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Associations between Human Aldosterone Synthase (CYP11B2) and Angiotensin II type 1 Receptor (ATR1) Gene Polymorphisms with End Stage Renal Disease In hypertensive Egyptian Patients on Maintenance Hemodialysis

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Abstract

Many people with an advanced form of kidney disease do not know they have weak or failing kidneys, but early detection and treatment can help prevent the progression of kidney disease to kidney failure. The resulting costs of treatment of ESRD are enormous. ESRD is a complex phenotype, which results from the presence of underlying kidney disease, and superimposing inherited and environmental factors. Among the predisposing genetic factors, renin-angiotensin-aldosterone system (RAAS) disruption is clearly involved in ESRD development. The aim of this study is to evaluate the association between *CYP11B*2 C-344T and *ATR*1 A1166C gene polymorphisms with increased risk for ESRD in hypertensive Egyptian patients on maintenance hemodialysis.

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This study included 70 ESRD patients on maintenance hemodialysis (32 males and 28 females, mean age 54.5± 9.5 years, recruited from El Doaah and El Rayan hospitals, Cairo, Egypt. 70 healthy individuals, of matching age and sex (30 males and 40 females, mean age 50.2 ± 13.1 years), were also included in the study. All subjects were genotyped for both CYP11B2 C-344T and ATR1 A1166C gene polymorphisms. Serum aldosterone was also measured for all subjects. Concerning the *CYP11B2* gene, HD patients showed increased frequency of the TT genotype (68.57%) as compared to controls (only 12.85%), with no significant differences in T allele distribution between the 2 groups. In contrast, HD patients had low frequency of the CC genotype (5.71%) as compared to controls (32.85%), with a significant difference in C allele distribution between the HD patients (28.56%) and controls (70.7%). Comparing serum aldosterone levels in various *CYP11B2* genotypes in HD patients revealed that patients with TT genotype had statistically higher aldosterone levels (121.583 ± 43.311) than those with the TC (72.055 ± 11.709) or CC genotype (68.75 ± 13.145). On the other hand, for *AT1R A1166C* genotype frequency, HD patients showed significantly ($p<0.05$) higher frequency of CC genotype (70%) than controls (7.1%), with lower AA genotype frequency (5.71%) compared to controls (57%). Moreover, there was significant differences in A allele distribution between the HD patients (27.13%) and controls (64.25%). The same was true for the C allele, with a frequency of (59.28%) in HD patients compared to a frequency of (39.25%) in controls. On the other hand, studying *ATR1 A1166C* gene polymorphisms revealed that patients with the CC genotype had significantly higher aldosterone levels (121.25 ± 43.006) than those with AA (87.6 ± 25.4) genotype.

Keywords: End stage renal disease; CYP11B2 C-344T; ATR1 A1166C; gene polymorphisms.

1. Introduction

Nephropathies of any etiology tend to progress to end-stage renal disease (ESRD) more or less rapidly over time. Many people with an advanced form of kidney disease do not know they have weak or failing kidneys, but early detection and treatment can help prevent the progression of kidney disease to kidney failure. The resulting are enormous. ESRD is a complex phenotype, which results from the presence of underlying kidney disease, and superimposing inherited and environmental factors [1]. Among the predisposing genetic factors, renin-angiotensin-aldosterone system (RAAS) disruption is clearly involved in ESRD development [2]. The RAAS is a key blood pressure, renal hemodynamics, and volume homeostasis regulator [3]. Thus, genes that encode RAAS components are candidates for evaluating predisposition to renal disease development and progression [4].

Several studies have identified RAAS gene mutations and polymorphisms that affected host susceptibility to several diseases, including type 2 diabetes [5], myocardial infarction [6], chronic kidney disease, ESRD and hypertension [7]. Hypertension is the major contributor in the progression of renal failure in patients with renal disease both with and without proteinuria [8]. Moreover, hypertension per se is a risk factor for the development of ESRD [9-10]. On the other hand, the prevalence of hypertension increases with decreasing renal function [11]. This results from both a decreased sodium excretion and an activation of the renin-angiotensin-aldosterone system (RAS). Therefore it is possible that a genetic predisposition to salt-dependent hypertension or over activation of the RAS may: 1-predispose to the development of renal failure and 2-promote

a more rapid loss of glomerular filtration rate in patients suffering from renal diseases. Thus genes that regulate renal sodium reabsorption or genes of the RAS may be extremely important in patients suffering from ESRD. Among the candidate genes of the RAS, the aldosterone synthase gene (*CYP11B2*) and angiotensin II type I receptor (*ATR1*) are of particular interest [12].

