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Angiotensinogen Gene M235T Polymorphism and Serum Renin, Aldosterone Hormones Levels among Sudanese patients with Essential Hypertension

التباين في جين الأنجيوتنسين M235T ومستوى هرموني الرينين والألدستيرون في مصل الدم لدى المرضي السودانيين المصابين بإرتفاع ضغط الدم الأساسي

A thesis submitted in fulfillment for M.Sc. degree in Medical Laboratory Science (Clinical Chemistry).

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November 2016

Dedication

To my parents,

to my husband,

to my children,

to my family

and

my colleagues

who all had encouraged me to proceed with the study

Rinan

Acknowledgements

My thanks goes to all whom supported me with this research. Especially those who closely provided me with valuable inputs; Dr. Ghada AElfadil,Dr. Ammar Ismail whom are kindly guide me to accomplish this research and gave very good advice in perfect research methodology and data analysis, Dr. Salah EldeinElzaki, who help me in molecular practical, Mr. Mohamed Karar who help me in ELISA practical, Mrs,Suheer Ramadan in SUST research laboratory . Also I would like to thank my colleagues and partners; Mrs. Lemya Modawi Port Sudan university hospital lab, Dr. Abdelrahman Eltahir who help me to prepared and print my research.

Abstract

Background and objectives: Essential hypertension (EHT) is a major risk factor for several cardiovascular diseases. It is a complex trait resulting from the interactions of multiple genetic and environmental factors. EHT is common in the general population by its increasing incidences, thus recently researchers suggested that Angiotensingen gen (M235T) polymorphism may be the functional genotypes in hypertensive patients. Therefore the aimed of the study to evaluate Angiotensinogen polymorphisms (M235T) as well as its relation to serum levels of renin and aldosterone hormones among Sudanese hypertensive patients.

Materials and Methods: In a case control study, 96 patients with EHT and 79 healthy apparently controls were enrolled. The clinical data were obtained and serum renin and aldosterone levels were measured using ELISA technique. The AGT gene polymorphism (M235T) was identified by PCR–RFLP analysis.

Results: Serum Renin was differ significantly between the hypertensive group (94.3±43.8) Pg/mL and the control group (16.3±14.4) Pg/mL with p=0.00. However, Serum Aldosterone was differ significantly between hypertensive group (26.7±12.9) Pg/mL and the control group (19.5±10.5) Pg/mL with p=0.05.Analysis of allele frequency showed that, MM, MT and TT genotypes of AGT gene polymorphism (M235T) for hypertensive patients were 91.7%, 8.3%, and 0% respectively and that for the normotensive group was 96.2%, 3.8%, and 0%. Statistically no significant difference was found (p=0.219).

Conclusion: The study concluded that there was no association between the polymorphism (M235T) of the AGT gene and EHT, whereas association observed with serum renin and aldosterone levels in hypertensive patients. Also there was significant association between family history and Main Arterial Blood Pressure(MAP) in essential hypertensive patients.

المستخلص

الخلفية والأهداف: يعتبر ارتفاع ضغط الدم الأساسي عامل رئيسي للعديد من أمراض القلب. كما يعتبر نتيجة لتفاعلات معقدة بين العوامل الوراثية والبيئية المتعددة. وعلاوة على ذلك، ليس فقط الجينية، ولكن أيضا الميراث الجيني يلعب دورا هاما. ارتفاع ضغط الدم الأساسي هو شائع في عموم السكان وتتزايد نسبة حدوث المرض سنوياً، اقترح الباحثون مؤخرا أن جين AGT (M235T) في التركيب الوراثي الوظيفي قد يكون سبباً في حدوث ارتفاع ضغط الدم. لذلك هدفت الدراسة إلى تقييم الأنجيوتنسين (AGT) (M235T)، وكذلك علاقته بمستوى هرموني الرينين والألدوستيرون بين مرضى ارتفاع ضغط الدم الأساسي السودانيين.

المواد وطرق: في دراسة مراقبة الحالة، تم اختيار 96 حالة كعينة من الذين يعانون من ارتفاع ضغط الدم الأساسي و 79 حالة كعينة من الاصحاء. تم الحصول على البيانات السريرية وتم قياس مستوى الرينين والألدوستيرون في الدم باستخدام ELISA. تم اكتشاف جين AGT (M235T) للأليلات والأنماط الجينية عن طريق تحليل PCR-RFLP.

النتائج:هناك زيادة كبيرة في كل مستويات هرموني الرينين والألدوستيرون بين مرضى ارتفاع ضغط الدم الأساسي بقيمة أساسية (0.00 و 0.05) =pعلى التوالي مقارنة مع الأصحاء . و أظهر تحليل معامل اختلاف الأليل التالي، نسبة كل من الجينات MM، MT و TT المورثات من مولد الأنجيوتنسين (AGT) والتحول الجيني (M235T) لمرضى ارتفاع ضغط الدم الأساسي 91.7٪، 8.3٪، و 0٪ على التوالي، وكانت النسبة لمجموعة الأصحاء 96.2٪، 3.8٪ و0٪ على التوالي. إحصائياً لم يكن هنالك اختلاف معنوي (p=0.219).

الخلاصة: خلصت الدراسة إلى أنه لا يوجد أي ارتباط بين جين (AGT (M235T وارتفاع ضغط الدم الأساسي، في حين لاحظت الدراسة علاقة بين مستويات هرمون الرينين وهرمون الألدوستيرون في الدم في مرضى ارتفاع ضغط الدم. أيضا كان هناك ارتباط كبير بين التاريخ العائلي للمرض وMAP (ضغط الدم الرئيسي الشرياني) في مرضى ارتفاع ضغط الدم الأساسي.

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List of Abbreviations

ACTH	Adrenocorticotropic Hormone
AGT	Angiotensin
BP	Blood Pressure
cAMP	Cyclic Adonsine Mono Phosphate
cGMP	Cyclic Guanosine Mono Phosphate
EHT	Essential Hypertension
ELISA	Enzyme Linked Immunosorbent Assay
HT	Hypertension
JGCs	Jutaglomerular Cells
MAP	Main Arterial Pressure
Pg/mL	Pico gram per Milliliters
RAAS	Renin Angiotensin Aldosterone System
RAS	Renin Angiotensin System
rpm	Round Per Minute
SD	Standard Deviation
WHO	World Health Organization
µL	Microliters

Chapter One

Introduction

1.1. General introduction

Hypertension (HPT) or high blood pressure, sometimes called arterial hypertension, is a chronic medical condition in which the blood pressure in the arteries is elevated (Chobanian*et al*., 2003). This requires the heart to work harder than normal to circulate blood through the blood vessels. Blood pressure (BP) is summarized by two measurements, systolic and diastolic, which depend on whether the heart muscle is contracting (systole) or relaxed between beats (diastole). Normal blood pressure at rest is within the range of 100-140mmHg systolic (top reading) and 60-90mmHg diastolic (bottom reading). HPT is said to be present if it is persistently at or above 140/90 mmHg. HPT is classified as either primary (essential) hypertension (EHT) or secondary hypertension; about 90–95% of cases are categorized as "primary hypertension" which means HPT with no obvious underlying medical cause (Carreteroand Oparil., 2000).

The remaining 5–10% of cases EHT are caused by other conditions that affect the kidneys, arteries, heart or endocrine system. HPT is a major risk factor for stroke, myocardial infarction (heart attacks), heart failure, aneurysms of the arteries (e.g. aortic aneurysm), peripheral arterial disease and is a cause of chronic kidney disease. Even moderate elevation of arterial blood pressure (APR) is associated with a shortened life expectancy. Dietary and lifestyle changes can improve blood pressure control and decrease the risk of associated health complications, although drug treatment is often necessary in people for whom lifestyle changes prove ineffective or insufficient.Angiotensin is a peptide hormone that causes vasoconstriction and a subsequent increase in blood pressure. It is part of the renin-angiotensin system (RAS), which is a major target for drugs that lower blood pressure (Basso and Terragno., 2001).

Angiotensin also stimulates the release of aldosterone, another hormone, from the adrenal cortex. Aldosterone promotes sodium retention in the distal nephron, in the kidney, which also drives blood pressure up (Williams *et al*., 2008).

AGT is an α-2-globulin produced constitutively and released into the circulation mainly by the liver. Plasma AGT levels are increased by plasma corticosteroid, estrogen, thyroid hormone, and angiotensin II levels. AGT is also known as renin substrate. Human AGT is 452 amino acids long, but other species have AGT of varying sizes. The first 12 amino acids are the most important forimportantactivity. Gene duplication allows diversification by providing redundancy: one gene can mutate and lose its original function without harming the organism. During the process of DNA replication, errors occasionally occur in the polymerization of the second strand (Tewksbury *et al*., 2000).

