



ORIGINAL ARTICLE

WILEY

Asthma and Rhinitis

DNA methylation down-regulates integrin $\beta 4$ expression in asthmatic airway epithelial cellsMengping Wu¹ | Yu Yang¹ | Lin Yuan¹ | Ming Yang² | Leyuan Wang¹ | Xizi Du¹ | Ling Qin³ | Shuangyan Wu¹ | Yang Xiang¹ | Xiangping Qu¹ | Huijun Liu¹ | Xiaoqun Qin¹ | Chi Liu^{1,4} ¹Department of Physiology, School of Basic Medicine Science, Central South University, Changsha, China²Faculty of Health and Medicine, Centre for Asthma and Respiratory Disease, School of Biomedical Sciences and Pharmacy, University of Newcastle and Hunter Medical Research Institute, Callaghan, NSW, Australia³Department of Respiratory Medicine, National Clinical Research Center for Respiratory Diseases, Xiangya Hospital, Central South University, Changsha, China⁴Research Center of China-Africa Infectious Diseases, Xiangya School of Medicine Central South University, Changsha, China

Correspondence

Chi Liu, Department of Physiology, Xiangya School of Medicine, Central South University at Changsha, Hunan 410078, China.

Email: liu.chi@csu.edu.cn

Funding information

This work was funded by grants #81970033, #31900424, #81670002 and #316871188 from the NSFC; grants #2019JJ50760, #2020JJ4776, #2020JJ4688 from the Hunan Natural Science Foundation; grant #16K097 from open Foundation of Hunan College Innovation Program and grant #2018zzts813, #2018zzts812, #2019zzts327, #2019zzts1008 from the Fundamental Research Funds for the Central Universities of Central South University.

Abstract

Background: Integrin $\beta 4$ (ITGB4) is a hemi-desmosome protein which is downregulated in the airway epithelial cells of asthma patients. The proximal promoters and exons of ITGB4 contain CpG islands or multiple CpG sites both in human and mice, which indicated the possible methylation regulation of ITGB4 in airway epithelial cells.**Objective:** We sought to unveil that DNA methylation regulates the decreased ITGB4 during the pathogenesis of asthma.**Methods:** Mice were exposed to house dust mite (HDM) extracts to construct an asthma model. 5-Aza-2'-deoxycytidine (5-AZA) or dexamethasone (DEX) were added in the last two weeks. Besides, the primary human bronchial epithelial (HBE) cells were incubated for the detection of ITGB4 expression and methylation status after HDM stress. Furthermore, DNA methylation of ITGB4 in peripheral blood was measured in asthma patients. Logistic regression was employed to evaluate the association between methylation sites and asthma patients' ages in the control of potential confounders. Moreover, the correlations between differentially methylated sites (DMSs) and clinical parameters in asthma patients were assessed. Finally, the ability of candidate DMSs to predict asthma was evaluated by receiver operating characteristic (ROC) analysis and principal component analysis (PCA).**Results:** We found that in HDM-stressed asthma model, DNA methylation regulated the reduced ITGB4 expression in airway epithelial cells. Moreover, alteration in the specific CpG sites (chr17:73717720 and chr17:73717636) of ITGB4 may regulate ITGB4 expression and further may be associated with the clinically phenotypic of asthma. The specific DMSs of ITGB4 in peripheral blood can distinguish asthma patients from healthy controls (HCs) effectively.**Conclusions and Clinical Relevance:** This study confirmed that DNA methylation regulates the decreased expression of ITGB4 in the airway epithelial cells of asthma patients. These results supply some useful insights to the mechanism of the decreased ITGB4 in asthmatic airway epithelial and provide possible targets for early prediction and screening of asthma.

KEYWORDS

airway epithelial cells, asthma, DNA methylation, epigenetic modification, Integrin $\beta 4$

1 | INTRODUCTION

Asthma is a common chronic airway inflammatory disease characterized by epithelial shedding, bronchial hyperresponsiveness, chronic lung inflammation and airway remodelling.^{1,2} Airway epithelial cells act as the first barrier to contact the outside world, and also the target to various external stimuli. It has been verified that the structural and functional disruption of airway epithelial cells are critical to immune responses and airway remodelling in the pathogenesis of asthma.³⁻⁵ Integrins are a kind of adhesion molecules which play an important role in the structural adhesion, tissue repair and functional homeostasis of airway epithelial cells.⁶ Among these molecules, ITGB4 has been known to regulate the adhesion of airway epithelial cells on basal membrane through hemi-desmosome structure. Notably, ITGB4 is implicated in the pathogenesis of allergic asthma,⁷ and its level is significantly decreased in the airway epithelial cells of allergic asthma patients.^{8,9} The exacerbation of airway inflammation may be related to the reduced expression of ITGB4 in the airway epithelium as TSLP expression is up-regulated when airway epithelial barrier integrity is compromised.¹⁰ However, little is known about the mechanism of decreased ITGB4 expression in the airway epithelial cell of asthma patients.

Our previous work also demonstrated that both oxidative stress and inflammatory stimuli could induce a significant reduction of ITGB4 expression in airway epithelial cells in a time and dose-dependent manner.¹¹ In addition, the expression of ITGB4 decreased significantly after ozone challenge, which could gradually recover after ozone challenge retreat.¹² This sensitive regulation of ITGB4 expression after external environmental stimulation suggested the possible epigenetic regulation of ITGB4 expression in airway epithelia during the pathogenesis of asthma. Epigenetic modifications, including DNA methylation and histone modifications, are emerging as a dynamic and efficacious mechanism that regulates gene expression during disease pathogenesis.¹³ DNA methylation is the most embedded study of epigenetic phenomenon in asthma-related researches.^{14,15} Differential methylation not only plays an important role in gene regulation, but also is related to the clinical phenotype changes of asthma patients.^{16,17} Furthermore, previous studies have shown that DNA methylation can promote the pathogenesis of asthma through well-known pathways such as T cell differentiation and cytokine production.^{18,19} Analysis of ITGB4 promoter (including TSS) and exons revealed that the proximal promoter and exons of ITGB4 contained CpG islands or multiple CpG sites both in human and mice, which are targets of methylation. Moreover, the regulation of ITGB4 expression by DNA methylation has been confirmed in mammary epithelial cells.²⁰ However, whether DNA methylation modifies the decreased expression of epithelial ITGB4 in the pathogenesis of asthma is still unclear. In this study, we detected the expression and DNA methylation of ITGB4 in airway epithelial

cells from HDM-stressed mice model. Then, the inhibitor of DNA methylation 5-AZA was used to verify the methylation regulation of ITGB4 expression in the HBE cells. Moreover, the correlation between differentially methylated sites and lung function was analysed in asthma patients. Finally, we evaluated the possible feasibility of ITGB4 methylation as blood biomarker of asthma.

2 | METHODS

2.1 | Animal

This animal research was carried out with the approval No.2017JJ2402 of Xiangya Animal Protection and Utilization Committee of Central South University. All methods are implemented in accordance with relevant clauses. The mice were placed under the condition of an air-filtered temperature control unit, where the light and dark cycled alternately for 12 hours, with sufficient food and water supply. The construction of ITGB4 conditional knockout mice was based on our previous publications.¹⁰ Briefly, the Dox treatment was continued throughout the whole experimental system. The CCSP-rtTA^{tg/-}/TetO-Cre^{tg/-}/ITGB4^{fl/fl} mice were used as ITGB4^{-/-} mice by Dox induction. The Dox-treated ITGB4^{fl/fl} male littermates lacking either CCSP-rtTA, TetO-Cre or both were used as ITGB4^{+/+} mice.

2.2 | Sensitization and challenge protocols

The construction of HDM-induced asthma model was on the basis of previous publications.²¹ In the last two weeks, within 1 hour before HDM challenge 5-AZA (Sigma-Aldrich) (1 mg/kg body weight) or DEX (Sigma-Aldrich) (2 mg/kg body weight) were injected intraperitoneally, respectively. All mice were killed 48 hours after the last challenge.

2.3 | Immunohistochemical staining

Fresh lungs were fixed by 4% paraformaldehyde, and then they were embedded in paraffin according to routine histochemical procedure. HE staining of five micron sections was conducted as mentioned before.²² Quantitative analysis of lung inflammation was performed blindly.²³ Immunofluorescent staining was performed with different antibodies: CC10 (sc-365992, Santa Cruze). ITGB4 (Ab182120, Abcam). We employed Zeiss Discovery.V8 Stereo or Zeiss Axio Scope.A1 microscopes (Carl Zeiss MicroImaging GmbH) integrated with an Axio-Cam ICc3 camera (Spectra Service) for microscopy. Images were obtained from Zeiss through the axoview rel.4.7 software.

