The DNA methylation profile of liver tumors in C3H mice and identification of differentially methylated regions involved in the regulation of tumorigenic genes

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Abstract

Background: C3H mice have been frequently used in cancer studies as animal models of spontaneous liver tumors and chemically induced hepatocellular carcinoma (HCC). Epigenetic modifications, including DNA methylation, are among pivotal control mechanisms of gene expression leading to carcinogenesis. Although information on somatic mutations in liver tumors of C3H mice is available, epigenetic aspects are yet to be clarified.

Methods: We performed next generation sequencing-based analysis of DNA methylation and microarray analysis of gene expression to explore genes regulated by DNA methylation in spontaneous liver tumors of C3H mice. Overlaying these data, we selected cancer-related genes whose expressions are inversely correlated with DNA methylation levels in the associated differentially methylated regions (DMRs) located around transcription start sites (TSSs) (promoter DMRs). We further assessed mutuality of the selected genes for expression and DNA methylation in human HCC using the Cancer Genome Atlas (TCGA) database.

Results: We obtained data on genome-wide DNA methylation profiles in the normal and tumor livers of C3H mice. We identified promoter DMRs of genes which are reported to be related to cancer and whose expressions are inversely correlated with the DNA methylation, including Mst1r, Slpi and Extl1. The association between DNA methylation and gene expression was confirmed using a DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) in Hepa1c1c7 cells and Hepa1-6 cells. Overexpression of Mst1r in Hepa1c1c7 cells illuminated a novel downstream pathway via IL-33 upregulation. Database search indicated that gene expressions of Mst1r and Slpi are upregulated and the TSS upstream regions are hypomethylated also in human HCC. These results suggest that DMRs, including those of Mst1r and Slpi, are involved in liver tumorigenesis in C3H mice, and also possibly in human HCC.

Conclusions: Our study clarified genome wide DNA methylation landscape of C3H mice. The data provide useful information for further epigenetic studies of mouse models of HCC. The present study particularly proposed novel DNA methylation-regulated pathways for Mst1r and Slpi, which may be applied not only to mouse HCC but also to human HCC.

Keywords: C3H mice, DNA methylation, Liver tumors, Reduced representation bisulfite sequencing (RRBS), 5-aza-2′-deoxycytidine
Background

C3H mice have been employed for numerous studies of carcinogenesis. The mice, particularly their males, are predisposed to spontaneously develop liver tumors in adulthood and have been used as an animal model of spontaneous hepatocellular carcinoma (HCC) [1, 2]. They are also often used as chemically induced HCC models, such as diethylnitrosamine (DEN)- or DEN and phenobarbital (DEN/PB)-induced HCC models [1, 3]. A recent study established an HCC model with liver cirrhosis, a key feature of human HCC, by administering carbon tetrachloride (CCl4) to C3H mice [6].

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Somatic point mutations of ras-genes (Ha-ras, K-ras, and N-ras) were also detected in a variety of human and animal tumors [7]. These somatic mutations activate Ras proteins and lead to activation of tumor augmenting pathways, including the RAF/MEK/ERK kinase cascade [7]. Another somatic mutation was reported in B-raf, a member of the RAF kinase family in about 20% of liver tumors in C3H mice administered DEN at 2 weeks of age [8]. B-raf mutation also augments the kinase activity leading to activation of the MEK/ERK cascade [5, 8].

On the other hand, some of the spontaneous liver tumors of C3H mice do not have Ha-ras or B-raf mutations [8, 9]. Epigenetic modifications, such as DNA methylation and histone modifications, are pivotal posteriori control mechanisms of gene expression and are closely involved in tumorigenesis [10]. A recent study performed next-generation sequencing of DNA methylation of human non-cirrhotic HCC and fibrolamellar hepatocellular carcinoma (FLC) and found distinctive epigenetic signatures of the two types of tumors [11]. While we previously studied methylation status in the spontaneous liver tumors of control C3H mice and liver tumors of gestationally arsenate exposed C3H mice by a methylated DNA immunoprecipitation (MeDIP)–CpG island microarray method [12], precise single base resolution analysis of DNA methylation has not been performed for the liver tumors of C3H mice.

Recent progress in genomic study using next generation sequencing enabled DNA methylation analysis at one-base pair resolution and revealed more precise modes of action of DNA methylation, beyond gene silencing by canonical CpG islands. DNA methylation is involved in suppression/activation of gene expression, transcription factor binding, splicing and nucleosome positioning [13]. In the present study, we clarified differentially methylated CpGs (DMCs) and differentially methylated regions (DMRs), where DNA in the spontaneous liver tumors of C3H mice is hyper- or hypomethylated compared to the normal livers, by reduced representation bisulfite sequencing (RRBS) method, a sequencing-based genome-wide DNA methylation analysis [14, 15]. According to the recent reports showing that DNA methylation levels within ±1000 bp regions [13] or ±2000 bp regions [11, 16, 17] of TSS showed a strong correlation with gene repression, we focused on DMRs within ±2000 bp of TSS (promoter DMRs). Using the data of promoter DMRs and by in vitro experiments and PubMed search, we identified genes whose expressions are closely associated with the methylation of proximal promoter DMRs and which are involved in tumorigenesis. We also assessed the gene expression and DNA methylation status of these genes in human HCC using TCGA database to explore similarities in tumorigenic regulation in human.

