Identification of methylated genes in BALB/c mice liver using monoclonal antibody combined with the high-throughput cDNA microarray approach

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We have employed a microarray-based genome-wide methylation detection method to analyse methylation in internal coding regions of genes in a sequence-independent manner in liver tissue of BALB/c mouse. Using mouse 7.4k cDNA microarray, methylation status of genes spotted on the array was analysed. We detected methylation in coding and its proximal regions of 1415 genes, 70% of which contained CpG islands. Gene ontology analyses of these 1415 genes revealed that they were mainly involved in nucleic acid metabolism and signal transduction processes. Molecular function analysis revealed that these genes were mainly transcription factors, DNA repair proteins, chromatin remodelling enzymes and receptor molecules. The set of methylated genes also contained 11 imprinted genes and 9 genes known to be hypermethylated in liver cancers. Our analysis has led to the identification of methylation in many new regions. The analysis thus provides a methylation landscape in normal mouse liver. The platform can be used to analyse epigenetic alterations during oncogenesis, ageing, in response to environmental stimuli, etc.

Keywords: DNA methylation, epigenetics, imprinting, microarray.

DNA methylation is an important epigenetic change in eukaryotes and is known to play a significant role in a variety of processes like development, gene silencing and chromatin remodelling[1–3]. In mammals, it is mainly found at cytosine residues, especially in the CpG dinucleotides which are unevenly distributed in the mammalian genome[4–6]. DNA methylation levels vary in a cell, tissue and gene-specific manner and can be modulated by the environment[7,8]. Changes in methylation levels need to be monitored in several situations such as ageing, oncogenesis, development and diseases[8–10]. Several environmental factors and signals appear to influence the phenotype through alterations in the epigenetic footprints in the genome.

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Techniques like high-throughput microarrays and pyrosequencing have provided valuable insights into the distribution and role of DNA methylation in eukaryotes[11,12]. These studies have revealed new roles of DNA methylation in evolution, cellular differentiation and have identified potential links to tumourigenesis[13,14]. It is well established that methylation of CpG islands present within a promoter region of genes is involved in regulating gene expression[15]. Aberrant methylation occurring at the CpG-rich regulatory regions can result in various diseases like cancer[16]. However, there is growing evidence that in normal tissues, along with promoter methylation, genes are methylated at intragenic as well as intergenic regions[17–19]. This is also shown to be part of tissue-specific methylation and plays a role in the regulation of tissue-specific genes and transcription factors[18]. Although it is known that the repetitive and non-coding DNA is often extensively methylated, it is often important to examine DNA methylation in the genes.

We have used a microarray-based methylation detection method reported earlier by Kelkar and Deobagkar[20] for detecting and analysing specific methylation present in the internal regions of the genes along with promoter methylation. Using this method we have identified and analysed methylation in and near the coding regions of the genes in BALB/c mouse liver. The study provides the methylation landscape in BALB/c mouse liver and leads to the identification of several methylated genes. Since cDNA microarray can be used to analyse gene expression, methylation analysis can be directly correlated with gene expression.

Four-week-old female BALB/c mice were used for the study. The animals were bred and maintained at the animal house facility of the Bhabha Atomic Research Centre (BARC), Mumbai. The guidelines issued by the institutional animal ethics committee of BARC, were meticulously followed.

Total genomic DNA from liver tissue of BALB/c mice was isolated using phenol : chloroform method[21]. Briefly, the mouse liver (150–200 mg) tissue was crushed into a fine powder in liquid nitrogen. The powder was then homogenized in $T_{10}E_1$ buffer with NP-40. The homogenate was digested with proteinase K (10 µg/ml) in the presence of 0.2% sodium dodecyl sulphate (SDS) and incubated at 37°C for 3 h. DNA was isolated by phenol : chloroform extraction and subsequent RNAase A treatment at 37°C for 1 h was given. This was followed by extraction of DNA by phenol : chloroform and ethanol precipitation.

Mouse 7.4k cDNA microarrays purchased from University Health Network, Canada Microarray Centre, Toronto, Canada were used for the experiments.

