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中文摘要

糖尿病及高血壓是造成腎衰竭的最主要的原因之一，有相當多的因子可使糖尿病或高血壓的病人更快進展至腎衰竭。在過去兩年我們探討了自由基代謝基因及腎病症候群相關基因在腎衰竭所扮演的角色。而本年度的計劃則主要探討藥物代謝基因在腎衰竭發展過程所扮演的角色，我們利用 PCR-RFLP 及直接定序的方法，分析了 CYP1A1、CYP2E1、GSTM1、GSTT1、MTHFR、APOV6 及 CCR5 等基因的變異型與腎衰竭的關係，結果顯示，CYP2E1 的 Wild type 與高血壓進展至腎衰竭有關，(p＝0.007)，而 GSTT1 與糖尿病進展至腎衰竭有關（p＝0.004)。

關鍵詞：高血壓、糖尿病、腎衰竭、藥物代謝基因、變異型、PCR-RFLP 。

Abstract

DM and Hypertension play important roles in the development of chronic renal insufficiency（CRI）.There are a number of factors which may promote the development of CRI in DM and Hypertension patients.In the past two years，we focused on the studies of free radical metabolized-related genes and nephritic ryndrome-related genes to explore their roles in the development of CRI.In this study，we explored the relationshy's letueen the dmg-metabolized-related t genes and direct sequencing methods.We analyzed the vaiant forms of CYP1A1、CYP2E1、GSTM1、GSTT1、MTHFR、APOV6、CCR5 and the results showed that CYP2E1 and GSTT1 play a role in the development of CRI from DM and Hypertension.

Keywords: DM、Hypertension、chronic renal insufficiency 、dmg-metabolized-related genes 、vaiant forms、PCR-RFLP

Introduction

Reactive oxygen metabolites (ROS) are the product of partial reduction of oxygen in biological systems and include superoxide anions, hydrogen peroxides, hydroxyl radicals and hypochlorous acid (1-5). Many evidences have shown that ROS plays a role in the pathogenesis of a variety of diseases (1-6). In addition, ROS plays a role in neutrophil-dependent and macrophage-dependent or -independent glomerular injury (7-8), and also in the development of acute renal failure caused by endotoxin, glycerol, gentamicin, adriamycin, lupus nephritis, puromycin aminonucleoside, and the institution of ischemia (9-11). In addition to invading cells (macrophages, neutrophils), ROS may be produced by isolated glomeruli and by cultured mesangial cells in response to diverse stimuli.

Hyperglycermia has been shown to associate with the production of ROS in diabetic patients (12-14). The production of ROS is considered to be one of the major causes of diabetic complications, including nephropathy (15-16). There are a number of endogenous antioxidants that provide protection against the harmful effects of ROS. For instance, superoxide can be scavenged by superoxide dismutase (SOD), and hydrogen peroxide can be degraded by catalase or glutathione peroxidase, resulting in the formation of water (17-18). Glutathione S-transferases (GSTs) are a family of multifunctional enzymes that play an important role in the cellular detoxification and excretion of numerous physiological and xenobiotic substances (19-22). GSTs also work as antioxidants by catalyzing the conjugation of electrophilic compounds including carcinogens, cytotoxic drugs and organic hydroperoxides with reduced glutathione (19-21). Therefore, diminished expression of GSTs may result

in a reduced capacity of defense against oxidative stress, followed by the development of diabetic nephropathy.

Human GSTs contain at least five classes: α, π, μ, θ and κ (19,21,23,24). In human kidney, GST μ class is mainly localized in tubuli (25). Human μ class GSTs are thought to be products of M1, M2, M3, M4 and M5 gene loci (21, 26-27). The expression of M1 membrane of human GST μ class (GSTM1) is detected only in about 50% of all populations. The genetic locus encoding human GSTM1 is polymorphic and the absence of GSTM1 has been ascribed to the homozygous deletion of the gene (null genotype) (28-30). GST θ class is highly expressed in liver and kidney, and there are two types: GSTT1 and GSTT2. The genetic locus encoding human GSTT1 is also polymorphic, and the absence of the GSTT1 has been found in 15%-30% of Caucasians and over 50% of Chinese (31-32).