Angiotensin II is the major biologically active product of RAAS, formed from its original precursor, angiotensinogen by two successive enzymatic cleavages. Angiotensin II acts as a potent vasoconstrictor and exerts its effects through two structurally different receptor subtypes: angiotensin II type 1 receptor and angiotensin II type 2 receptor. Angiotensin II type 1 receptor mediates most of the known biological effects of angiotensin II. ANG II acts on ANG II type 1 receptors in the adrenal gland and enhances Aldosterone secretion [13].

The gene coding for angiotensin II type 1 receptor is localized to chromosome 3q21q25, spans 45.123 kb and comprises 5 exons, the first four exons represent the 5′-UTR, whereas exon 5 harbored coding region [14]. Several polymorphic sequence variants have been found on angiotensin II receptor type 1 (*ATR1*) gene. The most well studied polymorphism is A1166C (rs5186), a transversion at position 1166 which is located in the 3′-UTR of the *ATR1* gene [15]. The *ATR1* 1166-C allele has been associated with increased risk for coronary artery disease, ischemic stroke, heart failure, and hypertension [16].

Another important component of the Raas system is aldosterone synthase (18-hydroxylase or cytochrome P450 11B2). It is a mineralocorticoid synthesized from deoxycorticosterone in the zona golmerulosa of the adrenal cortex by a mitochondrial cytochrome P450 enzyme [17]. Aldosterone synthase plays a major role in the regulation of sodium-water homeostasis, intravascular volume and blood pressure [18]. Several lines of evidence have demonstrated the role of high plasma aldosterone level in hypertension and progression of kidney diseases [19]. The gene coding for aldosterone synthase (*CYP11B2*) is located on chromosome 8q and contain 9 exons. Several polymorphic variants have been identified in the *CYP11B*2 gene [20]. The promoter polymorphism (-344T/C: rs1799998), has been identified to alter aldosterone production, leading to sodium wasting and decreased excretion of potassium [21]. Initial studies have reported a positive association between -344T allele and essential hypertension [22].

The aim of this study is to evaluate the association between *CYP11B*2 and *ATR*1 gene polymorphisms with increased risk for ESRD in hypertensive Egyptian patients on maintenance hemodialysis.

2. Patients

This study included 70 ESRD patients on maintenance hemodialysis, recruited from El Doaah and El Rayan hospitals, Cairo, Egypt. The work was done after taking acceptance of all patients and controls to share in the study as well as acceptance of ethics committee of Ain Shams University. The work has been carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The controls (n=70) were individuals who have had no medical history of kidney disease and were normal in blood pressure, blood chemistry, urinalysis, and electrocardiogram (EKG). Basic demographic

data, current blood pressure, information on underlying renal disease, and current antihypertensive medication (data not shown) were obtained for all ESRD subjects.

3. Methods

3.1. Genotyping of the CYP11B2 C-344T gene polymorphism

DNA was extracted from whole blood using a QIAamp Blood mini-prep Kit (QIAGEN, Germany) according to manufacturer's instructions. Genotypes were determined by polymerase chain reaction (PCR) amplification of the promoter region of the *CYP11B*2 gene using the oligonucleotide primers (upstream: 5'-CAG GAGGAGACCCCATGTGAC-3'; downstream: 5'-CCTCC ACCCTGTTCAGCCC-3'). PCR conditions were: initial denaturation at 94˚C for 3 min; then 32 cycles at 94 ˚C for 1 min, at 60˚ C (annealing) for 1 min, and at 72 ˚C (extension) for 1 min. Restriction fragment length polymorphism (RFLP) was performed by adding 10 U of restriction endonuclease *Hae*III site in the appropriated buffer to 5 μL from each reaction (a 537 bp product) and by incubating at 37 C for 2 hours. The digested samples then underwent gel electrophoresis, stained with ethidium bromide, and analyzed under UV lights. Since the (-344) T allele lacks an *Hae*III site (GGCC) present in the (-344) C allele, the (-344) T alleles are detected as fragments of 273 bp and (-344) C alleles as fragments of 202 bp (plus smaller fragment in each case).

3.2. Genotyping of the ATR1 A1166C gene polymorphism

The A1166C variant of the AT1R gene was identified with primers: forward: 5'-GCAGCACTTCACTACCAAATGGGC-3' and reverse: 5'-CAGGACAAAAGCAGGCTAGGGAGA-3'. The reaction conditions were: The initial denaturation at 95˚C was followed by 35 cycles of denaturation at 94˚C, annealing at 55˚C and elongation at 72˚C, 1 min each. Final extension was at 72˚C for 7 min. The PCR products were digested with BsuRI restriction enzyme (MBI Fermentas, St. Leon-Rot, Germany). In the presence of cytosine there is a restriction site for this enzyme, resulting in a fragment 231 bp (C allele). Undigested 255 bp fragment indicates the presence of the A allele.

