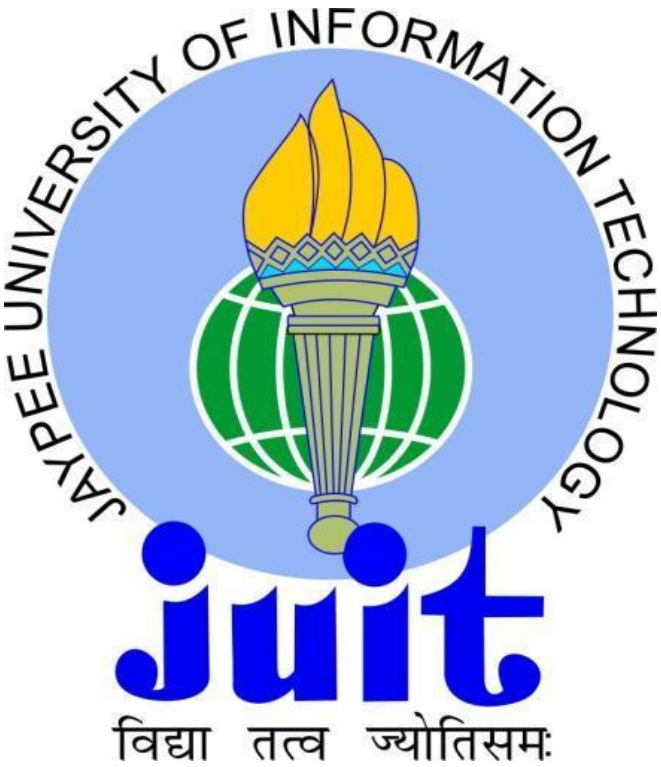
Role of SNP rs510432 of autophagy related gene ATG5 in Asthma

Dissertation submitted in partial fulfillment of the requirement for the degree of

Masters of Technology
In
Biotechnology

Submitted by: Ashish Chauhan (162555)

Under the supervision of: Dr. Harish Changotra



Department of Biotechnology and Bioinformatics
Jaypee University of Information Technology Waknaghat Solan-173234, Himachal Pradesh

CONTENTS

CERTIFICATE

This is to state that the work titled "**Role of SNP rs510432 of autophagy related gene ATG5 in Asthma**", submitted by "**Ashish Chauhan**" in partial indulgence for the award degree of **Masters of Biotechnology** of **Jaypee University of Information Technology, Waknaghat** has been conceded out under my direction. This work has not been submitted moderately or exclusively to any other Institute for the honor of this or any other degree of diploma.

Name of Supervisor:

Harish Changotra, Ph.D.

(Associate Professor)

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology

Waknaghat Solan, Himachal Pradesh 173234

ACKNOWLEDGEMENT

All praise belongs to the almighty lord to whom I thank for the strength, courage and perseverance bestowed upon to me to undertake the course of the study. I express our sincere gratitude and indebtedness to my guide Dr. Harish Changotra for his exemplary guidance, valuable feedback and constant encouragement throughout the duration of the project. I am highly grateful to him for providing me the opportunity to do my research work in my interest area. Thank you sir for being a wonderful supervisor.

I am also highly thankful to Mr. Sanjay Singh, Ms. Avni Vij and Ms. Ambika Sharma for their constant support and motivation during my project work for their continuous guidance, inspiration, presence and blessings. They have always helped me throughout my work. I am highly grateful to them.

Very special thanks to almighty, my parents for their constant moral support and their encouragement that helped me throughout my work

ASHISH CHAUHAN

DATE:

SUMMARY

Asthma, a common respiratory disease characterized by chronic inflammation of bronchioles and lungs [1], caused by various genetic as well as environmental factors [2, 3]. Though there may be various genetic cause of asthma, SNP rs510432 of a gene named ATG5 (autophagy-related gene 5) was selected for this study.

There has already been the reporting of significance of ATG5 in the formation of autophagosome during autophagy [4]. In addition to this, SNP rs510432 has also been shown to have association with asthma, mainly childhood asthma [5, 6].

The association of ATG5 gene polymorphism rs510432 with asthma susceptibility among north India population was investigated in this study. Genotyping of single nucleotide polymorphism (SNP) rs510432 of ATG5 gene was performed in 91 asthmatic patients and 90 healthy individuals with no history of asthma, all from the north India region.

Several molecular biology techniques were used to make our work convenient and effective. In this study we, used PCR-RFLP technique to genotype control and case DNA samples.

We observed that the allele G (mutant) of rs510432 SNP was found in 44.5% of cases and 36.6% of control samples, while the wild type allele A of the same SNP was found in 55.5% of cases and 63.3% of control samples. The results obtained indicated towards the role of the SNP rs510432 in the autophagy related ATG5 gene in asthma susceptibility with the heterozygous model.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATION

ABBREVIATION	
Bp	Base pair
CI	Confidence interval
ºC	Degree Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium Bromide
Ml	Microlitre
Mins	Minutes
mM	Millimolar
M	Molar
OR	Odds ratio
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RFLP	Restriction fragment length polymorphism
Secs	Seconds
SNP	Single nucleotide polymorphism
TE buffer	Tris – EDTA buffer
UVR	Ultraviolet rays

CHAPTER- 1

INTRODUCTION

INTRODUCTION

Asthma is an inflammatory disease that affects the respiratory system which causes episodes of breathlessness, wheezing, cough and chest pain [1, 7]. It is a very common disease affecting millions of people worldwide every year. According to World Health Organization, around 250-300 million people are globally affected by asthma, with around 15-20 million people in India including 10-15% children between the age of 5-11 years [8].

The exact cause for asthma is currently not known although it is thought to be induced by various environmental as well as genetic factors [9, 10]. These all factors collectively are responsible for disease prevalence in an individual [11]. We conducted this study on the north Indian population to find out the significance of one such genetic factor responsible for asthma susceptibility.

The objective of this work was to identify potential asthma susceptibility in the SNP rs510432 of autophagy related gene ATG5 in the expectation that this data may show the way to an improved understanding of disease pathogenesis. This experiment is an approach towards the new discoveries about asthma pathology. Mostly aiming upon the genetics of this disease, we are presently, looking into the association of the SNP rs510432 of ATG5 with asthma susceptibility among the north Indian population.

Significance of the study:

In India, around 15-20 million people including 10-15% children between the age of 5-11 years are affected by asthma [8]. The treatments that are provided to the patients are not very efficient, as the disease is no completely treatable [1, 7].

Scientists are trying to find out the exact reason/cause of asthma susceptibility. In order to do so, various environmental as well as genetic factors needs to be further explored to open up the way for ultimately curing asthma. Hence more and more genetic studies on asthma are essential for cracking up the disease cure.

In this study we chose a gene called as autophagy related ATG5 gene for our study. This gene has been previously linked with asthma in various ways [4]. DNA samples from asthmatic as well as non-asthmatic patients were taken. These samples were then further subjected to PCR RFLP genotyping. After the genotyping was done, various statistical analyses were conducted using various online tools [12, 13, 14, 15]. The autophagy related 5 gene (ATG5) was selected because ATG5 is necessary for autophagosome formation [3], and has already been shown to have association with childhood asthma [4]. To test this we are genotyping tagging single nucleotide polymorphisms (SNPs) in 91 asthmatic patients and 90 non-asthmatic healthy individuals with no history of asthma.

CHAPTER- 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Asthma

Asthma is a long term disease which is typically characterized by chronic airway inflammation. Person of all ages can be vulnerable to asthma [1].

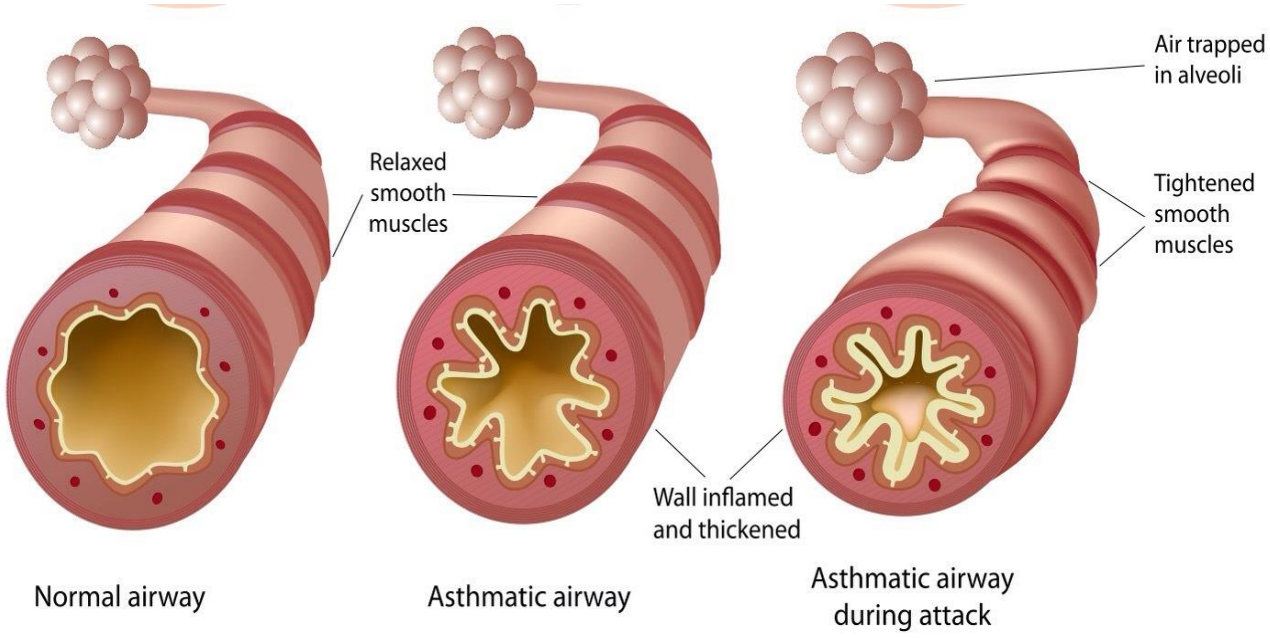


Figure 2.1: Bronchiole pathway of a normal individual vs. asthmatic patient.

