Study of Pharmacogenetic Association of Candidate Gene Polymorphism in Pakistani Hypertensive Patients

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Hypertension

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Amlodipine.

Thesis Title

Study of Pharmacogenetic Association of Candidate Gene Polymorphism in Pakistani Hypertensive Patients

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FINAL APPROVAL

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DECLARATION

I hereby declare that the work present in the following thesis is my own effort except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

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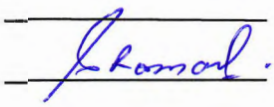

CONTENTS

CONTENTS

CONTENTS

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LIST OF ABBREVIATIONS

Acronym	Abbreviations
HTN	Hypertension
CA	Cardiac arrest
JNC7	Joint National Committee 7
SBP	Systolic Blood pressure
DBP	Diastolic Blood pressure
CVD	Cardio Vascular Diseases
PKD	Polycystic Kidney Disease
CKD	Chronic Kidney Disease
ICU	Intensive Care Unit
IDH	Isolated Diastolic Hypertension
WCH	White coat hypertension
RAAS	Renin-Angiotensin-Aldosterone System
ADH	Antidiuretic hormone
DASH	Dietary approach to stop hypertension
mmHg	Millimeter Mercury
VGCC	Voltage Gated Calcium channel
L-VGCC	Long lasting VGCC
CCB	Calcium channel blocker
DHP	Dihydropyridine
ATR	Angiotensin Type receptor
ACE	Angiotensin converting enzyme
LOS-K	Losartan potassium
BMI	Body mass index
M	Molar
mM	Mill molar
µl	Microliter

mL	Milliliter
Rpm	Resolution per minute
PCR	Polymerase chain reaction
EDTA	Ethylene Diamine Triacetic Acid
TBE	Tris Borate EDTA
UV	Ultra violet
°C	Degree Celsius
RFLP	Restriction Fragment length Polymorphism
DMSO	Dimethyl Sulfoxide
Bp	Base Pair
T	Temperature
U	Unit
P	P value

LIST OF FIGURE

LIST OF TABLES

Abstract

Hypertension is major cause of cardiac disease, renal failure, diabetes mellitus and ultimately death. It is known as silent killer because it may damages vital organs without clear symptoms. Hypertension prevalence is increasing globally. Prevalence rate of hypertension is also very high in developed countries. In underdeveloped countries including Pakistan prevalence of hypertension is very high. Despite of fact antihypertensive medications are available but prevalence rate of hypertension in Pakistan is increasing gradually.

Hypertension is caused by integration of genetic and environmental factors. Polymorphisms of genes are also associated with hypertension so identification of genes is decisive. It is important to identify causes because of which antihypertensive drugs do not shows progressive response.CACNA1C gene codes for alpha subunit of voltage gated calcium channels. Polymorphism of CACNA1C gene and its association with hypertension is well studies in different populations of world. It is very important to analyze CACNA1C gene in Pakistani population and response of antihypertensive drugs in hypertensive patients.

Aim of study was to evaluate variants of CACNA1C gene and its association with antihypertensive drugs. Samples of hypertensive patients were collected and out of 285 samples 185 patients were using amlodipine only and 100 patients were using amlodipine + losartan both drugs. rs2239050 C/G polymorphism and rs2239127 C/T polymorphism of CACNA1C gene was studied. According to genotypic distribution and allele frequency of rs2239050 C/G polymorphism GG genotype of risk allele p value was less than 0.05 ($P < 0.05$) which shows that rs2239050 is associated with hypertension. Patients with GG (risk) genotype were good responders of amlodipine monotherapy and carrier genotype GC was good responders of combine medication amlodipine+losartan.

According to genotypic distribution and allele frequency of rs2239127 C/T polymorphism p value was greater than 0.05 ($P > 0.05$) and results are statistically non-significant. A non-significant result indicates that rs2239127 C/T polymorphism is not associated with hypertension. Patients with CC (risk) genotype were good responders of amlodipine monotherapy and carrier genotype CT was good responders of combine medication amlodipine+losartan.

CHAPTER 1

Hypertension

Hypertension (HTN) is an imperative communal health problem globally and for majority recognized as amendable risk factor for cardiac arrests (CA), vascular dementia and renal dysfunction. Hypertension is exceedingly prevailing and in year 2025 around 26.4 % of grownups had hypertension. It is predicted that 60 % peoples globally will be affected from hypertension in 2025 (Doulougou *et al.*, 2013).

William Harvey first time enlightened multifaceted mechanism of hypertension in 17th century (Padmanabhan *et al.*, 2015). The force by which blood moves in arteries is termed blood pressure. There is optimal level of blood pressure and if it surpasses from optimal level it is called hypertension. Hypertension causes several damages and affects organs such as brain, kidney, heart and eyes. Risk of hypertension development is more in individuals elder than 50 years (Park *et al.*, 2015).

The Joint National Committee on Prevention, Detection, Evaluation and Treatment seventh report (JNC7) explicates hypertension as pressure of blood 140/90 mmHg. Individuals having blood pressure (SBP 120-139 mm Hg) and (DBP 80-89 mm Hg) are categories as pre-hypertensive. Hypertension development risk is more in pre-hypertensive individuals then those with normal or low blood pressure levels (Yadav *et al.*, 2008).

Hypertension is recognized as silent killer as it eventually damages organs and affects blood vessels (Popescu *et al.*, 2013). For diagnosis of hypertension systolic blood pressure is important to point out. Systolic blood pressure 150 mmHg at age of 80 years is well thought out as satisfactory (Weber *et al.*, 2014). Hypertension is allied with visual impairment, olfactory, hearing and learning detriment, Congestive heart failure and kidney failure (Wolf *et al.*, 2004).Minor rise in SBP increases cardiac diseases, for instance elevation of 2 mmHg SBP in hypertensive patients 10 % increases risk of stroke (Neutal *et al.*, 2005).

It is anticipated that 60 % individuals will be hypertensive by year 2025.Hypertension is caused by integration of genetic as well as environmental factors, Integration of organs, cellular level and molecular level. In most cases it damages' more than one organ and due to intricacy of hypertension pathophysiology it is not controlled effectually (Touyz, 2012).

1.1. Hypertension Types

Hypertension is leading cause of CVD and death worldwide. High blood pressure can be cause of blood vessels eruption (Chen and Yang, 2013). Hypertension is categorized in following sub-categories.

1.1.1. Primary Hypertension

Utmost communal type of hypertension is primary hypertension also renowned as Essential hypertension affecting 90-95 % patients. Elementary origin of primary hypertension is not recognized, there are many factors linked to advance primary hypertension. Vitamin D insufficiencies, consumption of liquor, discrepancy of potassium, intuitive plumpness, anxiety, and deskbound life are risk factors of essential hypertension. Congenital transfigurations are other possible causes of essential hypertension (Kesari *et al.*, 2014). Epigenetics of EH shows that it is caused by interface of genomic and ecological factors.

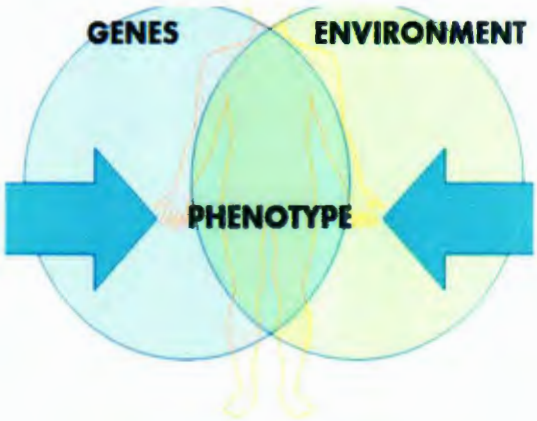


Figure No 1.1: Epigenetics of hypertension (Wise and Charchar, 2016)

1.1.2. Secondary Hypertension

Hypertension is chief cause of many diseases worldwide. Even though majority of patients have essential hypertension that does not have discernible genesis, 10 % of individuals have secondary hypertension. In general elderly patients have secondary hypertension (Puar *et al.*, 2016).Secondary hypertension instigated due to irregularity of imperative paths (Nahida and Feroz, 2001).

1.1.2.1. Endocrinal engenders

- Hyperadrenocorticism
- Idiopathic aldosterone's
- Glandular disorder
- Hypopituitarism/Hyperpituitarism (Tougorti *et al.*, 2016) (Kayser *et al.*, 2016).

1.1.2.2 Nephritic engenders

- Nephritis
- PKD
- Aortic stenosis
- CKD (Mengena *et al.*, 2016).

1.1.3. Resistant Hypertension

Resistant hypertension is discrete category of hypertension where in patients not attain optimal BP even using at least three antihypertensive drugs (Judd *et al.*, 2014).Patients with resistant hypertension are hyperaldosteronism, Heavy liquor consumption, salt sensitivity, potassium ions disproportion. Assimilation of large serving of fruitlets and vegetables, homogenized dairy products are means to achieve normal blood pressure alongside use of antihypertensive drugs (Calhoun *et al.*, 2008).

1.1.4. Malignant hypertension

Malignant hypertension also recognized as hypertension emergency well defined as adversity of with SBP 180 mm Hg and DBP 120 mmHg. In MH one or more than one organ is damaged particularly kidney, nervous system, cardiac system. Damage of organ is irretrievable. Blood pressure must be controlled by antihypertensive medications and about 10 % blood pressure level must be reduced in first hour and patients should be treated in ICU (intensive care unit) (Vaidya and Ouellette, 2007). Mechanism of hypertension emergency comprises of blood pressure elevation, vasoconstriction, RAAS (Januszewicz *et al.*, 2016).

1.1.5. Isolated diastolic Hypertension

IDH is defined as hypertension with DBP > 90 mm Hg and SBP < 140 mm Hg. IDH is prominent in Youngers as well as elderly peoples. Family history, high glucose level, BMI excess intake of tea are risks associated with IDH (Yang *et al.*, 2016). In elders IDH is caused by arterial stiffening and its affective can be Youngers as well as elders. About 90 % Patients above 70 years have IDH. Antihypertensive drugs are not effective in most cases as patients are resistant towards drugs (Franklin, 2008).

1.1.6. White coat hypertension

Another type of hypertension is WCH (white coat hypertension) in which blood pressure of individual in office is high but remains normal at home. White coat hypertensive individuals do not use antihypertensive medications to attain optimum blood pressure. WCH is more conjoint in elders and pervasiveness ratio is 15-20 %. WCH can be treated by routine amendments and healthy dietary plans (Aronow, 2015).

1.2. Hypertension Risk Factors

Blood pressure is not curable but lifestyle modifications & antihypertensive medications can effectively overcome adverse effects of hypertension and can reduce cardiovascular events. For Most of times cause of blood pressure are not clear so it's sometimes known as silent killer. There are certain factors that increase risk of hypertension. Among these some are modifiable risks and some are out of individuals control (Joffres *et al.*, 2013).

1.2.1. Risk factors outside of control

- Most imperative non-modifiable risk factor of hypertension is family history. Genetic variability in RAAS results high secretion of ADH, reabsorption of renal sodium and ultimately vasoconstriction causes elevation of blood pressure. High levels of insulin in plasma, oxidative anxiety & BMI are risk factors of hypertension out of individual control (Ranasinghe *et al.*, 2015).
- Agedness is one more non-changeable aspect of hypertension. An incidence of cardiovascular diseases surges with age (Higashi *et al.*, 2012).

- Na/K-ATPase activity decreases in elderly age. As a result intracellular sodium concentration raises triggering reduction in interchange of sodium-calcium. Intracellular level of calcium increases and will cause vascular resistance (Yan and Shapiro, 2016).
- Dark skin color is another non-modifiable risk factor of hypertension. Dark population in Americans is more hypertensive because of stress, poverty and social discrimination (Gravlee *et al.*, 2005).