These errors, called mutations, can have an impact on the phenotype of an organism, especially if they occur within the protein coding sequence of a gene. Error rates are usually very low-1 error in every 10–100 million bases—due to the "proofreading" ability of polymerases (Inoue *et al*., 1997). The relevance of the RAS in arterial pressure (AP) regulation and the association of the T235 allele with higher plasma AGT levels suggested that, rather than serving as a marker for a neighboring gene, molecular variation in the AGT gene could mediate individual predisposition to EHT (Gimenez., *et al*. 1998). It remains conceivable that the T235/M235 (the substitution of threonine [Thr] for methionine [Met] at codon 235) polymorphism affects AGT function indirectly, by affecting protein conformation or glycosylation. There is general consensus that genetic variation accounts in part for individual susceptibilities to EHT. Investigations of the link between AGT gene and EHT illustrate this point (Celerier*et al*., 2000).

1.2. Rationale

Essential hypertension is a major risk factor for several cardiovascular diseases. It is a complex trait resulting from the interactions of multiple genetic and environmental factors. Moreover, not only genetic but also epigenetic inheritance plays a significant role. About 90–95% of cases are categorized as EHT "primary hypertension" which means high blood pressure with no obvious underlying medical cause. Most reports have focused on India, Japanese, North Africa, and Chinese individuals while no published studies on Sudan, a population concerning incidence, prevalence and genetic basis of EHT.

One can speculate that hypertension develops as a consequence of ‘‘errors’’ in the well-coordinated regulatorysystems of blood pressure (KunesandZicha., 2009). Researchers have estimated that raised blood pressure currently kills nine million people every year (WHO, 2013). This study aims to insight the light about this point mutation and its effect on serum renin and aldosterone activity among Sudanese population with EHT.

1.3. Objectives

1.3.1. General Objective

To detect AGT gene (M235T) polymorphism and serum renin, aldosterone hormones levels among Sudanese patients with EHT.

1.3.2. Specific Objectives

1. To estimate serum renin and serum aldosterone in the study group (essential hypertension patients + healthy control).
2. To detect the frequency of AGT gene polymorphism (M235T) in the study group.
3. To compare between AGT genotypes and mean of serum renin, aldosterone hormones levels in the study group.
4. To correlatebetween Main Arterial Pressure (MAP) and family history patient.

Chapter Two

Literature Review

1.4. Hypertension

Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure. It is estimated that hypertension affects 50 million Americans with a prevalence rate of 25–30% in the adult Caucasian population. Hypertension is a polygenic disease, and it has been estimated by segregation analysis and twin studies that approximately, 45% of the inter individual differences in blood pressure (BP) can be accounted by genetic differences (Luft., 2002). In the past two decades, many genes that were implicated in simple (Mundelein) diseases have been identified using genetic linkage and positional cloning methods (Liftone*t al*., 2001).

Although these methods have been remarkably successful in identifying high relative risk genes that have not been successful in identifying genes that are involved in the complex forms of disease such as hypertension and diabetes type II. This failure is the result of three main features of complex diseases. First, such diseases typically vary in severity of symptoms and age of onset, which results in difficulty in defining an appropriate phenotype and selecting the best population to study. Second, they can vary in the etiological mechanisms, which might involve various biological pathways. Third, and perhaps most importantly, complex diseases are more likely to be caused by several and even numerous genes, each with a small overall contribution and relative risk. In addition, hypertension is an arbitrary definition and not a quantitative trait that appears relatively late in life. Nothing is known about the number of genes involved, their mode of transmission, their quantitative effect on BP, their interaction with other genes, or their modulation by environmental factors. Parameters such as ethnicity and body weight increase the genetic heterogeneity and the difficulty of replication from one study to another. Heart disease, stroke, and renal failure are leading causes of death with

hypertension being the predominant risk factor. hypertension diagnosed when blood pressure is ≥ 140/90mmhg or more according to WHO classification (WHO,2003), as five categories: Hypotension (Systolic mmHg 90 or less, or Diastolic mmHg 60 or less), normotensive (Systolic mmHg 90-119, and Diastolic mmHg 60-79), prehypertension (Systolic mmHg 120-139, and Diastolic mmHg 80-89),stage 1 hypertension (Systolic mmHg 140-159, and Diastolic mmHg 90-99),stage 2 hypertension (Systolic mmHg over 160, and Diastolic mmHg over 100). When referring to the causes of high blood pressure, it is divided into two categories (Silverberg *et al.*,2002): EHT (primary high blood pressure) - no cause has been identified and Secondary high blood pressure - the high blood pressure has an underlying cause, such as kidney disease, or a specific medication of the patient is taking. Even though there is no identifiable cause for EHT, there is strong evidence linking some risk factors to the likelihood of developing the condition: Age, family history temperature, ethnic background, obesity/overweight, some aspects of gender in general, physical inactivity lack of exercise, smoking, alcohol intake, high salt intake, high fat diet and mental stress (Nordqvist., 2015).

2.1.1. Normal regulation of blood pressure

To control body blood pressure, must adjust how much blood is pumped through the heart. Your body must also adjust the amount of pressure in blood vessels. Blood pressure begins to fall too low, a signal is sent to brain. Brain responds by producing chemicals called neurotransmitters. Certain neurotransmitters, called catecholamines, cause heart to beat faster and more forcefully and cause blood vessels to tighten. These actions increase blood pressure. The change in the rate and force of your beating heart also causes a change in the amount of blood flowing through kidneys. Your kidneys react to low blood pressure by producing a chemical called renin, which causes blood vessels to tighten. Renin is converted to a chemical called angiotensin II, which further tightens blood vessels. In addition, neurotransmitters are thought to make the kidneys produce more renin, increasing blood pressure even more. Kidneys also regulate blood pressure in another way. When there is not enough fluid in body as in cases where you are dehydrated or have experienced very heavy bleeding, your kidneys will absorb salt, or sodium, and fluid from

your urine. This causes an increase in the amount of fluid in blood vessels and raises blood pressure. This is how the body attempts to keep the blood pressure at a normal level. Blood pressure has to be high enough for enough blood to reach all the organs. In contrast, when there is too much fluid in body, kidneys will flush sodium and excess water out of body in the urine. This reduces the amount of fluid or blood plasma in blood vessels and lowers blood pressure (Elizabeth.,1969).

2.1.1.1. Role of renin in normal regulation of blood pressure

Renin is a central hormone in the control of blood pressure and various other physiological functions. In spite of the very early discovery of renin over 100 years ago, only last years ago gained a deeper understanding of the origin of renin-producing cells and of the mechanisms responsible for renin synthesis and secretion. The main source of renin is the juxtaglomerular cells (JGCs), which release renin from storage granules. Besides the RAS in the JGCs, there exist local RASs in various tissues. JGCs originate in situ within the metanephric kidney from mesenchymal cells that are not related to smooth muscle lineages, as hitherto assumed. The previous notion that JGCs stem from vascular smooth muscle cells may be explained by JGC differentiation: they acquire smooth muscle markers that are maintained throughout adulthood. It has become clear that increasing intracellular free [Ca2+] inhibits renin secretion in JGCs. In contrast, Cyclic Adenosine Monophosphate (cAMP) stimulates renin release. Over the last decade, numerous studies on isolated JGCs and intact animals have provided contradictory results as to whether cyclic guanosine monophosphate (cGMP) has a stimulatory or inhibitory action on renin release. More results strongly suggest that the effects of cGMP on renin release from JGCs involve the degradation of cAMP, which is modulated by cGMP. Finally, it has been found that not only is the production of renin modulated by enhancing or attenuating renin transcription, but renin messenger RNA (mRNA) stability is controlled by various proteins present in renin-producing cells. This hormone enzyme initiates the enzymatic cascade generating the angiotensin peptides that regulate blood pressure, cell growth, apoptosis and electrolyte balance, to mention only some of the foremost-recognized functions. Renin is rate limiting in the production of angiotensin II (Ang II), a hormone that ultimately

integrates cardiovascular and renal function in the control of blood pressure as well as salt and volume homeostasis. Once the RAS is experimentally kept constant, salt sensitivity of blood pressure regulation becomes apparent (Cholewaand Mattson, 2001).

The juxtaglomerular cells (JGCs) constitute the most important source of circulating renin. Generally it is assumed that JGCs are metaplastically modified smooth muscle cells, because adult mammal JGCs contain myofilaments (TaugnerandHackenthal,1989). Besides the circulating RAS, there exist several local ones within various tissues including the heart, brain and adrenal glands. Even though all components of the RAS are expressed in these tissues, the mode of action can be quite different (Peters*and*Clausmeyer.,2002-*a*), as underscored by the remarkable discovery of renin in inclusion bodies of the mitochondria. Although the exact function of mitochondrial renin remains to be fully elucidated, the fact that aldosterone production takes place in adrenal mitochondria indicates a potential role of mitochodrial renin in its control. This functional link between mitochondrial renin and aldosterone has been shown experimentally after bilateral nephrectomy (Peters *et al*., 1999-*b*), where the amount of mitochodria containing renin increases with the augmented aldosterone production. It seems that only a truncated form of prorenin can be imported into the mitochondria. This truncated prorenin is synthesized by an alternative transcript characterized by an alternative exon 1A. Interestingly, this is the only renin transcript expressed in the heart. The classical mRNA coding for preprorenin does not respond to stimuli of the cardiac RAS such as hypertrophy or myocardial ischaemia. Conversely, exon 1A renin transcript increases promptly (Pontus*et al*.,2003).