At present time, there is no proper treatment for asthma, but its symptoms can be somehow restricted with suitable medications or medical treatment, self-awareness and by avoiding the patient's contact with those irritants and allergens that can set off an asthmatic attack [1, 7].

During asthma the person's bronchial airways becomes inflamed, narrow and swell

+

produces extra mucus

The respiratory airway shrinks causing lesser inflow of air into the lungs

2.1.1 Incidence Rate

WHO has estimated that around 235 million people are currently being affected from asthma all over the globe and this number is rising.

In India, approximately 15-20 million people are at present affected from asthma. Rough estimates indicate an occurrence between 10% and 15% in 5-11 year old children [8].

Annually the global death of people due to asthma has reached over 180000. In India, the patient death rate because of asthma is 17.16 per 100000 asthmatic patients.

2.1.2 Symptoms

Symptoms of asthma generally include various factors such as:

- Cough, mainly at night
- Tightening of chest
- Breathlessness
- Wheezing
- Trouble in sleeping

These are all the common symptoms that can tell us about a person if he is suffering from asthma or not [16].

Figure 2.2: Common symptoms of asthma.

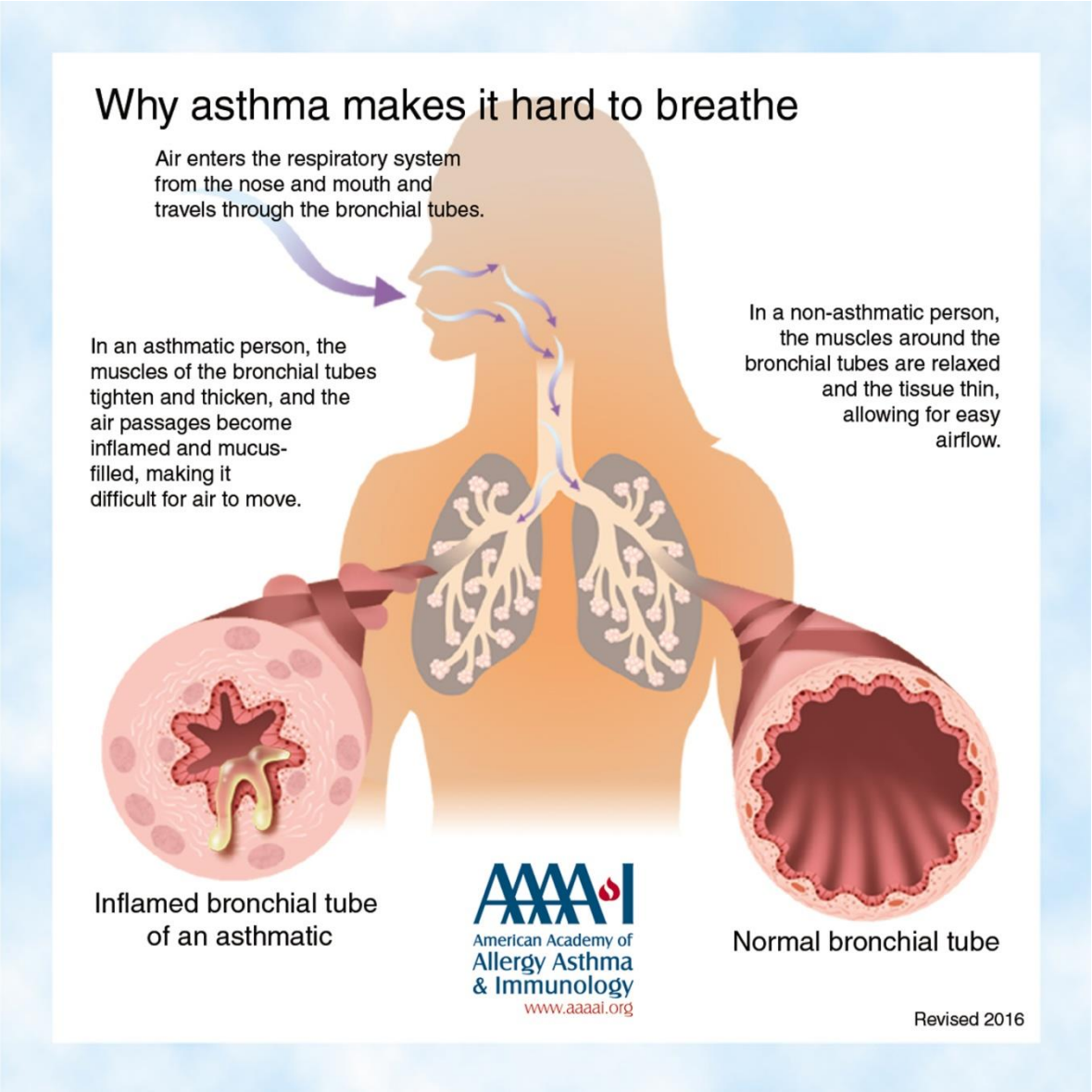


Figure 2.3: Response of body when a person is exposed to various allergens which lead to asthmatic attack.

2.1.3 Causes of Asthma

There is no complete and detailed knowledge about the elementary cause of Asthma. Asthma can be developed through the combination of various genetic factors and exposure

to several environmental factors which can enter the body by inhaling them where they interact with the airway tract of the patient [16].

Some of these factors can be:

- Allergy causing materials
- Environment allergens (example: pollen grains etc.)
- Smoke from tobacco
- Air pollution in surrounding environment
- Genetic causes

The topic “Genetic causes of asthma” is very diverse in itself. Various genes have been reported in the literature which can directly or indirectly affect asthma or triggers its common symptoms [9, 11].

2.2 Autophagy

A lot of molecular mechanisms occur simultaneously inside a cell. These mechanisms work together in order to maintain the normal functioning of the cell which finally leads to the maintaining of the cell integrity [17, 18].

Autophagy is one of the key mechanisms concerned with the immune responses, inflammations as well as immunity against viruses [19]. Autophagy naturally, regulates the destruction of several internal components of cell cytoplasm. Its aim is to remodel the internal structure of cell for cell differentiation and recycling the cell’s macromolecules [20].

One of the most important roles of autophagy is to play a cleaning role in destroying misfolded proteins or mass of proteins, clearing damaged cellular organs, such as peroxisomes, mitochondria, endoplasmic reticulum etc. In addition to this it is also involved in killing the intracellular pathogens from the cell [21].

2.2.1 Molecular mechanism of Autophagy

Autophagy is primarily activated by intervention of two very important conjugation systems: 2 ubiquitin systems are present that play a major role in autophagy [22, 23] starting from the conjugation of Atg5–Atg12 and ends at towards the processing step of LC3.

Conjugation of ATG5–ATG12 protein: In this system, Atg12 is activated by Atg7 which functions as an E1 ubiquitin activating enzyme in an ATP-dependent manner. It binds to one of its residue which is known as carboxyterminal glycine residue. Atg12 now gets transported to Atg10 that creates covalent bonding of Atg12 with position 130 in lysine of Atg5. Atg5–Atg12 molecule now pairs up with the Atg16L dimers which lead to the formation of a multimeric Atg5– Atg12–Atg16L complex that functions in the phagophore extension. [21, 24].

Lipidation of LC3 protein: The second step involved in the formation of autophagosome is the dispensation of a protein which is associated with microtubule's light chain 3. LC3B is synthesized by a mammalian homologue of Atg8. It is produced in almost all types of cell. Upon start of process of autophagy, it is cleaved by Atg4 protein which give rise to the formation of LC3B-I. LC3B-I is now activated by Atg4 in an ATP-dependent manner. This LC3B-I (activated) is now migrated to another protein named Atg3 (E2-like carrier protein) and then conjugation of phosphatidylethanolamine occurs to the carboxyl glycine in order to form LC3B-II. Integration & recruitment of LC3B-II with the

phagophore is totally reliant on Atg5–Atg12 complex. Internal and external surfaces of the autophagosome are found to have LC3B-II. The major function of LC3B-II is in the selection of cargo for degradation & membrane's hemifusion [21].

