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Polymorphic Study on Exon-19 (G>C) of ADAM33 gene and its association with Asthma in Vindhyan population (India)

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Abstract

Asthma is a multifactorial issue, principally coming about because of collaborations among hereditary and ecological variables. ADAM33 gene (situated on chromosome 20p13) has been accounted for to assume an imperative job in asthma. This survey article is proposed to incorporate the majority of the productions, until this point, which have evaluated the relationship of ADAM33 gene polymorphisms just as have demonstrated the job of ADAM33 gene in aviation route rebuilding and their demeanor with asthma. Our investigation on "ADAM33 gene and asthma, reveales "ADAM33 gene polymorphisms" at exon 19 G>C is related with asthma.

The genotype dispersion of ADAM33 (rs528557) was significantly different on the off chance that and control ($\chi 2$ = 18.67, P<0.0001). HC group showed a significant increase in 'GG' genotype when contrasted with asthma patients (59.5% versus 39.44.0%). The heterozygous genotype 'GC' was fundamentally dispersed in HC group when contrasted with cases (34% in control versus 43.88% on the off chance that). Genotype 'TT' was available on the off chance that 16.6% and 6.5% in charge and significantly different. An odds ratio of 0.443 in separately for 'GG' genotype demonstrated a defensive impact of this regular genotype 'GG' in our populace. By and large allele 'G' was observed to be in altogether low frequency in asthma patients bunch when contrasted with HC gathering (61.38% versus 76.5%) while allele 'C' was available in higher frequency in the ailment gathering ($\chi 2$ =20.35, P<0.0001). G allele was discovered defensive with chances proportion of 0.4884 in the interim C allele demonstrates chances proportion 2.047 which shows its solid relationship with athma defenselessness. The example of genotype, allele distribution and carriage rate in ailment and control bunch recommended a critical relationship of ADAM33 (rs528557) (carriage of 'CC' and 'GC') in asthma susceptibility.

Key- words: Asthma, ADAM33, Genotype, Allele frequency.

Introduction

Asthma, a chronic inflammatory disease of the airways, mediated by a Th2 dependent inflammation is an important cause of morbidity in both children and adults worldwide[1]. It is associated with recurrent bouts of cough and wheezing and some of the main concerns are non-responsiveness to steroids, progressive and accelerated lung function decline in a sub-set of patients[1]. The prevalence of asthma in many countries around the world is around 5 per cent in adults and 10 per cent in children[1]. In a large multicenter study in India, the prevalence of asthma was lower and estimated to be about 2.4 per cent among the Indian adults[2]. The smaller percentage still translates to a huge burden of disease in the community taking into account the population of India.

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Asthma is a genetically complex disease and multiple genes and environmental factors play a role in its development. Recently, chromosome 20p13 was significantly linked to asthma and airway hyper-responsiveness in families with asthma from the United Kingdom (UK) and the United States (USA). ADAM33 was identified in this chromosomal region as a candidate gene for asthma and airway hyper-responsiveness and this has been replicated in our Dutch and USApopulations. ADAM33 is a member of the family of proteins known as ADAMs (A Disintegrin And Metalloproteinase). ADAM proteins play a role in cell fusion, cell adhesion, cell signalling and proteolysis [1-5].

The latter includes the shedding of cytokines, growth factors or their receptors from the cell surface. Since ADAM33 is expressed in airway smooth muscle cells and lung fibroblasts, it seems a plausible candidate for airway remodelling. We hypothesise that polymorphisms of the ADAM33 gene are associated with accelerated lung function decline in patients with asthma. it is defined as ‘a chronic inflammatory

disorder of the conducting airways and is characterized by the airway hyper responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or early morning' (Global Initiative for Asthma. It is a complex, chronic inflammatory disorder of airways of the lungs resulting in airflow obstruction.It is a major global public health problem. In asthma, the airways show features of acute and chronic inflammation and associated airway remodelling, which include thickening of the airway wall, subepithelial fibrosis and increased smooth muscle mass. These structural changes may contribute to the development of airflow limitation by increasing airway resistance. Although the majority of individuals with asthma have a normal level of lung function over time, a subgroup has accelerated lung function loss [6,7].

Chronic asthma is a result of abnormal repair and remodeling processes of the airways, characterized by epithelial damage, smooth muscle hyperplasia, and matrix depositions. It continues to have a severe impact on global public health problem, affecting an estimated 300 million people worldwide. The major obstacle in preventing and treating asthma has been our incomplete understanding of its etiology and biological mechanisms. Asthma is one of the most serious allergic diseases and the most common chronic childhood disease in developed nations . It has been characterized by increased responsiveness of the tracheobronchial tree to a multiplicity of stimuli, increased infiltration of various inflammatory cells especially eosinophils into the airway, epithelial damage, airway smoothmuscle hypertrophyconstriction, variable airway obstruction usually associated with inflammation in the conducting airways of the lungs and mucous hypersecretion in the bronchiolar walls of the lung. It is critically dependent on a series of cell adhesion moleculemediated interactions between vascular endothelium and leukocytes, leading to symptoms and elevation in total serum IgE Although environmental factors are important in the origins and progression of asthma, it is widely recognized that asthma has a strong genetic component and is the result of complex interactions between genes and environment [8-12].

