Aberrant methylation of aging-related genes in asthma

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March 30, 2022

Abstract

Background: Asthma is a complex pulmonary inflammatory disease which is common among older adults.Aging-related alterations have also been found in structural cells and immune cells of asthma patients. Of note, DNA methylation have been proven to be play a critical mechanism for age-related gene expression changes However, the methylation changes of aging-related genes in asthma patients is still obscure. Methods: First, changes in DNAm and gene expression were detected with multiple targeted bisulfite enrichment sequencing and qPCR in peripheral blood of 51 healthy controls and 55 asthmatic patients. Secondly, the correlation between the DNAm levels of specific altered CpG sites and the pulmonary function indicators of asthma patients was evaluated. Lastly, ROC curve and PCA were used to identify the feasibility of the candidate CpG sites as biomarkers for asthma. Results: Compared with HCs, there was a differential mRNA expression for 9 aging-related gene in peripheral blood of asthma patients. Besides, the methylation level of the 9 aging-related genes were also altered, and a total of 68 CpG sites were associated with the severity of asthma. Moreover, ROC curve and PCA analysis showed that the candidate differential methylation sites can be used as potential biomarkers for asthma. Conclusions: In summary, this study confirmed the differentially expressed mRNA and aberrant DNAm level of aging-related genes in asthma patients. The differential DMSs are associated with the clinical evaluation indicators of asthma, which indicate the involvement of aging-related genes in the pathogenesis of asthma and provide some new possible biomarker for asthma.

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Abstract

Background:

Asthma is a complex pulmonary inflammatory disease which is common among older adults. Aging-related alterations have also been found in structural cells and immune cells of asthma patients. Nonetheless, the underlying mechanism by which differenced aging-related gene contributes to asthma pathology remains unclear. Of note, DNA methylation (DNAm) have been proven to be play a critical mechanism for age-related gene expression changes However, the methylation changes of aging-related genes in asthma patients is still obscure.

Methods:

First, changes in DNAm and gene expression were detected with multiple targeted bisulfite enrichment sequencing (MethTarget) and qPCR in peripheral blood of 51 healthy controls (HCs) and 55 asthmatic patients. Secondly, the correlation between the DNAm levels of specific altered CpG sites and the pulmonary function indicators of asthma patients was evaluated. Lastly, the Receiver Operator Characteristic (ROC) curve and Principal Component Analysis (PCA) were used to identify the feasibility of the candidate CpG sites as biomarkers for asthma.

Results:

Compared with HCs, there was a differential mRNA expression for 9 aging-related gene in peripheral blood of asthma patients. Besides, the methylation level of the 9 aging-related genes were also altered in asthma patients, and a total of 68 CpG sites were associated with the severity of asthma. Notably, 10 of the 68 CpG sites were significantly associated with pulmonary function parameters. Moreover, ROC curve and PCA analysis showed that the candidate differential methylation sites (DMSs) can be used as potential biomarkers for asthma.

Conclusions:

In summary, this study confirmed the differentially expressed mRNA and aberrant DNAm level of aging-related genes in asthma patients. The differential DMSs are associated with the clinical evaluation indicators of asthma, which indicate the involvement of aging-related genes in the pathogenesis of asthma and provide some new possible biomarker for asthma.

Keywords: Aging-related genes, DNA methylation, Aging, Asthma,

Introduction

Asthma is a chronic and complex pulmonary inflammation disease which is characterized by aberrant immune responses to allergen, reversible airflow obstruction, and airway hyper-responsiveness (AHR). Although bronchodilators and inhaled/systemic corticosteroids are highly effective in most asthma patients, approximately 5-10% asthma patients are still steroid-refractory which always have lower lung function and higher mortality [1, 2]. Classical "allergic constitution" or "airway inflammation" cannot fully explain the occurrence and development of asthma. Thus, accumulating studies are attempted to further elucidate the inner pathogenesis of asthma and identify novel therapeutic targets.

Intriguingly, asthma is common among older adults (age over 65 years) which is usually more severe, with little opportunities of remission [3]. Accumulative studies have demonstrated the involvement of aging in the parthenogenesis of chronic pulmonary diseases, including idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary diseases (COPD). As is known that the pathological changes in asthma resemble COPD, such as airway remodeling, chronic inflammation and decreased lung function [4, 5]. It is feasible to speculate the possible involvement of aging in the development of asthma. Indeed, some valuable evidences have implicated that aging is a vital dangerous factor for the development of asthma [6]. Aging-related

changes have also been found in structural cells and immune cells of asthma patients. Of particular note is that the hallmarks of aging such as telomere attrition, epigenetic alterations, loss of proteostasis, and altered intercellular communication have also been detected in asthma patients [7]. Besides, aging can affects the asthma severity along with its diagnosis and management which is significant for the treatment of asthma [6]. The aging of different targeted cells can also contribute to the pathobiology of asthma, including airway inflammation, airway remodeling and decreased lung function [8]. Furthermore, it has been confirmed that anti-aging strategies can improve pathological processes such as airway inflammation and airway remodeling in asthma patients [9].

