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APPLICATION OF MOLECULAR BEACON BASED BIOSENSOR AGAINST rs699 SNP IN HYPERTENSIVE AND NON- HYPERTENSIVE PUNJABI POPULATION

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ABSTRACT: Molecular beacons have shown their topnotch potential in a variety of basic research, biomedical detection and clinical diagnosis. Their excellent selectivity, sensitivity, detection without separation have made them widely accepted tool for nucleic acid analysis. MBs present high-throughput screening of SNPs. The use of SNPs in the detection of genetic disorders is facilitated by the recent discovery of more than 4 million SNPs in the human genome. The objective of this study was to explore the association of rs699 SNP with essential hypertension in Punjabi Population. MB based biosensor developed by Verma *et al.*, in 2016 was chosen for analysis. These hypertensive subjects tested positive against hypertensive biosensor (against allele C) were associated with essential hypertension. C4072T or rs699 polymorphism was genotyped in 50 hypertensive and 50 normotensive subjects. The p-value for the C allele in hypertensive patients was 0.71, which concludes susceptibility to hypertension in this population when the CC genotype is present. The implementation was cross-validated by applying the same samples to Normotensive Biosensor (against T allele). The presence of fluorescence with hypertensive biosensor confirms the presence of a mutation in hypertensive patients or patients that are clinically naive but can develop hypertension in the forthcoming due to hypertensive allele presence. Hence it can help in the future pharmacogenetic based treatment of patients.

INTRODUCTION: Molecular Beacons (MBs) are single-stranded nucleic acid probes which are composed of three different functional domains: a stem, a loop, and a fluorophore/quencher pair ¹⁻⁴. The fluorophore/quencher pair is the signaling unit that produces the on / off signals as per the conformation state of MBs.

MBs open up only when perfectly matched target sequences are present, thus strongly discriminating even at the single base pair mismatch ⁵. Opening up of MB upon hybridization to the target relays the fluorescence signal and hence can be used for SNP detection ¹⁻³.

Various conventional techniques such as RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism), and SSR (Single Sequence Repeat) was used for SNP analysis. The development of high throughput techniques such as Taqman probes Amplifluor, Genome resequencing, SNP-arrays, MALDI-TOF, allele-specific PCR, *etc.* not only

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helped in reaction speed but also with large sample size. These techniques have a high economic cost. MBs on contrary present many advantages such as ease of synthesis, molecular specificity, unique functionality, structural tolerance to various modifications and inherent signal transduction mechanism. Also, the reusability of MBs when immobilized lowers the economic cost, thus aiding in the development of MB based SNP arrays. They can differentiate between even single mismatch nucleotide due to their unique thermodynamic and structural properties, thus providing them a high degree of molecular specificity in SNP detection. MBs can be used in conjunction with amplified target DNA and simultaneously detect amplified products in real-time PCR. One of the most potent applications is that it is not necessary to isolate the probe-target hybrids as non-hybridized MBs do not fluoresce⁶. SNPs are variations present in 1% population and are randomly distributed throughout the genome contributing to our uniqueness.

An SNP in a protein-coding sequence induces amino acid change, thus leads to functional changes in the protein. Some of these variations are directly linked to human diseases. These genetic differences can be medically relevant, which are not only health conquering, but also descends into our progeny. SNPs are useful markers for human genetic studies because of their high density throughout human genome⁷. Moreover, they make it easy to perform large-scale genetic analysis by high-throughput techniques⁸.

Furthermore, the occurrence of SNPs in the coding region (coding SNP; cSNP) and promoter region (regulatory SNP; rSNP) of genes cannot only alter protein function but also gene expression^{9, 10}. Various diseases such as autoimmune diseases, genetic diseases, cancers, neurodegenerative diseases, cardiovascular diseases have been linked to SNPs. Hypertension is one of the cardiovascular diseases that tops the chart because of modern lifestyle as it hits 25% of the adult population. Genetics of hypertension is complex, and here multiple genes interact and react to different environmental stimuli. Various studies have confirmed the presence of a biallelic and multiallelic polymorphism in genes responsible for hypertension¹¹⁻¹⁴. The risk of stroke is lowered by 40% if blood pressure is controlled.

Renin-angiotensin-aldosterone system (RAAS) controls the blood pressure, a system that includes kidney, cardiovascular system, lungs and central nervous system¹⁵⁻¹⁸. AGT gene is one of the regulators of blood pressure. The AGT code for angiotensinogen peptide that is a precursor to angiotensin II where latter regulates BP by multiple mechanisms that include sodium retention in blood and its re-absorption in the kidney^{15, 18, 19}. One of the first-line agents for the treatment of hypertension are angiotensin-converting enzyme inhibitors (ACEI). These inhibitors block the active site of angiotensin-converting enzyme, thereby, decrease in angiotensin levels, hence lowering BP¹⁹ **Fig. 1**.

However, the BP management response varies among an individual in different ethnic groups²⁰⁻²², that concludes the genetic constitution is the key in BP response to ACEI therapy. Pregenetic screening of presence disease-causing SNP, in particular, the ethnic group may help with pharmacogenetic analysis and thus recommend much-needed complimentary therapy. The molecular linkage of the angiotensinogen gene in human hypertension was first established in 1992¹⁵. Until then, there have been various studies that linked AGT polymorphism to essential hypertension^{15, 23-42}. The various ethnic groups that were reported with mutated rs699 are Polish Population, Northern Han Chinese population, Older Australians, Female subjects in Japan, Mexican - mestizo women, Chinese Coronary patients, Japanese male workers²³⁻⁴¹.

Our present research is based on the application of pre-reported developed biosensor against rs699 SNP in Punjabi Population⁴². rs699 is angiotensinogen SNP that encodes a functional change. This is known as M235T or C4072T. C allele instead of T encodes threonine in protein, which contributes to higher plasma angiotensin levels thus elevating blood pressure. The surface immobilized molecular beacons can distinguish a C/T mutation in gene sequence detected by G/C allele at 28th nucleotide position. C allele in a mutated sample of target oligonucleotides binds to the central position (28th) of the loop of the molecular beacon (G allele), and 3 fold enhancement of fluorescence was obtained⁴². The working of Biosensor is shown in **Fig. 2**.

