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Methylation Silencing of Transforming Growth Factor-β Receptor Type II in Rat Prostate Cancers

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Abstract

To identify methylation-silenced genes in prostate cancers, a microarray analysis for genes up-regulated by treatment with a demethylating agent, 5-aza-2′-deoxycytidine, was performed using three rat prostate cancer cell lines. Eight genes (*Aebp1, Dysf, Gas6, LOC361288, Nnat, Ocm, RGD1308119*, and *Tgfbr2*) were re-expressed at 16-fold or more, and their promoter CpG islands were shown to be densely methylated in the cancer cell lines. From the eight genes, *Tgfbr2*, a key mediator of transforming growth factor-β (TGF-β) signaling that has been strongly implicated in human and rat prostate carcinogenesis, was selected, and its silencing in primary samples was analyzed further. *Tgfbr2* was methylated and markedly down-regulated in three of seven 3,2′-dimethyl-4-aminobiphenyl–induced invasive adenocarcinomas in the dorsolateral lobe of the rat prostate. In humans, marked down-regulation of TGFBR2 protein was observed in 12 of 20 high-grade prostatic intraepithelial neoplasia and 36 of 60 prostate cancers. DNA methylation of the human *TGFBR2* promoter CpG islands repressed transcription, if present, but neither methylation nor mutation were detected in 27 human prostate cancers analyzed. Methylation silencing of rat *Tgfbr2* was associated with histone H3 lysine 9 trimethylation, whereas decreased expression of human *TGFBR2* was mainly due to decreased transcription activity, sometimes in concert with histone deacetylation and H3 lysine 27 trimethylation. The identification of methylation silencing of *Tgfbr2* in rat prostate cancers, in accordance with *TGFBR2* down-regulation in human prostate cancers, will enable us to analyze how aberrant methylation is induced *in vivo* and identify factors that promote and suppress the induction of aberrant methylation. [Cancer Res 2008;68(7):2112–21]

Introduction

Gene silencing due to DNA methylation of promoter CpG islands (CGIs) is one of the major mechanisms of tumor-suppressor gene inactivation, along with mutations and loss of heterozygosity (1). Many methylation-silenced tumor-suppressor genes have been identified, and more will be revealed by genome-wide procedures (2). In contrast, limited information is available on the mechanism of how methylation silencing is induced *in vivo* and on the factors that promote or suppress aberrant methylation. For example, although chronic inflammation is known to be an inducer of aberrant methylation in humans (3), the exact effector cells and molecular changes in target cells are unknown. To address these questions, animal models are indispensable. However, because we select models by the presence of dense methylation of a promoter CGI in cancer and by the meaningful expression of its downstream gene in the corresponding normal tissue, only a limited number of methylation-silenced genes have thus far been identified in animal models (4–7).

Prostate cancer is one of the leading causes of cancer death in men in most developed countries (8). To analyze molecular, cellular, and physiologic events in prostate carcinogenesis, rodent models have been used. Particularly in rats, prostate cancers can be induced in an age-dependent manner in ACI/Seg and Lobound-Wistar strains, or by chemical carcinogens, and the effects of androgens have been clearly shown (9). If methylation-silenced genes involved in prostate carcinogenesis are found in rat prostate cancers, they will enable us to analyze the molecular processes of how aberrant methylation is induced *in vivo* as well as the factors, including hormones, that influence the process.

To identify methylation-silenced genes in rat prostate cancers, a chemical genomic screening method (2) was adopted for its efficiency. This method screens genes re-expressed after treatment with the demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC), using a microarray. It is technically simple, and effective in identifying methylation-silenced genes using cell lines. Three rat prostate cancer cell lines, PLS10, PLS20, and PLS30 have been established from three prostate cancers in the dorsolateral lobes independently induced by 3,2′-dimethyl-4-aminobiphenyl (DMAB) plus testosterone in male F344 rats (10, 11).

Here, we report the results of a chemical genomic screening using PLS10, PLS20, and PLS30 cell lines. Among the genes whose methylation silencing was confirmed, the transforming growth factor-β (TGF-β) receptor type II gene (*Tgfbr2*), a key mediator of TGF-β signaling that has been strongly implicated in human and rat prostate carcinogenesis (12–19), was identified. We further analyzed *Tgfbr2* methylation and expression both in rat and human prostate cancers.

Materials and Methods

Cell lines and their 5-aza-dC or trichostatin A treatment. PLS10 (well-differentiated adenocarcinoma), PLS20 (poorly differentiated adenocarcinoma), and PLS30 (well-differentiated adenocarcinoma) were established from three independent transplantable tumor lines induced by DMAB plus testosterone propionate in the dorsolateral lobes of F344 rats, and maintained as reported (11). Human prostate cancer cell lines (PC3, LNCaP, DU145, MDA-PCa-2b, and 22Rv1) and prostatic epithelial cells immortalized by papillomavirus 18 (RWPE-1) were purchased from the American Type Culture Collection.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

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doi:10.1158/0008-5472.CAN-07-5282

Table 1. Eight genes silenced in rat prostate cancer cell lines

Gene symbol	CpG island (bp)	Methylation and expression induction			Gene title
		PLS-10	PLS-20	PLS-30	
Aebp1	500	M*	M	M	AE binding protein 1 (predicted)
Dysf	300	M	M*	M	Dysferlin (predicted)
Gas6	500	M*	M	M†	Growth arrest–specific 6
LOC361288	500	M*	U	U	Similar to FUN14 domain containing 2 (predicted)
Nnat	500	M*	M†	M†	Neuronatin
Ocm	300	M*	M*	M/U*	Oncomodulin
RGD1308119	500	M*	M	M	Similar to F-box protein FBL2
Tgfbr2	500	M/U	M*	M†	TGF-β receptor II

Abbreviations: M, methylated; U, unmethylated.
*≥16-fold increase.
† ≥4-fold increase.

For treatment with 5-aza-dC, 2×10^5 cells (1×10^5 cells for PLS-10)/10 cm dish were seeded on day 0, and exposed to freshly prepared 10 μmol/L 5-aza-dC (Sigma) for 24 h on days 1 and 3. This dose suppressed cellular growth rates to approximately half of nontreated cells. After each treatment, the cells were placed in fresh medium and harvested on day 4. For treatment with trichostatin A (TSA), cells were seeded at a half-confluent density, and exposed to 100, 300, and 1,000 nmol/L of TSA (Sigma) for 24 h until harvest. Genomic DNA was extracted by standard phenol/chloroform procedures. Total RNA was extracted using ISOGEN (Nippon Gene) and purified using an RNeasy Mini kit (Qiagen).

Primary prostate cancers and immunohistochemistry. To induce prostate cancers, 6-week-old male F344 rats underwent subcutaneous injection of 100 mg/kg of testosterone propionate and 50 mg/kg of DMAB, which was repeated 10 times in a 2-week cycle, followed by the subcutaneous implantation of a Silastic tube containing 40 mg of testosterone propionate (10). The prostate was resected *en bloc*, examined for gross abnormalities, and fixed in 10% buffered formalin. One sagittal slice was prepared for each lobe, and embedded in paraffin. A 4-μm-thick section was stained with H&E. Organ-confined prostate cancers were obtained from 60 patients (ages 49–77, stage II–IV, Gleason pattern 2–5) who underwent prostatectomy. None of these cancer patients had previously undergone chemotherapy, radiotherapy, or hormonal therapy. All histologic diagnoses were made by experienced pathologists (S. Takahashi and T. Shirai). DNA from formalin-fixed, paraffin-embedded tissue sections was extracted by heating the sections at 100°C for 20 min under pH 12 (20). The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center.

TGFBR2 immunohistochemistry in rat and human prostate cancers was performed using polyclonal anti-TGFBR2 antibody (L-21, Santa Cruz Biotechnology). The areas with TGFBR2 protein expression were quantitatively measured by an Image Processor for Analytical Pathology (IPAP-WIN, Sumika Technoservice), and regions that had an absorbance of one-third or less of the normal prostate were considered to have TGFBR2 down-regulation.

Oligonucleotide microarray analysis and database search. Oligonucleotide microarray analysis was performed using a GeneChip Rat Genome 230 2.0 Array (Affymetrix) and GeneChip Operating Software as in our previous studies (21, 22). Database searches were carried out at a GenBank web site, and CGI were searched for based on (*a*) CpG score ≥ 0.65, (*b*) G + C content ≥ 55%, and (*c*) length (≥200, ≥300, or ≥500 bp).

Methylation-specific PCR and bisulfite sequencing. DNA from cell lines was digested by *Bam*HI and 1 μg of digested DNA was denatured in 0.3 N NaOH at 37°C for 15 min. DNA from formalin-fixed, paraffin-embedded tissue sections was used without digestion (0.2–0.5 μg each). The samples in 3.6 N sodium bisulfite (pH 5.0) and 0.6 mmol/L of hydroquinone underwent 15 cycles of 30-s denaturation at 95°C and 15-min incubation at 50°C, desalted and desulfonated with Zymo-Spin IC Columns (Zymo Research), and were dissolved in 16 to 40 μL of TE buffer.

Methylation-specific PCR (MSP) was performed with a primer set specific to the methylated or unmethylated sequence (M or U set), using 0.5 μL (2.0 μL for DNA from formalin-fixed tissue) of the sodium bisulfite–treated DNA. DNA methylated with *Sss*I methylase (New England Biolabs) and DNA amplified by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences) was used as fully methylated and unmethylated control DNA, respectively (23). Bisulfite sequencing was performed with primers common to methylated and unmethylated DNA sequences, using 0.5 μL (1.0 μL for DNA from formalin-fixed tissue) of the sodium bisulfite–treated DNA (22). Primer sequences are shown in Supplementary Table S1.

Quantitative reverse transcription-PCR and 5′-rapid amplification of cDNA ends. cDNA was synthesized from 1 μg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen) with a random primer. Real-time PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems) with SYBR Green Real-Time PCR Master Mix (Toyobo; ref. 22). The copy number of a target gene was normalized to that of *GAPDH* in human and cyclophilin A (*Ppia*) in rat (24). Primer sequences are shown in Supplementary Table S2.

Rapid amplification of 5′ complementary DNA ends (5′ RACE) was performed using a GeneRacer kit (Invitrogen) on cDNA from AT6.3 and MAT-LyLu rat prostate cancer cell lines that abundantly expressed *Tgfbr2*. After the first and second PCR using LA Taq (Takara Bio), the PCR product was cloned into a pGEM-T Easy Vector (Promega), and a total of 54 clones were sequenced using a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare Bio-Sciences) and an ABI310 DNA sequencer (Applied Biosystems).

Chromatin immunoprecipitation analysis. Cells (1.5×10^6) were treated with 1% formaldehyde for 10 min at room temperature for cross-linking, and the reaction was quenched by adding glycine. Cells were lysed in the SDS lysis buffer containing protease inhibitors (Upstate), and DNA was sonicated to a size of 100 to 3,000 bp by Bioruptor UCD-250 (Cosmo Bio). To the sonicated solution, anti-K4 dimethylated histone H3 (H3K4me2, Upstate), anti-K9 trimethylated histone H3 (H3K9me3, Upstate), or anti-K27 trimethylated histone H3 (H3K27me3, Upstate) was added, and the mixture was incubated at 4°C overnight with rotation. The resultant immune complexes were collected using Dynabeads protein G (Invitrogen Dynal AS), and washed with Immune Complex Wash Buffer (Upstate). The cross-link was reversed by incubation for 5 h at 65°C in the presence of 0.3 mol/L of NaCl. DNA was recovered by treatment with RNase and proteinase K,

phenol/chloroform extraction, and isopropanol precipitation. The number of DNA molecules precipitated from a specific starting volume of the sonicated solution was compared with the number of DNA molecules in the same volume of the sonicated solution (whole cell extract). The number of DNA molecules was quantified by real-time PCR (primer sequences in Supplementary Table S1).