Although more and more undeniable studies have evidenced the association between aging and asthma. It is still obscure about the role of aging and the mechanism

behind differential expression of aging-related genes remains unknown. A serious of recent researches have confirmed that epigenetic mechanisms are involved in the regulation of the expression of aging-related gene [10, 11]. Epigenetic mechanisms containing DNAm, microRNAs expression and histone modifications could regulate the transcription activities of target genes without alteration of nucleotide sequence. In particularly, DNAm is the most deeply studied epigenetic regulation, which have been proven to play a crucial role in the regulation of aging-related genes [12]. Specifically, it has been verified that cytosine methylation at the CpG site affected multiple regulatory mechanisms of aging-related genes during transcription [13, 14] and further participated in aging-related disease such as asthma and COPD [15-17]. However, there is still no definitive literature on the DNAm variations of aging-related genes in asthma patients.

Our previous study screened and evaluated the differentially mRNA expression and altered methylation levels of 9 aging-related genes (AREG, ATG3, E2F1, FOXO3, HDAC1, MMP2, NUF2, TGFB1and TP53) in COPD patients [18]. It is found that DNAm was involved in regulating the expression of 9 aging-related genes in peripheral venous blood of COPD patients. Besides, the methylation level of certain special CpG sites was associated with the incidence and severity of COPD [18]. In this study, we further aim to probe the potential involvement of these previous screened 9 aging-related genes in the parthenogenesis of asthma. Firstly, we inspected the changes in DNAm and mRNA expression of the 9 aging-related genes in peripheral venous blood of HCs and asthmatic patients. Then, we analyzed the correlation between DMSs and clinical indicators in asthmatic patients. Finally, we assessed the feasibility of the candidate CpG sites as biomarkers for asthma.

Methods

Subjects and data collection

The study was approved by No. 20180308 of the Xiangya Hospital Ethics Review Committee. From October 2018 to January 2019, 51 HCs and 55 asthma patients were chosen from the Respiratory Department and Physical Examination Center of Xiangya Hospital, China. FEV_1/FVC ratio <0.7 and $FEV_1\%$ <70% was defined as the presence of asthma. The inclusive standards for the patient group were between the age of 40 and 70 with a clear diagnosis of asthma (according to the criteria of 2018 Global Strategy for Asthma Management and Prevention) but without other respiratory or other diseases [19]. The HCs had no differences in age and gender without asthma or other organic mental diseases, including smoking and non-smoking controls. Quality control methods were strictly enforced.

After obtaining the written informed consent from each subject, we collected questionnaire information (general condition, smoking history and other respiratory diseases), pulmonary function testing and peripheral blood samples. For our analysis, pulmonary function parameters were adopted including forced expiratory volume in one second as percentage of predicted volume ($FEV_1\%$), the spirometric values of forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), peak expiratory force (PEF) and forced expiratory flow (FEF). Certified staff performed all interviews and examinations. Moreover, feedback on work quality would be regularly provided to field staff during the data collection process, and secondary training would be conducted when necessary.

Sample collection

A total of 106 whole blood samples were collected from the enrolled 51 HCs and 55 asthma patients, respectively. Then,collected peripheral blood was placed into 5 ml EDTA anticoagulation tubes and transferred to a centrifuge tube. After adding 2 volumes of erythrocyte lysate and lysing for 5 minutes, peripheral blood cells were pelleted by centrifugation and stored at -80.

RNA extraction and quantitative RT-PCR

Total mRNA was purified from peripheral blood cells using Trizol (Invitrogen) and quantified by an ultraviolet spectrophotometer (Thermo Fisher Scientific, USA) [20]. 1μg RNA was reverse transcribed into cDNA using Reverse Transcriptase Kit (Qiagen, Netherlands) accordance to the manufacturer's instructions [21]. Then, quantitative RT-PCR was executed using SYBR® Premix Ex Taq^{TM}II system (TaKaRa, Japan) with the CFX96 Touch^{TM}Real-Time PCR Detection System (Bio-Rad, USA). 1μl of the reverse-transcript was added to a 30 μl PCR mixture for 40 cycles. Each cycle included 93 for 30s and 54 for 60s. By the comparison between the copy numbers of target gene and β-actin, the normalization of mRNA expression data for sample-to-sample variability in RNA input, RNA quality and reverse transcription efficiency was completed. Primer sequences were described in Table 1.

DNA Extraction, Bisulfite Treatment, Methylation Array Methods

A commercially available kit (TIANGEN Biotech, Beijing, China) was used to extract genomic DNA from whole blood according to previous publications [22]. Genesky Biotechnologies Inc. performed bisulfite processing, methylation library construction, high-throughput sequencing and quality control [23]. CpG islands located between 2K upstream of the gene transcription start site and 1K downstream of the first exon were selected to measure methylation level. 18 CpG islands from the 9 screened aging-related genes were selected (2 from AREG, 2 from ATG3, 1 from E2F1, 3 from FOXO3, 1 from HDAC1, 3 from MMP2, 1 from NUF2, 3 from TGFB1 and 2 from TP53) according to our previous publications [18]. Then, bisulfite modification of DNA sample, methylation library construction and MethTarget were performed [18]. 856 CpG sites from 9 distinguishingly expressed aging-related genes in the methylation assay were detected. We only selected the original data with a sequencing quality value of Q>40 (basic sequencing error rate <0.1%), and the methylation percentage of each CpG site was presented.