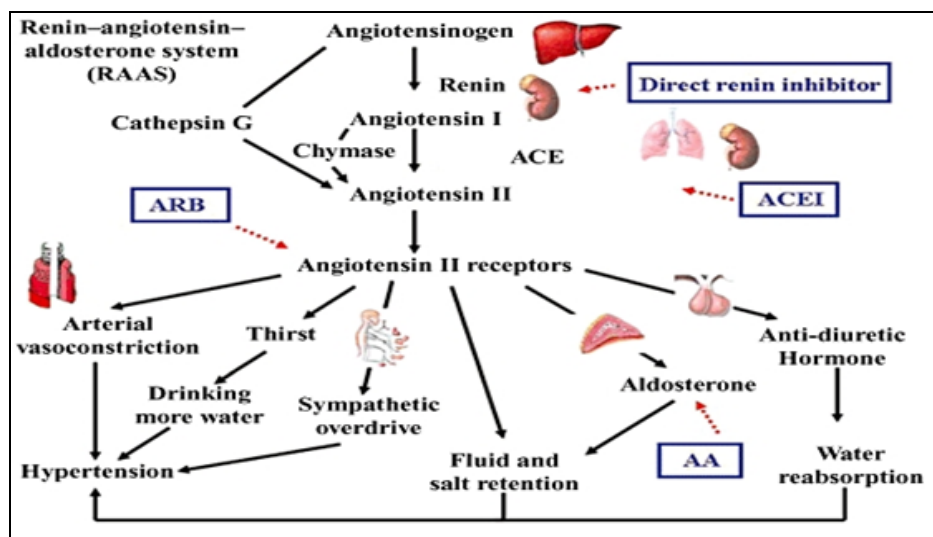


FIG. 1: RAAS. RENIN, PRODUCED BY THE JUXTAGLOMERULAR CELLS OF THE KIDNEY CONVERTS ANGIOTENSINOGEN TO A-I. ANGIOTENSINOGEN IS AN ALPHA-2-GLOBULIN MAINLY PRODUCED BY THE LIVER. A-I IS BIOLOGICALLY INACTIVE AND IS ACTIVATED BY ACE, MAINLY PRODUCED BY THE LUNGS TO FORM A-II. A-II ACTS ON A-II RECEPTORS. THE ANGIOTENSIN TYPE 1 (AT1) RECEPTOR GOVERNS MOST PHYSIOLOGICAL EFFECTS. THE NET EFFECTS OF ACTIVATION OF THE RAAS INCLUDE VASOCONSTRICTION, INCREASED ARTERIAL BLOOD PRESSURE, INCREASED MYOCARDIAL CONTRACTILITY, SODIUM AND WATER RETENTION WHICH SUBSEQUENTLY INCREASES THE EFFECTIVE CIRCULATING VOLUME. RENIN-ANGIOTENSIN-ALDOSTERONE BLOCKADE CAN BE ACHIEVED BY DIRECT RENIN INHIBITOR, ACEI, ARB, AND AA. A-II CAN ALSO BE PRODUCED BY ALTERNATIVE PATHWAYS BY ENZYMES LIKE CHYMASE AND CATHEPSIN G, WHICH FORM THE BASIS OF 'A-II ESCAPE.' THIS IS ALSO THE RATIONALE FOR USING THE DUAL BLOCKADE OF THE SYSTEM BY ACEI AND ARB¹⁹

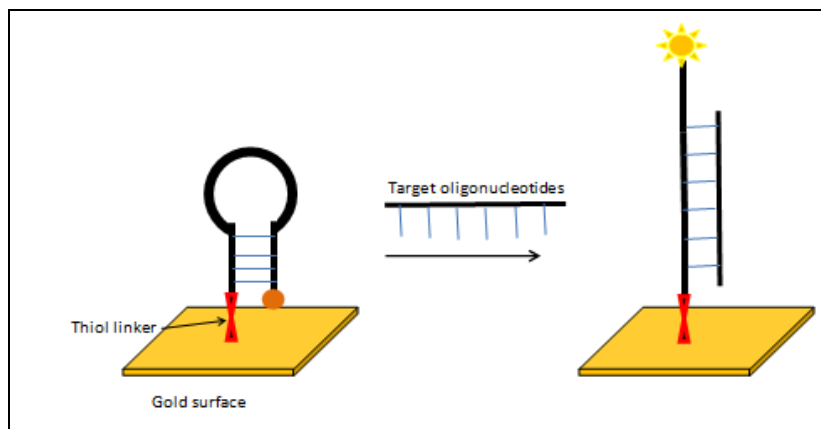


FIG. 2: HYBRIDIZATION OF MOLECULAR BEACON TO TARGET SEQUENCE⁴²

MATERIALS AND METHODS:

Chemicals: Hypertensive and non-hypertensive blood samples were procured from Muktsar and Patiala. PCR solutions of molecular grade were procured from Himedia technologies. The primers used for DNA amplification were procured from Regeneration Technologies, Chandigarh, India are shown in **Table 1**. Silicon wafer with gold-coated was procured from IIT Delhi, India. The oligonucleotides sequence of Molecular Beacons is shown in **Table 2**, purchased from Regeneration Technologies, Chandigarh, India. Molecular

Beacons (MBs) were functionalized at 5' end with the thiol group and 3' end with Tetramethylrhodamine (TMR). All other chemicals used were of molecular biology grade, without any further purification. Ultrapure water with a resistivity of 18.2 MΩ as produced by the WaterPro water purification system (Labconco Corporation, Kansas City, MO) was used in the preparation of buffers and rinsing solution. The buffered saline used has a composition of 20 mM cacodylic acid, 0.5 M NaCl and 0.5 mM EDTA, pH = 7.

TABLE 1: FORWARD AND REVERSE PRIMER SEQUENCES USED IN PCR AMPLIFICATION

1	Forward primer	5'TGACAGGATGGAAGACTGGC-3'
2	Reverse primer	5'CTAAGTCCTAGGGCCAGAGC-3'

TABLE 2: MOLECULAR BEACON PROBES WITH THEIR SEQUENCE

S. no.	Sequence Designation	Oligonucleotide Sequence
1	Molecular Beacon Sequence 1 (MB1) SNP	5'-C-6-thiol- TGGAAGACTGTGTCCACACTGGCTCCCGTCAGGGAGCA GCCAGTCTTCCA-3' Amino-C7-TMR-3'
2	Molecular Beacon Sequence 2 (MB2) wild type	5'-C-6-thiol- TGGAAGACTGTGTCCACACTGGCTCCCATCAGGGAGCAG CCAGTCTTCCA-3' Amino-C7-TMR-3'

Methods:

Hypertensive Blood Samples: Blood samples were procured from the Muktsar district in Punjab, India. The patient consent contains the following data, the name of a patient, age, sex, blood pressure stats, hereditary of disease, and drug recommended. Ethical clearance for the procurement of blood samples from human subjects was obtained from the Institutional Ethics Committee (IEC) and file no. is 141/DLS/HG.

