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Study the Association between Erythropoietin Resistance and Polymorphisms in two genes, *IL-1B* and *ACE* in Egyptian Patients on Maintenance Hemodialysis

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Abstract
The introduction of recombinant human erythropoietin (EPO) is effective treatment for anemia in chronic kidney disease patients The causes of inadequate response to EPO include iron deficiency, infection, chronic inflammation, and uremia. IL-1B-511C/T polymorphism has been associated with inflammation that plays important role in EPO resistance also the ACE gene polymorphism may display erythropoietic resistance. The study was conducted on 100 chronic kidney disease patients treated with hemodialysis. All participants gave informed consent .Clinical data were evaluated, including use of ACE inhibitors or angiotensin receptor blocker (ARB) prior to the time of the study. Lab investigations as Hb levels, serum albumin, total iron-binding capacity, high sensitivity C-reactive protein and Intact parathyroid hormone (PTH) levels.the aim of the study was to evaluate the association between(IL-1B-511C/T and ACE I/D gene polymorphisms) and EPO resistance index (ERI)
We found that Patients with the ACE D/D genotype had lower ERI (6.4318± 3.43 IU/kg weight/g Hb) if compared with other two genotypes (ACE I/I, 8.248±2.35 IU/kg weight/g Hb; ACE I/D, 7.30±2.92 IU/kg weight/g Hb) also ERI Values were lower in IL-1B-511C/T individuals (6.64±2.43IU/kg weight/g Hb) than in IL-1B-511CC (7.27±3.03 IU/kg weight/g Hb) and IL-1B-511TT (8.50±3.32 IU/kg weight/g Hb) and this difference was significant .we concluded that ACE genotype has predictive_value when determining the EPO dosage, as the II/ID genotypes may be associated with a suboptimal_response to EPO. Also patients with IL-IB C/T may require less EPO than other two genotypes IL B T/T and IL B C/C
Key words *Erythropoietin resistance, IL-1B , ACE gene, hemodialysis*

Introduction

Progression of chronic kidney disease (CKD) is associated with high prevalence of anemia. Many factors play a role in this sitting but it is mainly due to impaired production of erythropiotein (EPO). Several well known factors may contribute to the increased erythropiotein resistance like vitamins and iron deficiency, chronic inflammation, hyperparathyroidism and chronic infection [(1)].Early diagnosing and management of this resistance is important. This will avoid the hazards of increasing the dose of EPO or the

effect of the anemia itself on morbidity (mainly on cardiovascular system) and mortality [2]

EPO is a multi-functional cytokine, which exerts erythropoietic and immune-modulatory effects upon binding to two distinct receptors, which are expressed on erythroid, parenchymal and immune cells The bond between EPO-α and EPO receptor (EPOR) in peripheral blood mononuclear cells (PBMCs) leads to a reduction in inflammation[3]

Chronic inflammation is a common feature of many heamodialysis patients and a list of endogenous and exogenous factors that induce this inflammation is growing, due to the reduced excretion of immunoactive proteins by the kidneys and the dialysis procedure itself. Elevated levels of C-reactive protein (CRP) is an important finding among these patients. The production of CRP is induced by several cytokines among them most prominently interleukin-6 (IL-6), which is a mediator that is secreted by macrophages, monocytes and lymphocytes. Elevated CRP and cytokine levels are correlated with reduced erythropoetin efficacy and elevated erythropiotein dose requirements in these patients. The IL-1 gene cluster on chromosome 2q contains 3 related genes within a 430-kb region, *IL-1A*, *IL-1B*, and *IL-1RN*, that encode the pro-inflammatory cytokines which are IL-1α and IL-1β [4]

Pro-inflammatory cytokines can affect erythropoiesis at several levels, and manifest their effects as inflammatory anaemia and hypo-responsiveness to EPO treatment. Pro-inflammatory cytokines can cause EPO resistance, by interfering with iron homeostasis and inhibiting the *in vitro* growth of erythroid progenitors [5]. In particular, tumor necrosis factor (TNF-α), IL-1 and IL-6 they increase the expression of divalent metal transporter-1, induce ferritin synthesis, reduce ferroportin expression and stimulate the transferrin-bound iron reuptake by increasing the transferrin receptor expression in macrophages . All these processes promote the reducing iron bioavailability leading to intracellular accumulation of iron; reduce the production of endogenous EPO as well as their effect on bone marrow response to the erythropoietic stimulus, and ending by keeping patients anemic[6].

