Role of genetic variants in Autophagy related gene 7 (Atg 7)

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CERTIFICATE

This is to certify that the work titled, "**ROLE OF GENETIC VARIANTS IN AUTOPHAGY RELATED GENE 7 (ATG 7)**" by Tapish Dogra and Sudip Mukherjee in partial fulfilment for the award of degree of 5 year Dual degree programme B.tech- M.Tech in Biotechnology of Jaypee University of Information Technology has been carried out 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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27th May 2015

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Tapish Dogra

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Summary

Asthma is a chronic inflammatory disease mainly recognised by the airway inflammation affecting around 300 million people worldwide. It affects all age groups but mostly starts in the childhood and the frequency and severity of the recurrent attacks of breathlessness and wheezing vary from person to person due to inflammation of air passage in the lungs.

Autophagy is an evolutionarily conserved pathway which involves the sequestration of the cytoplasmic contents by the cell so as to remain in homeostasis during the periods of starvation.

Autophagy, the sequestration pathway has been directly linked to asthma, by various researchers.Polymorphism in Autophagy related gene 5 (Atg5) has been shown to be directly linked to asthma in various populations.

In this study, we try to find association of Autophagy related gene 7 (Atg7) to asthma, in north Indian population. SNP rs35807939 (G/A) was studied in this study.

Tapish Dogra

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27th May 2015

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macrophages also release transforming growth factor-β (TGFβ), which stimulates fibroblast proliferation, resulting in fibrosis in the small airways.

Figure 4 Interactions between TH1 and TH2 cells in asthma. The transcription factor GATA3 (GATA-binding protein 3) is regulated by interleukin-4 (IL-4) via STAT6 (signal transducer and activator of transcription 6) and regulates the expression of IL-4, IL-5, IL-9 and IL-13 from T helper 2 (TH2) cells and also inhibits the expression of T-bet via inhibition of STAT4. IL-33 enhances the actions of GATA3. T-bet regulates T helper 1 (TH1)-cell secretion of IL-2 and interferon-γ (IFNγ) and also has an inhibitory action on GATA3. T-bet is regulated by IL-12 via STAT4 and by IL-27 via STAT1. This demonstrates the complex interplay of cytokines and transcription factors in asthma.

Figure 5 Schematic showing the action of restriction enzymes on the double stranded DNA.

Figure 6 Schematic showing the action of blunt end cutters and sticky end cutters.

Figure 7 the digestion pattern of restriction enzyme Pf1M1.

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Chapter-1

Introduction

Asthma

Asthma is a chronic inflammatory disorder of the airways estimated to affect 300 million individuals worldwide. People of all ages in countries throughout the world are affected by this chronic airway disorder that can place severe limits on daily life and is sometimes fatal.

Asthma attacks all age groups but mostly starts in the childhood and the frequency and severity of the recurrent attacks of breathlessness and wheezing vary from person to person. This condition is due to the inflammation of the air passages in the lungs and affects the sensitivity of the nerve endings in the airways so they become easily irritated. In an episode, the lining of the passages swell causing the airways to narrow and reducing the flow of air in and out of the lungs.

The strongest risk factors for developing asthma are exposure to indoor allergens and a family history of asthma or allergy. Exposure to tobacco smoke and exposure to chemical irritants in the workplace are additional risk factors. Climatic changes such as a thunderstorm which breaks the pollen grains, releasing the starch granules can also trigger attacks.

According to the WHO report, In India, rough estimates indicate a prevalence of 10 – 15% in 5-11 year old children. India has an estimated 15-20 million asthmatics. According to the same report, the world wide deaths have reached over 180,000 annually.

The human and economic burden associated with this condition is severe. World-wide, the economic costs associated with asthma are estimated to exceed those of TB and HIV/AIDS combined. For instance, in US the annual asthma care costs exceeds US$6 billion.

There are various types of asthma such as allergic asthma (because of exposure to allergen), cough variant asthma (dry/non-productive cough), occupational asthma (exposure to a chemical at workplace) and nocturnal asthma (continuous coughing and wheezing during nights) [1][2][3].

Autophagy

Autophagy is an evolutionarily conserved pathway which involves the sequestration of the cytoplasmic contents by the cell so as to remain in homeostasis during the periods of starvation. The autophagy process can be divided into three main steps, the initiation of isolation membrane,

elongation of the isolation membrane and formation of autophagosomes and autophagosome-lysosome fusion and degradation [4].

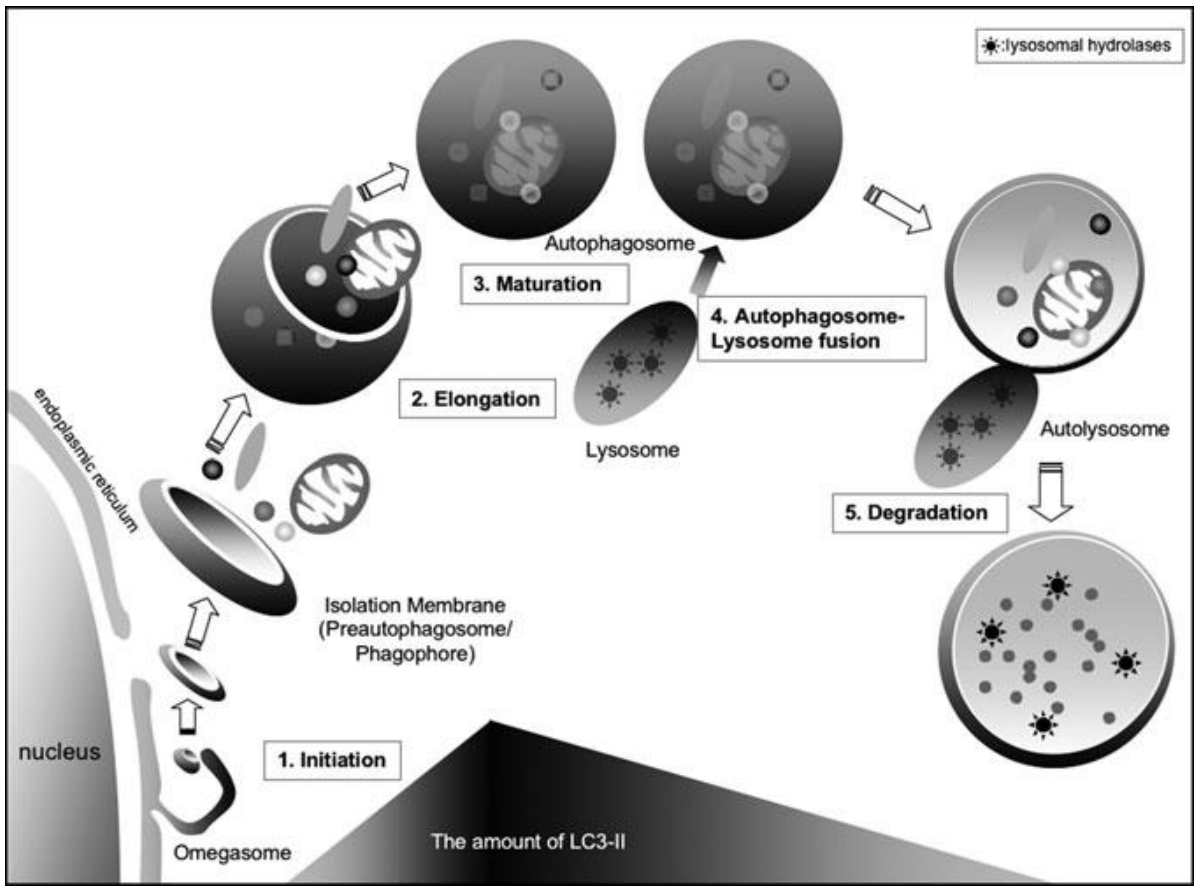


Figure 1

Autophagy is initiated by the formation of crescent-shaped autophagic isolation membranes (phagophores) that expand to form a double membrane vesicle, the autophagosome. The initiation of autophagy activation involves the formation of beclin 1 complex and its associated proteins, such as vacuolar sorting protein 34 (VPS34), a class III phosphatidylinositol-3 kinase (PI-3K), UV irradiation resistance-associated gene (UVRAG) and Atg14L. In the elongation step, two ubiquitin-like conjugation cascades are proposed to play a key role in the extension of autophagic vesicle membranes. The Atg12-Atg5 conjugate interacts non-covalently with Atg16L and forms a large multimeric complex called the Atg16L complex, which acts as an E3-like enzyme. The Atg5/12/16 complex drives the lipidation and conjugation of LC3/Atg8 to the autophagosomal membrane in a reaction that requires Atg3/Atg7 complex. The lipidated form of LC3 (LC3-II) remains within the autophagosome lumen, but is removed from the autophagosome membrane after fusion with lysosomes; LC3-II is then degraded. Finally, the maturation of the autophagosome occurs through the fusion of the autophagic membrane with the late endosomal and lysosomal organelles. This results in the dissolution of the inner membrane of the autophagosome and the formation of the

autolysosome. The autophagosome/endosome maturation and degradation of endocytic captured material can be mediated through the interaction of the Beclin1-binding autophagic tumour suppressor, UVRAG, with the class C Vps complex [5].

