Mol Biol Rep (2013) 40:2357–2368
DOI 10.1007/s11033-012-2317-1

Epigenetic regulation of DNMT1 gene in mouse model of asthma disease

M. Verma · B. D. Chattopadhyay · B. N. Paul

Received: 10 August 2012 / Accepted: 19 November 2012 / Published online: 30 November 2012

Abstract Asthma is a complex genetic disease, which arises from the interaction of multiple genes and environmental stimuli. These influences are important to asthma pathogenesis. These can be mechanically explained by the Epigenetic phenomenon, which consists of the chromatin and its modifications, as well as a covalent modification of cytosines residing at the dinucleotide sequence CG in DNA by methylation. This reaction is catalyzed by a family of DNA methyltransferase enzyme (DNMTs). DNMT1 is one of them which maintained the methylation status during replication and also critical for the development, differentiation and regulation of Th1 and Th2 cells. Therefore we studied the DNMT1 mRNA expression profiling as well as CpG methylation status in promoter region. For these studies we developed asthma mouse model, and used Flow cytometer, qRT^2-PCR, Methylation specific PCR, bisulfate conversion and BiQ analyzer. We found that DNMT1 expression level was low in all the tissues (lung, trachea and BALF cells) of asthmatic in comparison to normal mice. This was due to the methylation of regulatory sites of DNMT1 promoter region at cytosine residue. As the incidence of asthma is increasing globally and in world, this study assumes greater significance in designing and developing therapeutic means.

M. Verma (✉) · B. N. Paul
Immunobiology Division, CSIR-Indian Institute of Toxicology Research, M.G. Marg, Lucknow 226001, India
e-mail: mviitr@gmail.com; mukeshverma82@iitr.res.in

B. N. Paul
e-mail: bholanathpaul@yahoo.com

B. D. Chattopadhyay
Department of Physics, Jadavpur University, Kolkata, India
e-mail: bdc_physics@yahoo.co.in

Keywords Asthma · Epigenetic · DNMT1 · CpG methylation

Introduction

There is a widespread concern that the prevalence and incidence of asthma is still rising in developed countries, but the economic and humanitarian effects of asthma are greater in the developing world. According to Centers for Disease Control and Prevention (CDC) report in 2009, nearly 8.2 % of Americans surveyed had asthma, an increase of 0.4 % over 2008. However Asthma and allergic diseases are believed to be a complex genetic disease, which result from the interaction of multiple genes and environmental stimuli [1, 2]. The genetic and environmental influences are important to asthma pathogenesis. These gene-by-environment interactions can be mechanically explained by the epigenetic phenomenon. Epigenetic regulation provided an attractive mechanistic explanation for some of the molecular events, link to early-life environmental exposures with the subsequent development of disease [3].

Epigenetic is an important, alternative mechanism of gene regulation that is independent of the nucleotide sequence of DNA, and it has been shown to be related to the development of several diseases [4, 5]; including cancer, syndromes involving chromosomal instabilities, mental retardation, immunodeficiency and autoimmune diseases (i.e. systemic lupus erythematosus). Epigenetics consists of the chromatin and its modifications, as well as a covalent modification of cytosines residue by DNA methylation at the dinucleotide sequence CG [6]. Cytosine methylation of the regulatory sequences of DNA is a dominant epigenetic mechanism that is associated with the transcriptional inactivation of genes, where as decreased

methylation (hypomethylation) has been considered to be related to hyper responsiveness against autoantigen and exogenous antigen in certain autoimmune as well as allergic disorders [7]. The expression and activity of enzymes that regulate this epigenetic modification have been reported to be abnormal in airways of patient with respiratory disease [8]. Two types of normal methylation processes are known in eukaryotic cells. First is de novo methylation which is involved in the rearrangement of methylation pattern during embryogenesis or differentiation processes in adult cells [9]. This DNA methylation patterns are established and maintained by three major DNA methyltransferases (DNMTs): DNMT1, DNMT3A, and DNMT3B enzymes. First a family of enzymes was described in human, containing methyltransferases DNMT3a and DNMT3b which show the de novo methylation activity [10, 11]. The same homologous genes were also identified in mouse [12]. The gene targeting experiments showed that both DNMT3a and DNMT3b are important for de novo methylation and have no consequence of maintenance methylation [13]. The second methylation activity in eukaryotic cell is the maintenance methylation which is responsible for maintaining the methylation pattern as well as de novo methylation and known as DNMT1. During DNA replication DNMT1 is located in the replication complex where it recognizes the normally methylated CpG sites in the parent strand and catalyses the addition of the methyl group in the corresponding CpG site in the daughter strand. DNMT1 is the only mammalian DNMT that has a preference for hemimethylated CpG sites [14, 15]; and localizes at both replication foci and repair sites because of its interaction with the proliferating cell nuclear antigen [16, 17]. Active localization of the enzyme to sites of DNA replication in dividing cells may facilitate a maintenance role of DNMT1 [16]. In mammals, DNA methylation is significantly involved in controlling gene expression, cell differentiation, silencing of transposable elements, X chromosome inactivation, imprinting, and neoplastic transformation [18].

As a result of these observations and data from genetic manipulations in the mouse, DNMT1 is thought to be the major enzyme responsible for post replicative maintenance of DNA methylation. Homozygous null deletions of mouse *DNMT1* are lethal early in development and result in an 80 % reduction of global genomic methylation in embryonic stem cells and embryos [19]. These and other studies also showed that despite growing normally, *DNMT1* −/− embryonic stem cells have reduced differentiation potential both in vivo and in vitro [20]. Functional analysis of this enzyme showed that it has maintenance methylation activity and is important for the development, function and survival of T cell [21].

Here we found that mRNA levels of DNMT1, STAT3, IFN-y and IL-6 were significantly low in lung, trachea tissues and BALF cells of asthmatic as compared to normal mice along with heightened mRNA expression of SOCS3, IL-4 and IL-5. We know that DNMT1 is essential for the development and differentiation of the T cell. For this study we hypothesized that the low expression of DNMT1 might be DNA methylation mediated down regulation. Our finding suggested that reduced expression of dnmt1 is due to the cytosine methylation in the regulatory sites of promoter region. This study assumes greater significance in designing and developing to therapeutic means.

Methods and materials

Animal sensitization and challenge

Balb/c mice available in the Institutional Animal Facilities were used for experimentation with consent from the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), India. Ten-to twelve-week-old female BALB/c mice were sensitized with intraperitoneal (i.p.) injection of 100 μg ovalbumin (OVA) (Grade V; Aldrich-Sigma Chemical) emulsified in 2 % alum solution on day 0 and 14 or sham sensitized with saline (SAL). 30 min post OVA sensitization on day 14, the animals were challenged with OVA through the nasal route. A total of 50 μg OVA in sterile SAL was applied on the snout of the nostril in 5 cycles, 10 μL containing 10 μg OVA in SAL at each cycle at an interval of 2 min. On days 21, 22, 23 and 26, mice were challenged through the nasal route with similar dose. On day 26, 30 min after last nasal challenge, lung function was measured by evaluating airway hyper responsiveness (AHR) to Methacholine chloride (Mch) (Sigma Chemical Co., St Louis, MO, USA) aerosol by using double chamber whole body plethysmography. Twenty-four hours after the last nasal challenged, the mice were anaesthetized by ketamine-HCl (0.15 mL i.p. injection per 25 g mouse). There after animals were sacrificed, for collection of blood and bronchoalveolar lavage fluids (BALF), trachea and lung tissues dissected out for isolation of RNA and DNA.

