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DETECTION OF SNPs OF TNF –ALPHA AT -308 POSITION (G/A) IN HIMACHAL PRADESH POPULATION

BY

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Thesis submitted in partial fulfillment of the Degree of

Bachelor of Technology

DEPARTMENT OF

BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION

TECHNOLOGY, WAKNAGHAT

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CERTIFICATE

This is to certify that the work entitled, "**Detection of SNP (G/A) of TNF alpha at -308 ;position in Himachal Pradesh population** " submitted by **Shivranjani Sharma (081555)** in partial fulfillment of the Degree of Bachelor of Technology.

It is submitted in the fulfillment for the award of degree of Bachelors of Technology in Biotechnology of Jaypee University of Information Technology and has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or institute.

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To all the individuals who have helped me complete my experiments on time, I again express my appreciation. I am obligated to all those who provided reviews and suggestions for improving the results and the topics covered in this project, and extend my apologies to anyone I may have failed to mention.

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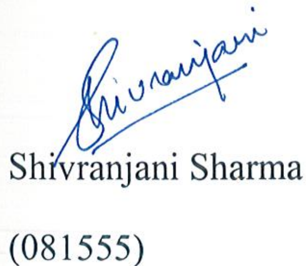
(081555)

SUMMARY

Many factors are present in the body that regulate and maintain its normal functioning. Out of these a very important factor is the Tumor Necrosis Factor alpha. It is a protein that plays a vast number of roles in the body. These roles range from that of a cytokine that elicits the bodies immune response, to a necrosis factor, that destroys the bodies own cells as well. It possesses both growth stimulatory as well as growth inhibitory properties. Required in a large number of pathways, this cytokine clearly has an integral role in maintaining the body's internal homeostasis. It is produced by the TNF alpha gene. But mutations upon this gene result in abnormal functioning of the body.

Various studies have shown that a mutation at the -308 locus of the TNF-alpha gene results in many diseases. This mutation results in replacement of the G allele by the A allele. The aim of my study is to determine the frequency of occurance of this mutation in the Himachal Pradesh population. I have taken up Himachal, as previously no such study has been conducted here.



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LIST OF ABBREVIATIONS

- **SDS**-Sodium dodecyl sulphate
- **%age-** Percentage
- µl- Microlitre
- **G**-Gram
- **Mg**-Micrograms
- **L**- Litre
- **M**- Molarity
- **Min** –Minute
- **Ml**- Millilitre

CHAPTER-1

INTRODUCTION

TUMOR NECROSIS FACTOR-ALPHA

Tumor necrosis factor is a 17kDa protein secreted by macrophages and classified as a cytokine. It binds to high-affinity receptors on the cell surface and is involved in a wide variety of biological responses.Tumor necrosis factor-alpha (TNF-a) is the best-known member of this class.Tumor necrosis factor-alpha (TNF-a) is a pleiotropic inflammatory cytokine. It was first isolated by Carswell et al. in 1975. Most organs of the body appear to be affected by TNF-a, and the cytokine serves a variety of functions, many of which are not yet fully understood.

The cytokine possesses both growth stimulating properties and growth inhibitory processes, and it appears to have self regulatory properties as well. The cytokine is produced by several types of cells, but especially by macrophage.TNF-a is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. TNF-a secreted by the macrophage causes blood clotting which serves to contain the infection.

GENE

The human TNF gene was cloned in 1985.It maps to chromosome 6p21.3, spans about 3 kilobases and contains 4 exons. The primary transcript has a length of 2762 nucleotides and encodes a precursor protein of 233 amino acids.

SOURCES

TNF is secreted by macrophages, monocytes, neutrophils, T-cells, NK-cells following their stimulation by bacterial lipopolysaccharides. Cells expressing CD4 secrete TNF-alpha while CD8(+) cells secrete little or no TNF-alpha.

The synthesis of TNF-alpha is induced by many different stimuli including interferons, Immune complexes and PAF (plateletactivating factor).

The production of TNF is inhibited by IL6, vitamin D3, prostaglandin E2 and dexamethasone.

STRUCTURE

TNF is primarily produced as a type II transmembrane protein arranged in stable homotrimers. From this membrane-integrated form the soluble homotrimeric cytokine (sTNF) is released via proteolytic cleavage by the metalloprotease TNF alpha converting enzyme (TACE, also called ADAM17).

The 17-kilodalton (kDa) TNF protomers (185-amino acid-long) are composed of two antiparallel β-pleated sheets with antiparallel β-strands, forming a 'jelly roll' β-structure, typical for the TNF family.

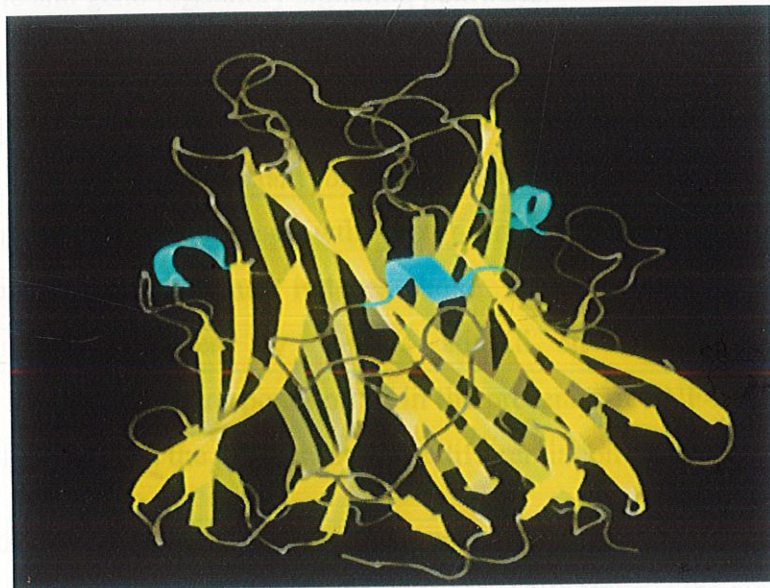

Figure 1: Structure of TNF-α

PROTEIN CHARACTERISTICS

Human TNF-alpha is a non-glycosylated protein of 157 amino acids. Murine TNF-alpha is N-glycosylated. Homology with TNF-beta is approximately 30 %. TNF-alpha forms dimers and trimers.

The 17 kDa form of the factor is produced by processing of a precursor protein of 233 amino acids. A TNF-alpha converting enzyme has been shown to mediate this conversion.

TNF-alpha contains a single disulfide bond that can be destroyed without altering the biological activity of the factor. These sites are involved in receptor binding. Most of the inflammatory and proviral effects of tumor necrosis factor (TNF) are mediated through the activation of the nuclear transcription factor NF-?B.

BIOLOGICAL ACTIVITIES OF TNF ALPHA IN THE BODY

In general, TNF-alpha and TNF-beta display similar spectra of biological activities in in vitro systems. TNF-alpha shows a wide spectrum of biological activities. It causes cytolysis and cytostasis of many tumor cell lines in vitro. Sensitive cells die within hours after exposure to picomolar concentrations of the factor and this involves, at least in part, mitochondria-derived second messenger molecules serving as common mediators of TNF cytotoxic and gene-regulatory signaling pathways.

The factor induces hemorrhagic necrosis of transplanted tumors. Within hours after injection TNF-alpha leads to the destruction of small blood vessels within malignant tumors. TNF-alpha is a potent chemoattractant for neutrophils and also increases their adherence to the endothelium .TNF-alpha is a growth factor for normal human diploid fibroblasts. TNF activates osteoclasts and thus induces bone resorption. TNF-alpha enhances the proliferation of T-cells induced by various stimuli in the absence of IL2. Some subpopulations of T-cells only respond to IL2 in the presence of TNF-alpha. In The presence of IL2 TNF-alpha promotes the proliferation and differentiation of B-cells.

