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Genome-wide analysis of DNA methylation changes induced by gestational arsenic exposure in liver tumors

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Inorganic arsenic is known to be a human carcinogen. Previous studies have reported that DNA methylation changes are involved in arsenic-induced carcinogenesis, therefore, DNA methylation changes that are specific to arsenic-induced tumors would be useful to distinguish tumors induced by arsenic from tumors caused by other factors and to dissect arsenic carcinogenesis. Previous studies have shown that gestational arsenic exposure of C3H mice, which tend to spontaneously develop liver tumors, increases the incidence of tumors in male offspring. In this study we used the same experimental protocol as in those previous studies and searched for DNA regions where methylation status was specifically altered in the liver tumors of arsenic-exposed offspring by using methylated DNA immunoprecipitation–CpG island microarrays. The methylation levels of the DNA regions selected were measured by quantitative methylation-specific PCR and bisulfite sequencing. The results of this study clarified a number of regions where DNA methylation status was altered in the liver tumors in the C3H mice compared to normal liver tissues. Among such regions, we showed that a gene body region of the oncogene *Fosb* underwent alteration in DNA methylation by gestational arsenic exposure. We also showed that *Fosb* expression significantly increased corresponding to the DNA methylation level of the gene body in the arsenic-exposed group. These findings suggest that the DNA methylation status can be used to identify tumors increased by gestational arsenic exposure. (*Cancer Sci* 2013; 104: 1575–1585)

Naturally occurring inorganic arsenic is known to cause serious health problems.[1,2] In many areas in the world, exposure to high concentrations of inorganic arsenic in drinking water has been found to increase the risk of cancer in the skin and several other organs, including the urinary bladder, lung, and liver.[1] Epidemiological studies have also indicated that gestational arsenic exposure is associated with increased incidence of cancers in several organs, including the bladder and liver, in adulthood.[3,4] Despite its high carcinogenicity, arsenic has been documented to be a relatively weak mutagen.[5] Consistent with the notion of arsenic being a weak mutagen, many studies have shown that arsenic induces aberrant epigenetic changes, mainly in DNA methylation and histone modification, and the arsenic-induced epigenetic effects have been implicated in carcinogenesis.[6,7] However, consensus on the epigenetic effects of arsenic remains to be established, as some of these studies are still contradictory and mostly descriptive.[6]

The most studied epigenetic changes in cancer have included global DNA hypomethylation and site-specific DNA hypermethylation.[8] Global DNA hypomethylation is thought to play a role in inducing genome instability and thereby promoting carcinogenesis.[9] Hypermethylation of CpG islands in promoter regions generally results in the transcriptional silencing of genes, and many different tumor suppressor genes have been found to be silenced by promoter hypermethylation in many types of cancers.[8] Alterations in DNA methylation have already been applied as biomarkers that predict patient outcome and to choose treatments that take advantage of many unique features that are different from mutations.[10,11] If there exist alterations in DNA methylation that are specific to arsenic-induced cancers, they should also be useful markers to distinguish arsenic-induced cancers, and provide important clues to the selection of suitable treatments.

The C3H mouse has a high incidence of spontaneous liver tumors and chemically induced liver cancers,[12] so this strain is often used in studies of induction of liver carcinogenesis by chemicals, including arsenic.[12] Giving pregnant mice water containing a 42.5 or 85 ppm concentration of sodium arsenite to drink from days 8–18 of gestation has been found to dose-dependently increase the incidence and numbers of liver tumors in their male offspring when examined at 74 weeks of age.[13] Hypomethylation of the promoter region of *ER*α and upregulation of *ER*α expression have been found in the normal liver tissues of tumor-bearing livers in a gestationally arsenic-exposed group of C3H mice in comparison with the normal liver tissues of control mice.[14] In a study in which we used the same experimental protocol we also detected an increase in liver tumors in C3H male mice,[15] but we did not detect hypomethylation of the promoter region of *ER*α or upregulation of *ER*α expression.[15] These findings indicated that changes in DNA methylation and *ER*α expression are not necessary for liver tumors to increase as a result of gestational arsenic exposure. Thus, it remains to be clarified whether there is an association between DNA methylation changes and increases in liver tumors after gestational arsenic exposure.

In this study we used MeDIP–CpG island microarray assays to identify DNA regions where methylation status is altered in the liver tumors of C3H mice in an experimental model of liver tumor induction by gestational arsenic exposure.[13–15] We then searched for DNA regions where methylation status was specifically altered in the liver tumors of arsenic-exposed C3H mice compared to the liver tumors of control mice by MSP and bisulfite sequencing. We also investigated the expression of genes that contained regions where DNA methylation was altered in the liver tumors of arsenic-exposed C3H mice.

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Materials and Methods

Sample preparation. The normal tissues and tumor tissues were obtained in our previous study[15] from the livers of male 74–84-week-old offspring born to C3H mice mothers given drinking water or water containing 85 ppm sodium arsenite *ad libitum* from day 8 to 18 of gestation. The incidence of liver tumors in the offspring in the control group and arsenic-exposed group was 51% and 41%, respectively. The predominant histological type of tumor was adenoma in both groups.[15] The previous study revealed that the liver tumors in the arsenic-exposed group had a higher rate of Ha-*ras* mutation (71%) compared to the liver tumors in the control group (47%).[15] The mice were handled in a humane manner in accordance with the National Institute for Environmental Studies (Tsukuba, Japan) guidelines for animal experiments.

Genomic DNA extraction. The liver tissues were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 20 mM EDTA, 1% SDS, 0.3 mg/mL proteinase K). After RNase treatment, genomic DNA was purified with phenol chloroform mixture, precipitated with ethanol, dried, and resuspended in buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0.

Methylated DNA immunoprecipitation–CpG island microarray analysis. The MeDIP-CpG island microarray analysis was carried out as previously described.[16] Briefly, genome DNAs were prepared from pooled normal liver homogenates of three control mice and pooled tumor tissue homogenates of three arsenic-exposed mice. Five micrograms of genome DNA of each group was immunoprecipitated with an anti-5-methylcytidine antibody (Diagenode, Liége, Belgium), and the precipitated DNA and the input DNA were labeled with Cy5 and Cy3, respectively. A mouse CpG island microarray that contains 97 652 probes covering 12 573 genes (Agilent Technologies, Santa Clara, CA, USA) was hybridized with the labeled probes and scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). Scanned data were processed with Feature Extraction version 9.1 and Agilent G4477AA ChIP Analytics 1.3 software (both Agilent Technologies). A signal of a probe was converted into a ''Me-value'' which represented the methylation level as a value from 0 (unmethylated) to 1 (methylated).[16] Differentially methylated regions were detected by comparison of the Me-values of the two groups.

