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Relationship between *eNOS* 894G>T polymorphism and the antihypertensive efficiency of two dihydropyridine calcium channel blocker drugs, azelnidipine and nitrendipine, in Chinese EH patients

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Abstract: *Background:* Dihydropyridine calcium-channel blockers (dCCBs) were widely used in anithypertensive treatment. The aim of this study was to examine the effect of polymorphisms of CACNA1C, eNOS and RGS2 on the antihypertensive efficiency of dihydropyridine calcium channel blocks (dCCBs) in Chinese patients with essential hypertension (EH). *Methods:* A total of 107 untreated Chinese mild to moderate EH patients were enrolled in this study, and had been prescribed azelnidipine or nitrendipine as monotherapy. All patients who had gave informed consent for genetic research were divided into two groups: treated with azelnidipine or nitrendipine for at leaset 6 weeks. Five polymorphisms of three blood pressure (BP) and hypertension susceptible genes were studied in our research, and these polymorphisms were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing. Every patients' BP and heart rate were measured at 0 week, 2 weeks, 4 weeks and 6 weeks. The biochemical parameters of blood were detected before and 6 weeks after the administration. Adverse effects were evaluated at the last visitation. *Results:* Both the systolic and diastolic BP levels were significanlty decreased after six weeks of dCCBs treatment, from 149.3 ± 9.2 mmHg to 132.2 ± 11.7 mmHg and form 97.9 ± 3.0 mmHg to 85.5 ± 7.5 mmHg, as well as the levels of TP, TBIL, CHO and LDL, the P-values were P=0.017, P=0.045, P=0.039, P=0.041 respectivley. As 11 of 75 patients appeared adverse reactions, the rate of adverse effects showed no difference in various genotypes. There were significant interactions between eNOS G894T polymorphism and ΔDBP, ΔMBP on azelnidipine therapy patients, but not in nitrendipine, the GG genotype carriers were more sensitive in blood decrease than GT/TT genotype carriers (P<0.05). *Conclusion:* CCBs had potential hepatoprotective and antiatherosclerosis effects for Chinese EH paitents. And the eNOS G894T polymorphism is associated with the hypotensive effect of azelnidipine.

Keywords: Hypertension, Calciumchannel Blockers, Zelnidipine, Nitrendipine, eNOS, Polymorphism

1. Introduction

Hypertension, which is considered to be present when at least two times subsequent measurement of systolic blood pressure (SBP) ≥ 140 mmHg or diastolic blood pressure (DBP) ≥ 90 mmHg, is a critical risk factor for cardiovascular diseases and blood pressure (BP) control in hypertensive patients is the most effective intervention for reducing cardiovascular diseases risk[1]. Although one in six Chinese adults have been bitterly suffered from hypertension, the awareness, treatment and control rate of hypertension is relatively low in China [2].

Calcium-channel blocker drugs (CCBs), especially the dihydropyridine (dCCBs) subclass, are widely used in the treatment of essential hypertension (EH)[3, 4]. As we known, the antihypertensive response rate of patients to dCCBs monotherapy is just a little above 50% in stage I or II hypertension patients; however, there are not reliable predictors to predict the hypotensive efficiency of dCCBs[5]. Previous researches have evidenced that gender is not an important factor to antihypertensive effect of CCBs when compared with age and initial BP; moreover, genetic factors can significantly influence the antihypertensive sensitivity of CCBs [6-9].

The α1C subunit of the L-type calcium channel (CACNA1C) is involved in encoding the drug target of CCBs, the polymorphisms of which are widely recognized to be the most critical genetic factor of the response of dCCBs[10-12]. The key DHP-sensing residues of CACNA1C are defined in transmembrane segments IS6, IIIS5, IIIS6, IVS6 and the pore helix IIIP[5, 13]. By screening the data of HapMap website, we find that a mutation in the IS6, CACNA1C I391L (rs1051256), has never been studied before. And another mutataion (rs2239101) was found associated with SBP in a genome-wide association meta-analysis study [14].

Endothelial nitric oxide synthase (eNOS) is a limiting-velocity factor in producing nitric oxide (NO). Both clinical and animal studies have suggested that abnormal NO synthesis is involved the pathogenesis of hypertension[15, 16]. Previous article has proved that dCCBs can up-regulate eNOS gene expression in brain and further lower BP by increasing the production of NO[17]. Some studies also have mentioned that polymorphism of eNOS G894T associated with the complex pathogenesis of EH[18, 19], NO production deficiency[20] and also played an important role in resistance to the conventional antihypertensive therapy[21].