In addition to renin located in the mitochondria, the cardiac cells can also internalize circulating renin. There are two proposed mechanisms by which this can occur, one by mannose-6-phosphate receptor-mediated endocytosis, the other by the internalization of nonglycosylatedprorenin. The mannose-6-phosphate receptor-mediated uptake may simply constitute a clearance mechanism, i.e. it inactivates the circulating RAS. The second form of internalization may be crucial for the intracardiac RAS and its various functions (Peters and Clausmeyer., 2002-*a*).

The release of active renin from JGCs is considered to be the main rate-limiting step in providing circulating renin. Remarkably, the stimuli that inhibit renin secretion, e.g. increased arterial pressure or Ang II, increase intracellular free calcium. This is in contrast to the other secretory cells of the organism, in which augmented free calcium levels lead to enhanced depletion of secretary granules. Thus, this unique feature of renin secretion is commonly referred to as the calcium paradox. The reason for the opposite effect of calcium on renin secretion can be found in the origin of the renin storage granules that appear to be modified lysosomes .The membrane potential of JGCs acts in concert with the various mechanisms controlling renin secretion. Again, calcium seems to play an important role in this context. For instance, voltage-dependent L-type calcium channels and calcium-sensitive voltage-gated calcium channels have been identified in JGCs. The latter channels are sensitive to cAMP as they belong to the ZERO variant, and play an important role in determining membrane potential. However, they are not directly responsible for renin secretion, as their blockade does not affect renin secretion. Conversely, activating the L-type calcium channels inhibits cAMP-mediated renin secretion, thus providing evidence for a functional link between these channels and the control of renin secretion (Friis*et al.* 2003).

Transcriptional control depends on the DNA located immediately upstream of the gene itself. As for other peptides, transcription of renin RNA requires the binding of RNA polymerase II to the basic promoter region of the gene. For the renin gene, there are additional regulatory elements considerably further upstream of the cap site which activate or repress transcription. These regulatory elements are found in areas where remarkable interspecies homology can occur (Mrowka*et al.* 2003; Persson*et al.* 2003).

Interestingly, vitamin D3 and its receptor seem to play an important role in the complex regulation of renin transcription. The vitamin D receptor belongs to the thyroid hormone (T3) subfamily of nuclear hormone receptor transcription factors. These also include the retinoic acid receptor. The latter shares a binding site with the vitamin D receptor. As an example of the clinical importance of this binding site, it has been shown by observations in patients that there is an inverse relationship between plasma vitamin D3 levels and plasma renin activity as well as with blood pressure. Moreover, it has been known for

some time that vitamin D3 supplementation lowers blood pressure in hypertensive subjects. Thus, the vitamin D3 receptor may constitute an important negative regulator of renin expression (Lind *et al.*, 1989)

2.1.1.2. Role of Aldosterone in normal regulation of blood pressure

Aldosterone is a mineralcorticoid hormone, a type of hormone that is essential to life because it regulates the amounts of electrolytes in the body. Aldosterone is secreted by the adrenal cortex and responsible for the reabsorption of sodium into the bloodstream. Aldosterone also stimulates the excretion of potassium. Aldosteone is the chief regulator of sodium, potassium, and chloride metabolism, thus controlling the body's water and electrolyte balances. The adrenal cortex, where aldosterone is produced, is part of the adrenal gland. Aldosterone regulates sodium and potassium levels in animals, helping to maintain both blood pressure and bodily fluids. If aldosterone levels in the body are out of sync, symptoms can result (Yang and Ma., 2009).

High levels of aldosterone can cause high blood pressure, muscle cramps and weakness. Low levels may indicate disease, such as diabetes. Often, aldosterone levels vary between the sexes and may be affected by the amount of sodium in a person's diet. Women often have significantly higher levels of aldosterone when pregnant. The hormone renin, which is produced in kidney, helps to regulate the release of aldosterone, and renin levels are often compared with aldosterone levels for diagnostic purposes. An aldosterone test may be performed to determine the cause of high or low blood potassium or of certain conditions, such as heart failure or kidney disease. Aldosterone is an important regulator of Na^+ and K^+ transport in the distal nephron modulating the surface expression of transporters through the action of the mineralocorticoid receptor as a ligand-dependent transcription factor. Aldosterone stimulates the rapid activation of protein kinase-based signaling cascades that modulate the genomic effects of the hormone. Evidence is accumulating about the multi-factorial regulation of the epithelial sodium channel (ENaC) by aldosterone (Thomas and Eneaney ., 2008).

Aldosterone is a hormone that increases the reabsorption of sodium and water and the release of potassium in the kidneys. This increases blood volume and therefore, increases

blood pressure. Many drugs, such as spironolactone, lower blood pressure by blocking the aldosterone receptor. Aldosterone is part of the renin-angiotensin system. Jerome Conn first described the syndrome of autonomous and excessive aldosterone secretion or "primary aldosteronism." Aldosterone is a steroid hormone produced by the outer-section of the adrenal cortex in the adrenal gland, and acts on the distal tubules and collecting ducts of the kidney to cause the conservation of sodium, secretion of potassium, increased water retention, and increased blood pressure. The overall effect of aldosterone is to increase reabsorption of ions and water in the kidney (Yang and Ma., 2009).

Aldosterone is an adrenal hormone that regulates sodium, fluid, and potassium balance. Contrary to the historical belief, recent studies indicate that primary aldosteronism is a common cause of hypertension with a prevalence of 5-10% among general hypertensive patients. Various animal models have demonstrated that aldosterone in association with a high salt diet results in target-organ inflammation and fibrosis. Similarly, cross-sectional and observational human studies have demonstrated the association of aldosterone with development and severity of hypertension, congestive heart failure, coronary artery disease, chronic kidney disease, and metabolic syndrome. Several interventional studies have also demonstrated the beneficial effects of mineralocorticoid receptor antagonists in these disease processes, particularly hypertension, heart failure, and post myocardial infarction, further supporting the role of aldosterone in their pathogenesis (Gaddam and Pimenta., 2009).

When sodium intake diminishes, both the kidney and distal colon contribute directly to sodium homeostasis. In response to a diet with low amounts of sodium, the body hormonal profile changes to produce different effects on crypt-colon permeability and absorption and in the pericryptal sheath surrounding distal colonic crypts. This adaptation produces an increase in Na absorption, a decreased crypt-wall permeability, and an activation of the growth of pericryptalmyofibroblasts. The separate roles of the 2 main hormones implicated in the process, aldosterone and angiotensin II, until now have been unclear. Experiments conducted on adrenalectomized rats on low- and high-sodium diets, implanted with osmotic pumps perfusing either aldosterone or angiotensin II, allow us to

discriminate between the effects of these hormones. In the distal colon, aldosterone acts as a trophic agent on the myofibroblasts layer and is the key hormone controlling colonic permeability, but angiotensin II alone has no discernable direct role in the process (Cristia and Moreto., 2007).

Aldosterone plays a pivotal role in sodium and water homeostasis, in particular in patients with heart failure or high blood pressure. These medications, when used on top of a standard therapy, improve the outcome of patients with heart failure and are also effective in lowering blood pressure of hypertensive patients. The major risk associated with the use of these antagonists is hyperkalemia, which can be prevented in avoiding their prescription in patients with impaired renal function. Eplerenone has the advantage, compared with spironolactone, to be better tolerated in terms of "hormonal" adverse effects (Waeber., 2006).

The adrenal cortex is the outer layer of the adrenal gland, a component of the endocrine system of the body which regulates and produces hormones. The inside of the adrenal gland is known as the adrenal medulla or simply medulla. The medulla and the cortex perform very different functions, and each is critical to healthy life. A variety of medical conditions can interfere with the function of the adrenal cortex, including Cushing's syndrome and Addison's disease. Using cholesterol as a base, the adrenal cortex creates a number of compounds with a variety of uses, many of which play a role in metabolism and blood chemistry. The adrenal glands are located on top of the kidneys. The cortex is yellow in healthy individuals, and the gland itself has a star-like shape. There are three separate layers in the adrenal cortex, each of which is responsible for synthesizing different chemicals for use by the body. The cells in each layer have slightly different structures, reflecting their different functions, and the difference can clearly be seen with the assistance of a high powered microscope. On the outside of the adrenal cortex, the zonaglomerulosa makes mineralcorticoids such as aldosterone. The next layer, the zonafasciculata, makes glucocorticoids like cortisol, while the inner layer, known as the zonareticularis, makes androgens such as testosterone. The levels of production are varied, depending on the person and his or her physical condition. Men, for example, tend to produce more testosterone than women, and this hormone plays a critical role in physical

development, and people under stress make more cortisol. Dysfunction in either area of the adrenal gland can lead to a variety of symptoms, including fatigue, weight changes, hirsutism, vomiting, nausea, specific food cravings, hypoglycemia, and low blood pressure. In some cases, multiple parts of the endocrine system are involved, creating a cascading effect as the body's overall hormonal balance is severely disrupted, and in other instances, problems occur with the adrenal gland alone. Patients who suffer from adrenal insufficiency or overproduction have a number of treatment options, depending on the cause of the condition. The corticosteroids are synthesized from cholesterol within the adrenal cortex. Most steroidogenic reactions are catalysed by enzymes of the cytochrome P450 family. They are located within the mitochondria and require adrenodoxin as a cofactor. Aldosterone and corticosterone share the first part of their biosynthetic pathway. The last part is either mediated by the aldosterone synthase (for aldosterone) or by the 11β-hydroxylase (Yang and Ma., 2009).

