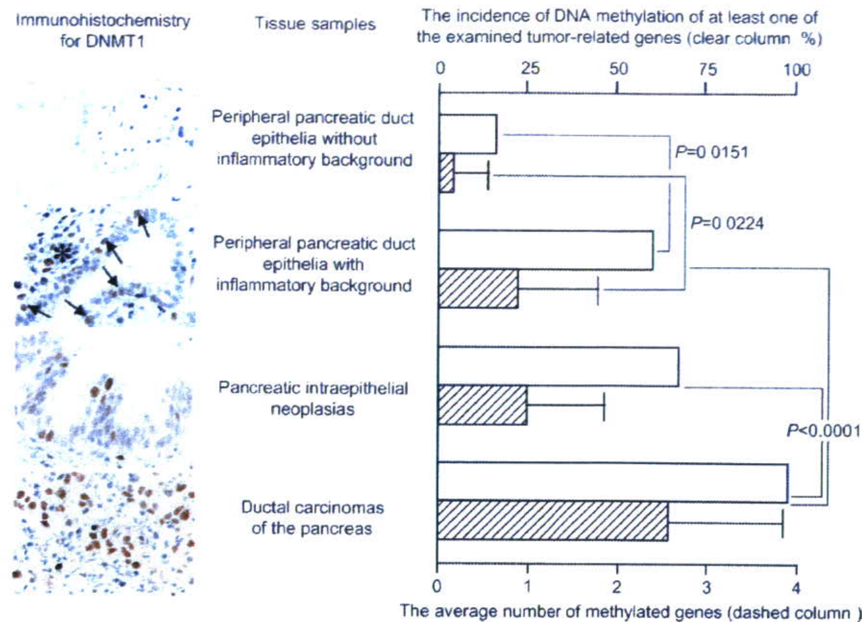

Fig. 1. DNMT1 protein expression and DNA methylation status of CpG islands in tumor-related genes during multistage carcinogenesis of the pancreas. Immunohistochemical examination for DNMT1 was performed in peripheral pancreatic duct epithelia without an inflammatory background, peripheral pancreatic duct epithelia with an inflammatory background (arrows), PanINs and ductal carcinomas (49). Infiltrating lymphocytes (asterisk) were used as an internal positive control for DNMT1 immunoreactivity. Tissue samples were microdissected from surgically resected materials, embedded in agarose beads and subjected to methylation-specific PCR to evaluate the DNA methylation status of the *p14, p15, p16, p73, APC, hMLH1, MGMT, BRCA1, GSTP1, TIMP-3, CDH1 and DAPK-1* genes (50). The incidence of DNMT1 nuclear immunoreactivity, the incidence of DNA methylation of at least one of the 12 genes and the average number of methylated genes increased progressively during multistage carcinogenesis of the pancreas. The average number of methylated genes in ductal carcinomas was significantly correlated with DNMT1 protein expression level ($P = 0.0093$).

who were current smokers was significantly higher than in patients who had never smoked (52). The incidence of DNA hypermethylation in non-cancerous lung tissues obtained from patients with non-small cell lung cancers was significantly correlated with the extent of pulmonary anthracosis, as an index for the cumulative effects of smoking (Figure 2; K. Eguchi, Y. Kanai, K. Kobayashi and S. Hirohashi, unpublished data). Cigarette smoking may participate in alteration of DNA methylation during the development of non-small cell lung cancers. The molecular mechanisms by which carcinogens related to cigarette smoking affect DNA methylation status are still unclear and warrant further investigation.

The incidence of DNA hypermethylation at multiple C-type CpG islands in non-cancerous tissues and cancers from various organs is summarized in Table I. For example, the methylated in tumor-25 clone is methylated in normal renal tissues obtained from patients without renal cancers as frequently as in non-cancerous renal tissues showing no marked histological findings obtained from patients with renal cancers or in renal cancers (39), although it is never methylated in normal liver tissues. DNA methylation profiles of normal tissues tend to be organ specific. Moreover, hot spots of DNA hypermethylation vary among cancers arising in different organs and may reflect the influence of various carcinogenetic factors. The molecular mechanisms responsible for determination of target genes of the CpG island methylator phenotype (CIMP), defined by frequent DNA hypermethylation of C-type CpG islands (53), should be further clarified.

Alterations of DNA methylation are a hallmark of precancerous conditions even in histologically normal tissues

Alterations of DNA methylation are considered to participate in the precancerous stage in various organs, in association with obvious etiological factors, e.g. chronic inflammation, persistent infection with viruses or other pathogenic microorganisms, as mentioned above. Unlike cancers derived from such organs, precancerous conditions in the kidney have been rarely described: pathologists hardly ever observe histological changes in non-cancerous renal tissues obtained from patients with renal cancers. Surprisingly, even in non-cancerous renal tissues showing no marked histological findings obtained from patients with renal cancers, the average number of methylated CpG islands was significantly higher than that in normal renal tissues obtained from patients without renal cancers, regardless of patient age and smoking history (39). The average number of methylated CpG islands was even higher in renal cancers. From the viewpoint of alterations of DNA methylation, the presence of precancerous conditions can be recognized even in the kidney. In other words, regional DNA hypermethylation participates in the early and precancerous stage of multistage renal carcinogenesis. More surprisingly, the average number of methylated CpG islands in non-cancerous renal tissues showing no marked histological change obtained from patients with conventional renal cell carcinomas (RCCs) was significantly correlated with a higher histological grade of corresponding RCCs developing in individual patients (Figure 3; 39), indicating that precancerous conditions showing accumulation of DNA methylation may generate more malignant RCCs.

Regional DNA hypermethylation has a prognostic impact on patients with cancers

Accumulation of DNA methylation at CpG islands in conventional RCCs is significantly correlated with higher histological grade, an infiltrating growth pattern and vascular involvement (39), suggesting that regional DNA hypermethylation is continuously involved in multistage renal carcinogenesis from precancerous conditions to malignant progression. The recurrence-free survival rate of patients with RCCs showing accumulated DNA methylation of CpG islands was significantly lower than that of patients with RCCs not showing this feature (39).

The incidence of increased DNMT1 protein expression in HCCs is significantly correlated with poorer tumor differentiation and portal

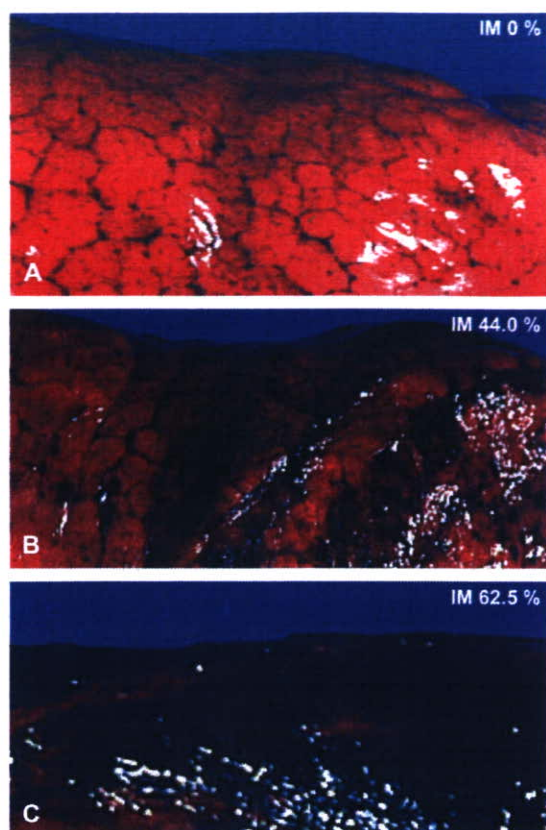

Fig. 2. Correlations between DNA hypermethylation at the D17S5 locus and anthracosis in non-cancerous lung tissues from patients with non-small cell lung cancers. The extent of pulmonary anthracosis in each resected lung was graded macroscopically: grade 1, slight accumulation of charcoal particles in the intra-lobular lymphatics forming a fine reticular pattern scattered in the visceral pleura (**A**); grade 2, the reticular pattern due to charcoal particle accumulation was denser and showed fusion in places (**B**) and grade 3, dense accumulation of charcoal particles was present throughout most of the visceral pleura (**C**). The incidence of DNA hypermethylation at the D17S5 locus (IM) analyzed by Southern blotting using a DNA methylation-sensitive restriction enzyme (NotI) in non-cancerous lung tissues showing grade 3 anthracosis (62.5%) was higher than that in those showing grade 2 (44.0%) or 1 (0%) anthracosis ($P = 0.0011$).

vein involvement (54). Moreover, the recurrence-free and overall survival rates of patients with HCCs showing increased DNMT1 protein expression are significantly lower than those of patients with HCCs that do not (54). Increased DNMT1 protein expression in ductal carcinomas of the pancreas is significantly correlated with the extent of cancer invasion to the anterior pancreatic capsule, retroperitoneal tissue and other surrounding organs and with advanced stage (49), suggesting that DNMT1 over-expression is associated with aggressiveness of pancreatic cancers. Moreover, patients with ductal carcinomas of the pancreas showing increased DNMT1 protein expression have a poorer prognosis (49).

Regional DNA hypermethylation and increased DNMT1 protein expression participate not only in the precancerous stage but also in malignant progression, and have a prognostic impact on patients with cancers. Analysis of DNA methylation status at multiple CpG islands and/or immunohistochemical examination for DNMT1 in biopsy specimens obtained for histological diagnostic purposes and/or surgically resected materials may become a useful tool for prognostication in individual clinical cases.