In order to determine whether the GSTM1 and GSTT1 null genotypes are associated with the development of diabetic or hypertensive related end-stage renal disease (ESRD), we investigated the genetic polymorhisms in type 2 diabetic patients with ESRD and in hypertension patients with ESRD as well as patients without ESRD.

Materials and Methods

Patients

We recruited 119 type 2 DM patients with diabetic-related ESRD and 111 type 2 DM patients without diabetic ESRD, microalbuminuria or proteinuria. We also recruited 101 patients with hypertension-related ESRD and 374 patients of hypertension without ESRD. All patients were Taiwanese of Han-Origin. The diagnostic criterion for ESRD was chronic renal failure with serum creatinine >8.0 mg/dl or creatinine clearance <5 ml/min, need start dialysis. Ethnic approvals were obtained from the institutional review board of China Medical University Hospital and Changhua Christian Hospital, and informed consent was received from all participants.

GSTM1 and GSTT1 genotyping

Genomic DNA was extracted from peripheral white blood cells as in our previous studies (33). Genotyping of GSTM1 and GSTT1 was performed by multiplex polymerase chain reaction (PCR) using primers from the protocols of Comstock et al (34) and Pemble et al (35) with some modifications. The primers used were as follows: for detection of GSTM1, the forward primer was 5'-CTG CCC TAC TTG ATT GAT GGG-3' and the reverse primer was 5'-CTG GAT TGT AGC AGA TCA TGC; for detection of GSTT1, the forward primer was 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' and the reverse primer was 5'-TCA CCG GAT CAT GGC CAG CA-3'. In order to confirm that the PCR had worked in subjects homozygous for the GSTM1 or GSTT1 gene deletion, one pair of primers was used as an internal control to amplify a 100 bp fragment of the β-globin gene: the forward primer was 5'-ACA CAA CTG TGT TCA CTA GC-3', the reverse primer was 5'-CAA CTT CAT CCA CGT TCA CC-3'. The PCR mixture (100 μl) contained 200-500 ng of genomic DNA, 10 pmol of each primer, 2.5mM of each dNTP, 1.5mM $MgCl_2$, 500 mM KCl, 10 mM Tris HCl (pH8.3) and 2.5 units of Taq polymerase (Protech, Taipei, Taiwan). Amplification was performed in a thermal cycler (Perkin Elmer, Foster City, CA, USA) for 35 cycles with steps of denaturing at 94 ℃ for 1min, annealing at 58℃ for 1 min and extension at 72℃ for 5 min. The PCR products were analyzed by electrophoresis in 3% agarose gels and visualized by UV light.

Statistical analysis

The differences in distribution of the GSTM1 and GSTT1 genotypes between ESRD patients with diabetes and ESRD patients with hypertension, and controls were determined by chi-square test. Probability values of <0.05 were regarded as statistically significant. Odds ratios (ORs) with a 95% confidence interval (CI) calculated using unconditional logistic regression and adjusted for age and gender

were computed to estimate the association between certain genotypes and diseases. All of the statistical analyses were performed by Statistical Analysis System software (SAS Institute, Cary, NC, USA).

Results

The clinical characteristics of the subjects in this study are shown in the Tables 1 and 2. The diabetic (119 with ESRD, 111 without ESRD) and hypertensive (101 with ESRD, 374 without ESRD) groups were well-matched in regard to age, cholesterol, and triglyceride levels. As expected, serum creatine concentrations of patients with ESRD were significantly high. The results of the multiplex PCR analysis of GSTM1 and GSTT1 are shown in Fig. 1. The PCR product of the GSTM1 and GSTT1 genes are a fragment of 273 bp and a fragment of 480 bp, respectively. The amplican of the β-globin gene for internal control showed a 100 bp fragment. In total, 705 cases were analyzed.