Methylated control DNA and unmethylated control DNA. Fully methylated control DNA (CpG methylated NIH3T3 genomic DNA) was purchased from New England Biolabs (Ipswich, MA, USA), and fully unmethylated control DNA was prepared by amplifying NIH3T3 genomic DNA using GenomiPhi DNA Amplification Kit (GE Healthcare Bio-Sciences, Little Chalfont, UK).

Bisulfite treatment. Bisulfite treatment was carried out as previously described.[17] Briefly, genomic DNA, methylated control DNA, and unmethylated control DNA, prepared as described above, were digested with *Eco*RI and incubated with freshly prepared 0.3 M NaOH for 15 min. To this solution was added sodium metabisulfite, pH 5.0, and hydroquinone to give final concentrations of 2.0 M and 0.5 mM, respectively. The mixture was incubated for 16 h at 50°C in the dark. The samples were desalted with the Wizard DNA Clean-up System (Promega, Madison, WI, USA), and the bisulfite reaction was terminated by adding NaOH to give a final concentration of 0.3 M and incubating for 15 min at 37°C. The DNA was then precipitated with ethanol, dried, and resuspended in buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0.

Methylation-specific PCR. Methylation-specific PCR was carried out by using bisulfite-treated DNA and specific primers. The primer sequences and annealing temperatures used for

Table 1. Oligonucleotide primers for methylation-specific PCR, bisulfite sequencing, and real-time RT-PCR

Gene symbol	Status	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temp. (°C)
Fosb	M	tagagttggagtcggagatcgtc	gaccaacgaacctaaccccg	60
	U	tagagttggagttggagattg	ccaaaaccatcttccttaaca	60
	BS	ttgttgggatttattaggaaattga	aaacttaaacttcactatatataaaaaaaa	52
	RT	gtgcgagcttccttgttttc	gtctccaacagccagaggag	60
Btd	M	aagttttagcgttaaatatatc	aacaaactcgatatttacctcg	56
	U	gaattgtgtttaaaatttagttgt	aaaacaaactcaatatttacctca	56
	BS	agggagagagtttttatggttgtt	aactaaacttcaaaacatttccaaaac	56
Mab21l2	M	atatgtttcgttttcgtttatc	ataatctcgcccaccgaattcg	60
	U	agatatgttttgtttttgtttatt	aaataatctcacccaccaaattca	60
	BS	tttgggttattagtgtttgg	caaactaatttaccaactcaataaa	52
Fam43a	M	agatgaagttgatcgtgagtgc	ctcataccgatatacccaaacg	56
	U	ttaaagtttttgtttgggtatatt	aaacatttaaactctaccaaaaca	56
Hspa2	M	agagtattaatttcgacgaggc	cataacaccgccaactatttcg	56
	U	taagagtattaattttgatgaggt	atcataacaccaccaactatttca	56
Il1r1	M	ttcggttgttttcggtttgaac	ttctcctacctaaacgtacccg	56
	U	gttttggttgtttttggtttgaat	ttttctcctacctaaacataccca	56
Cyp26a1	M	tgcggagtcggagtaggggaac	actctataacctaaaaccgacg	56
	U	gttggagtaggggaatgtaagttt	aacctaaaaccaacaaatcttaaca	56
Adam11	M	tattgggaggcgttcgagtttc	taaataaaaaacgtcgcttacg	52
	U	gttattgggaggtgtttgagtttt	aataaataaaaaacatcacttaca	56
B4galt6	M	attagagagcgattggagattc	ttaaaacctaaaccgtaacccg	56
	U	taattagagagtgattggagattt	atttaaaacctaaaccataaccca	56
Cdh8	M	gtggagtttggtgcggggatgc	ataaacctaaacgtcaacaccg	60
	U	ttgtggagtttggtgtggggatgt	acataaacctaaacatcaacacca	60
Tram1l1	M	ttttgattgatcggtcggtagc	aattcctaacgttcttcttacg	52
	U	gtttttgattgattggttggtagt	aaaattcctaacattcttcttaca	52
18S rRNA	RT	taccacatccaaggaaggcag	tgccctccaatggatcctc	64

BS, primers for bisulfite sequencing; M, primers specific to methylated DNA; RT, primers for real-time RT-PCR; temp., temperature; U, primers specific to unmethylated DNA.

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MSP are shown in Table 1. The gel images were captured and visualized as described previously.[18]

Real-time MSP. Real-time MSP was carried out by using bisulfite-treated DNA and specific primers. The primers were designed for the gene body region in which Me-value changes were detected by MeDIP–CpG island array assays. The approximate positions from transcription start site of the primers of *Fosb*, *Btd*, and *Mab21l2* are indicated by arrows in Figures 2–4. The primer sequences and annealing temperatures used for real-time MSP are shown in Table 1. LightCycler 480 software, version 1.5 (Roche Diagnostic, Basel, Switzerland) was used to compare amplification in bisulfite-treated tissue samples during the log-linear phase of the standard curve based on the results obtained in a dilution series of bisulfite-treated control DNA. The PCR efficiencies were calculated from measurements of bisulfite-treated fully methylated DNA by using M primers, or measurements of bisulfite-treated fully unmethylated DNA by using U primers. All of the primers yielded similar efficiencies. DNA methylation levels were calculated as percentages as follows: (amount of DNA amplified with M primers) / [(amount of DNA amplified with M primers) + (amount of DNA amplified with U primers)] × 100.

Bisulfite sequencing analysis. Bisulfite sequencing was carried out as previously described.[17] Briefly, the bisulfite-treated DNA was amplified with the primers shown in Table 1. The PCR products were cloned into pGEM-T Easy vector (Promega). Fourteen to 16 clones from each sample were cycle sequenced with M13RV primers and a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and analyzed with an Applied Biosystems 3730 DNA analyzer (Applied Biosystems).

Preparation of cDNA and real-time PCR. Total RNA of livers was prepared with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The purity of RNA was checked by confirming no amplification of target DNA occurs before reverse transcription. Reverse transcription reaction was carried out using the TaKaRa RNA PCR Kit (AMV) version 3.0 (TaKaRa Bio, Shiga, Japan). Quantitative real-time PCR analysis was carried out on a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland) as described previously.[19] The primer sequences and annealing temperatures used for real-time PCR are shown in Table 1.

