Altered expressions of DNA methyltranferases in biomass using rural women in West Bengal

Banani Bindhani[1], Hirak Saha[2], Bidisha Mukherjee[2], Manas Ranjan Ray[2]

Abstract-- **Unprocessed solid biomass such as wood, coal, dung and agricultural residues are still being used in many rural households in India as main source of domestic energy for cooking, boiling and heating of water. Combustion of biomass releases a considerable amount of particulate matters (PM) and toxic pollutants. Therefore, use of biomass as fuels cause very high level of indoor air pollution (IAP) in rural households and the women who do most of the daily household cooking with these fuels, receive the maximum exposure. Thus, the cells of the nasopharynx, oral cavity, airways and the lungs in these women get severely affected and undergo harmful changes. The present study is conducted to appraise the effects of IAP generated PMs and/or carcinogens on epigenetic changes in airway epithelial cells as little information is known and available about it. In the present study LPG using rural women were used as control against biomass using rural women and both these groups comprise of non-smokers and non-chewer of tobacco and betel nut. Significantly enhanced production of ROS was evident in biomass fuel users with depletion of SOD, a major scavenger enzyme for oxidants in comparison to LPG using control women. Furthermore, Immunocytochemical evaluation showed significantly increased expressions of DNMT1 and DNMT3a enzymes and reduced expression of SET7, an inhibitor of DNMT1, in airway epithelial cells of biomass-using rural women in comparison to LPG using control women. The findings indicate major epigenetic changes in airway epithelial cells of biomass users due to long-term exposure to particulate pollution which also increases the risk of lung cancer in these women.**

Key words - Indoor Air Pollution (IAP), Particulate Pollution, Biomass fuel, DNA methyltransferases, SET7, Epigenetic Changes, Reactive Oxygen Species (ROS)

I. INTRODUCTION

Air pollution is one of the most important causes of various respiratory diseases including different types of cancer. It is generally perceived as an urban problem associated with toxic pollutants emitted from motor vehicles and industries (Zhang and Smith 2003). However, developing nations of Asia, Africa and Latin America have another major source of air pollution (Smith 2002). Around 50% of the world's population, particularly in developing countries, uses solid fuels, including biomass (wood, dung and agricultural residues) and coal, to meet their most basic energy needs: cooking, boiling water and heating (Bruce et al., 2000). According to the report of the World Health Organization, a significant portion of the population of rural areas in India still uses unprocessed solid biomass such as wood, dung and agricultural residues as their primary source of domestic energy (Bruce et al., 2000). Recent studies have shown that indoor biomass combustion causes very high level of indoor air pollution (IAP) by releasing a considerable amount of pollutants, including carbon monoxide (CO), nitric oxides (NO_x), sulphur dioxide (SO_2), formaldehyde (HCHO), volatile organic compounds (VOC), particulate matter (PM) and polycyclic aromatic hydrocarbons (PAHs) (Rehfuess 2006; Gennarro et al., 2015). Moreover, several factors such as occupant's behavior, microclimatic and ventilation condition and outdoor intrusion can also influence indoor pollution levels.
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IAP resulted from combustion of biomass in rural households often exceeds the level of ambient (outdoor) air pollution in the cities (Balachandran et al., 2000; Ramachandran et al., 2003).

Like cigarette smoke, biomass smoke also contains a number of harmful chemicals including oxides of nitrogen and sulphur, thousands of organic compounds such as volatile organic compounds (VOCs) like benzene, benzene 1, 3-butadiene, benzo(a)pyrene which itself is a well-established carcinogen, transition metals, coarse (diameter <10 µm, PM_{10}), fine (diameter < 2.5 µm , $PM_{2.5}$) and ultra fine particles (UFP) (diameter < 0.1 µm) (Zhang and Smith, 2003; Danielsen et al., 2009).

Poverty and easy availability are the main reasons behind dependence on biomass for cooking. Biomass fuels are not energy-efficient, and incomplete combustion of biomass emits a very high level of smoke that remains in the cooking area for a longer time because of poor kitchen ventilation in most poor households. In many cases, kitchens are altogether absent and women cook in a place adjacent to the living room (Gennaro et al., 2015). As a result, every member of the family is exposed to high levels of indoor air pollution, and women, who bear the burden of daily household cooking with these fuels, receive the maximum exposure. It is reasonable to assume therefore that cumulative exposures to biomass smoke may cause harmful changes especially in cells that are present at the direct route of exposure such as cells of the

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nasopharynx, oral cavity, airways and the lung (Zhang and Smith 2003; Rehfuess 2006).

Genomic DNA is often methylated at the fifth position of the cytosine base in CpG sequences (Ehrlich et al., 1982). DNA methylation is a common mechanism for gene silencing and plays a crucial role in development, genome stability, genomic imprinting, X-chromosome inactivation, and silencing of retrotransposons (Reik and Lewis 2005; Goll and Bestor 2005). DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group to the fifth position of cytosine bases in DNA. Mammalian genomes carry three distinct active Dnmt genes. Dnmt3a and Dnmt3b are highly expressed in embryonic tissues and undifferentiated ES cells and down regulated in differentiated cells. Two of the three genes, Dnmt3a and Dnmt3b, encode enzymes that show activity toward unmethylated DNA (Okano et al 1998; Aoki et al 2001) and are responsible for creating global DNA methylation patterns during embryogenesis and gametogenesis (Okano et al., 1999; Kaneda et al., 2004). Once the DNA methylation patterns are established, they are maintained by DNMT1 encoded by the Dnmt1 gene, which ensures the transmission of lineage-specific DNA methylation patterns during replication (Li et al., 1992). DNMT1 preferentially methylates the hemimethylated state of DNA that appears just after replication or repair & plays a crucial and major role in epigenetic changes in mammals (Takeshita et al., 2011).

DNA methylation is a potential mechanism linking indoor air pollution to adverse health effects. Recent studies have established associations between DNA methylation and PM exposure. ROS generated by transitional metals or other PM-contents can damage DNA. PM- containing chemical components may exert direct or indirect influences on DNA methylation patterns (Wang et al., 2012). Oxidative DNA damage can interfere with the ability of DNMT to interact with the DNA, thus resulting in a generalized altered methylation of cytosine residues at CpG sites (Wang et al., 2012). Exposure to ambient PM leads to a consistently reduced global DNA methylation in both human and experimental animal (Baccarelli et al., 2009; Tarantini et al., 2009).

