RFLP POLYMORPHISM IN *ADD1* GENE ASSOCIATED WITH ESSENTIAL HYPERTENSION IN NORTH INDIAN POPULATION

Project Report

Submitted in partial fulfilment of the requirements for the degree of

Master of Science (Biotechnology Hons.)

Submitted By

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Under the guidance of

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ABSTRACT

Hypertension, also known as high blood pressure is a major health problem which affects large population worldwide. Essential hypertension does not have known secondary cause. The association between α-adducin (α-ADD1) and essential hypertension is not yet sorted. This study was carried out to observe the possible association between α-adducin gene polymorphism and hypertension in north Indian population. A number of patients with hypertension and a number of normotensive subjects i.e. control group were selected and genotyped for the single nucleotide polymorphism of α-adducin gene by PCR-RFLP technique. Various other biochemical parameters i.e. systolic blood pressure, diastolic blood pressure, total cholesterol, LDL cholesterol, stearin triglycerides were also recorded for control and hypertensive patients and found to be associated with essential hypertension whereas HDL cholesterol, drinking, and smoking didn't show any impact. A significant association between α-ADD1 gene and hypertension was observed in north Indian population. Further studies are needed to be carried on large sample size to confirm these results.

ACKNOWLEDGEMENT

I wish to express my deep sense of thanks and gratitude to my mentor and guide Dr. Mohammad Amin-ul Mannan, Assistant professor, Lovely Professional University, Phagwara. His dedication, scientific approach, keen interest and attitude to help his students encouraged me to do the work. I owe a deep sense of gratitude to Dr. Umesh Gautam, Head of Department of Molecular Biology and Genetic Engineering for his continuous encouragement and guidance. Also, I sincerely thank all the faculty members who helped me during my work. My thanks also go out to Er. Chirag Chopra, Head of Laboratory.

I would like to express my gratitude towards my parents and siblings for their kind co-operation and encouragement.

I thank God for providing me with everything I required.

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DECLARATION

I hereby declare that the project entitled ***"****RFLP* ***Polymorphism in ADD1 Gene Associated with essential hypertension in North Indian Population"*** is an authentic record of my own work carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, for the partial fulfilment of the award of Master of Science in Biotechnology (Hons.) under the guidance of Dr. Mohammad Amin-ul Mannan.

This work is my original work and has not been submitted for any degree/diploma in this or any other University. The information furnished in this report is genuine to the best of my knowledge and belief.

Place: Lovely Professional university Shikha Dhial (11510782)
Date:

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CERTIFICATE

This is to certify that **Shikha Dhial (11510782)** have completed the project, *entitled* ***"RFLP Polymorphism in ADD1 Gene Associated with essential hypertension in North Indian Population"*** under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study.

No part of the report has ever been submitted for any other degree at any University. The report is fit for submission and the partial fulfilment of the conditions for the award of **M.Sc. Biotechnology (Hons.)**.

Date: Supervisor Signature:

CHAPTER -1

INTRODUCTION

Hypertension which is also known as high blood pressure is a major health problem which effects large population worldwide. If caused by another disease or condition is called as secondary hypertension. Blood pressure (BP) is basically the force which is exerted by the blood against the walls of arteries when it is pumped through the body. BP is measured as systolic blood pressure which is the maximum arterial blood pressure during the contraction of the heart (specifically of left ventricle) and diastolic blood pressure which is the force exerted in the arteries when the heart relax between the beats.

Primarily hypertension is subdivided into various forms i.e., essential or primary, and secondary (Carretero.et.al. 2016). Furthermore, hypertension is basically classified according to its cause, essential hypertension means when the cause of hypertension is not known. It usually disappears once the conditions is cured or controlled. Some of the conditions include kidney or endocrine diseases, sleep apnea, cocaine use, smoking, pregnancy, stress, very exhausting exercise, and long term overuse of alcohol etc. On the other hand hypertension is also classified according to its response in an individual as malignant or accelerated hypertension, labile or transient hypertension, isolated systolic hypertension (ISH) and resistant hypertension.

Essential hypertension is recently perceived as a multifactorial disease due to its polygenic inheritance pattern and association with various environmental factors. (Shin,et.al.2004). Essential hypertension is a major risk factor for cardiovascular disease (CVD). It is a most common cardiovascular risk factor in the industrialized world (Naz, et.al.2015). Essential hypertension (EH) is responsible for 24% of coronary heart failure and 57% of all cerebral hemorrhage deaths in India (Gupta.2004). Moreover not only genetic but also epigenetic inheritance is involved in essential hypertension. Hypertension develops an altered situation in well-coordinated regulatory systems of blood pressure. Alterations in the functioning of molecular, biochemical and genetic processes, which control blood pressure, probably result in

hypertension. Numbers of genetic markers have been spotted in the regulation of hypertension. The occurrence of EH may be associated with a variety of gene mutations and variations including adducin gene (ADD) (Wang, et.al.2014).

Adducin (ADD) is a heterodimeric structural protein which consists of a α-subunit with either a β- or a γ-subunit, and they are known by three different genes *ADD1*, *ADD2*, and *ADD3*, respectively. In Present study, we will focus on α-ADD1 gene. The α-adducin (α-ADD1) is ubiquitous and structural protein that is involved in various functions such as cell-to-cell contact, cell membrane ion transport, and signal transduction. Hypertension can also be caused due to abnormalities of membrane sodium transport in the kidney. Adducin promotes spectrin and actin association which is regulated by calcium/calmodulin. In Human species, the location of *ADD1* gene is on chromosome 4p16.3 i.e. position 16.3 and consists of 16 exons (Zhang, et.al.2013). In humans, there are two types of polymorphisms related to *ADD1* gene which leads to amino acid substitutions: Gly460Trp and Ser586Cys (Cusi et al., 1997). The G614T polymorphism in nucleotide results in the substitution of glycine amino acid by tryptophan amino acid (Gly460Trp). The amino acid substitution of tryptophan for glycine occurs at amino acid positioned 460. The α-adducin gene is located on the 4^{th} chromosome in *Homo sapiens* and a Guanine →Thymine polymorphism in exon 10 at position 217 of the gene results in the substitution of glycine to tryptophan at amino acid position 460 (Gly460Trp) in the protein. Gly460Trp polymorphism of gene *ADD1* was related with a salt-sensitive hypertension.