Two microgram of genomic DNA was sonicated to an average size of 1.5–3 kb in TE buffer (10 mM Tris, 1 mM EDTA). The sonicated DNA was then denatured at 94°C for 2 min. The denatured DNA was then mixed with 4×

hybridization buffer (Amersham Biosciences) and water to make a total volume of 300 uL. This hybridization mixture was then allowed to hybridize onto the microarray slide. The slide was covered with a coverslip and incubated at 42°C for 14 h in a hybridization unit (Quantifoil). The excess unhybridized DNA was washed from the slide using high and medium-stringency wash buffers from GeneTAC biosolutions (Perkin Elmer Ltd.) After washing the slide was incubated with primary antibody solution consisting of anti-5-methyl cytosine (anti-5 mC) monoclonal antibodies (raised and checked for their sensitivity and specificity in mouse as described by Kelkar and Deobagkar[20]) in TNT buffer (1 mM Tris (pH 7.5), 100 mM NaCl, 0.01% Tween 20) at a dilution of 1 : 1000. The incubation was carried out for 1 h at 25°C. Excess primary antibodies were washed with TNT buffer for 20 min with 5 changes of 5 min each. After washing the excess unbound primary antibodies, the slide was incubated with secondary antibody solution consisting of goat anti-mouse secondary antibody labelled with Cy3 in TNT buffer at a dilution of 1 : 500. The incubation was carried out for 1 h at 25°C. The excess antibody was washed with TNT buffer. The slide was finally washed with water and dried by centrifugation at 1500 rpm for 2 min. The dried slide was scanned at 543 nm wavelength using Perkin Elmer Scan array express scanner at a resolution of 5 μm. The microarray experiment was repeated thrice using DNA samples from three different animals individually on three separate slides (Figure 1).

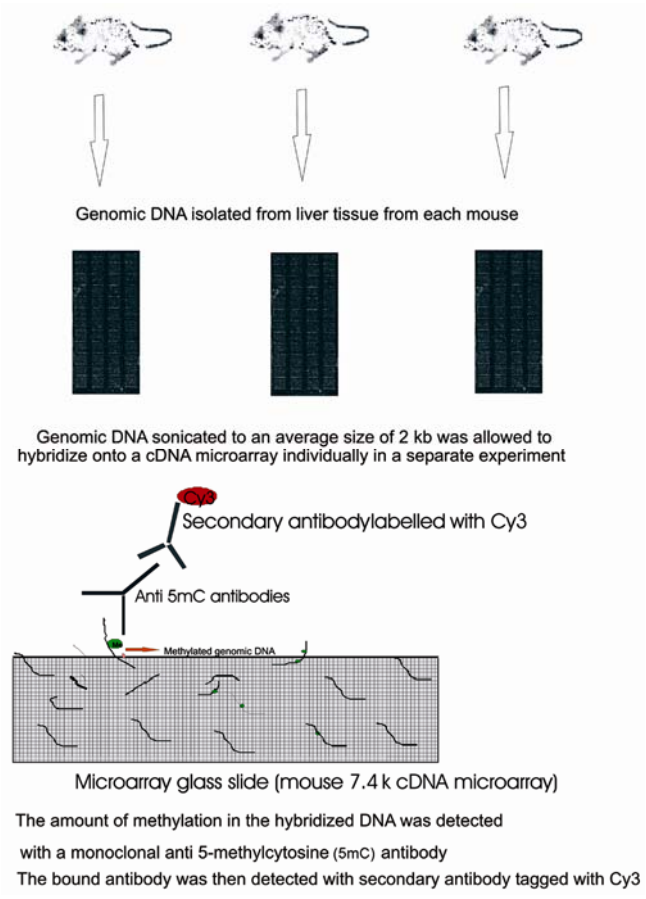

Figure 1. Schematic representation of the microarray protocol used in the experiment.

The mean fluorescent intensity value of each spot subtracted from the background fluorescent intensity value of each spot on the array was used as a signal to carry out data analysis. The signal intensities were LOWESS normalized and a signal-to-noise ratio of 2 was used as the quality criterion. To identify the 'true positive spots' which contained cytosine methylation, fluorescent signals of 3XSSC and *Arabidopsis thaliana* spots, present on the array, were used as 'controls'. As a check, a *t*-test (unpaired, $P < 0.05$) between the intensities of these spots with the blank spots present on the array revealed no significant difference between the intensities, suggesting that the binding efficiency of the antibodies to control DNA is equal to the binding efficiency with the glass slide. Hence, the florescence values from these spots were considered to be representatives of non-specific binding of the genomic DNA and antibodies to the slides. Assuming an error of 10–20% in the ability to detect true positives, the fluorescence intensities of the spots which were 10% and 20% higher than the control values were considered as errors and were removed from the analysis. Any gene spot which showed a fluorescent signal higher than the cut-off was considered as 'true positive'. A comparison of fluorescence intensities between control spots and true positive gene spots is depicted in Figure 2.