Methods

Animals

Male C3H/HeN mice around 74 weeks of age were obtained from F2 pups of the control group of a project for investigating the effects of gestational arsenic exposure on the F2. The design of animal breeding was the same as described previously [9, 18], Briefly, pregnant F0 C3H/HeN mice were purchased from CLEA Japan (Tokyo, Japan) and F1 and F2 pups in the control group were given free access to a standard diet (CA-1; CLEA Japan) and tap water (the control group). The mice were handled in a humane manner in accordance with the National Institute for Environmental Studies (NIES) guidelines for animal experiments.

Cell lines and treatment

Hepa1c1c7 cells were kindly provided by Dr. Y. Fuji-Kuriyama (University of Tsukuba) in 2005 [19] and cultured in the Dulbecco’s Modified Eagle’s Medium (DMEM, SIGMA-ALDRICH, D5796) containing 10% FBS and 1% penicillin-streptomycin. Hepa1-6 cells provided by RIKEN BRC CELL BANK were cultured in DMEM containing 10% FBS, 1% penicillin-streptomycin and 5% sodium pyruvate. 5-Aza-dC was purchased from Santa Cruz (CA, USA). After attaching to the dish, cells were incubated in a culture medium containing 5-aza-dC for 72 h. For overexpression study, mouse ORF clones Mst1r (NM_001287261) and Slpi (NM_011414), were incubated in a culture medium containing 5-aza-dC for 72 h. For overexpression study, mouse ORF clones Mst1r (NM_001287261) and Slpi (NM_011414), and pCMV6-Kan/Neo (pCMV6KN) as an empty vector were purchased from ORIGENE Inc. (MD, USA). Hepa1c1c7 cells were transfected with 2 µg of vectors using HilyMax (Dojindo, Japan) according to the
manufacturer’s instructions and gene expression changes were examined after culturing for 24 h.

Genomic DNA extraction
Genomic DNA was prepared as previously described [18] from the normal livers and macroscopic liver tumor tissues of C3H mice and cell lines. Briefly tissues or cells were lysed in lysis buffer, treated with RNase, and purified with a phenol-chloroform mixture.

RRBS analysis
RRBS libraries were prepared from genomic DNA of 3 normal liver tissues and 3 tumor tissues having a Ha-ras mutation according to the protocol reported by Boyle et al. [15], with some modifications. Briefly, 100 ng of genomic DNA was digested with MspI, subjected to gap-filling and A-tailing, and ligated with TruSeq adaptors included in the TruSeq DNA Sample Prep Kit (FC-121-1001, Illumina). After DNA size selection and ligation efficiency were checked, adaptor-ligated DNA was subjected to bisulfite conversion and amplified by PCR. The PCR products were cleaned-up using Agencourt AMPure XP beads and sequenced on an Illumina HiSeq2500. The sequencing Omnibus and are accessible through GEO Series accession number GSE111420 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111420).

Alignment of RRBS data and identification of DMCs and DMRs
Bisulfite-treated sequencing reads were aligned by a paired-end alignment method for a unique best hit to the mouse reference genome (NCBI/mm10) using the Bismark program [20], and adapter trimming and quality control was performed using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The aligned data were used for generating and analyzing DNA methylation profiles using the methylKit package [21]. Briefly, %methylation scores were determined for CpG sites using the read.bismark function of methylKit as SAM format alignments files created with the Bismark aligner as input files. For each CpG site, the read.bismark function counts the numbers of reads with C (methylated and remained) and with T (unmethylated and converted) in both strands separately, and calculates %methylation. The obtained %methylation profiles were evaluated for their overall statistics and extent of overall similarities among samples using methylKit functions such as getMethylationStats and getCorrelation [21].

DMCs were extracted using methylKit for the cytosines in CpG sites with < 0.01 q-value by the logistic regression method, ≥10 reads in coverage and ≥25% methylation differences between normal and tumor tissues. Under the condition, 2,331,643 CpG sites were identified. DMRs were detected for regions containing ≥3 CpG sites and at least 1 DMC and with ≥30% absolute mean CpG methylation difference by using an eDMR package on R. eDMR implements statistical analysis [22]. Promoter DMRs, which were defined as regions within ±2000 bp from TSSs for Refseq transcripts [11, 16, 17], were extracted using bedtools 2.25.0 (bedtools.readthedocs.io). A summary of the detailed sequencing and alignment characteristics for the samples used in this study will be published elsewhere with the data of arsenite-exposed F2 mice (Okamura et al., in preparation).