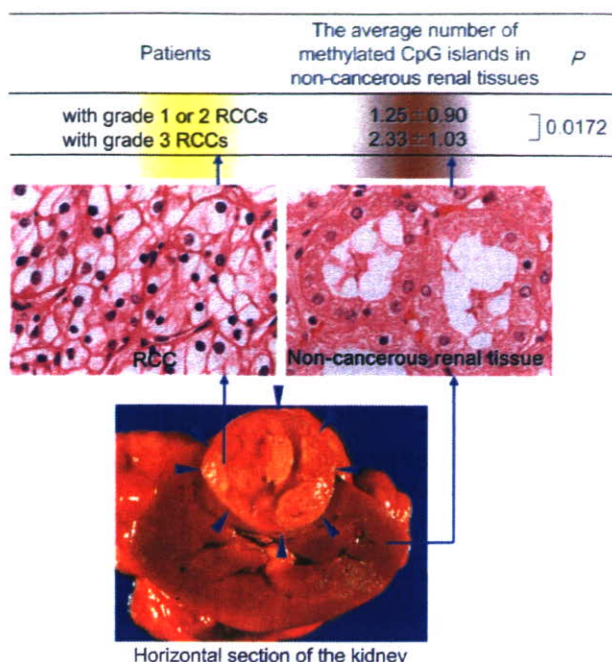

Fig. 3. DNA methylation status in non-cancerous renal tissues obtained from patients with RCC (arrowheads). The average number of methylated CpG islands in non-cancerous renal tissues obtained from patients with histological grade 3 RCCs was significantly higher than that in equivalent tissues obtained from patients with histological grade 1 or 2 RCCs (39): accumulation of DNA methylation of CpG islands in non-cancerous renal tissues was significantly correlated with a higher histological grade of the corresponding RCCs, indicating that precancerous conditions showing regional DNA hypermethylation may generate more malignant RCCs.

DNMT1 over-expression is not always a secondary result of increased cell proliferative activity but is significantly correlated with regional DNA hypermethylation

We focused on abnormalities of DNMTs underlying alterations of DNA methylation in human cancers. The expression level of DNMT1 mRNA was first examined by quantitative reverse transcription–PCR analysis, and found to be significantly higher even in non-cancerous liver tissues showing chronic hepatitis or cirrhosis than in normal liver tissues and was even higher in HCCs (55,56). With respect to multistage carcinogenesis of the pancreas, the incidence of nuclear DNMT1 immunoreactivity was significantly elevated in peripheral pancreatic ductal epithelia with an inflammatory background and PanINs than in peripheral pancreatic ductal epithelia without an inflammatory background (Figure 1; 49). The incidence of nuclear DNMT1 immunoreactivity was significantly associated with the degree of PanIN dysplasia. The incidence of nuclear DNMT1 immunoreactivity was significantly higher in invasive ductal carcinomas of the pancreas than in PanINs (Figure 1; 49). The average number of methylated tumor-related genes in microdissected specimens of ductal carcinomas of the pancreas was significantly correlated with the expression level of DNMT1 protein examined immunohistochemically in the precisely microdissected areas (50).

The level of DNMT1 mRNA expression was higher in colorectal and stomach cancers than in the corresponding non-cancerous mucosae (38). The average ratio of DNMT1 expression in tumor tissue relative to the corresponding non-cancerous mucosa was significantly higher in CIMP-positive colorectal and stomach cancers than in CIMP-negative colorectal and stomach cancers, but no such association was observed for the expression of DNMT2, DNMT3a or DNMT3b (38). Immunohistochemical examination showed that increased DNMT1 protein expression was significantly associated

with poorer differentiation of stomach cancers, whereas none of the examined non-cancerous stomach epithelia exhibited DNMT1 immunoreactivity (except in the proliferative zones) (37). Significant correlation between DNMT1 over-expression and CIMP in stomach cancers was also confirmed by immunohistochemistry at the protein level (37). The *hMLH1, THBS-1 and E-cadherin* genes may be targets for over-expressed DNMT1 in stomach cancers (37).

Thus, DNMT1 may be responsible for *de novo* methylation of CpG islands during multistage carcinogenesis. The maintenance activities of DNMT1 are related to its preference for hemimethylated substrates *in vitro*. A theoretical explanation for the role of DNMT1 in *de novo* DNA methylation in human cancers with dysfunction of p21WAF1, which competes with DNMT1 for binding with proliferating cell nuclear antigen (PCNA), has been proposed (57). Moreover, it has recently been suggested that DNMT1 is capable of *de novo* DNA-methylating activity *in vivo* as well as having a maintenance function: *de novo* methylation of CpG islands has actually been observed in human fibroblasts over-expressing DNMT1 (58,59). Therefore, it is feasible that, in cancers, DNMT1 over-expression participates in regional DNA hypermethylation.

Transitional cell carcinomas (TCCs) of the urinary bladder are clinically remarkable because of their multicentricity and tendency to recur: synchronously or metachronously multifocal TCCs 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 showing no remarkable histological features obtained from patients with urinary bladder cancers can be considered precancerous because they may be exposed to carcinogens in the urine. On the other hand, DNMT1 mRNA is expressed mainly during 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 independently of cell proliferative activity in non-cancerous urothelia showing no marked histological features obtained from patients with urinary bladder cancers, where the PCNA-labeling index had not yet increased, compared with that in normal urothelia obtained from patients without urinary bladder cancers, indicating that DNMT1 over-expression preceded increased cell proliferative activity during multistage urothelial carcinogenesis (60). A similar discrepancy between DNMT1 immunoreactivity and the PCNA-labeling index was also observed in liver (54), stomach (37) and uterine cervix cancers (46). Excessive amounts of DNMT1 compared with PCNA, which targets DNMT1 to replication foci, may participate in *de novo* methylation of CpG islands. Further examinations are required to clarify whether or not unknown functional protein complexes recruit DNMT1 to specific DNA sequences during carcinogenesis. The incidence of nuclear DNMT1 immunoreactivity was even higher in dysplastic urothelia and TCCs than in non-cancerous urothelia showing no marked histological features obtained from patients with urinary bladder cancers (60). Among all examined microdissected specimens of non-cancerous urothelia showing no marked histological features obtained from patients with urinary bladder cancers, dysplastic urothelia and TCCs, concurrent DNA hypermethylation of three or more examined C-type CpG islands was significantly correlated with increased DNMT1 protein expression (40).

Splicing alteration of DNMT3b may result in chromosomal instability through DNA hypomethylation of pericentromeric satellite regions

Satellite regions are abundant in pericentromeric heterochromatin DNA on chromosomes 1, 9 and 16 and are heavily methylated in normal cells. DNA hypomethylation of these regions is known to result in centromeric decondensation and enhanced chromosome recombination. In 18% of examined non-cancerous liver tissues showing chronic hepatitis or cirrhosis and 67% of examined HCCs, satellites 2 and 3 were hypomethylated (56). Frequent chromosome 1q copy gain with a pericentromeric breakpoint has been reported in HCCs showing DNA hypomethylation of satellite 2 (61). In TCCs of the urinary bladder, the ureter or the renal pelvis, DNA hypomethylation of satellites 2 and 3 was significantly correlated with LOH on chromosome 9 (62). Therefore, DNA hypomethylation of pericentromeric satellite regions may induce chromosomal instability during multistage carcinogenesis.

DNMT3b is specifically required for DNA methylation of pericentromeric satellite regions in embryonic stem cells and early mouse embryos (11). Germ line mutations of the *DNMT3b* gene have been reported in patients with immunodeficiency, centromeric instability and facial anomalies syndrome, a rare recessive autosomal disorder characterized by DNA hypomethylation of pericentromeric satellite regions (63). However, no mutation of any coding exon of the *DNMT3b* gene was detected in examined HCCs (64). When examined by quantitative reverse transcription–PCR analysis using a primer set not discriminating splice variants of DNMT3b, the total level of DNMT3b mRNA was higher in HCCs than in the corresponding non-cancerous liver tissues (64). Thus, it is unlikely that reduced expression of DNMT3b simply causes DNA hypomethylation of 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. DNMT activity of human DNMT3b3 has been confirmed *in vitro* (65), and DNMT3b3 is expressed ubiquitously in normal human tissues. Our data obtained by splice variant-specific quantitative reverse transcription–PCR has also indicated that the major variant in normal liver tissues is DNMT3b3 (64). On the other hand, DNMT3b4 probably does not show DNMT activity because it lacks the conserved methyltransferase motifs IX and X, although it retains the N-terminal domain required for targeting to heterochromatin sites through binding to RP58. We have confirmed that normal liver tissues show only a trace level of DNMT3b4 expression (64). The level of DNMT3b4 mRNA in non-cancerous liver tissues obtained from patients with HCCs and in HCCs was significantly correlated with the degree of DNA hypomethylation of pericentromeric satellite regions (64). In addition, the ratio of DNMT3b4 mRNA to DNMT3b3 mRNA in non-cancerous liver tissues obtained from patients with HCCs and in HCCs was also significantly correlated with the degree of DNA hypomethylation of pericentromeric satellite regions (64). DNMT3b4 lacking DNMT activity may compete with the major variant, DNMT3b3, for targeting to pericentromeric satellite regions. This may be the reason why DNMT3b4 over-expression results in DNA hypomethylation of pericentromeric satellite regions in precancerous conditions and HCCs. To confirm this possibility, we introduced DNMT3b4 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 DNMT3b4 transfectants, depending on the expression level of myc-tagged DNMT3b4 (64). DNMT3b4 over-expression may lead to chromosomal instability through induction of DNA hypomethylation of pericentromeric satellite regions during hepatocarcinogenesis.

The growth rate of DNMT3b4 transfectants was approximately double that of mock transfectants soon after the introduction of DNMT3b4, when chromosomal instability may not yet have accumulated (66). We assumed that this change was caused by altered gene expression. A majority of the genes that were up-regulated in DNMT3b4 transfectants but not in mock transfectants were implicated in interferon signaling (66). Although genes that encoded interferons themselves were not up-regulated, *signal transducer and activator of transcription (STAT) 1*, which acts as an effector of interferon signaling, has been listed as one of the up-regulated genes in DNMT3b4 transfectants (66). It had been reported previously that inhibition of DNA methylation in cultured human cancer cells by 5-aza-2′-deoxycytidine induces a set of genes implicated in interferon signaling primarily via over-expression of STAT1, 2 and 3 (67). DNMT3b may act to maintain the DNA methylation status of not only pericentromeric

satellite regions but also specific genes, probably in cooperation with DNMT1, in cancer cells, and this may explain why inhibition of DNMT3b activity by induction of DNMT3b4 produced a similar result to the general inhibition of DNA methylation obtained with 5-aza-2′-deoxycytidine. There is a significant correlation between the mRNA expression levels of DNMT3b4 and STAT1 in HCCs (66). Over-expression of DNMT3b4 is involved in multistage carcinogenesis not only by inducing chromosomal instability but also by affecting the expression of specific genes.

