ASSOCIATION OF TGF-β 509C>T POLYMORPHISMS IN ASTHMA

Dissertation submitted to

In partial fulfilment of the award of the degree of

MASTER OF SCIENCE IN GENETICS

By

ABBAGALLA RAVITEJA (1007-17-517-015)

Under the guidance of

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TELANGANA STATE

CERTIFICATE

This is to certify that the dissertation report entitled "ASSOCIATION OF TGF-β POLYMORPHISMS IN ASTHMA**"** is the result of project work carried out by **Abbagalla Raviteja (H.T.NO.1007-17-517-015**) at the Department of Genetics, Osmania University, Hyderabad 500007 in partial fulfilment for the award of Msc-Genetics under my supervision and guidance during the academic year of 2017-2019.

Approved by:

Dr.G. Sumanlatha	Chairperson	Head
(Supervisor)	Board of Studies (Genetics)	Department of Genetics

Evaluated by:

Internal Examiner External Examiner

DECLARATION

I hereby, declare that the project work entitled "ASSOCIATION OF TGF-β 509C>T POLYMORPHISMS IN ASTHMA**"** has been carried out by **Abbagalla Raviteja (H.T.NO.1007-17-517-015)** is an original work done and submitted by me in partial fulfilment for the degree **MASTER OF GENETICS IN OSMANIA UNIVERSITY**, HYDERABAD is a project work done by me under the guidance of **DR.G. SUMANLATHA** Associate Professor, Department of Genetics, Osmania University, Hyderabad, during the course of year 2017-2019.

DATE:

PLACE:

(ABBAGALLARAVITEJA)

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ABSTRACT

INTRODUCTION: Asthma is a chronic inflammatory disorder of the airways characterized by airway inflammation, airway constriction and bronchial hyper responsiveness. The pathophysiology of asthma is deemed to be a result of Th1-Th2 imbalance, skewed towards increased Th2 responses. TGFβ is a recently identified cytokine thought to play a role in asthma pathophysiology.

AIM: The present study was designed to analyse the association of TGFβ–509C>T Polymorphism with asthma as well as quantitate the serum levels of TGFβ in asthma subjects and healthy controls.

METHODS: A total of 50 asthma patients and 50 healthy controls were recruited for the study. TGFβ -509 C>T genotyping was done using PCR. Serum levels were quantitated using Sandwich ELISA. Appropriate statistical tools were employed to analyse the results.

RESULTS: Patients with TT genotype was found as susceptible genotype and TGF-β cytokine serum levels were found high in patients than healthy controls**.**

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INTRODUCTION:

Asthma is a chronic inflammatory disorder characterized by airway inflammation caused by many cells and cellular elements, in particular mast cells, eosinophils, T-lymphocytes macrophages, neutrophils and epithelial cells. This inflammation is usually associated with widespread but variable obstruction this often reversible either spontaneously or with treatment. The inflammation and air flow obstruction are responsible for the manifestation of asthma symptoms like recurrent episode lung wheezing, chest tightness, shortness of breath, coughing, particularly at night or early in the morning in susceptible individual. The inflammation is also responsible for associated increase in the existing bronchial responsiveness to a variety of stimuli [1].

Asthma is considered to be result of allergic inflammation leading to the production of immunoglobulin E (IgE).Allergen that enter the airway are presented by antigen presenting cells (APC) to the T cells which differentiates into T helper type 2 cells(TH2) in the presences of certain cytokines [2]. TH2 cells secrete number of cytokines including (IL)-4 and IL-13 acting on different target cells such as mast cells eosinophils, epithelial cells, smooth muscle cells and lymphocytes. The stimulated b cells start synthesizing IgE .All this changes in the airway in the form of inflammation lead to airflow obstruction and thus asthmatic symptoms.

TYPES OF ASTHMA:

Intrinsic asthma represents a small amount of all cases. It usually develops after the age of 30 and is not typically associated with allergies. Women are more frequently affected and many cases seem to follow a respiratory tract infection. Obesity also appears to be a risk factor for this type of asthma. Intrinsic asthma can be difficult to treat and symptoms are often chronic and year-round[3].

The different types of asthma:

1.Allergic asthma [extrinsic asthma]

Extrinsic, or allergic asthma, is more common and typically develops in childhood. Approximately 70%-80% of children with asthma also have documented allergies[3]. Typically, there is a family history of allergies. Additionally, other allergic conditions, such as nasal allergies or eczema, are often also present. Allergic asthma often goes into remission in early adulthood. However, in many cases, the asthma reappears later.

This type occurs when an allergy sets off an asthma flare up. Mold, roaches, pollens and pet dander are common allergies but the list can be endless.

Patients may be prescribed inhaled corticosteroids depending on the severity of their asthma.

2.Asthma without allergies [intrinsic asthma]

People may also have asthma not triggered by allergies. Usually an upper respiratory infection (cold, flu, and rhinovirus) sets off their asthma. As soon as cold or flu symptoms appear patients are typically prescribed a short course of inhaled corticosteroids for 10-14 days [4].

3.Aspirin Exacerbated Respiratory Disease (AERD)

Aspirin-exacerbated respiratory disease (AERD) is characterized by adult onset of asthma, chronic rhinosinusitis (CRS), nasal polyposis, and aspirin sensitivity. In this syndrome, each disease component has deleterious effects on the patient's health and quality of life. Latest figures from the Center for Disease Control indicate 8.2% of the U.S. population has asthma and among adult asthmatic patients, up to 9% have AERD. Approximately 13% of the population suffers from CRS and 15% of patients with CRS with nasal polyposis have AERD. A review of the impact that each component of AERD has on patients will delineate the considerable burden of AERD, especially when considering the cumulative effects of the tetrad [9].

4.Exercise induced asthma:

For these asthmatics, any type of physical exertion or sports leads to coughing, difficulty breathing and chest tightness that improves when they stop the exertion. Typical treatment is an inhaled bronchodilator medication to open their airway taken about fifteen minutes before exercise [5].

There are multiple studies that say taking 2000 mg of vitamin C before exercise can relieve exercise induced asthma. Some folks also have cold weather induced asthma. Cold air can be a lung irritant just like perfume or cigarette smoke. This generally occurs in winter.

5.Cough variant

Cough variant is asthma that is characterized by a dry hacking cough. It can occur while awake or asleep and affect both adults and children.[6] Patients usually respond well to inhale corticosteroids. Vitamin D has also been shown to improve asthma. Studies show there is less incidence of asthma in the south, which may be related to people having less sun exposure and lower vitamin D levels in northern climates.

6. Occupational asthma

Occupational asthma occurs when something on the job sets off an asthma attack. Irritant induced asthma is usually from smoke or inhaled irritants like chlorine. It's not related to an allergy; the irritant is inhaled and triggers an attack[6].

In occupations that deal with chemicals like paint or lab animals like rats or mice, patients may also be allergic to their trigger. If you can't get away from your trigger, you may have to use a corticosteroid inhaler to ease symptoms. Pescatore also likes vitamin A has been shown to help get rid of the mucus in the respiratory tract, which can be an irritant.

The pine tree bark extract Pycnogenol is an anti-inflammatory and antioxidant that clinical research shows helps to open the bronchial tubes and reduces asthma symptoms.