Luciferase reporter assay using a promoter with DNA methylation of a specific region. The 5′ region of human *TGFBR2* was amplified using an upper primer (5′-CCAGGAATGTCTTGGGCAAA-3′) and a lower primer (5′-CCAGCGCAGCGGACG-3′) and cloned into a *Sma*I site of the pGL3-Basic vector (Promega). To methylate a specific region within the reporter plasmid, the region was excised and methylated twice by *Sss*I methylase. The methylated DNA fragment and the mock-treated DNA fragment (treatment without *S*-adenosylmethionine) were ligated back into the remaining arm using Ligation high (Toyobo). Nonessential regions within the reporter plasmid were digested with *Sac*I, *Bam*HI, and *Fsp*I. Then,

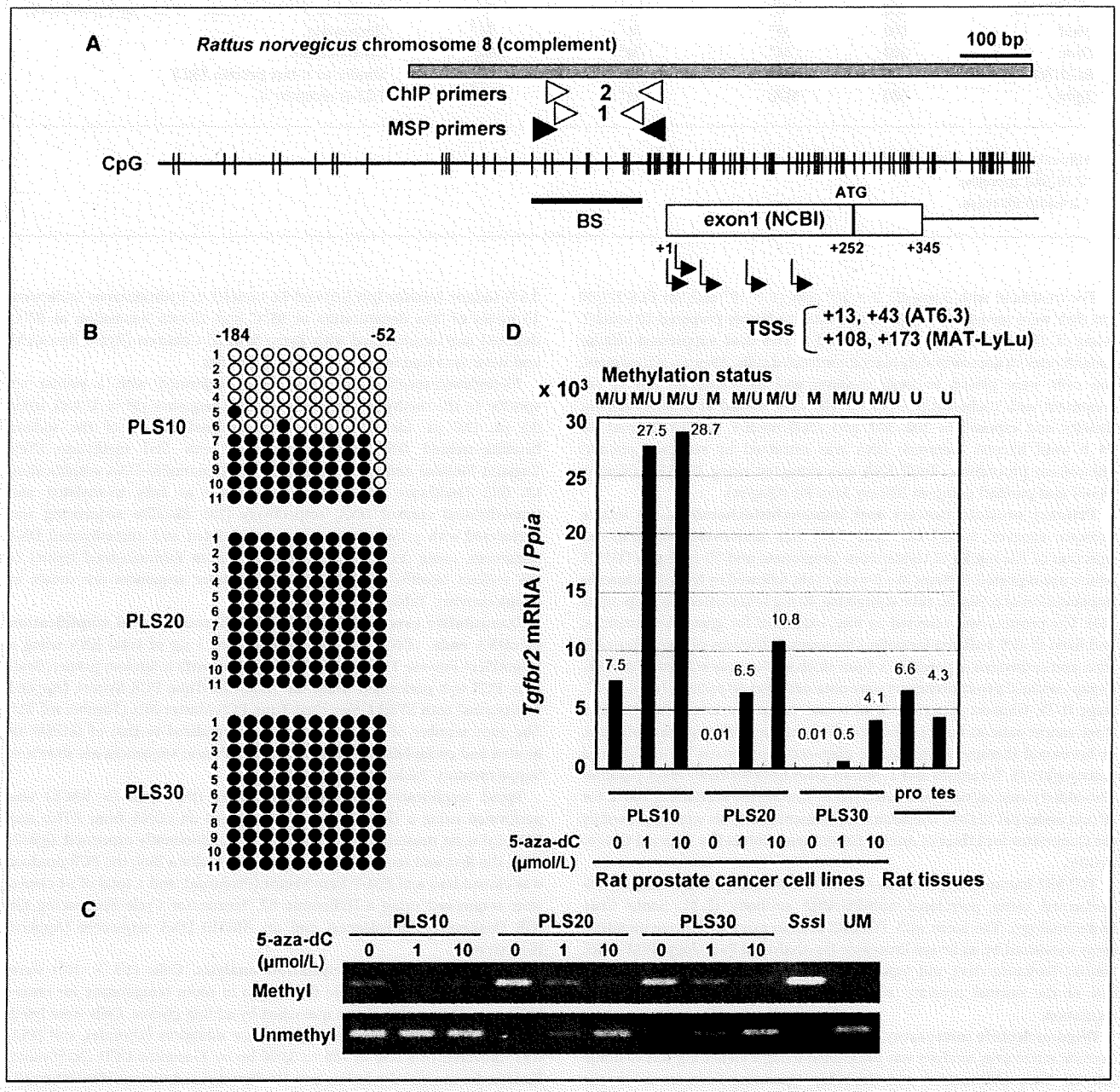

Figure 1. Methylation-silencing of *Tgfbr2* in rat prostate cancer cell lines induced by DMAB and testosterone. *A,* map of a promoter CGI, TSSs, and exon 1 of rat *Tgfbr2*. The TSSs were identified by 5′ RACE of AT6.3 and MAT-LyLu cell lines. *+1, Tgfbr2* TSS in the National Center for Biotechnology Information database (NC_005107.2, 120680453). *Vertical lines,* individual CpG sites; *gray box,* CGI region; *open boxes,* noncoding and coding exons; *arrows,* TSSs; *thick line,* the area analyzed by bisulfite sequencing; *arrowheads,* positions of MSP and ChIP primers. *B,* results of bisulfite sequencing in rat prostate cancer cell lines. The presence of dense methylation of the promoter CGI was confirmed for PLS20 and PLS30. *C, Tgfbr2* methylation status in rat prostate cancer cell lines analyzed by MSP. Demethylation was induced by 5-aza-dC in PLS20 and PLS30. *SssI,* genomic DNA methylated with *Sss*I methylase; *UM,* unmethylated control. *D,* quantitative mRNA expression analysis of rat *Tgfbr2*. *Tgfbr2* was expressed in the normal prostate (*pro*), testes (*tes*), and PLS10 that had unmethylated DNA molecules.

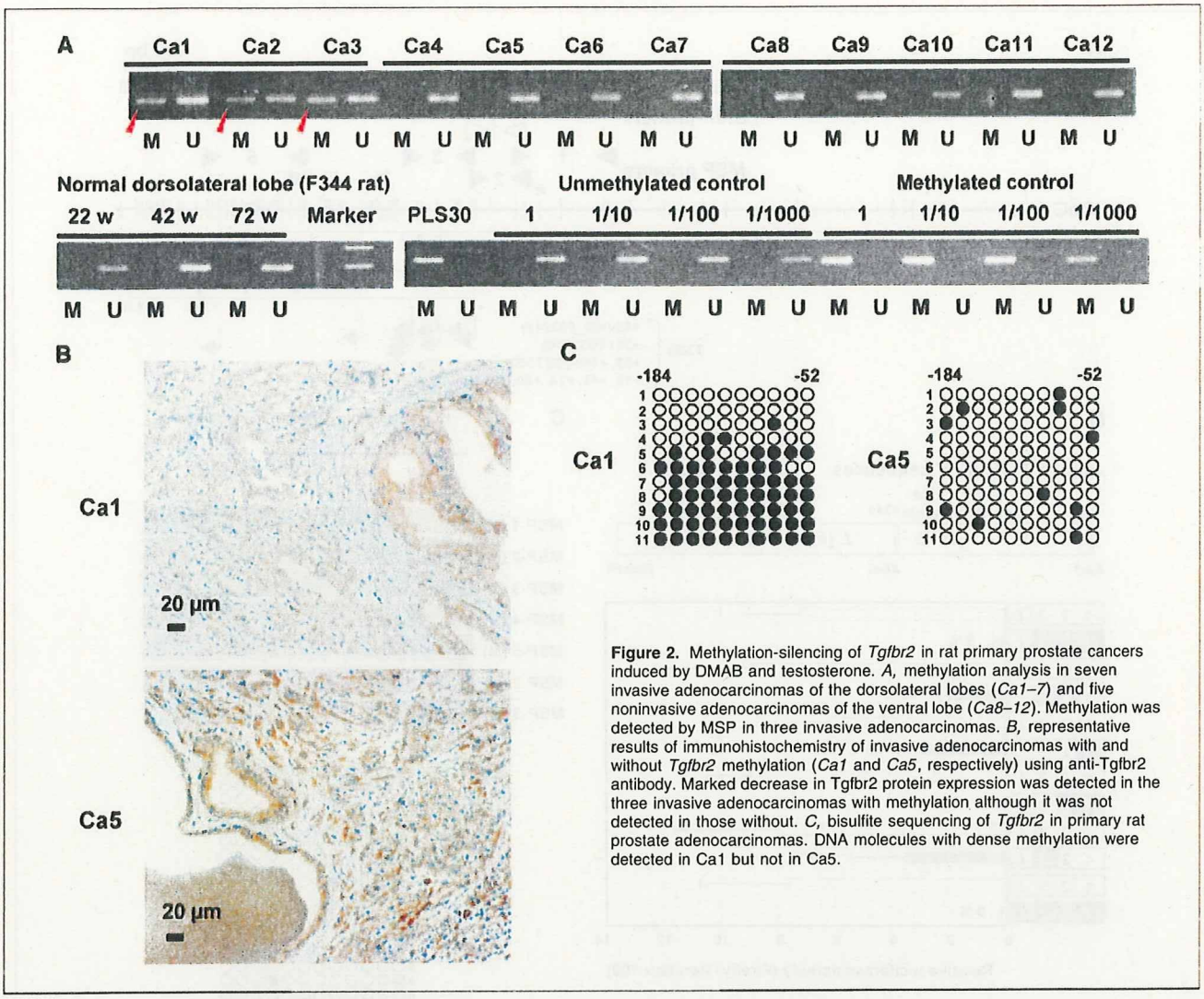

Figure 2. Methylation-silencing of *Tgfbr2* in rat primary prostate cancers induced by DMAB and testosterone. *A,* methylation analysis in seven invasive adenocarcinomas of the dorsolateral lobes (*Ca1–7*) and five noninvasive adenocarcinomas of the ventral lobe (*Ca8–12*). Methylation was detected by MSP in three invasive adenocarcinomas. *B,* representative results of immunohistochemistry of invasive adenocarcinomas with and without *Tgfbr2* methylation (*Ca1* and *Ca5*, respectively) using anti-Tgfbr2 antibody. Marked decrease in Tgfbr2 protein expression was detected in the three invasive adenocarcinomas with methylation although it was not detected in those without. *C,* bisulfite sequencing of *Tgfbr2* in primary rat prostate adenocarcinomas. DNA molecules with dense methylation were detected in Ca1 but not in Ca5.

a *Sac*I-*Bam*HI fragment that contained *TGFBR2* promoter and luciferase cDNA was recovered after electrophoresis in an agarose gel.

Along with a control plasmid for transfection efficiency (3 ng phRL-TK; Promega), 30 ng of the *Sac*I-*Bam*HI fragment was transiently transfected into PC3 cells using Lipofectamine 2000 transfection reagent (Invitrogen) with Opti-MEM I Reduced-Serum Medium (Invitrogen) in a 96-well format. At 24 h after transfection, cells were harvested, and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) in a Lumat LB 9507 (Berthold Technologies). Each transfection and measurement was performed in triplicate.

Sequencing analysis for mutation analysis of human *TGFBR2*. The polyadenylic acid tract in exon 3 of *TGFBR2* (nucleotides 831–840 of NM_001024847), the target region of microsatellite instability (25), was amplified using Phusion high-fidelity DNA polymerase with HF Buffer (New England Biolabs). The product was sequenced with inner primers (Supplementary Table S2).

Results

Genes up-regulated by 5-aza-dC treatment and their methylation analysis. Three rat prostate cancer cell lines (PLS10, PLS20, and PLS30) were treated with 10 μmol/L of 5-aza-dC, and up-regulated genes were searched for using an oligonucleotide microarray. Among >28,000 genes and expressed sequence tags analyzed by the microarray, 47, 13, and 10 annotated genes (59 nonredundant annotated genes), respectively, were up-regulated at 16-fold or more (signal log ratio ≥4) in the three cell lines (Supplementary Tables S3 and S4). The presence of a putative promoter CGI was examined by a database search, and 10, 3, and 1 genes (12 nonredundant genes) were found to have CGIs that spanned 300 bp or more (Supplementary Table S3). Genes with these CGIs were considered as candidates for novel methylation-silenced genes in rat prostate cancers.