Statistical analysis

The characteristic data of all recruited HCs and asthma patients were showed as Mean ± SD, p -value < 0.05, analyzed by unpaired T test. T test and nonparametric test (Mann-Whitney U test) were used to analyze the mRNA expression and the methylation array of AREG, ATG3, E2F1, FOXO3, HDAC1, MMP2, NUF2, TGFB1 and TP53. We used the Benjamin Hochberg method to control the false discovery rate (FDR). The selection of distinguishingly expressed CpG sites was performed by Logistic regression analysis, with latent risk factors of age and gender [24]. The correlation between the percentage of methylation of candidate CpG sites and successive variables for instance FEV_1%, FVC, FEV_1 and PEF was assessed by Pearson's correlation or Spearman's correlation. ROC analysis was obtained to elucidate the accuracy of candidate DMSs or methylation change rate s in predicting asthma. For each candidate DMS, the optimal cutoff value for predicting asthma and corresponding sensitivity and specificity were defined by the maximum Youden index value (sensitivity + specificity-1) [25]. The methylation percentage of candidate DMSs or the methylation status (change or not change) were used for PCA to identify asthma. For each candidate DMS, the change in methylation status was defined by its optimal threshold [26]. The methylation change rate in each sample mainly referred to the probability that the methylation status of the candidate DMSs changed. The statistical analyses were implemented using SPSS version 22.0 (IBM Corporation, Armonk, NY, USA). A two-tailed p -value < 0.05 was considered statistically significant, **** $p < 0.0001$; * $p < 0.05$.

Results

Differential expression of the 9 screened aging-related genes in peripheral blood of asthma patients

In order to detect the expression of the previous screened 9 aging-related genes in asthma patients, peripheral blood was collected from 51 HCs and 55 asthma patients, respectively. The demographic characteristics of the all subjects was shown in Table 2. There was no significant difference between in age between asthma patients and HCs. Compared with HCs, the mRNA expression of AREG, ATG3, E2F1, FOXO3, HDAC1, MMP2, NUF2, TGFB1 and TP53 in the asthma group changed significantly (Figure 1).

Altered methylation levels of the 9 aging-related genes in peripheral blood of asthma patients

As the mRNA expression of the 9 aging-related genes altered significantly in asthma patients, we further determined the methylation levels of the 9 aging-related genes in asthma patients. We analyzed the total 856 CpG sites in the CpG islands of the 9 aging-related genes. The methylation analysis result was showed via volcano maps (Figure 2). It is showed that the methylation levels of 68 CpG sites were related to asthma at FDR $< 5\%$. The detailed information of all the differential 68 DMSs were demonstrated in Table S1.

Potential correlation between DMSs of aging-related genes and clinical index of asthma

To further assess whether the differential methylation of the 9 aging-related genes is related to the occurrence and severity of asthma, we detected the correlation between the differential 68 DMSs in aging-related genes and the lung function indicators of asthma patients. The results demonstrated that there were 10 DMSs significantly were associated with lung function. The maximum correlation coefficient for each DMSs was presented in Figure 3. The remaining correlation analysis data was showed in Figure S1. For these 10 DMSs, three DMSs (Chr4:75310649, Chr6:108883024, Chr17:7591672) were closely related to at least three clinical indicators. In addition, other three DMSs (Chr4:75310649, Chr20:32274088, Chr6:108882977) were related to two clinical indicators. It has also been shown that the correlation coefficients of the 10 DMSs were all greater than 0.38 with$p$ -value ¡ 0.05. It was also particularly noteworthy that Chr17:7591672 was closely related to four lung function indicators (FVC, FEV_1, PEF, FEF_{25}), with a correlation coefficient of 0.671 and a p -value equal to 0.0001. These data strongly suggested that theses differential DNAm of the specific aging-related DMSs may influence the occurrence and severity of asthma. The complete data for the 10 DMSs and clinical indicators were showed in Table 3.

Feasibility ofcandidate DMSs as biomarkers of asthma

Since the differential 10 DMSs have been confirmed to be closely associated to the clinical lung function of asthma patients, we further evaluated their potential as biomarkers for asthma patients. First, ROC analysis of the methylation levels of each candidate DMS was performed. The areas under the curve (AUC) of 9 DMSs (p -value$< 5\%$) were between 65.3% and 76.3%, and the AUC of 6 DMSs was greater than 70% (Figure 4A and Table 4). Besides, logistic regression was conducted and the ROC of 9 candidate DMSs showed that the AUC of the predicted probability of the 9 candidate DMSs was as high as 95.4%, and the result was statistically significantly (p -value $< 0.1\%$, Figure 4B). These results indicated that the 9 candidate DMSs had the potential value to be the biomarkers for asthma. Meanwhile, to verify the above results, PCA analysis consisting of 9 candidate DMSs was executed. The result revealed that the methylation levels of the total 9 DMSs could effectively distinguish asthma patients from HCs (Figure 4C).