Significance of DNA hypomethylation in human cancers

In addition to the above-mentioned satellites 2 and 3, DNA hypomethylation of other tandem repeats such as NBL2 and D4Z4 and retrotransposons such as LINE-1 and Alu was frequently observed in various human cancers (68). Hypomethylation of repeated DNA sequences can disrupt the functions of neighboring genes through transcriptional interference by either sense or anti-sense transcripts (68). Activation of transposable elements can potentially lead to insertional mutagenesis (68). Although global DNA hypomethylation, i.e. reduction of the total level of 5-methylcytosine examined by high-performance liquid chromatography of DNA digested to mononucleotides, is considered to mainly reflect the above-mentioned hypomethylation of repeated DNA sequences, individual genes are also hypomethylated in human cancers. In earlier days, DNA hypomethylation of oncogenes such as *c-myc* in human cancers was frequently reported (69). However, such DNA hypomethylation affects the body or 3′ end of genes whose DNA methylation status is usually not related to gene expression. Recently, significant correlation between over-expression and DNA hypomethylation of tumor-related genes such as *maspin* (70) and *synuclein* γ (71) and cancer/testis antigens such as *melanoma and germ cell-expressed* genes (72) has been reported in human cancers.

We focused on the possibility that functional disruption of DNMT1 due to gene mutations might induce hypomethylation of repeated DNA sequences and individual genes in human cancers. Mutations of the *DNMT1* gene, including a one-base deletion resulting in deletion of the whole catalytic domain due to a premature stop codon, were detected in 7% of examined colorectal cancers (73). This was the first evidence of *DNMT1* gene mutations in human cancers. However, no stomach cancers or HCCs showed mutations in any of the 40 coding exons of the *DNMT1* gene (73). Mutational inactivation of the *DNMT1* gene may be a rare event during human carcinogenesis. Critical dietary components leading to synthesis of the methyl group donor, *S*-adenosylmethionine, include folate, vitamins B6 and B12, methionine and choline. Diets devoid of folate and choline and low in methionine are sufficient to independently induce hepatocarcinogenesis in rats (74). Non-coding RNAs such as anti-sense transcripts of individual genes may be involved in demethylation of the genes (75).

Although global DNA hypomethylation as an early event during colorectal carcinogenesis was noticed in the early days of molecular cancer research (76), the relative timing of global DNA hypomethylation has been revealed to differ between cancers derived from various organs (68). Researchers focusing on cancer epigenetics generally pay more attention to DNA hypermethylation than to DNA hypomethylation, and the clinicopathological significance of DNA hypomethylation during multistage carcinogenesis is, if anything, less well understood. DNA hypomethylation around the promoter regions of individual genes is usually correlated with global DNA hypomethylation (77), but not with DNA hypermethylation of C-type CpG islands in human cancers (78). Hypomethylation of both repeat DNA sequences and individual genes and regional DNA hypermethylation seem to be distinct consequences and are not mutually exclusive in individual patients with cancer.

Altered expression of methyl-CpG-binding proteins in human cancers

Until 1998, MeCP2 had been the only functionally defined methyl-CpG-binding protein (MBD). When MeCP2 binds to methylated CpG dinucleotide, its transcriptional repression domain recruits a co-repressor complex containing Sin 3A and histone deacetylases, resulting in compaction of the chromatin and stable repression of the target gene (79,80). Later, MBD1, MBD2, MBD3 and MBD4 were identified. MBD2 is a transcriptional repressor involved in the MeCP1 complex, identified in mammalian nuclear extracts, and represses transcription from the methylated promoter (81). MBD3 is involved in another histone deacetylase complex, Mi-2/NuRD (82). MBD4 is thought to act as a thymine DNA glycosylase, repairing G:T or G:U mismatches at CpG sites (83).

The expression level of MeCP2 mRNA in HCCs with portal vein involvement is significantly lower than that in HCCs without such involvement, suggesting that reduced expression of MeCP2 may be associated with malignant progression of HCCs (56). Reduced MBD2 mRNA expression has been observed in HCCs (56), colorectal and stomach cancers (84), suggesting that reduced MBD2 expression may be associated with a particular step in human carcinogenesis. The expression level of MBD4 mRNA in HCCs is significantly lower than that in the corresponding non-cancerous liver tissues and is significantly correlated with poorer tumor differentiation and involvement of the portal vein (56). Reduced MBD4 expression may result in frequent C–T transitions in tumor suppressor genes. Although many researchers have focused on crosstalk between DNA methylation and histone modification, abnormalities of MBDs in human cancers do not seem to have attracted much attention, and the implications of these proteins in carcinogenesis need to be further clarified.

Perspectives

Alterations of DNA methylation are associated with multistage carcinogenesis from precancerous conditions to malignant progression. Therefore, estimation of carcinogenetic risk and early diagnosis of cancers using alterations of DNA methylation as indicators are promising approaches for mass screening of clinical samples. For such purposes, non- or less invasive methodologies for detecting subtle alterations of DNA methylation have been developed for serum, urine, sputum and other body fluid samples. Moreover, recently developed array-based technology for accessing genome-wide DNA methylation status (85) will be useful for identifying the DNA methylation profile that is the optimum indicator for risk estimation and early diagnosis. Analysis of DNA methylation status in biopsy specimens and/or surgically resected materials may also become a useful tool for prognostication. Clinical trials of DNA demethylation agents are underway and many DNA demethylation agents are now being developed (86). However, global DNA hypomethylation and regional DNA hypermethylation are commonly observed during multistage carcinogenesis. In some patients, global DNA hypomethylation will have greater significance than regional DNA hypermethylation during carcinogenesis. Therefore, before using DNA demethylation agents for prevention or therapy of cancers, it will be necessary to carefully identify patients who might benefit most from this type of demethylation strategy. It may also be necessary to develop sequence-specific demethylation agents to reduce any severe side effects. In order to apply correction of DNA methylation status to practical prevention and therapy of cancers, the full picture and molecular mechanisms of DNA methylation alterations corresponding to specific carcinogenetic factors should be further clarified for each organ.

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Increased expression of DNA methyltransferase 1 (DNMT1) protein in uterine cervix squamous cell carcinoma and its precursor lesion

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Abstract

Aberrant DNA methylation has been shown to play important roles during multistage carcinogenesis in various human organs. The aim of this study was to evaluate the significance of DNA methyltransferase 1 (DNMT1) protein expression during cervical carcinogenesis. We carried out an immunohistochemical examination for DNMT1 in 34 samples of histologically normal squamous epithelium, 36 samples of low-grade cervical intraepithelial neoplasia (CIN), 61 samples of higher-grade CIN and 30 samples of squamous cell carcinoma of the uterine cervix. The DNMT1 protein expression score, reflecting the intensity and incidence of DNMT1 nuclear immunoreactivity, was increased even in low-grade CIN ($P < 0.0001$) in comparison with histologically normal squamous epithelium and was further increased in higher-grade CIN ($P < 0.0001$ compared to low-grade CIN). The DNMT1 protein expression score remained at a plateau in microinvasive carcinoma (Stage IA, $P = 0.0690$ compared to higher-grade CIN) and then decreased with cancer invasion (Stage IB or more, $P = 0.0176$ compared to Stage IA), whereas the proliferating cell nuclear antigen (PCNA) labeling index did not decrease with cancer invasion ($P = 0.8259$ between Stage IA and Stage IB or more). Thus, the DNMT1 protein expression score and the PCNA labeling index were not mutually correlated in squamous cell carcinoma of the uterine cervix ($P = 0.2304$). These data suggest that progressively increasing expression of DNMT1 protein is not entirely a secondary result of increased cell proliferative activity, but is associated with an early step of multistage cervical carcinogenesis.
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Keywords: DNA methylation; DNA methyltransferase 1 (DNMT1); Proliferating cell nuclear antigen (PCNA); Cervical intraepithelial neoplasia (CIN); Squamous cell carcinoma; Uterine cervix

1. Introduction

DNA methylation plays important roles in transcriptional regulation, chromatin remodeling and genetic stability. Overall DNA hypomethylation and regional DNA hypermethylation are commonly

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observed in human cancers of various organs [1–3]. Aberrant DNA methylation may play roles in carcinogenesis as a result of (a) increased gene mutagenicity due to deamination of 5-methylcytosine to thymine; (b) possible association of aberrant DNA methylation with genetic instability; and (c) silencing of tumor-related genes through DNA methylation on CpG islands in cooperation with histone modification [1]. Furthermore, accumulating evidence suggests that aberrant DNA methylation is involved even in the early and precancerous stages of human carcinogenesis [4,5]. Precancerous conditions showing aberrant DNA methylation may rapidly progress and generate more malignant cancers [6,7].

DNA methyltransferase 1 (DNMT1) is the major human DNMT [1]. As DNMT1 shows a preference for hemimethylated rather than unmethylated substrates *in vitro* [8], and targets replication foci by binding to proliferating cell nuclear antigen (PCNA) [9], it has been considered to be a maintenance form of DNMT that copies methylation patterns after DNA replication. However, some workers have proposed that DNMT1 possesses both maintenance and *de novo* DNA methylation activity *in vivo* [10,11], regardless of its *in vitro* substrate preference. We have reported that DNMT1 protein overexpression precedes an increase of the PCNA labeling index in precancerous conditions in the urinary bladder [12], and is significantly correlated with poorer differentiation of liver [13], stomach [14] and pancreatic [15] cancers and a poorer prognosis of patients with liver [13] and pancreatic [15] cancers. Moreover, in stomach [14], colorectal [16], urinary bladder [17] and pancreatic [18] cancers, DNMT1 overexpression is significantly correlated with the CpG island methylator phenotype (CIMP) [19], which is defined as frequent DNA hypermethylation on C-type CpG islands that are usually methylated in a cancer-specific (not age-dependent) manner, or accumulation of DNA hypermethylation of multiple tumor-related genes.

With respect to cervical carcinogenesis, an early study demonstrated global DNA hypomethylation in squamous cell carcinoma and its precursor lesion [20], and some subsequent studies have revealed accumulation of DNA hypermethylation of tumor-related genes in squamous cell carcinoma [21–24]. However, to our knowledge, expression of DNMT1 has never been reported during multistage cervical carcinogenesis to date. In this study we carried out an immunohistochemical examination of DNMT1 in a series of tissue samples of uterine cervix squamous cell carcinoma and its precursor lesion.