Global DNA methylation analysis. Global DNA methylation analysis was carried out as previously described.[20] Genomic DNA was denatured by heating at 98°C for 3 min. The solution was mixed with 2 U nuclease P1 (Wako, Osaka, Japan) and 100 mM ammonium acetate to a final concentration of 10 mM and incubated at 45°C for 2 h. Next, the solution was supplemented with 0.002 U phosphodiesterase I (Worthington Biochemical, Lakewood, NJ, USA) and 1 M ammonium bicarbonate to a final concentration of 100 mM and incubated at 37°C for 2 h and then 0.5 U alkaline phosphatase (Promega) was added and incubation was continued at 37°C for an additional 1 h. A 100 mM ammonium bicarbonate solution was added to make a total volume of 100 μL, and the mixture was filtered with a Microcon Ultracel YM-10 cartridge (Millipore, Billerica, MA, USA). The LC–MS analyses were carried out by using an LC/MS-2010A mass spectrometer and ESI ionization probe (Shimadzu, Kyoto, Japan). Deoxycitidine and 5medC were separated by using a reversed-phase column (Atlantis dC18, 2.1 × 150 mm, 5 μm; Waters, Milford, MA, USA) in isocratic mode with a mobile phase of methanol : 10 mM ammonium acetate (2:98, v/v) at a flow rate of 0.2 mL/min. The prepared internal standards ($^{13}C_9$,$^{15}N_3$-2′-deoxycytidine, $^{13}C_{10}$,$^{15}N_2$-5- methyl-2′-deoxycytidine, 100 ng each) were added to 15 μL of the hydrolyzed sample, and the mixture was diluted to 500 μL with H_2O. A 10 μL volume of the mixture was injected into HPLC–MS, and dC and 5medC were analyzed on a SIM mode.

Statistical analysis. Comparisons were carried out using a two-tailed paired Student's *t*-test. A *P*-value <0.05 was considered to be statistically significant. A ROC curve was plotted for detection of liver tumors that were affected by gestational arsenic exposure, and Youden index (sensitivity + specificity−1) was calculated.[21]

Results

Microarray analysis of DNA regions where methylation status was altered in liver tumors of C3H mice. Although DNA methylation patterns are generally fairly stable,[10] DNA methylation

Table 2. List of primary annotation identified by methylated DNA immunoprecipitation–CpG island microarray as having different methylation status between normal livers of mice in control group and liver tumors of mice in arsenic-exposed group

Location		Primary annotation	Primary annotation type	Average Me-value		
Chr	nt number			Tumor tissue	Normal tissue	Difference
1	111810301–111810709	Cdh7	PROMOTER	0.52	0.19	0.33
1	040211362–040211474	Il1r1	INSIDE	0.18	0.54	−0.35
3	086633247–086633682	Mab21l2	INSIDE	0.64	0.17	0.47
3	124313191–124313473	Tram1l1	INSIDE	0.55	0.19	0.36
6	118559274–118559490	ENSMUST00000078320.4	Unknown	0.49	0.16	0.34
6	030889400–030889519	ENS MUST00000101589.1	Unknown	0.54	0.12	0.41
6	110611869–110612025	Grm7	INSIDE	0.48	0.17	0.32
7	018463460–018463833	Fosb	INSIDE	0.60	0.18	0.42
8	102305418–102305602	Cdh8	INSIDE	0.50	0.16	0.34
11	102590006–102590400	Adam11	INSIDE	0.18	0.71	−0.54
11	033413037–033413188	Ranbp17	INSIDE	0.48	0.18	0.31
12	077324539–077324770	Hspa2	INSIDE	0.53	0.17	0.36
14	030490675–030491319	Btd	INSIDE	0.62	0.18	0.44
16	030520656–030520955	Fam43a	INSIDE	0.20	0.69	−0.49
18	020888422–020888579	B4galt6	INSIDE	0.17	0.51	−0.34
19	037764225–037764481	Cyp26a1	INSIDE	0.54	0.18	0.35

Chr, chromosome number; INSIDE, probe position is downstream within 10 kb of transcription start site and in translated region; Normal liver, normal livers of mice in control group; nt number, nucleotide number in the NCBI database (NCBI36/mm8); PROMOTER, probe position is within 10 kb upstream of transcription start site; Tumor tissue, tumor tissue from tumor-bearing livers of mice in arsenic-exposed group.

