

Analysis of T344C Genetic Polymorphism of *CYP11B2* Gene in Malaysian End Stage Renal Disease Subjects

R. Vasudevan, Aisyah B.T. Ali, Mimi Sooraya Mansoor, Nurul Fasihah Zulkifli and Patimah Ismail
Genetic Research Group, Molecular Biology and Bioinformatics Unit,
Department of Biomedical Science, Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia, 43400 Selangor, Malaysia

Abstract: The T344C polymorphism of aldosterone synthase *CYP11B2* gene at promoter region was vastly studied in various populations with conflicting results in relation to End-stage Renal Disease (ESRD). In this study, researchers aimed to know the association of T344C polymorphism of *CYP11B2* gene in Malaysian ESRD subjects. This study involved 165 Malaysian ESRD subjects and 165 healthy individuals as control subjects. Using commercially available kits, genomic DNA was extracted from the subjects using their buccal cells and the blood. The 152 bp product of *CYP11B2* gene polymorphism were amplified by Polymerase Chain Reaction (PCR) and digested with Hae III restriction enzyme using Restriction Fragment Length Polymorphism (RFLP) method. The restricted fragments were separated by metaphor agarose gel electrophoresis and showed 152 bp represent TT allele (wild type), 152 and 97 bp represent the TC allele (heterozygous) and 97 and 56 bp represent CC allele (mutant type) as the genotypes for T344C polymorphism. The frequency of TT, TC and CC genotypes of T344C in *CYP11B2* gene in ESRD subjects were 98 (59.39), 60 (36.36%) and 7 (4.24%) while 92 (55.76), 70 (42.42) and 3 (1.82%) were found in control subjects, respectively. The genotypic and allelic frequencies of T344C polymorphism of *CYP11B2* gene show no significant differences as compared to control subjects ($p>0.05$). The T allele of T344C polymorphism of *CYP11B2* gene might not be considered as a possible genetic marker or predisposing risk factor for ESRD in Malaysian subjects. However, this study has to be further continued with more subjects to confirm the association of *CYP11B2* gene mutation with ESRD.

Key words: Polymorphism, T344C, *CYP11B2*, ESRD, DNA, Malaysia

INTRODUCTION

End Stage Renal Disease (ESRD) a stage 5 Chronic Renal Disease where the Glomerulus Filtration Rate (GFR) is gradually decline to $<15 \text{ mL/min/1.73 m}^2$ and the ESRD patients will be under the renal replacement therapy or dialysis (Shlush *et al.*, 2010). According to United States Renal Data System in 2010 and Annual Data Report in 2010, the prevalence of ESRD was high along with the cost of its treatment.

The incidents of ESRD in United State increased from 342 per million in 2000 to 351 per million in 2008. From the 17th report of Malaysian Dialysis and Transplant Registry in 2009, the incidents of ESRD increased rapidly and the prevalence of Type 2 Diabetes Mellitus (T2DM) had risen to 14.9 from 8.3% in 10 years (Letchuman *et al.*, 2010). The leading etiologies of ESRD are hypertension and diabetes mellitus are strongly independent with factors for ESRD progression with or without presence of protienuria for

hypertension (Shlush *et al.*, 2010; Abbasi *et al.*, 2010). However, other factors also consider as predisposing factor ESRD development which are smoking (Orth, 2002), races, male, hyperlipidemia, recreational drug use, prenatal factor and genetic polymorphism. ESRD is multi-factorial disease which has both genetic and environment influent in which recent study had shown the genetic polymorphism that contributes to ESRD (Lovati *et al.*, 2001; Shlush *et al.*, 2010).