Although TNF-alpha is required also for normal immune responses the overexpression has severe pathological consequences. TNF-alpha is the major mediator of cachexia observed in tumor patients (hence its name: Cachectin).

CLINICAL USE

Extensive preclinical studies have documented a direct cytostatic and cytotoxic effect of TNF-alpha against subcutaneous human xenografts and lymph node metastases. However, clinical trials on the whole have unfortunately so far failed to demonstrate significant improvements in cancer treatment. The combined use of TNF and cytotoxic or immune modulatory agents may be of advantage in the treatment of some tumors.

There are some indications that inhibitors of TNF may be of advantage. Since TNF is found in the synovial fluid of patients suffering from arthritis, these inhibitors may be helpful in curing the disease.

TNF-alpha appears to be an important autocrine modulator promoting the survival of hairy cell leukemia cells. It may be important, therefore, in the pathogenesis of this disease.

WHAT IS AN SNP?

A **single-nucleotide polymorphism** (SNP) is a DNA sequence variation occurring when a single nucleotide — A, T, Cor G — in the genome differs between members of a biological species or paired chromosomes in an individual. Single-nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions (regions between genes). A SNP in which both alleles produce the same polypeptide sequence is called a *synonymous polymorphism* (silent mutation). If a different polypeptide sequence is produced the polymorphism is a *replacement polymorphism*. A replacement polymorphism change may be either missense, which results in a different amino acid, or nonsense, which results in a premature stop codon.

Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens, chemicals, drugs, vaccines, and other agents.

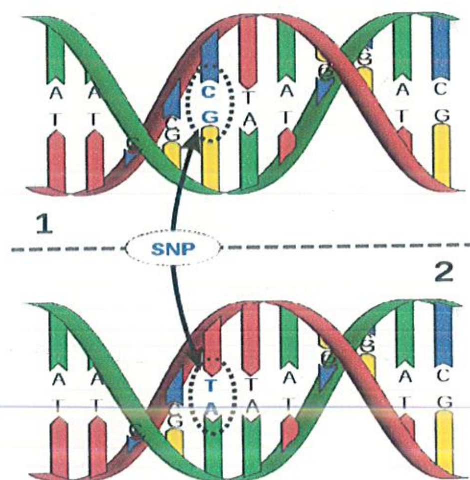

Figure 2: single nucleotide polymorphism

OBJECTIVES

1. To assess the frequency of occurrence of Snp at -308 locus of the TNF-alpha gene in Himachal population.
2. To co relate presence of mutation with occurrence of various diseases in his population.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE IN THE PROPOSED AREA

As of yet no studies relating to the occurrence of this polymorphism have been conducted in the Himachal Pradesh region. Therefore we found it significant to conduct the study in this region.

As seen above, TNF- alpha plays a vital role in the normal functioning of the body. The variations in the normal functioning of the TNF-alpha often lead to some disease in the body. It has been observed that mutations at the -308 locus of the gene cause many of these diseases. Though SNPs do occur at other loci in the TNF alpha gene also, but it is observed that the most widely studied mutations are the ones that occur at -308.Various experiments have been carried out around the world to try and obtain the exact frequency of occurrence of theses mutations.

The importance of the normal functioning of the TNF-a gene in the body can easily be observed by studying the mutations of the gene in diseased organisms. Since TNF-a plays a role in several diseases, a substantial amount of research has been conducted concerning TNF-a therapies and anti-TNF-a therapies. Because TNF-a exhibits anti tumor activity research has been conducted to determine the protein's effectiveness against certain forms of cancers. Utilizing TNF-a tumoricidal activities has proved problematic, especially due to the cytotoxin's systematic toxicity. While higher doses of TNF-a may exhibit higher cytotoxicity, high doses also lead to systematic toxicity (National Cancer Institute, 1995). Some studies involving TNFR-75 and TNFR-55 mutants have suggested that the TNFR-75 receptor plays a role in systematic toxicity, while TNFR-75 mutants will exhibit cytotoxicity but not systematic toxicity (Van Ostade et al., 1993). Additionally, a mutant form of TNF-A which exists only in the transmembrane form acts only by cell-to-cell contact and may result only in cytotoxicity (Perez et al.,1990), suggesting that mutant forms of TNF-A might effectively be used therapeutically as against specific types of cancers.

Other research has focused upon inhibiting the effects of TNF-a in such diseases as Rheumatoid Arthritis, Crohn's Disease, AIDS, bacterial septic shock (caused by certain gram negative bacteria), and bacterial toxic shock (caused by super antigens) as well as in prevention of alloreactivity and graft rejection. Mutant mice that lack TNF-A are resistant to gram-negative bacteria induced sepsis (Janeway et al. 1999), and anti-TNF monoclonal antibodies have been used to effectively reduce or inhibit TNF-a activity (Beutler et al., 1985b). One hypothetical advantage of treatment with anti-TNF-a antibodies results from its role in multiple types of inflammation. It is often difficult to determine that inflammation in burn and trauma victims are of infectious etiology and warrant treatment with antibiotics; therefore another treatment strategy might involve anti-TNF-a therapy (Strieter, et al., 1993). Strategies for preventing TNF-a activity include neutralization of the cytokine via either anti-TNF antibodies, soluble receptors, or receptor fusion proteins; supression of TNF-a synthesis via drugs such as cyclosporine A, glucocorticoides, or cytokine IL-10; reduction of responsiveness to TNF-A via repeated low dose stimulation; and lastly, by inhibition of secondary mediators such as IL-1, IL-6, or nitric oxide (Tracey et al., 1993).

Pharmaceutical companies such as Peptech Limited have developed different antibodies to TNF-a, some of which inhibit various TNF-a function and others which do not affect protein activity. For instance, Remicade (TM) is a chimeric Igk monoclonal anti-TNF antibody manufactured by Centocor which has been used to treat Crohn's disease--a chronic inflammatory disease of the intestines (Centocor 2000). Soluble TNF-R will also neutralize TNF-A before it can bind to its target cell receptor. Another drug, Enbrel (TM) , developed by Immunex Corporation, is a fusion of two soluble TNF receptors and a human immunoglobulin (Immunex Corporation, Nov. 1999). It has been approved for treatment of rheumatoid arthritis. Additionally, Chloroquine inhibits transcription of the protein in macrophage (Zhu et al. 1993). However, the efficacy of preventing septic shock has been questioned as a result of recent research which suggests that, in the absence of TNF-a, other cytokines will eventually initiate the inflammatory response. The authors of this study speculate that TNF-a production may instead play a key kinetic role by amplifying release of cytokines IL-A, IL-B, and IL-6 and thereby affecting the severity of a response to LPS (Amiot et al., 1997). Additionally, eliminating the stimulatory affects of TNF-a in diseases

such as AIDS presents problems because inactivation of TNF-a leaves the host at even greater risk for bacterial infections normally countered by TNF-a activity.

In many diseases the TNF-a gene mutations occur. They disrupt the normal functioning of the body, and may act to either promote the disease or to get the body to elicit a response against the disease. Some such diseases are:

Cancer:

The current use of TNF in cancer is in the regional treatment of locally advanced soft tissue sarcomas and metastatic melanomas and other tumors of any histology to avoid amputation of the limb. It has been demonstrated in the isolated limb perfusion setting that TNF-alpha acts synergistically with cytostatic drugs. The interaction of TNF-alpha with TNF receptor 1 and receptor 2 (TNFR-1, TNFR-2) activates several signal transduction pathways, leading to the diverse functions of TNF-alpha.