2. Materials and methods

2.1. Patients and samples

Thirty-four samples of histologically normal squamous epithelium, 36 samples of low-grade CIN (CIN1) [25], 61 samples of higher-grade CIN (CIN2 and CIN3) [25] and 30 samples of squamous cell carcinoma (10 samples of microinvasive caricinoma [Stage IA] [26] and 20 samples of invasive carcinoma [Stage IB or more] [26]) of the uterine cervix were used for immunohistochemistry. Histopathological diagnosis and clinical staging were performed on the basis of previously described criteria [25,26]. All 161 tissue samples were obtained from 49 patients (mean age ± SD, 46.88 ± 11.72 years [range, 26–74 years]) who underwent conization or hysterectomy because of higher-grade CIN or squamous cell carcinoma of the uterine cervix at the National Cancer Center Hospital, Tokyo. With respect to 10 samples of microinvasive carcinoma, their microinvasive components, not non-invasive components, were examined immunohistochemically. There were no patients from whom multiple tissue samples were obtained metachronously. This study was approved by the Ethics Committee of the National Cancer Center, Tokyo.

2.2. Immunohistochemistry

Five-micrometer-thick sections of formalin-fixed, paraffin-embedded tissue specimens were deparaffinized and dehydrated. For antigen retrieval, the sections were heated for 10 min at 120 °C in an autoclave. Non-specific reactions were blocked with 2% normal swine serum. All sections were incubated with specific primary antibodies directed against DNMT1 (goat polyclonal antibody, sc-10219; Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:1000) and PCNA (mouse monoclonal antibody, p56720; Transduction Laboratories, Lexington, KY; dilution 1:200). We had previously confirmed the specificity of the goat anti-human DNMT1 polyclonal antibody by Western blotting analysis: an immunoreactive band of about 193.5 kDa, corresponding to the molecular mass of DNMT1, was detected in human cancer cells, but no non-specific bands were detected [13]. Both primary antibody incubations were conducted at 4 °C overnight, and were followed by incubation with biotinylated secondary antibodies (anti-goat IgG or anti-mouse IgG, Vector Laboratories, Burlingame, CA; dilution 1:200) at room temperature for 30 min. The sections were then treated with Vectastain Elite ABC reagent (Vector Laboratories).

3.3′-Diaminobenzidine tetrahydrochloride was used as the chromogen, and all sections were counterstained with hematoxylin. As a negative control, the primary antibodies were omitted from the reaction sequence.

In all examined patients without exception, uniform intensity of DNMT1 immunoreactivity was detected in the nucleus of parabasal cells of the histologically normal squamous epithelium of the uterine cervix. Therefore, parabasal cells were used as internal positive controls in all samples. Without exception, DNMT1 immunoreactivity was detected only in the nucleus, and never in the cytoplasm or cell membrane. The intensity of DNMT1 nuclear immunoreactivity in each sample was graded as 0 (less than the internal positive control), 1 (equal to the internal positive control) and 2 (more than the internal positive control). For each sample, at least 500 cells were randomly counted. If the lesion was small with less than 500 cells, all the cells were counted. The incidence of DNMT1 nuclear immunoreactivity in each sample was graded as 0 (less than 20% of the counted cells), 1 (20% or more, and less than 50%) and 2 (50% or more). The DNMT1 protein expression score (0, 1, 2 or 4) was determined as the product of the intensity grade (0, 1 or 2) and the incidence grade (0, 1 or 2). The PCNA labeling index in each sample was expressed as a percentage of all the cells counted.

2.3. Statistics

Comparisons of the DNMT1 protein expression score and the PCNA labeling index between sample groups were analyzed by the chi-squared test and the Mann–Whitney *U*-test, respectively. Comparisons of mean patient age among sample groups were analyzed by the Kruskal–Wallis test. Correlations of the DNMT1 protein expression score on the one hand and patient age or the PCNA labeling index on the other were analyzed by the chi-squared test and the Mann–Whitney *U*-test, respectively. For all tests, $P < 0.05$ was considered to be the level of significance.

3. Results

3.1. DNMT1 protein expression score in uterine cervix squamous cell carcinoma and its precursor lesion

Fig. 1 shows examples of immunohistochemical staining for DNMT1 in tissue samples. The distribution of the DNMT1 protein expression score in each sample group is summarized in Table 1. The DNMT1 protein expression score increased progressively from histologically normal squamous epithelium, to low-grade CIN ($P < 0.0001$ compared to histologically normal squamous epithelium), and to higher-grade CIN ($P < 0.0001$ compared to low-grade CIN). DNMT1 protein expression peaked in higher-grade CIN, and there was no significant increase of the DNMT1

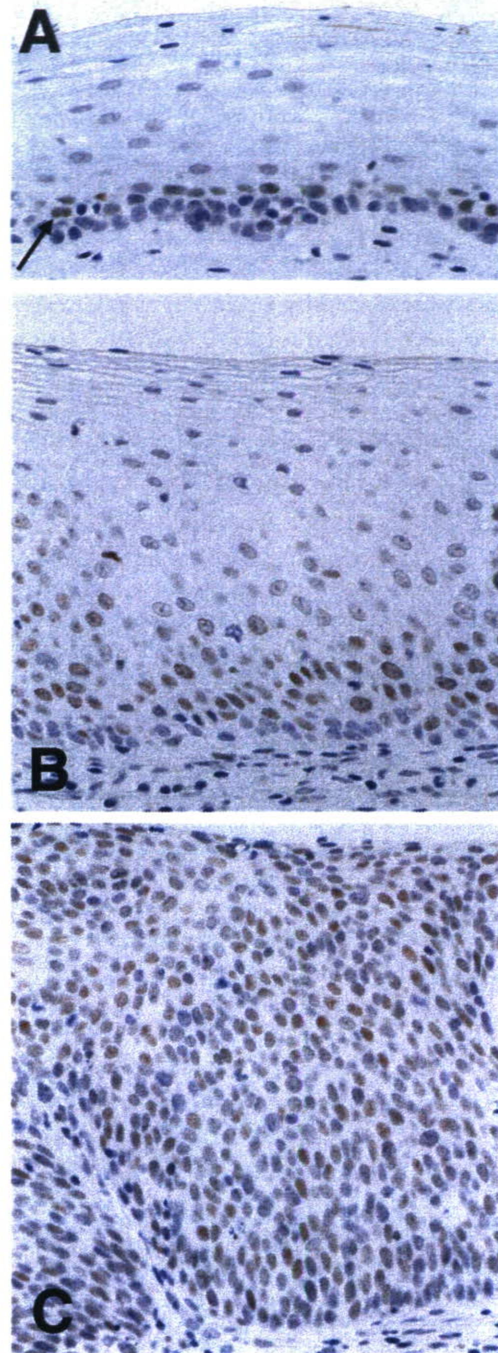

Fig. 1. Immunohistochemical examination for DNMT1 in histologically normal squamous epithelium (A) and CINs (B and C) of the uterine cervix. (A) Parabasal cells (arrow) of histologically normal squamous epithelium served as positive controls. The DNMT1 protein expression score was 0 in this sample. (B) DNMT1-positive cells were observed in low-grade CIN (DNMT1 protein expression score: 1). (C) The incidence of DNMT1 nuclear immunoreactivity was further increased in higher-grade CIN (DNMT1 protein expression score: 2) 280×.

Table 1
Immunohistochemical examination for DNA methyltransferase (DNMT) 1 in uterine cervix squamous cell carcinoma and its precursor lesion

Tissue samples	Number of specimens (%)					P^d
	Analyzed	DNMT1 protein expression score[c]				
		0	1	2	4	
Histologically normal squamous epithelium	34	34 (100)	0 (0)	0 (0)	0 (0)	<0.0001
Low-grade cervical intraepithelial neoplasia (CIN1)[a]	36	16 (44.4)	18 (50)	2 (5.6)	0 (0)	<0.0001
Higher-grade CIN (CIN2 and CIN3)[a]	61	3 (4.9)	29 (47.5)	29 (47.5)	0 (0)	0.0690
Microinvasive carcinoma (Stage IA)[b]	10	1 (10)	5 (50)	3 (30)	1 (10)	0.0176
Invasive carcinoma (Stage IB or more)[b]	20	8 (40)	12 (60)	0 (0)	0 (0)	

[a] According to Ref. [25].
[b] According to Ref. [26].
[c] The DNMT1 protein expression score (0, 1, 2 or 4) was determined as the product of the intensity grade (0, 1 or 2) and the incidence grade (0, 1 or 2).
[d] Chi-squared test.

protein expression score from higher-grade CIN to microinvasive carcinoma (Stage IA, $P = 0.0690$ compared to higher-grade CIN). With cancer invasion and extent, from microinvasive carcinoma to invasive carcinoma (Stage IB or more), the DNMT1 protein expression score decreased significantly ($P = 0.0176$ between Stage IA and Stage IB or more). Even in invasive carcinoma, the DNMT1 protein expression score was significantly higher than that in histologically normal squamous epithelium ($P < 0.0001$).

The patient age (mean age ± SD) in each sample group (histologically normal squamous epithelium, low-grade CIN, higher-grade CIN, microinvasive carcinoma and invasive carcinoma) was 45.53 ± 11.70, 45.89 ± 11.97, 44.21 ± 11.69, 42.40 ± 8.86 and 48.25 ± 13.47, respectively, and there were no significant differences in patient age among the sample groups ($P = 0.7682$). The DNMT1 protein expression score in histologically normal squamous epithelium, low-grade CIN, higher-grade CIN and squamous cell carcinoma each did not correlate with patient age (Table 2). The DNMT1 protein expression score in squamous cell carcinoma ($n = 30$) did not correlate with the clinicopathological parameters (presence or absence of lymphatic vessel involvement [$P = 0.2954$], venous involvement [$P = 0.7370$] and lymph node metastasis [$P = 0.6163$], data not shown).