The fluorescent intensity of the true spot divided by the fluorescent intensity of the highest control spot was used as a measure for the level or degree of methylation. This gave a quantifiable positive value in the form of a fold change as a measure of methylation. These fold intensity values were compared between replicates and similar fold intensities were identified using the *t*-test. All gene spots

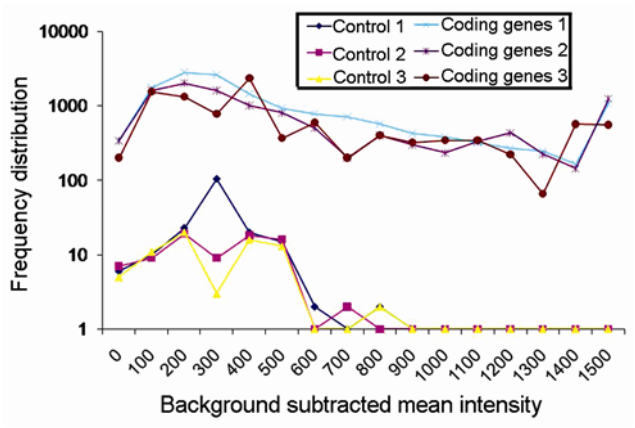

Figure 2. Frequency distribution of fluorescent intensity of control spots compared with actual gene spots identified as methylated in three array replicates. A clear difference in the fluorescent intensity between the controls and genes identified as methylated has been observed.

which were identified as true positives (methylated genes) in at least two out of the three array replicates were used for further analysis. Gene ontology of these methylated genes was further carried out using DAVID[22] and PANTHER[23] software. CpG island predication was carried out using DBTSS[24].

A cDNA microarray-based platform was used to assess methylation of genes (7.4k array) in liver tissue of BALB/c mice. The experiment involves hybridization of genomic DNA onto a cDNA array, followed by extensive washing. The main objective of using cDNA arrays is to identify DNA methylation in the coding regions other than the conventional reported methylated regions like CpG islands and gene promoters. Also, a similar platform can be used to study the expression of the same genes. The methylation in hybridized DNA was detected by anti-5mC antibody and secondary antibody labelled with Cy3. The intensity of Cy3 was used as an indicator of methylation, which was normalized using control or blank spot intensity present on the array[20]. The binding of Cy3 represents localization of anti-5mC-antibody, which in turn represents methylation in the hybridized genomic DNA. Since the fragments spotted on the microarray are 5′ and 3′ cDNA regions representing the coding region of the genes, methylation detected will be part of gene-body cytosine methylation and could be present at the internal coding region or the promoter region, if the promoter is located 3–6 kb away from the site of binding. This analysis provides a methylation landscape in the given set of genes.

Using the method described above the methylation of DNA in BALB/c mice was determined. Out of 3893 known gene ESTs (expressed sequence tags) present on the array, we observed positive signal for methylation in 1415 ESTs. These 1415 genes were positive in any two out of the three arrays used. Totally 231 genes showed positive signal for methylation consistently in all the array replicates (Table 1). It is known that methylation patterns are subject to variation. It has been reported that DNA methylation levels change in a tissue, cell, and developmental stage-specific fashion[10,25]. The variation in methylation can be allele-specific, clone or cell-specific; hence genes which showed methylation signals in at least two out of the three array replicates were considered as harbouring methylation and used for further analysis. Gene ontology analysis revealed that a large number of the methylated genes (12%) were involved in nucleic acid metabolism and signal transduction biological processes (Figure 3 *a*). Genes in the nucleic acid metabolism category were mainly involved in chromatin packaging (e.g. *H3f3a* [Entrez ID:15081], *H2afy* [26914], *Hat1* [107435], *H2afx* [Entrez ID: 15270], *Dnmt3a* [13435]); transcription (e.g. *Tcerg1l* [70571], *Ruvbl2* [20174]); RNA splicing and processing (e.g. *Rcl1* 59028 Sf3b1 81898); cell cycle (e.g. *Cdkn2b* [12579], *Ccni* [12453], *Cdk6* [12571], *Cdk8* [264064]) and DNA repair (e.g. *Ercc6*, Polk [27065], *Fancc* [14088]) processes (Table 2). It is noteworthy that chromatin remodelling genes such as those encoding histones, histone-modifying enzymes and methyltransferases were identified to carry methylation. This indicates that the expression of genes involved in the epigenetic machinery appears to be regulated by epigenetic mechanisms like DNA methylation. It has been reported that *Dnmt3a* has an internal promoter region which regulates the expression of *Dnmt3a* isoforms[26]. Our analysis identified the presence of methylation in the internal coding region of *Dnmt3a*. It would be interesting to study whether the methylation observed in our study might be involved in regulating/suppressing the expression of *Dnmt3a* isoforms. Molecular function analysis revealed that maximum number of methylated genes (11.2%) show nucleic acid-binding characteristics (Figure 3 *b*).