Signs and Symptoms of Bronchial Asthma: Shortness of breath

According to the National Institutes of Health, shortness of breath is one of the most common signs of bronchial asthma, in addition to coughing and wheezing. Shortness of breath may be most noticeable in bronchial asthma sufferers during exercise or other strenuous activity[7]. The Nemours Foundation compares the shortness of breath you experience with asthma to the feeling you would get trying to suck air through a straw--it is difficult to breathe in and out and fill your lungs with oxygen.

Coughing & Wheezing

Repeated coughing and wheezing is also another common symptom of Bronchial asthma. The National Institutes of Health finds that wheezing in asthma sufferers is likely be at its worst early in the morning or late at night, and will usually occur without warning before it goes away on its own or is relieved by and inhaler.

Chest Tightness

A tight feeling in the chest can be caused by asthma and is usually associated with shortness of breath. There are a number of medical conditions that can cause chest tightness, so if you are experiencing chest pain or tightness you should see your doctor or an emergency physician right away.

Emergency Symptoms

Bronchial asthma attacks can cause an emergency situation if they are severe and not treated right away. If you or someone you know seems to be having trouble breathing and cannot speak, is sweating or is turning blue, go to the emergency room immediately.

Bronchial Asthma Causes

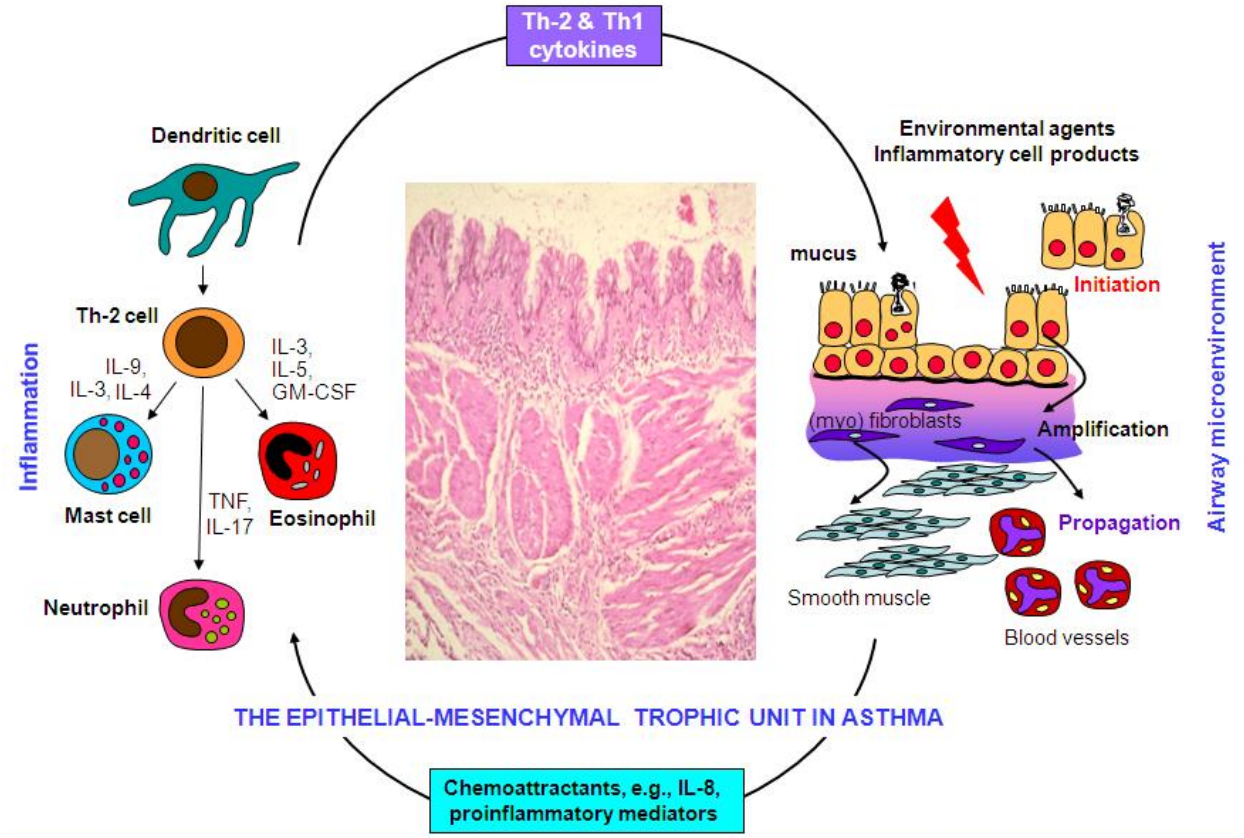
Asthma is primarily associated with allergy but allergy is not the only to get asthma. Allergies are also associated with genetic inheritance and so does asthma. Mast cells, eosinophils, and T lymphocytes in human body are responsible for causing bronchial asthma. Mast cells (the allergy causing cells) release chemicals called histamine which in turn is responsible for nasal stuffiness and constriction of airways. Eosinophils and T lymphocytes are a type of white blood cells that

are associated with allergic diseases and inflammation. Thus, the causes of bronchial asthma can be listed as under.

- Allergies
- Genetic factors
- Increased numbers of Eosinophils
- Tobacco smoke
- Infections like colds, flu, pneumonia
- Allergens like food, pollen, mold, dust mites, and pet dander (skin flakes)
- Exercise
- Air pollution, toxins
- Extreme changes in temperature
- Certain drugs like aspirin, NSAID, and beta-blockers
- Food additives like MSG flavour enhancers
- Emotional stress and anxiety
- Hyper activities like singing, laughing, or crying
- Perfumes or sprays
- Acid reflux