- 50 Hypertensive samples and 50 normotensive samples with less than 60 years and more than 20 years were selected.
- As per JNC, SBP was not less than 140 mmHg, and DBP was not less than 90 mmHg.

DNA Isolation, Qualification and Quantification: DNA was extracted from samples by salting out technique⁴³. Nucleic acid precipitation is used to purify and concentrate DNA. Precipitation is based upon the fact that DNA is less soluble in alcohol than in polar water. Ethanol is the most widely used alcohol for DNA purification.

The extracted DNA was analyzed on 1% agarose gel to view the genomic DNA. Genomic DNA was quantitatively analyzed on an optical fiber spectrophotometer. All the 100 isolated genomic DNA samples were analyzed by measuring absorbance at wavelengths of 260, 280 and 320 nm. After UV quantification the concentration of DNA was calculated as below:

$$\text{Conc. of DNA} = \text{OD}_{260} \times 50 \mu\text{g/ml} \times \text{DF}$$

Where DF = Dilution Factor, OD_{260} of 1 = 50 μg of DNA/ml

PCR Amplification and Purification of Amplified Targets: PCR reaction was optimized at different concentrations of MgCl_2 and range of temperature. One with best bands was selected for the amplification. The 125bp sequence from rs699 SNP (AGT gene) and an ancestral gene sequence was amplified *via* PCR by using primer pairs as listed in **Table 1**. Various chemicals used in reactions are summed up in **Table 3**, and **Table 4** contains different phases of PCR Cycles where numbers of cycles were 35

TABLE 3: DIFFERENT COMPONENTS OF PCR REACTION MIXTURE

S. no.	Contents	Optimum concentration range	Concentration used	Volume used (μl)
1	Template DNA	1ng-1 μg	In mg as per sample	1
2	Forward Primer	0.05-1 μM	1 μM	1
3	Reverse Primer	0.05-1 μM	1 μM	1
4	Buffer S	10x	1x	2.5
5	MgCl_2	1.5- 4mM	2mM	1
6	dNTPs	200 μM	200 μM	2
7	IP water			16
8	Taq polymerase	0.125-2.5 units	2.5 units	0.5

TABLE 4: PCR PHASE FOR 25 μL SOLUTION

S. no.	PCR Phases	Temperature	Time	No. of cycles
1	Initial Denaturation	94 °C	5 min	01
2	Denaturation	94 °C	45 sec	35
	Annealing	55.6 °C	30 sec	
	Extension	72 °C	30 sec	
3	Final extension	72 °C	10 min	01

The total volume of reaction: 25 μl , Lid Temperature 105 °C, Hold at 4 °C forever.

Application of Amplified Targets to rs699 Based Biosensor:

Preparation of Self-assembled Oligonucleotides on Gold Surface: ⁴² Gold coated silicon wafers were cleaned with a piranha solution (4:1 concentrated H₂SO₄ / 30% H₂O₂) overnight at room temperature and then rinsed with ultra-pure water. The self-assembly process of hairpin oligonucleotides on the gold surface was done by pouring the mixture of hairpin oligonucleotides and 3-mercaptopropanol in a ratio of 1:1. After two hours the modified chip was thoroughly rinsed with hot water (90 °C or higher) to remove any unbound oligonucleotides. Next, the gold chip carrying the mixed monolayer was immersed in buffered saline for hairpin formation. Hybridization with the target (amplified DNA) to the hairpin probes on the gold was performed at room temperature under the same conditions.

Determination of Fluorescence: Hybridization was analyzed at 35 °C using Mini Opticon, BioRad, USA, set with a 435 ± 5 nm excitation filter and a 556 ± 5 nm emission filter after 5 mins of hybridization.

Analysis of Population by Applying Hardy-Weinberg Law: The Hardy-Weinberg equation is a mathematical equation which is used to calculate the genetic variations of a population at equilibrium. It states the amount of genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors. The equation is given as:

$$p^2 + 2pq + q^2 = 1$$

Where p represents the "A" allele frequency and q is the frequency of the "a" allele in the population. Here in the equation, p² is the frequency of the homozygous genotype AA, q² is the frequency of the homozygous genotype aa, and 2pq is the frequency of the heterozygous genotype Aa. Also, the sum of the allele frequencies of all the alleles at the locus must be 1, so p + q = 1. If the p and q allele frequencies are known, then the frequencies of the three genotypes may be calculated using the Hardy-Weinberg equation.

RESULTS AND DISCUSSION:

Hypertensive Blood Samples: The blood samples procured from hypertensive and normotensive

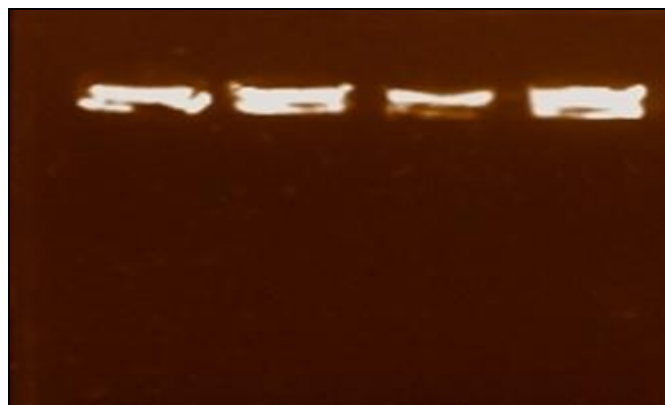
patients have the following statistics as presented in Table 5.

