

The Methylation Patterns of a Disintegrin and Metalloproteinase 33 Gene (ADAM33) in Adult Asthma

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Key Words

Adult asthma · Methylation · Epigenetics · ADAM33 · Gene

Abstract

Background: Asthma is a common chronic inflammatory respiratory disease. Previous studies have suggested that the pathogenesis of asthma may be affected by epigenetic regulation. The purpose of this study is to characterize the effect of the methylation of each CpG site in the ADAM33 (a disintegrin and metalloproteinase 33) gene in adult asthma. **Methods:** A human CpG island microarray was used to examine 4 asthmatic cases and 4 healthy controls, and the results suggested that there might be differences in methylation within exon 9 of the ADAM33 gene. Therefore, we designed a case-control study with 50 asthmatic patients and 50 age- and sex-matched healthy controls to examine the relationship between the CpG methylation of the ADAM33 gene and asthma using bisulfite deoxyribonucleic acid modification and sequencing. **Results:** Bisulfite sequencing experiments showed that the 14 CpG sites in exon 9 of the ADAM33 gene were highly methylated (100%) in all individuals.

The proportions of methylation of the 14 CpG sites in ADAM33 in the case group were not different from those of the control group. The methylation of exon 9 of this locus was not associated with age, sex, IgE levels, or lung function. This study found no association between the methylation of CpG sites in exon 9 of the ADAM33 gene and adult asthma. **Conclusions:** The 14 CpG sites were highly methylated in the case and control groups. Further investigation of exon 9 in ADAM33 in a larger population is needed to evaluate its role in asthma.

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Introduction

Asthma is one of the most common chronic diseases in children and adults. A disintegrin and metalloproteinase 33 gene (ADAM33), one of the members of the ADAM family, is located on chromosome 20p13. ADAM33 is ex-

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1018–2438/13/1611–0074\$38.00/0

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pressed in lung fibroblasts and bronchial smooth muscle cells, and several single-nucleotide polymorphisms in ADAM33 are associated with bronchial hyperresponsiveness (BHR) and asthma severity in China [1, 2]. In addition, ADAM33 is composed of 812 amino acid residues and 22 exons [3].

Human DNA mainly consists of adenine, guanine, cytosine, and thymine; however, the DNA of humans and other mammals also shows the covalent modification of cytosine with a methyl group without alterations in sequence, which is known as epigenetic regulation [4]. Previous studies have suggested that epigenetic regulation may partially regulate genes and cause asthma in response to exposure to environmental factors [5, 6]. Yang et al. [7] indicated that the hypermethylation of CpG sites in the ADAM33 promoter resulted in a loss of ADAM33 expression in bronchial epithelial cells. However, the hypomethylation of CpG sites in the ADAM33 promoter of bronchial fibroblasts leads to ADAM33 expression. In addition, no significant difference was identified in the expression of ADAM33 mRNA in bronchial biopsy specimens between healthy subjects and severe asthma patients [7].

Our study utilized a CpG island microarray to evaluate asthma-related target genes, and a ratio >1.5 indicated that CpG sites displayed methylation. We further examined the DNA methylation status of exon 9 of ADAM33 in DNA extracted from peripheral blood leukocytes in the asthma and control groups. We performed methylation-specific PCR (MSP) and bisulfite sequencing to investigate the association between the methylation status of ADAM33 and asthma.

Materials and Methods

Subjects and Clinical Characteristics

A total of 228 hospital-based asthmatic adults between the ages of 20 and 65 years were recruited from the Division of Pulmonary and Critical Care Medicine of Kaohsiung Medical University in Southern Taiwan. The participants were diagnosed with asthma by chest physicians according to Global Initiative for Asthma (GINA) guidelines and/or spirometry demonstrating that the postbronchodilator value increased by at least 12% and ≥ 200 ml of forced expiratory volume in the first second (FEV₁) compared with the prebronchodilator value [8, 9]. In total, 178 asthmatic patients were excluded as follows: (1) 18 asthmatic patients suffered from chronic obstructive pulmonary disease (COPD), tuberculosis, emphysema, or lung cancer; (2) 44 asthmatic patients were current smokers, and (3) 116 asthmatic patients were regularly taking oral corticosteroids. Only 50 patients were selected for further analysis of their methylation patterns.