Fetal and early-life environmental exposures have been associated with altered DNA methylation and play a critical role in progression of various diseases in adulthood (Tao et al., 2014). Furthermore, DNA methylation has emerged as a promising biomarker for environmental-related diseases, including lung cancer (Hou et al., 2014).

SET7 is a methyltransferase that methylates lysine residues in its target proteins. SET7 mediated methylation of mammalian DNMT1 at Lys142 leads to its proteasomal degradation. Therefore, the enzyme SET7 act as a negative regulator of DNA methyltransferase-1 (DNMT1) activity in mammalian cells by promoting degradation of DNMT1 and thus allows epigenetic changes to occur via DNA demethylation (Estève et al., 2011). Thus SET7 plays a critical role as a regulator of gene expression by controlling the stability of DNMT1.

Presence of high level of DNMT1 can hypermethylate the promoter regions of different genes thereby leading to transcriptional inactivation of these genes. For example, presence of high amount of particulate matters in air may lead to DNMT1 mediated hypermethylation of p16 promoter resulting its subsequent inactivation (Soberanes et al., 2012). Moreover, studies have shown that exposure to tobacco specific carcinogen NKK also leads to high level of DNMT1 expression in cells and causes hypermethylation of various cancer-critical genes in lung cancer patients (Lin et al., 2010). Several studies have documented altered expression of DNMTs following the exposure of test subjects to high level of particulate matters (Wang et al., 2012; Soberanes et al., 2012). Benzo(a)pyrene also regulates the expression level of DNMTs (Ye and Xu 2010). Smoke resulted from incomplete combustion of biomass contributes particulate matter and several carcinogens like benzo(a)pyrene in breathing air. However, very little information has been generated about the effects of IAP generated PMs and/or carcinogens on epigenetic changes through regulation of DNMTs in airway epithelial cells. In view of this, the current study has been undertaken to appraise the changes in the expressions of DNMT1, DNMT3a, DNMT3b and SET7 in airway epithelial cells of biomass users exposed to IAP in contrast to LPG users.

II. MATERIALS & METHODOLOGY

Participants

For the study, a total number of 128 premenopausal women aged between 27 and 42 years from rural areas of West Bengal were enrolled. Among the participants, 83 women (age 27–42 years, median 34 years) used to cook daily about 5 hours exclusively with wood, cow dung and agricultural wastes, such as bamboo, jute stick, paddy husk, hay and dried leaves for the past 5 years or more. They were considered as biomass users. The remaining 45 women were aged 27–41 years [median age 32 years] from same locality and cooked with cleaner fuel LPG and were considered as control.

Inclusion and Exclusion criteria

The inclusion criteria were apparently healthy premenopausal married women with husbands, actively engaged in daily household cooking for the past 5 years or more, non-smoker and non- chewer of tobacco and betel nut and having a body mass index > 15 and < 30 kg/m^2. Exclusion criteria were mixed fuel (biomass + LPG/kerosene) users, pregnant or breastfeeding, using oral contraceptive pills, had recent or past history of malignancy and currently under medication. Information about age, habits, education, family size and income, number of smokers in family, cooking time per day, years of cooking, fuel and oven type, and location of kitchen, was obtained during personal interview with female researchers of the study team. The Ethics Committee of Chittaranjan National Cancer Institute, Kolkata approved the study protocol.

Collection of background data

During personal interview, each participant was requested to furnish information about age, education, family size and income, habit, cooking time per day, years of cooking, fuel and oven type, location and ventilation of kitchen, health problems in past 3 months and last one year. As most of the participants were poorly educated, the researchers entered their responses in structured questionnaire forms on their behalf. The Ethics Committee of Chittaranjan National Cancer Institute, Kolkata approved the study protocol.

Measurement of air pollution

The concentrations of particulate matter with a diameter of less than 10 and 2.5 □m (PM_{10} and $PM_{2.5}$, respectively) were measured by real-time aerosol monitor (DustTrak™, model 8520, TSI Inc., MN, USA). The instrument contains 10-mm nylon Dor-Oliver cyclone, operates at a flow rate of 1.7 L/min and measures particle load in the concentration range of 1 mg-100 mg/m^3. The monitor was calibrated to the standard ISO 12103-1 A1 test dust. Monitoring was carried out for 3 consecutive days, 8 hours/day (07:00-15:00 hours). For biomass-using women who cook in a sitting position 2-3 ft away from the open chullah (oven), the monitor was placed in the breathing zone of the cook, 2.5 ft above floor level on a wooden stool, 3 ft away from the chullah. LPG users, on the other hand, cook in a standing position and the monitor was placed accordingly at a height of 4.5 ft.

Collection of Sputum sample

For the collection of sputum sample, the participants were instructed to cough vigorously after awakening in the morning and to collect the expectorated sputum in sterile plastic cups given to them. For better collection of cells from the deeper airways, early morning sputum samples of each participant were collected for three consecutive days (Erkilic et al., 2003). Highly viscous parts of the sputa were collected in 30 ml of PBS that contained 0.1% dithiotheritol. Collected samples were transported from the villages to the laboratory under dry ice.

Flow cytometric measurement of ROS (Reactive Oxygen Species) generation

Flow cytometric measurement of ROS Generation of reactive oxygen species (ROS) was measured in cells present in expectorated sputum by flow cytometry following the procedure of Rothe and Valet (1990) with some modifications. In brief, an aliquot of 200 µl of sputum cell suspension was diluted with 1 ml of HBSS containing 0.15 M NaCl and 5 mM HEPES, pH 7.35. Thereafter, 20 µl of 0.5 mM 2, 7-dichlorofluorescein diacetate (DCF-DA, Sigma Chem, USA) solution in dimethyl formamide was added to the cell suspension and incubated at 37^0C for 30 min in darkness. After that, 10,000 events were acquired immediately in flow cytometer (FACS Calibur with sorter, Becton Dickinson [BD], San Jose, CA, USA) using Cell Quest software (BD, USA). Respiratory burst and generation of ROS by cells present in sputum resulted in green fluorescence that was recorded in fluorescence channel and was expressed as mean fluorescence intensity (MFI) in arbitrary unit.

