UNIVERSITI PUTRA MALAYSIA

GENETIC POLYMORPHISM OF T344C OF CYP11B2 GENE IN MALAYSIAN END STAGE RENAL DISEASE (ESRD) SUBJECTS

BY
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ABSTRACT

Polymorphism of CYP11B2 gene at T344C of promoter region of CYP11B2 gene was vastly studied in various populations in relation to end-stage renal disease (ESRD). The objective of this study was to determine the T344C polymorphism of CYP11B2 gene in Malaysian ESRD subjects. This study involving 165 Malaysian ESRD subjects and 165 controls. Genomic DNA was extracted from buccal cells of 165 samples using commercially available kits. For T344C gene of CYP11B2, the 152bp products were amplified by Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP) method. The 152bp PCR products were digested with *Hea III* restriction enzyme for 3 hours at 37ºC. The restricted fragments showed 152 bp, 56bp and 97bp. The fragments produced were separated by metaphor agarose gel electrophoresis. The frequency of the allele was counted based on the band showed by the gel. The restricted fragments of 152bp represent TT allele (wild type), 152bp and 97bp represent the TC allele (heterozygous) and 97bp and 56bp represent CC allele (mutant). 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%), and 92(55.76%), 70(42.42%) and 3(1.82%) for control subjects, respectively. T344C polymorphism of CYP11B2 gene show no significant when compared to control subjects. The T allele of T344C polymorphism of CYP11B2 gene cannot 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 cases and control subjects to confirm the association of CYP11B2 gene mutation with ESRD.

POLIMORFISMA T344C PADA GEN CYP11B2 DIKALANGAN PESAKIT PENYAKIT BUAH PINGGANG PERINGKAT AKHIR (ESRD) DI MALAYSIA

NURUL FASIHAH ZULKIFLI

ABSTRAK

Polimorfisma gen T344C pada promoter gen CYP11B2 telah dikaji secara meluas dikalangan pelbagai penduduk tentang kaitan polimorfisma ini dengan penyakit buah pinggang peringkat akhir(ESRD). Tujuan kajian ini adalah untuk menentukan polimorfisma T344C dalam gen CYP11B2 dan kaitannya dikalangan pesakit ESRD di Malaysia. Kajian ini melibatkan 165 pesakit ESRD warganegara Malaysia dan 165 kontrol. DNA diekstrak daripada sel pipi dari 165 sampel menggunakan kit komersil. Untuk polimorfisme T344C dari gen CYP11B2, hasil produk 152bp yang telah diamplifikasi dengan Polymerase Chain Reaction (PCR) diikuti oleh teknik Restriction Fragment Length Polymorphism (RFLP). Produk PCR, 152bp yang dibertindakbalas dengan enzim *Hea* III selama 3 jam pada 37 ° C, akan menghasilkan 3 fragmen; 152 bp, 56bp dan 97bp. Fragmen yang dihasilkan dipisahkan dengan teknik metaphor agarose gel electrophoresis. Frekuensi alel tersebut dikira berdasarkan jalur yang ditunjukkan pada gel. Fragmen 152bp menunjukkan TT alel (wild type), 152bp dan 97bp mewakili alel TC (heterozigot) dan 97bp dan 56bp menunjukkan alel CC (mutan). Frekuensi TT, TC dan CC pada polimorfisma T344C pada gen CYP11B2 dikalangan pesakit ESRD di Malaysia adalah 98 (59.39%), 60 (36.36%) dan 7 (4.24%), dan 92 (55.76%), 70 (42.42%) dan 3 (1.82%) untuk subjek normal. Polimorfisma T344C gen CYP11B2 C tidak menunjukkan perbezaan signifikasi apabila dibandingkan dengan subjek normal. T alel pada polimorfisma T344C gen CYP11B2 C tidak boleh dianggap sebagai penanda genetik atau faktor risiko awal untuk ESRD untuk penduduk Malaysia. Namun, kajian ini harus dilanjutkan dengan lebih banyak subjek bagi kes dan kontrol untuk mengesahkan hubungan antara mutasi gen CYP11B2 dengan ESRD.

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Thank you very much.

APPROVAL

It is hereby certified that I have read this paper project entitled, **"Genetic Polymorphism of T344C of CYP11B2 Gene in Malaysian End Stage Renal Disease (ESRD) Subjects"** by Nurul Fasihah Zulkifli, and in my opinion it is satisfactory in terms of scope, quality and presentation as fulfillment of the requirement for SBP3999 course.

…….
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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged.

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LIST OF ABBREVIATIONS

CKD	Chronic Kidney Disease
CYP11B2	Aldosterone Synthase gene
DBP	Diastolic Blood Pressure
ESRD	End-Stage Renal Disease
GFR	Glomerular Filtration Rate
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
PCR	Polymerase Chain Reaction
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
RRT	Renal Replacement Therapy
SBP	Systolic Blood Pressure
SF-1	Steroidogenic Transcription Factor
SNP	Single Nucleotide Polymorphism

SPSS	Statistical Package for the Social Sciences
TBE	Tris-Borate-EDTA
TC	Total Cholesterol
TG	Triglycerides

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

End Stage Renal Disease (ESRD) is a permanent disease where kidney is damaged and retained less than 10% of its normal function. ESRD progress from Chronic Renal Disease stage 1 to stage 5 where the glomerulus filtration rate (GFR) is progressively decline to less than 15 ml/min/1.73m^3. As a result, the kidney is unable to secrete waste and regulate other body function. ESRD patients have decline quality of life which required the Renal Replacement Therapy (RRT) or dialysis, because without it, ESRD can be fatal (Slush *et al*., 2010).

According to United States Renal Data System 2010 Annual Data Report (USRD) 2010, the prevalence of ESRD is 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. In Malaysia, the incidents of ESRD also increase when the 17th report of Malaysian Dialysis and Transplant Registry 2009 reported the number patients undergoing dialysis is triple to 21, 159 patients in 2009 compared to 6, 689 patients in 2000. The prevalence of type 2 diabetes (T2DM) has risen to 14.9% from 8.3% ten years earlier, which showed that diabetic nephropathy would still account for the majority of ESRD for many years (3rd National Health and Morbidity Survey, 2006).

According to the previous studies, the leading etiologies of ESRD are hypertension and diabetes mellitus (Slush *et al.,* 2010, Maaz Abbasi *et al*., 2010). Diabetes and hypertension are strongly independent with factors for ESRD progression, with or without presence of protienuria for hypertension (Maaz Abbasi *et al.,* 2010, Bracanti *et al.,* 97). However, other factors also consider as pre-disposing factor ESRD development which are smoking (Orth, 2002), races, male, hyperlipidemia, recreational drug use, prenatal factor and genetic polymorphism (Yu, 2003).

As ESRD is multi-factorial disease which have both genetic and environment influent, in which recent study had shown the genetic polymorphism that contributes to ESRD (Lovati *et al.*, 2001, Slush *et al.,* 2010). Genetic polymorphism is the genetic variation within individuals, group and populations that may have a single change of nucleotides that resulting in ESRD development. The candidate genes suspected to be

pre-disposing factor of ESRD is actively being studied based on their physical functions (Wong, 2008, Bowden, 2003).