It is known that CpG methylation, particularly in the CpG islands, has been implicated in epigenetic regulation of a large number of genes in mouse. CpG island prediction using DBTSS revealed the presence of CpG island in 70% of the genes identified to be methylated in this analysis (99 genes)[24]. BLAT analysis revealed that CpG islands of histones Hat1 and H2afx, and imprinted genes *Cdkn2b*, *Polk*, *Nnat*, *Snrpn* and *Nqo1*, were found to be located within a span of 2 kb from the probe binding site (Table 3; Figure 4). However, probe binding sites of genes *Cdk6*, *Cdk8*, *Sf3b1*, *Dact1*, *Ccni* and *H2afy* were found to

Table 1. Summary of methylation microarray data for BALB/c mouse (control)

Number of genes spotted on mouse 7.4 K array	3893
Number of genes positive for methylation signal (all three replicates)	231
Number of genes positive for methylation signal (two out of the three replicates)	1413
Number of genes annotated in the NCBI database	1327
Number of imprinted genes	11
Number of genes known to be hypermethylated in liver tumours	9
Number of genes involved in nucleic acid metabolism	247
Number of genes involved in signal transduction	246
Number of genes involved in apoptotic mech.	14
Number of genes involved in transcription	127
Number of genes involved in replication	13
Number of DNA methyltransferases	1
Number of RNA methyltranferases	2
Number of genes with CpG islands	99