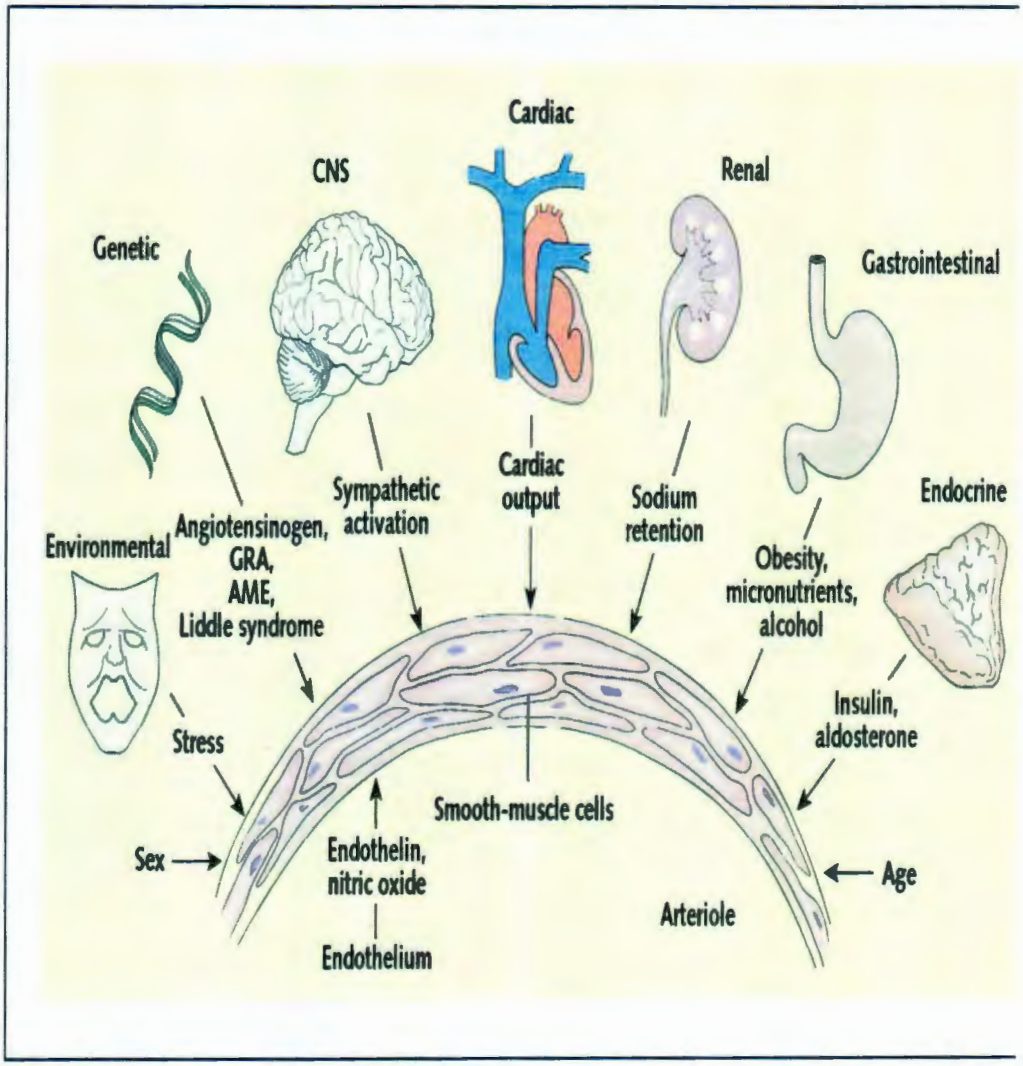
1.2.2. Risk factors under control

- Alcohol consumption has evident influence on blood pressure. It is a modifiable risk factor and hypertension can be controlled by adopting healthy life style (Chen *et al.*, 2008).
- Physical activity is important to sustain average body weight. Losing weight regularizes both systolic and diastolic blood pressures. Exercise is a key for optimal blood pressure and peripheral resistance declines (Huai *et al.*, 2013).
- DASH (Dietary approaches to stop Hypertension) is effective way to control non-optimized blood pressure, improves autonomic functions. DASH is a slant that can diminish LVM (Blumenthal *et al.*, 2010).
- Consumption of excess sodium salt is allied with hypertension. Excess intake of sodium salt causing reabsorption of Na^+ and causes water retention. Reducing intake of sodium rich diet prevents from arterial toughness and minimizes risk of hypertension (Hamlyn *et al.*, 2013).
- Diet with low potassium is also modifiable factor of hypertension. Potassium has antagonistic effect for sodium so prevents water retention and reduces blood pressure (Adrogue and Madias, 2007).

1.3. Pathophysiology of Hypertension

Hypertension is distinguished cause for ill health & deaths allied with cardiac diseases and renal failure. Blood pressure is multifaceted and along environmental reasons genetic aspects are also responsible for hypertension. More than a few mechanisms have been recognized and well-studied in animal models (Sarkar and Singh, 2015).

Pathophysiology of hypertension is multifactorial mechanism integrating genetic and ecological causes. Long lasting raise of blood pressure causes organ damage and ultimately results in augmented complaint and deaths. Key aspects of hypertension are cardiac output and vessels resistance. Over stimulation of RAAS causes vascular toning. An increase in calcium level is tangled in vascular narrowing as a result blood pressure increases (Foex and Sear, 2004).Though quite few aspects evidently contribute in pathogenesis of hypertension RAAS plays prime role.



(Oparil *et al.*, 2003)

Figure 1.2: Pathophysiological Mechanism of Hypertension

Table No 1.1: Classification of blood pressure (BP) levels (mmHg)

Classification of blood pressure (BP) levels (mmHg)

Classification	Systolic	Diastolic
Optimum	120	80
Standard	120–129	80–84
High normal	130–139	85–89
Rank 1 hypertension	140–159	90–99
Rank 2 hypertension	160–179	100–109
Rank 3 hypertension	180	110
Isolated systolic Hypertension	140	90

(Illyas, 2009)

1.4. Prevalence of Hypertension worldwide

Major causes of hypertension are renal failure, cardiac diseases and deaths. Health of population can be determined by checking blood pressure. Out of 3 individuals 1 is hypertensive in USA. American Heart association is trying to overcome hypertension prevalence by 2020 in population. Hypertension is most important risk factor of cardiovascular disease (CVD) and untimely deaths. According to 2009-2010 appraisal in USA prevalence of hypertension was 28.5 % in females and 30.5 % in males and 69.7 % individuals were aware of hypertension (Guo *et al.*, 2012).

To estimate prevalence of hypertension in Malaysia a study was accompanied in 2016. Around 11288 participants (18 town and 22 country communities) included in this study. Hypertension prevalence was 42 %, less in females (41 %) and higher in males (43 %). Rural areas participants were less hypertensive then urban areas (Abdul-Razak *et al.*, 2016). In 2016 medical data of 29740 was evaluated in New Zealand to analyze prevalence of hypertension. Almost 96 % of patients had abandoned blood pressure using antihypertensive medications and around 16 % patients were suffering from diabetic, Cardiac and kidney failure (Gu *et al.*, 2016).

In 2016 a survey was conducted in India to estimate prevalence of hypertension in Indian rural residents. According to this survey HTN prevalence rate was 16.8 % among 495 participants and hypertension was more prevalent among individuals above 40 years age (Premkumar *et al.*, 2016). A survey was conducted in 2015 to access the prevalence of hypertension in South Asian populaces living in UAE. Among 1375 participants hypertension was prevalent in 30 % and about 76 % of participants were unaware of their state of hypertension. Few were mindful of hypertension and were taking antihypertensive treatment (Shah *et al.*, 2015).

To access the prevalence of hypertension in China, survey was conducted in 2013.Data of 46239 hypertensive Chines were collected. Hypertension was more prevalent in occupants of rural areas occupants then in urban areas. About 26.6 % of adults were hypertensive and prevalence rate of hypertension was greater in men's (29.2 %) then (24.1%) in females (Gao *et al.*, 2013).

In 2008 prevalence of hypertension in Iranian population was studied. Data of 70981 was collected and 68250 participants were under age of 25-64 years. Around 25 % adults were hypertensive and 46 % participants were diagnosed pre-hypertensive (Esteghamati *et al.*, 2008).

In 2008 a survey was conducted in Turkey to evaluate prevalence of hypertension. Among 4809 participants 2601 were females and 2208 were males. Further hypertension was more prevalent in females (46.1 %) then males (41.6 %) and 54.5 % patients were using antihypertensive drugs but only 24.3 % of patients had standard blood pressure (Erem *et al.*, 2008). To study prevalence of hypertension a population based survey of 26913 was steered in Greek. Hypertension was more prevalent among males (40.2 %) and (38.9%) in females. Although hypertension is more prevalent in males but prevalence ratio rises in women's after age of 55 years (Psaltopoulou *et al.*, 2004).

In 2001 an analysis was directed to estimate pervasiveness of hypertension in Korean population. Among participants 1948 were females and 2278 males. Hypertension prevalence rate was 33.7 % and females were less hypertensive (24.5 %) then males (41.5 %) Jo *et al.*, 2001.

1.5. Prevalence of hypertension in Pakistan

Hypertension prevalence in Pakistan is very disquieting aggregating gradually. The rate of hypertension prevalence at juvenile phase is about 3-4 % and surges progressively. Hypertension associated causes are genetic and environmental factors, deskbound lifestyle, industrialization and sluggish living standard of females (Aziz, 2015).

Hypertension is a devastating challenge worldwide. The situation of HTN prevalence is tormenting. An international survey was conducted to access HTN prevalence in non-European countries citizens (Pakistan, Algeria, Ukraine, Egypt, and Venezuela) and out of 2185 participants 40 % patients' blood pressure was optimal. Unrestrained blood pressure was perceived in patients in taking salt rich foods and family history (Aoun *et al.*, 2015).

To study hypertension prevalence in Karachi tenant's survey was conducted in 2014. Among 1336 patients 28.6 % patients had uncontrolled blood pressure and prevalence of hypertension was 56.3 %. Renal failure was most conjoint complaints among hypertensive patients. To evaluate prevalence of hypertension in Pakistan; NHSP (National health survey of Pakistan) was

conducted in 2010. According to this assessment hypertension affects 18 % adults amongst 33 % adults ages were exceeding 45 years. About 50 % patients were treated and 50 % individuals had never used any antihypertensive medications (Saleem *et al.*, 2010).

A study from 2012-2013 was conducted in Southern Punjab to evaluate prevalence of cardiac disease risk factors. Cardiac diseases risk factors were more prevalent in females (65.53 %) and (34.46 %) in males. In Punjab residents' hypertension (52 %) is chief factor for cardiac diseases. Adopting healthy standard of living can decrease prevalence of cardiac diseases (Khan *et al.*, 2016).

In 2013 a survey was systemized to study prevalence of hypertension in 661 Pakistani school children's. Age of children's was 13-14 years and BMI was 16-5-19.5. Among 661 children's 81.8 % were non-hypertensive, 15 % were pre-hypertensive and 3 % were observed hypertensive. The pattern of hypertension prevalence in Pakistani children's was similar to that of Iranians and Chines children's (Rahman *et al.*, 2013).

To determine prevalence of hypertension in Pakistani inhabitants a study was accompanied by National Health Survey of Pakistan in 2005. In this survey 18135 individuals were evaluated. According to this survey 35.6 % participants were hypertensive and females were more prevalent (41.3 %) and (29.0 %) in males. Degree of blood pressure airing among Pakistanis was very low and HTN prevalence in provinces was as follow, Punjab (0.45 %), KPK (0.47 %), Baluchistan (0.47 %) and (0.80 %) in Sindh (Ahmad *et al.*, 2005).

In 2004 prevalence of hypertension among low wages public was studied. A total of 857 participants were scrutinized and prevalence rate was 26 % and males were more prevalent (34 %) then females (24 %). Average age of participants was 14-35 years and this survey revealed that HTN prevalence upturns with advancement of age (Safdar *et al.*, 2004).