TABLE 5: SUMMARY OF PATIENT SAMPLES

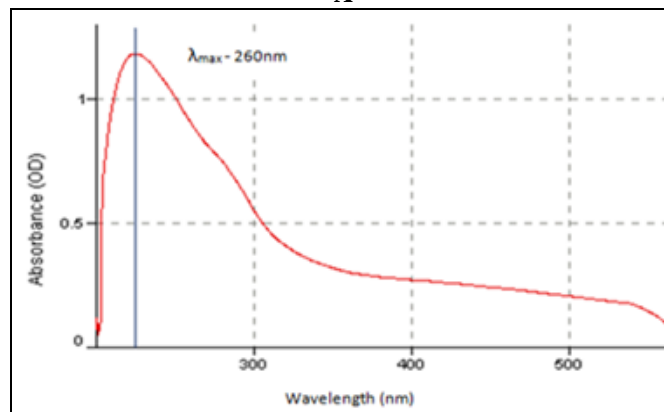
No. of Patients	100 (Hypertensive subjects -50; Normal Subjects- 50)
Hypertensive Subjects (50)	Men -19; Women- 31
Normotensive Subjects (50)	Men-27; Women-23
Blood Pressure Statistics	Systolic-above 140mm of Hg Diasystolic-above 130mm of Hg
Hereditary Occurrence	Yes, Men -14; Women -23
Therapies Used	Diuretics, Beta Blockers, ACEI, ARB
1 st choice of therapy	ACEI

DNA Isolation, Qualification, and Quantification:

The DNA extracted from blood samples was analyzed for the presence of intact genomic DNA, which was confirmed by its presence in wells of 1% agarose gel Fig. 3. The presence of lambda peak at 260 confirms purified DNA and hence less protein and RNA. The derived $\lambda_{260} / \lambda_{280}$ for most of the samples lies in between 1.68 to 1.77.



A



B

FIG. 3: A) INTACT GENOMIC DNA IN WELLS WITHOUT SHEERING. B) THE SCANNING SPECTRA FROM THE 200nm - 600nm

PCR Amplification and Purification of Amplified Targets: The amplified 125bp target sequence were analyzed on 10% polyacrylamide gel along with 100bp DNA ladder⁴⁴ and the bands were visualized *via* silver staining⁴⁵. The amplified 125bp bands are shown in **Fig. 4**.

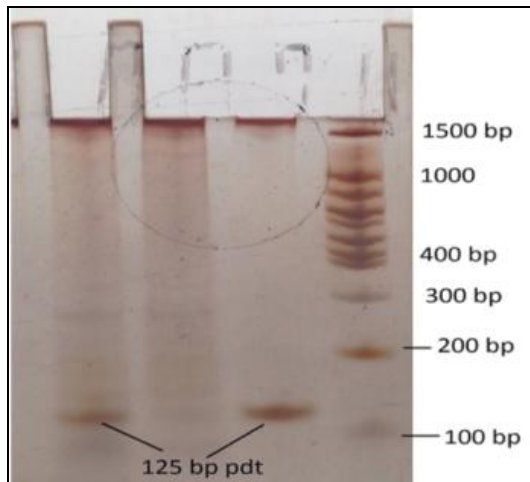


FIG. 4: 125BP AMPLIFIED BP PRODUCT VISUALIZED ON POLYACRYLAMIDE GEL

Application of Amplified Targets to rs699 Based Biosensor: The amplified 125bp target DNA was applied to immobilize MB onto gold chips (Hypertensive Biosensor). The fluorescence was observed in the presence of the SNP. Schematic illustration for MBs against SNP/Wild type sequence is depicted in **Fig. 5**. Amplified DNA from 50 hypertensive samples was applied to immobilized biosensor based on MB1 (Hypertensive Biosensor). As per the results, there should be an increment in fluorescence value since amplified PCR products are of hypertensive subjects. 38 samples (H1, H2, H6, H7, H8, H10, H11, H13, H16, H18, H19, H20, H21, H22, H25, H26, H27,

H28, H29, H30, H31, H32, H34, H35, H37, H38, H39, H40, H41, H42, H43, H44, H45, H46, H47, H48, H49, and H50) registered increment in fluorescence thus indicating mutation in rs699 leading to hypertension **Table 6**. While in others there was no increment in fluorescence hence indicating the absence of mutation rs699 but here hypertension may be present due to a mutation in any other gene hence leading to the condition.

For cross-validation, amplified, hypertensive samples were applied to the biosensor with immobilized MB2 (complementary to the wild-type gene of AGT gene sequence) or Normotensive Biosensor. The above-derived results were validated as PCR amplified products were applied to the biosensor as there should be no increment. 38 samples that fluoresced with MB1 based biosensor did not register increment here **Table 7**. The increment was registered in 20 samples (H2, H3, H4, H5, H8, H9, H10, H12, H14, H15, H16, H17, H23, H24, H31, H32, H33, H35, H36, and H39). These samples have wild-type rs699 SNP since the increment was registered against MB2. In these hypertensive subjects, the condition may prevail due to a mutation in some other gene than rs699.

Eight samples (H2, H8, H10, H16, H31, H32, H35 and H39) registered fluorescence enhancement with both Hypertensive and Normotensive Biosensor **Table 6** and **Table 7** indicating the presence of heterozygous gene with one wild-type allele and one mutated allele suggesting the clinical presence of hypertension is due to dominant expression of mutated gene due to environmental stimuli. For example, patients may have a high dietary salt intake which could elevate BP.

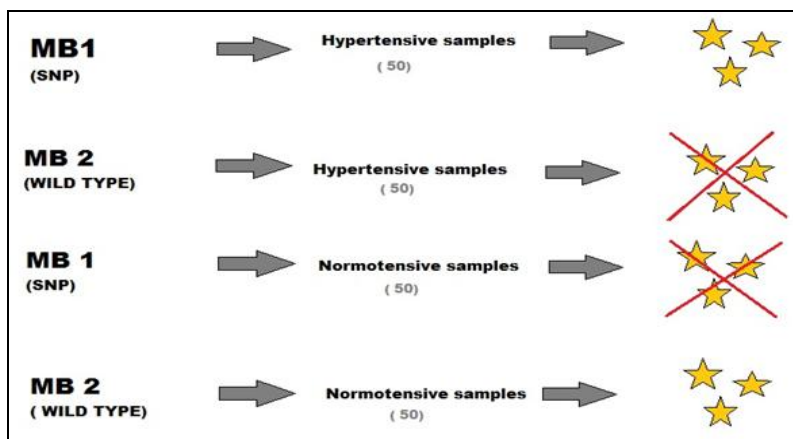


FIG. 5: SCHEMATIC ILLUSTRATION FOR MBS AGAINST SNP / WILD TYPE SEQUENCE IS DEPICTED

TABLE 6: FLUORESCENCE ENHANCEMENT IN HYPERTENSIVE SAMPLES AGAINST MB1 (HYPERTENSIVE BIOSENSOR)