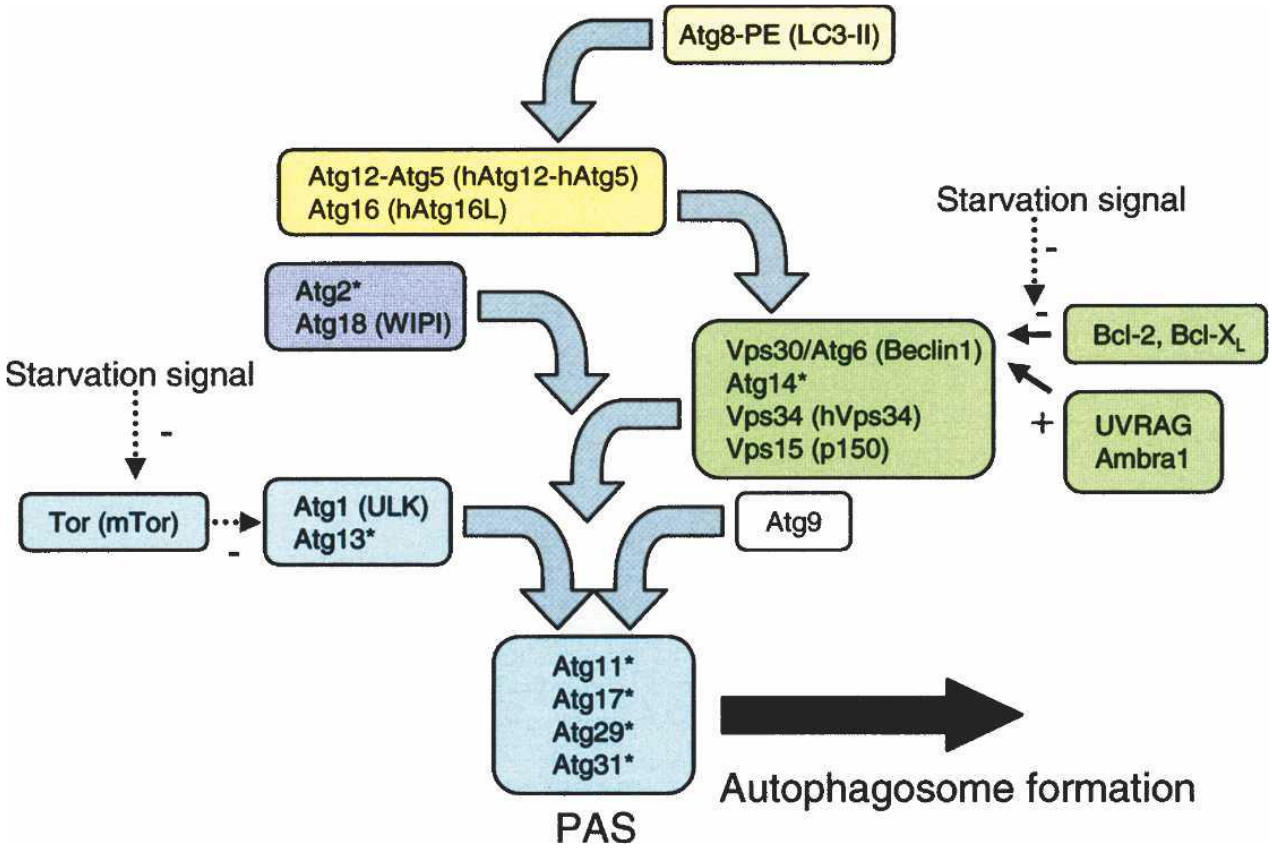


Figure 2.4: All the Atg proteins and their interdependence for localization of PAS [22].

The above figure represents the analysis of hierarchical classification which was originally performed in yeast [25].

- Arrows with blue colour specifies the interdependence of Atg for targeting of PAS.
- Arrows with black colour represents +ve and -ve regulation.
- Parentheses represent the nomenclature of mammals.

- Symbols such as asterisks (*) represents various factors which have already been analyzed (exclusively in yeast).

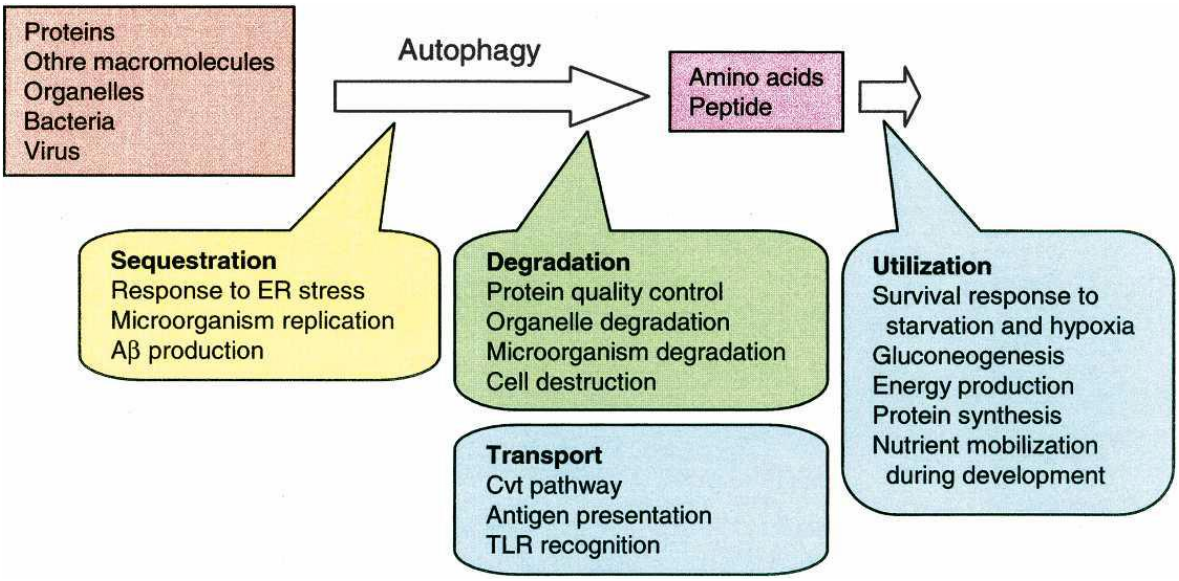


Figure 2.5: Autophagy and its functions at each and every step in the formation of autophagosome [22].

It has briefly been described in the following figure.

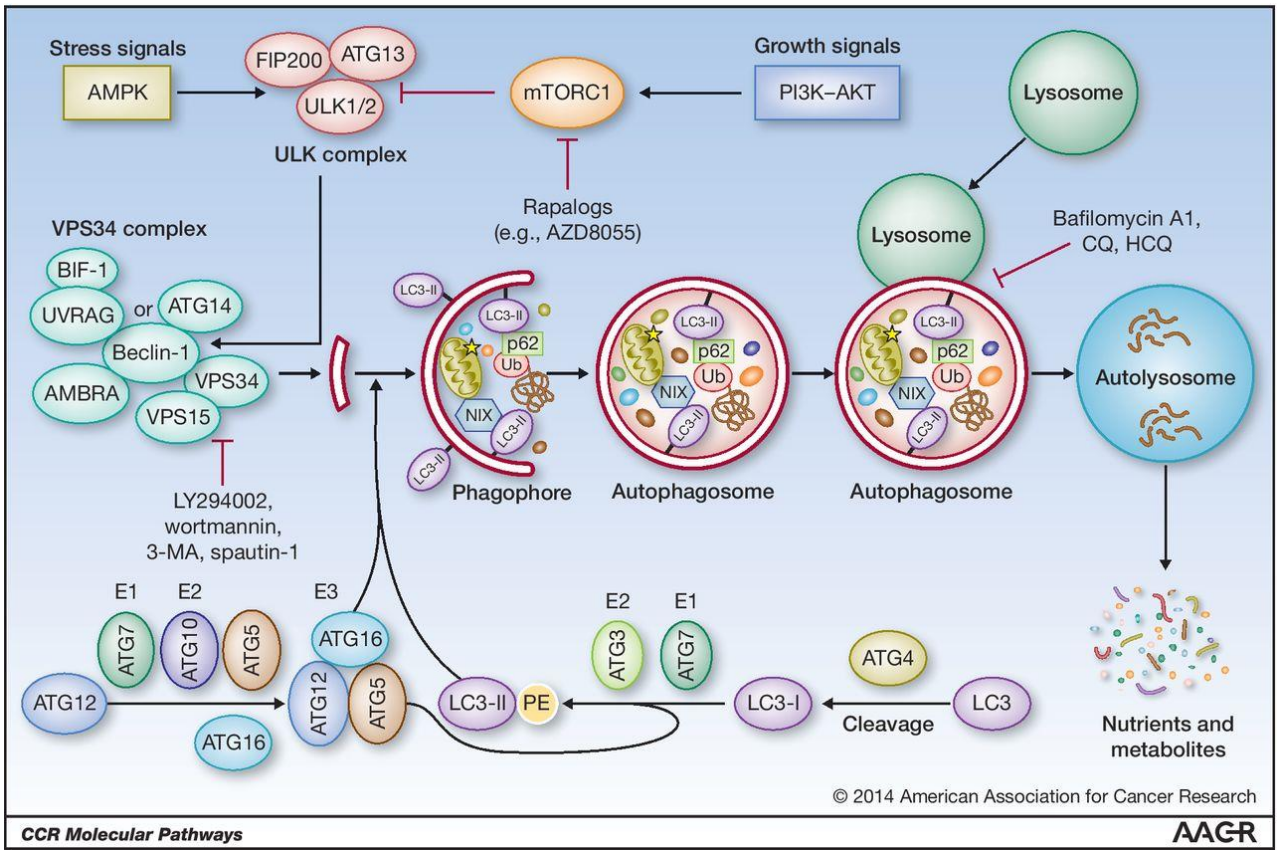


Figure 2.6: Molecular mechanism depicting steps of autophagy from the cconjugation of ATG5–ATG12 protein & lipidation of LC3 protein to the formation of autophagosome [26].