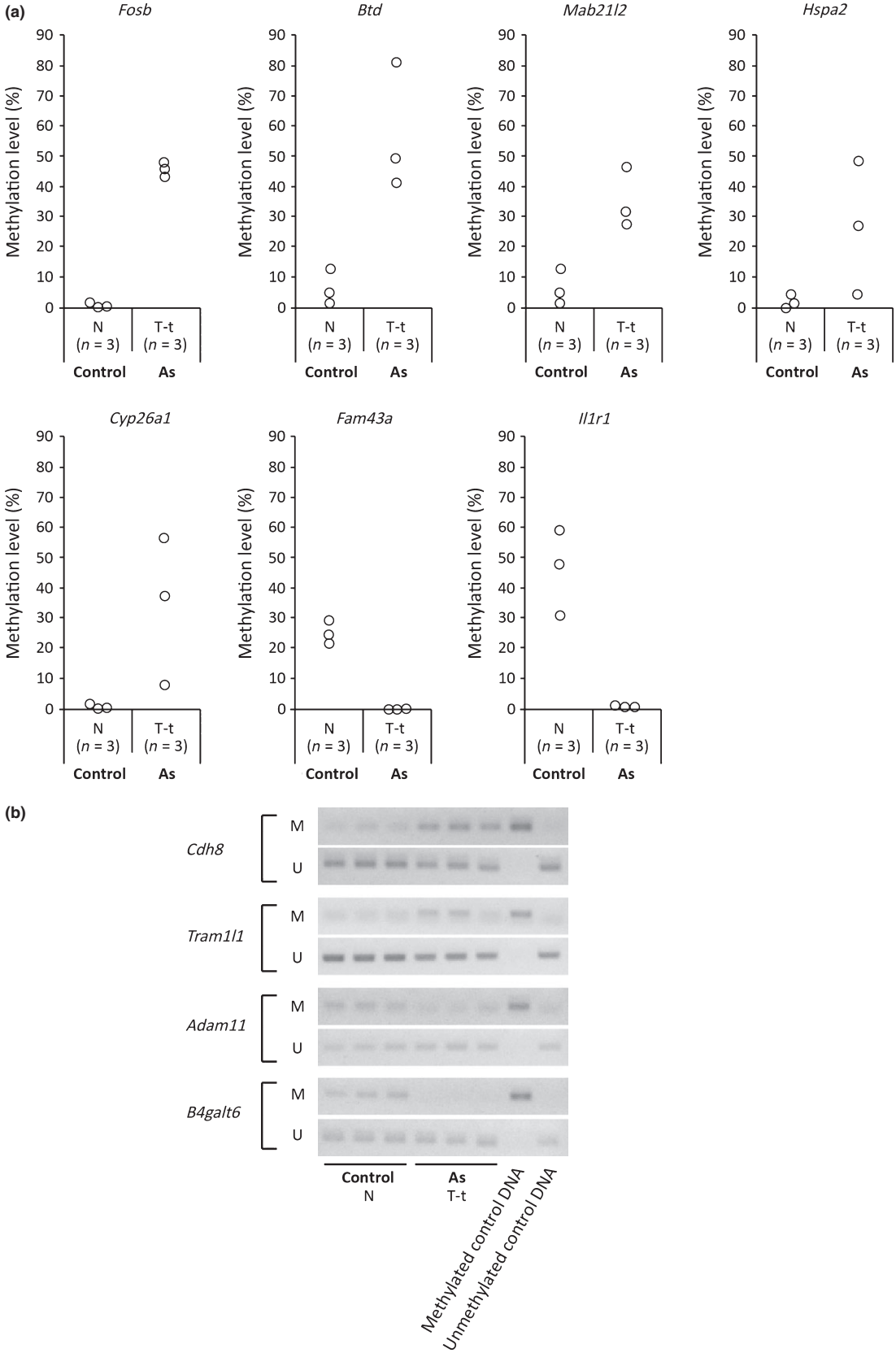

Fig. 1. Validation of data obtained by methylated DNA immunoprecipitation–CpG island microarray analysis by real-time methylation-specific PCR (MSP). Individual genome samples that were used for the microarray analyses (obtained from normal livers of three control mice [Control-N] and tumor tissues from tumor-bearing livers of three gestationally arsenic-exposed mice [As T-t]) were examined by real-time MSP. (a) Methylation levels of DNA regions were measured by real-time MSP. (b) Methylation changes of DNA regions were measured by MSP. Electrophoresis patterns of each DNA region are shown. M, M primer set; U, U primer set.

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changes have been shown to be involved in carcinogenesis and tumorigenesis.[8–11] We assumed that arsenic exposure may target, by changing the status of tumor, the DNA methylation regions that are affected by tumorigenesis. Thus, we first detected DNA regions where methylation status was altered in the liver tumors by using MeDIP–CpG island microarray analysis. To do so, we compared the DNA methylation status of genome DNAs prepared from pooled normal liver homogenates of three control mice and pooled tumor tissues homogenates of three arsenic-exposed mice. The Me-values were used to represent methylation levels.

We selected regions where two or more consecutive probes in the control group or arsenic group yielded Me-values of 0.20 or less and where the differences in the Me-values of corresponding probes in the two groups were 0.30 or more. According to the criteria, 16 regions were selected, as shown in Table 2. We found that the DNA methylation level in 12 regions was higher, and the level in four regions was lower, in the tumors of the arsenic group than in normal livers of the control group. The results also showed that DNA methylation was mainly altered in the gene body (annotated as "INSIDE" in Table 2).

In order to validate the data obtained by the MeDIP–CpG island microarray analyses and to measure the methylation levels of those regions in each liver tissue, we established real-time MSP primers for seven regions (*Il1r1*, *Mab21l2*, *Fosb*, *Hspa2*, *Btd*, *Fam43a*, and *Cyp26a1*) and measured the methylation levels of individual genome samples. The DNA methyla-

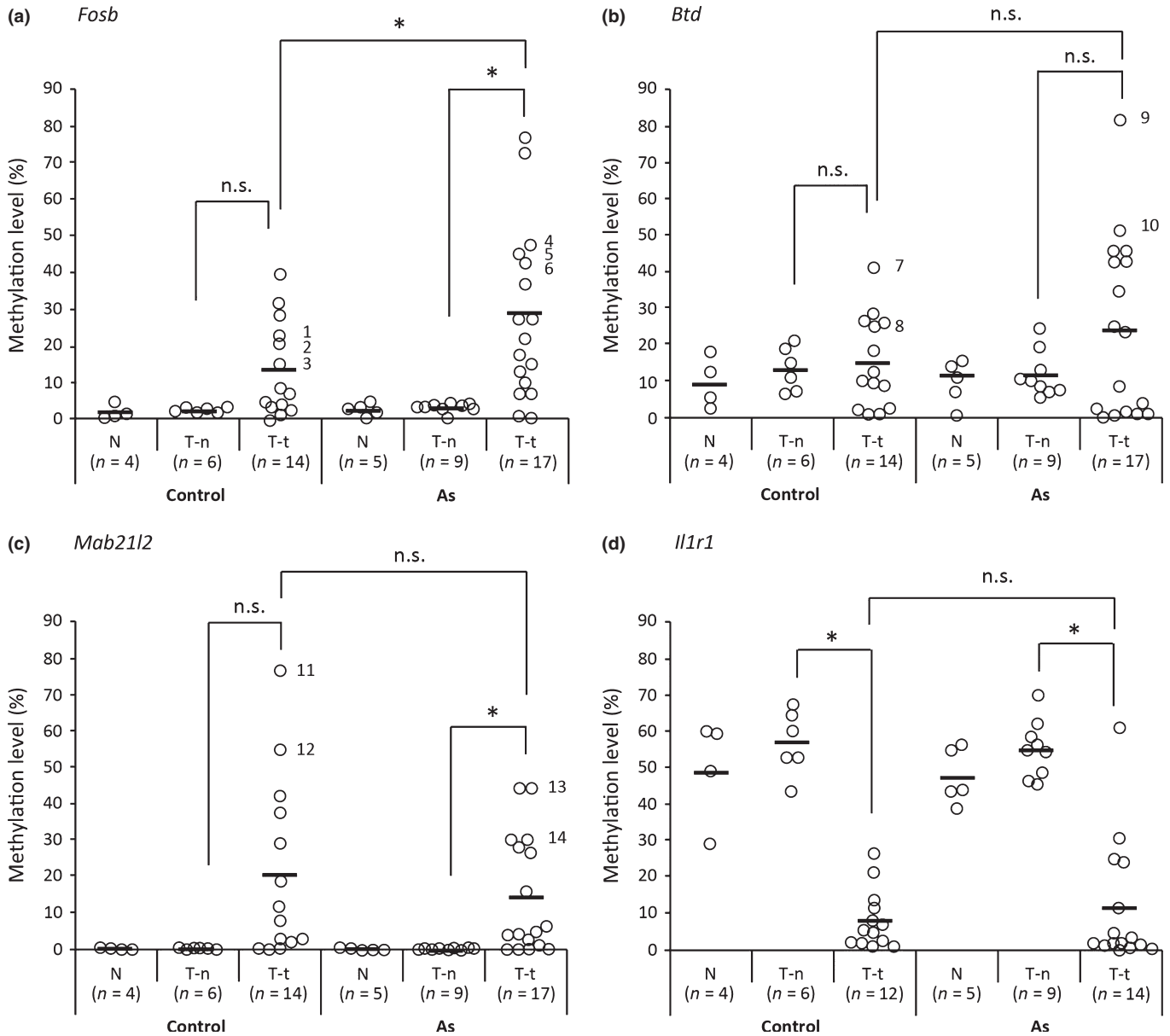

Fig. 2. Methylation levels of DNA regions in liver tissues from control mice and gestationally arsenic-exposed mice. Methylation levels of DNA regions of *Fosb*, *Btd*, *Mab21l2*, and *Il1r1* in tissues from normal livers (Control-N, As-N), normal tissues from tumor-bearing livers (Control T-n, As T-n), and tumor tissues from tumor-bearing livers (Control T-t, As T-t) were measured by real-time methylation-specific PCR. The distributions of the methylation levels of the DNA region of *Fosb* (a), *Btd* (b), *Mab21l2* (c), and *Il1r1* (d) are shown, and the horizontal lines in the chart represent the average methylation level of the liver tissues from each source. Statistically significant differences between the two groups (Control T-n *vs.* Control T-t, As T-n *vs.* As T-t, or Control T-t *vs.* As T-t) were analyzed by Student's *t*-test. *$P < 0.05$ was considered statistically significant. n.s., not significant. 1–14, sample numbers.