TABLE 1 Primer sequence for target genes

Primer genus	Gene	Primer sequence
Mouse	ITGB4	Forward: 5' - CCGCTTCCAAGTCCAACCTCA - 3' Reverse: 5' - TGAGACTCCTGTCCGTTTCA - 3'
Mouse	IL-4	Forward 5' - CCAAACGTCCTCACAGCAAC - 3' Reverse 5' - AGGCATCGAAAAGCCCGAA - 3'
Mouse	IL-5	Forward 5' - AAGCAATGAGACGATGAGGCT - 3' Reverse 5' - CCCACGGACAGTTTGATTCT - 3'
Mouse	IL-13	Forward 5' - CTTGCTTGCCTTGGTGGTCT - 3' Reverse 5' - TCCATACCATGCTGCCGTTG - 3'
Mouse	IL-17A	Forward 5' - TACCTCAACCGTTCCACGTC - 3' Reverse 5' - TTTCCCTCCGATTGACACA - 3'
Mouse	IL-1 β	Forward 5' - GCAACTGTTCTGAACTCAACT - 3' Reverse 5' - ATCTTTTGGGGTCCGTCAACT - 3'
Mouse	TNF- α	Forward 5' - CCCTCACACTCAGATCATCTTCT - 3' Reverse 5' - GCTACGACGTGGGCTACAG - 3'
Mouse	β -actin	Forward 5' - TTGCAGCTCCTTCGTTGCC - 3' Reverse 5' - GACCCATTCCCACCATCACA - 3'
Human	ITGB4	Forward: 5' - CACCTCCGTCTCCTCCAC - 3' Reverse: 5' - GTTGGGGATGTTGAGCCGA - 3'
Human	ITGB5	Forward: 5' - GCCTTTCTGTGAGTGCGACAAC - 3' Reverse: 5' - CCGATGTAACCTGCATGGCACT - 3'
Human	ITGB8	Forward: 5' - CTGTTTGCAGTGGTCGAGGAGT - 3' Reverse: 5' - TGCCTGCTTCACACTCTCCATG - 3'
Human	β -actin	Forward 5' - TTCCAGCCTTCCTTCCTGGG - 3' Reverse 5' - TTGCGCTCAGGAGGAGCAAT - 3'

2.4 | BAL fluid and phenotyping

Bronchoalveolar lavage fluid (BALF) was collected for cells counting as previously described.²⁴

removed the supernatant, resuspended the bottom cell mass in 2 mL basal medium (Ham's F-12 medium, penicillin 100 μ L/mL, streptomycin 100 μ L/mL, 5%FBS), and cultured in a 6 cm tissue culture dish at 37°C, 5% CO₂.

2.5 | Primary airway epithelial cells

Mouse primary airway epithelial cells were extracted according to previously existing procedures.^{25,26} Mice were killed by use of excessive sodium pentobarbital, then immersed in 75% ethanol solution for 5 minutes, and placed on a sterile workbench. Under sterile conditions, the trachea was cut from the main trachea to the bronchus bifurcation. Then, the trachea was placed in a cold sterile PBS solution, and other tissues attached to the trachea were carefully removed. In the cold sterile collection medium (Ham's F-12 medium, penicillin 100 μ L/mL, streptomycin 100 μ L/mL), the trachea was cut open longitudinally, gently moved to a test tube of freshly configured digestion medium (collection medium, 10 μ L/mL Streptomycin, 0.01 mol/L CaCl₂, 0.5 mg/mL crude pancreatic DNAaseI, 10 mg/mL bovine serum albumin), and digested at 4°C for 12 hours. The digested trachea was ground and filtered. The filtered liquid was centrifuged at 500 g, 4°C for 10 minutes. The supernatant was discarded, and the bottom cell mass was resuspended and incubated in 2 mL incubation medium (Ham's F-12 medium, penicillin 100 μ L/mL, streptomycin 100 μ L/mL, 10% FBS) for 5 hours. Collected the supernatant in a new test tube, centrifuged at 500 g, 4°C for 10 minutes,

TABLE 2 Demographic characteristics of asthma patients and HCs

	HCs	Asthma patients
Number of subjects	20	19
Age	50.50 $\pm$ 5.95	47 $\pm$ 10.77
FEV1	/	2.02 $\pm$ 0.53
FEV1%	/	0.76 $\pm$ 0.20
FVC	/	3.05 $\pm$ 0.80
FEV1/FVC	/	0.67 $\pm$ 0.09
PEF	/	5.34 $\pm$ 1.66
FEF75	/	0.54 $\pm$ 0.18
FEF50	/	0.42 $\pm$ 0.16
FEF25	/	0.31 $\pm$ 0.12
FEF75/25	/	0.37 $\pm$ 0.14

Note: Data are presented as Mean $\pm$ SD.

Abbreviations: FEF, forced expiratory flow; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; HCs, healthy controls; PEF, maximum expiratory flow.

*P < .05 was considered statistically significant, asthma patients vs HCs.

2.6 | Quantitative RT-PCR

We used Trizol (Invitrogen) to lyse primary airway epithelial cells to obtain total RNA.¹² 1 mg RNA was reverse transcribed into cDNA at 40°C for 50 minutes, and then heated at 90°C for 5 minutes. Then, 1 µL of reverse transcriptase was added to 30 µL of the PCR mixture for 40 cycles, and each cycle consisted of two sessions: 93°C for 30 seconds and 54°C for 60 seconds. By comparing the copy number of the target gene with the copy number of β -actin, the standardized mRNA expression data were realized for RNA input, RNA quality and reverse transcription efficiency. The sequences of the primers are listed in the Table 1.

2.7 | Cell culture and treatment

The HBE cells from healthy smokeless donors were purchased from Lifeline Cell Technology. Cells were incubated in humidified 5% CO₂/

95% air at 37°C, and the culture medium was changed every two days. HBE cells were inoculated in a 6-well tissue culture dish and cultured in a conditioned medium to sub-confluence. When the cell density reached 50%, DMEM containing 10% FBS was used to replace the previous medium. After 48 hours, HDM was used to stimulate HBE cells (100 µg/mL) for 24 hours. In the 5-AZA intervention group, the culture system was treated with 5-AZA (10 µmol/L) for 72 hours.²⁷

2.8 | Western blot analysis

In the presence of protease inhibitors and phosphatase inhibitors, RIPA lysis buffer was used to collect protein from HBE cells. The protein concentration was determined by BCA protein assay (Beyotime). Then, Western blot analysis was conducted in accordance with previously procedures.²⁸ Briefly, 10% SDS-PAGE isolated 50 µg protein in HBE cells, which was transferred to a polyvinylidene difluoride

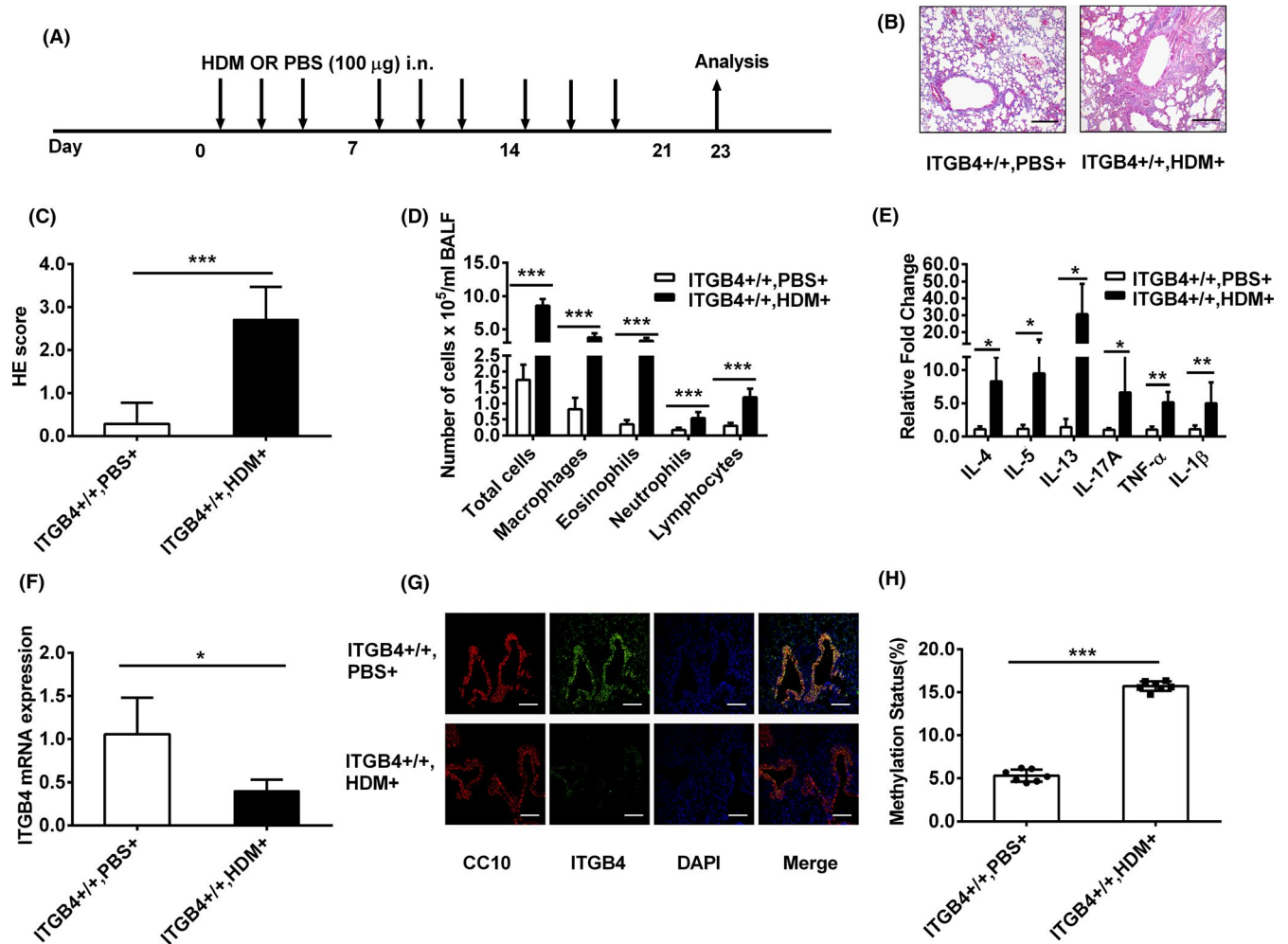


FIGURE 1 Decreased expression and hypermethylation modification of ITGB4 in airway epithelial cells of HDM-stressed asthma model. A, Mice were sensitized to HDM on days 0, 7, 14 or 21. Control non-sensitized mice were treated with PBS. B, HE-stained lung sections for analysis of pulmonary inflammatory infiltration (magnification, 100×; scale bar, 50 µm). C, Lung inflammation was assessed. ****P* < .001. D, BALF inflammatory cell was counted. ****P* < .001. E, 48 h after the final challenge, the levels of IL-4, IL-5, IL-13, IL-17A, TNF-α and IL-1β in lung tissue (*n* = 6) were examined by qPCR. **P* < .05 and ***P* < .01. F, Airway epithelial cells were isolated from lung. The mRNA expression of ITGB4 was quantified with qPCR. **P* < .05. G, Immunofluorescent staining for ITGB4 in lung sections (magnification, 40×; scale bar, 50 µm). H, Statistical analysis of the mean percentage of methylation of ITGB4 CpG sites in primary airway epithelial cells. ****P* < .001