Airway hyper responsiveness

Methacholine induced AHR was assessed by double chamber whole body plethysmograph, using the Buxco system (Buxco Electronics, Sharon, CT, USA). The regularity of the breathing patterns was first assessed by qualitatively monitoring the constancy of peak flows. Irregularities in the breathing pattern were often encountered due to movement of the neck in most cases and to a

lesser extent, sigh-breath and cough. Data arising from these irregularities in breathing pattern were not considered for quantitative analysis. Before the start of the experiments, the animals were conditioned to stay in the plethysmograph chamber for at least three or four sessions of 5 min duration each. It was necessary to precondition the animals to minimize irregularities in the breathing pattern during data acquisition and achieve constancy in peak thoracic and nasal flows in the shortest possible time. After a brief acclimatization to the chamber, the mice received an initial baseline challenge of saline, followed by aerosolized methacholine in varying concentrations, 3.125, 12.5 and 50 mg/mL and AHR were measured in term of specific resistance airway (sRaw) of each dose.

Estimation of total immunoglobulin-E levels in serum

After measurement of in vivo airway responsiveness, mice were used for the blood collection. Blood was collected from the retro-orbital sinus using non heparinised glass capillaries, allowed to clot for 1 h and centrifuged to separate the serum. Subsequently, serum was collected and stored at −20 °C until analysis. Total IgE in the serum measured by mouse IgE ELISA quantitation kit (Bethyl laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's protocol.

Analysis of the cellular composition in the bronchoalveolar lavage fluid

After blood collection, mice were sacrificed by injection of 100 μL ketamine-HCl. The sacrificed animals were used for measured to infiltrated cells, bronchoalveolar lavage fluid (BALF) was performed as previously described [22]. Cytokines (IL-4 & IL-5 & IFN-y) in the supernatant of the BALF were determined by Sandwich ELISA (R&D System, Minneapolis, MN, USA) according to the manufacturer's protocol.

Analysis of intracellular cytokines from whole blood by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation on Ficoll-Hypaque cushion Hi-Media, India. A total of 6×10^6 cells/well were cultured in a 6-well culture plate containing DMEM supplemented with 10 % (v/v) FCS, 1 % (v/v) non essential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 μg/mL gentamycin and Brefeldin A (10 μg/mL; Sigma, St. Louis, MO, USA) to block secretion of intracellular proteins. Aliquots of PBMC were either kept in medium or stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL) and ionomycin (1 μM; both Sigma) to provoke cytokine production. Following incubation for 4 h in a CO2 incubator, the cells were used for either surface staining or permeabilised and stained for intracellular molecules. 5 μL of CD4-FITC antibody was used for surface staining. For intracellular labeling cytokine-specific antibodies (20 μL, IFN-y-FITC, IL-4-PE and IL-5PE at a dilution of 1:2,000 (PBS) (Becton–Dickinson) were added to the cells and incubated for 30 min at room temperature in the dark. After one wash, cells were resuspended in 1 % paraformaldehyde (500 μL) and saved at 4 °C till flow cytometric analysis. Cells were gated using a PASIII Partec flow cytometer and data were analyzed using FloMax software. Percentages of Th1 and Th2 cytokine-producing cells were identified as the number of IFN-γ positive or IL-4-positive cells respectively, in a total population of $CD4^+$ T-helper cells. A minimum of 5,000 $CD4^+$ cells was counted from each sample. Fluorochrome-equivalent IgG2 isotype controls (Becton–Dickinson) served to detect nonspecific binding.

Real-time quantitative polymerase chain reaction (RQ-PCR) analysis

Total mRNA was isolated from BALF, lung and trachea tissues using RNeasy mini kit as per manufacturer's instruction (RNeasy mini kit; QIAGEN, Germany). Then cDNA was obtained from the purified RNA using SYBR green one step RT-PCR kit (QIAGEN, Germany); as described previously [22]. Quantitative analysis of data of the samples was obtained with the Light Cycler software. The Primers used in this study for Socs3 and β-actin were obtained from earlier published paper [22] and for DNMT1 primer was designed by using the Oligo 6.6 primer Analysis Software and the sequences accessible in the GenBank (Acc. No. 13433) database and procured from Operon Biotechnologies GmbH, Germany and used for RT-PCR. The primer sequence are: Socs3: 5′GAAGACCAAGTTCA TCTGTGTG3′ (forward); 5′GTAGCACACTC CGAGG TCAGAT3′ (reverse); Stat3: 5′GAAGACCAAGTTCATC TGTGTG3′(forward); 5′GTAGCACACTCCGAGGTCAG AT3′ (reverse); DNMT1: 5′GCAAGGTCAAGGTCATCT AC3′ (forward); 5′CAGCCGGATACAAGATAGG3′ (reverse); IL4: 5′GACAAAAATCACTTGAGAGAGA3′ (forward); 5′ACGAGTAATCCATTTGCATGAT3′ (reverse); IL5: 5′AAGGATGCTTCTGCACTTGA3′ (forward); 5′ACA CCAAGGAACTCTTGC3′ (reverse); IL6: 5′TTGCCTTCTT GGGACTGATGCT3′ (forward); 5′GTATCTCTCTGAAGG ACTCTGG3′ (reverse); IFNy 5′ACTGG CAAAAGGATG GTGAC3′ (forward); 5′TGAGCTCATTGAATGCTTGG3′ (reverse) and βactin: 5′GACATGGAGAAGATCTGGCAC3′ (forward); 5′TCCGACGCAGGATGGCGTGA3′ (reverse).

Pathology

Tissues of normal (saline sensitized and challenged) and asthmatic (OVA sensitized and challenged) mice were fixed in 10 % neutral buffered formalin, paraffin embedded, cut into 10-µm sections, and stained with PAS, hematoxylin and eosin, to evaluate epithelial basement thickening, bronchiole constriction alveoli depicted emphysema and mucus exudates in airways Lumina.

Bisulfite sequencing

Genomic DNA of the lung tissues was extracted by using DNeasy blood and tissue kit (Qiagen GmbH, Hilden) according to the manufacturer's protocol. Genomic DNA was subjected to bisulphite modification by using Epi-Tect Bisulphite Kit (Qiagen GmbH, Hilden) according to the manufacturer's protocol. 2 µg of genomic DNA was mixed with sodium bisulfite and DNA protection buffer and denaturation performed in a thermal cycler. The thermal cycler conditions were :(1)Denaturation at 99 °C for 5 min; (2) Incubation at 60 °C for 25 min; (3) Denaturation at 99 °C for 5 min; (4) Incubation at 60 °C for 85 min; (5) Denaturation at 99 °C for 5 min; (6) incubation at 60 °C for 175 min. The modified DNA was transferred on to the membrane of an EpiTect spin column where washing and desulphonations were carried out and eluted with RNAse and DNAse free water. 50 ng DNA was used as templates for methylation specific PCR (MSP) using HotStar Taq Master Mix (Qiagen GmbH). MSP was performed using both methylation(M) and unmethylation (U) specific primers DNMT1:M; 5′GGGTG GCGTTAGTCGTAGTAC3′ (left primer); 5′AACGACAA AACAACTCTAAACGAA3′ (right primer) and DNMT1:U; 5′GTGGGTGGTGTTAGTTGTAGTATG3′ (left primer); 5′AAACAACAAAACAACTCTAAACAAA3′ (rightprimer) under the following conditions: Initial activation at 95 °C for 15 min followed by three step cycling (denaturation at 94 °C for 45 s; annealing at 58 °C for 45 s; extension at 72 °C for 1 min) for 30 cycles and final extension at 72 °C for 10 min. Specific primers for CpG islands, located near or inside the promoter region of studied genes, were designed by *Meth-Primer* program. Data were analyzed by using BiQ analyzer software and clones showing less than 85 % conversion or 90 % homology to the reference sequence were not incorporated in succeeding analysis.

