ROLE OF GENETIC VARIANT OF AUTOPHAGY RELATED GENE 5 (ATG5) (rs17587319 C\G) IN ASTHMA IN NORTH INDIAN POPULATION

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CERTIFICATE

This is to certify that the work titled, "**Role of genetic variant of Autophagy related gene 5(Atg5) (rs17587319 C\G) in Asthma in North Indian population.**" submitted by **Durga Jha (111803)** for partial fulfilment for the award of degree of B. Tech (dual degree) 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.

Dr. Harish Changotra

Assistant Professor, Department of Biotechnology and Bioinformatics, JUIT

ACKNOWLEDGEMENT

I would like to extend my sincere & heartfelt gratitude towards all the people who extended their support and provided motivation during the continual work done over period of last one year on this project. Without their active guidance, help, cooperation & encouragement, this project might not have reached its completion.

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……

Signature of Student

Name

Date

SUMMARY

Asthma is a common inflammatory respiratory disease which causes airway hypersensitiveness. Several factors including both environmental and genetic factors have been found to be associated with occurrence of Asthma. Many molecular components such as cytokines and autophagy related genes have been found to play a role in Asthma. Atg5 (Autophagy Related Gene 5) is involved in autophagy pathway for autophagosome formation. Its role has been associated with asthma in different populations. It has been found that its concentration is quite high in bronchial tissues during acute exacerbations and it also plays a paradoxical role of delaying viral clearance in asthmatic individuals. Based on these observations the study was undertaken to determine the role of a polymorphism (rs17587319 C/G) of Atg5 gene in asthma in North Indian Population. A case control study using blood samples from 93 healthy individuals and 94 asthmatic individuals was performed in this project. The genotypes were determined using Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP). We found no significant association of Atg5 polymorphism (rs17587319 C/G) with increased asthma risk.

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Signature of Student	Signature of Supervisor
Name	Name
Date	Date

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LIST OF ABBREVIATIONS

1. ATG – Autophagy Related Gene
2. ROS – Reactive oxygen species
3. SNP – Single Nucleotide Polymorphism
4. IFN – Interferon
5. RANTES – Regulated on Activation, Normal T Expressed and Secreted
6. IL – Interleukin
7. LC3 – Microtubule associated protein 1 Light chain 3
8. mTOR – Mammalian target of rapamycin
9. STAT – Signal Transducers and Activators of Transcription
10. IgE – Immunoglobulin E
11. EDTA – Ethylenediaminetetraacetate
12. SDS – Sodium Dodecyl Sulphate
13. Tris-Cl – TrisaminomethaneChloride
14. Tris-EDTA – Trisaminomethane Ethylenediaminetetraacetate
15. PCR-RFLP – Polymerase Chain Reaction – Restriction Fragement Length Polymorphism
16. TRANSFAC database - TRANScription FACtor database

CHAPTER -1

INTRODUCTION

ASTHMA

Asthma is a chronic lung disease in which inflammation of the lung's airways and a tightening of the muscles occur around them, causing reversible airway obstruction and wheezing which is difficulty in breathing [1]. Some degree of airway obstruction is often constantly present in those with asthma, but are severe reactions can occur due to exposure to a variety of triggers. Asthma triggers vary depending upon person and environment, but some known triggers include cigarette and other smoke, mold, pollen, dust, animal dander, exercise, cold air, household and industrial products, air pollutants, and infections. Asthma symptoms include coughing, wheezing and shortness of breath [2]. During an asthma attack, these symptoms worsen and a person feels like they cannot breathe. An asthma attack is often the result of exposure to one or more asthma triggers.

Adequate healthcare is integral to reducing the burden of asthma. Without proper treatment, asthma can become life threatening. The issue of access to healthcare for minority populations or people of less developed countries has become an area of concern in recent years. Scientists have proposed varying explanations for the high rates of asthma in some ethnic and racial populations. One theory is that these groups have a genetic predisposition to asthma. A second theory is that environmental influences, such as poverty, stress, living in inner cities, and decreased access to healthcare contribute to the high prevalence of asthma among these populations. Some researchers believe that both genetic and environmental factors combine and interact to explain the higher asthma rates in some communities. Often, these other factors may be the true cause of a disparity, with race or ethnicity being the factor that is easier to detect between different populations [3]. Research and interventions based on and comparing the contributions of these theories is ongoing.

Asthma research has grown, but the complexity of the disease continues to challenge progress. One problem is the lack of sufficiently large studies of minority populations [4]. Others include the number of asthma-related environmental factors, the different levels of exposure possible, and the many interactions between these factors and genetic influences.

AUTOPHAGY

Autophagy is a process of lysosomal self-degradation that helps to maintain the homeostatic balance between the synthesis, degradation and recycling of cellular proteins and organelles. Autophagy does not simply function as the machinery for supplying amino acids in response to energy demands, it is an adaptive pathway of cytoprotection against cellular stressors, including starvation, reactive oxygen species (ROS), endoplasmic reticulum (ER) stress and microbial infection [5]. Accordingly, autophagy is considered to be the mediator of a variety of cellular processes and cell fates, including cell survival and death, cellular senescence and immune responses [5,6]. Due to the organ-specific role of gas exchange, various cell types within the lungs are serially exposed to a diverse array of cellular stressors, and growing evidence has revealed the crucial involvement of autophagy in the pathogenic processes underlying pulmonary diseases like asthma.

Autophagy occurs at low basal levels in virtually all cells to perform homeostatic functions such as protein and organelle turnover. It is rapidly upregulated when cells need to generate intracellular nutrients and energy, for example, during starvation, growth factor withdrawal, or high bioenergetic demands. Autophagy is also upregulated when cells are preparing to undergo structural remodeling such as during developmental transitions or to rid themselves of damaging cytoplasmic components, for example, during oxidative stress, infection, or protein aggregate accumulation [7]. Nutritional status, hormonal factors, and other cues like temperature, oxygen concentrations, and cell density are important in the control of autophagy [8].

The autophagic machinery is highly conserved in organisms as diverse as plants, animals, and yeast. Recent studies have demonstrated a role for autophagy in both promoting and preventing human diseases. In the last few years, tremendous advances have been made in identifying the molecular components required for macroautophagy through genetic studies in yeast. Combining these analyses with the pharmacological and biochemical information that is available from work in mammalian systems has begun to provide a detailed understanding of the mechanisms that underlie each stage of the autophagic process [9]. Furthermore, workers in both plant and animal systems are exploiting the genomic data now available to identify homologs of the yeast genes for directed studies in these multicellular

systems. Such studies should allow researchers to address tissue- and development-specific roles for autophagic degradation [10].

AUTOPHAGY RELATED GENE 5 (ATG5)

The ATG5 gene is located on chromosomal region 6q21 and is approximately 141 kilobase pairs in length. The common ATG5 mRNA transcript encodes a 276 amino acid ATG5 protein, and during autophagy it is covalently conjugated with ATG12 and interacts with ATG16 to form the ATG12–ATG5-ATG16 complex. This complex is associated with membrane destined to form an autophagosome. It is also involved in mitochondrial quality control after oxidative damage, and in subsequent cellular longevity. The ATG12-ATG5 conjugate also negatively regulates the innate antiviral immune response by blocking the type I IFN production. It has been found in a study that it plays a role in translation or delivery of incoming viral RNA to the translation apparatus [11]. It is also said to play a critical role in multiple aspects of lymphocyte development and is essential for both B and T lymphocyte survival and proliferation [12].