The healthy controls came from communities in Southern Taiwan. The control subjects were excluded if they were current

smokers and had physician-diagnosed asthma, COPD, tuberculosis, emphysema, or lung cancer. The 50 community controls were matched 1:1 to the case groups by age (± 5 years old) and sex. The asthma patients and healthy controls all agreed to: complete a questionnaire; accept an assessment of lung function; provide measurements of weight, height, and waist and hip circumferences, and provide blood samples. Pulmonary function tests mainly included FEV₁ and forced vital capacity (FVC). The study subjects performed pulmonary function tests 3 times, and the highest score was recorded. The study protocol was approved by the Institutional Review Board of Kaohsiung Medical University. Written informed consent was obtained from each study subject.

Bioinformatics Analysis

CpG Island Microarray

DNA was extracted using the commercial Purge DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, Minn., USA). Only 4 asthmatic patients and 4 matched healthy controls were randomly selected for CpG island microarray analysis. The microarray used in this study was a customized 8×15 K human CpG island microarray with 243 genes that contained asthma-related genes from the Welgene Biotech Company (Agilent Methylation Microarray Service, Welgene Biotech Co., USA). The tiled arrays consisted of 60 oligomer probes spaced approximately 100 bp apart. Each gene contained 1–39 probes corresponding to different gene lengths (<http://www.welgene.com.tw>). Immunoprecipitated methylated DNA (IP DNA) was labeled with the Cy5 fluorophore (red), and the input genomic DNA (IN DNA) without immunoprecipitation was labeled with the Cy3 fluorophore (green). The IP DNA and IN DNA are competitively hybridized to the Agilent Human CpG Island Microarray [10, 11]. Feature extraction 10.5.1.1 software was used to analyze the microarray data. To ensure high reliability of our microarray data, we replicated 5 random experiments for each CpG site. The maximum and minimum ratios were deleted, and the ratios of the remaining 3 replicates were averaged for these CpG sites. A ratio >1.5 indicated that the CpG sites displayed methylation [12, 13]. In the Human CpG Island array, there are 22 probes in the ADAM33 gene. Only 5 probes (A_17_P17169189, A_17_P17169190, A_17_P11026245, A_17_P17169191, and A_17_P11026246) among the 22 probes have higher methylation levels in asthma cases than in controls. The expression ratio values of the above 5 probes were more than 1.5 in the asthma group, and the p values were less than 0.05. These probes were then used to design the MSP primers.

Investigation of CpG Islands and Methylation Analysis

The CpG islands in the ADAM33 gene [retrieved from the National Center for Biotechnology Information (NCBI) website] were identified using the CpG Islands Searcher website (<http://www.cpgislands.com/>). Our criteria for screening the CpG islands was ObsCpG/ExpCpG = 0.7, %GC = 60%, and minimum length of a CpG island = 500. The design of MSP primers was performed using an online biological information website (<http://urogene.org/methprimer/index1.html>). The MSP product includes 14 CpG sites and partly maps to exon 9 of ADAM33. The size of the product amplified with the unmethylation (UM) primer was similar to the size of the product amplified with the methylation (M) primer [14, 15]. The BISMA (bisulfite sequencing DNA methylation analysis) website was used to perform the DNA

methylation sequencing analysis (http://biochem.jacobs-university.de/BDPCBISMA/manual_unique.php).

MSP and Bisulfite Sequencing

Methylation bisulfate conversion and MSP were performed for our 100 study participants. Genomic DNA (200 ng) from the asthma and control subjects was modified with sodium bisulfite using the MethylCode™ Bisulfite Conversion Kit (Invitrogen Corporation, Carlsbad, Calif., USA) before conducting PCR. The bisulfite conversion process was performed using MSP. The M primer pair (methylated condition) of MSP resulted in a 186-bp product and included the forward primer 5'-GCGTTTACGAAGGATTATC-GGC-3' and the reverse primer 5'-GCCACTACAAAAA-CCCAAACG-3'. The UM primer pair of MSP for the unmethylated condition resulted in a 189-bp product and included the forward primer 5'-GGGTGTTTATGAAGGATTATTGGT-3' and the reverse primer 5'-CACCCTACAAAAA-CCCAAACA-3'. The MSP conditions were: 1 cycle of 95°C for 5 min, and 40 cycles of 95°C for 30 s, 63°C for 1 min, and 72°C for 30 s, and finally 72°C for 7 min. MSP was performed using the Universal Methylated Human DNA Standard Kit (Zymo Research Corporation, Irvine, Calif., USA). Control primers were used as a positive control for bisulfite conversion processes, and MSP was performed without DNA as a negative control. The products of MSP were sequenced for all of the case and control subjects. Bisulfite sequencing was performed with the ABI Reaction Kit (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit 3.1; Applied Biosystems, USA) and analyzed with an ABI Sequencer (ABI 3730 XL DNA Analyzer; Applied Biosystems).

