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# Increased expression of *ADAM33* protein in asthmatic patients as compared to non-asthmatic controls

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***Background & objectives*: *ADAM33* is a member of a family of genes that encode membrane-anchored proteins with a disintegrin and a metalloprotease domain, primarily expressed in lung fibroblasts and bronchial smooth muscle cells. *ADAM33* has been identified as a risk factor for asthma and is known as a gene associated with airway remodelling. The present study was conducted with the aims to investigate the expression of *ADAM33* protein in patients of asthma and non-asthmatic controls, and to assess if the expression of *ADAM33* protein relates with severity of asthma.**

***Methods*: A total of 35 subjects, including 27 patients with asthma and eight non-asthmatic controls were included using Global Initiative for Asthma guidelines 2005. Bronchial biopsy tissues were collected and paraffin sections were made to store all study samples. Immunohistochemistry was performed using standardized protocol.**

***Results*: An increase in expression of *ADAM33* protein was observed in the epithelium, smooth muscle and mesenchymal cells of asthma cases when compared to controls but there was no relationship with severity of asthma.**

***Interpretation & conclusions*: A higher expression of *ADAM 33* protein was seen in asthma patients compared to controls. Large prospective studies need to be done with adequate study design to confirm these preliminary finding.**

**Key words** *ADAM33* protein expression - asthma - bronchial biopsy - immunohistochemistry - severity of asthma

Asthma is one the most common chronic disorders with several overlapping phenotypes[1], characterized by symptoms of intermittent airway obstruction[2]. In asthma, the airways show features of acute and chronic inflammation that are associated with airway remodelling which include thickening of the airway wall, subepithelial fibrosis, increased smooth muscle mass that may contribute to the development of airflow limitation by increasing airway resistance.

The *ADAM* gene family is a sub group of the zinc-dependent metalloproteinase having surface proteins with adhesion and protease activity[3]. *ADAM33* is the member of the *ADAM* family and is most closely

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related to human *ADAM12, 15, 19* and *Xenopus ADAM13*, all of which possess proteolytic activity[4-7]. *ADAM33* represents a complex domain structure, also contains a transmembrane and cytoplasmic domain. The domain encodes signal sequence, pro, catalytic (metalloproteinase), disintegrin-like, cysteine-rich, and epidermal growth factor (EGF)[3,4,8]. This complex domain construction allows ADAM proteins to be implicated in diverse functions including cell proliferation, differentiation, migration, and embryogenesis via ecto-domain shedding of growth factors, ligands and receptors, membrane fusion, and cell adhesion via interactions with integrins and extracellular matrix proteins[9-13]. From a functional standpoint, these different domains interpret different functions of *ADAM33*, which include role in cell-cell and cell-matrix interactions[14], cell migration[12,15], cell-cell adhesion[16] and signal transduction[17]. ADAM proteins are membrane anchored proteins and can function as a sheddases, and can "release a variety of cell-surface proteins, including growth factors, cytokines, cell adhesion molecules and receptors"[3,14,18]. Additionally, soluble form of ADAM33 promotes angiogenesis which has been described as the first biological function[19].

Van Eerdewegh *et al*[20] identified *ADAM33* as an asthma susceptibility gene with the help of positional cloning method on an outbred Caucasian population. Linkage analysis and association studies of single nucleotide polymorphisms (SNPs) and haplotypes provide strong evidence for the involvement of several SNPs of *ADAM33* for susceptibility to asthma and bronchial hyperresponsiveness (BHR)[21]. Many association studies have confirmed association of *ADAM33* gene polymorphisms with asthma susceptibility and airway hyperreactivity, whereas other studies have shown absence of association[21].

Studies on the expression of *ADAM33* mRNA in human cells and tissues have revealed exclusive distribution of this protein in cells of mesenchymal origin, such as fibroblasts and smooth muscle cells, which indicates a possible role of *ADAM33* in airway remodelling[22-24]. Studies show *ADAM33* gene as a main challenge for asthma development and bronchial hyperreactivity. Therefore, the objectives of the present study were to investigate the expression of *ADAM33* protein in patients of asthma and controls, and to assess if the expression of *ADAM33* protein relates with severity of asthma.

