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The relation of adiponectin and tumor necrosis factor α levels between endothelial nitric oxide synthase, angiotensin-converting enzyme, transforming growth factor β, and tumor necrosis factor α gene polymorphism in adrenal incidentalomas

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ABSTRACT. ***Objective:*** **The aim of our study was to demonstrate demographic characteristics, presence of inflammatory markers, distribution of angiotensin-converting enzyme (ACE), tumor necrosis factor (TNF), endothelial nitric oxide synthase (eNOS) genotypes and relations among these parameters in these patients and control subjects.** ***Research design and methods:*** **Study samples were collected from 50 patients with adrenal mass and 30 control groups. The eNOS, ACE, TNF-α, transforming growth factor (TGF)-β genes polymorphisms, TNF-α, adiponectin levels were analysed in 50 unrelated Turkish patients with a diagnosis of adrenal incidentaloma (AI).** ***Results:*** **There was statistically significant difference between TNF-α levels of patient and controls (*p*=0.048). We have not detected the connection between TGF-β, TNF-α, ACE, eNOS gene polymorphism with serum TNF-α and adiponectin levels. In this study, we demonstrated that there were significant differences for ACE genotypes in the patients when compared to the controls (*p*<0.05). The percentages of the ID, DD, II genotypes for ACE gene polymorphism in the patients group were 30.0, 13.0, 7.0%, respectively.** ***Conclusions:*** **According to different cases of eNOS, TGF-β, ACE, and TNF-α gene genotypes; no statistical significant difference was found between basal cortisol, ACTH, DHEAS, metanephrine, renin, aldosterone, normetanephrine, 17-hydroxyprogesterone, 1 mg low-dose dexamethasone suppression test-cortisol response and AI size. In this study, I/D genotype was determined to be statistically higher in ACE gene in patients with AI (*p*=0.014).**
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INTRODUCTION

An adrenal incidentaloma (AI) is a mass lesion >1 cm in diameter, serendipitously discovered by radiologic examination (1). Clinically asymptomatic adrenal tumors occurred more frequently in overweight or obese women, 51-70 yr old, with lipid disorders and hypertension (2). In a study of patients with AI researchers found that higher insulin levels in the fasting state or after a glucose challenge (3). The potent cell growth promoting activity of insulin explains these studies. The act of insulin on the adrenal cortex stimulates steroidogenesis and cell increase (4-6). Cushing's syndrome, Conn syndrome and pheochromocytoma have been related with a higher incidence of cardiovascular risk factors. But there is less data related to adipocytokines and atherosclerosis in AI.
Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine mostly activated by monocytes and macrophages in response to tissue damage, inflammation. TNF-α induces genes involved in inflammatory responses and suppression of apoptosis (7, 8). TNF-α increases CRH release from the hypothalamus that is leading to increased ACTH creation from the pituitary gland and subsequent stimulation of adrenal steroidogenesis (9, 10). TNF-α mRNA and/or protein expression showed adult adrenals (11). TNF-α has also been suggested for an important intra-adrenal regulator of steroidogenesis (12). It is a potent inducer of ACTH secretion but, at the adrenal level, it seems to be mainly inhibiting ACTH-induced steroidogenesis and also IGF II expression. It is produced in adrenocortical steroidogenic cells in addition to macrophages, which suggests that TNF-α may have some autocrine/paracrine functions in the adrenal cortex (13).
Per definition, no clinical symptoms or signs of adrenal disease should be present at the time of diagnosis in patients with incidentally detected adrenal masses. It seems to be an association of incidentalomas with evidence of subtle hormone excess (such as recent weight gain, skin atrophy, episodic headaches, *etc.*) and features of metabolic syndrome [arterial hypertension, obesity, Type 2 diabetes mellitus (DM), dyslipidemia, dyscoagulation]. Some gene polymorphisms may also contribute to pro-inflammatory activities. The association of the TNF-α gene polymorphisms with DM and its related conditions, such as insulin resistance and obesity, has been extensively examined (14-16). Nitric oxide (NO) facilitates the uptake and metabolism of glucose in skeletal muscle (17, 18). The functional variation in NO synthase (NOS) genes may play role in insulin resistance, Type 2 DM, hypertension, dyslipidemia, coronary heart disease (19). The ACE gene known to be associated with cardiovascular disorders and hypercortisolism through ACE is assumed to influence

Key-words: ACE, adiponectin, adrenal incidentalomas, eNOS, TGF-β, TNF-α gene polymorphism, TNF-α levels.
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the activity of the hypothalamic-pituitary-adrenocortical (HPA) system (20).
The study by Sciarretta and et al. has shown that cardiovascular damage is more frequent in hypertensives with metabolic syndrome than in hypertensives without metabolic syndrome, and this is significantly related to the increased levels of inflammation and fibrosis. They found TNF-α and transforming growth factor-β (TGF-β) levels higher in hypertensives with metabolic syndrome than in hypertensives without metabolic syndrome (21).
We have analyzed in 50 unrelated Turkish patients with adrenal mass which were discovered during abdominal ultrasound or computerized tomography (CT) performed for unrelated reasons. The aim of our study was to demonstrate demographic characteristics, the presence of inflammatory markers, distribution of ACE, TNF-α, endothelial NOS (eNOS) genotypes and relations among these parameters in these patients and control subjects. This study is different from other studies because we differently the evaluated association between cytokine and gene.

