MATRIX METALLOPROTEINASE- 8 GENE PROMOTER POLYMORPHISM IN ASTHMA

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CERTIFICATE

This is to certify that the work entitled "**Matrix metalloproteases 8 gene promoter polymorphisms in Asthma**" submitted by **Sonalee Mehta** to the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat in the partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology is a record of bonafide research work carried out by them under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Signature of Student

Sonalee Mehta

Dated :

SUMMARY

According to the second National Family Health Survey (NFHS 2), conducted in the year 1998-1999, asthma is the most common disease in India, both in urban as well as rural areas, as compared to tuberculosis (TB), diabetes, malaria, thyroid and other diseases. Asthma has a strong genetic component and the final disease phenotype results from complex interactions between environment and multiple genes of small-to-modest effects. Previous studies showed that an excessive MMP activity plays an important role in the pathogenesis of several respiratory diseases including asthma, chronic obstructive pulmonary disease (COPD), adult respiratory distress syndrome (ARDS), and pulmonary fibrosis. Amongst them, MMP-8, in particular, is reported to be overly expressed in the pulmonary tissue and lavage fluid from patients with asthma to suggest its potential role in the development of asthma. Matrix metalloproteinases (MMPs) form a family of enzymes that mediate multiple functions both in the tissue destruction and immune responses related to asthma inflammation.

Various studies have shown the role of MMP-8 -381A/G promoter single nucleotide polymorphism (SNP) in asthma pathogenesis as mutation results in the replacement of A allele by G allele. The aim of my study is to determine the frequency of wild allele and mutant allele in North Indian population, as previously no such study has been conducted in this region and to see whether particular allele is associated with the protective role in the disease or have role in susceptibility to the disease.

Signature of Student

Name: Sonalee Mehta

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Dated:

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LIST OF ABBREVIATIONS

- **SDS-** Sodium dodecyl sulphate
- **µl-** microlitre
- **g-**gram
- **mg-**microgram
- **l-**litre
- **M-**molarity
- **min-**minute
- **ml-**mililitre
- **SNP-** Single nucleotide polymorphism
- **MMP-** Matrix metalloprotease

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INTRODUCTION

1.1 ASTHMA

Asthma is a chronic (long-term) lung disease that inflames and narrows the airways. Asthma causes recurring periods of wheezing (a whistling sound when you breathe), chest tightness, shortness of breath, and coughing. People who suffer from this chronic condition (long-lasting or recurrent) are said to be asthmatic.

The airways in the lungs are the tubes which carry air in and out of the lungs. The inflammation makes the airways swollen and very sensitive. The airways tend to react strongly to certain inhaled substances. When the airways react, the muscles contract tightly and less air passes through the lungs as a consequence of narrowed airways. If the airways swell more, the airways narrows even more. In some cases the cells in the airways might make more mucus than usual worsening the situation as the mucus is a sticky, thick liquid which can further contribute to the narrowing of the airways. These chain reactions can result in asthma symptoms. Symptoms can be observed each time the airways are inflamed. Clinically, asthma can be described based on symptoms that are either intermittent or persistent, and these symptoms are further classified in terms of severity (i.e., mild, moderate, or severe).

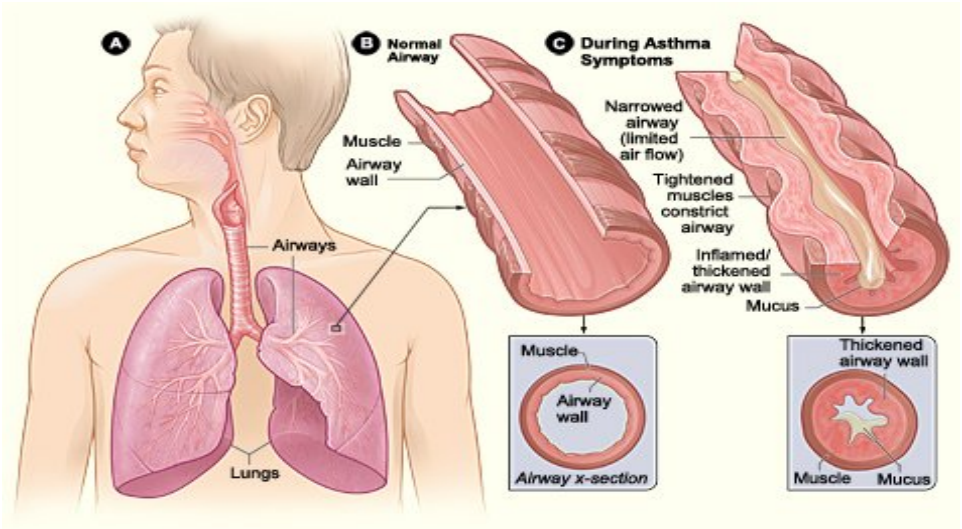

Figure 1 : Pathophysiology of Asthma

1.2 GENE

MMP8 (matrix metallopeptidase 8 (neutrophil collagenase)) is a protein-coding gene. Matrix metalloproteases also called matrixins and then are zinc-dependent endopeptidases. The gene is part of a cluster of MMP genes which localize to chromosome 11q22.3. The different proteins of this family matrix metalloproteinase(MMP) are responsible for the breakdown of extracellular matrix in normal physiological processes such as tissue remodelling, embryonic development and reproduction. Secretion of MMPs mostly is done in inactive form as proproteins which are activated when cleaved by extracellular proteinases. But however, the enzyme encoded by this gene is stored in secondary granules within neutrophils which are further activated by autolytic cleavage. Type I , II , III collagens are basically involved. Alternative splicing results in multiple transcript variants. Till now 24 matrixin genes have been identified which are involved in the basic metabolism in human body, which can be categorised into six classes based on their domain organisation and substrate preference. The six classes are as follows : Collagenases (MMP-1, -8 and -13), Gelatinases (MMP-2 and MMP-9), Stromelysins (MMP-3, -10 and-11), Matrilysin (MMP-7 and MMP-26), Membrane-type (MT)-MMPs (MMP-14, -15, -16, -17, -24 and -25) and others(MMP-12, -19, -20, -21, -23, -27 and -28).

Catalytic activity of MMPs is strongly controlled at 4 different levels : Gene regulation with the transcriptional and post transcriptional regulation, extracellular localization, pro-enzyme activation by removal of prodomain and inhibition by specific inhibitors i.e. TIMPs and alpha2-macroglobulin. MMPs play a central role in cell proliferation, migration, differentiation, angiogenesis, apoptosis and host defences.

Balance between the production of active enzymes and their inhibition is critical to avoid the conditions of uncontrolled ECM turnover, inflammation and dysregulated cell growth and migration. Dysregulatoin of MMPs has been implicated in many diseases including arthritis, lung diseases, chronic ulcers, encephalomyelitis and cancer.

1.3 WHAT IS POLYMORPHISM?

Genetic Polymorphism is a difference in DNA sequence among individuals, groups, or populations. Whereas genetic mutation is a change in the nucleotide sequence of a DNA molecule. Genetic mutations are a kind of genetic polymorphism. Sources include SNPs, sequence repeats, insertions, deletions and recombination. Genetic polymorphisms may be

the result of chance processes, or may have been induced by external agents (such as viruses or radiation). A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide –A,T,C or 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 , noncoding regions of genes, or in the intergenic regions (regions between genes). A SNP in which both the alleles produce the same polypeptide sequence is called a *synonymous polymorphism*(silent mutation). If a different polypeptide sequence is produced the polymorphism is a *non-synonymous 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. In the human genome there are at least 3.1 million single nucleotide polymorphisms (SNPs), or about 1 SNP per kilobase of sequence.

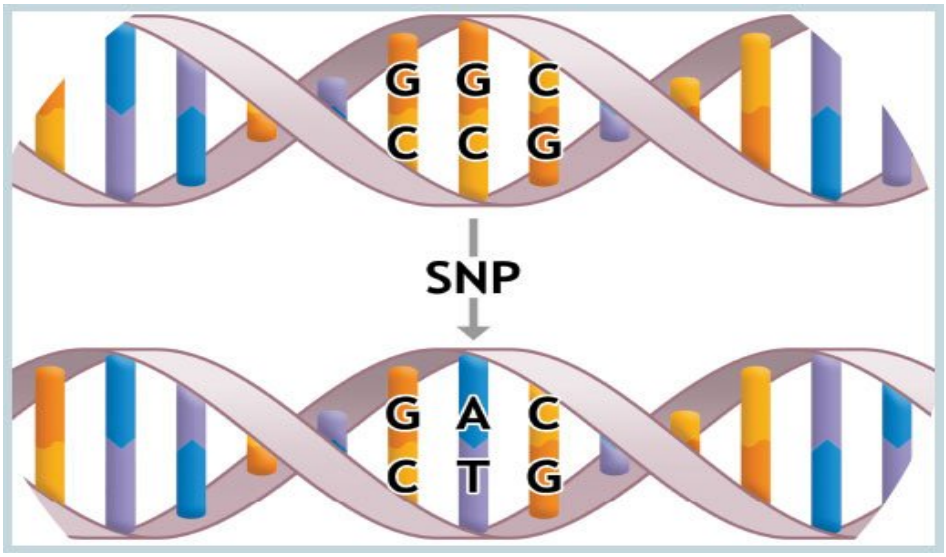

Figure 2 : Single nucleotide polymorphism

There are three SNPs identified in the case of MMP8 at -799C/T , -381A/G AND +17C/G.