Sample no.	Before hybridization	After hybridization	Fluorescence enhancement
H1	0.404 ± 0.0024	0.627 ± 0.0005	1.55 ± 0.009
H2	0.679 ± 0.0031	0.750 ± 0.003	1.10 ± 0.002
H3	0.450 ± 0.004	0.313 ± 0.004	0.695 ± 0.003
H4	0.652 ± 0.007	0.559 ± 0.003	0.85 ± 0.0064
H5	0.267 ± 0.004	0.279 ± 0.005	1.04 ± 0.002
H6	0.370 ± 0.002	0.724 ± 0.002	1.95 ± 0.0055
H7	0.177 ± 0.005	0.386 ± 0.006	2.180 ± 0.028
H8	0.240 ± 0.004	0.356 ± 0.004	1.48 ± 0.001
H9	0.273 ± 0.006	0.283 ± 0.003	1.036 ± 0.0115
H10	0.209 ± 0.003	0.264 ± 0.002	1.26 ± 0.01
H11	0.336 ± 0.0057	0.429 ± 0.023	1.27 ± 0.05
H12	0.592 ± 0.006	0.598 ± 0.04	1.01 ± 0.002
H13	0.544 ± 0.007	1.26 ± 0.03	2.31 ± 0.025
H14	0.233 ± 0.004	0.197 ± 0.04	0.84 ± 0.016
H15	0.171 ± 0.003	0.173 ± 0.03	1.01 ± 0.15
H16	0.769 ± 0.005	1.40 ± 0.002	1.82 ± 0.0057
H17	0.667 ± 0.002	0.715 ± 0.005	1.07 ± 0.0057
H18	0.305 ± 0.005	1.43 ± 0.005	4.68 ± 0.065
H19	0.243 ± 0.003	0.489 ± 0.007	1.72 ± 0.16
H20	0.205 ± 0.004	0.301 ± 0.003	1.46 ± 0.015
H21	0.286 ± 0.006	0.498 ± 0.002	1.74 ± 0.03
H22	0.308 ± 0.002	0.683 ± 0.03	2.22 ± 0.085
H23	0.255 ± 0.005	0.232 ± 0.041	0.90 ± 0.143
H24	0.145 ± 0.003	0.248 ± 0.0032	1.02 ± 0.005
H25	0.300 ± 0.003	0.417 ± 0.0013	1.39 ± 0.0057
H26	0.198 ± 0.00	0.334 ± 0.00	1.68 ± 0.0057
H27	0.412 ± 0.00	0.578 ± 0.00	1.40 ± 0.0057
H28	0.371 ± 0.032	0.474 ± 0.008	1.277 ± 0.089
H29	0.347 ± 0.034	0.561 ± 0.004	1.61 ± 0.145
H30	0.204 ± 0.02	0.298 ± 0.003	1.46 ± 0.13
H31	0.331 ± 0.0035	1.648 ± 0.07	4.98 ± 0.17
H32	0.191 ± 0.023	0.268 ± 0.0045	1.40 ± 0.082
H33	0.203 ± 0.007	0.190 ± 0.005	0.94 ± 0.011
H34	0.348 ± 0.0039	0.496 ± 0.003	1.43 ± 0.011
H35	0.103 ± 0.003	0.166 ± 0.004	1.61 ± 0.01
H36	0.161 ± 0.04	0.159 ± 0.004	1.00 ± 0.23
H37	0.219 ± 0.076	0.332 ± 0.004	1.64 ± 0.59
H38	0.217 ± 0.004	0.453 ± 0.004	2.08 ± 0.02
H39	0.35 ± 0.004	0.391 ± 0.0056	1.18 ± 0.037
H40	0.228 ± 0.006	0.352 ± 0.0076	1.54 ± 0.01
H41	0.318 ± 0.003	0.395 ± 0.004	1.24 ± 0.0
H42	0.210 ± 0.002	0.327 ± 0.005	1.55 ± 0.01
H43	0.160 ± 0.004	0.218 ± 0.003	1.36 ± 0.015
H44	0.172 ± 0.006	0.352 ± 0.07	2.04 ± 0.34
H45	0.187 ± 0.003	0.247 ± 0.0025	1.32 ± 0.0057
H46	0.181 ± 0.005	0.299 ± 0.006	1.65 ± 0.015
H47	0.19 ± 0.004	0.647 ± 0.034	3.40 ± 0.15
H48	0.158 ± 0.002	0.218 ± 0.005	1.38 ± 0.015
H49	0.151 ± 0.004	0.667 ± 0.043	4.42 ± 0.17
H50	0.180 ± 0.003	0.309 ± 0.0012	1.72 ± 0.02

TABLE 7: FLUORESCENCE ENHANCEMENT IN HYPERTENSIVE SAMPLES AGAINST MB1 (NORMOTENSIVE BIOSENSOR)