To better understand the possible value of the 9 DMSs, we further calculate the methylation change rate of the 9 DMSs in HCs and asthma patients, which is a description of the possibility of methylation status alteration. Then, the status of the changed methylation or unchanged methylation was determined using the optimal cutoff value. The optimal cutoffs of the 9 DMSs were calculated according to the Youden index which was presented in Table 3. The methylation change rate of HCs and asthmatic patients were included in Figure 5. Specially, the methylation change rate of the total 9 DMSs in HCs showed a significant decreasing trend, whereas significantly increased methylation change rate was observed in asthma patients (Figure 5A). The methylation change rate of the total 9 DMSs in asthma was 33.3% ˜ 100%, and the rate in HCs was only 0 ˜ 55.6%. Notably, the change rate of a single DMS in asthma patients was between 47.27% and 89.09%, while it was 1.96% ˜ 41.17% in HCs (Figure 5B). Similarly, asthma patients had a higher rate of methylation change. Statistical results showed that the methylation change rate of the total 9 DMSs was significantly increased in asthma patients (p -value $< 0.1\%$, Figure 6A). In addition, ROC analysis was implemented

according to the methylation change rate of the 9 DMSs in all samples (Figure 6B) and there was a higher AUC compared to previous method (AUC=0.98). Moreover, the PCA analysis results also indicated that the methylation change rate of 9 DMSs could better distinguish asthma patients from HCs (Figure 6C).

Discussion

Asthma is a common chronic pulmonary disease, and the incidence of asthma has increased in the last few decades [27]. With the increased incidence of asthma, new preventive strategies and therapies for asthma are urgently needed to further reduce the morbidity and mortality of asthma. Of particular note is the potential causal role of aging in the asthma pathogenesis [28, 29]. Several relevant studies have identified the altered expression of aging-related genes (such as TP53 and FOXO3) in respiratory diseases [30, 31]. The polymorphism of transcription factor FOXO3 was confirmed to regulate the overactivation of mast cells, down-regulation of anti-inflammatory factors and production of cytokines during the pathogenesis of COPD and asthma [32]. FOXO3 deficiency has been confirmed to play an important role in regulating lung inflammation of COPD/emphysema, which has emerged as a new approach to address the development of pulmonary inflammatory diseases [33]. Similarly, TP53 has been implicated in COPD pathogens is by mediating the senescence of multiple lung cells, and the overexpression of TP53 also could promote the progression of emphysema in COPD patients [31, 34].

Not only that, as a stable epigenetic marker, aging-related CpG sites were either hypo- or hyper-methylated in COPD and other aging-related diseases [35, 36]. Our previous research identified that DNAm was involved in regulating the expression of 9 aging-related genes in peripheral venous blood of COPD patients [18]. As asthma and COPD have similar even overlapping clinical phenotypes in chronic inflammation and decreased lung function. In this study, we further explored the methylation change of the previous screened aging-related genes in peripheral venous blood of asthma patients. Indeed, the association between these screened 9 aging-related genes and asthma have been extensively studied by previous literatures [37-44]. AREG, E2F1, FOXO3,

HDAC1, MMP2, TGFB1 and TP53 have been confirmed as crucial signaling molecules in asthma [30, 45-51]. Although ATG3 is a key central regulator in autophagy induction during aging [52], and NUF2 is closely related closely associated with lung cell senescence [53], their specific role in asthma has rarely been studied. The differential expression of ATG3, FOXO3, NUF2 and TP53 in asthma patients were also aligned with former studies [30, 53-55]. In addition, excessive secretion of AREG in the airway after acute asthma attack promote airway remodeling [51]. However, AREG is downregulated in peripheral blood of elderly asthma patients, which may be attributed to the different disease stages. It is particularly worth noting that the decreased expression of E2F1 in asthma patients is consistent with what we have previously observed in COPD patients [18], which is different from that in lung cancer patients [55]. One possible reason is the specificity of the sample tissue and pathogenic genes in different diseases. MMP2, as a member of the matrix metalloproteinase family, shows an increasing trend in the acute and chronic phases of lung disease. Our results observed the increased expression of MMP2 in asthma patients which is consistent with previous literatures [56].