MATERIALS AND METHODS

Patients

The eNOS, ACE, TNF-α, TGF-β genes polymorphisms, TNF-α, adiponectin levels were analysed in 50 unrelated Turkish patients with a diagnosis of adrenal mass who were admitted to Department of Endocrinology, Medical Faculty, Ege University, Turkey. Thirty consecutive healthy control subjects without a history of adrenal mass were recruited among those who presented to the check-up clinics in same hospital January and July 2008. The clinical characteristics and imaging and laboratory findings were recorded. All individuals and patients gave their informed consent for inclusion in the study, which was conducted according to the Helsinki Agreement.
Fifty patients (39 females and 11 males, mean age 56.86±9.08 yr, had adrenal mass which were discovered during abdominal ultrasound or CT performed for unrelated reasons. In patients, CT scan showed adrenal lesions whose mean size 25.62±19.48 mm. None of the patients underwent adrenal surgery.

Biochemical and hormonal parameters

Blood samples were collected from patients and controls after an overnight fast to determine basal plasma levels of interleukin 6 (IL-6), adiponectin, TNF-α. For homonal evaluation, fractionated urinary or plasma metanephrines (normetanephrine and metanephrine) performed in all patients with AI (1). Metanephrine and normetanephrine levels were measured with high performance liquid chromatography methods (Agilent 1100 series, HP1049A electrochemical detector, 2002). Before the test, various medications were questioned (i.e. methyldopa, levodopa, labetalol, sotalol, tricyclic antidepressants, benzodiazepines, drugs containing catecholamines, amphetamines, withdrawal from clonidine and ethanol). A ratio >20 is highly indicative, while a ratio >30 in a patient whose previous plasma aldosterone concentration (PAC)/plasma renin activity (PRA) ratio was high confirms the diagnosis of primary aldosteronism (22). We excluded patients with higher PAC/PRA (23).
The possibility of malignancy was evaluated in each case. Pheochromocytoma and Cushing's syndrome were excluded by determination of urinary excretion of catecholamines and an overnight dexamethasone suppression test. Furthermore, in all patients, hormonal evaluation included measurements of plasma cortisol, ACTH, 17-hydroxyprogesterone (17-OHP), DHEAS levels at 08:00 h under resting conditions. All patients underwent the following endocrine work-up aimed to study the HPA axis: 1) measurement of serum cortisol at 08:00 h, 2) measurement of plasma ACTH at 08:00 h, 3) overnight low-dose dexamethasone suppression test (LDDST) (1 mg, po, at 23:00 h with measurement of serum cortisol at 08:00 h the following morning) (Table 1). The suppression was adequate when morning cortisol fell below 1.8 μg/dl (24). 17-OHP and aldosterone were measured by radioimmuno assays (Packard gammacounter). Plasma cortisol and DHEAS levels were detected using E 170 Roche Chemilucence Method. Plasma ACTH level was measured by Immunulite 1000 Roche Chemilucence Method. PRA and PAC were also determined under resting conditions and in an upright posture after furosemide following exercises. PRA was measured manually by radioimmunoassays (CIS BioInternational, France). Thus, primary aldosteronism and hyperandrogenism were also excluded. Urinary vanillylmandelic acid (VMA) and 5-hydroxyindoleacetic acid (5-HIAA) were determined by reversed-phase chromatography. The mobile phase was a mixture of tartaric acid and EDTA at a flow rate of 0.8 ml/min. Fluorometric detection was performed at an excitation wavelength of 280 nm and emission at 320 nm. A Shimadzu LC 10A HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of a Shimadzu LC-10AD pump, a Shimadzu SIL-10AXL autoinjector with a 20 μl loop and a Shimadzu RF-10AXL fluorescence detector was used. The system was controlled through a Shimadzu CBM-10A communication module and a personal computer. None of the patients had clinical evidence of hormonal overproduction.