Sample no.	Before hybridization	After hybridization (5min read)	Fluorescence enhancement
H1	0.253 ± 0.023	0.249 ± 0.003	0.98 ± 0.075
H2	0.273 ± 0.004	0.312 ± 0.006	1.14 ± 0.005
H3	0.466 ± 0.061	0.554 ± 0.004	1.18 ± 0.015
H4	0.343 ± 0.0044	0.426 ± 0.003	1.24 ± 0.0057
H5	0.240 ± 0.03	0.354 ± 0.0056	1.48 ± 0.160
H6	0.264 ± 0.039	0.284 ± 0.0062	1.07 ± 0.14
H7	0.250 ± 0.005	0.234 ± 0.0029	0.93 ± 0.01
H8	0.190 ± 0.0074	0.234 ± 0.004	1.23 ± 0.025
H9	0.213 ± 0.005	0.334 ± 0.004	1.57 ± 0.152
H10	0.265 ± 0.003	0.292 ± 0.005	1.10 ± 0.0057
H11	0.676 ± 0.01	0.723 ± 0.003	1.06 ± 0.015
H12	0.258 ± 0.004	0.675 ± 0.005	2.62 ± 0.02
H13	0.267 ± 0.007	0.284 ± 0.002	1.06 ± 0.02
H14	0.257 ± 0.005	0.340 ± 0.005	1.32 ± 0.006
H15	0.456 ± 0.003	0.559 ± 0.003	1.22 ± 0.0
H16	0.677 ± 0.006	1.50 ± 0.004	2.22 ± 0.01
H17	0.187 ± 0.004	0.277 ± 0.002	1.48 ± 0.02
H18	0.301 ± 0.005	0.287 ± 0.005	0.95 ± 0.0
H19	0.167 ± 0.004	0.182 ± 0.00	1.08 ± 0.025
H20	0.210 ± 0.003	0.215 ± 0.006	1.02 ± 0.015
H21	0.496 ± 0.002	0.350 ± 0.002	0.70 ± 0.0
H22	0.225 ± 0.001	0.191 ± 0.006	0.84 ± 0.025
H23	0.176 ± 0.006	0.250 ± 0.003	1.42 ± 0.03
H24	0.143 ± 0.003	0.264 ± 0.004	1.86 ± 0.0057
H25	0.180 ± 0.008	0.179 ± 0.006	0.99 ± 0.01
H26	0.603 ± 0.003	0.561 ± 0.005	0.93 ± 0.0057
H27	0.318 ± 0.004	0.345 ± 0.004	1.08 ± 0.0
H28	0.406 ± 0.002	0.376 ± 0.002	0.92 ± 0.0
H29	0.205 ± 0.004	0.185 ± 0.0045	0.90 ± 0.015
H30	0.135 ± 0.003	0.145 ± 0.03	1.07 ± 0.195
H31	0.875 ± 0.005	2.164 ± 0.06	2.47 ± 0.055
H32	0.163 ± 0.002	0.188 ± 0.05	1.15 ± 0.29
H33	0.299 ± 0.0034	0.386 ± 0.0045	1.29 ± 0.0
H34	0.188 ± 0.005	0.204 ± 0.0067	1.08 ± 0.01
H35	0.165 ± 0.0032	0.378 ± 0.0034	2.28 ± 0.025
H36	0.95 ± 0.004	1.26 ± 0.0023	1.32 ± 0.002
H37	1.00 ± 0.003	0.173 ± 0.0023	0.172 ± 0.001
H38	0.677 ± 0.006	0.598 ± 0.0023	0.88 ± 0.004
H39	0.397 ± 0.004	1.50 ± 0.002	3.77 ± 0.035
H40	0.187 ± 0.007	0.177 ± 0.002	0.94 ± 0.025
H41	0.198 ± 0.00	0.191 ± 0.001	0.95 ± 0.0057
H42	0.178 ± 0.008	0.146 ± 0.004	0.81 ± 0.15
H43	0.252 ± 0.004	0.201 ± 0.003	0.79 ± 0.0
H44	0.393 ± 0.009	0.350 ± 0.004	0.89 ± 0.01
H45	0.298 ± 0.003	0.271 ± 0.003	0.90 ± 0.0057
H46	0.175 ± 0.004	0.165 ± 0.004	0.94 ± 0.0
H47	0.225 ± 0.002	0.210 ± 0.005	0.92 ± 0.015
H48	0.176 ± 0.004	0.191 ± 0.002	1.08 ± 0.01
H49	0.143 ± 0.003	0.146 ± 0.004	1.01 ± 0.0057
H50	0.180 ± 0.005	0.191 ± 0.003	1.05 ± 0.011

Application of Hypertensive Biosensor and Normotensive Biosensor on Normotensive Samples: PCR amplified normal samples (50) tested against Hypertensive Biosensor. Since they are non-complementary to each other, there should be no fluorescence increment in this analysis. But here 14 (N2, N3, N4, N5, N6, N7, N9, N10, N12, N15, N16, N24, N32, and N44) samples out of 50 registered fluorescence increment **Table 8**. These samples are clinically normotensive, but since rs699 SNP associated with hypertension is present, these patients in the future may have a higher risk of disease occurrence.

For cross-validation, normal samples were tested against Normotensive Biosensor (against the wild gene). Here 42 (N1, N6, N8, N11, N12, N13, N14,

N15, N16, N17, N18, N19, N20, N21, N22, N23, N25, N26, N27, N28, N29, N30, N31, N32, N33, N34, N35, N36, N37, N38, N39, N40, N41, N42, N43, N44, N45, N46, N47, N48, N49 and N50) samples registered fluorescence enhancement thus validating biosensor cross results **Table 9**.

6 samples (N6, N12, N15, N16, N32, and N44) fluoresced with both Hypertensive Biosensor and Normotensive Biosensor, indicating these are heterozygous samples with both alleles. Since clinically hypertension does not persist in these samples, the expression of the mutant gene here masked by wild gene, but these patients could develop hypertension in the future when exposed to a certain environment which could trigger a gene **Table 8** and **Table 9**.

