic precipitation. Studying of allele frequency of TNF gene showed that G allele is mostly associated with BPH smokers (90.6%) than non smokers (80%) and more than cancer patients either smokers or non smokers (79% and 79.9% respectively) in Table 6 & Graph 2.

DISCUSSION

Genetic variation likely underlies a significant proportion of the individual variation in human susceptibility to toxicants by influencing processes such as metabolism, oxidative stress, DNA damage response, and repair. Characterization of this genetic variability, which is currently not well-understood, will enable more accurate chemical exposure risk assessment and the identification of subgroups of individuals at greater risk of disease resulting from exposure to toxicants. The main approaches to

Table 6. Genotype distribution and allele frequency of TNF 308 polymorphism in the studied groups

Group 4 Cancer smokers No (%)	Group 3 Cancer No (%)	Group 2 BPH smokers No (%)	Group 1 BPH No (%)	Control No (%)	
85 (59.4%)	67 (48.2%)	65 (60.7%)	52 (49.5%)	77 (67%)	Genotype 1–1 (G / G)
28 (19.6%)	44 (31.7%)	32 (29.9%)	32 (30.5%)	25 (21.7%)	Genotype 2–1 (G / A)
30 (21%)	28 (20.1%)	10 (9.3%)	21 (20%)	13 (11.3%)	Genotype 2–2 (A / A)
(n=143)	(n=139)	(n=107)	(n=105)	(n=115)	Total No.
58 (40.6%)	72 (51.8%)	42 (39.2%)	53 (50.5%)	38 (33%)	A allele
113 (79%)	111 (79.9%)	97 (90.6%)	84 (80%)	102 (88.7%)	G allele



Graph 2: The genotype distribution and allele frequency of TNF 308 polymorphism in the studied groups.
Genotype 1-1 (G / G), Genotype 2-1 (G / A), Genotype 2-2 (A / A)

Table 7. A study of PSA, TNF and Conitine in different Genotypes in the studied groups

Genotype 2-2 (A / A)			Genotype 2-1 (G / A)			Genotype 1-1 (G / G)				
Cotinine	TNF	PSA	Cotinine	TNF	PSA	Cotinine	TNF	PSA		
13	13	13	25	25	25	77	77	77	No.	Control group
373.40	16.13	3.50	373.40	16.13	3.50	373.40	16.13	3.50	Range	
.03	13.37	2.17	.03	12.47	2.18	.03	12.37	2.23	Mean	
.01	5.07	1.02	.94	5.21	1.06	±.01	±5.20	±1.10	± SD	
21	21	21	32	32	32	52	52	52	No.	BPH
91.90	1.95	4.50	374.00	5.23	8.50	325.30	12.30	8.80	Range	
431.16	20.58	11.06	401.30	20.167	16.04	339.55	15.14	9.40	Mean	
47.03	.997	2.30	158.72	2.18	1.78	125.65	4.50	2.95	± SD	
10	10	10	32	32	32	65	65	65	No.	Smokers BPH
.00	.00	.00	650.50	8.39	4.00	746.50	13.37	6.03	Range	
952.90	21.41	13.23	1485.77	22.73	11.59	1392.96	17.56	9.87	Mean	
.00	.00	.00	268.08	3.70	1.83	191.41	5.47	1.93	± SD	
28	28	28	44	44	44	67	67	67	No.	Cancer
48.70	.30	28.00	403.00	17.78	36.90	300.60	6.48	36.90	Range	
290.25	39.05	30.20	539.92	42.56	34.30	407.87	29.85	31.34	Mean	
24.80	.15	.14	90.59	3.39	6.92	101.94	2.35	.12	± SD	
30	30	30	28	28	28	85	85	85	No.	Smokers Cancer
47.90	16.80	.20	66.90	4.80	4.00	829.30	8.20	10.40	Range	
1697.35	46.50	61.30	1800.75	53.70	53.90	1760.99	35.81	54.89	Mean	
24.36	8.54	.10	34.06	2.44	2.04	295.65	2.73	4.29	± SD	

identifying gene–environment interactions in toxicant-mediated disease are candidate gene association studies and genome-wide association studies (GWAS), which test for an association of a subset of genes or pathways or all genes, respectively, with a toxicant-related phenotypic outcome (Zhang *et al*., 2014). TNF-α -308 G/A polymorphism was associated with increased hepatocellular carcinoma (HCC) risk in a Han Chinese population (Feng *et al*., 2014). Cigarette smoking had been shown to be a risk factor for prostate cancer (Hsing *et al*., 1990, Coughlin *et al*., 1996; Plaskon *et al*., 2003). This study was designed to investigate whether 308 G/A single nucleotide polymorphism of tumor necrosis factor-α (TNF-α) gene promoter region was associated with outcomes of prostate cancer and to analyze the gene environment interaction between 308 G/A TNF polymorphism and cigarette smoking. Results concluded that prostate cancer is significantly associated with TNF G/G genotype and this is accompanied by elevated plasma TNF, PSA and urinary Cotinine. Cancer smokers showed a high frequency of TNF-α 308 G allele compared with other patient groups associated with increased TNF levels. In contrary Oh *et al* (2000) observed that the relative risks of incidence for prostate cancer was 14-fold higher in people with genotype GA at -308 region of TNF-alpha and the genotype GA at -308 of TNF-alpha was related to higher clinical tumour stage of prostate cancer than genotype G (Oh *et al*., 2000). On the other hand, in meta-analysis included 14 studies with 5,757 patients and 6,137 control subjects for the TNF-α-308G/A polymorphism and 1,967 patients and 2,004 control subjects for the TNF-α-238C/T polymorphism. A significantly increased prostate cancer risk was found to be associated with the TNF-α-308C/T polymorphism in studies with healthy volunteers. No significant association was found between the TNF-α-238G/A polymorphism and prostate cancer risk in the overall or subgroup analyses Ma *et al* (2014). Being on different racial groups may explain the difference in results. Also, this analysis did not study the effect of cigarette smoking in those patients. Up to authors knowledge, this study is the only "until now" that studied both the effect of smoking and genetic polymorphism together in both benign and malignant prostatic disorders. Results of this study support the hypothesis that polymorphism in proinflammatory genes may be important in prostate cancer development and the sequence variants in these inflammatory genes may interact with environmental modifiers such as cigarette smoking to increase prostate cancer risk. Yet, further prospective studies on large and different ethnic populations will be necessary to confirm these findings and elucidate the underlying molecular effects of cigarette smoking to induce these prostatic disorders whether directly or through genetic polymorphism or combined effect.

ACKNOWLEDGMENTS

We wish to thank all staff of Clinical Toxicology Center, Mansoura University, Egypt for making this research possible especially Prof Dr Mona El Harouny and Prof Dr. Sahar Eldakrory for their continuous support and advice.

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