This phase can be mild, with or without superimposed severe episodes, or can be much more serious, with severe obstruction persisting for days or weeks; the latter condition is known as "acute severe asthma". In unusual circumstances, acute episodes can cause death(McFadden *et al* 2005) . Asthma exacerbations are characteristically worse at night and can progress to severe airflow obstruction, shortness of breath, and respiratory distress and insufficiency. Rarely, severe sequel such as hypoxic seizures, respiratory failure, and death can occur(Liu AH *et al.*, 2004). Asthma is a chronic (long-term) lung disease that inflames and narrows the airways. Asthma causes recurring periods of wheezing (a whistling sound when you breathe), chest tightness, shortness of breath, and coughing. The coughing often occurs at night or early in the morning. Asthma affects people of all ages, but it most often starts during childhood. In the United States, more than 22 million people are known to have asthma. Nearly 6 million of these people are children. The prevalence of asthma has increased significantly since the 1970s. As of 2010, 300 million people were affected worldwide. In 2009 asthma caused 250,000 deaths globally. Despite this, with proper control of asthma with step down therapy, prognosis is generally good. While asthma is classified based on severity, at the moment there is no clear method for classifying different subgroups of asthma beyond this system. Finding ways to identify subgroups that respond well to different types of treatments is a current critical goal of asthma research [13-17].

Asthma is a genetically complex disease and multiple genes and environmental factors play a role in its development. Recently, chromosome 20p13 was significantly linked to asthma and airway hyper-responsiveness in families with asthma from the United Kingdom (UK) and the United States (USA). ADAM33 was identified in this chromosomal region as a candidate gene for asthma and airway hyper-responsiveness and this has been replicated in our Dutch and USApopulations [3]. ADAM33 is a member of the family of proteins known as ADAMs (A Disintegrin And Metalloproteinase). ADAM proteins play a role in cell fusion, cell adhesion, cell signalling and proteolysis. The latter includes the shedding of cytokines, growth factors or their receptors from the cell surface. Since ADAM33 is expressed in airway smooth muscle cells and lung fibroblasts, it seems a plausible candidate for airway remodelling [4]. We hypothesise that polymorphisms of the ADAM33 gene are associated with accelerated lung function decline in patients with asthma. it is defined as 'a chronic inflammatory disorder of the conducting airways and is characterized by the airway hyper responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or early morning'[1-5].

Although the majority of individuals with asthma have a normal level of lung function over time, a subgroup has accelerated lung function loss. Chronic asthma is a result of abnormal repair and remodeling processes of the airways, characterized by epithelial damage, smooth muscle hyperplasia, and matrix depositions. It continues to have a severe impact on global public health problem, affecting an estimated 300 million people worldwide. The major obstacle in preventing and treating asthma has been our incomplete understanding of its etiology and biological mechanisms. Asthma is one of the most serious allergic diseases and the most common chronic childhood disease in developed nations. It has been characterized by increased responsiveness of the tracheobronchial tree to a multiplicity of stimuli, increased infiltration of various inflammatory cells especially eosinophils into the airway, epithelial damage, airway smoothmuscle hypertrophy constriction, variable airway obstruction usually associated with inflammation in the conducting airways of the lungs and mucous hypersecretion in the bronchiolar walls of the lung [17-21].Heritability estimates of asthma lie between 36 and 79 Recently, positional cloning and candidate genes approach have been widely used to identify genes for asthma and associated phenotypes. A large number of asthma candidate genes have been found on chromosomal regions 2q33, 5q23-31, 6p24-21,11q 21-13, 12q24-12, and 13q14-12. It has been established that inflammation of airways leads to its remodeling and is presumedly related to asthma and associated phenotypes(Cakebread JA 2004) Since the 1990s, various genes associated with inflammation have also been identified using new molecular techniques. Several genome wide screens have identified linkage of certain chromosomal regions with both asthma and inflammation such as 5q23-31, 5p15, 6p21.3-23, 11p15, 12q14-24.2, 13q21.3,14q11.2-13, 17p11.1-q11.2, 19q13, and 21q21. Among them, 5q23-31, 5p15, and 12q14-24.2 are constantly replicated, which contain genes such as IL-3, IL-4, IL-5, IL-9, IL-12b, IL-13, interferon (IFN) γ, iNOS, and FcεRIβ. Most of these genes influence cytokine regulation and activity of eosinophils, mast cells, and neutrophils. ADAM33 is one of many genes that influence the onset and progression of asthma. Identification of ADAM33 gene changed the belief that airway remodeling was solely the result of persistent inflammation and established that it is influenced by other genetic factors also. Its potential in shaping multiple phenotypes such as early life lung functions and chronic obstructive pulmonary disorder makes ADAM33 an important asthma susceptibility gene. Genetic origin of BHR has also been linked with ADAM33. Likewise, another gene, dipeptidyl peptidase 10 (DPP10), was found associated with BHR and IgE. Expression of various genes, such as G-protein coupled receptor for asthma (GPRA) and serine protease inhibitors of the Kazal type (SPINK5), have also been found to have a role in airway remodeling and asthma. [22-27].

Case-control and family based association studies have confirmed a link between *ADAM33* and asthma. Its restricted expression to mesenchymal cells as well as its association with bronchial hyper responsiveness and accelerated decline in lung functions over time strongly point to its involvement in the structural airway components of asthma. Extensive alternative splicing, expression during branching morphogenesis in the developing foetus, impaired lung function in childhood, the production of a soluble form linked to chronic asthma, and tight epigenetic regulation indicate a level of complexity in the way *ADAM33* influences the disease phenotype. *ADAM33* function includes activation, proteolysis, adhesion, fusion, and intracellular signaling. The crystal structure of the catalytic domain of ADAM33 has been resolved around the nonselective matrix metalloproteinase inhibitor (marimastat) in addition to the zinc binding site. *ADAM33* polymorphisms influence lung function in early life and epithelial mesenchymal dysfunction in the airways may predispose individuals toward asthma, being present in early childhood before asthma becomes clinically expressed. *ADAM33* contains over 55 SNPs, some of which play an important role in asthma and related traits. Polymorphisms in the *ADAM33* are associated with an accelerated decline in forced expiratory volume in the first second (FEV1) in the spirometery of general population and these are not only risk factors for the development of asthma, but also for COPD. Thus, polymorphisms in *ADAM33* constitute important risk factors for the development of respiratory diseases in a large subset of the general population [28-31].