2.2.2 Autophagy related ATG5 Gene

ATG5 is one of the major gene involved in autophagy. It is also known by various other names such as:

- **ASP**
- **APG5**
- **APG5L**
- **APG5-LIKE**

This gene synthesizes a protein called as ATG5 protein. This protein is concerned with in various cellular processes, which includes autophagosome vesicle formation, post quality control check of mitochondria following oxidative damage, opposite regulation of the innate immune response against viruses, growth and proliferation of lymphocyte, antigen presentation of MHC II [27, 28, 29, 30].

2.2.3 Location of ATG5

Cytogenetic Location: 6q21- It is the long (q) arm of chromosome 6 (position 21).
Molecular Location: chromosome 6 (bp from 106,184,476 to 106,325,820)

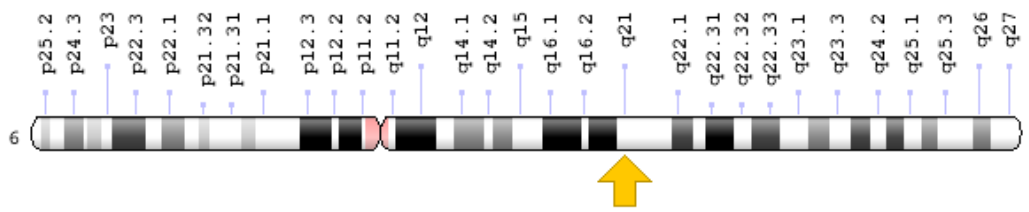


Figure 2.7: Chromosome number 6 of *Homo sapiens* depicting the location of ATG5 gene [NCBI].

[Source: http://www.genecards.org/cgi-bin/carddisp.pl?gene=ATG5]

2.3 Relation between ATG5 and Asthma

We have focused our research on ATG5 gene in this study. This is because this gene has been linked with the pathogenesis of asthma in certain ways.

Some of them are as follows:

Extreme synthesis of Reactive Oxygen Species is considered to take part in the occurrence of asthma. Exhaled level of ROS is shown to positively influence the disease harshness [31, 32].

- Autophagy is very vital for innate immunity defense in opposition to bacteria and virus infections [33].

- Expression of mRNA of ATG5 is increased in human nasal epithelial cell when a patient suffers from a severe asthma attack [32].

- Several genetic variants in ATG5 gene, along with a useful promoter SNP (rs510432) plays a very significant role in the association of Asthma [32, 34].

Human rhinovirus (HRV)	HRV2 stimulates autophagy in Hela, 293T cells; HRV2 viral replication is dependent on autophagy
Respiratory syncytial virus (RSV)	RSV stimulates autophagy in murine dendritic cells; post RSV infection cytokine production in murine dendritic cells dependent on autophagy; autophagy facilitates dendritic cell and T-cell interaction for cytokine production in mice
Coronavirus	Nonstructural protein 6 (ns6) induces autophagy
Adenovirus	Adenoviral protein E1B19K interacts with beclin 1 and induces autophagy
Influenza A virus	H5N1-infected cadaveric lungs show increased autophagosomes; induction of autophagy and autophagic cell death in human A549 cells and murine lungs by H5N1; H5N1 induced ALI severity decreased by inhibition of autophagy in murine ALI model

Table 2.1: Autophagy and respiratory viruses linked with asthma [35].

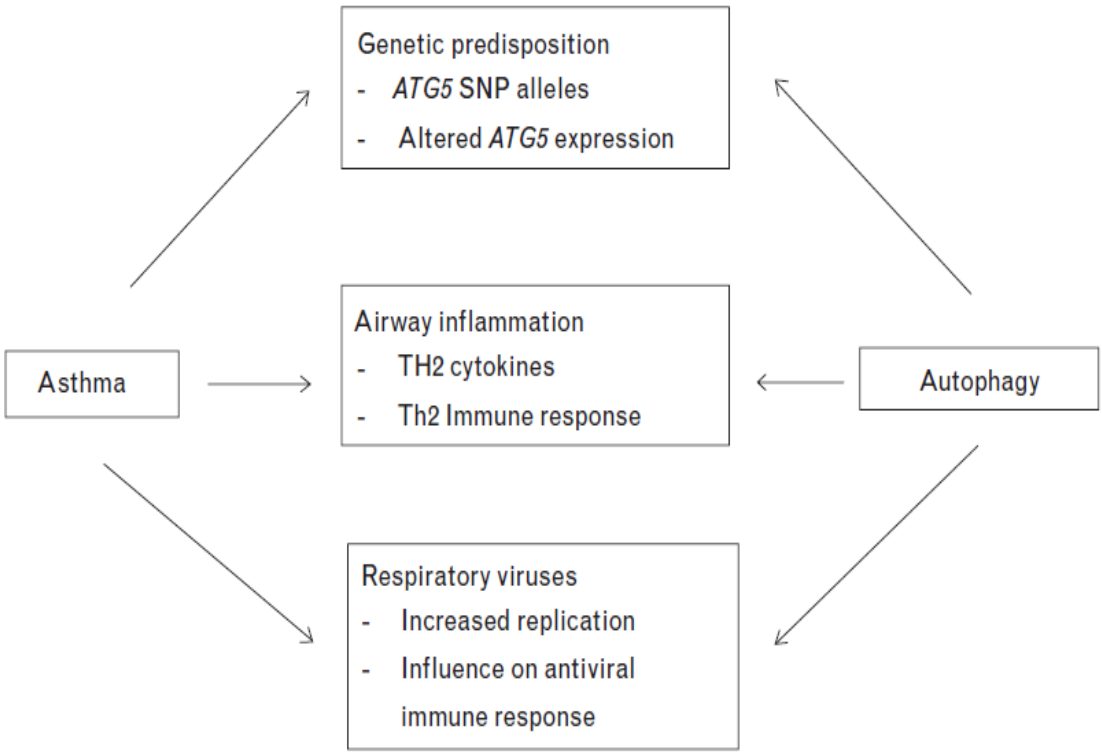


Table 2.2: Asthma and its potential relation with autophagy [35].

2.4 Significance of rs510432 SNP of Autophagy Related Gene (ATG5)

- **rs510432 SNP of Autophagy Related Gene** is located in the promoter region of the gene [36].

- The SNP is strongly associated with asthma occurrence [32, 34].

- The SNP has not been studied for its role in Asthma in North Indian population till date.

2.5 Single Nucleotide Polymorphism

Single Nucleotide polymorphisms are a single change in the nucleotide of a DNA sequence in a genome of an organism. In a population they are present at an incidence rate of more than 1%. These are present in the coding regions or non-coding regions with a single base variation that can generate a replacement of an amino acid in the correspondent proteins. These nonsense variants make up the majority of identical groups of SNPs. Through the information provided by the utilization of SNP genotyping technique, researchers can examine those genes that are associated with any particular disease and finally examine the genetic makeup of an individual.

SNPs can be present in coding and non-coding region of the genome. Hence, proper records of diverse variations in a lot of genes and the examination of their effects can show the way to the improved perception of their effect on the function of gene.

We have chosen one SNP (rs510432) in order to genotype its frequency in the north Indian population by using PCR-RFLP method and to assess the association of this SNP with asthma susceptibility.

2.6 SNP Genotyping by using PCR RFLP

Genotyping of SNP is typically achieved by several ways which can comprise of hybridization, allele definite PCR, endonuclease cleavage, sequencing of DNA strand and ligation of nucleotide. All of these methods have some advantages as well as some disadvantages. To have a restriction site in the DNA template is the most important thing in order to carry out the genotyping of SNPs by the use of PCR-RFLP. There should always be restriction site for the restriction enzyme at the SNP position in the DNA strand. This is the most simple and economical technique and can be usually in small research laboratories. The PCR-RFLP method always allows the very simple way of pointing out small mutations such as SNPs.

PCR-RFLP includes these following steps:

- **PCR:** DNA amplification by the use of taq polymerase.
- **RFLP:** Recognize the genotype of the amplified DNA with the SNP.

2.7 Polymerase chain reaction/PCR

The polymerase chain reaction or the PCR is a method of DNA amplification or amplification of a particular part of the DNA strand which is very frequently used in molecular biology laboratories. This technique is dependent on the cyclic heating which starts with the repetitive cycles of heating up and cooling down of reaction mixture. Main aim of PCR is to ultimately attain the melting and amplification of DNA strand with the help of thermostable enzymes.