Spectrophotometric measurement of SOD (Superoxide dismutase)

The activity of the antioxidant enzyme superoxide dismutase (SOD) was assayed in whole sputum cell lysates spectrophotometrically following the procedure of Paoletti et al. (1986). Presence of SOD in sample was reflected by proportionate inhibition of the rate of NADH oxidation. This was calculated after measuring the absorbance of the samples and standard at 340 nm in a UV-spectrophotometer (Shimadzu, Japan) at 1 min intervals up to 5 min. The absorbance (OD) values were graphically plotted against time after mercaptoethanol addition (at 0, 1, 2, 3, 4, and 5 min). SOD activity in lysates was calculated from the standard curve and was expressed as U/ml.

Collection of sputum samples for Immunocytochemistry

The participants were asked to cough vigorously and the expectorated sputum was collected in sterile plastic cup for three consecutive days. The thick viscous parts of the sputa were smeared on clean glass slides. Ten slides were prepared from sputa of each participant. Slides containing airway cells were air dried and then fixed with cold methanol at the site of collection for immunocytochemistry.

Immunocytochemical detection of DNMT1, DNMT3a, DNMT3b and SET7 in airway epithelial cells

Expressions of DNMT1, DNMT3a, DNMT3b and SET7 proteins, actively involved in epigenetic changes were detected by immunocytochemistry (ICC) using the primary antibody by Abcam, UK following the established staining protocol (Ghosh et al, 2009).

Reagents used

For preparation of 1x phosphate buffered saline (PBS, pH 7.2), chemicals like NaCl (Merck, Mumbai, India), KCl (Merck, Mumbai, India), $Na_2HPO_4.7H_2O$ (Merck, Mumbai, India), KH_2PO_4 (Merck, Mumbai, India) were used. BSA solutions (Sigma–Aldrich Chemicals, Saint Louis, MO, USA) for making of 3% (w/v) BSA in 1X PBS 1% (w/v) BSA in 1X PBS were required for non-specific site blocking and for antibody dilution respectively. Primary antibodies, Rabbit polyclonal DNMT1 (dilution 1:50) (ab19905) and Mouse monoclonal SET7 (dilution 1:50) (ab14820) were purchased from Abcam, UK; and Rabbit polyclonal DNMT3a (dilution 1:50) (sc20703) and Rabbit polyclonal DNMT3b (dilution 1:50) (sc130740) were purchased from Santacruz, USA. Secondary antibodies, Goat anti-rabbit IgG, $F(ab')_2$- HRP (dilution 1:500) (sc3837) was purchased from Santa Cruz Biotechnology, Inc., USA) and Rabbit anti-mouse IgG, $F(ab')_2$- HRP (dilution 1:500) (ab6728) was purchased from Abcam, UK. Tris powder was purchased from SISCO Research Laboratories, India, for preparation of Tris-HCl

solution (pH 7.6). HRP substrate mixture was prepared by using DAB (Santa Cruz Biotechnology, Inc., USA), 50% H_2O_2 (Merck, Mumbai, India) and 1M Tris-HCl.

Procedure

The slides containing exfoliated airway epithelial cells (AEC) in spontaneously expectorated sputum, after fixation, were air dried, washed in PBS thrice and blocked in 3% bovine serum albumin (BSA) for 1 hour at room temperature. Thereafter, rabbit polyclonal DNMT1, DNMT3a and DNMT3b and mouse monoclonal SET7 primary antibodies (diluted both 1:50 in 1% BSA) were added separately to each slide. The slides were placed in a humid box at 4^{o}C and kept overnight in darkness. After washing with PBS, both anti-rabbit IgG, F(ab')$_2$- HRP and anti-mouse IgG , F(ab')$_2$- HRP secondary antibodies (diluted 1:500 in 1% BSA) was added to the respected slides and kept for 90 min. After washing with PBS the HRP substrate mixture was added to the slides and kept for 60 min in darkness. Then the slides were washed with distilled water and counterstained with hematoxylin, dehydrated in graded ethanol and mounted in distrene plasticizer xylene (DPX) and examined under light microscope (model BX50, Olympus, Japan). Slides were evaluated for the presence of DAB-stained golden brown nuclei. At least 300 AEC were scored from each slide and the results were expressed as percentage of positive (stained) cells.

Statistical evaluation of data

Results were expressed as mean ± standard deviation (SD). Data were processed and analyzed in EPI info 6.0 and SPSS 10.0 (IL, USA) statistical software. The collected data were statistically analyzed by Chi-square test and Student's t-test and $p<0.05$ was considered significant. Logistic regression analysis for odds ratios (OR) at 95% confidence interval (CI) and Spearman's rank correlation test was done to examine the relationship between respiratory symptoms, spirometric lung function measurements, cellular lung responses and air pollution exposure. Then, the cumulative impact of these factors on the expression of DNA methylation proteins was evaluated by stepwise multivariate logistic regression analysis. Statistical significance was assigned at $p < 0.05$.

III. RESULTS

Demographic characteristics

Demographic and socio-economic characteristics of rural women exposed to biomass smoke have been compared with LPG using control women from same locality, with respect to age, body mass index, cooking years, cooking hours per day, having separate kitchen, food habit, use of mosquito repellent, number of family members and environmental tobacco smoke for the presence of smokers in the family. However, the three groups differed significantly with respect to education ($p<0.0002$), family income ($p< 0.0001$) which were lower in biomass users, and lack of a separate kitchen ($p=0.0477$) which was more prevalent among biomass users. The findings are tabulated in Table 1.