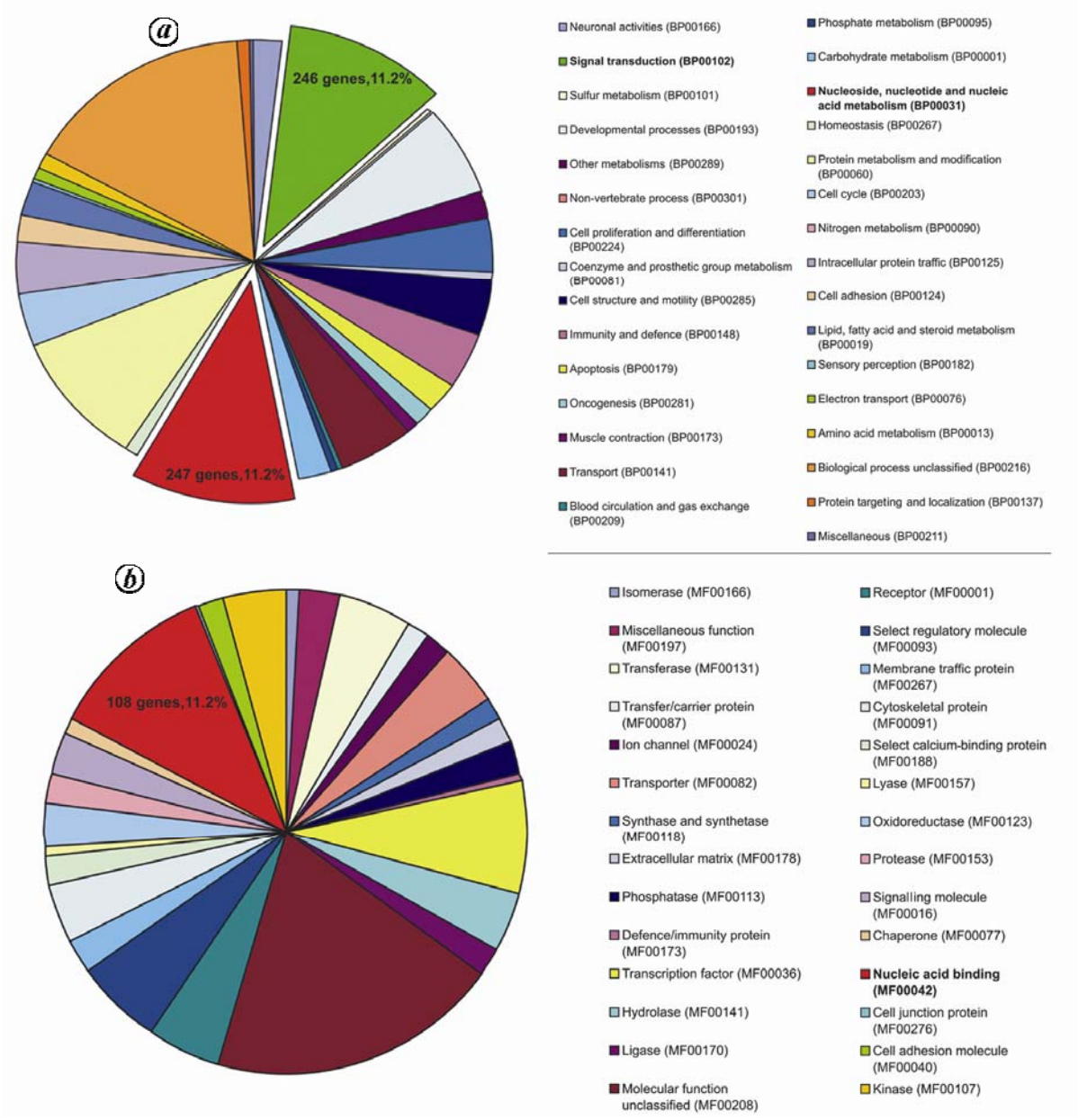

Figure 3. The 1415 genes that were found to be consistently methylated were analysed for their gene ontology. (***a***) Pie chart showing percentage of genes involved in various biological processes. (***b***) Distribution of genes on the basis of their molecular functions.

be more than 4 kb away from known CpG islands of the genes (Table 3; Figure 4).

Overall the distribution of methylated genes on mouse chromosomes was found to be uniform, except chromosomes 2 and 11 where the number of methylated genes was comparatively more, and chromosomes 6 and 10 where the number of methylated genes was comparatively less (Figure 5).

Our analysis led to the identification of 11 imprinted genes as methylated, viz. *Asb4* [65255], *Copg2as2* [100044236], *Impact* [16210], *Magel2* [27835], *Meg3* [17263], *Nnat* [18111], *Ppp1r9a* [243725], *Sgce* [20392], *Snrpn* [20646], *Xist* [213742] and *Zrsr1* [22183][27]. *Xist* is a canonical case of imprinted gene and is known to play a significant role in X-chromosome inactivation in mammals[28]. Interestingly, nine genes known to be hypermethylated in liver cancer[29], viz. *Cdkn2b* [12579], *Col1a2* [12843], *Dact1* [59036], *Dapk1* [69635], *Dlc1* [50768], *Fn1* [14268], *Nqo1* [18104], *Ptgs2* [19225] and *Thbs1* [21825] were detected as methylated in the internal coding regions in our study. This observation could have implication in cancer diagnosis and predisposition. *Cdkn2b*, which belong to a family of cyclin-dependent kinase 4 inhibitors (INK4) and is known to control cell

Table 2. Functional classification of genes having (A) nucleic acid binding molecular function and involved in (B) nucleic acid metabolism biological process