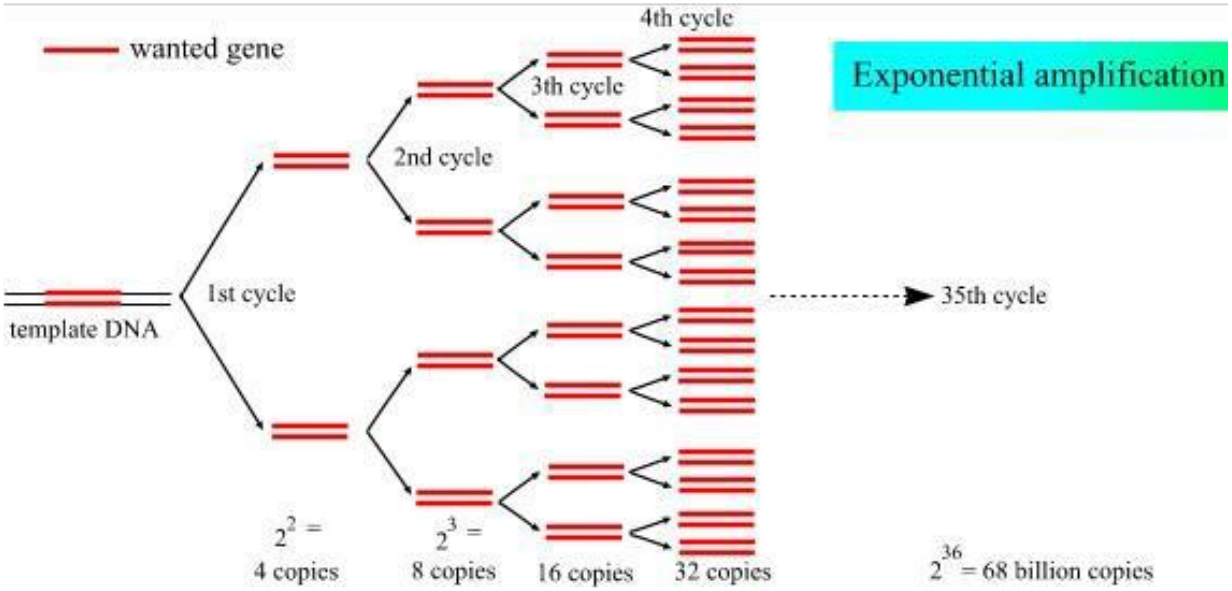
On the whole there are only three key steps in the polymerase chain reaction, which can be repeated for infinite number of cycles. It is performed on a thermocycler/PCR machine, which can increase or decrease temperature of the PCR tubes in a very short interval of time. These tubes contain the reaction mixture which is essentially required for the PCR.

2.7.1 Detailed description of the 3 steps of PCR

- **DENATURATION:** This is the first step of PCR. It involves heating the reaction mixture to a temperature of around 94ºC for 15-20 seconds. Its outcome is the melting of DNA via breakage of hydrogen bonds between the complementary bases, hence finally obtaining 2 ssDNA molecules from 1 dsDNA molecule.

- **ANNEALING:** As the temperature of reaction mixture reaches down to the annealing temperature of the DNA, the DNA primers which are complementary to the ssDNA anneal themselves together. This sticky and complementary region of the DNA comes together and finally forms a very stable bond.

- **EXTENSION:** This is the final stage of PCR. It involves the use of an enzyme called as Taq polymerase which functions best at an ideal temperature of 72ºC. The Taq polymerase is a thermostable enzyme that is basically a DNA polymerase which can add dNTPs to the ssDNA molecule from the position of 5' to 3' and while interpreting the DNA strand from position 3' to 5'. Initiation of the synthesis of DNA starts from the 3'-hydroxyl end of every primer. Major role is played by Taq polymerase in extending the DNA primers by combining the dNTPs that are complementary to the DNA strand.



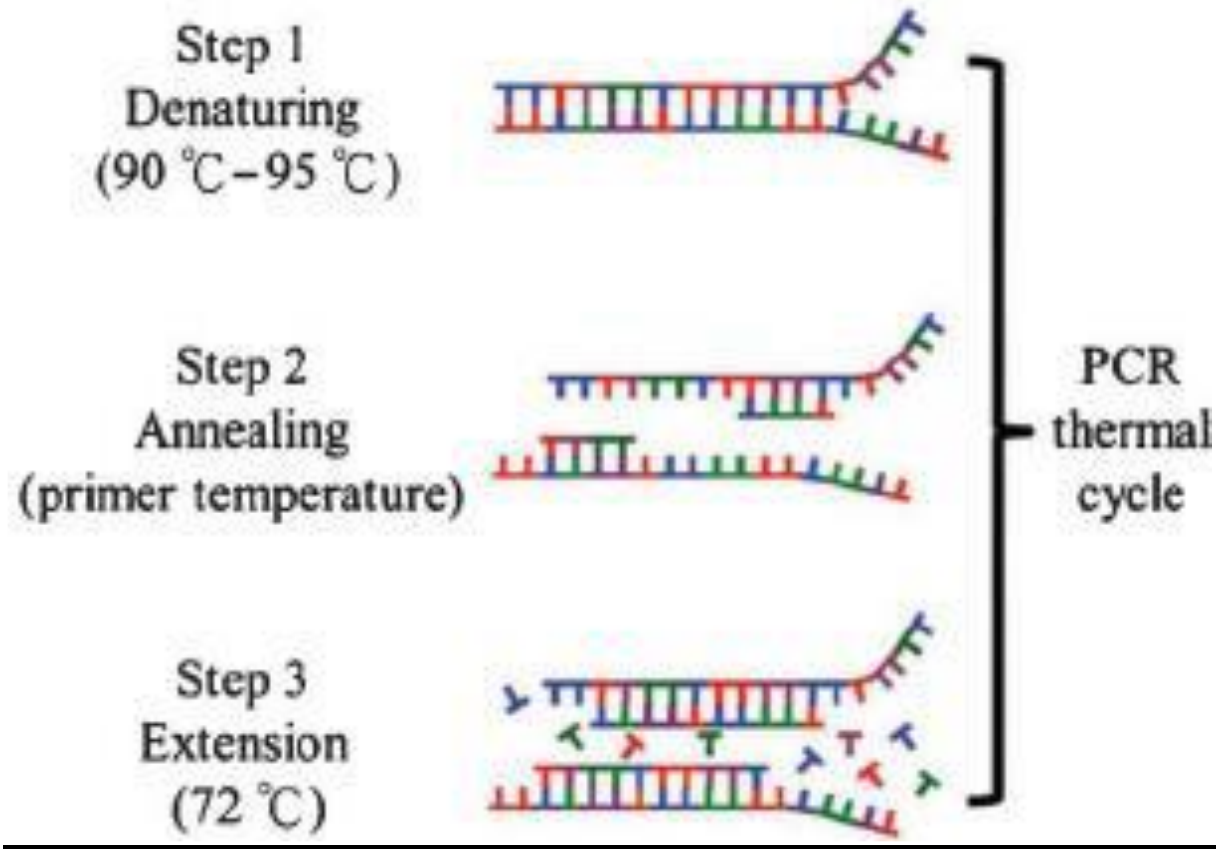


Figure 2.8: Major steps in the PCR Amplification.

2.8 Restriction fragment length Polymorphism (RFLP)

The basic principle of RFLP is that a single change in the base pair (bp) in a DNA strand can easily create or terminate the sites that are being identified by various specific restriction enzyme(s). This change will always lead to the distinction between the persons in the formed fragment's length from similar location of genome. A sole base pair (bp) alteration i.e. SNPs are very noticeable genetic markers by the use of RFLP since a site that has been mutated cannot be cleaved by the same enzyme anymore. Hence two chromosomes are easily distinguishable from each other on the basis of RFLP if they consist of such mutations, which occur due to the existence of a particular cleavage site in the DNA molecule.

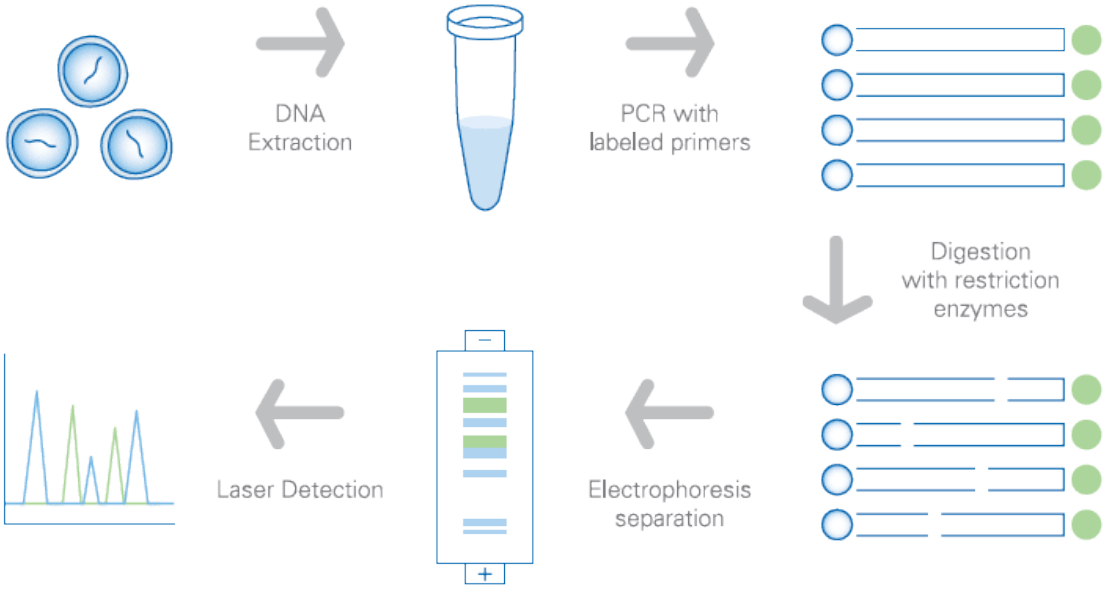


Figure 2.9: Different steps of a common RFLP technique.

CHAPTER - 3

OBJECTIVES

OBJECTIVES

In this study, the genetic relationship between the polymorphisms of autophagy related gene ATG5 (rs510432) and a common respiratory disease asthma was studied which may be capable of using as a prognostic marker for asthma susceptibility in the North Indian population.