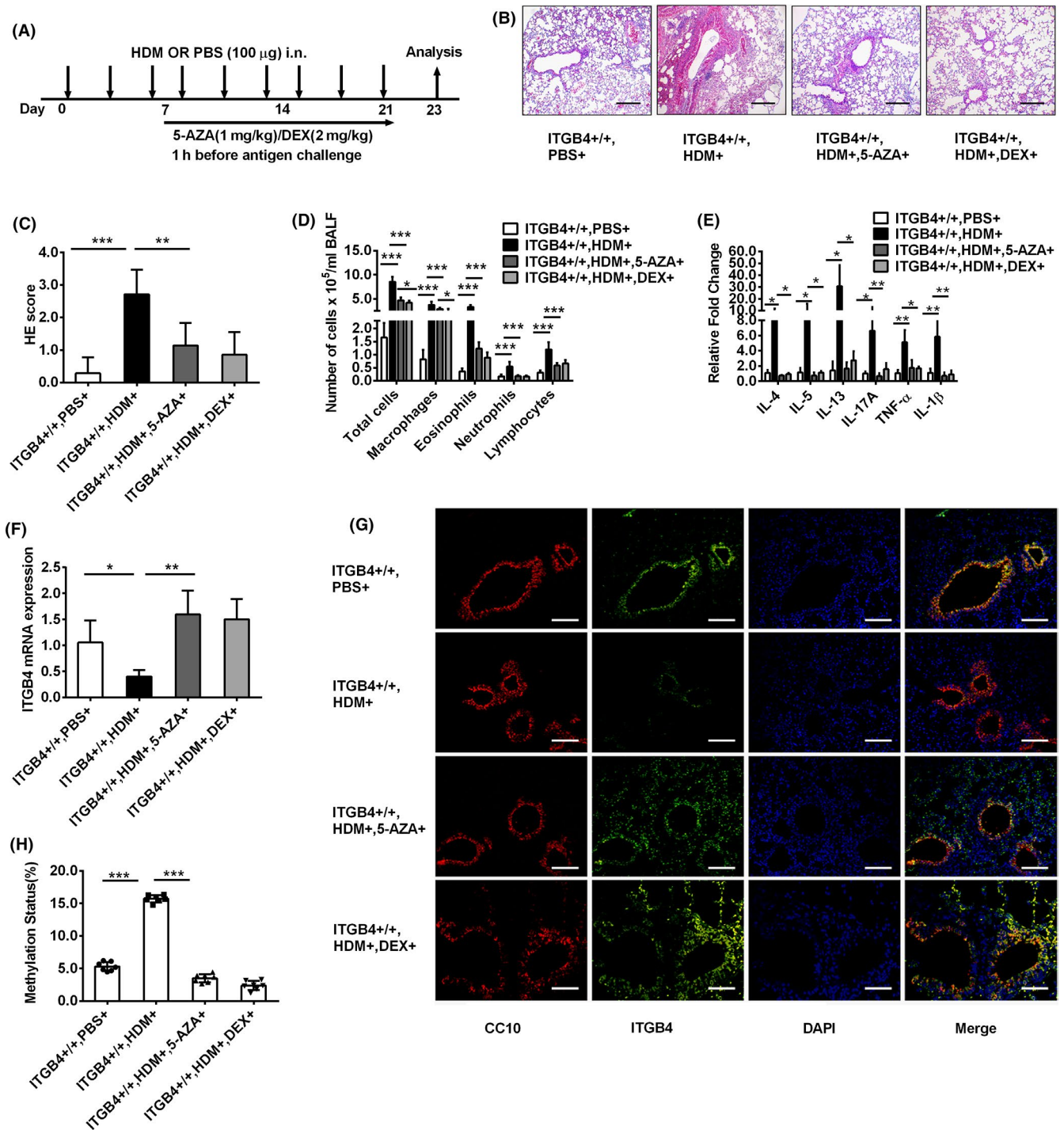


FIGURE 2 DNA demethylation suppressed pulmonary inflammation and restored ITGB4 expression in HDM-stressed asthmatic mice. A, Mice were sensitized to HDM on days 0, 7, 14 or 21. Some group were treated with 5-AZA to block DNA methylation. Control non-sensitized mice were treated with PBS. B, HE-stained lung sections for analysis of pulmonary inflammatory infiltration (magnification, 100x; scale bar, 50 µm). C, Lung inflammation was assessed. $^{*}P < .01$ and $^{***}P < .001$. D, BALF inflammatory cell was counted. $^{*}P < .05$ and $^{***}P < .001$. E, 48 h after final challenge, the levels of IL-4, IL-5, IL-13, IL-17A, TNF-α and IL-1β in lung tissue (n = 6) were examined with qPCR. $^{*}P < .05$ and $^{**}P < .01$. F, The mRNA expression of ITGB4 was quantified with qPCR in primary airway epithelial cells. $^{*}P < .05$ and $^{**}P < .01$. G, Immunofluorescent staining for ITGB4 in lung sections (magnification, 40x; scale bar, 50 µm). H, Statistical analysis of the mean percentage of methylation of ITGB4 CpG sites in primary airway epithelial cells. $^{***}P < .001$

(PVDF) membrane. Primary antibody incubated PVDF membrane overnight at 4°C, then, it was incubated with secondary antibody combined with HRP. ITGB4 antibody (Ab182120, Abcam) and β -actin antibody (Ab6301, Abcam) were used in Western blot analysis.

2.9 | Recruitment of participants and data collection

From June 2018 to August 2019, we selected 19 asthma patients and 20 HCS from the Respiratory Department and physical examination centre of Xiangya Hospital. Questionnaire survey was conducted on the volunteers, including general situation, clinical symptoms of asthma, other respiratory diseases and pulmonary function test results. In addition, peripheral blood samples were collected from 39 volunteers. The inclusion criteria of the case group were 27- to 60-year-old men or women, who were diagnosed with asthma for the first time and had no other diseases (such as diabetes, other respiratory or cardiovascular diseases). The control group was without asthma or other acute or chronic diseases, and there was no difference in gender or age compared to the asthma group. The demographic characteristics of the asthma patients and HCs were shown in Table 2. In the collected data, pulmonary function phenotypes included forced expiratory volume in one second (FEV1), forced expiratory volume in one second as a percentage of predicted volume (FEV1%), forced vital capacity (FVC), ratio of FEV1 to FVC, maximum expiratory flow (PEF), forced expiratory flow (FEF) 75, FEF50, FEF25 and FEF75/25. The scheme was authorized by approval No. 20180308 of Ethics Review Committee of Xiangya Hospital, and every subject signed a written consent.

2.10 | DNA extraction, bisulphite treatment, methylation Array methods

Bisulphite sequencing PCR (BSP) was performed in Genesky Biotechnologies Inc Shanghai, including DNA extraction and quality control, bisulphite treatment, primer design, and cloning and sequencing.²⁹ In short, genomic DNA was extracted from whole blood with commercial kits (TIANGEN Biotech).^{30,31} The extracted DNA was modified with EZ DNA Methylation-Gold Kit (ZYMO). After DNA purification, primer 3 (<http://primer3.ut.ee/>) was used to design the primer sequence of PCR. CpG islands or dense CpG sites located in the promoter and exons proximal to the target gene were selected for measurement in the following conditions: (a) 200 bp minimum length; (b) 50% or higher GC content; (c) 0.60 or higher ratio of observed/expected dinucleotides CpG. The Illumina MiSeq Benchtop Sequencer

sequenced the product. There is one CpG island in the proximal promoter and the first exon of human ITGB4. A total of 50 CpG sites from ITGB4 were detected by methylation detection. The original data with a sequencing quality value $Q > 40$ (basic sequencing error rate $< 0.1\%$) were kept and the methylation percentage of each CpG site was recorded. For mouse samples, ITGB4 proximal promoter and the first exon contain 74 CpG sites; we have also adopted the same method for multiple targeted bisulphite enrichment sequencing.

