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Polymorphisms in two genes, *IL-1B* and *ACE*, are associated with erythropoietin resistance in Korean patients on maintenance hemodialysis

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Abbreviations: ACE, angiotensin converting enzyme; EPO, erythropoietin; ERI, erythropoietin resistance index; ESRD, end stage renal disease; Hb, hemoglobin; hs-CRP, high sensitivity C-reactive protein; iPTH, intact parathyroid hormone; TIBC, total iron binding capacity

Abstract

Genetic polymorphisms may be linked to inter-individual differences in erythropoietin (EPO) resistance. We investigated the -511C/T polymorphism of the *IL-1B* gene and the I/D polymorphism of the *ACE* gene for any association with EPO resistance index (ERI) in maintenance hemodialysis patients (n = 167). Because EPO responsiveness is multi-factorial, we also included other possible influences (age, sex, time on dialysis, ACE inhibitor or angiotensin receptor blocker use, ferritin, transferrin saturation, intact PTH, high sensitivity C-reactive protein, albumin, Kt/V, and presence of diabetes mellitus) on ERI in our analyses. Multiple regression analysis showed significant association of the *IL-1B*-511CC and *ACE* DD polymorphisms with ERI (P = 0.038 and P = 0.004 in the recessive model, respectively). The combination (C) of alleles of two loci showed that C1 (I-T) was significantly associated with ERI in the co-dominant and recessive models (P = 0.005 and P = 0.0001, respectively). Subjects who did not carry C1 showed significantly decreased ERI (10.10 ± 5.15 IU/kg weight/g hemoglobin) compared to other study subjects (C1/C1 and C1/-; 12.97 ± 4.90 and 15.12 ± 7.43 IU/kg weight/g hemoglobin, respectively). Our study indicates that the *IL-1B*-511C/T and *ACE* I/D polymorphisms may be useful genetic markers of EPO requirement in hemodialysis patients. These findings might also provide a new perspective on therapeutic approaches to the treatment of end stage renal disease patients with anemia.

Keywords: end stage renal disease; erythropoietin; interleukin-1β; kidney failure, chronic; peptidyl-dipeptidase A; polymorphism, genetic

Introduction

Since the introduction of recombinant human erythropoietin (EPO) in 1986, it has been an effective treatment for renal anemia in the majority of patients with end-stage renal disease (ESRD) (Winearls *et al*., 1986; Schaefer *et al*., 1989). The most common cause of inadequate response to EPO is generally thought to be absolute or functional iron deficiency (Tarng *et al*., 1995). Various clinical conditions may also cause EPO resistance, including infections (Muirhead and Hodsman, 1990), chronic inflammation (Schardin, 1990), secondary hyperparathyroidism (Rao *et al*., 1993), and uremia.

Chronic inflammation can modify the process of erythropoiesis, probably via various inflammatory cytokines (Danielson, 1995; Cooper *et al*., 2003). Pro-inflammatory cytokines, which are under some degree of genetic control, may be associated with EPO resistance. Recent studies have shown that genetic polymorphisms in cytokine genes may influence the level of corresponding cytokines, which also play an important role in the pathogenesis of anemia (Maury *et al*., 2004). The IL-1 gene cluster on chromosome 2q contains 3 related genes within a 430-kb region, *IL-1A, IL-1B*, and *IL-1RN*, which encode the pro-infammatory cytokines IL-1α and IL-1β as well as their endogenous receptor antagonist IL-1ra (Dinarello, 1996). Moreover, the *IL-1B*-511C/T (rs1143627) single nucleotide polymorphism (SNP) has been associated with a variety of diseases in which inflammation plays an important role (Hurme *et al*., 1998). To date, however, there has been no published study on the relationship between *IL-1B*-511C/T polymorphism and EPO resistance in hemodialysis patients.

In 1990, the *ACE* gene polymorphism (rs1799752)

characterized by the insertion or deletion of a 287-base pair fragment in the 17q23 chromosome was identified (Rigat *et al*., 1990). Mean angiotensin converting enzyme (ACE) levels in *ACE* DD carriers were approximately twice that found in *ACE* II genotype individuals (Rigat *et al*., 1990). ACE is a key enzyme in the production of angiotensin II; thus, *ACE* DD carriers show the highest ACE and angiotensin II levels (Rigat *et al*., 1990). Angiotensin II stimulates proliferation of early erythroid progenitors *in vitro* (Mrug *et al*., 1997). As a result, *ACE* DD individuals may display greater erythropoietic activity. Some investigators have observed an increase in EPO requirement in patients with the *ACE* II genotype undergoing continuous ambulatory peritoneal dialysis (CAPD) (Varagunam *et al*., 2003), but others have found no effect (Hatano *et al*., 2000).