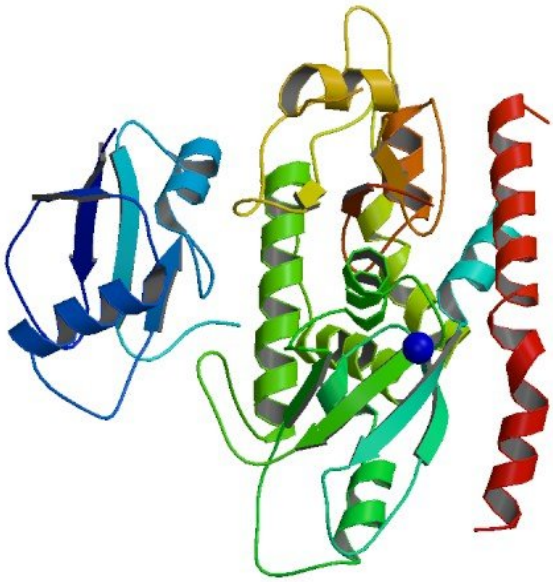


Figure 1: Crystal Structure of Human Atg12~Atg5 Conjugate in Complex with an N-terminal Fragment of Atg16L1. Protein chains are colored from the N-terminal to the C-terminal using a rainbow (spectral) color gradient

OBJECTIVES

1. To optimize PCR-RFLP assay to genotype rs17587319 C/G of Atg5 gene.
2. To genotype and assess the frequency of occurrence of SNP of Atg5 gene in North Indian population.
3. To co-relate presence of polymorphism with occurrence of asthma in this population.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE IN PROPOSED AREA

ASTHMA

Asthma is a disease characterized by variable airway inflammation and airflow obstruction. Asthma management was revolutionized by the advent of inhaled corticosteroids, which greatly improved asthma control and decreased morbidity and mortality. But despite advances in medical therapy and early diagnosis, increase in asthma related mortality is a big concern.

In the acute inflammatory aspects of asthma, allergen-IgE– directed processes are predominant features of airway pathology, withmast cells, TH_2 lymphocytes, and eosinophils the predominant histologic features. The cytokine network associated with these processes often includes IL-3, IL-4, IL-5, IL-9, and IL-13. Mast cells are important contributors both to the initiation of asthma with release of acute-phase mediators, including cysteinyl leukotrienes, and also inflammatory cytokines, which serve to perpetuate inflammatory events in the airway. Subpopulations of lymphocytes polarized toward a TH_2 profile further the inflammatory process by release of cytokines, including IL-4, IL-5, and IL- 13. It is these factors that serve to drive inflammation (eg, recruitment of eosinophils) and also regulate IgE production. Eosinophils are a characteristic feature of allergic inflammation. The biology of eosinophils is well designed to cause airway inflammation, enhancement of airway hyperresponsiveness, and airflow obstruction. Eosinophils are recruited to the airway in asthmatic subjects by families of cytokines, and chemokines (eg, IL-5, RANTES, and eotaxin) undergo cell activation through processes not fully identified and release highly inflammatory granule-associated substances, the actions of which injure the airway and cause persistent inflammation. Eosinophils are also a rich source for leukotrienes, products of oxidative metabolism, and inflammatory cytokines and growth factors [13]. Although the eosinophil is a prominent feature of airway pathology in asthmatic subjects, its precise contribution to airway pathophysiology is undergoing re-evaluation.

Asthma most commonly develops in early childhood, and more than three-quarters of children who develop asthma symptoms before age 7 no longer have symptoms by age 16. However, asthma can develop at any stage in life, including adulthood. The most recent revised global estimate of asthma suggests that as many as 334 million people have asthma, and that the burden of disability is high [14]. The historical view of asthma being a disease of high-income countries no longer holds: most people affected are in low- and middle-income countries, and its prevalence is estimated to be increasing fastest in those countries. Ongoing monitoring is needed to follow the epidemic of asthma and its management [15].

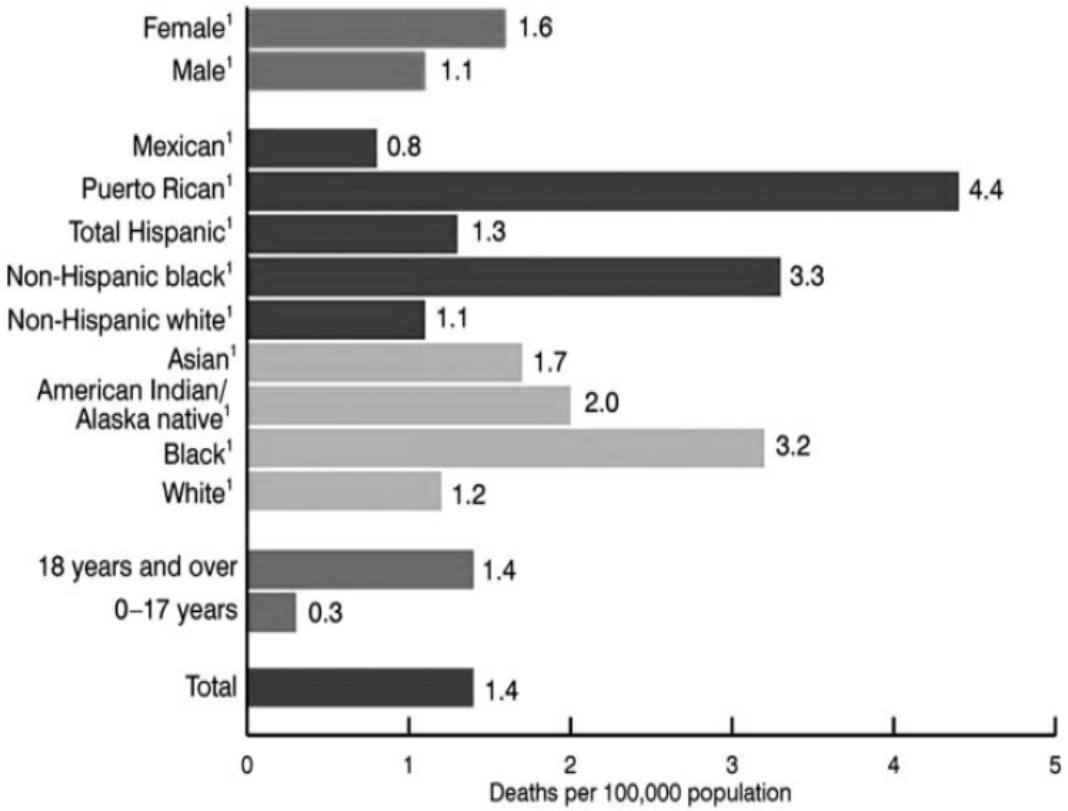


Figure 2: Asthma mortality rate (2003) (Asthma prevalence, health care use, and mortality: 2003-05. National Center for
Health Statistics.)