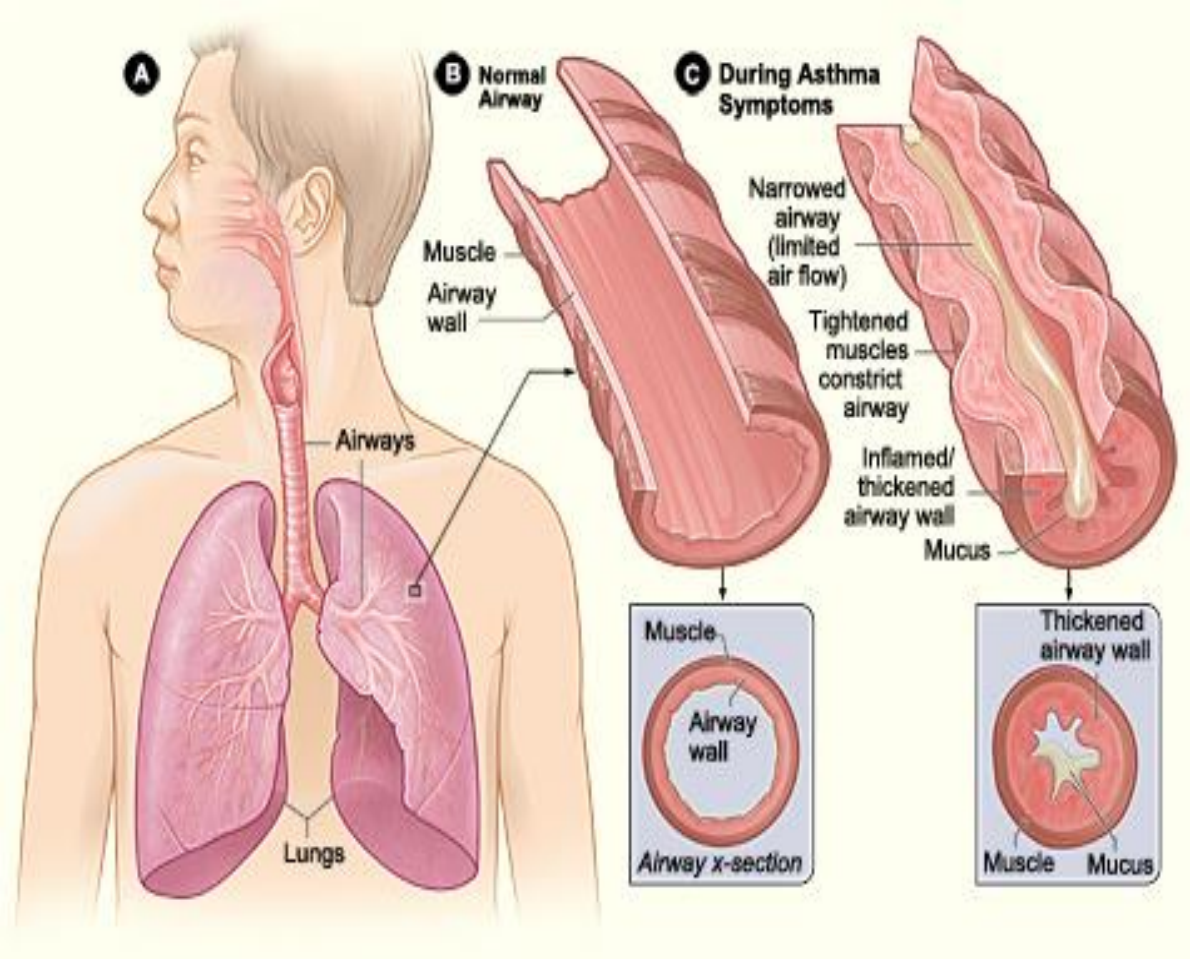
PATHOGENISIS OF ASTHMA:

Asthma is an airway disease that can be classified physiologically as a variable and partially reversible obstruction to air flow, and pathologically with overdeveloped mucus glands, airway thickening due to scarring and inflammation, and bronchoconstriction the narrowing of the airways in the lungs due to the tightening of surrounding smooth muscle[8]. Bronchial inflammation also causes narrowing due to edema and swelling caused by an immune response to allergens.



Airflow limitation in asthma is recurrent and caused by a variety of changes in the airway. These include:

Bronchoconstriction: In asthma, the dominant physiological event leading to clinical symptoms is airway narrowing and a subsequent interference with airflow. Allergen-induced acute bronchoconstriction results from an IgE-dependent release of mediators from mast cells that includes histamine, tryptase, leukotrienes, and prostaglandins that directly contract airway smooth muscle[1].



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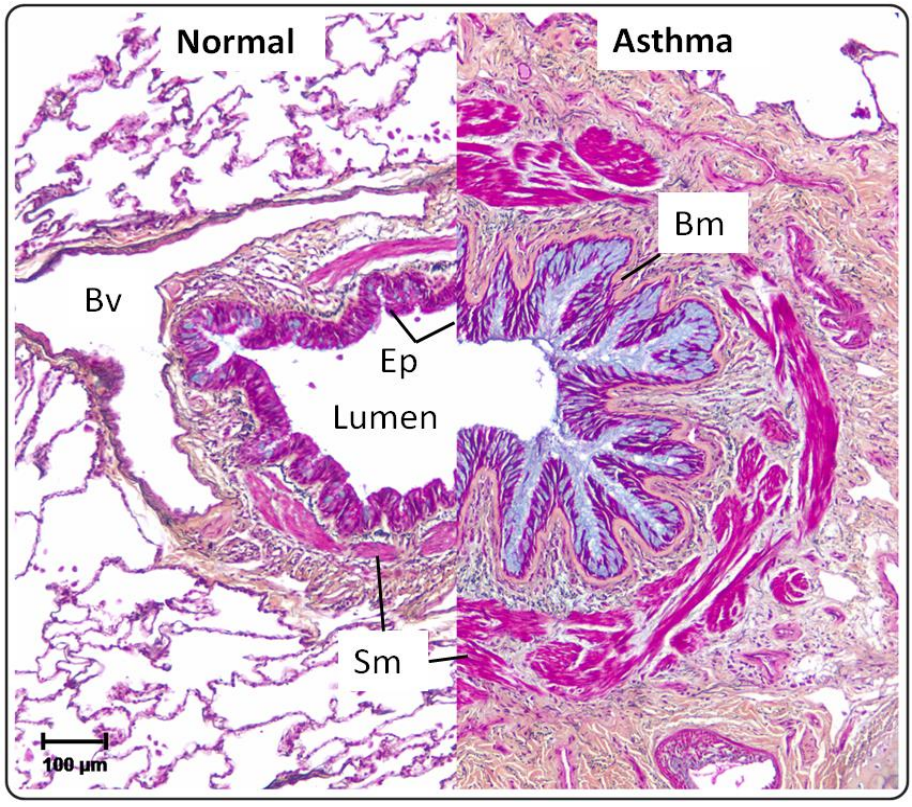
Airway edema: As the disease becomes more persistent and inflammation more progressive, other factors further limit airflow. These include edema, inflammation, mucus hyper secretion and the formation of inspissated mucus plugs, as well as structural changes including hypertrophy and hyperplasia of the airway smooth muscle [1].

Airway hyper-responsiveness: The airway hyper-responsiveness can be defined by contractile responses to challenges with methacholine correlates with the clinical severity of asthma. The mechanisms influencing airway hyperresponsiveness are multiple and include inflammation, dysfunctional neuroregulation, and structural changes; inflammation appears to be a major factor in determining the degree of airway hyperresponsiveness. Treatment directed toward reducing inflammation can reduce airway hyperresponsiveness and improve asthma control.Airway remodeling: Airway remodeling involves an activation of many of the structural cells, with consequent permanent changes in the airway that increase airflow obstruction and airway responsiveness and render the patient less responsive to therapy.

FEATURES OF AIRWAY REMODELING:

- Inflammation
- Mucus hyper secretion
- Sub epithelial fibrosis
- Airway smooth muscle hypertrophy
- Angiogenesis



The airways in asthma undergo significant structural remodeling. Medium-sized airways from a normal and severe asthmatic patient were sectioned and stained using Movat'spentachrome stain. The epithelium (Ep) in asthma shows mucous hyperplasia and hyper secretion (blue), and significant basement membrane (Bm) thickenin .Smooth muscle (Sm) volume is also increased in asthma.

SIGNS AND SYMPTOMS:

Signs and symptoms of asthma include the following:

 Wheezing

 Coughing

 Shortness of breath

 Chest tightness/pain

Other nonspecific symptoms in infants or young children may be a history of recurrent bronchitis, bronchiolitis, or pneumonia; a persistent cough with colds; and/or recurrent croup or chest rattling [31].

Asthma symptoms, also called asthma flare-ups or asthma attacks, are often caused by allergies and exposure to allergens such as pet dander, dust mites, pollen or mold. Non-allergic triggers include smoke, pollution or cold air or changes in weather.