The genotypic frequencies of the GSTM1 and GSTT1 polymorphisms in DM patients with or without ESRD and in hypertension patients with or without ESRD are shown in Tables 3 and 4, respectively. The homozygous deletion of the GSTM1 gene was observed in 58.0% (69/119) of diabetic patients with ESRD versus 50.5% (56/111) of those without ESRD, and in 53.4% of hypertensive patients with ESRD versus 54.8% of those without ESRD. The presence of the GSTM1 gene was identified in 42.0% of diabetic patients with ESRD versus 49.5% of those without ESRD, and in 46.6% of hypertensive patients with ESRD versus 45.2% of those without ESRD. No significant difference in genotypic frequencies was found in these two groups (P=0.25 for the diabetic group, p=0.81 for the hypertensive group) (Table 3 and Table 4). The homozygous deletion mutation of the GSTT1 gene was observed in 58.0% of diabetic patients with ESRD versus 38.8% without ESRD, and in 40.6% of hypertensive patients with ESRD versus 36.6% without ESRD. The presence of the GSTT1 gene was identified in 42.0% of diabetic patients with ESRD versus 61.2% without ESRD, and in 59.4% of hypertensive patients with ESRD versus 63.4% without ESRD. Genotypic frequencies were significantly different between the diabetic groups (P=0.004, OR=2.18, 95% CI=1.29-3.70), but no significant difference was observed between the hypertensive groups (P=0.47).

The frequencies of homozygous deletion of both GSTM1 and GSTT1 genes was much higher in diabetic patients (32.8%) than those without ESRD (19.9%). The difference in frequencies was statistically significant (Table 3). However, the homozygous deletion of both GSTM1 and GSTT1 genes was observed in 17.8% of hypertensive patients with ESRD versus 12.0% without ESRD. There was no statistical significance in this group (Table 4)

Discussion

ROS participates in the pathogenesis of various renal diseases, including inflammatory lesions such as glomerulonephritis and interstitial nephritis, ischemic reperfusion injury, hemolytic uremic syndrome, toxic nephropathies, and possibly chronic renal failure (7,9). Several studies have repected that antioxidants may improve renal function of the pationts with these diseases (17-19). Recent studies also demonstrated that vitamin E and glutathione can prevent the development of renal complications in diabetic animals and patients (36-37). These results suggest that the levels of endogenous antioxidants may be a determinant of the susceptibility of diabetic patients to renal complications in.

The activity of GSTM1, one of the endogenous antioxidant enzyme, is determined genetically (24,30). Several studies have demonstrated that the genotype of homozygous *GSTM1* deletion GSTM1 activity (24, 29-30), and no association of this genotype with diabetic complications was found in the study by Fujita et al (38). Our data are consistent with their results.

However, another endogenous antioxidant, GSTT1, showed its effect on diabetic complications. The homozygous deletion genotype, which reduced the GSTT1 activity, was associated with diabetic ESRD.

Little has been known about the role of ROS in the development of ESRD in hypertensive patients. Our study revealed that lower activities of antioxidant enzymes GSTM1 and GSTT1 were not associated with the development of ESRD in hypertensive patients. Therefore, ROS may not play an important role in the development of ESRD in hypertensive patients.

The mechanism of development of ESRD in DM patients and hypertensive patients is different. DM may produce much more oxidative stress than hypertension so that it requires more antioxidant to reduce the stress. This is the reason for the association between GSTT1-null genotype and DM in patients with ESRD, but not in hypersensitive patients with ESRD. GSTM1 is also an important endogenous antioxidant enzyme, but it does not play an important role in the development of ESRD in DM or hypertensive patients. This suggests that GSTT1 plays a more important role than GSTM1 in the process of reducing oxidative stress in the kidney.