Rennin angiotensin system play an important role in erythropiosis .This was proved in vitro studies that Angiotensin II and angiotensin II receptors AT II could accelerate the iron incorporation in erythrocytes which enhance erythropiosis also it is important on regulation of haemopiotic stem cell proliferation leading to acceleration of stem cell mitosis and differentiation to erythroid progression.[7]

A single gene is responsible for the expression of both sACE and tACE. It is located at locus 17q23. *ACE* gene is 21 kb long and composed of 26 exons and 25 introns, a polymorphism involving insertion of 287 bp sequence resulting in insertion (I) allele, whereas deletion (D) allele is present in the absence of insertion [8]. This polymorphism is responsible for the ACE activity level, which increases 2-fold in homozygous deletion carriers (D/D), as compared to homozygous insertion carriers (I/I). I/D carriers show intermediate ACE activity.

Therefore It seems possible that polymorphisms of the two genes (*IL-1B* and *ACE*) play an important role in the development of EPO resistance in ESRD patients. We evaluated the *IL-1B*-511C/T and *ACE* I/D polymorphisms in Egyptian CKD patients on hemodialysis to determine the association between various polymorphisms and EPO resistance.

Subjects

100 CKD patients on maintenance hemodialysis. All participants gave informed consent according to local ethics committee consent procedures. Inclusion criteria: (1) treatment with hemodialysis for three months or more; (2) injections of either EPO α or β for renal anemia (3) age 18 years or above.

Exclusion criteria (1) hypothyroidism (2) symptoms and signs of bleeding less than two months before inclusion; (3) malignancy (4) hematologic disease; (5) acute infectious disease. Clinical data were evaluated, including age, and

use of ACE inhibitors or angiotensin receptor blocker (ARB).

Methods

Five milliliter venous blood was withdrawn from every patient in the morning after overnight fasting. 1000 µl whole blood was separated into an eppendorf containing ethylenediamine tetraacetic acid (EDTA) 5% and stored at –20°C for genotyping ACE I/D and IL-1B-511C/T polymorphisms.

ELISA for ACE [9] and IL-1β[10]: To determine ACE and IL-1β levels, serum ACE concentration was measured by ELISA (R&D systems, Inc., Minneapolis, MN) . Intra- and inter-assay coefficients of variation were 3.7% and 5.9%, respectively. Serum IL-1β concentrations were also assayed by ELISA The intra- and inter-assay coefficients of variation were 6.7 and 8.9%, respectively.

DNA extraction and ACE genotyping: Genomic DNA was extracted from whole blood by an established method for extraction of DNA. [11]

ACE I/D polymorphism was carried out using polymerase reaction (PCR) [12], using site specific primers forward 5`-CTGGAGACCACTCCCATCCTTTCT-3` and reverse: 5`-GATGTGGCCATCACATTCGTCAGAT-3` which amplify the intron 16 region where the I/D fragment is located. Amplification with this primer pair results in 490 bp and 190 bp amplification products corresponding to the I and D alleles respectively. PCR amplification products were obtained using 25 µl reactions [0.5 µg genomic DNA, 200 p mol of each primer, 0.5 mM each of deoxy- ATP, GTP, CTP AND TPP nucleotides, 3 mM Mg Cl_2, 1 unit of Taq DNA polymerase and 2.5 µl 10 x PCR buffer (50 mmol / l kCl, 0.001% gelatin and 10 mmol/ l Tris- HCl, pH 8.3)]. The amplification was carried out using thermal cycler. The PCR product is a 190 bp fragment in the absence of the insertion and a 490 bp fragment in the presence of insertion.

IL-1B -511 C/T [13]: The promoter region polymorphism in IL-1B was amplified using site specific primers; Forward primer 5`- TGG CAT TGA TCT GGT TCA TC -3`, Reverse primer 5`- GTT TAG GAA TCT TCC CAC TT -3` PCR conditions included denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 1min, annealing at 54°C for 1min and extension at 72°C for 1min. The PCR was completed with a final extension step of 5 min at 72°C. PCR product of 304 bp was size matched on 1.5% agarose gel before subjecting to 2.5 U of *Ava*1 restriction enzyme (New England Biolabs) at 37°C overnight. Allele C which did not contain the AvaI restriction enzyme site remained undigested as 304 bp fragments, whereas, allele T yields 190 bp and 114 bp fragments

Routine Laboratory methods were used to measure serum albumin, total iron-binding capacity, high sensitivity C-reactive protein (hs-CRP), and intact parathyroid hormone (PTH) levels (every three months) while hemoglobin (Hb) levels monthly measured .