(A)		(B)	
Primase (MF00058)	1	Pyrimidine metabolism (BP00033)	1
DNA methyltransferase (MF00050)	1	Regulation of nucleoside, nucleotide metabolism (BP00057)	1
DNA photolyase (MF00047)	1	tRNA metabolism (BP00054)	2
Replication origin-binding protein (MF00059)	2	RNA localization (BP00053)	2
DNA topoisomerase (MF00057)	2	Other nucleoside, nucleotide and nucleic acid metabolisms (BP00059)	3
RNA methyltransferase (MF00054)	3		
Double-stranded DNA-binding protein (MF00294)	3	Nucleoside, nucleotide and nucleic acid transport (BP00058)	3
Other nucleic acid binding (MF00076)	3	Metabolism of cyclic nucleotides (BP00056)	4
Ribosomal protein (MF00075)	3	RNA catabolism (BP00256)	4
Single-stranded DNA-binding protein (MF00055)	4	rRNA metabolism (BP00055)	5
Histone (MF00063)	4	Purine metabolism (BP00032)	9
Damaged DNA-binding protein (MF00060)	4	Chromatin packaging and remodelling (BP00273)	24
Ribonucleoprotein (MF00069)	5	Pre-mRNA processing (BP00047)	31
DNA-directed DNA polymerase (MF00043)	7	DNA metabolism (BP00034)	41
Translation factor (MF00071)	7	mRNA transcription (BP00040)	127
Other RNA-binding proteins (MF00053)	8		
Other DNA-binding proteins (MF00286)	15		
Nuclease (MF00044)	15		
Chromatin/chromatin-binding protein (MF00070)	16		
Helicase (MF00051)	18		
mRNA processing factor (MF00065)	19		

Figure 4. Schematic representation of the locations of the probes identified as methylated as depicted in Table 3 on mouse chromosomes.

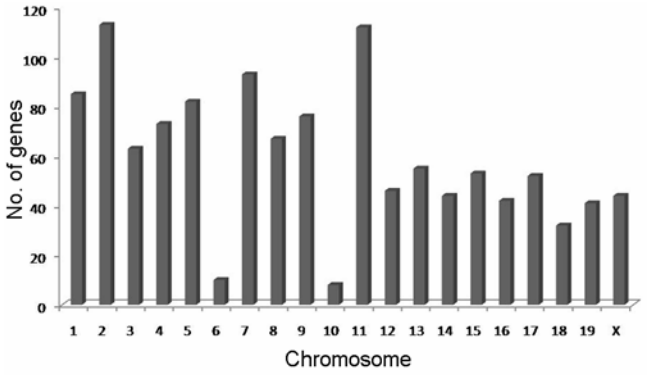

Figure 5. Chromosome-wide distribution of genes identified as methylated in the study (1415 genes).

proliferation during the G1 phase of the cell cycle has been found to be hypermethylated in the promoter regions in hepatic and many other cancers[30].

Thus our method could detect methylation in genes which are known to harbour imprinting signals or are subject to epigenetic silencing via DNA methylation in various situations.

We have used a method to analyse methylation of genes on a genome-wide scale that is not restricted to detecting methylation in specific sequences like CpGs. Data from the BALB/c liver DNA enabled us to derive a methylation profile of a mouse strain in a sequence-independent but gene-specific manner. It has led to the localization of methylated regions in the genome and generation of a methylation landscape of coding regions of the mouse genome.

We propose that the methylation identified in our analysis is part of the gene-body methylation along with promoter methylation. This observation is in accordance with earlier studies, where it has been shown most of the tissue-specific methylated regions, excluding those associated with genomic imprinting, were present at non-repetitive intergenic and intragenic regions located away from CpG islands[31–36]. Most of the earlier studies have focused on the methylation patterns of promoters and CpG islands. Hence it is important to analyse methylation in the coding region of the genes, especially at the intergenic and intragenic regions.

In addition to identifying several regions which are known to harbour methylation, this study resulted in the identification of a large number of genes which were