To examine whether the induction of these genes by 5-aza-dC treatment was due to demethylation of promoter CGIs, the methylation statuses of the putative promoter CGIs were analyzed by MSP. The CGIs of eight genes (*Aebp1, Dysf, Gas6, LOC361288, Nnat, Ocm, RGD1308119*, and *Tgfbr2*) were completely methylated before the treatment and demethylated after the treatment in at least one of the three rat prostate cancer cell lines (Table 1). The up-regulation of mRNA expression of these eight genes detected by

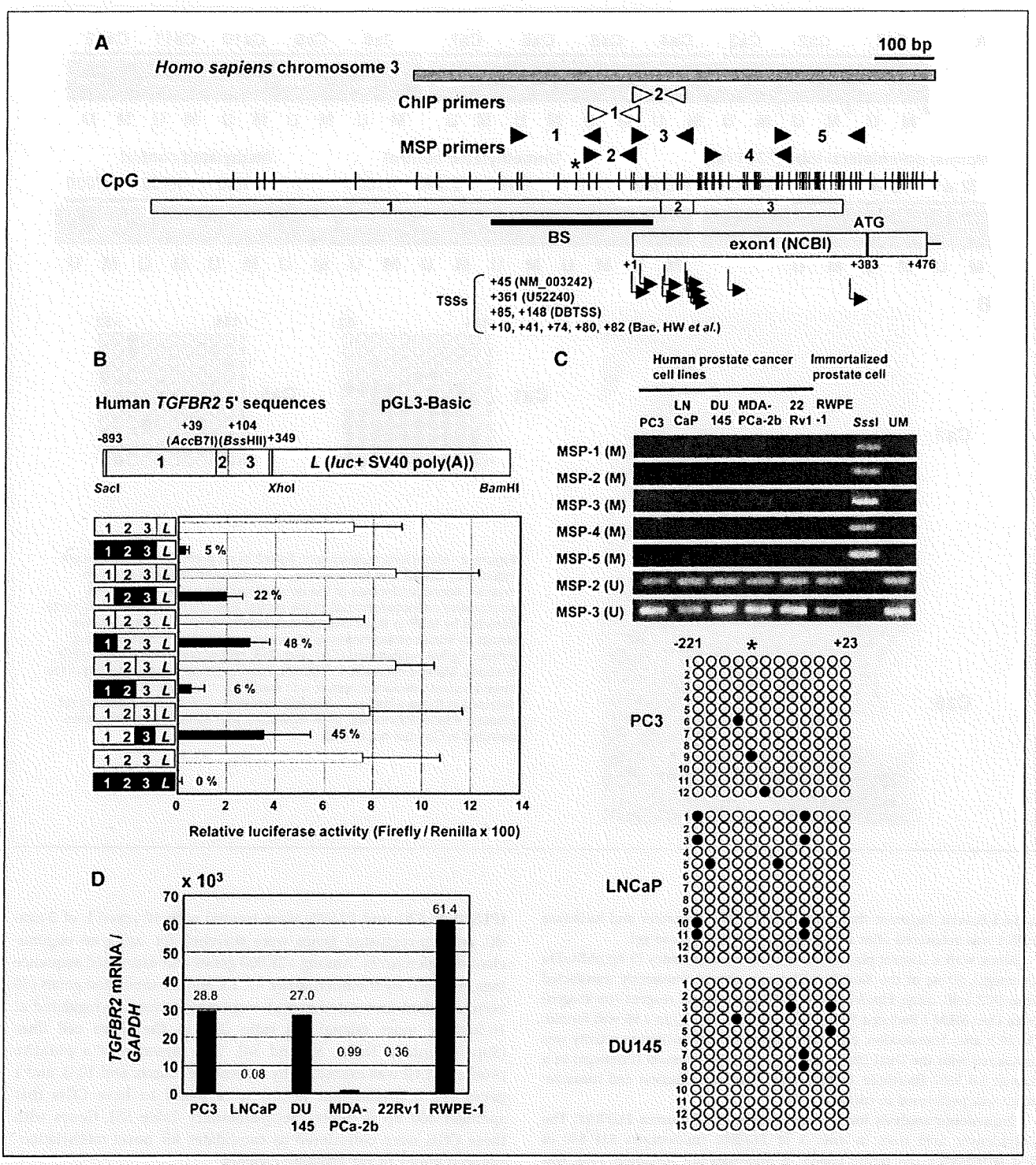

Figure 3. Human *TGFBR2* silencing due to dense methylation of its CGI, and its rare occurrence among human cancer cell lines. *A,* map of the promoter region and a CGI overlapping human *TGFBR2. +1,* TSS from the National Center for Biotechnology Information database (NC_000003.10, 30622998). Multiple TSSs reported (30) are also shown. *, a specific CpG site at nucleotide −140 (nucleotide −96 in this article) reported in ref. 36. *B,* structure of the *SacI-BamHI* DNA fragment used for the luciferase reporter assay (*top*). A DNA fragment spanning from −893 to +349 of human *TGFBR2* was ligated to the luciferase reporter gene. *Bottom*, luciferase activity of the reporter constructs with and without methylation of specific regions of the *TGFBR2* promoter. *Open* and *closed boxes,* unmethylated and methylated regions, respectively. The promoter activities were normalized to the activity of the cotransfected phRL-TK vector. Compared with the control without methylation, a reporter construct with methylation of regions 1 and 2 showed a marked decrease in luciferase activity. A fragment that had methylation of the entire reporter plasmid showed no transcription activity. *Columns,* mean; *bars,* SD. *C,* MSP of *TGFBR2* in five human prostate cancer cell lines and immortalized prostate epithelial cells (*RWPE-1*). Screening of 33 human cancer cell lines in the same manner showed that *TGFBR2* methylation was rare. *D,* real-time reverse transcription-PCR analysis of *TGFBR2* mRNA expression in human prostate cancer cell lines and RWPE-1. The expression was down-regulated to $<10^{-4}$ of that of *GAPDH* in LNCaP and to $<10^{-3}$ in MDA-PCa-2b and 22Rv1.

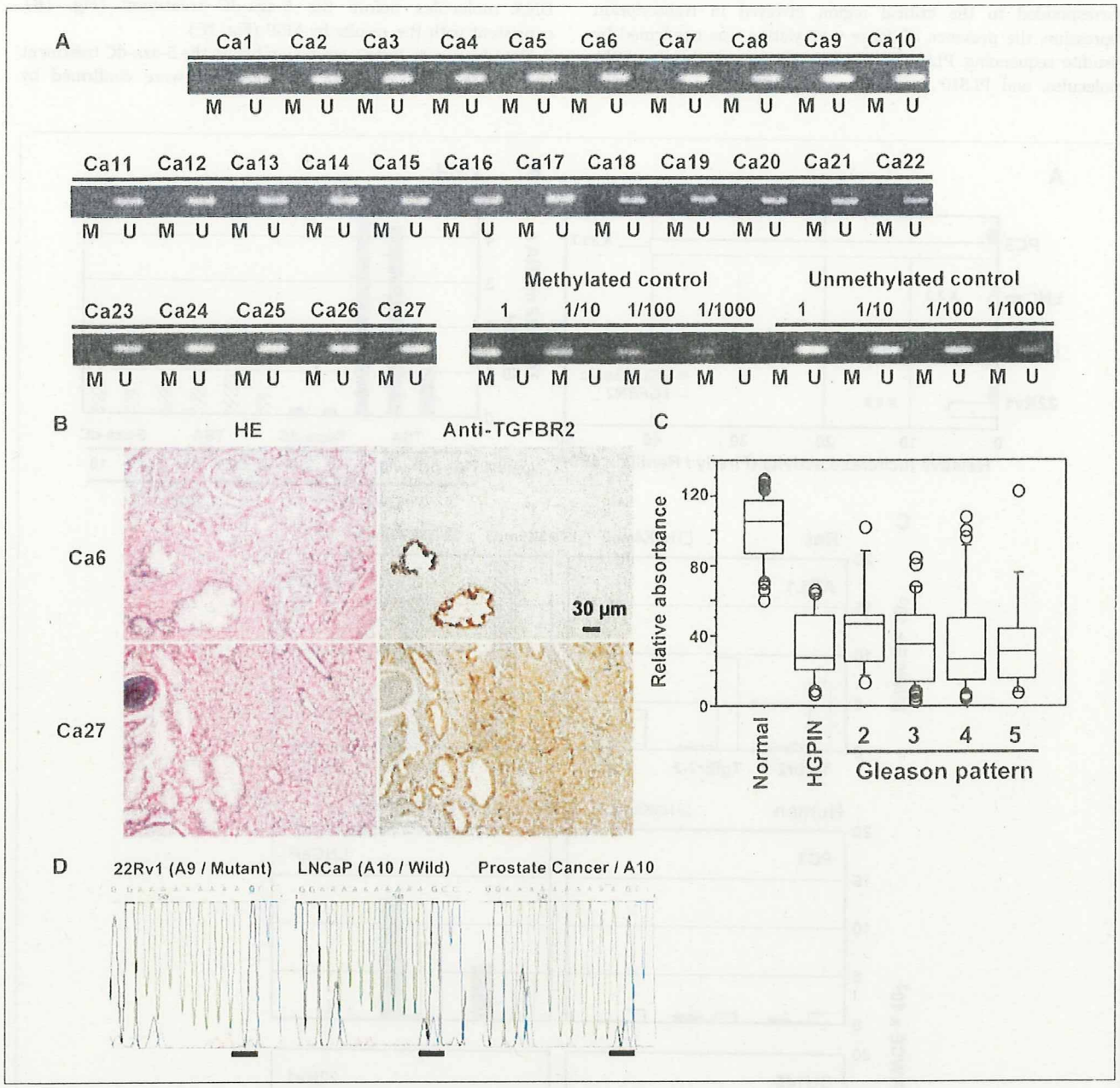

Figure 4. Absence of *TGFBR2* methylation and decreased TGFBR2 protein expression in primary human prostate cancers. *A,* methylation analysis of 27 primary human prostate cancers by MSP (MSP-3). PCR conditions were adjusted to allow detection of methylation of DNA molecules to as little as 1% of the positive control, but methylation was not detected. *B,* representative results of immunohistochemistry using anti-TGFBR2 antibody. *Ca6,* a cancer with decreased expression (*top*); *Ca27,* a cancer with positive expression (*bottom*). Decreased immunoreactivity was observed in 36 of 60 cases. *C,* TGFBR2 expression levels in HGPIN and prostate cancers with different Gleason patterns. Decreased TGFBR2 expression was already detected in HGPIN. *D,* representative results of mutation analysis. A10 (normal sequence) was deleted to A9 only in 22Rv1 and MDA-PCa-2b cell lines, and none of the 27 primary prostate cancers had a mutation.

the microarray was confirmed by quantitative reverse transcription-PCR (Supplementary Table S5). These eight genes were considered to be methylation-silenced in the rat prostate cancer cell lines.

Methylation silencing of *Tgfbr2* in rat prostate cancer cell lines. Interestingly, among the eight genes, *Tgfbr2*, a key mediator of TGF-β signaling that has been implicated in human and rat prostate carcinogenesis (12–19), was present. The methylation silencing of *Tgfbr2* was analyzed further. Generally, for methylation silencing, a dense DNA methylation of a region within a promoter CGI, specifically a region devoid of nucleosome just upstream of a transcription start site (TSS; nucleosome-devoid region), is critical (26, 27). To search for any *Tgfbr2* TSSs additional to the one reported in osteoblasts (28), we performed the 5′ RACE method using rat prostate cancer cell lines. Several TSSs were found to be located from +13 to +173 of the reported *Tgfbr2* TSS (NM_031132.3, National Center for Biotechnology Information; Fig. 1*A*), and the region analyzed by MSP was located at −178 to −22 of the multiple TSSs. Because this region

corresponded to the critical region involved in transcription repression, the presence of dense methylation was confirmed by bisulfite sequencing. PLS20 and PLS30 had only methylated DNA molecules, and PLS10 had both methylated and unmethylated DNA molecules before the 5-aza-dC treatment (Fig. 1*B*), consistent with the results by MSP (Fig. 1*C*).

The absence of *Tgfbr2* expression before the 5-aza-dC treatment and its re-expression after the treatment were confirmed by