1.4 ASSOCIATION OF MMP8 & ASTHMA

Asthma is a highly prevalent disease that involves a complex interplay of environmental factors, airflow obstruction, bronchial hyperresponsiveness, and inflammation. The dominant feature that leads to clinical symptoms is smooth muscle contraction and inflammation, which results in narrowing of the airway and obstruction. Epithelial cells, endothelial cells and fibroblasts participate in the regulation of inflammation. This occur by the activity of

variety of MMPs produced by these cells. Fibrillar collagen constitutes pulmonary extracellular matrix which is degraded by the matrix metalloproteases. Neutrophils are the first cells recruited to the site of the allergic reaction. Patients with symptomatic asthma have elevated levels of peripheral neutrophils that show signs of being activated. MMPs are released by neutrophils which function for the degradation of extracellular matrix, hence resulting in inflammation.

1.5 OBJECTIVES

1. To standardise the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism assay for the detection of SNP at -381 A/G locus of MMP-8 gene.
2. To determine the frequency of occurrence of SNP at -381 locus of MMP-8 gene in North Indian population (Asthmatic and healthy control groups).
3. To correlate association of -381 A/G locus of MMP-8 gene polymorphism with the occurrence of Asthma in this population.

CHAPTER-2

REVIEW OF LITERATURE

As yet no studies related to the occurrence of -381A/G polymorphism have been conducted in Himachal Pradesh region; therefore we found it significant to conduct the study in this region. As seen in the previous chapter, MMP-8 plays a vital role in the normal functioning of the body. The variations in the normal functioning of the MMP-8 gene lead diseases like asthma, cancer, and periodontitis and so on.

2.1 GENETICS OF ASTHMA

Asthma belongs to the category of classical allergic diseases which generally arise due to IgE mediated hypersensitivity to environmental triggers. Due to its increased prevalence among related individuals, it was understood quite long back that it is a genetic disorder. Asthma is a chronic pulmonary disorder often characterized by airway inflammation and remodeling that leads to reversible airway obstruction. Inflammation is mostly found in the larger conducting airways; however in severe forms of asthma even the smaller airways show infiltration of immune cells . The various symptoms of asthma are wheezing and breathing difficulty; cough, running nose and eyes, dyspnoea etc. with variable degree and frequency**.[1]**

Inherent genetic factors interact with environmental triggers, to bring about its pathogenesis and depending upon the trigger asthma can be categorised into intrinsic or extrinsic. On one hand extrinsic asthma can be caused due to hypersensitivity reactions which further lead to increased serum IgE and bronchial hyper-responsiveness to specific or non-specific inhaled allergens. On the other hand intrinsic asthma is caused due to non-immune and without any atopic background. **[1, 2]**

Approaches to identify genetic components in asthma

Population genetic studies like association studies and linkage studies are used majorly in the identification of the causative genes for most of the complex disorders including asthma. It is seen that in population genetic studies, it could be either hypothesis driven, which is the case in candidate gene studies, or with no prior hypothesis such as linkage studies. In candidate gene studies, genes are selected from the pathways shown or expected to play role in asthma

pathogenesis. Candidate gene studies could be based on allele frequency differences between affected (cases) and non-affected (control) individuals known as case-control studies or based on transmission distortion or disequilibrium of allele(s) as in family based association studies. Candidate gene studies are supposed to have high sensitivity to detect alleles or variants playing minor role in disease pathogenesis. On the contrary, linkage studies are usually carried out with motivation to identify novel disease loci/genes by genotyping evenly spaced markers in the entire genome, in large extended families. Since large fractions of genome are shared among individuals in a family, it is expected that loci with large effects on the phenotypes could be detected easily and fine mapped to fish out the susceptibility genes. In each case sensitivity and specificity are two vital issues when adopting any of the two approaches. There are other approaches which can be used for disease identification like microarrays, which have the advantage that the transcripts of several genes can be assayed at large scale simultaneously. With the help of animal models as well, this technology have been successful in the identification of genes/molecules involved in various complex diseases including asthma. Animal models are suitable as confounding environmental factors can be better controlled and tissue samples can be harvested sufficiently with ease. Also, identical genetic background of the inbred animal strains allow for dissection of environmental factors in influencing gene regulation in different pathological conditions. It is unlikely that any single factor, genetic or environmental, could account for asthma pathogenesis, therefore statistical tools are being designed to carryout multifactorial analysis. After obtaining the data it is necessary to divide the data into two categories; genes that affect inflammation and genes that play critical role in airway remodeling events**.[1]**

Genes influencing the inflammatory pathways

Human chromosomal regons were first found to be linked with allergy or asthma in the late 1980s and early 1990s. Several genome-wide screens have found linkage to chromosomal regions, such as, *5q23-31, 5p15, 6p21.3-23, 11p13, 11p15, 12q14-24.2, 13q21.3, 14q11.2-13, 17p11.1-q11.2, 19q13, 21q21* etc. The most consistently replicated among them are *5q23-31, 5p15* and *12q14-24.2* containing genes like *IL-3, IL-4, IL-5, IL-9, IL-12b, IL-13, IFNγ, iNOS, FCεRI*β, MMP9, MMP8 etc. Most of these affect the T cell development/polarization towards Th1 or Th2 and also affecting other features like recruitment of eosinophils, mast cells, neutrophils etc. to the site of inflammation. **[1]**

Genes involved in airway remodelling

Airway remodelling component has not received much attention as earlier it was believed that it appears late in disease process, resulting from persistent inflammation. What we really understand by airway remodelling is that these are the structural changes of the surface of the airway that lead to its narrowing and constriction. Identification of ADAM33, a disintegrin matrix metalloproteinase 33, lead researchers to believe that airway remodeling events are quite distinct and are influenced by genetic factors. *ADAM 33*, which is present on chromosome 20, was identified by positional cloning approach, using linkage studies in a Caucasian population. It is expressed by lung fibroblasts and bronchial smooth muscles but not by bronchial epithelial or immune cells. *DPP10* (dipeptidyl peptidase 10) is another gene that was identified, using positional cloning approach in mouse and human, to be associated with brochial hyperresponsiveness and IgE production . This gene is located on chromosome *2p14. GPRA* (G protein coupled receptor for asthma), which is located on chromosome *7p15* also shows consistent association with asthma after its initial linkage to asthma related traits. *SPINK5*, on chromosome *5q23-31*, is another gene that might play an important role in airway remodeling as it is highly expressed in bronchial epithelium and consistently shows association with asthma. Taken together, these data suggest a vital role of tissue remodeling, in asthma pathogenesis, which is brought about by complex interaction of tissue components like epithelium, smooth muscles etc**.[1]**

Copy number variation/polymorphisms

The genomic variation in the human genome ranges from single nucleotide variation to large microscopically detectable variations that have also been shown to be associated with many disorders. The advancement in the genotyping technology have led to identification of structural variation that fall in between these two extremes, known as copy number variations (CNVs). Currently all genomic variations larger than 1 kb of DNA are termed as structural variations. Structural variants could lead to change in gene dosage in case of deletion or duplication etc. or with any change in gene dosage as in inversions or balanced translocation. **[1]**

2.2 BIOLOGICAL ROLE OF MMPs

Members of the matrix metalloproteinase (MMP) family of zinc-dependent proteolytic enzymes regulate physiological and pathophysiological events in development, injury and repair, such as morphogenesis,vasculogenesis, remodelling of the extracellular matrix, cell migration, cleavage of cytokines, and activation of mediators and defensins. MMPs are usually minimally expressed in normal physiological conditions and thus homeostasis is maintained. They are synthesised as secreted or transmembrane proenzymes and processed to their active forms by the removal of amino-terminal propeptides. Their activation by the cleavage of the prodomain from the latent proenzyme and the presence of natural inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), are important mechanisms that restrict the action of secreted MMPs to the micromilieu of their origin. MMP activity is tightly controlled at the level of transcription, pro-peptide activation and inhibition by tissue inhibitors of MMPs. Dysregulated MMP activity leads to pathological conditions such as arthritis, inflammation, asthma and Cancer. In most tissues, their constitutive expression is low and their overexpression at sites of inflammation or other pathogenic events was thought to be due the classical role of extracellular matrix degradation.[3,9]

At least 25 different vertebrate MMPs have been characterised up to now. In humans, 24 MMPs have been identified and a cluster of nine MMP genes is located on chromosome 11q22. Although the activity of MMPs has been shown to be essential in cell biological processes and many fundamental physiological events involving tissue remodelling, such as angiogenesis, bone development, wound healing and mammary involution , the increasing interest in MMP function mainly stems from their role in several pathological conditions, such as cancer or chronic inflammatory diseases. Fibrillar collagens, which are highly resistant to enzymatic cleavage, are important constituents of the pulmonary extracellular matrix. However, collectively, MMPs are able to degrade all components of the extracellular matrix, resulting in cavities. Matrix metalloproteinases are excreted by a variety of connective tissue and pro-inflammatory cells including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes. These enzymes are expressed as zymogens, which are subsequently processed by other proteolytic enzymes to generate the active forms. Under normal physiological conditions, the proteolytic activity of the MMPs is controlled at any of the following three known stages: activation of the zymogens, transcription, and inhibition of the active forms by various tissue inhibitors of MMPs (TIMPs). In pathological conditions this equilibrium is shifted toward increased MMP activity leading to tissue degradation. Since uncontrolled MMP activity can easily become

destructive and lead to breakdown of homeostasis, their activity has to be tightly regulated. the catalytic activity of MMPs is strongly controlled at four different levels: 1) gene expression with transcriptional and post-transcriptional regulation; 2) extracellular localisation and tissue or cell type of MMP release, termed compartmentalisation; 3) pro-enzyme activation by removal of the pro-domain; and 4) inhibition by specific inhibitors, i.e. tissue inhibitors of matrix metalloproteinases (TIMPs), and by non-specific proteinase inhibitors, e.g. a2-macroglobulin. Human a2-macroglobulin is a broad spectrum proteinase inhibitor of tissue fluids and blood. This homotetrameric macromolecule of 725 kDa inhibits almost all classes of endopeptidases by entrapping the whole enzyme. The naturally occurring inhibitors of human MMP activity are four members of the TIMPs. Each TIMP molecule consists of around 190 amino acids composed of two distinct domains, a larger N-terminal and a smaller C-terminal domain, each one stabilised by three conserved disulfide bonds. In general all TIMPs are broad spectrum inhibitors of MMPs, but there are differences in their specificity. For example TIMP-1 has been shown to have low inhibitory activity against MMP-19 and membrane bound MMP-14, -16 and -24, while it is more potent for MMP-3 and MMP-7 than TIMP-2 and TIMP-3. **[5]**