To evaluate HTN prevalence in Pakistani racial groups among 9442 participants a survey was conducted in 2003. Racial groups were Punjabis, Mohajirs, Balouchi and Pashtun's. HTN prevalence was highest in Balouchis (41.4 % in females and 25.3 % in males) similarly in Pashtun's (28.4 % in females and 23.7 % in males) Muhajirs (24.6 % in females and 24.1 % in males) in Sindh (19.0 % in males and 9.9 % in females) & lowest prevalent rate was observed in

Punjabis (17.3 % in males and 16.4 % in females).HTN was less prevalent in residents of countryside areas (18.1 %) and (22.7 %) in residents of cities (Jafar *et al.*, 2003).

1.6. Voltage Gated Calcium Channels

VGCC (voltage gated calcium channels) are involved in many important pathophysiological processes. Paul Fatt & Bernard Katz identified voltage gated calcium channels (Dolphin, 2006).VGCC serves as imperative intermediaries of many processes. VGCC are involved in hormones secretions, transmitter's exudations, and contraction of muscles and is complex of 5 subunits.

- Alpha 1 subunit (170 kDd)
- Alpha 2 subunit (150 kDd)
- Beta subunit (52 kDd)
- Delta subunit (17-25 kDd)
- Gamma subunit (32 kDd) Zamponi *et al.*, 2015

There are six sub-categories' of VGCC and each has distinct role. They are as follow: L-type VGCC, N-type, P-type, Q-type, R-type and T-type VGCC.

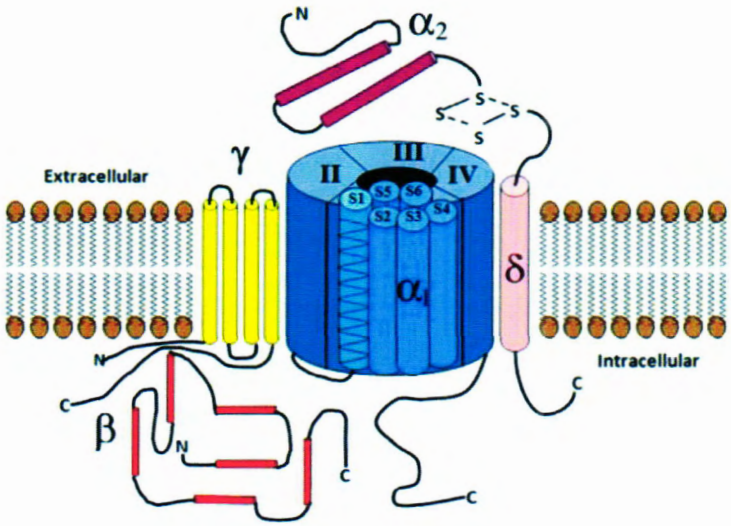


Figure No1.3: VGCC and its Subunits (Gurkoff *et al.*, 2013)

1.7. L-type Voltage gated calcium channels

In plasma membrane ions channels are embedded that permits movement of different ions. Ions channels have several subunits and are encoded by specific genes. These ions channels have important functions such as pore formation enabling ions flow. Disruption of ions channels current balance is associated with a mutation that affects sodium (Na^+), potassium (K^+) and calcium (Ca^{2+} ions) fluxes steadiness (Campuzano *et al.*, 2010).

L-type voltage gated calcium channels are present in various kinds of impulsive cells and are compulsory for accurate functions of many organs. There are four sub-categories of L-type VGCC but among four alpha Cav 1.2 & Cav 1.3 are most important present in cardiac cells and neurons. Alpha Cav 1.2 and Cav 1.3 possess structural resemblance but hold diverse activities and interact with distinct proteins (Ortner and Striessnig, 2016).

L-type VGCC Cav 1.2 a pore forming sub-unit regulates on cell membrane of nerve cells and skeletal muscle cells and many mechanisms are associated with Cav 1.2 subunit such as expression of genes, muscles contraction - relaxations & reminiscence. Few Cav 1.2 channels can causes inflow of calcium ions at higher level (Navedo *et al.*, 2010).

1.8. CACNA1C Gene

The chief pore making subunit of L-type voltage gated calcium channel is alpha subunit (α1c). CANCNA1C is gene that codes alpha subunit of L-type voltage gated calcium channels. CACNA1C is hefty gene positioned on sort arm of chromosome 12p 13.3 with 49 exons and 44 introns. CACNA1C gene size is 300 kb and exonic region is around 8 kb. This α1c is very imperative as it makes available binding site for calcium channels blockers (Beitelshees *et al.*, 2010).

CACNA1C gene expressed in brain, cardiac cells, and children's as well as in elders and in arteries. The Cav 1.2 channel activates many processes including muscles contraction, endocrinal secretion of hormones, and synapsis of sensory cells. Calcium channels blockers also bind with cav 1.2 subunit and blocks' the entry of calcium ions (Puckerin *et al.*, 2016). CACNA1C polymorphism is major cause for onset of hypertension. Over expression of CACNA1C gene in arteries can cause valves complications (Olarte *et al.*, 2015).

1.9. Hypertension & Antihypertensive drugs

Hypertension is communal intricate condition that is concomitants along genetic and ecological facets. Hypertension is hereditary as if affects several genetic factors and hypertension transmissible ratio is 20-60 %. Identifying hypertension vulnerability genes and their mechanism, recognizing hypertensive individuals and improvement of antihypertensive drugs can reduces prevalence of hypertension (Yang *et al.*, 2013).Hypertension is associated with arterial stenosis, over activity of RAAS. The objective of antihypertensive drugs is to eradicate possibility of hypertension in addition to improving blood pressure levels without distressing normal life and minimal side effects (Chen *et al.*, 2015).

Antihypertensive drugs

ACE Inhibitors	Beta Blockers	Calcium Channel Blockers	Diuretic	
• Inhibition of ACE • Decrease s activity of RAAS	• Decrease in cardiac output • Decrease in plasma renin activity	• Decrease of free intracellular calcium ions • Decrease contraction & vasodilation • Aldosterone secretion inhibition	Thiazide	• Retention of water
			Loop of diuretics	• Inhibition of sodium reabsorption
			K+ diuretics	• Inhibition of K+ secretion

Figure No 1.4: Antihypertensive drugs classification (Okhuelegbe *et al.*, 2015)

1.10. Calcium Channels Blockers (CCB, s)

Voltage gated calcium channels allow influx of calcium ions into cells. Calcium ions have many physiological roles in body. In cardiac and smooth muscles increase in cytosolic calcium level causes contraction of muscles, In endocrine cells influx of calcium ions cause hormones secretion, genes expression and enzymes activity are also related with calcium ions (Catteral, 2011).

Calcium channels blockers are drugs that blocks voltage gated calcium channels present in heart, muscles and blood vessels. An excess of calcium ions causes contractility of smooth muscles and cardiac cells. CCB.s overcomes contractility and reduces cardiac output and in smooth muscles reduces contraction (Arnett and Claas, 2009).

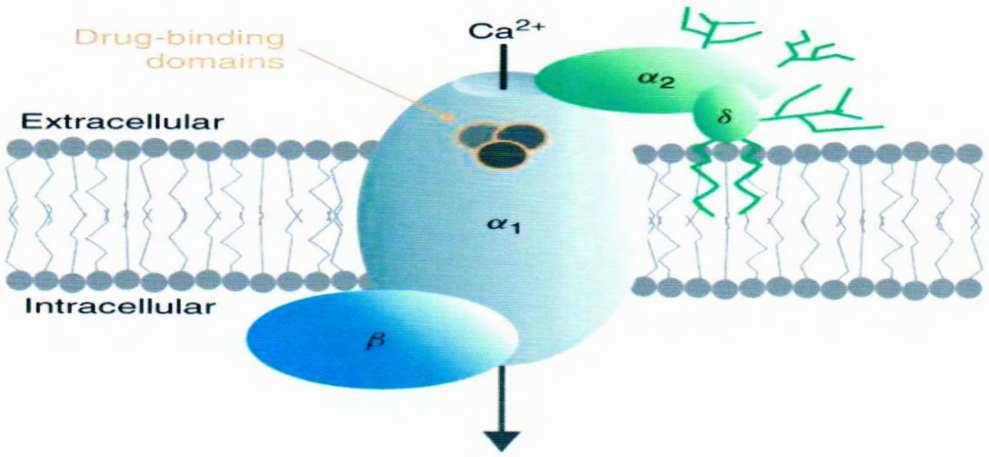


Figure No 1.5: Mechanism of Calcium Channels Blockers (Striessnig *et al.*, 2014)

There are two types of CCB, s. Dihydropyridines such as amlodipine, felodipine, and nifedipine. Non-dihydropyridines CCB include diltiazem and verapamil. These calcium channels are involved in vessels dilation as well as blocks calcium ions influx (Schifrin, 2010). DHP are calcium channels blockers that blocks L-type calcium channels. They also blocks T type VGCC and are affective to treat angina and hypertension (Bladen *et al.*, 2014).

1.11. Amlodipine

Hypertension is most important risk factor of many diseases including cardio vascular disease and kidney failure. Many classes of antihypertensive medications are available to achieve normal SBP and DBP such as ATR blockers, ACE inhibitors, CCB, and Beta blockers (Johnson, 2010).

DHP, s (1, 4-Dihydropyridines) is nitrogen containing compounds. DHP, s has many therapeutic protagonists such as calcium channel blockers. Most communal medications of DHP, s is amlodipine, nifidipine, felodipine, nicardipine used for treatment of hypertension and cardiovascular diseases (Arslan *et al.*, 2009).

Amlodipine is a calcium channel blocker mostly recommended to hypertensive patients globally. It causes vasodilation by inhibiting calcium ions influx from L-type voltage gated calcium channels. Amlodipine is metabolized in liver by cytochrome CYP-3A that belongs to cytochrome P450 in liver. Effectiveness and antagonistic retorts of amlodipine are allied with CYP-3A. CYP-3A and POR genes polymorphisms badly reduce efficacy of amlodipine (Guo *et al.*, 2015). Binding of Amlodipine that is located on outer surface causes conformational changes and blocks pore forming subunit (Tang *et al.*, 2016).

Table No 1.2: Pharmacokinetic properties of Amlodipine

Drug	Bioavailability	Food effect	Inactive metabolite excretion	Half Life	Protein Binding	Normalize BP (days)	Drug absorption
Amlodipine	64-80 %	No	60 %	37-50 (h)	97 %	7-8 days	6-12 (h)

(Billecke and Marcovitz, 2013), (Mascoli *et al.*, 2013)

1.12. Angiotensin II

Ang II is important key of RAAS as it binds with Angiotensin II type 1 receptors on cell surface and cause's secretion of aldosterone hormone from adrenal cortex. Aldosterone causes reabsorption of sodium and causes water retention as result volume of fluid increases and causes elevation of blood pressure (Nabeshima *et al.*, 2009).There are two types of Angiotensin receptors, Angiotensin II type 1 receptor & Angiotensin II type 2 receptors. These receptors are present in almost all tissues, organs and are associated in hypertension & circulatory maladies (Dasgupta & Zhang, 2011).

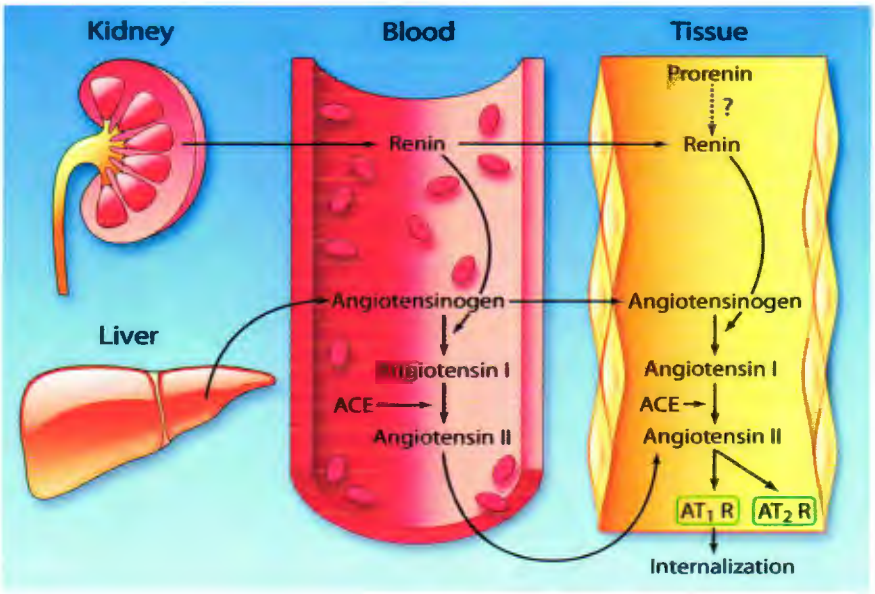


Figure No 1.6: Role of Angiotensin II in hypertension (Riet *et al.*, 2015)

1.13. Angiotensin II type 1 Receptor Blockers

ARB, s is non-peptide, very selective and is used for treatment of high blood pressure. Seven types of ARB are available such as Losartan, Candesartan, Eprosartan, Ibesartan, Olmesartan, Telmisartan (Ribeiro and Gavras, 2006).