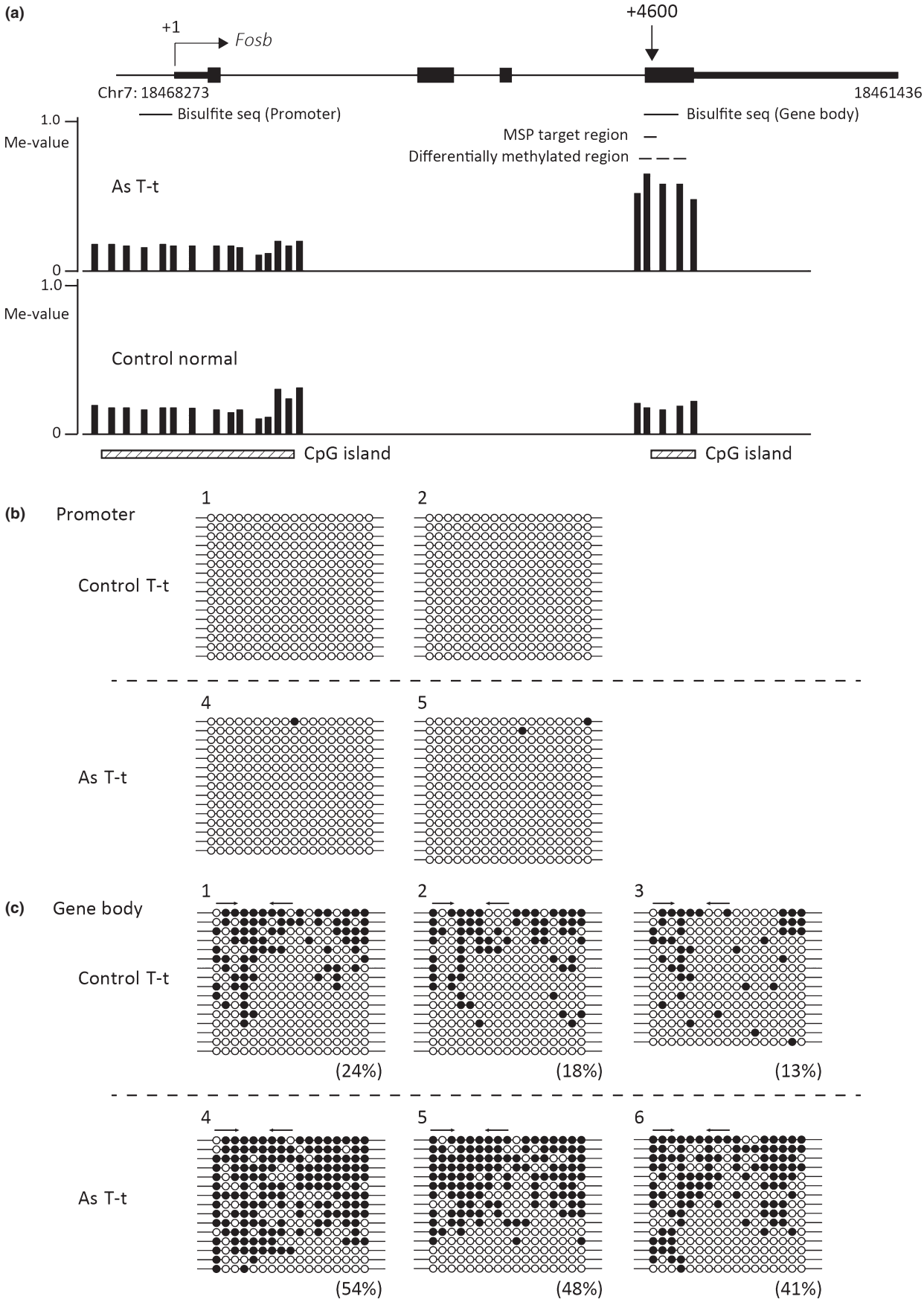

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Fig. 3. (Shown on previous page) Bisulfite sequencing (seq) analysis of the *Fosb* region in tumor tissues from tumor-bearing livers of control mice (Control T-t) and gestationally arsenic-exposed mice (As T-t). (a) Schematic representation of the *Fosb* locus. The vertical arrow indicates the approximate position of methylation-specific PCR (MSP) primers from the transcription start site (+1). The black boxes indicate the transcribed region of *Fosb*. Vertical bars show methylation levels (Me-values) of each probe. Dashed lines show the areas where two or more consecutive probes in the control group or arsenic group yielded Me-values of 0.20 or less and where the differences in the Me-values of corresponding probes in the two groups were 0.30 or more. The MSP primers were designed in the area shown by a black line. This figure was drawn by the UCSC Genome Browser (http://genome.ucsc.edu/) on NCBI36/mm8 assembly that shows the *Fosb* locus on chromosome 7 (Chr7). The bisulfite sequencing data of samples 1, 2, 4, and 5 in the promoter region (b) and samples 1–6 in the gene body region (c) are shown. The percentages in parentheses are the DNA methylation levels of each MSP primer region calculated from the data of bisulfite sequencing. The arrows indicate the positions of the MSP primers. ○, Unmethylated cytosine; ●, methylated cytosine.

tion levels were calculated as described in "Materials and Methods" and are summarized in Figure 1. The results showed that the methylation levels of the seven DNA regions were consistent with the data obtained by the MeDIP–CpG island microarray analysis (Fig. 1a). We also obtained ordinary MSP primers for four additional regions and analyzed the MSP products by electrophoresis (Fig. 1b). The results also supported those of the MeDIP–CpG island microarray analyses.

Real-time MSP analyses of individual samples. We then analyzed the DNA methylation levels of individual genomic samples obtained from the normal livers and the normal tissues and the tumor tissues from tumor-bearing livers of the control group and arsenic group by real-time MSP. We investigated the DNA regions of *Fosb, Btd, Mab21l2*, and *Il1r1,* where MeDIP–CpG island microarray analyses showed higher Me-values in the tumor tissues than in the normal tissues, and the region of *Il1r1*, where the microarray analysis showed lower Me-values in the tumor tissues than in the normal tissues (Fig. 2). The comparison between the normal tissues and the tumor tissues in tumor-bearing livers showed that the DNA methylation levels of the *Fosb* and *Mab21l2* regions in the arsenic group and *Il1r1* region in both the control and arsenic groups were significantly different (Fig. 2).