MMPs trigger bone growth and modelling : The formation of the skeleton in humans or mice depends on two major ossification processes which require extensive matrix remodelling. MMPs that show bone marrow remodelling are : MMP-2, MMP-9, MMP-13, MT1- MMP and MT3-MMP**.[3]**

MMPs in angiogenesis and vascular development : Angiogenic phenotype of MMP-9 null mice during long bone growth, MMP-2 and MT1-MMP knockout mice also show modifications in angiogenesis, especially under pathological conditions, e.g. tumour-induced angiogenic sprouting. **[3]**

MMPs in the immune response and innate immunity: MMP-7 forms a chemoattractant gradient necessary for neutrophil guidance in damaged lung epithelia. the expression pattern of the MMPs -8, -2 and -9 in innate immune cells, such as monocytes, activated macrophages, alveolar macrophages and neutrophils, these enzymes have been linked to pathologies associated with innate immune dysregulation**.[3]**

MMPs have essential roles in wound healing and cell migration: Many secreted and membrane bound MMPs contribute either directly or indirectly to the process of wound healing and neovascularisation. Upon injury most, if not all, MMPs are induced and expressed in almost any involved cell types, including mesenchymal, epithelial and immune cells. The induction of MMP-1 expression by keratinocytes at the wound edge, for example, is mediated by the binding of a2b1 integrin to the dermal collagen type I. Another recently identified role of MMP activity in wound healing is the recruitment of immune cells since neutrophil recruitment requires the presence and activity of MMP-7. **[3]**

2.3 ROLE OF MMPs IN ASTHMA

Asthma is an Ig E mediated immune hypersensitivity response to environmental triggers. An increased prevalence of this disease is seen among related individuals. It is a multifactorial disorder of airways by complex interaction between genetic and environmental factors. It is a chronic pulmonary disorder in which the airways in the lungs get inflamed making the airways swollen and very sensitive to certain inhaled substances. When the airways react, the muscles around them tighten. This narrows the airways, causing less air to flow into the lungs. It may also lead to mucus hyperproduction and thickening of sub-mucosa. To be more precise asthma leads to bronchial remodelling which includes shedding of airway epithelial cells,increase number of goblet cells,mucus gland hypertrophy, increased fibroblast number,Inflammed smooth muscle mass, degradation of ECM and neovascularity and increased collagen deposition**.[16]**

MMP8 is derived from degranulation of PMNs and certain non-PMN lineages. It is usually expressed on the surface of neutrophils. MMP8 plays a protective role in asthma since its deficiency promotes allergen induced airway inflammation.When the lungs cells are exposed to allergens, neutrophils, eosinophil,mastcells along with IgE antibodies are recruited in order destroy these allergens. Degranulation of PMNs leads to secretion of inflammation mediators resulting in inflamed cells. It is here that MNP8 shows suppressing effect on asthma development. 3 single nucleotide polymorphisms 799 C\T, 17 C\G, 381 A\G were seen in patients suffering from asthma. Suppression of inflammation was lost in patients with major C allele whereas the minor A allele patients showed suppressing effect of inflammation. MMP8 also acts as a diagnostic marker,as an increased expression of this enzyme seen in patients with severe asthma. Acute asthma is correlated with increased immune cell trafficking including components such as cleaved fibrin collagen,

inhibiters,immune cells and chemokines.Delayed clearance of these components results in the chronic effects of asthma. Production of MMP 8 is done in the form of secreted or transmembrane proenzymes which are further activated by the removal of an amino terminal pro-peptide. The property of maintain the latent state of the enzyme is attained by the interaction of the cysteine residue present in this peptide with the zinc molecule in the enzyme active site. When the interaction is disturbed it triggers the cysteine switch mechanism and the enzyme is activated. The various functions of MMPs are during the developmental stages and in normal physiology which can be stated as follows; (1) disruption of ECM molecules which further results in cell migration; (2) modifying the microenvironment of ECM and hence alter the cellular behaviour; (3) it activates the biological molecules by direct cleavage releasing them from bound stores, or, modulating of the activity of their inhibitors**.[9,11,12]**

2.4 SNP GENOTYPING

Single nucleotide polymorphisms (SNPs) have emerged as genetic markers of choice because of their high-density and relatively even distribution in the human genomes. When a disease gene has been mapped to a chromosomal region, a high-density SNP mapping or candidate gene association studies are logical steps to follow. Typically, genotyping protocols start with target amplification and follow with restriction digestion using a restriction enzyme. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is useful in small-scale basic research studies of complex genetic diseases that are associated with single nucleotide polymorphism (SNP). Designing a feasible primer pair is an important work before performing PCR-RFLP for SNP genotyping and an available restriction enzyme for discriminating a target SNP, are required. **[7]**

Target DNA Amplifications

Human genome has about 3 billion base pairs. The target of genotyping is usually a few hundreds base pairs. The challenge of assaying such a tiny fraction of the genome is a key reason why most genotyping techniques begin with target amplification. Almost all techniques use the polymerase chain reaction (PCR) for amplification

In brief, PCR works by using heat to separate a duplex DNA molecule into two single-stranded molecules and then copying each of the two single-stranded molecules with thermostable DNA polymerases and an oligonucleotide primer. When the DNA polymerases

finish copying the single stranded molecules, each becomes a new duplex DNA molecule. This process can be repeated 30–40 times, after each round of amplification the amount of targeted DNA fragment doubles. By the end of the process, the amount of the targeted DNA fragment has increased 10^9 times or more**.[15]**

For genotyping purposes target DNA amplification has to be specific—an amplicon amplifies one and only one locus in the genome. Primers that amplify multiple loci can lead to serious genotype errors. Pseudogenes, conserved sequences within gene families and repetitive sequences are common features of our genome and they can cause nonspecific amplifications. To avoid this problem known repeat sequences should be filtered out before designing PCR primers, and PCR products should be tested before genotyping. **[14,15]**

Restriction fragment length polymorphism

In molecular biology, restriction fragment length polymorphism, or RFLP (commonly pronounced "rif-lip"), is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated. In RFLP analysis, the DNA sample is broken into pieces and (digested) by restriction enzymes and the resulting *restriction fragments* are separated according to their lengths by gel electrophoresis. Although now largely obsolete due to the rise of inexpensive DNA sequencing technologies, RFLP analysis was the first DNA profiling technique inexpensive enough to see widespread application. RFLP analysis was an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing. Restriction fragment length polymorphism (RFLP) is considered to be the simplest and earliest method to detect SNPs. SNP-RFLP makes use of the many different restriction endonucleases and their high affinity to unique and specific restriction sites. By performing a digestion on a genomic sample and determining fragment lengths through a gel assay it is possible to ascertain whether or not the enzymes cut the expected restriction sites. A failure to cut the genomic sample results in an identifiably larger than expected fragment implying that there is a mutation at the point of the restriction site which is rendering it protected from nuclease activity.

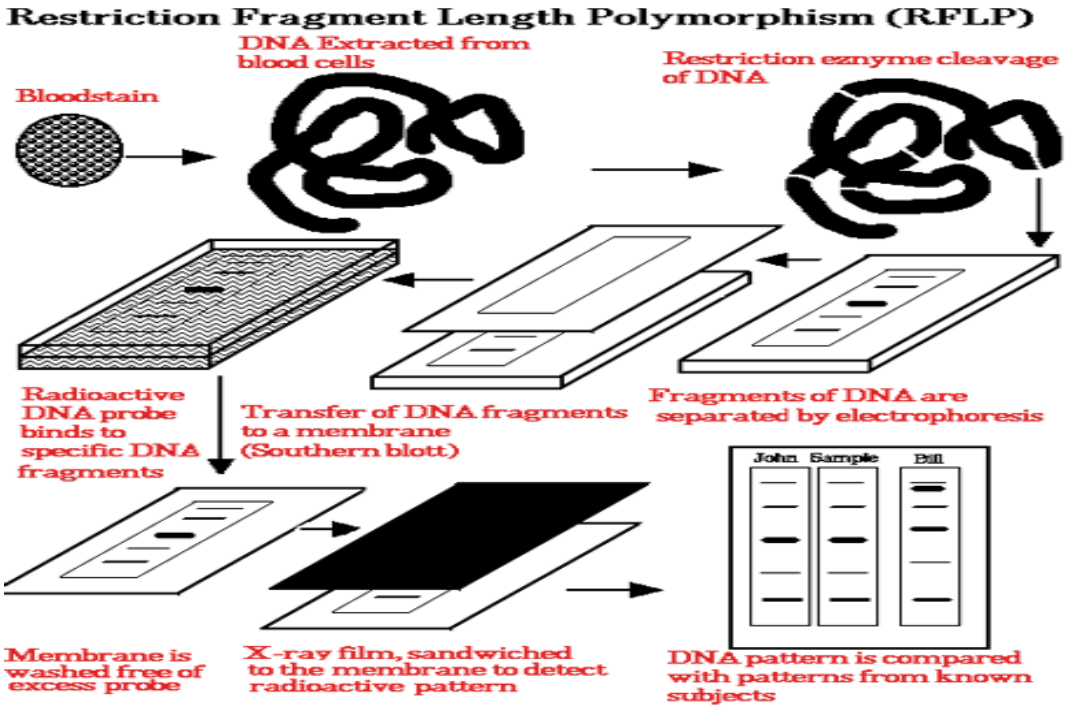

Figure 3 : RFLP in DNA fingerprinting.