Why study association of Atg7 with Asthma?

In a study conducted in Canada, to check the genetic evidence of autophagy in Asthma, double membraned autophagosomes where identified in the bronchial biopsys of patients with moderate asthma. These results suggest that autophagy does has a role in asthma [6].

In the similar study, an association was found in the Autophagy related gene 5 (Atg5) and asthma. Atg5 is a key gene involved in the autophagosome elongation and it also forms a complex with Atg12 and Atg17L1 and these complexes are found on the outer surface of the forming autophagosome. Atg7 is required in the formation of the Atg5-Atg12-Atg16L1 complex.

Thus, Atg7 is indespensible in the formataion and elongation of the autophagosome, thus a key in the autophagy process. Till date there has been no significant research associating Atg7 to asthma. Thus, this study has been undertaken so as to gain more knowledge about the evolutionary conserved autophagy pathway and a very severe disease Asthma.

Chapter-2

Review/ Background material

Asthma

The term 'Asthma' is derived from the Greek verb *aazein* meaning to pant, to exhale with the open mouth, sharp breath. The earliest use of the word asthma was done by Hippocrates in The *Corpus Hippocratium*, as a medical term, where he described spams linked to asthma likely to occur among tailors and metalworkers.

At the beginning of the 20th centuryasthma was recognised as a psychosomatic disease, it was even known as the holy seven psychosomatic illnesses. Psychoanalysts treated asthma patients for depression, though this psychiatric theory was eventually refuted and by 1960s asthma was recognised as an inflammatory disease, a physical condition.

Types of Asthma

1. Child onset Asthma

   This type of asthma begins during the childhood, as the child becomes sensitized to common allergens in the environment, most likely due to genetic reasons. An allergen can be any substance capable of triggering an immune reponse. An allergen varies between individuals, these can be animal proteins, fungi, pollen, house dust mites and certain kinds of dust. The airways of children are very sensitive to particular materials thus causing a severe asthmatic episode if exposed to certain amount of an allergen.

2. Adult onset Asthma

   When a person debvelops asthma after the age of 20 years, it is refered to as adult onset asthma. It is a lesser common phenomenon as compared to child onset asthma and affects woman more as compared to men. It can be triggered either by an allergen (more common asthma type) or by exposure to a particle or chemical (intrinsic asthma or non allergic adult onset asthma). Although 50% of adult onset asthma are because of allergies, a substantial proportion of it is caused by non allergens such as chemicals in certain plastics, metals, medications or wood dust.

3. Exercise induced Asthma

   When a person coughs, wheeze or feels out of breath during or after exercise, this is refered to as exercise induced asthma. A person feels difficulty in breathing during exercise because of the

inflammation of the bronchial tubes and extra mucus. People with exercise induced asthma do not have symptoms while they are not physically exerting themselves.

4. Cough induced Asthma

   It is one of the most difficult type of Asthma to be diagnosed, as there is only coughing without any other symptoms. The coughing occurs at any time of the day and it also includes hay fever or sinus disease.

5. Occupational Asthma

   When a person triggers an asthma attack by something in the work place, it is referd to as occupational asthma. The substance triggering an episode can be a chemical, vapors, gases, smoke, dust, fumes,or even viruses, molds, pollen or even temperature.

6. Nocturnal Asthma

   When a person suffers form an asthma episode between midnight and 8 AM, it is refered to as Nocturnal or nighttime asthma. This episode may be triggered by allergens in the home, and it is known by wheezing or short breath when lying down and it only gets noticed once the person gets awoken by shortness of breath.

7. Steroid resistance Asthma

   When a person does not respond to the regular steroid therapy it is refered to as Steroid resistance asthma. These people have higher levels of immune activation in their airways leading to an increased airway inflammation [1].

Causes Of Asthma

Asthma is the most common chronic disorder, it effects all age groups from children to adults. The risk of asthma starts from early age, studies state that babies born by Caesarean sections have a 20% increase in asthma prevalence compared to those born by vaginal birth. Young boys are more likely to develop asthma than young girls, but this trend reverses during adulthood.

Allergens contribute the maximum towards asthma, the allergic reactions triggered by antibodies in the blood lead to the airway inflammation that is associated with asthma. Tobacco smoke is also a leading cause of asthma and deaths related to asthma [7].