CYP11B2 gene is one of the candidate genes that are suspected to be as pre-disposing factor of ESRD (Lovati *et al.,* 2001, Lee e*t al.,* 2009). Aside for ESRD, CYP11B2 is hypothesize as pre-disposing factor of hypertension and T2DM, which are the risk factor for ESRD (Pan *et al.,* 2010). Nevertheless, the data regarding the association of CYP11B2 with ESRD are inconsistent. There are previous studies had failed to show the association of ESRD and CYP11B2 (Hung *et al.,* 2010, Lovati *et al.,* 2001, Lee *et al.*, 2009). In spite of that, Prasad *et al.*, (2006), reported that T344C CYP11B2 is associated with diabetes specific renal disease. There are few studies on the CYP11B2 and its association with the ESRD. However, many studies shown association of CYP11B2 gene with hypertension and T2DM which lead to ESRD eventually.

1.2 Problem Statement

The numbers of ESRD patients requiring maintenance dialysis being increasing more than 2 million by year 2010. As the demand of dialysis and other treatment is increasing, it associated with cost which placing unsustainable financial burden on health related sector in countries which are new to the world (Philip *et al.*, 2006). In Malaysia, the increasing number of dialysis patient increase with 699 per

million prevalence. Peritoneal dialysis and hemodylisis, give annual death rate of 14.5% and 6-9% respectively, as the cost of dialysis was estimated RM 30 million in 2005 (17th report of the Malaysia Dialysis And Transplant Registry 2008, Hooi, 2006).

In addition, the lack of study of CYP11B2 gene with T344C polymorphism in Malaysia, as risk factor of ESRD. As CYP11B2 gene with T344C have been variously studied with association of hypertension and T2DM, only few been have directly shown the association with ESRD, even though both, hypertension and T2DM are major contributor of ESRD development. Since the CYP11B2 gene play an important role in kidney function, this study will help to know the association of CYP11B2 gene polymorphism with ESRD among Malaysian subjects.

1.3 Significance of the Study

Although, the major contributors of ESRD are hypertension and T2DM, there is also an evidence of genetic factor which contribute to ESRD development. In this study, we will determine the genotype and allele frequency and the possibility of CYP11B2 gene with T344C polymorphism as ESRD genetic-marker.

The studies of association of CYP11B2 gene with T344C polymorphism have been done, with or without association with hypertension and T2DM in other populations in Korea, British, French, African, Japanese, Finnish and selected group

of Italian subjects (Casiglia *et al.,* 2005). Therefore, this study was 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.

1.4 HYPOTHESIS

There is an association of T344C polymorphism in CYP11B2 gene with ESRD among Malaysian subjects.

1.5 OBJECTIVES

MAIN OBJECTIVE

The main objective of this study is to determine the genetic polymorphism of CYP11B2 gene in Malaysian ESRD subjects.

SPECIFIC OBJECTIVES

To determine the genotypic and allele frequency of T344C gene polymorphism of CYP11B2 gene among Malaysian subjects.

CHAPTER TWO

LITERATURE REVIEW

2.1 Kidney and its role

Kidney plays an important role in human life. Human have a pair of kidney and is bean in shape, located on either side of spine in lower middle back. Kidneys are made of millions of nephron which is from glomerules and tubules. Each kidney will connect to a pair of urethra to flow urine to bladder. The main function of the kidney is to secrete waste and excessive water from blood. Besides that, kidney also plays roles in regulating mineral aside from produce certain hormones which are alcitriol, erythropoietin and renin. Each hormone produced by kidneys has different roles in human body.

2.2 End-stage Renal Disease (ESRD)

ESRD is permanent diseases which characterize by damaged or severely damaged kidney that it retains less than 10% of its normal functions. The progression of ESRD starts from development of CKD. The development of CKD is classes by the GFR. The stages of CKD as below:

Stage	Description	GFR (mL/min/1.73m)
1	Kidney damage with normal or elevated GFR	90 or Greater
2	Kidney damage with mildly reduced GFR	60 - 89
3	Moderately reduced GFR	30 - 59
4	Severely reduced GFR	15 - 29
5	Kidney failure (ESRD)	Less than 15 or dialysis

Adopted from National Kidney Foundation, 2002

Table 2.1 : Stages of CKD

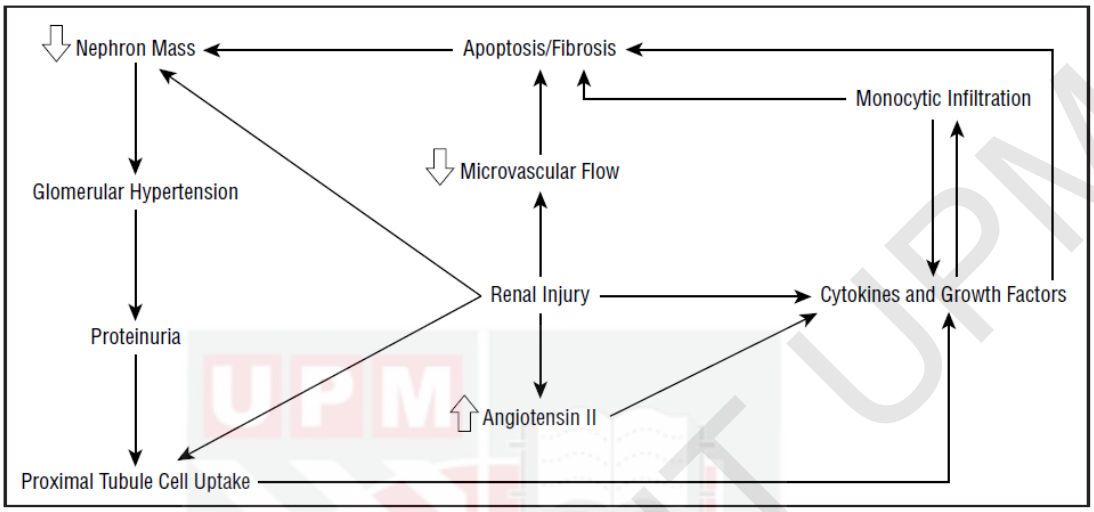
As stage of CKD progress from stage 1 to stage 5, the 5th stage is when the kidney fails to function as it called ESRD or kidney failure. As kidney failed to function to secrete waste, regulate pH and electrolytes, the requirement of treatment is severely needed as ESRD can be fatal without treatment. The RRT and dialysis can be burden to patient because of highly cost, even though dialysis in Malaysia is mainly sponsor by government (17th Report of the Malaysian Dialysis and Transplant Registry 2009).

Kidney function is analyzed by calculating GFR from blood creatinine level, proteinuria and blood pressure. Even though kidney disease is classes into two group –acute kidney diseases and chronic kidney disease, both diseases can be developed into ESRD when kidney function is progressively decline.