TABLE 1
Demographic and socio-economic characteristics of biomass and LPG-using rural women

Variable	LPG-using control (n = 45)	Biomass user (n = 83)	P value
Age in years, median (range)	32.0 (27-41)	34.0 (27- 42)	=0.2173
Body mass index (kg/m^2), median (range)	23.1 (19.4-25.5)	22.7 (18.2-25.4)	=1.0000
Cooking years, median (range)	9 (5-21)	10 (5-27)	=0.1750
Cooking hours per day, median (range)	3.0 (2-5)	4.0 (3-6)	=0.4804
Cooking hours per day, (mean ± SD)	3.3 ± 1.1	3.8 ± 1.2	=0.4804
Years of schooling, median (range)	10(5–16)	2(0–8)	<0.0002**
Homes with separate kitchen (%)	84.5	66.3	=0.0477*
Smoker in family (%)	42.3	42.2	=0.1213
Use of mosquito repellent at home (%)	57.8	57.8	=1.000
Food habit, mixed (%)	100	100	
Members in family, median (range)	5 (3-6)	5 (4-6)	=0.0842
Family income per month in US $ (mean ± SD)	104 ±16	54 ± 12	< 0.0001***

n, number of subjects; NS, statistically not significant; Significantly different from control in *, Chi-square test; **,Mann-Whitney U test' ***, Student's t-test

Indoor air quality of the biomass using households

Concentrations of particulate pollutants in cooking areas during cooking and non-cooking time are presented in Table 2 and Figure 1. During cooking hours, the mean concentration of PM_{10} in biomass-using kitchen was 482±112 (SD) □g/m^3 in contrast to 134±39 □g/m^3 in LPG-using kitchen. Thus, 3.6 times more PM_{10} was recorded in indoor air of biomass-using kitchen when compared to that of LPG-using kitchen ($p<0.0035$). Even in non-cooking hours, PM_{10} level in indoor air of biomass-using kitchen was 1.8 times higher than that of LPG-using kitchen (134±42vs. 73±29, $p<0.0035$).

Like PM_{10}, the concentration of $PM_{2.5}$ in indoor air of biomass-using kitchen was significantly higher than LPG-using kitchen (Table 2, Figure 1). During cooking time, $PM_{2.5}$ level in indoor air of biomass-using kitchen was 272±69 μg/m^3 against 74±21 μg/m^3 in LPG-using kitchen, showing 3.7 times more fine particulate matter in kitchen where biomass is used as cooking fuel ($p<0.001$). In non-cooking time also, $PM_{2.5}$ level in indoor air of biomass-using kitchen was 2 times higher than that of LPG-using kitchen (78±39 μg/m^3 vs. 39±14 μg/m^3, $p<0.0217$).

TABLE 2
Comparison of particulate pollution in indoor air of cooking areas between LPG and biomass using Households

	LPG-using Households	Biomass-using Households
PM_{10} (µg/m^3)		
Cooking time	134±39	482±112*
Non-cooking time	73±29	134±42*
$PM_{2.5}$ (µg/m^3)		
Cooking time	74±21	272±69*
Non-cooking time	39±14	78±39*

Significantly different from control in *, Student's t-test

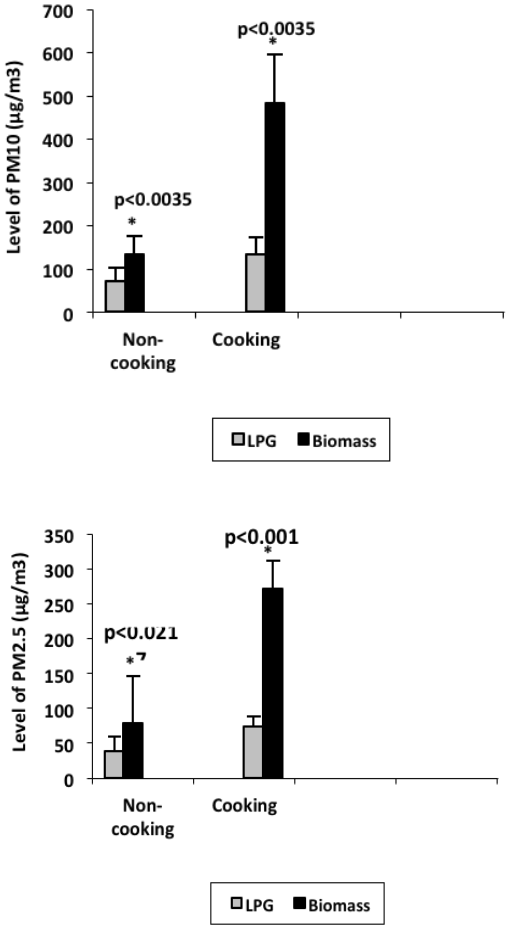


Fig. 1 The levels of particulate pollution in cooking areas of biomass- and LPG-using households during cooking and non-cooking hours

Generation of ROS in Airway Epithelial cells of Biomass Users

Flow cytometric analysis showed appreciable rise in ROS generation ($p<0.0001$) in airway epithelial cell exfoliated in sputum of biomass using women than LPG users. The MFI of DCFH-DA was increased by 36% in airway epithelial cells (62.5 ± 13.2 vs. 31.7 ± 8.5 in control, $p<0.0001$, Figure 2).

Depletion of SOD level in Airway Epithelial cells of Biomass Users

Since, air pollutants elicits oxidative stress that could be harmful for the body, antioxidant defense state was evaluated by measuring the activity of antioxidant enzyme superoxide dismutase (SOD). Significant decline ($p<0.0011$) in antioxidant enzyme levels ($p<0.0011$) was recorded in indoor air pollution-exposed subjects (859.8 ± 45.9 in LPG user vs. 706.5 ± 96.7 U/ml in biomass smoke-exposed), implying greater oxidative stress that may lead to cellular damage (Figure 3).

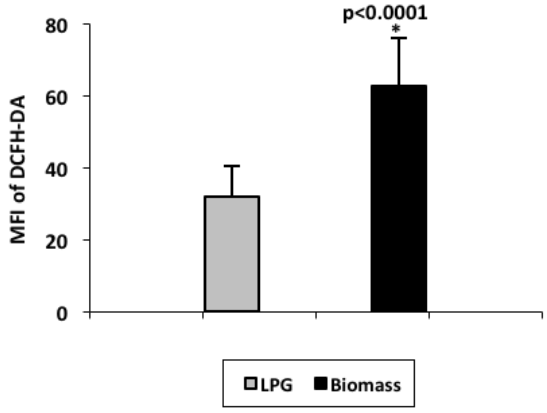


Fig. 2 Significant generation of ROS in Airway epithelial cells of Biomass user women

Association between Particulate Matter Exposure and Generation of ROS

Particulate matter exposure during cooking with Biomass fuel was significantly correlated with generation of Reactive Oxygen Species in Airway Epithelial cells. The value of Pearson Correlation was 0.851, $p<0.0001$ was highly significant. Kendall's tau-b value was 0.782, $p=0.001$ was significant and Spearman ρ value was 0.94, $p<0.0001$ was highly significant.