Statistics

Results are expressed as mean ± SE ($n = 5$). Significance of the difference between mean values was determined by one way analysis of variance (ANOVA) followed by Student's t test. $P < 0.05$ were considered significant.

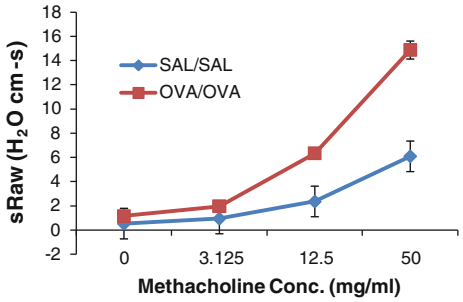

Fig. 1 Measurement of airway hyper responsiveness (AHR) in term of specific resistance of airways (sRaw) at various concentration of methacholine chloride (Mch) in control (SAL/SAL) and treated (OVA/OVA) control mice

Results

Airway inflammation and BALF cellular composition

The pattern of varying doses of Methacholine chloride (Mch) on specific resistance of airways was monitored by a non-invasive technique. Ovalbumin/Ovalbumin (OVA/OVA) (OVA sensitized and challenged) mice showed significantly ($P < 0.05$) higher AHR as compared to control SAL/SAL (Saline sensitized and challenged) mice (Fig. 1). In control mice Mch-induced sRaw was comparatively low in comparison to OVA sensitized and challenged mice. Increased sRaw indicated that mice have became asthmatic. Histopathology of lungs revealed bronchial walls thickened by marked submucosal edema, moderate mucosal and muscular hypertrophy. Lung tissues from OVA/OVA (sensitized and challenged) mice were characterized by gross alterations in the structural integrity of the airway walls and parenchyma, epithelial cell shedding, microvascular leakage and extensive mucosal edema. Increased tissue cellularity and particulate exudates in the airways lumina and alveolar septa were also observed. Asthmatic lung sections stained with H&E showed epithelial basement membrane thickening and emphysema. PAS hematoxylin staining further revealed the presence of mucous exudates in the lumen of the bronchiole (Fig. 2c, d). These features were absent in normal lung (Fig. 2a, b). The cytokines IL-4 and IL-5 in BALF were measured by ELISA after 24 h of the last challenge and significantly ($P < 0.05$) increased level of IgE, IL-4, IL-5, eosinophil and neutrophil were found in BALF of OVA/OVA mice (Table 1). Thus OVA sensitized and challenged mice were found to be asthmatic based on lung function, cytokine profile and lung architecture. Airways tissues from these animals were used for all downstream studies.

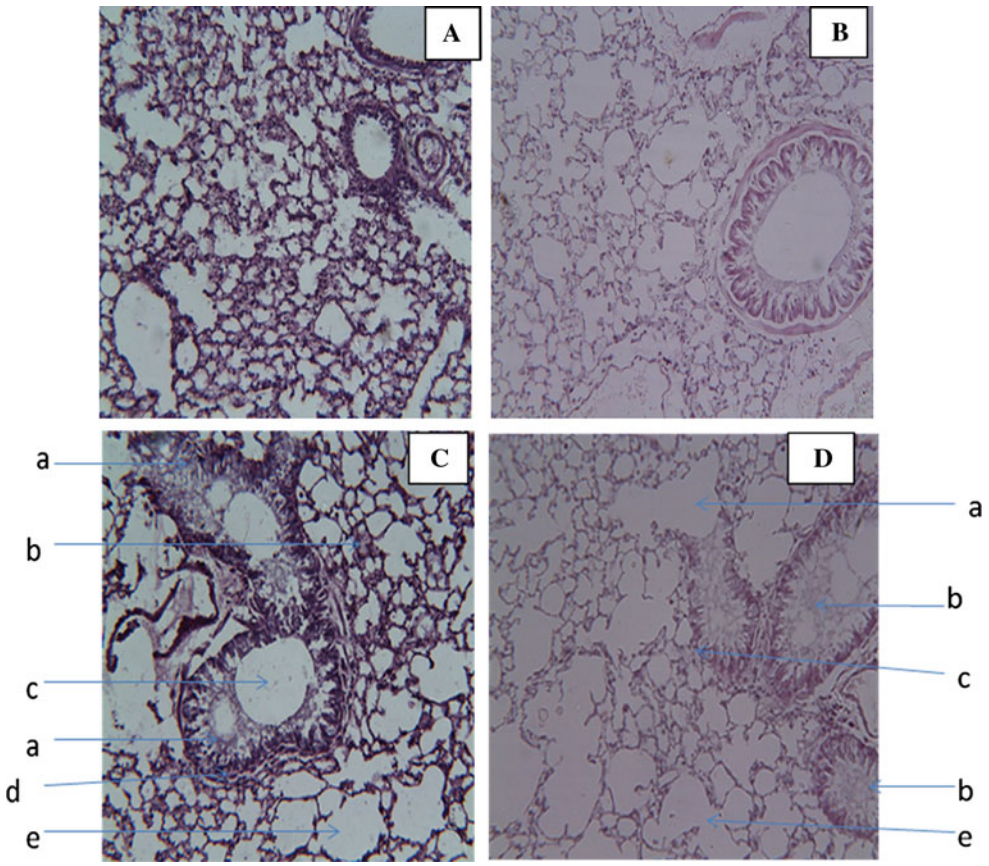

Fig. 2 Representative PAS (**a**, **c**) and H&E (**b**, **d**) stained lung histology sections of control and OVA-sensitized and challenged BALB/c. Control showed normal bronchiole structure and alveoli (Fig. **a**, **b**). OVA treated showed bronchiole filled with mucus exudates, few alveoli depicted emphysema and bronchiolar constriction. The airways of BALB/c mice contained significantly more PAS-stained mucus than H&E stained. *a* Bronchiole filled with mucus. *b* Alveoli with mucus. *c* Bronchiole. *d* Bronchiole thickening. *e* Alveoli depicted emphysema

Table 1 Different parameters (IgE, IL-4, IL-5, eosinophil and neutrophil percentage) in BALF of OVA sensitized and challenged mice to reflect the asthmatic symptoms in mice ($n = 5$)

S. no.	Antibodies	Saline (pg/mL)	OVA (pg/mL)
1.	IgE	251 ± 55.26	1,208 ± 254
2.	IL-4	14.34 ± 3.2	87.77 ± 6.9
3.	IL-5	84 ± 14.5	268 ± 16.34
4.	Eosinophil (%)	1.8 ± 0.86	5.5 ± 3.23
5.	Neutrophil (%)	2.0 ± 0.78	ND

Table 2 Percentage of Th1 and Th2 Cytokines IL-4, IL-5 (Th2) and IFN-γ (Th1) in normal (SAL/SAL) and asthmatic (OVA/OVA) mice ($n = 5$)

Cytokine	Sample	Mean ± SE
IL-4	SAL/SAL	4.384 ± 1.15076
	OVA/OVA	11.752 ± 0.89176
IL-5	SAL/SAL	4.306 ± 1.03847
	OVA/OVA	11.028 ± 1.29576
IFN-γ	SAL/SAL	9.706 ± 0.79762
	OVA/OVA	11.614 ± 1.37403

Flow cytometric profiling of cytokines

We analyzed Th1/Th2 profile in the PBMC of asthmatic and normal mice by flow cytometry. Based on cytokine profile presence of IFN-γ represents Th1 cells and IL-4 and IL-5 represents Th2 cells. In asthmatic mice there was an increased percentage of Th2 cells producing IL-4 and IL-5 compared to normal (Table 2). In contrast, Th1 cells producing IFN-γ decreases in asthmatic mice compared to normal mice. The Th1/Th2 profile of PBMC is shown in (Fig. 3a, b). The percentage of both Th2 cytokines (IL-4 and IL-5) were found to be significantly ($P < 0.05$) higher in the asthmatic (OVA/OVA) group than in the normal (SAL/SAL) mice but insignificant in case of IFN-γ. We further analyzed the ratio of Th1 and Th2 cells in terms of IFN-γ/IL-4 ratio and found significantly low in OVA/OVA in comparison to SAL/SAL mice are shown in Fig. 3c, d).