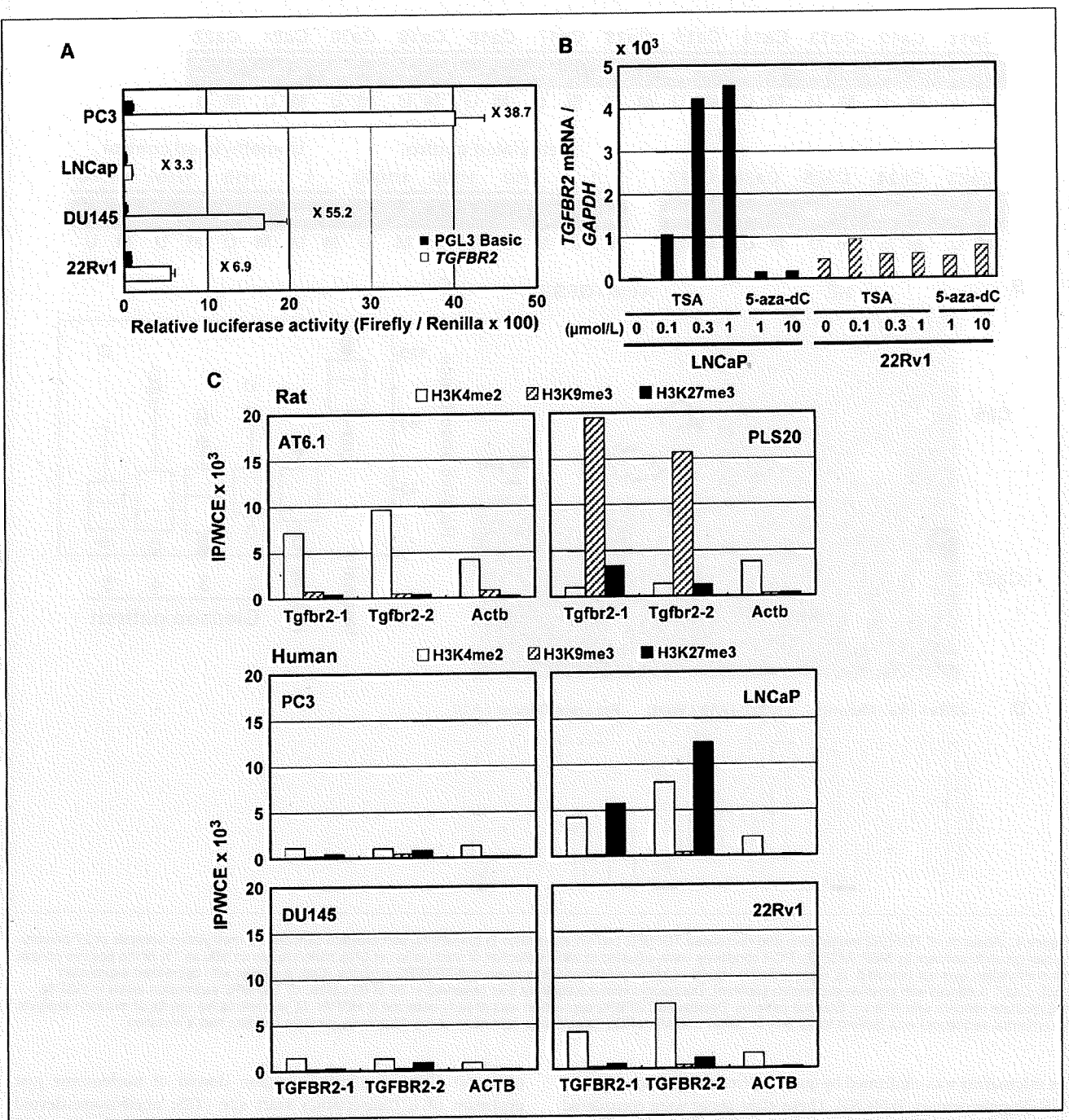

Figure 5. Transcriptional capacities and histone modifications of the *Tgfbr2* (*TGFBR2*) promoter in rat and human prostate cancer cell lines. *A*, luciferase reporter assay using a 1,242 bp DNA fragment covering the human *TGFBR2* promoter and TSSs. The DNA fragment had a 38.7-fold, 3.3-fold, 55.2-fold, and 6.9-fold higher luciferase activity in PC3, LNCaP, DU145 and 22Rv1, respectively, compared with control (pGL3-Basic vector without an inserted promoter DNA fragment). *B*, real-time reverse transcription-PCR analysis of *TGFBR2* mRNA expression in LNCaP and 22Rv1 cells with TSA or 5-aza-dC treatment. *C*, ChIP analysis of histone modifications at the *TGFBR2* and *ACTB* promoters in rat and human prostate cancer cell lines. At the *ACTB* promoter, increased H3K4me2 was observed in all rat and human prostate cancer cell lines. At the *Tgfbr2* promoter, a rat cell line with *Tgfbr2* expression (*AT6.1*) had increased H3K4me2 whereas another rat cell line without (*PLS20*) had increased H3K9me3. In contrast, human prostate cancer cell lines had both H3K4me2 and H3K27me3. Especially, a cell line with decreased *TGFBR2* expression (*LNCaP*) had H3K27me3. *IP*, immunoprecipitated; *WCE*, whole cell extract (input).

quantitative reverse transcription-PCR in PLS20 and PLS30 cells (Fig. 1*D*). The re-expression was associated with the appearance of unmethylated DNA molecules by MSP (Fig. 1*C*). Expression of *Tgfbr2* in the normal prostate, which is important for functional gene silencing in cancer, was confirmed. We concluded that *Tgfbr2* was methylation-silenced in PLS20 and PLS30 rat prostate cancer cell lines.

***Tgfbr2* silencing in rat primary prostate cancers.** DNA methylation of the *Tgfbr2* promoter CGI and its decreased protein expression were analyzed in rat primary prostate cancers induced by DMAB and testosterone. Using MSP, methylation was detected in three of seven invasive adenocarcinomas in the dorsolateral lobe, but in none of five noninvasive adenocarcinomas in the ventral lobe (Fig. 2*A*). Using immunohistochemistry, protein expression was found to be markedly decreased in the three invasive adenocarcinomas with methylation, but was not decreased in the remaining four invasive adenocarcinomas or in any of the five noninvasive adenocarcinomas (Fig. 2*B*). The results obtained by MSP were confirmed by bisulfite sequencing of two representative samples with and without methylation (Fig. 2*C*). These data showed that the *Tgfbr2* gene was methylation-silenced in rat primary prostate cancers induced by DMAB and testosterone.

Methylation silencing of human *TGFBR2*, and its rare occurrence. In humans, silencing of *TGFBR2* due to dense DNA methylation of its promoter CGI has been reported in a limited number of cell lines (29), but has not been found in any primary cancers. Unlike that of rat *Tgfbr2*, the "promoter CGI" of human *TGFBR2* is located mainly in its first exon, based on its well-documented multiple TSSs (Fig. 3*A*; ref. 30), and CpG density becomes lower in the promoter region. It is now known that DNA methylation of a nucleosome-devoid region is critical for gene silencing (26, 27), and that methylation of a promoter region with intermediate or low CpG density does not necessarily repress the transcription of its downstream gene (31). Therefore, we decided to examine whether or not dense methylation of various regions of human *TGFBR2* could cause its silencing.

First, a 1,242 bp DNA fragment covering the human *TGFBR2* TSSs and its promoter region was cloned. Then, the entire fragment or three regions (regions 1, 2, and 3; shown in Fig. 3*A* and *B*) and their combinations were specifically methylated, and the effect was analyzed by a reporter assay. Methylation of region 1 only (−893 to +39) and methylation of region 3 only (+104 to +349) reduced the promoter activity to half of their unmethylated controls (Fig. 3*B*). On the other hand, methylation of regions 1 and 2 (−893 to +104) reduced the activity to 6% of its unmethylated control. These data showed that dense methylation of the human *TGFBR2* CGI, if present, can repress transcription, and indicated that methylation of region 2 is indispensable. Due to the short size of region 2, data on methylation of region 2 only could not be prepared.

MSP primers were designed in region 2 (MSP-3), the possible nucleosome-devoid region, encompassing most TSSs, and methylation status was screened in 33 human cancer cell lines (five prostate, six ovarian, six lung, seven pancreatic, and nine stomach cancer cell lines; data for prostate cancers in Fig. 3*C*). Only TYK-nu (ovarian) and MIAPaCa-2 (pancreas) had *TGFBR2* methylation (data not shown). The lack of methylation in three prostate cancer cell lines (PC3, LNCaP, and DU145) was confirmed by bisulfite sequencing (Fig. 3*C*). When *TGFBR2* mRNA expression was examined, it was down-regulated to $<10^{-4}$ of that of *GAPDH* in LNCaP and to $<10^{-3}$ in MDA-PCa-2b and 22Rv1 (Fig. 3*D*). These data showed that silencing of *TGFBR2* due to dense methylation of its promoter region was rare among human cancer cell lines and absent in the five human prostate cancer cell lines analyzed.

Lack of methylation of the *TGFBR2* nucleosome-devoid region, but its frequent down-regulation in primary human prostate cancers. Methylation of the possible nucleosome-devoid region (MSP-3) was analyzed in 27 primary human prostate cancers by MSP. However, none of them showed methylation (Fig. 4*A*). It has been reported that TGFBR2 expression is markedly down-regulated in human prostate cancers (14–16), and we confirmed this. Following immunohistochemical analysis of the 20 high-grade prostatic intraepithelial neoplasia (HGPIN), the 27 cancers, and an additional 33 cancers, down-regulation of TGFBR2 protein was observed in 12 of 20 HGPIN and 36 of 60 prostate cancers (Fig. 4*B* and *C*). There was no correlation between expression levels of TGFBR2 protein and histologic grade in human prostate cancers. Finally, as a possible mechanism for decreased TGFBR2 expression, its mutations were searched for. Mutations were only detected in two prostate cancer cell lines (22Rv1 and MDA-PCa-2b), but not in the 27 primary prostate cancers analyzed (Fig. 4*D*).

Contrastive histone modifications in the rat and human prostate cancer cell line with down-regulated *Tgfbr2* expression. To analyze the molecular mechanisms causing down-regulation of *TGFBR2* in human prostate cancer cell lines (LNCaP and 22Rv1), we first analyzed their transcriptional capacity by a luciferase reporter assay using a 1,242 bp DNA fragment covering the human *TGFBR2* promoter and TSSs. The transcriptional capacity of LNCaP and 22Rv1 was significantly lower than that of PC3 and DU145 (Fig. 5*A*), although precise comparison of transcription activities among different cell lines was difficult because transfection and/or luminescence efficiencies were highly variable (Supplementary Table S6). We also analyzed the histone acetylation status in rat and prostate cancer cell lines by observing the effect of 5-aza-dC or TSA, a histone deacetylase inhibitor. LNCaP showed marked re-expression of *TGFBR2* mRNA after TSA treatment whereas 22Rv1 did not (Fig. 5*B*). This showed that, in addition to decreased transcription capacity, histone deacetylation was involved in the decreased *TGFBR2* expression in LNCaP, but not in 22Rv1.

Histone methylation status was further analyzed by chromatin immunoprecipitation (ChIP) assays in the rat and human prostate cancer cell lines. A rat cell line with *Tgfbr2* methylation silencing (PLS20) had increased H3K9me3, a typical mark for inactive chromatin (32), whereas another rat cell line with *Tgfbr2* expression (AT6.1) had increased H3K4me2, a typical mark for active chromatin (ref. 32; Fig. 5*C*). In contrast, human prostate cancer cell lines had both H3K4me2 and H3K27me3, and LNCaP, which had histone deacetylation, had a marked increase of H3K27me3 (Fig. 5*C*). These suggested that the loss of *Tgfbr2* expression in a rat prostate cancer cell line (PLS20) was due to DNA methylation, accompanied by the H3K9me3 modification, and that the decreased *TGFBR2* expression in a human prostate cancer cell line was due to decreased transcriptional capacity in concert with (LNCaP) or without (22Rv1) histone deacetylation and H3K27 trimethylation.

Discussion

Silencing of *Tgfbr2* was identified in invasive adenocarcinomas of the dorsolateral lobe of the rat prostate. This is the first report

of *Tgfbr2* silencing in animal cancers of any tissue, and of gene silencing in rat prostate cancers. In animal models, only a limited number of genes are known to be silenced by dense methylation of a region just upstream of a TSS, within a CGI, a nucleosome-devoid region (26, 27), in skin, lung, hematologic, and renal cancers (4–7). Our finding of *Tgfbr2* silencing in prostate cancers will enable us to analyze the processes of how aberrant methylation is induced *in vivo* and the factors that promote and suppress the induction of aberrant methylation, including testosterone. Mouse prostate cancers induced by the SV40 polyoma virus early region are known to be prevented by a demethylating agent, 5-aza-dC (33), but the genes responsible are still indefinite.

Functional involvement of *Tgfbr2* (TGFBR2) down-regulation in rodent and human prostate carcinogenesis is strongly supported in the literature. In rats, loss of TGF-β responsiveness in prostate epithelial cells causes malignant transformation (18), and prostate cancer sublines with high metastatic potential, MAT-LyLu and AT-3, show loss of Tgfbr2 protein (19). In mice, dominant negative Tgfbr2 mutant expression increased metastasis in the prostate of the TRAMP model (34), and conditional inactivation of *Tgfbr2* in fibroblasts resulted in intraepithelial neoplasia in the mouse prostate (35). In human prostate cancers, impaired TGF-β signaling, for which TGFBR2 is a key mediator, is likely to be deeply involved (12). Factors supporting this include, first, that TGF-β functions as an inducer of apoptosis in the normal prostate (12, 13); second, *TGFBR2* expression is reduced or lost in prostate cancers (14–16), as confirmed in this study; and third, overexpression of *TGFBR2* restores sensitivity of prostate cancer cells to apoptosis (12, 17). All these strongly indicate that *Tgfbr2* silencing is causally involved in rat prostate carcinogenesis, and suggest that *TGFBR2* down-regulation could be causally involved in human prostate carcinogenesis.

Human *TGFBR2* silencing due to dense methylation of its promoter region was first reported in lung cancer cell lines (29). Here, we showed that a critical region for its silencing was located just upstream of the human *TGFBR2* multiple TSSs (region 2, MSP-3), and that dense methylation of the region can repress its transcription. However, in human primary prostate cancers, *TGFBR2* silencing by dense methylation was not detected. The initial report on human *TGFBR2* silencing did not analyze primary cancers (29). These findings suggest that *TGFBR2* methylation silencing is very rare in human primary cancers. Methylation of a specific CpG site at −96 (nucleotide −140 in the original report) was reported to correlate with reduced *TGFBR2* expression in prostate cancer cell lines (36). However, we were not able to observe the correlation between methylation of the specific CpG site and transcription, or to detect dense methylation in any regions around the TSSs (MSP primers 1–5; Fig. 3*A* and *C*).