The signaling molecules of TNFR-1 have been elucidated quite well, but regulation of the signaling remains unclear. Besides these molecular insights, laboratory experiments in the past decade have shed light upon TNF-alpha action during tumor treatment. Besides extravasation of erythrocytes and lymphocytes, leading to hemorrhagic necrosis, TNF-alpha targets the tumor-associated vasculature (TAV) by inducing hyperpermeability and destruction of the vascular lining. This results in an immediate effect of selective accumulation of cytostatic drugs inside the tumor and a late effect of destruction of the tumor vasculature. In this review, covering TNF-alpha from the molecule to the clinic, we provide an overview of the use of TNF-alpha in cancer starting with molecular insights into TNFR-1 signaling and cellular mechanisms of the antitumor activities of TNF-alpha and ending with clinical response. In addition, possible factors modulating TNF-alpha actions are discussed.

Obesity:

Obesity, an epidemic of our times with rates rising to alarming levels, is associated with co morbidities including cardiovascular diseases, arthritis, certain cancers, and degenerative diseases of the brain and other organs. Importantly, obesity is a leading cause of insulin resistance and type 2 diabetes. As emerging evidence has shown over the last decade, inflammation is one of the critical processes associated with the development of insulin

resistance, diabetes and related diseases, and obesity is now considered as a state of chronic low-grade inflammation Obesity leads to infiltration of the expanded adipose tissue by macrophages and increased levels in proinflammatory cytokines. The first indication for increased cytokine release in obesity was provided by the identification of increased expression of TNF-alpha, a proinflammatory cytokine, in the adipose tissue of obese mice in the early 1990s. TNF-alpha is expressed in and secreted by adipose tissue, its levels correlating with the degree of adiposity and the associated insulin resistance. Targeting TNF-alpha and/or its receptors has been suggested as a promising treatment for insulin resistance and type 2 diabetes.

This review will summarize the available knowledge on the role of TNF-alpha in obesity and related processes and the potential implications of the above in the development of new therapeutic approaches for obesity and insulin resistance. Recent data from clinical studies will also be described together with late findings on the pathogenesis of obesity and insulin resistance.

Asthama:

Although only 5-10% of patients with asthma are relatively unresponsive to treatment with inhaled corticosteroids, refractory asthma represents an important condition. Treatment options are limited and there is a large unmet clinical need for additional therapies. Tumor necrosis factor (TNF)-alpha is a pro-inflammatory cytokine that has been implicated in many aspects of the airway pathology in asthma, and which has recently been highlighted as potentially important in refractory asthma.

The development of neutralizing biological agents against TNF-alpha has allowed us to test the role of this cytokine in vivo. Preliminary studies have demonstrated an improvement in lung function, airway hyper responsiveness and asthma quality-of-life, together with a reduction in exacerbation frequency, in patients treated with anti-TNF-alpha therapy.

Atherosclerosis:

TNF-alpha is actively involved in the progression of atherosclerosis. Accordingly, TNF-alpha represents a possible target for prevention of atherosclerosis. This may be of

particular importance in rheumatoid arthritis because these patients have an increased risk for cardiovascular disease.

SNP genotyping is the measurement of genetic variations of single nucleotide polymorphisms (SNPs) between members of a species. It is a form of genotyping, which is the measurement of more general genetic variation. SNPs are one of the most common types of genetic variation. A SNP is a single base pair mutation at a specific locus, usually consisting of two alleles (where the rare allele frequency is >1%). SNPs are found to be involved in the etiology of many human diseases and are becoming of particular interest in pharmacogenetics. Because SNPs are conserved during evolution, they have been proposed as markers for use in quantitative trait loci (QTL) analysis and in association studies in place of microsatellites.

The use of SNPs is being extended in the Hap Map project, which aims to provide the minimal set of SNPs needed to genotype the human genome. SNPs can also provide a genetic fingerprint for use in identity testing (Rapley & Harbron 2004). The increase in interest in SNPs has been reflected by the furious development of a diverse range of SNP genotyping methods.

Of the many studies carried out world wide a few significant ones were:

- SNP frequency measurement in the Japanese population: genome-wide association study by genotyping 94 individuals of Japanese descent with using the high-throughput PCR-Invader assay methods was done.
- In the populations of Colorado and Columbia in USA it was seen that a study with 76 and 98 people of the population respectively was such as done.
- Countries such as African countries and European countries also have these studies.

The results of these studies are mentioned in Table 1.

POPULATION	GG	GA	AA	G allele	A allele
England	96(62.7)	52(33.9)	5(3.2)	0.797	0.202
California	212(78.0)	55(20.0)	6(2.0)	0.877	0.122
Colorado	27(94.5)	21(3.5)	0(0.0)	0.983	0.017
Netherlands	50(57.0)	34(34.0)	4(2.0)	0.761	0.238
Japan	23(95.8)	1.(4.1)	0(0.0)	0.980	0.020
Russia	12(85.7)	2(14.3)	0(0.0)	0.843	0.157
Africa	7(63.6)	4(36.4)	0(0.00)	0.818	0.182
Germany	143(66.2)	63(29.2)	10(4.2)	0.810	0.190

Table1: Frequency of occurrence of SNP at -308 locus of TNF-α gene, world wide

Also various such surveys have been conducted in India. Most states have had such studies conducted. Yet there has been no such analysis in Himachal Pradesh. The frequency of occurance in Indian states is given in table 3.

POPULATION	GG	GA	AA	G allele	A allele
Punjab	165(86.8)	24(12.6)	1(0.5)	0.932	0.068
Hyderabad	26(15.2)	14(4.6)	1(0.6)	0.945	0.033
Bengal	122(73.8)	21(11.3)	1(0.4)	0.889	0.072
Kerala	43(47.9)	39(20.2)	4(2.3)	0.802	0.094
Delhi	58(69.9)	24(28.9)	1(1.2)	0.843	0.157

Table 2: Frequency of occurrence of SNP at -308 locus of TNF-α gene, India

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CHAPTER 3

MATERIALS AND METHODS

COLLECTION OF SAMPLE

A total of 45 samples were collected from the residents of Himachal Pradesh of unbiased age and sex. The samples were collected from the arm with the help of a sterile needle. These samples essentially are the normal control samples. DNA was then isolated from these samples using inorganic methods.

DNA ISOLATION

Genomic DNA from human blood was isolated using modified inorganic methods.

REAGENTS UTILISED:

- ***TrisaminomethaneChloride** (Tris-Cl)(pH 8.0):* 75 ml of sterile MQ water was used to dissolve 12.11gm of trisbase.pH was set to 8.0 with 1N HCl and the final volume was made 100ml with MQ water.
- ***Tris-***Cl(pH 7.3): 12.11 gm of Tris base was dissolved in 75 ml of sterile MQ water and the pH was set to 7.3 with 1N HCl. Final solution made 100 ml with MQ water.
- ***Ammonium Chloride(1 M):*** Ammonium Chloride(5.35 gm) was dissolved in 80 ml of MQ water and final solution was named 100 ml.
- ***Di-Sodium ethylene diamine tetra acetate***: Added 18.61 gm of EDTA salt to 50 ml of MQ water in a 250ml flask and placed it on a magnetic stirrer. Simultaneously, supplemented the solution with 10 M NaOHdropwise, until Ph reached 8.0. Allowed the salt to dissolve and then made the final volume of the solution 100 ml with MQ water.
- ***Red blood cell lysis buffer:*** (10mM Tris, pH 8.0; EDTA,1mM; NH_4Cl, 125mM,pH 8.0) : 10 ml Tris, 2 ml of EDTA and 125 ml of NH_4Cl were mixed in MQ water to obtain a final volume of 1000ml of RBC lysis buffer.
- ***Tris-EDTA***(TE buffer, pH 8.0): TE buffer was prepared by mixing 10 ml Tris-Cl and 2 ml of EDTA in 700 ml MQ water and the final volume was made 1000 ml.
- ***Tris-EDTA***(TE buffer, pH 7.3): 10 ml of Tris-Cl and 2 mlof EDTA were mixed in MQ water to obtain a final volume of 1000ml.