ADAM33 belongs to the ADAM12, ADAM15, ADAM19, and ADAM28 subfamily, it is expected to have similar activities.(Howard L *et al* 2000) From a wide range of substrates for ADAM33 only four, *viz.*, stem cell factor (C- kit), β-amyloid precursor protein, TNF-related activation–induced cytokine, and insulin β, were cleaved. However, it is not certain if any of these are natural substrates. A crystallization study of catalytic domain of ADAM33 revealed that the catalytic site shares the feature of other matrix

metalloproteinases having a Zn2_ binding site but differs in structure of substrate pocket. ADAM33 may have cytokine stimulating effects, given that other ADAM proteins (ADAM10 and ADAM17) also appear to interact with inflammatory cytokines. Cysteinerich and EGF domains of ADAM33 have been identified to have a role in cell adhesion and membrane fusion events. These properties of ADAM33 suggest that it might play a role in progression of asthma. Various studies have reported a cluster of single nucleotide polymorphisms (SNPs) in the ADAM33 gene that have significant association with asthma and related phenotypes. Selective expression of the ADAM33 gene in mesenchymal cells suggests its potential to affect the epithelial mesenchymal tropic unit (EMTU) along with TH2 cytokines. In the last decade, tremendous progress has been made in the genetic study of asthma with many genes identified as asthma-susceptible genes. ADAM33 gene is the first novel susceptibility gene for asthma and airway hyperresponsiveness (AHR) identified by positional cloning and has been replicated in over 33 different population samples worldwide. The *ADAM33* protein harbours several domains which include prometalloproteaselike, disintegrin-like, cysteine-rich, epidermal growth factorlike, transmembrane, and cytoplasmic domains, and many specific functions of these domains have already been discovered [32-34].

It is highly expressed in lung, heart, and brain of adults. However, the specific process by which *ADAM33* variants could cause asthma is unknown. One of the hypotheses suggests that*ADAM33* may be involved in airway remodelling. The *ADAM33* is indeed expressed in airway fibroblasts, myofibroblasts, and smooth muscle. An association with *ADAM33* variants in adult-onset asthma has been observed in several. However, most such studies on *ADAM33* in asthma have been carried out on Caucasian populations; very few reports on *ADAM33* SNPs in asthma are available for Asian populations [27].

Material and Methods

Sample Collection:

(a) Patient recruitment

Medically certified Hypertensive and Stroke patients were recruited from medicine department (OPD) of Shyam Shah medical college Rewa, District Hospital Rewa, and Krishna Pathology Rewa, Madhya Pradesh during the July to November 2017. 210 patients were enrolled for cases and 230 healthy controls were recruited for the study.

(b) Healthy controls

230 randomly selected healthy controls (HC) for each disease were enrolled in the study. They consist of medical staff and healthy volunteers from Rewa as well as individuals residing in central region of India. Hence, control group was drawn from same area assuming similar environmental and social factors with same mean age and sex ratio.

(c) Sample collection strategy

Approximately 3-5 ml. of blood sample was collected in 0.5 M EDTA tubes from each Hypertensive and Stroke patients as well as from healthy controls. These samples were stored frozen at -80˚C until DNA was extracted from them. While taking the samples, the following life style factors of the patients and control persons will be recorded: Habit of smoking, tobacco chewing and alcohol consumption, height and weight, occupation type, physical exercise and mental work, family history for disease. The clinical profile other information were filled in a detailed Performa.

Method for DNA isolation

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller et al. 1988)Frozen blood sample was thawed at room temperature. 0.5 ml of whole blood sample was suspended in 1.0ml of lysis buffer(0.32M sucrose, 1mM Mgcl2, 12 mM Tris,and 1% Triton X 100) in a 1.5ml microcentrifuge tubes. This mixtures mixed gently by inverting the tube upside down for 1 min. The mixture was then allowed to stand for 10 min at room temperature to ensure proper lysis of cells.The mixture was centrifuged at 11000 rpm for 5 min at 4 ^{0}C to pellet the nuclie. The supernatant was discarded carefully in a jar containing disinfectant, as pallet formed is losely adheres to the bottom of centrifuge tube. The pallete was resuspended in 0.2 ml of lysis buffer and recentrifuged at 11000 RPM for 5 minutes. The pallet was then dissoved in 0.2 ml of deionised autoclaved water and mixed thourughly on vorteser. The mixture was recentrifuged at 14000 RPM for 1 min at 4 ^{0}C. Supernatent was discarded to gain an intaccr pallet. To the above pallet, 80 micro littre of proteinase K buffer (0.375 M NaCl, 0.12 M EDTA, pH 8.0) and 10 micto littre of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was wrll frothed with the help of micro tip to allow proper lysis of palleted nuclei. After digestion was complete, 100 micro littre of saturated cold 5 M NaCl was added and shaken rigourously for 15 s. To the above mixture 0.2 ml of d-ionised autoclaved water and 0.4 ml of Phenol-chloroform (4:2 v/v) was added to

remove most of the non-nucleic acid organic moleccules. Mocro centrifuge tube was inverted upsided own untill the silution turned milkey. Phases were separated by centrifuging the above mixtute at 12000 RPM for 10 min at 4 ^{0}C. Aqueous (top) layer was saved and transferred in another micro centrifuge tube. Transferring of any interface layer was avoided. To the aqueous layer, 1 ml chilled absolute ethonol was added and the tube was inverted several times untill the DNA precipitated. DNA precipitates like thread. This was centrifuged at 14000 RPM for 4 min at 4^{0}C to pallete the DNA thread. Supernatent was discarded. The pallet was washed twice with 1 ml of 70% alcohol. The mixture was again centrifuged at 14000 RPM for 1 min 4^{0}C. Supernantent was discarded and pallet was air dried for 10-20 mins. The palletted DNA was re-hydrated in 100-200 micro littre of TE buffer, pH-7.4 (10 mM Tris-HCl pH-7.4, 1 mM EDTA, pH-8.0) DNA was allowed to dissolved overnight at 37^{0}C before quantitating.