The rare occurrence of *TGFBR2* methylation silencing in human primary cancers was in sharp contrast with the frequent occurrence of *Tgfbr2* methylation silencing in rat invasive prostate cancers. Methylation silencing of genes other than *TGFBR2* are frequently observed in human prostate cancers (37). As a mechanism for the decreased *TGFBR2* expression, we first looked for *TGFBR2* mutations, but could not observe any. Then we analyzed transcriptional capacity and histone modifications, and revealed the presence of contrastive mechanisms between rats and humans. In the rat prostate cancer cell lines with *Tgfbr2* methylation silencing (PLS20 and PLS30), *Tgfbr2* expression levels were almost zero (Fig. 1*D*), their promoter regions were densely methylated, and had histone modification (H3K9me3) typical for inactive chromatin. In contrast, the human prostate cancer cell lines with decreased *TGFBR2* expression (LNCaP and 22Rv1) had very low levels of expression (Fig. 5*B*), decreased transcriptional capacity, and histone deacetylation and H3K27 trimethylation (LNCaP). The relative location of a CGI against the TSSs was markedly different between human and rat sequences, the human CGI mainly in exon 1 and the rat CGI mainly in the promoter region, and could be responsible for the contrastive mechanisms for the decreased *Tgfbr2* (*TGFBR2*) expression.

The induction mechanism of rat *Tgfbr2* silencing in the prostate is an interesting issue. Androgen exposure, a critical promoting factor of prostate cancers, is known to down-regulate *Tgfbr2* expression at the transcriptional level (38, 39), and transcriptional repression is known to trigger aberrant DNA methylation (3). In the rat prostate cancer model used here, a combination of an androgen (testosterone) and DMAB is important in inducing invasive prostate cancers, and thus *Tgfbr2* silencing. This suggests that not only the reduced *Tgfbr2* transcription but also some abnormality, required for induction of *Tgfbr2* silencing, is induced by testosterone and DMAB.

As for other methylation-silenced genes in the PLS rat prostate cancer cell lines, *Aebp1* is a binding partner for tumor-suppressor PTEN (40). *Gas6* and *Ocm* have oncogenic functions (41, 42). *Nnat* is known as an imprinting gene and its aberrant hypermethylation occurs frequently in pediatric acute leukemia (43). There is a possibility that silencing of these genes is related to the development and progression of rat prostate carcinoma. In human prostate cancers, two studies reported genomic screening of methylation-silenced genes (44, 45). No common genes were present between the genes identified in the two studies and the eight genes identified here. However, if we adopted a more relaxed criterion for screening of up-regulated genes in this study, *Tgfb3* (11-fold up-regulation in PLS10) was commonly identified (45). *Tgfb3* is also involved in TGF-β signaling, and is a candidate for a gene commonly methylation-silenced in both rat and human prostate cancers. Considering the number of methylation-silenced genes, it is likely that the majority of the genes silenced in PLS cells do not have causal roles in carcinogenesis.

In summary, we found *Tgfbr2* silencing due to dense DNA methylation of its promoter CGI in rat prostate cancers. This will enable us to analyze mechanisms of how methylation silencing is induced *in vivo* and identify factors that affect its induction.

Acknowledgments

Received 9/12/2007; revised 12/31/2007; accepted 1/18/2008.

Grant support: Grant-in-Aid for the Third-term Cancer Control Strategy Program from the Ministry of Health, Labour and Welfare, Japan. S. Yamashita is a recipient of a travel award from the Sankyo Foundation of Life Science, and N. McDonell is a recipient of a Foreign Scientist Fellowship from the Foundation for Promotion of Cancer Research.

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We thank Dr. Yutaka Kondo, Aichi Cancer Center Research Institute, for his helpful advice in ChIP assays.

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Pathology International 2008; **58**: 544–558 doi:10.1111/j.1440-1827.2008.02270.x

Review Article

Alterations of DNA methylation and clinicopathological diversity of human cancers

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Alterations of DNA methylation can account for the histological heterogeneity, reflected in the stepwise progression and complex biological characteristics of human cancers, that genetic alterations alone cannot explain. Analysis of DNA methylation status in tissue samples can be an aid to understanding the molecular mechanisms of multistage carcinogenesis. Human cancer cells show a drastic change in DNA methylation status, that is, overall DNA hypomethylation and regional DNA hypermethylation, which results in chromosomal instability and silencing of tumor-suppressor genes. Overexpression of DNA methyltransferase (DNMT) 1 is not a secondary result of increased cell proliferative activity but may underline the CpG island methylator phenotype of cancers. Splicing alteration of DNMT3B may result in chromosomal instability through DNA hypomethylation of pericentromeric satellite regions. Alterations of DNA methylation are observed even in the precancerous stage frequently associated with chronic inflammation and/or persistent viral infection or with cigarette smoking. Precancerous conditions showing alterations of DNA methylation may generate more malignant cancers. Aberrant DNA methylation is significantly associated with aggressiveness of cancers and poorer outcome of cancer patients. Genome-wide analysis of DNA methylation status based on array-based technology may identify DNA methylation profiles that can be used as appropriate indicators for carcinogenetic risk estimation and prognostication.

Key words: chromosomal instability, chronic inflammation, DNA methylation, DNMT1, DNMT3B, hepatocellular carcinoma, multistage carcinogenesis, precancerous condition, renal cell carcinoma, urothelial carcinoma

Microscopy of human cancers, which are considered to be genetically clonal lesions, frequently indicates histological heterogeneity (e.g. well, moderately or poorly differentiated carcinoma components are simultaneously observed even in tissue sections from a single patient). Such histological heterogeneity reflects the stepwise progression and complex biological characteristics of each tumor. Genetic alterations causing activation of oncogenes and inactivation of tumor suppressor genes cannot solely explain such histological heterogeneity of human cancers. Epigenetics has been defined as 'heritable changes in gene expression that are not due to any alteration in the DNA sequence'[1] and normally accounts for the diversity of phenotypes within cloned animals, monozygotic twins and single populations that genetics alone cannot explain.[2] Analysis of epigenetic alterations in tissue samples, in connection with the histological features of each cancer, may aid understanding of the molecular background of clinocopathological diversity in human cancers. DNA methylation is one of the most consistent and best-known epigenetic events in human cancers.

DNA methylation, a covalent chemical modification resulting in addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring in CpG dinucleotides (Fig. 1a), plays important roles in chromatin organization and gene expression.[3] DNA methylation can directly impede the binding of transcription factors to their target sites, thus prohibiting the transcription of specific genes. Moreover, DNA methylation normally promotes a highly condensed heterochromatin structure, where active transcription does not occur, through recruitment of DNA-organizing proteins (Fig. 1b). DNA methylation is a stable modification that is inherited throughout cell divisions (Fig. 1c). When found within the promoter regions, DNA methylation prevents the reactivation of silent genes. This allows the daughter cells to retain the same expression pattern as the parent cells and is important for inactivation of the X chromosome and imprinting. Transposons and other parasitic elements have been acquired in the mammalian genome over time, and make up the repetitive sequences in the intergenic and intragenic regions of DNA. The activation of these parasitic elements can allow for the movement of these elements within the

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Received 22 April 2008. Accepted for publication 20 May 2008.

genome. To preserve the integrity of the genome, DNA methylation persistently silences such parasitic elements.[4]

Murine *DNA methyltransferase 1 (Dnmt 1)* cDNA was cloned in 1988 and its C-terminal domain was found to show striking similarities to the catalytic methyltransferase domain of bacterial type II DNA cytosine methyltransferases.[5] Homologs of DNMT1 have been found in nearly all eukaryotes that have DNA bearing 5-methylcytosine, but not in those that lack it. Until the identification of DNA methyltransferase (DNMT) 2,[6] DNMT3A and DNMT3B[7] in 1998, DNMT1 (EC2.1.1.37) had been the only known DNMT, and is currently the major and best-known of this enzyme family. Embryos of *Dnmt1 –/–* mice, which have genome-wide DNA hypomethylation, are stunted, show delayed development, and do not survive past mid-gestation,[8] indicating that DNA methylation is essential for the development of mammals.

The C-terminal catalytic domain of DNMT transfers methyl groups from S-adenosyl-L-methionine (AdoMet) to cytosines (Fig. 1a).[9] Critical dietary components leading to synthesis of AdoMet include folate, vitamins B_6 and B_{12}, methionine and choline. The C-terminal catalytic domain of DNMT is characterized by the presence of five conserved amino acid motifs, namely I, IV, VI, IX and X (Fig. 1d).[9] Motifs I and X are filed together to form most of the binding site for AdoMet. Motif IV contains the prolylcysteinyl dipeptide that provides the thiolate at the active site. Motif VI contains the glutamyl residue that protonates the 3 position of the target cytosine. Motif IX has a role in maintaining the structure of the target recognition domain.

The N-terminal regulatory domain of DNMT1 contains a proliferating cell nuclear antigen (PCNA)-binding domain, a nuclear localization signal, a cysteine-rich alpha thalassemia and retardation on the X (ATRX) zinc finger DNA-binding motif, and a polybromo homology domain targeting DNMT1 to the replication foci (Fig. 1d).[10] Thus DNMT1 forms the core of the DNA replication machinery complex. In addition to methyltransferase activity, interaction with DNMT1-associated protein (DMAP) 1,[11] E2F1,[12] histone deacetylase (HDAC) 1 and 2 and methyl CpG binding proteins (MBD)[13] through the N-terminal regulatory domain makes DNMT1 a crucial element of the transcription suppression complex.

The preference of DNMT1 for hemi-methylated over unmethylated substrates *in vitro*[14] and its targeting of replication foci[15] are believed to allow copying of the methylation pattern of the parental strand to the newly synthesized daughter DNA strand. Thus, DNMT1 has been recognized as the 'maintenance' DNMT (Fig. 1c). Although DNMT2 contains the full set of conserved motifs of the C-terminal catalytic domain, it lacks the N-terminal regulatory domain characteristic of eukaryotic DNMT (Fig. 1d). The methyltransferase activity of the recombinant DNMT2 protein is weak *in vitro* and *in vivo*. DNMT3A and DNMT3B also contain the full set of conserved motifs of the C-terminal catalytic domain, and their N-terminal regulatory domains are divergent on the N-terminal side of the cysteine-rich ATRX zinc finger DNA-binding motif (Fig. 1d). DNMT3A and DNMT3B show *de novo* DNA methylation activity (Fig. 1c) *in vitro*.[16] Pericentromeric satellite regions are considered to be one of the specific targets of DNMT3B, because Dnmt3B–/– mice lack DNA methylation in such regions and die *in utero*.[16] Germline mutations of the *DNMT3B* gene have been reported in patients with immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, a rare recessive autosomal disorder characterized by DNA hypomethylation on pericentromeric satellite regions.[17] Because *de novo* methylation of CpG islands has actually been observed in human fibroblasts overexpressing DNMT1,[18] DNMT1 is capable of *de novo* DNA methylation activity *in vivo* as well as having a maintenance function, and DNA methylation status may be determined on the basis of cooperation between DNMT1 and the DNMT3 family *in vivo*.[19] DNMT3L lacks conserved motifs of the catalytic domain but is otherwise closely related to the N-terminal regulatory domain of DNMT3A and DNMT3B (Fig. 1d) and cooperates with the DNMT3 family to establish an imprinting pattern.[20]

MBD are one of mediators of cross-talk between DNA methylation and another major epigenetic event, histone modification. Until 1998, MeCP2 had been the only functionally defined MBD. When MeCP2 binds to methylated CpG dinucleotide, its transcriptional repression domain recruits a co-repressor complex containing Sin 3A and HDAC, resulting in compaction of the chromatin and stable repression of the target gene.[21,22] Later, MBD1, MBD2, MBD3 and MBD4 were identified. MBD1 interacts with histone H3 methyltransferase SETDB1.[23] MBD2[24] and MBD3[25] are involved in another HDAC complex, Mi-2/NuRD. MBD4 is thought to act as a thymine DNA glycosylase, repairing G:T or G:U mismatches at CpG sites.[26]

DNA METHYLATION AND HUMAN CANCERS

In comparison with normal cells, human cancer cells show a drastic change in DNA methylation status, generally exhibiting global DNA hypomethylation and accompanying region-specific hypermethylation.[27–30] Because 5-methylcytosine is deaminated to thymine, DNA hypermethylation facilitates gene mutation in human cancers. DNA hypomethylation in cancer cells causes chromatin decondensation and chromosomal rearrangements that may result in chromosomal instability. Moreover, DNA hypermethylation of CpG islands near the promoter regions silences specific genes including tumor suppressor genes in cooperation with histone modification:[31] hypermethylation of CpG islands in the promoter regions of tumor-suppressor genes in cancer cells is associated with

deacetylation of histones H3 and H4, loss of histone H3, lysine 4 (H3K4) methylation, and gain of H3K9 methylation (Fig. 1b).