## Material & Methods

From August 2007 to December 2010 a case-control study was conducted in the Departments of Pulmonary Medicine, Pediatrics and Pathology of Chattrapati Shahuji Maharaj Medical University, a tertiary care, referral center at Lucknow, Uttar Pradesh, India. The study protocol was approved by the institutional ethics committee and written informed consent for participation was obtained from all the patients and controls.

*Characterization of phenotype*: All subjects who underwent bronchoscopic examination from August 2007 to December 2010 were screened for suitability for inclusion in the study. Of the total 859 subjects, 27 subjects as asthma cases and eight as non-asthmatic controls satisfied the inclusion and exclusion criteria. Inclusion criteria for cases were *(i)* diagnosis of asthma according to the treating physician, *(ii)* symptoms, *(iii)* use of medications for asthma, *(iv)* reversible airflow limitation according to GINA (Global Initiative for Asthma) guidelines; FEV1 reversibility >12 per cent and 200 ml after a post-bronchodilator spirometry, *(v)* on any regular medication for asthma such as corticosteroids, β-2 agonist, methylxanthines, leukotriene modifiers and cromones, *(vi)* at least one urgent care visit in the previous 12 months, and *(vii)* at least 3 year records of medically diagnosed asthma. Additionally, all considered patients had positive family history of asthma. Asthma severity was classified according to GINA guidelines[2].

The inclusion criteria for controls were: *(i)* no past or present diagnosis of asthma and other pulmonary diseases, *(ii)* no history of wheezing, shortness of breath, and other symptoms of allergic diseases such as nasal and skin symptoms, *(iii)* no use of medications for asthma, and *(iv)* absence of first-degree relatives with a history of asthma.

*Spirometry*: Pulmonary function test (PFT) was done using a spirometer (Spiro lab II, MIT II, Longfian Scitech Company, Korea) of all cases and controls before they underwent a bronchoscopic examination according to standard guidelines[25]. Post-bronchodilator reversibility testing was performed 15-30 min after 2 puffs of salbutamol. Controls underwent only a pre-bronchodilator study.

*Bronchosopy*: Flexible bronchoscope (Model-PENTAX FB-18V (South India Surgical Co. Ltd., New Delhi Bronchoscope from Japan) was used for performing

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bronchoscopy. Accessories for tissue sampling used were bronchial brushes [Bigger (Model-CS6015ST); Medium (Model-CSC3S) and Small (Model-CS6002SN)], aspiration needle and endobronchial biopsy forceps (Model-KW24159).

All bronchial biopsies were fixed in 10 per cent buffered formalin and embedded in paraffin. The whole tissue sample was blocked and 3-µm sections were taken for the immunohistochemistry analysis.

*Immunohistochemistry*: The sections were dewaxed in xylene and dehydrated through graded alcohol concentration and washed in tris buffer (*p*H 7.2). Endogenous peroxidase activity was blocked using 3 per cent hydrogen peroxide in methanol for 30 min; antigen retrieval was done with citrate buffer (*p*H 6.2) at $90^0$ C for 90 min in a water bath. After washing in tris-buffer (*p*H 7.4), sections were incubated with the primary polyclonal ADAM33 antibody (Rb pAb –abcam ab39193-100) (*Source*: Allied Scientific Products, Kolkata) at a dilution range of 1:100 and left overnight at $4^oC$ in a humid chamber. The next day, sections were brought to room temperature and further procedures were performed involving the following steps; washing in tris-buffer, incubation with secondary antibody directly for 15 min [MACH 4 Universal HRP Polymer Kit with DAB (*Source*: Biocare Medical, USA)] again washing in tris-buffer, 3'diaminobenzidine tetrahydrochloride was used to reveal the peroxidase complex. The sections were counterstained with hematoxylin and dehydrated, cleared, and mounted. Parallel control slides were run with all batches including positive and negative controls. Negative controls were those sections on which the primary antibodies were absent and positive controls were those sections on which standardization was done. All buffers and reagents used for the experiment were either from MERCK (Merck Limited, Mumbai, India) or SRL (SISCO Research Laboratories Pvt. Ltd., Mumbai, India).