2.5 MMP8 and its SNPs

Matrix metalloproteinase 8 (MMP8), an enzyme that degrades fibrillar collagens imparting strength to the fetal membranes, is expressed by leukocytes and chorionic cytotrophoblast cells. We identified three single nucleotide polymorphisms (SNPs) at 799C/T, 381A/G and 17C/G from the major transcription start site in the MMP8 gene, and determined the functional significance of these SNPs by analyzing their impact upon MMP8 promoter activity and their association with preterm premature rupture of membranes(PPROM).

Jiwon Lee et.al. observed that human matrix metalloproteinase (MMP)-8 is over-expressed in ectatic bronchi in patients with bronchiectasis suggests that polymorphisms altering the expression of MMP-8 may contribute to the susceptibility to development of bronchiectasis. We evaluated the association between the presence of bronchiectasis in a Korean population and two single nucleotide polymorphisms (SNPs) (-799C/T and -381A/G) on the promoter region of the MMP-8 gene that are reported to alter the promoter activity and thereby the gene expression. Genotyping through polymerase chain reaction (PCR) and subsequent

automatic sequencing was done in 167 patients with bronchiectasis and their age-, sex matched healthy controls to reveal that only -799C/T is polymorphic among Koreans. In the patient group with bronchiectasis, the frequency of -799C/C, C/T, and T/T genotypes were 41.9%, 49.7%, and 8.4%, respectively. A similar distribution was observed in the control group: C/C (49.7%), C/T (43.1%), and T/T (7.2%) (p=0.36). In subgroup analysis, no significant difference was observed among the patients according to; the extent of disease (p=0.76), colonization of microorganisms (p=0.56), or association of mycobacteria (p=0.17).

From these results, we conclude that -799C/T on the promoter region of MMP-8 lacks association with development of bronchiectasis in Koreans.

Since no association was found with SNP 799C/T on the promoter region of MMP8 , hence therefore in the assigned project, SNP 381A/G and its prevalence in asthma in the Northern Indian region is analyzed. **[7,11]**

CHAPTER-3

MATERIALS AND METHODS

3.1 COLLECTION OF SAMPLE

A total of 94 asthma and 93 control samples were collected from the residents of Northern India of unbiased sex and age. Venous blood was collected with the help of a sterile needle. DNA was then isolated from these samples using inorganic method.

3.2 DNA ISOLATION

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

REAGENTS USED:

- ***Tris Aceticacid-EDTA (TAE) buffer (50x):***242 gm of tris base was dissolved in 500ml of distilled water. This solution was then supplemented with 57.1ml of glacial acetic acid followed by 100ml of 0.5 M EDTA (ph 8.0). Final volume of the solution was made to 1000ml with the distilled water and the same was filtered and diluted to a working concentration of 1X before use.
- ***Agarose*** : 1.2% agarose gel was prepared in 1XTAE buffer for qualitative analysis of genomic DNA samples.
- ***Ethidium bromide*** (10mg/10ml) : 10mg of ethidium bromide was added to 1ml of distilled water and dissolved by gentle mixing before storing in an amber bottle at room temperature.
- ***Gel loading dye (6x):***0.025% bromophenol blue (BPB) with 40%sucrose in water was used for analysis of genomic DNA.
- ***Gel loading dye (10x):*** 0.5%(w/v) xylene cyanol, dissolved in distilled water was mixed with equal volume of glycerol. The same was used for PCR product analysis.
- ***Tris EDTA(TE) buffer*** (Tris, 10mM; EDTA, 1mM ;pH8.0) : TE buffer was prepared by mixing 10ml Tris-Cl (1M) and 2ml of EDTA (0.5 M) in 700ml distilled water and the final volume was made 1000ml.

- ***Tris EDTA(TE) buffer*** (Tris, 10mM; EDTA, 1mM ;pH 7.3) : As above, 10ml TrisCl and 2ml of EDTA (0.5M) were mixed in distilled water to obtain a final volume of 1000ml.
- ***Sodium dodecyl Sulphate***(SDS) 10% :Dissolved 10gm of SDS salt in 70ml of warm distilled water and the final volume was made 100ml.
- ***Ammonium acetate (7.5M)*** : Dissolved 28.9 gm of ammonium acetate salt in 20ml of distilled water and the final volume was adjusted to 50ml.
- ***Chilled dehydrated ethyl alcohol*** : Undiluted dehydrated ethyl alcohol stored in -20°C deep freezer.
- ***Ethanol (70%)*** : 70ml of dehydrated ethanol was added to 30ml of distilled water to obtain a final volume of 100ml.

PROTOCOL FOR DNA ISOLATION

1. To 400µl of blood sample, added RBC lysis buffer (3times the volume of the blood sample taken) and kept for incubation on a rocker, to permit perpetual shaking at room temperature until the RBCs completely lysed.
2. Centrifuged the solution at 13000RPM for 1min to obtain a creamish white WBC pellet.
3. The supernatant was discarded and the WBC pellet suspended in 400µl TE buffer (pH 8.0) using a vortexing machine. There after 22µl of 10% of SDS solution was added to above solution and the mixture was incubated at 56°C for 30minuteson a dry bath.
4. Subsequently added 150µl of 7.5M ammonium acetate and mixed vigorously for about 1minute per sample, on vortexer. Centrifuged the mixture at 13000 RPM @ room temperature for 15minutes there by resulting in separation of the precipitated proteins as a pellet.
5. The clear supernatant was transferred to a fresh sterile micro centrifuge tube. To this added chilled absolute ethyl alcohol ethyl alcohol (twice the volume of clear supernatant). The tube was gently rocked a couple of time to allow the precipitation of genomic DNA.
6. The genomic DNA precipitates were centrifuged at 13000RPM for 10minutes to get pellet at the bottom of the tube. The latter subsequently washed in 160µl of 70% ethanol and air dried at roo temperature for about 15minutes.

7. 100µl of TE buffer (pH 7.3) was used to dissolve the dried DNA pellet by incubating at 65°C for 10minutes. The dissolved DNA was finally stored at -20° C till further use.

3.3 QUANTIFICATION OF DNA USING NANODROP

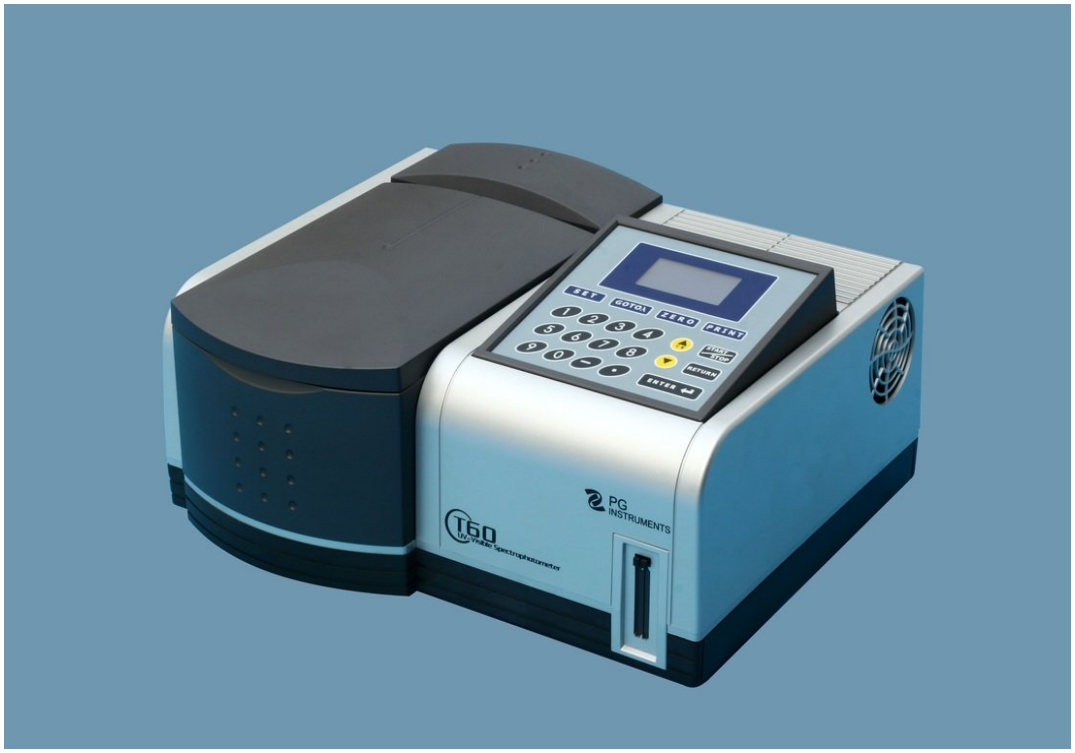

Figure 4 : Nanodrop

ANALYSIS OF DNA

If sample is pure (i.e. without significant amounts of contaminants such as a proteins, phenol, agarose, or other nucleic acids), can use spectrophotometer to measure amount of UV irradiation absorbed by the bases. For quantifying DNA and RNA, readings should be taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample.