Table 3. List of selected genes detected as methylated in the study

Gene	Gene ID	Chromosome	Probe position on the chromosome		CpG island	TSS location	CpG location	Distance from probe (bp)
H3f3a	15081	1	182732698	182733160	Yes	182743738	182743738	
H2afy	26914	13	56175570	56197553	Yes	56236876	56236876	39323
Hat1	**107435**	**2**	**71228020**	**71259340**	**Yes**	**71227317**	**71227317**	**703**
H2afx	**15270**	**9**	**44143702**	**44144154**	**Yes**	**44142789**	**44142700–44143700**	**454**
Sf3b1	81898	1	55042015	55042475	Yes	55084322	55084322	41847
Cdkn2b	**12579**	**4**	**88953169**	**88956905**	**Yes**	**88956863**	**88956850**	**55**
Ccni	12453	5	93616495	93617076	Yes	93635054	93635054	17978
Cdk6	12571	5	3522659	3523208	Yes	3344312	3344312	178347
Cdk8	264064	5	147106519	147106874	Yes	147043251	147043251	63623
Polk	**27065**	**13**	**97271483**	**97312848**	**Yes**	**97312440**	**97312440**	**408**
Fancc	14088	13	63441638	63453480	Yes	63533053	63533053	79573
Asb4	65255	6	5379802	5381362	No	5333386	–	46416
Nnat	**18111**	**2**	**157385858**	**157387801**	**Yes**	**157385850**	**157385850**	**8**
*Ppp1r9*a	243725	6	5114419	5115015	Yes	4853320	4853320	261099
Snrpn	**20646**	**7**	**67132360**	**67149980**	**No**	**67150042**	**67150042**	**62**
Dact1	59036	12	72419312	72419766	Yes	72410971	72410971	8641
Fn1	14268	1	71673860	71676288	Yes	71699745	71699745	23457
Nqo1	**18104**	**8**	**109916062**	**109927086**	**No**	**109927105**	**–**	**19**
Dapk1	69635	13	60855467	60862116	Yes	60703572	60703572	158544

The position of the probe on the array to which genomic DNA got hybridized has been indicated. The position of the transcription start site (TSS) and CpG island present, if any, has been shown. Highlighted are the genes where probe-binding site was found to be within a span of 2 kb from a known CpG island on that gene.

hitherto not reported to be methylated (either at the promoter or any other region) in the mouse genome.

We observed a strong correlation between the methylation status of the genes observed in our experiment and the presence of CpG island in the upstream region. CpG islands are known methylatable sites and methylation is known to occur in clusters and spread across a region[37]. Identification of the presence of methylation in these genes in our analysis could be due to gene-specific CpG island methylation, which could have spread to the intragenic regions. This methylation may have a role in liver-specific regulation of gene expression. However, many genes were observed to be methylated at regions distant from CpG islands. This suggests that it may be a part of the non-CpG island-specific methylation present in the internal coding regions of the genes. Shen *et al.*[18] have reported that CpG richness is not the only criterion for methylation, which indicates that cytosine in any other combination can get methylated and may play an important role in methylation-mediated gene silencing. It has been reported that methylation of gene promoter region is not always a pre-requisite for gene silencing[14] and additional sequence features may be involved in methylation-mediated gene silencing. Hence, internal regions of the genes are potential sites for DNA methylation.

Gene ontology analysis of the methylated genes observed in our study revealed that a maximum number of them were involved in transcription and chromatin remodelling. It thus appears that the epigenetic machinery itself may be regulated by DNA methylation. Since DNA methylation is known to be heritable and has a capacity to modulate gene expression patterns during development, differentiation and in response to many external stimuli, identification of gene-body methylation in genes which regulate epigenetic imprints has important implications.

Our analysis identified 11 out of the 36 imprinted genes spotted on the microarray as methylated. This clearly indicates that our method has implications in identifying other potentially methylated sites in and around the coding region of genes that might be playing a role in gene regulation. *Asb4* and *Copg2as2* imprinted genes are known to be maternally methylated in liver tissue[38,39]. This substantiates our results as female BALB/c mice were used for the study. *Asb4* is reported to inhibit cytokine signalling by interacting with JAK-kinase.

All the genomic locations of the methylated sequences were confirmed using BLAT. The genomic locations of the methylated regions of many genes were found to be within a range of 2–4 kb from transcription start site (TSS) or known CpG island. This further substantiates our earlier observation and highlights the role of the observed methylation in gene expression regulation (Table 2).

In summary, using cDNA arrays we could assess the methylation that is physically associated with genes rather than repeat DNA and satellite DNA. This can provide a methylation landscape which can be monitored under various stress and disease conditions. The cDNA platform can be used to correlate the DNA methylation pattern with gene expression analysis. It can provide methylation status of the genes which show expression changes under certain conditions using similar cDNA arrays. This work has identified 1415 genes that were methylated in the BALB/c mouse under normal conditions, many of which have been reported to harbour promoter

methylation or potential methylatable sites. Many new genes were also identified which were earlier not reported to be methylated at the promoter or any other region. Thus, the study has enabled us to provide the methylation landscape in and around genes in the BALB/c mouse strain.

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