2.11 | Data analysis and statistics

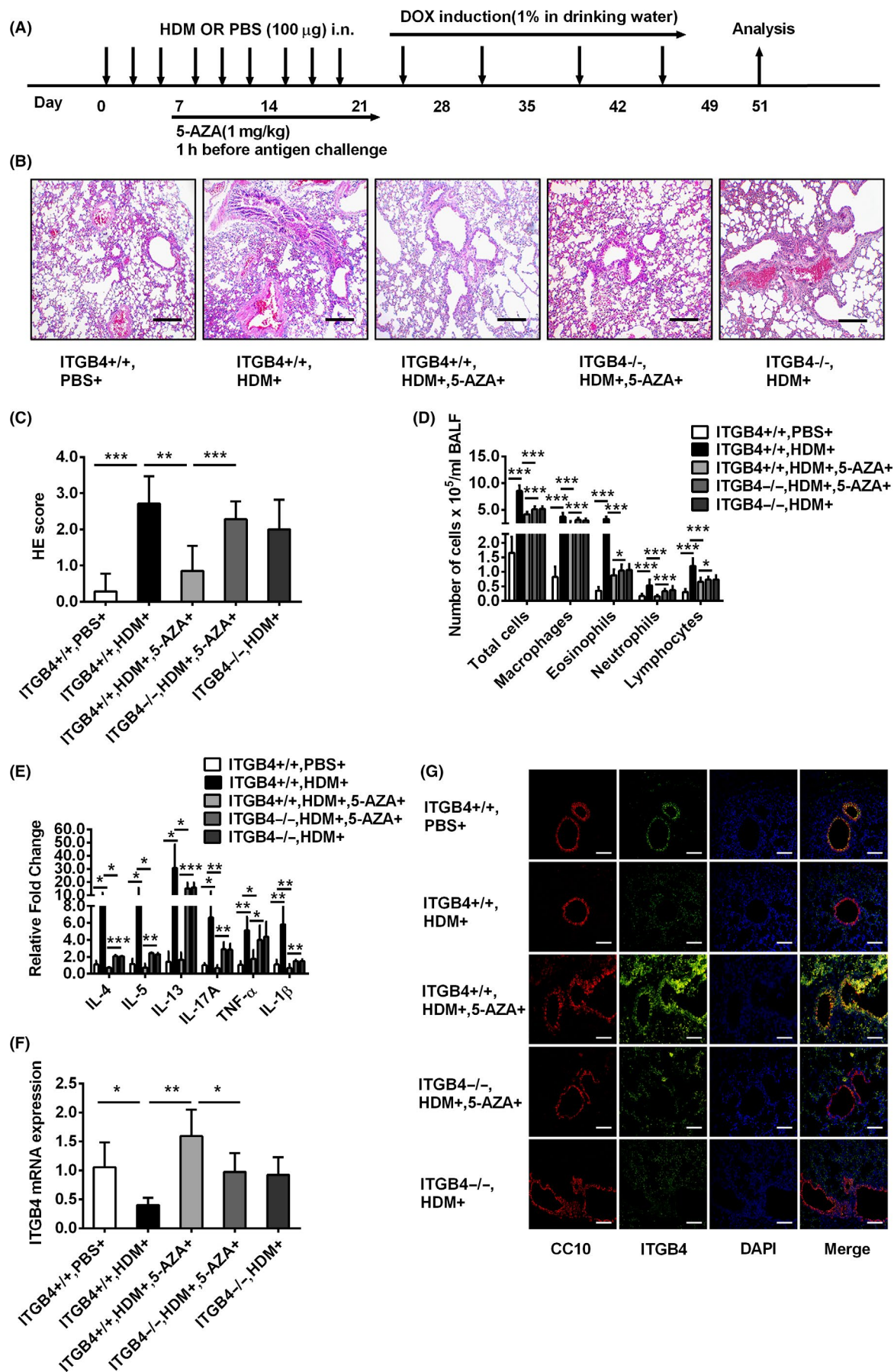
Statistical analyses were conducted with SPSS version 22.0 (IBM Corporation). The characteristic data of recruited asthma patients and controls were presented as Mean $\pm$ SD and analysed with unpaired *t* test. *t* Test and non-parametric test (Mann-Whitney *U* test) were used to analyse the methylation sequence of ITGB4. We employed Benjamin Hochberg method to control the false discovery rate (FDR). In order to exclude the potential of age and gender from affecting the data, logistic regression analysis was carried out on the CpG sites of the selected differential expression.³² As for the correlation between CpG methylation rate and continuous variables, we used Pearson's correlation or Spearman's correlation to evaluate. Continuous variables refer to FEV1, FEV1% predicted, FVC, the ratio of FEV1 to the FVC, PEF, FEF75, FEF50, FEF25 and FEF75/25. ROC curves were acquired to elucidate the veracity of differentially expressed CpG sites in calculating asthma. The methylation percentage of candidate CpG sites were used in PCA to identify asthma. Differences were considered statistically significant for $*P < .05$, $**P < .01$ and $***P < .001$.

3 | RESULTS

3.1 | The expression of ITGB4 in airway epithelial cells decreased significantly along with hypermethylation in HDM-stressed asthma model

The construction of HDM-stressed asthma model was displayed on a graph timeline (Figure 1A). The pulmonary inflammation was assessed by HE staining and BALF phenotyping, respectively. Compared to control mice, the infiltration of inflammation cells was obviously increased after HDM stress (Figure 1B,C). The infiltration of inflammatory cells such as lymphocytes and eosinophils in BALF of HDM-induced mice was increased, which was consistent with the enhanced inflammation in lung tissue (Figure 1D). Likewise, the transcription levels of IL-4,

FIGURE 3 Effects of ITGB4 deficiency on pulmonary inflammation in 5-AZA-treated mice. A, Mice were sensitized to HDM on days 0, 7, 14 or 21. Some group were treated with 5-AZA to block DNA methylation. Control non-sensitized mice were treated with PBS. B, HE-stained lung sections for analysis of pulmonary inflammatory infiltration (magnification, 100 $\times$; scale bar, 50 μ m). C, Lung inflammation was assessed. $**P < .01$ and $***P < .001$. D, BALF inflammatory cell was counted. $*P < .05$, $**P < .01$ and $***P < .001$. E, 48 hours after final challenge, the levels of IL-4, IL-5, IL-13, IL-17A, TNF- α and IL-1 β in lung tissue ($n = 6$) were examined with qPCR. $*P < .05$, $**P < .01$ and $***P < .001$. F, The mRNA expression of ITGB4 quantified with qPCR in primary airway epithelial cells $*P < .05$ and $**P < .01$. G, Immunofluorescent staining for ITGB4 in lung sections (magnification, 40 $\times$; scale bar, 50 μ m)



IL-5, IL-13 and IL-17A were significantly elevated as demonstrated in the lung after HDM stress (Figure 1E). Meanwhile, mRNA expression of ITGB4 in primary airway epithelial cells was also found to be significantly decreased after HDM stress (Figure 1F). Immunofluorescence staining showed the specific localization of ITGB4 expression in airway epithelial cells (Figure 1G). To further investigate the aberrant CpG island methylation of ITGB4 after HDM stress, bisulphite conversion of genomic DNA and PCR-based MiSeq analysis were performed. Compared to control mice, the percentage of ITGB4 methylation increased significantly after HDM stress (Figure 1H).

3.2 | DNA demethylation suppress pulmonary inflammation and restored ITGB4 expression in HDM-stressed asthmatic mice

To estimate whether a representative DNA-demethylating agent (5-AZA) could suppress HDM-induced pulmonary inflammation based on restoration of ITGB4 expression, we processed HDM-stressed mouse model with 5-AZA and used DEX as positive control. The treatment timeline of 5-AZA or DEX was shown in Figure 2A. Compared to HDM-stressed model, the administration of 5-AZA inhibited pulmonary inflammation (Figure 2B,C) and decreased total inflammatory cells in BALF which were mainly eosinophils, neutrophils and lymphocytes (Figure 2D). Likewise, the transcription levels of IL-4, IL-5, IL-13 and IL-17A were also reduced significantly after 5-AZA treatment (Figure 2E). QPCR detection and immunofluorescence staining demonstrated that 5-AZA treatment could restore ITGB4 expression, which is consistent with the DEX group (Figure 2F,G). The PCR-based MiSeq analysis also showed that the 5-AZA treatment induced demethylation of ITGB4 (Figure 2H).

3.3 | Effects of ITGB4 deficiency on pulmonary inflammation in 5-AZA-treated mice

To further verify the significant role of epithelial ITGB4 in 5-AZA-treated mice, we assessed whether ITGB4 deficiency in airway

epithelial cells could abolish the effect of 5-AZA on lung inflammation. Specifically, we knocked out ITGB4 in airway epithelium via Dox induction after 5-AZA treatment. The specific Dox-induced timeline was shown in Figure 3A. The results revealed that the remissive pulmonary inflammation in 5-AZA-treated mice was reversed by Dox induction (Figure 3B-E). Also, the knock-out efficiency of ITGB4 in airway epithelia was detected by qPCR and immunofluorescence staining (Figure 3F,G). These results further support the above observation that the restoration of ITGB4 expression by 5-AZA could suppress lung inflammation after HDM stress. These results strongly indicate the involvement of decreased ITGB4 by hypermethylation regulation in the occurrence and development of airway inflammation after HDM stress.

3.4 | Involvement of DNA methylation in the regulation of ITGB4 expression in HDM-stressed HBE cells

We next evaluated the effect of HDM on the methylation of ITGB4 in primary normal HBE cells. Compared to control group, both the mRNA and protein expression of ITGB4 in HDM-stimulated HBE cells were reduced, which was partially restored by 5-AZA treatment (Figure 4A,B). In addition, we also observed that the methylation level of HBE cells was increased after HDM stimulation (Figure 4C). Meanwhile, the increased methylation of ITGB4 in HDM-stimulated HBE cells was partially restored after 5-AZA treatment. These results strongly indicated the involvement of DNA methylation in the regulation of ITGB4 expression in HDM-stressed HBE cells.