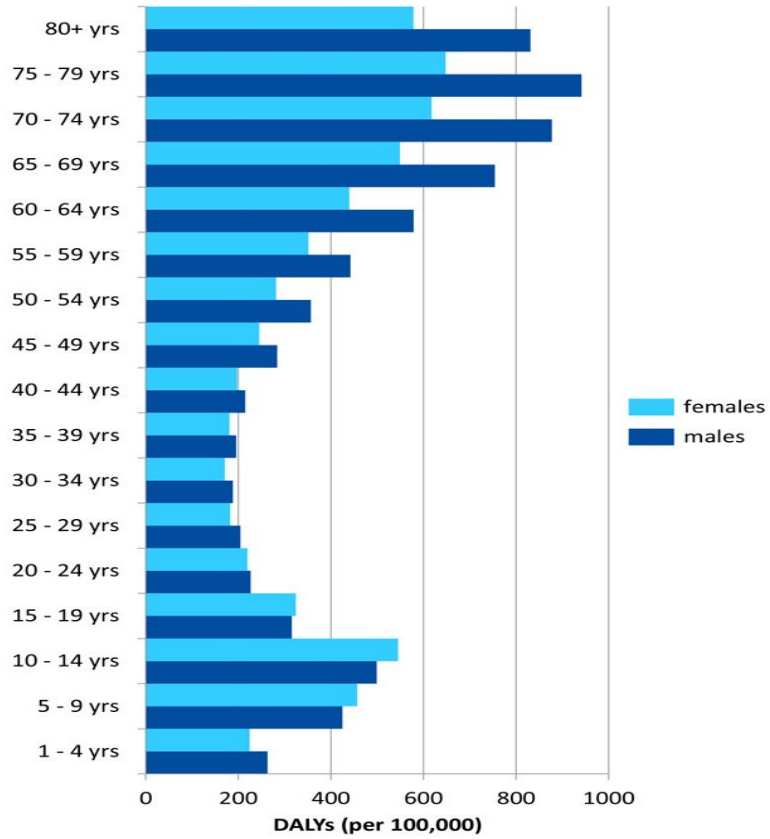


Figure 3: Burden of disease, measured by disability adjusted life years (DALYs) per 100,000 population attributed to asthma by age group and sex.(The global asthma report, WHO , 2010)

AUTOPHAGY

Autophagy (from the Greek, "auto" oneself, "phagy" to eat) refers to any cellular degradative pathway that involves the delivery of cytoplasmic cargo to the lysosome. At least three forms have been identified: chaperone-mediated autophagy, microautophagy, and macroautophagy—that differ with respect to their physiological functions and the mode of cargo delivery to the lysosome.

In macroautophagy pathway, Mammalian target of rapamycin (mTOR) signaling pathway plays a crucial role in control of the pathway. mTOR is a serine/threonine protein kinase and suppresses autophagy in normal physiological conditions. Nutrient starvation and rapamycin suppress mTOR activity and initiate autophagy [16]. Execution of autophagy is mediated by

two key conjugation systems: ATG5– ATG12 conjugation and lipidation of LC3 protein. Atg12 is activated by transient covalent linkage to Atg7 followed by Atg10 before being covalently linked to Atg5. Atg16 binding generates an Atg5– Atg12–Atg16 complex that is present in the autophagy isolation membrane. The terminal amino acid at the carboxyl terminus of microtubule-associated protein light chain 3 (LC3, also known as Atg8) is cleaved by the cysteine protease Atg4. Cleaved LC3 is then transiently linked to Atg7 followed by Atg3, and finally to phosphatidylethanolamine. Both these pathways are crucial for formation of double membrane structured autophagosomes and are used as markers of autophagy. Maturation occurs by fusion with endosomal vesicles forming intermediate autophagosomes and then fusion with lysosomes. This fusion creates autolysosomes containing lysosomal enzymes leading to degradation of cellular cargo [17].

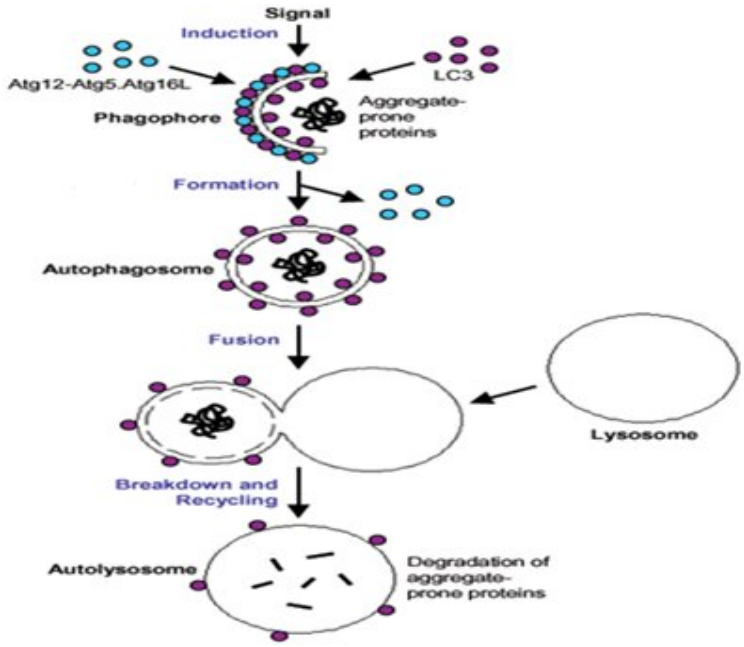


Figure 4: A schematic representation of Autophagy Pathway

AUTOPHAGY AND DISEASE

Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation, development, and homeostasis. Autophagy principally serves an adaptive role to protect organisms against diverse pathologies, including infections, cancer, neurodegeneration, aging, and heart disease. However, in certain experimental disease settings, the self-cannibalistic or, paradoxically, even the prosurvival functions of autophagy may be deleterious [18].

AUTOPHAGY AND ASTHMA

Bronchial asthma is considered to be a chronic allergic inflammatory disease with Th2-type cytokine dominance. In the development of bronchial asthma, the initial Th1-type immune response to viral infection is recognized to be a prerequisite for progression to the subsequent dominant Th2-type response. Therefore, it is not surprising that autophagy is implicated in the pathogenic sequence of bronchial asthma [19].

In terms of the regulation of immunity and viral clearance. autophagy is modulated by both Th1 and Th2-type cytokines. IFN-γ, a Th1 cytokine, has been demonstrated to induce autophagy, while the Th2 cytokines IL-4 and IL-13 inhibit starvation-induced autophagy in macrophages. However, the role of Th2-type cytokinemediated autophagy inhibition in bronchial asthma remains unclear [20]. Airway hyperresponsiveness to cholinergic stimuli an underlying pathology for the development of bronchoconstriction in patients with bronchial asthma, is achieved by conditional *Atg7* knockout in airway epithelial cells [21]. Epithelial cell damage resulting from autophagy inhibition induced by *Atg7* deletion is proposed to be a potential mechanism for this hyperresponsiveness.

Autophagy is also involved in the regulation of ROS (Reactive oxygen species) production and the elimination of oxidized proteins in order to minimize tissue damage. Oxidative stress is associated with airway inflammation in patients with bronchial asthma, and the levels of exhaled hydrogen peroxide (H_2O_2) and nitric oxide (NO) are associated with the severity of asthma. Accordingly, further examinations are warranted to clarify the clinical implications; however, there are several plausible mechanisms underlying the involvement of autophagy in

the pathogenesis and severity of bronchial asthma, including the modulation of immune responses, viral clearance and ROS regulation [22].

ATG5 AND DISEASE

Atg5 gene is located on chromosome 6q21. The protein formed by this gene is approximately of 141Kb in length. Atg5 is important in autophagy mechanism for formation of autophagosome. It role in proliferation and survival of T cells has been identified. In a recent study, this function was used to correlate it with occurrence of Multiple sclerosis (MS) which is an inflammatory central nervous system (CNS) disorder in which T cell mediated demyelination occurs. They used blood sample to perform Quantitative real-time PCR analysis of RNA isolated from it which showed increade Atg5 expression. They also utilised brain sample of mice with multiple sclerosis and found that T cells are present in large number which is a function related to Atg5 [23]. In an other study, Atg5 polymorhism has been associated with occurrence of Crohn's disease. In this study they utilised mice model and transcriptional analysis of tissue from Paneth cell which is a specialized epithelial cell whose main function is the delivery of antimicrobial factors into the intestinal lumen. The presence of genetic variant led to reduction in gene expression [24]. Polymorphisms of Atg5 has also been associated with occurrence of Systemic Lupus Erythematosus. A case control study with control healthy individuals and patients suffereing from SLE was performed in Chinese population. A total of nine single nucleotide polymorphisms association with SLE was studied [25].