Bisulfite sequencing
Bisulfite sequencing analysis was carried out as previously described with minor modifications [23]. Genomic DNA (1 μg) was digested with EcoRI and subjected to bisulfite modification using EZ DNA Methylation-Gold Kit (Zymo Research, CA, USA). The bisulfite-treated DNA (2 ng) was amplified with the primers shown in Additional file 1: Table S1. The PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega) and cloned into pGEM-T Easy Vector (Promega). Obtained clones were cycle sequenced with M13RV primers (5′-CAG GAAAACAGCTATGAC-3′) and a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), and analyzed with an Applied Biosystems 3730 DNA analyzer.

Microarray analysis of gene expression
Total RNA was prepared from the 3 normal liver tissues, 3 tumor tissues having Ha-ras mutation and Hepa1c1c7 cells with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). After the quality of total RNA was checked using a 2100 Bioanalyzer (Agilent Technologies), the gene expression profile was analyzed using SurePrint G3 Mouse GE arrays (Agilent Technologies) according to the manufacturer’s protocol for one-color microarray-based gene expression analysis. The scanned images were analyzed with Feature Extraction Software 9.1 to obtain background subtracted and spatially detrended Processed Signal intensities. The microarray data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE104627 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104627) and GSE104626 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104626).

The expression data from each chip were then normalized to the 75th percentile of all measurement using Agilent Gene-Spring GX. Genes with an expression value of more than 1.0, in either normal tissues or tumor tissues or in both, were considered to be expressed above the threshold and utilized for analysis. The genes
with a two- or more fold increase or decrease in mean expression values \( (n = 3) \) were judged to have changed. For Hepa1c1c7 cells with or without overexpression of \( \text{Mst1r} \) or \( \text{Slpi} \), genes with raw expression values of more than 50, either after upregulation or before downregulation or in both cases, were utilized for further analysis.

![Fig. 1 Outline of DNA methylation status of normal and tumor liver tissues in C3H mice.](image)