Determination of TNF-α by enzyme-linked immunosorbent assay

Study samples were collected from 50 patients with adrenal mass and 30 control groups. Study samples were stored in the deep freeze (−86 C) until use. To reduce interassay variance, all samples were analyzed in one assay. The repeated freeze-thaw cycles were avoided. The mean value of the triplicate readings for each standard, patients with adrenal mass and control group was calculated.
Quantitative detection of human TNF-α was determined by using an enzyme-linked immunosorbent assay (ELISA) method (Bender MedSystems) in the sera samples of 50 patients with adrenal mass and 30 control group. In this method, the microwell plates were coated with TNF-α monoclonal antibody. TNF-α present in test or standard sample binds to antibodies adsorbed to the microwells. A biotin-conjugated TNF-α antibody was added to detect TNF-α captured by the first coated antibody. Following incubation, the unbound biotin-conjugated anti-TNF-α was removed during a wash step. Streptavidin-horseradish peroxidase-(HRP) was added for binding biotin-conjugated anti-TNF-α. Following incubation, unbound Streptavidin-HRP was removed during a subsequent wash step. Substrate solution reactive with HRP was finally added to the wells. A colored product was formed in proportion to the amount of TNF-α present in the sample. The reaction was terminated by adding phosphoric acid and absorbance of each microwell was measured on a spectro-photometer using 450 nm as the primary wave length. Absorbance of both, the samples and the TNF-α standarts were determined. A standard curve was prepared from 7 geometric TNF-α standard dilution to have it extrapolated to test samples.

Table 1 - *Clinical and laboratory parameters in patients and controls.*

	Patients (% no.)		Controls (% no.)		p
Genus					>0.05
Woman	66	39	66.6	20	
Man	34	11	33.3	10	
Age (yr)	56.86±9.087		53.9±6.782		>0.05
BMI (kg/m²)	27.70±3.270		26.65±4.89		>0.05
Size of incidentaloma (mm)	25.62±19.48		–		
Adiponectin levels (ng/ml)	73.63±30.03		65.73±30.38		>0.05
TNF levels (pg/ml)	3.38±2.71		2.21±2.01		>0.05
Urine VMA (1.4-6.5) mg/24 h	4.103±1.305		–		
Urine metanephrine levels (52-341 µg/day)	116.89±63.088		–		
Urine normetanephrine levels (84-444 µg/day)	200.254±125.89		–		
Renin activity resting (0.2-3.4 ng/ml/h)	1.72±6.27		–		
Renin activity after exercise (0.2-3.4 ng/ml/h)	1.507±0.868		–		
Aldosteron levels resting (10-180 pg/ml)	97.62±50.50		–		
Aldosteron levels after exercise (50-300 pg/ml)	155.360±71.54		–		
ACTH levels (pg/ml)	24.30±11.75		–		
Cortisol levels (µg/dl)	16.22±4.35		–		
DHEAS (µg/dl)	119.27±70.33		–		
17-OHP (ng/ml)	1.39±0.63		–		
1 mg LDDST	1.02±0.32		–		
5-HIAA (2-7) mg/24 h	3.32±1.40		–		

BMI: body mass index; TNF: tumor necrosis factor; VMA: vanillylmandelic acid; 17-OHP: 17-hydroxyprogesterone; LDDST: low-dose dexamethasone suppression test; 5-HIAA: 5-hydroxyindoleacetic acid.

Determination of serum adiponectin concentration

The serum adiponectin was specifically assayed with a commercially available enzyme-linked immunosorbent assay kit [AssayMax Human Adiponectin (Acrp30) ELISA Kit]. This assay employs a quantitative sandwich enzyme immunoassay technique that measures adiponectin. A polyclonal antibody specific for adiponectin has been pre-coated onto a microplate. Adiponectin in standards, samples and control group is sandwiched by the immobilized antibody specific for adiponectin, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured by reading the absorbance on a microplate reader at a wavelength of 450 nm.

Genotypes

For mutation analysis in these patients, genomic DNA was extracted from 200 µl of EDTA-anti-coagulated peripheral blood leucocytes using the QUIAmp Bood Kit (Quiagen, Ontaria Canada, Cat. no.51106). Amplification of DNA for genotyping, the ACE I/D polymorphism was carried out by PCR in a final volume of 15 µl containing 200 µM dNTP mix, 1.5 mM MgCl2, 1x Buffer, 1unit of AmpliTaq polymerase (PE Applied Biosystems) and 10 pmol of each primer. The primers used to encompass the polymorphic region of the ACE were 5'-CTGGAGACCACTCCCATCCTTTCT-3' and 5'-ATGTGGCCATCACATTCGTCAGAT-3' (25).