- ***Sodium dodecyl sulphate:*** Dissolved 10 gm of SDS salt in 70 ml of warm MQ water and the final volume was made 100ml.
- ***Ammonium acetate:*** *Dissolved 28.9gm of ammonium acetate salt in 20 ml MQ water and final volume was adjusted to 50 ml.*
- ***Chilled dehydrated ethyl alcohol:*** *Undiluted dehydrated ethyl alcohol stored in a -20^0C deep freezer.*
- ***Ethanol:*** *70 ml of dehydrated ethanol was added to 30 ml of sterile MQ water o obtain a final volume of 100 ml.*

PROTOCOL OF ISOLATION OF DNA

- Two 300 μl of blood sample, added RBC lysis buffer(3 time s the volume of the blood samle taken) and kept for incubation on a rocker, to permit perpetual shaking at room temperature until the RBCs completely lysed.
- Centrifuged the solution at 1300 RPM for 1 minute to obtain a creamish white WBC pellet.
- The super natant was discarded and the WBC pellet suspended in 300 TE buffer(ph 8.0) using a vortexing machine. There after 20 μl of 10% of SDS solution was added to the above solution and the mixture was incubated at 56^0 C for 30 minutes on a dry bath.
- Subsequently added 150 μl of 7.5M ammonium acetate and mixed vigorously for about 1 minute per sample, on vortexer. Centrifuged the mixture at 1300 RPM @ room temperature for 15 minutes there by resulting in separation of the precipitated proteins as a pellet.
- The clear supernatant was transferred to a fresh sterile micro centrifuge tube. To this added chilled absolute ethyl alcohol(twice the volume of clear supernatant). The tube was gently rocked a couple of time to allow the precipitation of genomic DNA.
- The genomic DNA precipitates were centrifuged at 1300 RPM for 10 minutes to get pellet at the bottom of the tube. The latter subsequently washed in 150 μl of 70% ethanol and air dried at room temperature for about 15 minutes.
- 100 μl of TE buffer (ph 7.3) was used to dissolve the dried DNA pallet by incubating at 65 C for 10 minutes. The dissolved DNA was finally stored at -20^0 C till further use.

QUANTIFICATION OF DNA USING SPECTROPHOTOMETRE

In chemistry, **spectrophotometry** is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.[1] It is more specific than the general term electromagnetic spectroscopy in that spectrophotometry deals with visible light, near-ultraviolet, and near-infrared, but does not cover time-resolved spectroscopic techniques. Branch of spectroscopy dealing with measurement of radiant energy transmitted or reflected by a body as a function of wavelength. The measurement is usually compared to that transmitted or reflected by a system that serves as a standard. In chemistry and physics, different types of spectrophotometers cover wide ranges of the electromagnetic spectrum: ultraviolet (UV), visible light, infrared (IR), or microwave. UV spectrophotometry is particularly useful in detecting and quantifying colourless substances in solution. IR spectrophotometry is used mostly to study the molecular structures of complex organic compounds. In astronomy and astrophysics, spectrophotometric studies also include the X-ray and gamma ray ranges of the spectrum. colorimetry.

The use of spectrophotometers spans various scientific fields, such as physics, materials science, chemistry, biochemistry, and molecular biology.[4] They are widely used in many industries including semiconductors, laser and optical manufacturing, printing and forensic examination, as well in laboratories for the study of chemical substances. Ultimately, a spectrophotometer is able to determine, depending on the control or calibration, what substances are present in a target and exactly how much through calculations of observed wavelengths.

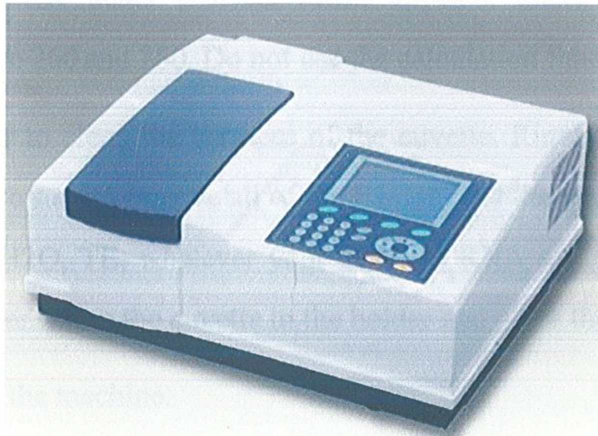

Figure 3: uv-vis spectrophotometre

ANALYSIS OF DNA

The concentration of an RNA or DNA sample can be checked by the use of UV spectrophotometry. Both RNA and DNA absorb UV light very efficiently making it possible to detect and quantify either at concentrations as low as 2.5 ng/µl. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm

DNA concentration (µg/ml) = (OD_{260}) x (dilution factor) x (50 µg DNA/ml)/(1 OD_{260} unit)

Procedure for using the spectrophotometre to measure OD at 260nm and 280nm

1. Turn the machine on using the switch in the back of the machine (lower right-hand side as you face the machine, turn on the monitor screen and printer. Wait for the machine to go through its start up routine.

2. Open the lid on the top of the machine. Choose the small cuvette holder and place it in the holder in front of the light source. The word FRONT should be toward the front of the machine.

3. Click on the UV light key to turn the UV on. It takes about a minute for the UV lamp to warm up. Quit the diagnostic screen by clicking on the word QUIT in the upper

right hand corner. The Main Menu will appear Choose Nucleic Acid from the menu. When the nucleic acids analysis menu appears, make sure that the measured absorbances are 260 and 280. Do not use the calculation functions.

4. Use lens paper to clean the surfaces of the cuvette. Rinse the cuvette chamber with 70% ETOH. Be sure to remove all of the ETOH after the wash. Place 100μl sample of your blank (nH2O, TE, whatever your DNA or RNA sample is dissolved in) in the cuvette chamber. Place the cuvette in the holder and place the lid on the holder.

5. Shut the lid of the machine.

6. Click READ BLANK in the bottom left corner of the screen.

7. Prepare a 1:100 dilution of the sample you want to read.

8. After the machine has read the blank, remove the cuvette, remove the blanking solution from the chamber, rinse and dry the chamber and place your diluted sample in it.

9. Click on READ SAMPLES in the upper left hand of the screen.

10. After the machine has read your sample, the data will appear on the screen. You do not need to print this each time you measure a sample. A machine will collect data for you.

11. Between samples clean the cuvette as described above. You do not need to blank each time unless your samples are dissolved in different solutions. If you use a different cuvette, you must run a blank.

12. When you are finished reading samples, remove and clean the cuvette and put it away. Print your results by clicking on PRINT at the top right of the screen. QUIT the data screen. This will take you back to the main menu. Turn off the UV. You can turn off the machine while the Main Menu screen is active. Turn off the machine the monitor and the printer.