Determination of Quality and Quantity of isolated DNA:

The isolated DNA is to be used for PCR based study. Therefore its suitability for PCR along with its size, heterogeneity is among the most important criteria for purity. As a matter of general practice all DNA preparations were tested for quality and quantity measures as described in the following paragraph.

Quantitation by UV spectrophotometry

The isolated genomic DNAs were then tested for purity measuring their absorbance values at 230 nM, 260 nM, 280 nM and 300 nM and chilled using a UV visible spectrophotometer (Systronic, India). A DNA preparation was considered to be good if it had a 260 nM/ 280 nM ratio as approximately 1.8 and a 300 nM was 0.1 or lesser. The absorbance at 260 nM was used to calculate the amount of DNA, using the relationship that double standard DNA at 50 microgram/ml concentration has an absorbance = 1.0 at 260 nM.

Agarose Gel Electrophoresis

Gel Electrosphoresis of the genomic DNA was carried out for qualitative estimation of samples prepared. A good DNA preparation appears as single band. A horizontal Agarose slab gel electro-phoresis apparatus (Bangalore Genei, Bangalore, India) was used. In brief, 2 micro litter of each genomic DNA was loaded on 0.8 agarose (0.8% w/v, Sigma) containing Ethidium Bromide solution (0.5 micro gram/ml) and electro phoresis was done at 80 V in 1X TAE buffer (40 mM Tris, 20 mM Acetic, 1 mM EDTA). Lambda DNA Eco R1/Hind III double digest (Bangalore, Genei, Bangalore, india) was used as molecular waight marker after completion of electro phoresis, the DNA bands were visualized and photographed using an UV trans illuminator (312 nM) and Gel documentation system (Vilber Lourmate, Cedex 1, France) respectively.

Polymorphism Screening

In general the genomic DNA extracted from peripheral blood of healthy individuals and diseased individuals was subjected to PCR followed by restriction digestion and electrophoresis to genotyte both the groups for relevant gene of interest.

All the PCRs were carried out in a PTC 200 thermo cycler (MJ Research Inc, USA). PCR is a rapid, inexpensive and simple means of producing relatively large copy number of DNA molecules from the small amount of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amount of DNA, which could serve as an unwanted template. Appropriate negative control was included in each PCR run carried out for each gene, to monitor decontamination of PCR mix to avoid any false positive results. The negative control used for PCR contained whole PCR reaction mix except target DNA, which was replaced by HPLC purified water, free of RNAs, DNAs and any contamination from any other source, resembling the gene sequence.

Subsequently restriction enzyme digestion was performed by incubating the double standard DNA with appropriate amount of restriction enzyme in its respective buffer as recommended by the supplier and at optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per micro gram of starting DNA. One enzyme unit is usually defined as the amount of enzyme needed to completely digest one micro gram of double standard DNA stranded in one hour at the appropriate temperature. There bio chemical activity of the restriction enzyme is the hydrolysis of phosphodiester backbone at specific sites in a DNA sequence. Precaution was taken to avoid star activity of restriction enzymes. When DNA is digested with certain restriction enzyme under non-standard condition, cleavage can occur at sites different form the normal recognition sequence. Such aberrant cutting is called “Star Activity” which can be due to high pH (>8.0) or low ionic strength, extremely high concentration of enzyme (>100 U/ microgram of DNA) and presence of organic solvants in the reaction (e.g. Ethanol, DNSO).

The PCRand restriction digestion conditions were optimized for specific locus of a relevant segment of

the gene to be studied. The PCR produces as well as the digested products were separated on either agarose gel or polyacrylamide gel, depending on their size. Gels were stained with ethidium bromide solution (0.5 microgram/ml) and subsequently visualized and photographed under UV trans-illuminator.

Detection of Genetic Polymorphism In ADAM33:

PCR amplification with specific primers gave 211bp product which was digested with NarI enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The common genotype (GG) was digested in to two fragments of 147bp and 64bp, whereas the mutated homozygous genotype (CC) was not digested and fount in a single fragment of 211bp..

Primer Sequences

Forward primer (F) 5'AGAGCTCTGAGGAGGGGAACCG3'

Reverse primer: (R) 5'GCAGACCATGACACCTTCCTGCTG3'

PCR Mix

The PCR was carried out in afinal volume of 25 micro litre, containing 100 ngof genomic DNA(4-5micro litre), 2.5 microlitre of 10X buffer(10 mM Tris HCl pH8.8,50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin,0.005% NP 40; final concentration1 X; Genetix Biotech Asia pvt. Ltd.) 1microlitre of 10mM dNTPs(Banglore Geneie , Banglore, india) 1 microlitre of 25pmol/microlitre of forward and reverse primer specific for and 1 microlitre of unit of 1U/microlitre Red Taq DNA polymerase (Banglore Geneie).

PCR Thermal Programm

After an initial denaturation of 5 min. at 94oC, the samples were subjected to 35 cycles at 94oC for 1 min. at 55 for 40 s, and 72oCfor 40s, with a final extension of 10 min at 72oC in a thermal cycler . A 100 bp ladder with amplified product has been run under agarose gel electrophoresis.