*Quantitative measurement of ADAM33 expression*: Slides were examined and images were acquired by a ZEISS, Imager Z2 attached to a digital camera Axiocam HRc using the DELL PRECISION T3500 system. Scoring of epithelial staining (which refers to the percentage of the epithelium stained positively) was performed blindly. The scoring system was as follows: 0 = no staining, 1=1 to 12.5%, 2=12.5 to 25%, 3=25 to 37.5%, 4=37.5 to 50%, 5=50 to 62.5%, 6=62.5 to 75%, 7=75 to 87.5%, and 8=87.5 to 100 per cent. All positive stained epithelial cells were counted independent of intensity.

*Statistical analysis*: Data were analyzed using SPSS 11.5 (SPSS Inc., Chicago, IL). Demographic characteristics of patients and controls were described as frequencies and percentages, whereas descriptive statistics of patients and controls were presented as mean and standard deviations for continuous measures. Categorical data were compared by Chi square ($\chi 2$) test and Fisher's exact test, and student's *t* test was applied for continuous variables. To find out association of epithelial staining, single (1-way) ANOVA was used to analyze differences in between the groups followed by Tukey post-hoc multiple comparison test.

## Results

A total of eight non-asthmatic controls and 27 asthma patients were selected during the study period. GINA grading[2] was used to classify asthma severity and the subjects with the following asthma severity were included: *(i)* mild persistent [n=14 (51.9%)], *(ii)* moderate persistent [n=13 (48.1%)]. Table I illustrates the demographic characteristics of the study population. Eleven patients (31.4%) were current smokers and the remaining 68.6 per cent were ex-smokers. All eight controls had stopped smoking; however, were ex-smokers. In controls, exposure to second hand smoke was 100 per cent.

Higher expression of *ADAM33* was identified in the epithelium, subepithelium, submucosal inflammatory cells, and smooth muscles of the subjects with asthma as compared with controls (Figure). The epithelium stained positively for *ADAM33* protein in subjects with asthma and the staining was between 75-100 per cent. The epithelium of control biopsies stained positive weakly (staining between 0-62.5%). The scores for epithelial staining of ADAM33 are shown in Table II. No significant difference was found between mild persistent and moderate persistent subgroups of asthma. However, both mild and moderate persistent subgroups were found to be significantly associated with highly positive epithelium cells compared with control ($P<0.001$ and 0.001, respectively).

## Discussion

In the current study, bronchial biopsies were taken from 35 subjects (27 asthma cases and 8 non-asthmatic controls). An increase in expression of *ADAM33* protein was seen in the epithelium, smooth muscle and

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**Table I.** Demographic profile of cases and controls