Normal vs. Asthmatic Breathing:

When a person without asthma breathes in, the air:

. Enters through the nose or mouth

. Goes down the trachea, or windpipe

. Enters the bronchioles, or airways of the lung

. Blood is oxygenated at the alveoli

. Goes back out again

However, for asthmatics, this process is different and more difficult. In asthma patients, the airways are very sensitive and may react to a number of different triggers, such as smoke, pollens and infections, leading to constriction and inflammation of the airways. Constriction and inflammation cause airflow obstruction, making it difficult to breathe. Asthma symptoms will wax and wane over time with treatment focused on both the prevention and control of symptoms and the reduction of inflammation [32].

TREATMENT AND MANAGEMENT:

There is no cure for asthma, but symptoms can be controlled with effective asthma treatment and management. This involves taking patients medications as directed and learning to avoid triggers that cause patient asthma symptoms. Allergist will prescribe the best medications for patient condition and provide specific instructions for using. The aim of asthma treatment is to avoid the substances that trigger your symptoms and control airway inflammation [33]. There are two basic kinds of medication for treating asthma:

DIAGNOSIS AND PREVENTION:

Asthma is diagnosed based on the patient's medical history, physical examination and laboratory test results. Your doctor will take a detailed medical history and ask you about your asthma symptoms and allergy triggers.

Your doctor may use one or more of the following asthma tests to diagnose asthma, to assess your breathing and to monitor the effectiveness of asthma treatment:

Blood and Sputum analysis – show an increase in the number of eosinophils. The level of a certain antibodies can be elevated.

Chest X-ray – may show abnormality in the airway.

Arterial blood gas analysis – shows decreased oxygen concentration in blood.

Lung function testing – The two most common pulmonary function tests for asthma are spirometry and methacholine challenge tests

Spirometry - helpful in judging severity of airway obstruction. It measures how much air you can breathe in and out and how fast you can blow air out [34].

Some of the common values looked at are:

a. Forced Vital Capacity or FVC – total volume of air one can exhale after maximum breathing in.

b. Forced Expiratory Volume in One Second, or FEV1- measures the volume of air one can exhale in the first second.

Methacholine challenge test – commonly used in adults. Performed when your symptoms and spirometry do not convincingly diagnose asthma. Methacholine is an agent that, when inhaled,

causes the airways to spasm (contract involuntarily) and narrow if asthma is present.Allergy test- to identify any allergies that trigger asthma symptoms. Skin tests and blood tests help in detecting allergens.

REVIEW OF LITERATURE

Chronic inflammation and airway remodeling are two key steps in asthma pathophysiology [37]. Transforming growth factor-beta1 (TGF-beta1) is a multifunctional cytokine induced in pro- and anti- inflammatory pathways [38]. It is produced by many types of cells that are activated in the asthmatic response. Recent studies highlighted this cytokine as an important negative regulator in an experimental model of asthma [39]. In addition, TGF- beta1 is responsible for subepithelial fibrosis and airway smooth muscle cell (ASMC) hypertrophy, the principle features of airway wall remodeling in asthma [38, 40].

Bronchial asthma requires early pharmacological treatment and long-term management. Anti-inflammatory agents, particularly inhaled corticosteroids, are currently the most effective long-term preventive medication. Moreover, early intervention with inhaled corticosteroids plays an important role in airway remodelling [41]. Despite the fact that the role of TGF-beta1 in human asthma remains obscure, data derived from animal models encouraged further investigation of its suppressive mechanisms in order to develop novel therapies for asthma

Transforming growth factor-β (TGF-β) plays an essential role in promoting the structural changes of airway remodeling. It is an extremely potent stimulus for the formation of the extracellular matrix (ECM) by fibroblast such as collagen types I and III, fibronectin, vitronectin, tenascin, and proteoglycan. Furthermore, it decreases synthesis of enzymes that degrade the ECM, namely matrix metalloproteinase (MMPs) and increases the production of proteins that inhibit enzymes that degrade the ECM (tissue inhibi- tor of matrix metalloproteinase)[35]. There are three TGF-β isoforms identified in mammals, namely TGF-β1, TGF-β2 and TGF-β3. In asthmatic airway, there were abundant TGF-β1 ex- pressions in epithelium [36] and increased TGF-β1 levels in the bronchoalveolar lavage fluids [41]. Additionally, TGF-β1 and TGF-β2 are increased in BAL fluid fol- lowing segmental allergen challenge.[41,42] The TGF- β1 immunostaining has been localized to the subepi- thelial airway.[43]

Furthermore, it has been reported that ele- vated level of plasma TGF-β1 are a predictor of lung fibrosis.[44] Serum TGF-β1 has also been found to be associated with fibrosis in several diseases such as liver and cardiac fibrosis.[45,46] To our knowledge, there have been only few reports regarding the role of serum TGF-β in atopic asthmatic patients.[47,48]

TGF-B AND ASTHMA IMMUNE RESPONSE

Allergic diseases are caused by inappropriate immunological responses to allergens [10]. Several studies have shown that regulatory T cells (Treg cells) have a key role in controlling allergic diseases and chronic inflammation in asthma. A defect on immune regulation leads to an exacerbation of Th2 response [11,12]. Treg cells are essential in the maintenance of immunological tolerance to self-antIg-Ens and in the regulation of the immune response to infectious organisms and represent a major pathway proposed to keep immune homeostasis in the airways. Thus, recently, attention has been given to Treg cells producing IL-10 and TGF-β.These immune modulatory cytokines can down regulate the production of both Th1 and Th2 cytokines and suppress the inflammatory response on asthma[13-15].

Accordingly, TGF-β is crucial in the development and function of CD4+CD25+ T-reg and induces expression of the master regulatory transcription factor Foxp3, a critical gene regulator for T-reg cells development. TGF-βinhibits Ig-E, the main antibody associated with allergic diseases and asthma26 and has been shown to induce IL-10 expression in T cells[17].

Hansen et al. have demonstrated that T cells producing TGF-β are able to reduce inflammation and airway hyperreactivity in a mouse model [18]. It was also demonstrated that blocking the TGF-β signalling pathway in T lymphocytes, an increasing inflammation and airway hyperreactivity is observed, suggesting that TGF-β-induced immune regulation reduces the pulmonary inflammatory response *in vivo*[13]. In contrast, several studies have shown that TGF-β is associated to an increased airway remodelling by inducing apoptosis of airway epithelial cells and is potentially involved in the regulation of epithelial cells adhesion leading to tissue damage [19]. The neutralisation of TGF-β in two different models of chronic allergen challenge-reduced airway remodelling [20]. Asthmatic patients showed increased TGF-βexpression in both bronchial biopsy sections and broncho-alveolar lavage in comparison with normal subjects and expression correlated with the lung fibrosis degree [21].

These data reinforce the idea that TGF-β acts to regulate immune response in the lungs and that perturbations in the level of expression of that cytokine or even in one of the TGF-β signalling pathway molecules may have severe consequences for maintenance of pulmonary homeostasis. However, many studies have investigated the rationale that increased TGF-β expression may be associated with asthma severity by increasing airway remodelling.