2.3 Pathophysiology of ESRD

ESRD is starts to develop when nephrons are damaged and replaced by fibrous scar. The mediators of the injury of the kidney are classes into hemodynamics, hypoxia, proteinuria, systemic hypertension, complement activation, angiotensin II and other chemical mediators as all these factors will eventually lead to development of CKD and ESRD. It occurs when compensatory hyperfiltration of spared nephrons occurs to maintain the overall GFR. Even so, it will leads to glomerular hypertension, proteinuria and progressive chronic renal failure as shown by early diebetic nephropathy as it known to be high elevated GFR. Increase of the glomerular capillary hydraulic pressure and hyperfiltration propagates chronic GFR decline lead to protein leaking, called proteinuria. This will result in the activation of mediator, inflammatory cytokines and various pathways to occur thus resulting in progressive declining of GFR.

As hypertension frequent accompany ESRD, it affect as the stimuli from kidney may activate the sympathetic nervous system thus elevated the pressure due to sodium intake, excessive volume and activation of RAAS. Hypertension can accelerate the declining of the kidney functions likely due to the increased glomerular capillary hypertension (Yu, 2003).



Adapted from Yu, 2003

Figure 2.1 : Pathophysiologic pathway of chronic renal failure.

2.4 Prevalence of ESRD

In Malaysia, the number of patients undergoing dialysis was increased from 6, 689 patients in 2000 to 21, 159 patients in 2009 as the number is triple in duration of 10 years (17th Report of the Malaysian Dialysis and Transplant Registry 2009). The number of prevalent rate also increased as it doubled over the last 10 years into 699 per million 2009, compared to year 1999, where the prevalent is 285 per million. Besides the prevalence of patients also increase from 79 per million into 146 per million 2009. The increased of ESRD incident in worldwide from 75 to 350 million in developed countries causing the increase in the dialysis and RRT (Yap, 2007).

2.5 Risk Factors of ESRD

Numerous studies had shown that, the leads of risk factor of ESRD are hypertension, diabetes mellitus and glomerulopathies (Wong, 2008, Agrawal *et al.*, 2010). Metabolic syndromes in which hypertension and T2DM are present are and growing cause for renal damage (Foley *et al*., 2005, Lucove *et al*., 2008). Other factors posing pre-disposing factors for ESRD are races (American African), age (ESRD incident group), proteinuria, smoking, gender, hyperlipidemia, prenatal factors, and recreational drugs used (Yu, 2003). Agrawal *et. al*., (2010) reported, there was four fold higher risk of kidney failure among African Americans, whereas the risk is three fold in native Americans, and two fold for Hispanic Americans. Genetic factors like DNA single nucleotide polymorphism, also been suggested as contributor in the increasing risk factor developing ESRD (Nordfors *et al.*, 2005)

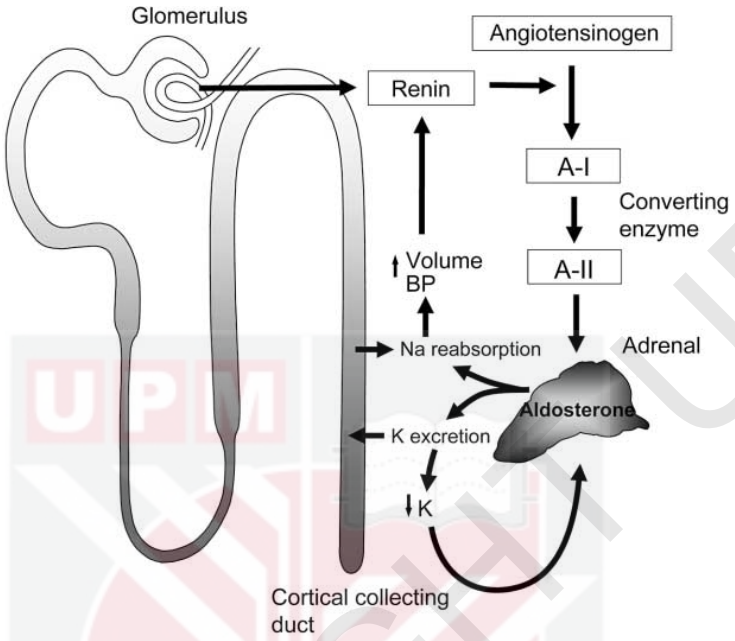
2.6 Genetic factors

Various studies had report that genetic polymorphism plays roles in development of ESRD. As RAAS plays important roles in human physiology, the system also plays role in promoting kidney failure. Genetic association between RAAS and homogenous group of renal failure cases, provide some information association of genetics of renal failure in general. As RAAS composed of renin, angiotensinogen

(AGT), angiotensin I-converting enzyme (ACE), chymase (CMA), angiotensin II type I receptor (AT1R), and aldosterone synthase (CYP11B2), 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).

2.7 Aldosterone synthase (CYP11B2) Gene and Mechanism

CYP11B2 is a gene that responsible to encode a steroid 11/18-beta-hydroxylase (aldosterone synthase) enzyme that functions in mitochondria in the zona glomerulosa of the adrenal cortex to synthesize the mineral corticoid aldosterone (Pascoe *et al.,* 1992). CYP11B2 gene located at promoter region in chromosome 8q24.3, play roles in major organs which are kidney and heart (Chang, 2009, Tsukada *et. al*., 2002). Aldosrerone is synthesize in adrenal zona glomerulosa is stimulates by Angiotensin II, potassium or corticotrophin (ACTH) (Annis and Nanc, 2007). Being included in RAAS, makes CYP11B2 one of the gene that are responsible for regulating system of sodium and potassium balance in human body (Kuhnle *et. al.,* 2004). 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 (NA, K-ATPase) (Kuhnle *et. al.,* 2004). CYP11B2 occupy the intracellular receptor (mineral corticoid receptor) which binds DNA, this influence transcription of various genes (White, 2004). As genetic variation occurs in this gene, it may result in diseases that feature salt retention or salt loss as its characteristic.



Adapted from Kuhnle, 2004.

Figure 2.2 : The function of CYP11B2 in regulating sodium and potassium homeostasis system.

Various studies had related CYP11B2 with cardiovascular disease, especially hypertension besides relate it with kidney disease (Agrawal *et al*., 2010). Some studies reported that increased concentration of aldosterone are associated with endothelial dysfunction in human hypertension and acute aldosterone administration causes endothelial dysfunction in healthy individual (Annis and Nancy, 2007). According to Kuhnle *et al.,* (2004) the disturbance in CYP11B2 gene resulting in sodium loss which associates with acid and potassium retention that leads to metabolic acidosis that may cause in cardiac standstill, as aldosterone increase Na^+-K^+-$2Cl^-$ co-transporter activity and decrease Na^+/K^+ pump activity through a non-genomic PKCε –dependent mechanism in heart. Thus, inappropriate high aldosterone concentration will promote

cardiovascular remodelling and renal injury by stimulating oxidative stress, endothelial dysfunction, inflammation and fibrosis during highly salt intake (Annis and Nancy, 2007).

2.8 Single Nucleotide Polymorphism (SNP)

A SNP is a DNA sequence variation occurring when a single nucleotide – A, T, C, and G - differs between members of a biological species or chromosome in an individual. SNP is the most common type of genetic variation among people as humans are estimated to have one SNP every 300 base pair of nucleotides (Ke *et al.,* 2008). It commonly found in the DNA between genes. When it occurs within a gene or in regulatory near a gene, they can play role by affecting the genes function. SNP can act as genetic marker in identifying loci of susceptibility of common disease or loci defining drugs sensitivity. However, SNP might not be appropriate in identifying genes involved studies (Haga *et a*l., 2002).