- **a** Hierarchical clustering of methylation profiles of normal and tumor tissues using 1-Pearson’s correlation distance.
- **b** Horizontal bar plots showing the percentage of hyper- and hypo-DMCs in all DMCc (left) and in all CpG (right) per chromosome.
- **c** Pie charts showing percentage of DMCs in the region between –5000 bp and TSS, exon, intron and intergenic.
- **d** Pie charts showing percentage of DMCs in CpG islands, CpG island shores (defined as 2 kb flanks of CpG islands) and other regions.
- **e** The scatter plot of DNA methylation differences of DMRs and gene expression ratios between the normal and tumor tissues. The data of 118 expressing genes with the promoter DMRs were used. The relationship between DNA methylation differences and gene expression ratios of normal and liver tumors was determined by Spearman correlation analysis. 28 DMRs whose associated genes are up- or downregulated by hypo- or hyper-DMRs more than 2-fold in tumor tissues and appear in a PubMed search for the term “cancer” were shown in square, and listed in Table 1. Triangle plots indicate other DMRs.
| Associated gene | Description | DMR position | DMR Distance from TSS | DMCs number | Methylation difference (Tumor - Normal) | Gene expression (Tumor / Normal) | p-value |
|-----------------|-------------|--------------|----------------------|-------------|----------------------------------------|-------------------------------|---------|
| **Downstream**  |             |              |                      |             |                                        |                               |         |
| Slpi            | secretory leukocyte protease inhibitor | chr2: 164355520–164,355,812 | + 697      | 9   | −33.83                                | 408.37                        | 0.0090  |
| Cela1           | chymotrypsin-like elastase, family, member 1 | chr15: 100687184–100,687,240 | + 682      | 3   | −31.23                                | 71.51                         | 0.0003  |
| Mst1r           | macrophage stimulating 1 receptor | chr9: 107908081–107,908,516 | + 1192     | 13  | −46.34                                | 67.47                         | 0.0005  |
| Btg2            | B cell translocation gene 2 | chr1: 134077506–134,077,566 | + 1591     | 3   | −46.61                                | 18.30                         | 0.0050  |
| Fgl2            | fibrinogen-like protein 2 | chr5: 21373036–21,373,583 | + 366      | 10  | −31.73                                | 11.88                         | 0.0002  |
| P2rx7           | purinergic receptor P2X | chr5: 122644473–122,644,655 | + 562      | 4   | −43.12                                | 8.23                          | 0.0031  |
| Lss             | lanosterol synthase | chr10: 76532531–76,532,879 | + 904      | 8   | −59.93                                | 6.17                          | 0.0000  |
| Grh1            | grainyhead-like 1 | chr12: 24573292–24,573,626 | + 1005     | 3   | −39.22                                | 6.06                          | 0.0020  |
| Itm2c           | integral membrane protein 2c | chr1: 85895741–85,895,786 | + 1231     | 3   | −31.78                                | 3.91                          | 0.0015  |
| Chmp6           | charged multivesicular body protein 6 | chr11: 119915542–119,915,667 | + 1732     | 6   | −53.11                                | 2.88                          | 0.0002  |
| Vasp            | vasodilator-stimulated phosphoprotein | chr7: 19269155–192,699,927 | + 1929     | 14  | −40.96                                | 2.27                          | 0.0016  |
| Mmp14           | matrix metalloproteinase 14 | chr14: 54433543–54,434,231 | + 1939     | 13  | −39.11                                | 2.19                          | 0.0012  |
| Marveld2        | MARVEL domain containing 2 | chr13: 100615542–100,615,803 | + 1143     | 2   | −34.10                                | 2.06                          | 0.0238  |
| Pdk2            | pyruvate dehydrogenase kinase, isoenzyme 2 | chr11: 95039447–95,039,657 | + 1716     | 7   | 38.16                                 | 0.48                          | 0.0034  |
| Ext1f1          | exostoses-1 | chr4: 134371099–134,371,620 | + 929      | 44  | 48.12                                 | 0.39                          | 0.0216  |
| Grrm8           | glutamate receptor, metabotropic 8 | chr6: 28133156–28,133,165 | + 1206     | 3   | 33.66                                 | 0.32                          | 0.0016  |
| Ppp1r14a        | protein phosphatase 1, regulatory inhibitor subunit 14A | chr7: 29291240–29,291,527 | + 1920     | 8   | 36.03                                 | 0.25                          | 0.0052  |
| Snc29           | sorting nexin 29 | chr16: 11405750–11,405,827 | + 102      | 3   | 60.92                                 | 0.22                          | 0.0218  |
| C3b             | complement component B, beta polypeptide | chr4: 104767260–104,767,325 | + 943      | 7   | 54.51                                 | 0.18                          | 0.0066  |
| Igfals           | insulin-like growth factor binding protein, acid labile subunit | chr17: 24880338–24,882,131 | + 1568     | 57  | 38.73                                 | 0.17                          | 0.0003  |
| Adam11          | a disintegrin and metalloproteinase domain 11 | chr11: 102762268–102,762,503 | + 829      | 16  | 54.16                                 | 0.16                          | 0.0002  |
| CypBb1          | cytochrome P450, family B, subfamily b, polypeptide 1 | chr9: 121914943–121,915,025 | + 1279     | 6   | 63.26                                 | 0.10                          | 0.0231  |
| **Upstream**    |             |              |                      |             |                                        |                               |         |
| Ltb             | lymphotixin B | chr17: 35194367–35,194,502 | −4         | 3   | −32.55                                | 20.94                         | 0.0410  |
| Nfe2            | nuclear factor, erythroid derived 2 | chr15: 103259509–103,259,588 | −1105      | 4   | −36.04                                | 12.39                         | 0.0045  |
| Hmgcr           | 3-hydroxy-3-methylglutaryl-coenzyme A reductase | chr13: 96671738–96,671,790 | −801       | 1   | −30.40                                | 4.80                          | 0.0144  |
Real-time PCR analysis
Total RNA was prepared from tissues and cells with an RNeasy Mini Kit (Qiagen). cDNA synthesis and real-time PCR was carried out as described previously [24]. The primers used are shown in Additional file 1: Table S1.

Prediction of the transcription factor binding site
Consensus regions of transcription factor binding sites were detected using the JASPAR database (http://jaspar.genereg.net/) [25] with the relative score threshold set at >90% and also by using published literature.

TCGA analysis
Forty one paired datasets of normal and HCC tissues of human livers for RNA-seq and DNA methylation levels were obtained from the TCGA database (http://cancer-genome.nih.gov/). They were downloaded and used to find genes with significant expression differences and differences in DNA methylation within ±2000 bp from TSS between normal and HCC tissues.

Statistical analysis
The differences in gene expression between the two groups were analyzed by a two-tailed paired Student’s t-test. A p-value < 0.05 was considered to be statistically significant. The differences in gene expression among three groups were analyzed by one-way ANOVA followed by the Turkey-Kramer test as a post hoc comparison. Spearman correlation analysis was used to determine the relationship between DNA methylation differences and gene expression ratios of normal and liver tumors in C3H mice. For multiple comparisons to identify DNA methylation differences, paired t-test with Bonferroni correction was performed.

Results
Genome wide analysis of DNA methylation and gene expression in normal and tumor livers of C3H mice
We conducted genome wide DNA methylation analysis for normal liver tissues and tumor tissues of male C3H mice by RRBS. Approximately 22–55 million sequence read pairs were obtained for the RRBS libraries and 63–71% of the reads were aligned to unique genomic locations. Hierarchical clustering analysis showed that DNA methylation status was distinctly separated between the normal and tumor tissues (Fig. 1a). Scatter plots of methylation values show high correlation coefficient value for each pair (Additional file 2: Figure S1).