It seems possible, therefore, that polymorphisms of the *IL-1B* and *ACE* genes play a role in the development of EPO resistance in ESRD patients. We evaluated the *IL-1B*-511C/T and *ACE* I/D polymorphisms in patients in hemodialysis to determine the association between various polymorphisms and EPO resistance. Because EPO responsiveness is multi-factorial, we also included other possible influences on EPO resistance in our analyses.

Materials and Methods

Subjects

End-stage renal disease patients treated with maintenance hemodialysis from Kyung Hee University Medical Center were included in this study. All participants gave informed consent according to local ethics committee consent procedures. Those who satisfied the following criteria were recruited in our study population: (1) treatment with hemodialysis for three months or more; (2) age 18 years or above; and (3) injections of either EPO α or β for renal anemia. Exclusion criteria were: (1) symptoms and signs of bleeding less than two months before inclusion; (2) hypothyroidism (euthyrotic patients on thyroid hormone replacement therapy were included); (3) malignant disease; (4) hematologic disease; and (5) acute infectious disease. Relevant clinical data were evaluated, including age, primary cause of kidney disease, dry body weight, and use of ACE inhibitors or angiotensin receptor blocker (ARB).

Methods

Clinical data: The following clinical and laboratory data were obtained for three months prior to the time of the study, and average values were used for analysis: serum albumin, iron, total iron-binding capacity, ferritin, high sensitivity C-reactive protein (hs-CRP), Kt/V (monthly), and intact parathyroid hormone (PTH) levels (every three months). The precision of these laboratory findings was analyzed with 20 repeated measurements in 3 different levels of controls. The coefficient of variation was usually less than 5.0%. We measured hematocrit and hemoglobin (Hb) levels bimonthly and evaluated EPO responsiveness to the EPO therapy. 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. Although there was a dose-response relationship between EPO and Hb, some patients required higher doses of EPO to reach a target Hb level. To quantitatively assess EPO responsiveness, an 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). We defined the weekly EPO dose as the average value over three months.

ELISA for ACE and IL-1β: To determine ACE and IL-1β levels, fasting blood samples were obtained between 08 : 00 and 10 : 00. Samples were centrifuged and stored at -80°C. Plasma ACE concentration was measured by ELISA (R&D systems, Inc., Minneapolis, MN) (Danilov *et al*., 1996). Intra- and inter-assay coefficients of variation were 3.7% and 5.9%, respectively. IL-1β concentrations were also assayed in plasma by ELISA according to the manufacturer's instructions (R&D systems, Inc., Minneapolis, MN). The intra- and inter-assay coefficients of variation were 6.7 and 8.9%, respectively.

DNA preparation and genotyping: Genomic DNA was isolated from peripheral blood samples. PCR-based genotyping of *IL-1B*-511 C/T and *ACE* I/D polymorphisms was carried out as previously described (di Giovine *et al*., 1992; Varagunam *et al*., 2003; Sharples *et al*., 2006). The following primers were used: *IL-1B*, 5'-TGGCATTGATCTGGTTC-ATC-3' and 5'-GTTTAGGAATCTTCCCACTT-3'; and *ACE*, 5'-CTGGAGACCACTCCCATCCTTTCT-3' and 5'-GATGTGGCCATCACATTCGTCAGAT-3'. The PCR products for *IL-1B* were digested with *Ava*I (NEB, Beverly, MA) at 37°C, analyzed by electrophoresis on 1.5% agarose gels, and visualized with ethidium bromide under UV light. Amplified *ACE* products were run on 2% agarose gels. *Ava*I digested the amplified products from the *IL-1B*-511C allele into two fragments, and products amplified from the *IL-1B*-511T allele remained undigested. The *ACE* I/D polymorphism was evi-

dent as a 490-bp fragment in the presence of the insertion (I allele) and as a 190-bp product in the absence of the insertion (D allele). Twenty percent of the subjects were randomly selected for DNA sequencing to confirm the accuracy of the analysis.

Statistical analysis: The SPSS statistical package, version 13.0 (SPSS; Chicago, IL), was used to compare all clinical and laboratory parameters. Differences between group means were tested using one-way ANOVA (with Tukey's post hoc test when ANOVA was significant), and differences in proportion were assessed by the chi-square test. All values are expressed as mean ± SD, with statistical significance defined as $P < 0.05$. Multiple regressions adjusted for age, sex, time on dialysis, ACE inhibitor or ARB use, ferritin, transferrin saturation, intact PTH, hs-CRP, albumin, Kt/V, and presence of diabetes mellitus were used for association analyses with ERI.