EPO resistance index (ERI) was calculated as weekly EPO dose per kg of body weight, divided by the Hb concentration (weekly EPO dose/kg weight/g Hb). The dose of EPO was titrated by 25% every two weeks in an attempt to maintain a target Hb level between 10 and 11 g/dl

Results

Subjects characteristics

Table 1 - Demographic characteristics of participants.

sex	
Male	54%
Female	46%
Age	53.56±13.77 years
Time on dialysis	33.4±32.7 months

Table (2) Laboratories characteristics of participants

	Minimum	Maximum	Mean	Std. Deviation
Albumin g/L	3.20	4.50	3.8310	.29600
TIBC ug/ml	1.60	5.00	2.9750	1.06376
CRP mg/L	2.50	11.00	5.8290	2.22294
PTH pmol/L	.21	1600.00	557.7211	339.80091
Hb g/dL	8.00	12.00	9.8100	.79411
ERI IU/kg weight/g Hb	2.20	13.70	7.3650	2.94904

Genotype distributions of ACE I/D and IL-1B-511C/T polymorphism

The distributions of the *ACE* II, *ACE* ID, and *ACE* DD genotypes among patients (27%), (51%), and (22%), respectively. The distributions of *IL-1B*-511C/T genotypes were 20 % ,in the *IL-1B*-511CC group , 48% in the *IL-1B*-511CT group and 32% in the *IL-1B*-511TT group (*20 %*)..

Table 3, shows that Patients with the *ACE* D/D genotype had lower ERI (6.4318± 3.43 IU/kg weight/g Hb) if compared with other two genotypes (*ACE* I/I, 8.248±2.35 IU/kg weight/g Hb; *ACE* I/D, 7.30±2.92 IU/kg weight/g Hb) but this difference was without statistical significant difference.

Table (3): Relation between ACE gene polymorphism and ERI.

ERI	N	Mean	Std. Deviation	Minimum	Maximum
ID	51	7.3000	2.92793	2.20	13.70
II	27	8.2481	2.35639	4.30	12.70
DD	22	6.4318	3.43336	2.30	12.60
Total	100	7.3650	2.94904	2.20	13.70
F		2.390			
p		.097			

Table (4) shows lower Values for ERI in *IL-1B*-511C/T individuals (6.64±2.43IU/kg weight/g Hb) than in *IL-1B*-511CC (7.27±3.03 IU/kg weight/g Hb) and *IL-1B*-511TT (8.50±3.32 IU/kg weight/g Hb) and this difference was statistician significant.

Table (4): Relation between IL-1B gene polymorphism and ERI.

ERI	N	Mean	Std. Deviation	Minimum	Maximum
CC	20	7.2700	3.03577	2.30	13.00
TT	32	8.5000	3.32012	2.30	13.70
CT	48	6.6479	2.43240	2.20	11.00
Total	100	7.3650	2.94904	2.20	13.70
F		4.032			
p		.021*			

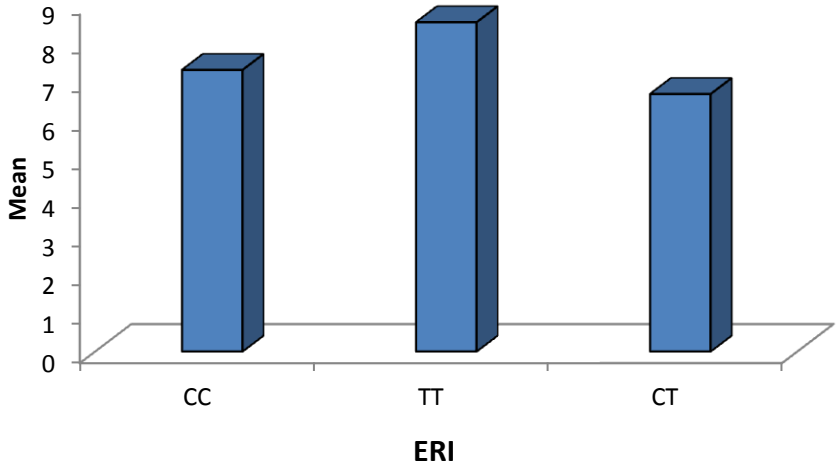

Figure (1): Relation between IL-1B gene polymorphism and ERI

Table (5) shows statistical significant difference between the three ACE genotypes and Hb levels. The highest Hb level was associated with *ACE* D/D(10.3682±.75994 g/dL).