We identified 103,965 DMCs (4.5% in total CpGs) with higher ratios of hypo-methylated CpGs in tumor tissues (hypo-DMCs), such as 60–80% in total DMCs in autosomal chromosomes and more than 90% in sex chromosomes (Fig. 1b). Annotation analysis showed that the majority of DMCs were in intron and intergenic regions (Fig. 1c) and in the region other than CpG island (CGI) or CGI shore (Fig. 1d). Higher ratio of hyper-DMCs, compared with hypo-DMCs, were found in the region between ~5000 bp upstream and TSS (Fig. 1c) and in CGI and CGI shore (Fig. 1d).

We then identified 3337 DMRs (Additional file 3: Table S2) including 225 promoter DMRs. Among the 225 genes driven by the differentially methylated promoters, 118 genes are expressed (see Methods). The methylation status of the 118 promoter DMRs was inversely associated with gene expression [13, 16, 17]. The scatter plot of DNA methylation differences of DMRs and gene expression ratios between the normal and tumor tissues of the 118 genes showed moderate inverse correlation (Fig. 1e). From the 118 genes, we shortlisted 27 genes which show more than 2-fold expression differences (p < 0.05) between normal and tumor tissues and inverse association with methylation status of the DMR, and which appear in a PubMed search for the term “cancer” (Table 1 and gray square in Fig. 1e).

| Associated gene | description | DMR position | DMR Distance from TSS | DMCs number | Methylation difference (Tumor / Normal) | Gene expression ratio (Tumor / Normal) | Gene expression | p-value |
|-----------------|-------------|--------------|-----------------------|-------------|----------------------------------------|----------------------------------------|----------------|---------|
| Slc29a1         | solute carrier family 29 member 1 | chr17: 45600404–46,600,454 | − 800 | 2 | 37.50 | 0.37 | 0.0010 |
| Slc17a8         | solute carrier family 17 | chr10: 89621306–89,622,039 | −60 | 7 | 51.72 | 0.11 | 0.0142 |

*These genes are hit by PubMed search for the term “cancer” and whose expressions are up- or down-regulated more than 2-fold in an inverse manner compared to the methylation rates between the normal and tumor tissues. Chr: chromosome, DMR: differentially methylated region, DMC: differentially methylated cytosine, TSS: transcription start site. P-values were analyzed by Student's t-test.

Expressions of Mst1r, Slpi and Ext1 are associated with DNA methylation alterations in the liver tumors of C3H mice
Among the genes shown in Table 1, we focused on Mst1r and Slpi, which are greatly up-regulated (67- and 408-fold, p-value 0.0005 and 0.009, respectively) and have relatively large numbers of DMCs (13 and 9) in the DMRs, and Ext1, which have a large number of DMCs (44 DMCs) in the DMR. Mst1r is a receptor tyrosine kinase activated by the binding of the ligand, Mst1 (macrophage-stimulating 1, also known as hepatocyte growth factor-like; HGFL) [26, 27].
Fig. 2 Mst1r associated with hypo-DMR is up-regulated in the liver tumor tissues of C3H mice. a Schematic view of the Mst1r locus. Methylation levels of CpG sites were visualized using Integrative Genomics Viewer (IGV) software. The position of TSS, DMR and the positions assessed by bisulfite sequencing (BS-1 and BS-2) were indicated. The magnified view indicates 8 CpG positions in the DMR and predicted transcription factor binding sites. b The average methylation values of the CpG sites in normal and tumor tissues. The CpG sites with ≥ 10 reads on both strands (≥ 20 reads in total) are indicated. Statistical significance between normal and tumor tissue was analyzed by Student’s t-test. **, *** Significantly different at p < 0.01 and 0.001, respectively. c Validation of RRBS data for normal and tumor liver tissues of C3H mice by bisulfite sequencing. Methylated and unmethylated cytosine are shown as ● and ○ respectively. The numbers above the circles indicate the position of CpG shown in Fig. 1a. d Validation of gene expression levels of Mst1r by real-time PCR in the normal (n = 6) and tumor tissues (n = 11) of C3H mice. The expression of Mst1r is normalized to the expression of rRNA. Statistical significance between the two groups was analyzed by Student’s t-test. *** Significantly different at p < 0.001.
Overexpression of Mst1r activates the molecule without the need of the ligands [27]. Such overexpression and activation of Mst1r are found in many types of human cancers and often correlate with poor prognosis in various cancers [27]. Slpi has activity as a serine protease inhibitor and is overexpressed in various types of cancers [28], including mouse liver tumors induced by CCl₄ or DEN [4]. Extl1 is a putative tumor-suppressor gene found in neuroblastoma patients [29].