2.1.2. Hypertension and renin–angiotensin–aldosterone system (RAAS)

The RAAS has drawn substantial attention because of its physiological magnitude in cardiovascular homeostasis and the genes that regulate the system may contribute to the development of hypertension and end-organ damage. At the beginning of the RAAS cascade is renin, a protease syn¬thesized by the kidneys, cleaving the precursor AGT to release angiotensin I. This is further converted into angiotensin II, a potent vasoconstrictor and stimulator of aldosterone release, and initiating a cascade of reactions that produce an elevation of blood pressure and increased sodium retention by the kidney. From the biological and functional points of view, renin exerts a powerful effect on electrolyte homeostasis and blood pressure regulation Thus well-characterizing the role of renin may be a clue to elucidating the genetic makeup of EHT (Mohana*et al*., 2013).

Renin protein and its gene, located at chromosome 1q32, spans 12.5 kb in length and encodes 10 exons.4 Renin, an aspartyl protease, is synthesized primarily w juxtaglomerular (JG) cells of the kidney. Several lines of evidence have shown that renin is also produced in a vari¬ety of extra renal tissues, including adrenal gland, ovary, testis, brain, heart, submandibular gland (SMG) and mac¬rophage/monocyte cells. Synthesis and

secretion of renin by kidney JG cells is up-regulated by cAMP (mainly through beta 1 adrenergic receptor agonism), and down regulated by angiotensin II and intracellular calcium, in response to salt load, renal perfusion pressure, noradrena¬line and renal prostaglandins. Human renin is secreted by at least two cellular pathways: a constitutive pathway for the secretion of prorenin and a regulated pathway for the secretion of mature renin. The precursor of the renin gene consists of 406 amino acids with a pre- and a pro-segment carrying 20 and 46 amino acids respectively. Mature renin contains 340 amino acids and has a mass of 37 kDa(Mohanaet al 2013).

Identification of molecular factors that influence the sus¬ceptibility of an individual to EHT is one of the main issues to address in the management of cardio¬vascular diseases. In this context the RAAS is considered as an important component of blood pressure regulation, maintaining sodium homeostasis and modulating vascular tone and cardiovascular structure.Animal (such as rat) model studies have demonstrated that variations in the renin gene affect blood pressure and that the renin gene is directly involved in the development of EHT. In humans also association between REN gene polymorphisms and EHT have been studied extensively based on linkage and sib-pair linkage analysis and association studies. Individual REN poly¬morphisms have associated with plasma renin activity (PRA), BP and susceptibility to hypertension in a variety of ethnic groups, although often with incon¬sistent results. Structure and is poor so the electrolyte feedbacks predominate short term (Mohanaet al 2013).

2.1.3. Role of AGT in Hypertension

The RAS plays an important role in the regulation of BP. The octapeptide, angiotensin II (Ang II), is one of the most active vasopressor agents and is obtained by the proteolyticcleavage of a larger precursor molecule AGT, which is primarily synthesized in the liver and to a lesser extent in the kidney, brain, heart, adrenal, fat, and vascular walls. The human AGT cDNA is 1455 nucleotides long and codes for a 485- amino acid protein (Kageyama et al 1984). AGT is first converted by renin to produce a decapeptide, Ang I, and is then converted to Ang II by the removal of a C-terminal dipeptide by ACE (Corvol et al 1997). In experimental as well as clinical studies, chronic administration of renin-

angiotensin inhibitors has proven effective in lowering BP in hypertension. Genes that encode components of the RAS are therefore potential candidate genes that may play a role in the regulation of BP. The plasma concentration of AGT is close to the Michael is constant of the enzymatic reaction between renin and AGT For this reason, a rise in plasma AGT levels can lead to a parallel increase in the formation of Ang II that may ultimately result in hypertension (Gould et al 1970).

2.1.4. Aldosteronism and Hypertension

Historically, primary aldosteronism has been thought to be an uncommon cause of hypertension. Recent studies, however, suggest that 10 to 15% of individuals with hypertension fulfill the biochemical criteria for primary aldosteronism. Demonstration of such a high prevalence of primary aldosteronism in patients with presumed primary hypertension suggests that aldosterone excess is a common contributing cause to the development of hypertension. Primary aldosteronism is particularly common in patients with resistant hypertension, with prevalence of approximately 20%. An important role of hyperaldosteronism in contributing to the development of treatment resistance is suggested further by the broad antihypertensive benefit of aldosterone antagonists as add-on therapy in patients who are resistant to multidrug regimens. The anti-hypertensive benefit in this setting is not limited to patients with classically defined primary aldosteronism, suggesting aldosterone excess as a cause of resistant hypertension to a degree that is greater than is indicated by measurement of either plasma or urinary aldosterone levels (David et al 2006).

Multiple, independent studies suggest that aldosterone contributes broadly to the development of hypertension separate from cases of classically defined primary aldosteronism. The role of aldosterone in causing hypertension is supported by cross-sectional studies that relate plasma aldosterone levels to ambulatory BP measurements, by prospective analyses that indicate that aldosterone levels predict development of hypertension, and by studies that confirm the broad antihypertensive efficacy of aldosterone antagonists in treating presumed primary hypertension. Importantly, in these studies, indices of renin activity were not related to BP levels, suggesting an autonomous

role of aldosterone in causing hypertension that is independent of renin–angiotensin II. Cross-sectional studies demonstrate a significant correlation between plasma aldosterone and 24-h ambulatory BP levels. The relation is particularly strong in black individuals. In an analysis of black American and white French Canadian patients with primary hypertension, supine and standing plasma aldosterone levels were significantly correlated with daytime and nighttime systolic and diastolic BP levels in the black patients (El-Gharbawy*et al*., 2001 andGrim*et al*., 2005).

Primary aldosteronism as described by (Conn.,1955) had been thought to be an uncommon cause of hypertension, with a prevalence of _1% among general hypertensive patients(Kaplan.,1969). However, beginning in the early 1990s with reports from Gordon and associates in Brisbane, Australia, the prevalence of primary aldosteronism has been found to be considerably higher. In the earliest study, Gordon*et al., 1993-a).* Screened 52 hypertensive individuals who responded to a newspaper advertisement for participation in a hypertensive drug trial and found that 12% of the individuals were positive for primary aldosteronism. In a subsequent evaluation of 199 individuals who were referred to the hypertension clinic in Brisbane, the prevalence of primary aldosteronism was found to be at least 8.5% and probably 12% (Gordon et al .,1994-b). These results were remarkable in suggesting that primary aldosteronism was not rare but rather a common cause of hypertension. Since these earlier studies by Gordon, multiple investigators worldwide have confirmed a prevalence of primary aldosteronism of 5 to 15% in general or selected hypertensive populations (Nishizaka*et al*., 2005)

2.1.5. Hypertension with family history and race

Hypertension is a major risk factor for heart disease and stroke, the first and third-leading causes of death in the United States, the prevalence of hypertension is 50% greater in blacks than in whites. Both genetic and environmental factors may contribute to the higher prevalence of hypertension in blacks. In addition, a genetic contribution to the increased prevalence of renal disease among hypertensive blacks has been proposed on the basis of family studies and studies of associated histocompatibility antigens. This review discusses the magnitude of the problem, its epidemiology, and the evaluation and management of

hypertension as recommended by the reports of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. Activities related to the control of this disorder are also highlighted. Data from the Third National Health and Nutrition Examination Survey, 1998-1994, (NHANESIII) suggest approximately three-quarters (75%) of Black hypertensives are aware of their diagnosis, but only 57% are treated and just 25% have their blood pressure under control (<140 mm Hg systolic and <90 mm Hg diastolic). Although substantial evidence indicates a significant increase in awareness of hypertension over the past three decades, control rates are remarkably low, particularly among Blacks. This review serves to emphasize and reiterate the burden of hypertension among Blacks and acts as a reminder of the need for additional research to determine if culturally competent interventions are appropriate to prevent, treat, and control this disease within this population (Ashayeand Gile.,2003).