1 O.D. at 260 nm for double-stranded DNA = 50 ng/µl of dsDNA

1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/µl of ssDNA

1 O.D. at 260 nm for RNA molecules = 40 ng/µl of RNA

The reading at 280 nm gives the amount of protein in the sample. Pure preparations of DNA and RNA have OD260/OD280 values of 1.8 to 2.0, respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible. So typically, dilute sample 1 µl in 50 µl so the dilution factor is 50. Put whole 50 µl in spectrophotometer cuvette.

The DNA concentration read : (OD260)x (50 ng DNA /µl) x (dilution factor)/(1 OD260 unit)

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

1. With the sampling arm open, loaded 1-2 µL of de-ionized water onto the lower measurement pedestal
2. When the software starts, we should saw this message: a. "Ensure pedestals are clean…Click OK to initialized instrument." b.Clicked OK. c.The messages "Initializing Spectrometer- please wait" appeared.
3. With the sampling arm open, wiped both pedestals with a laboratory wipe.
4. Loaded a reference blank (the buffer, solvent, or carrier liquid used with your samples) onto the lower measurement pedestal.
5. Clicked on the Blank (F3) button to store the blank reference.
6. Analyzed a fresh replicate of the blanking solution as though it were a sample by selecting Measure (F1).
7. Analyzed our sample.

AMPLIFICATION 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. The polymerase chain reaction was operationalized by Kary Mullis and colleagues at Cetus Corporation in the early 1980's. There are three major steps involved in the PCR technique: denaturation, annealing, and extension. PCR is based on the mechanism of DNA replication in vivo: dsDNA is unwound to ssDNA, duplicated, and rewound. This technique consists of repetitive cycles of: denaturation of the DNA through melting at elevated temperature to convert double-stranded DNA to single-stranded DNA, annealing (hybridisation) of two

oligonucleotides used as primers to the target DNA and extension of the DNA chain by nucleotide addition from the primers using DNA polymerase as catalyst in the presence of Mg2+ ions.

PCR is useful in the investigation and diagnosis of a growing number of diseases. Qualitative PCR can be used to detect not only human genes but also genes of bacteria and viruses. PCR is also used in forensics laboratories and is especially useful because only a tiny amount of original DNA is required.

Despite the numerous variations on the basic theme of PCR, the reaction itself is composed of only a few components. These are as follows: Water, PCR Buffer, MgCl2, dNTPs ,Forward Primer, Reverse Primer, Target DNA Polymerase.

Water is present to provide the liquid environment for the reaction to take place. The next component is the PCR Reaction Buffer. This reagent is supplied with commercial polymerase and most often as a 10x concentrate. The primary purpose of this component is to provide an optimal pH and monovalent salt environment for the final reaction volume. Many commercially supplied PCR buffers already contain magnesium chloride (MgCl2). MgCl2 supplies the Mg++ divalent cations required as a cofactor for Type II enzymes, which include restriction endonucleases and the polymerases used in PCR. The purpose of the deoxynucleotide triphosphates (dNTPs) is to supply the “bricks.” Since the idea behind PCR is to synthesize a virtually unlimited amount of a specific stretch of double-stranded DNA, the individual DNA bases must be supplied to the polymerase enzyme. 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, 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 selectively of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

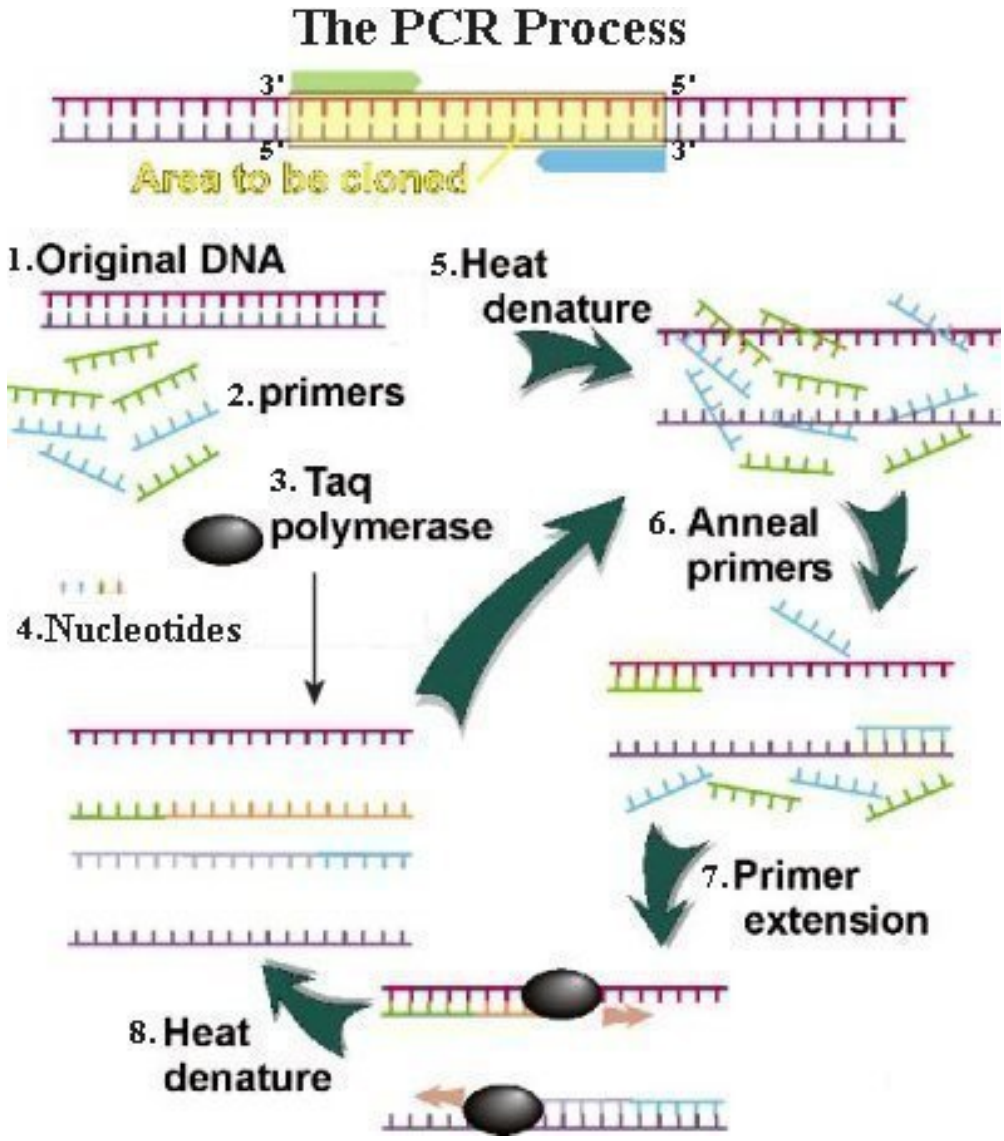

Figure 5 : Depiction of Polymerase Chain Reaction

PRIMER DESIGN

Primers used for MMP8 promoter gene were :

- **MMP8 (F) 5'-AAGTGGGAGACTACCATGCAAAGC-3'**
- **MMP8 (R) 5'-GTCACATGTTTCATTTGTGGAGGG-3'**

Tm = 4 (G+C) + 2 (A+T)

MMP 8(F) Tm=57°C

GC Content=47.8%

MMP 8(R) Tm=56.5°C

GC Content=45.8%

PCR STANDARDISATION

Gradient PCR used to optimize the PCR to know the suitable annealing temperatures. The machine is able to set several different temperatures over the run. The gradient machine will run each row at a different temperature. Running a gradient PCR for new primer set allows you to identify a single annealing temperature that will provide efficient, specific amplification of all target.

Following were the reaction conditions used for standardisation :

94°C -2 mins

94°C – 30 secs

50°C,52°C,54°C,56°C,58°C,60°C– 30 secs (35cycles)

72°C – 1 mins

72°C – 5 mins

MATERIALS REQUIRED FOR PCR

1. **PRIMERS:** Short strands of DNA that adhere to the target segment. They identify the portion of DNA to be multiplied and provide a starting place for replication. MMP8 forward and reverse primers are used.
2. **TAQ POLYMERASE** : This is the enzyme that is in charge of replicating DNA. This is the polymerase part of the name polymerase chain reaction.
3. **NUCLEOTIDES** : You'll need to add nucleotides (dNTPs) so the DNA polymerase has building blocks to work with.
4. **BUFFER** : The primary purpose of this component is to provide an optimal pH and monovalent salt environment for the final reaction volume.
5. **DISTILLED WATER** : Water is present to provide the liquid environment for the reaction to take place.
6. **MgCl2** : MgCl2 supplies the Mg++ divalent cations required as a cofactor for Type II enzymes, which include restriction endonucleases and the polymerases used in PCR.

METHOD OF PCR

1. Prepare a reaction mixture of 15 µl : Buffer 1.5µl , distilled water 11.5µl , primer (forward) 0.3µl , primer (reverse) 0.3µl, dNTPs 0.3µl, taq polymerase 0.1µl.
2. Add 1µl of genomic DNA.
3. Mix thoroughly and place in thermal cycler.
4. Amplify for 35 cycles as follows : 2mins at 94°C , 30 secs at 94°C , 30 secs at 60°C , 1min at 72°C and a final extension period of 5min at 72°C following the 35^{th} cycle.
5. Remove the tubes from thermal cycler and add 1 µl loading dye. Mix properly.
6. Load 3µl aliquot onto a 1.2% agarose gel and run at 100V for approximately 20-30mins in TAE buffer.
7. Stain the gel with ethidium bromide solution, visualise bands on a UV light box and save the picture of the gel for further analysis.