To further prove the specificity of the ITGB4 methylation after HDM stimulation, we tested the mRNA expression and methylation levels of integrin $\beta 5$ (ITGB5) and integrin $\beta 8$ (ITGB8) under the same conditions in HBE cells. Compared to control group, there was no significant change in the mRNA expression of ITGB5 and ITGB8 in HDM-stimulated HBE cells. Meanwhile, no statistical changes have been detected in the mRNA expression levels of ITGB5 and ITGB8 in HBE cells after 5-AZA treatment (Figure S1A,B). In addition, no

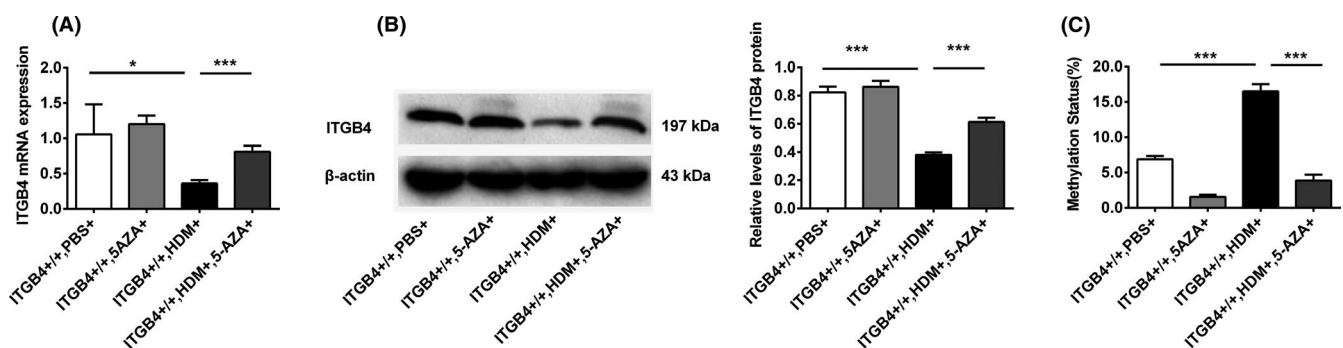


FIGURE 4 Involvement of DNA methylation in the regulation of decreased ITGB4 expression in HDM-stressed HBE cells. A, The mRNA expression of ITGB4 was quantified with qPCR in HBE cells. * $P < .05$ and *** $P < .001$. B, Western blot staining for ITGB4 in HBE cells and the density quantification of ITGB4 was expressed as a ratio relative to β -actin. *** $P < .001$. C, Statistical analysis of the mean percentage of methylation of ITGB4 CpG sites in HBE cells. *** $P < .001$

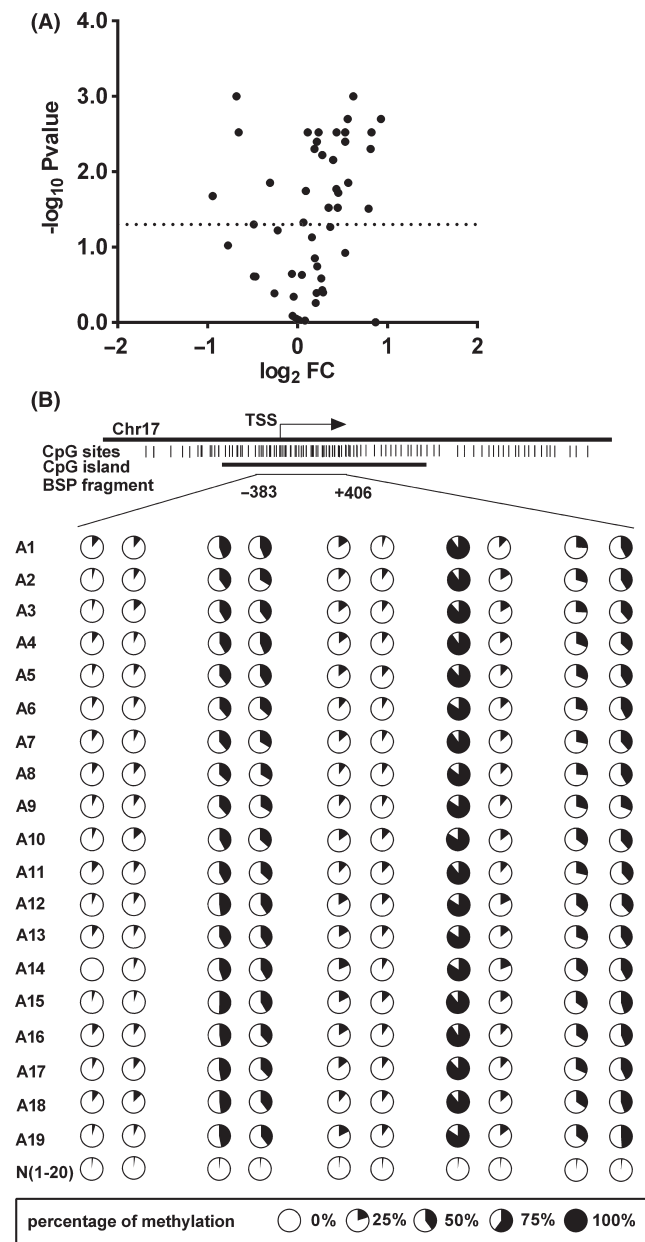


FIGURE 5 DNA methylation of ITGB4 in peripheral blood of asthma patients and HCs. A, Volcano plot of differential methylation CpG sites between asthma patients and HCs. Black dots refer to genes that have difference between asthma patients and HCs, and grey dots are points where there is no difference between asthma patients and HCs. $P < .05$. B, Schematic representation of the location of the CpG island in the transcriptional region of human ITGB4 genes. CpG dinucleotides (CpG sites) are indicated by vertical lines, and the grey box shows the CpG island. The black horizontal line under the grey box indicates the region examined by bisulphite next-generation sequencing. Methylation status of specific ITGB4 CpG sites in healthy controls and asthma patients are represented by circles, and the mean percentage of methylation at each CpG site is represented by the fraction of dark shading inside

matter after HDM stress or 5-AZA treatment, no significant changes have been found in the methylation levels of ITGB5 and ITGB8 in HBE cells (Figure S1C,D).

3.5 | DNA methylation of ITGB4 in the peripheral blood of asthma patients

To further determine the relationship between ITGB4 methylation and asthma, we collected peripheral blood from asthma patients and HCs to detect the methylation level of ITGB4 CpG island by PCR-based MiSeq analysis. Bounded by P -value $< .05$ of logistic regression analysis, 26 DMSs in asthma patients were screened compared with HCs (Figure 5A). In addition, by comparing between the methylation level of asthma patients and HCs, the normalization of methylation level data for HCs was achieved. We selected the top 10 of the 26 CpG sites as the main methylation analysis objects based on methylation difference (Figure 5B). Of note, the methylation of ITGB4's CpG islands in HCs was very low, whereas dense methylation was detected in asthma patients.

3.6 | Correlation analysis between DMSs of ITGB4 and clinical indicators of asthma patients

To further verify the involvement of DNA methylation of ITGB4 in the development of asthma, we analysed the correlation between 26 DMSs of ITGB4 and lung function indicators of asthma patients. Screening was based on P -value $< .05$, 10 methylation sites of ITGB4 were associated with asthmatic lung function (Table 3). The absolute value of the methylation difference of the 10 DMSs was between 0.3% and 9.1% (Table 4). Notably, 2 of the 10 DMSs were closely related to asthma. Specifically, the methylation level of chr17:73717720 was negatively related to FEV1%, FEV1, FVC%, PEF, FEF25 and FEF75/25 (Figure 6A). And the methylation level of chr17:73717636 was also negatively correlated with FEV1/FVC%, FEF50, FEF25 and FEF75/25 (Figure 6B).

3.7 | DNA methylation levels of chr17:73717720 and chr17:73717636 as a possible biomarker of asthma

As the two DMSs (chr17:73717720 and chr17:73717636) were related to lung function indicators, we further analysed whether the methylation level of the two DMSs could predict asthma. Analysis of the ROC curve showed that the area under curve (AUC) of chr17:73717720 and chr17:73717636 were 0.937 and 0.913, respectively (Figure 7A and Table 4). In the ROC analysis of chr17:73717720 and chr17:73717636, the AUC value increased to 0.955 (Figure 7B). A PCA analysis composed of the two DMSs showed that methylation levels of chr17:73717720 and chr17:73717636 could distinguish asthma patients from HCs effectively (Figure 7C).

4 | DISCUSSION

Asthma is a common irreversible chronic lung disease which is associated with increasing global health burden.³³ Our previous researches

TABLE 3 Correlation between clinical parameters and DNA methylation levels of asthma patients

CpG site	P-value								
	FEV1	FEV1%	FEV1/ FVC	FVC	PEF	FEF75	FEF50	FEF25	FEF75/25
Chr17:73717024	.026 [*]	.117	.896	.044 [*]	.056	.744	.612	.069	.212
Chr17:73717193	.574	.166	.126	.283	.909	.322	.037 [*]	.185	.104
Chr17:73717189	.077	.034 [*]	.884	.066	.080	.646	.532	.421	.363
Chr17:73717282	.649	.040 [*]	.630	.657	.888	.963	.420	.771	.476
Chr17:73717808	.076	.683	.568	.025 [*]	.130	.279	.211	.139	.238
Chr17:73717793	.039 [*]	.216	.966	.004 [*]	.057	.498	.380	.301	.358
Chr17:73717722	.016 [*]	.059	.593	.018 [*]	.061	.555	.272	.110	.137
Chr17:73717720	.011 [*]	.001 [*]	.177	.030 [*]	.114	.469	.054	.007 [*]	.011 [*]
Chr17:73717661	.084	.069	.067	.111	.619	.096	.162	.006 [*]	.030 [*]
Chr17:73717636	.111	.613	.008 [*]	.059	.759	.104	.004 [*]	.002 [*]	.006 [*]

Abbreviations: FEF, forced expiratory flow; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; PEF, maximum expiratory flow.