Statistical Analysis

Our study used an independent t test to analyze the differences in age, IgE levels, body mass index (BMI), waist-hip ratio, lung function, and the results of the microarray between the case and control groups. The χ^2 test was performed to evaluate the percentage difference in the risk factors between the asthma and control groups. All of the statistical analyses were performed with Statistical Package for the Social Sciences (SPSS) software (version 14).

Results

Participant Characteristics

The age and sex distributions of the case group were the same as those of the control group due to 1:1 matching for age and sex. The IgE concentrations and the predictive values of FEV₁% and FVC% of the case group were significantly different from those of the control group ($p < 0.05$). The BMI ($p = 0.212$) and waist-hip ratios ($p = 0.123$) of the case group were not different from those of the control group (table 1).

CpG Island Microarray

The 22 probes were located on exons 1, 8, 9, 11, 12, and 13 of the ADAM33 gene. Five out of 22 probes in the CpG islands of the ADAM33 gene (A_17_P17169189, A_17_

Table 1. Basic characteristics of the asthma and control groups

	Case group (n = 50)	Control group (n = 50)	p value
Age, years	46.04 ± 12.50	46.04 ± 12.50	
Sex			
Male	20 (40%)	20 (40%)	
Female	30 (60%)	30 (60%)	
IgE ^a , IU/ml	299.87 ± 701.19	74.35 ± 154.39	<0.001*
BMI	24.86 ± 3.94	23.89 ± 3.77	0.212
Waist-hip ratio	0.84 ± 0.09	0.82 ± 0.07	0.123
Lung functions			
FEV ₁ , % predicted	87.84 ± 17.01	93.96 ± 12.94	<0.05
FVC, % predicted	89.85 ± 19.40	96.56 ± 13.88	<0.05
Passive smoker			
No	35 (74.5%)	38 (79.2%)	0.765
Yes	12 (25.5%)	10 (20.8%)	

Values are presented as means ± SD. * $p < 0.05$. ^a The p value was determined after the data were logarithmically transformed.

P17169190, A_17_P11026245, A_17_P17169191, and A_17_P11026246) had a ratio value greater than 1.5 in the asthma group, which indicates the higher methylation levels (table 2) (fig. 1a). Two probes, A_17_P17169191 and A_17_P11026245, contained CpG sites 1–5 and sites 9–10 of MSP products, respectively. The MSP amplification product includes CpG sites 1–14 and partially contains exon 9 of the ADAM33 gene. The amplification lengths of the M primer (yellow) and the UM primer (red) are 186 and 189 bp, respectively (fig. 1a, b).

Methylation Profiles

We found that CpG sites 1–14 were 100% methylated in the case and control groups by bisulfite sequencing. We found that the methylation status of all 14 CpG sites of the ADAM33 gene was not associated with asthma (fig. 2).

Discussion

Many studies have found that polymorphisms within the ADAM33 gene are strongly associated with asthma and BHR [16–19]. To date, only one study has discussed the association between ADAM33 DNA methylation and asthma [7]. In the present study, we evaluated peripheral blood leukocyte methylation patterns in exon 9 of ADAM33 in adult asthmatics and controls. We found that the 14 CpG sites in exon 9 of the ADAM33 gene showed complete methylation in the case and control

Table 2. Means of 3 probe ratios of ADAM33 in asthma patients and controls (UCSC, hg18)