1.14. Losartan Potassium

LOS-K (Losartan potassium) is type of ARB used as antihypertensive drug. It prevents binding of angiotensin II on AT1 receptor so reduces casual of blood pressure elevation (Singh and Karnik, 2016).

Losartan is absorbed rapidly and is converted into E 3174 that is active form of losartan and has low interaction with other drugs. Cytochrome P450 is involved in metabolism of losartan. It has minimal side effects as compare to other angiotensin receptors blockers (Sica *et al.*, 2005).

Table No 1.3: Pharmacokinetic Properties of Losartan Potassium

Drug	Bioavailability	Food effect	Active metabolite	Half life	Protein Binding	Daily Dosage(g)
LOS-K	33 %	No	Yes	6-9 (h)	99.8 %	50-100 g

(Yang *et al.*, 2012, Patel *et al.*, 2013)

1.15. Aim of study

We have conducted this study to explore CACNA1C gene polymorphism in intronic regions. It includes rs2239127 of C/T polymorphism and rs2239050 of C/G polymorphism. Samples of patients were taken and are divided into two groups. One group was using calcium channel blocker (Amlodipine) and second group was using calcium channel blocker and angiotensin receptor blocker both (Amlodipine + Losartan).

This study includes

- Determination of rs2239127 C/T polymorphism and rs2239050 C/G polymorphism.
- To assess genotype and allele frequency of patients using amlodipine and other group using amlodipine and Losartan both.
- Comparison of obtained results with other studies accomplished on diverse populaces.

CHAPTER 2

Study Approval

This study has been ratified by International Islamic University Islamabad Bioscience committee. This study is conducted to assess CACNA1C gene single polymorphism in hypertensive Pakistani population and role of CACNA1C gene variants in hypertensive patients and to evaluate effect of Amlodipine and Amlodipine + Losartan in hypertensive patients

2. Subjects

Study subjects comprise of 285 hypertensive patients and blood samples were taken through coalitions of hospitals. Age of patients was from 20-85 years.

Table No 2.1 Sample size of hypertensive patients and Controls

Amlodipine	Amlodipine + Losartan	Control
185	100	100

2.1. Patient enclosure Criteria

Those patients were included in this study who is taking amlodipine as antihypertensive drug. Questioner was given to patients and all data of patient such as age, disease span, family history, occupation, BMI and marital status was noted.

2.2. Patient Exclusion Criteria

All those patients who were hypertensive but not using amlodipine were excluded from this study. A patient suffering from any other disease was also excluded from this study e.g., pregnant women's, diabetic, asthma, renal failure etc.

2.3. Control group

In this group blood sample of individuals is taken who were not suffering from any disease and are normal and hale and hearty.

- They must not be diabetic or renal failure.
- Age of control members must be similar to that of patients chosen for this study.

2.4. Blood samples collection

All blood samples of hypertensive patients were collected from POF Hospital WAH CANTT.5ml of peripheral venous blood was drawn from the median cubital vein at elbow joint using a sterile 5 ml sterile syringe. Blood was shifted in ACD vacutainers immediately containing anticoagulant. The vacutainer tubes were inverted too and blood samples were set a side at temperature 4°C till the extraction of DNA.

Table No: 2.2 (Equipment's & Accessories)

Weigh Balance	Mettler Toledo international
Beakers (Various sizes)	NALGENE
Centrifuge (eppendrof)	MSE MISTRAL USA
Falcon tubes	Corning 430791
Flasks (100,200,500 ml)	Pyrex
Glass pipettes	Preciculer HBG.W.Germany
PH meter	Jenway GB, UK
Vacutainers	BD FRANKLINWAY USA

Table No: 2.3 (Composition of solutions used in Genomic DNA extraction)

Solutions	Composition
Solution A	Sucrose (0.32M) $MgCl_2$ (5Mm) Tris Base (10Mm, 7.5 pH) 1 % V/V Titron X-100
Solution B	EDTA (2Mm, pH 8.8) Nacl (400M) Tris base (10Mm) pH 7.5
Solution C	Saturated phenol
Solution D	Chloroform & Isoamylalcohol (24:1)
10 % SDS	10 g in 50 ml water
DNA dissolving buffer	10mM tris & 0.1 mM EDTA

2.5. DNA extraction protocol

2.5.1. Day 1

Phenol-chloroform extraction method was used for extraction of DNA. This method comprises of following steps.

- Take 750 µl of blood in eppendrof tube and 750 µl of solution A.
- Mix solution A and blood by inverting eppendrof and left at room temperature for 15-20 minutes.
- Centrifuge for 1 minute at 13000 rpm.
- Afterward centrifuge throw away the supernatant.
- Resuspend the nuclear pallet in 400 µl of solution A and dissolve pellet by hitting.
- Centrifuge for 1 minute at 13000 rpm.
- Now discard supernatant and add 400 µl of solution B, 28 µl of 20 % SDS & 15 µl of chilled proteinase kinase.
- Incubate at 37 °C for overnight.

2.5.2. Day 2

- First of all make fresh mixture of solution C and solution D (250 µl phenol & 250 µl chloroform and isoamylalcohol).
- Add 500 µl solutions C+D, invert and centrifuge for 10 minutes at 13000 rpm.
- Collect aqueous layer because DNA is present in upper layer and shift it into new eppendrof.
- Now add 500 µl of solution D and centrifuge for 10 minutes at 13000 rpm.
- Again pick aqueous layer and shift into new eppendrof. Now add 55 µl sodium acetate (3M, 6 pH) for DNA precipitation.
- Add 500 µl chilled Iso-propanol, DNA will be visible now.
- Centrifuge again at 13000 rpm for 10 minutes.
- Discard supernatant, DNA pellet will be present.
- Now add 200 µl (70%) ethanol and centrifuge at 13000 rpm for 7 minutes.
- After centrifuge discard ethanol and invert eppendrof on tissue paper

- Place eppendrofs containing DNA pellet in drying oven. Let DNA pellet for 30 minutes to dry.
- Dissolve DNA in 200 µl T.E buffer and place eppendrofs in water bath at 55 °C (at least overnight).
- Store DNA at -20 °C temperature.

2.6. Gel electrophoresis of genomic DNA

Before running PCR, 1 % agarose gel was used in order to guesstimate presence of DNA and quantity of DNA. 0.8 grams agarose dissolved in 100 ml of 10X TBE buffer and placed in oven for 2 minutes to gel agarose. Then 8µl ethidium bromide is added and gel is poured into gel tank (E.C Apparatus Corporation, St. Petersburg Florida USA) and left the gel for 20-30 minutes to solidify at room temperature. DNA samples were vortex and then short spine at 8000 rpm for 30 seconds. After solidification of gel casting tray is removed and gel is shifted into loading tank containing gel running buffer.4 µl bromophenol blue and 4 µl DNA and laden in gel wells. Electrophoresis was performed for 30-35 minutes at 200 volts. Gel was envisaged underneath UV light and image was taken.

Table No: 2.4 (Composition of Gel electrophoresis solutions)

Solutions	Compositions
Gel loading dye	40 g sucrose 0.25 g Bromophenol Blue
10X TBE Buffer	0.89 M Tris 0.025 M Boric acid EDTA 0.5 M (pH 8.3)

2.7. PCR analysis of CACNA1C gene rs2239050 C/G polymorphism

PCR reaction was carried out to appraise rs2239050 C/G polymorphism in CACNA1C gene. Details of primers used for PCR are given below. Primers used in PCR are shown in table no 2.5. Reagents used are shown in table no 2.6 and thermal profile of PCR is shown in table no 2.7.

Table No: 2.5 (Primers used for rs2239050 C/G polymorphism)

Gene	CACNA1C	
Forward Primer	GGTTTGGTCTTTGCCTCTGG	
Reverse Primer	ACATTTCTGGTATGCAGCCAC	
No of Bases	Forward Primer	20bp
	Reverse Primer	21bp
Product Size	587bp	
Restriction Enzyme	Tail	
Temperature	51°C	

2.7.1. PCR Methodology

After optimization of all conditions samples were amplified under optimized PCR conditions to produce 587bp amplicons. Master Mix of PCR was prepared in 1.5 ml eppendrof tube with specific primers. Total of 25 µL reaction volume was used for single PCR. Primers were vortex and spun down for few seconds.

2.7.2. Agarose Gel Electrophoresis

The digested samples were analyzed by 2 % agarose gel electrophoresis and stained with ethidium bromide. Bands were visualized under UV light.

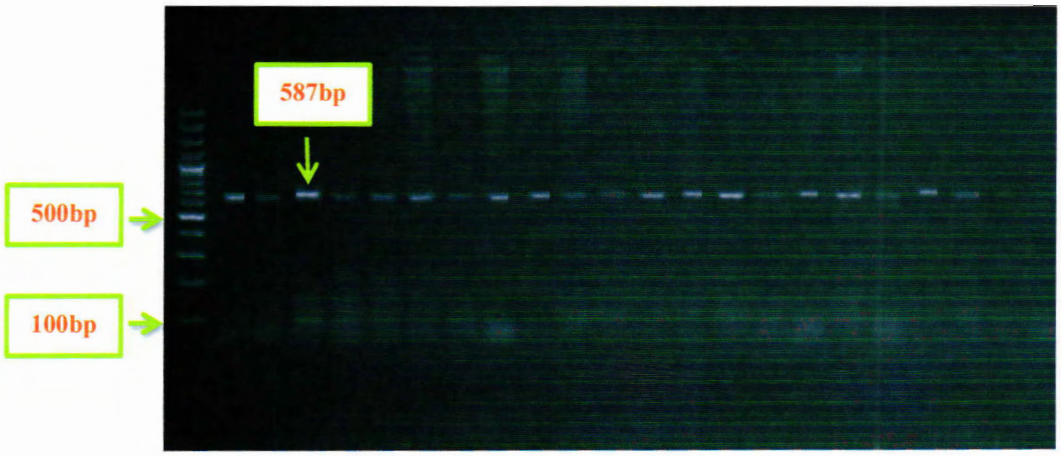


Figure No 2.1: Gel image of rs2239050 PCR Product

2.7.3. PCR-RFLP of rs2239050 C/G Polymorphism

Genotyping was done by RFLP (restriction fragment length polymorphism) analysis. Tail restriction enzyme was used for digestion of PCR product. All samples were digested by adding 0.5 µL of tail, 6.5 µL dH_2O and 3 µL of tail buffer in 20 µL PCR reaction mixtures. It was mixed by short spin (15 seconds).Reaction mixture was incubated for 16 hours at 37 °C.