Regulator of G-protein signaling 2 (RGS2), a protein of the RGS family, mediates the actions of many important vasoconstrictors by interfering with signaling through receptors that couple to Gqa subclass of G proteins[22]. Reduced RGS2 expression contributes to resistance to antihypertensive agents through poor negative feedback on the effects of aldosterone and of other vasoactive agents[23]. Recently, a report showed that A-638G polymorphism of RGS2 could affect the therapeutic performance of Azelnipine[24]. And there are evidences that two common mutations of RGS2, RGS2 1891-1892del TC (rs3053226)[24], and C1114G (rs4606) is respectively associated with hypertension and RGS2 expression[25].

The purposes of our study is to investigated the influence of genetic polymorphisms of CACNA1C (rs1051356, rs2239101), eNOS (rs1799983), and RGS2 (rs3053226, rs4606) on the antihypertensive efficiency of dCCBs in Chinese Han EH patients.

2. Methods

2.1. Subjects

The protocol of this research was approved by the Ethics Committee of Xiangya hospital. We enrolled 107 EH patients, according to the inclusion and exclusion criteria, but only 75 patients returen visit on time. Peripheral blood samples for genetic analysis were collected after signature of informed consent. All of the subjects were untreated before and had been prescribed dCCBs as monotherapy. The clinical data could be obtained from records of 4 consecutive outpatient visits before and after 2 weeks, 4 weeks and 6 weeks of treatment. Blood pressure and heart rate were mesured by trained nurses in quiet conditions after the patients had rested at least 10 minutes. Automatic blood pressure monitor with intellisense was used to in our study. Both the systolic blood pressure (SBP) and diastolic blood pressure (DBP) were the resultes of the means of 3 times measurements in 10 minutes. The patients who took azelnidpine were 8 mmg/day (50/75) or 10 mmg nitrendipine every day (25/75).

The biochemical parameters of blood were evaluated before and 6 weeks after the administration, which including four liver function related indexes (total protein, albumin, total bilirubin and glutamic-pyruvic transaminase), three renal function related indexes (blood urea, serum creatinine and serum uric acid), four lipid level related indexes (triglyceride, total cholesterol, high-density lipoprotein, low-density lipoprotein) and fasting blood-glucose. And the adverse effects were evaluated by clinical doctors at the last return visitation. The adverse effects investigated in this study included respiratory adverse reactions (cough, respiratory syndrome), common cardiovascular system adverse reactions (palpitation, edema and orthostatic hypotension), common gastrointestinal system adverse reactions, common adverse reactions of the skin and common adverse reactions of the nervous system (dizziness).

2.2. Inclusion and Exclusion Criteria

Inclusion criteria: men or women; diagnosed with EH, confirmed with two or more times BPs, SBP＞140mm Hg or DBP ＞90mm Hg; never received antihypertensive therapy before; with entire 6 weeks of clinical follow up date and blood sample.

Exclusion criteria were secondary hypertension, heart failure, myocardial infarction, stroke, renal or liver dysfunction, malignant tumor or taking any medication that would further affect BP during CCBs monotherapy.

2.3. Genotyping

The polymerase chain reaction (PCR) was performed using oligonucleotide primers, which were designed based on published gene sequence and synthesized by Invitrogen Trading (Shanghai) Co., Ltd. The PCR reactions were carried out with a total volume of 25μL, the reaction mixture contains as follows:2.5μL 10×dNTPs (2.5μM each), 2.5μL 10×PCR buffer, 0.5μL both forward and reverse primers, 0.3μL Taq DNA polymerase (5U/μL), 1.0μL of genomic DNA sample and 17.7μL of PCR grade water. All the PCR reaction processes forenamed were carried out with the silver tank PCR instrucment (eppendorf AG, Germany). All of the digestion enzymes for the PCR products were designed according to the criteria.

The genotypes of CACNA1C (rs1051356, rs2239101), eNOS (rs1799983) were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and the restriction enzymes are HpyF31, BsuRI, and Mbol respectively. Genotypes of GRS2 (rs3053226, rs4606) were directly sequenced by shanghai majorbio company. The genotype results of PCR-RFLP were verified by sanger sequencing.