Probe name	Gene name	Chromosome	Start	End	Case group		Control group		p value
					mean ^a	SD	mean ^a	SD	
A_17_P17169180	ADAM33	chr20	3653087	3653131	0.748	0.038	0.601	0.024	<0.0001
A_17_P11026242	ADAM33	chr20	3653142	3653186	1.295	0.109	1.281	0.096	0.8182
A_17_P17169181	ADAM33	chr20	3653242	3653286	1.069	0.008	1.055	0.025	0.2474
A_17_P17169182	ADAM33	chr20	3653359	3653403	1.010	0.031	0.957	0.078	0.1515
A_17_P17169184	ADAM33	chr20	3653523	3653567	0.528	0.010	0.623	0.081	0.0344
A_17_P11026243	ADAM33	chr20	3653774	3653827	1.135	0.094	0.808	0.027	0.0002
A_17_P11026244	ADAM33	chr20	3653927	3653971	0.905	0.038	0.957	0.074	0.1560
A_17_P17169186	ADAM33	chr20	3654094	3654138	1.153	0.084	1.169	0.086	0.7462
A_17_P17169187	ADAM33	chr20	3654154	3654198	1.307	0.125	1.205	0.024	0.1014
A_17_P17169189	ADAM33	chr20	3654370	3654414	1.538	0.136	1.453	0.056	0.1841
A_17_P17169190	ADAM33	chr20	3654449	3654493	2.147	0.049	1.807	0.366	0.0723
A_17_P11026245	ADAM33	chr20	3654525	3654569	1.888	0.119	1.377	0.319	0.0093
A_17_P17169191	ADAM33	chr20	3654599	3654643	1.496	0.037	1.314	0.057	<0.0001
A_17_P11026246	ADAM33	chr20	3654705	3654749	1.868	0.150	1.531	0.200	0.0081
A_17_P17169192	ADAM33	chr20	3654778	3654822	1.146	0.036	1.038	0.032	0.0003
A_17_P11026310	ADAM33	chr20	3662203	3662247	0.829	0.133	0.800	0.077	0.6532
A_17_P17169204	ADAM33	chr20	3662297	3662341	1.204	0.104	1.010	0.098	0.0079
A_17_P17169205	ADAM33	chr20	3662404	3662448	1.129	0.021	1.140	0.004	0.2755
A_17_P11026311	ADAM33	chr20	3662648	3662692	1.083	0.139	1.065	0.080	0.7868
A_17_P11026312	ADAM33	chr20	3662983	3663027	1.479	0.120	1.302	0.143	0.0434
A_17_P17169208	ADAM33	chr20	3663071	3663115	0.850	0.012	1.038	0.137	0.0199
A_17_P17169209	ADAM33	chr20	3663188	3663232	0.940	0.028	1.016	0.117	0.1763

All of the probe variables were evaluated with an independent t test. ^a The means were calculated after excluding the maximum and minimum ratios of five repeated experiments.

groups. The methylation of the CpG sites in exon 9 of the ADAM33 gene is not associated with adult asthma, age, sex, IgE levels, or lung function.

Based on CpG island microarray data in our study, we chose 5 probes on exon 9 of the ADAM33 gene to perform MSP and bisulfite sequencing based on a ratio >1.5. Previous studies have shown that the methylation status of an exon is correlated with malignancy and tumor stage [20, 21]. Therefore, we hypothesized that the methylation status of the exon of the ADAM33 gene may be associated with its related diseases; the methylation level of the exon is noteworthy. However, our study showed that 14 CpG sites on exon 9 in ADAM33 were highly methylated, and there were no significant differences between the case and control groups. Similarly, Yang et al. [7] found that the methylation status of the promoter of the ADAM33 gene was not associated with asthma.

Previous studies have shown that hypomethylation of the promoter of the ADAM33 gene drives the transcription and translation of ADAM33 and induces ADAM33 expression in airway fibroblasts, myofibroblasts, and

smooth muscles [22–25]. Van Eerdewegh et al. [1] found that ADAM33 expression in lung fibroblasts and bronchial smooth muscle cells resulted in airway structural changes. Therefore, it could be speculated that an association exists between airway remodeling in asthma patients and ADAM33 [1, 23]. A previous study also found that a demethylating agent (5-aza-2'-deoxycytidine) used to treat epithelial cells induced CpG island demethylation and ADAM33 expression [7].

Previous studies have indicated that tobacco can modify asthma airway inflammation and BHR in adult asthma patients [26], and smokers have reduced FEV₁ compared with nonsmokers [27]. Parental smoke exposure affects DNA methylation [28]. Therefore, we excluded the subjects who were smokers in the case and control groups to control for the confounding effect of smoking. Our study is a case-control study in which the temporal causality is not clear. Therefore, a prospective cohort study and confounding factor adjustment, such as diet and occupational exposure, should be considered in further studies.