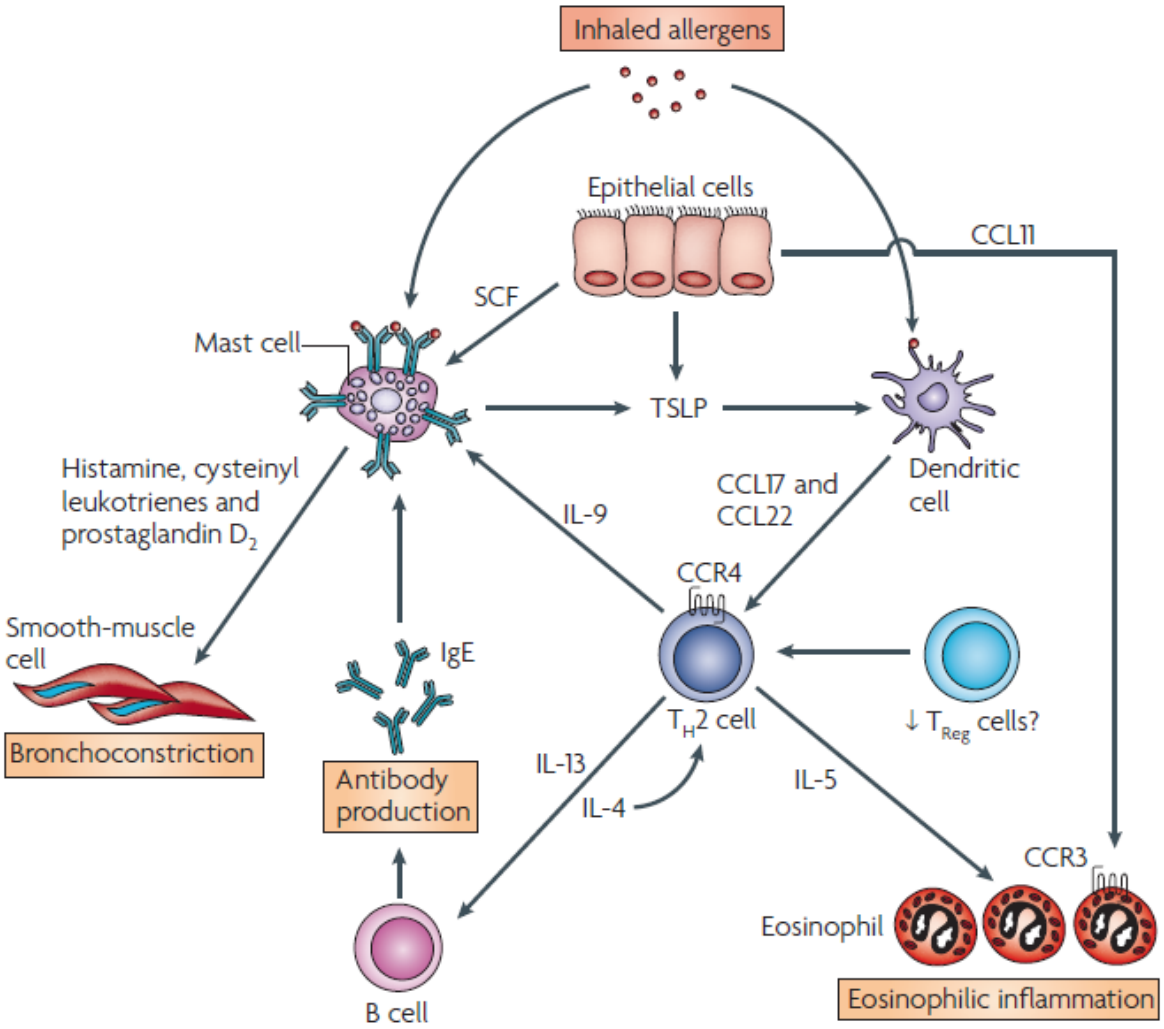


Figure 2

The environmental factors are also a great contributor to asthma, people often get exposed to certain allergens within household. People cooking with gas are more prone to falling into asthma. Some individuals have been shown susceptible to pollution, sulphur dioxide, nitrogen oxide, cold temperatures and high humidity, inappropriate exposure triggering an asthmatic episode. Stress is also a cause of asthma, this can be only explained by habbits taken over because of stress (smoking) [8].

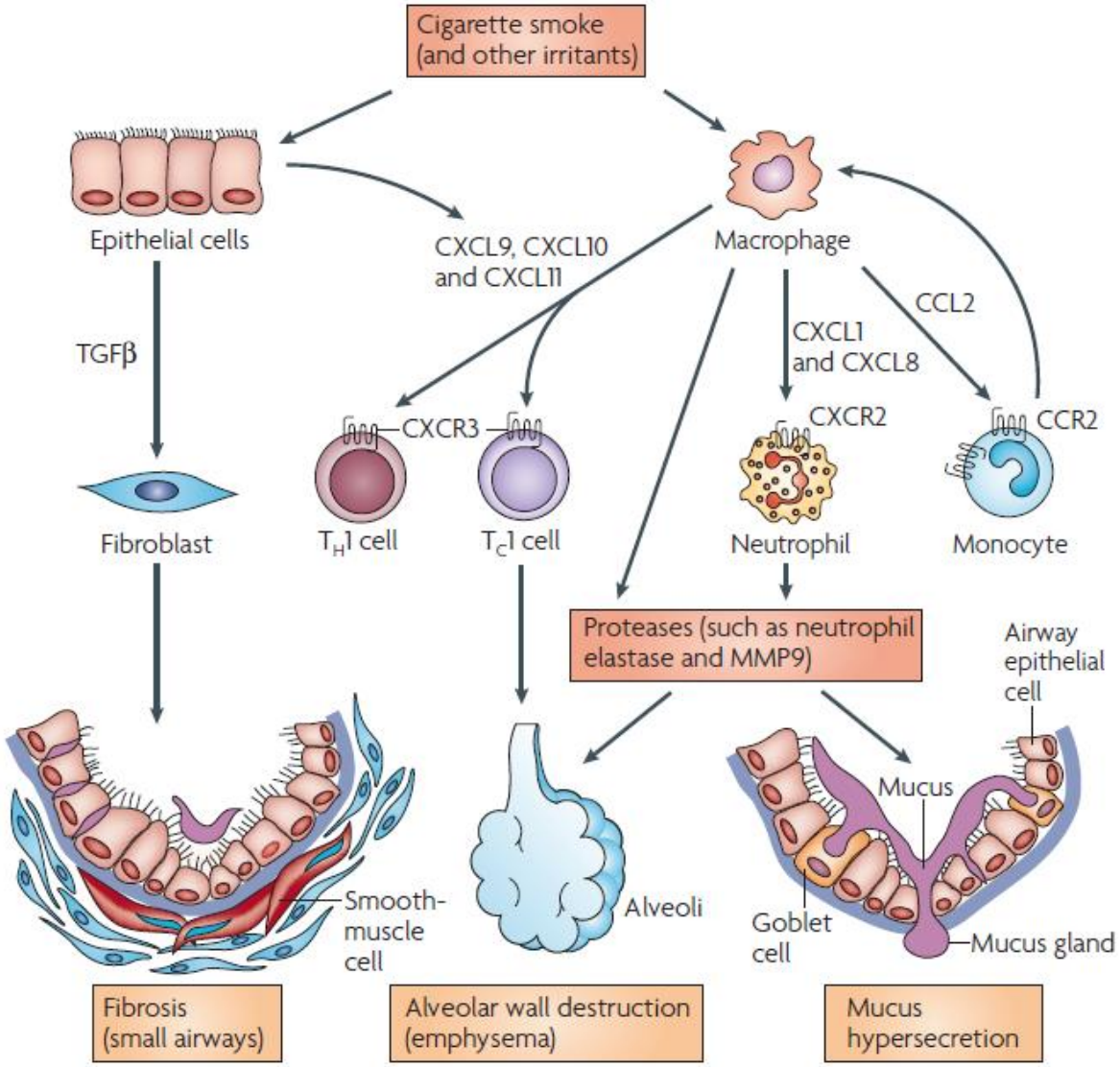


Figure 3

Genes play an equal role in causng asthma. It is estimated that over 100 genes are linked to asthma. Genes linked to asthma manage the immune system and inflammation. About three fifths of all asthma cases are hereditary and the Centre for Disease Control (CDC) has reported that a person is six times at risk of asthma if any of the parent has asthma. Genetics interacting with environmental factors is also associated with asthma, for instance the exposure to bacterial endotoxin and having the genetic trait CD 14 (SNP C- 159T) is associated with asthma [6].

Symptoms

Common symptoms of Asthma include wheezing, coughing, breathing difficulty, tightness in chest, breathing problems during night and the mentioned symptoms on exposure to an allergen.

Diagnosis Of Asthma

Asthma is diagnosed based on three components. These are the medical history, a physical examination and results from breathing tests. If a person is diagnosed with asthma, in that case it is classified as intermittent, mild, moderate or severe.

A detailed family medical history of asthma and allergies is the first component. An individuals personal history of allergies is important is diagnosis.

The physical examination mainly focuses on the upper respiratory tract, chest and skin. Although physical symptoms are not always present in asthma sufferers, and it is also possible to have asthma without presenting any physical symptoms. A high pitched whistling sound while exhaling or wheezing means obstructed airway meaning aasthma.

The breathing tests or the lung function tests help in measuring the severity of asthma. A spirometer is used to measure how much air one can breathe in and out and the quickness in which a person can blow air out. The spirometer displays two key measurements, Forced vital capacity (the maximum amount of air one can inhale and exhale) and Forced expiratory volume (the maximum amount of air exhaled in one second). These are then measured with the standards based on the person's age and measurements below the standards reflect the obstruction in the airways.

A new test kit has been developed which measures the amounts of a chemical marker, nitric oxide whose levels are directly linked to the severity of asthma.

Treatment

Asthma which is a chronic long term disease, as such can not be treated, but is controlled. The best way to tackle with asthma is to control the symptoms and be cautious and get routine check ups.

Medication for asthma is broadly classified as long term control medicine or quick relief medicines or rescue medicines. These medications are mostly administered using inhalers.

Long term control medicines are designed to prevent asthma symptoms and are taken daily. Corticosteroids are the most effective long term control medicine at relieving airway inflammation and swelling.

Quick relief medicines are administered to relieve the asthma symptoms when they occur. Inhaled short acting B2 agonists bronchodilators are the most commonly used recue medicines as they relax tight muscles around the airways, thus allowing air to flow through them.

Asthma medicines are mainly classified as bronchodialators and anti-inflammatories. Bronchodialators are used during an asthma attack. They relax the muscles in the air tubes, forcing them to open up enabling the person to breath. These also help clear mucus from the lungs allowing it to move freely and easily get coughed out. Anti-inflammatories on the other hand prevent asthma attacks by keeping the air tubes open all the time, they are designed to reduce swelling in the air tubes and decrease the amounts of mucus.