* $P < .05$ was considered statistically significant.

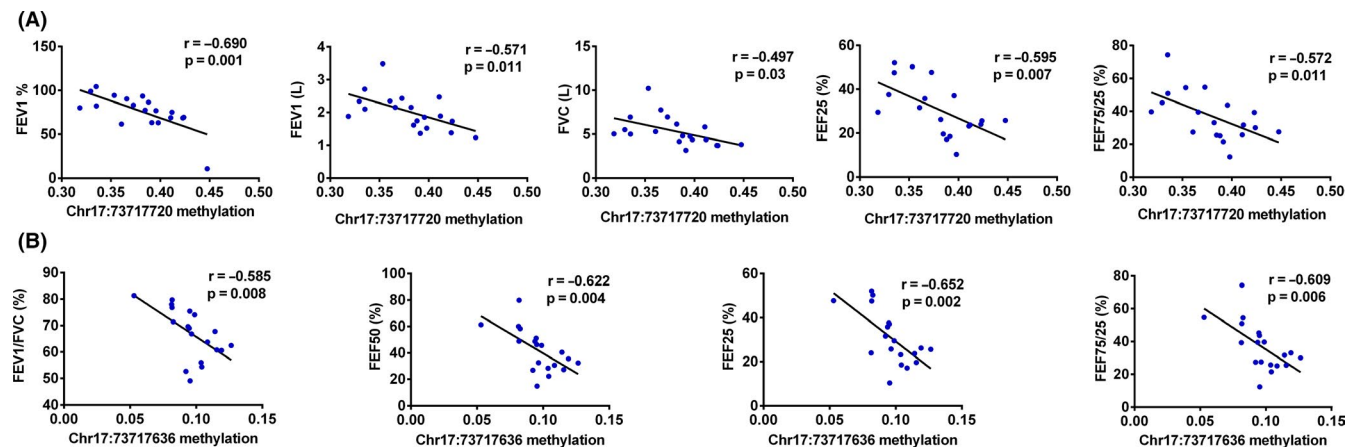
CpG site	Mean difference methylation	FDR adjusted P-value	P-value (logit)	AUC	P-value (AUC)
Chr17:73717024	0.031	.000 [*]	.005 [*]	0.842	.029 [*]
Chr17:73717193	-0.031	.000 [*]	.021 [*]	0.966	.000 [*]
Chr17:73717189	-0.011	.005 [*]	.014 [*]	0.797	.002 [*]
Chr17:73717282	0.003	.054	.019 [*]	0.713	.023 [*]
Chr17:73717808	0.031	.057	.047 [*]	0.679	.056
Chr17:73717793	0.046	.016 [*]	.018 [*]	0.779	.003 [*]
Chr17:73717722	0.059	.000 [*]	.004 [*]	0.858	.000 [*]
Chr17:73717720	0.091	.000 [*]	.007 [*]	0.937	.000 [*]
Chr17:73717661	0.036	.000 [*]	.003 [*]	0.863	.000 [*]
Chr17:73717636	0.034	.000 [*]	.001 [*]	0.913	.001 [*]

TABLE 4 Differentially methylated sites of ITGB4 associated with asthma

Note: Differential methylation analysis was conducted between asthma patients and controls in blood samples. The method of Benjamin Hochberg was used to control the FDR.

Abbreviations: AUC, area under curve; ITGB4, Integrin $\beta 4$.

* $P < .05$ was considered statistically significant.

**FIGURE 6** Correlation analysis between chr17:73717720, chr17:73717636 and clinical lung function, respectively. A, The methylation level of chr17:73717720 was negatively correlated with FEV1%, FEV1, FVC%, PEF, FEF25 and FEF75/25. B, The methylation level of chr17:73717636 was negatively correlated with FEV1/FVC%, FEF50, FEF25 and FEF75/25

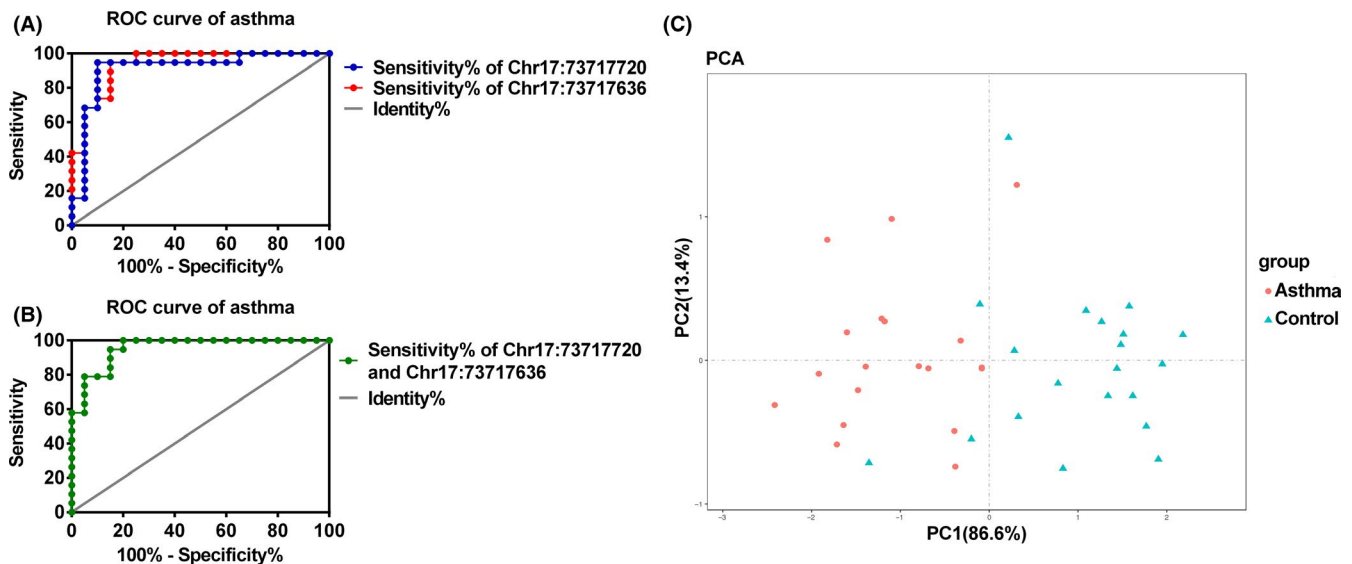


FIGURE 7 The accuracy of chr17:73717720 and chr17:73717636 in predicting asthma. A, ROC curve of chr17:73717720 and chr17:73717636, respectively. The red dotted line indicates chr17:73717720, and the blue dotted line indicates chr17:73717636. B, ROC curve of chr17:73717720 and chr17:73717636 together. C, A PCA plot consisting of the chr17:73717720 and chr17:73717636, red origin indicates asthma patients, blue triangle indicates HCs

have shown that ITGB4 defect in airway epithelial cells leads to elevated Th2 airway inflammatory response and exaggerated AHR.^{8,9,12} In this study, we further demonstrated that the expression of ITGB4 in airway epithelial cells was downregulated significantly in HDM-stressed asthma mice model along with hypermethylation of ITGB4. 5-AZA reversed the decreased expression of ITGB4 and alleviated the airway inflammation after HDM stress. Meanwhile, ITGB5 and ITGB8 were also used to further elucidate the specificity of ITGB4 methylation after HDM stress in HBE cells. And there were 10 CpG sites associated with asthma in the CpG islands of human ITGB4. Among them, chr17:73717720 and chr17:73717636 were closely related to the clinical indicators of asthma. Moreover, the ROC curve analysis and PCA suggested that the DNA methylation levels of chr17:73717720 and chr17:73717636 in the peripheral blood can effectively distinguish asthma patients from HCs. This result indicates that the methylation levels of chr17:73717720 and chr17:73717636 are highly correlated to asthma, which may help the investigation of blood biomarkers for asthma in the future.