As mentioned above, the average DNMT1 protein expression score in invasive carcinoma (Stage IB or more) was lower than that in microinvasive carcinoma or non-invasive higher-grade CIN. In fact, the incidence of DNMT1 nuclear immunoreactivity was lower in the central portion of large nests of invading cancer cells than that in non-invasive lesions. However, even in such invasive carcinoma, cancer cells often showed particularly strong DNMT1 nuclear immunoreactivity at the leading front of deep invasion (Fig. 2).

Table 2
Lack of significant correlation between patient age and expression level of DNA methyltransferase (DNMT) 1 protein in uterine cervix squamous cell carcinoma and its precursor lesion

Tissue samples	Age (y)	Number of patients (%)					P^c
		Analyzed	DNMT1 protein expression score[b]				
			0	1	2	4	
Histologically normal squamous epithelium	Less than 47	18	18 (100)	0 (0)	0 (0)	0 (0)	
	47 or more	16	16 (100)	0 (0)	0 (0)	0 (0)	
Low-grade cervical intraepithelial neoplasia (CIN1)[a]	Less than 47	19	10 (52.6)	7 (36.8)	2 (10.5)	0 (0)	0.1504
	47 or more	17	6 (35.3)	11 (64.7)	0 (0)	0 (0)	
Higher-grade CIN (CIN2 and CIN3)[a]	Less than 47	35	1 (2.9)	19 (54.3)	15 (42.9)	0 (0)	0.3918
	47 or more	26	2 (7.7)	10 (38.5)	14 (53.8)	0 (0)	
Squamous cell carcinoma	Less than 47	15	4 (26.7)	9 (60)	2 (13.3)	0 (0)	0.6815
	47 or more	15	5 (33.3)	8 (53.3)	1 (6.7)	1 (6.7)	

[a] According to Ref. [25].
[b] The DNMT1 protein expression score (0, 1, 2 or 4) was determined as the product of the intensity grade (0, 1 or 2) and the incidence grade (0, 1 or 2).
[c] Chi-squared test.

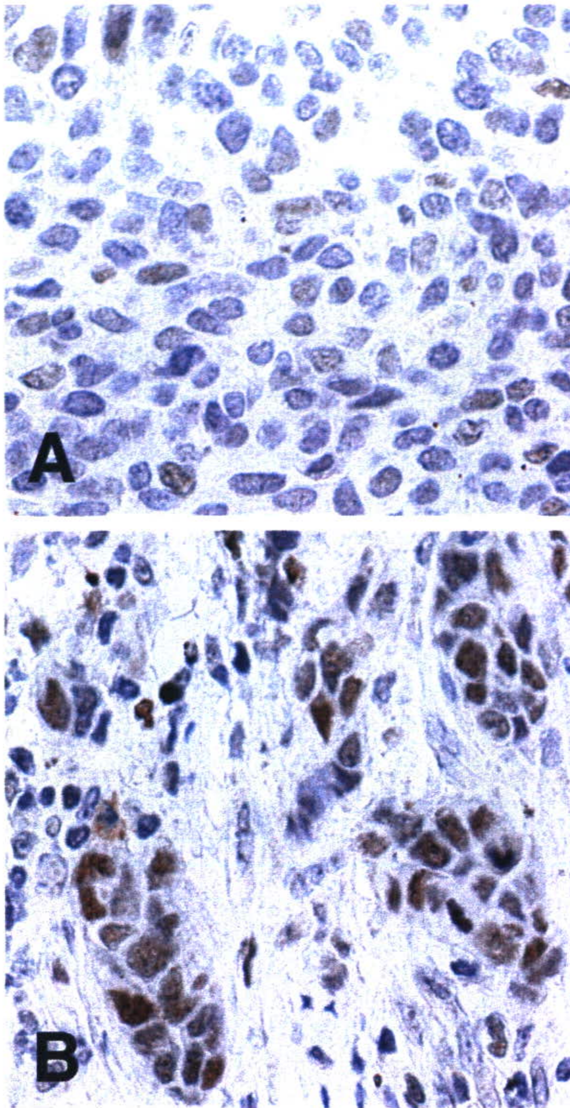

Fig. 2. Immunohistochemical examination for DNMT1 in invasive squamous cell carcinoma of the uterine cervix. Although only weak DNMT1 nuclear immunoreactivity (DNMT1 protein expression score: 1) was detected in the center of a large nest of invasive carcinoma (A), cancer cells showed particularly strong nuclear DNMT1 immunoreactivity at the invading front (B) 560×.

3.2. *PCNA lebeling index in uterine cervix squamous cell carcinoma and its precursor lesion*

Fig. 3 shows examples of immunohistochemical staining for PCNA in tissue samples. The PCNA labeling index of each sample group (a mean ± SD) is summarized in Table 3. Unlike the DNMT1 protein expression score, the PCNA labeling index increased progressively from histologically normal squamous epithelium, to low-grade CIN ($P < 0.0001$ compared to histologically normal squamous epithelium), higher-grade CIN ($P < 0.0001$ compared to low-grade CIN) and microinvasive carcinoma (Stage IA, $P = 0.0112$ compared to higher-grade CIN). The PCNA labeling index did not decrease with cancer invasion and remained at a plateau in invasive carcinoma (Stage IB or more, $P = 0.8259$ to Stage IA). Thus, the DNMT1 protein expression score and the PCNA labeling index did not show mutual correlation in squamous cell carcinoma of the uterine cervix ($n = 30$, $P = 0.2304$, Fig. 4).

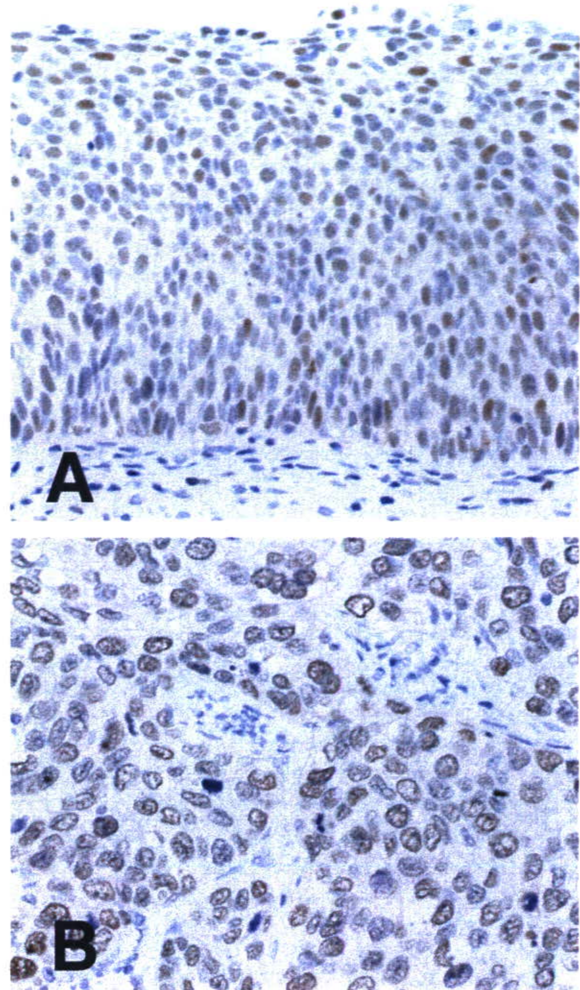

Fig. 3. Immunohistochemical examination for PCNA in higher-grade CIN (A) and invasive squamous cell carcinoma (B) of the uterine cervix. The PCNA labeling index did not decrease with cancer invasion. 280×.

4. Discussion

We believe that this is the first report to describe DNMT1 protein expression in uterine cervix squamous cell carcinoma and its precursor lesion.

Table 3
Immunohistochemical determination of proliferating cell nuclear antigen (PCNA) in uterine cervix squamous cell carcinoma and its precursor lesion

Tissue samples	Analyzed (number of specimens)	PCNA labeling index (mean ± SD)	P^c
Histologically normal squamous epithelium	34	4.03 ± 8.581	<0.0001
Low-grade cervical intraepithelial neoplasia (CIN1)[a]	36	37.419 ± 13.595	<0.0001
Higher-grade CIN (CIN2 and CIN3)[a]	61	74.711 ± 20.716	0.0112
Microinvasive squamous cell carcinoma (Stage IA)[b]	10	89.190 ± 9.563	0.8259
Invasive squamous cell carcinoma (StageIB or more)[b]	20	87.310 ± 13.601	

[a] According to Ref. [25].
[b] According to Ref. [26].
[c] Mann–Whitney U-test.

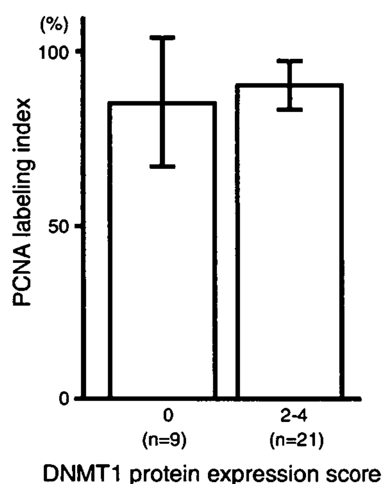

Fig. 4. DNMT1 protein expression score and PCNA labeling index in squamous cell carcinom of the uterine cervix. The PCNA labeling index is expressed as mean ± SD. DNMT1 protein expression level and cell proliferative activity were not mutually correlated ($P = 0.2304$).

DNMT1 protein expression was found to be significantly increased in squamous cell carcinoma in comparison with histologically normal squamous epithelium. There were no significant differences in patient age between the sample groups. Therefore, DNMT1 protein overexpression was not attributable to aging, but showed a possible association with cervical carcinogenesis.