Immunological responses in Asthma

Asthma is mainly associated with airway inflammation, which leads to the feeling of breathlessness. Adaptive immunity has a crucial role in airway inflammation, it is an antigen dependent response where T helper cells (CD4+) are the main executors. Various cytokines such as INF-γ, TNF-β, IL-4, IL-5,IL-10 and IL-13 are involved in the response. Results from study on murine subjects states that asthma arises with the imbalance in the Th1 and Th2 pathways and the over driven Th2 inflammation leads to airway inflammation and asthma [9].

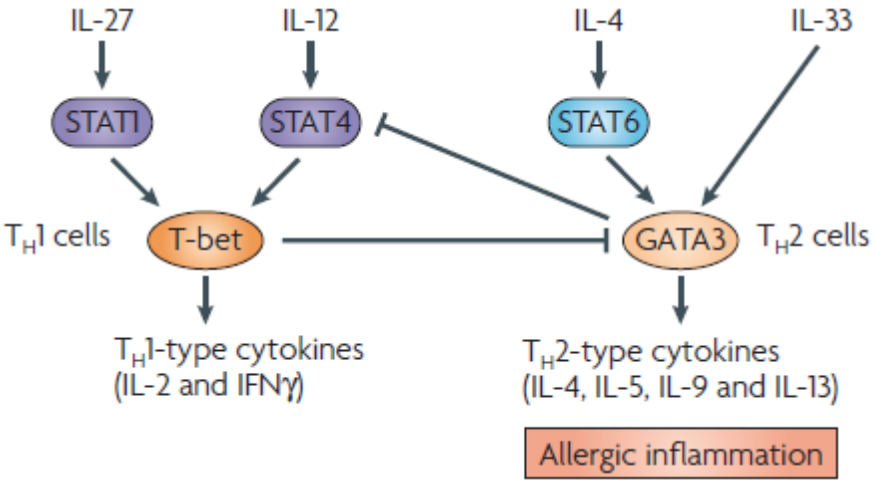


Figure 4

Autophagy is an evolutionary conserved pathway involving the sequestration of cytoplasmic components during starvation. The execution of autophagy is mediated by two key conjugation systems, ATG5-ATG12 conjugation and the lipidation of LC3 protein.

ATG12 is activated by covalent linkage to Atg7 followed by Atg10 and then convalently likned to ATG5. Then Atg16 binds to the complex and this Atg12-Atg5-Atg16 complex is found in the autophagy isolation membrane [10]. The terminal amino acid at the carbonyl terminus of microbulue associated protein light chain 3 (LC3/ Atg8) is cleaved by the cysteine protease Atg4. This cleaved LC3 is then linked to Atg7 followed by Atg3, and then finally to phosphatidylethanolamine. These two pathways are crucial in autophagy and are used as markers for autophagy.

Genetic susceptibility to asthma has been long recognised and with all the latest technology and equipment researchers have found several gene which are associated to asthma. Gene polymorphism or single nucleotide polymorphism (SNP) have been found which link the genes directly to asthma. In a study, conducted in Canada (the SLSJ study) , single nucleotide polymorphism was found in Atg5 gene (SNP- rs12212740) directly linking it to asthma. Electron microscopy analysis of bronchial tissues of asthmatic patients as well as control, show that double membrane autophagosomes present in the fibroblast and epithelial cells in asthmatic patients compared to very less or none in controls [6].

In another study done on pediatric cohort, analysis revealed that allele (A) of Atg5 rs12201458 was associated with a decreased risk of asthma while another allele (G) of Atg5 rs510432 to be associated with increased risk of asthma. It was also reported using expression studies that the G allele enhance the promoter activity by 23% compared to the other allele A. It was also found that the variation in the alleles affects the gene transcription, as two transcription factors, STAT1 and C-Fos have their binding site in the region where the polymorphism is found [11].

Chapter-3

Materials and Methods

Sample collection:

A total of 93 asthmatic patients and 87 controls were recruited into the study from northern India. Blood was drawn by the method of venipuncture from left or right arm with the use of sterile disposable needle with the help of doctors present at the camp.

The blood samples were immediately mixed with 15µl Na_2EDTA (2.5mM concentration) to prevent coagulation (anti-coagulating agent) and transferred to -20°C through providing cold condition or ice cubes.

Consent from all the individuals was collected.

Isolation of Genomic DNA

Recent research advances in genomic disorders have necessitated the collection of large amounts of good quality DNA that needs to be obtained from different sample sources. DNA typing is currently the most validated method for the personal identification of human bodily fluid stains found at crime scenes. In a wide variety of genetic studies, the commonly used method is to obtain genomic DNA from nucleated cells of peripheral blood; as a result of the invasiveness of this approach, it may be difficult to obtain samples from the study subjects. DNA isolation is a process of purification of genomic DNA from blood sample using a combination of physical and chemical methods. The first isolation of DNA was done in 1869 by Friedrich Miescher. Currently it's the routine procedure in molecular biology or forensic analysis [12] [13][14].

Reagents Required:

Tris (hydroxymethyl) aminomethane-chloride (Tris-Cl)(1M;pH 8.0): 75ml of sterile MQ water was used to dissolve 12.11g of Tris base pH was set to 8.0 with 1N HCL and final volume was made 100ml with sterile MQ water. The solution was filtered through whatmann filter paper and stored in a reagent bottle.

Tris-Cl (1M;pH 7.3): As prepared above, 12.11g of Tris base was dissolved in 75ml of sterile MQ water and pH was set to 7.3 with 1N HCL. The final volume of the solution was made 100ml with sterile MQ water. The solution was filtered and stored in a sterile tight screw capped reagent bottle.

Ammonium Chloride (NH_4Cl) (1M): Ammonium chloride (5.35g) was dissolved in 80 ml of sterile MQ water and the final volume of the solution was raised to 100ml.

Di-sodium ethylene di-amine tetra acetate (Na_2EDTA) (0.5M; pH 8.0): added 18.61g of EDTA salt to 50ml of MQ water in a 250 ml flask and placed it one magnetic stirrer. Simultaneously, supplemented the solution with 10M NaOH drop-wise, until the pH reached at 8. Allowed the salt to dissolve and then made the final volume of the solution 100ml with MQ water.

RBC lysis buffer (10nM Tris, pH 8.0; EDTA, 1mM NH_4Cl2, 125mM; pH 8.0): 10ml Tris (1M; pH 8), 2ml of EDTA (0.5M) and 125ml NH_4Cl (1M) were mixed in MQ water to obtain a final volume of 1000ml RBC lysis buffer.

Tris-EDTA (TE) buffer (Tris, 10mM; EDTA, 1mM; pH 7.3): As above, 10ml Tris-Cl (1M; pH 7.3) and 2ml of EDTA (0.5M) were mixed in MQ water to obtain a final volume of 1000ml.

Tris-EDTA (TE) buffer (Tris, 10mM; EDTA, 1mM; pH 8.0): TE buffer was prepared by mixing 10ml Tris-Cl (1M) and 2ml of EDTA (0.5M) in 700ml MQ water and the final volume was made 1000ml.

Sodium Dodecyl Sulphate (SDS) 10%: Dissolve 10g of SDS salt in 70ml of warm MQ water and the final volume was made 100ml.

Ammonium acetate (7.5M): Dissolve 28.9g of ammonium acetate salt in 20ml of MQ water the final volume is adjusted to 50ml.

Chilled dehydrate ethyl alcohol: Undiluted dehydrated ethyl alcohol stored in -20°C deep freezer.

Ethanol (70%): 70ml of dehydrated ethanol was added to 30ml of sterile MQ water to obtain a final volume of 100ml.