Table No: 2.6 (PCR Components & Concentration for rs2239050 C/G Polymorphism)

Serial No	Reagents & Concentration	Quantity Used (µL)
01	10x PCR buffer (NH_4Cl) Fermantas, Lithuania	2.5 µL
02	$MgCl_2$ (Fermentas, Lithuania)	1.8 µL
03	2.0 mM dNTPs	1.0 µL
04	5U Taq DNA Polymerase (Fermantas, Lithuania)	0.2 µL
05	20 µM Forward Primer	1 µL
06	20 µM Reverse Primer	1 µL
07	40 ng Genomic DNA Sample	5 µL
08	DMSO	1 µL
09	Triton X	1 µL
10	dH_2O	10.5 µL

Table No: 2.7 (Thermal Profile of PCR for rs2239050)

Stage No	Step No	Temperature (°C)	Time	Cycles No
1st Stage	1st Step	95 °C	5 Minutes	1
2nd Stage	Step A	95°C	45 seconds	35
	Step B	51°C	45 seconds	
	Step C	72°C	45 seconds	
3rd Stage	1st Step	72°C	10 Minutes	1

2.8. PCR analysis of CACNA1C gene for SNP rs2239127 C/T

Primers for CACNA1C gene polymorphism was designed by using Primer 3 (Bioinformatics tool). PCR reaction was carried out for amplification of CACNA1C gene in hypertensive patients for rs2239127 C/T polymorphism in intronic region. Primers for rs2239127 C/T polymorphism is shown in table no 2.8.

Table No: 2.8 (Primers for rs2239127 C/T polymorphism)

Gene	CACNA1C	
Forward primer	5'- AACACAGACCCCACACTCAT-3'	
Reverse primer	3'- TTCTCTCCCAGTGGCTTGTT-5'	
No of bases	Forward primer	20bp
	Reverse primer	20bp
Product size	490bp	
Restriction enzyme	HinfI	
Temperature	56 °C	

2.8.1. Reconstitution of primers

Before opening primers tube vortex and centrifuge the tube. Calculated amount of dis H_2O and lyophilized primers are mixed. From 100 µM stock primer solution 20 µM working solution of primer was prepared (20 µM stock solution & 80 µM dH_2O) and for PCR amplification 20 µM primer solution was used.

2.8.2. PCR methodology

After optimization of PCR conditions with reproducible results all samples were amplified producing 490bp amplicon. PCR master mix was prepared with specific primers in 1.5 mL eppendrof tube. DNA will act as template in two PCR reactions, one primer matches to wild type allele and second matches with mutant allele. Total reaction volume for a single PCR reaction was 25 µL. Reagents used in pcr are shown in table 2.9. Thermal profile of pcr reaction is shown in table 2.10.

2.8.3. Agarose Gel Electrophoresis

The digested samples were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide. Bands were visualized under UV light.

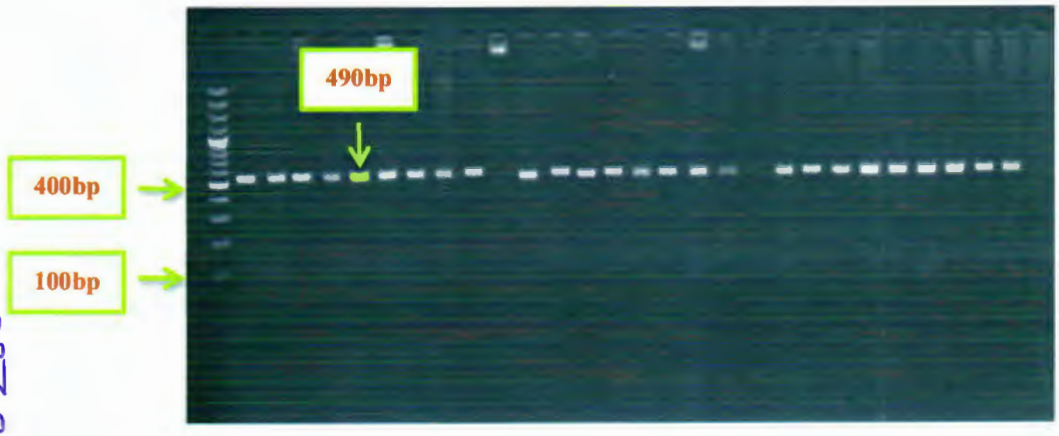


Figure 2.2: Gel Image of rs2239127 PCR Product

2.8.4. PCR-RFLP of rs2239127 C/T Polymorphism

Genotyping was done by RFLP (restriction fragment length polymorphism) analysis. HinfI restriction enzyme was used for digestion of PCR product. All samples were digested by adding 0.5 µL of HinfI, 6.5 µL dH_2O and 3 µL of HinfI buffer G in 20 µL PCR reaction mixtures. It was mixed by short spin (15 seconds).Reaction mixture was incubated for 16 hours at 37 °C.

Table No: 2.9 (PCR Reagents of rs2239127 and their concentrations)

Sr. No	Reagents with initial concentrations	Quantity used (µL)
1	10x PCR buffer (NH_4) (Fermentas, Lithuania)	2.5 µL
2	$MgCl_2$ (Fermentas, Lithuania)	1.5 µL
3	2.0 mM dNTPs	1.0 µL
4	5U Taq DNA Polymerase (Fermentas, Lithuania)	0.2 µL
5	20 µM Forward primer	1 µL
6	20 µM Reverse primer	1 µL
7	40 ng DNA sample	5 µL
8	dH_2O	12.8 µL

Table No: 2.10 (PCR Thermal Profile for rs2239127)

Stage No	Step No	Temperature (°C)	Time duration	Cycles No
1st Stage	1st Step	95 °C	5 Minutes	1
2nd Stage	Step A	95 °C	45 seconds	35
	Step B	56 °C	45 seconds	
	Step C	72 °C	45 seconds	
3rd Stage	1st Step	72 °C	10 Minutes	1

RESULTS

Hypertension is very prevalent worldwide and is risk factor for other ailments. Various genes are associated in regularization of blood pressure. Polymorphism of genes is major factor for onset of hypertension. Various studies have been carried out to study genetic polymorphism of genes associated with high blood pressure. Various categories of antihypertensive drugs are available. It is very important to analyze drugs association with genes involved in hypertension.

This study was carried out to analyze pharmacogenomic association of antihypertensive drugs, rs2239050 and rs2239127 in populates of POF Wah Cantt, Hari-pur, Khan- Pur, Hawaliyan, Hassan- Abdal and Taxila. Blood samples were taken after taking detailed information of patients. All subjects were hypertensive, using two types of antihypertensive drugs calcium channel blocker and angiotensin receptor blocker.

Subjects were divided into two groups. In first group 185 samples of patients, who were using amlodipine (calcium channel blocker) as antihypertensive drug. In second group 100 samples of patients were using two antihypertensive drugs amlodipine and losartan potassium (calcium channel blocker and angiotensin receptor blocker) for treatment of hypertension because monotherapy was not sufficient for control of high blood pressure. Distribution of subjects is shown in table 1.

Table No 3.1 (Distribution of Subjects)

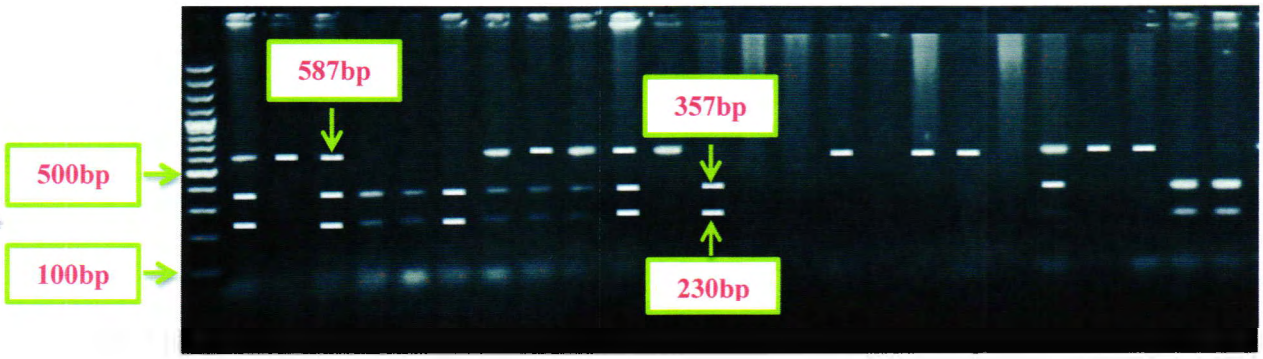
Group	Number of Subjects	Mean Age
Amlodipine	185	55±15
Amlodipine + Losartan	100	60±10

3. SNP Genotyping:

3.1. rs2239050 C/G polymorphism of CACNA1C gene:

We have studied rs2239050 in intronic region of CACNA1C gene. PCR-RFLP was carried out and bands of minor allele, major allele and heterozygous allele was produced.GG shows diseased homozygous allele with one band (587bp), GC for heterozygous 3 bands (587bp, 357bp, 230bp) and CC for wild allele 2 band (357bp and 230bp) . Restriction enzyme has produced fragments seen as bands. Bands of respective allele are shown in figure below. Genetic distribution and allele frequency of rs2239050 C/G polymorphism is shown in table no 2. PCR-RFLP image is shown in (figure no 3.1).

Figure No 3.1: PCR-RFLP image of rs2239050 C/G polymorphism



- **Risk allele GG (1 band) = 587bp**
- **GC allele(3 bands) = 587bp, 357bp,230bp**
- **CC allele(2 band) = 357bp, 230bp**

CHAPTER 3

CHAPTER 3

RESULTS

Hypertension is very prevalent worldwide and is risk factor for other ailments. Various genes are associated in regularization of blood pressure. Polymorphism of genes is major factor for onset of hypertension. Various studies have been carried out to study genetic polymorphism of genes associated with high blood pressure. Various categories of antihypertensive drugs are available. It is very important to analyze drugs association with genes involved in hypertension.

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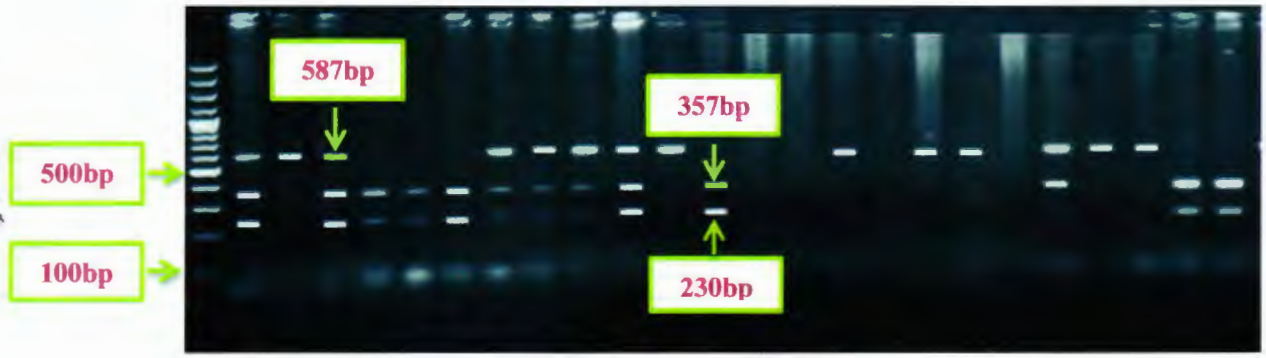
Figure No 3.1: PCR-RFLP image of rs2239050 C/G polymorphism



- **Risk allele GG (1 band) = 587bp**
- **GC allele(3 bands) = 587bp, 357bp,230bp**
- **CC allele(2 band) = 357bp, 230bp**

3.1.1.Genetic distribution & allele frequency of rs2239050 C/G polymorphism of CACNA1C gene b/w amlodipine, amlodipine + losartan & control group

Genetic distribution and allele frequency of rs2239127 C/T polymorphism of CACNA1C gene is calculated from which association of polymorphism with disease is analyzed. Difference of genetic distribution between amlodipine group, amlodipine + losartan and healthy control group was very clear. Genetic distribution of GG (Risk allele) was 53% in amlodipine group greater than amlodipine + losartan group (22 %) and 74% in control group. Similarly in GC genotype (Heterozygous carriers) was 41% in amlodipine group less than amlodipine + losartan group 74% and 24% in control group. Genotypic distribution in major allele CC (Ancestral allele) was highest in amlodipine group (6%), in amlodipine + losartan group is 4% and less in control group 2%. Allele frequency of G genotype in amlodipine group is 0.7283. In amlodipine +losartan group is 0.6869 and in control group is 0.864. Similarly allele frequency of C genotype in amlodipine group is 0.2717, 0.4139 in amlodipine + losartan group and 0.125 in control group. Genetic distribution and allele frequency of rs2239050 C/G polymorphism of three groups (amlodipine, amlodipine + losartan and control) are shown in table no 3.2.