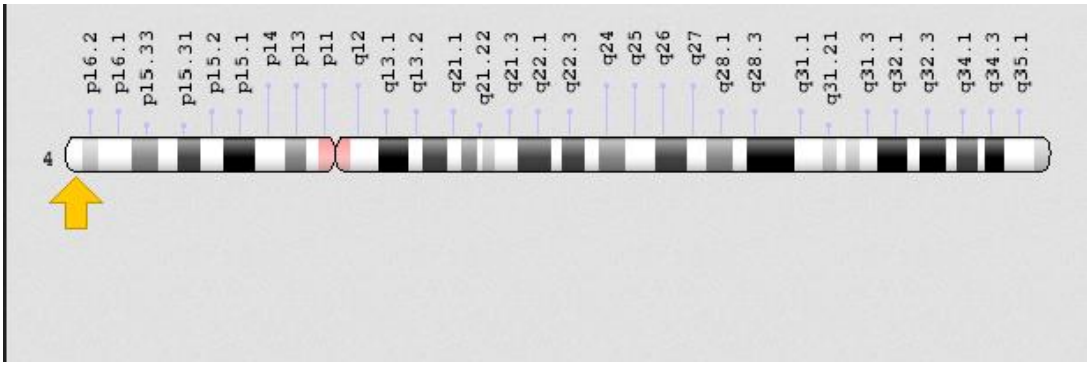


Figure1. Cytogenetic location of *ADD1* gene: Located on the short (p) arm of chromosome 4 specifically at position 16.3 i.e. 4p16.3. Molecular location is from base pairs 2,843,732 to 2,930,076 on chromosome 4 (image source: NCBI)

ADD1 gene polymorphism G614T (rs4961) is found to result in an increased enzymatic activity of the outer medulla Na^+-K^+-ATPase prior to the development of hypertension in the Milan hypertensive strain of rats (MHS) (Wang, et.al.2014). *ADD1* gene polymorphism at Ser586Cys is related to colorectal cancer (CRC) and is not further considered. ADD1 can act as a 'renal hypertensive gene' that disturbs the potential of the tubular epithelial cell to transport sodium and thus, affects blood pressure (Kundu, et.al; 2013). Besides genetic factors, environmental factors also play the major role in the regulation and maintenance of blood pressure. One can decrease the chances of hypertension by maintaining proper body weight, doing good amount of physical work, restriction of stress, supplement healthy food and vegetable consumption (Naz et.al.2015).

CHAPTER -2

TERMINOLOGY

Genomic: - Study of the whole set of genes present in a cell of an organism.

Hypertension: - The extended medical condition in which the blood pressure is persistently high.

Essential Hypertension: - It refers to a condition of high blood pressure also known as primary hypertension which doesn't have known secondary cause such as reno-vascular disease, renal failure, Aldosteronism etc. It is a heterogeneous disorder with a different causal factor in different patients that leads to high blood pressure.

Normotensive: -Representing normal blood pressure.

Calmodulin: - Ubiquitous calcium- binding protein that can bind to different protein targets, affecting many different cellular functions.

PCR: - Polymerase Chain Reaction, molecular biology technique used to amplify a segment of DNA.

RFLP: - Restriction Fragment Length Polymorphism, a technique used to differentiate between organisms on the basis of unique pattern in DNA fragments when restricted with a particular restriction enzyme.

SNP: - Single Nucleotide Polymorphism, Genetic variation (deletion, insertion etc.) which leads to alterations causing diseases.

ADD1: - *ADD1* is a gene which codes for alpha-adducin protein in humans.

Polymorphism: - The presence of DNA sequence at a particular locus in different forms within the population.

SBP: - Systolic Blood pressure (120mmHg).

DBP: - Diastolic Blood Pressure (80mmHg).

LDL: Low-density lipoprotein is also known as bad cholesterol.

HDL: High-Density lipoprotein is also known as good cholesterol.

S. Cholesterol: Serum Cholesterol is total amount of cholesterol in blood.

***Taq* Polymerase**: - Enzyme used in replication of DNA in thermocycler.

Phenol: Chloroform: - Denature proteins and facilitate the separation of aqueous and organic phase.

Isoamyl alcohol: - It is a transparent, colorless alcohol that reduces foaming.

Ethanol: - Ethanol without dilution is used to precipitate DNA and when diluted (70%) it is used to wash DNA.

TE buffer: - consist of Tris-Cl and EDTA and maintain pH. Used for storing DNA.

Proteinase K: - Serine protease, digests protein.

SDS: Sodium Dodecyl Sulfate is an anionic detergent that reduces surface tension.

CHAPTER -3

REVIEW OF LITERATURE

There are multifactorial factors responsible for hypertension which means combined effect of several factors produce hypertension. It is influenced by inherited genetic factors, environmental factors, and lifestyle of individuals. Hypertension causes 4.5% of current global diseases such as cardiovascular, neural, and cardiac.(Kaplan et.al, 2003) and is responsible for deaths and diseases occurring in the India (Gupta, 2004)

HYPERTENSION AND ITS TYPES:

High BP is also linked to hypertension. Blood pressure is the measure of pressure which is forced by blood against the sides of the arteries when it is pumped through the body. Blood pressure is generally recorded as two numbers which are: (i) systolic BP (ii) diastolic BP.

(i) Systolic BP: It is the maximum atrial blood pressure during contraction of the heart (specifically of the left ventricle). Normal systolic blood pressure is 120 mm Hg (millimeters of mercury)

(ii) Diastolic BP: It is the arterial pressure when the heart rests between the beats.

BP categories defined by American heart association (AHA):

- In Normal BP: systolic BP: 120 and diastolic BP: 80
- Prehypertension: systolic: 120-139 and diastolic: 80-89.
- High BP (Hypertension): Systolic blood pressure (SBP) is ≥140mmHg and/or diastolic blood pressure (DBP) is ≥90mmHg (Li-naZhang et.al; 2012).

Stages of hypertension:

- Stage1- systolic: 140-159 and diastolic: 90-99.
- Stage 2- systolic: 160 or high and diastolic: 100 or high.