Table (5): Relation between ACE gene polymorphism and Hb.

		N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
Hb g/dL	ID	51	9.7980	.69957	8.00	11.90	11.429	.0001*
	II	27	9.3778	.73188	8.00	11.20		
	DD	22	10.3682	.75994	9.40	12.00		

Table (6) shows that, *IL-1B*-511CC patients were having highest Hb level (10.09±0.82 g/dL) when compared with *IL-1B*-511CT (9.7479±.67002 g/dL) *and IL-1B*-511TT (9.7281±.92431 g/dL) but without statistical significance.

Table (6): Relation between IL-IB gene polymorphism and Hb level.

		N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
Hb g/dL	CC	20	10.0900	.82328	9.30	12.00	1.578	.212
	TT	32	9.7281	.92431	8.00	12.00		
	CT	48	9.7479	.67002	8.00	11.40		

Table (7) shows significant low Hb levels in, *IL-1B*-C/C genotype patients receiving ACEi .

Table (7) the relation between the Hb levels, ACEi and IL-BI gene polymorphism

			Hb g/dL	
IL-BI gene		ACEi	Mean	Std. Deviation
dimension1	CC	Negative	10.2467	.88226
		Positive	9.6200	.35637
		p	0.031*	
	TT	Negative	9.7120	.95364

		Positive	9.7857	.87831
		p	0.725	
	CT	Negative	9.7489	.69040
		Positive	9.7333	.25166
		p	0.822	

Table (8) shows no statistical significant difference in Hb levels among patients (either with using or without using ACEi) with different ACE gene polymorphism

Table (8) the relation between the Hb levels, ACEi and ACE gene polymorphism

			Hb g/dL	
ACE gene		ACEi	Mean	Std. Deviation
dimension1	ID	Negative	9.8293	.76819
		Positive	9.6700	.27101
		p	0.236	
	II	Negative	9.2773	.62177
		Positive	9.8200	1.07331
		p	0.144	
	DD	Negative	10.3682	.75994
		Positive	10.3682	.75994
		p	0.652	

Table (9) represents a significant negative statistical correlation between the Hb levels and ERI, and between the ERI levels and serum albumin.

Table (9): Correlation between ERI and different studied parameters.

		Hb	Albumin	TIBC	CRP	PTH
ERI	Pearson Correlation	-.251*	-.289**	.014	.092	-.073
	Sig. (2-tailed)	.012	.004	.891	.362	.471

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

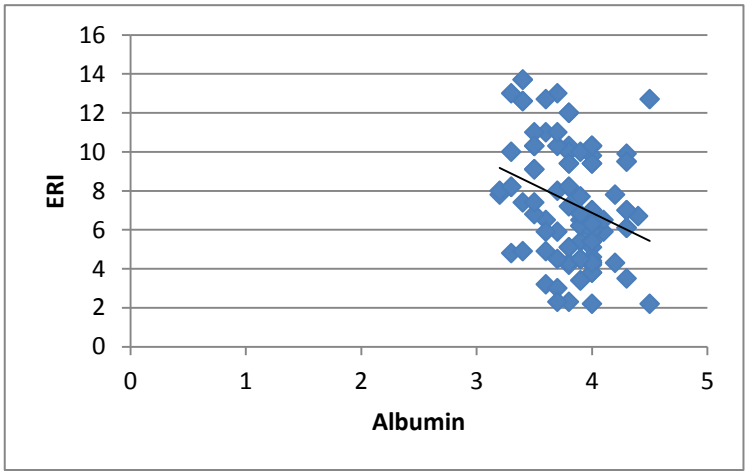

Figure (2) represent the negative correlation between the serum albumin levels and ERI.