Protocol for isolation of genomic DNA [12]:

a) To 400µl of blood sample, added RBC lysis buffer (3 times the volume of blood sample taken) and kept for incubation on a rocker, to permit perpetual shaking at room temperature until the RBCs completely lysed.
b) Centrifuged the solution at 13,000 rpm for 1min to obtain a creamish white WBC pellet.
c) The supernatant was discarded and the WBC pellet was thoroughly suspended in 400µl of TE buffer (pH 8.0) using a vortexing machine. Thereafter 22µl of 10% SDS solution was added to the above solution and the mixture was incubated at 56°C for 30 minutes on a water bath.
d) Added 160µl of 7.5M ammonium acetate and mixed vigorously for about (minutes per sample, on a vortexer). Centrifuge the mixture at 13,000 rpm at RT for 15minutes, resulting in separation of precipitated proteins as pellets.
e) The supernatant was transformed to a fresh sterile micro-centrifuge tube. To this added chilled absolute ethyl alcohol (2x the volume of clear supernatant) this tube was gently rocked a couple of times to allow precipitation of genomic DNA.
f) The genomic DNA precipitates were centrifuge at 13,000 rpm for 10 minutes to pellet at the bottom of the tube. The lattes were subsequently washed in 150µl of 70% ethanol & air dried at RT for about 10-15minutes.
g) 40µl of TE buffer (7.3) was used to dissolve the dried DNA pellet by incubating at 65°C for 10 minutes. The dissolve RNA was stored at -20°C till further use.

Agarose Gel Electrophoresis (1.2% PCR amplification and 2% RD):

Agarose gel electrophoresis is the easiest and most popular way of separating and analyzing DNA. Here DNA molecules are separated on the basis of charge by applying an electric field to the electrophoretic apparatus. Shorter molecules migrate more easily and move faster than longer than molecules through the pores of the gel and this process is called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of ethidium bromide.

Agarose is a polysaccharide obtained from the red algae *porphyra umbilicalis*. Its systematic name is (14)-3, 6-anhydro-a-L-galactopyranosyl-β-D-galactopyranan. Agarose makes an inter matrix. Most agarose gels are made between 0.7% and 2% of agarose. A 0.7%gel will show good separation for larger DNA fragments (5-10kb) and a 2% gel will show good resolution for small fragments with size range of (0.1-1kb). Low percentage gels are very weak but high percentage gels are usually brittle and do not set evenly. The volume of agarose required for a minimal gel preparation is 50-70ml but for PCR amplification cheek low volume gel can be prepared.

Factors Affecting the Movement of DNA:

a) Voltage Applied: The migration rate of the liner DNA fragments through agarose gel is proportional to the voltage applied to the system. As voltage increase, the speed of DNA also increases but voltage should be limited because it heats and finally causes the gel to melt.
b) Ethidium Bromide (EtBr): It is an intercalating agent which intercalates between nucleic acid bases and allows the convenient detection of DNA fragments in gel. When exposed to UV light, it will fluoresce with an orange colour. When the gel is not cooled or not to hot in that condition EtBr should be added to the gel.
c) Buffers: several different buffers have been recommended for electrophoresis of DNA. The most commonly used buffers are Tris-acetate-EDTA (TAE) and Tris-borate-EDTA.

Preparation of Reagents:

Tris-Acetic acid-EDTA (TAE) buffer (50x): 242g of Tris base was dissolved in 500ml of MQ water. This solution was than supplemented with 57.1 ml of glacial acetic acid followed by 100ml of same was filtered and diluted to a working concentration of 1X before use.

Agarose: 1.2% and 2% agarose gel was prepared in 1x TAE buffer for qualitative analysis of genomic DNA samples.

Ethidium bromide (10mg/ml): 10mg of ethidium bromide was added to 1ml MQ water and dissolve by gentle mixing before storing in an amber bottle at room temperature.

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.

Pouring a Standard 1% Agarose Gel: Agarose gel are commonly used in concentration of 0.7% to 2% depending on the size of bands needed to be separated. Simply adjust the amount of starting agarose to g/100ml TAE (i.e.2g/100ml =2% gel).

A) Measured 1g of agarose.
B) Poured agarose powder into microwave flask along with 100ml of 1x TAE.
C) Microwave for 2minutes (until the agarose was completely dissolved and there was a nice rolling boil).
D) Let agarose solution cool down for 5minutes.
E) Added ethidium bromide (EtBr) to the final concentration of approximately 0.5-0.7µg/ml (usually about 5-7µl of lab stock solution per 100ml gel). EtBr binds to DNA and allows visualizing the DNA under UV light.
F) Poured the agarose into a gel tray with the well comb in place.
G) Placed newly poured gel at 4°C for 10-15minutes or let it solidify in room temperature for 20-30minutes.

Loading sample and Running an Agarose Gel:

1) Added loading buffer to each of digest samples.
2) Once solidified, placed the agarose gel into the gel matrix (electrophoresis unit).
3) Filled gel matrix with 1x TAE until the gel fully marched.
4) Carefully load the molecular weight ladder into the first lane of the gel and thus undigested and samples.
5) Connect the plugs and ran the gel at 100V until the dye cross the well and thus increased the voltage to 150V for faster movement of genomic DNA fragment.
6) When the dye moved to the middle of the gel carefully observe the gel image under gel doc system.
7) Save the pictures and note down the genotype.

Polymerase Chain Reaction (PCR)

Kary Mulis invented PCR technique in 1985 while working as a chemist at the Cetus Corporation, a biothechnology firm in Emeryville, California. The procedure requires placing a small amount of the DNA containg the desire gene into tube. A large batch of loose nucliotides, which link into extract copies of the original gene, is also added to the tube. A pair of synthesized short DNA segments, that match segments on each side of the desired gene is added. These "primers" find the right portion of DNA, and serve as starting points for DNA coyping. When the enzyme Thermus aquaticus is added, the loose nucleotides look into a DNA sequence dictated by the sequence of that target gene located between two points.

When Mulis' completed PCR technique manually it was slow and labor-investive. Therefor, Cetus scientists began looking for ways to automate the process. Before the discovery of the thermostable Taq enzyme, scientists needed to add fresh enzyme to each cycle. The first thermocycling machine, "Mr. Cycle" was developed by Cetus Engineers to address that need to add fresh enzyme to each test tube after the heating and cooling process and the purification of the Taq polymerase resulted in the need for machine to cycle more rapidly among different temperatures. In 1985 Cetus and Perkin-Elmer corporation jointly introduced the DNA Themal cycler.

The polymerase chain reaction is a biochemical technology in molecular biology to amplify a single or a few nanogram concentration of DNA across several orders of magnitude, generating thousand to millions of copies of a particular DNA sequence.[15]

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragment) containing sequence complementary to the target region along with a DNA polymerase (after which the method is named) is key components to enable selective and repeated amplification. As PCR progress, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponently amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

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 in a DNA double helix at a high temperature steps. The first step is to physically separate the strands in a DNA double helix at a

high temperature, the process called DNA melting. The second step is to lower the temperature. Then 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.

Primer sequences used for amplification of rs35807939**:**

- Forward: 5'-AAGCCCTCAGTAGTGGGTAGA-3'
- Reverse: 5'-AGTCGGTCTGCCTCAGTGTA-3'

Table 1: Reaction mixture (1x PCR amplification reaction):

Component	Volume (µl)
i-Taq	0.125
Buffer	1
dNTP	0.25
Forward Primer	0.2
Reverse Primer	0.2
MQ water	9.75
DNA	1

Table 2: Condition for PCR:

Component	Volume (µl)
i-Taq	0.125
Buffer	1
dNTP	0.25
Forward Primer	0.2
Reverse Primer	0.2
MQ water	9.75
DNA	1

Restriction Digestion:

Restriction enzymes are Nucleases which can cleave the sugar-phosphate backbone of DNA, found in bacteria. As they cut within the molecule, they are commonly called restriction endonucleases. They specifically cleave the nucleic acids at specific nucleotide sequence called Restriction sites to generate a set of smaller fragments.