DNA is amplified for 35 cycles, each cycle comprising denaturation at 94 C for 30 sec, annealing at 50 C for 30 sec, extension at 72 C for 1 min with final extension time of 7 min. The initial denaturation stage was carried out at 95 C for 5 min. The PCR products are separated on 2.5% agarose gel and identified by ethidium-bromide staining. Each DD genotype was confirmed through a second PCR with primers specific for the insertion sequence (26). Endothelial NOS genotyping for the Glu298Asp mutation was performed as described by Hingorani et al. (27).

The primers used were 5'-CATGAGGCTCAGCCCCAGAAC-3' (forward) and 5'-AGTCAATCCCTTTGGTGCTCAC-3' (reverse). DNA is amplified for 30 cycles, each cycle comprising denaturation at 95 C for 1 min, annealing at 60 C for 1 min, extension at 70 C for 1 min with final extension time of 5 min at 70 C. The initial denaturation stage was carried out at 95 C for 5 min.

PCR products were digested with the restriction enzyme MboI at 37 C for 16 h. In presence of T at nucleotide 894 which corresponds to Asp 298, the 206-bp PCR products is cleaved into two fragments of 119 and 87 bp. The PCR products are separated on 2.5% agarose gel and identified by ethidium-bromide staining.

DNA purification for the TNF-α genotyping was performed. Total genomic DNA from patients and healthy controls was extracted from peripheral blood using QIAamp DNA Blood Mini Kits 50 (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. TNF-α-308 G/A polymorphisms were genotyped by the allele-specific oligonucleotide PCR. Using specific primer and probe sequences, PCR amplification was carried out separately for the polymorphism (28).

PCR analysis was performed on a Perkin Elmer 9600 thermal cycler (PE Biosystems, Foster City, CA, USA). The products were separated on a 1.5% agarose gel, stained with ethidium bromide. Quality control of genotyping data was verified by repeat testing of 10% of randomly selected individuals for each of the polymorphisms tested. There were no discrepancies encountered in these analyses.

Furthermore, DNA preparation and polymorphism screening were performed for TGF-β. Genomic DNA was prepared from whole blood using QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden Germany) according to manufacturer's instructions. DNA concentration was determined by the PicoGreen ds-

DNA quantization kit (Molecular Probes Inc., Eugene, OR) following the manufacturer's instructions. Genotyping of the TGF-β1 exon 1' polymorphism (G915C) was detected also with an adapted mutagenically separated PCR (MS-PCR) through the method of Rust et al. (29).
Briefly, different length allele specific primer sets (TGFB1-915G; 5'-TGCTGCTACCGCTGCGTTGGCTACTGGTGCTGACGCCTGGCTG-3' and TGFB1-915C; 5'-TGTGGCTACTGGTGCTGACGCCTGGTCC-3') together with an allele-unspecific complementary strand primer (TGFB1-CS; 5'-TTGGACAGGATCTGGCCGCGGATG-3') were used in a one tube reaction assay. At least one PCR product was found in single reaction, thus avoiding false negative results. Additional base substitutions at different mutagenic positions were introduced into the allele-specific primers as described by Rust et al. This allowed a clear-cut separation between the two alleles during the subsequent amplification steps through the reduction of cross reactions. Amplification was performed on GeneAmp PCR System 9700 (PE Applied Biosystems), 1 mM MgCl2, 50 µl M each of dGTP , dATP, dTTP and dCTP 1.0 Units AmpliTaq Gold polymerase (PE Applied Biosystems) and primers in the following concentrations: 0.1 µM TGFB1-915G; 0.2 µM TGFB1-915C; 0.2 µlM TGFB1-CS. The cycling conditions were: hot start at 95 C for 10 min; 35 cycles of 95 C for 30 sec, 67 C for 45 sec, 72 C for 45 sec; and final extension at 72 C for 7 min. Aliquots of the PCR products were separated by size on 4% agarose gels. After staining of the gels with ethidium bromide, PCR products were visualized under ultraviolet light. The PCR products with 136bp (915 G) and 121bp (915 C) in length could readily be distinguished.

Statistical analysis

SPSS 14.0 for Windows (SPSS Inc. Chicago USA) was used for statistical analysis of the results. $p<0.05$ values were accepted as statistically significant. The characteristics of the patients with AI and the mean plasma hormone concentrations were compared by Student's t test for unpaired data and between and within the different groups of eNOS, ACE, TNF-α, and TGFβ genotypes with the analysis of variance. Allelic and genotypic frequencies were determined from observed genotype counts, and the expectations of the Hardy-Weinberg equilibrium were evaluated by 2 analysis. Differences in the genotype distribution between different groups were assessed by Pearson's 2 test of heterogenity.