Restriction digestion

The obtained DNA product has been subjected to restriction digestion with NarI endonuclease at 37`c for 8 hrs. The common genotype (GG) was digested in to two fragments of 147bp and 64bp, whereas the mutated homozygous genotype (CC) was not digested and fount in a single fragment of 211bp.

Results and Discussion

Anthropometric results:

The descriptive data and comparison of anthropometric parameters of asthma patients versus controls are presented in Table No.-3. The age, sex, BMI were the parameters. The asthma patients had slightly low levels of weight for women (P=0.0573), Men (P=0.4301) and BMI of Women (P=0.0388*), Men (P=0.4301). In women BMI were slightly significant with Asthma.

Table 1: Comparison of anthropometric parameters of Asthma patients and controls

Characteristics	*Cases*	*Controls*	*P-value*
n(Men/Women)	180(123/57)	200(130/70)	
Age(years)	52.5±12.5	53.0±14.2	0.7100, ns
Height(m)	162.6±8.40	164±9.60	0.1330, ns
Weight (Kg)			
Women	60.5 ±5.70	61.5 ± 4.50	0.0573, ns
Men	64.2±5.60	64.3±7.1	0.8798, ns
BMI (kg/m^2)			
Women	26.4±3.1	25.1 ± 4.3	0.0388*
Men	24.6±4.7	24.1± 5.1	0.4301, ns

*denotes level of significant change between case and control

DNA quality assessment by agarose gel electrophoresis:

Qualitative analysis of isolated DNAs were routinely carried out by visual observation of ethidium bromide stained gels. When genomic DNA was electrophoresed through 0.8% agarose gels, a distinct bright DNA band migrated equivalent to or slower than the 21.1 kb band of Eco RI/ Hind III double digested lambda DNA. This showed that the isolated DNA was of high molecular weight and was also undegraded as inferred from the lack of or minimal smearing seen. Figure.9 shows the gel image having DNA samples of RA patients and HC group.

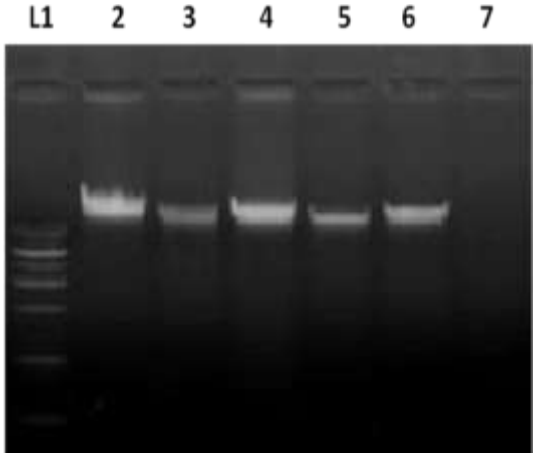


Fig. 1: Representative gel image of isolated DNA of case and control (L1 – Eco RI/ Hind III double digested lambda DNA and L2-6 contains isolated DNA, 7 is negative control)

Detection of Genetic Polymorphism in ADAM33:
PCR amplification with specific primers gave 211bp product which was digested with NarI enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The common genotype (GG) was digested in to two fragments of 147bp and 64bp, whereas the mutated homozygous genotype (CC) was not digested and fount in a single fragment of 211bp. The heterozygous genotype (GC) was represented as 3 fragments of 211, 147, and 64 bp as depicted in gel picture 15.

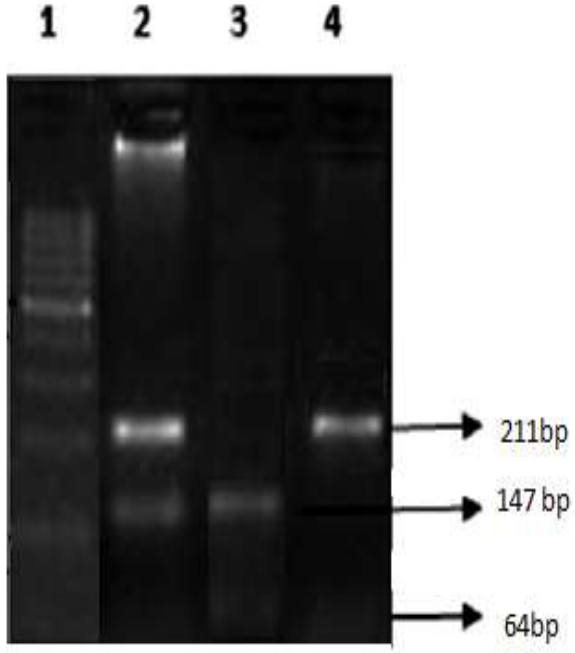


Fig. 2: Gel 2: Representative gel picture of ADAM33 (rs528557) polymorphism (Lane 1: 100bp DNA ladder , Lane 2: heterozygous GC having three bands of 211bp, 147bp, 64bp, Lane 3: GG two bands of 147 and 64bp , Lane 4: CC one band of 211bp.)