Histopathological and clinical observations have revealed that CIN is a precursor lesion for squamous cell carcinoma of the uterine cervix [25]. Even in low-grade CIN, significant elevation of the DNMT1 protein expression score was detected in comparison with histologically normal squamous epithelium, and the DNMT1 protein expression score reached a peak in higher-grade CIN, suggesting that DNMT1 protein overexpression is an early event during multistage cervical carcinogenesis. It is well known that CIN of the uterine cervix is closely associated with human papillomavirus (HPV) infection [27,28]. We have frequently observed DNMT1 protein overexpression in precancerous conditions with chronic inflammation and/or persistent infection with viruses or other pathogenic microorganisms, such as chronic hepatitis and liver cirrhosis associated with hepatitis B or C virus infection [6,29–35]. The present data suggest that CIN of the uterine cervix can be ranked as a precancerous lesion showing DNMT1 protein overexpression associated with persistent viral infection. When PCR detection of HPV-16, which is known to be frequently associated with cervical carcinogensis, was performed in specimens microscopically dissected from tissue samples in which we had carried out immunohistochemical examination for DNMT1, the DNMT1 protein expression score did not correlate with the presence or absence of HPV-16 infection in low-grade CIN ($P = 0.1715$), higher-grade CIN ($P = 0.4569$) and squamous cell carcinoma ($P = 0.5836$, data not shown). Although HPV-16 infection may not directly affect the DNMT1 expression level, HPV-16 E7 protein has been reported to directly associate with DNMT1 and stimulate the methyltransferase activity of DNMT1 *in vitro* [36]. Overexpressed DNMT1 may be activated by HPV-16 E7 protein during cervical carcinogenesis. Since DNMT1 protein overexpression is a very early event during multistage cervical carcinogenesis, it is not surprising that the DNMT1 protein expression score did not correlate with clinicopathological parameters reflecting tumor aggressiveness

or progression, such as lymphatic and venous involvement, and lymph node metastasis.

DNMT1 mRNA is expressed mainly during S-phase in normal cells, and because tumor tissue presumably contains a greater proportion of dividing cells than normal tissue, there has been some debate as to whether increased DNMT1 mRNA expression is due to an increase in the proportion of dividing cells or to an acute increase in DNMT1 expression per individual cell [37]. This continuing discussion prompted us to compare the DNMT1 protein expression score and the PCNA labeling index in tissue samples. DNMT1 protein overexpression reached a peak in higher-grade CIN and microinvasive carcinoma, and the peak of the DNMT1 protein expression score preceded that of the PCNA labeling index. The DNMT1 protein expression score was decreased in invasive carcinoma, whereas the PCNA labeling index was retained at the same level. Thus, the DNMT1 protein expression level did not correlate with cell proliferative activity in tissue specimens, suggesting that DNMT1 protein overexpression does not result entirely from increased numbers of dividing cells during cervical carcinogenesis. We have previously observed a similar discrepancy between DNMT1 protein expression level and cell proliferative activity in precancerous conditions in the urinary bladder [12] and in certain subgroups of stomach [14] and renal [7] cancers. Several mechanisms for regulation of DNMT1 expression have been proposed in human cancers. Dysfunction of p53 tumor suppressor protein results in DNMT1 mRNA overexpression [38] and DNMT1 protein is stabilized in cultured cancer cells [39]. Aberrant DNA methylation is closely associated with cancers preceded by chronic inflammation and/or persistent viral infection, and cytokine interleukin-6 treatment [40] and induction of latent membrane protein 1 of Epstein–Barr virus [41], which is associated with nasopharyngeal and stomach cancers, induce DNMT1 overexpression in cultured cancer cells. The molecular mechanisms responsible for DNMT1 overexpression during multistage cervical carcinogeneis should be clarified.

DNMT1 targets replication foci, where DNA methylation patterns are copied from the mother strand, by binding to PCNA [9]. However, targeting of the substrate DNA by DNMT1 may be disrupted by mechanisms such as dysfunction of p21WAF1, which competes with DNMT1 for binding to PCNA, in cancer cells [42]. Moreover, it has recently been suggested that DNMT1 is capable of *de novo* methylation activity as well as having a maintenance function [10,11]. Therefore, it is feasible that, in cancers, DNMT1 overexpression participates in the *de novo* methylation of CpG islands. In fact, we have already reported that DNMT1 mRNA or protein overexpression is significantly correlated with CIMP of stomach [14], colorectal [16], and urinary bladder [17] cancers, and with accumulation of DNA hypermethylation of multiple tumor-related genes in pancreatic cancer [18]. DNMT1 protein overexpression may thus also become a background factor associated with silencing of tumor-related genes during multistage cervical carcinogenesis. In fact, reduction of DNMT1 expression results in re-expression of the E-cadherin gene in cultured cervical cancer cells [43]. We tried to evaluate the DNA methylation status of the promoter regions of multiple tumor-related genes in specimens microscopically dissected from formalin-fixed and paraffin-embedded tissue samples in which we had performed immunohistochemical examination for DNMT1. However, we were able to examine only a small number of genes and specimens because the microdissected specimens were tiny and the DNA was degraded. Further technical improvement will be needed in order to identify tumor-related genes that may be targeted by overexpressed DNMT1 in our cohort.

The level of DNMT1 protein expression initially increased until higher-grade CIN and microinvasive carcinoma, and then decreased in invasive carcinoma. In our previous study of the urinary bladder, although DNMT1 protein expression was lower in invasive carcinoma than in carcinoma *in situ*, CIMP was continuously maintained during progression from carcinoma *in situ* to invasive carcinoma [17]. DNMT1 overexpressed at an early stage may be related not only to DNA hypermethylation of tumor-related genes, but also to silencing of genes by recruiting the transcriptional repressor protein complex including DMAP1, DNMT1-associated protein, and histone deacetylases [44,45]. DNA hypermethylation on multiple CpG islands may be maintained even if DNMT1 expression is diminished to some extent during malignant progression.

Even after the peak of DNMT1 protein overexpression, heterogeneous expression was observed in some invasive carcinomas: cancer cells often showed particularly strong DNMT1 nuclear immunoreactivity at the invading front (Fig. 2). There appears to be a mechanism regulating DNMT1 protein expression, possibly one that depends on cancer–stromal interactions and/or the

microenvironment of cancer cells. Such findings are consistent with the particularly strong DNMT1 expression at the invading front of urinary bladder cancers with unfavorable clinical outcome [12]. DNMT1 reactivated at the invading front may locally silence an additional set of genes with anti-invasive function.

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Promoter Hypermethylation Contributes to Frequent Inactivation of a Putative Conditional Tumor Suppressor Gene *Connective Tissue Growth Factor* in Ovarian Cancer

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Abstract

Connective tissue growth factor (CTGF) is a secreted protein belonging to the CCN family, members of which are implicated in various biological processes. We identified a homozygous loss of *CTGF* (6q23.2) in the course of screening a panel of ovarian cancer cell lines for genomic copy number aberrations using in-house array-based comparative genomic hybridization. *CTGF* mRNA expression was observed in normal ovarian tissue and immortalized ovarian epithelial cells but was reduced in many ovarian cancer cell lines without its homozygous deletion (12 of 23 lines) and restored after treatment with 5-aza 2′-deoxycytidine. The methylation status around the *CTGF* CpG island correlated inversely with the expression, and a putative target region for methylation showed promoter activity. *CTGF* methylation was frequently observed in primary ovarian cancer tissues (39 of 66, 59%) and inversely correlated with *CTGF* mRNA expression. In an immunohistochemical analysis of primary ovarian cancers, CTGF protein expression was frequently reduced (84 of 103 cases, 82%). Ovarian cancer tended to lack CTGF expression more frequently in the earlier stages (stages I and II) than the advanced stages (stages III and IV). CTGF protein was also differentially expressed among histologic subtypes. Exogenous restoration of CTGF expression or treatment with recombinant CTGF inhibited the growth of ovarian cancer cells lacking its expression, whereas knockdown of endogenous CTGF accelerated growth of ovarian cancer cells with expression of this gene. These results suggest that epigenetic silencing by hypermethylation of the *CTGF* promoter leads to a loss of CTGF function, which may be a factor in the carcinogenesis of ovarian cancer in a stage-dependent and/or histologic subtype-dependent manner. [Cancer Res 2007;67(15):7095–105]

Introduction

Epithelial ovarian cancer is the leading cause of death from gynecologic tumors (1), due to its aggressive nature and the fact that the majority of patients are diagnosed in advanced stages of the disease. The lack of preventive strategies, early diagnostic methods, and effective therapies to treat recurrent ovarian cancer creates a pressing need to understand the molecular mechanisms responsible for the development and progression of ovarian cancer and to identify molecular markers and targets for diagnosis as well as therapy (2). Sporadic ovarian cancers display defects in many genes, including *AKT, EGFR, ERBB2, RAS, PIK3CA, MYC, DOC-2/DAB2, SNCG*, and *TP53*, as well as a myriad of cytogenetic abnormalities (3). These defects result from both genetic and epigenetic changes and can occur at varying frequencies in different pathologic subtypes, which are morphologically and biologically heterogeneous, both early and late in the transformation process (2). Because there have been no known tumor suppressor genes (TSG) other than *TP53* showing high frequencies of somatic mutations in ovarian cancer, further efforts for the identification of putative TSGs are needed.

Several typical TSGs were originally pinpointed by mapping regions of biallelic loss in cancer cells (4–6), although the homozygous deletion of those genes is a rare event and other mechanisms, including aberrant methylation of CpG sites within the promoter region (7), may predominantly contribute to their functional inactivation. Therefore, scanning the entire genome for homozygous deletions with high resolution is believed to be useful for a precise and rapid identification of tumor suppressors. Indeed, we have applied in-house bacterial artificial chromosome (BAC)–based arrays (8) for an array-based comparative genomic hybridization (array-CGH) analysis of various human cancers and identified candidate TSGs mainly inactivated through homozygous loss or promoter hypermethylation from homozygously deleted regions (9, 10).

In ovarian cancer, the aberrant DNA methylation of known TSGs, such as *p16INK4A* (11), *RASSFIA* (12), *BRCA1* (13), and *hMLH1* (14), has been reported. However, the importance of epigenetic changes to TSGs in ovarian cancer remains largely unknown, and it is possible that more genes frequently inactivated through DNA methylation and involved in the pathogenesis of ovarian cancer will be identified. In the report presented here, during the course of a program to screen a panel of ovarian cancer cell lines for copy number aberrations in a genome-wide manner using our in-house BAC array (8), we have identified a homozygous loss of *connective tissue growth factor* (*CTGF/CCN2*) at 6q23.1, whose expression was absent in some ovarian cancer cell lines without homozygous loss, although it

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

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was present in normal ovary. Because (*a*) reduced expression of CTGF and its clinicopathologic significance (15, 16) and DNA methylation of the genomic sequence around *CTGF* (17–19) in several cancers were reported but (*b*) a detailed target sequence for DNA methylation contributing gene silencing has never been shown and (*c*) the effect of down-regulated CTGF expression on ovarian carcinogenesis remains unknown, we further determined the expression and methylation status of *CTGF* and their clinicopathologic and functional significance using cell lines and primary tumors of ovarian cancer.