3.5 RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

A restriction fragment length polymorphism is defined by the existence of alternative alleles associated with restriction fragments that differ in size from each other. RFLPs are visualized by digesting DNA from different individuals with a restriction enzyme, followed by gel electrophoresis to separate fragments according to size, then blotting and hybridization to a labeled probe that identifies the locus under investigation. RFLP methodology involves cutting a particular region of DNA with known variability, with restriction enzymes, then separating the DNA fragments by agarose gel electrophoresis and determining the number of fragments and relative sizes. The pattern of fragment sizes will differ for each individual tested. Restriction Fragment Length Polymorphism (RFLP) is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination. Analysis of RFLP variation in genomes was a vital tool in genome mapping and genetic disease analysis. In our experiment we used Hind III as the restriction enzyme for the process.

OPTIMISATION OF PCR-RFLP ASSAY

The Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is a relatively simple and inexpensive method for genotyping single nucleotide polymorphisms (SNPs).Hind III enzyme with original concentration of 20,000 units/ml was obtained. In initial RFLP 4 units i.e 0.2µl was used. Further 2units i.e. 1µl was used. Better resolution bands were obtained in both the cases but to make the process cost effective 2units/µl enzyme was used.

PROCEDURE FOR RESTRICTION DIGESTION

1. Prepare a reaction mix of Buffer 1.5µl , Hind III enzyme 0.1µl in 2units, distilled water 3.45µl.
2. Add 10µl PCR product to the reaction mix.
3. Mix thoroughly and place in thermal cycler.
4. Set the incubation temperature at 37°C for 60mins and inactivation temperature 80°C for 20mins.
5. Remove the tubes from thermal cycler and add 1 µl loading dye. Mix properly.
6. Load 10µl aliquot onto a 3.5% agarose gel and run at 100V for approximately 45-60mins in TAE buffer.
7. Stain the gel with ethidium bromide solution, visualise bands on a UV light box and save the picture of the gel for further analysis.

CHAPTER -4

RESULT AND DISCUSSION

After performing the various protocols, the following results were obtained.

Sample collection

93 control and 94 asthma samples were collected with consent form of each patient. Family history for any genetic or medical disorder was also noted down.

DNA isolation

First the DNA isolation was carried out in replicates, for all the samples that had been collected. The reagents used in this process were all made from scratch in the lab. The method of isolation described by Miller et.al is used. The DNA was successfully isolated from each sample and was stored at -20°C .

Quantification using Nanodrop

Next the samples were taken and their optical densities were measure at 260nm as well as 280nm. This measuring process was carried out in Microplate reader. Multiple samples were loaded in the microplate reader and OD was taken simulatneuosly. A large table was made evaluating the OD at 260nm and 280nm for each of the samples.

SAMPLE NO.	260nm/280nm	CONCENTRATION (ng)
3.1	1.58	143
4.1	1.33	148
5.1	1.75	151
6.1	1.9	133
7.1	1.48	101

Table 1 : Examples of readings obtained from spectrophotometer

Based on these numerous readings it was seen that a range was obtained. The values ranged from 1.2-2.0 .Out of the total samples, it was observed that a few were showing small quantities of protein impurities. These samples were then made to undergo the quantification process again, after thoroughly cleaning out the apparatus.

PCR Standardisation

After all the various sample sets were diluted to stable concentration, gradient PCR was carried out for standardisation of annealing temperatures.

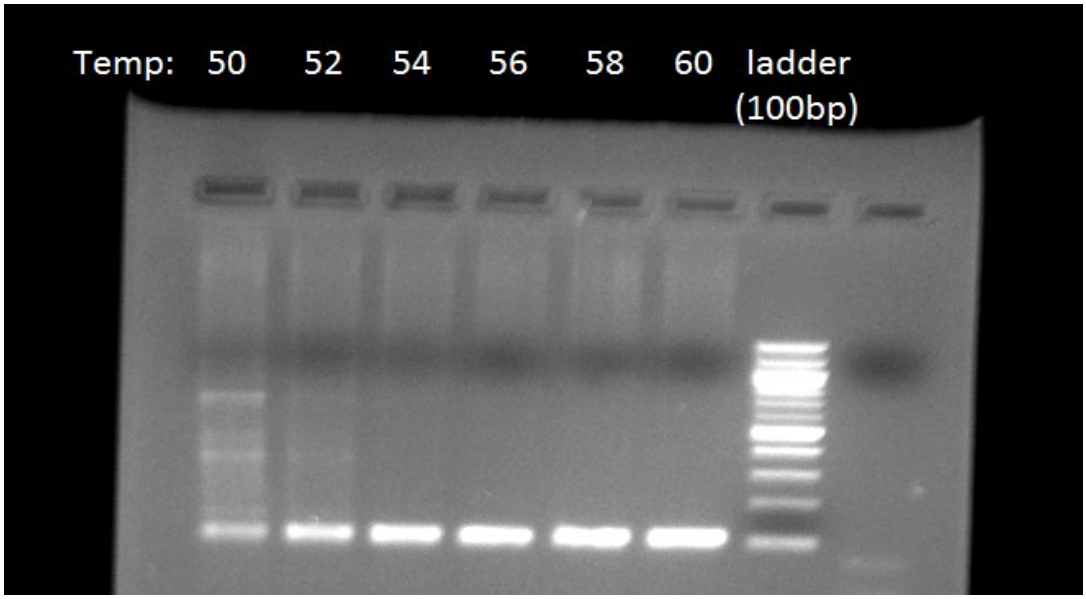

Figure 6 : Gel image showing annealing temperatures.

Sample loaded were run on 5 different temperatures . From the above picture, it is can be deduced that 60°C gives the most optimal amplification since we get the sharpest band at 60°C.

Lane 1	:At 50°C same sample loaded
Lane 2	: At 52° C same sample loaded
Lane 3	: At 54° C same sample loaded
Lane 4	: At 56°C same sample loaded
Lane 5	: At 58°C same sample loaded
Lane 6	: At 60° C same sample loaded
Lane 7	: 100bp ladder
Lane 8	: Negative control

DNA Amplification

1.2% agarose gel electrophoresis was carried out to check the DNA amplification. In this technique a 100bp ladder was taken and the various 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.

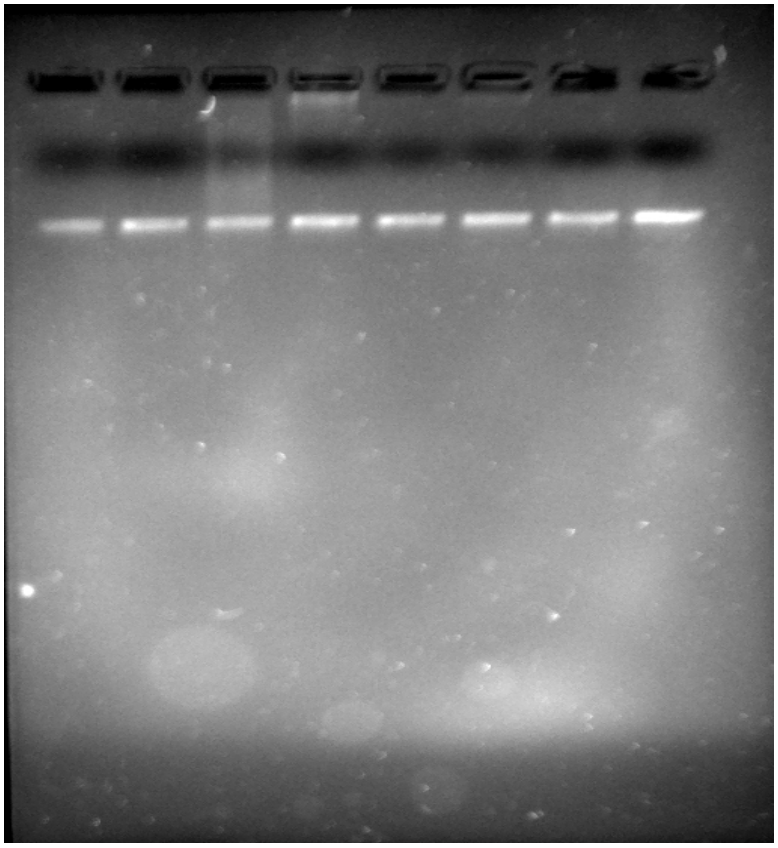

Figure 7 : Gel image showing amplification in Control samples.

Lane 1 : Control sample 1
Lane 2 : Control sample 2
Lane 3 : Control sample 3
Lane 4 : Control sample 4
Lane 5 : Control sample 5
Lane 6 : Control sample 6
Lane 7 : Control sample 7
Lane 8 : Amplified DNA used as marker.