TABLE 8: NORMAL SAMPLES AGAINST HYPERTENSIVE BIOSENSOR

Sample no.	Before Hybridization	After Hybridization	Fluorescence enhancement
N1	0.362 ± 0.02	0.356 ± 0.0013	0.98 ± 0.05
N2	0.240 ± 0.043	0.298 ± 0.02	1.25 ± 0.14
N3	0.226 ± 0.04	0.342 ± 0.030	1.52 ± 0.14
N4	0.147 ± 0.003	0.164 ± 0.002	1.11 ± 0.011
N5	0.230 ± 0.003	0.308 ± 0.027	1.33 ± 0.1
N6	0.210 ± 0.004	0.260 ± 0.0024	1.23 ± 0.0152
N7	0.180 ± 0.004	0.222 ± 0.0056	1.23 ± 0.0057
N8	0.136 ± 0.0034	0.137 ± 0.0023	1.00 ± 0.01
N9	0.163 ± 0.0056	0.240 ± 0.0045	1.47 ± 0.02
N10	0.414 ± 0.023	0.690 ± 0.0034	1.66 ± 0.085
N11	0.312 ± 0.0045	0.307 ± 0.002	0.98 ± 0.01
N12	0.230 ± 0.0034	0.288 ± 0.004	1.25 ± 0.0
N13	0.184 ± 0.0034	0.192 ± 0.003	1.04 ± 0.0
N14	0.165 ± 0.04	0.172 ± 0.004	1.07 ± 0.24
N15	0.281 ± 0.006	0.453 ± 0.002	1.61 ± 0.03
N16	0.246 ± 0.003	0.285 ± 0.0056	1.15 ± 0.01
N17	0.186 ± 0.004	0.192 ± 0.003	1.02 ± 0.0057
N18	0.178 ± 0.005	0.175 ± 0.004	0.97 ± 0.0057
N19	0.193 ± 0.045	0.188 ± 0.003	1.0 ± 0.22
N20	0.230 ± 0.003	0.244 ± 0.004	1.05 ± 0.01
N21	0.312 ± 0.004	0.275 ± 0.002	0.87 ± 0.057
N22	0.203 ± 0.002	0.201 ± 0.006	0.98 ± 0.0152
N23	0.284 ± 0.004	0.271 ± 0.004	0.95 ± 0.00
N24	0.344 ± 0.003	0.392 ± 0.0034	1.13 ± 0.0
N25	0.295 ± 0.004	0.259 ± 0.003	0.87 ± 0.0
N26	0.204 ± 0.003	0.203 ± 0.002	0.99 ± 0.0057
N27	0.299 ± 0.045	0.250 ± 0.0031	0.84 ± 0.12
N28	0.362 ± 0.06	0.274 ± 0.005	0.76 ± 0.115
N29	0.278 ± 0.0036	0.274 ± 0.002	0.98 ± 0.0057
N30	0.949 ± 0.0042	0.739 ± 0.004	0.77 ± 0.0
N31	0.309 ± 0.002	0.308 ± 0.007	0.99 ± 0.015
N32	0.211 ± 0.006	0.246 ± 0.003	1.16 ± 0.02
N33	0.179 ± 0.003	0.181 ± 0.006	1.00 ± 0.015
N34	0.172 ± 0.004	0.166 ± 0.003	0.96 ± 0.0057
N35	0.274 ± 0.005	0.274 ± 0.005	1.00 ± 0.0
N36	0.185 ± 0.0042	0.186 ± 0.004	1.00 ± 0.0
N37	0.165 ± 0.008	0.168 ± 0.039	1.01 ± 0.185

N38	0.152 ± 0.0067	0.140 ± 0.056	0.89 ± 0.315
N39	0.138 ± 0.008	0.145 ± 0.003	1.05 ± 0.04
N40	0.200 ± 0.0074	0.188 ± 0.0023	0.93 ± 0.025
N41	0.254 ± 0.0045	0.251 ± 0.0045	0.98 ± 0.0
N42	0.235 ± 0.0022	0.240 ± 0.023	1.01 ± 0.085
N43	0.202 ± 0.0032	0.175 ± 0.04	0.85 ± 0.185
N44	0.224 ± 0.0054	0.277 ± 0.023	1.23 ± 0.07
N45	0.160 ± 0.0039	0.161 ± 0.0034	1.0 ± 0.0057
N46	0.344 ± 0.0045	0.311 ± 0.0012	0.91 ± 0.025
N47	0.168 ± 0.0023	0.150 ± 0.0041	0.89 ± 0.01
N48	0.150 ± 0.0067	0.137 ± 0.030	0.90 ± 0.16
N49	0.136 ± 0.0022	0.132 ± 0.0056	0.96 ± 0.025
N50	0.120 ± 0.0023	0.106 ± 0.06	0.87 ± 0.48

TABLE 9: NORMAL SAMPLES AGAINST NORMOTENSIVE BIOSENSOR

Sample no.	Before Hybridization	After Hybridization	Fluorescence enhancement
N1	0.300 ± 0.0034	0.369 ± 0.0023	1.22 ± 0.0057
N2	0.212 ± 0.0074	0.222 ± 0.004	1.04 ± 0.0152
N3	0.177 ± 0.0054	0.176 ± 0.0023	0.99 ± 0.02
N4	0.167 ± 0.007	0.164 ± 0.004	0.98 ± 0.02
N5	0.180 ± 0.008	0.183 ± 0.002	1.01 ± 0.035
N6	0.216 ± 0.004	0.291 ± 0.005	1.34 ± 0.0
N7	0.152 ± 0.006	0.162 ± 0.003	1.06 ± 0.02
N8	0.161 ± 0.003	0.269 ± 0.001	1.66 ± 0.025
N9	0.159 ± 0.007	0.145 ± 0.033	0.90 ± 0.170
N10	0.198 ± 0.005	0.199 ± 0.067	0.99 ± 0.315
N11	0.351 ± 0.034	0.439 ± 0.003	1.25 ± 0.115
N12	0.242 ± 0.030	0.361 ± 0.00 2	1.5 ± 0.180
N13	0.166 ± 0.004	0.231 ± 0.0045	1.38 ± 0.0057
N14	0.163 ± 0.005	0.252 ± 0.0023	1.54 ± 0.035
N15	0.213 ± 0.048	0.339 ± 0.0023	1.64 ± 0.372
N16	0.170 ± 0.004	0.291 ± 0.002	1.71 ± 0.03
N17	0.158 ± 0.00 3	0.299 ± 0.0023	1.89 ± 0.02
N18	0.134 ± 0.002	0.262 ± 0.002	1.95 ± 0.01
N19	0.160 ± 0.004	0.229 ± 0.004	1.57 ± 0.24
N20	0.234 ± 0.002	0.351 ± 0.0012	1.49 ± 0.0057
N21	0.246 ± 0.004	0.343 ± 0.012	1.39 ± 0.02
N22	0.200 ± 0.002	0.301 ± 0.003	1.50 ± 0.0
N23	0.310 ± 0.006	0.363 ± 0.004	1.17 ± 0.01
N24	0.147 ± 0.003	0.145 ± 0.005	0.98 ± 0.0152
N25	0.137 ± 0.005	0.261 ± 0.005	1.90 ± 0.03
N26	0.121 ± 0.004	0.217 ± 0.003	1.79 ± 0.03
N27	0.105 ± 0.005	0.242 ± 0.002	2.30 ± 0.095
N28	0.719 ± 0.003	0.999 ± 0.003	1.38 ± 0.0057
N29	0.226 ± 0.006	0.353 ± 0.005	1.56 ± 0.02
N30	0.124 ± 0.006	0.231 ± 0.002	1.86 ± 0.075
N31	0.114 ± 0.003	0.331 ± 0.0032	2.88 ± 0.028
N32	0.114 ± 0.004	0.201 ± 0.004	1.76 ± 0.03
N33	0.109 ± 0.002	0.217 ± 0.0052	1.98 ± 0.015
N34	0.136 ± 0.007	0.257 ± 0.0083	1.88 ± 0.035
N35	0.136 ± 0.004	0.239 ± 0.0067	1.75 ± 0.0
N36	0.110 ± 0.005	0.207 ± 0.0045	1.87 ± 0.045
N37	0.101 ± 0.003	0.187 ± 0.05	1.83 ± 0.44
N38	0.108 ± 0.002	0.231 ± 0.00 2	2.13 ± 0.025
N39	0.132 ± 0.008	0.239 ± 0.005	1.81 ± 0.07
N40	0.129 ± 0.004	0.239 ± 0.003	1.84 ± 0.035
N41	0.235 ± 0.007	0.305 ± 0.005	1.29 ± 0.015
N42	0.127 ± 0.004	0.308 ± 0.004	2.42 ± 0.045
N43	0.113 ± 0.007	0.183 ± 0.00 3	1.61 ± 0.07
N44	0.126 ± 0.0044	0.198 ± 0.006	1.56 ± 0.0057