Table no: 3.2. Genetic distribution and allele frequency of rs2239050 C/G Polymorphism of CACNA1C gene

Group	Genotype			Allele frequency	
	GG(Risk)	GC	CC(Major)	G	C
Amlodipine	53	41	6	0.7283	0.2717
Amlodipine +Losartan	22	74	4	0.5869	0.4139
Control	74	24	2	0.864	0.135

3.1.2.Genotypic distribution of rs2239050 C/G polymorphism between Amlodipine and Amlodipine + Losartan group and association with hypertension

This Statistical analysis shows rs2239050 C/G polymorphism of CACNA1C gene b/w amlodipine and amlodipine + losartan group and its association with hypertension in Pakistani population. Genetic distribution was significant between amlodipine and amlodipine + losartan group. According to genotypic distribution and allele frequency of rs2239050 C/G polymorphism $P < 0.05$ for risk allele GG and results are which shows that polymorphism of C/G in CACNA1C gene causes hypertension. Similarly for heterozygous risk allele carrier genotype CG $P < 0.05$ indicating results significant which shows hypertensive patients are carriers of risk allele that is responsible for hypertension. Chi square value for GG genotype is 8.231. For wild type CC genotype $P > 0.05$ showing statistically non-significant results. Chi square value for GG genotype is 8.231, similarly 4.388 for GC genotype and 0.485 for CC genotype. Online chi square contingency table calculator was used to evaluate p value.

Table No 3.3. Association of rs2239050 C/G polymorphism of CACNA1C gene with Hypertension b/w Amlodipine & Amlodipine +losartan group

rs2239050 C/G polymorphism			
Group	GG (Risk)	CG	CC (Wild)
Amlodipine	53	41	6
Amlodipine + losartan	22	74	4
P value	P < 0.05	P < 0.05	P > 0.05
Chi square	8.231	4.388	0.485

3.1.3.Genotypic distribution of rs2239050 C/G polymorphism between Amlodipine and Control group and association with hypertension

This Statistical analysis shows rs2239050 C/G polymorphism of CACNA1C gene b/w amlodipine and control group and its association with hypertension in Pakistani population. Results were non- significant between amlodipine and control group. According to genotypic distribution and allele frequency of rs2239050 C/G polymorphism $P > 0.05$ for risk allele GG showing statistically non-significant results. Similarly for heterozygous risk allele carriers genotype CG $P > 0.05$ indicating results non- significant. For wild type CC genotype $P > 0.05$ showing statistically non-significant results. Chi square value for GG genotype is 8.231, similarly 4.388 for GC genotype and 0.485 for CC genotype. Online chi square contingency table calculator was used to evaluate p value.

Table No 3.4. Association of rs2239050 C/G polymorphism of CACNA1C gene with Hypertension b/w Amlodipine & Control group

rs2239050 C/G polymorphism			
Group	GG (Risk)	CG	CC (Wild)
Amlodipine	53	41	6
Control	74	24	2
P value	P > 0.05	P > 0.05	P > 0.05
Chi square	8.231	4.388	0.485

3.1.4.Genotypic distribution of rs2239050 C/G polymorphism between Amlodipine + losartan group, Control group & its association with hypertension

This Statistical analysis shows rs2239050 C/G polymorphism of CACNA1C gene b/w amlodipine + losartan and control group and its association with hypertension in Pakistani population. Results were non- significant between amlodipine and control group. According to genotypic distribution and allele frequency of rs2239050 C/G polymorphism $P < 0.05$ for risk allele GG showing statistically significant results. Statistical results indicate association of rs2239050 C/G polymorphism with hypertension in Pakistani population. Similarly for heterozygous risk allele carriers genotype CG $P < 0.05$ indicating results significant. Statistically significant results show that hypertensive patients are carriers of risk allele which is responsible for high blood pressure. For wild type CC genotype $P > 0.05$ showing statistically non-significant results. Chi square value for GG genotype is 8.231, similarly 4.388 for GC genotype and 0.485 for CC genotype. Online chi square contingency table calculator was used to evaluate p value.

Table No 3.5. Association of rs2239050 C/G polymorphism of CACNA1C gene with Hypertension b/w Amlodipine + Losartan & Control group

rs2239050 C/G polymorphism			
Group	GG (Risk Allele)	CG	CC (Wild Allele)
Amlodipine + Losartan	22	74	4
Control	74	24	2
P value	P < 0.05	P < 0.05	P > 0.05
Chi square	8.231	4.388	0.485

3.2.rs2239050 C/G polymorphism and drugs response

3.2.1.rs2239050 GG genotype & Drug response between Amlodipine, Amlodipine+ Losartan group

Antihypertensive drugs are used to normalize high blood pressure. All patients do not respond in same way towards drugs. Some are good responders towards specific drug and in some patients that drug do not shows any affirmative impact. Response of patients towards drugs is also associated with their genotypes. In our study subjects were divided into two groups. Patients of group one were using amlodipine only (calcium channel blocker) and patients of second group were using two drugs to achieve normal blood pressure.

Patients with GG genotype (risk allele) were good responders of amlodipine (53%). Their blood pressure was under control using a single drug. Patients with GG genotype were not good responders of combined medication amlodipine + losartan (22%) group.

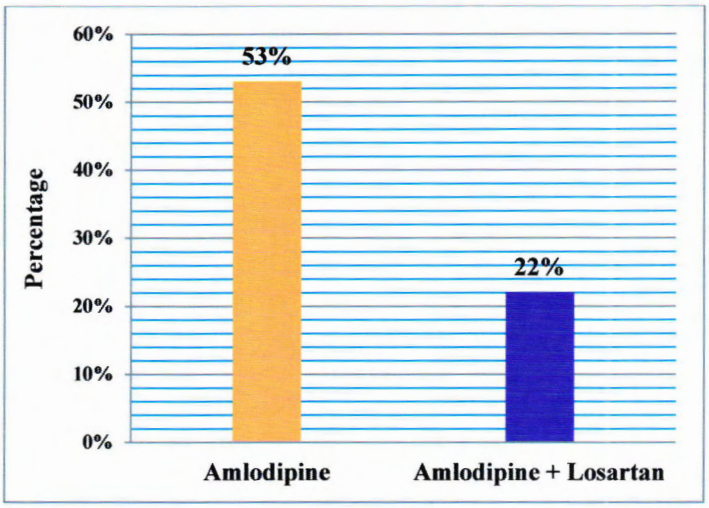


Figure no. 3.2.1. rs2239050 GG genotype & Drug response

This figure show that patients of GG genotype are good responders of amlodipine and combine medication amlodipine + losartan was not very effective among them for control of hypertension.

3.2.2. rs2239050 GC genotype & Drug response between Amlodipine, Amlodipine + Losartan group

Antihypertensive drugs are used for treatment of hypertension. Many types of drugs are available but all patients do not respond in same way towards that drug. Variability in drug response is associated with genetics. Variability in drug response is associated with genetic variability. In our study subjects were divided into two groups. Patients of one group were using amlodipine (calcium channel blocker) and second group was using two antihypertensive drugs amlodipine +losartan for treatment of hypertension.

Patients of GC genotype (Risk allele carriers) were not good responders of amlodipine (41%). High blood pressure was not under control by antihypertensive monotherapy. They were good responders of combine medication amlodipine + losartan (74%). Patients of GC genotype have optimal blood pressure level and combine medication was effective for treatment of hypertension among them.

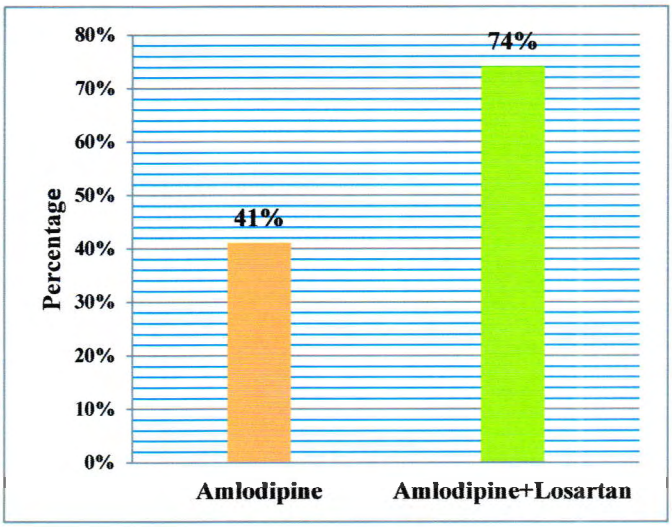


Figure no. 3.2.2. rs2239050 GG genotype & Drug response

This figure shows that patients with GC genotype are not good responders of amlodipine (41%). They were good responders of amlodipine + losartan (74%).

3.2.3.rs2239050 CC genotype & Drug response between Amlodipine, Amlodipine+ Losartan group

Hypertension is very prevalent globally and is termed as silent killer. Antihypertensive drugs are available for treatment of high blood pressure. All patients do not respond in same way towards same drug. Genetic variability is responsible for difference of same drugs response among patients. In our study subjects were divided into two groups. Patients of one group were using amlodipine (calcium channel blocker) and second group was using two antihypertensive drugs amlodipine +losartan for treatment of hypertension.

Patients of CC genotype (major allele) were good responders of amlodipine (6%). They were not good responders of combine medication amlodipine + losartan. Antihypertensive monotherapy was effective among patients of genotype CC and have optimal blood pressure levels by using amlodipine only.

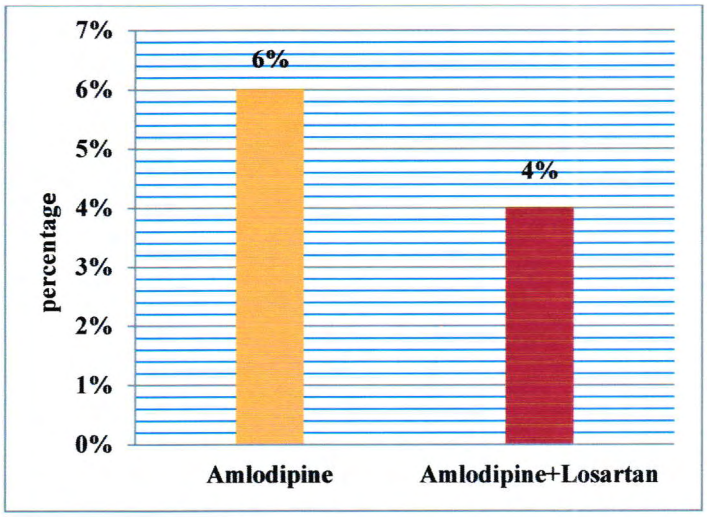


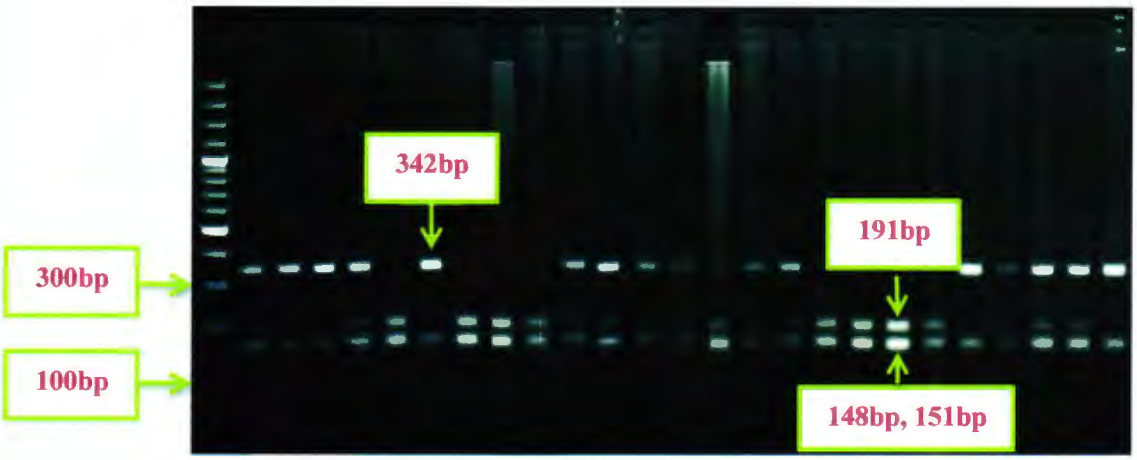
Figure no. 3.2.3. rs2239050 CC genotype & Drug response

The figure shows that patients with CC genotype were good responders of amlodipine only (6%) and combine medication amlodipine + losartan (4%) was less effective to attain optimal blood pressure.