Objectives of this study are:

- To collect the blood sample from asthma and healthy individuals alongside the DNA isolation from the same.

- To standardized the PCR-RFLP condition for rs510432 polymorphism of ATG5 gene.

- To identify that whether any genetic association is present between the selected SNP for asthma susceptibility or not by the exploitation of statistical analysis.

CHAPTER - 4

MATERIALS AND METHODS

MATERIALS AND METHODS

4.1 Population Study

The population group which was taken under study comprised of 91 Asthma patients till now who had already referred to a medical college for their treatment i.e. Doaba Collage, Jalandhar. Along with 91 asthmatic patients, 90 healthy samples of individuals with no previous reported history of Asthma were also included as control samples.

4.2 Sampling

3 ml venous blood sample was collected from the patients and healthy subjects in EDTA coated tubes. Blood samples are stored at -20^{O}C till further use.

4.2.1 Isolation of DNA from whole blood sample

1. Blood sample of 300 µl quantity was uniformly mixed along with RBC lysis buffer of exactly 1000 µl of in a autoclaved centrifuge tube of 2ml. This mixture was kept undisturbed for incubation on rocker shaker at room temperature for 30-50mins till the point it turned shiny.
2. A white pellet was obtained after the centrifugation was done for 1 min at13000 rpm.
3. All the obtained supernatant was thrown away/discarded & the pellet obtained was carefully resuspended in buffer media i.e. TE Buffer (pH 8.0) of 300 µl quantity by vortexing in a vortex machine.
4. 20 µl SDS solution (10%) was supplemented in the mixture, and the mixture was incubated for 30mins at 56^{O}C in the water bath.
5. 160 µl ammonium acetate solution (7.5M) was added and it was uniformly and forcefully mixed well by using vortex machine for duration of 1min.

Proteins were separated as a pellet in the bottom of the tube after centrifugation was done at 13,000 rpm for 15mins.

6. Ethyl alcohol (chilled) was added to the supernatant in the quantity double than that of the volume of the supernatant. The tube was slowly and gently rocked for 3-4 times to allow the genomic DNA to get completely precipitated.
7. Centrifugation was again performed for the duration of 10mins at 13,000 rpm in order to pellet down the genomic DNA. The washing of pellet was done with 150 µl of ethyl alcohol (70%) & was air dried at RT for 9-10min.
8. DNA pellet was completely dissolved in 60 µl of 7.3pH TE Buffer via incubation for 10mins at 65^{O}C. Storing of DNA was done at -20^{O}C for future use.
9. The genomic DNA that has been isolated was visualized on 0.8% agarose gel and it was quantified by using NanoDrop.

4.3 Mutagenic Primer Designing

Both forward primers as well as the reverse primers were manually designed for the SNP rs510432 of autophagy related ATG5 gene. It was kept im mind that no self complementary binding takes place in the primers in order to avoid false results or no results.

The primers formed were:

ATG5 SNP	Type	Primer sequences 5'– 3'	Size of amplified DNA (bp)	Restriction enzyme	Genotypes (bp)
rs510432 A/G	promotor variant	**Forward primer** $A_{(15)}C_{(15)}$TCCAACAAAGTAGAGAA GAAGATCATAT **Reverse primer** TCTACCCTCTTCTGAGAATCTTG	260	*NdeI* 5′...CA▼TATG...3′ 3′...GTAT▲AC...5′	**GG**-205, 55 **GA**-260, 205, 55 **AA**-260

Table 4.1: Forward and reverse primers used for this study along with the Restriction enzyme.

4.4 Genotyping SNP rs510432

Polymerase chain reaction was carried out for the total volume of 12.5µl for the SNP rs510432 of autophagy related ATG5 gene.

The annealing temperature for the primers was kept 57ºC as per the gradient PCR. Total volume of reaction mixture was 12.5µl. Total time of the PCR reaction was around 1 hour and 35mins.

All the components utilized in the reaction mixture are mentioned in the table below:

Components of reaction mixture	Reaction component per reaction volume(µl)
Master mix	5µl
Forward(F) Primer	0.25µl
Reverse(R) Primer	0.25µl
DNase free Water	6µl
DNA template	1µl
Total	12.5µl

Table 4.2: Constituents used in PCR reaction for the amplification of DNA.

4.5 PCR Cycling Conditions

PCR cycling conditions utilized during the experiment for DNA amplification are as follows.

STEPS	TEMPERATURE	TIME INTERVAL	NUMBER OF CYCLES
Initial Denaturation	95ºC	2min	
Denaturation	95ºC	30sec	30cycles
Annealing	57ºC	40sec	
Extension	72ºC	30sec	
Final Extension	72ºC	5min	
Final Hold	4ºC	∞	

Table 4.3: PCR cycling conditions for the amplification of genomic DNA.

4.6 Analyzing the PCR products

Analyzing of amplified PCR products was done by using a very reliable method of DNA visualization called as agarose gel electrophoresis. It was conducted on a 1.5% agarose gel containing a dye commonly known as ethidium bromide/EtBr (0.5mg\ml).

100bp DNA ladder was run alongside the amplified PCR products. Electrophoresis was performed for 45-50mins at 150 volts in electrophoresis chamber/unit which was filled with 1X TAE buffer.

Clear DNA bands were seen under the UV GelDoc/U.V. transilluminator.

4.7 Restriction fragment length polymorphism (RFLP)

The resulting amplified DNA products obtained after PCR were cleaved using a particular restriction enzyme to detect the type of genotype in all the samples. The restriction enzyme used was *NdeI*. Reaction conditions of RFLP for the SNP rs510432 is given below in Table 4.

SNP	**Enzyme Used**	**Restriction Site**	**PCR Product(µl)**	**Buffer(µl)**	**Restriction Enzyme(µl)**	**Nuclease Free Water(µl)**
rs510432	*NdeI*	5′...CA▼TATG...3′ 3′...GTAT▲AC...5′	10µl	1.5µl	0.1µl	3.4µl

Table 4.4: Components used for RFLP with restriction enzyme.

rs510432 (A/G)	
Genotype	**Band Size (bp)**
GG	205bp + 55bp
GA	260bp + 205bp + 55bp
AA	260bp

Table 4.5: Band pattern of digested SNP rs510432.

4.8 Statistical Analysis

An online tool (https://www.medcalc.org/calc/odds_ratio.php) was used to perform the statistical analysis and to measure the relationship between the asthma susceptibility and ATG5 gene polymorphism i.e. SNP rs510432 [14, 15].

Fisher exact test was utilized to learn the important differences between the allele & genotype frequencies in the control as well as diseased individuals. (http://www.quantpsy.org/fisher/fisher.html).

If $p<0.05$ observed frequency is well thought-out to be not in equilibrium, then the odds ratios and 95%CI were thought to calculate the risk linked with the variant allele.

The significance of the odds ratio was determined by Z test and $p<0.05$ was measured statistically significant [15].

CHAPTER - 5

OBSERVATIONS AND RESULTS

OBSERVATIONS AND RESULTS

"91 diseased patients suffering from asthma and 90 control subjects" with no history of asthma were subjected to genotyping for the SNP rs510432 in autophagy related A*T*G5 gene. The genotype distribution for the SNP revealed no deviation from the Hardy-Weinberg equilibrium in control population ($p>0.05$).

After the genotyping of the SNP rs510432 was done, it was found out that the allele G (mutant) of SNP rs510432 was found in 44.5% of cases and 36.6% of control samples, while the wild type allele A of the same SNP was found in 55.5% of cases and 63.3% of control samples.

The allelic frequencies for this SNP between various genotypic models are as follows:

- The p-value of heterozygous genotype GA model (p-value = 0.0363, OR=2.1866, 95% CI=1.0510 to 4.5494) suggested the significant association of this genotype with asthma.

- The p-value of homozygous genotype GG model was 0.1571, OR=3.7143 and 95% CI value=0.6033 to 22.8671, which suggested no significant association of this genotype with the asthma susceptibility.

- The allelic model also showed no significant association with asthma as its p-value was 1.3852, OR=1.3852 and 95% CI value was 0.9091 to 2.1107

The genotype model having p-value<0.05 is considered to be showing any significant association with asthma.

Genotype /Allele	Asthma (n/N[%])	Control (n/N[%])	Odds ratio (OR)	95% CI	p-value
rs510432 (A>G)					
AA	14/91[15]	26/90[29]	Ref		
GA	73/91[80]	62/90[69]	2.1866	1.0510 to 4.5494	0.0363
GG	4/91[5]	2/90[2]	3.7143	0.6033 to 22.8671	0.1571
A	101/182[55.5]	114/180[63.3]	Ref		
G	81/182[44.5]	66/180[36.6]	1.3852	0.9091 to 2.1107	0.1294

Table 5.1: Genotype and allele distribution of the ATG5 polymorphism in cases (N=91) & control (N=90).