RESULTS

A total of 80 subjects were enrolled in this study. We analyzed 50 Turkish patients with adrenal mass and 30 healthy control subjects without a history of adrenal mass. Average age with body mass index (BMI) of the controls (no.=30) and the patients (no.=50) was comparable (Table 1). Biochemical parameters of the patients and the control subjects were demonstrated (Table 1). Urinary metanephrine was 116.89±63.088 µg/day, normetanephrine levels were 200.254 ±125.89 µg/day. Urinary VMA level 4.103±1.305 mg/24 h. There were no significant difference in adiponectin levels, mean age, BMI between 2 groups ($p>0.05$) (Table 1). There was statistically significant difference between TNF-α levels of patient and controls ($p=0.048$).
We have not detected the connection between TGF-β, TNF-α, ACE, eNOS gene polymorphism with serum TNF-α and adiponectin levels (Tables 2-5). Furthermore, there was no relation between the presence of diabetes mellitus or hypertension with serum adiponectin, TNF-α levels.
Association of eNOS, TNF-α, ACE polymorphism according to the patients and the control subjects was compared (Table 6). The distribution of genotypes and allele frequency for eNOS, TNF-α gene polymorphism in the controls and patients did not differ significantly ($p>0.05$). The percentages of the GG, GT, TT genotypes for eNOS gene polymorphism in the patients group were 30.0, 11.0, and 9.0%, respectively. The distributions of allele frequency in the patients for G and T alleles were 71.0, 29.0%, respectively. The percentages of the GG, GA, AA genotypes for TNF-α gene polymorphism in the patients group were 29.0, 18.0, 3.0%, respectively (Table 6). The distributions of allele frequency in the patients for G and A alleles were 76.0, 24.0%, respectively.
On the other hand, we demonstrated that there were significant differences for ACE genotypes in the patients when compared to the controls ($p<0.05$). The percentages of the ID, DD, II genotypes for ACE gene polymorphism in the patients group were 30.0, 13.0, 7.0%, respectively (Table 6). In the control subjects, these percentages for the ID, DD, II genotypes were 8.0, 13.0, 9.0%, respectively (Table 6). The observed difference according to the distribution of alleles for ACE gene polymorphism between the patients and the control subjects was not statistically significant ($p>0.05$).
The association among clinical, biochemical parameters with eNOS, ACE, TNF-α, TGF-β gene polymorphisms was not significant (Tables 2-5). But we demonstrated increase frequency of DM and hypertension in the patients with TGF-β gene mutations ($p=0.013$). Moreover, in the patients with TGFβ gene mutations were found to be in statistically significant association with diagnosis of bilateral adrenal mass ($p=0.013$).
There were no statistically significant differences between the levels of urine VMA (1.4-6.5) mg/24 h, urine metanephrin levels (52-341 µg/day), urine normetanephrin levels (84-444 µg/day), PRA resting (0.2-3.4 ng/ml/h), PRA after exercise (0.2-3.4 ng/ml/h), PAC resting (10-180 pg/ml), PAC after exercise (50-300 pg/ml), ACTH levels (pg/ml), ACTH levels (pg/ml), DHEAS (µg/dl), 17 OHP (ng/ml), 1 mg LDDST, 5 HIAA (2-7) mg/24 h according to different TGF-β, eNOS, ACE, and TNF-α genotypes.

DISCUSSION

The presence of an AI has been associated with higher incidence of different cardiovascular risks. Actuality, patients with AI can show a high prevalence of obesity, hypertension, DM, glucose intolerance, and dyslipidemia (30-34). Abnormalities in adiponectin and TNF-α cytokine levels may be altered in AI patients. In the present study, we examined and compared the clinical, biochemical, and hormonal characteristics of 50 patients with non-functional AI.
In the adrenal, the effects of TNF-α have been variable depending on the species or the developmental stage of the adrenal. TNF-α stimulated corticosterone production in normal rats in experiments conducted *in vi-*

Table 2 - *Biochemical and hormonal parameters between endothelial nitric oxide synthase (eNOS) genotypes in patient group.*