3.3. Measurement of serum aldosterone

Serum aldosterone was measured by radioimmunoassay for the in vitro determination of aldosterone in human serum, plasma and urine (Immunotech, A Beckman Coulter Company, IM1664). The radioimmunoassay of aldosterone is a competition assay. Samples and calibrators were incubated with an 125I-labeled aldosterone, as tracer, in antibody-coated tubes. After incubation, the liquid contents of the tube were aspirated and bound radioactivity is measured. A standard curve was established and unknown values were determined by interpolation from a standard curve.

3.4. Statistical analyses

The results were analyzed using the Statistical Package of Social Sciences (SPSS) computer software program, version 21.0 (Chicago, IL, USA). Quantitative data were presented as mean ± SD for normally distributed data. Qualitative data were presented in the form of frequencies and percentages. Differences among groups were

tested using Pearson's chi-square test (χ^2). A *P* value less than 0.05 was considered statistically significant.

4. Results

Table 1: Baseline characteristics of controls and ESRD patients

parameter	Age (years) (mean± SD)	Gender Male/female	Serum Aldosterone (pg/ml)	Serum creatinine (mg/dL)	Blood urea (mg/dL)
Controls (n=70)	50.2 ± 13.1	30/40	80.79 ± 17.37	0.82 ± 0.35	16.5±2.3
HD patients (n=70)	54.5± 9.5	32/28	105.83 ± 43.23	7.5±2.9	140.5±33.1
P- value	NS	NS	P< 0.001	P< 0.001	P< 0.001

Table 2: *CYP11B*2 genotype frequency in patients and controls

	ESRD	Control	P value
TT	48 (68.57%)	9 (12.85%)	p<0.001
TC	18 (25.71%)	38 (54.28%)	p<0.001
CC	4 (5.71%)	23 (32.85%)	p<0.001

Table 3: *CYP11B*2 allele frequency in patients and controls

	ESRD	Control	P value
T allele	42 (59.995 %)	43 (60.68 %)	NS
C allele	20 (28.565 %)	50 (70.7 %)	p<0.05

Table 4: *ATR*1 Genotype frequency in patients and controls

	ESRD	Control	P value
AA	4 (5.71%)	40 (57%)	p<0.05
AC	17 (24.28%)	25 (35.7%)	NS
CC	49 (70%)	5 (7.1%)	p<0.05

Table 5: *ATR*1 allele frequency in patients and controls

	ESRD	control	P value
A allele	19 (27.135%)	45 (64.25%)	p<0.05
C allele	42 (59.28%)	27 (39.25%)	p<0.05

Table 6: Comparison between mean serum aldosterone levels in different *CYP11B*2 genotypes in ESRD patients

		N	Mean±SD	vs. TT	vs. TC
serum aldosterone	TT	48	121.583 ± 43.311		
	TC	18	72.055 ± 11.709	p<0.001**	
	CC	4	68.75 ± 13.145	p<0.05*	NS

*, ** = Significant and highly significant difference, respectively.

Table 7: Comparison between mean serum aldosterone levels in different *ATR*1 genotypes in ESRD patients

		N	Mean±SD	vs. AA	vs. AC
serum aldosterone	AA	4	87.6 ± 25.4		
	AC	17	99.024± 43.177	NS	
	CC	49	121.25± 43.006	p<0.05*	NS

* = significant difference

5. Discussion

In the present study, there was a statistical evidence for significant interactions between genetic polymorphisms in *ATR*1 and *CYP11B*2 gene polymorphisms with ESRD risk.

The present study was designed to investigate the association of *CYP11B*2 and *ATR1 A1166C* gene polymorphisms with ESRD. In this case-control study, significant differences could be observed in genotype and allele frequency of the *CYP11B*2 -344C/T as well as the *AT1R A1166C* polymorphisms between controls and ESRD patients. Concerning the *CYP11B*2 gene, HD patients showed increased frequency of the TT genotype (68.57%) as compared to controls (only 12.85%), with no significant differences in T allele distribution between the 2 groups. In contrast, HD patients had low frequency of the CC genotype (5.71%) as compared to controls (32.85%), with a significant difference in C allele distribution between the HD patients (28.56%) and controls (70.7%). Comparing serum aldosterone levels in various *CYP11B*2 genotypes in HD patients revealed that patients with TT genotype had statistically higher aldosterone levels (121.583 ± 43.311) than those with the TC (72.055 ± 11.709) or CC genotype (68.75 ± 13.145). This might explain the significant association between the TT genotype with ESRD.