The DNA methylation status of these genes are visualized in Figs. 2a, 3a, and 4a. Mst1r and Slpi, which are upregulated in the tumors, have hypo-DMRs starting from +1192 and +697 bps from TSS, respectively. Extl1, whose

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**Fig. 3** Slpi associated with hypo-DMR is up-regulated in the liver tumor tissues of C3H mice. **a** Schematic view of the Slpi locus. The position of TSS, DMR and the position assessed by bisulfite sequencing (BS) were indicated. The magnified view indicates 7 CpG positions in the DMR and predicted transcription factor binding sites. **b** The average methylation values of the CpG sites in normal and tumor tissues. The CpG sites with ≥10 reads on both strands are indicated. Statistical significance between normal and tumor tissue was analyzed by Student’s t-test. * Significantly different at p < 0.05. **c** Validation of RRBS data for normal and tumor liver tissues of C3H mice by bisulfite sequencing. Methylated and unmethylated cytosine are shown as ● and ○ respectively. **d** Validation of gene expression levels of Slpi by real-time PCR in the normal (n = 6) and tumor tissues (n = 11) of C3H mice. The expression of Slpi is normalized to the expression of rRNA. Statistical significance between the two groups was analyzed by the Student’s t-test. *** Significantly different at p < 0.001.
expression is downregulated in the tumors, has hyper-DMR starting from +929 bp from TSS. We validated the DNA methylation status of these DMRs by bisulfite sequencing (Figs. 2b, 3b, and 4b) and confirmed that the RRBS data correlated well with the bisulfite sequencing data. We also measured gene expression levels of the normal and tumor tissues by real-time PCR and confirmed up-regulation of Mst1r and Slpi and down-regulation of Extl1 in the tumor tissues (Figs. 2c, 3c, and 4c). The expression levels of these genes did not differ between tumor tissues with and without Ha-ras mutation (Additional file 2: Figure S2).

Expressions of Mst1r, Slpi, and Extl1 are upregulated in cancer cell lines by 5-aza-dC treatment
To determine whether DNA methylation alterations in the DMRs of Mst1r, Slpi, and Extl1 are involved in the expression of these genes, we assessed the effects of the DNA methylation inhibitor 5-aza-dC on the DNA methylation statuses and gene expressions in mouse liver cancer cell lines (Fig. 5). Mst1r and Slpi were up-regulated and
hypomethylated at DMRs by culturing Hepa1c1c7 cells with 50 μM and 100 μM of 5-aza-dC (Fig. 5a, c). Expression of Extl1 was not detected in Hepa1c1c7 cells. In Hepa1-6 cells, the three genes were up-regulated and their DMRs were hypomethylated by culturing with 0.1 and 1 μM of 5-aza-dC (Fig. 5b, d, e). These data support the notion that expressions of Mst1r, Slpi, and Extl1 are up-regulated by hypomethylation of DMRs.

Interestingly, susceptibility against 5-aza-dC largely differed between the two cell lines. Measurement of gene expression levels of DNA methyltransferase Dnmt1/3a/3b and DNA demethylation enzymes Tet1/2/3, the major enzymes determining DNA methylation level, clarified that Hepa1-6 cells have significantly lower expression levels of Dnmts in comparison with Hepa1c1c7 cells (Additional file 2: Figure S3). Expression levels of Tet2 was also significantly lower in Hepa1-6 cells compared to Hepa1c1c7 cells (p < 0.01). Tet1 expression was not detected in either cell line. These results suggest that the lowered expression of DNA methyltransferases is involved in the different sensitivity to 5-aza-dC between Hepa1-6 and Hepa1c1c7 cells.

Transcription factor (TF) binding sites in the DMRs of Mst1r and Slpi
DNA methylation of TF binding sites or their proximity near the TSS can affect the binding of transcription factors and regulate transcription of genes [13, 30]. We searched for TF binding sites in the DMRs of Mst1r, Slpi, and Extl1 using the JASPAR database [25] and literature. We found a CpG site overlapping NF-κB binding site in the DMR of Mst1r (Fig. 2a) and one overlapping AhR binding site in the DMR of Slpi (Fig. 3a). Methylation of CpG in the binding sequence for NF-κB [31] and AhR [32, 33] have been reported to inhibit their binding affinities. No CpG site overlapping the TF binding site was detected in the DMR of Extl1 (Fig. 4a).

Overexpression of Mst1r induces robust expression of Il33
We further assessed whether upregulation of Mst1r and Slpi play causal roles in tumorigenesis by overexpressing these genes and analyzing gene expression changes using microarrays in hepa1c1c7 cells.

We selected genes which are statistically significantly up or down regulated more than 10 fold by
overexpression of Mst1r or Slpi, also show the same direction of significant expression changes in the liver tumors of C3H mice, and are found to be related with “cancer” by a PubMed search.