Genetic polymorphism is the genetic variation occurs within individuals, group and populations that may have a single change of nucleotides that resulting in the development of diseases. The candidate genes suspected to be pre-disposing factor of ESRD are actively being studied based on their physical functions in various populations (Ali *et al.*, 2011; Wong *et al.*, 2008; Bowden, 2003). Aldosterone synthase *CYP11B2* gene is one of the candidate genes that are suspected to be as a pre-disposing factor of ESRD (Lovati *et al.*, 2001;

Corresponding Author: Patimah Ismail, Molecular Biology and Bioinformatics Unit, Genetic Research Group,
Department of Biomedical Science, Faculty of Medical and Health Sciences,
Universiti Putra Malaysia, 43400 Serdang, Selangor DE, Malaysia

Lee *et al.*, 2009). Aside for ESRD, CYP11B2 is hypothesized as a pre-disposing factor for ESRD (Pan *et al.*, 2010) and also in Malaysian hypertensive and T2DM subjects (Vasudevan *et al.*, 2008).

Nevertheless, the data regarding the association of CYP11B2 with ESRD are inconsistent. There are previous studies had failed to show an association of ESRD with CYP11B2 genetic polymorphism (Lovati *et al.*, 2001; Lee *et al.*, 2009). Various studies have performed in order to provide better the understanding of *CYP11B2* gene with T344C polymorphism in renal function and the data of its association with ESRD (Prasad *et al.*, 2006).

The conflicting results found in many populations in relation to CYP11B2 genetic polymorphism and ESRD initiated us to determine the association of T344C gene polymorphism of *CYP11B2* gene among Malaysian ESRD subjects.

MATERIALS AND METHODS

Ethics: The study protocol was approved by the Ethical Committee of the Faculty of Medical and Health Science (UPM/FPSK/PADS/T7-MJKEtikaPPer/F01); Universiti Putra Malaysia (UPM). Written informed consent was obtained from all the respondents.

Subjects: During 2009-2010 year period, a total of 330 subjects were recruited under this study among them 165 were ESRD patients who were under the dialysis treatment and diagnosed as stage 5 chronic kidney disease and 165 subjects were healthy individuals considered as control subjects at the time of recruitment. Buccal cells were collected from the ESRD patients whereas 4-5 mL blood was drawn from the healthy individuals to know their creatinine, glucose levels and lipid profiles.

Biochemical analysis: Blood pressure, creatinine, glucose levels and the lipid profiles of ESRD patients were obtained from their medical records whereas for control subjects, the biochemical analysis such as creatinine, High Density Lipoprotein Cholesterol (HDL-C), Low Density Lipoprotein Cholesterol (LDL-C), Total Cholesterol (TC) and Triglycerides (TG) were measured using Hitachi-912 Autoanalyser (Hitachi, Germany).

Genomic DNA extraction: DNA extraction kit from Qiagen (Germany) was used to extract DNA from buccal cell samples. Whereas for the extraction of DNA from the blood, we used Accuprep genomic DNA extraction kit (Bioneer, Korea). Protocols had been followed under the instructions provided by the manufacturer's kit. DNA

from both sources was quantified using Biophotometer (Eppendorf, Germany) against TE buffer as blank at 260 nm wavelength.

Genotyping *CYP11B2* gene polymorphism: A final volume of 25 μ L PCR mixture consisted of 10 pm of forward primer 5'-CAGGGCTGAGAGGAGTAAAA-3', reverse primers: 5'-CAGGGGGTACGTGGACATTT-3', (synthesised by Nexgene, Malaysia), 0.4 mmol L⁻¹ each dNTP, 2 mmol L⁻¹ MgCl₂, 1 \times Taq buffer and 1 unit of NEB Taq DNA polymerase (New England Biolabs, Beverly, MA and USA) and the template DNA was amplified under the cycling conditions with an initial denaturation step of 3 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 30 sec followed by a final extension for 5 min at 72°C before the storage of the samples at 4°C were carried out on an iCycler machine (BioRad Laboratories, Hercules, CA and USA). The amplified PCR products (152 bp) were separated by agarose gel electrophoresis (Promega, Madison and USA). DNA fragments were stained in GelRed (Biotium, Hayward, CA) and visualized under the UV light using Alpha imager (Alpha Innotech, San Leandro, CA, USA). The PCR products were kept in the freezer at 4°C for further analysis. In RFLP method, Hae III restriction enzyme was used to cut at specific restriction (GGCC) site. The Hae III enzyme (New England Biolabs, Beverly, MA, USA) was used with 1 \times NEBuffer 4, provided with the enzyme, BSA and distilled water in a total volume of 20 μ L. The mixture will be centrifuged and incubated at 37°C for 3 h. After that the mixture is centrifuged before inactivation was done at 80°C for 20 min. Restricted fragments were separated at 3% of metaphor agarose gel. The fragments showed three genotypes; the wild type produced 152 bp band, heterozygous produced 152 and 97 bp bands and mutant produced 97 and 56 bp bands. Identical results were obtained when genotyping was performed on two separate occasions for 10% of the samples.