| Basic demographic characteristics | Control (n=8) | Total cases (N=27) | Mild persistent (N=14) | Moderate persistent (N=13) |
|---|---|---|---|---|
| Gender (F) N (%) | 3 (37.5) | 7 (25.9) | 4 (28.6) | 3 (23.1) |
| Age (yr) mean ± SD | 37.7 ± 6.8 | 41.8 ± 9.3 | 36.8 ± 9.9 | 47.2 ± 4.2 |
| Weight (kg) mean ± SD | 60.7 ± 10.2 | 64.7 ± 7.0 | 61.9 ± 9.6 | 59.5 ± 11.1 |
| Height (cm) mean ± SD | 165.5 ± 6.2 | 161.4 ± 7.6 | 162.0 ± 7.3 | 160.8 ± 8.1 |
| BMI mean ± SD (kg/m$^2$) | 23.7 ± 2.5 | 23.4 ± 4.1 | 23.6 ± 3.6 | 23.1 ± 4.7 |
| Family history for asthma N (%) | 0 (0) | 12 (44.4) | 6 (42.9) | 6 (46.2) |
| FEV1 % predicted | 105.4 ± 5.1 | 81.8 ± 8.1 | 88.6 ± 4.2 | 74.5 ± 3.2 |
| Smoking status (No. of cigarettes/*bidis* smoked/day) | | | | |
| | | Subject N (%) | | |
| 1-2 | - | 4 (11.4) | 4 (28.6) | (0) |
| 3-10 | - | 3 (8.6) | 2 (14.3) | 1 (7.7) |
| >10 | - | 4 (11.4) | 3 (21.4) | 1 (7.7) |
| Ex-smoker N (%) | 8 (100) | 16 (68.6) | 5 (35.7) | 11 (84.6) |
| Father/other family member | | | | |
| 1-2 | 2 (25.0) | 15 (55.6) | 10 (71.4) | 5 (38.5) |
| 3-10 | 4 (50.0) | 6 (22.2) | 2 (14.3) | 4 (30.8) |
| >10 | 2 (25.0) | 3 (11.1) | 0 (0) | 3 (23.1) |
| Ex-smoker | - | 3 (8.6) | 2 (14.3) | 1 (7.7) |
| Distribution of various anti-asthma drugs received by the patients | | | | |
| Daily controller Medications used by patients | - | - | ICS (<500 mg or equivalent) | ICS (200-1000 mg or equivalent) + LABA |
| Other received treatment in past | | | *(i)* Sustained-release theophylline or *(ii)* Cromone or *(iii)* leukotriene modifier | *(i)* ICS (500-1000 mg or equivalent) + sustained –release theophylline *(ii)* ICS (500-1000 mg or equivalent) + oral LABA *(iii)* ICS at higher doses (> 1000 mg or equivalent) *(iv)* ICS (500-100 mg or equivalent) + leukotriene modifier |

ICS, inhaled corticosteroids; LABA, long acting ß-2 agonist; Ex-smokers, subjects who had quit smoking at least 2 years before the time of recruitment

mesenchymal cells of cases when compared to controls but there was no relationship with severity of asthma. Controls also showed weak positivity of expression of *ADAM33* protein in epithelium cells. The reason may be that all controls had past history of smoking and had exposure to second hand smoke, and earlier studies have confirmed that smoking is responsible for airway damage[26,27]. Table III shows expression of *ADAM33* in patients of asthma compared with controls and relation with severity of asthma in earlier studies along with the present results.

Bronchial hyperresponsiveness (BHR) is the marker that distinguishes asthma from other diseases of the airways. It has also been suggested that the increase in adventitial thickness could cause an uncoupling of the airway smooth muscle from the load provided by surrounding parenchymal recoil to cause embroidered

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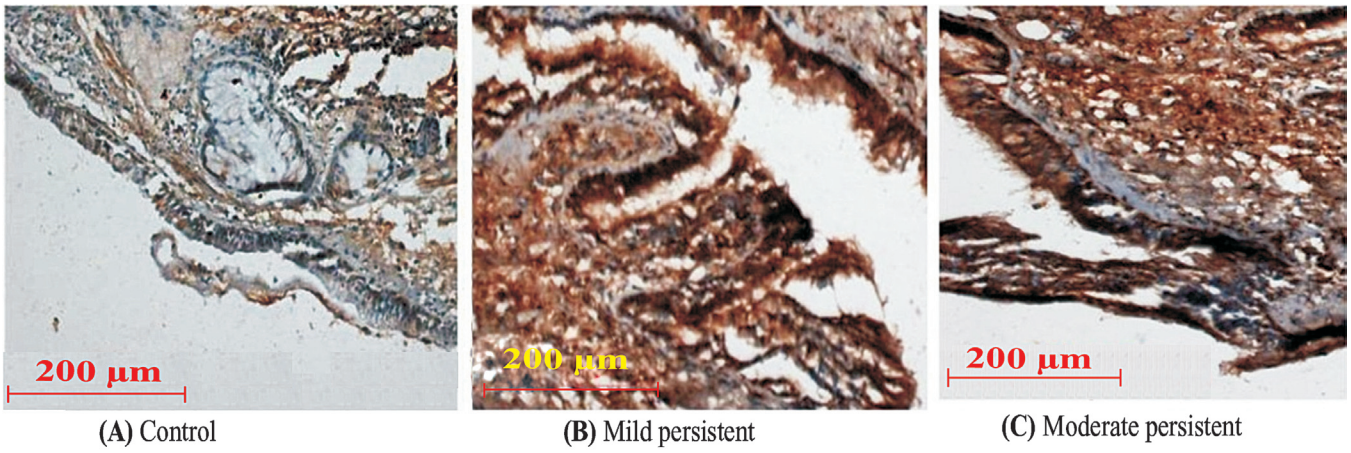