eNOS genotype	GG	GT	TT	p
Age (yr)	57.0±9.68	57.55±9.36	55.56±7.31	0.885
BMI (kg/m2)	27.91±3.58	27.24±2.48	27.59±3.27	0.846
Incidentaloma size (mm)	27.1±24.7	23.36±6.65	23.44±6.04	0.812
Adiponectin concentration (ng/ml)	76.85±29.73	78.62±29.15	56.803±29.4	0.177
TNF levels (pg/ml)	3.29±3.12	3.19±1.6	3.89±2.45	0.822
Urine VMA (1.4-6.5) mg/24 h	4.27±1.32	4.24±1.36	3.36±1.00	0.174
Urine metanephrine levels (52-341 μg/day)	116.67±66.77	138.36±66.88	91.38±35.44	0.258
Urine normetanephrine levels (84-444 μg/day)	203.35±139.13	199.9±100.66	191.55±118.95	0.970
Renin activity resting (0.2-3.4 ng/ml/h)	0.88±0.7	4.85±13.32	1.06±0.703	0.186
Renin activity after exercise (0.2-3.4 ng/ml/h)	1.45±0.90	1.45±0.67	1.74±1.01	0.674
Aldosterone levels resting (10-180 pg/ml)	90.10±54.99	101.36±30.01	118.11±53.18	0.339
Aldosterone levels after exercise (50-300 pg/ml)	135.83±63.79	161.54±58.43	212.88±84.41	0.014
ACTH levels (pg/ml)	25.43±12.93	20.66±8.34	24.97±11.40	0.517
Cortisol levels (μg/dl)	16.18±4.7	15.85±3.74	16.83±4.18	0.882
DHEAS (μg/dl)	122.58±69.32	128.48±76.27	97.01±69.96	0.571
17-OHP (ng/ml)	1.50±0.71	1.38±0.55	1.04±0.28	0.170
1 mg LDDST	1.00±0.31	1.1±0.42	0.97±0.26	0.628

BMI: body mass index; TNF: tumor necrosis factor; VMA: vanillylmandelic acid; 17-OHP: 17-hydroxyprogesterone; LDDST: low-dose dexamethasone suppression test.

vo (35, 36), but inhibited ACTH-stimulated corticosterone and aldosterone release in *in vitro* conditions (37). In cultured human adrenal lines, TNF-α decreased basal and ACTH-stimulated cortisol secretion (38). Also, there was statically significant difference between patients and controls in terms of sera TNF-α level in this clinical study (Table 1).

In our study, mean adrenal mass size is 25.62±19.48. The maximum diameter of the adrenal mass is predictive of malignancy. This was illustrated in a study of 887 patients with AI from the National Italian Study Group on Adrenal Tumors (39). Adrenocortical carcinomas were significantly associated with mass size, with 90% being >4 cm in diameter when discovered.

Another report compared 28 patients with subclinical Cushing's syndrome due to an AI and 100 normal subjects and found differences in cardiovascular risk profiles between the two groups (40). Those with subclinical Cushing's syndrome were more likely to have hypertension, dyslipidemia, impaired glucose tolerance or Type 2 DM, and evidence of atherosclerosis. We determined that there was Type 2 DM in 17 patients (34%) and hy-

Table 3 - *Biochemical and hormonal parameters between ACE genotypes in patient group.*

ACE Genotype	ID	DD	II	p
Age (yr)	55.67±8,37	59.92±9.49	56.29 ±11.28	0.371
BMI (kg/m2)	28.56±3.69	26.22±1.89	26.80 ±2.19	0.069
Incidentaloma size (mm)	23.47±6.90	31.54 ±36.61	23.86 ±9.22	0.453
Adiponectin concentration ng/ml	72.46 ±30.09	82.26 ±16.94	62.62 ±45.88	0.365
TNF levels (pg/ml)	3.47 ±3.02	3.16 ±2.38	3.37 ±2.06	0.946
Urine VMA (1.4-6.5) mg/24 hh	4.17±1.23	3.73 ±1.07	4.50±1.92	0.417
Urine metanephrine levels (52-341 μg/day)	121.21 ±67.36	105.97±56.40	118.64±62.08	0.772
Urine normetanephrine levels (84-444 μg/day)	210.59±148.73	197.61±82.60	160.85±80.69	0.649
Renin activity resting (0.2-3.4 ng/ml/h)	2.31±8.08	1.12±0.72	0.76±0.62	0.769
Renin activity after exercise (0.2-3.4 ng/ml/h)	1.35 ±0.75	1.97 ±0.94	1.29±1.01	0.78
Aldosteron levels resting (10-180 pg/ml)	96.43±58.57	101.84±28.14	94.85±51.10	0.940
Aldosteron levels after exercise (50-300 pg/ml)	143.80±70.18	177.00±60.24	164.71±94.63	0.359
ACTH levels (pg/ml)	23.74±11.86	27.88±10.36	20.02 ±13.53	0.340
Cortisol levels (μg/dl)	15.94±4.36	17.35 ±4.79	15.35 ±3.56	0.537
DHEAS (μg/dl)	107.50±60.40	134.61±84.06	141.26±82.87	0.350
17-OHP (ng/ml)	1.27±0.66	1.61±0.65	1.53±0.38	0.224
1 mg LDDST	1.02 ±0.34	0.99±0.26	1.09±0.39	0.800
5-HIAA (2-7) mg/24 h	3.22±1.52	3.25±1.03	3.88±1.52	0.530

BMI: body mass index; TNF: tumor necrosis factor; VMA: vanillylmandelic acid; 17-OHP: 17-hydroxyprogesterone; LDDST: low-dose dexamethasone suppression test; 5-HIAA: 5-hydroxyindoleacetic acid.