Statistical analysis: Clinical characteristics of all the subjects were expressed as mean \pm SD. All the statistical analyses were carried out using SPSS (Chicago, IL, USA) software Version 15.0 for Microsoft Windows. Allelic frequencies were calculated using the gene counting method and the genotype distribution with Hardy-Weinberg expectations by a Chi-square (χ^2) test. Continuous variables were compared between the groups by using two-tailed Student's t-test. Odds Ratios (OR) with 95% confidence intervals were estimated for the effects of high-risk alleles. A level of p<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In this cross sectional study, 165 case subjects were compared with 165 control subjects to detect the genotype and allelic for T344C polymorphism of CYP11B2 in Malaysian population. For the ESRD case subject, 53.3% were males and 46.7% female were recruited with distribution of 41.2% from Malays, 43.0% from Chinese and 15.8% from Indians. Whereas for the control subjects consisted of 47.3% males and 52.7% females.

For ethnic distribution, the most dominant samples were from Malay (46.7%) followed by Chinese (42.4%) and the least were from Indians which was 10.9%. Means age for case samples was higher than control samples. The case subjects had the highest number in range of 50-70 years old (56.4%) followed by range of 30-49 years old (33.9%), age >70 years old (10.3%) and the least was from range of 18-29 years old (1.8%).

For control samples, age range 30-49 years old gave the highest samples which was 45.5% followed by range 50-70 years old (35.2%), range 18-29 years old (14.5%) and the least was age >70 years old which was 4.8% and differed significantly as compared with the control subjects (p<0.05).

Clinical characteristics of all subjects: Table 1 showed the clinical characteristic of case and control samples. The independent t-test had been done in order to find the means and significant difference of the entire clinical characteristics. Similarly, the SBP, triglycerides, total cholesterol and creatinine level were higher compared to case samples.

However, some characteristics had slighter higher means reading in control samples compared to case samples which were DBP, HDL and LDL. The significant difference was observed in SBP, triglycerides, total cholesterol and creatinine level as the p<0.05. In contrast, there was no significant difference was observed DBP, HDL and LDL (p>0.05).

Table 1: Clinical characteristics among ESRD and controls

Parameters	ESRD n = 165	Control n = 165	p-values
Gender (M/F)	88/77	87/78	-
Age (years)	55.03±11.89	45.99±13.83	0.028*
Creatinine	843.43±260.05	0.64±0.23	0.000*
SBP	149.32±28.43	133.39±19.18	0.000*
DBP	77.69±16.87	78.74±10.81	0.502
HDL	1.11±0.34	1.15±0.46	0.399
LDL	3.01±1.15	3.12±1.09	0.398
Triglycerides	2.37±1.75	1.25±0.92	0.000*
Total cholesterol	5.12±1.30	4.76±1.32	0.013*

* Significant p<0.05

Genotypic and allelic frequency of T344C polymorphism:

Figure 1 and 2 shows the PCR amplification products and the restricted fragments size products of T344C polymorphism of CYP11B2 gene, respectively. The distribution of genotype in case samples were higher than in control sample with the highest percentage was TT (59.4%) followed by TC (36.4%) and the least was CC (4.2%). In spite of that the TT and CC were low in control, 55.8 and 1.8%, respectively the percentage of TC in control sample was higher than case samples (36.4%) in control as shown in Table 2.