Numerous researchers have found that genetic factors play an important role in hypertension. Patients who had family history (FH) of hypertension would have a 2 to 4 fold higher risk of getting this disease (Winnicki et al 2006). Also, there were studies found that the prevalence of hypertension increased with the number of affected relatives. Other studies showed that mothers with hypertension contributed more than fathers, (Goldstein et al 2006) and first-degree relatives with hypertension were linked to higher risk of getting hypertension, compared with second-degree relatives (Ranasinghe et al. 2015).

The prevalence of hypertension was mainly from the American and European countries. There was noliterature for the related research about Chinese people, who are undergoing huge social-economical changes and rapidly increasing prevalence of hypertension. The study aims to examine the association of FH risk categories and prevalence of hypertension in a rural community elderly population of Beijing, China, and to provide evidence for different FH categories and its influence on prevalence of hypertension. The study, which was conducted in a representative metropolitan area of rural Beijing, had trict training process and high response rate. The results showed that FH risk categories of first-degree relatives are strongly associated with the prevalence of hypertension and blood

pressure level, even in the normal range. Most studies have revealed the association between FH and hypertension; but the study did an in-depth study of the number of first-degree relatives affected, and showed not only positive or negative but also different FH risk categories are related with prevalence of hypertension. Third, explored the gender difference between the association of FH risk categories and hypertension, and a stronger association was revealed in women (Miao et al 2015).

2.1.6. M235T polymorphism of AGT gene and hypertension

The AGT gene contains five exons and four introns, which span 13 kb (Gaillard *et al*., 1989) An extensive study of the potential role of the AGT gene in human EHT was performed on two large series of hypertensive siblingships yielding 379 sibling pairs. The highly polymorphic CA dinucleotide repeat marker located in the 3'-region of the AGT gene and the powerful affected sibling pair methodology were used to obtain evidence of a genetic linkage between the AGT gene and hypertension in this study (Jeunemaitre*et al*., 1992-*a*). A 17% excess of AGT allele sharing was found in severely hypertensive sibling pairs. Whereas significant linkage was obtained in male pairs in both the Utah and Paris groups, no excess of shared AGT alleles was observed in female subjects, suggesting the influence of an epistatic hormonal phenomenon. These studies also showed that variants 235T and 174M of the AGT gene are associated with hypertension. From these studies, it was estimated that mutations at the AGT locus might be a predisposing factor in at least 3–6% of hypertensive individuals younger than 60 years of age in the Caucasian population. When the same methodology was used to analyze the same hypertensive sibling pairs from Utah or Paris, it showed no linkage with other genes of the renin system: namely renin, ACE, or Ang II type I receptor (Bonnardeaux*et al*., 1994). Two other linkage studies also indicate a relationship between the AGT locus and hypertension. This linkage was observed in the subgroup of patients with diastolic pressure above 100 mmHg, but there was no difference among female–female pairs. However, there was no association between hypertension and the 235T AGT variant in this population (Caulfield et al 1996). The same group also found linkage and association of the AGT locus with high BP in 63 affected sibling pairs of African-Caribbean origin, suggesting some similarity in the

genetic basis of EHT in populations of different ethnicity (Caulfield *et al*., 1996). The corroboration of these linkage studies indicate that molecular variants of the AGT gene, such as M235T, or those in linkage disequilibrium with this variant are inherited predispositions to EHT in humans. However, results of these studies must be interpreted with caution for several reasons. First, the frequency of the 235T AGT allele varies in different ethnic groups. The 235T allele is more frequent in the Asian than in the Caucasian population and is by far the predominant allele in the African population. As a consequence, positive results may arise from population admixture, and negative results in populations in which this allele is predominant may result from the constraint of limited statistical power. Second, differences in the design of each study and the choice of the control group make any overall comparison difficult. Finally, most of the results reported to date have been obtained with relatively small numbers of patients, which can also generate false negative or false positive results. There are divergent results on the association between variants in AGT at position 235 (235T) or 174 (174M) and hypertension in Caucasians. In their original study, Jeunemaitre et al. found that the M235T variant was more frequent in hypertensive probands from Utah and Paris, especially in the more severe index cases (Jeunemaitre*et al* 1992-b). A subsequent study by the same group on 136 mild to moderate hypertensive subjects also found that the frequency of the 235T allele was increased, although the increase was significant only for patients with a family history of hypertension. Schmidt also found a higher frequency of the 235T allele in subjects with hypertension, a family history of the disease, and early onset of hypertension (Schmidt *et al*., 1995). However, other studies have not found any association between the 235T variant and hypertension. Hingorani et al. found no difference in the frequency of M235T in 223 hypertensive subjects and 187 normotensive individuals in Anglia in the United Kingdom (Hingorani*et al*., 1996). A more powerful study performed in Finland by Kiema gave negative results with 508 mild hypertensives and 523 population-based controls (Kiema*et al*., 1996). On the other hand, four large studies recently showed a positive association between the M235T allele and EHT. An association with severe hypertension with stronger relationship for men than for women was found in a sample from the Framingham Heart study and from the Atherosclerosis

Risk in Community (ARIC) study, when the effect of body mass index and triglycerides were taken into account (Boreckiet *al*., 1997).

The proportions of cases attributable to the 235 allele were 8% in the ARIC population and 20% in the Framingham population. In a study testing a large number of AGT alleles, the frequency of the 235Tallele was 0.47 in the 477 probands of hypertensive families and 0.38 in the 364 Caucasian controls (Jeunemaitreet *al*., 1997-*c*). In a cross-section sample of 634 middle-aged subjects from the MONIC Angsburg cohort, Schunkert found that individuals carrying at least one copy of the T235allele had high SBP and diastolic BP(DBP)s and were more likely to use antihypertensive drugs (Schunkertet *al*., 1997). Finally, another case-control study involving 802 hypertensive subjects and 658 Caucasian controls has shown a significant increase in the frequency of the T235 allele in men and in women whose hypertension was diagnosed before they were 45 yr of age (Tiretet *al*., 1998). These studies emphasize the importance of sample size testing for susceptibility locus in a complex disease such as hypertension (Kunz *et al*., 1997).The frequency of the T235 allele is invariably high in the Japanese population, ranging from 0.65 to 0.75. An association between hypertension and molecular variants of AGT in the Japanese population has been found in several independent studies (Iwai *et al*., 1995). All of the studies reported so far indicate that frequency of the 235T variant is higher in Japanese hypertensive subjects. A more homogeneous genetic and environmental background may explain why the results reported for Japanese populations are more uniform. However, (Ishigamiet *al*., 1997) showed that the –20C variant of the human AGT gene plays an important role in hypertension in Japanese population. Walker et al. Showed a remarkably high correlation between plasma AGT concentration and elevated BP ($p < 0.0001$) in a large study involving 574 black subjects (Hunt *et al*., 2002). (Bloemet *al*., 1997) showed that (1) plasma AGT level is about 19% higher in black children as compared with white children; and (2) BP is normally higher and increases faster over time in black children as compared with white children. (Caulfield *et al*., 1995) found an association between the AGT gene locus and high BP in 63 affected sibling pairs of African-Carribean origin using CA dinucleotide marker. However, these workers could not find an association between variants M235T and hypertension in the African-American population. Various studies

have suggested that although the frequency of 235T allele is increased in the African-American population (0.8–0.9), there is no association between 235T allele and hypertension in this population (Larson *et al.*, 2000).

Chapter Three

Materials and Methods

2.1. Study design

This is an analytical case-control, hospital based study, which was carried out during the period from June 2013 to June 2016.

2.2. Study area

The study was carried out in the "Red Sea" State at Port Sudan University Hospital.

2.3. Study population

The current study included ninety six patients who were diagnosed as EHT (newly discovered) according to WHO criteria and, seventy nine healthy subjects were included as control group. Sex and age were matched.

2.4. Inclusion criteria

Study group: Sudanese patients who were diagnosed with EHT.

2.5. Exclusion criteria

Patients with clinical history of diabetes, renal disease, cardiac disease, secondary hypertension and non-Sudanese nationality were not included in this study.

2.6. Ethical considerations

The study approval was obtained from the authorities of Port Sudan University Hospital, Port Sudan, Sudan. The objectives of this research were explained to all participants in this study and a verbal informed consent was obtained (Appendix I).

2.7. Data collection

First: An interview to obtain the clinical data was done for each participant in this study and a questionnaire (Appendix II) was specifically designed by the researcher to obtain data which help in either including or excluding certain individuals in or from the study, respectively.

Second: Clinical history and examination of the test group and the controls were done by physicians to help in exclusion or inclusion of the study subject.

2.8. Sampling

Under aseptic condition, a venous blood sample (5 ml) was collected from each participant, and then divided into 2.5 mL in EDTA container for DNA extraction, and 2.5 ml in a plain container, for measurement of serum rennin and serum aldosterone levels. Serum was obtained by centrifugation at 3000 rpm for 5 minutes, collected and stored ineppendorf tube tubes at - 20°C till used.