Comparison of the DNA methylation level of tumor tissues between the control group and the arsenic-exposed group for the seven regions showed that the DNA methylation level of the *Fosb* region was significantly different in the two groups (Figs. 2,S1). We further plotted the ROC curve for detection of liver tumors that were affected by gestational arsenic exposure. The area under the ROC curve was 0.69, and the cut-off value at which the Youden index showed maximum (sensitivity = 0.65, specificity = 0.64, and Youden index = 0.29) was 0.15.

Analysis of DNA methylation status by bisulfite sequencing. Additionally, we carried out bisulfite sequencing to evaluate the methylation pattern of the DNA regions of *Fosb*, *Btd*, and *Mab21l2*. The results showed that the DNA regions of *Fosb* (Fig. 3) and *Mab21l2* (Fig. 5) exhibited a mosaic pattern of DNA methylation and that the DNA region of *Btd* consisted of densely methylated DNA and unmethylated DNA (Fig. 4). The DNA methylation levels measured by real-time MSP almost completely paralleled the DNA methylation levels calculated by bisulfite sequencing (Figs 2–5).

Upregulation of *Fosb* mRNA in liver tumors of gestationally arsenic-exposed mice. As the DNA methylation level of *Fosb* was found to be altered between the tumor tissues in the control group and arsenic group, we measured the expression of *Fosb* by real-time RT-PCR. The results showed that the mRNA level of *Fosb* in tumors of the arsenic-exposed mice was approximately 10 times higher than in other tissues of the control and arsenic groups (Fig. 6).

It was recently reported that gene body DNA methylation corresponded to upregulation of gene expression.[22–24] In this study, the increase in DNA methylation in the gene body of *Fosb* was accompanied by an increase in expression of *Fosb* mRNA in the liver tumors of the gestationally arsenic-exposed mice (Figs 3,6).

Global DNA methylation analysis based on LC/ESI–MS measurements. Aberrant DNA methylations, such as global DNA hypomethylation (lower percentage of 5-methylcytosine) and region-specific hypermethylation, are often associated with cancer.[8] To determine the global DNA methylation status of liver tissues, we precisely measured the level of 5medC in liver genome DNA by LC/ESI–MS and calculated 5 methylcytosine (5meC) content as a percentage of total cytosine. The results showed a significantly lower percentage of 5meC in the tumor tissues than in the tissues from normal livers and the normal tissues from tumor-bearing livers in the control group (Table 3), but we did not observe any significant difference among those tissues in the arsenic group (Table 3).

Discussion

In this study the results of MeDIP–CpG island microarray analyses and real-time MSP indicated that there exist several DNA regions where methylation levels were significantly different between the non-tumor tissues and tumor tissues in control C3H mice and/or C3H mice gestationally exposed to arsenic (Table 2, Fig. 2). This is the first report on the genome-wide DNA methylation analysis of liver tumors in C3H mice. A study by Cui *et al.* reported that chronic exposure of male A/J mice to inorganic arsenic increased their incidence of lung tumors compared with control mice.[25] They also reported that MSP analyses of the promoter region of the tumor suppressor genes $p16^{Ink4a}$ and *RASSF1A* showed higher methylation in lung tumors of the arsenic-exposed group than in the control group.[25] In this study, we newly clarified that the methylation level of the gene body region of *Fosb* was significantly higher in the liver tumor tissues of C3H mice gestationally exposed to arsenic than in the tumor tissues of control mice (Fig. 2a).

Real-time PCR analyses of tumor tissue revealed significantly increased expression of the oncogene *Fosb* in gestationally arsenic-exposed mice (Fig. 6). Hypermethylation of CpG islands in promoter regions generally results in transcriptional silencing, however, increased DNA methylation of the CpG island of gene bodies is reported to be associated with increased gene expression.[22–24] In this study, the increase in DNA methylation in the gene body of *Fosb* was found to be associated with increased expression of *Fosb* in tumor tissues of the arsenic-exposed group (Fig. 2). In contrast, the CpG island of the *Fosb* promoter region was completely unmethylated in normal tissues from the tumor-bearing livers and tumor tissues of both the control group and arsenic group (Fig. 3 and data not shown). Thus, the upregulation of *Fosb* in the tumor tissues of the arsenic group may be associated with increased DNA methylation in the gene body. Previous studies suggested several explanations for the linkage between increased DNA methylation in the gene body and increased gene expression. An interpretation is that upregulated transcription may facilitate de novo DNA methylation in the gene body, and an alternative explanation is that gene body methylation represses the expression of antisense transcripts that would downregulate the expression of sense transcripts.[23,24,26] However, experimental evidence is yet to be obtained.