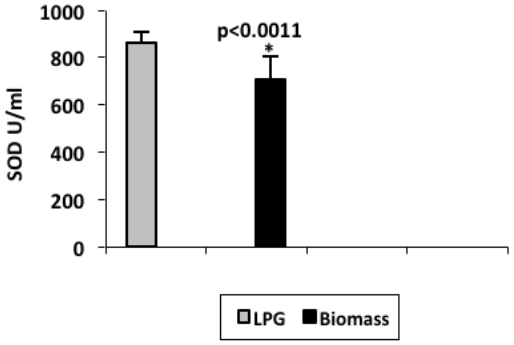


Fig. 3 Significant decline antioxidant enzyme levels in women chronically exposed to indoor air pollution compared with age and sex-matched controls.

Concentration of trans, trans–muconic acid (t, t-MA) in urine

The concentration of t, t-MA in urine, a biomarker of benzene exposure, was 8.24 ± 6.74mg/l in biomass-using women in contrast to 1.19 ± 0.8 mg/l in control subjects (Figure 4, p<0.0001 in Student's t-test). The range of urinary t, t-MA was 0.3-2.9 mg/l with a median of 0.9 mg/l in control women whereas biomass using women had a range of 2.4-20.5 mg/l with a median of 6.8 mg/l (p<0.0001 in Mann-Whitney U-test).

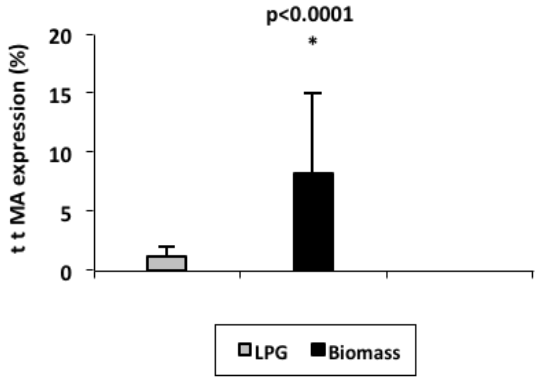


Fig. 4 Histogram showing increase level of t,t-MA in exposed group

Expression of DNMT1 and SET7 in airway epithelial cells

In ICC, the expressions of DNMT1 and SET7 proteins were found mainly in the nuclei of airway epithelial cells, especially in the basal and parabasal cells (Plate 1 & 2, Table 3, Figure 5). The percentages of airway epithelial cells expressing DNMT1 and SET7 were significantly higher and lower respectively in women who were chronically exposed to biomass smoke (Table 3). For instance, as the frequencies of DNMT1 expressing epithelial cells exfoliated in sputam varied considerably between biomass users and control women, we converted the relative (%) values to absolute ones (cells/hpf). Still, expression of DNMT1 (67.6 ± 7.3 vs. 28.6 ± 4.2 cells/hpf in control, p<0.0001) was significantly higher in biomass users than LPG users. However, SET7 expressing cells (15.7 ± 6.3 vs. 28.8 ± 9.2cells/hpf in control, p<0.0001) in biomass-using women were significantly lower than the control.

Expressions of DNMT1 and SET7 in Airway epithelial cells of Biomass users

Expressions of DNMT1 and SET7 in Airway epithelial cells of biomass user women show negative correlation. DNMT1 expression was significantly higher whereas SET7 expression was diminished in the nuclei of Airway epithelial cells of biomass users. The value of Pearson Correlation was -0.581, p=0.04 was significant. But Kendall's tau-b value was -0.492, p=0.055 was not significant and Spearman ρ value was -0.54, p=0.062 was not significant.

TABLE 3
Immunocytochemical expression of DNMT1 and SET7 in airway epithelial cells (AEC) of biomass- and LPG-using women (mean±SD)

Parameters	LPG using control (n = 45)	Biomass users (n = 83)	P value
Percentage of DNMT1-expressing cells			
Mean ± SD	28.6 ± 4.2	67.6 ± 7.3	<0.0001*
Median (range)	30 (24-32)	66.5 (52-83)	<0.0001**
Percentage of SET7-expressing cells			
Mean ± SD	28.8 ± 9.2	15.7 ± 6.3	<0.0001*
Median (range)	26 (19-43)	15.5 (8-27)	<0.0001**

Significantly different from control in **, Mann-Whitney U test and *, Student's t-test

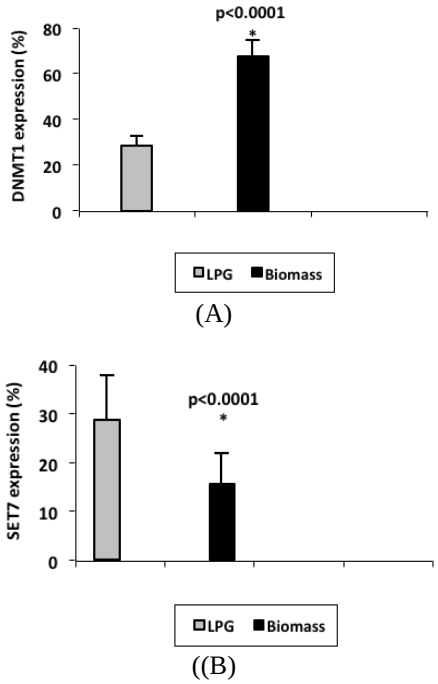


Fig. 5 Immunocytochemical expression of DNMT1 (A) and SET7 (B) in Airway Epithelial cells of LPG and Biomass - users rural women