13. Calculate the concentration of your RNA or DNA sample and the OD_{260}/OD_{280}

AMPLIFICTION USING POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

PCR (**P**olymerase **C**hain **R**eaction) is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary first to physically separate the two strands in a DNA double helix at a high temperature in a process called DNA melting. At a lower temperature, each strand is then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on

human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsar.[18]

Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample—a technique often applied to quantitatively determine levels of gene expression. Real-time PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

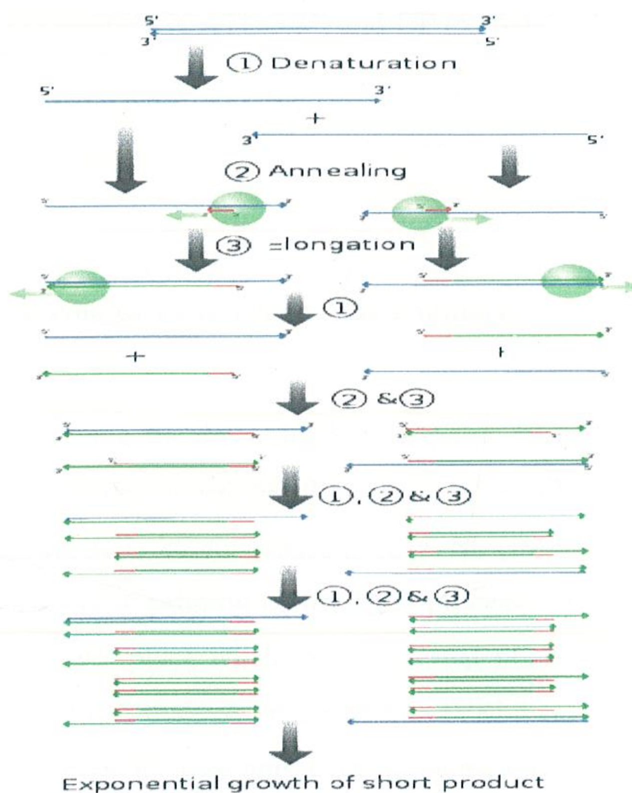

Figure 4: Depiction of polymerase chain reaction

THE MOLECULAR MARKERS

Information on genetic relationships among individuals is of tremendous importance to breeders for hybrid development, conservation and breeding program. An estimate on the genetic similarity of breeding materials is best obtained using DNA markers. The development of DNA based genetic markers has had a revolutionary impact on crop genetic. With DNA markers, it is possible to observe and exploit genetic variation in the entire genome. The application of DNA markers has allowed rapid progress in the investigation of genetic variability and inbreeding, species and strain identification, and construction of high resolution genetic linkage maps for eukaryotic species.

There are basically two types of molecular markers:

a). Sequence based

b). Non-Sequence based

Primer Design

Here primers against the TNF- alpha gene -308 position were obtained. The primers used were

AP-1(5'ATAGGTTTTGAGGGGCATCG 3') and **CP** (5' AAGAATCATTCAACCAGCGG 3')

AP-2(5'ATAGGTTTTGAGGGGCATCA3') AND **CP**(5'AAGAATCATTCAACCAGCGG 3')

ARMS-PCR

The method or type of PCR that we have utilized is called as the ARMS-PCR i.e Amplification refractory mutation system.

The ARMS technique for detecting known point mutations was first described by Newton et al. It has been developed for the diagnosis of all the common ß-thalassaemia mutations found in all the main ethnic groups (Old, J. M. (1996)). The technique is based on the principle of allele-specific priming of the PCR process, i.e. a specific primer will only permit amplification to take place when its 3' terminal nucleotide matches with its target sequence. Thus to detect the ß-thalassaemia mutation IVSI-5 (G->C), the 3' nucleotide of the ARMS primer is G in order to base pair with the substituted C in the mutant DNA. The primer forms a G-G mismatch with normal DNA, but this is a weak mismatch and will not prohibit extension of the primer by itself. Only strong mismatches (C-C, G-A and A-A) were found to reduce priming efficiency to zero or below-5%, and to prevent amplification, a further mismatch with the target sequence had to be introduced at the second, third or fourth nucleotide from the 3' end of the primer (Kwok, S., Kellogg, D. E.1990).

As a general rule for ARMS primer design, if the 3' terminal mismatch is a weak one, a strong secondary mismatch is engineered. If it is a strong one, a weak secondary mismatch is introduced. Try putting the mismatch at the second nucleotide in the first instance and test the primer for specificity and generation of product. The position of the mismatch can be altered if the primer does not work, or the strength of the mismatch increased if non-specific bands are observed. According to Little in Current Protocols in Human Genetics (Little S,

(1994)), the strength of mismatch pairings are; maximum, GA, CT, TT; strong, CC; medium, AA, GG; Weak, CA, GT; none, AT, GC.

The mutation-specific ARMS primers used in the Oxford laboratory to diagnose the 25 most common ß-thalassaemia mutations, plus the hemoglobin variants HbS, HbC and HbE, are listed in Table 3. All are 30 bases long so that they can all be used at a single high annealing temperature (65 °C).

A typical ARMS test for a single mutation consists of two amplifications in the same reaction mixture using the same genomic DNA as substrate. One amplification product results from the specific ARMS primer and its primer pair (when the mutation is present in the genomic DNA) and the other amplification results from two primers that generate a control fragment in all cases. The generation of control product indicates the reaction mixture and thermal cycler is working optimally. The strategy is to screen for the common mutations expected in the country of the ethnic origin of the patient first and then to screen for the rarer mutations. After which, uncharacterized mutations are identified by genomic sequencing.