**Fig.** Representative examples of immunohistochemistry of bronchial biopsies showing staining for ADAM33 in a subject; **(A)** Control, **(B)** Mild persistent, **(C)** Moderate persistent. Magnification X 200.

airway narrowing. These alterations in smooth muscle mass relative to disease severity are thought to be the most likely explanations for BHR and the unnecessary airway narrowing observed in the established disease[27]. The role of the epithelium in airway remodelling has previously been suggested[28]. Earlier studies have confirmed *ADAM33* gene as a newly identified protease involved in airway remodelling[30,31].

Expression of *ADAM33* was first observed in cells of mesenchymal origin, such as fibroblasts and smooth muscle cells, indicating a possible role in airway remodelling[20]. Another study demonstrated the existence of multiple novel isoforms of the *ADAM33* in human airway fibroblasts[32] and gene to be selectively expressed by mesenchymal cells[18].

**Table II.** Epithelium scoring of subjects

| Scoring* | Asthma patients (n=27) | | Controls (n=8) |
|---|---|---|---|
| | Mild persistent (n= 14) | Moderate persistent (n=13) | (n=8) |
| 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 5 (62.5) |
| 2 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 |
| 5 | 0 | 0 | 3 (37.5) |
| 6 | 0 | 0 | 0 |
| 7 | 4 (28.6) | 3 (23.1) | 0 |
| 8 | 10 (71.4) | 10 (76.9) | 0 |

*The scoring was done according to Foley *et al* 2007[24]
Values in parentheses are percentages

Similar to our study, Foley *et al*[24] found increased expression of *ADAM33* epithelial staining in more than 80 per cent of biopsies from subjects with asthma with weak staining in control epithelium. Unlike our results, they observed a statistically significant increase in the epithelial staining as asthma severity progressed from mild to severe[24]. One possible reason for this difference may be due to the fact that in our study patients in intermittent and severe persistent subgroups of asthma could not be included. Cases of asthma recruited by us were somewhat homogeneous as far as clinical severity of disease was concerned and in accordance to GINA guidelines[2].

Another study reported no difference in *ADAM33* mRNA expression between bronchial biopsies from subjects with asthma and control as well as reported weak immunoreactivity in bronchial epithelial cells, attributing this to nonspecific cross-reactivity with epithelial cytokeratins[22]. Lee *et al*[23] have previously reported a significant negative correlation between protein expression of *ADAM33* in bronchoalveolar lavage samples and FEV1% predicted in asthma and observed positive epithelial staining expression of *ADAM33* protein, though at a frequency of less than 50 per cent in the subjects with asthma. Ito *et al*[28] also showed association of *ADAM33* protein expression in airway smooth muscle cells, but not defined positivity of epithelium cell with cases of asthma and controls. Dijkstra *et al*[30] assessed the expression of various