The distribution of the polymorphisms of ADAM33 (rs528557) was consistent with Hardy- Weinberg equilibrium (HWE) in both asthma patients and healthy controls. The observed genotype frequencies, allele frequencies and carriage rates for ADAM33 exon 19 G>C polymorphism are depicted in table 4 and table 5 nd Graph 1, 2, 3. The genotype distribution of ADAM33 (rs528557) was significantly different in case and control (χ^2 = 18.67, P<0.0001). HC group showed a significant increase in 'GG' genotype as compared to asthma patients (59.5% vs 39.44.0%). The heterozygous genotype 'GC' was significantly distributed in HC group as compared to cases (34% in control vs 43.88% in case). Genotype 'TT' was present in case 16.6% and 6.5% in control and significantly different. An odds ratio of 0.443 in respectively for 'GG' genotype indicated a protective effect of this common genotype 'GG' in our population. An odds ratio of 2.80 of less common genotype 'CC" showed the possible role of 'CC' genotype in pathophysiology of athma. Heterozygous genotype showed odds ratio of 1.518 and may also be associated with disease susceptibility. Overall allele 'G' was found to be in significantly low frequency in asthma patients group as compared to HC group (61.38% vs. 76.5%) whereas allele 'C' was present in higher frequency in the disease group (χ^2 =20.35, P<0.0001). G allele was found protective with odds ratio of 0.4884 meanwhile C allele shows odds ratio 2.047 which indicates its strong association with athma susceptibility. Carriage rate of allele 'G' was slightly higher in HC, whereas carriage rate of allele 'C' was significantly higher in disease group as compare to control (60.5% in case vs 40,5% in control) and difference was significant (χ^2 = 8.037, 0.0046). The pattern of genotype, allele distribution and carriage rate in disease and control group suggested a significant association of ADAM33 (rs528557) carriage (carriage of 'CC' and 'GC') in asthma susceptibility. CC Genotype, C allele carriage has shown strong susceptibility to RA (P value- 0.0020 OR- 2.877, 95% CI-1.449 to 5.710, P Value <0.0001 OR-2.047, 95% CI-1.496 to 2.802 respectively) meanwhile GG Genotype and G allele Carriage was significantly protective (P value< 0.0001 OR- 0.4434, 95% CI-0.2939 to 0.6689, P Value < 0.0001 OR-0.4884, 95% CI- 0.3569 - 0.6685respectively).

Table 2: Frequency distribution and association of Genotype, allele frequency and carriage rate of ADAM33 G<C polymorphism in population of Vindhyan region using Chi Square Test

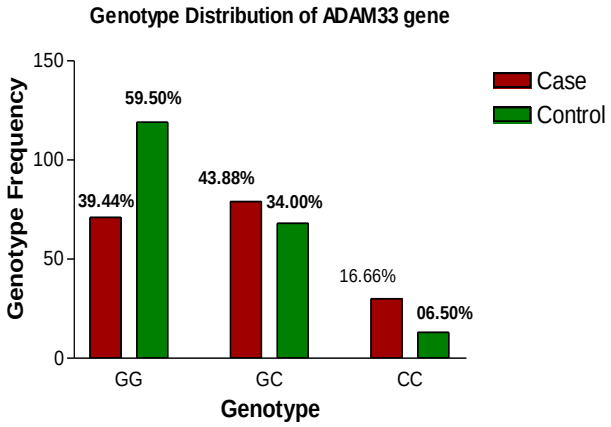
ADAM33 Genotype	CASE N= 180 n %	CONTROL N=200 n %	CHI SQUARE VALUE χ^2 (P Value)
Genotype			
GG	71 39.44	119 59.50	18.67, (P<0.0001***)
GC	79 43.88	68 34.00	
CC	30 16.66	13 06.50	
Allele			
G	221 61.38	306 76.50	20.35, (P<0.0001***)
C	139 38.62	9 4 23.50	
Carriage Rate			
G	150 57.91	187 69.77	8.037, (P=0.0046**)
C	109 42.08	81 30.22	

N – Number of individuals in study group
%- Genotype allele frequency and carriage rate expressed in percentage
* denotes the level of significant association between case and control

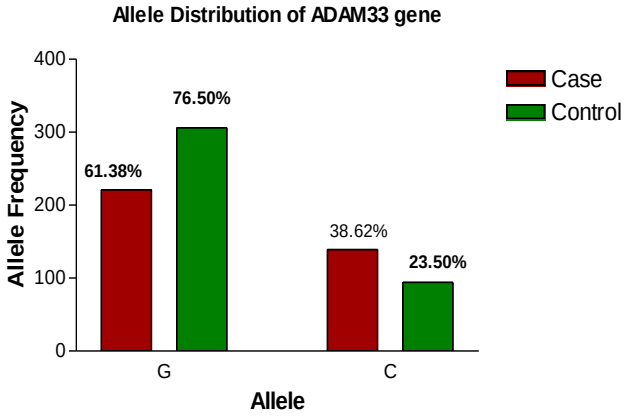
Table 3: Fisher Exact Test values ADAM33 G<C polymorphism.

ADAM33 Genotype	CASE N= 180 n %	CONTROL N=200 n %	P Value	Odds Ratio
GG	71 39.44	119 59.5	0.0001***	0.4434 (0.2939 to 0.6689)
GC	79 43.88	68 34	0.0575	1.518 (1.002 to 2.300)
CC	30 16.66	13 6.5	0.0020**	2.877 (1.449 to 5.710)
Allele				
G	221 61.38	306 76.5	P<0.0001***	0.4884 (0.3569 - 0.6685)
C	139 38.62	94 23.5		2.047 (1.496 to 2.802)
Carriage Rate				
G	150 57.91	187 69.77	0.0050**	0.5961 (0.4163 to 0.8535)
C	109 42.08	81 30.22		1.678 (1.172 to 2.402)

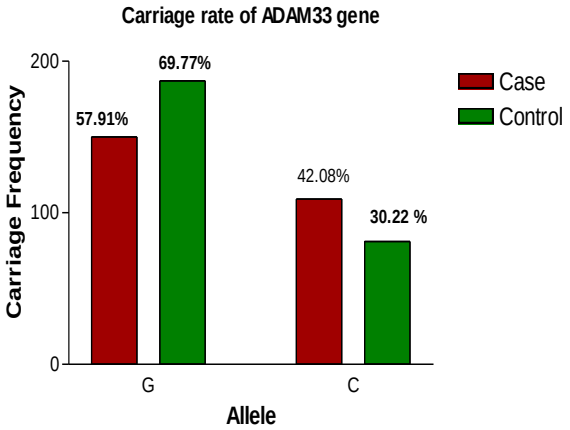
(* denotes the level of significant association between case and control)
N – Number of individuals in study group
%- Genotype allele frequency and carriage rate expressed in percentage



Graph No.1: Genotypic Distribution of ADAM33 gene in population.