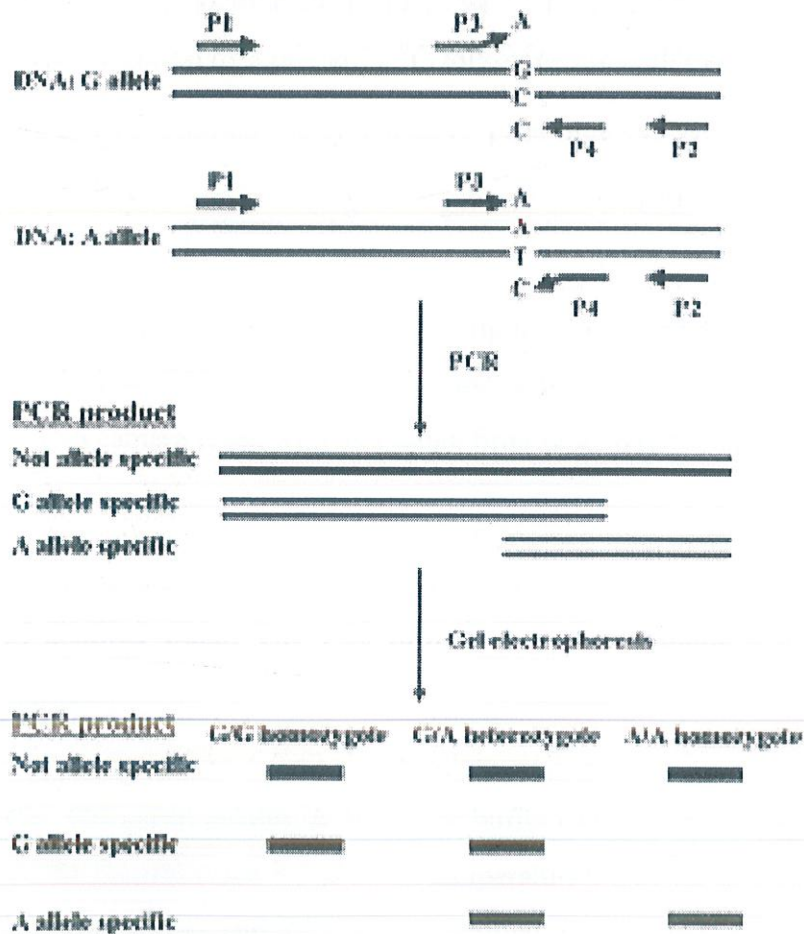

Figure 5: Depicting ARMS-PCR principle

Method of pcr

1. Prepare a reaction mixture (4 ml) comprising of: 0.5 ml of 10x ARMS PCR buffer;1.25 ml of 1.35 mM dNTP mixture; 2.65 ml of sterile distilled water
2. Pipette 20 µl of PCR reaction mixture into a 0.5 µl tube.
3. Add 1 µl of each primer (1 OD unit/ml).
4. Add 0.05 µl of Taq DNA polymerase (5u/µl).
5. When more than one test is being performed, a primer and the enzyme can be mixed together in a separate tube before addition to the reaction mix. This decreases pipetting errors as larger quantities are used.
6. Add 1 µl of genomic DNA (100 ng/µl).
7. Overlay with 25 µl of mineral oil.
8. Mix, centrifuge and place in thermal cycler.
9. Amplify for 25 cycles as follows: 1 min at 94 ^{o}C/1 min at 65 ^{o}C/1.5 min at 72 ^{o}C with a final extension period of 3 min at 72 ^{o}C following the 25th cycle.
10. Remove tubes from thermal cycler and add 5 µl of blue dye. Mix and centrifuge.
11. Load a 20 µl aliquot onto a 3% agarose gel and run at 100 V for approx. 45 min in TBE
12. Stain gel in ethidium bromide solution (0.5 µg/ml) for 15-30 minutes, visualise bands on a UV light box (312 nm) and photograph with an electronic camera system or a Polaroid CU-5 camera fitted with an orange filter (e.g. Wratten 22A).

Materials

1. DNTPs: Add together 50 µl of a 100 mM solution of each dNTP (as purchased) and 3.8 ml of distilled water. The 1.25 mM dNTP stock solution should be stored in frozen aliquots.
2. ARMS PCR buffer: 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at room temperature), 1.5 mM $MgCl_2$, 100 µg/ml gelatin. A 10x stock buffer can be prepared by adding together 0.5 ml of 1 M Tris-HCl (pH 8.3 at room temperature), 1.25 ml of 2 M KCl, 75 µl of 1 M $MgCl_2$, 5 mg gelatin, and 3.275 ml of distilled water. The stock buffer is heated at 37 ^{0}C until the gelatin dissolves and then frozen in aliquots.

3. Taq polymerases: suggested ones are as follows, AmpliTaq Gold (PE Biosystems) works best for ARMS-PCR/RE digestion assays and Platinum Taq (Gibco Life Technologies) for gap-PCR. (Tris-borate -EDTA (TBE) buffer: 89 mM Tris-borate, 89 mM boric acid,10 mM EDTA PH 8.0).

In examining the results, if a genomic sample is homozygous, then the PCR products that result will be from the primer which matches the SNP location to the outer, opposite strand primer as well from the two opposite, outer primers. If the genomic sample is heterozygous, then products will result from the primer of each allele to their respective outer primer counterparts as well as from the two opposite, outer primers. The two primer pairs are also designed such that their PCR products are of a significantly different length allowing for easily distinguishable bands by gel electrophoresis.

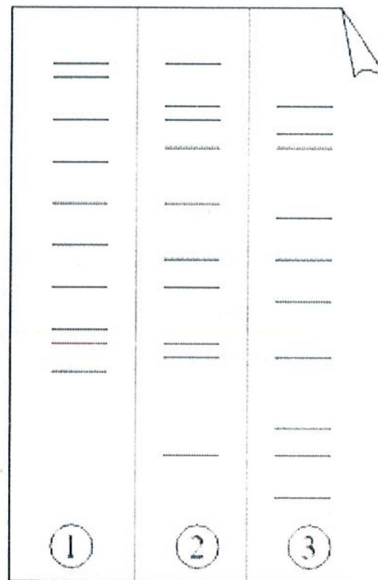

Figure 6: Analysis of pcr products using Gel Electrophoresis

CHAPTER 4

RESULT AND DISCUSSION

The various methods mentioned above were utilized upon the samples that had been collected from the Himachal Pradesh population.

Sample collection

45 samples were collected and forms were filled out by all contributors, recording their name, address etc as well as family history regarding any disease in family.

Dna isolation

First the DNA isolation was carried out in two sets each, for all the samples that had been collected. The reagents used in this process were all made from scratch in the lab. The above mentioned protocol was used for this isolation.

The Dna was successfully isolated from each of the 45 samples and was stored in sets of two at -20C

Quantification using spectrophotometer

Next the samples were taken and their optical densities were measured at 260nm as well as 280 nm. This mesuring process was carried out in Quartz cuvettes. The spectrophotometer used, was a UV-VIS one. The samples were the carefuly diluted and their OD was measured. A large table was made evaluating the OD at 260 and 280nm for each of the samples. A few are mentioned below:

SAMPLE NO.	260nm	280nm	260nm/280nm
1B	0.0022	0.0015	1.40
8A	0.0049	0.0044	1.12
12B	0.0022	0.0023	0.96
26A	0.0022	0.0022	1

Table 3 : Examples of readings obtained from spectrophotometer

Based on these numerous readings it was seen that a range was obtained. The values ranged all the way from 0.92 to 2.3. Out of the 45 samples, it was observed that few were showing small quantities of protein impurities. These samples were then made to undergo the quantification process again, after thoroughly cleaning out the cuvettes and other apparatus.

<u>Using the formula</u>

DNA concentration (μg/ml) = (OD_{260}) x (dilution factor) x (50 μg DNA/ml)/(1 OD_{260} unit)

The concentration of the DNA samples at hand was found out. The concentrations seen varied a lot. For the next step i.e. amplification of the DNA samples using ARMS-PCR we required a stable concentration in all the samples. It is essential that an even concentration be kept while carrying out the PCR or else how would the primers attach and how would we get to know the actual presence or absence of the occurrence the of the mutation within the gene. Only then can the fragment size be properly observed in the electrophoresis stage. All the sets of the samples were then diluted to gain a concentration on 10ng/μl.

Amplification using ARMS-PCR

After all the various sample sets were diluted to a stable concentration, the ARMS-PCR was carried out. As discussed above, this pcr technique is the best in the case of measurement of the SNPs in a particular gene. Much more effective than the other forms of PCR. The only disadvantage can be that a large number of primers have to be designed for the ARMS-PCR.