2.4. Statistical Analysis

Statistical analyses were performed using SPSS ver. 19.0, measurement data results are expressed as the mean ± SD. Baseline characteristics between different groups were analyzed with ANOVA. Hardy-Weinberg equilibrium was evaluated by pearsons $\chi 2$ test, allele-frequencies were determined by gene counting. Comparison of BP variance between genotypes was performed with multivariate analysis of variance (MANOVA), adjusting for confounding factors, which including aging, sex and BMI. A two-tailed P-value <0.05 was considered statistically significant.

3. Result

3.1. Antihypertensive Effects of dCCBs in EH Patients

As showed in Table 1, both the systolic and diastolic blood pressure levels were significanlty decreased after six weeks of dCCBs treatment, from 149.3 ± 9.2 mmHg to 132.2 ± 11.7 mmHg and form 97.9 ± 3.0 mmHg to 85.5 ± 7.5 mmHg respectively, (both P-values were lower than 0.000). No significant change was observed in the levels of heart rate aftre 6 weeks treatment. After compare the biochemical parameters of blood before and after antihypertensive treatmeent, we found the TP level (74.2 ± 4.9 mg/dL and 70.9 ± 4.6 mg/dL before and after the treatment; P=0.017), TBIL level (15.6±5.3 mg/dL and 13.9±4.7 mg/dL before and after treatment; P=0.045), CHO level (5.4±1.1 mg/dL and 5.0±1.0 mg/dL before and after treatment; P=0.039) and LDL level (3.1±0.8 mg/dL and 2.8±0.8 mg/dL before and after treatment; P=0.041) decreased by big margin. Both three renal function indexes and FBG had no significant change (P > 0.05).

3.2. Genotypic analysis

The genotypes of 5 mutations of the three genes were successfully determined in the study population. The allelic frequencies and genotype distributions of the patients were presented in Table 2. In our study population, two genotype frequencies of RGS2 gene (rs3053226 and rs4606) in this study population were not accorded with Hardy-Weinberg equilibrium, P-values were 0.768, 0.9191 and 0.855 respectively. So, we did not take these two SNPs of RGS2 into consideration in the further investigation.

3.3. Adverse Effects after dCCB Drugs Treatment

The adverse effects were evaluate after patients taken at least six weeks of CCBs hypotension treatment. Six of 75 patients appeared dizziness symptom (2 of nitrendipine gourp and 4 of azelnidpline group), 3 patients of azelnidipin showed cardiovascular adverse reactions and 2 patients of azelnidpine group had respiratory adverse reactions. The incidences of adverse effects showed no significant difference between azelnidipine (14%) and nitrendipine (8%) groups (P>0.05). We further compare the rate of adverse effects in different genotypes, and we did not found statistic relationship between the occurrence of adverse effects and gene polymorhpisms, (all P-values >0.05).

3.4. Pharmacogenetics Study of Antihypertensive Efficiency of dCCB Drugs and Gene Polymorphisms

After analysis the relationship between these gene polymorphisms and the change of BP at each time point (2, 4, 6 weeks), we found only the polymorphism of the eNOS (G894T) showed a significant association with the change of BP (ΔBP: BP at 6 week – BP at 0 week). The eNOS 894GG genotype carriers had much more decrease of DBP and MBP than 894GT/TT genotype carriers (ΔSBP: GG -18.92±11.32 mmHg, GT/TT -13.78±5.69 mmHg, P = 0.104; ΔDBP: GG -13.78±5.69 mmHg, GT/TT -8.85±9.02 mmHg, P = 0.008; ΔMBP: GG -15.49±6.61 mmHg, GT/TT -10.37±10.55 mmHg, P=0.018). After adjustment for gender, age and BMI, the P-values for ΔSBP, ΔDBP and ΔMBP were 0.23, 0.012 and 0.031 between GG and GT/TT genotype carriers respectively as shown in Figure 1. Interestingly, after examined the effect of genetic factors in different drug groups, the relationship between eNOS G894T polymorphism and BP decrease only showed in azelnidipine, but not in subject with nitrendipine, as showen in Table 3. (ΔSBP: GG -16.2±8.9 mmHg, GT/TT -9.5±13.0 mmHg, P = 0.073; ΔDBP: GG -12.5±5.1 mmHg, GT/TT -5.0±7.6 mmHg, P = 0.001; ΔMBP: GG -13.7±5.0 mmHg, GT/TT -6.5±9.1 mmHg, P=0.002). There was no gene polymorphisms had relationship with the change of heart rate and the biochemical parameters of blood or adverse effects.