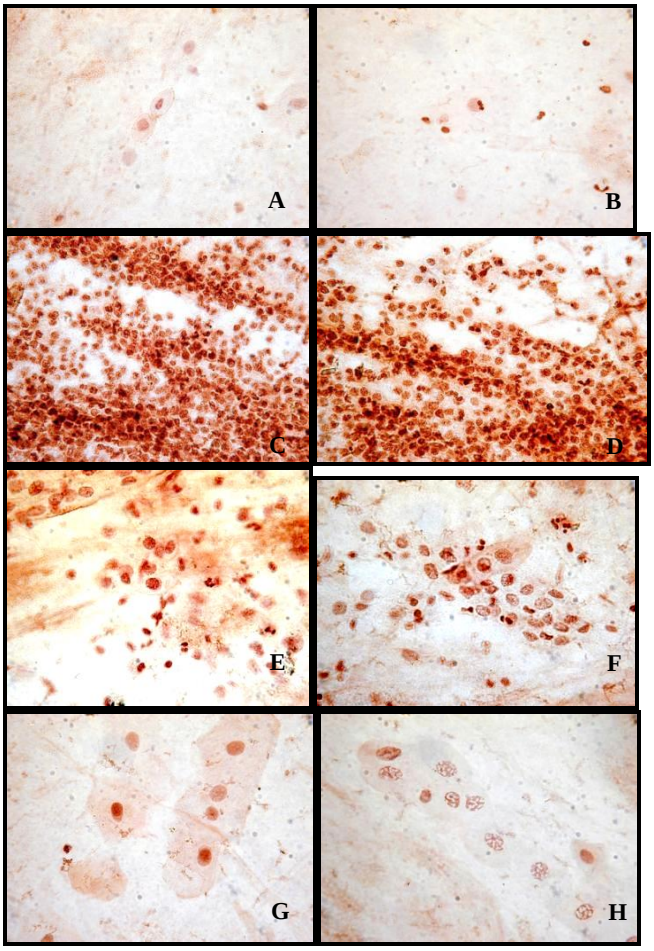


Plate 1 Immunocytochemical expression of DNMT1 – Airway Epithelial cells of LPG users showing very low expression (A,B); Airway Epithelial cells of Biomass users showing very high expression (C,D); Strong expression in nucleus of epithelial cell of Biomass users (E,F,G,H). The cell nuclei were counterstained with hematoxylin, original magnification, 1000x.

Generation of ROS and Expression of DNMT1

Generation of ROS due to chronic exposure of particulate matter in indoor air pollution was highly correlated with DNMT1 expression in Airway epithelial cells of Biomass user women. The value of Pearson Correlation was 0.871, $p<0.0001$ was highly significant. Also Kendall's tau-b value was 0.776, $p=0.001$ was significant and Spearman ρ value was 0.90, $p<0.0001$ was highly significant.

Depletion of SOD and Expression of DNMT1

Depletion of SOD was negatively correlated with high level of DNMT1 expression in airway epithelial cells of Biomass user women. The value of Pearson Correlation was -0.591, $p=0.043$ was significant. Also Kendall's tau-b value was -0.492, $p=0.039$ was significant but Spearman ρ value was -0.563, $p=0.057$ was not significant.

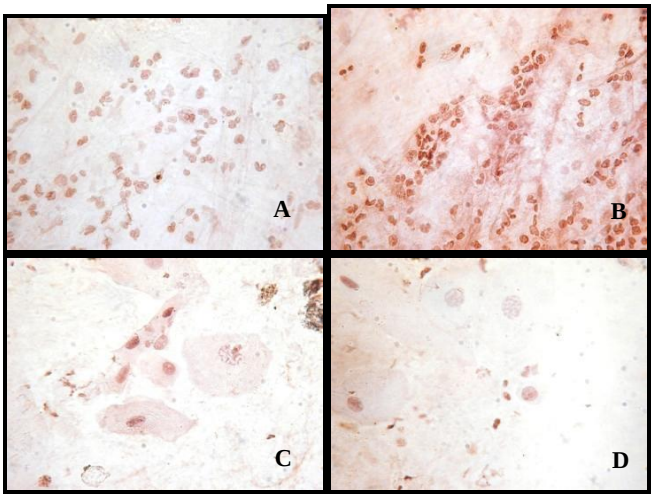


Plate 2 Immunocytochemical expression of SET7 – Airway Epithelial cells of LPG users showing very high expression (A,B); Strong expression in nucleus of epithelial cell of LPG users (C); Airway Epithelial cells of Biomass users showing low expression (D). The cell nuclei were counterstained with hematoxylin, original magnification 1000x.

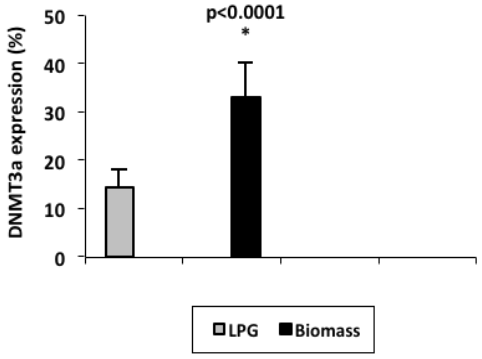
Expression of DNMT3a and DNMT3b in airway epithelial cells

In ICC, the expressions of DNMT3a and DNMT3b proteins were found mainly in the nuclei of airway epithelial cells, especially the basal and parabasal cells (Plate 3, 4; Table 4, Figure 6). The percentages of airway epithelial cells expressing DNMT3a and DNMT3b were significantly higher and lower respectively in women who were chronically exposed to biomass smoke (Table 4). For instance, as the frequencies of DNMT3a expressing epithelial cells exfoliated in sputam varied considerably between biomass users and control women, we converted the relative (%) values to absolute ones (cells/hpf). Still, expression of DNMT3a (32.9 ± 7.3 vs. 14.3 ± 3.7 cells/hpf in control, $p<0.0001$) was significantly higher in biomass users than LPG users. However, DNMT3b expressing cells (11.8 ± 2.9 vs. 11.2 ± 2.2 cells/hpf in control, $p=0.2282$) in biomass-using women were not significantly higher than the control.