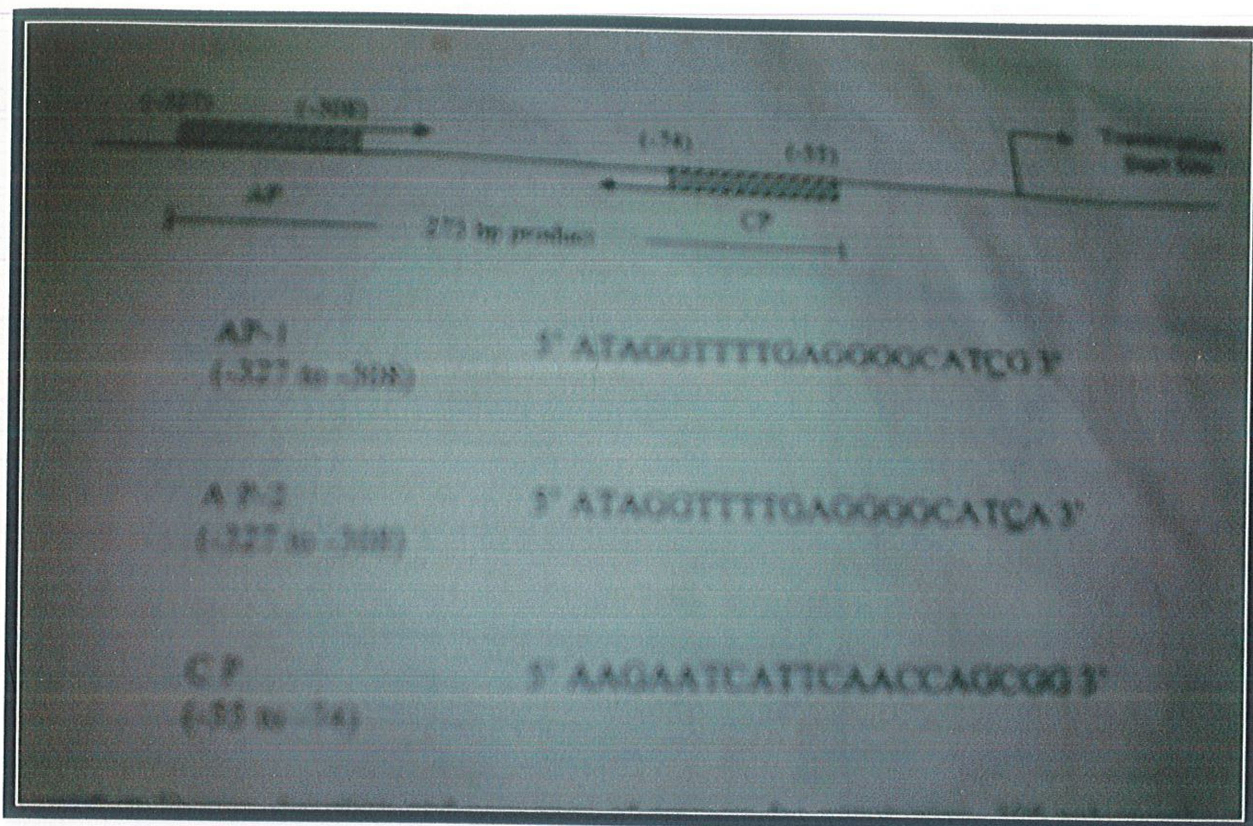

Figure 7: Design ,location and sequence of primers for genotyping -308 polymorphism in TNF-α gene promoter. The penultimate base in the primers AP-1 andAP-2 were mutated from G to C.

Gel Electrophoresis

Then the agarose gel electrophoresis was carried out. In this technique a certain ladder was taken and the various large number of samples were run against this ladder on the gel. The gel was then viewed in a gel-doc system. The standard procedure of gel electrophoresis was used.

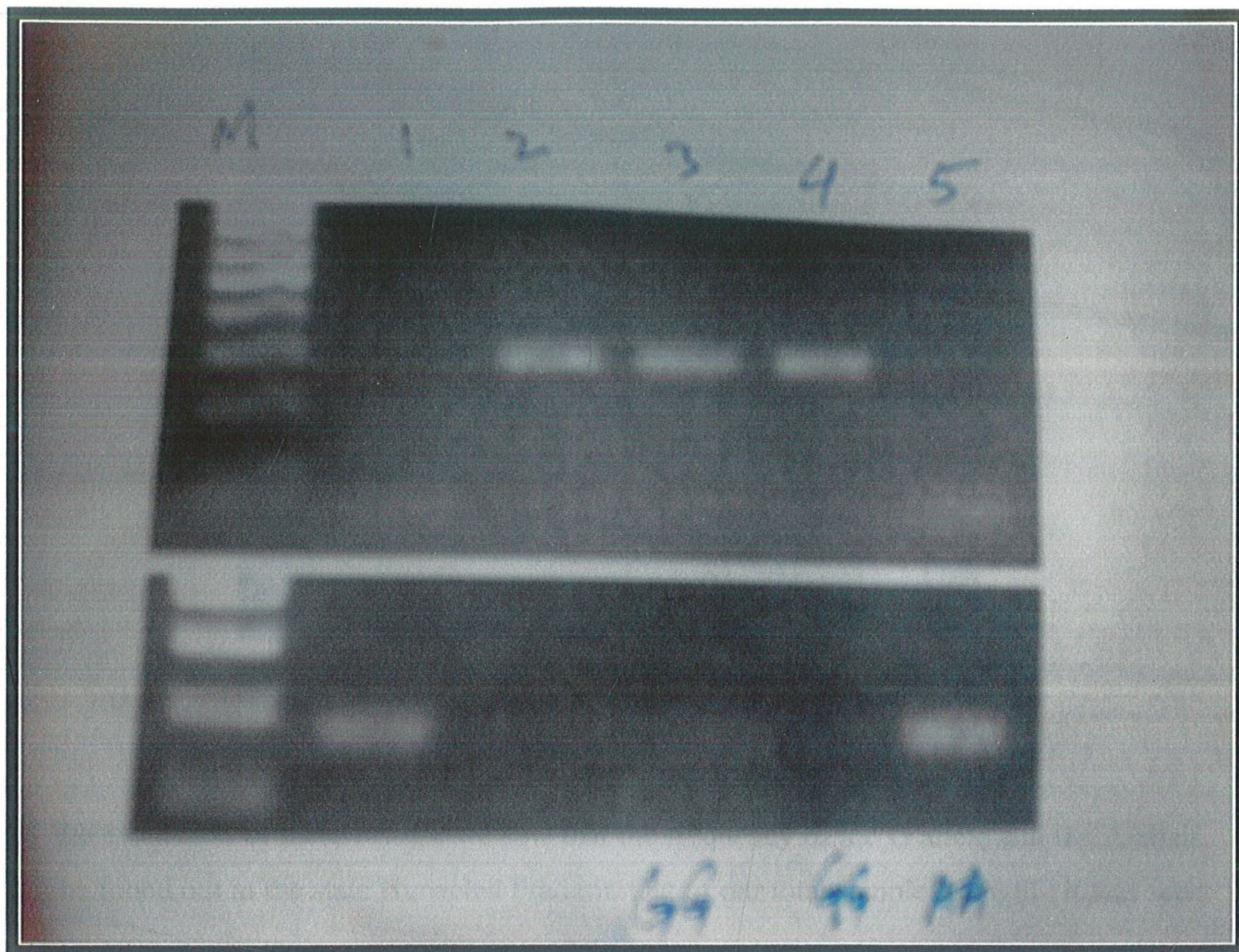

Figure 8: Allele bands as seen in gel-doc system

Allelic frequency of -308 Tnf alpha polymorphism in normal control population of Himachal Pradesh was the found out.