Table 4 - *Biochemical and hormonal parameters between tumor necrosis facor (TNF)-α genotypes in patient group.*

TNF-α genotype	GG	GA	AA	p
Age (yr)	55.34±9.21	59.00±8.90	58.67±9.01	0.390
BMI (kg/m2)	27.50±3.66	28.01±2.79	27.89±2.37	0.875
Incidentaloma size (mm)	22.97±7.43	30.00±31.10	25.00±5.00	0.493
Adiponectin concentration ng/ml	74.18±29.83	75.40±29.31	57.68±43.82	0.641
TNF levels (pg/ml)	3.02±2.25	3.84±3.42	4.04±2.22	0.559
Urine VMA (1.4-6.5) mg/24 h	4.12±1.22	4.01±1.30	4.46±2.45	0.859
Urine metanephrin levels (52-341 μg/day)	126.54±65.88	109.01±61.00	70.86±4.80	0.284
Urine normetanephrin levels (84-444 μg/day)	220.65±139.00	179.20±106.37	129.33±63.95	0.337
Renin activity resting (0.2-3.4 ng/ml/h)	2.37±8.22	1.00±0.62	0.82±0.85	0.746
Renin activity after exercise (0.2-3.4 ng/ml/h)	1.44±0.84	1.69±0.90	0.99±0.92	0.366
Aldosteron levels resting (10-180 pg/ml)	102.44±56.61	96.55±40.68	57.33±27.75	0.343
Aldosteron levels after exercise (50-300 pg/ml)	149.62±64.10	169.72±82.75	124.66±77.02	0.490
ACTH levels (pg/ml)	24.52±11.94	24.33±10.68	21.93±20.23	0.939
Cortisol levels (μg/dl)	16.41±3.98	16.00±5.28	15.80±1.52	0.939
DHEAS (μg/dl)	108.56±50.61	125.25±92.41	186.96±62.45	0.168
17-OHP (ng/ml)	1.41±0.64	1.41±0.68	1.14±0.27	0.779
1 mg LDDST	1.08 ±0.34	0.94±0.30	0.86±0.29	0.253
5-HIAA (2-7) mg/24 h	3.28±1.53	3.42±1.29	3.13±1.90	0.924

pertension in 42 patients (84%) among 50 patients in the study that we conducted.

Tunny et al. also compared the ACE gene I/D polymorphism allelic pattern in matched adenoma and peripheral blood DNA in the 55 patients with aldosterone-producing adenoma. The frequency of the D allele was similarly in patients and control subjects. They found the deletion of the insertion allele of the ACE gene I/D polymorphism in 16% of aldosterone-producing adenomas (41). In another study, cortisol area under the curve values were higher in those with ACE gene D/D genotype and lowest in those with I/I genotype (42). We determined in this study that DD (p=0.013) and II (p=0.006) genotypes were higher in control group in which ACE gene I/D polymorphism were studied. ACE gene ID genotype was determined to be higher in patients with AI (p=0.014) (Table 6). However, no statistical significant difference was found between basal plasma cortisol, ACTH, DHEAS, metanephrine, renin, aldosteron, normetanephrine, 17-OHP, and 1 mg LDDST-cortisol levels according to ACE genotype difference. At the same time, no statistical difference was found between AI sizes according to ACE genotype.

Hanke et al. showed that adenovirus-mediated gene transfer of eNOS in adrenal zona glomerulosa cells re-

Table 5 - *Biochemical and Hormonal parameters between TGFβ genotypes in patient group.*

TGFβ codon 10 genotype	AA	AG	GG	p
Age (years)	57.71±9.09	56.50±8.73	45.00±4.24	0.156
BMI (kg/m2)	27.54±3.23	28.44±3.51	25.39±0.16	0.415
Incidentaloma size (mm)	26.44±23.25	23.57±6.99	26.00±5.65	0.901
Adiponectin concentration ng/ml	72.68±30.52	77.11±23.74	65.36±76,07	0.835
TNF levels (pg/ml)	3.68±3.07	2.46±1.27	4.65±3.12	0.302
Urine VMA (1.4-6.5) mg/24 h	4.42±1.32	3.43±0.94	3.35±1.76	0.037
Urine metanephrin levels (52-341 μg/day)	120.07±62.87	112.69±68.57	92.25±40.65	0.804
Urine normetanephrin levels (84-444 μg/day)	198.25±132.35	213.14±120.74	144.00±16.97	0.765
Renin activity resting (0.2-3.4 ng/ml/h)	0.99±0.72	3.85 ±11.84	0.82±0.67	0.355
Renin activity after exercise (0.2-3.4 ng/ml/h)	1.61±0.94	1.23±0.53	1.65±1.54	0.385
Aldosterone levels resting (10-180 pg/ml)	98.82±52.36	91.57±46.65	119.50±68.58	0.750
Aldosterone levels after exercise (50-300 pg/ml)	158.00±70.70	140.28±70.70	216.00±100.40	0.356
ACTH levels (pg/ml)	24.59±11.33	25.88±12.28	8.25±4.59	0.135
Cortisol levels (μg/dl)	15.44±4.71	18.18±2.62	15.88±5.49	0.139
DHEAS (μg/dl)	121.94±78.11	116.10±55.08	96.12±12.55	0.868
17-OHP (ng/ml)	1.36±0.49	1.45±0.93	1.60±0.53	0.827
1 mg LDDST	1.02±0.30	0.99±0.39	1.21±0.15	0.695
5-HIAA (2-7)	3.47±1.36	3.00±1.57	3.05±1.06	0.561