2.9 T344C Polymorphism of CYP11B2 Gene

It is believe that the T344C polymorphism 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.,* 2009). The T344C polymorphism is located at the promoter region of CYP11B2 gene at 344bp, where the nucleotide change from C to T, influence the

transcriptional regulatory protein, (steroidogenic transcription factor) SF-1 binding. The T344C polymorphism could modify the response of the CYP11B2 gene to the SF-1 as C allele binds to SF-1 more strongly than T allele as it is reported to be more represented in hypertensive than in normotensive subject (Casiglia *et al.,* 2005). Although the evidence of the association of T344C polymorphism is varied, it is believed that the T344C polymorphism alter the sensitivity of CYP11B2 gene to angiotensin II, resulting in inappropriate aldosterone production for the prevailing renin level (Nicod *et al.,* 2003). It is likely that the T344C polymorphism has a negligible influence as other factor may affect such as dietary salt, level of potassium and angiotensin II, it is seen clear strand in the T-carriers towards higher values of aldosterone (pg/ml) to renin (pg/ml) ratio (ARR) (Casiglia *et al.,* 2005, Tamaki *et al.,* 1999). Even though the lack of direct association of T344C with ESRD, the various studies done with association of T344C with hypertension showed that the T344C may give influence to the development of ESRD with association of hypertension as it affect the blood pressure regulation (Russo *et al.,* 2002).

2.10 DNA Quantification

DNA quantification is a technique used to determine the concentration of the DNA in a sample and to ensure the optimal yield when the DNA is amplified. There a few methods developed to quantify DNA which are UV spectrometry, SYBR-green dye staining, slotblot hybridization and TaqMan real-time PCR assays, according to the purpose of each technique served (Nielsen *et al.,* 2006). In spite of many

sophisticated techniques had been developed, UV spectrometry has been traditional used to quantify DNA eventhough the technique is not sensitive enough to detect small amount of DNA as it need at least 3ng/µl of DNA (Nielsen *et al.,* 2008). A. biophotometer is used to measure the DNA absorbance at 260nm under UV light. The OD was counted by comparing the DNA absorbance under 260nm and 280nm (best wavelength to absorb protein). The OD ratio range between 1.7 to 2.0 showed the relatively purity of DNA free from protein.

2.11 Polymerase chain reaction (PCR)

PCR (polymerase chain reaction), is a technique to amplify a single or several DNA by in vitro enzymatic replication. This method depends on thermal cycling, which repeated heated and cooling cycles for DNA melting and replication. Along with the DNA polymerase, primer of containing complimentary sequence enables for selective and repetitive amplification. In chain reaction motion, the DNA was replicated started from original molecules and doubling the replication. Thus, it will create many copies of original DNA sequences. Basic step involve in PCR are denaturation of double strand DNA into single strands, annealing of specific primer into single-stranded DNA and elongation of the primer, creating new double strand DNA. PCR is used in various purposes such as DNA cloning procedure, southern blotting, DNA sequencing and recombinant DNA.

2.12 RFLP

RFLP, restriction fragment line polymorphism is a technique to detect the differences in homologous DNA sequence that are detected by presence of fragment after digestion of the DNA by restriction enzyme. By using restriction enzyme, the DNA is cut into short fragments at restriction site, the short fragment of different length are detected by electrophoresis. The digestive fragments results from the enzyme digestion represent allele and useful for genetic analysis (Ganguly *et al.,* 2010).

2.13 Gel Electrophoresis

AGE is a process of separating DNA, RNA or protein molecules using an electrical field. By using gel as medium for molecules to be sorted, negatively charge molecules will moves through the gel to positively charges when electric is applied. Large molecules move slowly than smaller molecules. The distinct band appear on the gel because of the differences of the size of the molecules

2.14 Gene Counting

Principle of Hardy-Weinberg equilibrium stated that gene and genotype frequencies are constant from generation to generation in a large population with randomly mating. Equilibrium will be re-established after one generation of random mating when disequilibrium occurred. However, this principle only applies when the

populations are natural selection, there is no mutation or migration, the infinite of population size and individuals in the population mate randomly (Andrews, C., 2010). Dominant allele is denoted with capital letter: - A, while recessive allele is denoted with small letter: - a. in this equilibrium, the frequencies are represent by *p* and *q*, both are represent the dominant and recessive alleles – A and a, respectively. The equation of the frequency is $p + q = 1$. When the population is in equilibrium, then frequency of AA is denoted as p2 for the AA homozygous in a population. The aa heterozygous is denoted by q2 in the population. To calculate the allele frequency, the formula as below was used :

Total number of A alleles in a population = [(the number of Aa heterozygous) + (2 x the number of AA homozygous])

2.14 DNA Sequencing

DNA sequencing is a process of determine the order of the nucleotide bases- adenine, cytosine, guanine and thymidine in DNA molecule. The DNA is served as template in order to generate a set of fragment that differ if length from each other by a single base. The fragments are then separated by size, and the basest the end are identified, recreating the original sequence of DNA.

2.15 Statistical analysis

SPSS **(**Statistical Package for the Social Sciences) is a statistical analysis program used to analyze data. All variable studies such as socio-demographic characteristic, genotype and allele frequencies are analyzed by SPSS. Case and control samples are compared their significant as the significant will represent the association of the polymorphism towards the disease.

CHAPTER THREE

METHODOLOGY

3.1 Study Design

This study is a cross-sectional study where the comparisons were done between 165 case and 165 control subjects for T344C polymorphism of CYP11B2 gene among Malaysian subjects.

3.2 Sampling

A number of 330 samples for this study are taken with 165 samples each for case and control samples. All the samples taken were followed the inclusion and exclusion from the RUGS project as stated below:

	Inclusion Criteria	Exclusion Criteria
Case Samples	1. ESRD patients 2. Undergoing dialysis 3. Age above 21 years old 4. Having high creatinine levels	1. Any subjects without ESRD nor undergoing dialysis are excluded from this study 2. Age below 21 years old 3. Subjects refuse to give consent

Table 3.1 : Inclusion and Exclusion Criteria of Case Subjects.

3.3 Biochemical Analysis

All information of biochemical analysis for case and control subjects was obtained from previous study information as the samples were submitted to clinical laboratories. The information of biochemical analysis was level of creatinine, High Density Lipoprotein Cholesterol (HDL-C), Low Density Lipoprotein Cholesterol (LCL-C), Total Cholesterol (TC) and Triglycerides (TG).

3.4 Genomic DNA Extraction

DNA Extraction Kit from Qiagen (German) was used to extract DNA from samples. All the protocol for DNA extraction is based on protocol provided by manufacturer.