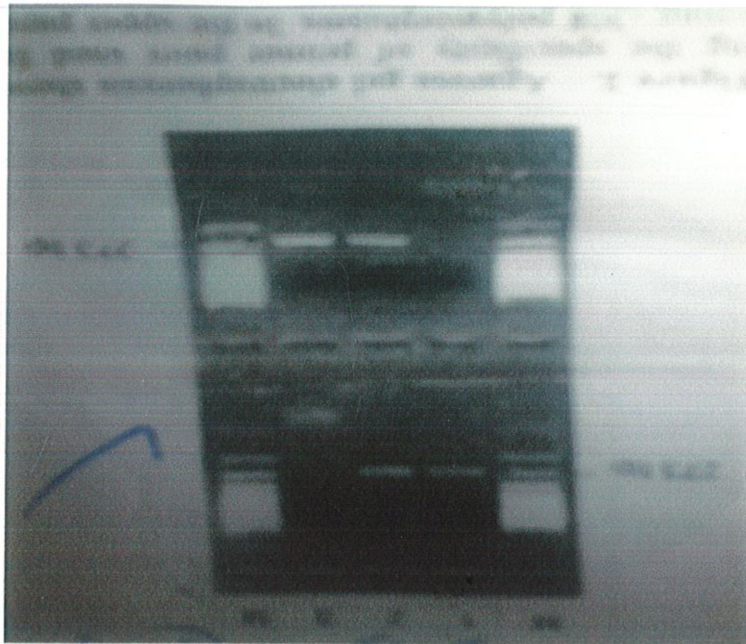

Figure 9: Gel doc images showing specificity of primer pairs

By the evaluation of these results, we saw that the frequency of the G allele and the A allele can be found out in the state Himachal Pradesh. Out of our total sample strength , it was seen that on running the gel:-

Total number of samples: 30

Samples with GG allele: 25

Samples with GA allele: 4

Samples with AA allele: 1

Based on this result, we calculate the frequency of occurance of each G and A in the Himachal Pradesh population. These calculations are done using the Hardy-Weinberg equation :

Which follows the basic principle that p+q=1

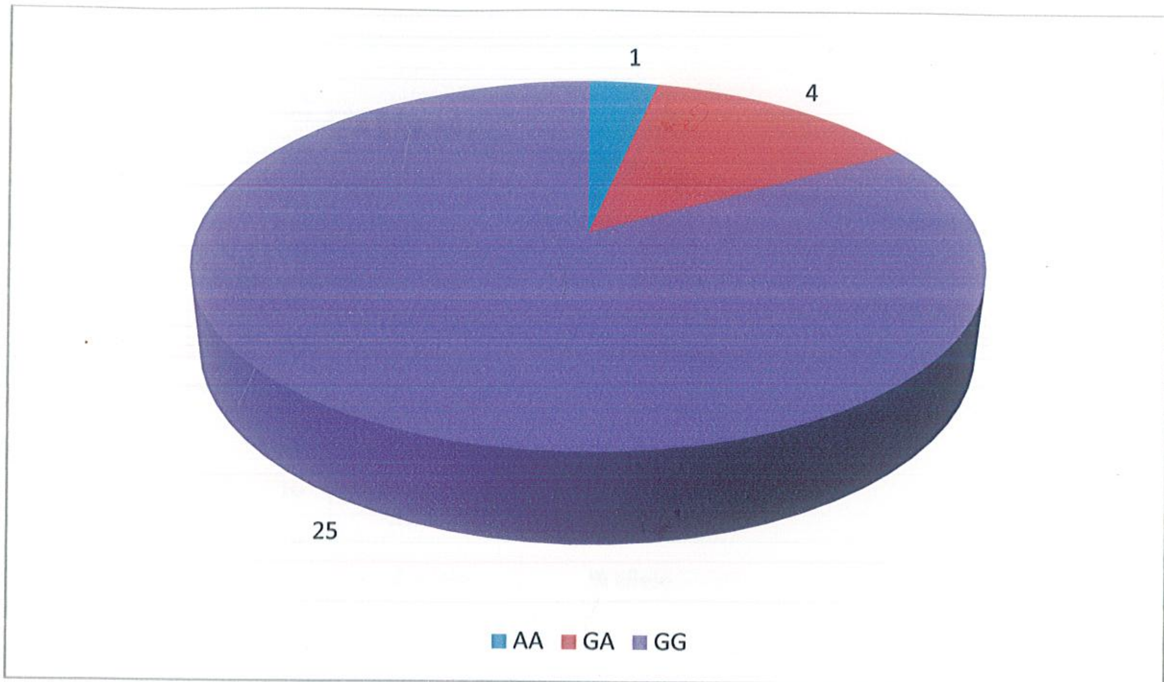

Figure 10: Representation of pcr and gel results

POPULATION	GG	GA	AA	G allele	A allele
HIMACHAL PRADESH	25(83.3)	4(13.3)	1(3.3)	0.100	0.900

Table 4: Results

Calculation of frequency of occurance:

TOTAL=60

G= 50 +4=54

54/60 x 100 = 90% or 90/100= 0.9

A= 2+4=6

6/60 x 100 = 10% or 10/100= 0.1

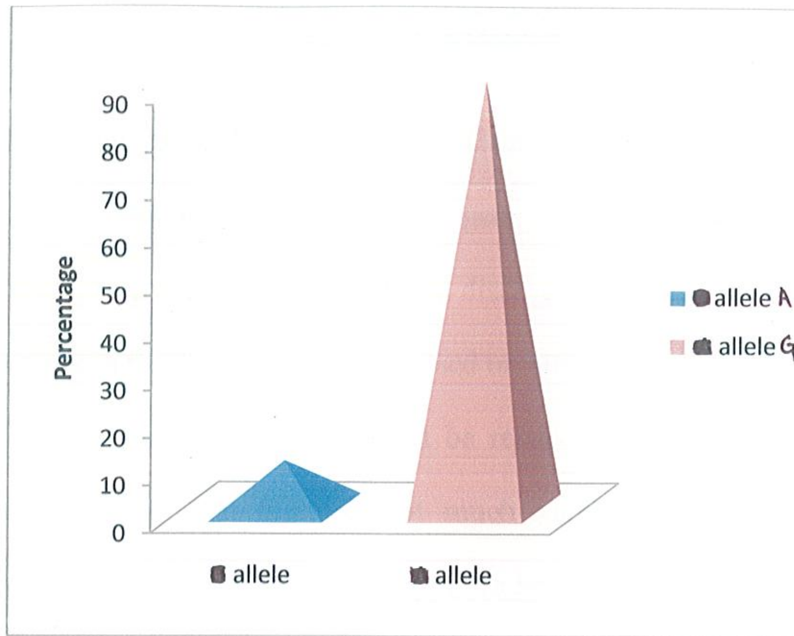

Figure 11: Allele occurrence in percentage

Discussion

Based on our results, we clearly observe that the variation in the frequency of occurrence of the SNP at -308 locus in the TNF –α gene, between people living in Himachal Pradesh and those residing in other parts of India is very less. So the incidence of this mutation at -308, where G gets replaced by A is rarely seen in Himachal Pradesh. The wild type in this region is also the G allele i.e.GG.

CHAPTER 5

CONCLUSION

This study was essentially taken up to evaluate the rate and frequency of occurrence of SNP (G/A) at the -308 locus of TNF- alpha gene in the residents of Himachal Pradesh. This would help us determine how many of the diseases caused in the Himachal population are due to mutations in the TNF-alpha gene. Based on our results, I may conclude that a small percentage of the diseases in this area might be related to the -308 (G/A) mutation. The interesting observation is that there is not much variation between the frequency of occurrence of this specific mutation in Himachal Pradesh and in other parts of the country(as seen in the review of literature). To make certain if the ideas mentioned above are valid or not there is a requirement of a more number of samples. Also samples taken from diseases individuals need to be analyzed for discrepancies in the TNF-alpha gene.

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