Hypertensive crises: May leads to death. Systolic is higher than 180 and diastolic is lower than 110. Increased blood pressure also significantly elevates the risk of cardiovascular diseases (Carretero et.al; 2000)

Hypertension is classified into different forms i.e. according to cause and according to response in an individual. According to cause hypertension is classified into two which is primary or essential hypertension and secondary hypertension. Essential hypertension is a state where the cause of hypertension is not known. It is more often present in the population and hypertension caused by another disease or condition is called secondary hypertension (Fardella et al; 2000). Secondary hypertension disappears in controlled conditions. On the basis of response hypertension is classified into malignant or accelerated hypertension, Labile or transient hypertension, Isolated Systolic Hypertension (ISH) and resistant hypertension. Malignant or accelerated hypertension is the sudden rise in blood pressure that may be deadly (Mohan, 2005), Labile or transient hypertension is temporary and occur during stressful conditions (Mancia et al. 1996), isolated systolic hypertension occur in older people due to only rise in systolic blood pressure (Bulpitt et al. 1996) and resistant hypertension does not respond to treatment and is difficult to control (Tobe et al. 2007).

DIAGNOSIS AND SYMPTOMS:

Blood pressure is a silent disease and needs to be regularly monitored. Blood pressure is measured with a device reading called mercury sphygmomanometer which consists of a stethoscope, arm cuff, valve, and pump. Blood pressure is helpful in diagnosing hypertension.

EFFECTS AND SIDE-EFFECTS OF HYPERTENSION:

Previous studies have predicted 60% increase in the number of adults with hypertension by the year 2025; totaling to 1.56 billion populations. Hypertension is also a root cause of many other diseases. It's different effects or side-effects have been discussed here.

Hypertension is likely to occur in diabetic patients and affects approximately 70% of patients with diabetes. Approximately it is twice as common in persons with diabetes as in those without (Lago. et.al, 2007). hypertension makes heart working complicated as it makes the heart work too hard and causes atherosclerosis (hardening of arteries) and also increases the chances of

stroke, heart diseases, blindness, kidney disease and congestive heart failure (Tabassum. et.al,2011).

CAUSES OF HYPERTENSION:

Since hypertension and blood pressure are related, so many factors are known to increase BP namely, stress, insulin resistance, high alcohol intake, obesity, aging, inactive lifestyle, high salt intake (in salt-sensitive patients), low calcium intake, and low potassium intake. Moreover, obesity and alcohol intake are some of the additive factors. (A Drewnowski.et al. 2015).

METHODS OF PREVENTION:

Previous studies on prevention of hypertension concluded that there are six efficiently proven perspectives for prevention of hypertension: involve in physical activity, maintain normal body weight and stay fit, reduce sodium intake, limited alcohol consumption, maintain adequate consumption of potassium, and eat a healthy diet rich in vegetables and fruits and low-fat dairy eatables and reduce in saturated and total fat. (Whelton.et.al.2002).

TREATMENT:

In developing countries, nearly 75-80% of the world's population rely on herbal medicine mainly for primary health care treatment because of better adaptability & lesser side effects for human body, easy availability, and cheap (Nahida Tabassum.et.al., 2011)

Previous studies on clinical review in the management of hypersensitive crises suggest several anti-hypersensitive agents available including labetalol, fenoldopam, esmolol, and nicardipine. Sodium nitroprusside can also be suggested in selected circumstances (Joseph Varon.et.al.2003).

GENE POLYMORPHISMS RESPONSIBLE FOR HYPERTENSION

The various gene polymorphisms which are responsible for hypertension which are:

(i) α-Adducin Gene Polymorphism (*ADD1*): The α-adducin gene is located on chromosome number 4 in homo sapiens. A single nucleotide substitution (G→T, Guanine to Thymine) at position 217 in exon 10 of this gene results in the substitution

of glycine to tryptophan at 460 amino acid position (Gly460Trp) in the protein. Gly460Trp polymorphism of *ADD1* was related with a salt-sensitive kind of hypertension (Katsuya.T,et.al; 2003). *ADD1* gene polymorphism G614T (rs4961) is found to accelerate enzymatic activity of the outer medulla Na^+K^+-ATPase before the development of hypertension in the Milan hypertensive strain(MHS) of rats (Lifang Wang,et.al)

(ii) Angiotensinogen gene polymorphism (*AGT*): previous studies suggest that the T235 allele in exon 2 or C31 allele in exon 1 of AGT has been related with increased chances for hypertension, positive family background of hypertension , coronary heart disease and lacunar infarction(Katsuya et al.2003).

(iii) Aldosterone Synthase Gene Polymorphism (*CYP11B2*): aldosterone synthase gene (*CYP11B2*) aldosterone synthase gene is located on 8q21 and encodes steroid 18-hydroxylase, it is different from *CYP11B1* sequence of genome of their exons are 93% identical but *CYP11B2*, it plays an important role in the biosynthesis of aldosterone, and it is expressed in both adrenal fasciculata and glomerulosa. Polymorphism in the 5′- region of the *CYP11B2*, T(− 344)C, is reported to be associated with hypertension and plasma aldosterone levels in a Caucasian population(Katsuya et al.2003)

(iv) Other than *AGT*, *ADD1* and *CYP11B2* the gene which is encoding for the G-Protein β3 subunit, *GNB* β3 subunit, *GNB3* have also being shown to be associated with salt hypersensitive hypertension (Katsuya et.al.2003).

HYPERTENSION AND ITS ASSOCIATION WITH ADDUCIN GENE POLYMORPHISM:

The occurrence of EH may be associated with a variety of gene mutations and variations including mutation in adducin gene (*ADD1*) (Lifang Wang, et.al.). we will study about *ADD1* gene and we will be able to determine other effects and diseases related to the *ADD1* gene polymorphism. The α-adducin (*α-ADD1*) is a structural protein that is known to have function in cell-to-cell contact, cell membrane ion transport, and signal transfer. Hypertension can also be caused due to abnormalities of membrane sodium transport in the kidney. Adducin promotes spectrin and actin association which is regulated by calcium/calmodulin.

ADD1 gene polymorphism G614T (rs4961) is found to result in an accelerated enzymatic activity of the outer medulla Na+-K+-ATPase prior to the development of hypertension in the Milan hypertensive strain of rats (MHS). Further, by using PCR-RFLP technique the study analyzed and reported significant relation between α-adducin gene G614T mutation and essential hypertension observed in hypertensive patients (170) in Chinese population in comparison to 154 normotensive individuals (Control group) (Lifang Wang, et.al 2014).