Materials and Methods

Cell lines and primary tumors. Of the 24 ovarian cancer cell lines used (Supplementary Table S1), ES-2 was obtained from the American Type Culture Collection; OVISE, OVMANA, OVTOKO, OVKATE, OVSAHO, and RMUG-S were from the Japanese Collection of Research Bioresources (Osaka, Japan); and HT, MH, KK, KF28, and KFr13 were from the National Defense Medical College (20, 21). Other lines (HTOA, HUOA, HMKOA, MCAS, HMOA, HNOA, RMG-I, RMG-II, RMUG-L, W3UF, HIOAnu, and HTBOA) were described previously (22). As a control, the normal ovarian epithelial cell–derived cell line OSE-2a (23) was kindly provided by Dr. Hidetaka Katabuchi (Kumamoto University School of Medicine, Kumamoto, Japan). All cell lines were maintained in appropriate medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The status of the *TP53* gene (exons 5–8) mutation was determined as described previously (24). To analyze restoration of genes of interest, cells were cultured with or without various concentrations of 5-aza 2′-deoxycytidine (5-aza-dCyd) for 5 days and/or 100 ng/mL trichostatin A (TSA) for the last 12 h.

Primary tumor samples were obtained during surgery from 114 patients being treated at the National Cancer Center Hospital in Tokyo, with written consent from each patient in the formal style and after approval by the local ethics committees. Samples from 66 of these patients were frozen immediately in liquid nitrogen and stored at −80°C until required, whereas samples from 103 of the patients were embedded in paraffin for immunohistochemistry. None of the patients had received preoperative radiation or immunotherapy. All patients underwent complete surgical staging, including i.p. cytology, bilateral salpingo-oophorectomy, hysterectomy, omentectomy, and pelvic/para-aortic lymphadenectomy. Aggressive cytoreductive surgery was done in patients with advanced disease. Surgical staging was based on the International Federation of Gynecology and Obstetrics (FIGO) staging system: stage I, 57 patients; stage II, 11 patients; stage III, 34 patients; and stage IV, 12 patients.

Array-CGH analysis. A MCG Cancer Array-800 (8) was used for the array-CGH. Hybridizations were carried out as described elsewhere (9). Hybridized slides were scanned with a GenePix 4000B (Axon Instruments), and acquired images were analyzed with GenePix Pro 6.0 imaging software (Axon Instruments). Average ratios that deviated significantly (>2 SD) from 0 (log 2 ratio, <−0.4 and >0.4) were considered abnormal.

Screening for homozygous deletions by genomic PCR using cell lines. We screened DNAs from 24 ovarian cancer cell lines for homozygous losses by genomic PCR. All primer sequences used in this study are listed in Supplementary Table S2.

Reverse transcription-PCR and quantitative real-time reverse transcription-PCR. Single-stranded cDNAs were generated from total RNAs and amplified with primers specific for each gene (Supplementary Table S2). The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was amplified at the same time to allow estimation of the efficiency of cDNA synthesis. For reverse transcription-PCR (RT-PCR), PCR products were electrophoresed in 3% agarose gels (9). Quantitative real-time RT-PCR experiments were done with an ABI Prism 7900 Sequence Detection System (Applied Biosystems) as described previously (24). Each assay was done in triplicate.

Methylation analysis. Genomic DNAs were treated with sodium bisulfite and subjected to PCR using primer sets designed to amplify regions of interest (Supplementary Table S2). For the combined bisulfite restriction analysis (COBRA; ref. 26), PCR products were digested with *Bst*UI and electrophoresed. For bisulfite sequencing, PCR products were subcloned and then sequenced.

For the methylation-specific PCR (MSP) analysis, sodium bisulfite-treated DNAs were subjected to PCR using primers specific to the methylated (MSP) and unmethylated (unmethylated specific PCR) forms of DNA sequences of interest (Supplementary Table S2), and PCR products were visualized on 3% agarose gels. DNAs from cell lines recognized as unmethylated by bisulfite sequencing were used as negative controls for methylated alleles, whereas those from cell lines recognized as methylated or CpGenome Universal Methylated DNA (Chemicon International) were used as positive controls.

Promoter reporter assay. DNA fragments around the *CTGF* CpG island were obtained by PCR and ligated into the vector pGL3-Basic (Promega). Reporter assay was done as described elsewhere (9) using each construct or a control empty vector and an internal control pRL-hTK vector (Promega).

Immunohistochemistry and scoring method. Indirect immunohistochemistry was done with formalin-fixed, paraffin-embedded tissue sections as described elsewhere (26). Briefly, antigens were retrieved by autoclave pretreatment in high pH buffer (DAKO) for 10 min at 95°C. After blocking in 2% normal swine serum, the slides were incubated with an anti-CTGF antibody (1:100 dilution; Santa Cruz Biotechnology) overnight at 4°C and then reacted with a Histofine simple stain, MAX PO(G) (Nichirei). Antigen-antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. The slides were counterstained with Mayer's hematoxylin.

A formalin-fixed HTBOA cell line expressing *CTGF* mRNA, in which >50% of cells showed cytoplasmic staining of CTGF protein, and RMUG-S cell line lacking *CTGF* mRNA expression, in which none of the cells showed cytoplasmic staining of CTGF protein, were used as positive and negative controls, respectively. Specificity of the antibody was verified by Western blotting (9) as well as an absorption test using synthetic peptide (Santa Cruz Biotechnology; Supplementary Fig. S1). The percentage of the total cell population that expressed CTGF was evaluated for each case at ×200 magnification. Expression of CTGF was graded as either positive (≥10% of tumor cell cytoplasm showing immunopositivity) or negative (<10% of tumor cell cytoplasm showing immunopositivity or no staining). Two observers, who were blinded to the clinical outcomes of the patients, evaluated the slides independently; if a significant discrepancy emerged between their judgments, a consensus was reached after discussion.

Transient transfection, Western blotting, and colony formation assay. A plasmid expressing COOH-terminal 3× Myc-tagged CTGF (pCMV-3Tag-4-CTGF) was obtained by cloning the PCR product of the full coding sequence of *CTGF* in-frame along with the Myc epitopes into the vector pCMV-3Tag-4 (Stratagene). pCMV-3Tag-4-CTGF, or the empty vector (pCMV-3Tag-4-mock), was transfected into cells for colony formation assays (9). The expression of CTGF protein in transiently transfected cells was confirmed 48 h after transfection by Western blotting as described elsewhere (9). Cells were stained with crystal violet after 2 weeks of incubation in six-well plates with appropriate concentrations of G418.

Treatment with recombinant CTGF. To assess the effect of CTGF on growth of ovarian cancer cell lines, cells were treated with 2.5 μg/mL of recombinant human CTGF (Peprotech EC) or PBS for 72 h. The numbers of viable cells after transfection were assessed by a colorimetric water-soluble tetrazolium salt (WST) assay (24). The cell cycle in CTGF-treated cells was analyzed using fluorescence-activated cell sorting (FACS) as described elsewhere (24). For Western blotting, 24-h serum-starved cells were pretreated with or without 2.5 μg/mL CTGF for 1 h and then stimulated with 25 ng/mL of recombinant human epidermal growth factor (EGF; Sigma) for 15 min. Phosphorylation status of extracellular signal-regulated kinase 1/2 (ERK1/2) was evaluated using anti–phospho-ERK1/2 (P-ERK1/2) and anti-ERK1/2 antibodies (Cell signaling Technology).

Transfection with synthetic small interfering RNA. *CTGF*-specific small interfering RNA (siRNA; *CTGF*-siRNA) was purchased from Santa Cruz Biotechnology. A control siRNA for the luciferase gene (CGUACGCG-GAAUACUUCGA, *Luc*-siRNA) was synthesized by Sigma. Each siRNA

(50 nmol/L) was transfected into ovarian cancer cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The numbers of viable cells 24 to 96 h after transfection were assessed by WST assay.

Statistical analysis. Differences between subgroups were tested by the Mann-Whitney *U* test. Correlations between *CTGF* methylation or expression in primary ovarian cancers and the clinicopathologic variables pertaining to the corresponding patients were analyzed for statistical significance by χ^2 or Fisher's exact test. For the analysis of survival, Kaplan-Meier survival curves were constructed for groups based on univariate predictors, and differences between the groups were tested with the log-rank test. Differences were assessed with a two-sided test and considered significant at the $P < 0.05$ level.

Results

Array-CGH analysis of ovarian cancer cell lines. We assessed copy number alterations among the 24 ovarian cancer cell lines by array-CGH using the same batch of MCG Cancer Array-800 slides for all of them. Copy number gains and losses were seen to some degree in all 24 lines (data not shown). Figure 1*A* documents the frequencies of copy number gains and losses across the entire genome of each cell line. Our array-CGH analysis predicted frequent copy number gains for 3q and 20q and frequent losses for 4q, 13q, 15q, 17p, 18q, Xp, and Xq (Supplementary Table S3), which were mostly consistent with those of our earlier conventional CGH analysis of ovarian cancer cell lines (22) and were similar to published results of conventional CGH analyses of primary ovarian cancers (27–29).

Because the most common genetic aberrations had already been identified in ovarian cancer cell lines and primary tumors, we paid attention to more remarkable patterns of chromosomal abnormalities, such as high-level amplifications (log 2 ratio, >2) and homozygous deletions (log 2 ratio, <−2), which are likely to be landmarks of oncogenes and TSGs, respectively (Table 1). High-level amplifications were detected in two cell lines, and three clones (genes) were presented. Homozygous deletions were detected in three cell lines, and five clones (genes) were presented. Among those genes, *MTAP* and *CDKN2A/p16* located of 9p21.2, *TGFBR2* at 3p24.1, and *SMAD4* at 18q21.1 are known as TSGs inactivated in various human cancers. On the other hand, the homozygous loss at 6q23, the location of *CTGF* (Fig. 1*B*), observed in RMUG-S cells had not been documented in ovarian cancer before, prompting us to examine whether genes, including *CTGF*, located within this region might be involved in the pathogenesis of ovarian cancer.