A reduction of DNMT1 activity in ApcMin mice due to heterozygosity of the *Dnmt1* gene, in conjunction with treatment using the DNMT inhibitor 5-aza-deoxycytidine, reduces the average number of intestinal adenomas.[32] In contrast, genomic hypomethylation in *Nf1+/– p53+/–* (NPcis) mice due to the introduction of a hypomorphic allele of *Dnmt 1* (Dnmt1Chip/–) induces sarcomas at an earlier age in comparison with NPcis littermates possessing normal levels of DNA methylation (Dnmt1Chip/+).[33] The loss of heterozygosity (LOH) rate is increased in hypomethylated cells in Dnmt1Chip/– mice. Chromosomal instability accompanied by activation of endogenous retroviral elements has also been observed in Dnmt1Chip/– mice.[34] These observations in genetically engineered animals clearly demonstrate a causal relationship between alterations of DNA methylation and human cancers. Correlation between the etiological backgrounds of human cancers and alterations of DNA methylation, however, can be clarified only by analysis of clinical samples.

In order to determine the significance of DNA methylation alterations during multistage carcinogenesis, DNA methylation status should be analyzed in a range of tissue samples from precancerous conditions to malignant states (Fig. 2). Such empirical data are indispensable for clinical application of DNA methylation to carcinogenetic risk estimation, early diagnosis, prognostication, prevention and therapy. Therefore the following sections describe the results obtained by analysis of DNA methylation status in tissue samples for which the clinicopathological characteristics have been strictly determined.

HEPATOCARCINOGENESIS IN LIVERS DAMAGED BY HEPATITIS VIRUS INFECTION

Alterations of DNA methylation in precancerous conditions

The majority of hepatocellular carcinomas (HCC) are associated with HBV or HCV infection. Clonal expansion of hepatocytes is initiated during the regeneration process in damaged livers; a clonal integration pattern of HBV is evident in each cirrhotic nodule. Therefore, chronic hepatitis and liver cirrhosis are considered to be precancerous conditions. Small nodular lesions of early-stage HCC first develop in livers with chronic hepatitis and cirrhosis, and then progressed HCC often emerge within early-stage HCC nodules (nodule-in-nodule-type HCC). Thus, macro- and microscopically, HCC represent a typical scenario of multistage carcinogenesis.[36]

The LOH on chromosome 16 has been frequently detected on classic restriction fragment length polymorphism using Southern blot in HCC that are poorly differentiated, large in size, and associated with metastasis.[37] Therefore, this seems to be a late event during multistage hepatocarcinogenesis. At the time of these discoveries, only a few molecular events in the earlier stage of hepatocarcinogenesis were known. But studies using classic Southern blot with a DNA methylation-sensitive restriction enzyme frequently showed alterations of DNA methylation at multiple loci on chromosome 16 even in non-cancerous liver tissues with chronic hepatitis or cirrhosis, unlike normal liver tissues obtained from patients with liver metastases from primary colon cancer.[38] This was one of the earliest reports of alterations of DNA methylation in the precancerous stage. Because the molecular weight of DNA fragments digested using a DNA methylation-sensitive restriction enzyme in HCC was higher than that in precancerous conditions, and the intensity of larger-sized bands was increased in HCC in comparison with precancerous conditions, the numbers of methylated CpG dinucleotides and cells having DNA hypermethylation may increase progressively as precancerous conditions develop into HCC.[38] The incidence of DNA hypermethylation on chromosome 16 was significantly correlated with higher histological grade, portal vein involvement and intrahepatic metastasis of HCC.[38] The presence of DNA hypermethylation in both precancerous conditions and progressed HCC suggests that aberrant DNA methylation is one of the earliest molecular events during hepatocarcinogenesis and also participates in malignant progression.

Silencing of tumor suppressor genes

The *E-cadherin* gene is located on 16q22.1 near the aforementioned hot spots of both DNA hypermethylation and LOH in HCC. E-cadherin acts as a Ca^{2+}-dependent cell–cell adhesion molecule in the adherens junctions of epithelial cells.[39] Cell–cell adhesion determines cell polarity and participates in histogenesis. The mutual adhesiveness of cancer cells is significantly weaker than that of normal cells, and this allows cancer cells to disobey the social order, resulting in destruction of histological architecture, which is a morphological hallmark of malignant tumors. The *E-cadherin* gene is a tumor suppressor gene that can be silenced by a two-hit mechanism consisting of LOH and gene mutation in cancers such as signet-ring cell carcinoma of the stomach[40] and lobular carcinoma of the breast,[41] in which cancer cells completely lose their mutual adhesiveness even in the *in situ* carcinoma stage. In contrast, reduced expression of E-cadherin is believed to trigger the release of cancer cells from primary cancer nests, resulting in cancer invasion and metastasis.[42] Significant correlations between reduced expression of E-cadherin and poor prognosis have been

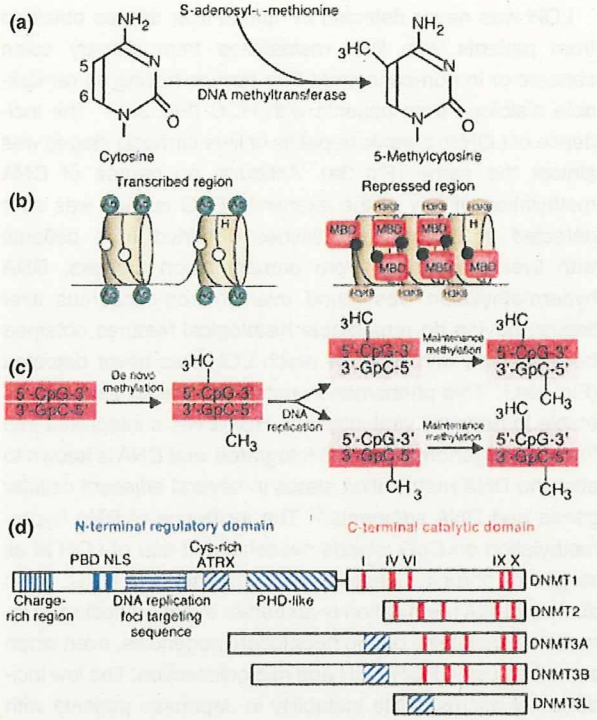

Figure 1 DNA methylation in mammals. (**a**) DNA methylation is a covalent chemical modification resulting in addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring in CpG dinucleotides. DNA methyltransferases (DNMT) transfer methyl groups from S-adenosyl-L-methionine to cytosines. (**b**) DNA methylation normally promotes a highly condensed heterochromatin structure, in which active transcription does not occur, through recruitment of DNA-organizing proteins including histone deacetylase complex and methyl CpG binding proteins (MBD). The repressed regions are associated with deacetylation of histones H3 and H4 and gain of histone H3, lysine 9 (H3K9) methylation. H, histone octamer; (○) unmethylated CpG dinucleotides; (●) methylated CpG dinucleotides. (**c**) DNA methylation is a stable modification that is inherited throughout cell divisions (maintenance methylation). The preference of maintenance DNMT for hemi-methylated over unmethylated substrates and its targeting of replication foci are believed to allow copying of the methylation pattern of the parental strand to the newly synthesized daughter DNA strand. *De novo* methylation occurs by *de novo* DNMT during the development of mammals and carcinogenesis. (**d**) Structure of DNMT. The C-terminal catalytic domain is characterized by the presence of conserved motifs I, IV, VI, IX and X. The N-terminal regulatory domain of DNMT1 contains a proliferating cell nuclear antigen-binding domain (PBD), a nuclear localization signal (NLS), a cysteine-rich alpha thalassemia and retardation on the X (ATRX) zinc finger DNA-binding motif, and a polybromo homology domain (PHD) targeting DNMT1 to the replication foci.

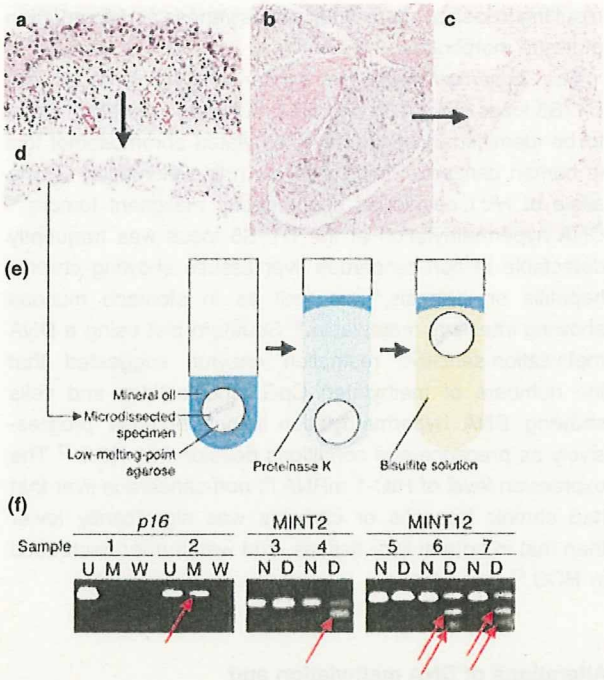

Figure 2 Analysis of DNA methylation status in tissue specimens. Tissue sample of carcinoma *in situ* (**a**) before and (**d**) during microdissection, and that of invasive carcinoma (**b**) before and (**c**) after microdissection. (**e**) Microdissected specimens were subjected to agarose bead-embedded methylation-specific polymerase chain reaction (MSP),[35] which was originally developed for analysis of DNA methylation in tiny tissue samples. (**f**) Examples of the results of MSP for the *p16* gene and combined bisulfite restriction enzyme analysis (COBRA) for MINT 2 and 12 clones. Polymerase chain reaction products yielded by primer sets for methylated (M), unmethylated (U) and unmodified wild-type (W) DNA and digested (D) and non-digested (N) DNA fragments using methylation-sensitive restriction enzymes are shown. Arrows, methylated DNA fragments.

reported in patients with cancers.[42] The promoter region of the *E-cadherin* gene contained DNA methylation in human cancer cell lines lacking E-cadherin expression, and E-cadherin expression was induced after treatment with the DNMT inhibitor 5-azacytidine in such cell lines.[43] Thus, following the *retinoblastoma* (*RB*) and *von Hippel-Lindau* (*VHL*) genes, the *E-cadherin* gene became the third example of a tumor suppressor gene that is silenced by DNA hypermethylation.[43] When assessed on Southern blot, DNA hypermethylation around the promoter region of the *E-cadherin* gene can be frequently detected even in non-cancerous liver tissues showing chronic hepatitis or cirrhosis.[44] Heterogeneous E-cadherin expression in non-cancerous liver tissues showing chronic hepatitis or cirrhosis, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity, might be due, at least partly, to DNA hypermethylation.[44] A significant correlation between DNA hypermethylation around the promoter region and reduced expression of E-cadherin was found in HCC.[44] This was the first demonstration of a significant correlation between DNA hypermethylation and reduced expression in a cohort of clinical tissue samples. DNA hypermethylation around the promoter region may participate in hepatocarcinogenesis through reduction of E-cadherin expression,

resulting in loss of intercellular adhesiveness and destruction of tissue morphology.

The *hypermethylated in cancer (HIC)-1* gene at the D17S5 locus (17q13.3) was the first tumor suppressor gene to be identified in commonly methylated chromosomal loci in human cancers;[45] mice with germ line disruption of one allele of *Hic1* developed spontaneous malignant tumors.[46] DNA hypermethylation at the D17S5 locus was frequently detectable in non-cancerous liver tissues showing chronic hepatitis or cirrhosis,[47] as well as in stomach mucosa showing intestinal metaplasia.[48] Southern blot using a DNA methylation-sensitive restriction enzyme suggested that the numbers of methylated CpG dinucleotides and cells showing DNA hypermethylation might increase progressively as precancerous conditions develop into HCC.[47] The expression level of HIC-1 mRNA in non-cancerous liver that had chronic hepatitis or cirrhosis was significantly lower than that in normal liver tissues, and was further decreased in HCC.[47]

Alterations of DNA methylation and chromosomal instability

The hot spot for DNA hypermethylation in HCC corresponds to a previously reported hot spot of LOH on chromosome 16.[38] It remains to be clarified whether alterations of DNA methylation might predispose the locus to allelic loss, or whether common or different causes facilitate both alterations of DNA methylation and LOH at certain loci. But classic Southern blot clearly showed that DNA hypermethylation precedes LOH at the same chromosomal loci during hepatocarcinogenesis: DNA hypermethylation was detected in bulk non-cancerous liver tissues showing chronic hepatitis or cirrhosis, in which LOH has never been detected using the same method.