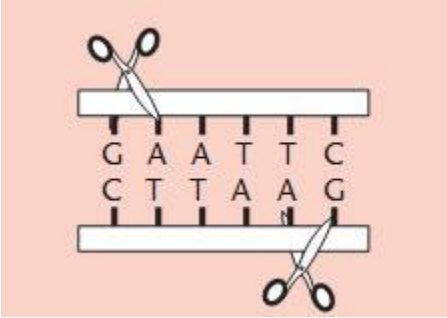


Figure 5

Restriction enzymes form part of the restriction-modification system of bacterial cells that provides protection against invasion of the cell by foreign DNA – especially bacteriophage DNA. But the cells own DNA is not cleaved by these Restriction enzymes. This self-protection is achieved by the help of the specific DNA methyltransferase enzyme which will methylates the specific DNA sequence for its respective restriction enzymes by transferring methyl groups to adenine or cytosine residues to produce N6-methyladenine or 5-methylcytosine. An interesting feature of restriction endonuclease is that they commonly recognize recognition sequences that are mostly palindromes - they shows the same forward (5' to 3' on the top strand) and backward (5' to 3' on the bottom strand) sequences. In other words, they are nucleotide sequences or complimentary strands that read the same in opposite direction.[16]

In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzyme and the resulting restriction fragments are separated according to their lengths by gel electrophoresis. RFLP analysis was in important tool in genome mapping, localization of genes for genetic disorder, determination of risk for disease and paternity testing.

Restriction Enzyme:

Restriction enzymes are DNA-cutting enzymes found in bacteria (and harvested from them for use). Because they cut within the molecule, they are often called restriction endonucleases. In order to be able to sequence DNA, it is first necessary to cut it into smaller fragments. Many DNA-digesting enzymes (like those in your pancreatic fluid) can do this, but most of them are no use for sequence work because they cut each molecule randomly. This produces a heterogeneous collection of fragments of varying sizes. What is needed is a way to cleave the DNA molecule at a few precisely-located sites so that a small set of homogeneous fragments are produced. The tools for this are the restriction endonucleases. The rarer the site it recognizes, the smaller the number of pieces produced by a given restriction endonuclease.A restriction enzyme recognizes and cuts DNA only at a particular sequence of nucleotides. For example, the bacterium *Hemophilus aegypticus* produces an enzyme named HaeIII that cuts DNA wherever it encounters the sequence

5'GGCC3'

3'CCGG5'

Figure 6

The cut is made between the adjacent G and C. This particular sequence occurs at 11 places in the circular DNA molecule of the virus φX174. Thus treatment of this DNA with the enzyme produces 11 fragments, each with a precise length and nucleotide sequence. These fragments can be separated from one another and the sequence of each determined.[17]

HaeIII and AluI cut straight across the double helix producing "blunt" ends. However, many restriction enzymes cut in an offset fashion. The ends of the cut have an overhanging piece of single-stranded DNA. These are called "sticky ends" because they are able to form base pairs with any DNA molecule that contains the complementary sticky end. Any other source of DNA treated with the same enzyme will produce such molecules.

Mixed together, these molecules can join with each other by the base pairing between their sticky ends. The union can be made permanent by another enzyme, a DNA ligase that forms covalent bonds along the backbone of each strand. The result is a molecule of recombinant DNA (rDNA).

The ability to produce recombinant DNA molecules has not only revolutionized the study of genetics, but has laid the foundation for much of the biotechnology industry. The availability of human insulin (for diabetics), human factor VIII (for males with hemophilia_A), and other proteins used in human therapy all were made possible by recombinant DNA.

For rs35807939:

- Enzyme used Pf1M1
- Incubation temperature 37°C overnight.
- Digestion pattern: - 2 bands of 271 bp and 163 bp each.

Restriction Enzyme sequence:

Figure 7

Atg 7 restriction sequence:

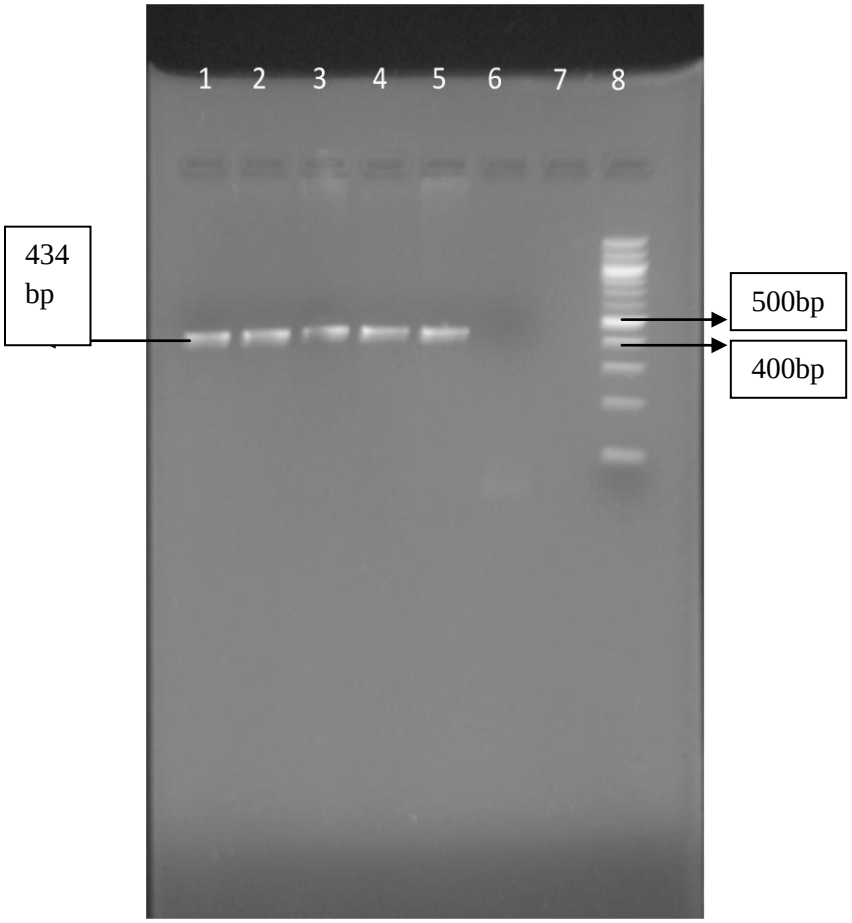
CCACCCTGTGGCATCTG

Table 3: Reaction for Restriction Digestion

Component	Volume (µl)
i-Taq	0.125
Buffer	1
dNTP	0.25
Forward Primer	0.2
Reverse Primer	0.2
MQ water	9.75
DNA	1

Chapter-4

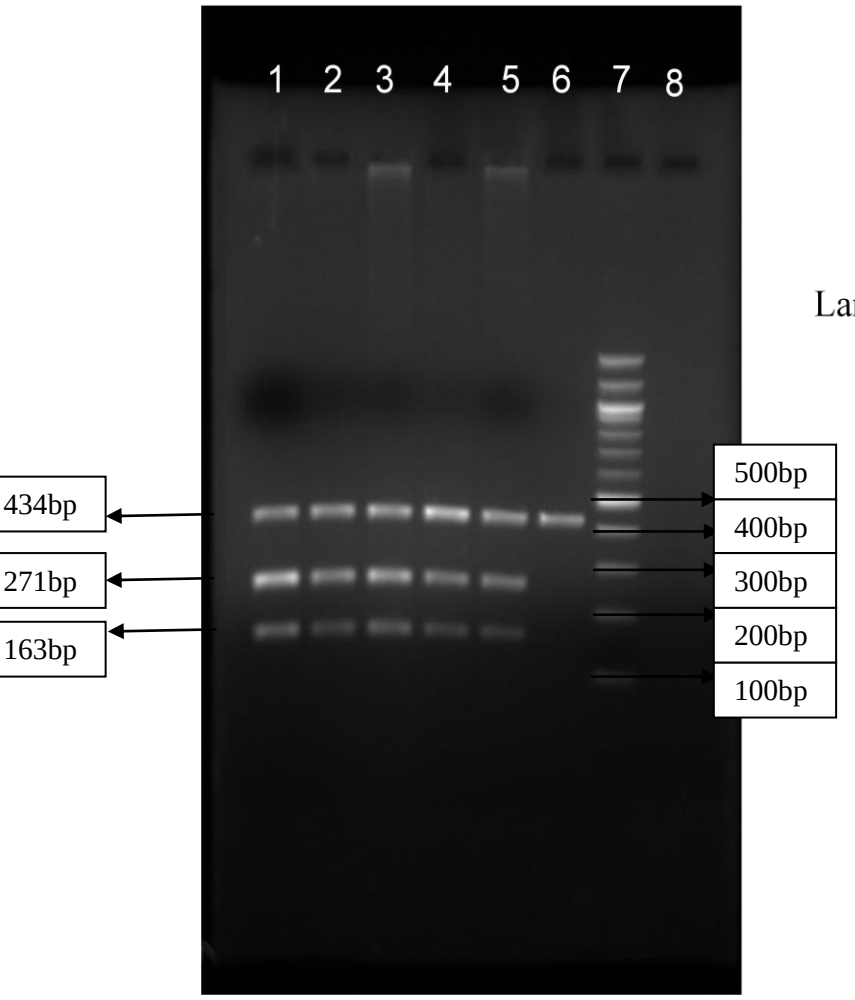
Result and Discussion



Results PCR Amplification

Lane – 1	Control sample 1
2	Control sample 2
3	Control sample 3
4	Control sample 4
5	Control sample 7
6	Negative
7	Empty
8	Ladder 100bp