Results

Subject characteristics

Of the 167 hemodialysis patients included in this study, 89 were male (53.3%). Table 1 shows clinical profiles of participants. Mean age was 54.56 ± 13.77 years (range, 23-88 years) and mean time on dialysis was 34.40 ± 33.37 months (range, 3-139 months). The mean Hb concentration, ferritin, intact PTH, hs-CRP, and ERI of the subjects were 9.97 ± 0.97 g/dL, 122.89 ± 135.13 μg/L, 96.83 ± 117.28 pmol/L, 3.41 ± 5.33 mg/L and 12.99 ± 6.81 IU/kg weight/g Hb, respectively. The enter method of multiple regression analysis was used to detect those clinical variables with the most influence on EPO responsiveness. Based on this analysis, no significant association with ERI level was detected (data not shown).

Table 1. Demographic and clinical characteristics of participants.

Age	54.56 ± 13.77 years
Male : Female (%)	89 : 78 (53.3% : 46.7%)
Time on dialysis	34.40 ± 33.37 months
Hemoglobin concentration	9.97 ± 0.97 g/dL
Ferritin	122.89 ± 135.13 μg/L
Transferrin saturation	29.39 ± 12.24%
Parathyroid hormone	96.83 ± 117.28 pmol/L
High sensitivity C-reactive protein	3.41 ± 5.33 mg/L
Albumin	3.72 ± 0.68 g/L
Kt/V	1.62 ± 0.30
Erythropoietin resistance index	12.99 ± 6.81 IU/kg weight/g Hb
ACEi/ARB therapy	76 patients
Presence of DM	75 patients

ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; DM, diabetes mellitus.

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

Genotype distributions of the two loci studied are shown in Table 2. The distributions of the *ACE* II, *ACE* ID, and *ACE* DD genotypes among patients were 25.1% (n = 42), 54.5% (n = 91), and 20.4% (n = 34), respectively. The distributions of *IL-1B*-511C/T genotypes were 21.6% in the *IL-1B*-511CC group (n = 36), 43.1% in the *IL-1B*-511CT group (n = 72), and 35.3% in the *IL-1B*-511TT group (n = 59). In this study the *ACE* I and *IL-1B*-511C polymorphisms had allele frequencies of 52.4% and 43.1%, respectively. The possible combinations of alleles of two loci studied were C1 (I-T), C2 (D-T), C3 (I-C), and C4 (D-C). The frequencies of the observed combinations are presented in Table 3.

Association with ERI

Associations between *ACE* I/D and *IL-1B*-511C/T polymorphisms and ERI were analysed using multiple regressions, adjusting for age, sex, time on dialysis, ACE inhibitor or ARB use, ferritin, transferrin saturation, intact PTH, hs-CRP, albumin, Kt/V, and presence of diabetes mellitus. Two SNPs (*ACE* DD, *IL-1B*- 511CC) showed significant association with ERI (P = 0.038 and P = 0.004 in the recessive model, respectively). Patients with the *ACE* DD genotype (10.02 ± 5.14 IU/kg weight/g Hb) had lower ERI compared with those without (*ACE* I/I, 13.26 ± 5.52 IU/kg weight/g Hb; *ACE* I/D, 13.97 ± 7.60 IU/kg weight/g Hb). ERI values were also lower in *IL-1B*-511CC individuals (9.66 ± 5.16

Table 2. Frequencies of the *ACE* I/D and *IL-1B*-511C/T polymorphisms.

Genotype	Genotype frequency (n/%)			Allele frequency (n/%)	
ACE I/D	II (42/25.1)	ID (91/54.5)	DD (34/20.4)	I (175/52.4)	D (159/47.6)
IL-1B-511C/T	CC (36/21.6)	CT (72/43.1)	TT (59/35.3)	C (144/43.1)	T (190/56.9)

n, number of patients.

IU/kg weight/g Hb) than in *IL-1B*-511CT (15.25 ± 7.58 IU/kg weight/g Hb) and *IL-1B*-511TT (12.25 ± 5.71 IU/kg weight/g Hb) genotype patients.

Four combinations of alleles were constructed using the two SNPs (Table 4). We found a significant association between C1 (I-T) and ERI values in both co-dominant and recessive models ($P = 0.005$ and $P = 0.0001$, respectively); e.g., individuals who did not carry C1 showed significantly decreased ERI (10.10 ± 5.15 IU/kg weight/g Hb) compared to those with C1/C1 and C1/- (12.97 ± 4.90 and 15.12 ± 7.43 IU/kg weight/g Hb, respectively). Other combinations (C2, C3, and C4) were not associated with ERI.