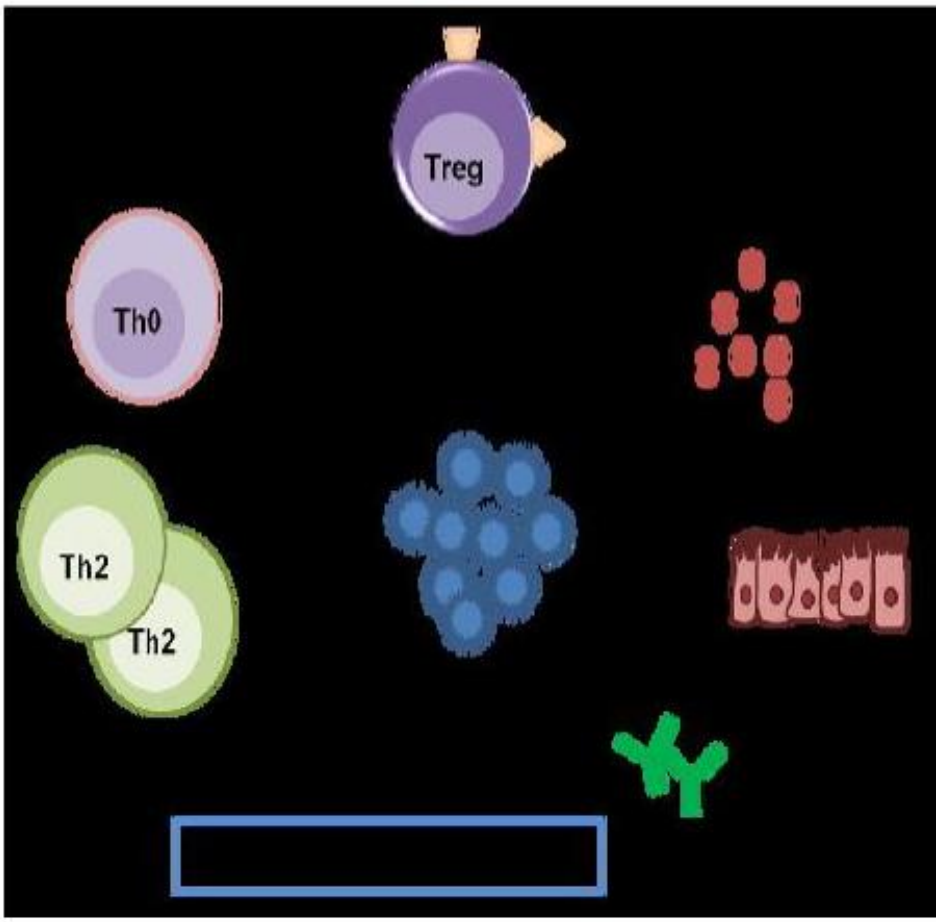


Figure 1: TGF-β on asthma immune response. TGF-β is crucial to induce expression of the main regulatory transcription factor Foxp3 and to regulate the development, function and IL-10 production from of CD4+CD25+ T-reg cells. Moreover, TGF-β can down-regulates the activation of Th2 lymphocytes, suppress the inflammatory response and airway hyperreactivity and inhibits Ig-E release. TGF-β also seems to be involved in the airway remodelling and, consequently, severity of asthma.

TGF-B GENE POLYMORPHISMS AND ALLERGIC DISEASE RISK

Polymorphisms in gene sequences can affect the expression of proteins in various ways: levels of gene transcription, splicing, stability and levels of mRNA translation [22]. Polymorphisms in genes that participate on immunity may influence the development of several diseases. Susceptibility to many diseases is associated with a particular 'pro-inflammatory 'profile, which can be explained by individual genetic determinants. TGF-β1 is an important replicated asthma candidate gene, and few studies have evaluated the direct association of *TGF*-β polymorphisms and risk to allergic diseases, in particular, asthma [23,24]. The TGF-β gene is located on chromosome 19q13.1– 13.335 [25], and some polymorphisms were shown in this gene and can be found in exons, introns and promoter gene sequences.

rs1800469 (-509 C > T)

The -509 C > T polymorphism is located in the promoter region of *TGFB1* and can modulate TGF-β1 function and circulating TGF-β1levels [24]. The T allele has been associated with higher TGF-β1 plasma levels [26]. An interesting Genome-wide association study (GWAS) previously reported that -509 C > T was associated with asthma in a Mexican population [27]. These results were consistent with several authors [24,28]. In contrast, other authors found no association between -509 polymorphisms and risk to clinically manifest atopic asthma or other allergies [23,29]. Although these data seem controversial, this single-nucleotide polymorphism (SNP) can be an interesting marker for allergic diseases. However, more studies are needed to better under- stand the results obtained so far. Lack of associations can be achieved when sample size is small and therefore may have lacked power to detect statistically significant associations. Moreover this polymorphism was associated with Ig-E levels. One study described phenotypic association between total Ig-E levels in serum and -509 C > T and also association with persistent Ig-E mediated cow's milk allergy in children [30].

Several SNPs on TGF-β1 have been studied in asthma diseases. Some SNPs were positively associated with asthma but not always replicated by all authors. Although these data seem controversial, polymorphisms on TGF-β1 may be an interesting marker for asthma since it is related to an increase on TGF-β1 levels, and it may be related to tissue remodelling. However, more studies are required to better understand the results observed so far. It is important to point out that in some studies, lack of associations can be related to small sample size and

consequently no power to detect statistically significant associations. Moreover, the prevalence of these polymorphisms may vary from population to population, which can also interfere with these controversial results. Greater sample size and studies using a considerable number of informative ancestry markers are required to confirm these findings and also to identify populations, where TGF-β1 variants are mediating the casual pathway of allergic diseases, in special, asthma.

JUSTIFICATION/HYPOTHESIS:

Transforming growth factor beta-1 (TGFB1) is a multifunctional cytokine with proinflammatory effects in some settings and anti-inflammatory effects in others. It is expressed in many cell types including inflammatory cells and structural cells, such as airway epithelial and smooth muscle cells. TGFB1 levels in bronchoalveolar lavage (BAL) fluid are higher in asthma patients and increase further in response to allergen exposure compared with healthy control subjects. TGFB1 may modulate the development of allergic inflammation and airway remodelling in asthma. The C-509T SNP in the TGFB1 promoter appear to influence TGFB1 blood levels and gene expression in the lungs. Associations of C-509T and other TGFB1 SNPs with wheezing illness in infants asthma diagnosis severity and increased Ig-E in asthmatic children have been found in some studies. Few data have addressed TGFB1 haplotypes and the risk of asthma or atopy. So C 509 T may be associated with asthma and it may play a role in progress of asthma.

OBJECTIVES:

The aim of the current study is to explore association of TGF-β 509c/t polymorphism in asthma patients.

- Isolation of genomic DNA from whole blood using salting out method.
- PCR amplification of TGF-β1 using specific primers from isolated genomic DNA.
- RFLP of amplified PCR product using Xmni I.
- Ig-E serum levels evaluation by ELISA.
- Statistical analysis of RFLP data.

METHODOLOGY

BLOOD SAMPLE COLLECTION:

A total of 100 samples comprising of 50 patients and 50 controls are involved in the present study. Blood sample were collected from all the individuals. 5 ml of Blood sample was collected from. With the approval of ethical committee and the informed consent, recruited from local hospital, Hyderabad.