Graph No.2: Allele Distribution of ADAM33 gene in population.



Graph No.3: Genotypic Distribution of ADAM33 gene in population.

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or early in the morning. These episodes are usually associated with widespread, but variable airflow obstruction within the lung that is often reversible either spontaneously or with treatment. The symptoms of asthma are due to bronchoconstriction, excess mucus production and airway inflammation. Episodes of worsening symptoms, or asthma exacerbations, are an exaggerated lower airway response to an environmental exposure, such as respiratory virus infections, allergens, pollutants, medications or other irritants (Wark PA and Gibson PG; 2006). Structural changes reported in the airways of patients with asthma include epithelial fragility, goblet cell hyperplasia, enlarged submucosal mucus glands, angiogenesis, increased matrix deposition in the airway wall, increased airway smooth muscle mass, wall thickening and abnormalities in elastin. These alterations are thought to be due to genetic influences, early life exposures, duration of disease and long-term uncontrolled inflammation [33].Asthma is multi-factorial diseases with genetic as well as environmental factors influencing disease progression. A gene encoding a disintegrin and metalloprotease (ADAM) 33 located on chromosome 20p13 was identified by positional cloning as a putative candidate gene for the development of asthma and bronchial hyper-responsiveness (BHR) [23,40].

Although asthma is a chronic obstructive condition, it is not considered as a part of chronic obstructive pulmonary disease as this term refers specifically to combinations of disease that are irreversible such as bronchiectasis, chronic bronchitis, and emphysema. Over 100 genes have been associated with asthma in at least one genetic association study. However, such studies must be repeated to ensure the findings are not due to chance. ADAMs are membrane-anchored proteins belonging to the zinc protease super family [23,26,31]. They play a role in cell adhesion, cell migration and proteolysis and thus are fundamental to many control processes in development and homeostasis [36]. ADAM33 might be associated with overall mortality through its link to “inflamm-ageing”. This phenomenon refers to the fact that ageing is associated with chronic, low grade inflammatory activity leading to long-term tissue damage and systemic chronic inflammation which

contribute to increased mortality in elderly individuals [38]. In 2002, Van Eerdewegh et al. identified ADAM33 as a susceptibility gene for asthma and airway hyper responsiveness (Van Eerdewegh P *et al*., 2002). Subsequent studies have linked polymorphisms in ADAM33 to airway hyper responsiveness and airway inflammation in Chronic Obstructive Pulmonary Disease (COPD), and to accelerated lung function decline and COPD development in the general population [39].

Our study revealed anthropometric association with asthma. We selected 180 asthma patent and 200 control population. In which age, sex, BMI were the parameters. The asthma patients had slightly low levels of weight for women (P=0.0573), Men (P=0.4301) and BMI of Women (P=0.0388*), Men (P=0.4301). In women BMI were slightly significant with Asthma. Qualitative analysis of isolated DNAs were routinely carried out by visual observation of ethidium bromide stained gels. When genomic DNA was electrophoresed through 0.8% agarose gels, a distinct bright DNA band migrated equivalent to or slower than the 21.1 kb band of Eco RI/ Hind III double digested lambda DNA [12].This showed that the isolated DNA was of high molecular weight and was also undegraded as inferred from the lack of or minimal smearing seen. Figure.9 shows the gel image having DNA samples of RA patients and HC group. We calculated hardy-Weinberg value of ADAM33 and TNF-α 308 promoter for case and control population was below the tabulated value (3.84). In asthma patient ADAM33 (0.99) and TNF-α 308 promoter (0.14) thus in control population ADAM33 [9, 24].

PCR amplification with specific primers gave 211bp product which was2 digested with NarI enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The common genotype (GG) was digested in to two fragments of 147bp and 64bp, whereas the mutated homozygous genotype (CC) was not digested and fount in a single fragment of 211bp. [25].The heterozygous genotype (GC) was represented as 3 fragments of 211, 147, and 64 bp. The genotype distribution of ADAM33 (rs528557) was significantly different in case and control (χ^2 = 18.67, P<0.0001). HC group showed a significant increase in 'GG' genotype as compared to asthma patients (59.5% vs 39.44.0%). The heterozygous genotype 'GC' was significantly distributed in HC group as compared to cases (34% in control vs 43.88% in case). Genotype 'TT' was present in case 16.6% and 6.5% in control and significantly different. An odds ratio of 0.443 in respectively for 'GG' genotype indicated a protective effect of this common genotype 'GG' in our population. An odds ratio of 2.80 of less common genotype 'CC" showed the possible role of 'CC' genotype in pathophysiology of asthma. Heterozygous genotype showed odds ratio of 1.518 and may also be associated with disease susceptibility. Our study is similar to findings of Awasthi S,et. al.[9] who revealed ADAM33 gene and asthma in Indian children has been examined using a case–control study. Five SNPs of the ADAM33 gene, F+1(rs511898) G/A, S2 (rs528557) G/C, ST+4 (rs44707) A/C, ST+5 (rs597980) C/T and V4 (rs2787094) C/G, were analyzed in 211 asthma cases and 137 controls aged 1–15 years using the PCR–restriction fragment length polymorphism method. genotypes and mutant alleles were significantly associated with increased asthma risk. A positive Taken together, out results suggest that ADAM33 gene polymorphisms may modify individual susceptibility to develop childhoodasthma in the Indian population[17,28,30].