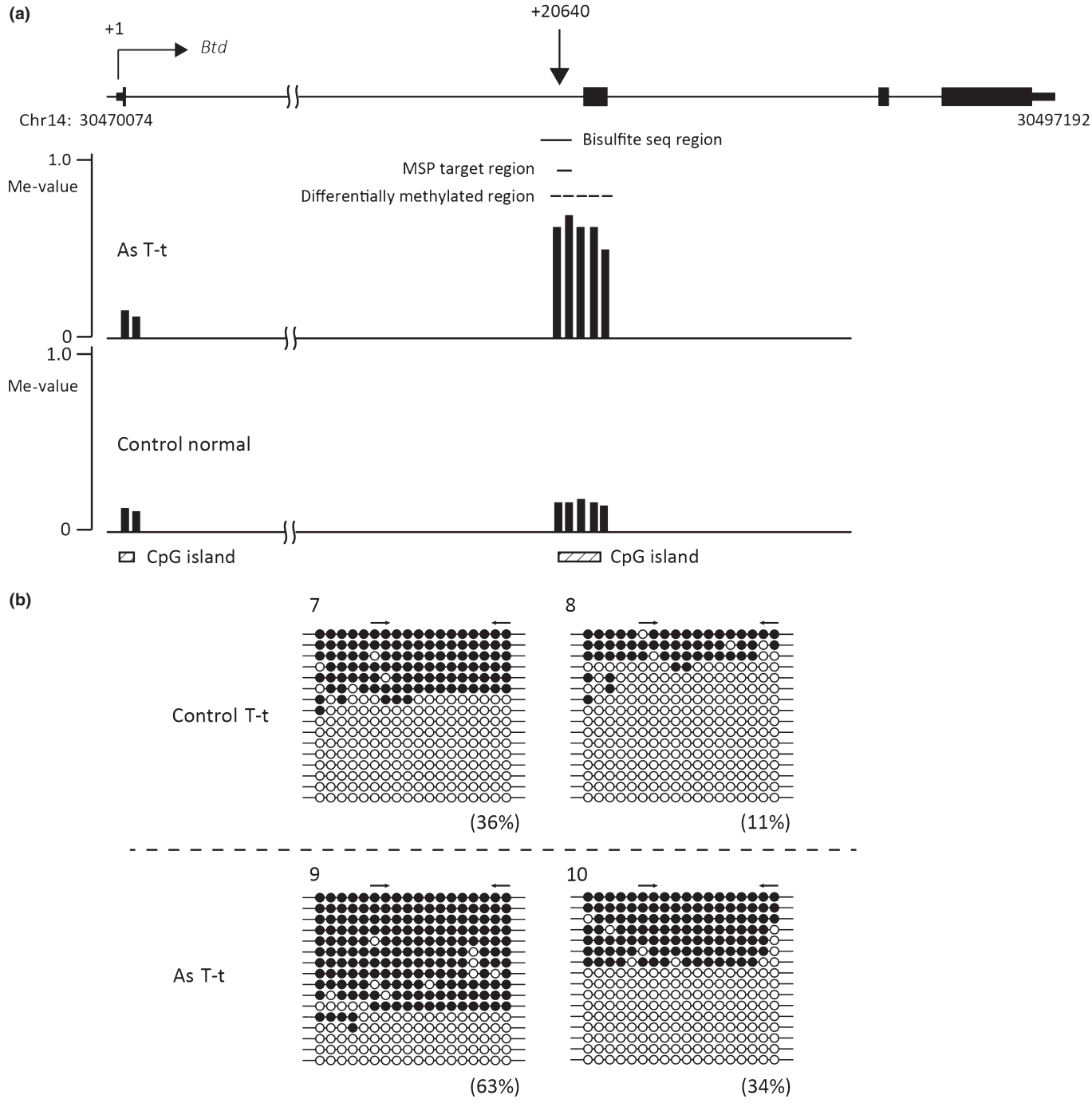

Fig. 4. Bisulfite sequencing (seq) analysis of the *Btd* region of tumor tissues from tumor-bearing livers of control mice (Control T-t) and gestationally arsenic-exposed mice (As T-t). (a) Schematic representation of the *Btd* locus. The vertical arrow indicates the approximate position of methylation-specific PCR (MSP) primers from the transcription start site (+1). The black boxes indicate the transcribed region of *Btd*. Vertical bars show methylation levels (Me-values) of each probe. Dashed lines show the areas where two or more consecutive probes in the control group or arsenic group yielded Me-values of 0.20 or less and where the differences in the Me-values of corresponding probes in the two groups were 0.30 or more. The MSP primers were designed in the area shown by a black line. This figure was drawn by the UCSC Genome Browser (http://genome.ucsc.edu/) on NCBI36/mm8 assembly that shows the *Fosb* locus on chromosome 14 (Chr14). (b) Bisulfite sequencing data of samples 7–10. The percentages in parentheses are the DNA methylation levels of each MSP primer region calculated from the data of bisulfite sequencing. The arrows indicate the positions of the MSP primers. ○, Unmethylated cytosine; ●, methylated cytosine.

Fosb is a member of the Fos family of proteins, which constitutes transcription factor complex AP1. AP1 is activated by ERK1/2-mediated phosphorylation and is involved in tumor formation.[27] Our recent study, carried out in the same model as used in this study, revealed that liver tumors in mice gestationally exposed to arsenic harbored a higher rate of Ha-*ras* mutation than tumors of control mice.[15] Ha-*ras* is activated on mutation and in turn activates the Raf–ERK signaling pathway,[28] which leads to AP1 activation.[29] The upregulation of *Fosb* seems to be a downstream event of Ha-*ras* mutation and to be involved in the increase in liver tumors as a result of gestational arsenic exposure. As the area under the ROC curve

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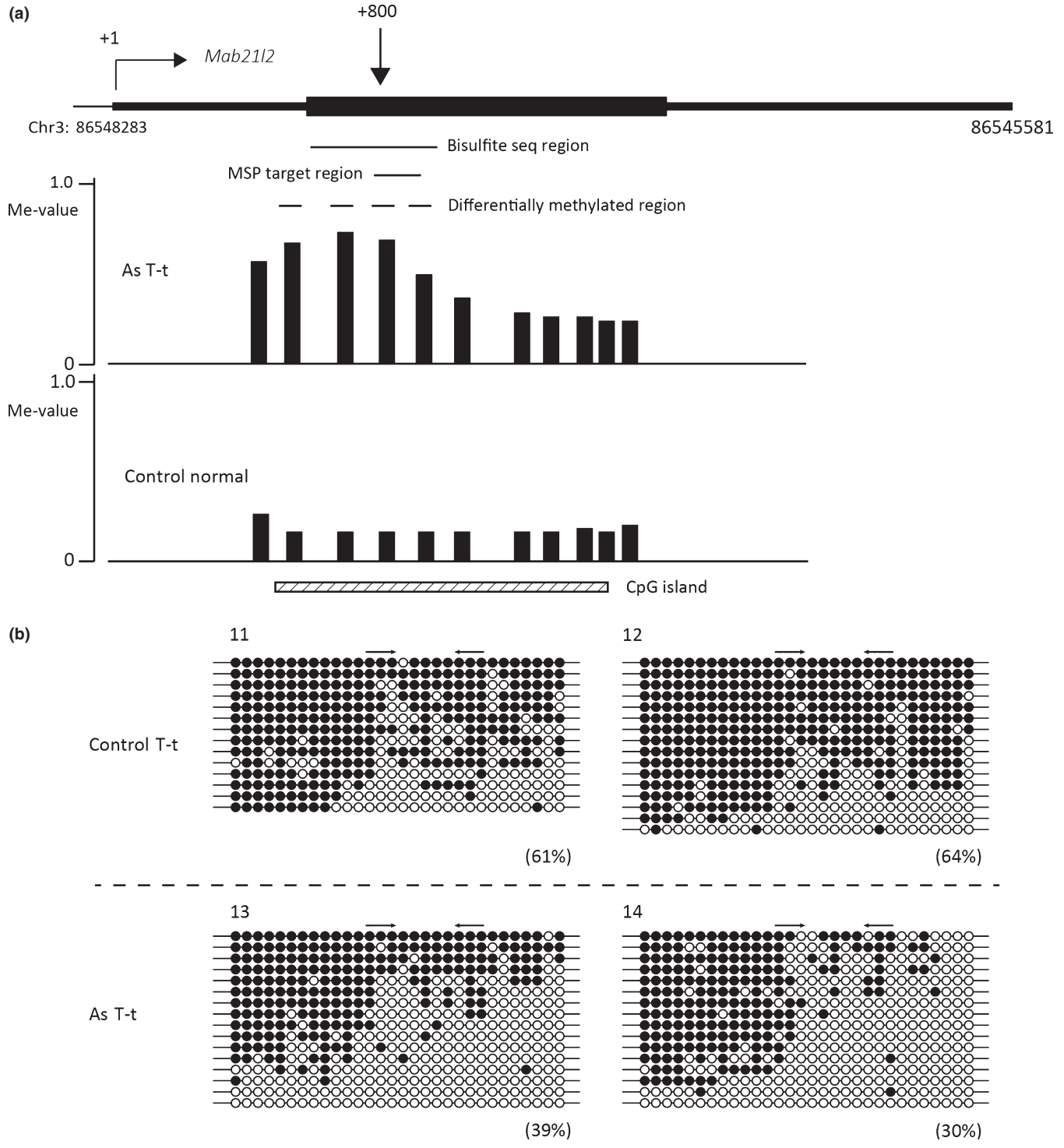