PCR AMPLIFIED PRODUCT:

rs510432

The amplified DNA product after the PCR was visualized on the UV gel doc system. Following is the gel doc image of the amplified DNA product:

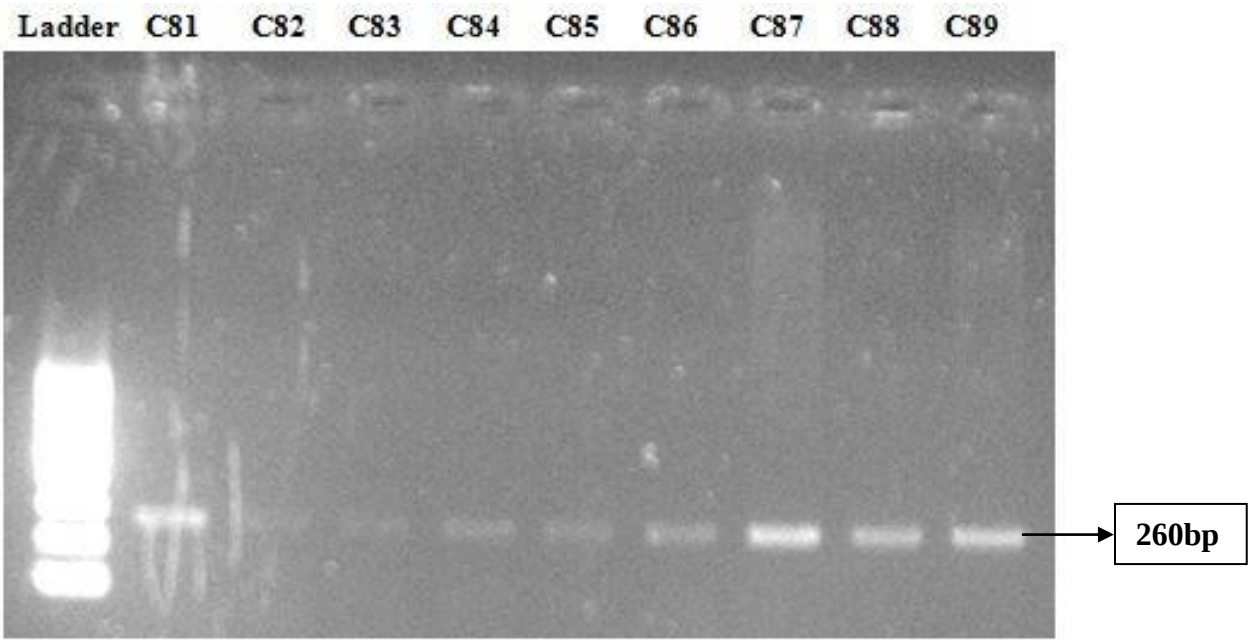


Figure 5.1: DNA amplification of 9 DNA samples at 57ºC annealing temperature.
Lane 1: 100kb DNA ladder.
Lane 2 - **Lane 10:** DNA samples: C81-C89.

The DNA band pattern was obtained as expected. All the DNA bands obtained were of 260bp length in size. This was further confirmed as all the DNA bands were located between the 200kb and 300kb DNA ladder.

RESTRICTION DIGESTION GEL IMAGES

rs510432

The amplified DNA product was kept for overnight incubation after the addition of restriction enzyme *NdeI* in it.

The restriction enzyme cleaved the DNA into different bands, each having different lengths. The length of these DNA products depends upon the genotype of the DNA sample.

The banding pattern found after RFLP was:

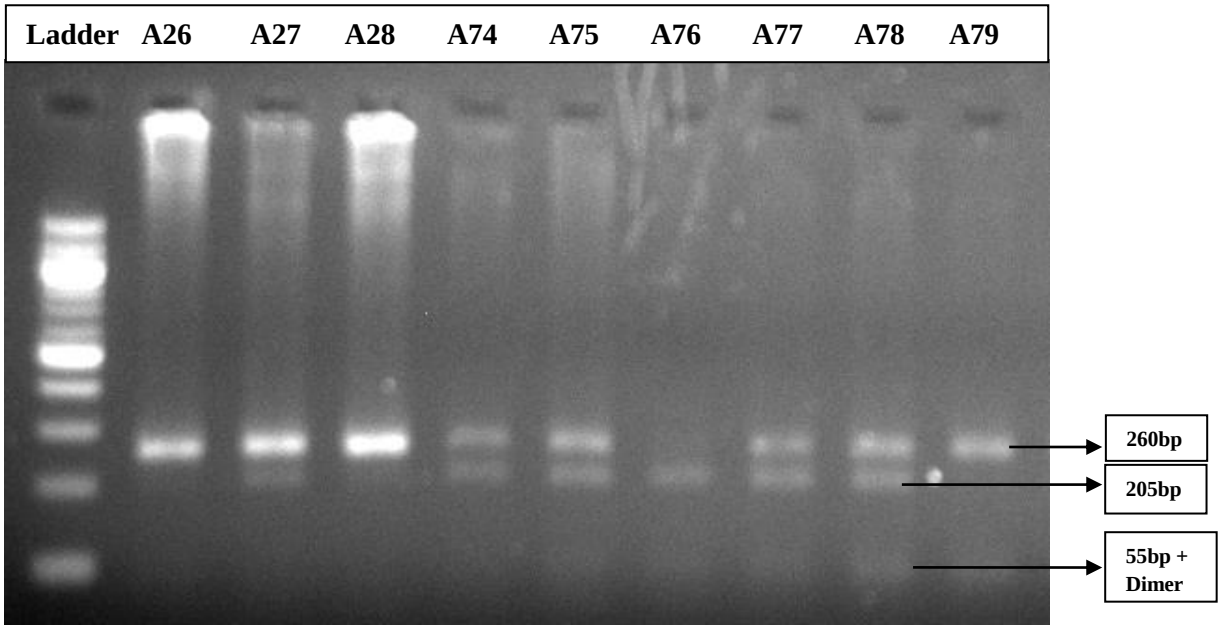


Figure 5.2: Restriction digestion of 13 DNA samples at 57ºC annealing temperature.

Lane 1: 100kb DNA ladder.

Lane 2-Lane 10: DNA sample A26-A28, A74-A79.

CHAPTER - 6

DISCUSSION

DISCUSSION

Asthma is an inflammatory disease that affects the respiratory system which causes episodes of breathlessness, wheezing, cough and chest pain. It is a common respiratory disease which can be caused by environmental factors as well as multiple genes playing a vital role in triggering the disease expression. Various genetic studies of asthma pathogenesis tell us that genetic factors are regarded to be one of the major determinants in asthma susceptibility.

In this study, we have examined the asthma patients for genetic variation. Main focus was on the association of SNP rs510432 which is present in the autophagy related ATG5 gene with the danger of asthma in North Indian population.

The SNP which is under study was chosen on the basis of earlier identified genetic relations of the same SNP in different population studies.

We showed that the one variant of the autophagy related ATG5 gene has a significant role with the susceptibility of asthma in the North Indian population, while the other two genotype models does not have any significant role in asthma susceptibility as observed from the obtained odds ratio and p-value. To the best of my knowledge, no previous report on association of autophagy related ATG5 gene variant (rs510432) with asthma in North Indian population has been done. This report is the first to report the link between the autophagy related ATG5 gene and its SNP rs510432 with asthma in north India population.

The help of various molecular biology techniques was taken in order to make our work very convenient. Along with the advances of polymerase chain reaction – Restriction fragment length polymorphism technique, every genotype was efficiently found out for both the control and cases. Accordingly, appropriate and correct statistical analysis methods were applied to explain the relation of obtained genetic variants with the disease susceptibility.

The obtained statistical parameters as given below;

Heterozygous genotype GA model: p-value = 0.0363; odds ratio (OR) =2.1866; 95% CI=1.0510 to 4.5494

Homozygous genotype GG model: p-value=0.1571; odds ratio (OR) =3.7143 and CI=0.3530 to 1.788

Allelic model: p-value=1.3852; odds ratio (OR) =1.3852 and 95% CI=0.9091 to 2.1107

Therefore, the results obtained indicated towards the role of the SNP rs510432in the autophagy related ATG5 gene in asthma susceptibility with the heterozygous model.

CHAPTER – 7

CONCLUSION & FUTURE PROSPECTS

CONCLUSION & FUTURE PROSPECTS

Genotyping of the predicted SNP rs510432 in 91 asthmatic samples and 90 healthy samples was carried out to discover its role in asthma pathogenesis. The result from this study suggested the role of the SNP rs510432in the autophagy related ATG5 gene in asthma susceptibility with the heterozygous genotype GA model.

Proper documentation and reporting of those genes that are related towards asthma susceptibility would be of great assistance and can easily act as one of the prognostic marker that can definitely help us in the future to identifying those individual who are at the risk of asthma at a very early stage of the disease. In addition to this, this will also aid to control suitable therapeutic approaches for disease treatment.

Our work provides and indicates the support for supplementary prospective studies in order to confirm the contribution of autophagy related ATG5 gene variants in asthma. It has been found that the result obtained indicates the role of the SNP rs510432 in the autophagy related ATG5 gene in asthma susceptibility with the heterozygous model.
This can pave the way for several researchers in identifying and short listing candidate ns-SNPs as well as prioritizing various SNPs for further confirmatory analysis.

Moreover, as asthma is caused due to various genetic and environmental factors, both gene environment relations and gene-gene interactions may occur. Hence a solitary genetic variation is always very questionable to be sufficient enough to predict down on the whole information about the disease risk.

Hence, further genetic studies are required to explain on a whole about the possible role of other functional SNPs of autophagy related ATG5 gene and other genes that are knotted in a parallel biological pathway which can be involved in susceptibility of asthma.