3.3.Genotyping of rs2239127 C/T polymorphism of CACNA1C gene

We have studied rs2239127 in intronic region of CACNA1C gene. PCR-RFLP was carried out and bands of minor allele, major allele and heterozygous allele was produced.CC shows diseased homozygous allele with two bands 342bp and 148bp, CT for heterozygous 4 bands 342bp, 191bp, 151bp, 148bp and TT for wild allele 3 bands 191bp, 151bp, and 148bp. Restriction enzyme has produced fragments seen as bands. Bands of respective allele are shown in figure below. Genetic distribution and allele frequency of rs2239127 C/T polymorphism is shown in table no 2. PCR-RFLP image is shown in (figure no 3.2).

Figure No 3.2: PCR-RFLP Image of rs2239127 C/T polymorphism



- **Risk allele CC (2 fragments) = 342bp, 148bp**
- **Heterozygous allele CT (4 fragments) = 342bp, 191bp, 151bp, 148bp**
- **TT allele (3 fragments) = 191bp. 151bp. 148bp**

3.3.1.Genetic distribution & allele frequency of rs2239127 C/T polymorphism of CACNA1C gene b/w amlodipine, amlodipine + losartan & control group

Genetic distribution and allele frequency of rs2239127 C/T polymorphism of CACNA1C gene is calculated from which association of polymorphism with disease is analyzed. Difference of genetic distribution between amlodipine group, amlodipine + losartan and healthy control group was very clear. Genetic distribution of CC (Risk allele) was 52% in amlodipine group greater than amlodipine + losartan group (36 %) and 52% in control group. Similarly in CT genotype (Heterozygous carriers) was 42% in amlodipine group less than amlodipine + losartan group 48% and 40% in control group. Genotypic distribution in major allele TT (Ancestral allele) was lowest in amlodipine group (6%), in amlodipine + losartan group is 16% and less in control group 8%. Allele frequency of C genotype in amlodipine group is 0.726. In amlodipine +losartan group is 0.6 and in control group is 0.718. Similarly allele frequency of T genotype in amlodipine group is 0.274, 0.4 in amlodipine + losartan group and 0.282 in control group. Genetic distribution and allele frequency of rs2239127 C/T polymorphism of three groups (amlodipine, amlodipine + losartan and control) are shown in table no 3.6.

Table no: 3.6. Genetic distribution and allele frequency of rs2239127 C/T polymorphism of CACNA1C gene

Group	Genotype			Allele frequency	
	CC(Risk)	CT	TT(Major)	C	T
Amlodipine	52	42	6	0.726	0.274
Amlo+Los	36	48	16	0.6	0.4
Control	52	40	8	0.718	0.282

3.3.2.Genotypic distribution of rs2239127 C/T polymorphism between Amlodipine and Amlodipine + Losartan group and association with hypertension

This Statistical analysis shows rs2239127 C/T polymorphism of CACNA1C gene b/w amlodipine and amlodipine +losartan group and its association with hypertension in Pakistani population. Genetic distribution was non-significant between amlodipine and amlodipine + losartan group. According to genotypic distribution and allele frequency of rs2239127 C/T polymorphism P > 0.05 for risk allele CC and results are which shows that polymorphism of C/T in CACNA1C gene is not responsible for hypertension in Pakistani population. Similarly for heterozygous risk allele carrier CT genotype P > 0.05 indicating results non-significant which shows hypertensive patients are non-carriers of risk allele that is responsible for hypertension. For wild type CC genotype P > 0.05 showing statistically non-significant results. Chi square value for GG genotype is 1.732, similarly 0.21 for CT genotype and 3.751 for TT genotype. Online chi square contingency table calculator was used to evaluate P value.

Table No 3.7. Association of rs2239127 C/T polymorphism of CACNA1C gene with Hypertension b/w Amlodipine & Amlodipine + losartan group

rs2239127 C/T polymorphism			
Group	CC (Risk)	CT	TT (Wild)
Amlodipine	52	42	6
Amlodipine + Losartan	36	48	16
P value	P > 0.05	P > 0.05	P > 0.05
Chi square	1.732	0.21	3.751

3.3.3.Genotypic distribution of rs2239127 C/T polymorphism between Amlodipine and Control group and association with hypertension

This Statistical analysis shows rs2239127 C/T polymorphism of CACNA1C gene b/w amlodipine and control group and its association with hypertension in Pakistani population. Results were non- significant between amlodipine and control group. According to genotypic distribution and allele frequency of rs2239127 C/T polymorphism $P > 0.05$ for risk allele CC showing statistically non-significant results. Similarly for heterozygous risk allele carrier genotype CT $P > 0.05$ indicating results non- significant. For wild type TT genotype $P > 0.05$ showing statistically non-significant results. Chi square value for CC genotype is 0.01, similarly 0.046 for CT genotype and 0.377 for TT genotype. Online chi square contingency table calculator was used to evaluate p value.

Table No 3.8. Association of rs2239127 C/T polymorphism of CACNA1C gene with Hypertension b/w Amlodipine & Control group

rs2239127 C/T polymorphism			
Group	CC (Risk)	CT	TT (Wild)
Amlodipine	52	42	6
Control	52	40	8
P value	P > 0.05	P > 0.05	P > 0.05
Chi square	0.01	0.046	0.377

3.3.4.Genotypic distribution of rs2239127 C/T polymorphism between Amlodipine+ losartan group, Control group and association with hypertension

This Statistical analysis shows rs2239127 C/T polymorphism of CACNA1C gene b/w amlodipine + losartan and control group and its association with hypertension in Pakistani population. Results were statistically non- significant between amlodipine + losartan and control group. According to genotypic distribution and allele frequency of rs2239127 C/T polymorphism $P > 0.05$ for risk allele CC showing statistically non-significant results. Statistical results indicate non-association of rs2239127 C/T polymorphism with hypertension in Pakistani population. Similarly for heterozygous risk allele carrier CT genotype $P > 0.05$ indicating results non-significant. Statistically non-significant results show that hypertensive patients are carriers of risk allele and risk allele is not responsible for high blood pressure. For wild type TT genotype $P > 0.05$ showing statistically non-significant results. Chi square value for CC genotype is 8.231, similarly 4.388 for CT genotype and 0.485 for TT genotype. Online chi square contingency table calculator was used to evaluate p value.

Table No 3.9. Association of rs2239127 C/T polymorphism of CACNA1C gene with Hypertension b/w Amlodipine + Losartan & Control group

rs2239127 C/T polymorphism			
Group	CC (Risk)	CT	TT(Wild)
Amlodipine + Losartan	36	48	16
Control	52	40	8
P value	P > 0.05	P > 0.05	P > 0.05
Chi square	8.231	4.388	0.485

3.4.1.rs223127 CC genotype & Drug response between Amlodipine, Amlodipine + Losartan group

Antihypertensive drugs are used to normalize high blood pressure that is major risk factor of many diseases. Patients respond differently towards same drugs. This difference in response towards drugs is due to genetic variability. Genetic variability is changes in genes that are involved in regulatory mechanisms. In our study subjects were divided into two groups. Patients of one group were using amlodipine (calcium channel blocker) and second group was using two antihypertensive drugs amlodipine +losartan for treatment of hypertension.

Patients of CC genotype (risk allele) were good responders of amlodipine (53%). They were not good responders of combine medication amlodipine + losartan. Percentage of drug response in amlodipine + Losartan group is 36% lower than amlodipine group. Antihypertensive monotherapy was effective among patients of genotype CC and have optimal blood pressure levels by using amlodipine only.

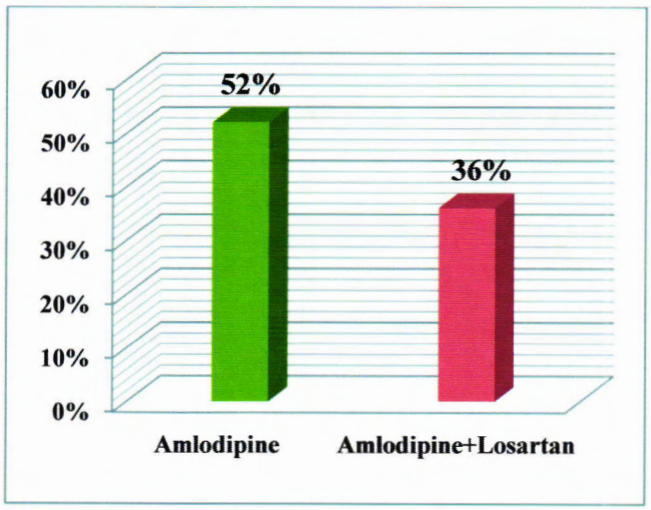


Figure no. 3.3.1 CC genotype & Drug response

This figure shows that patients of genotype CC risk allele were good responders of amlodipine a calcium channel blocker (53%). Patients of CC genotype were not good responders of amlodipine + losartan (36%). Combined medication was not effective among CC genotype patients.

3.4.2. rs223127 CT genotype & Drug response between Amlodipine, Amlodipine + Losartan group

All patients do not respond in same way towards drugs. Genetic variability is associated with it. To analyze drugs response among patients it is very important to study their genetics. In our study subjects were divided into two groups. Patients of one group were using amlodipine (calcium channel blocker) and second group was using two antihypertensive drugs amlodipine +losartan for treatment of hypertension.

Patients of CT genotype (carriers of risk allele) were not very good responders of amlodipine (42%).They were good responders of combine medication amlodipine + losartan. Percentage of drug response in amlodipine + Losartan group is 48% greater than amlodipine group. Antihypertensive monotherapy was not very effective among patients of genotype CT risk allele carriers so two drugs are more effective among them to achieve optimal blood pressure levels.

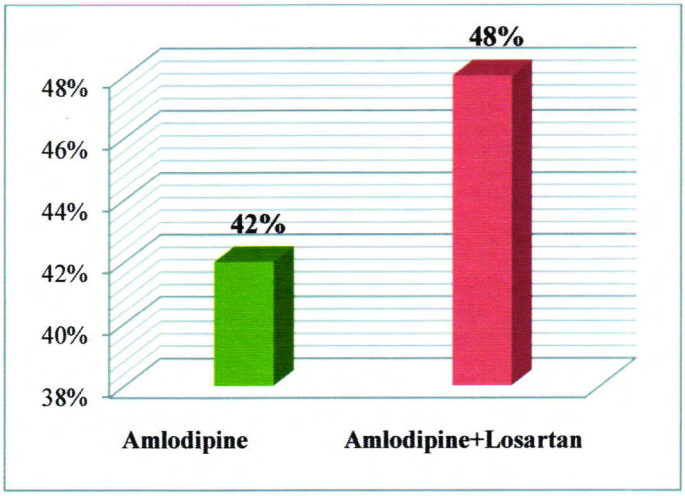


Figure no. 3.3.2. CT genotype & Drug response

This graph shows that patients with CT genotype were not good responders of amlodipine (42%). They were good responders of combine medication (48%) amlodipine + losartan for effective control of high blood pressure.

3.4.3. rs2239127 TT genotype and Drug response between Amlodipine, Amlodipine + Losartan group

Hypertension is risk factor of cardiac disease and renal failure. Antihypertensive drugs are available for normalization of high blood pressure. Genetic variability is factor associated with response of drugs. All patients do not respond in same way towards drugs. To analyze drugs response among patients it is very important to study their genetics. In our study subjects were divided into two groups. Patients of one group were using amlodipine (calcium channel blocker) and second group was using two antihypertensive drugs amlodipine + losartan for treatment of hypertension.