2.9. Laboratory investigations

3.9.1. Serum rennin levels

The DEMEDITEC Renin ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle.

3.9.1.1. Principle

The microtiter wells are coated with a monoclonal (mouse) antibody directed towards a unique antigenic site of the human active Renin molecule. An aliquot of patient sample containing endogenous. Renin is incubated in the coated well together with Assay Buffer. After incubation, unbound components are washed off. Finally, Enzyme Conjugate, which is a monoclonal anti-Renin antibody conjugated with horseradish peroxidase, is added, and after incubation, unbound enzyme conjugate is washed off. The amount of bound peroxidase is proportional to the concentration of Renin in the sample. Having added the substrate solution, the intensity of colour developed is proportional to the concentration of active Renin in the patient sample (Carey andPadia., 2008)**.**

3.9.1.2. Procedure

Brief according to manufacturer, all reagents and samples were allowed to reach room temperature. The desired numbers of microtiter wells were secured in the frame holder and 150µl of the assay buffer were dispensed in all wells, then 50 µl of each standards, controls and samples were added into appropriate labeled well and then incubated for 90

minutes at room temperature after 700rpm shaking. Microtiter plate was washed 3times with 300 µl diluted washing solution, enzyme conjugate (100 µl) was added into each well and incubated for 90 minutes at room temperature on a plate shaker with 700rpm. After the incubation time was completed the microtiter plate was washed with 300 µl diluted wash buffer, then100 µl of substrate solution was added to each well and incubated for 15 minutes at room temperature. In order to stop the enzymatic reaction 100 µl of stop solution was added to each well. Finally the optical density of each well was measured at 450nm, using microtiter plate reader within 10 minutes. Results were calculated from blotting standards (Appendix III).

3.9.2. Serum Aldosterone levels

Serum Aldosterone Activity was measure by Aldosterone ELISA DE4128 Kit, enzyme immunoassay test followed the typical competitive binding scenario principle.

3.9.2.1. Principle

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in Calibrators, control and patient samples) and anenzyme-labeled antigen (conjugate) for a limited number of antibody binding sites on the micro well plate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a micro titer plate reader. The intensity of the color formed is inversely proportional to the concentration of aldosterone in the sample. A set of Calibrators is used to plot a Calibrator curve from which the amount of aldosterone in patient samples and healthy controls can be directly read.(Cartledgeand Lawson., 2000).

3.9.2.2. Procedure

In brief according to manufacturer, all reagents and samples were allowed to reach room temperature. Each of standard, control and specimen sample into correspondingly labelled wells in duplicate 50 µl was added. Conjugate working solution into each well 100 µl was

added. Putted on a plate shaker 200 rpm for 1 hour at room temperature was incubated. Washed the wells 3 times with 300 µl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry. TMB substrate into each well 150 µl was added at timed intervals. Incubated on a plate shaker for 15 minutes at room temperature. Then added stop solution into each well by 50 µlthen incubateded for 15 minutes at room temperature. The plate on a micro well plate reader at 450nm within 20 minutes after addition of the stopping solution was read. (Appendix IV). The test levels have been calculated automatically using a 4 Parameter Logistics curve fit. The concentration of the samples can be read directly from the standard curve.

3.9.3. DNA Extraction

Genomic DNA was extracted using DNA Pure Link™ Genomic DNA Kits.

3.9.3.1. Procedure

About 2.5 ml of blood K2EDTA centrifuged at 3000 rpm for 5 minutes (eppendrof 5430). Buffy coat was collected carefully. Blood Lysate was prepared with 200 µL frozen Buffy coat sample was added to a sterile 1.5 microcentrifuge tube .20 µL Proteinase K and. 20 µLRNAase was added mixed well by brief vortexing, and incubated at room temperature for 2 minutes. 200 µLPureLink® Genomic Lysis/Binding Buffer was added mixed well by brief vortexing and Incubated at 55°C for 10 minutes. 200 µL 100% ethanol was added to the lysate. Mixed well by vortexing for 5 seconds.

Centrifuged the column at 10,000 rpm for 1 minute at room temperature then the collection tube was discarded. 500 µL Wash Buffer 1 was added and centrifuged in Spin column at room temperature at 10,000 rpm for 1 minute then discarded the collection tube. 500 µL Wash Buffer 2 was added and centrifuged the column at 100,000 rpm for 3 minutes at room temperature then discarded collection tube, placed the spin column in a sterile 1.5-mL micro centrifuge tube. Added 100 µL of Pure Link® Genomic Elution Buffer to the column. Incubated at room temperature for one minute. Centrifuged the column at 100,000 rpm for one minute at room temperature. Removed and discarded the

column the tube contains purified genomic DNA. The purified stored DNA at –20°C till use (appendix V).

3.9.4. Principle of Gel Electrophoresis

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. (Kryndushkin*et al*., 2003). Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive)pole. The migration flow is determined solely by the molecular weight where small weightmolecules migrate faster than larger ones. In addition to sizeseparation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step forfurther purification of a band of interest.The ethidium bromide dye in the gel intercalates between the base pairs of the dsDNA resulting in an intense orange fluorescence. This fluorescence may be visualized as bands, under UV light; at the point the DNA has migrated (Sharp *et al*., 1973) (Appendix VI).

3.9.4.1. Procedure of Gel Electrophoresis

Three micro litters of extracted DNA was mixed with 2µL (6X) loading dye and loaded on 1.5% Agarose gel. The gel was prepared by dissolved 1.5 gram of agarose (promega, USA) in 100ml (1X) tris borate EDTA (TBE) and heated using a microwave oven. Electrophoresis was performed using (1X) TBE buffer at 100 volt at constant voltage, for 20 minutes (Appendix VI). The gel was stained with 4 µL ethidium bromide (0.5 µg/ mL) and visualized by UV transillminator. The gel was photographed by gel documentation system. The size of the DNA bands was determined by comparing with 1 Kb DNA ladder (Appendix VII).

3.9.5. PCR amplification

Polymerase chain reaction (PCR) was used to amplify a 303 base pair fragment in AGT gene (exon 2) in chromosome (1) using synthetic olignucleotide primers complementary to the DNA sequence of these regions. The forward primer strand5′-GAT GCG CAC AAG GTC CTG TC-3′ 3’, and-reverse-strand 5′-CAG GGT GCT GTC CAC ACT GGA CCC C-3′ (Laura et al., 2008) appendix were synthesized by the Trilink Biotechnology (San

Diego California, USA). In Mini-V/ PCR TELstar under sterile condition amplification was performed in 25µL a total reaction volume. Each reaction 25µL PCR reaction contained 5.5µL nuclease free water,12.5 green master mix (promga) ,1 µL from each primer and 5 µL from genomic DNA in 0.2 sterile PCR tube and mixed well then loaded in thermocycler BIO-RAD.

PCR was carried out under the following conditions: denaturing at 94ºC for 2min, followed by 30 cycles at 94ºC for 20 s, annealing at 57ºC for 30 s, extension at 72ºC for 30 s, and a final extension at 72º C for 5 min. The amplified fragments of 303bp electrophoresis on 1.5% Agrose gel, and visualized under UV transilluminator. (Appendix VIII).

3.9.6. Restriction fragment length polymorphism (RFLP)

A standard restriction enzyme analysis consist of the following component to a final volume of 50 µL; 10 µL of PCR product, 1µL (10 IU) restriction enzyme PflFI (New England Biolabs, UK), 5 µL of 1X buffer and 34 µL from DW. The mixture was incubated at 65C° for 1 hour (AppendixIX).

3.9.7. RFLP product gel electrophoresis

After incubation was completed the RFLP product was seperated out in 2 % Agarose gel electrophoresis with 1X TBE buffer and 5 µl ethidiumbromide staining. 20 µL of RFLP products was disposed in the wells. 5 µL molecular weight markers 100 bp also was disposed in one well. A voltage of 120 volts was applied during 30 min of the run. Gel was observed under UV and the size of RFLP product was compared with the molecular marker and photographed.

2.10. Quality control

The precision and accuracy of all methods used in this study were checked each time a batch was analyzed by including commercially prepared control sera.

For PCR reaction positive and negative control were run parallel to each test sample. Positive control was performed by applying all reaction mixture except for the template

DNA, and used other known DNA template. Negative control was performed by applying all reaction mixture except for the template DNA to ensure the amplification quality.

2.11. Statistical analysis

SPSS software (version 16) was used for analysis of clinical variables. Descriptive statistics were used to analyze all study variables such as the demographic characteristics data were summarized as mean±SD or present. Student's *t* test was used for comparison of mean concentration of study parameters. Correlation analysis between quantitative study parameters. Genotype and allele frequencies in control and EHT groups were compared by Chi-square ($\chi 2$) analysis. Statistical significance was accepted at *p*-value ≤ 0.05.