APPENDIX

GLASSWARES AND INSTRUMENTS

GLASSWARES

- Beaker - 1000ml, 500ml, 100ml
- Eppendorfs (autoclaved) - 2ml, 1.5ml, 0.5ml, 0.2ml
- Autoclaved micro tips (100-1000ul,20-200ul,0.1-10ul)
- PCR Tube stand
- Capped Bottles
- Measuring cylinder - 500ml, 100ml, 10ml
- Eppendorfs stand

INSTRUMENTS

- PCR Tube stand
- Micro pipette
- Thermo-cycler
- Spinner
- Weighing balance
- Autoclave
- Incubator
- Laminar Air Flow

- Hot air oven
- pH meter
- Rocker
- Refrigerator (-80ºC,-20ºC,-4ºC)
- Vortex
- Centrifuge
- Water bath
- Agarose Gel Electrophoresis chamber
- UV trans illuminator

REAGENTS

Di-sodium ethylene diamine tetra acetate (0.5M,Ph 8.0)

- Take 186.1g of Na2EDTA and add to it 800ml of milliQ water.
- Stir vigorously on magnetic stirrer.
- Adjust ph to 8.0 with 10M NaOH.
- Make up the volume to 1000ml.

Tris (hydroxymethyl) aminomethane-chloride, Tris-Cl (1M,pH8.0)

- Take 121.2g Tris Base in 800ml of distilled water.
- pH 8 was adjusted by adding 1N HCl.
- Make up the volume to 1L by adding MQ water.

Tris-Cl (1M, pH 7.3)

- In 800ml of distilled water add 121.2g of Tris base.
- pH 7.3 was adjusted by adding 1N HCl.
- Make up the volume to 1L.

Ammonium Chloride, NH4Cl (1M)

- In 800 MQ water dissolve 53.5g of ammonium chloride.
- Make up the volume to 1L.

10% SDS

- In 70ml of distilled water dissolve 10g of SDS.
- Heat to 68*C to mix the solution.
- Make up the volume to 1L.

Red Blood Cell Lysis Buffer

COMPOSITION: Tris 10mM , ph – 8.0;EDTA 1mM ; NH4Cl 125mM, ph 8.0

i. EDTA (0.5M) 2ml

ii. Tris (1M,ph-8.0) 10ml

iii. NH4Cl(1M) 125ml

Mix the above reagent in MQ water to obtain final volume of 1L.

Tris-EDTA (TE) buffer (pH 8.0)

COMPOSITION: Tris 10Mm; EDTA 1mM , pH 8.0

i. EDTA (0.5M) 2ml

ii. Tris (1M , pH8.0) 10ML

Mix the above reagent in distilled water to obtain absolute volume of 1L

Tris – EDTA (TE) buffer (pH7.3)

COMPOSITION: Tris 10Mm; EDTA 1Mm,pH7.3

i. EDTA(0.5M) 2ml

ii. Tris (1M, pH7.3) 10ml

Mix the above reagents in distilled water to obtain absolute volume 1L.

Ammonium Acetate (7.5M)

- In 20ml of MQ water dissolve 28.9g of ammonium acetate salt.
- Final volume was adjusted to 50ml.

REFERENCES

REFERENCES

[1] Centers for Disease Control and Prevention (CDC. "Vital signs: asthma prevalence, disease characteristics, and self-management education: United States, 2001--2009." *MMWR. Morbidity and mortality weekly report* 60.17 (2011): 547.

[2] Antó, Josep M. "The causes of asthma: the need to look at the data with different eyes." *Allergy* 59.2 (2004): 121-123.

[3] Eggleston, Peyton A. "Environmental causes of asthma in inner city children." *Clinical reviews in allergy & immunology* 18.3 (2000): 311-324.

[4] Jounai, Nao, et al. "The Atg5–Atg12 conjugate associates with innate antiviral immune responses." *Proceedings of the National Academy of Sciences* 104.35 (2007): 14050-14055.

[5] Martin, Lisa J., et al. "Functional variant in the autophagy-related 5 gene promotor is associated with childhood asthma." *PLoS one* 7.4 (2012): e33454.

[6] Poon, Audrey, et al. "ATG5, autophagy and lung function in asthma." *Autophagy* 8.4 (2012): 694-695.

[7] National Asthma Education, et al. *Expert panel report 2: Guidelines for the diagnosis and management of asthma*. DIANE Publishing, 1998.

[8] Asher, I., and N. Pearce. "Global burden of asthma among children." *The international journal of tuberculosis and lung disease* 18.11 (2014): 1269-1278.

[9] Bijanzadeh, Mahdi, Padukudru A. Mahesh, and Nallur B. Ramachandra. "An understanding of the genetic basis of asthma." *The Indian journal of medical research* 134.2 (2011): 149.

[10] Cookson, William OC, and Miriam F. Moffatt. "Genetics of asthma and allergic disease." *Human molecular genetics* 9.16 (2000): 2359-2364.

[11] Mukherjee, Anil B., and Zhongjian Zhang. "Allergic asthma: influence of genetic and environmental factors." *Journal of Biological Chemistry* 286.38 (2011): 32883-32889.

[12] Altman, Douglas G., Jonathon J. Deeks, and David L. Sackett. "Odds ratios should be avoided when events are common." *BMJ: British Medical Journal* 317.7168 (1998): 1318.

[13] Pagano, M., and K. Gauvreau. "Principles of biostatistics, Brooks." *Cole: USA* (2000).

[14] Sheskin, D. J. "Inferential statistical tests employed with two or more dependent samples (and related measures of association/correlation)." *Handbook of parametric and nonparametric statistical procedures.* 4 (2004): 1021-1116.

[15] Deeks, Jonathan J., and Julian PT Higgins. "Statistical algorithms in review manager 5." *Statistical Methods Group of The Cochrane Collaboration* (2010): 1-11.

[16] Kim, Harold, and Jorge Mazza. "REVIEW Open Access." *group* 3 (2011): 5.

[17] Lifton, Richard P., Ali G. Gharavi, and David S. Geller. "Molecular mechanisms of human hypertension." *Cell* 104.4 (2001): 545-556.

[18] Levine, Beth, and Daniel J. Klionsky. "Development by self-digestion: molecular mechanisms and biological functions of autophagy." *Developmental cell* 6.4 (2004): 463-477.

[19] Marino, G., and Carlos López-Otín. "Autophagy: molecular mechanisms, physiological functions and relevance in human pathology." *Cellular and Molecular Life Sciences CMLS* 61.12 (2004): 1439-1454.

[20] Klionsky, Daniel J. "Autophagy revisited: a conversation with Christian de Duve." *Autophagy* 4.6 (2008): 740-743.

[21] Glick, Danielle, Sandra Barth, and Kay F. Macleod. "Autophagy: cellular and molecular mechanisms." *The Journal of pathology* 221.1 (2010): 3-12.

[22] Mizushima, Noboru. "Autophagy: process and function." *Genes & development* 21.22 (2007): 2861-2873.

[23] Kirkin, Vladimir, et al. "A role for ubiquitin in selective autophagy." *Molecular cell* 34.3 (2009): 259-269.

[24] Barth, Sandra, Danielle Glick, and Kay F. Macleod. "Autophagy: assays and artifacts." *The Journal of pathology*221.2 (2010): 117-124.

[25] Kabeya, Yukiko, et al. "Cis1/Atg31 is required for autophagosome formation in Saccharomyces cerevisiae." *Biochemical and biophysical research communications* 356.2 (2007): 405-410.

[26] Cicchini, Michelle, Vassiliki Karantza, and Bing Xia. "Molecular pathways: autophagy in cancer—a matter of timing and context." *Clinical cancer research* 21.3 (2015): 498-504.

[27] Tanida, Isei, et al. "Mammalian Apg12p, but not the Apg12p· Apg5p conjugate, facilitates LC3 processing." *Biochemical and biophysical research communications* 296.5 (2002): 1164-1170.

[28] Guévin, Carl, et al. "Autophagy protein ATG5 interacts transiently with the hepatitis C virus RNA polymerase (NS5B) early during infection." *Virology* 405.1 (2010): 1-7.

[29] Mai, Sören, et al. "Autophagy proteins LC3B, ATG5 and ATG12 participate in quality control after mitochondrial damage and influence lifespan." *Autophagy* 8.1 (2012): 47-62.

[30] Kim, Myungjin, et al. "Mutation in ATG5 reduces autophagy and leads to ataxia with developmental delay." *Elife* 5 (2016).

[31] Poon, Audrey H., et al. "Genetic and histological evidence for autophagy in asthma pathogenesis." *The Journal of allergy and clinical immunology* 129.2(2012): 569.

[32] Martin, Lisa J., et al. "Functional variant in the autophagy-related 5 gene promotor is associated with childhood asthma." *PLoS one* 7.4 (2012): e33454.

[33] Xu, Yi, and N. Tony Eissa. "Autophagy in innate and adaptive immunity." *Proceedings of the American Thoracic Society* 7.1 (2010): 22-28.

[34] Poon, Audrey, et al. "ATG5, autophagy and lung function in asthma." *Autophagy* 8.4 (2012): 694-695.

[35] Jyothula, Soma SSK, and N. Tony Eissa. "Autophagy and role in asthma." *Current opinion in pulmonary medicine* 19.1 (2013): 30-35.

[36] Suzuki, Kuninori, et al. "Hierarchy of Atg proteins in pre-autophagosomal structure organization." *Genes to Cells* 12.2 (2007): 209-218.