BMI: body mass index; TNF: tumor necrosis factor; VMA: vanillylmandelic acid; 17-OHP: 17-hydroxyprogesterone; LDDST: low-dose dexamethasone suppression test; 5-HIAA: 5-hydroxyindoleacetic acid.

Table 6 - *Distrubution of haplotypes and genotypes.*

eNOS	Patients (% no.)		Controls (% no.)		*p*
Genotypes					
GG	60.0	30	53.3	16	>0.05
GT	22.0	11	20.0	6	>0.05
TT	18.0	9	26.7	8	>0.05
Alleles					
G	71.0	71	63.0	38	>0.05
T	29.0	29	37.0	24	>0.05
TNF					
Genotypes					
GG	58.0	29	63.3	19	>0.05
GA	36.0	18	33.3	10	>0.05
AA	6.0	3	3.3	1	>0.05
Alleles					
G	76.0	76	80.0	48	>0.05
A	24.0	24	20.0	12	>0.05
ACE					
Genotypes					
ID	60.0	30	26.7	8	0.014
DD	26.0	13	43.3	13	0.013
II	14.0	7	30.0	9	0.006
Alleles					>0.05
D	56.0	56	57.0	34	>0.05
I	44.0	44	43.0	26	>0.05

eNOS: endothelial nitric oxide synthase; TNF: tumor necrosis factor.

sults in the expression of active eNOS enzyme and that this endogenous NO making by zona glomerulosa cells decreases aldosterone synthesis (43). In the zona reticularis, eNOS may control C (19) steroid production at the level of 17α-hydroxylase (44).

No statistical significant difference was found between eNOS gene polymorphism genotypes and alleles in patient and control groups in the study that we carried out. At the same time, no statistical significant difference was determined between hormonal, adrenal size, adiponectin, and TNF-α level according to eNOS genotype.

The orphan nuclear receptor, steroidogenic factor 1 (SF-1), plays a major role in adrenal and gonadal development. In another study adrenocortical cells TGF-β inhibits the expression of SF-1 gene at a transcriptional level, and researchers were considered that the inhibitory effect of TGF-β on steroid hormone synthesis in the adrenal cortex can be due to an attenuated transcription of SF-1 (45). In this study, we worked on TGF-β1 exon 1 polymorphism (G915C) genetic analysis in patients with AI. No statistically significant difference was found in terms of TGF-β1 genotypes and alleles between AI patient and control groups. It is determined in this study that VMA levels in patient group with GG genotype in patient group where TGF-β1 gene was examined were at normal range levels but higher than other genotypes (p=0.037) (Table 5). However, as in ACE genotype and eNOS gene analysis, no statistically significant difference was determined between hormonal, adrenal size, adiponectin, and TNF-α levels according to TGF-β1 genotype.

We also worked on TNF-α-308 G/A polymorphisms genetic analysis in patients with AI in this study. No statistically significant difference was found in terms of TNF-α genotype and alleles between AI patient and control groups. No statistical significant difference was found between hormonal parameters, adrenal size, adiponectin, and TNF-α levels according to TNF-α genotype.

As a result, no significant statistical difference was determined in patient and control groups in terms of TNF-α and adiponectin levels. At the same time, no statistical significant difference was found according to TNF-α and adiponectin levels, eNOS, TGF-β, ACE, and TNF-α polymorphisms in patients with AI. However, I/D genotype was determined to be statistically higher in ACE gene in patients with AI (p=0.014). According to different cases of eNOS, TGF-β, ACE, and TNF-α gene genotypes; no statistical significant difference was found between basal cortisol, ACTH, DHEAS, metanephrine, renin, aldosteron, normetanephrine, 17-OHP, 1 mg LDDST-cortisol response and AI size.

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