N45	0.116 ± 0.040	0.208 ± 0.003	1.49 ± 0.68
N46	0.143 ± 0.003	0.283 ± 0.005	1.97 ± 0.0057
N47	0.118 ± 0.002	0.263 ± 0.004	2.22 ± 0.0057
N48	0.098 ± 0.0032	0.214 ± 0.002	2.18 ± 0.05
N49	0.113 ± 0.004	0.233 ± 0.0032	2.05 ± 0.045
N50	0.140 ± 0.0023	0.262 ± 0.0045	1.87 ± 0.0

Applying Hardy - Weinberg's Law: Hardy-Weinberg equation helps to calculate the genotype frequency from allele frequency in a population as given in **Table 10** and **Table 11** include derived allele frequencies where p is 0.68 for the C allele, and q is 0.32 for the T allele in hypertensive patients. In normotensive patients it is p is 0.78 for T allele and q is 0.22 for C allele. Hence, patients with CC genotype are associated with a higher risk

of hypertension in Punjabi Population. Similar results were found in Northern Han Chinese population study where 4 polymorphisms of angiotensinogen gene, *i.e.*, rs5050 (A-20C), rs5051 (A-6G), rs4762 (C3889T), and rs699 (C4072T) were genotyped. Here patients with CC genotypes, *i.e.* (C4072T or rs699) had a higher risk of hypertension³³.

TABLE 10: SAMPLE SIZE WITH HOMOZYGOUS rs699 MUTATION AND HETEROZYGOUS GENE

	Hypertensive Patients	Normotensive Patients	Total
rs699 ⁺ (SNP, Homo)	30	8	38
rs699 ⁻ (Wild, Homo)	12	36	48
rs699 ⁺ / rs699 ⁻	8	6	14
Total	50	50	100

TABLE 11: GENOTYPES AND ALLELE FREQUENCY IN AGT GENE M235T WHERE C ALLELE IS HYPERTENSIVE AND T ALLELE IS NORMOTENSIVE. C/C ARE HYPERTENSIVE HOMOZYGOTES, T/T ARE NORMOTENSIVE HOMOZYGOTES, AND C/T ARE HETEROZYGOTES

Polymorphism	Type and number of patients	Allele frequency
AGT gene, C4072T mutation or rs699	Hypertensive subjects	
	30 C/C	0.68 C
	12 T/T	0.32 T
	08 C/T	
	Normotensive subjects	
	08 C/C	0.22 C
	36 T/T	0.78 T
	06 C/T	
	Total samples (Hypertensive + Normotensive)	
	38 C/C	
48 T/T	0.45 C	
14 C/T	0.55 T	

CONCLUSION: Verma *et al.*, developed MB based biosensor against rs699 angiotensin SNP associated with Hypertension in certain populations. This biosensor detected 'C' allele in presynthesized target oligonucleotides at 35 °C with 5 min response time. For most of the molecular beacon assays on a solid surface, the specific/nonspecific signal ratios obtained are in the range of only 2 - 6^{46 - 48}. By comparison, our surface immobilized molecular beacons can distinguish a G/C mismatch of its 28th nucleotide position. Here 3 fold enhancement was observed²³.

This biosensor was successfully applied to 100 blood samples (50 hypertensive and 50 normotensives). Here hypertensive subjects tested

positive against Hypertensive Biosensor were associated with essential hypertension. The p-value for hypertensive patients was 0.68 which was estimated by Hardy-Weinberg Law. This proved that rs699 SNP is associated with hypertension in Punjabi Population. However, certain samples that were clinically hypertensive but tested negative against hypertensive biosensor had hypertension because of some other gene mutation and interaction.

The application was cross-validated by applying hypertensive and normotensive samples to Normotensive Biosensor. Here normotensive samples fluoresced and hypertensive does not. But there were certain samples that were clinically

naive but fluoresced. These can develop hypertension in the future due to hypertensive allele presence. Hence can help in the future pharmacogenetic based treatment of patients. Certain samples that fluoresced with both hypertensive and normotensive biosensor were heterozygous. Here presence or absence of hypertension depends upon gene interaction to environmental stimuli.

Significance: Although, evolution is a common occurrence in natural populations, allele frequencies remain unaltered unless any evolutionary mechanisms such as natural selection or mutation cause them to change. Before Hardy and Weinberg, the theory genophagy (gene eating) was thought to be correct which means that dominant alleles must, over time, inevitably swamp recessive alleles out of existence. In other words, dominant alleles always increase in frequency from generation to generation. Hardy and Weinberg demonstrated that dominant alleles could also decrease in frequency.

Here in our results, the frequency of a dominant wild T allele is 0.32 which is less than mutant C allele, *i.e.* 0.68 in a case of hypertensive subjects. Thus, there is no such deviation in results from Hardy-Weinberg's Law.

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