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**Table III.** Expression of ADAM33 in patients of asthma compared with controls and protein relation with severity of asthma in other previous published studies and with our results

| Reference | Study sample | Sample size CC=case/control AS=M/Mo/Se | Association between CC = case control | Association in terms of severity of asthma |
|---|---|---|---|---|
| Foley *et al,* 2007[24] | Bronchial biopsies | CC=37/7 M/Mo/Se=NS | Epithelium, subepithelium, submucosal inflammatory cells, and smooth muscle of subjects with asthma.<br>Smooth muscle: weakly positive in controls.<br>Epithelial staining: Cases vs controls- *P*<0.001 | Smooth muscle: identified in all biopsy specimens and stained positively, 100% of severe and moderate cases, one third of mild biopsies. (SND)<br>Epithelial cells: identified in all biopsy, Expression Results: Severe vs mild: *P*<0.001;<br>moderate vs mild: *P*<0.001; severe vs moderate: *P*=0.14 |
| Ito *et al*, 2007[28] | Bronchial biopsy | CC=10/10 | Expression in ASM: Cases vs Control- *P*<0.002.<br>Other cells (Eosinophilic inflammation and increased smooth muscle mass): expression observed in patients. *P* value NS)<br>Epithelium cells= NS | ND |
| Haitchi *et al*, 2005[22] | Bronchial biopsies | CC=19/21 | Expression in alpha-smooth muscle bundles (*P*<0.05)<br>Submucosa and around vessels: positively stained but SND (immunostaining of the muscle and sub mucosal cells was blocked in the presence of the immunizing peptide)<br>Epithelial cells: not fully blocked by the immunizing peptide and probably represents nonspecific cross-reactivity with epithelial cytokeratins | ND |
| Lee *et al*, 2006[23] | Bronchoalveolar lavage (BAL) fluids | CC=35/10 AS=M/Mo and Se merged: 21/14 | Expression was found in the smooth muscle cells and submucosal gland and basement membrane. However, SND<br>Epithelium cells: Noted in the bronchial ciliated epithelium from 30% of the patients (SND) | ADAM33 levels were increased significantly in patients with moderate to severe asthma (*P*< 0.001).<br>Epithelium cells, smooth muscle cells: NS |
| Present study | Bronchial Biopsies | CC=27/8 AS=M/Mo=14/13 | Expression in submucosal inflammatory cells, and smooth muscles of the subjects with asthma: (100%) in cases, controls were weakly positive. However, SND.<br>Epithelium cells: cases vs controls- *P*=0.001 | Smooth muscle: identified in all biopsy specimens and stained positively was 100% in mild persistent and moderate persistent subgroups of asthma.<br>Epithelium cells: mild persistent vs moderate persistent subgroups of asthma (*P*=0.990) |

ASM, airway smooth muscle cells; CC, case-control; NS, not specified; AS, types of asthma severity; M/Mo/Se, Mild/Moderate/Severe; ND, not done; SND, significance not defined statistically; *P* values represent statistical association between cases and controls (cases vs controls) and among severity subgroup of asthma

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*ADAMs (ADAM8, ADAM9, ADAM10, ADAM17, ADAM19* and *ADAM33)* in human lung tissue and found that all were usually distributed over the bronchial epithelium.

Our study had the following limitations: *(i)* sample size determination was not performed, *(ii)* controls were only eight which may not be sufficient to obtain robust conclusions, *(iii)* due to the difficulty in obtaining healthy controls for the study only controls who were not healthy, were smokers and suffered from lung cancer, were included, *(iv)* due to low sample size further sub-group analysis to evaluate the impact of medications such as ICS (inhaled corticosteroids) on *ADAM33* expression could not be performed, *(v)* post-bronchodilator study could not performed in controls and therefore, the exclusion of diagnosis of asthma in this group could not be confirmed, *(vi)* a significant proportion of subjects was current smokers and the rest were ex-smokers whereas all controls were ex-smokers. Since smoking is known to be associated with increased *ADAM33* expression, the effect of smoking and asthma in the increased expression of *ADAM33* could not be differentiated.

It is already known that epithelium cells play an important role in airway remodelling[28]. Significantly unregulated expression of *ADAM33* protein in bronchial epithelium cells has also been reported earlier[23,24,31] as observed in the present study also.

In conclusion, a higher expression of *ADAM33* protein was observed in bronchial epithelium of asthma patients compared to controls. This observation and its clinical significance in the Indian population need to be confirmed in larger, prospective studies.

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