The samples were spun at 1, 300 rpm for 2 minutes before the supernatant was discarded. 30 µl cell lysis buffer was added to the tubes and the tubes were vortexed and incubated at 65 °C for 15 minutes. During the incubation time, the tubes were vortexed at every 5 minutes interval. Then, 1.5 µl Proteinease K was added and the tubes were inverted for 25 times. All the tubes were incubated at 55 °C for 1 hour. 1.5 µl RNAse was added to the tubes, before the tubes being inverted for 25 times and incubated for 15 minutes at 37 °C. After that, the tubes were incubated in ice for 1 minute and 100 µl protein precipitation solutions were added. The tubes were vortexed at high speed for 12 seconds. The tubes were incubated in ice for 5 minutes and

centrifuged at 1, 300 rpm for 5 minutes. Supernatant was transferred into new tubes contained 300 µl isopropanol and 1 µl glycogen solution. The tubes were inverted gently for 50 times for mixing purpose. Then, the tubes were centrifuged for 10 minutes at 1, 300 rpm and the supernatant was discarded and the pallet was dried. 300 µl 70% ethanol was added, before the tubes were inverted, vortex and centrifuged at 1, 300 rpm for 1 minute. The supernatant was discarding and the pallet was dried. 20 µl DNA hydration solutions was added, vortex for 5 minutes, and incubated at 65 °C for 1 hour. All the extracted DNA is kept under -20°C for further analysis.

3.5 Quantification of Genomic DNA

The purity of extracted DNA was determined by using biophotometer (Eppendorf, Germany) at 260 nm. Distilled water was used as blank against A_{260} and A_{280} of genomic DNA, is obtained. The DNA absorbed the UV light at 260 nm while protein at 280 nm. The standard measurement of the purity genomic DNA was calculated by dividing the amount of UV absorption at 260 nm by the absorption at 280 nm. The ratio of optical density (OD) between 1.7 and 2.0 was considered relatively free of protein impurity.

3.6 Polymerase Chain Reaction (PCR) of the CYP11B2 gene

PCR is a technique of amplification of DNA for further analysis. This study used PCR-RFLP method to amplify and digest the gene of interest. Each PCR tubes contain 25 µl mixing according to the table below. The primers reverse and forward, were from AITbiotech (Singapore) while the master mix is from iDNA. This calculation was derived from the PCR optimization to yield the optimum result. Each primer is diluted with 20 µl TE buffer. After all the PCR mixture is prepared, the mixture is vortexed and centrifuged before being inserted in PCR machine. The PCR technique is done by using iCycler PCR machine (Biorad Laboratories, USA). All the PCR conditions, primer are stated as below. The PCR products were kept in the freezer at 4°C for further analysis.

PCR mixing	1X (µl)
Distilled water	12.4
Master Mix	10.0
Reverse Primer	0.3
Forward Primer	0.3

Extracted DNA	2.0
Total	25 µl

Table 3.2 : PCR mixture

Gene • Polymorphism • Location • Roles	**CYP11B2 (Aldosterone synthase)** T344C Promoter region, Chromosome 8q24.3 Regulate sodium and potassium balance		
Method	PCR	Primers • Forwards • Reverse	5'-CAGGGGG TACGTGGACATTT-3' 5'-CAGGGCTGA GAGGAGTAAAA-3'
		Condition • Initial denaturation • Denaturation • Annealing • Extension	94 °C, for 3 minutes 94 °C for 30 seconds 52 °C for 30 seconds 72 °C for 30 seconds
		Cycle	35 cycles
		Product size	152 bp
	RFLP	RE	*Hae III*

		Product size	152bp 97bp 56bp
Reference	Lovati *et al*., 2001 .		

Table 3.3 : PCR and RFLP condition and product sizes for T344C polymorphism

3.7 Optimization of PCR

PCR optimization was done by using 3 samples and 3 different concentration of mixture. The master mix was added to each samples are 7.50 µl, 10.0 µl and 12.5 µl in order to get the optimum yield. The optimum result was yield with 10.0 µl master mix.

3.8 Determination of PCR Product

PCR product was detected by using 3% Agarose gel. 0.8gram of Agarose powder was mixed with, 40 ml TBE buffer and 3 µl of Gel red (EZ-Version, Ohio) for staining purpose. Samples were loaded into respective wells and connected to electricity. The tank was run about 30 minutes. The gel was reviewed under Alpha Imager 2.0.

3.9 Analysis of PCR Products

In RFLP method, restriction enzyme was used to cut at specific restriction site in order to produce several band. This study used *Hae III* enzyme to cut the PCR product at GGCC site. The *Hae III* enzyme is used with 1xNEBuffer 4, provided with the enzyme, BSA and distilled water with the respective volume as below:

Mixing	Volume
Hae III enzyme	0.75 µl
1 x NEBuffer 4	3.00 µl
BSA	1.00 µl
Distilled water	9.40 µl
PCR product	6.00 µl
Total	20.00 µl

Table 3.4 : RFLP mixture

The total volume for this method is 20 µl. The mixture will be centrifuged and incubated at 37°C for 3 hours. After that, the mixture is centrifuged before inactivation was done at 80°C for 20 minutes.

3.10 Metaphor Agarose Gel Electrophoresis

RFLP products were detected using Metaphor agarose gel. The 3% Metaphor agarose gel was produced by using 0.6 gram agarose powder and 0.6 gram Metaphor powder along with 40 ml TBE buffer and 5 µl gel red for staining purpose. The electrophoresis was run for 30 minutes. The gel was previewed under Alpha Imager 2.0 UV light. there bands were produced which are 152bp, 97bp and 56bp. The wild type produced 152bp band, heterozygous produced 152bp and 97bp bands and mutant produced 97bp and 56bp bands.

3.11 Purification of PCR product

PCR product needed to be purified before it can be sequenced. The presence of unspecific primers and excess dNTPS may interfere the sequencing process. The Silica Bead DNA Gel Extraction Kit (Fermentas, America) was used in purification method and the protocol used was from manufacturer's protocol.

3.12 DNA Sequencing

The T344C polymorphism genotype confirmation was done by DNA sequencing, (Research BioLabs). The result was analyzed by using BLAST (www.ncbi.nlm.nih.gov/blast).

3.13 Data Analysis

SPSS software version 17.0 was used for data analysis. All variables studies such as socio-demographic background, anthropometric measurements, and biochemical parameters were analyzed by using descriptive statistic. Genotype frequency for both, case and control were compared by using Chi-Square test while means was compared by using independent T-test. The statistical probability of $P<0.05$ was considered significant.

CHAPTER FOUR

RESULTS

4.1 Demographic Distribution of Subjects

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 (Figure 4.1).

The 165 control samples consist of 47.3% from male and 52.7% from female. For ethnic distribution, the most dominant samples were from Malay (46.7%), followed by Chinese (42.4%) and the least was from Indians, which was 10.9%. Figure 4.2 showed the percentage of social-demographic distribution of control samples.

All 330 samples were grouped into 4 classes of age which were 18 years old to 29 years old, 30 years old to 49 years old. 50 years old to 70 years old, and the last group was age more than 70 years old. The case subjects had the highest number in range of 50 to 70 years old (56.4%), followed by range of 30 to 49 years old (33.9%),

age more than 70 years old (10.3%) and the least was from range of 18 to 29 years old (1.8%). For control samples, age range 30 to 49 years old gave the highest samples which was 45.5%, followed by range 50 t0 70 years old (35.2%), range 18 to 29 years old (14.5%) and the least was age more than 70 years old which was 4.8%. Figure 4.3 showed the age range distribution for both, case and control, respectively.