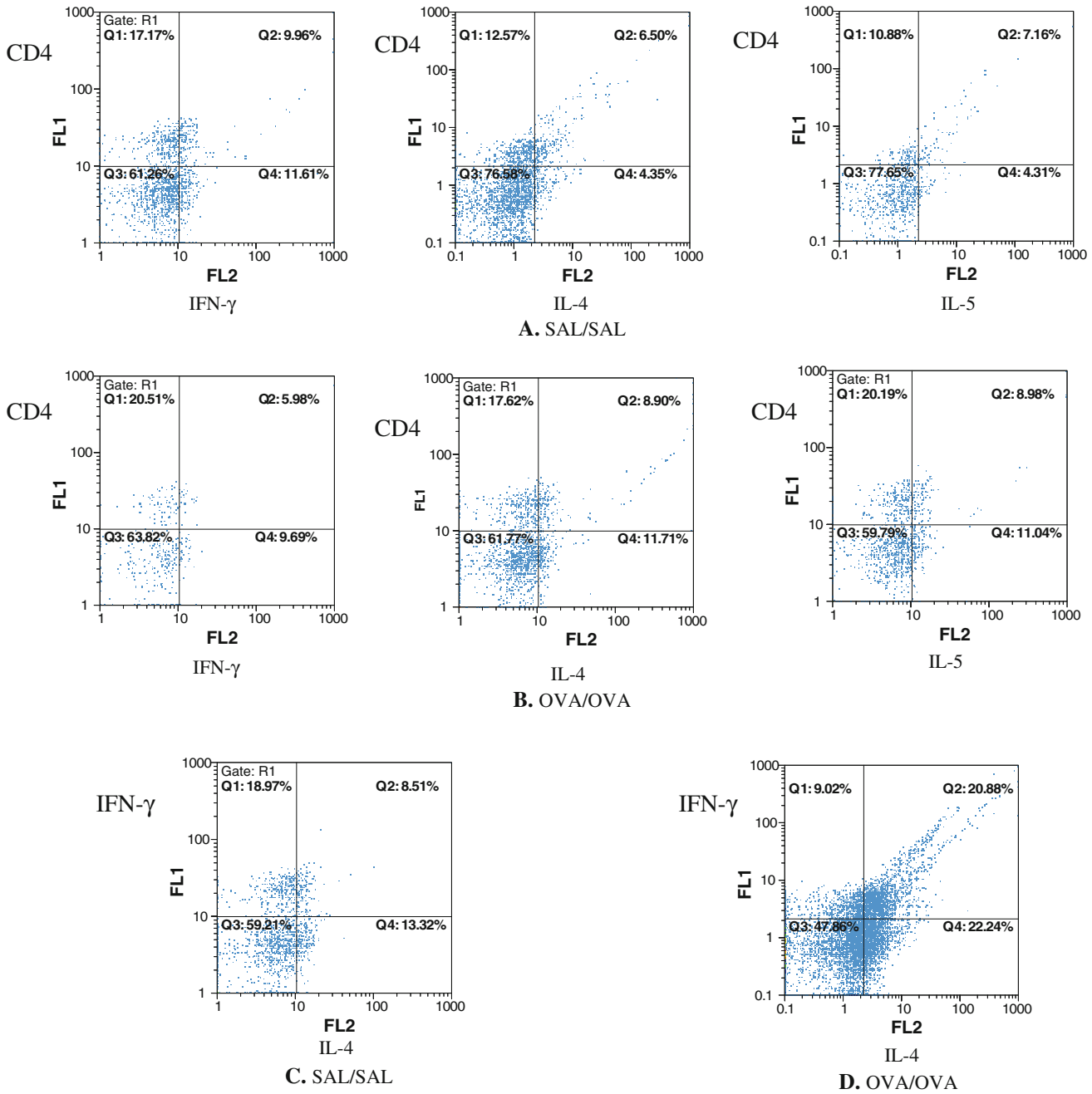

Fig. 3 Representative flow cytometric data are shown as *dot plot* Fig. **a** SAL/SAL and **b** OVA/OVA. Quadrant *left top* represents surface CD4 and *right bottom* represents either intracellular IFN-γ, IL-4 or IL-5 secreting cells. **c** SAL/SAL and **d** OVA/OVA. Quadrant *left top* represents intracellular IFN-γ and *right bottom* represents IL-4 secreting cells

mRNA expression of DNMT1, Socs3, STAT3, IFN-y, IL-4, IL-5 and IL-6

To understanding the role of DNMT1 in development of asthma/allergy we did the mRNA profiling of DNMT1, Socs3, STAT3 with cytokines IL-4, IL-5 and IL-6 in OVA/OVA and SAL/SAL mice. For this study we performed qRT^2-PCR, using lung, trachea tissues and BALF cells. The mRNA expression levels were normalized with respected normal (SAL/SAL) tissues and β-actin was used as an endogenous control to normalize expression data. The data were evaluated and validated according to Chen et al. [23]. We found the expression level of DNMT1, STAT3, IFN-y, and IL-6 were low and Socs3, IL-4 and IL-5 were high in all the tissues (Lung, trachea) and BALF cells of OVA/OVA mice in comparison to normal tissues showed the least (Fig. 4). Cytosine (CpG) methylation of the regulatory sequences of DNA is a dominant epigenetic

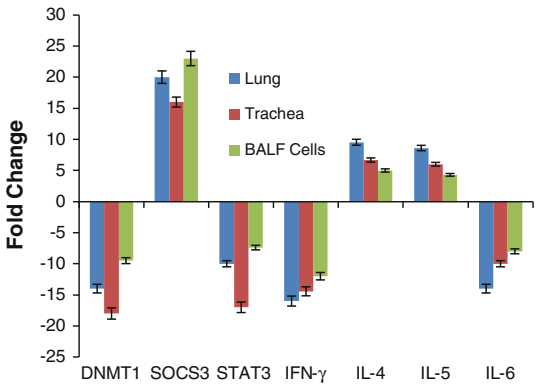

Fig. 4 mRNA profile in lung, trachea and BALF cells of asthmatic (OVA/OVA) mice. Relative quantification results are shown in relation to control (SAL/SAL) mice

mechanism that is associated with the transcriptional inactivation of genes but till date nobody reported the role of DNA methylation in regulation of DNMT1 gene expression in asthma/allergy disease. Therefore we studied the status of CpG methylation in the promoter region of DNMT1 gene and found the Low expression of DNMT1 gene in different airways tissues was due to methylation of DNMT1 promoter region.