Figure 8



Restriction Digestion Results Control samples

Lane – 1	Control sample 1
2	Control sample 2
3	Control sample 3
4	Control sample 4
5	Control sample 7
6	Undigested
7	Ladder 100bp
8	Empty

Figure 9

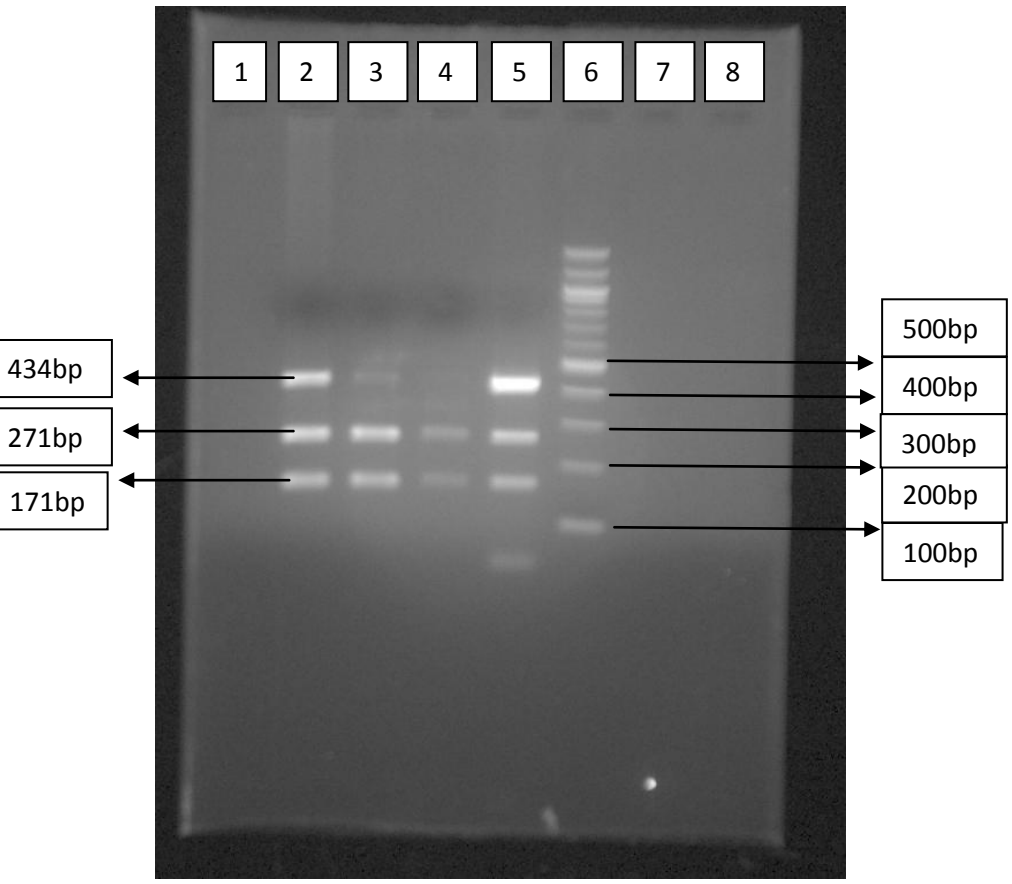


Figure 10

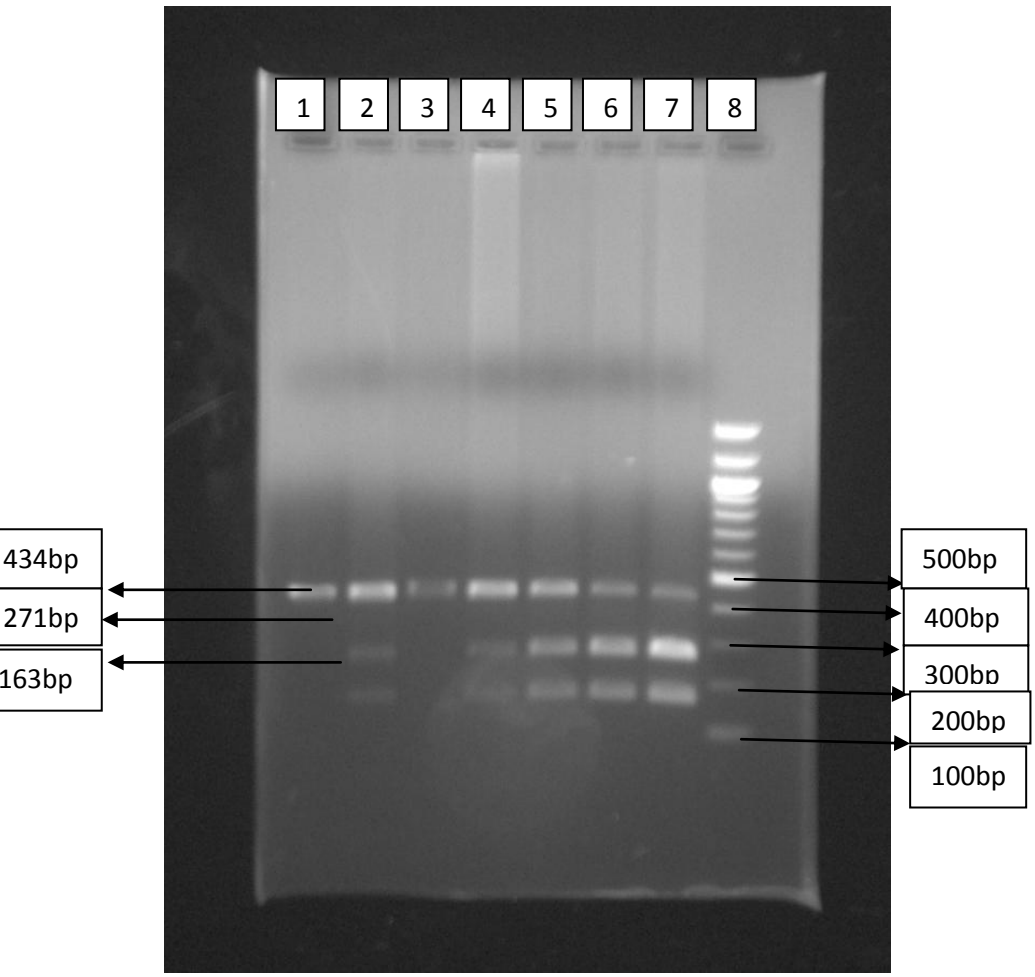


Figure 11

Analysis of Result:

In these restriction digestion gel electrophoresis pictures SNP rs35807939, genotype AG(heterozygous) has three bands 434bp, 271bp, 163bp, genotype GG(homozygous dominent) has two bands 271bp and 163bp where AA(homozygous recesive) genotype has only 1 band size of 434bp.