Overexpression of Mst1r induced IL33 which meets all the above criteria (Fig. 6a, b). Real-time PCR confirmed up-regulation of IL33 in the liver tumors of C3H mice (Fig. 6c). No gene was identified under the criteria in the cells overexpressing Slpi.

Mst1r and Slpi are upregulated in human HCC with altered DNA methylation status around the TSS

We analyzed the association between gene expression and DNA methylation status of human HCC by use of TCGA database. Data from 41 pairs of normal livers and HCC tissues were used for each analysis (Additional file 4: Table S3). Expression of Mst1r and Slpi are upregulated in the human HCC dataset we selected (Fig. 7a and b), as reported previously [27, 28]. Similar to the methylation status of Mst1r and Slpi in C3H mice, the downstream regions of TSS in both genes were weakly but significantly hypomethylated in human HCC compared to the normal liver tissues (Fig. 7c). These results showed the possibility that both the genes are regulated by DNA methylation, not only in the liver tumors of C3H mice, but also in human HCC. Furthermore, the expression of IL-33 strongly (r = 0.807), although not significantly (p < 0.1), correlated with MST1R expression in human HCC tissues highly expressing MST1R (Fig. 7d). Correlation was not observed between the expressions of the two genes when all the 41 pairs were analyzed (r = 0.095).

Discussion

In the present study, we investigated the DNA methylation landscape of liver tumors of C3H mice by RRBS analysis. Hypo-DNA methylation is a well-known feature of tumors [34]. The present study clarified a large increase in the number of hypo-DMCs in the liver tumors of C3H mice (Fig. 1b), well illustrating the features of tumors. The present study further identified tumor-specific DMRs, including promoter DMRs for cancer-related genes.

Among the genes with promoter DMRs, we focused on three genes, Mst1r, Slpi and Extl1 (Table 1). Mst1r activation is involved in tumor augmenting signaling pathways, such as the PI3K/AKT and RAS/ERK pathways [27]. Slpi is overexpressed in various types of cancers [28]. While there are studies reporting anti-metastatic activity of Slpi, a large body of studies reported overexpression of Slpi augments or associates with tumor proliferation and invasion in cancers of many origins [28, 35]. Extl1 is reported as a putative tumor-suppressor gene [29]. The present study showed hypo-DMR around +1000 bp from TSS and robust upregulation in Mst1r and Slpi in the tumors (Figs. 2 and 3) and hyper-DMR around +1000 bp...
Human database searches showed hypomethylation of downstream regions of \( MST1R \) and \( SLPI \) with upregulation of their expressions. RNA-seq dataset and DNA methylation dataset of 41 paired normal and tumor tissues of human livers were downloaded from TCGA. a, b Average gene expression ratio (tumor tissues/normal tissues) was calculated using 41 paired data from TCGA. * * Significantly different at \( P < 0.05 \), and \( P < 0.01 \), respectively. c The difference in DNA methylation \( \beta \)-value (tumor – normal) for each CpG around TSS was calculated using 41 paired data from TCGA. The correlation between expressions of \( MST1R \) and \( IL33 \) in the HCC tissues highly expressing \( MST1R \) is highly expressed.
from TSS and downregulation of Ext1 in the tumors (Fig. 4). These DMRs, which are not overlapped with CpG island, were not detected in our previous study on the spontaneous liver tumors of C3H mice using MeDIP-CpG microarrays [12]. Furthermore, this study firstly reported an association between expression changes of these genes and DMRs by an in vitro experiment using 5-aza-dC (Fig. 5).

We analyzed TCGA data for 41 pairs of normal and HCC tissues from patients and found overexpression of MST1R and SLPI and lowered DNA methylation of TSS down-stream region also in human HCC (Fig. 7). On the other hand, a recent study reported downregulation of MST1R and hypermethylation of the TSS upstream region in human hepatoblastomas [36]. The TSS upstream region of MST1R in the human HCC we analyzed was hypermethylated with transcription upregulation (Fig. 7c). Thus, MST1R expression and the association of DNA methylation status differ between HCC and hepatoblastomas. Aiming to target MST1R for cancer therapy, drugs such as tyrosine kinase inhibitors against the tyrosine kinase activity of MST1R and monoclonal antibodies against MST1R are being developed and some of these are under clinical trials or in research phases [27]. The results of the present study encourage further study on DNA methylation status around TSS of MST1R to develop epigenetic therapies.

In the present study, we further identified IL33 as the putative down-stream targets of Mst1r by overexpression of Mst1r into hep1c1c7 cells (Fig. 6c). IL33 was also up-regulated in the tumor tissues of C3H mice (Fig. 6e) and its expression is associated with MST1R expression in higher MST1R expressing group (Fig. 7d). IL33 activates NF-kB and ERK signaling pathways and is implicated in HCC [37]. The present study newly demonstrates the possibility that IL33 is a down-stream target of Mst1r and is involved in the liver tumors of C3H mice and in human HCC.