There was no significant difference observed in both genotypic and allelic frequency of T344C Polymorphism of CYP11B2 gene between ESRD and control subjects (p>0.05). Various studies had reported that genetic polymorphism plays an important role in the

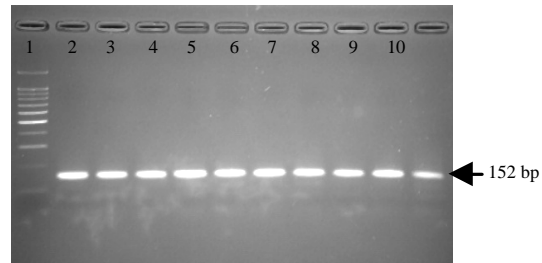


Fig. 1: Amplification of PCR product in 3.0% agarose gel electrophoresis. It shows the amplification of PCR products (152 bp) of the T344C gene polymorphism in 3.0% agarose gel electrophoresis

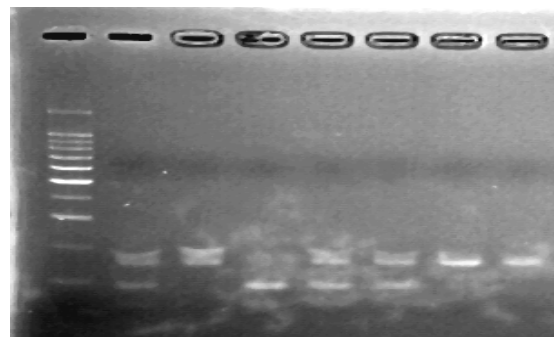


Fig. 2: Restriction enzyme fragments (RFLP) using Hae III of T344C gene polymorphism in 3.0% metaphor agarose gel electrophoresis. Lane 6 and 7 shows the wild-type fragments (152 bp), lane 4 and 5 shows the heterozygous fragments (152 and 97 bp), lane 3 show mutant fragment (97 and 56 bp, the 56 bp was not seen) in 3.0% metaphor agarose

Table 2: Distribution of genotypic and allelic frequencies of T344C polymorphism of *CYP11B2* gene

Genotypes	Case (n = 165) (%)	Control (n = 165) (%)
TT	98 (59.39)	92 (55.76)
TC	60 (36.36)	70 (42.42)
CC	7 (4.24)	3 (1.82)
p-value	0.278*	-
Alleles		
T	256 (77.58%)	254 (76.97%)
C	74 (22.42%)	76 (23.03%)
p-value	0.853*	-
Odds ratio (95% CI)	1.035 (0.719-1.490)	-

Data were evaluated by Pearson Chi-square (χ^2) test. *p>0.05

development of ESRD (Tripathi *et al.*, 2008). Any blockage in RAAS show slow renal function decline in individuals with renal disease, providing evidence that activation of the RAS may promote a more rapid loss of GFR (Worobey *et al.*, 2009). Genetic association between RAAS and homogenous group of renal failure cases are associated with the genetic polymorphisms of angiotensinogen (Gumprecht *et al.*, 2000), angiotensin I-converting enzyme (Ali *et al.*, 2011), angiotensin II type I receptor (Buraczynska *et al.*, 2006) and aldosterone synthase (Lee *et al.*, 2009) genes. *CYP11B2* gene located at promoter region in chromosome 8q24.3 responsible to encode a steroid 11/18-beta-hydroxylase enzyme that functions in mitochondria in the zona glomerulosa of the adrenal cortex to synthesize the mineral corticoid aldosterone (Annis and Nancy, 2007). Aldosterone regulates sodium homeostasis system by stimulating sodium reabsorption in distal nephron and distal colon which is radiated via amiloride-sensitive sodium channel and energy-depend sodium potassium pump (Kuhnlea *et al.*, 2004).