But it has several other roles apart from its role in autophagy, which includes its role in apoptosis and Interferon response regulation during cases of viral infection. In a rather unusual function of Atg5, it has been found that it negatively regulates the the type 1 Interferon pathway as usually autophagy has antipathogenic roles too. It was found out that Atg5 has the capacity to promote Replication of vesicular stomatitis virus (ssRNA virus) by suppressing innate anti viral immune response. It has been found that there is increase Atg5 expression in nasal epithelium cells in acute asthma exacerbations. Thus, it has been proposed that due to its non canonical role, increased level of Atg5 can cause increased viral RNA replication and thus increased virus production which would lead to slower viral clearance [26]. It is in sync with previous studies which stated that asthmatics have slower viral clearance.

In other study role of a genetic variant of Atg5 gene in asthma was determined by performing a genetic association study and testing the human bronchial biopsy tissue samples for the presence of double membrane of autophagosomes utilisisng electron microscopy. Two different asthma populations were taken. The SNP was rs12212740 (G<A) which is present in the intron 3 of the gene. G allele was found to be present more frequently in first population [P value 0.0002; odds ratio (OR) 1.35; 95% confidence interval (CI) 1.01–1.89] and it increased the risk of low prebronchiodialtor forced expiratory volume in 1 second (FEV1) but this association was not present in the second one. The reason for this was cited that the first population had patients with different asthma severities but second one had only mild severity asthmatics. FEV1 is low in high severity cases and thus, risk might not have been detected. Double membrane autophagosomes were more easily found in bronchial biopsy tissue in a subject with increased asthma severity compared to healthy subjects. However, the autophagy markers level was not quantified [27].

In another study, association of ATG5 SNP rs510432 (G<A) with asthma was determined. This SNP is present upstream of the first exon of ATG5 gene in the putative promoter region. Amplification of DNA resulted in formation of promoter fragments and their activity was determined using luciferase activity. The promoter fragment was used as a template and base change from A to G was done. The ‘‘G’’ allele resulted in significantly enhanced promotor activity by 23% compared to the ‘‘A’’ allele (p, 0.007). Query from TRANSFAC database for transcription factor binding sites which include rs510432, identified two transcription factors, STAT1 and C-Fos, which have been associated previously with asthma [28].

ROLE OF rs17587319 (C/G) IN VARIOUS DISEASES

rs17587319 (C/G) is located on the intron region of Atg5 gene. The ancestral allele is C. In a study in Han population of the Western Guangdong province in China, positive association of the single-nucleotide polymorphism (rs17587319C/G) of Atg5 (autophagy-related gene 5) and occurrence of Parkinson's disease was found. They performed a case control study using healthy control samples and patients with Parkinson’s disease. They utilised Polymerase Chain Reaction- Restriction Fragment Length Polymorhism to determine the presence of SNP [29]. In another study, positive association between this single nucleotide polymorphism

and Crohn's disease has been found. Till now no study discussing its role in asthma has been done [30].

POPULATION	GENOTYPIC FREQUENCIES			ALLELIC FREQENCIES	
	CC	CG	GG	C	G
European	0.875	0.125	0	0.938	0.062
African American	0.957	0.043	0	0.978	0.022
Asian	0.733	0.267	0	0.867	0.133
Sub-Saharan African	1.000	0	0	1.000	0
Australian	0.667	0.311	0.022	0.822	0.178

Table 1: Frequency of occurrence of genotypes of rs17587319 in population over the world

CHAPTER 3

MATERIALS AND METHODS

COLLECTION OF SAMPLE

A total of 93 control samples were collected along with 94 asthmatic samples. These samples were received from Doaba College, Jalandhar. The samples were from North Indian residents of unbiased age and sex with their consent.

DNA ISOLATION

Genomic DNA was isolated from blood using modified inorganic methods.

REAGENTS UTILISED:

1. **TrisaminomethaneChloride** (Tris-Cl)(pH 8.0): 75 ml of sterile MQ water was used to dissolve 12.11 gm of trisbase. pH was set to 8.0 with 1N HCl and the final volume was made 100 ml with MQ water.
2. **Tris-Cl** (pH 7.3): 12.11 gm of Tris base was dissolved in 75 ml of sterile MQ water and the pH was set to 7.3 with 1N HCl. Final solution made 100 ml with MQ water.
3. **Ammonium Chloride** (1M): Ammonium Chloride (5.35 gm) was dissolved in 80 ml of MQ water and final solution was made 100 ml.
4. **Di-Sodium ethylene diamine tetra acetate**: 18.61 gm of EDTA salt was added to 50 ml of MQ water in a 250 ml flask and placed it on a magnetic stirrer. Simultaneously, the solution was supplemented with 10M NaOH dropwise, until pH reached 8.0. The salt was allowed to dissolve and the final volume was made up to 100 ml with MQ water.
5. **Red blood cell lysis buffer**: (10 mM Tris, pH 8.0, EDTA 1mM, NH_4Cl, 125mM, pH 8.0): 10 mM Tris, 2 ml of EDTA and 125 ml of NH_4Cl were mixed in MQ water to obtain a final volume of 1000ml of RBC lysis buffer.
6. **Tris-EDTA** (TE buffer, pH 8.0): TE buffer was prepared by mixing 10 ml Tris-Cl and 2 ml of EDTA in 700 ml of MQ water and the final volume was made 1000ml. pH was set at 8.
7. **Tris-EDTA** (TE buffer, pH 7.3): TE buffer was prepared by mixing 10 ml Tris-Cl and 2 ml of EDTA in 700 ml of MQ water and the final volume was made 1000ml. pH was set at 7.3.

8. **Sodium dodecyl sulphate**: 10 gm of SDS salt was dissolved in 70 ml of warm MQ water and the final volume was raised to 100 ml.
9. **Ammonium acetate**: 28.9 gm of ammonium acetate salt was dissolved in 20 ml MQ water and final volume was raised to 50 ml.
10. **Chilled dehydrated ethyl alcohol**: Undiluted absolute ethyl alcohol was stored in a -20℃ deep freezer.
11. **Ethanol** (70% EtOH): 70 ml of absolute ethyl alcohol was added to 30 ml of sterile MQ water to obtain a final volume of 100 ml.

PROTOCOL OF ISOLATION OF DNA

1. 400μl of blood sample was taken in a vial to which RBC lysis buffer (3 times the volume of the blood sample taken) was added and kept for incubation on a rocker, to permit perpetual shaking at room temperature until the RBC's completely naked.
2. The solution was centrifuged at 1300 RPM for 1 minute to obtain a creamish white WBC pellet.
3. The supernatant was discarded and the WBC pellet was suspended in 300 μl TE buffer (pH 8.0) using a vortexing machine. Then 20 μl of 10% of SDS solution was added to the above solution and the mixture was incubated at 56℃ for 30 minutes on a dry bath.
4. Subsequently 150 μl of 7.5mM ammonium acetate was added and mixed vigorously for about 1 minute per sample, on vortexer. The mixture was centrifuged at 1300 RPM at room temperature for 15 minutes 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 chilled absolute ethyl alcohol (twice the volume of clear supernatant) was added. The tube was gently rocked a couple of time to allow the precipitation of genomic DNA.
6. The genomic DNA precipitates were centrifuged at 1300 RPM for 10 minutes to get pellet at the time of the tube. The later subsequently was washed in 150 μl of 70% ethanol and air dried at room temperature for about 15 minutes.
7. 100 μl of TE buffer (pH 7.3) was used to dissolve the dried DNA pellet by incubating at 65℃ for 10 minutes. The dissolved DNA was finally stored at -20℃ till further use.