CpG methylation status of DNMT1 promoter

The promoter region of DNMT1 gene (DDBJ accession no. AB056446) was used for the study of CpG methylation (Fig. 5). This sequence contained the start codon (ATG) and transcription factors binding (AP1, c-fos, E2F-Rb, Fli-1, Sp1 and Sp3) site towards the upstream [24]; along with CpG island. We found the similar transcription factor with transcription factor binding by using online software (Proscan: version 1.7) Table 4. The CpG island was analyzed by using the online software CpG island searcher (cpgislands.usc.edu/) which predicted three CpG island (Fig. 6). The third CpG island was analyzed by methylation specific PCR (MSP) and bisulphite sequencing, which have the transcription factor binding sites/sequences (Table 4; Fig. 5) whereas rest of the two CpG islands have no transcription binding site. Also the promoter region of DNMT1 relative to transcription start sites, chromosome coordinates, location of our selected sequence region (MDMN_1), GC %, DNMT1 gene, CpG island and the conservative region were represented by UCSC genome browser on Mouse July 2007 (NCBI37/mm9) assembly (Fig. 7). Our selected region contains 21CpG motif in both control and asthmatic mice (Table 3). We found that these motifs were not methylated in normal tissues, since only the unmethylated DNA template was amplified (Fig. 8). Prominent methylated amplicon was seen in all the asthmatic tissues (lung, trachea and BALF cells) following PCR amplification using methylated set of primers. This trend was not seen in airways tissues of normal mice. We further performed bisulphite sequencing to reconfirm the methylation status of the DNMT1 promoter and observed >85 % CpG methylation in the DNMT1 promoter of lung, trachea and BALF cells (Fig. 9a). Where as in normal mice methylation was not observed in lung (Fig. 9b). Similar results were observed in trachea and BALF cells (data not shown).

Discussion

In this study, we investigated the role of DNMT1 in development of allergic airway inflammation in a mouse

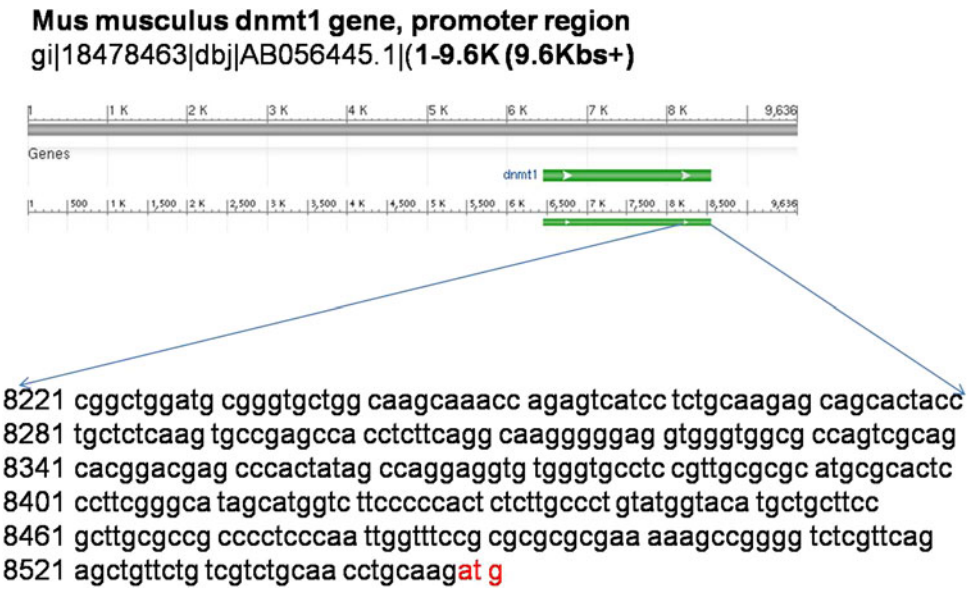

Fig. 5 Display of *Mus musculus* dnmt1 gene, promoter region (gi|18478463|dbj|AB056445.1|) sequence contain transcription factors binding site like Sp1, Sp3, AP1, AP2, c-fos, Fli-1 and E2F

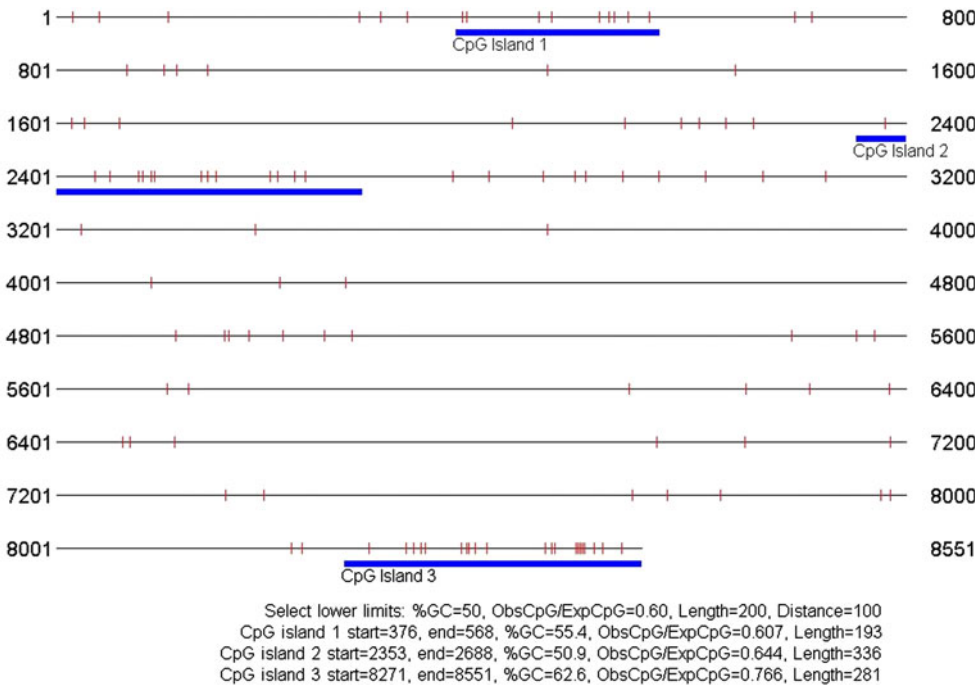

Fig. 6 Showed CpG island which predicted by online software CpG island searcher (cpgislands.usc.edu/)

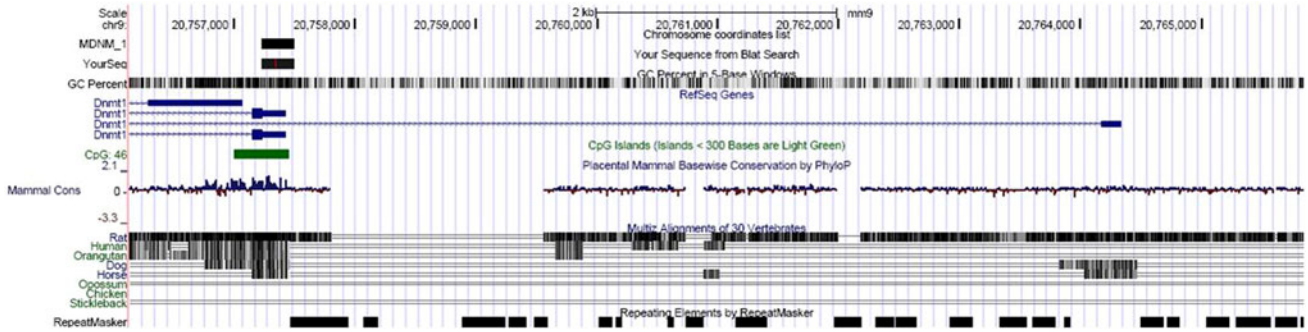

Fig. 7 Display of UCSC genome browser on Mouse July 2007 (NCBI37/mm9) assembly chromosome 9 (promoter of Dnmt1) is shown. The picture was generated by uploading the 'ucsc_upload.txt' file as a custom track at http://genome.ucsc.edu/cgi-bin/. From *top* to *bottom* the figure shows chromosome coordinates, location of our selected sequence (MDMN_1), GC %, DNMT1 gene, CpG island and the conservative region