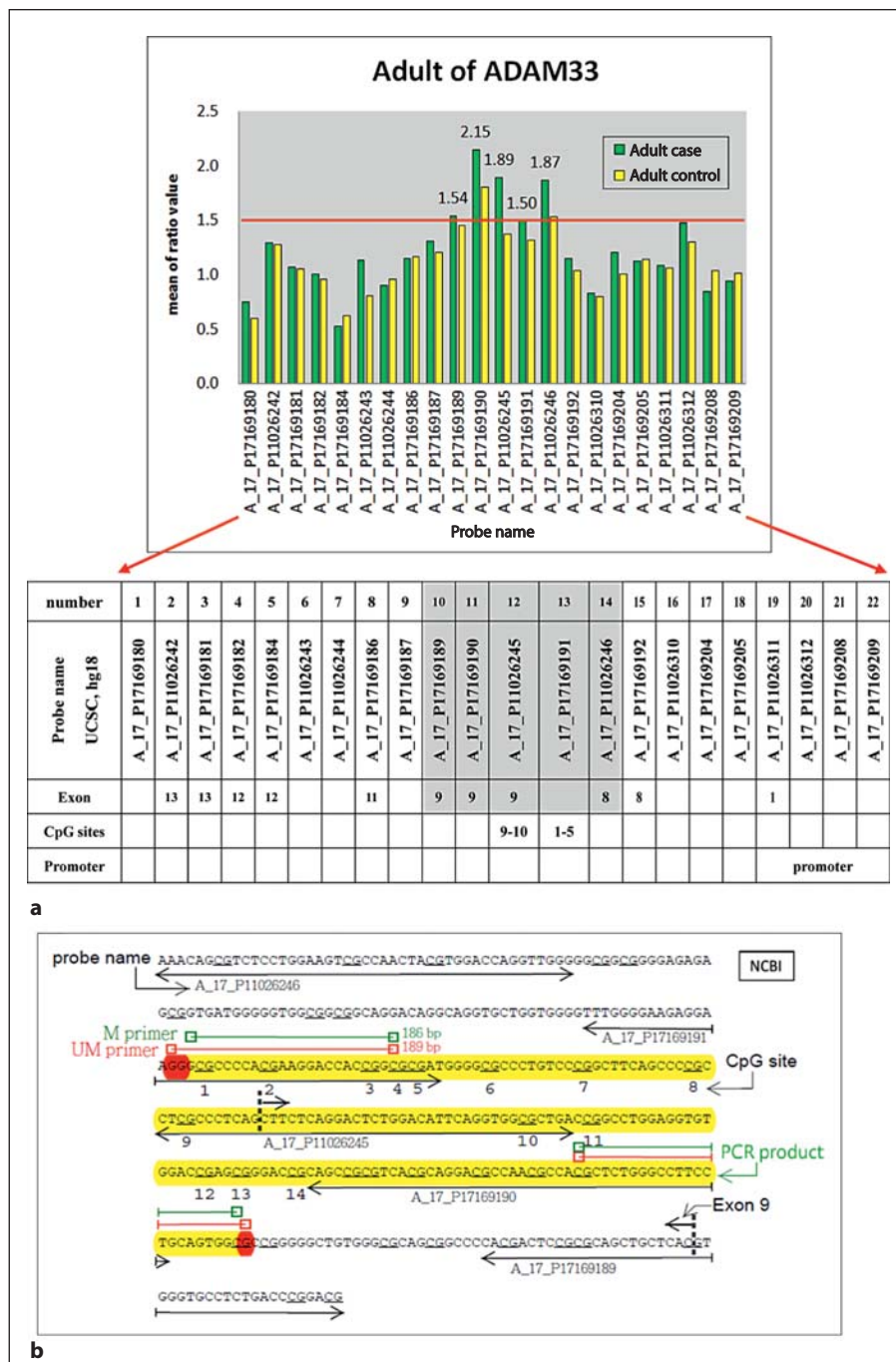


Fig. 1. a The 14 CpG sites, the 5 selected probes in the microarray, and **b** the 2 primers for MSP in exon 9 of ADAM33 (colors are shown online only).

There are some limitations of this study. This study lacks consensus regarding the ideal tissue for analyzing methylation patterns in studies of respiratory diseases. Due to the difficulty of collecting other tissue types, we obtained DNA from peripheral blood leukocytes but not from airway epithelia. We could not ascertain whether the methylation patterns would reflect the patterns present in

the airway; therefore, the selection of relevant tissue for the trait should be of high priority in the planning of future epigenetic studies. However, several studies have suggested that the detection of genetic alterations and methylation patterns in the peripheral blood DNA of cancer patients may serve as noninvasive markers for monitoring and diagnosing cancers in high-risk populations [29, 30].