CYP11B2 occupy the intracellular receptor which binds DNA, this influence transcription of various genes and the genetic variation occurs in this gene, it may result in diseases that feature salt retention or salt loss as its characteristic (White and Slutsker, 1995). T344C polymorphism of *CYP11B2* gene is responsible to ESRD (Lovati *et al.*, 2001) and the essential hypertension as the *CYP11B2* plays important role in controlling sodium balance, intravascular volume and regulating blood pressure (Vasudevan *et al.*, 2008).

The T344C (nucleotide change from C-T) polymorphism is located at the promoter region of *CYP11B2* gene at 344 bp, influence the Steroidogenic transcription Factor (SF-1) binding and it is reported to be more represented in hypertensive subjects (Casiglia *et al.*, 2005).

Creatinine level, age, SBP, triglycerides and total cholesterol were significant while DBP, HDL and LDL were not significant when compared to control subjects. From this study, older age can be said to be more

susceptible to get ESRD as the means and standard deviant of ESRD case samples showed higher value (55.03±11.89) than control value (45.99±13.83).

Among ESRD case samples, the significant difference of SBP showed that increase in SBP was likely to develop ESRD and supports the findings of Gu *et al.* (2004), Hautanen *et al.* (1988) and Fabris *et al.* (2005) as they were all reported that the association of T344C polymorphism and renal failure in the hypertensive population. However, there was no significant difference was found in DBP (p>0.05). Besides that, there are also significant difference showed by triglyceride and total cholesterol indicated that both characteristic play roles in ESRD. Diepeveen *et al.* (2008) reported where high cholesterol was associated with increased mortality in ESRD.

From this study, there was no association of T344C polymorphism of *CYP11B2* gene observed among Malaysian ESRD subjects. There was no significant difference for genotype and alleles of T344C polymorphism with ESRD as both values, genotype value was 0.278 and alleles value was 0.853 (p>0.05). The TT genotype had been dominating when the percentage was 59.39% which was the highest among the 3 genotype while CC genotype only has 4.24% among ESRD case subjects. However, the control subjects also did not showed too much different result than ESRD case subjects.

The T allele exist in most of the subjects when it has the highest percentage (77.58% for case and 76.97% for control) compared to C allele. There also no significant difference can be observed on alleles. This may result from sampling bias, population, stratification and ethnics. This finding was consistent with findings by Lovati *et al.* (2001) and Lee *et al.* (2009) as there was no association of T344C polymorphism of *CYP11B2* with ESRD. However, this is contradicted with finding by Fabris *et al.* (2005) reported that significant association was found between the *CYP11B2* gene polymorphism and renal insufficiency in the hypertensive population. However, the data was contradicted with Tang *et al.* (2006) and Munshi *et al.* (2010).

Nevertheless, it is still debatable as some studies have oppose and some are agree with the association with T344C with hypertension and the association of T344C polymorphism and renal failure in the hypertensive population but not the direct effect of the T344C polymorphism of *CYP11B2* gene with ESRD (Fabris *et al.*, 2005). The present study had got some limitations as researchers did not focus on gene expression of mRNA at protein level or the mechanism of gene itself. Besides that the population study was not homogenous as the

subjects on each ethnic were not balance and it contributed to difference genetic make-up in Malaysia as the inconsistency data that may contributed from sampling bias, population, stratification and ethnics. This study should be further continued with larger size of samples to confirm the association of *CYP11B2* gene polymorphisms among ESRD subjects in Malaysian population.

CONCLUSION

The T allele of 344T/C polymorphism of *CYP11B2* gene lacks an association with ESRD in Malaysian subjects. Therefore, CYP11B2 with T344C polymorphism might not considered as a genetic risk factor related to ESRD in Malaysian population.

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