ACE and IL-1β level

We measured ACE and IL-1β levels directly to show the biological functions of the two SNPs in this study. Seventy-six patients in this study were taking ACE inhibitors or ARB, drugs known to affect ACE or angiotensin II levels. For this reason, we evaluated ACE levels only ninety-one patients (*ACE* II, $n = 24$; *ACE* ID, $n = 46$; *ACE* DD, $n = 21$) who were not taking ACE inhibitors or ARB. The *ACE* I/D polymorphism had a significant effect on on plasma ACE levels (Figure 1). Patients with the *ACE* DD genotype had the highest plasma ACE levels compared to patients with *ACE* ID or II genotypes (187.2 ± 46.2, 137.1 ± 59.5, 125.0 ± 29.66 ng/ml, respectively; P=0.0005). Patients with the *IL-1B*-511TT genotype also showed a significantly higher mean IL-1β level (0.9 ± 0.6 pg/mL), compared to those with the *IL-1B*-511CC genotype (0.3 ± 0.3 pg/mL; $P = 0.02$) (Figure 2).

Table 3. Combinations of alleles of the *ACE* I/D, *IL-1B*-511C/T polymorphisms and their frequencies.

Combination	*ACE* I/D	*IL-1B*-511C/T	Frequency (%)
C1	I	T	30.8
C2	D	T	26.0
C3	I	C	21.5
C4	D	C	21.5

C, combination.

Discussion

EPO production is markedly lower in patients with ESRD, resulting in the development of renal anemia. The use of EPO in ESRD patients results

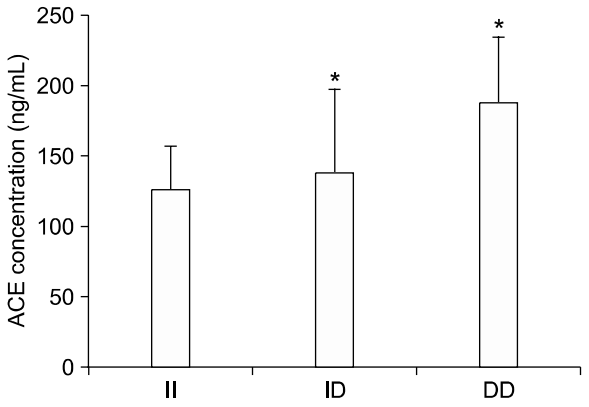

Figure 1. Association of plasma ACE level with the *ACE* I/D polymorphism. Plasma ACE levels determined by sandwich ELISA (Data are means ± SD. *$P < 0.05$, versus the II group).

Table 4. Regression analysis of ERI in hemodialysis patients according to the *ACE* I/D, *IL-1B*-511C/T polymorphisms and combinations of alleles of two loci.

Genotype	C/C*	C/R*	R/R*	*Pa*	*Pb*	*Pc*
ACE I/D	13.26 ± 5.52[a]	13.97 ± 7.60	10.02 ± 5.14	0.087	0.437	**0.038**
IL-1B-511C/T	12.25 ± 5.71	15.25 ± 7.58	9.66 ± 5.16	0.262	0.446	**0.004**
C1	12.97 ± 4.90	15.12 ± 7.43	10.10 ± 5.15	**0.005**	0.881	**0.0001**
C2	9.05 ± 4.97	14.59 ± 7.61	11.83 ± 5.59	0.733	0.090	0.229
C3	8.20 ± 5.55	13.46 ± 6.84	9.05 ± 4.97	0.085	0.165	0.090
C4	7.03 ± 4.17	13.88 ± 7.77	12.61 ± 5.78	0.676	0.256	0.939

ERI, erythropoietin resistance index; C, combination

*C/C, C/R and R/R represent homozygotes for the common allele, heterozygotes and homozygotes for the rarer allele, respectively.

[a]Mean ± standard deviation of ERI (IU/kg weight/g Hb).

Pa, Pb and *Pc* are *P* values of codominant (minor allele homozygotes vs. heterozygotes vs. major allele homozygotes), dominant (minor allele homozygotes plus heterozygotes vs. major allele homozygotes) and recessive (minor allele homozygotes vs. heterozygotes plus major allele homozygotes) models for multiple regression analysis controlling for age, sex, time on dialysis, ACE inhibitor or ARB use, ferritin, transferrin saturation, intact PTH, hs-CRP, albumin, Kt/V and presence of diabetes mellitus as covariates.