ITGB4 has multiple functions in normal epithelial homeostasis,³⁴⁻³⁶ and certain effects on cancer cell function.^{37,38} However, there are only a few researches involved in the regulation mechanism of ITGB4 expression. ITGB4 proximal promoter and the first exon have a high GC content,³⁹ and it was illustrated that ITGB4 expression in breast epithelial cells is regulated by complex epigenetic modifications.^{20,40} Epigenetic modification, such as DNA methylation, histone modification and non-coding RNA, involves alterations of genome information and interpretation, without alterations of nucleotide sequence. Under the stimulation of TGF- β , the expression of ITGB4 is decreased which is also regulated by DNA methylation during EMT in breast epithelial cells. When TGF- β is withdrawn, it promotes the occurrence of MET, and ITGB4 is up-regulated by

histone modification. In addition, the low expression of ITGB4 by hypermethylation has been also reported in leukaemia.⁴¹ Moreover, the expression changes of ITGB4 caused by methylation in angiogenesis transformation have been explored.⁴² These studies above suggest that the expression of epithelial ITGB4 is sensitive to external stimulation which can be regulated by DNA methylation or histone modifications.⁴³

The methylation regulatory region of ITGB4 gene was detected in some related literatures of ITGB4.^{44,45} Consistent with these previous findings, our results further verified the methylation regulation of epithelial ITGB4 during the pathogenesis of asthma. In addition, previous study also found that some drugs such as glucocorticoids have an effect on DNA methylation during the treatment of asthma.⁴⁶ However, the relationship between oral or inhaled glucocorticoids and DNA methylation still need further work to verify.⁴⁷⁻⁴⁹

Additionally, this study further explored that there were 2 critical differential methylation sites of ITGB4 in asthma patients which had a strong correlation with clinical lung function indicators. The ROC curve and PCA indicate that these two sites can distinguish asthma patients from HCs. Current traditional methods for diagnosing asthma, such as methacholine challenge tests, have significant false positives and negative results.⁵⁰⁻⁵² Therefore, many studies are devoted to breaking through the routine diagnosis of asthma. Chatkin and Deykin et al demonstrated that the measurement of FeNO can distinguish between asthmatics and healthy subjects.^{53,54} In addition, studies have also shown extracting periostin from serum and extracting certain differential genes from sputum as new biomarkers for diagnosis of asthma.^{55,56} Compared with the traditional diagnostic methods, DNA methylation detection from peripheral blood is more convenient and cost-effective, which is an ideal choice for early asthma screening. Moreover, our AUC of

ROC curve reaches 0.955, indicating that the ITGB4 gene has the potential to be a biomarker of early risk prediction of asthma than other measures.

Although our results have demonstrated the DNA methylation regulation of ITGB4 expression in asthmatic airway epithelial cells may provide new possible blood biomarker to predict and screen asthma, there are also some limitations. The first one is the relatively small number of samples. More samples are still needed to further explore the key CpG methylation sites of ITGB4. Besides, there may be differences of methylation level between whole blood and lung as DNA methylation is tissue specific.⁵⁷ Moreover, the potential regulation mechanism of ITGB4 expression by DNA methylation is still obscure. At last, primary airway epithelial cells from asthma patients would provide a more convincing result. We will consider to collect primary airway epithelial cells to detect the DNA methylation of ITGB4 in our future work.

5 | CONCLUSIONS

In summary, this study confirmed the effect of DNA methylation on the decreased expression of ITGB4 for the first time in airway epithelial cells of asthma patients. Besides, alteration in the specific CpG sites of ITGB4 may regulate ITGB4 expression and may be related to the change of clinical phenotype of asthma. The specific DMSs of ITGB4 in peripheral blood may be used as effective blood biomarkers of asthma. This study offers some serviceable insights for the molecular mechanism of the decrease of ITGB4 expression in asthmatic airway epithelia which may also provide a useful target for early prediction and screening of asthma.

ACKNOWLEDGEMENTS

We appreciate the Key laboratory of Basic and Clinical Respiratory Diseases in Hunan Province for offering the equipment and experiment condition.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

WM carried out the experiments, analysed and interpreted the data and drafted the manuscript. WM, WS, YL, YY and DX collected clinical samples. WM, DX and YL: performed the experiments and statistical analysis. XY, QX, LH, QX and LC analysed and interpreted the data, provided the project funding and revised the manuscript. LC analysed and interpreted the data, revised the manuscript and finally approved the version of the manuscript for publication. All authors provided critical feedback and helped shape the research, analysis and manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this article.

ORCID

Ming Yang  <https://orcid.org/0000-0002-6367-8199>

Chi Liu  <https://orcid.org/0000-0003-2872-9576>

REFERENCES

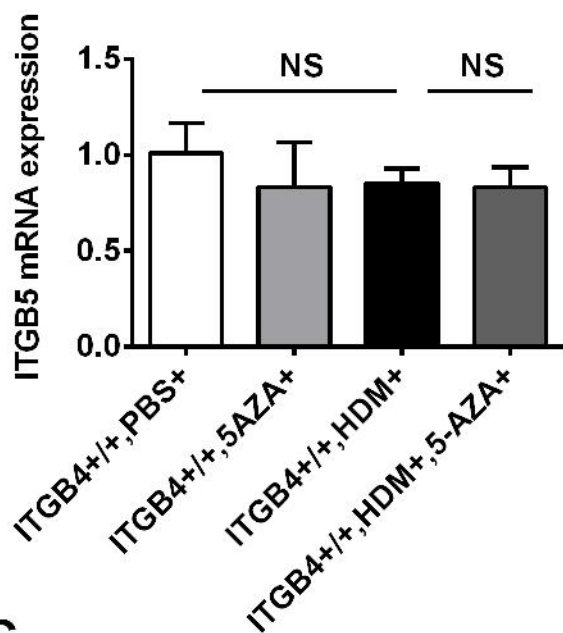
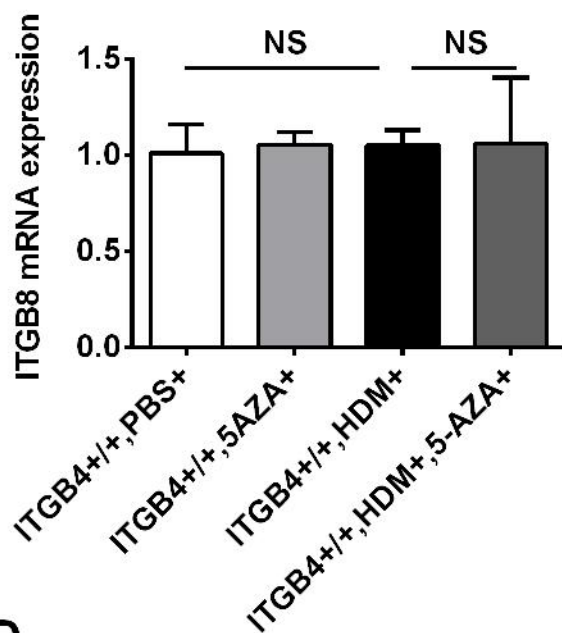
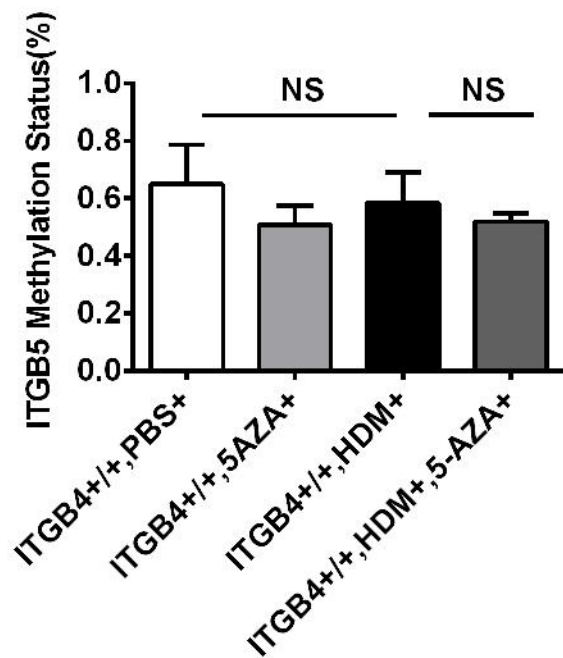
1. Bel EH, ten Brinke A. New anti-eosinophil drugs for asthma and COPD targeting the trait!. *Chest*. 2017;152(6):1276-1282.
2. Ellwood P, Asher MI, Billo NE, et al. The Global Asthma Network rationale and methods for Phase I global surveillance: prevalence, severity, management and risk factors. *Eur Respir J*. 2017;49(1).
3. Malmstrom K, Pelkonen AS, Malmberg LP, et al. Lung function, airway remodelling and inflammation in symptomatic infants: outcome at 3 years. *Thorax*. 2011;66(2):157-162.
4. Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol Rev*. 2011;242:205-219.
5. Tai A, Tran H, Roberts M, et al. Outcomes of childhood asthma to the age of 50 years. *J Allerg Clin Immunol*. 2014;133(6):1572-1578.
6. Sheppard D, Cohen DS, Wang A, Busk M. Transforming growth factor beta differentially regulates expression of integrin subunits in guinea pig airway epithelial cells. *J Biol Chem*. 1992;267(24):17409-17414.
7. Yuan L, Du X, Tang S, et al. ITGB4 deficiency induces senescence of airway epithelial cells through p53 activation. *FEBS J*. 2019;286(6):1191-1203.
8. Liu C, Xiang Y, Liu H, et al. Integrin beta 4 was downregulated on the airway epithelia of asthma patients. *Acta Biochim Biophys Sin*. 2010;42(8):538-547.
9. Liu C, Qin X, Liu H, Xiang Y. Downregulation of integrin beta 4 decreases the ability of airway epithelial cells to present antigens. *PLoS One*. 2012;7(4), e32060.
10. Liu C, Yuan L, Zou Y, et al. ITGB4 is essential for containing HDM-induced airway inflammation and airway hyperresponsiveness. *J Leukoc Biol*. 2018;103(5):897-908.
11. Liu C, Liu H-J, Xiang Y, et al. Wound repair and anti-oxidative capacity is regulated by ITGB4 in airway epithelial cells. *Mol Cell Biochem*. 2010;341(1-2):259-269.
12. Liu C, Xiang Y, Liu HJ, et al. Involvement of integrin beta 4 in ozone stress-induced airway hyperresponsiveness. *Biochem Biophys Res Comm*. 2010;397(2):290-295.
13. Sun B, Hu L, Luo Z-Y, et al. DNA methylation perspectives in the pathogenesis of autoimmune diseases. *Clin Immunol*. 2016;164:21-27.
14. Dumasia K, Kumar A, Deshpande S, et al. Estrogen, through estrogen receptor 1, regulates histone modifications and chromatin remodeling during spermatogenesis in adult rats. *Epigenetics*. 2017;12(11):953-963.
15. Stirzaker C, Song JZ, Davidson B, et al. Transcriptional gene silencing promotes DNA hypermethylation through a sequential change in chromatin modifications in cancer cells. *Can Res*. 2004;64(11):3871-3877.
16. Du X, Yuan L, Wu M, et al. Variable DNA methylation of aging-related genes is associated with male COPD. *Resp Res*. 2019;20(1):243.
17. Rastogi D, Suzuki M, Grealley JM. Differential epigenome-wide DNA methylation patterns in childhood obesity-associated asthma. *Sci Rep*. 2013;3:2164.
18. Jones B, Chen J. Inhibition of IFN-gamma transcription by site-specific methylation during T helper cell development. *EMBO J*. 2006;25(11):2443-2452.
19. Sominen HK, Zhang X, Biagini Myers JM, et al. Ten-eleven translocation 1 (TET1) methylation is associated with childhood asthma and traffic-related air pollution. *J Allerg Clin Immunol*. 2016;137(3):797-805.
20. Yang X, Pursell B, Lu S, Chang TK, Mercurio AM. Regulation of beta 4-integrin expression by epigenetic modifications in the mammary