Patients: 50

Controls: 50

DNA ISOLATION

Materials required

- ✓ Eppendorf tubes (required).
- ✓ 200µℓ tips and micropipette.
- ✓ 1000µℓ tips and micropipette.
- ✓ Floating trays for incubation.

Chemicals used

- ✓ TKM1-(10mM Tris-HCl; pH 7.6; 10mM KCl; 4 mM MgCI2).
- ✓ TKM2-(10mM Tris-HCl; pH 7.6; 10mM KCl; 4 mM MgCI2; NaCl).
- ✓ Triton X
- ✓ SDS (buffer)
- ✓ 6M NaCl
- ✓ (100%) Absolute Ethanol and 70% Ethanol.

PROCEDURE:

Step 1: Transfer 300µℓ of blood sample into eppendorf.

Step 2: Add 1mℓ of TKM1 and 100µℓ of 1% Triton X to the blood sample.

Step 3: Now Vortex the sample and centrifuge at 5000 rpm for 10 min.

Step 4: Discard the supernatant. Repeat Step 2 & Step 3 for 3 to 4 times, then white pellet is obtained or formed.

Step 5: After discarding the supernatant, to the white pellet add 300µℓ of TKM2 and 100µℓ of 10% SDS

Step 6: Incubate at 55ºC for 30 min.

Step 7: After incubation, add 80µℓ of 6M NaCl and centrifuge immediately at 10000 rpm for 5 min.

Step 8: Collect the supernatant to a fresh eppendorf tube and add twice the volume of chilled 100% ethanol.

Step 9: Invert the tube till DNA precipitates and centrifuge at 10000 rpm for 5 min.

Step10: Discard the supernatant and to the pellet add 300µℓ of 70% Ethanol, then centrifuge at 12000 rpm for 5 min.

Step 11: Discard the supernatant and air dry over night.

Step12: Add 80µl of TE buffer to the eppendorf and incubate for 30 minutes.

PCR AMPLIFICATION:

The polymerase chain reaction is an invitro technique for generating large quantities of a specified DNA, developed by Karry Mullis in 1984. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.

THERMOCYCLER FOR PCR

Principle:

The double stranded DNA of interest is denatured to separate in to two individual strands. Each strand is then allowed to hybridize with a primer. The primer-template duplex is used for DNA synthesis (the enzyme DNA polymerase). The three steps denaturation, renaturation and synthesis are repeated again and again to generate multiple forms of target DNA.

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three (Figure below). The cycling is often preceded by a single temperature step at a high temperature (>90 °C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.

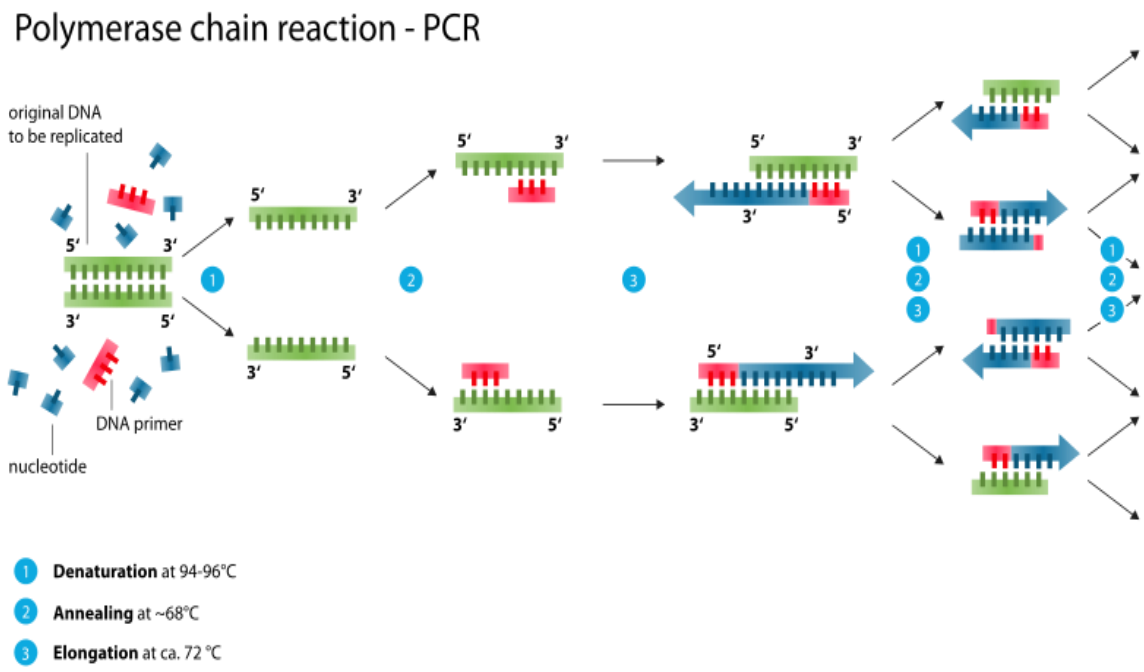
$$Tm = [4(G+C)] + [2(A+T)]$$

$$\text{Annealing temperature} = Tm-(5 \text{ to } 10)\ ^{o}C$$

- ***Initialization step*** (Only required for DNA polymerases that require heat activation by hot-start PCR: This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes.
- ***Denaturation step*****:** This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
- ***Annealing step*****:** The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3–5 °C below the Tm of the primers used the polymerase binds to the primer-template hybrid and begins DNA formation.
- ***Extension/elongation step*****:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-

phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified.

- ***Final elongation*:** This single step is occasionally performed at a temperature of 70–74 °C (this is the temperature needed for optimal activity for most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
- ***Final hold*:** This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.



The essential requirements of PCR are:

- **DNA template:**

The sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.

- **Two primers:**

Primers (17-30 nucleotides length) are complimentary to the flanking regions of target DNA and used for the amplification of desired DNA segment.

- **Deoxy nucleoside triphosphate:**

The four deoxy nucleoside triphosphate (dATP, dCTP, dGTP and dTTP) are the single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands.

- **DNA polymerase:**

A type of enzyme that synthesizes new strands of DNA complementary to the target sequence by extending the primers. The most commonly used enzyme is Taq DNA polymerase (from Thermusaquaticus)

- **Buffer:**

Provide a suitable chemical environment for optimum activity and stability of the DNA polymerase. The reaction mixture contains the target DNA, two primers, a thermo stable DNA polymerase (Taq polymerase) and four deoxyribo nucleosides.

The technique of PCR involves repeated cycles for amplification of target DNA. Steps involved in PCR

PRIMERS FOR PCR AMPLIFICATION:

Novel Primers of SNP locus rs1800469 which was generally designed for PCR-RFLP technique.

rs1800469 Primers:
Forward primer: 5'CAGACTCTAGAGACTGTCAG 3'
Reverse primer: 5'GTCACCAGAGAAAGAGGAC 3'

PROCEDURE:

Preparation of PCR mix for 10µL

Template	0.5 µl
Buffer	1.20 µl
Forward Primer	0.15 µl
Reverse Primer	0.15 µl
dNTP's	0.15 µl
Taq polymerase	0.15 µl
Distilled water	7.70 µl

- After mixing all the contents, the tubes were placed in a thermocycler and the following conditions were setup:

PCR CONDITIONS:

TEMPERATURE	TIME
95°	5′
95°	30″
59°	45″
72°	30 ″
REPEAT 32 CYCLES	
72°	10 ′

NOTE: use clean work surface for all PCR mix preparations preferable to use laminar air flow. Each time micro tips should be changed to avoid cross contamination.