Fig. 5. Bisulfite sequencing (seq) analysis of the *Mab21l2* region of tumor tissues from the tumor-bearing livers of control mice (Control T-t) and gestationally arsenic-exposed mice (As T-t). (a) Schematic representation of the *Mab21l2* locus. The vertical arrow indicates the approximate position of methylation-specific PCR (MSP) primers from the transcription start site (+1). The black boxes indicate the transcribed region of *Mab21l2*. Vertical bars show methylation levels (Me-values) of each probe. Dashed lines show the areas where two or more consecutive probes in the control group or arsenic group yielded Me-values of 0.20 or less and where the differences in the Me-values of corresponding probes in the two groups were 0.30 or more. The MSP primers were designed in the area shown by a black line. This figure was drawn by the UCSC Genome Browser (http://genome.ucsc.edu/) on NCBI36/mm8 assembly that shows the *Mab21l2* locus on chromosome 3 (Chr3). (b) Bisulfite sequencing data of samples 11–14. The percentages in parentheses are the DNA methylation levels of each MSP primer region calculated from the data of bisulfite sequencing. The arrows indicate the positions of the MSP primers. ○, Unmethylated cytosine; ●, methylated cytosine.

for the DNA methylation status of the *Fosb* region in tumors was not large, that region alone may not be a potent marker to distinguish tumors affected by arsenic. However, the gene body methylation status in conjunction with the gene expression of *Fosb* would give a promising clue to diagnose those tumors affected by gestational arsenic exposure.

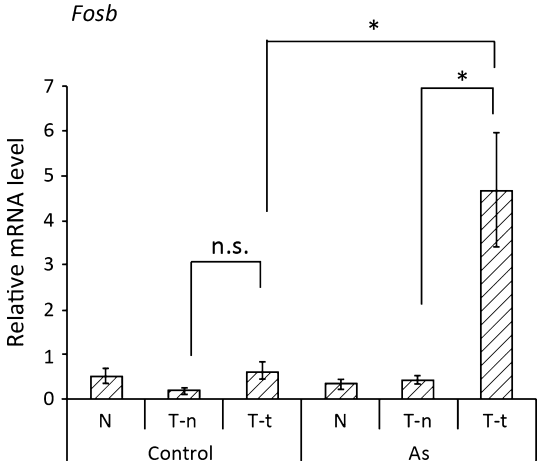

Fig. 6. Increased *Fosb* expression in the liver tumors of gestationally arsenic-exposed mice. Expression of *Fosb* in tissues from normal livers (Control-N, As-N), normal tissues from tumor-bearing livers (Control T-n, As T-n), and tumor tissues from tumor-bearing livers (Control T-t, As T-t) was measured by real-time PCR. The *Fosb* expression levels were normalized to the levels of *18S rRNA* expression. Results are reported as means ± SE ($n = 6$). Statistical significance between the two groups (Control T-n *vs*. Control T-t, As T-n *vs*. As T-t, or Control T-t *vs*. As T-t) was analyzed by Student's *t*-test. *$P < 0.05$ was considered statistically significant. n.s., not significant.

Table 3. Proportion (%) of 5-methylcytosine in total cytosine (cytosine + 5-methylcytosine) from normal livers (Normal), normal tissues from tumor-bearing livers (T-n), and tumor tissues from tumor-bearing livers (T-t) in control and gestationally arsenic-exposed mice (As)

	Normal	T-n	T-t
Control (%)	4.63 ± 0.04 (100)	4.65 ± 0.07 (100.4)	4.29 ± 0.09* (92.7)
As (%)	4.61 ± 0.06 (100)	4.62 ± 0.06 (100.2)	4.46 ± 0.07 (96.7)

*Significant differences from Control normal and Control T-n at *$P < 0.01$. The number in the parenthesis indicates the value of each sample compared to the value in the Normal groups, in which it was set as 100 for both Control and As mice. Results are reported as means ± SE ($n = 6$).

In the present study, we also measured the contents of 5meC in the liver tissues. Although the tumor tissues of the control group showed significantly lower levels of 5meC compared to normal tissues, as reported in previous studies,[8] the 5meC level of tumor tissues in the arsenic group was not significantly reduced. These results may be attributable to the effects of arsenic on the function of machineries involved in the maintenance of DNA methylation. Another explanation would be that arsenic affects the status of tumor, which indirectly leads to the alteration in the level of 5meC.

In summary, we carried out MeDIP–CpG island microarray analyses to dissect the DNA methylation status in liver tumors of C3H mice gestationally exposed to arsenic. Validation of the results of microarray analyses by real-time MSP indicated that the microarray method correctly depicted the changes in DNA methylation in the genome. As a result of the analyses, we revealed that the gene body region of the oncogene *Fosb* is highly methylated and its expression is greatly increased in the tumors of gestationally arsenic-exposed mice, compared with the tumors of control mice. These results suggest that DNA methylation changes are involved in the alteration in tumor phenotype by gestational arsenic exposure.

The study first compared the DNA methylation statuses of normal tissues from the control group and tumor tissues from the arsenic group by MeDIP–CpG island microarray analyses. However, the combination may not have identified the DNA regions where the methylation statuses were largely altered in tumors from the control group. Thus, further precise study would potentially clarify additional DNA methylation regions that discriminate arsenic-induced tumors from other tumors. Application of next generation sequencing-based methods, such as reduced representation bisulfite sequencing[30] and whole genome bisulfite sequencing,[31] would be useful to improve the resolving power and sensitivity of analysis of the present study.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

5meC	5-methylcytosine
5medC	5-methyldeoxycytidine
Btd	biotinidase
Cyp26a1	cytochrome P450, family 26, subfamily a, polypeptide 1
dC	deoxycytidine
ERα	estrogen receptor α
ESI	electrospray ionization
Fam43a	family with sequence similarity 43, member A
Fosb	FBJ osteosarcoma oncogene B
Hspa2	heat shock protein 2
Il1r1	interleukin 1 receptor, type I
LC	liquid chromatography
Mab21l2	Mab-21-like 2
MeDIP	methylated DNA immunoprecipitation
MS	mass spectrometry
MSP	methylation-specific PCR
ROC	receiver operating characteristic

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Methylation levels of the DNA region of *Hspa2*, *Fam43a*, and *Cyp26a1* in liver tumor tissues from control mice and gestationally arsenic-exposed mice.