ADD1 gene polymorphism at Ser586Cys is related to colorectal cancer (CRC). *ADD1* may be examined as a 'renal hypertensive gene' that influences the potential of the tubular epithelial cell to transfer Na and hence, influence blood pressure. In their study they committed to the non-synonymous single nucleotide polymorphisms of a adducin 1 (*ADD1*) gene and to understand its role in hypertension its variations in different populations were analyzed and amino acid substitution for rs4961 was observed from glycine to tryptophan, which means from an alkyl amino acid to an aromatic amino acid and these variations may be the cause of hypertension (Kundu, et.al).

Liu and coworkers 2010 worked on alpha-adducin polymorphism Gly460Trp and risk of hypertension by performing a Meta-Analysis consisting of 22 Studies Including 14303 Cases and 15961 Controls. They used systematic and computerized literature search from PubMed and EMBASE databases (up to May 2010) and Medline for their study and considered human subjects without any country restrictions by limiting search to articles in English and failed to show evidences for the genetic association between α-adducin gene and hypertension in their meta-analysis.

Gly460Trp polymorphism related to *ADD1* gene was determined using PCR (polymerase chain reaction) and study provided no significant difference between the hypertensive patients and normotensive individuals, which suggests that there is no relation between Gly460Trp polymorphism of *ADD1* and hypertension. Participants of the study were 903 individuals and all of them participated in a population-based study in Jangseong County, Korea, in August 2000. The frequency of the Gly460Trp allele was 61.1% in hypertensive patients and 59.4% in normotensives; this showed no significant difference between the hypertensive and normotensive groups and it suggested that the Gly460Trp polymorphism of ADD-1 was not associated with hypertension. (M.-H. Shin.et.al.2004).

The study based on relationship between α-adducin gene polymorphism (Gly460Trp) and essential hypertension was conducted in North Indian population living in the same geographic region with a similar socioeconomic level was done using PCR-RFLP (restriction fragment length polymorphism) considering randomly recruited subjects including 151 healthy people and 101 patients of essential hypertension. It was found that the rs 4961 polymorphism of the ADD1 gene is having association with essential hypertension which suggests that the alpha adducin might be a susceptible gene to essential hypertension (Naz.et.al.2015).

Essential hypertension (EH) is responsible for 24% of coronary heart failure and 57% of all cerebral hemorrhage deaths in India. This study was based on Trends in the hypertension epidemiology in India (Gupta, 2004).Shen and colleagues, 2015 studied on an association between ADD1 and Colorectal Cancer risk and genetics of colorectal cancer. Correlation of ADD1-rs4963 was also investigated with smoking or drinking exposure and found that variant in ADD1 significantly conferred susceptibility to CRC.Appel and coworkers, 2006 presented their studies on the 'Dietary Approaches to Prevent and cure Hypertension' and reported number of dietary factors the contribute to high blood pressure. Further, modifying the dietary habits viz. increasing the potassium intake, reducing the salt intake and limiting the alcohol consumption supplemented by exercise and diet called the DASH diet (rich in vegetables and fruits) effectively lowers the blood pressure.

However previous studies were controversial and unable to clearly suggest the relation between *ADD1* gene and hypertension. Some of the studies have found polymorphism of *ADD1* not associated with hypertension and some previous works concluded that there is a significant association between *ADD1* gene polymorphism and essential hypertension. Despite in several studies the exact molecular mechanism in *ADD1* gene and hypertension is not clear, so in this study an attempt was made to find out the association of *ADD1* gene with hypertension.

CHAPTER -4

RATIONALE AND SCOPE OF THE STUDY

From previous studies we get to know that there are contradictory results on association between essential hypertension (EH) and α-adducin gene polymorphism and hence their association is not clear. A lot of study has been done in India and other countries but limit study is available on north Indians so, this study is based on clarifying the association of α-adducin gene polymorphism with essential hypertension by considering various parameters including blood pressure (BP). Single nucleotide polymorphism for α- adducing will be tested in hypersensitive patients against control from north Indian population (Punjab) (normotensive) by PCR-RFLP technique.

CHAPTER-5

OBJECTIVES OF STUDY

- ✓ To amplify adducin gene (*ADD1*) by polymerase chain reaction (PCR).
- ✓ To identify single nucleotide polymorphism (SNP) in α-adducin1 gene by RFLP analysis.
- ✓ To study the interdependence between α-adducin1 gene polymorphism and hypertension in the north Indian population.
- ✓ To study association of various biochemical parameters with α-ADD1 gene polymorphism.

CHAPTER -6

MATERIALS AND RESEARCH METHODOLOGY

Sample Collection: 2-3 ml of blood sample was collected from each participant in EDTA vacutainer and stored at -20ºC for Polymorphism study.

Sample of Patients were collected from Satyam Lab, S.S Memorial Satyam hospital, Pathankot from January – April 2017. Consent form and pre-information was given to participants with the help of physician and technician involved and the project title was approved by ethical committee, Lovely Professional University.

Overall 29 samples were collected; eight were normal and twenty-one from hypertensive individuals. Additional data including clinical characteristics (age, sex, drinking & smoking) and biochemical parameters (HDL, LDL, Serum cholestrol, and Serum triglycerides) were also collected.

DNA Isolation: Genomic DNA was extracted using two alternative methods: (1) by standard Phenol chloroform method (organic method) and by using Blood genome DNA extraction Kit (GeNei™) as per the manufacturer's instructions. DNA bands were observed on 1% Agarose gel and DNA concentration was determined by measuring OD using NanoDrop 2000 at GADVASU, Ludhiana.

Genotyping: The genotyping of G→T Polymorphism of *ADD1* gene at 460 Position was done by PCR-RFLP.

PCR: Amplification of DNA fragments was done in total volume of 20 µl PCR reaction mixture containing 10X buffer (with $MgCl_2$) 2µl, 2.5mM dNTP 3µl, forward Primer (5'-CTCCTTTGCTAGTGACGGTGATTC-3') 1µl, reverse primer (5'-GACTTGGCACTGCTTCCATTCGGC-3') 1µl, Nuclease free water 10 µl, Taq polymerase (Hi-media) 1µl and DNA 2µl. The reactions was amplified under following conditions: one cycle of 3 min at 94ºC, 30 cycles of 20 sec at 94ºC, 25 sec at 58ºC and 20 sec at 72ºC, followed by 5 min at 72ºC. The amplified products were loaded onto 2% Agarose gel.