Table 1. *BP, heart rate and biochemical parameters of blood change after antihypertensive treatment.*

Variables	Pre-treatment	Post-treatment	P
SBP (mmHG)	149.3±9.2	132.2±11.7*	0.000
DBP (mmHg)	97.9±3.0	85.5±7.5*	0.000
Heart Rate	72.7±7.2	71.5±6.9	0.276
Liver function indexes			
Total protein (TP) (mg/dL)	74.2±4.9	70.9±4.6	0.017
Albumin (ALB) (mg/dL)	43.1±2.9	42.0±2.9	0.842
Total bilirubin (TBIL) (mg/dL)	15.6±5.3	13.9±4.7	0.045
Glutamic-pyruvic transaminase (ALT) (U/L)	26.3±12.4	25.2±15.4	0.501
Renal function			
Blood urea (BU) (mg/dL)	5.1±1.3	5.0±1.1	0.639
Serum creatinine (Scr) (mg/dL)	74.5±17.3	74.6±15.8	0.983
Serum Uric Acid (UA) (mg/dL)	314.7±67.4	318.9±63.3	0.796
Blood glucose level			
Fasting blood-glucose (FBG) (mg/dL)	5.4±1.3	5.4±1.2	0.786

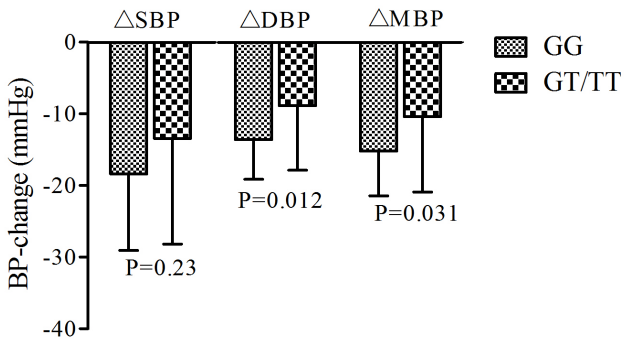
Variables	Pre-treatment	Post-treatment	P
Blood lipid levels			
Triglyceride (TG) (mg/dL)	1.9±1.4	2.0±1.4	0.627
Total cholesterol (CHO) (mg/dL)	5.4±1.1	5.0±1.0	0.039
High-density lipoprotein (HDL) (mg/dL)	1.5±0.3	1.4±0.4	0.255
Low-density lipoprotein (LDL)	3.1±0.8	2.8±0.8	0.041

Table 2. *Allelic frequency and genotype frequency distribution of eNOS, RGS2, CACNA1C in EH patients.*

gene	SNP	genotype	Frequency number (%)	Allele frequency	P (H-W)
eNOS	Rs1799983	GG	52(74.3%)	G (86.4%)	0.77
		GT	17(24.3%)	T (13.6%)	
		TT	1(1.4%)		
CACNA1C	Rs2239101	TT	55(78.6%)	T (88.6%)	0.92
		CT	14(20.0%)	C (11.4%)	
		CC	1(1.4%)		
CACNA1C	rs1051356	AA	67(95.7%)	A (97.9%)	0.85
		AT	3(4.3%)	T (2.1%)	
		TT	0(0%)		
*RGS*2	rs3053226	DD	8(11.9%)	D (44.0%)	0.013
		DI	43(64.2%)	I (56.0%)	
		II	16(23.9%)		
RGS2	Rs4606	CC	9(12.9%)	C (46.4%)	0.0034
		CG	47(67.1%)	G (53.6%)	
		GG	14(20%)		

Table 3. *Stratified analyses of the different hypotensive effects between eNOS G894T genotype and dCCBs interaction.*

Variables	GG-azelnidpine			GT/TT-azelnidipine			P (GG versus GT/TT)		
Vist (N)	2W (38)	4W (38)	6W (36)	2W (9)	4W (9)	6W (9)	2W	4W	6W
ΔSBP (mmHg)	-12.4±9.5	-12.7±8.6	-16.2±8.9	-14.2±9.3	-10.1±11.7	-9.5±13.0	0.603	0.464	0.073
ΔDBP (mmHg)	-9.4±5.4	-11.6±6.0	-12.5±5.1	-9.3±4.5	-6.6±5.1	-5.0±7.6	0.948	0.023	0.001
ΔMBP (mmHg)	-10.4±5.8	-12.0±6.0	-13.7±5.0	-10.9±5.3	-7.8±7.0	-6.5±9.1	0.803	0.072	0.002
Variables	**GG-nitrendipine**			**GT/TT-nitrendipine**			**P (GG versus GT/TT)**		
Vist (N)	2W (16)	4W (16)	6W (16)	2W (9)	4W (9)	6W (9)	2W	4W	6W
ΔSBP (mmHg)	-17.1±12.8	-18.3±15.8	-23.3±13.0	-19.1±13.8	-17.2±8.8	-17.3±16.1	0.721	0.851	0.322
ΔDBP (mmHg)	-15.9±7.1	-15.4±9.4	-16.1±5.8	-9.9±9.1	-11.6±6.1	-12.7±9.1	0.077	0.286	0.266
ΔMBP (mmHg)	-16.3±8.4	-16.4±11.0	-18.5±7.7	-13.0±10.5	-13.4±6.2	--14.2±11.0	0.387	0.476	0.267