Overall allele 'G' was found to be in significantly low frequency in asthma patients group as compared to HC group (61.38% vs. 76.5%) whereas allele 'C' was present in higher frequency in the disease group (χ^2 =20.35, P<0.0001). G allele was found protective with odds ratio of 0.4884 meanwhile C allele shows odds ratio 2.047 which indicates its strong association with asthma susceptibility. Carriage rate of allele 'G' was slightly higher in HC, whereas carriage rate of allele 'C' was significantly higher in disease group as compare to control (60.5% in case vs 40,5% in control) and difference was significant (χ^2 = 8.037, 0.0046). The pattern of genotype, allele distribution and carriage rate in disease and control group suggested a significant association of ADAM33 (rs528557) carriage (carriage of 'CC' and 'GC') in asthma susceptibility. CC Genotype, C allele carriage has shown strong susceptibility to RA (P value- 0.0020 OR- 2.877, 95% CI-1.449 to 5.710, P Value <0.0001 OR-2.047, 95% CI-1.496 to 2.802 respectively) meanwhile GG Genotype and G allele Carriage was significantly protective (P value< 0.0001 OR- 0.4434, 95% CI-0.2939 to 0.6689, P Value < 0.0001 OR-0.4884, 95% CI- 0.3569 - 0.6685respectively). SNP rs528557 was shown to be associated with asthma in a UK-based Caucasian population (Van Eerdewegh et al. 2002), a family based study in Germans in African-American [39,41] , US whites, Hispanic, Japanese (Hirota et al. 2006), and in Thai population (Thongngarm et al. 2008), besides the present study on Indian populations [9, 11].This study was done on the small population and

if we perform the analysis in large population then we can have significant result. Furthermore, we studied a life style factor like smoking which showed association with asthma between case and control population. We sorted smoker in study population (including case and control) and find smoking habit was a risk factor for asthma (χ^2 = 10.53, P=0.0012**).Overall we can conclude that ADAM33 gene polymorphism and smoking habit were associated with risk of asthma.

Conclusion

Asthma is a complex, chronic inflammatory disorder of airways of the lungs resulting in airflow obstruction; bronchial hyper responsiveness (BHR) to a variety of stimuli; and symptoms of wheeze, cough, and breathlessness. It is a major global public health problem. It is estimated that there are about 300 million asthmatic patients worldwide. Chronic asthma is a result of abnormal repair and remodeling processes of the airways, characterized by epithelial damage, smooth muscle hyperplasia, and matrix depositions. It has been postulated that the aforementioned processes are a result of complex interactions between genes and the environment, often beginning in utero and early infancy. The exact role of ADAM33 in causing asthma is unclear .Various studies have reported a cluster of single nucleotide polymorphisms (SNPs) in the ADAM33 gene that have significant association with asthma and related phenotypes. Selective expression of the ADAM33 gene in mesenchymal cells suggests its potential to affect the epithelial mesenchymal tropic unit (EMTU) along with TH2 cytokines.

The genotype frequencies of each gene in each study group were tested to be in accordance with Hardy Weinberg equilibrium using chi square (χ2) test for independence. When the calculated value of χ2 was less than tabulated value of χ2 at degree of freedom 1 (d.f. = 1) and level of significance (P = 0.05), the population is at equilibrium for the gene and vice versa. The standard tabulated value of χ2 at degree of freedom 1 and level of significance 0.05 is 3.84. All the tabulated χ2 values for all the genes were compared to this value. The genotype frequencies of all the study groups included in the study were in accordance with Hardy Weinberg equilibrium.PCR amplification with specific primers gave 211bp product which was digested with NarI enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The common genotype (GG) was digested in to two fragments of 147bp and 64bp, whereas the mutated homozygous genotype (CC) was not digested and fount in a single fragment of 211bp. The heterozygous genotype (KQ) was represented as 3 fragments of 211, 147, and 64 bp.

The genotype distribution of ADAM33 (rs528557) was significantly different in case and control (χ^2 = 18.67, P<0.0001). HC group showed a significant increase in 'GG' genotype as compared to asthma patients (59.5% vs 39.44.0%). The heterozygous genotype 'GC' was significantly distributed in HC group as compared to cases (34% in control vs 43.88% in case). Genotype 'TT' was present in case 16.6% and 6.5% in control and significantly different. An odds ratio of 0.443 in respectively for 'GG' genotype indicated a protective effect of this common genotype 'GG' in our population. An odds ratio of 2.80 of less common genotype 'CC" showed the possible role of 'CC' genotype in pathophysiology of asthma. Heterozygous genotype showed odds ratio of 1.518 and may also be associated with disease susceptibility. We selected some inflammatory genes also for the present investigation and found that polymorphism in ADAM 33 is strongly associated with asthma. Before our study only a single study supports that these genes may be associated with asthma. It has been widely accepted that in patients of asthma a chronic and low grade inflammation is present in bronchial airway. Finally I strongly recommend that both genetic as well as environmental factors are responsible for asthma. A complex interaction of these both factors is needed to cause asthma. Genome wide association study in large sample size in this area is needed to study the associated genes which are associated with pathophysiology of asthma and its related complication. Further proteomic (Functional Genomic) study is also recommended. Life style factors are those factors which could be modified and by modifying the life style the asthma management is possible so we strongly recommend that peoples must make a better life style is another better approach to minimize the risk of asthma.

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