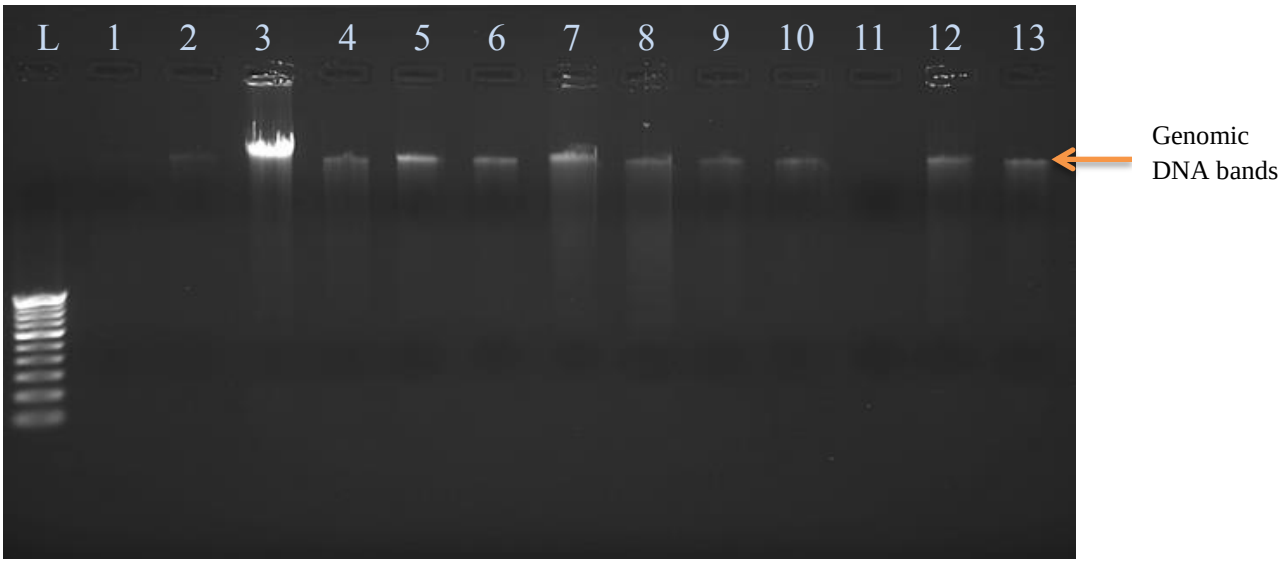
RFLP: Digestion of PCR amplified product was done using restriction enzyme Sau96I (New England Biolabs). Restriction was carried out in total volume of 20µl reaction mixture containing 10X NEB buffer (cutsmart) 10µl, 2µl of Sau96I enzyme and 8µl of PCR amplified at 37ºC for 1 hour. Products were loaded onto 4% agarose gel and electrophoresed. The product was 122bp and 147 bp for the 460Trp and 460Gly, respectively.

CHAPTER -7

RESULT AND DISCUSSIONS

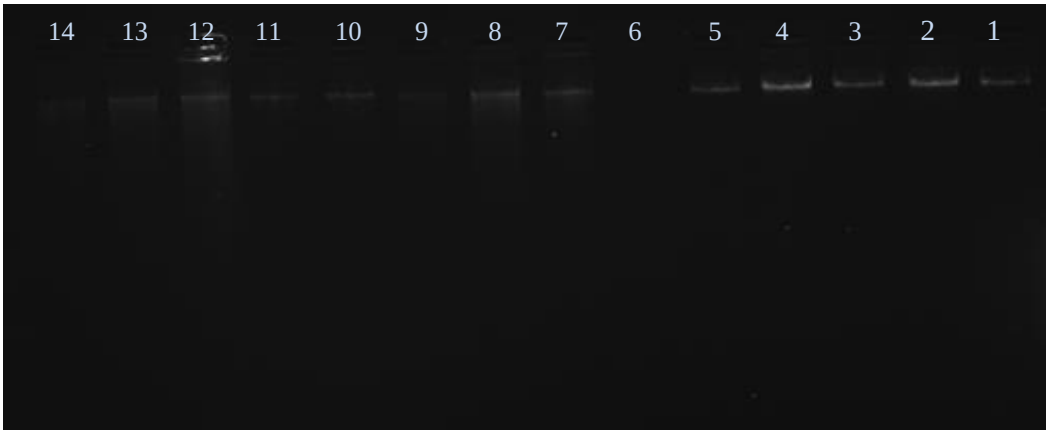
DNA ISOLATION:

DNA was isolated using two methods i.e. standard phenol chloroform method and genomic DNA isolation kit (GeNei^{TM}) and bands were observed on 1% agarose gel. Good quality DNA was observed of almost all samples except 2 or three.

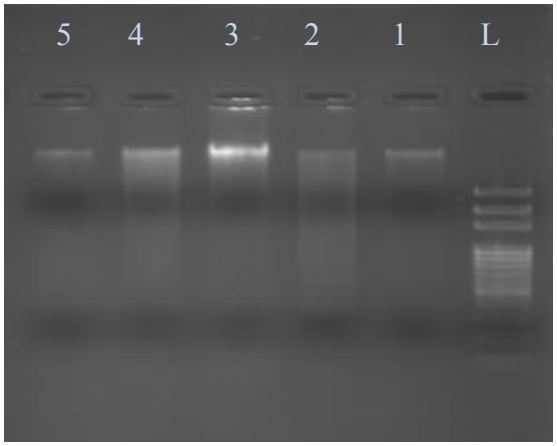


2 (a) L= Ladder 1kb

DNA samples= Lane1:HY1, Lane 2= HY2, Lane 3= HY3, Lane 4: HY4 Lane 5:HY5, Lane 6= HY6, Lane 7= HY7, Lane 8=HY8, Lane 9= HY9, Lane 10= HY10, Lane 11= HY11, Lane 12= HY12, Lane 13= HY13. The DNA of each sample is isolated using kit.



2 (b) DNA Samples = DNA of hypertension patients isolated using Phenol chloroform method: Lane 1=HY14, Lane 2= HY15, Lane 3= HY16, Lane 4= HY17, Lane 5= HY18, Lane 6= no DNA. DNA isolated from blood of normal participants using kit: Lane 7 =N1, Lane 8= N2, Lane 9= N3, Lane 10= N4, Lane 11= N5, Lane 12=N6, Lane 13= N7, Lane 14= N8.