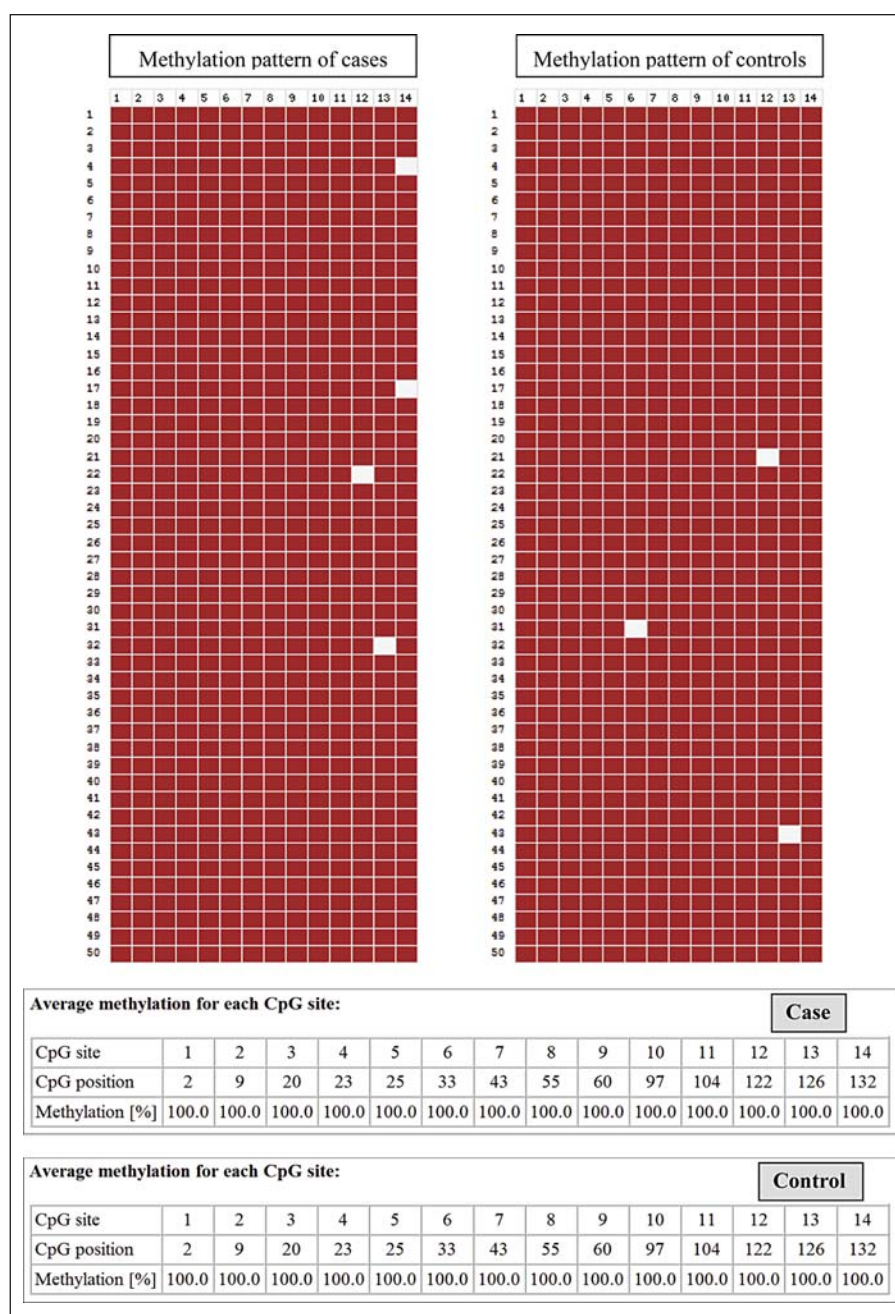


Fig. 2. The methylation profile of 14 CpG sites in ADAM33. The red boxes show methylation, and the white boxes show unknown data (colors are shown online only).

A recent study demonstrated that DNA methylation in blood was highly correlated with buccal cells ($r > 0.75$) [31]. Furthermore, the sample size of the study was only 100 subjects and quite small; therefore, the findings applied to the large population group still needed further investigation. Our study found that the methylation status of exon 9 in the ADAM33 gene was not associated with adult asthma, and all 14 CpG sites showed 100% methylation in the

study subjects. Exon 9 in ADAM33 was highly methylated, but further investigation of this region in a larger population is needed to evaluate its role in asthma.

Acknowledgments

This work was supported by grant NSC 99-2314-B-037-056-MY2 and NSC 100-2314-B-037-025-MY3, Taiwan.

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