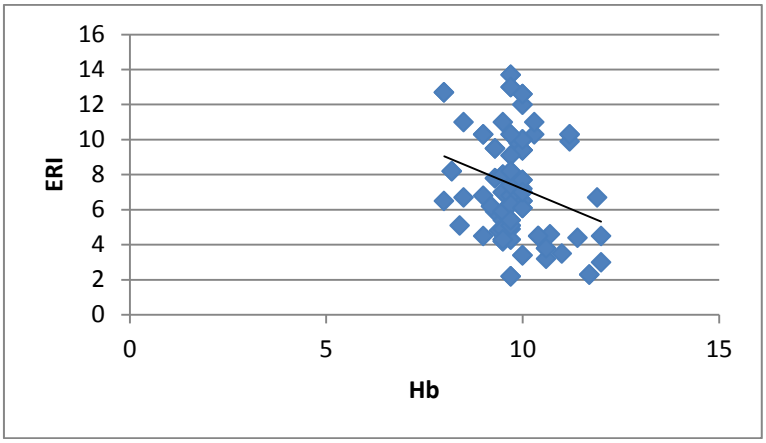

Figure (3) represent the negative correlation between the Hb levels and ERI

Discussion

Chronic kidney disease patients may respond insufficiency to recombinant human erythropio-tein due to erythropiotein resistance. In current study we found that this variability in response to EPO was linked in part to genetic polymorphisms. Patients with the *ACE* D/D genotype had lower ERI values compared to those with *ACE* I/D or *ACE* I/I, independent of other traditional factors. But this difference wasn't statistically significant and these findings were matching Jeong et al study (14) but on the other hand Zoltán Kiss1et al(15) did not find any direct association between erythropoietin resistance and ACE gene I/D polymorphism alone.

Jeong et al(14)and Zoltán Kiss1et al [15] found that *ACE* D/D genotype lowers the EPO requirement due to the relatively high level of angiotensin II, an important stimulus of erythropoiesis. This agreed the results of our study that shows that patients with ACE D/D polymorphism were having highest levels of Hb compared to the other two genotypes and this difference was statistically significant. Therefore, lower exogenous rHuEpo doses were required to achieve treatment targets in patients with D/D genotype.

Previous work by Hantano *et al.* [16] on hemodialysis patients found no significant effect of the *ACE* I/D polymorphism on EPO requirement. Lower rHuEpo dose requirement was associated with the D/D genotype in patients on peritoneal dialysis in the United Kingdom in two studies by Varagunam et al. and Sharples et al [17]. However, those patients used different dialysis modality; they had significant residual renal function and lower level of chronic inflammation. So they were unlike present study..

The current study showed a negative statistically correlation between ERI and Hb levels and negative statistically correlation between ERI and serum albumin. Although albumin concentration can be considered a marker of nutritional status, it is essentially a marker of inflammation, which acts as a negative acute phase reactant. In, Juan M López-Gómez et al [18] the intensity of the response to EPO was directly related to albumin concentration. A decrease in albumin level is usually accompanied by an increase in ERI. This situation means that underlying inflammatory processes can be ruled out as the cause of EPO resistance

Inflammation considers one of the major factors for resistance to EPO therapy. IL-1β suppresses the colony formation of bone marrow erythroid progenitors, and negatively influence iron utilization, thereby interfering with Hb synthesis. These data suggest that IL-1β, which is under some degree of genetic control, might be associated with EPO resistance. [19].

Jeong et al [14] found in their study that the IL-1B-511C/C genotype was significantly associated with lower ERI values in hemodialysis patients. But these findings were opposite to the current study findings which found that ERI values were lowest with hemodialysis patients with IL-1B-

511CT compared with the other genotypes *IL-1B*-511C/C and *IL-1B*-511T/T. Patients with IL-I B C/C were having highest Hb levels but the difference between the groups was not statistically significant.

The use of ACEi in our CKD patients showed that lowest Hb levels were found in patients with IL-1B C/C genotype compared to the patients with IL-1B C/T, IL-1B T/T genotype and this difference was statistically significant. Also lowest Hb level was found in patients receiving ACEi with ACE I/D polymorphism. Our results suggest that in patients with I/D genotype the pharmacological inhibition of ACE enzyme can increase erythropoietin resistance and worsen erythropoiesis as a result of fall of AT II levels and decrease endogenous EPO production. Zoltán Kiss1 et al ([16]) found in their study that patients with D/D genotype or with D allele receiving ACEi have the lowest Hb levels but they did not find any direct association between erythropoietin resistance and ACE gene I/D polymorphism alone. While some studies reported negative effect of ACEi on erythropoietin resistance and anaemia in haemodialysis others could not demonstrate this relationship.

Further studies is needed to find that holding ACEi therapy can potentially result in increased erythropoiein response in some patients, likely those with D allele in their ACE gene.

There are a number of limitations to this study: small sample size, we did not measure serum erythropoietin and AT-II levels these might have helped in understanding the pathophysiological background of our results, but are unlikely to affect the explored associations. Also patients were on a variety of ACE is and we did not capture its type, dosage or length of treatment.

More importantly, since we included only Egyptian patients (south Caucasians which includes Mediterranean and Middle East), our results might not be generalized to patients of other races.

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