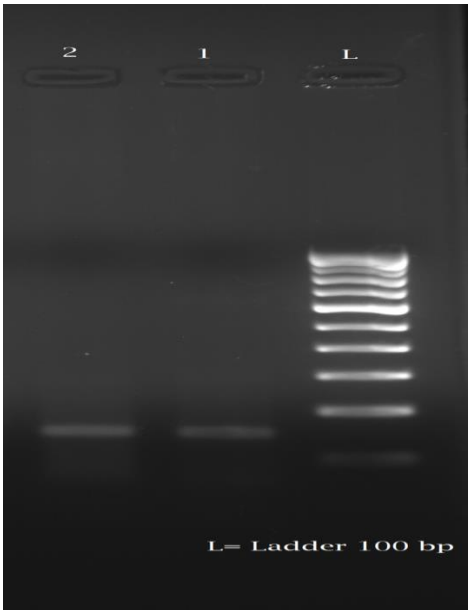


2 (c) DNA Samples of hypertension patients Isolated using kit: Lane1= HY19, Lane 2= HY20, Lane 3=HY21, Lane 4= HY22, Lane 5= HY23.

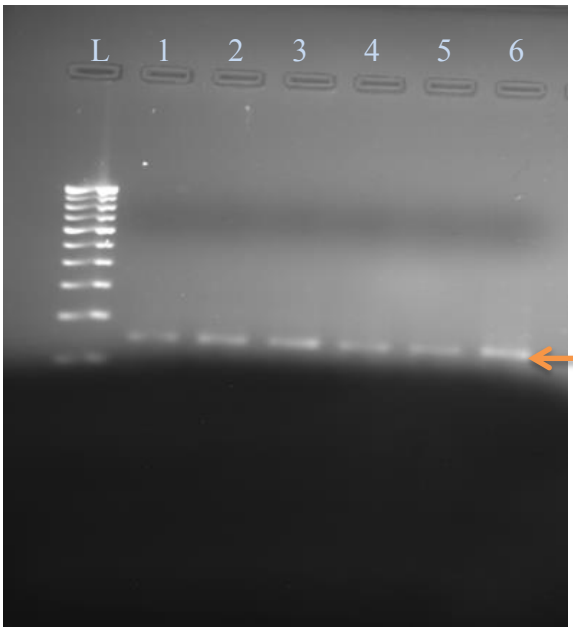
Figure 1: 1% Agarose gel showing genomic DNA in (a) & (c) isolated using kit method, (b) isolated using standard phenol chloroform method.

PCR:

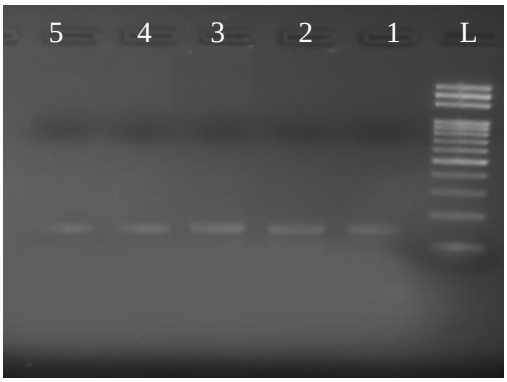
PCR conditions were standardized and DNA was amplified using protocol mentioned in material and methods. Results were observed on 2% Agarose gel.



3 (a) PCR
Lane 1= N1, Lane 2= N2



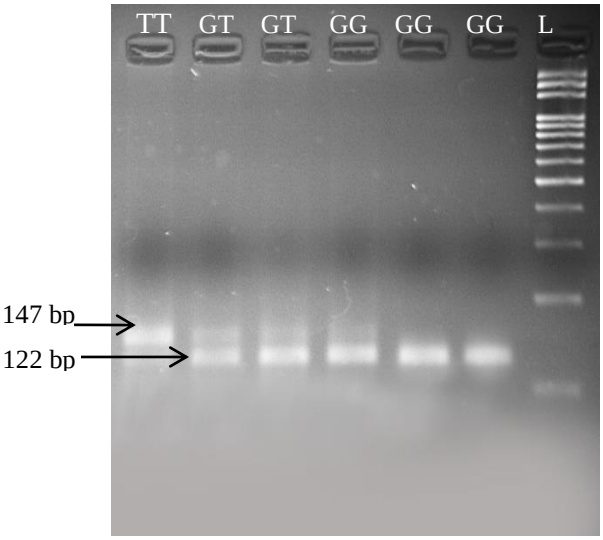
3 (b) PCR
L= 100 bp ladder. Lane 1=HY14, Lane 2= HY16, Lane 3= HY17, Lane 4 =HY15, Lane 5 = HY18, Lane 6= N6



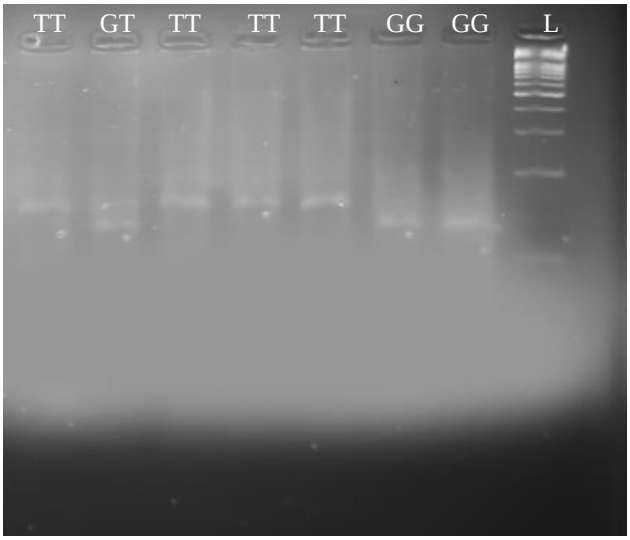
3(c) PCR
L= Ladder, Lane1=HY19, Lane2= HY20, Lane3=HY21, Lane4=HY22, Lane5= HY23

Figure 2: (a), (b) & (c) showing PCR amplified bands on 2% Agarose gel.