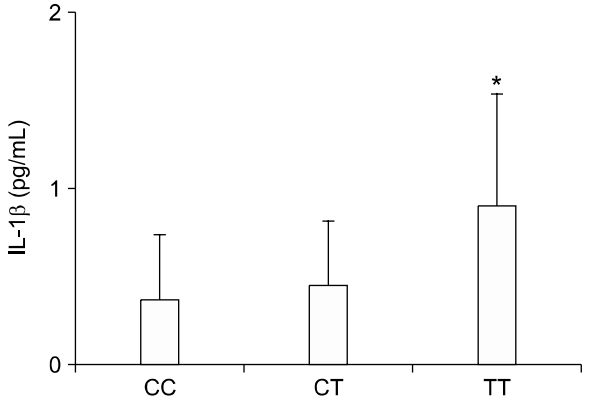

Figure 2. Association of plasma IL-1β levels with the *IL-1B*-511C/T polymorphism. Plasma IL-1β levels determined by sandwich ELISA (Data are means ± SD. **P* < 0.05, versus CC group).

in a significant increase in Hb concentration and improvements in quality of life for the majority of patients. Approximately 5-10% of ESRD patients receiving EPO, however, appear to be resistant to this drug (Priyadarshi and Shapiro, 2006).

In the current study we found that this variability was linked in part to genetic polymorphisms. Patients with the *ACE* DD genotype had significantly lower ERI values compared to those with *ACE* II or *ACE* ID, independent of other traditional factors. ACE is a key enzyme in circulatory homeostasis, where it catalyzes the conversion of angiotensin I to angiotensin II. The *ACE* I/D polymorphism affects plasma and tissue levels of ACE activity, and individuals with the *ACE* DD genotype show the highest levels of this enzyme (Rigat *et al*., 1990). We also evaluated plasma ACE levels in ninety-one patients who were not taking ACE inhibitors or ARB. There was a significant effect of *ACE* I/D polymorphism on plasma ACE levels. This study argues for a role of *ACE* I/D polymorphism in renal anemia, in which the *ACE* DD genotype lowers the EPO requirement due to the relatively high level of angiotensin II, an important stimulus of erythropoiesis. Previous work by Hantano *et al*. (2000) on hemodialysis patients found no significant effect of the *ACE* I/D polymorphism on EPO requirement. Our results are, however, in agreement with those from two recently published studies that included CAPD patients and demonstrated that the *ACE* II/ID genotypes seem to be associated with suboptimal EPO response (Varagunam *et al*., 2003; Sharples *et al*., 2006).

Inflammation is one of the major independent predictors of resistance to EPO therapy, and pro-inflammatory cytokines have been shown to inhibit erythropoiesis (Means and Krantz, 1991, 1993). For example, IL-1β suppresses the colony formation of bone marrow erythroid progenitors and inhibits EPO production (Jelkmann, 1998). In addition, pro-inflammatory cytokines may negatively influence iron utilization, thereby interfering with Hb synthesis (Nemeth *et al*., 2004). These data also suggest that the pro-inflammatory cytokine IL-1β, which is under some degree of genetic control, might be associated with EPO resistance. Recent research indicated that the *IL-1B*-511C/T polymorphism was associated with several inflammatory diseases (Hurme *et al*., 1998; Maury *et al*., 2004). Maury *et al*. (2004) reported that the occurrence of anemia in patients with AA amyloidosis was associated with the -511T allele of the *IL-1B* gene and high circulating levels of IL-1β. We found in this study that the *IL-1B*-511CC genotype was significantly associated with lower ERI values in hemodialysis patients.

This study was limited by its small sample size and single-center focus. Compared with those of more heterogeneous populations, Korean genotype frequencies are relatively even and similar regardless of center, making it reasonable to generalize the results of a single-center study to the greater population. To clarify the exact impact of polymorphisms in the *IL-1B* and *ACE* genes on EPO resistance in hemodialysis patients, a family-based association study or a longitudinal study including appropriately adjusted clinical variables would be helpful. There are several additional potential limitations of this study. We did not measure angiotensin II levels, and it is unclear from our study whether angiotensin II mediates the association between ACE genotype and erythropoietic response. In addition, we did not measure EPO levels, another possible explanation for differences in EPO resistance.

In summary, we found that the *IL-1B*-511CC genotype is associated with lower ERI values in hemodialysis patients. Furthermore, the *ACE* I/D polymorphism has predictive value when determining EPO responsiveness; the *ACE* DD genotype may be associated with lower ERI values. Anemia and resistance to recombinant EPO contribute to the excess morbidity and mortality associated with ESRD. Moreover, these genetic variations might contribute to patient-specific anemia risk profiling in ESRD in the future.

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