Chapter Four

Results

4.1Demographic data

This is an analytical cross sectional, hospital based study, which was carried out during the period from June 2013 to June 2016 in the "Red Sea" State. The current study included ninety six patients who were diagnosed as essential hypertension and seventy nine apparently healthy subjects as control group. Both sex and age were matching in study group.(Table 4.1)

Table 4.4: Anthropometric and biochemical parameters in patients versus control subjects

Characteristic	Hypertensive group (n=96)	Control group (n=79)	P value
Age/ years	52.98±9.9	50.19±11.5	0.09
Height (m)	1.6±0.09	1.4±0.3	0.000*
Weight (kg)	73.5±15	65.1±12.9	0.000*
Body mass index (kg/m^2)	27.7±4.9	24.4±5.1	0.000*
Systolic blood pressure (mmHg)	139.43±20.39	114.43±7.97	0.000*
Diastolic blood pressure (mmHg)	83.96±10.59	75.95±6.10	0.000*
Mean arterial pressure (MAP)	102.78±12.62	88.76±6.01	0.000*
Serum Renin (Pg/mL)	94.3±43.8	16.3±14.4	0.000*
Serum Aldosterone (Pg/mL)	26.7±12.9	19.5±10.5	0.05*

*****Result express as mean ±SD, and significant differences considered as *P* value ≤ 0.05

Table (4.1) showed demographic variables among participants. The mean age among the hypertensive group was (52.98 ±9.9) years, while the mean age among the normotensive was (50.19±11.5) years, with a statistically insignificant difference (p=0.09). The height mean among the hypertensive group was (1.6±0.09) meter, and (1.4±0.3) meter among the control group, with a statistically significant difference (p<0.001). The mean weight among the hypertensive group was (73.5±15) Kg, and (65.1±12.9) Kg for the control group, with a statistically significant difference (p<0.001).Body mass index was differed significantly between the hypertensive group (27.7±4.9) and the control group (24.4±5.1) with p<0.001. Systolic blood pressure was differed significantly between the hypertensive group (139.43±20.39) mmHg and the control group (114.43±7.97) with p<0.001. Diastolic blood pressure differed significantly between the hypertensive group (83.96±10.59) mmHg and the control group (75.95±6.10) with p<0.001. Mean Arterial Pressure (MAP) was differ significantly between the hypertensive group (102.78±12.62) and the control group (88.76±6.01) with p<0.001.Serum Renin was differed significantly Pg/mL between the hypertensive group (94.3±43.8) Pg/mL and the control group (16.3±14.4) Pg/mL with p<0.001. However, Serum Aldosterone was differed significantly between hypertensive group (26.7±12.9) Pg/mL and the control group (19.5±10.5) Pg/mL with p=0.05.

4.2 Relation between gender and study group:

In this study gender showed statically insignificant associated in both patients and healthy controls (Table 4.2)

Table 4.5: distribution of patients and healthy controls according to gender

Gender	Hypertensive group (n=96)	Control group (n=79)	P value
• Male	51.0% (n=49)	43.0% (n=34)	0.291
• Female	49.0% (n=47)	57.0% (n=45)	

Table (4.2) shows gender distribution with 51.0% males in hypertensive group vs 43.0% in control group. While 49.0% of females were in the hypertensive groupvs 57.0% females among the control group, with a statistically insignificant difference (p=0.291).

4.3 Age group distribution in patients and healthy control

The study showed the highest age group in patients and healthy control. Figure (4.1)

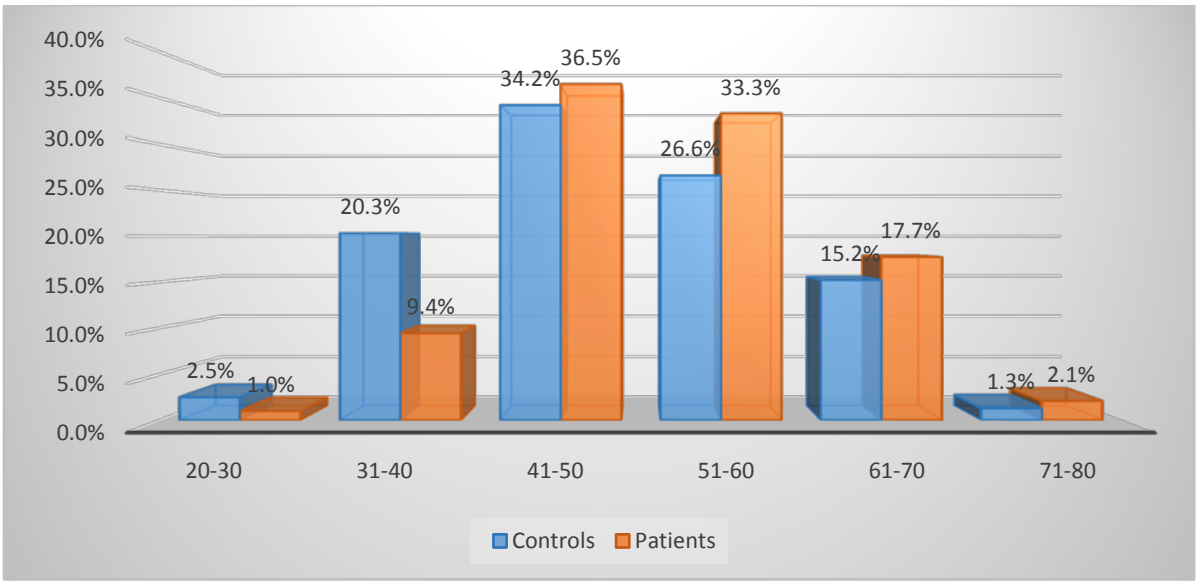

Figure 4.1distribution of patients and healthy controls according to age group.

The age were grouped into six categories from (20-30) year to(71 -80) years, with mean age of (52.98± 9.9) years in the EHT patients group(50.19 ±11.5) years in the controls .

The highest age range of (51-60) years has the highest frequency in the patientsthan in the controls group.

4.4 Family history and study group

The study showed significant differences in study group according family history, and also patients group with mean arterial pressure.(Table 4.3) and (Table 4.4).

Table 4.6: Distribution of the study group according to family history

Study Groups	Family History		Chi - Square	P Value
	No	Yes		
Hypertensive patients	42.7% (N= 41)	57.3% (N=55)	21.224	*0.00
Normotensive group	77.2% (N=61)	22.8% (N=18)		

Table (4.3) shows 57% (55/96) of the patients had positive family history (first degree relative),while 22.8% (18/79) in the control grouppositive family history(second degree relative) .There were significant correlation between family history and EHT occurrence.

Table 4.4: Comparison between MAP and family history in EHT patients

Family history	**MAP** Mean ±SD	**P value**
Yes	115.3±11.4	0.031*
No	100.2±13.1	

***** significant differences considered as *P* value ≤ 0.05

4.5 Genotypes and allelesin the study group

Study showed the distribution of genotypes and alleles among study group. (Figure 4.2) and (Table 4.4)

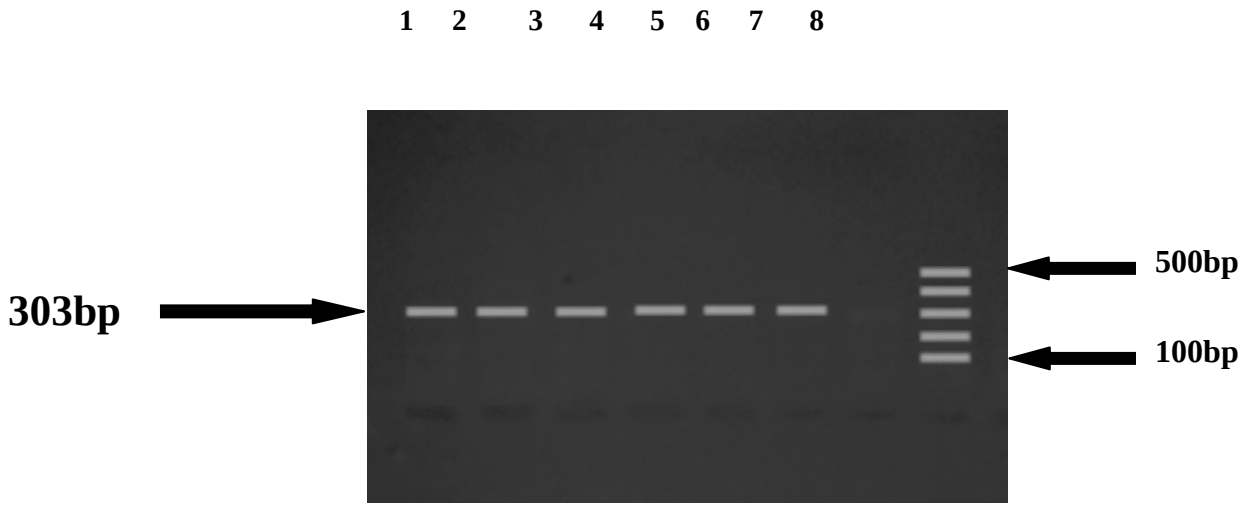

Figure 4.2:2% Agarose gel electrophoresis illustrating the amplification of 303 bp PCR product of AGT, Lanes (1,2,3,4,5) Samples PCR product, Lane(6) Positive Control, Lane(7) Negative Control, Lane (8) a100 bp DNA ladder (Solis BioDyne).