Recently, microdissection techniques and polymerase chain reaction (PCR) using microsatellite markers have been developed for detecting LOH in small numbers of cells from paraffin-embedded tissues. LOH has been reported even in microdissected specimens from dysplastic lesions adjacent to cancers. In order to re-examine whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis, in microdissected specimens obtained from pseudo-lobules and regenerative nodules in non-cancerous liver tissues having chronic hepatitis or cirrhosis and HCC, LOH and microsatellite instability were examined using multiple microsatellite markers, and the DNA methylation status of multiple C-type CpG islands[49] that are known to be methylated in a cancer-specific, but not age-dependent manner, was examined on methylation-specific PCR and combined bisulfite restriction enzyme analysis.[50,51]

LOH was never detected in normal liver tissues obtained from patients with liver metastases from primary colon cancers or in non-cancerous liver tissues having no remarkable histology from patients with HCC (Fig. 3a).[51] The incidence of LOH in chronic hepatitis or liver cirrhosis stages was almost the same (Fig. 3a). Although no degree of DNA methylation of any of the examined CpG islands was ever detected in normal liver tissues obtained from patients with liver metastases from primary colon cancers, DNA hypermethylation was found even in non-cancerous liver tissues having no remarkable histological features obtained from patients with HCC, in which LOH was never detected (Fig. 3a).[51] This phenomenon might be at least partly attributable to hepatitis viral infection. HBV-DNA is integrated into the cellular genome, and the integrated viral DNA is known to alter the DNA methylation status in several adjacent cellular genes and DNA segments.[52] The incidence of DNA hypermethylation on CpG islands overwhelmed that of LOH at all stages of chronic hepatitis, liver cirrhosis and HCC. Thus aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis, even when examined using PCR-LOH and microdissection. The low incidence of microsatellite instability in Japanese patients with HCC[53] (Fig. 3a) was compatible with absence of silencing of the *human MutL homologue 1* (*hMLH1*) gene by DNA hypermethylation during hepatocarcinogenesis.[51]

Overexpression of DNMT1

Abnormalities of DNMT underlying alterations of DNA methylation was examined during hepatocarcinogenesis. Mutational inactivation of the *DNMT1* gene that can potentially cause genome-wide alterations of DNA methylation was never detected in HCC or in stomach cancers, whereas colorectal cancers infrequently had mutations of the *DNMT1* gene, including a mutation resulting in deletion of the whole catalytic domain due to a premature stop codon.[54] Mutational inactivation of the *DNMT1* gene may be a rare event during human carcinogenesis. In contrast, the expression level of DNMT1 mRNA was significantly higher even in non-cancerous liver tissues having chronic hepatitis or cirrhosis than in normal liver tissues, and was even higher in HCC (Fig. 3b).[55,56] The incidence of DNMT1 protein overexpression in HCC is significantly correlated with poorer tumor differentiation and portal vein involvement (Fig. 3c).[57] Moreover, the recurrence-free and overall survival rates of patients with HCC that has overexpression of DNMT1 protein are significantly lower than those of patients with HCC that do not (Fig. 3d).[57] Immunohistochemistry of DNMT1 in liver biopsy specimens obtained for histological diagnostic purposes and/or hepatectomy specimens may become a useful tool for prognostication in individual clinical cases.

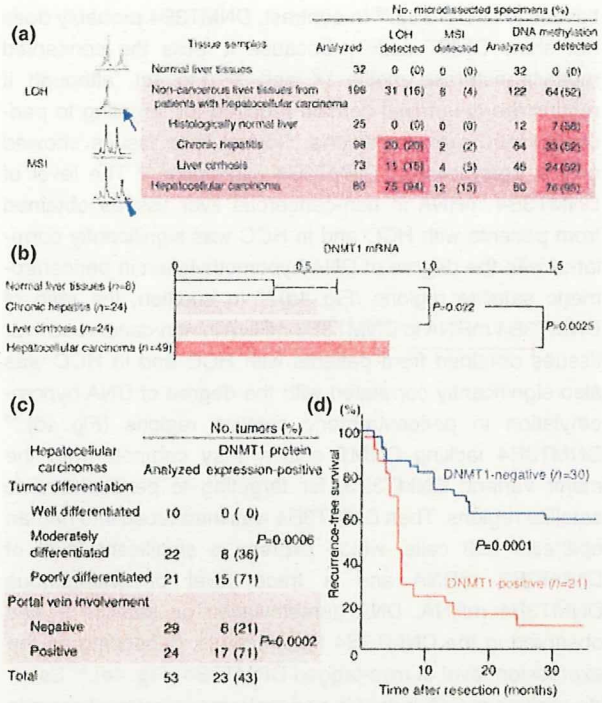

Figure 3 Alterations of DNA methylation during hepatocarcinogenesis. (**a**) Loss of heterozygosity (LOH; arrow) and microsatellite instability (MSI; arrowhead) were examined using 39 microsatellite markers; and DNA methylation status on 8 C-type CpG islands was examined on methylation-specific polymerase chain reaction and combined bisulfite restriction enzyme analysis of microdissected tissue specimens.[51] (**b**) The expression level of DNA methyltransferase 1 (DNMT1) mRNA was significantly higher even in non-cancerous liver tissues that had chronic hepatitis or cirrhosis than in normal liver tissues, and was even higher in hepatocellular carcinomas.[55,56] (**c**) DNMT1 protein expression in hepatocellular carcinomas was significantly correlated with poorer tumor differentiation and portal vein involvement.[57] (**d**) Recurrence-free survival rate of patients whose hepatocellular carcinomas had protein overexpression of DNMT1 was significantly lower than that of patients whose hepatocellular carcinomas did not.[57]

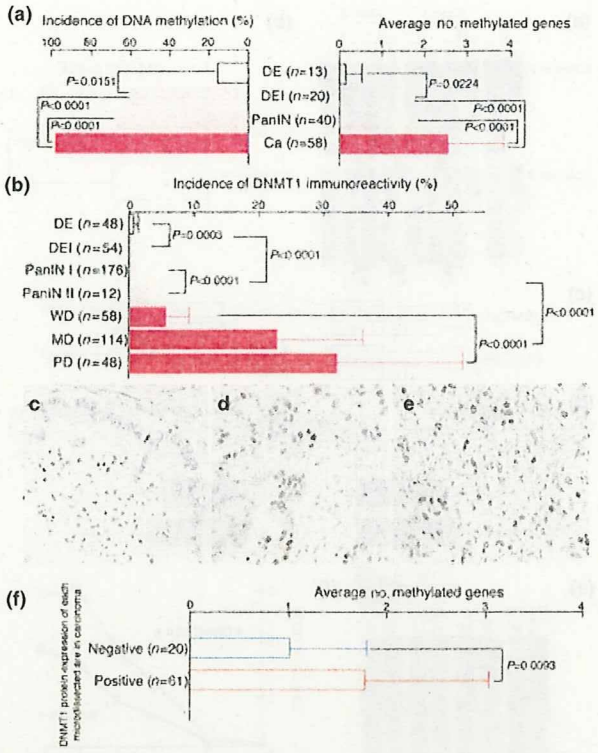

Figure 5 (**a**) Alterations of DNA methylation during pancreatic carcinogenesis. When DNA methylation status of the *p14*, *p15*, *p16*, *p73*, *APC*, *hMLH1*, *MGMT*, *BRCA1*, *GSTP1*, *TIMP-3*, *E-cadherin* and *DAPK-1* tumor-related genes was examined in microdissected specimens, the incidence of DNA hypermethylation of at least one of the genes and the average number of methylated genes were significantly higher in peripheral pancreatic duct epithelia with an inflammatory background (DEI) and pancreatic intra-epithelial neoplasia (PanIN) than in peripheral pancreatic duct epithelia without an inflammatory background (DE), and were further increased in ductal carcinomas (Ca).[70] (**b**) Incidence of nuclear DNA methyltransferase 1 (DNMT1) immunoreactivity was significantly elevated in DEI and PanIN than in DE.[71] The incidence of nuclear DNMT1 immunoreactivity was significantly associated with the degree of PanIN dysplasia (PanIN I vs Pan IN II). The incidence of nuclear DNMT1 immunoreactivity was significantly higher in ductal carcinomas than in PanIN, and was associated with poorer differentiation of ductal carcinomas (MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma; WD, well-differentiated adenocarcinoma). Heterogeneity of DNMT1 protein expression among components having different grades of histological differentiation was observed in a representative cancer from a single patient:[71] (**d**) Moderately and (**e**) poorly differentiated adenocarcinoma components had a higher incidence of DNMT1 immunoreactivity than (**c**) the well-differentiated adenocarcinoma component. (**f**) The average number of methylated tumor-related genes in microdissected specimens of ductal carcinomas was significantly correlated with the expression level of DNMT1 protein examined on immunohistochemistry in the precisely microdissected areas.[70]

Aberrant splicing of DNMT3B

Although DNA hypomethylation on pericentromeric satellite regions, such as satellites 2 and 3, was frequently detected in both non-cancerous liver tissues having chronic hepatitis or cirrhosis and HCC (Fig. 4a),[56] and such regions are one of the target sequences of DNMT3B, no mutation of any coding exon of the *DNMT3B* gene was detected in the examined HCC.[58] The total level of DNMT3B mRNA was higher in HCC than in the corresponding non-cancerous liver tissues (Fig. 4b).[56] Thus, it is unlikely that reduced expression of DNMT3B simply causes DNA hypomethylation in these regions during hepatocarcinogenesis. There are four splice variants in the C-terminal catalytic domain of DNMT3B. DNMT3B3 possesses the N-terminal region and conserved methyltransferase motifs I, IV, VI, IX and X (Fig. 4c) and its DNMT activity has been confirmed *in vitro*.[59] Data obtained on splice-variant-specific quantitative reverse transcription–PCR have indicated that the major variant in normal liver

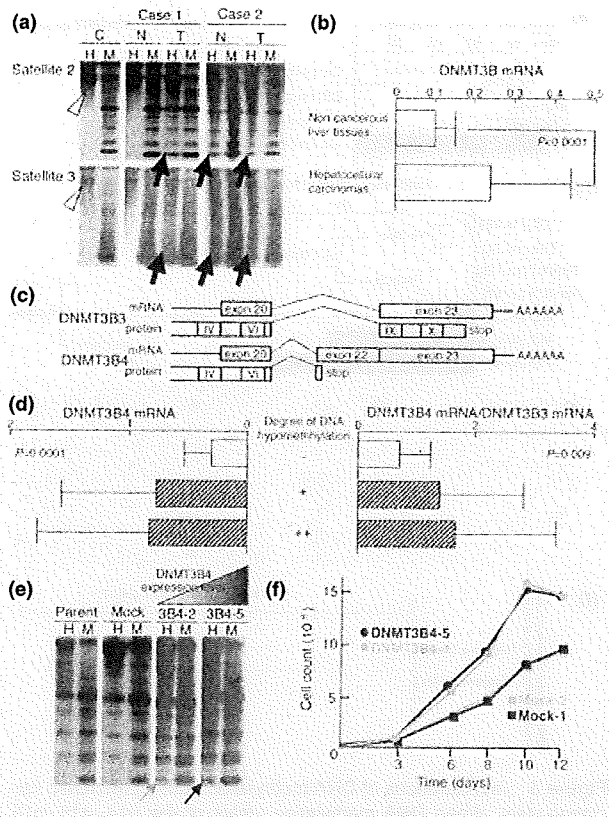

Figure 4 Overexpression of DNA methyltransferase 3B4 (DNMT3B4) associated with DNA hypomethylation in pericentromeric satellite regions during hepatocarcinogenesis. (**a**) Although satellites 2 and 3 were fully methylated in normal liver tissue obtained from a patient with liver metastasis from primary colon cancer (C, arrowheads), DNA hypomethylation on satellites 2 and 3 was frequently detected in both non-cancerous liver tissues that had chronic hepatitis or cirrhosis (N) and hepatocellular carcinomas (T).[56] H, methylation-sensitive restriction enzyme HpaII; M, methylation-non-sensitive restriction enzyme Msp I. Arrows, DNA hypomethylation. (**b**) The total level of DNMT3B mRNA was higher in hepatocellular carcinomas than in the corresponding non-cancerous liver tissue.[56] Thus, it is unlikely that reduced expression of DNMT3B causes DNA hypomethylation in these regions during hepatocarcinogenesis. (**c**) Structure of splice variants of DNMT3B, DNMT3B3 and DNMT3B4. (**d**) The level of DNMT3B4 mRNA and the ratio of DNMT3B4 mRNA to DNMT3B3 mRNA in non-cancerous liver tissues obtained from patients with hepatocellular carcinomas and in hepatocellular carcinomas were significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions.[58] +, smaller fragments detected in the *Hpa* II digest compared with the *Hpa* II digest of normal liver tissues; ++, *Hpa* II digest had the same hybridization pattern as the *Msp* I digest of its own and normal liver tissues. (**e**) DNA hypomethylation on satellite 2 was observed in transfection of human epithelial 293 cells with DNMT3B4 cDNA (clones 3B4-2 and 3B4-5) compared to mock transfectant (Mock) and parent 293 cells (Parent).[58] H; Hpa II digest; M, Msp I digest. (**f**) The growth rate of DNMT3B4 transfectants (clones DNMT3B4-4 and 3B4-5) was approximately double that of mock-transfectants (clones Mock-1 and Mock-2) soon after the introduction of DNMT3B4,[61] when chromosomal instability may not yet have accumulated.