Note: the P-values were adjusted wtih age, BMI and gender.

Figure 1. *Relationship between eNOS G874T polymorphism and the antihypertensive efficiency of dCCBs treatment after 6 weeks medication.*

4. Discussion

Our study indicates that both azelnidipine and nitrendipine monotherapy can successfully reduced both SPB and DBP, as well as the TP, TBIL, CHO and LDL levels after 6 weeks of dCCB treatment. And our data shows that eNOS G894T polymorphism associated with therapeutic heterogeneity of dCCB drugs.

Interesting, the resultes of our study suggest that CCBs treatment can significantly descease TP, TBIL, CHO and LDL levels (P=0.017, P=0.045, P=0.039, P=0.041, respectivley), which suggested that CCBs medication may have potential hepatoprotective activity and anti-atherosclerosis effects. Several researches had evidenced previously that azelnidipine exerts slight anti-atherogenic effect by reducing local oxidative stress[28, 29], and the results of our study suggested that CCBs showed potential antiatherosclerosis effects by lowing CHO and LDL levels. Previouse study evidenced that azelnidipine can significantly decrease both SU levels and the urinary uric acid to creatinine ration in hypertension patients [30]. And there also data showed that nitrendipine was effecitive in stabilising most parameters of renal function in incipient diabetic nephropathy[31] and had small but significant nephroprotective effect in renal-transplant patients [32, 33]. And the resultes of our study were in line with previous researches that BU, Scr and UA levels were decreased, and the decrease change of BU and Scr levles in nitrendipine treatment group were significantly reduced than azelnidipine groups (P=0.02 and P=0.04 respectively). Based on these results we can estimate that dCCBs do had potential renoprotection effect in hypertension patients, and the renoprotection effect of

nitrendipine was stronger than azelnidipine.

The pharmacogenetic results of this research found that, of the polymorphisms we studied, only eNOS G894T polymorphism associated with antihypertensive effect, and this relationship only presented in azelnidipine treatment group. The hypotensive effects of azelnidipine was more sensitive in GG genotypes than GT or TT genotype carriers. Endothelial nitric oxide synthase (*eNOS*) is a limiting-velocity factor for endothelial cell to produce nitric oxide (NO), and it is expressed in many organs, such as cardiovascular system, brain, lung, liver and so on, where NO produced by this enzyme plays an important physiological role[34, 35]. NO is a critical molecule in regulating the vascular system, and is involved in inhibiting the platelet aggregation and adhesion and reducing the cell proliferation of vascular smooth muscles[36]. Clinical and animal studies had suggested that NO synthesis abnormal is a contributing factor for the pathogenesis of hypertension[37, 38]. The eNOS G894T polymorphism, a coding region variant, results in a Glu298Asp substitution and decreases the NO levels [39]. Azelnidipine is a long-acting CCB, it dosen't elicit reflex tachycardia during antihypertensive therapy by inhibits sympathetic nerve activity directly or indirectly [40]. Evidence suggested that azelnidipine treatment could up-regulate the expression and activiation of eNOS, while reduced ROS simultanelously increase NO availability [28, 41]. Owing to the small number of subjects prescribed with nitrendipine in this study, we can not avoid the false negative errors of this result. The relationship between eNOS G874T polymorphism and nitrendipine hypotensive efficiency need further verification.

In summary, our study evidenced that dCCBs can successfully decrease BP and showed slight hepatoprotective activity and antiatheroscloresis effects. And our study evidanced for the first time that polymorphism of eNOS G894T was correlated with the antihypertensive efficiency of azelnidipine, the GG genotype carriers benenift more than GT/TT genotype carriers when under azelnidipine medication.

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