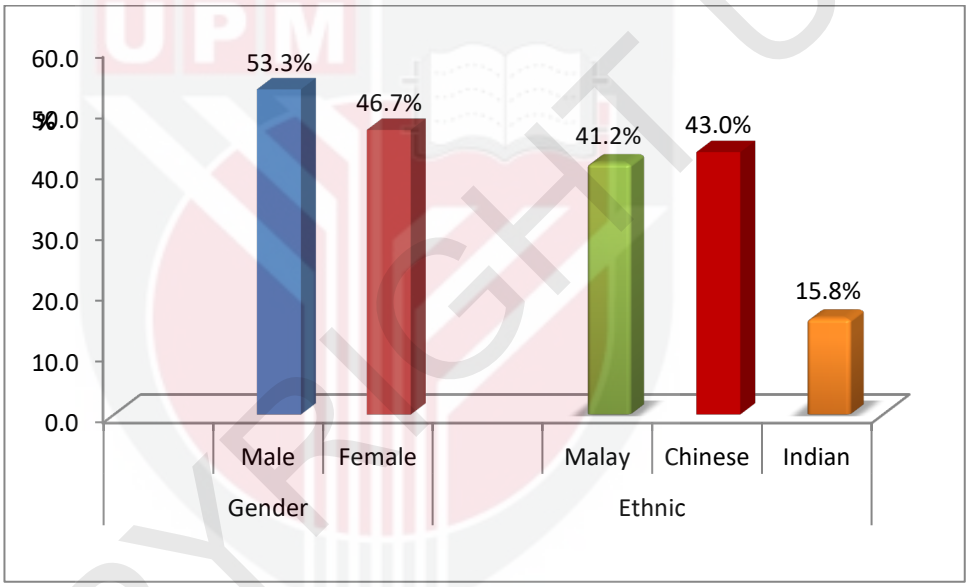


Figure 4.1: The distribution of case samples

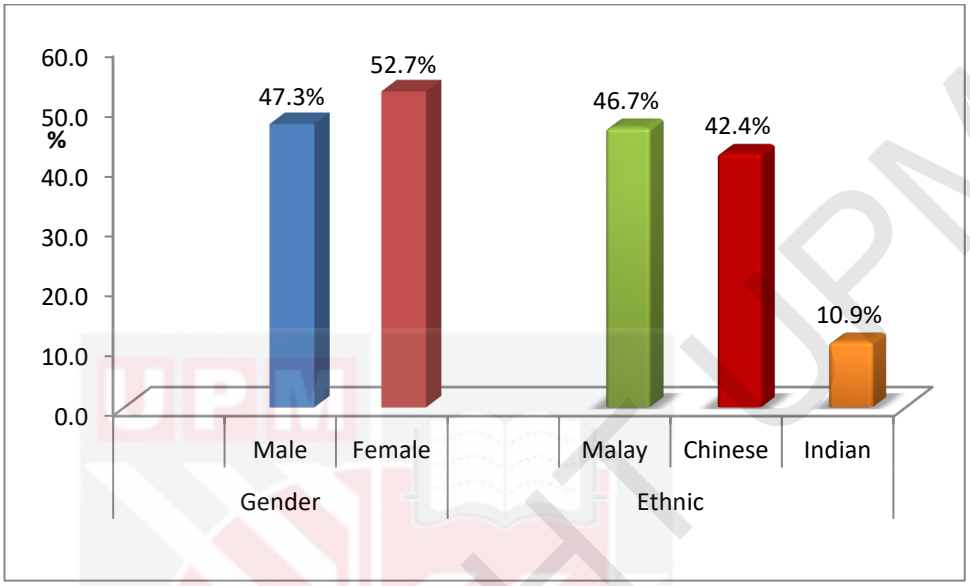


Figure 4.2: The distribution of control samples

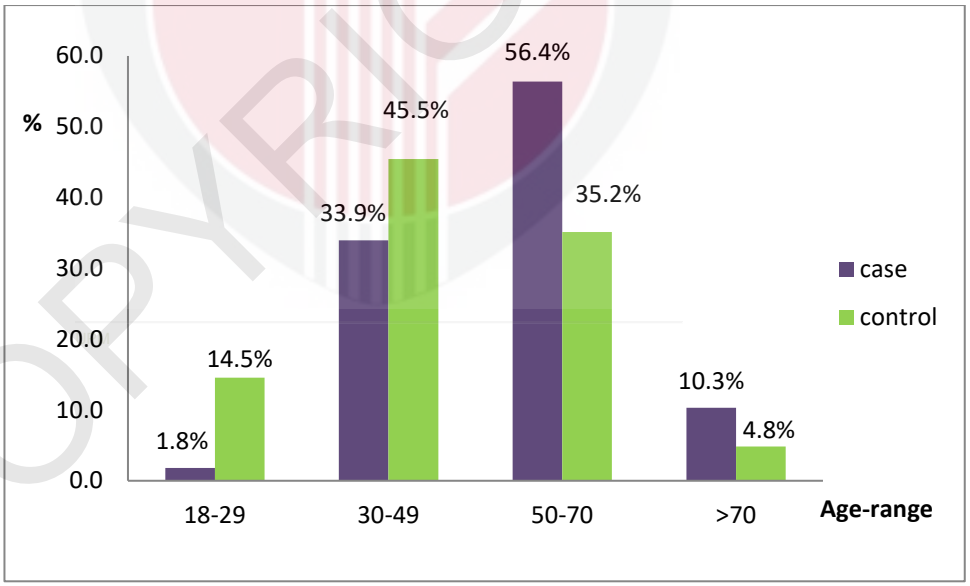


Figure 4.3: The distribution of age range for case and control samples

4.2 Clinical Characteristics of All Subjects

Table 4.1 showed the clinical characteristic of case and control samples. All clinical characteristic were obtained from RUGS project which consist of SBP, DBP, creatinine level, LDL, HDL, triglycerides and total cholesterol with significant value $P<0.05$.

The independent t-test had been done in order to find the means and significant difference of all the clinical characteristic. Means age for case samples was higher than control samples. 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 value is lower than 0.05. In contrast, there was no significant difference was observed DBP, HDL and LDL ($P>0.05$). The clinical characteristics were DBP, HDL, and LDL as shown in the table 4.1.

Parameter	**ESRD** **n = 165**	**Control** **n = 165**	***p*<0.05**
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 $P<0.05$

Table 4.1: Clinical characteristics of ESRD and control samples.

Gender of ESRD case samples were compared as in table 4.2. Between genders, means of creatinine level, DBP and LDL were higher in male. In contrast, female had higher means level in SBP, HDL, triglycerides and total cholesterol, even the difference were small. However, only creatinine level and HDL had significant difference while the other characteristic were not.