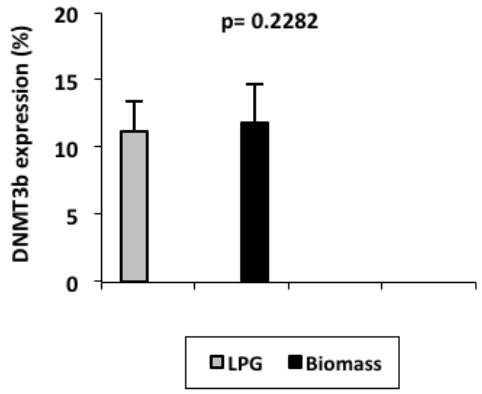
TABLE 4
Immunocytochemical expression of DNMT3a and DNMT3b in airway epithelial cells (AEC) of biomass- and LPG-using women (mean ± SD)

Parameters	LPG using control (n = 45)	Biomass users (n = 83)	P value
Percentage of DNMT3a-expressing cells			
Mean ± SD	14.3 ± 3.7	32.9 ± 7.3	<0.0001*
Median (range)	14.1 (11-34)	32.7 (31-64)	<0.0001**
Percentage of DNMT3b expressing cells			
Mean ± SD	11.2 ± 2.2	11.8 ± 2.9	0.2282
Median (range)	11 (4-18)	11.3 (6-19)	0.2282

Significantly different from control in **, Mann-Whitney U test and *, Student's t-test



(A)



(B)

Fig. 6 Immunocytochemical expression of DNMT3a (A) and DNMT3b (B) in Airway Epithelial cells of LPG and Biomass - users rural women

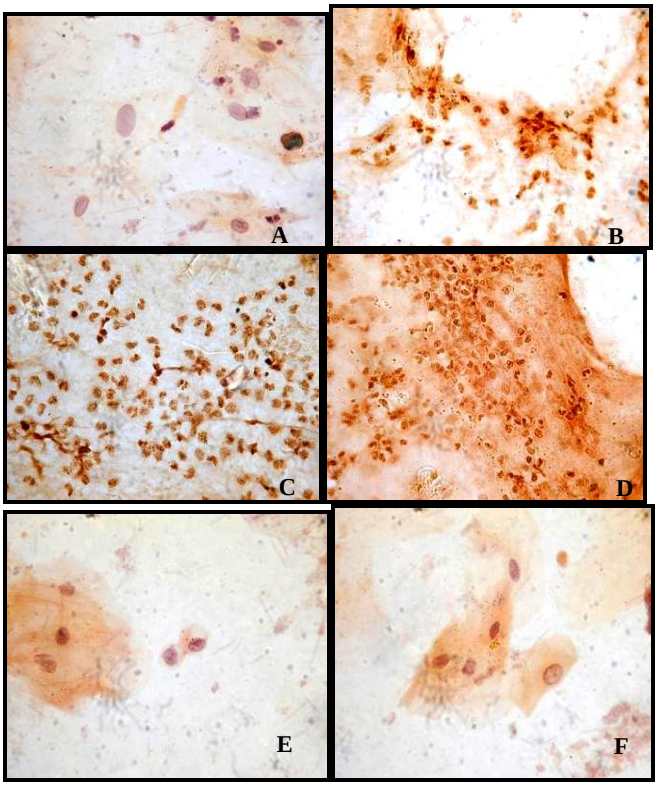


Plate 3 Immunocytochemical expression of DNMT3a – Airway Epithelial cells of LPG users showing very low expression (A); Airway Epithelial cells of Biomass users showing very high expression (B,C,D); Strong expression in nucleus of epithelial cell of Biomass users (E,F). The cell nuclei were counterstained with hematoxylin, original magnification, 1000x.

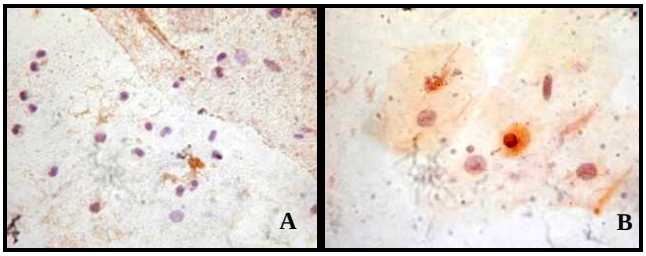


Plate 4 Immunocytochemical expression of DNMT3b – Airway Epithelial cells of LPG users showing low expression (A); Airway Epithelial cells of Biomass users showing nuclear expression (B). The cell nuclei were counterstained with hematoxylin, original magnification, 1000x.

Generation of ROS and Expression of DNMT3a

Generation of ROS due to chronic exposure of particulate matter in indoor air pollution, was well correlated with DNMT3a expression in Airway epithelial cells of Biomass user women. The value of Pearson Correlation was 0.714, $p<0.01$ was highly significant, Kendall's tau-b value was 0.654, $p<0.05$ was significant and Spearman ρ value was 0.651, $p<0.05$ was highly significant.

Depletion of SOD and Expression of DNMT3a

Depletion of SOD was negatively correlated with DNMT3a expression in airway epithelial cells of Biomass user women. The value of Pearson Correlation was -0.782, $p<0.01$ was significant, Kendall's tau-b value was -0.659, $p<0.05$ was significant and Spearman ρ value was -0.664, $p>0.05$ was not significant.

Association between ROS, SOD, t, t-MA and methylation protein expression

In univariate analysis, the reduction in the percentages of DNMT1 and DNMT3a-positive cells was positively associated with lower education and family income, increasing age and exposure years to biomass smoke, tobacco smoking habit of the husband, and cooking in a space adjacent to living room due to lack of separate kitchen. Even after controlling the influence of the confounders in multivariate logistic regression analysis, significant association was found between higher expression of DNMT1 and DNMT3a proteins and ROS generation, urinary t, t-MA and depletion of SOD (Table 5).

TABLE 5
Association between oxidative stress, expression of SOD, benzene exposure and lower expression of methylation proteins in airway epithelial cells of biomass-using women

Parameters	Higher expression of DNMT1 in cells	Higher expression of DNMT3a in cells
ROS generation	2.29 (1.46-4.59)	3.24 (1.83-5.61)
Depletion of SOD	2.04 (1.03-3.92)	1.74 (1.09-3.47)
t, t-MA in urine	1.71 (1.04-3.06)	1.75 (1.02-2.94)

Results are expressed as odds ratio with 95% confidence interval in parentheses after controlling education, family income, age, exposure years, husband's smoking habit and adjacent kitchen as potential confounders in multivariate logistic regression analysis.