model system. We have demonstrated that the first airway inhalation of allergen led to a marked neutrophil and macrophage recruitment in the lung of OVA-sensitized mice. This early neutrophil inflammation decreased during the progression and almost disappeared by the end of the protocol when the asthma-related Th2-type eosinophilic airway inflammation and hyper-reactivity developed, which was measured in term of sRaw (Fig. 1). These data suggest that the increased sRaw was due to the hyper responsiveness, has also been described in similar models of allergic airway inflammation [22]. The dominant physiological event in asthma is airway narrowing and a subsequent interference with airflow [1]. These result from bronchial smooth muscle contraction (bronchoconstriction), persistent and more progressive inflammation with airway edema, hyperemia of the airway mucosa and infiltration of mucosa with inflammatory cells, thickened airways wall (Fig. 2) with deposition of type III collagen and tenasein below the true basement membrane [25].

Local infiltrations of T helper 2(Th2) cells is response to allergen and cytokines (IL-4, IL-5, IL-9 and IL-13) by Th2 cells in the asthmatic airways is well defined [26]; and Th1 cells secreting IFN-y which cause airway inflammation [27]. The downregulation of IFN-y was due to the hypermethylation of CpG sites in the IFN-y promoter in the atopics and non-atopics disease has been reported [28]. But in the present study this was due to overexpression of Socs3 [22] and low expression of DNMT1. Interleukins (IL-4) and (IL-5) produced by infiltrated cells, have a central role in the induction of the IgE response and

Table 3 Average methylation for each CpG site start from the sequence 8,221 towards start sequence ATG (Chromosome 9: 20757229–20757500)

CpG site	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
CpG position	14	49	56	63	67	101	106	108	114	125	180	186	189	209	211	213	215	217	226	234	252
Methylation (%)	100.0	100.0	85.7	100.0	85.7	85.7	85.7	85.7	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

chronic inflammation including eosinophils [29]. These two genes are critical modulators of Th1/Th2 balance and play vital roles in asthma pathogenesis. But the development and differentiation of T helper cells are influenced by DNMT1 gene [21]. The downregulation of Stat3 was due to the feedback inhibition by induction of IL-6 mediated overexpression of Socs3 [22]. Stat3 expression is required for maintenance of DNMT1 expression and vice versa [30]. DNMT1 expression required to regulation of Socs3 by facilitating DNA methylation and, consequently, epigenetic gene silencing of Socs3.

Therefore the expression of DNMT1 is necessary for the regulation of IL-4, IL-5 and IFN-y. STAT3 and IL-6 both induces the transcription of DNMT1 gene, reverse function with STAT3 [30]; whereas IL-6 enhances the nuclear translocation of DNMT1 [31]. The promoters of many housekeeping genes have a number of common characteristics, such as the presence of multiple sites for initiation of transcription which, presumably, compensate for the absence of a TATA box and a CAAT box, and they often have an unusual high GC-content [32]. The DNMT1 gene is also a housekeeping gene but the 5′ end region of the DNMT1 gene in mouse is a TATA-less and GC-rich promoter [33]. The expression of DNMT1s requires a *cis*-element in the promoter region and is controlled by transcription factors like Sp1, Sp3, c-fos, Fli-1 and E2F residue in the promoter region, and these factors have been reported to control DNMT1 expression [24, 33–35]. However, specific *cis*- or *trans*-acting elements involved in the regulation of that promoter remain to be identified. A *cis*-element located between nucleotides -161 and -147 that appeared to be activated independently by Sp1 and Sp3 [24]. We have also found the similar result with our selected sequence when we analyzed the transcription factor binding sites with online software promoscan (Table 4). This selected sequences contains total 21 CpG motif in which 5 CpG present in E2F binding sites and other two motif present in other transcription factor bind sites, were found to be methylated in asthmatic mice. CpG methylation inhibits gene transcription by either blocking the ability of transcription factors to bind to the recognition sites on the CpG nucleotides or by facilitating the binding of transcription-inhibiting proteins [36]. Recently reported that hyper methylation in the promoter region of DNMT1 gene of human placenta is responsible for the down regulation of DNMT1 gene expression [37]. Moreover, the p300 coactivator appeared to be involved in the Sp3-mediated activation of the mouse Dnmt1 promoter in somatic cells [38]; its binding sites is also present in our selected sequence (Table 2 and Fig. 9). DNMT1 is considered to be a maintenance methyltransferase based on the in vitro enzyme assay in which it preferentially recognized hemimethylated DNA [15, 39] and on its localization at

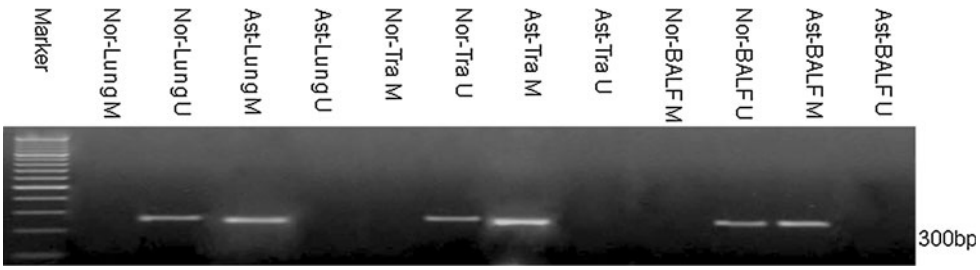

Fig. 8 Agarose gel electrophoresis of PCR products using methylation (M) and unmethylation (U) specific primers in lung, trachea and BALF cells respectively