QUANTIFICATION OF DNA

After isolation of DNA, quantification and analysis of quality are necessary to ascertain the approximate quantity of DNA obtained and the suitability of DNA sample for further analysis. This is important for many applications including digestion of DNA by restriction enzymes or PCR amplification of target DNA. The most commonly used methodologies for quantifying the amount of nucleic acid in a preparation are (A) electrophoretic run along with standard DNA (B) spectrophotometric estimation and (C) flourometric determination (D) DNA quantification using NanoDrop.

Spectrophotometry is designed to measure the degree of absorption of light by a substance, in a definite and narrow wavelength range. The absorption spectrum in the visible and ultraviolet regions of a substance in a solution is characteristic depending on its chemical structure. Therefore, Spectrophotometry is used to identify a substance by measuring the absorbances at various wavelengths. This method is applicable to identification tests, purity tests, and assays, in which the absorbance of a solution with a certain concentration is usually measured at the wavelength of the maximum absorption (λmax.) or the minimum absorption (λmin.).

Its use spans across various scientific fields, such as physics, material science, chemistry, biochemistry, and molecular biology. They are widely used in many industries including semiconductors, laser and optical manufacturing, as well as in laboratories for the study of chemical substances. Ultimately, a spectrophotometer is able to determine, depending on the control or caliberation, what substances are present in a target and exactly how much through calculation of observed wavelengths.

OPERATION OF NANODROP

The NanoDrop spectrophotometer uses a retention system that holds 1-2µl of sample without the need for traditional containment devices such as cuvettes and capillaries. Using fiber optics technology and surface tension, the sample is held in place between two optical sufaces that define the pathlength in vertical orientation. Removal of fixed containment devices from the system allows the pathlength to change in real time for a given sample. Direct coupling of the sample to the optics of the spectrophotometer removes interference caused by incident light and transmitted light passing through containment walls of traditional cuvettes and capillaries. Preparation of the next sample only requires wiping of both optical surfaces with a common laboratory wipe. Total measurement cycle time, is around ~30 sec and volume required is quite less too. Materials required for this process are:

1. Nucleic acid sample to be quantitated
2. Water or buffer in which sample is dissolved. (In this case, TE buffer pH 7.3)
3. NanoDrop spectrophotometer

PROCEDURE TO USE NANODROP

1. Clean the upper and lower optical surfaces of the microspecrrophotometer (ThermoScientific MultiScanner) sample retention plate. Pipet 1 to 2 µl of buffer or deionized water onto the fiirst row as they serve as blank. Rest of the samples are pipeted on the next rows. There are total 7 rows and thus at a time, 14 samples can be loaded.
2. The plate is placed in the NanoDrop.
3. Open the NanoDrop software and the nucleic acids module was selected.
4. Measure the nucleic acid content by selecting “Run”. Once the measurement is complete, the plate is ejected and cleaned with deionized water with a wipe.
5. The 260/280 ratio and concentration of the samples are provided on the software in a tabulated manner.

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. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. There are three major steps involved in the PCR technique: denaturation, annealing, and extension. PCR is useful in the investigation and diagnosis of a growing number of diseases.

The basic PCR principle is simple. As the name implies, it is a chain reaction: One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. PCR is a method used to acquire many copies of any particular strand of nucleic acids. It's a means of selectively amplifying a particular segment of DNA [31].

There are three major steps involved in the PCR technique: denaturation, annealing, and extension. In step one; the DNA is denatured at high temperatures (from 90 - 97 degrees Celsius). In step two, primers anneal to the DNA template strands to prime extension. In step three, extension occurs at the end of the annealed primers to create a complementary copy strand of DNA. This effectively doubles the DNA quantity through the third steps in the PCR cycle. To amplify a segment of DNA using PCR, the sample is first heated so the DNA single-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes - builds - two new strands of DNA, using the original strands as templates.

This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then each of these strands can be used to create two new copies, and so on, and so on. The annealing phase happens at a lower

temperature, 50-60°C. This allows the primers to hybridize to their respective complementary template strands, a very useful tool to forensic chemistry. The newly-formed DNA strand of primer attached to template is then used to create identical copies off the original template strands desired. Taq polymerase adds available nucleotides to the end of the annealed primers. The extension of the primers by Taq polymerase occurs at approx 72°C for 2-5 minutes [28].

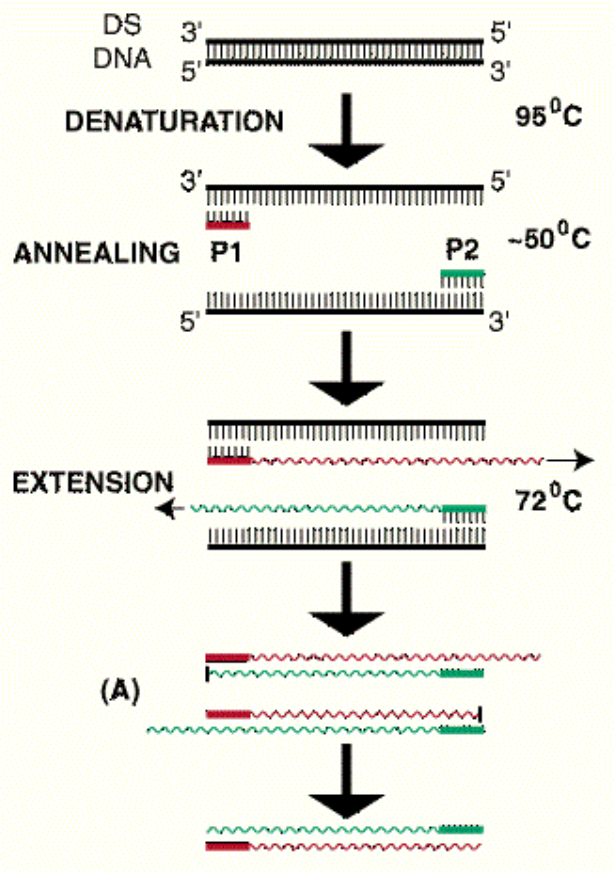


Figure 5: A schematic illustration of Polymerase Chain Reaction

Primer Design

Primer3 is an online tool which picks primers for PCR reactions, considering as criteria: oligonucleotide melting temperature, size, GC content, and primer-dimer possibilities, positional constraints within the source (template) sequence, PCR product size, etc. The steps for designing primers are:

1. Paste the sequence of ATG5 gene which is to be amplified in PCR into the window.
2. Setting parameters - The program has default parameters that are better to change such as product length.
3. Picking primers – click on the 'pick primers' button.

Here primers obtained for the ATG5 gene are following:

Forward primer: 5' – CAA CTG TAA TCT GAG GTC – 3' (Tm = 46.9℃)

Reverse primer: 5' – TAT CAC AAA TAA AAT CTT – 3' (Tm = 37.5℃)

Methods of PCR

Requirements:

1. Thermal cycler (thermocycler)
2. PCR amplification mix typically containing:
3. Sample dsDNA with a target sequence
4. Thermostable DNA polymerase
5. Two oligonucleotide primers
6. Deoxynucleotide triphosphates (dNTPs)
7. Reaction buffer containing magnesium ions and other components

Process:

1. A reaction mixture (20µl for each sample) was prepared based on total number of samples being amplified and thus, total volume was calculated. The volume of each component based on 1X of reaction were as: Taq Polymerase (0.2µl), Buffer (2µl),

Forward primer (0.5µl), Reverse primer (0.5µl), dNTP (0.4µl), distilled or MQ water (15.4µl) and DNA (1µl).