AGAROSE GEL ELECTROPHORESIS (AGE):

Gel electrophoresis is a routinely used analytical technique for the separation of specific DNA fragments. Agarose gel electrophoresis is convenient for the separation of DNA fragments from100 bp to 20 kbp. The migration rate of DNA is dependent on size and shape.

Materials Required:

- Agarose,
- 5XTBE buffer,
- Distilled Water,
- Bromophenol blue,
- tracking dye (40% sucrose+ Bromophenol blue),
- Ethidium bromide.

PREPERATION OF REAGENTS:

1) Bromophenol blue: To 25 mg of Bromophenol blue powder add 7ml of distilled water along with 3ml of glycerol.

2)40%Sucrose: 4gm of sucrose dissolved in 10ml of distilled water.

3) Loading dye: To 40µl of sucrose add 5µl of Bromophenol blue. Take 5µl of sample in each well.

4) Ethidium Bromide: 5mg of ethidium bromide is dissolved in 1ml of distilled water (5mg/ml)

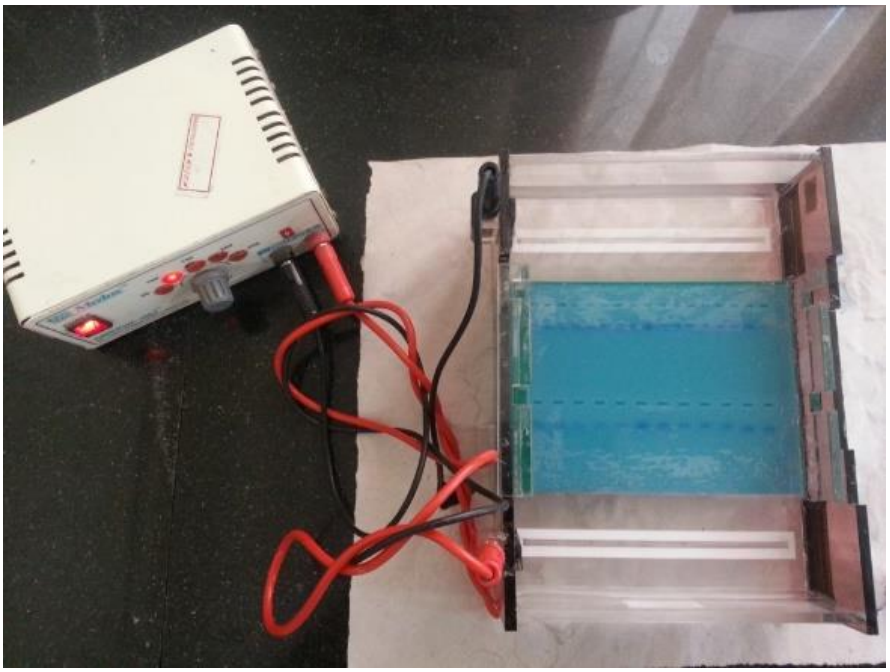
NOTE: Ethidium Bromide is a known mutagen and should be handled with care.

PROCEDURE:

- Rinse and dry the gel casting tray.
- Seal the ends of the casting tray and place the comb evenly in casting tray.
- Prepare 1%agarose gel by adding 0.25 gm of agarose in 25 ml of 1XTBE buffer.
- Boil the solution carefully till the agarose melts to get a clear solution.
- When the temperature of solution is around 45-50 ^{o}C , add 3µl of ethidium bromide and pour it in a gel casting tray and allow the gel to solidify

- After solidification remove comb carefully and insert the casting tray in to the electrophoresis chamber with the wells close to negative electrode.
- Take 2µl of genomic DNA sample and add 3µl of loading dye (Bromophenol blue)
- Mix well and load in to the wells.
- Make sure cords are correctly plugged in to the power supply (red to red, black to black)
- Plug in the power supply.
- Turn the power on and adjust the voltage to 100 volts.
- Run the agarose gel at appropriate volts until gel loading dye reaches the 3/4th of the gel.
- Gently transfer the gel from the electrophoresis chamber to UVtrans- illuminator to visualize the gel
- Capture the picture using a gel documentation unit

Visualization: The results can be analyzed quantitatively by visualizing the gel with UV light and a gel imaging device. The image is recorded with a computer operated camera, and the intensity of the band or spot of interest is measured and compared against standard or markers loaded on the same gel. The measurement and analysis are mostly done with specialized software.



Restriction fragment length polymorphism (RFLP):

Restriction fragment length polymorphism or RFLP analysis is used to identify a change in the genetic sequence that occurs at a site where a restriction enzyme cuts. RFLPs can be used to trace inheritance patterns, identify specific mutations, and for other molecular genetics techniques.

Restriction enzymes are proteins isolated from bacteria that recognize specific short sequences of DNA and cut the DNA at those sites.

Materials Required:

Restriction enzyme for rs1800469: **Xmni I**, Buffer.

SNP analysis for PCR-RFLP technique:

4µl of direct PCR products were combined with 1XNE buffer4 and 4 units of the restriction endonuclease, **Xmni I**, which recognizes the **GAANN** restriction site, in the 10µl total reaction volume and incubated at 37°for 3hrs .The total volume of the RE reactions were mixed with 2µl gel loading buffer. Multiple bands of RFLP were sized by horizontal electrophoresis on 2.5%mini-agarose gel with 1XTAE at 100 volts for 50-55 minutes, then stained in ethidium bromide solution for 10 minutes and visualized under UV transilluminator, the SNP alleles were examined so they could be distinguished from other SNP loci.

ENZYME LINKED IMMUNO SORBENT ASSAY (ELISA):

ELISA is used for the quantitative measurement of AMH in human serum. Measurement is done by using the Ultrasensitive **AMH/MIS ELISA**

Principle:

It is a quantitative three-step sandwich type immunoassay. The antibody –biotin conjugate binds to the solid phase antibody-ant Ig-En complex which in turn binds to the streptavidin-enzyme conjugate. The antibody –ant Ig-En-biotin conjugate-SHRP complex bound to the well is detected by enzyme-substrate reaction.

The degree of enzymatic turnover of the substrate is determined by the dual wavelength absorbance measured is directly proportional to the concentration of AMH in the samples and calibrators.

Materials required:

- AMH/MIS calibrators,
- coated microtitration strips,
- Assay buffer,
- Biotin conjugate,
- Streptavidin-enzyme conjugate,
- Tetra methyl benzidine (TMB) chromogen solution,
- stopping solution,
- wash concentrate,
- Deionized water.
- Microtitration plate reader,
- microplate orbital shaker,
- microplate washer,
- precision pipette,
- Vortexmixer.