Parameter	**Male**	**Female**	***p*<0.05**
	n = 88	**n = 77**	

Age (years)	2.72±0.64	2.79±0.68	0.459
Creatinine	919.79±285.24	756.17±195.77	**0.000***
SBP	147.90±26.93	150.95±30.14	0.493
DBP	77.86±18.08	77.49±15.50	0. 889
HDL	1.05±0.33	1.18±0.34	**0.019***
LDL	3.10±1.21	2.91±1.07	0. 299
Triglycerides	2.19±1.63	2.58±1.87	0.148
Total cholesterol	5.00±1.44	5.25±1.11	0.227

* Significant $P<0.05$

Table 4.2: Clinical characteristics of gender of ESRD case samples.

4.3 Genotypic and Allelic Analysis

The distribution of genotype in case sample 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%) percentage in control as showed in figure 4.4.

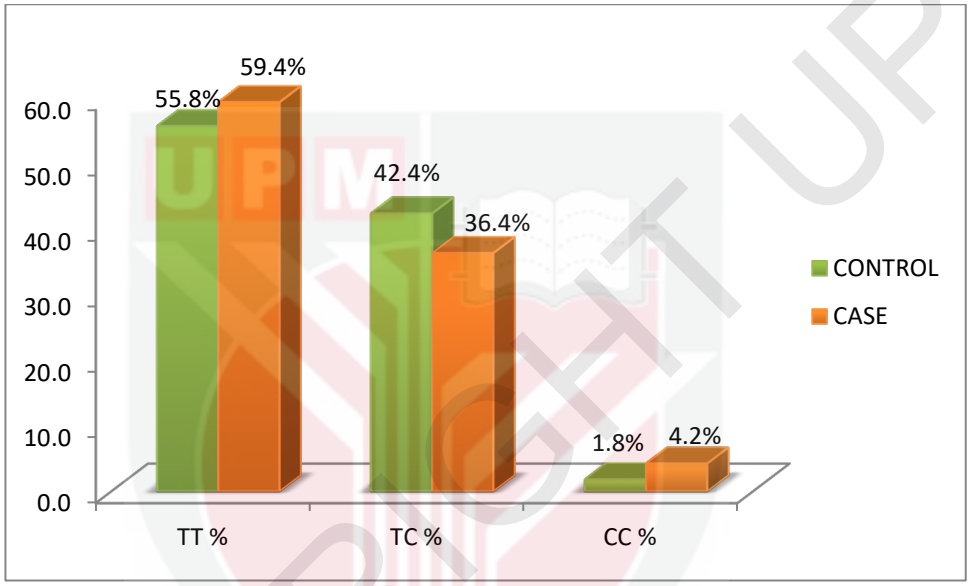


Figure 4.4: Distribution of genotypic distribution of T344C polymorphism of CYP11B2 gene

The ESRD case subjects were compared between the genders as shown in figure 4.5. Between genders, female had higher percentage of TT genotype with 61.0% compared to male (58.0%). However, male had higher percentage in TC genotype with 39.8% compared to female (32.5%). The percentage of CC was higher in female with 6.5% compared to male, 2.3%. There were no significant difference can be observed for both genotype and alleles as the significant value was $p<0.05$ shown in table 4.4.

Genotype	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 (.719 - 1.490)	

Data were evaluated by Pearson chi square test, $P<0.05$

Table 4.3: Genotypic and allelic distributions of T344C CYP11B2 gene polymorphism between ESRD subjects and controls.

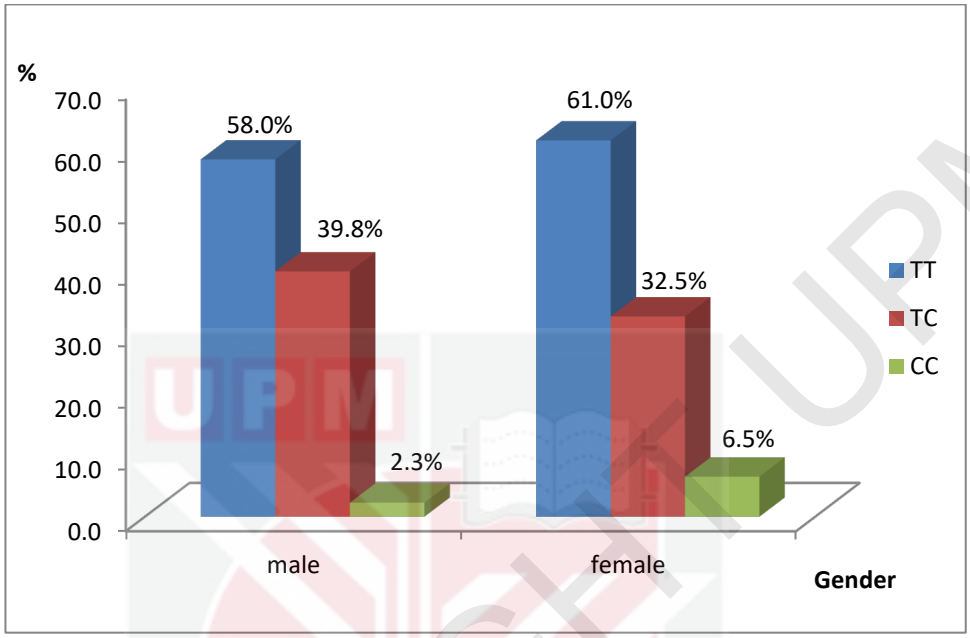


Figure 4.5: Distribution of genotype between genders in ESRD subjects

The ethnic genotype had been compared as in figure 4.6. It showed that Malay has the highest TT genotype (60%), followed by Chinese (59%), and the least was Indian (58%). However, Chinese had the highest TC genotype which was 41% and the least was Malay, with 32%. Indian has the highest CC genotype percentage with 8%, followed by Malay (7%), while Chinese has none.

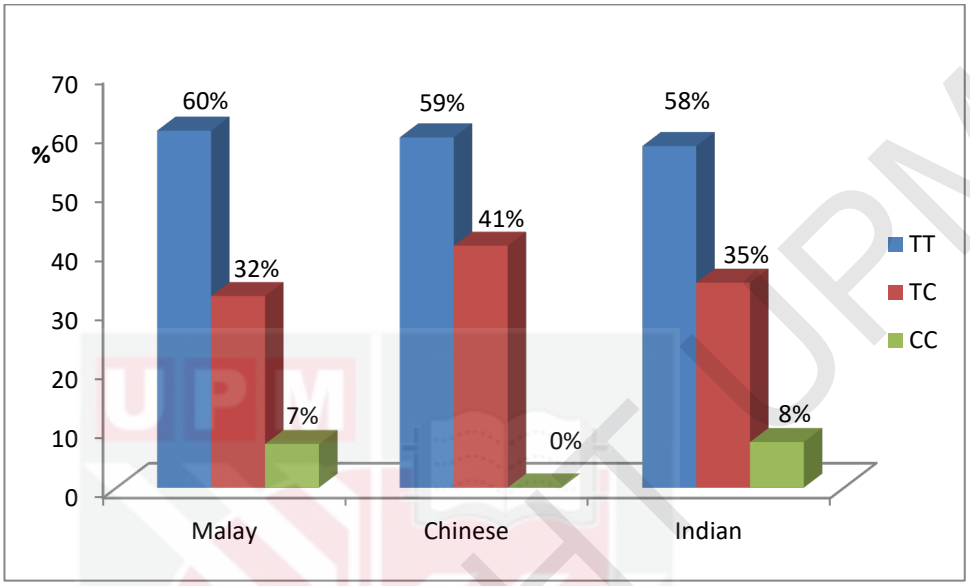


Figure 4.6: Distribution of genotypes among ethnics in ESRD subjects

4.3 PCR Amplification Product

Figure 4.7: 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.