2. From this stock solution, 20 µl of the mixture was taken ad added to 0.5 µl vials.
3. It was made imperative that Tag polymerase (5u/ µl) was added at the end of preparation of stock.
4. 1 µl of genomic DNA was added to each vial (10ng/ µl)
5. It was mixed, centrifuged and placed in thermal cycler.
6. The DNA was amplified under given conditions: 2 minutes at 94℃, 20 seconds at 94℃, 30 seconds at 44℃, 40 seconds at 72℃ and final extension for 7 minutes and 72℃. The number of cycles for the reaction was set at 35.
7. After the amplification, the vials were taken and used to perform gel electrophoresis. The size of the amplicon was nearly 108 bp.

The amplified DNA samples had the genetic polymorphism copied in large quantity. To determine genotype, a restriction enzyme was chosen using NebCutter ensuring that it cut in presence of wild genotype and has only single cut site. Thus, the enzyme utilised in this protocol is DdeI. The protocol for restriction digestion was:

1. The amplicon was taken and to this following components were added: Amplified DNA product (10µl), Restriction enzyme (0.1 µl, it is equivalent to 1 unit of enzyme), Buffer (1.5 µl), and distilled or MQ water (3.4 µl).
2. The mixture was centrifuged at placed at room temperature overnight as the restriction enzyme has not STAR activity.
3. After digestion, different genotypes have following banding pattern: CC (single band of 75bp), CG (single band of 33 bp) and GG (3 bands of 108bp, 75bp and 33 bp).

AGAROSE GEL ELECTROPHORESIS

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 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 gels are easy to cast and are particularly suitable for separating DNA of size range most often encountered in laboratories, which accounts for the popularity of its use.

Agarose Gel Electrophoresis for checking the presence of DNA was done on 1.2% agarose gel while to check results of restriction digestion was done o. 3.5% gel.

Preparation of Reagents

1. Tris-Acetic acid-EDTA (TAE) buffer (50X): 242 gm of Tris base was dissolved in 500 ml of MQ water. This solution was then supplemented with 57.1 ml of glacial acetic acid followed by 100 ml of 0.5M EDTA (pH 8.0). Final volume of the solution was made to 1000ml with MQ water and the same was filtered and diluted to a working concentration of 1X before use.
2. Agarose: 1.2% agarose gel to check the presence of DNA and 3.5% agarose gel to find out the results of restriction digestion was prepared in 1X TAE buffer for qualitative analysis of genomic DNA samples.
3. Ethidium Bromide (10mg/ml): 10 mg of ethidium bromide was added to 1 ml of MQ water and dissolved by gentle mixing before storing in an amber bottle at room temperature.
4. Gel loading dye (6X): 0.025% bromophenol blue (BPB) with 40% sucrose in water was used for analysis of genomic DNA.
5. Gel loading dye (10X): 0.5% (w/v) xylene cynol, dissolved in distilled water, was mixed with equal volume of glycerol. The same was used for PCR product analysis.

Protocol (Sambrook and Russell, 2001)

Pouring of agarose gel:

Note: Agarose gels are commonly used in concentrations off 0.7% to 2% depending on the size of bands needed to be separated. To do this the amount of starting agarose is adjusted to %g/100ml TAE (.eg. 2g/100ml will give 2%).

1. Agarose was measured (1.2% or 3.5% based on requirement).
2. Agarose powder was poured into microwavable flask along with 100ml of 1X TAE.
3. This mixture was microwaved for 1-3 minutes (till the agarose was completely dissolved and there was nice boil in solution which is noticeable as the solution becomes clear from hazy).
4. Agarose solution was allowed to cool down for 5 minutes.
5. Ethidium bromide (EtBr) was added to a final concentration od approximately 0.2-0.5µg/ml (usually about 2-3µl of lab stock solution per 100ml gel, but it can be increased if the concentration of agarose increases). EtBr binds to the DNA and allows to visualize the DNA under ultraviolet (UV) light.
6. The agarose was poured into a gel tray with the well comb in place.
7. The gel was allowed to sit at room temperature for 20-30 minutes, until it completely solidified.

Loading Samples and Running an Agarose Gel:

1. Loading dye was added to each DNA sample.
2. Once solidified, the agarose gel was placed into the electrophoresis unit.
3. The unit was filled with 1X TAE buffer until the gel was covered.
4. A molecular weight ladder was carefully loaded into the first lane (preferably but not necessary) of the gel.
5. The samples were carefully loaded into the rest of the wells of the gel.
6. The gel was run at 80-150V until the dye line was approximately 75-80% of the way down of the gel.
7. Power was turned OFF, the electrodes were disconnected from the power source, and then the gel was carefully removed from the electrophoresis tank.
8. Using a gel documentation system and placing it under UV light, DNA fragments were visualized.

CHAPTER 4
RESULT AND DISCUSSION

Sample Collection

93 blood samples from healthy individual and 94 blood samples from asthmatic individuals were collected and consent forms were filled out by all contributors, recording their name, address etc as well as family history regarding any disease in family.

DNA isolation

DNA isolation was carried in sets of two for each blood sample. The reagents used in this process were all made from scratch in the lab. The above mentioned protocol (Miller et al. 1988) was used for this collection.

The DNA was successfully isolated from each of the 94 samples from healthy individuals and asthmatic individuals and was later pooled together in a single set and was stored at -20℃.

Quantification using spectrophotometer

Next the samples were taken and their optical densities were measured at 260nm as well as at 280nm. The spectrophotometer used was ThermoScientific NanoDrop.. The advantage of using this equipment was that it utilises only 1-2µl of sample and directly provides the concentration of the DNA present in the sample. The blank used in the protocol was TE buffer (pH 7.3) as DNA pellet obtained is suspended. This way we eliminate any effect of the buffer on the optical density obtained. A large table was made with the sample number and their OD and concentration as found from spectrophotometer. A few are given below:

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 2: Examples of readings (Healthy individuals) obtained from spectrophotometer

Based on these numerous readings it was seen that a range was obtained. The values in general did not deviate heavily from the ideal ratio of 1.8-2.0, which meant that the DNA obtained was nearly pure.

These values were then used to prepare a standardised concentration of 10ng/µl of DNA by diluting the stock solution, to be used for the amplification process. It is essential that an even concentration be kept while carrying out the amplification to ensure that every sample was processed under similar conditions and the results of determining the presence or absence of occurrence of mutation was not affected by it. Only then the fragment size can be properly observed in the electrophoresis stage.

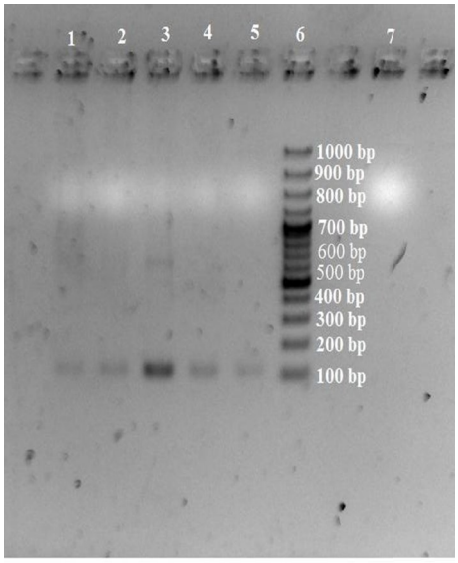
Amplification using Polymerase Chain Reaction

After diluting all the DNA samples to standardised concentration of 10ng/µl, PCR was carried out. As discussed in previous chapter, it is a simple and effective way to generate large number of copies of a particular DNA sequence and is an indispensable technique used for varied applications. The protocol mentioned above was utilised for this step.