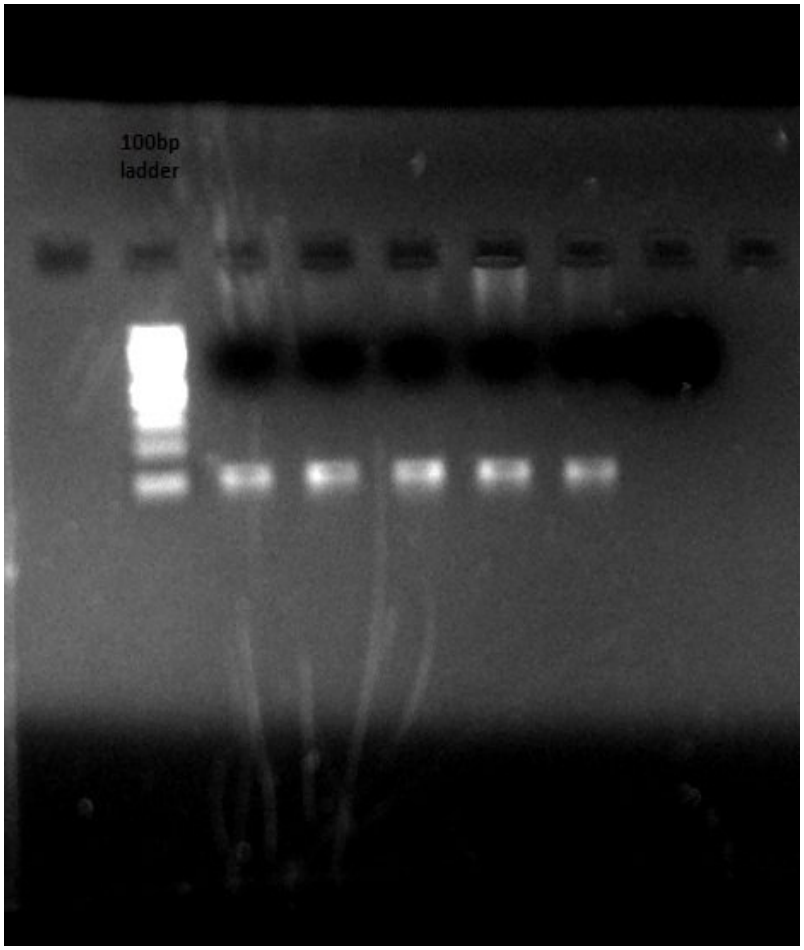

Figure 8 : Gel image showing amplification in Asthma samples.

Well-2 : 100bp ladder. Well 3-7 : shows the amplification of the samples .. Well-8 : Negative control hence no band

Lane 1 : Empty
Lane 2 : 100bp ladder
Lane 3 : Asthma sample 1
Lane 4 : Asthma sample 2
Lane 5 : Asthma sample 3
Lane 6 : Asthma sample 4
Lane 7 : Asthma sample 5

Restriction Digestion:

Restriction Fragment Length Polymorphism was used and enzyme is used for the restriction digestion was Hind III. Hind III recognizes A^AGCTT site and cuts best at 37°C in 60 minutes.

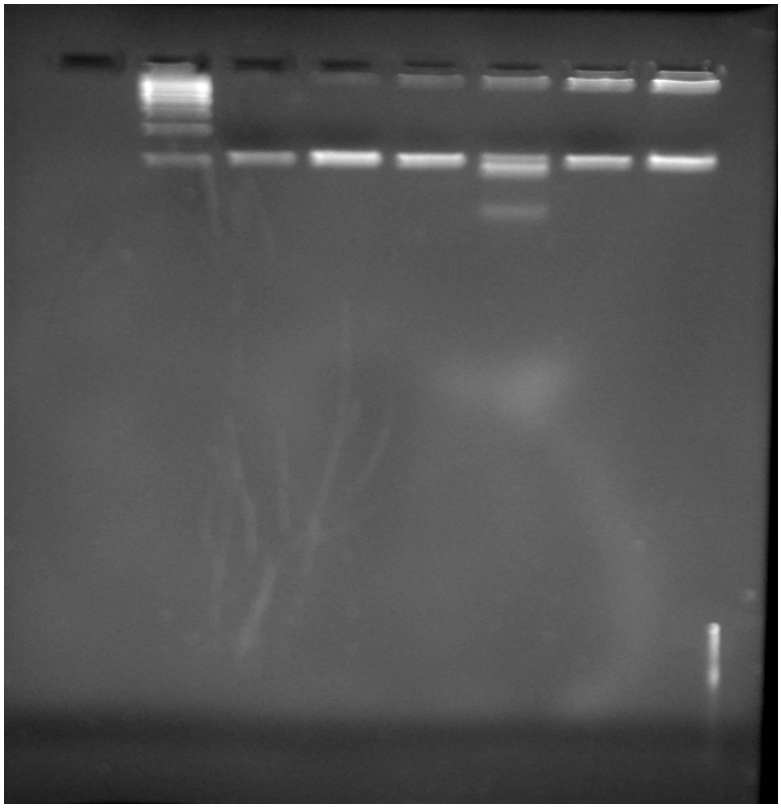

Figure 9: Allele bands as seen in gel image.

Lane 1 : Empty
Lane 2 : 100bp ladder
Lane 3 : Control sample 1 with GG genotype
Lane 4 : Control sample 2 with GG genotype
Lane 5 : Control sample 3 with GG genotype
Lane 6 : Control sample 4 with AG genotype
Lane 7 : Control sample 5 with GG genotype
Lane 8 : Control sample 6 with GG genotype

The band sizes of the alleles are as follows : GG- 105 bp ; AA- 80,25 bp ; AG- 105,80,25 bp. As clearly seen in the picture sample- 1,2,3,5,6 are GG with a single band. Sample-4 is heterozygous AG with three bands of band sizes 105bp, 80bp and 25bp.

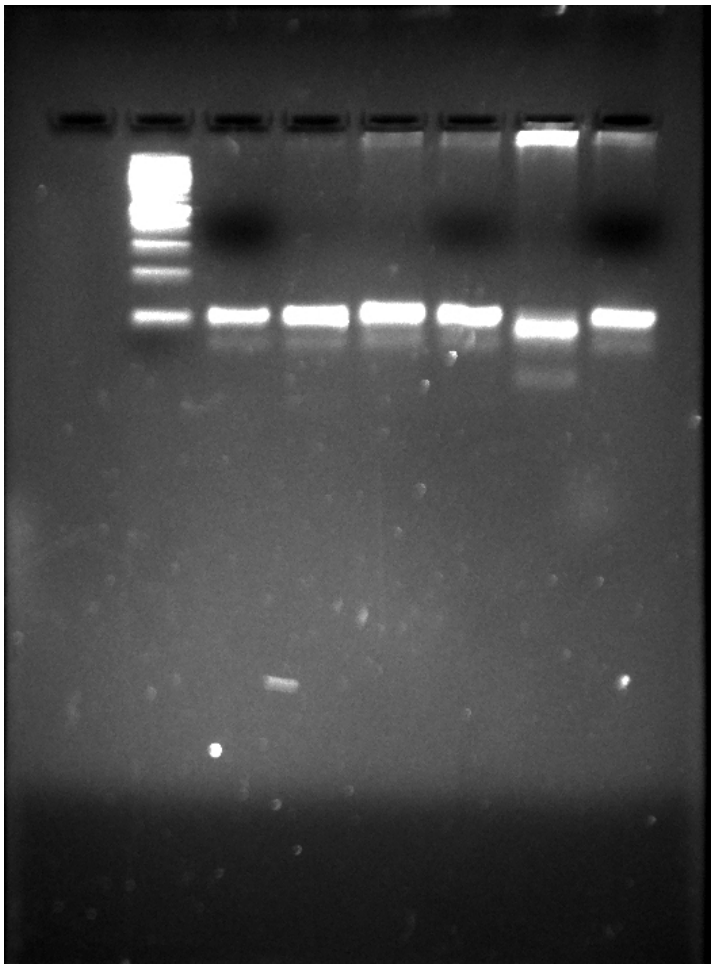

Figure 10: Allele bands are depicted in the gel image of Asthma Samples.

Lane 1 : Empty
Lane 2 : 100bp ladder
Lane 3 : Asthma sample 1with GG genotype
Lane 4 : Asthma sample 2 with GG genotype
Lane 5 : Asthma sample 3 with GG genotype
Lane 6 : Asthma sample 4 with GG genotype
Lane 7 : Asthma sample 5 with AA genotype
Lane 8 : Asthma sample 6 with GG genotype

Clearly it is seen that asthma samples loaded in well number 3,4,5,6 and 8 are homozygous for GG as only one band of 105bp is seen with faded primer dimers. But in well no.7 a homozygous allele AA can be seen as two bands of 80bp(Slightly down than the 100bp ladder) and 25bp can be seen.

CALCULATIONS

Total number of control samples : 93

Samples with GG allele : 85

Samples with AA allele : 7

Samples with AG allele : 1

Based on this result, we calculate the frequency of occurrence of each G and A allele in Control samples in North Indian population. These calculations are done using Hardy-Weinberg Equation which follows the basic principle that p+q=1

ALLELE	GG	AG	AA	G allele	A allele
FREQUENCY	85	1	7	0.919	0.081

Table 2: Allele Frequency of Control samples

Calculation of frequency of occurence :

TOTAL = 186

G=85+85+1=171

171/186 *100 = 0.919

A=1+7+7=15

15/186*100=0.081

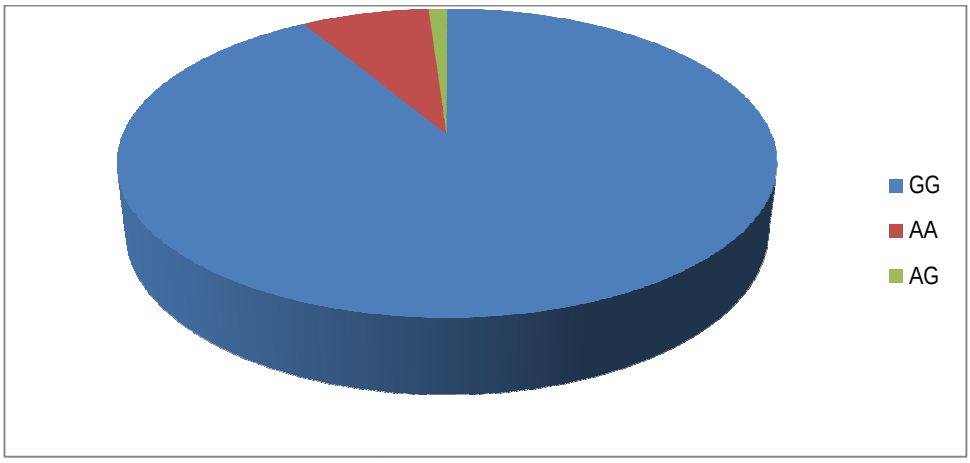

Figure 11 : Representation of Control allele frequency.