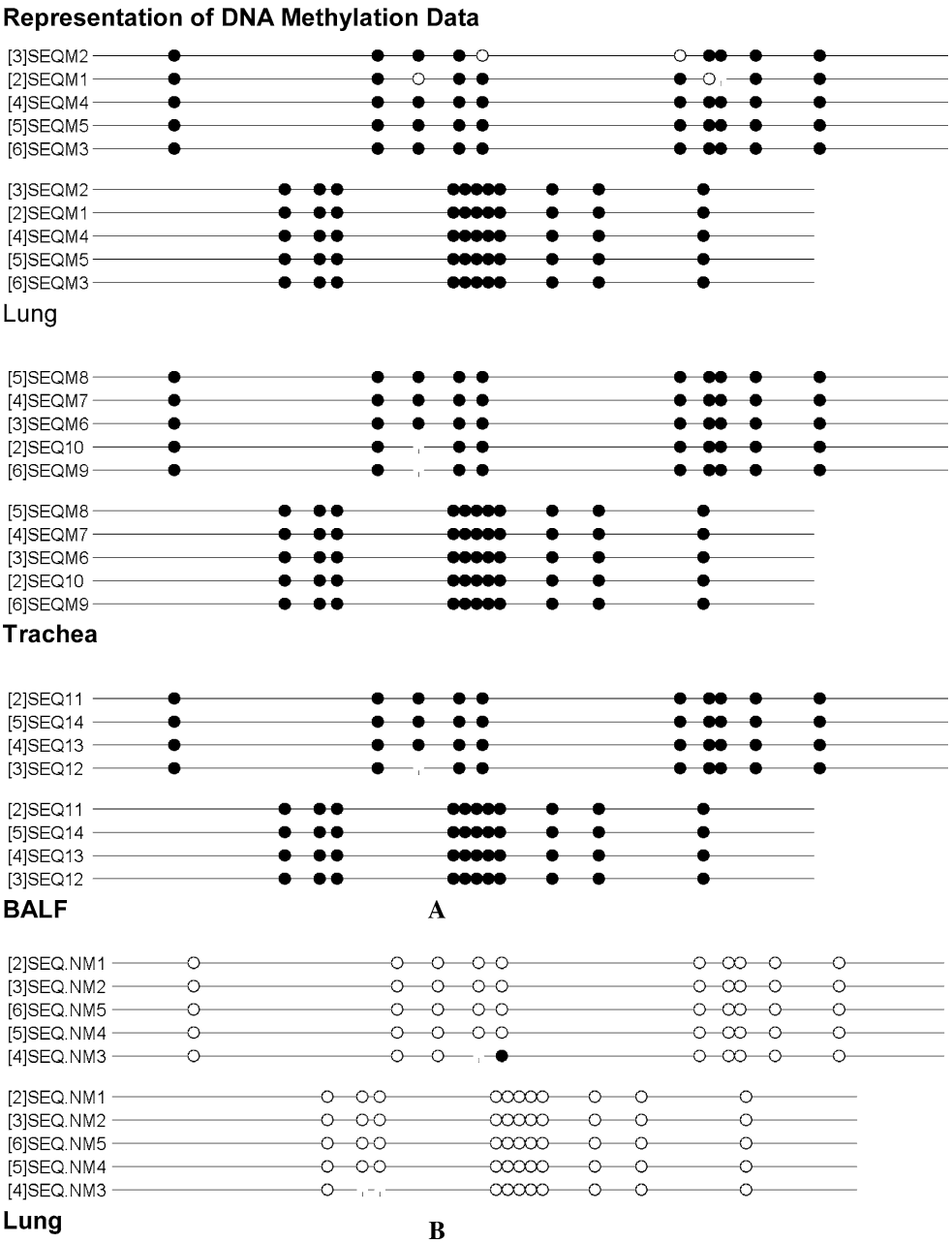

Fig. 9 Lollipop-style representation of DNA methylation data: this format is most often reported on DNA methylation data. It contains maximum information but it is sometimes not the most concise way to report DNA methylation data. *Filled* (*black*) *circles* correspond to methylated Cs, *unfilled* (*white*) *circles* correspond to unmethylated Cs, and small *vertical lines without a circle* correspond to non-CpG position where there is a CpG in the genomic sequence. **a** OVA/OVA and **b** SAL/SAL

replication foci in proliferating cells [16]. Inactivation of *DNMT1* gene in the mouse leads to global loss of methylation and biallelic expression or silencing of imprinted genes [40]. Mouse DNMT1 contains 4 transcription start sites, each sites are regulated by independent TATA less; promoter and enhancer element [33, 41]. A common trait of TATAA-less housekeeping gene promoter is that they are nested within a CG-rich area [42]. 1st promoters have

Table 4 Proscan: version 1.7, processed sequence: 9,636 base pairs, promoter region predicted on forward strand in 8,251–8,501, promoter score: 123.41 (promoter cutoff = 53.000000)

Significant signals				
Name	TFD #	Strand	Location	Weight
c-fos.5	S00675	–	8,331	1.912000
Sp1	S00802	+	8,468	3.292000
AP-2	S01936	+	8,471	1.108000
Sp1	S00978	–	8,473	3.361000
Sp1	S00333	–	8,474	3.442000
E2F	S01247	+	8,494	25.816999
EIIF	S00659	+	8,494	50.000000
E2F	S01244	+	8,494	50.000000
E2F	S00147	–	8,501	50.000000

rich CpG island and lies 5′ to the first exon, showed higher basal activity and can be replaced by the retinoblastoma protein. Other three promoters are downstream and located on 5′ to the 2nd, 3rd and 4th exon within poor CpG regions and have showed low basal activity and but can be induced by the transcription factor c-jun. In addition to these three enhancer elements were also found to be the either dependent or inducer on the ectopic expression of c-jun [33] and involved in the activation of all four promoter to enhance the transcription. In the murine system reported that DNMT1 is regulated by c-Jun [43, 44]. Here we found that the binding sites of c-Jun was heavily methylated therefore it was not bind with DNMT1 promoter region due to the inhibition of transcription factor binding sites and DNMT1 was not transcribed. It was demonstrated that the AP-1 element upstream to the 3rd exon of DNMT1 is heavily methylated in P-19 cells, but becomes demethylated upon five aza-cdR treatment. A CpG regions upstream the AP-1 element was shown to attract a different set of binding factors depending on its methylation status. It has therefore been proposed that demethylation of this CpG region inhibition of lack of DNMT1 leads the formation of different protein DNA complex; which in turn allow AP-1 binding, thus increasing DNMT1 expression [45]. The involvement of AP-1 in feedback regulation of DNMT1 promoter by methylation was also shown by another study in T cells, where it was found that treatment with DNA methylation inhibitor increases transcription regulated by a putative DNMT1 promoter and this process requires AP-1 sites DNMT1 [46, 47]. Th2 cytokines, loss of DNMT1 had more modest effects on IL-2 and IFN-y expression and did not alter the bias for greater expression of IL-2 by CD4 T cells and greater expression of IFN-y by CD8 T cells the silencing of cell autonomous Th2 cytokine expression in the CD8 T cell lineage is dependent on DNMT1 and DNA methylation.

Acknowledgments We thank Dr. K.C. Gupta, Director, Indian Institute of Toxicology Research for support and encouragement. We would like to express our gratitude to Dr. K.P. Singh, Dr. Dhirendra Singh, Dr. Mahadev Kumar, for encouragement and inspiration. I am obliged to technical staff of this institution who helped us to peruse this work. I thankful to Dr. S.L. Nagale Immunobiology division helped us lot. This work was financially supported by the Indian Council of Medical Research, New Delhi.

References

1. Meng JF, Rosenwasser LJ (2010) Unraveling the genetic basis of asthma and allergic diseases. Allergy Asthma Immunol Res 2(4):215–227
2. Le Souëf PN (2009) Gene-environmental interaction in the development of atopic asthma: new developments. Curr Opin Allergy Clin Immunol 9(2):123–127
3. Miller RL, Ho SM (2008) Environmental epigenetics and asthma: current concepts and call for studies. Am J Respir Crit Care Med 177(6):567–573
4. Bestor TH (2000) The DNA methyltransferases of mammals. Hum Mol Genet 9:2395–2402
5. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. Science 300(5618):489–492
6. Razin A (1998) CpG methylation, chromatin structure and gene silencing—a three-way connection. EMBO J 17(17):4905–4908
7. Sekigawa I, Kawasaki M, Ogasawara H, Kaneda K, Kaneko H, Takasaki Y, Ogawa H (2006) DNA methylation: its contribution to systemic lupus erythematosus. Clin Exp Med 6(3):99–106
8. Adcock IM, Ford P, Barnes PJ, Ito K (2006) Epigenetics and airways disease. Respir Res 7:21
9. Razin A, Cedar H (1993) DNA methylation and embryogenesis. EXS 64:343–357
10. Robertson KD, Uzvolgy E, Liang G, Talmadge C, Sumegi J, Gonzales FA, Jones PA (1999) The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res 27:2291–2298
11. Xie S, Wang Z, Okano M, Nogami M, Li Y, He WW, Okumura K, Li E (1999) Cloning, expression and chromosome locations of the human DNMT3 gene family. Gene 236:87–95
12. Hsieh CL (1999) In vivo activity of murine de novo methyltransferases, Dnmt3a and Dnmt3b. Mol Cell Biol 19:8211–8218
13. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99:247–257
14. Bestor T, Ingrain VM (1983) Two DNA methyltransferases from murine erythroleukemia cells: purification, sequence specificity, and mode of interaction with DNA. Proc Natl Acad Sci USA 80:5559–5563
15. Pradhan S, Bacolla A, Wells RD, Roberts RJ (1999) Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. J Biol Chem 274:33002–33010
16. Leonhardt H, Page AW, Weier HU, Bestor TH (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell 71:865–873
17. Mortusewicz O, Schermelleh L, Walter J, Cardoso MC, Leonhardt H (2005) Recruitment of DNA methyltransferase I to DNA repair sites. Proc Natl Acad Sci USA 102:8905–8909
18. Baylin SB, Ohm JE (2006) Epigenetic gene silencing in cancer-a mechanism for early oncogenic pathway addiction? Nat Rev Cancer 6(2):107–116

19. Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, Li E (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122:3195–3205
20. Jackson M, Krassowska A, Gilbert N, Chevassut T, Forrester L, Ansell J, Ramsahoye B (2004) Severe global DNA hypomethylation blocks differentiation and induces histone hyperacetylation in embryonic stem cells. Mol Cell Biol 24:8862–8871
21. Lee PP, Fitzpatrick DR, Beard C, Lehar S, Jessup, Makar KW, Perez-Melgosa M, Sweetser MT, Schlissel MS, Naguyen S, Cherry SR, Tsai JH, Tuckar SM, Weaver WM, Kelso A, Jaenisch R, Wilson CB (2001) A critical role for Dnmt1 and DNA methylation in T cell development, function and survival. Immunity 15:763–774
22. Paul B, Mishra V, Chaudhury B, Awasthi A, Das AB, Saxena A, Chauhan LK, Kumar P, Raisuddin S (2009) Status of Stat3 in an ovalbumin-induced mouse model of asthma: analysis of the role of Socs3 and IL-6. Int Arch Allergy Immunol 148:99–108
23. Chen YP, Higgins JA, Feldlaufer MF (2005) Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee(*Apis mellifera* L.). Appl Environ Microbiol 71:436–441
24. Kimura H, Nakamura T, Ogawa T, Tanaka S, Shiota K (2003) Transcription of mouse DNA methyltransferase1 (Dnmt1) is regulated by both E2FRb-HDAC-dependent and -independent pathways. Nucleic Acids Res 31:3101–3113
25. European respiratory review. 2007
26. Heijink IH, Van Oosterhout AJM (2006) Strategies for targeting T-cells in allergic diseases and asthma. Pharmacol Ther 112: 489–500
27. Koch M, Witzenrath M, Reuter C, Herma M, Schütte H, Suttorp N, Collins H, Kaufmann SH (2006) Role of local pulmonary IFN-gamma expression in murine allergic airway inflammation. Am J Respir Cell Mol Biol 35(2):211–219
28. White GP, Hollams EM, Yerkovich ST, Bosco A, Holt BJ, Bassami MR, Kusel M, Sly PD, Holt PG (2006) CpG methylation patterns in the IFNc promoter in naive T cells: variations during Th1 and Th2 differentiation and between atopics and non-atopics. Pediatr Allergy Immunol 17:557–564
29. Leung TF, Wong GWK, Ko FWS, Li CY, Yung E, Lam CWK et al (2005) Analysis of growth factors and inflammatory cytokines in exhaled breath condensate from asthmatic children. Int Arch Allergy Immunol 137(1):66
30. Zhang Q, Wang HY, Woetmann A, Raghunath PN, Odum N, Wasik MA (2006) STAT3 induces transcription of the DNA methyltransferase1 gene (DNMT1) in malignant T lymphocytes. Blood 108:1058–1064
31. Hodge DR, Cho E, Copeland TD, Guszczynski T, Yang E, Seth AK, Farrar WL (2007) IL-6 enhances the nuclear translocation of DNA cytosine-5- methyltransferase 1(DNMT1) via phosphorylation of the nuclear localization sequence by the AKT kinase. Cancer Genomics Proteomics 4(6):387–398
32. Latchman DS (2003) Eukaryotic transcription factors. Academic Press, London
33. Bigey P, Ramchandani S, Theberge J, Felipe D, Araujo Szyf M (2000) Transcriptional regulation of the human DNA Methyltransferase (dnmt1) gene. Gene 242:407–418
34. Claus R, Lubbert M (2003) Epigenetic targets in hematopoietic malignancies. Oncogene 22:6489–6496
35. Kantarjian HM, O'Brien S, Cortes J et al (2003) Results of decitabine (5-aza-2_deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. Cancer 98:522–528
36. Isidoro-Garc′ıa M, D′avila-Gonz′alez I, Pascual de Pedro M, Sanz-Lozano C, Lorente-Toledano F (2007) Interactions between genes and the environment. Epigenetics in allergy. Allergol Immunopathol 35:254–258
37. Novakovic B, Wong NC, Sibson M, Ng HK, Morley R, Manuelpillai U, Down T, Rakyan VK, Beck S, Hiendleder S, Roberts CT, Craig JM, Saffery R (2010) DNA methylation-mediated down-regulation of DNA methyltransferase-1 (DNMT1) is coincident with, but not essential for, global hypomethylation in human placenta. J Biol Chem 285(13):9583–9593
38. Kishikawa S, Murata T, Ugai H, Yamazaki T, Yokoyama KK (2003) Control elements of Dnmt1 gene are regulated in cell-cycle dependent manner. Nucleic Acids Res Suppl 3:307–308
39. Cho J, Kimura H, Minami T, Ohgane J, Hattori N, Tanaka S, Shiota K (2001) DNA methylation regulates placental lactogen I gene expression. Endocrinology 142:3389–3396
40. Caspary T, Cleary MA, Baker CC, Guan XJ, Tilghman SM (1998) Multiple mechanisms regulate imprinting of the mouse distal chromosome 7 gene cluster. Mol Cell Biol 18:3466–3674
41. Rouleau J, Tanigawa G, Szyf M (1992) The mouse DNA methyltransferase 5′ region. A unique housekeeping gene promoter. J Biol Chem 267:7368–7377
42. Bird AP (1986) CpG-rich islands and the function of DNA methylation. Nature 321:209–213
43. MacLeod AR, Szyf M (1995) Expression of an antisense to the DNA methyltransferase mRNA induces DNA demethylation and inhibits tumorigenesis. J Biol Chem 270:8037–8043
44. Rouleau J, MacLeod AR, Szyf M (1995) Regulation of the DNA methyltransferase by the Ras-AP-1 signaling pathway. J Biol Chem 270:1595–1601
45. Youngblood B, Reich NO (2008) The early expressed HIV-1 genes regulate DNMT1 expression. Epigenetics 3(3):149–156
46. Datta J, Kutay H, Nasser MW, Nuovo GJ, Wang B, Majumder S, Liu CG, Volinia S, Croce CM, Schmittgen TD, Ghoshal K, Jacob ST (2008) Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. Cancer Res 68(13):5049–5058
47. Qiang M, Denny A, Chen J, Ticku MK, Yan B, Henderson G (2010) The site specific demethylation in the 5′-regulatory area of NMDA receptor 2B subunit gene associated with CIE-induced up-regulation of transcription. PLoS ONE 5(1):e8798