Gel Electrophoresis

Then the agarose gel electrophoresis was carried out. The DNA sample used here was restricted with the help of restriction enzyme prior to use to ascertain its genotype. In this method a 100bp ladder was taken all the samples were taken and run against this ladder on the gel. The gel was then viewed in a gel documentation system. The standard procedure of gel electrophoresis was used.

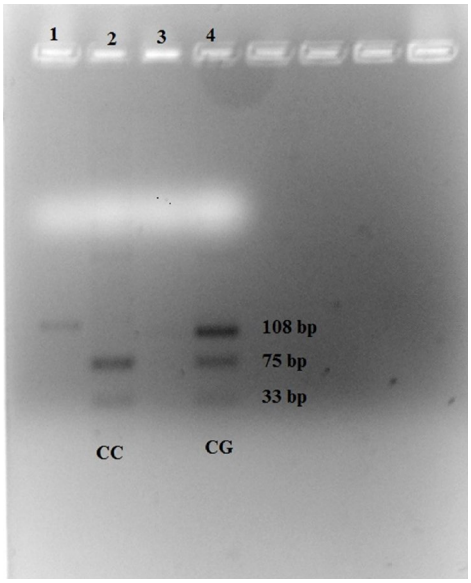
But prior to performing gel electrophoresis of all the samples, the conditions to perform polymerase chain reaction was determined. Ascertaining standardised condition is important as to find out at which different combinations of time and temperature, we can get optimal results. To perform this, different annealing temperatures based on the average of Tm of primers were done. The gel was run of the amplified DNA and the one with the most promising results was selected. Those conditions were applied to rest of the samples.



Lane 1: Sample 1 at annealing temperature of 40°C (Healthy);
Lane 2: Sample 1 at annealing temperature of 42°C (Healthy);
Lane 3: Sample 1 at annealing temperature of 44°C (Healthy);
Lane 4: Sample 1 at annealing temperature of 46°C (Healthy);
Lane 5: Sample 1 at annealing temperature of 48°C (Healthy);
Lane 6: DNA step-up ladder (100bp);
Lane 7: Negative control

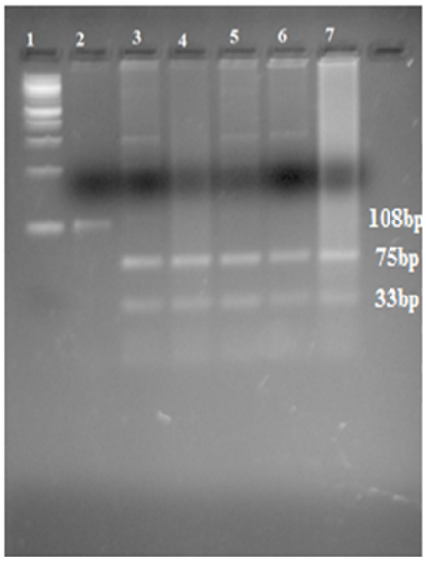
Figure 6: Agarose gel electrophoresis (1.2%) of a single sample at different annealing temperatures to find the optimal one (Expected band size = 108bp)

Thus, the annealing temperature of 44℃ was selected for further amplification of rest of the samples.



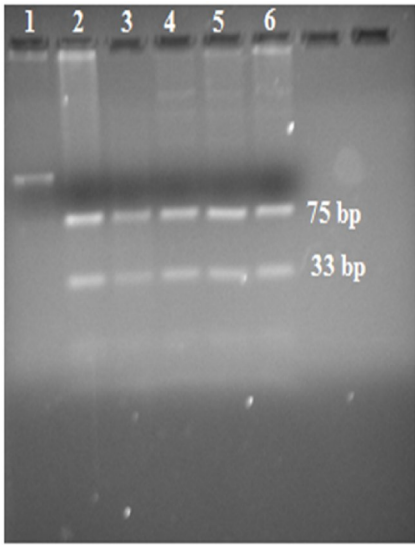
Lane 1: Sample 1 which is undigested DNA (Healthy);
Lane 2: Sample 2 of CC genotype (Healthy);
Lane 3: Blank
Lane 4: Sample 3 of CG genotype (Healthy)

Figure 7: Agarose gel electrophoresis (3.5%) of two samples after restriction digestion (Expected band size: CC= 75bp, CG= 33bp; GG= 3 bands of 108bp, 75bp, and 33 bp)



Lane 1: DNA step-up ladder of 100 bp;
Lane 2: Sample 2 is undigested DNA (Healthy);
Lane 3: Sample 3 of CC genotype (Healthy);
Lane 4: Sample 4 of CC genotype (Healthy);
Lane 5: Sample 5 of CC genotype (Healthy);
Lane 6: Sample 6 of CC genotype (Healthy);
Lane 7: Sample 7 of CC genotype (Healthy)

Figure 8: Agarose gel electrophoresis (3.5%) of five samples after restriction digestion (Expected band size: CC= 75bp, CG= 33bp; GG= 3 bands of 108bp, 75bp, and 33 bp)



Lane 1: Sample 1 is undigested DNA (Asthmatic);
Lane 2: Sample 2 of CC genotype (Asthmatic);
Lane 3: Sample 3 of CC genotype (Asthmatic);
Lane 4: Sample 4 of CC genotype (Asthmatic);
Lane 5: Sample 5 of CC genotype (Asthmatic);
Lane 6: Sample 6 of CC genotype (Asthmatic)

Figure 9: Agarose gel electrophoresis (3.5%) of five samples after restriction digestion (Expected band size: CC= 75bp, CG= 33bp; GG= 3 bands of 108bp, 75bp, and 33 bp)

After the completion of amplification and digestion of all the samples, both control and asthmatic, genotypic frequencies in both the samples were tabulated. Out of total sample, strength, frequency of different genotypes was found as:

GENOTYPE	ASTHMATIC SAMPLE	CONTROL SAMPLE
Samples with CC genotype	86	89
Samples with CG genotype	88	4
Samples with GG genotype	0	0
Total samples	94	93

Table 3: Result of genotyping of control and asthmatic samples.

STATISTICAL ANALYSIS:

The values from the control sample were used to find whether the population is in Hardy Weinberg Equilibrium. Online software was used to compute the value. The calculator gave a P value of 0.8321 which means that the data is significant and follows Hardy Weinberg Equilibrium.

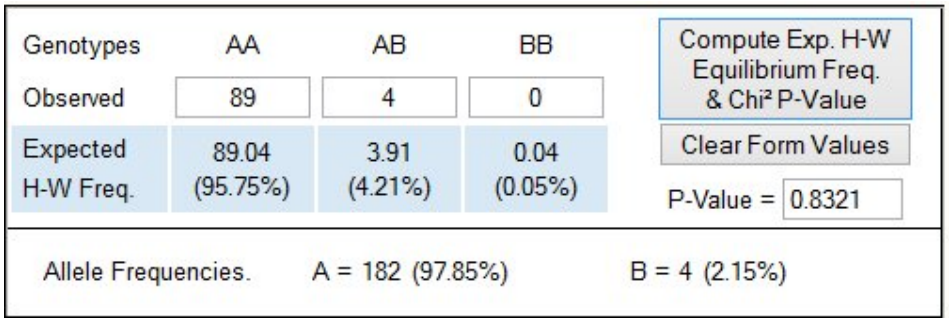


Figure 10: Result of the Hardy Weinberg Equilibrium Calculation

Based on the values of genotypic frequency, allelic frequencies of C and G allele in North Indian population were found.