Same can be done for Asthma Samples :

Total number of Asthma Samples : 94

Samples with GG allele : 78

Samples with AA allele : 14

Samples with AG allele : 2

ALLELE	GG allele	AG allele	AA allele	G allele	A allele
FREQUENCY	78	2	14	0.84	0.16

Table 3: Allele frequency of Asthma samples.

Calculation of frequency of occurence :

TOTAL = 188

G=78+78+2=158

158/188 *100 = .84

A=14+14+2=30

30/188*100=0.16

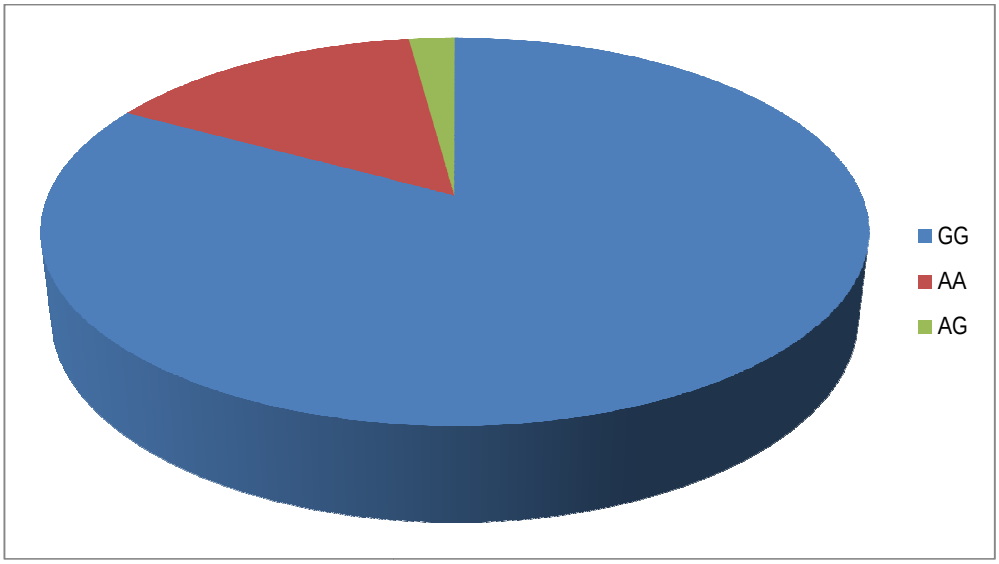

Figure 12: Representation of Asthma allele frequency.

Odds ratio

Cases with positive (bad) outcome

Number in exposed group:	158	*a*
Number in control group:	171	*c*

Cases with negative (good) outcome

Number in exposed group:	30	*b*
Number in control group:	15	*d*

Test

Results

Odds ratio	0.4620
95 % CI:	0.2396 to 0.8907
z statistic	2.306
Significance level	P = 0.0211

Figure 13 : Odds ratio results

DISCUSSION

Odds Ratio

An odds ratio is a relative measure of effect, which allows to determine association of the Polymorhism and disease by comparing presence of polymorphism along with disease (asthmatic group) and presence of poymorhism in absence of disease (control group). If the OR is < 1 then the polymorphism is associated with occurence of the disease. In our study, OR value is 0.4620 which means SNP 381 A/G might be associated with occurrence of Asthma.

The confidence interval indicates the level of uncertainty around the measure of effect which in this case is expressed as an OR. If the confidence interval crosses 1, it implies that there is no significant difference between the results obtained from both group of samples (control and asthmatic). 95% CI in our study came out to be 0.2396 to 0.8907 which means that there is no significant difference between the results obtained from control and asthmatic samples. $P < 0.05$ indicates a statistically significant difference between group while $P>0.05$ indicates there is not a statistically significant difference between groups. P-value for our study is 0.0211 which again indicates there is a significant difference in results. Thus, our results indicate that there is a statistical significance difference in between both the samples but no association of this SNP in asthma.

CHAPTER-5

CONCLUSION

To the best of our knowledge, this is the first epidemiological Study in India emphasizing the role of MMP-8 -381A/G promoter single nucleotide polymorphism (SNP) in the development of asthma. It is a well-researched fact that MMP8 promoter polymorphism has been associated with several diseases like asthma, arthritis, multiple sclerosis etc. MMP family of zinc-dependent proteolytic enzymes regulate physiological and pathophysiological events in development, injury and repair, such as morphogenesis, vasculogenesis, remodelling of the extracellular matrix, cell migration, cleavage of cytokines, and activation of mediators and defensins.

This study was essentially taken up to evaluate the rate and frequency of occurrence of SNP (A/G) at the -381 locus of MMP-8 gene in the residents of Northern India. This would help us determine how asthma is caused by the polymorphism in MMP-8 promoter gene. Based on our results, I may conclude that there is a statistical significance difference in between both the samples and no association of this SNP in asthma.

REFERENCES

1. Amrendra Kumar and Balaram Ghosh. **Genetics of asthma: a molecular biologist perspective,** Clinical and Molecular Allergy 2009, **7**:7

2. F.E. Hargreave and K. Parameswaran**. Asthma, COPD and bronchitis are just components of airway disease,** Eur Respir J 2006; 28: 264–267

3. Mark A. Birrell, Sissie Wong, Abdel Dekkak, Jorge De Alba, Saleem Haj-Yahia, and Maria G. Belvisi. **Role of Matrix Metalloproteinases in the Inflammatory Response in Human Airway Cell-Based Assays and in Rodent Models of Airway Disease,** The Journal of Pharmacology and Experimental Therapeutics 2006 ; 318:741–750

4. Tanya Tacheva, Dimo Dimov, Tatyana Vlaykova. **Biological Role Of Collagenases in Bronchial Asthma,** Science and technologies 2013 ; 3: 1-6

5. S. Lo¨ffek, O. Schilling and C-W. Franzke . **Biological role of matrix metalloproteinases: a critical balance.** Eur Respir J 2011; 38: 191–208

6. Rodrigo Athanazio**. Airway disease: similarities and differences between asthma, COPD and bronchiectasis .** CLINICS 2012;67(11):1335-1343

7. Hongyan Wang, Samuel Parry, George Macones, Mary D. Sammel, Pedro E. Ferrand, Helena Kuivaniemi, Gerard Tromp, Indrani Halder, Mark D. Shriver, Roberto Romero and Jerome F. Strauss . **Functionally significant SNP MMP8 promoter haplotypes and preterm premature rupture of membranes (PPROM).** Human Molecular Genetics, 2004, Vol. 13, No. 21 2659–2669

8. Loubna Akhabir and Andrew J. Sanford. **Genome-wide association studies for discovery of genes involved in asthma.** Respirology (2011) 16, 396–406

9. K. Kostamo, T. Sorsa, M. Leino,T. Tervahartiala, H. Alenius, M. Richardson, E. Toskala. **In vivo relationship between collagenase-2 and interleukin-8 but not tumour necrosis factor-a in chronic rhinosinusitis with nasal polyposis.** Allergy 2005: 60: 1275–1279

10. Terufumi Shimoda, Yasushi Obase, Reiko Kishikawa, and Tomoaki Iwanaga, **Association of matrix metalloproteinase 8 genetic polymorphisms with bronchial asthma in a Japanese population.** Allergy Rhinol , 2013 : Vol. 4, No. 3

11. Jiwon Lee, Hye-Ryoun Kim, Joo-Won Min, Jong Sun Park, Sang-Man Jin, Sung Koo Han,Young-Soo Shim, Jae-Joon Yim. **Lack of Association between Matrix**

Metalloproteinase 8 Promoter Polymorphism and Bronchiectasis in Koreans. J Korean Med Sci 2007; 22: 667-71

12. J. Mu¨ller-Quernheim. **MMPs are regulatory enzymes in pathways of inflammatory disorders, tissue injury, malignancies and remodelling of the lung.** Eur Respir J 2011; 38: 12–14

13. Monteseirín . **Neutrophils and Asthma.** J Investig Allergol Clin Immunol 2009; Vol. 19(5): 340-354.

14. John V. Fahy. **Eosinophilic and Neutrophilic Inflammation in Asthma:Insights from Clinical Studies.** Proceedings of the American Thoracic 2009 ;Vol 6 :109-121

15. Rajeshwar P. Verma and Corwin Hansch. **Matrix metalloproteinases (MMPs): Chemical–biological functions and (Q)SARs .** Bioorganic & Medicinal Chemistry 2007; 2223–2268

16. Alexander Bulinski, Oleg Butkovsky, Victor Sadovnichy, Alexey Shashkin, Pavel Yaskov, Alexander Balatskiy, Larisa Samokhodskaya, Vsevolod Tkachuk. **Statistical Methods of SNP Data Analysis and Applications.** Open Journal of Statistics, 2012 ; 2 ;73-87.

17. Pratikshya Pradhan-Palikhe. **Matrix Metalloproteinase-8 as a Diagnostic tool for the inflammatory and malignant diseases.** Acta Univ. Oul. D 1111, 2011 ; 1-96.