Patients of TT genotype (major allele) were not very good responders of amlodipine (6%).They were good responders of combine medication amlodipine + losartan. Percentage of drug response in amlodipine + Losartan group is 16% greater than amlodipine group. Antihypertensive monotherapy was not very effective among patients of genotype TT major allele carriers so two drugs are more effective among them to achieve optimal blood pressure levels.

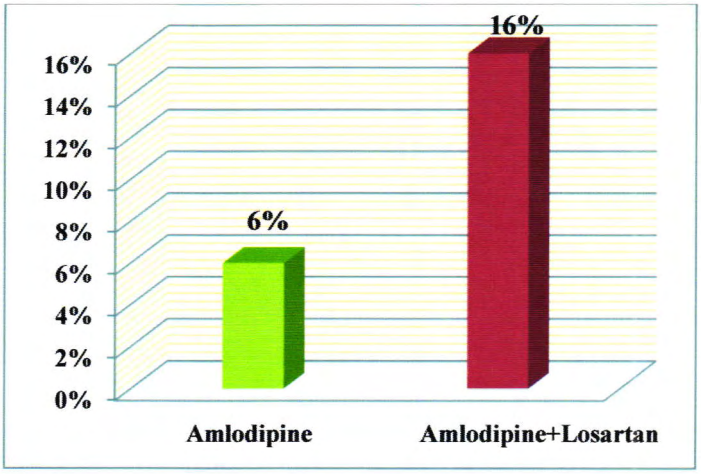


Figure no. 3.3.3. TT genotype & Drug response

This graph shows that patients with TT genotype were not good responders of amlodipine (6%).They were good responders of combine medication amlodipine + losartan (16%). High blood pressure was under control among those using two drugs.

3.5.Comparison of drug response between rs2239050 C/G polymorphism and rs2239127 C/T polymorphism

Genotype percentage among two variants 2239050 C/G polymorphism and rs2239127 C/T polymorphism is analyzed in this study. In rs2239050 major allele C is replaced by minor allele G. Percentage of GG genotype was 53% in amlodipine group and 22% in amlodipine + losartan group. Percentage of GG genotype was greater in amlodipine group. Percentage of GC genotype was less in amlodipine group 41% and greater in amlodipine + losartan group 74%. Similarly CC genotype percentage of CC was higher in amlodipine group (6%) and less in amlodipine +losartan group(4%).

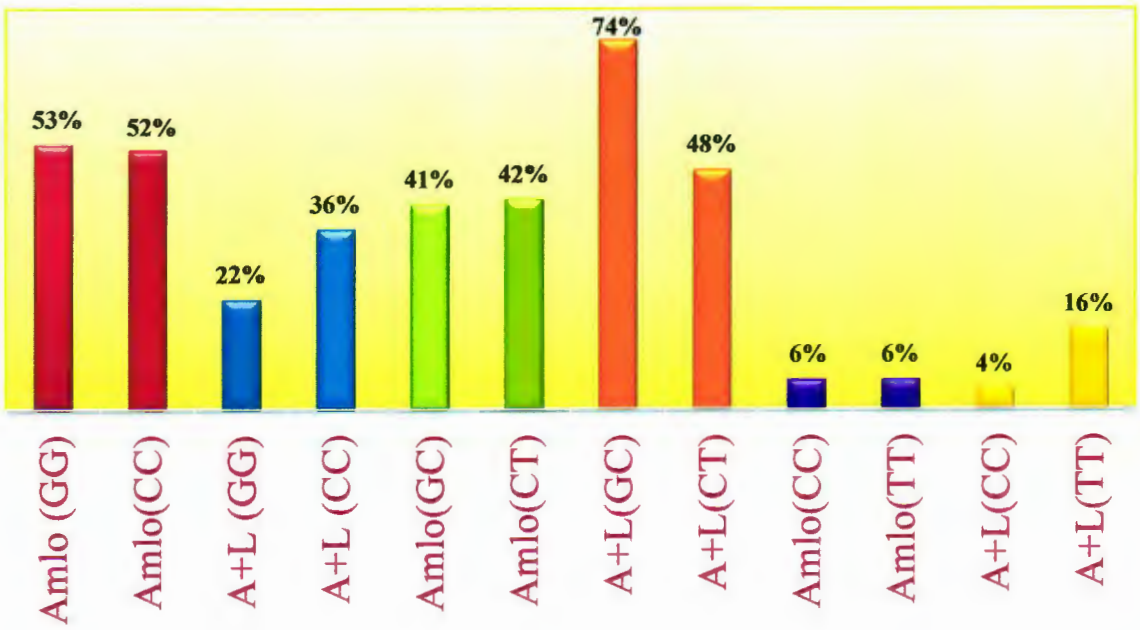
In rs2239127 C/T polymorphism T is major allele and C is risk allele. Percentage of CC genotype was higher in amlodipine group 53% and lower in amlodipine + losartan group (36%) similarly CT genotype percentage was lower in amlodipine group 42% and higher in amlodipine +losartan group 48 %. In TT genotype percentage was lower in amlodipine group 4% and higher in amlodipine +losartan group 6%.

In both SNP, s rs2239050 C/G and rs2239127 C/T polymorphism risk allele GG of rs-050 are good responders of amlodipine group and not good responders of amlodipine + losartan group. Similarly risk alleles CC of rs2239127 are good responders of amlodipine and not good responders of amlodipine + losartan group.

Similarly percentage of Heterozygous risk allele carriers GG genotype of rs2239050 are good responders of amlodipine +losartan group and are not good responders of amlodipine group. In rs2239127 heterozygous carrier genotype CT are good responders of amlodipine + losartan group and not good responders of amlodipine only.

Major alleles CC genotype in rs2239050 are good responders of amlodipine and not good responders of amlodipine and not good responders of amlodipine +losartan group. In rs2239127 major allele TT genotype are not good responders of amlodipine but are good responders of amlodipine + losartan group.

Figure No 3.4: GG (Risk allele) of rs2239050 C/G polymorphism and CC (Risk allele) of rs2239127 C/T polymorphism



CHAPTER 4

Hypertension is most common factor for cardiovascular diseases globally. High blood pressure is responsible for cardiac diseases and has affected 50 % men's and 30 % women are worldwide. Antihypertensive drugs are available to achieve optimal blood pressure. Hypertensive drugs reduce rate of mortality and morbidity and is responsible for modification of circulatory system. Pathophysiology shows complex mechanism of hypertension in which environmental as well as genetic factors are associated. Advancements in molecular studies have identified many genes of enzymes, receptors and channels are associated for onset of hypertension. Polymorphisms of genes have been identified which shows association of genes with high blood pressure.

Calcium ions are most important ion act as secondary messenger and are associated with crucial processes in body both at cellular level and organ level. Calcium ions are involved in contraction – relaxation of muscles, involved in apoptosis, expression of genes. Calcium ions are involved in many diseases.

CACNA1C gene codes alpha 1 subunit of L- VGCC that allows influx of calcium ions. GWAS (genome wide association studies) have shown that polymorphism of CACNA1C gene is involved in many disorders such as learning, memory, neuronal plasticity (Dietsche *et al.*, 2014). Polymorphism of CACNA1C gene that codes for alpha 1 subunit of VGCC increases bipolar disease risk (Green *et al.*, 2010).Studies have confirmed that CACNA1C gene polymorphism is responsible for high blood pressure (Kamide *et al.*, 2009).

Antihypertensive drugs are used worldwide for control of hypertension. Amlodipine that is a calcium channel blocker is used as mono therapy for effective control of high blood pressure (Miura and Saku, 2012). In some cases more than 1 drug is recommended for optimal blood pressure termed as combination therapy. Two antihypertensive drugs are given in combination such as calcium channels blocker (amlodipine) along angiotensin II receptor blocker (losartan) Frank, 2008.

Aim of our study was to evaluate polymorphism of CACNA1C gene in Pakistani population and antihypertensive drugs response among different genotypes. We have studied rs2239050 C > G polymorphism of CACNA1C gene in hypertensive Pakistanis which had shown significant association with hypertension. Majority of patients had GG genotype which shows that

Replacement of ancestral allele C to risk allele G and is associated with hypertension. Percentage of heterozygous carriers genotype GC was also greater then wild genotype CC.

According to genetic distribution patients of risk genotype GG were good responders of amlodipine monotherapy (53%) which shows that rs2239050 polymorphism of CACNA1C gene is associated with amlodipine efficacy. Patients with carrier genotype GC were good responders of combination therapy amlodipine+losartan (74 %) which shows efficacy of calcium channel blocker and angiotensin receptor blocker in treatment of hypertension among patients with GC genotype. Genetic distribution and allele frequency of rs2239050 C/G polymorphism of CACNA1C gene in Pakistani population was similar as observed by Lin in Chines population.

In 2012 seven SNPs of CACNA1C gene were studied to evaluate association of gene and efficacy of amlodipine as antihypertensive drug in 181 hypertensive chines. Among seven SNPs (rs2230128, rs2239127, rs2239127, rs2239050, rs2238032, rs1051375 and rs7311382) rs2239050 C/G polymorphism results showed that GC genotype was associated amlodipine (Lin *et al.*, 2012).

Similarly two genes CACNA1C and CACNA1D were studied to evaluate polymorphism of alpha 1C and alpha 1D subunits, association and efficacy of amlodipine among Japanese hypertensive patients. Results have shown efficacy of Dihydropyridines calcium channels blockers in hypertensive subjects (Watanabe *et al.*, 2009).

We have studied rs2239127 C/T polymorphism of CACNA1C gene. Patients with risk genotype CC were good responders of amlodipine (52 %) showing efficacy of amlodipine in hypertension treatment. Among carrier genotype CT were good responders of combine medication amlodipine + losartan (48 %) than patients using only amlodipine (42%). Combine medication is effective for control of blood when high blood pressure is not controlled by using only monotherapy. Genetic distribution and allele frequency observed in rs2239127 C/T polymorphism of CACNA1C gene in Pakistani population was similar as observed by Alleman *et al.*, 2008.

In polymorphism of CACNA1C gene and its association with calcium channels blocker amlodipine was studied. Similar results were found as in our study. Risk allele was good responders of monotherapy amlodipine. Blood pressure was normalized showing association of

Gene polymorphism and efficacy of amlodipine. Amlodipine as antihypertensive was not effective among patients with carrier genotype. Combine medication calcium channel blocker and angiotensin II receptor blocker was effective when monotherapy fails to normalize high blood pressure.

Our conclusion shows that CACNA1C gene is associated with hypertension in Pakistanis hypertensive population. Patients of risk allele are good responders of dihydropyridine calcium channels blocker amlodipine. Carriers of risk alleles shows efficacy of combine medication amlodipine and losartan both.

These results may divergent because of ethnic backgrounds, life style differences among other populations globally. These finding should be studied further to evaluate polymorphism of CACNA1C gene, its association with antihypertensive drugs and response of drugs among other populations.

Future Perspective

Prevalence of hypertension is increasing in Pakistani population. Need of time is to take thoughtful action for control of hypertension. Identifying genetic polymorphism and association of genes with efficacy of antihypertensive drugs. Human genome project information can be utilized to analyze pathophysiology of hypertension. Identification of variants and their response of drugs among population are helpful for development of therapeutics for hypertension. Modifying prescriptions for high blood pressure and if monotherapy is not effective for shifting towards combination pills for control of high blood pressure.

CHAPTER 5

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Abstract Hypertension is major cause of cardiac disease, renal failure, diabetes mellitus and ultimately death. It is known as silent killer because it may damages vital organs without clear symptoms. Hypertension prevalence is increasing globally. Prevalence rate of hypertension is also very high in developed countries. In underdeveloped countries including Pakistan prevalence of hypertension is very high. Despite of fact antihypertensive medications are available but prevalence rate of hypertension in Pakistan is increasing gradually. Hypertension is caused by integration of genetic and environmental factors. Polymorphisms of genes are also associated with hypertension so identification of genes is decisive. It is important to identify causes because of which antihypertensive drugs do not shows progressive response.