Calculation of frequency of occurrence:

Control sample

Total = 93

C = 89+89+4

= 182/186 = 97.85%

G = 4

= 4/186 = 2.15%

Asthmatic sample

Total = 94

C = 86+86+8

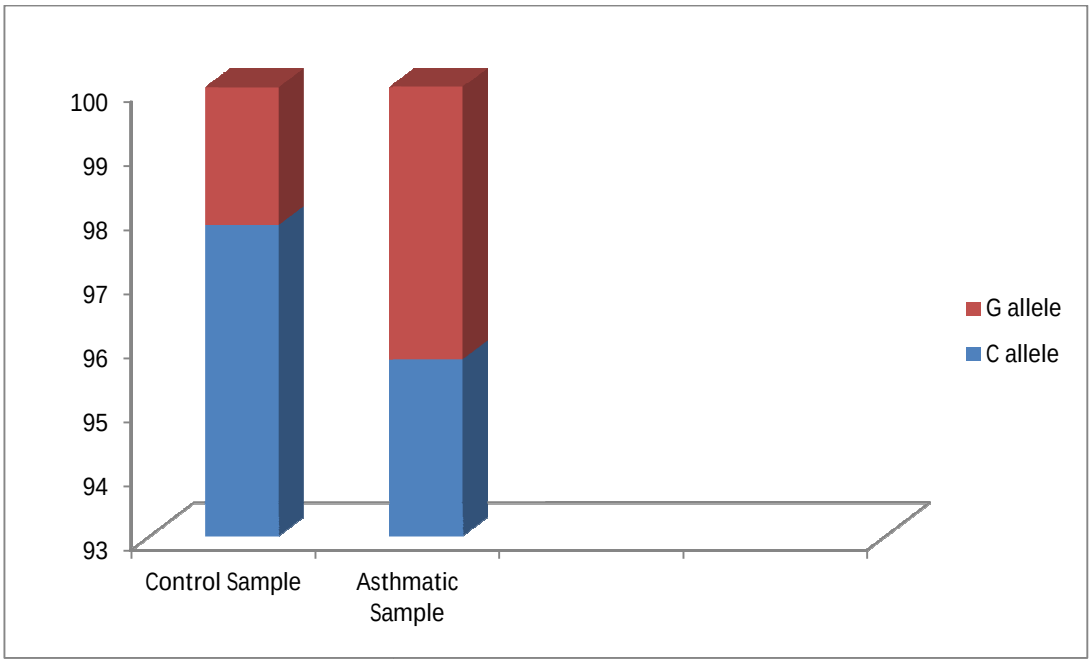
= 180/188 = 95.75%

G = 8

= 8/188 = 4.25%

SAMPLE	GENOTYPIC FREQUENCY			ALLELIC FREQUENCY	
	CC	CG	GG	C	G
ASTHMATIC SAMPLE	86	8	0	180 (95.75%)	8 (4.25%)
CONTROL SAMPPLE	89	4	0	182 (97.85%)	4 (2.15%)

Table 4: Genotypic and Allelic frequency in asthmatic and control samples of North Indian population.



Graph 1: Allele occurrence in percentage

Odds Ratio

The allelic frequency of C and G in both control and asthmatic samples were used to find out the Odds Ratio to determine whether there is any association between occurrence of an allele and asthma in North Indian population. Online software was used to compute Odds ratio. The calculation gave following result:

Odds ratio: **2.0222**

95% CI: **0.5983 to 6.346**

P value: **0.2571**

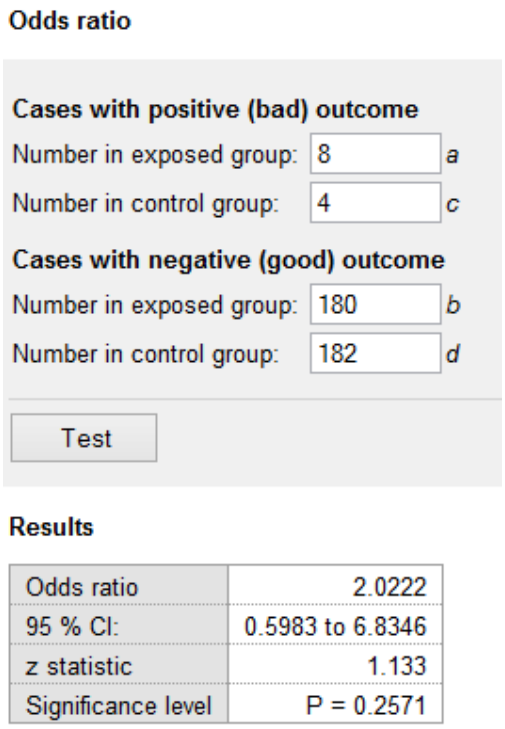


Figure 11: Results of odds ratio as obtained from online calculator.

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 polymorhism 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 2.0222 which means rs17587319 C/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 (controla and asthmatic). 95% CI in our study came out to be 0.5983-6.8346 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 0.2571 which again indicates there is no significant difference in results. Thus, our results indicate that there is no statistical significance difference in between both the samples.

DISCUSSION

Asthma is a chronic inflammatory diseases of airways and both environmental and genetic factors have been found to play a role in its occurrence. Various molecular components such as cytokines and autophagy related genes have been studied and their positive role has been identified. Atg5 is a key component of autophagy pathway and helps in the formation of autophagosome formation. Its autophagy independent functions such as lymphocyte development and in survival and proliferation of both B and T lymphocyte has been found in various studies. In several recent studies, certain genetic polymorphism of this gene present in the promoter and intron region, has been found to show positive association with occurrence of childhood asthma. In this study, our aim was to find the potential role of Atg5 polymorphism in association with asthma in North Indian population. The polymorphism selected (rs17587319 C/G) has been associated with Parkinson's disease and Crohn's disease. We performed a case control study using samples from healthy and asthmatic individuals. There are several method to genotype samples. For our study we selected Polymerase Chain Reaction – Restriction Fragement Length Polymorphism assay to determine genotypes because of its simplicity and efficiency. Optimisation of the Polymerase Chain Reaction – Restriction Fragement Length Polymorphism for rs17587319 C/G was done by determing the optimal annealing temperature and other standard conditions for the assay. The optimal annealing temperature found was 44℃. The frequency of occurrence of this allele in Asian population (Chinese Han population) as mentioned in Table 1 is 0.867 for C allele and 0.133 for G allele. In our study, we found the occurrence of this polymorphism in North Indian population in control samples as 0.9785 for C allele and 0.0215 for G allele and in asthmatic samples as 0.9575 for C allele and 0.0425 for G allele. We can observe that the occurrence of wild type allele C is way more than the mutated allele G compared to the frequency provided for Asian population. Based on our results, we clearly observe that the variation in the frequency of occurrence of the genetic polymorphism (rs17587319 C/G) of Atg5 gene, between healthy and individual individuals of North Indian population is very less.

CHAPTER 5

CONCLUSION

The aim of this study was find out the occurrence of the SNP in the North Indian population and determine whether its occurrence played a role in predisposing an individual to Asthma. This would have helped to find a potential molecular marker for diagnosis of Asthma. The occurrence of wild type allele is quite high compared to mutated allele which correlates with the frequency pattern found in other Asian population. We found no significant association of Atg5 polymorphism (rs17587319 C/G) with increased asthma risk. Thus, based on our results, we may conclude that this indicates that Atg5 (rs17587319 C/G) does not play a substantial role in genetic predisposition towards asthma in the North Indian population. Increasing the number of samples in the study is required to validate this conclusion.

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