Additionally, we identified the methylation status of the 9 aging-related genes in asthma patients. Most DMSs of asthma patients were hypermethylated, which was consistent with the differential expression of mRNA, indicating that DNA methylation regulating gene expression is related to aging. Moreover, except for ATG3, HDAC1, and TGFB1, correlation analysis showed that the expression of the aging-related genes in peripheral blood of asthma patients was associated with pulmonary function parameters (FEV_1%, FEV_1, FVC, PEF, FEF_{75}, FEF_{50}, FEF_{25}). It is known that TGFB1 was a key cytokine that directs airway remodeling [57] and HDAC1 played a critical role in the pathogenesis of asthma [58]. This partial difference may be due to the presence of single nucleotide polymorphism in asthma [59]. Chr16:55514392 located in the promoter region has a regulatory effect on gene expression, which is inversely associated with lung function index (FVC) [60]. Interestingly, Chr16:55514437 is also located at the transcription initiation site, but the specific molecular mechanism which regulate gene expression still needs further study [60]. Furthermore, there were 9 asthma-related CpG sites on the CpG islands of the differential aging-related genes. The

ROC curve and PCA analysis of methylation level showed that all the 9 DMSs could be used as potential biomarkers to distinguish asthma from HCs. Most notably, the methylation rate of either single DMS or total 9 DMSs in asthma patients were significantly higher than that of HCs. As population size and ethnicity may influence the methylation level, we assumed that a methylation marker

holds promise for better biomarker of asthma. Our analysis of the 9 DMSs methylation mutation rate also produced a better ROC specificity and sensitivity, suggesting that the combinatorial DMSs had a great potential to predict asthma. BALF (IL-25 and IL-33, etc.), induced sputum (eosinophils, Th2 cells, etc.) and airway remodeling could all be used as an useful indicators for asthma diagnosis [61, 62]. However, the detect of DNAm in peripheral blood has greater advantage of widespread access to samples and simple operation. Not only that, DNAm is an important cause of asthma exacerbation, the specific role of allergens and environmental exposure on the epigenetic modification during the exacerbation of asthma also deserved more attention [63].

Although our study provides potential value for diagnosis and treatment of asthma assessment, there are also some limitations. Firstly, asthma can be divided into different phenotypes which may have differential epigenetic modification. Besides, our previous work is not comprehensive enough to screen all the aging-related genes. Moreover, the sample size is relatively small.

Conclusions

In a word, this study demonstrated that DNAm may regulate the differential mRNA expression of aging-related genes in the peripheral blood of asthma patients. Besides, the specific DMSs in aging-related genes has strongly associated n with pulmonary function index of asthma patients. These results shed new light on DNAm that may be involved in regulating aging-related genes in asthma, which may also provide potential candidate biomarkers for the early diagnosis of asthma.

Acknowledgments

We appreciate the Key laboratory of Basic and Clinical Respiratory Diseases in

Hunan Province for offering the equipment and experiment condition.

Competing of interests

The authors declare that they have no competing interests.

Authors' contributions

YY: carried out the experiments, analyzed and interpreted the data and drafted the manuscript. YY, YL and WM, YM: collected clinical samples. WL, DX, QL, ZY and WS: performed the experiments and statistical analysis. XY, QX, LH, QX and LC: analyzed and interpreted the data, provided the project funding and revised the manuscript. LC: analyzed 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.

Ethics approval and consent to participate

This study was approved by Document No. 20180308 of the Xiangya Hospital Ethics

Review Committee. All necessary informed consents in writing were obtained from

all patients for permission to use their clinical information and samples for analysis.

Availability of data and materials

Not applicable.

Funding information

This work was funded by grants #82070034, #81970033, #31900424, #81670002 and #31671188 from the NSFC; grants #2020JJ4776, #2020JJ4688, #2019JJ50760 from the Hunan Natural Science Foundation; grant #20K142 from open Foundation of Hunan College Innovation Program and grants #2019zzts327, #2019zzts1008 from the Fundamental Research Funds for the Central Universities of Central South University.