- gland and during the epithelial-to-mesenchymal transition. *J Cell Sci.* 2009;122(14):2473-2480.
21. Perl A-K, Zhang L, Whitsett JA. Conditional expression of genes in the respiratory epithelium in transgenic mice cautionary notes and toward building a better mouse trap. *Am J Respir Cell Mol Biol.* 2009;40(1):1-3.
 22. Wang W, Li X, Zheng D, et al. Dynamic changes of peritoneal macrophages and subpopulations during ulcerative colitis to metastasis of colorectal carcinoma in a mouse model. *Inflamm Res.* 2013;62(7):669-680.
 23. Zhang J, Li C, Guo S. Effects of inhaled inactivated mycobacterium phlei on airway inflammation in mouse asthmatic models. *J Aerosol Med Pulm Drug Deliv.* 2012;25(2):96-103.
 24. Gregory LG, Causton B, Murdoch JR, et al. Inhaled house dust mite induces pulmonary T helper 2 cytokine production. *Clin Exp Allergy.* 2009;39(10):1597-1610.
 25. Lam HC, Choi AMK, Ryter SW. Isolation of mouse respiratory epithelial cells and exposure to experimental cigarette smoke at air liquid interface. *J Vis Exp.* 2011;48:e2513.
 26. Brockman-Schneider RA, Amineva SP, Bulat MV, et al. Serial culture of murine primary airway epithelial cells and ex vivo replication of human rhinoviruses. *J Immunol Methods.* 2008;339(2):264-269.
 27. Gao AI, Song S, Zuo X, et al. Epigenetic mediated transcriptional activation of PARP-1 participates in silica-associated malignant transformation of human bronchial epithelial cells. *Toxicol Lett.* 2010;193(3):236-241.
 28. Tang S, Du X, Yuan L, et al. Airway epithelial ITGB4 deficiency in early life mediates pulmonary spontaneous inflammation and enhanced allergic immune response. *J Cell Mol Med.* 2020;24(5):2761-2771.
 29. Li J-J, Li S, Zhu C-G, et al. Familial hypercholesterolemia phenotype in Chinese patients undergoing coronary angiography. *Arterioscler Thromb Vasc Biol.* 2017;37(3):570-579.
 30. Koshy L, Anju AL, Hari Krishnan S, et al. Evaluating genomic DNA extraction methods from human whole blood using endpoint and real-time PCR assays. *Mol Biol Rep.* 2017;44(1):97-108.
 31. Chacon-Cortes D, Haupt LM, Lea RA, et al. Comparison of genomic DNA extraction techniques from whole blood samples: a time, cost and quality evaluation study. *Mol Biol Rep.* 2012;39(5):5961-5966.
 32. Miravittles M, Guerrero T, Mayordomo C, et al. Factors associated with increased risk of exacerbation and hospital admission in a cohort of ambulatory COPD patients: A multiple logistic regression analysis. *Respiration.* 2000;67(5):495-501.
 33. Sears MR. Predicting asthma outcomes. *J Allergy Clin Immunol.* 2015;136(4):829-836.
 34. Dowling J, Yu QC, Fuchs E. Beta4 integrin is required for hemidesmosome formation, cell adhesion and cell survival. *J Cell Biol.* 1996;134(2):559-572.
 35. van der Neut R, Krimpenfort P, Calafat J, Niessen CM, Sonnenberg A. Epithelial detachment due to absence of hemidesmosomes in integrin beta 4 null mice. *Nat Genet.* 1996;13(3):366-369.
 36. Lipscomb EA, Mercurio AM. Mobilization and activation of a signaling competent alpha 6 beta 4 integrin underlies its contribution to carcinoma progression. *Cancer Metastasis Rev.* 2005;24(3):413-423.
 37. Nikolopoulos SN, Blaikie P, Yoshioka T, Guo W, Giancotti FG. Integrin beta 4 signaling promotes tumor angiogenesis. *Cancer Cell.* 2004;6(5):471-483.
 38. Wilhelmsen K, Litjens SHM, Sonnenberg A. Multiple functions of the integrin alpha 6 beta 4 in epidermal homeostasis and tumorigenesis. *Mol Cell Biol.* 2006;26(8):2877-2886.
 39. Zhao G, Zhou J, Gao J, et al. Genome-wide DNA methylation analysis in permanent atrial fibrillation. *Mol Med Rep.* 2017;16(4):5505-5514.
 40. Deb M, Sengupta D, Patra SK. Integrin-epigenetics: a system with imperative impact on cancer. *Cancer Metastasis Rev.* 2012;31(1-2):221-234.
 41. Phillips JL, Taberlay PC, Woodworth AM, et al. Distinct mechanisms of regulation of the ITGA6 and ITGB4 genes by RUNX1 in myeloid cells. *J Cell Physiol.* 2018;233(4):3439-3453.
 42. Goyal D, Goyal R. Angiogenic transformation in human brain micro endothelial cells: whole genome DNA methylation and transcriptomic analysis. *Front Physiol.* 2019;10:1502.
 43. Gutowska-Owsiak D, Schaupp AL, Salimi M, et al. IL-17 downregulates filaggrin and affects keratinocyte expression of genes associated with cellular adhesion. *Exp Dermatol.* 2012;21(2):104-110.
 44. Lee C, Zhang Q, Zi X, et al. TGF-beta mediated DNA methylation in prostate cancer. *Transl Androl Urol.* 2012;1(2):78-88.
 45. Li M, Jiang X, Wang G, et al. ITGB4 is a novel prognostic factor in colon cancer. *J Cancer.* 2019;10(21):5223-5233.
 46. Wan ES, Qiu W, Baccarelli A, et al. systemic steroid exposure is associated with differential methylation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2012;186(12):1248-1255.
 47. Wang AL, Qiu W, DeMeo DL, et al. DNA methylation is associated with improvement in lung function on inhaled corticosteroids in pediatric asthmatics. *Pharmacogenet Genom.* 2019;29(3):65-68.
 48. Galbally M, Watson SJ, van IJzendoorn M, et al. The role of glucocorticoid and mineralocorticoid receptor DNA methylation in antenatal depression and infant stress regulation. *Psychoneuroendocrinology.* 2020;115:104611.
 49. Wang AL, Gruziova O, Qiu W, et al. DNA methylation is associated with inhaled corticosteroid response in persistent childhood asthmatics. *Clin Exp Allergy.* 2019;49(9):1225-1234.
 50. Ungvári I, Hullám G, Antal P, et al. Evaluation of a partial genome screening of two asthma susceptibility regions using bayesian network based bayesian multilevel analysis of relevance. *PLoS One.* 2012;7(3):e33573.
 51. Konecny M, Markus J, Waczulikova I, et al. The value of SHOX2 methylation test in peripheral blood samples used for the differential diagnosis of lung cancer and other lung disorders. *Neoplasma.* 2016;63(2):246-253.
 52. Unnikrishnan A, Freeman WM, Jackson J, et al. The role of DNA methylation in epigenetics of aging. *Pharmacol Ther.* 2019;195:172-185.
 53. Heffler E, Guida G, Marsico P, et al. Exhaled nitric oxide as a diagnostic test for asthma in rhinitic patients with asthmatic symptoms. *Respir Med.* 2006;100(11):1981-1987.
 54. Berkman N, et al. Exhaled nitric oxide in the diagnosis of asthma: comparison with bronchial provocation tests. *Thorax.* 2005;60(5):383-388.
 55. Baines KJ, Simpson JL, Wood LG, et al. Sputum gene expression signature of 6 biomarkers discriminates asthma inflammatory phenotypes. *J Allergy Clin Immunol.* 2014;133(4):997-1007.
 56. Matsumoto H. Serum periostin: a novel biomarker for asthma management. *Allergol Int.* 2014;63(2):153-160.
 57. Cardenas A, Sordillo JE, Rifas-Shiman SL, et al. The nasal methylo-me as a biomarker of asthma and airway inflammation in children. *Nat Comm.* 2019;10:1-10.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Wu M, Yang Y, Yuan L, et al. DNA methylation down-regulates integrin β 4 expression in asthmatic airway epithelial cells. *Clin Exp Allergy.* 2020;00:1-13. <https://doi.org/10.1111/cea.13697>

A**B****C****D**