Table 4- Results for SNP rs35807939

Sample\ Genotype	**GG**	**GA**	**AA**
Control	1	80	6
Diseased	8	75	10

Table 5- Results

Control		Diseased	
Sample No	Genotype	Sample No	Genotype
1	AG	1	GG
2	AG	2	AG
3	AG	3	AG
4	GG	4	AG
5	AG	5	AG
6	AG	6	AG
7	AG	7	AG
8	AG	8	AG
9	AG	9	AG
10	AA	10	AG
11	AG	11	AG
12	AG	12	AG
13	AG	13	AG
14	AG	14	AA
15	AG	15	AG
16	AG	16	AA
17	AG	17	AG
18	AG	18	AA
19	AG	19	AG
20	AG	20	AA

21	AG	21	AG
22	AG	22	AG
23	AG	23	AG
24	AG	24	AG
25	AG	25	GG
26	AG	26	AG
27	AG	27	AG
28	AG	28	AA
29	AG	29	AG
30	AG	30	AG
31	AG	31	AG
32	AG	32	AG
33	AG	33	AG
34	AG	34	AG
35	AG	35	AG
36	AG	36	AG
37	AG	37	AG
38	AG	38	AG
39	AG	39	AG
40	AG	40	AG
41	AG	41	GG
42	AG	42	AG
43	AG	43	GG

44	AG	44	AG
45	AG	45	AG
46	AG	46	GG
47	-	47	AG
48	AG	48	AG
49	AG	49	AG
50	AG	50	AG
51	AG	51	AA
52	AG	52	AG
53	AG	53	AG
54	AA	54	AG
55	AG	55	AG
56	AG	56	AG
57	AG	57	AA
58	AG	58	GG
59	AG	59	AG
60	AG	60	AA
62	AG	61	AG
63	-	62	GG
64	AG	63	AG
65	AG	64	AA
66	AG	65	GG
67	AG	66	AG

68	AG	67	AG
69	AG	68	AG
70	AA	69	AG
71	AA	70	AG
72	-	71	AG
73	-	72	AG
74	-	73	AA
75	AG	74	AG
76	AG	75	AG
77	AG	76	AG
78	AG	77	AG
79	AG	78	AG
80	AG	79	AG
81	AG	80	AA
82	AG	81	AG
83	AG	82	AG
84	AG	83	AG
85	AG	84	AG
86	AG	85	AG
87	AG	86	AG
88	AA	87	AG
89	AA	88	AG
90	AG	89	AG

91	AG	90	AG
92	AG	91	AG
93	AG	92	AG
		93	AG

Chapter-5

Conclusion

After the study has been done, it can be concluded that there is no significant association of autophagy related gene 7 (Atg7) SNP rs35807939 with Asthma.

With the odd ratio 0.9305 and confidence interval of 0.6152- 1.4075, there isn't any significant association of Atg7 with asthma. Though as the literature says that autophagy plays a significant role in asthma pathogenesis, Atg7 is not playing a major role in asthma. Although SNPs associated with Atg5 have been associated to asthma, and presence of autophagosomes in the bronchial biopsy's strongly associate autophagy to asthma, just that this gene is not playing a hand in asthma.

Thus, in future researchers can target autophagy genes which have a more significant association with asthma.

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My main interest lies in studying the biology of a disease and then finding a diagnostic technique or a diagnostic marker (example, a protein, or a single nucleotide polymorphism). I am seeking opportunities in research areas. Currently a senior undergraduate at Jaypee University of Information Technology, I am looking forward to gaining more and more experience and hands on practice in laboratory works.

B.Tech PROJECT

Title : Role of genetic variants in Autophagy related gene 7 (Atg 7) in Asthma.

Description : Autophagy is an evolutionarily conserved lysosomal pathway of self digestion during starvation; it also has a role in degrading the intracellular and extracellular pathogens. The autophagosomes formed during the pathway have a physiology in case of airway inflammation in case of asthma. In this project, we are trying to find association of the autophagy related gene 7 with asthma.

Techniques : DNA isolation, PCR amplification, Gel Electrophoresis, Restriction fragment length polymorphism (RFLP)

SUMMER INTERNSHIP

Organisation : National Institute of Pathology, ICMR, Safdarjung Hospital, New Delhi

Title : Isolation of Exosomes and Characterisation using Transmission Electron Microscopy.

Duration : 2 Months

Description : Exosomes are the multi-vesicular bodies which have an important role in cell signalling in metastizing cancer. The project was on isolation of exosomes from gall bladder carcinoma samples, and then these exosomes were characterised using Transmission electron microscopy.

Techniques : Plasma isolation from blood, Ultracentrifugation, Protein isolation, SDS-PAGE, Protein digestion, Electron microscopy.

SCHOLASTICS

Present	B.Tech, Biotechnology, JUIT, Waknaghat, Solan, HP, and CGPA 6/10, till 7th semester
2011	**XII** from, N.K. Bagrodia Public School, New Delhi, CBSE Board. Secured 87.8%
2009	**X** from, N.K. Bagrodia Public School, New Delhi, CBSE Board. Secured 86%

TECHNIQUES KNOWN

- DNA isolation
- PCR amplification
- SDS-PAGE
- Gel Electrophoresis

IT FORTE

- Proficient with Microsoft Word, Excel, PowerPoint.
- C/C++

PERSONAL DOSSIER

Date of Birth	:	5th December, 1992
Contact Address	:	H.No-62, Pocket H-33, Sector-3, Rohini, Delhi- 110085
Languages known	:	English, Hindi, French and Pahari

Declaration:

I, the undersigned certify that to best of knowledge and belief, The Resume correctly describes my qualification and myself.

Tapish Dogra

Sudip Mukherjee

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

Waknaghat, P.O. Dumehar Bani, Distt.Solan (HP)

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OBJECTIVE:

Submitted in partially fulfilment of the Degree of 5 year Dual Degree Programme B.Tech-M.Tech

EDUCATIONAL QUALIFICATIONS:

Degree	College/School	Year	CGPA/Percentage
5 year B. Tech-M.Tech Degree (BioTechnology)	Jaypee University of Information Technology, Solan.	2011-2016	5.2 (60%) (Up till 7th semester)
12th (WBSE)	Shree Ramkrishna Vidyashram,W.B.	2011	65.4%
10th (WBSE)	Shree Ramkrishna Vidyashram,W.B.	2009	76.6%

TECHNICAL SKILLS:

- Programming Language : C (Beginner), Data structure
- Bioinformatics Tools : Blast, Fasta, ClustalX.
- PROFICIENCIES :

  - Genetic Engineering
  - Bioprocess Engineering
  - Genetics
  - Cell and Tissue Culture
  - GLP & Instrumentation
  - Molecular Biology
  - Microbiology
  - Immunology

EXPERIMENTAL TECHNIQUES:

- Microbiology
  - Streaking & Spreading, Growth Curve Estimation, Staining Technique, Evaluating anti-microbial activity by Disc Diffusion method.

- Biochemistry
  - Qualitative and Quantitative estimation of carbohydrates and protein, Qualitative estimation of lipids and nucleic acid, Paper chromatography.

- Molecular Biology and Genetic Engineering
  - Quantification of nucleic acid, DNA isolation from plant and blood, Southern Hybridization, PCR, Ligation, Transformation, SDS-PAGE, Pulse Field Gel Electrophoresis.

- Immunology
  - Radial Immuno Diffusion, Rocket Immuno Electrophoresis, Ouchterlony double diffusion, DOT ELISA.

Trainings:

1) 2 weeks industrial training in Albart David Ltd.
2) 6 weeks industrial training in Aurio Pharma Pvt. Ltd.

ACHIEVEMENTS:

- **District level School quiz champion** in 2010

Extra-Curricular Activities:

- Was part of Organizing committee of college's Bio Club ''Synapse".

HOBBIES AND OTHER AREAS OF INTEREST:

- Participating in social events
- Yoga
- Keen interest in playing Badminton, Cricket, Chess, Football
- Cooking
- Watching sensible movies and Discovery
- Writing and reading Quotes

PERSONAL DETAILS

Date of Birth : January 9, 1993

Gender : Male

Father's Name : Sukanta Mukherjee

Mother's Name : Papi Mukherjee

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Declaration:

I, the undersigned certify that to best of knowledge and belief, The Resume correctly describes my qualification and myself.

Sudip Mukherjee