DNA methylation exerts a variety of modes of action. DNA methylation of TF binding sequences affects the affinity of TFs to DNA [13, 30]. Hypermethylation of the Fas promoter, which contains 3 putative NF-kB binding sites inhibits NF-kB binding [31]. NF-kB pathway is deeply involved in HCC in human [38] as well as C3H mice [39, 40]. The DMR of Mst1r in the C3H liver tumors contained the putative NF-kB consensus sequence (5′-GTGGAGCCCC(G)-3′) and the CpG in the sequence is hypomethylated (Fig. 2b). Thus, hypomethylation of CpGs in the putative NF-kB site in the DMR of Mst1r may take a part in augmenting NF-kB binding and activating Mst1r. The DMR of Slpi includes the core sequence (5′-CGTG-3′) of xenobiotic-responsible element (XRE) and its hypomethylation inhibits the binding of AhR/Arnt complex [32, 33]. Thus, hypermethylation of CpG in the XRE core sequence in Slpi (Fig. 3b) may be involved in Slpi activation through AhR/Arnt binding, while the involvement of AhR/Arnt in spontaneous hepatic tumors is unknown. Contribution of the methylation status in TF binding sites require further studies.

During this study, we found that the sensitivity of Hepa1c1c7 cells and Hepa1-6 cells to 5-aza-dC is largely different (Fig. 5). 5-aza-dC is now utilized as epigenetic medicine to recover the expression of tumor suppressor genes by reducing DNA methylation [41]. 5-Aza-dC is transported into cells by nucleoside transporters, incorporated into DNA after being triphosphorylated by deoxy-cytidine kinase, and inhibits DNMT1 activity by forming a covalent bond with the enzyme or augmenting DNMT1 degradation. We measured the expression of DNA methylation and demethylation enzymes and found significantly lower expression of Dnmt1, Dnmt3a and Dnmt3b in Hepa-1 cells compared to Hepa1c1c7 cells. Thus the suppression of Dnmts may contribute to the difference in the sensitivity of the cells to 5-aza-dC.

Conclusions

The present study clarified the DNA methylation landscape in association with gene expression in the liver tumors of C3H mice. The data provide useful information for further genome-wide studies of mice models of HCC. The present study particularly proposed novel pathways regulated by DNA methylation for Mst1r and Slpi, which may be applied not only to mouse liver tumors but also to human HCC.

Additional files

**Additional file 1:** Table S1. Primers for bisulfite sequencing and qRT-PCR. (XLSX 10 kb)

**Additional file 2:** Figure S1. Scatter plots for sample pairs. Scatter plots of % methylation values for each pair in normal tissues (n = 3) and tumor tissues (n = 3) were obtained using methylKit. Numbers on upper right corner denote pair-wise Pearson’s correlation scores. The histograms on the diagonal are methylation distribution of CpG sites for each sample. (PPTX 296 kb)

**Additional file 3:** Table S2. List of DMRs position and methylation difference between normal and tumor liver tissues. (XLSX 317 kb)

**Additional file 4:** Table S3. Datasets of DNA methylation and gene expression downloaded from TCGA database. (XLSX 13 kb)

**Abbreviations**

5-aza-dC, 5-aza-2′-deoxycytidine; CC4: Carbon tetrachloride; DEN: N-nitrosodiethylnime; DMC: Differentially methylated cytosine; DMT1: Dulbecco’s modified Eagle’s medium; DMR: Differentially methylated region; FBS: Fetal bovine serum; FLC: Fibrolamellar hepatocellular carcinoma; HCC: Hepatocellular carcinoma; MeDIP: Methylated DNA immunoprecipitation; ORF: Open reading frame; RBSS: Reduced representation bisulfite sequencing; TCGA: The Cancer Genome Atlas; TF: Transcription factor; TSS: Transcription start site.
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Availability of data and materials

The datasets generated during the current study are available in the NCBI’s Gene Expression Omnibus, GSE111420 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111420), GSE104627 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104627) and GSE104626 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104626).

Authors’ contributions

KeN, JM and KO designed the study. JM, KO, TsA, ToS, YoH and Gi contributed to animal and cellular experiments. KaN, YuH, TK and iH contributed to next-generation sequencing. JM, KO, KeN and SY performed bioinformatic analysis. JM, KeN, KO and KaN wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal studies were permitted by the Animal Care and Use Committee of National Institute for Environmental Studies (NIES) and were performed in accordance with guideline for the Care and Use of Laboratory Animals of NIES. The results on human HCC shown in Fig. 7 are based on existing data generated by The Cancer Genome Atlas project established by the NCI and NHGRl http://cancergenomenavigov. New experimental material was not used in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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