IV. Discussion

Many families living in rural areas in India still use unprocessed solid biomass such as wood, coal, dung and agricultural residues as their main source of domestic energy for cooking, boiling water and heating. Biomass combustion releases a considerable amount of toxic pollutants, including carbon monoxide (CO), nitric oxides (NO_x), sulphur dioxide (SO_2), formaldehyde (HCHO), thousands of volatile organic compounds (VOC), particulate matters (PM) and polycyclic aromatic hydrocarbons (PAHs). Therefore, use of biomass as fuels causes very high level of indoor air pollution (IAP) in rural households. In these families usually it is the women who do most of the daily household cooking with these fuels and therefore receive the maximum exposure. Therefore, the cells that are present at the direct route of exposure of these harmful chemicals, such as cells of the nasopharynx, oral cavity, airways and the lungs in these women get severely affected and undergo harmful changes.

Little information is known about the effects of IAP generated PMs and/or carcinogens on epigenetic changes in airway epithelial cells. DNA methylation is one of the major epigenetic mechanisms exist in cells. DNA methyltransferases methylate cytosine residues in CpG sequences in promoter regions of different genes and thereby inhibit their expressions. Therefore, expressions of different genes can be controlled by regulating the stability and expressions of DNA methyltransferases. In view of this, the present study has been undertaken to evaluate the changes in the expression profiles of DNA methyltransferases [DNMT1, DNMT3a and DNMT3b] and SET7 in airway epithelial cells of biomass users exposed to IAP in contrast to LPG users.

The results show significantly high levels of DNMT1 and DNMT3a enzymes in airway epithelial cells of biomass-using rural women in comparison to LPG using women. Furthermore, the expression of another enzyme, SET7, an inhibitor of DNMT1, decreases significantly in airway epithelial cells of biomass-using rural women whereas its expression increases in LPG using control women. DNMT1 is involved in DNA methylation process and its elevated level in cells cause hypermethylation of DNA in airway cells. SET7 plays a key role in regulating the stability and function of DNMT1 by methylating Lys142 of DNMT1 and thereby leading to proteasomal degradation of DNMT1 (Estève et al 2009). Hence, reduced expression of SET7 enzyme in airway epithelial cells of biomass-using rural women corroborates well with the high expression of DNMT1 enzyme found in these cells of biomass users.

High levels of DNMT1 and DNMT3a enzymes in cells may lead to hypermethylation of the promoter regions of different genes resulting in transcriptional inactivation of these genes and thus leading to drastic changes in gene expression profiles of the cells.

Participants in this study, both LPG and biomass users were non-smokers and non-chewer of tobacco and betel nut. Therefore, increased expression of DNMT1 and reduced expression of SET7 in airway epithelial cells of biomass-using rural women cannot be explained by smoking or tobacco chewing habit. All participants are from same rural areas of West Bengal and in those rural areas effects of pollutants emitted from motor vehicles and industries are negligible due to absence of highway and industries within 5 km range of the study area. Here the LPG using control women as well as the biomass users are exposed in same ambient air, so ambient air pollution is not responsible for the changes in expressions of DNMT1, DNMT3a and SET7 enzymes. Both LPG and biomass users were apparently healthy and premenopausal women with very little variation and their lifestyle, food habit were more or less same. Therefore, it can be assumed that significant changes observed in the expressions of DNMT1, DNMT3a and SET7 enzymes in biomass users in contrast to LPG using women are not due to variations in physiological conditions.

The major difference between the control and the test groups was exposure to particulate matters generated from combustion of biomass fuels. One group of participants i.e. the biomass users were very much exposed to PM_{10} and $PM_{2.5}$ due to incomplete combustion of biomass fuels they used during cooking and these particulate matters, especially ultrafine particles which are abundant in biomass smoke, can produce ROS directly because of the presence of free radicals and oxidants adsorbed on their surface (Fubini et al., 2004). Thus, in the present investigation excess amount of ROS production in airway epithelial cells of biomass-using rural women can be explained as a result of higher exposure to particulate pollution due to incomplete combustion of biomass fuels they are using during household activities. Furthermore, reduced expression of antioxidant enzyme superoxide dismutase was recorded in indoor air pollution-exposed subjects indicating even greater oxidative stress that may lead to severe cellular damage. Finally, in multivariate logistic regression analysis, significant association was found between higher expression of DNMT1 and DNMT3a proteins and ROS generation, urinary t, t-MA [a biomarker of benzene exposure] and depletion of SOD in biomass using rural women.

In essence, our study has shown that exposure to particulate matter released into the breathing air during combustion of biomass fuel is associated with increase level of DNMT1 that can cause hypermethylation in sputum cells, and the action is probably mediated by oxidative stress. Participated women, same as rural women, became involved in cooking in their late teens when they started helping their mothers in the kitchen and after marriage they took the major role in cooking. As a result, they were more and more exposed in particulate matter during cooking with biomass fuel and the probability of epigenetic changes may increase. The consequences can be disastrous, if hypermethylation by DNMT1 occur in promoter region of necessary genes like DNA repair genes or may be in tumor suppressor gene then it should be lethal to health and increase the chances of cancer (White et al., 2015; Jacinto et al 2007; Lin et al 2010; Zhou et al 2012).

Recent *in vitro* and *in vivo* studies have demonstrated that exposure to PM significantly increased ROS production with enhanced expression of the DNA methyltransferase 1 (DNMT1) leading to hypermethylation of the p16 promoter. Furthermore, increased transcription of DNMT1 and methylation of the p16 promoter were inhibited by a mitochondrially targeted antioxidant and a JNK inhibitor (Soberanes et al., 2012). These previous results corroborate well with the current findings and provide a potential mechanism by which PM exposure increases the risk of lung cancer.

The present study may provide an epidemiological link to high incidence of lung cancer recorded among never-smokers and biomass user women in eastern India. In agreement with current study, a high incidence of lung cancer has been recorded among women in eastern India (where the present work was carried out) who were overwhelmingly never-smokers and biomass users (Nandakumar et al., 2004).

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