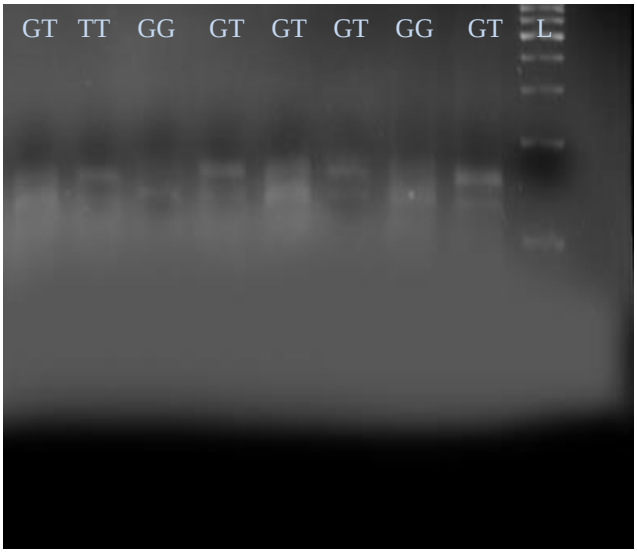
RESTRICTION: PCR amplified product was restricted using Sau96I enzyme



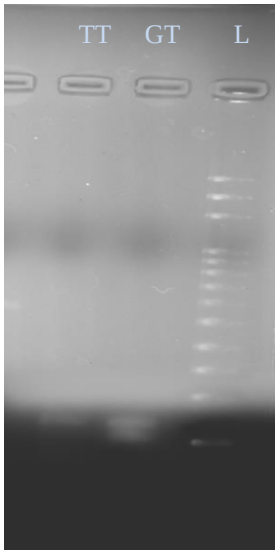
4(a) Restriction
Lane 1= HY14, Lane 2= HY16, , Lane 3= HY17, Lane 4 =HY15, Lane 5 = HY18, Lane 6= N6



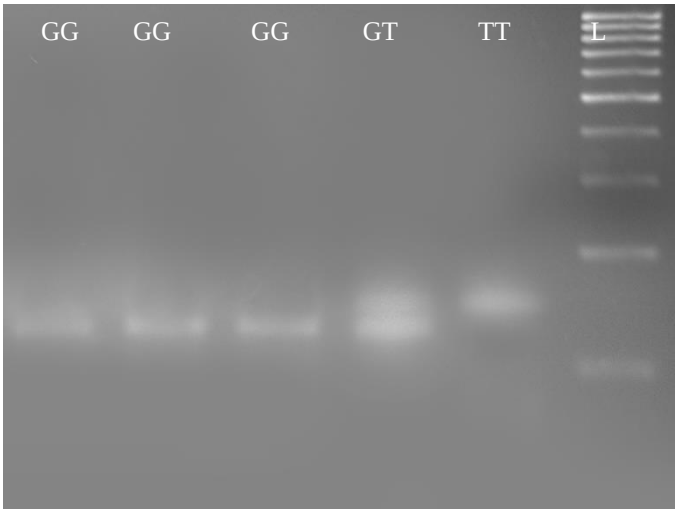
4(b) Restriction
L= 100 bp ladder, Lane 1= HY2, Lane 2= HY4, Lane 3=HY8, Lane 4 = HY9, Lane 5=HY10, Lane 6= HY12, Lane 7= HY13.



4(c) Restriction
Lane 1= HY3, Lane 2= HY5, Lane 3= HY6, Lane 4= HY7, Lane 5= N4, Lane 6= N5, Lane 7= N7, Lane 8= N8.



4(d) Restriction
Lane 1= N1, Lane 2= N2



4(e) Restriction
L= Ladder, Lane1=HY19, Lane2= HY20, Lane3=HY21, Lane4=HY22, Lane5= HY23

Figure 3: (a), (b), (c), (d) & (e) showing restriction digestion of PCR amplified product using Sau96I enzyme on 4% Agarose gel.

Statistical analysis were done for various biochemical parameters and no major difference was observed between smoking and drinking habits , HDL of normal controls and hypertension patients whereas values of SBP, DBP, LDL, serum cholesterol and serum triglycerides were higher in hypertension patients than normal controls as shown in Table 1. Therefore these biochemical parameters may be responsible for cause of essential hypertension.

Genotype GG, GT & TT percentage of α- adducin gene in normal control and hypertension patients was calculated, after observing bands in restriction digestion on 4% Agarose gel and significant association was observed between G614T association of *ADD1* gene and hypertension in north Indian population as shown in Table 2.

Table 1: Spectrophotometer readings of genomic DNA isolated from hypertensive patients.

Sample ID	A260	A280	A260/A280	Concentration (ng/µl)
HY1	0.893	0.590	1.51	44.67
HY2	0.965	0.626	1.54	48.24
HY3	4.334	2.341	1.85	216.72
HY4	7.088	3.883	1.83	354.39
HY5	1.551	0.863	1.80	77.53
HY6	1.373	0.847	1.62	68.66
HY7	1.384	0.800	1.73	69.19
HY8	1.399	0.812	1.72	69.96
HY9	1.042	0.723	1.44	52.08
HY10	0.740	0.439	1.69	37.02
HY11	0.371	0.432	0.86	18.55
HY12	2.377	1.755	1.35	118.84
HY13	1.029	0.630	1.63	51.46
HY14	1.164	0.659	1.77	58.21
HY15	1.226	0.879	1.40	61.32
HY16	0.595	0.372	1.60	29.73
HY17	2.116	1.187	1.78	105.80
HY18	0.349	0.223	1.57	17.47
HY19	1.312	1.053	1.25	65.58
HY20	0.865	0.464	1.86	43.26
HY21	2.635	1.416	1.86	131.77
HY22	6.234	3.471	1.80	311.69
HY23	1.855	0.980	1.89	92.73