4.4 Restriction Enzyme (RE) Product

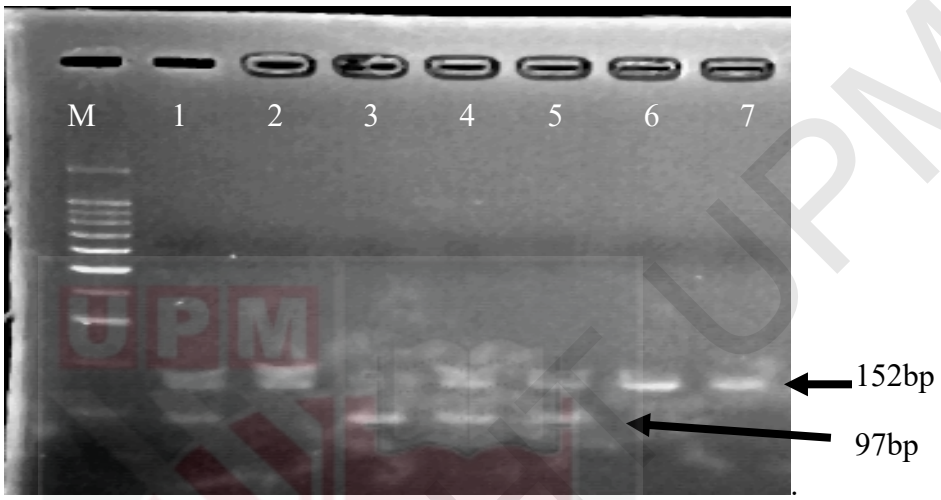


Figure 4.8: Restriction enzyme fragments (RFLP) using *Hae III* of T344C gene polymorphism in 3.0% Metaphor Agarose Gel Electrophoresis. Lane 6 and 7 shows the wild-type fragments (152 bp), lane 4 and 5 shows the heterozygous fragments (152 and 97 bp), lane 3 show mutant fragment (97bp and 56bp, the 56bp was not seen) in 3.0% Metaphor Agarose Gel Electrophoresis to determine the restriction digest products for RFLP analysis.

CHAPTER FIVE

DISCUSSION

5.1 Clinical Characteristics

In this study, not all clinical characteristics were significant. Only age, creatinine level, SBP, triglycerides and total control were significant while DBP, HDL and LDL were not significant, as the P value was small than 0.05. 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), even though there is no significant difference.

Among ESRD case samples, the significant difference of SBP showed that increase in SBP was likely to develop ESRD as Gu *et al.,* (2004), reported, the T344C polymorphism were associated with stage-2 hypertension in a sample of northern Han Chinese. Hautanena *et al*., (1988) reported the increase of SBP with association of T344C polymorphism. This study also is agreement with Febris *et al*., (2005) as they also reported the association of T344C polymorphism and renal failure in the hypertensive population. However, it unable showed the relation of DBP with T344C polymorphism as it has no significant difference.

However, this study fails to show the association gender with the T344C polymorphism and ESRD. It contradicts with the Gu *et al,.* (2004) study, when it reported that male gender has association with T344C polymorphism and hypertensive. Previous studies also showed the association of T344C polymorphism with male gender as this study has not associated it with male gender (Song *et al.,* 2003).

HDL and creatinine showed significant difference that associated with ESRD between case and control samples and also between genders. It demonstrates that HDL and creatinine have association with the ESRD. Besides that, there are also significant difference showed by triglyceride and total cholesterol indicated that both characteristic play roles in ESRD. This support the Diepeveen *et al*., (2008) reported where low cholesterol was associated with increased mortality in ESRD. However, the clinical characteristic comparison between genders had no much difference.

5.2 Genotype and Allele Frequencies

From this study, there was no association of T344C polymorphism of CYP11B2 gene with ESRD. 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, were bigger than 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 (Persu, 2006).

When gender of ESRD case subjects being compared, there was also not much difference between genders in the genotype distribution. It also applied to ethnics when the genotypes were equally distributed among races.

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.

According Casiglia *et al.,* (2005) the T allele in individuals had associated with elevated SBP, with increasing of age and body fat, than individuals has C allele. Previous study also showed that individuals have T allele more prone to get hypertension than C allele carrier (Brand et *al.,* 1998). Davies *et al.,* (1999) and Paillard *et al.,* (1999) had reported the association of plasma aldosterone in T allele than in C allele, thus the increase of risk having hypertension (Ponda and Hostetter,

2006). Increasing risk hypertension thus may develop into ESRD as elevated pressure may increase glomerular capillary hypertension.

However, the data was contradict with Tang *et al., (*2006) and Munshi *et al.,* (2010) when they reported that C allele binds to steroidogenic transcription factor 1 (SF-1) more strongly than the T allele and leads to increased aldosterone production and SF-1 binding site was required for basal and Angiotensin II or K^+, stimulated CYP11B2 transcription. They also indicated that diastolic blood pressure tended to be higher with the C allele subjects than T allele. Thus, suggested association with essential hypertension. 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).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

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

This study was not focused on gene expression of mRNA at protein level nor 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 inconsistence data that may contributed from sampling bias, population, stratification and ethnics (Persu, 2006). It also might be contributed from small sample size.

As some of previous studies of T344C have been contradicted with this project's result, more recommendation should be done to get the accurate result. As one of the ESRD factor is ethnic, subjects of each ethnic in Malaysia should be collected equally. Besides that, the samples collection between genders also should

be equal as gender may contribute to the genetic factor. Furthermore, more subjects with homogenous population should be participating in this project as large samples size will increase the accuracy of result. In addition, the control and case subjects range age should be equaled hence the age would not affect the accuracy of result. This study should be further continued with more case and control subjects to confirm the association of CYP11B2 gene mutation with ESRD.

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APPENDIX B

A) Preparation of Tris-Borate-EDTA (TBE)

TBE Buffer at pH 8.3

Stock = TBE buffer 50x, for 1000ml,

M1V1 = M2V2

(50)(V1) = (1)(1000ml)

V1 = 200ml TBE buffer

So, 200ml TBE + 800 ml dH_2O

B) Preparation of 3.0% Agarose Gel

1. Agarose Powder : 1.2 g
2. TBE buffer : 40 ml

(The mixture is microwaved, cooled down, and poured gently into the gel mould; solidification process will take 15 minutes).

C) Preparation of 3.0% Metaphor Agarose Gel

1. Metaphor powder : 0.6 g
2. Agarose powder : 0.6 g
3. TBE buffer : 40 ml

(The mixture is microwaved, cooled down, and poured gently into the gel mould; solidification process will take 15 minutes).