In conclusion, our results indicate that genetic variations of GSTT1 enzyme involving in free radical metabolism in DM are associated with the development of ESRD and may permit the targeting of preventative and early intervention strategies to high-risk individuals.

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Fig. 1. The results of representative PCR analysis of GSTM1 and GSTT1 genes are shown. M: marker. Lane 1: GSTM1 positive (273 bp fragment) with GSTM1 deletion. Lane 2: GSTT1 (480 bp fragment) and GSTM1 positive. Lane 3: GSTM1 positive with GSTT1 deletion. Lane 4: Both GSTM1 and GSTT1 deletion. The 100 bp fragment is the product of β-globin gene internal control.

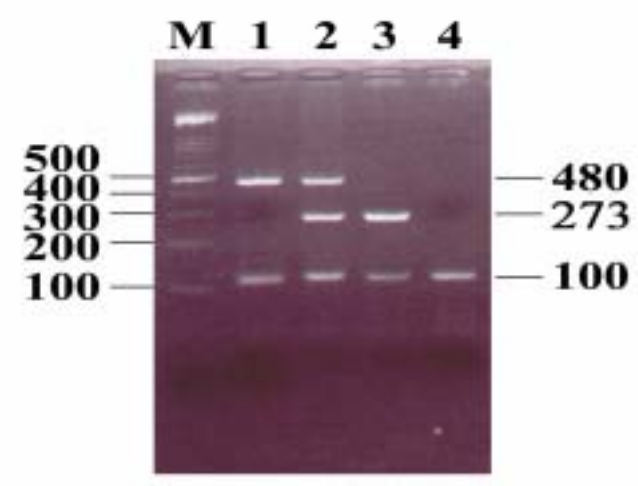

Table 1. The clinical data of type2 DM with or without ESRD

	DM (n=111)	DM with ESRD (n=119)	P-value
Gender (M/F)	53/58	57/62	
Age (years)	62.51±12.03	62.50±11.94	0.92
BMI (kg/m^2)	23.86±4.06	23.09±3.65	0.13
Cholesterol (mg/dL)	190.06±51.07	181.54±51.39	0.33
Triglyceride (mg/dL)	189.94±131.71	176.57±185.04	0.73
Creatinine (mg/L)	1.092±0.49	11.258±5.46	<0.05
HbA_1C (%)	7.86±1.85	7.08±2.19	<0.02

Data are means±SD. BMI: body mass index.

Table 2. The clinical data of hypertension with or without ESRD

	Hypertension (n=374)	Hypertension with ESRD (n=101)	P-value
Gender (M/F)	190/184	51/50	
Age (years)	62.57±13.04	60.76±13.74	0.45
BMI (kg/m^2)	23.03±4.11	22.72±3.94	0.52
Cholesterol (mg/dL)	204.26±38.99	183.08±40.21	0.027
Triglyceride (mg/dL)	193.76±155.94	177.36±202.62	0.798
Creatinine (mg/dL)	1.14±0.325	10.91±5.63	<0.05

Data are means±SD. BMI: body mass index.

Table 3. The genotype frequencies of GSTM1 and GSTT1 polymorphisms in DM with or without ESRD

Disease / Genotype	DM	DM+ESRD	P	OR	95%CI
GSTT1-positive	68	50	0.004	2.18	1.29-3.70
GSTT1-null	43	69			
GSTM1-positive	55	50	0.25	1.36	0.81-2.28
GSTM1-null	56	69			
Both null	22	39	0.029	1.95	1.07-3.57
Non-null	89	80			

Table 4. The genotype frequencies of GSTM1 and GSTT1 polymorphism hypertension with or without ESRD

Disease / Genotype	Hypertension	Hypertension+ESRD	P	OR	95%CI
GSTT1-positive	237	60	0.47	1.18	0.75-1.85
GSTT1-null	137	41			
GSTM1-positive	169	47	0.81	0.95	0.61-1.47
GSTM1-null	205	54			
Both null	45	18	0.21	1.46	0.82-2.49
Non-null	329	83			