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Figure Legends

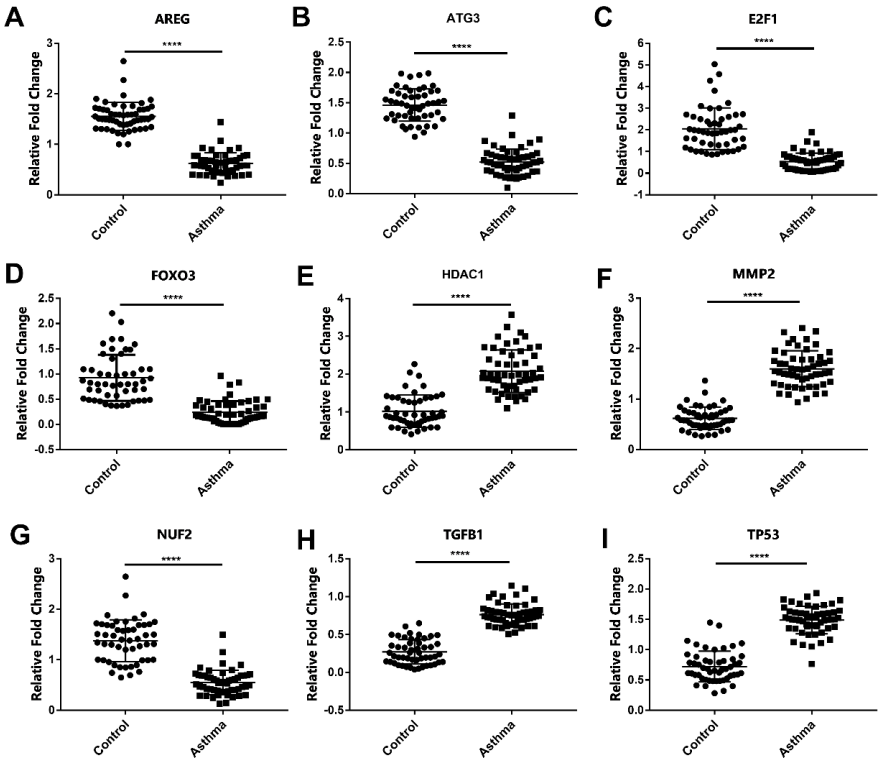

Figure 1. The mRNA levels of aging-related genes in HCs and asthma patients. (A-I) The mRNA expression of AREG, ATG3, E2F1, FOXO3, HDAC1, MMP2, NUF2, TGFB1, and TP53 in HCs and asthma patients. **** $p < 0.0001$.

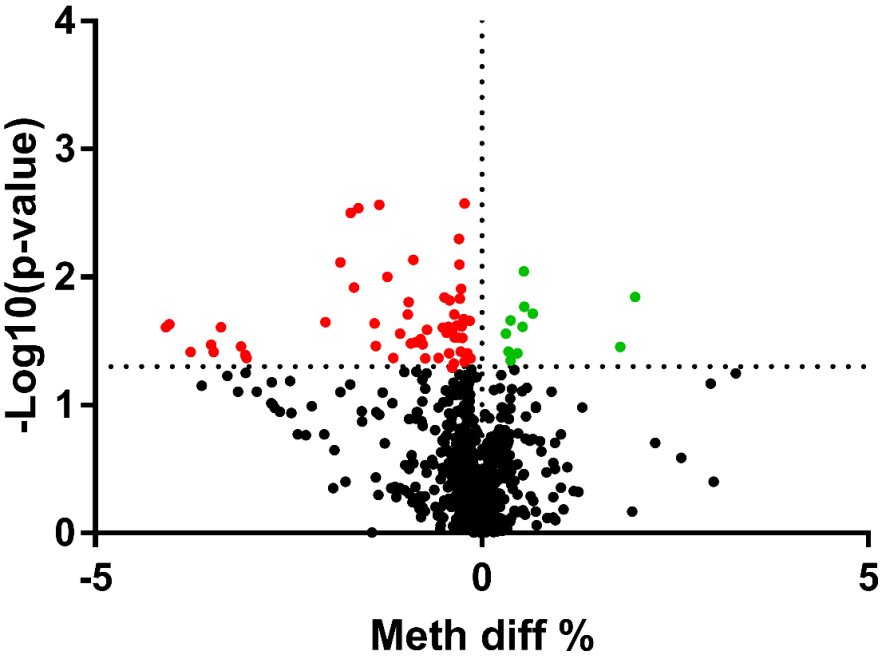

Figure 2. Volcano plot of the differential methylation CpG sites between HCs and asthma patients. The up-regulated sites were presented as green dots and down-regulated were presented as red dots. $*p$ ¡0.05 sites were presented above the dotted line.

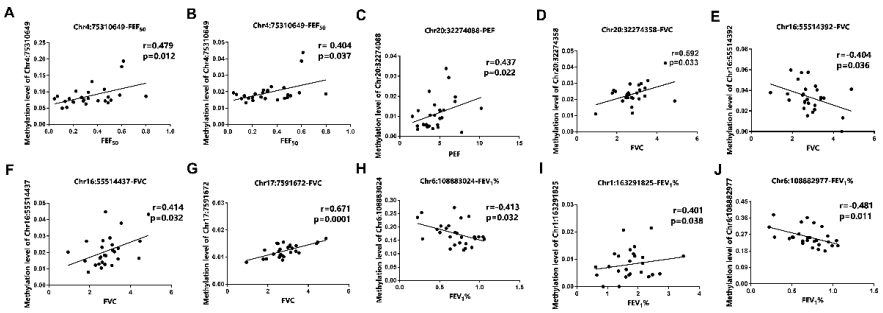

Figure 3. The correlation between DMSs and clinical parameters of asthma patients. (A-B) The methylation level of Chr4:75310649 and Chr4:75310649 were positively correlated with FEF_{50}. (C) The methylation level of Chr20:32274088 was positively correlated with PEF. (D-G). The methylation level of Chr20:32274358, Chr16:55514392, Chr16:55514437 and Chr17:7591672 were positively correlated with FVC. (H-J) The methylation levels of Chr6:108883024, Chr1:163291825 and Chr6:108882977 was negative correlated with $FEV_1\%$.