Table 4.5: AGT genotypes and allelic distribution among study group

Study Group	**Genotype distribution**			**Allele Frequency**		**P value**
	MM (n)%	MT (n)%	TT (n)%	M (n)%	T (n)%	0.219
Normotensive	76 96.2%	3 3.8%	0 0%	79 96.3%	3 3.7%	
Hypertension	88 91.7%	8 8.3%	0 0%	96 92.3%	8 7.6%	

MM: wild type genotype; TT: homozygous genotype; MT: heterozygous genotype .$p \leq 0.05$, statistical significant

4.6 Renin and aldosterone hormones levels and study group genotypes

The study showed statically associated between renin and aldosterone levels among study group genotypes.(Figure 4.3) and (Table 4.5).

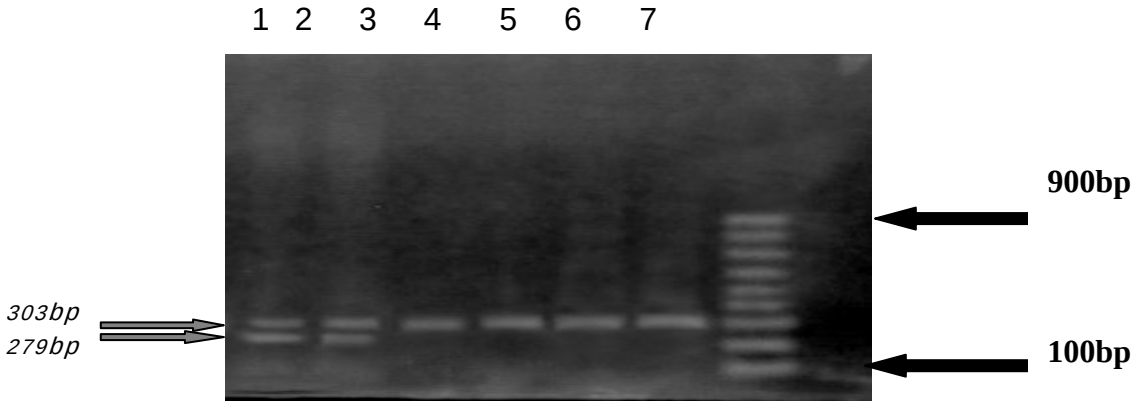

Figure 4.3:2% Agarose gel electrohoresis illustrating the amplification of 303 bp in the AGT gene and enzymatic digestion of this fragment with PflFI restrictions endonuclease enzyme. Lanes (1, 2, 3, 4, 5, 6) correspond to RFLP pattern.

Lane:

- heterozygous (MT), (fragments of 303bp and 279bp) lanes (1,2)
- homozygous wild-type (MM), (undigested fragment of 303bp) lanes (3,4,5,6)
- 100 bp DNA ladder (Solis BioDyne) lane (7).

Table 4.6: Comparison between mean of serum renin and Aldosterone level in hypertensive patients according togenotypes

Parameters	Genotype MM mean ±SD	Genotype MT mean ±SD	p-value
Serum renin	95.5±45.7	82.1±12.4	0.184
Serum Aldosterone	26.8±13.4	25.3±4.30	0.632

significant differences considered as p-value ≤0.05.

5. Chapter Five

5.1 Discussion

Essential hypertension (EHT) is a major risk factor for several cardiovascular diseases which was already affects one billion people worldwide, leading to heart attacks and strokes. It is a complex trait resulting from the interactions of multiple genetic and environmental factors. Moreover, not only genetic but also epigenetic inheritance plays a significant role. One can speculate that hypertension develops as a consequence of ‘‘errors’’ in the well-coordinated regulatory systems of blood pressure (Kunes*et al*., 2009). Researchers have estimated that raised blood pressure currently kills nine million people every year (WHO, 2013). The National Institutes of Health (NIH) estimates that about two-thirds of people over the age of 65 in the USA have high blood pressure. In the UK, The National Health Service, estimates that about 40% of British adults have the condition (Nordqvist., 2015).

This study aimed to assess serum renin activity and aldosterone activity as well as detection of polymorphism (M235T) in AGT gene among Sudanese patients with EHT.

The current study showed that serum renin was significantly higher among the hypertensive group compared with normotensive group (94.3±43.8)pg/mLvs.(16.3±14.4)pg/mL, respectively. This finding is in agreement with that reported in Australia by Gordon, who found a positive correlation between blood pressure measurements and renin concentrations among human beings as well as in experimental animals. This positive correlation between renin substrate and high levels of blood pressure suggests that increased renin substrate concentration(1500 to 2000) ng/ml, may be a causal factor in cases of hypertension (Gordon, et al.1983).

The study showed that mean of serum aldosterone levels was higher among essential hypertensive patients compared with those in the normotensive group

(26.7±12.9)pg/mLvs.(19.5±10.5)pg/mL, respectively). However, differences between both groups statistically significant. This finding is in accordance with Calhoun who stressed noted that there is a strong evidence that suggests that high serum aldosterone levels significantly contributes to the development and severity of hypertension as well as the resistance to antihypertensive therapy(Calhoun., 2006).

The distribution of M allelein AGT gene among normotensive and hypertensive were 96.3% (n=79) and 92.3% (n=96) respectively, while frequency of T allele was 3.7% (n=3) and 7.6% (n=8) for normotensive and hypertensive groups respectively, with no statistically significant differences. The study also showed that the frequency of MT genotype among the normotensive and hypertensive groups was 3.8% (n=3) and 8.3% (n=8) respectively, while the frequency of TT genotype among the normotensive and hypertensive groups was 0.0% (n=0) in both groups. This finding is similar to that study by Reiter in USA who showed (M235T) polymorphism was statistically insignificant with EHTwhich agreed with those(Rotimi*et.al.*, 1994),(Caulfiel*et al.*,1995) and (Whitfield et al.,2009)In the same lack of association between the M235T polymorphism and hypertension was seen (Reiter *et al.*, 2015).

Also the finding agreed with Mariam et.al. who also stated that there was no significant association between EHT and AGT MT and TT genotypes in England and Caucasians, in Mongolian population, Lebanese population and in India. It has been suggested that the population heterogeneity in the association of AGT (M235T) polymorphism with EHT may be due to significant variations of population backgrounds (Mariam et.al. 2015).

The current study disagreed with, Mariam et al who evaluated the frequency of AGT (M235T) polymorphism in relation to EHT in Egyptian population. The results showed that the frequency of T allele was significantly increased in hypertensive patients, with a positive risk of developing EH when having the T allele whether homozygous or heterozygous state. (Mariam et al. 2015).

Our finding disagreed with, Srivastava et al., stated that the AGT (M235T) gene polymorphism is significantly associated with EHT. They found that patients with“TT”

genotype had higher blood pressure lowering response when treated with ACE inhibitor, Enalapril than those carrying MM and MT genotypes. Therefore, the T allele may be a possible genetic marker for EHT (Srivastava et al. 2012).Shamaa et al., in Egypt, reported a positive risk of developing EHT when having the T allele whether in homozygous or heterozygous state. Therefore, they concluded that there is an association between AGT (M235T) gene polymorphism and the risk of developing EHT (Shamaa et al. 2015).

Moreover, In the current study, there were insignificant variation in renin and aldosterone among essential hypertensive patients according to different AGT genotype (MM, MT, TT).

The absence of a significant differences in renin and aldosterone serum levels among essential hypertensive patients according to their AGT (M235T) polymorphism may be due to the absence of (TT) genotype among Sudanese participants in this study, since all participants had either (MM) or (MT), genotypes but not (TT).This variation may be due to different ethnicity or due to other point of mutation still not detected.

In the current study showed that there was significant differences in MAP in essential hypertensive patient whom had positivefamily history and those whom had no positive family history, (115.3±11.4) and (100.2±13.1) respectively (P value = 0.031),this agree with the study of(Muldoon *etal*.,1993). and (Winnicki*etal*.,2006). Showed that patients who havepositive family history of hypertension would have a 2 to 4 fold higher risk of getting this disease.

5.2 Conclusion

The study concludes that, there is no association between the M235T polymorphism of the AGT gene and EHT, whereas association observed with serum renin and aldosterone levels. In addition, there was significant association between positivefamily history and main arterial blood pressure in EHT patients.

5.3 Recommendations

1. Studying different point of gene mutation throughout sequencing the whole exon of AGT gene.
2. Estimate component other than renin and aldosterone in RAAS cascade.
3. People should have healthy life style to prevent the risk of getting EHT, by minimize BMI, reduce salt dietary intake, avoid smoking, stress and reduce lipid intake.

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