Our results are in contrast to reference [23] whose findings didn't support the hypothesis that CYP11B2 polymorphism may be associated with prevalence of ESRD.

Previous studies performed in Europe showed inconsistent results. Lovati and his colleagues [24] reported that there was no association between the CYP11B2 genotype and progression of renal failure among the ESRD patients. On the other hand, reference [25] reported that significant association was found between the CYP11B2 gene polymorphism and renal insufficiency in the hypertensive population. They observed an increased proportion of CC genotype in hypertensive patients with renal damage compared with hypertensive patients without renal damage. In contrast, the current results are in agreement with a study by Su *and his colleagues* [26], where significant associations were found in the CYP11B2 C- 344T polymorphism between control and ESRD groups. For CYP11B2 C-344T, the CC genotype compared with the TT genotype was a protective factor for ESRD ($P = 0.007$).

Song *and his colleagues in* [27] have also found that CYP11B2 C-344T polymorphism was associated with renal dysfunction progression, but only in female patients with IgA nephropathy. The C-344T polymorphism was a risk factor for accelerated progression in a Polish population with primary chronic glomerulonephritis [28].

On the other hand, for *AT1R A1166C* genotype frequency, HD patients showed significantly ($p<0.05$) higher frequency of CC genotype (70%) than controls (7.1%), with lower AA genotype frequency (5.71%) compared to controls (57%). Moreover, there were significant differences in A allele distribution between the HD patients (27.13%) and controls (64.25%). The same was true for the C allele, with a frequency of (59.28%) in HD patients compared to a frequency of (39.25%) in controls.

On the other hand, studying *ATR1 A1166C* gene polymorphisms revealed that patients with the CC genotype

had significantly higher aldosterone levels (121.25 ± 43.006) than those with AA (87.6 ± 25.4) genotype, which explain the significantly increased frequency of CC genotype in HD patients than controls.

The current results are in agreement with the study by [12], on *AT1R A1166C*, in which HD patients showed decreased frequency of the wild (AA) genotype and increased frequency of heterotype (AC) and mutant (CC) type than the corresponding control values. The authors have also observed that in the whole HD patients, A allele had significantly lower frequency compared to controls, with significantly increased C frequency compared to controls.

In agreement with the current results is also the study by Hammady *and his colleagues* [29], where authors have found that *AT1R 1166 AC* genotype was significantly higher in hypertensives than normotensives, while AA was significantly higher in normotensive than hypertensives. Risk estimate for ATR1 A1166C AC genotype showed 8.5 times more risk for Hypertension than AA genotype. Authors have found that AC genotype frequency of *AT1R* gene is higher in CRF patients group versus control group while control group had higher AA genotype frequency versus CRF group, yet this difference did not reach a statistical significance. Authors have also found that C allele frequency was higher in CRF patients group versus control group, while A allele frequency was higher in control group versus CRF group, yet this difference did not reach a statistical significance.

The current results as well as previous results about ATR1 gene can be explained in light of the findings of Chandra *and his colleagues* [30], who revealed that the expression of ATR1 gene was higher in patients with CC genotypes as compared to AC and AA. Ceolotto *and his colleagues* [31], have also observed an increased protein expression (70%) in patients with CC genotype, which leads to increased aldosterone levels.

A1166C of ATR1 is located within the 3′ untranslated regions of the gene. Though this polymorphism does not lead to amino acid substitution, these 3′ untranslated regions may play a pivotal role in the genomic context of the genes and may influence their expression levels, since they could result in defects in messenger RNA (mRNA) processing, mRNA half-life, or affect the function of regulatory elements such enhancers and insulators [32-33]. Moreover, Sethupathy *and his colleagues* [34], have shown that there is a miRNA from chromosome 21, namely miR155, that down regulates the expression of the 1166A allele but not of the 1166C, hence, they hypothesize that the 1166C allele is associated with hypertension.

6. Conclusion

In conclusion, our findings support the hypothesis that both CYP11B2 TT and AT1R CC genotypes, are related to higher aldosterone levels and, hence, should be considered as risk factors for developing ESRD in hypertensive patients. Further studies are needed to investigate the relation between aldosterone levels and AGRT1 gene expression in relation to different ATR1 gene polymorphisms as well as to miR155.

7. Limitations of the study

Small sample size is a limitation in this study.

8. Recommendations

Further studies with a greater sample size, are needed. It is also recommended to investigate the association between miR155 and AGRT1 gene expression in different AGRT1 gene polymorphisms, and determine its impact on aldosterone levels.

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