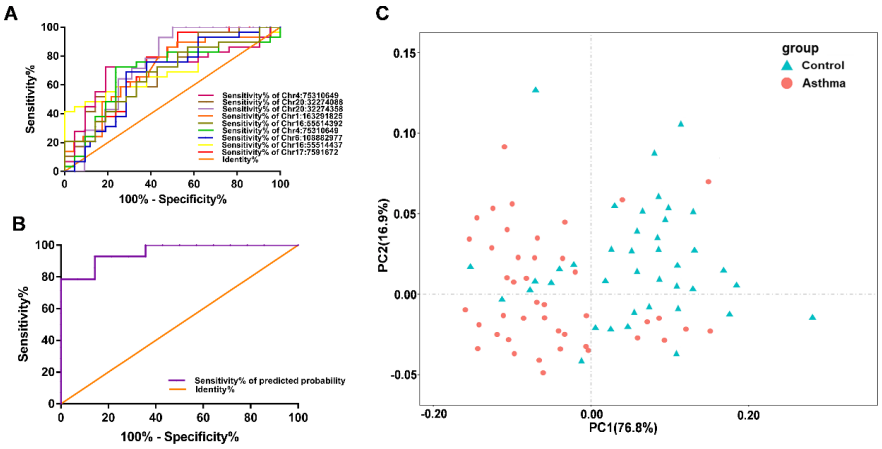

Figure 4. The accuracy of the methylation level of the 9 DMSs in distinguishing asthma patients from HCs. (A) ROC curve analysis of differential CpG sites Chr4:75310649, Chr4:75310649, Chr20:32274088, Chr20:32274358, Chr6:108882977, Chr16:55514392, Chr16:55514437, Chr1:163291825 and Chr17:7591672, respectively. (B) The ROC curve of the predicted probability of the 9 DMSs. (C) A PCA plot consisting of the methylation levels of the 9 DMSs in HCs and asthma patients.

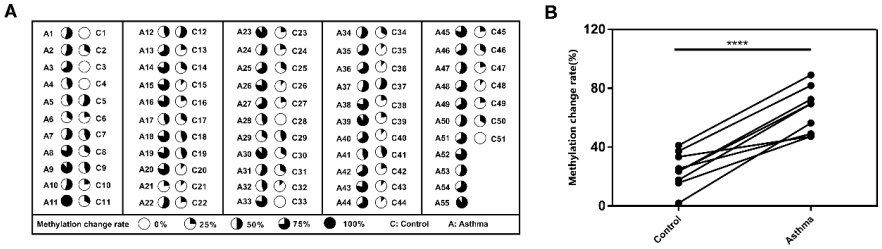

Figure 5. Methylation change rate for asthma patients. (A) The methylation change rate of the 9 DMSs in asthma patients and HCs is represented by pie chart, and the dark shades indicates the percentage of the methylation change rate. (B) Difference in methylation rate of single DMS in HCs and asthma patients.

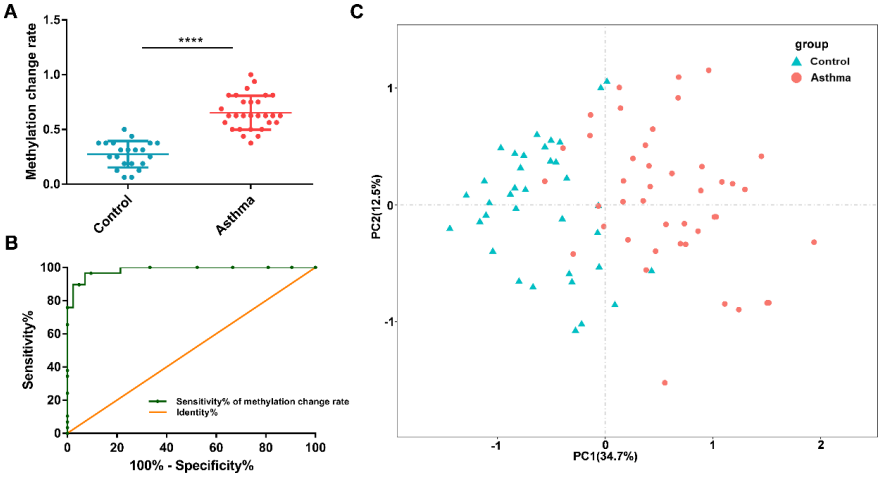

Figure 6. The accuracy of the 9 DMSs' methylation change rate in distinguishing asthma patients from

HCs. (A) Statistical analysis of the methylation change rate of the 9 DMSs in HCs and asthma patients. (B) ROC curve analysis of the methylation change rate in the 9 DMSs. (C) A PCA plot consisting of methylation change rate of the 9 DMSs in HCs and asthma patients.

Table 1. Primer sequence of aging-related genes for qPCR.