Procedure:

1. Reconstitute AMH/MIS calibrator and AMH/MIH controls I and II each with 1 ml deionized water. Solubilize for 10 minutes, mix well by gentle vortex.
2. Label the microtitration strips to be used.
3. Pipette 25µl of the calibrator, controls and unknown to the appropriate wells.
4. Add 100µl of the AMH/MIS assay buffer to each well using a repeater pipette.
5. Incubate the plate, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 90 minutes at room temperature.
6. Aspirate and wash each strip 5 times with wash solution using an automatic microplate washer.
7. Add 100µl of the antibody-biotin conjugate to each well using a repeater pipette.
8. Incubate the plate, shaking at a fast speed (600-800rpm) on an orbital microplate shaker, for 30 minutes at room temperature.
9. Aspirate and wash each strip 5 times with wash solution using an automatic microplate washer.
10. Add 100µl of the Streptavidin-enzyme-conjugate to each well using a repeater pipette.

11. Incubate the plate, shaking at a fast speed (600-800rpm) on an orbital microplate shaker, for 30 minutes at room temperature.
12. Aspirate and wash each strip 5 times with wash solution using an automatic microplate washer.
13. Add 100µl of the TMB chromogen solution to each well using a precision pipette. Avoid exposure to direct sunlight.
14. Incubate the wells, shaking at 600-800rpm on an orbital microplate shaker, for 8-12 minutes at room temperature.
15. Add 100µl of the stopping solution to each well using a precision pipette. Read the absorbance of the solution in the wells within 20 minutes, using a microplate reader set at 450 nm

Calculations:

Calculate the mean optical density (OD) for each calibrator, control, or unknown.

Plot the Log of the mean OD readings for each of the calibrators along the y-axis versus log of the AMH/MIS concentrations in ng/ml along the X-axis, using cubic-regression curve.

Determine the AMH/MIS concentrations of the controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding AMH/MIS concentrations.

RESULTS

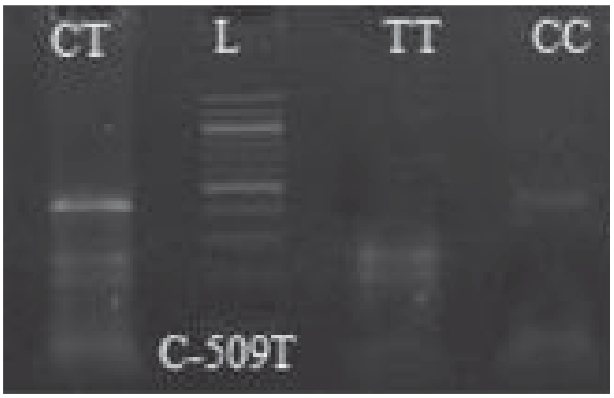
SAMPLE COLLECTION DATA

Table 1: Baseline charactersitics of the study participants

Charactereristics	**Asthma Patient (N=120) Mean ± SD**	**Healthy Control Subjects(N=40) Mean ±SD**	**P- value**
Sex(Male/Female)	57/63	17/23	Ns
Age (years)	40.14±10.76	36.06±17.03	Ns
BMI (kg/m2)	26.57±6.9	24.35±7.2	Ns
Total Serum IgE, IU/L	272.11±156.3	208.7±45.7	0.03
FEV1% Predicted	78.38±17.76		
FVC% Predicted post	82.55±15.68		
FVC % Predicted	74±22		
FVC % Predicted post	79.38±19.96		

PCR-RFLP OUTCOME:

There were 2 fragments (**228,190bp**) for the **TT** genotype and the pattern seemed to be two bands on 2.5%gel, the distinct pattern of the **C**C genotype appeared clearly in one band with **418bp** fragment and heterozygotes with **CT** genotype showed distinct pattern with three bands (**418,228,190bp**).



Genotype and allele frequencies of TGF-β 509C>T polymorphism among the study group

Genotype / Allele	Asthma Patients[n=50]	Healthy controls[n=50]	P =Value
CC [n, %]	11[22%]	16[32%]	control X^2 = 3.74 P= 0.62
CT [n, %]	22[44%]	30[60%]	
TT [n, %]	17[34%]	4[8%]	
C[n , %]	22[44%]	31[62%]	patients X^2=0.57 P = 0.44
T [n, %]	28[56%]	19[38%]	

TGF-β 509C/T genotypes and allilic frequencies are found to be significantly different between two study gropus.

Analysis of risk for the TGF-β 509C>T polymorphism by odds ratios

Genotype	Odds ratio and P-Value	95%CI
CC	**0.5994 [P=0.3678]**	**0.24-1.47**
CT	**0.5238[P=0.1608]**	**0.24-1.16**
TT	**5.9242 [P=0.0026]**	**1.82-19.23**

In TGF-β 509C/T, CC genotype was was found to be significantly protective factor as seen from the crude odds ratio 0.59 and TT genotype emerged as a susceptible genotype with odds ratio 5.92.

SERUM LEVELS EVALUATION BY ELISA

	Asthma Patients (n=50) Mean± SEM (ng/ml)	Normal Healthy Controls (n=50) Mean± SEM (ng/ml)	P Value
	172.35± 36.38	**25.58 ± 3.8**	**0.0215**

The **TGF-β** cytokine serum levels were found to be high in asthma patients (172.35 ± 36.38) $p<0.0215$ than healthy controls (25.58 ± 3.8).

DISCUSSION:

Asthma is a heterogenous disease. Its clinical picture is a result of interactions between different environmental factors and numerous genetic determinants leading to the development of inflammation, bronchial hyperreactivity, recurrent episodes of wheezing and dyspnea is determined by interactions between genetic and environmental factors. It has been estimated that approximately 100 genes are involved in the etiopathogenesis of asthma. It has been estimated that approximately 100 genes are involved in the etiopathogenesis of asthma. Many studies suggested that TGF-beta1 has a prominent role in progression and pathophysiology of asthma.

Transforming growth factor-beta1 (TGF-beta1) is a multifunctional cytokine induced in pro- and anti- inflammatory pathways. It is produced by many types of cells that are activated in the asthmatic response. Recent studies highlighted this cytokine as an important negative regulator in an experimental model of asthma.

Our observation on TGF-beta509C/T polymorphism has shown significance difference in allele frequencies and genotype frequencies. T allele frequency in asthma patients showed a significant increase from control population and C allele frequency has shown decrease in frequency from patients to controls. In TGF-β 509C/T, CC genotype was was found to be significantly protective factor as seen from the crude odds ratio 0.59 and TT genotype emerged as a susceptible genotype with odds ratio 5.92. It was found that serum igE levels are elevated in asthma patients when compared to control population. The TGF-β cytokine serum levels were found to be high in asthma patients (172.35 ± 36.38) $p<0.0215$ than healthy controls (25.58 ± 3.8).

CONCLUSION:

Asthma is common clinical syndrome resulting from several factors such as immunity, heredity and environment. A number of gene s has been proposed as causing are contributing to the development of asthma chronic inflammation coupled with the rise of cytokine TGF-β which may leads to airway remodelling which is a hallmark clinical feature of asthma

In TGF-β 509C/T, CC genotype was found to be significantly protective factor and TT genotype emerged as a susceptible genotype with odds ratio.

The TGF-β cytokine serum levels were found to be high in asthma patients than healthy controls.

The identification of TGF-β and its role in development of asthma provides a focus for the development of novel diagnostic therapeutic strategies, however genetic, epidemiological studies as well as functional analysis are required to fully elucidate the role of these gene in large sample size.

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