tissues is DNMT3B3.[58] In contrast, DNMT3B4 probably does not show DNMT activity because it lacks the conserved methyltransferase motifs IX and X (Fig. 4c), although it retains the N-terminal domain required for targeting to pericentromeric satellite regions. Normal liver tissues showed only a trace level of DNMT3B4 expression.[58] The level of DNMT3B4 mRNA in non-cancerous liver tissues obtained from patients with HCC and in HCC was significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions (Fig. 4d).[58] In addition, the ratio of DNMT3B4 mRNA to DNMT3B3 mRNA in non-cancerous liver tissues obtained from patients with HCC and in HCC was also significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions (Fig. 4d).[58] DNMT3B4 lacking DNMT activity may compete with the major variant, DNMT3B3, for targeting to pericentromeric satellite regions. Then DNMT3B4 was introduced into human epithelial 293 cells, which express a significant level of DNMT3B3 mRNA and a trace level of endogenous DNMT3B4 mRNA. DNA demethylation on satellite 2 was observed in the DNMT3B4 transfectants, depending on the expression level of myc-tagged DNMT3B4 (Fig. 4e).[58] Satellite regions are abundant in pericentromeric heterochromatin DNA on chromosomes 1, 9 and 16. In fact, frequent chromosome 1q copy gain with a pericentromeric breakpoint has been reported in HCC having DNA hypomethylation on satellite 2.[60] DNMT3B4 overexpression may lead to chromosomal instability through induction of DNA hypomethylation in pericentromeric satellite regions during hepatocarcinogenesis.

The growth rate of DNMT3B4 transfectants was approximately double that of mock-transfectants soon after the introduction of DNMT3B4 (Fig. 4f),[61] when chromosomal instability may not yet have accumulated. It was assumed that this change was caused by altered gene expression. Although the majority of the genes that were upregulated in DNMT3B4 transfectants were implicated in interferon signaling,[61] genes that encoded interferons themselves were not upregulated. Signal transducer and activator of transcription (STAT) 1,[61] which acts as an effector of interferon signaling, has been listed as one of the upregulated genes in DNMT3B4 transfectants. A significant correlation between the expression levels of DNMT3B4 and STAT1 mRNA was confirmed in tissue specimens of HCC.[61] Overexpression of DNMT3B4 is involved in multistage carcinogenesis not only by inducing chromosomal instability but also by affecting the expression of specific genes.

Altered expression of methyl CpG binding proteins

Although many researchers have focused on cross-talk between DNA methylation and histone modification,

abnormalities of MBD in human cancers do not seem to have attracted much attention. The expression level of MeCP2 mRNA in HCC with portal vein involvement is significantly lower than that in HCC without such involvement,[56] suggesting that reduced expression of MeCP2 may be associated with malignant progression of HCC. Reduced MBD2 mRNA expression has been observed in HCC,[56] as well as in colorectal and stomach cancers,[62] suggesting that reduced MBD2 expression may be associated with a particular step in human carcinogenesis. The expression level of MBD4 mRNA in HCC is significantly lower than that in the corresponding non-cancerous liver tissues and is significantly correlated with poorer tumor differentiation and portal vein involvement.[56] Reduced MBD4 expression may result in frequent C-T transitions in tumor suppressor genes.

VIRUS INFECTION-ASSOCIATED CARCINOGENESIS IN THE STOMACH AND THE UTERINE CERVIX

In immunohistochemistry for DNMT1, nuclear immunoreactivity was not detected in any of the non-cancerous epithelia, except in proliferative zones, but was frequently found in stomach cancers.[63] DNMT1 overexpression, at both the mRNA[64] and protein[63] levels, correlated significantly with poorer tumor differentiation and with CpG island methylator phenotype (CIMP), defined by frequent DNA hypermethylation of C-type CpG islands,[65] in stomach cancers. The *hMLH1*, *thrombospondin-1* (*THBS-1*) and *E-cadherin* genes may be targets for overexpressed DNMT1 in stomach cancers.[63] Four percent of the examined patients with stomach cancers had EBV infection, a potential etiological factor in gastric carcinogenesis, in their cancer cells, and all cancers with EBV infection had DNMT1 protein overexpression.[63] Induction of latent membrane protein 1 of EBV has been reported to induce overexpression of DNMT1 in cultured cancer cells.[66] EBV infection in stomach cancers was associated with marked accumulation of DNA hypermethylation of C-type CpG islands.[63] With respect to stomach carcinogenesis, *Helicobacter pylori* infection, another etiological factor, is known to strongly promote regional DNA hypermethylation.[67]

Cervical intra-epithelial neoplasia (CIN) is a precursor lesion for squamous cell carcinoma of the uterine cervix closely associated with HPV infection. DNMT1 protein expression is increased even in low-grade CIN relative to normal squamous epithelium, and further increased in higher-grade CIN and squamous cell carcinomas of the uterine cervix.[68] HPV-16 E7 protein has been reported to associate directly with DNMT1 and stimulate the enzyme activity of DNMT1 *in vitro*,[69] and accumulation of DNA hypermethylation on tumor-related genes has also been observed during cervical carcinogenesis.

PANCREATIC CARCINOGENESIS ASSOCIATED WITH PERSISTENT INFLAMMATION

Cumulative DNA methylation of tumor-related genes

In the same way that HCC are preceded by chronic hepatitis, ductal carcinomas frequently emerge in pancreases damaged by chronic pancreatitis. Therefore, at least a proportion of peripheral pancreatic duct epithelia with an inflammatory background may be at the precancerous stage. DNA methylation status of the *p14*, *p15*, *p16*, *p73*, *adenomatous polyposis coli* (*APC*), *hMLH1*, *O-6-methylguanine-DNA methyltransferase* (*MGMT*), *breast cancer 1* (*BRCA1*), *glutathione S-transferase pi* (*GSTP1*), *tissue inhibitor of metalloproteinase 3* (*TIMP-3*), *E-cadherin*, and *death-associated protein kinase 1* (*DAPK-1*) tumor-related genes was examined on agarose bead-embedded methylation-specific PCR, which had been developed for DNA methylation analysis of tiny microdissected tissue specimens (Fig. 2e).[35] The incidence of DNA hypermethylation of at least one of the genes and the average number of methylated genes were significantly higher in peripheral pancreatic duct epithelia with an inflammatory background and in another precancerous lesion, pancreatic intra-epithelial neoplasia (PanIN), than in peripheral pancreatic duct epithelia without an inflammatory background, and was further increased in ductal carcinomas (Fig. 5a).[70] The *BRCA1*, *APC*, *p16* and *TIMP-3* genes are frequently methylated in ductal carcinomas of the pancreas.[70]

Overexpression of DNMT1

When examined on immunohistochemistry, the incidence of nuclear DNMT1 immunoreactivity was significantly elevated in peripheral pancreatic ductal epithelia with an inflammatory background and PanIN than in peripheral pancreatic ductal epithelia without an inflammatory background (Fig. 5b).[71] With respect to inflammation-related carcinogenesis,[72] treatment with the cytokine interleukin-6 has been reported to induce overexpression of DNMT1 in cultured cells.[73] The incidence of nuclear DNMT1 immunoreactivity was significantly associated with the degree of PanIN dysplasia (Fig. 5b), being significantly higher in invasive ductal carcinomas than in PanIN, and was associated with poorer differentiation of invasive ductal carcinomas (Fig. 5b–e).[71] Protein overexpression of DNMT1 in ductal carcinomas is significantly correlated with the extent of cancer invasion to surrounding organs and with advanced stage,[71] suggesting that overexpression of DNMT1 is associated with aggressiveness of pancreatic cancers. Moreover, patients with ductal carcinomas of the pancreas

having overexpression of DNMT1 protein have a poorer outcome.[71]

The average number of methylated tumor-related genes in microdissected specimens of invasive ductal carcinoma was significantly correlated with the expression level of DNMT1 protein examined on immunohistochemistry in the precisely microdissected areas (Fig. 5f).[70] Thus DNMT1 may be responsible for *de novo* methylation of CpG islands during pancreatic carcinogenesis. A theoretical explanation for the role of DNMT1 in *de novo* DNA methylation in human cancers with dysfunction of p21WAF1,[74] which competes with DNMT1 for binding with PCNA, has been proposed.[15] Moreover, although maintenance activities of DNMT1 have been noticed *in vitro* in relation to its preference for hemi-methylated substrates, it has recently been suggested that DNMT1 is capable of *de novo* DNA methylating activity *in vivo*.[18,19] Therefore, it is feasible that, in cancers, overexpression of DNMT1 participates in regional DNA hypermethylation.

LUNG CARCINOGENESIS ASSOCIATED WITH CIGARETTE SMOKING

In addition to chronic inflammation and/or persistent infection with pathogenic microorganisms, cigarette smoking is another background factor associated with alterations of DNA methylation during multistage carcinogenesis. DNA hypermethylation at the D17S5 locus has been frequently observed in non-cancerous lung tissues, which may contain progenitor cells for cancers, obtained from patients with non-small-cell lung cancers, and in corresponding non-small-cell lung cancers.[75] The incidence of DNA hypermethylation at the D17S5 locus is significantly associated with poorer differentiation of lung adenocarcinomas.[75] The incidence of DNA hypermethylation in both non-cancerous lung tissues and non-small-cell lung cancers of patients who are current smokers is significantly higher than in patients who have never smoked.[75] The extent of pulmonary anthracosis is an index for the cumulative effects of smoking. The extent of pulmonary anthracosis in each resected lung has been graded macroscopically: grade 1, slight accumulation of charcoal particles in the intra-lobular lymphatics forming a fine reticular pattern scattered in the visceral pleura; grade 2, reticular pattern due to charcoal particle accumulation is denser and shows fusion in places; and grade 3, dense accumulation of charcoal particles is present throughout most of the visceral pleura. The incidence of DNA hypermethylation at the D17S5 locus analyzed on Southern blot using a DNA methylation-sensitive restriction enzyme in non-cancerous lung tissues showing grade 3 anthracosis obtained from patients with non-small-cell lung cancers was higher than that in patients with grade 2 or 1 anthracosis.[30] The molecular mechanisms by which carcinogens related to cigarette smoking affect DNA methylation status should be investigated.

UROTHELIAL CARCINOGENESIS SHOWING MULTICENTRICITY AND TENDENCY TO RECUR

DNMT1 overexpression is not always a secondary result of increased cell proliferative activity but correlated with regional DNA hypermethylation

Urothelial carcinomas of the urinary bladder are clinically remarkable because of their multicentricity and tendency to recur: synchronously or metachronously multifocal urothelial carcinomas often develop in individual patients. A possible mechanism for such multiplicity is the 'field effect', whereby carcinogenic agents in the urine cause malignant transformation of multiple urothelial cells. Even non-cancerous urothelia with no remarkable histology obtained from patients with urinary bladder cancers can be considered precancerous, because they may be exposed to carcinogens in the urine. DNMT1 protein expression is significantly higher in non-cancerous urothelia having no remarkable histology obtained from patients with urinary bladder cancers than in normal urothelia obtained from patients without urinary bladder cancers, and further increases from dysplastic urothelia to urothelial carcinoma.[76] Thus progressively increasing DNMT1 protein expression is associated with multistage urothelial carcinogenesis from precancerous stages.

In contrast, DNMT1 mRNA is expressed mainly during the S-phase and because tumor tissues of various organs generally contain a greater proportion of dividing cells than do normal tissues, it has been debatable whether increased DNMT1 expression is due to an increase in the proportion of dividing cells or to an acute increase of DNMT1 expression per individual cancer cell. This uncertainty prompted us to compare DMNT1 immunoreactivity and the PCNA labeling index during urothelial carcinogenesis. The incidence of nuclear DNMT1 immunoreactivity had already increased in non-cancerous urothelia having no remarkable histology obtained from patients with urinary bladder cancers, for which the PCNA labeling index had not yet increased, indicating that overexpression of DNMT1 is not a secondary result of increased cell proliferative activity but precedes increased cell proliferative activity during multistage urothelial carcinogenesis.[76] Excessive amounts of DNMT1 compared to PCNA, which targets DNMT1 to replication foci, may participate in *de novo* methylation of CpG islands. Indeed, among all examined microdissected specimens of non-cancerous urothelia having no remarkable histology obtained from patients with urinary bladder cancers, dysplastic urothe-