Table 2: Spectrophotometer readings of DNA isolated from normal participant

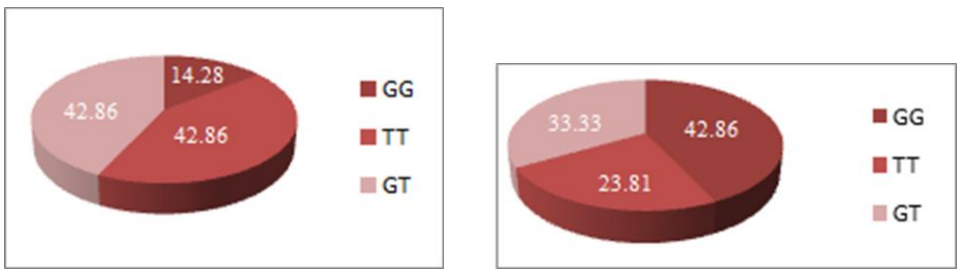
Sample ID	A260	A280	A260/A280	Concentration (ng/µl)
N1	1.742	.962	1.81	92.62
N2	1.454	.902	1.61	69.64
N3	1.264	.752	1.68	52.46
N4	.965	.882	1.09	49.26
N5	1.352	1.157	1.16	65.62
N6	1.086	.829	1.31	52.09
N7	1.054	.758	1.39	69.99
N8	1.515	.899	1.68	68.68

Table 3: Statistical analysis of biochemical parameters of normal control and HP patients

Statistical analysis of Biochemical Parameters of essential normotensive and hypertension group		
	Hypertension Patients (n±S.D)	**Normal control (n±S.D)**
Number	23	8
Age	25-75	25-70
SBP(mmHg)	205±25.37	117.5±12.81
DBP(mmHg)	99.13±9.960	81.25±6.41
LDL	91.25±35.87	79.625±17.93
HDL	48.65±4.97	47±3.74
S. TRIGLYCERIDES	189.38±100.9	153.41±36.45
S.Cholestrol	180.78±46.9	157.23±24.96

Table 4: Genotype percentage of α- adducin gene in normal control and hypertension patients

***ADD1* SNP**	**Hypertension Patients (%)**	**Normal Control (%)**
TT	23.81	42.86
GT	33.33	42.86
GG	42.86	14.28



(a) (b)

Figure 5: Pie chart showing allele and genotype frequency of α- adducin gene in (a) normal controls (b) hypertension patients

CHAPTER -8

EXPERIMENTAL WORK:

DNA ISOLATION:

Phenol Chloroform Method (Organic Method)

1X Lysis buffer **(**10X lysis buffer: NH_4Cl 155mM, $KHCO_3$ 10mM, EDTA .1mM, double distilled

Water) + 1ml Blood

(2ml) Keep at 4ºC for 20 min

Vortexed and centrifuged at 7000 rpm, 10 min

Minimum 3 washings with 1X lysis buffer were done (till pellet gets white/brown)

To the pellet added 405µl per sample of solution (5µl Proteinase K 10mg/ml,

25µl SDS 10%, 375µl NaOAC 2M) vortex to mix.

Keep at 56ºC for 30 min

Add Phenol: chloroform: isoamyl (25:24:1)-120µl

Vortex and centrifuge at 10,000 rpm for 5 min.

Transfer supernatant to fresh tube

Repeat

Add 120µl of chloroform: isoamyl (24:1) mix and centrifuge at 10,000 rpm for 5 min.

Transfer supernatant to fresh tube

Add 1 ml of chilled ethanol; keep at -20ºC overnight

Centrifuge at 10,000 rpm for 10 min, supernatant was discarded

Washing is done with 70% ethanol, Centrifuge at 10,000 rpm for 8-10 min, Discard supernatant

air dry at 37ºC

Dissolve DNA in 1X TE buffer and stored at -20ºC for further use (PCR-RFLP)

PCR PRIMERS

Table 5: PCR Primers used for amplification of ADD1 gene

Gene	Position	Primer sequence	Melt.T_m	Restriction Enzyme	Reference
ADD1	-460G/T	F-5'-CTCCTTTGCTAGTGACGGTGATTC-3' R-5'-GACTTGGCACTGCTTCCATTCGGC-3'	58ºC	Sau96I	Wang et al.2011

Table 6: PCR reaction conditions

COMPONENTS	VOLUME (µl)
DNA	2µl
Nuclease free water	10µl
dNTP	3µl
Forward Primer	1µl
Reverse Primer	1µl
Taq Polymerase	1µl
10X PCR buffer with $MgCl_2$	2µl

Table 7: PCR Conditions

PCR Conditions	Temperature	Time	Cycles
Initial Denaturation	94	3 min	1
Denaturation	94	20 sec	30
Annealing	58	25 sec	
Extension	72	20 sec	
Final Extension	72	5 min	1

Table 8: Restriction reaction

Restriction Reaction (20µl)	
DNA	8µl
Enzyme	2µl
Buffer	10µl

Restriction was done for 1 hour at 37ºC

CHAPTER -9

CONCLUSION AND FUTURE SCOPE:

On the basis of results it can be concluded that, a significant association was found between essential hypertension and G614T polymorphism related to α- adducin gene in north Indian population. Further studies can be done taking sample from other cities of north to strengthen the data as in this data samples are taken from the patients living in Pathankot or visiting to Satyam Hospital Pathankot from nearby areas.

Various other parameters can also be tested for association to find out other causes of essential hypertension, which may help to design a different and more efficient drug of essential hypertension.

CHAPTER-10

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CHAPTER-11

APPENDIX

Preparation of stock solutions:

EDTA 0.5M (pH-8) ,100ml

M= $W/mw \times 1000/V$

.5=W/292.25× 1000/100

W=.5×292.25/10

W=14.61g

NaCl 5M, 500ml

M= $W/mw \times 1000/V$

5=W/58.44×1000/500

W=58.44×5/2

W=146.1g

Tris-HCl, 500ml

M= $W/mw \times 1000/V$

1=W/157.60×1000/500

W=157.60/2

W=78.8g

10% SDS

10 g of SDS was dissolved in 100ml of distilled water

Sodium acetate 3M (pH-5.2), 500ml

M= $W/mw \times 1000/V$

3M=W/82.03×1000/500

W=3×82.03/2

W=123.045g

NaOH 5M, 100ml

M= $W/mw \times 1000/V$

5M=W/40×1000/100

W=20g

10X TAE buffer (500ml):

Tris base= 24.2g

0.5M EDTA=10 ml

Glacial acetic acid=5.72ml

Made the total volume up to 500ml

1X TAE, (1 L):

N1V1=N2V2

10X ×V1=1X ×1000ml

V1=1000/10

V1=100ml

100ml 10X TAE was added in 900ml distilled water.

$MgCl_2$ 1M, 50ml

M= $W/mw \times 1000/V$

1M=W/203.31×1000/50

W=203.31/20

W=10.1655