Gene	Primer	
AREG	forward	TGTCGCTCTTGATACTCGGC
	reverse	AGGCATTTCACTCACAGGGG
ATG3	forward	GTGTTCAGTTCACCCATGCAG
	reverse	TTAACAGCCATTTTGCCACTAATCT
E2F1	forward	CATCCCAGGAGGTCACTTCTG
	reverse	GACAACAGCGGTTCTTGCTC
FOXO3	forward	CGGACAAACGGCTCACTCT
	reverse	GGACCCGCATGAATCGACTAT
HDAC1	forward	TTTTTGGGTYGGAYGTTGAG
	reverse	CCCTCRCAACCTCCTCTCC
MMP2	forward	TGGCACCCATTTACACCTAC
	reverse	CCTCGTATACCGCATCAATC
NUF2	forward	TGTTAAGCAATACAAACGCACAG
	reverse	TGCCTTTTCAATACCGTCGTG
TGFB1	forward	CGACTCGCCAGAGTGGTTAT
	reverse	GCTAAGGCGAAAGCCCTCAA
TP53	forward	AAGTCTGTGACTTGCACGTACTCC
	reverse	GTCATGTGCTGTGACTGCTTGTAG
β-actin	forward	TTCCAGCCTTCCTTCCTGGG
	reverse	TTGCGCTCAGGAGGAGCAAT

Table 2. Demographic characteristics of asthma patients and HCs.

	Control	Asthma
Number of subjects	51	55
Age	53.83±6.84	46.72±10.41
Gender (f/m)	41/10	46/9
FEV_1	2.82±0.20	1.76±0.62*
FEV_1 % predicted	0.92±0.25	0.70±0.24*
FVC	4.02±0.65	2.84±0.85*
FEV_1/FVC	0.83±0.03	0.65±0.16*
PEF	8.34±0.92	4.65±0.85*
FEF_{75}	0.86±0.35	0.47±0.22*
FEF_{50}	0.83±0.34	0.37±0.16*
FEF_{25}	0.72±0.22	0.23±0.18*

Data are presented as Mean ± SD. *p -value < 0.05, asthma patients VS controls (Unpaired t test).

Table 3. Correlation analysis between DNA methylation levels and clinical parameters in asthma patients.

CpG site	Gene	p-value	p-value	p-value	p-value	p-value	p-value	p-value	p-value
		FEV_1	FEV_1%	FEV_1/FVC	PEF	FVC	FEF_{75}	FEF_{50}	FEF_{25}

CpG site	Gene	p-value	p-value	p-value	p-value	p-value	p-value	p-value	p-value
Chr4:75310649	AREG	0.309	0.105	0.093	0.33	0.933	0.025*	0.019*	0.030*
Chr4:75310649	AREG	0.465	0.115	0.135	0.512	0.687	0.044*	0.037*	0.079
Chr20:32274088	E2F1	0.035*	0.233	0.223	0.022*	0.051	0.05	0.05	0.068
Chr20:32274358	E2F1	0.113	0.059	0.968	0.182	0.033*	0.306	0.543	0.641
Chr6:108883024	FOXO3	0.044*	0.032*	0.063	0.038*	0.238	0.758	0.195	0.05
Chr6:108882977	FOXO3	0.063	0.011*	0.055	0.048*	0.366	0.949	0.147	0.051
Chr16:55514392	MMP2	0.064	0.243	0.424	0.104	0.036*	0.932	0.365	0.223
Chr16:55514437	MMP2	0.151	0.198	0.75	0.102	0.025*	0.343	0.489	0.246
Chr1:163291825	NUF2	0.508	0.038*	0.157	0.202	0.793	0.106	0.278	0.366
Chr17:7591672	TP53	0.001*	0.113	0.575	0.004*	0.000*	0.758	0.171	0.019*

A *p -value < 0.05 was considered statistically significant.

Table 4. The top 10 differentially methylated sites of the differential aging-related genes associated with asthma.

CpG site	Gene	AUC	p-value	Optimal diagnostic threshold	Sensitivity	Specificity
Chr4:75310649	AREG	0.716	0.009*	0.086	0.724	0.81
Chr4:75310649	AREG	0.691	0.022*	0.019	0.724	0.762
Chr20:32274088	E2F1	0.717	0.009*	0.009	0.517	0.857
Chr20:32274358	E2F1	0.746	0.022*	0.043	1	0.533
Chr6:108883024	FOXO3	0.653	0.066	0.166	0.909	0.667
Chr6:108882977	FOXO3	0.671	0.040*	0.263	0.69	0.714
Chr16:55514392	MMP2	0.763	0.038*	0.038	0.69	0.614
Chr16:55514437	MMP2	0.688	0.024*	0.017	0.414	1
Chr1:163291825	NUF2	0.708	0.010*	0.012	0.862	0.571
Chr17:7591672	TP53	0.721	0.008*	0.015	0.966	0.476

Statistics were done by spss 22.0, A P- value < 0.05 was considered statistically significant.

Additional file

Additional file 1:Figure S1. Correlation analysis between differential methylation sites and clinical parameters of asthma patients. (A-B) The methylation level of Chr4:75310649 and Chr17:7591672 were positively correlated with PEF_{25}. (C-D) The methylation level of Chr4:75310649 and Chr4: 75310649 were positively correlated with FEF_{75}. (E-G) The methylation level of Chr20:32274088, Chr6:108883024 and Chr17:7591672 were negatively correlated with FEV_1. (H-J) Correlation between the methylation levels of Chr6:108883024, Chr6:108882977, Chr17:7591672 and PEF.

Additional file 2:Table S1. The data of 68 CpG sites related to DNA methylation.