Identification of target genes involved in homozygous deletion at 6q23.1. To define the extent of the homozygous deletion in RMUG-S cells and to identify other cell lines harboring cryptic homozygous loss in this region, we did genomic PCR experiments with 10 genes, *MOXD1, CTGF, ENPP1, ENPP3, CRSP3, ARG1, AKAP7, EPB41L2, KIAA1913*, and *L3MBTL3* (Fig. 2*B*), which are located around RP11-69I8 (Fig. 1*C*) according to information archived by genome databases.[9] We detected a complete loss of *CTGF, ENPP1, ENPP3, CRSP3, ARG1, AKAP7, EPB41L2*, and *KIAA1913* only in RMUG-S cells (4.2%), whereas *MOXD1* and *L3MBTL3* were retained in this cell line, indicating that the size of the homozygous deletion is ~2.2 Mb at maximum.

[9] http://www.ncbi.nlm.nih.gov/ and http://genome.ucsc.edu/

Loss of *CTGF* expression and its restoration after DNA demethylation in ovarian cancer cell lines. Next, we determined mRNA expression levels of *CTGF, ENPP1, ENPP3, CRSP3, AKAP7, EPB41L2*, and *KIAA1913* by in all 24 ovarian cancer cell lines, normal ovary, and the normal ovarian epithelial cell–derived immortalized cell line OSE-2a. We excluded *ARG1* from the analysis because our preliminary experiment (data not shown) and the information archived by the genome databases[10] showed almost no expression of this gene in normal ovary. Among seven genes we tested, *CRSP3, EPB41L2*, and *AKAP7* were expressed in most of the ovarian cancer cell lines and normal ovary (Fig. 1*C*), suggesting that these genes are unlikely to be targets for inactivation in ovarian cancer cells. On the other hand, *CTGF, ENPP1, ENPP3*, and *KIAA1913* were frequently silenced even in ovarian cancer cell lines without their homozygous loss, suggesting that the loss of expression of those genes might result from mechanisms other than genomic deletion. Because aberrant methylation in 5′ regulatory region harboring a larger than expected number of CpG dinucleotides (CpG island) is a key mechanism by which TSGs can be silenced (7), we searched for the CpG island around transcription start sites of those genes using the CpGPLOT program[11] and identified it only in *CTGF* but not in the other three genes, prompting us to focus on *CTGF* for further analyses. *CTGF* showed a complete loss of expression in the RMUG-S cell line and a reduced expression in another 12 lines without its homozygous loss (12 of 23, 52%; Fig. 1*C*). The other 11 ovarian cancer lines, normal ovary, and OSE-2a cells did express *CTGF* mRNA. Only one of the five lines that had shown a hemizygous loss around *CTGF* in array-CGH analysis exhibited a decline in the expression of this gene (data not shown).

To investigate whether DNA demethylation could restore the expression of *CTGF* mRNA, we treated ovarian cancer cells lacking *CTGF* expression with 5-aza-dCyd for 5 days. Induction of *CTGF* mRNA expression occurred after treatment with 5 μmol/L 5-aza-dCyd in HNOA and in RMG-II cells (Fig. 1*D*). In addition, we observed an enhancement of *CTGF* mRNA expression by 5-aza-dCyd given along with TSA in both lines, although treatment with TSA alone had no effect on the expression, suggesting that histone deacetylation does play some role in the transcriptional silencing of *CTGF* among methylated ovarian cancer cells. Restoration of *CTGF* expression by the treatment with 5-aza-dCyd was also observed in the rest of the ovarian cancer cell lines with reduced expression of this gene except RMUG-S (Supplementary Fig. S2*A*).

Methylation of the *CTGF* CpG island in ovarian cancer cell lines. To show the potential role of the methylation within CpG island in silencing of *CTGF*, we first assessed the methylation status of each CpG site around the *CTGF* CpG island (regions 1–3 in Fig. 2*A*) in ovarian cancer cell lines with or without *CTGF* expression and the OSE-2a cells, by means of bisulfite sequencing (Fig. 2*A*). Regions 2 and 3 tended to be extensively methylated in *CTGF*-nonexpressing cell lines (HTOA, HUOA, RMUG-L, RMG-I, HNOA, and KF28), whereas region 1 was hypomethylated in almost all cell lines tested. In addition, regions 2B, the 3′ part of region 2, and 3 are extensively methylated in some *CTGF*-expressing ovarian cancer lines (KK and OVISE), whereas region 2A, the 5′ part of region 2, tended to be hypermethylated in the nonexpressing

[10] http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene and http://www.lsbm.org/database/index.html

[11] http://www.ebi.ac.uk/emboss/cpgplot/

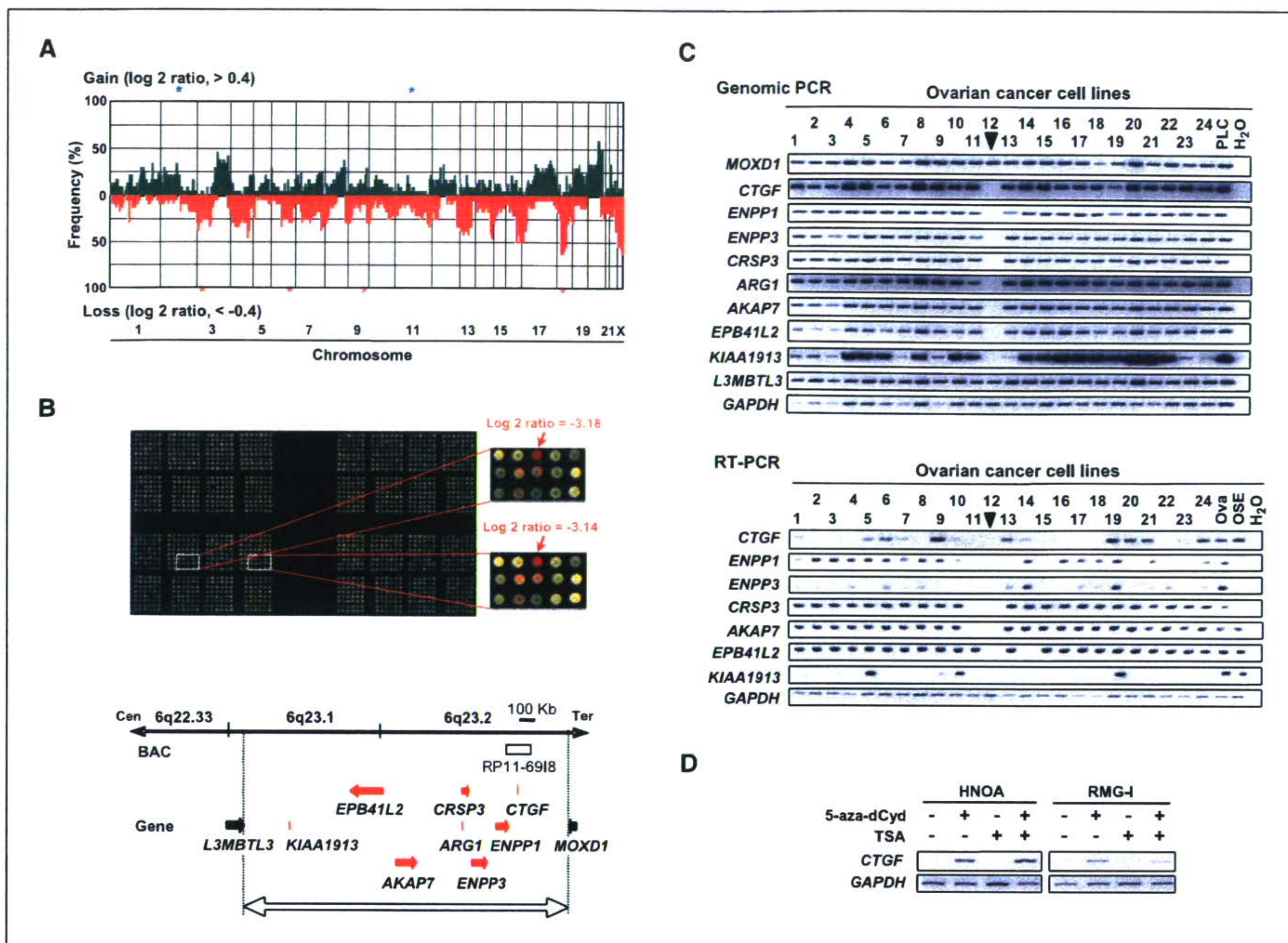

Figure 1. *A,* genome-wide frequencies of copy number gains (>0; *green*) and losses (<0; *red*) in 24 ovarian cancer cell lines. Clones are ordered as chromosomes 1 to 22 and X, and within each chromosome based on the University of California Santa Cruz (UCSC) mapping position (version May 2004). *Green asterisks,* clones with at least one high-level amplification; *red asterisks,* clones with at least one homozygous deletion (Table 1). *B,* identification of the 6q23.2 homozygous deletion in ovarian cancer cell line. *Top,* representative duplicate array-CGH image of the RMUG-S cell line. A homozygous deletion (copy number ratio as log 2 ratio) of the BAC clone at 6q23.2 was detected as a clear red signal (*red arrows*). *Bottom,* map of 6q23 covering the region homozygously deleted in the RMUG-S cell line. A BAC (RP11-69I8) was homozygously deleted in the array-CGH analysis (*vertical white bar*). Homozygously deleted region in RMUG-S cells, as determined by genomic PCR analysis (*vertical white closed arrow*). Ten genes located within this region (*red or black arrows*) show homozygously deleted or retained genes, respectively, and positions and directions of transcription. *C,* genomic and RT-PCR analyses of genes located around the 6q23 homozygously deleted region in ovarian cancer cell lines. *Top,* homozygous deletions of *CTGF, ENPP1, ENPP3, CRSP3, ARG1, AKAP7, EPB41L2,* and *KIAA1913,* but not *MOXD1* and *L3MBTL3,* were detected in one ovarian cancer cell line (RMUG-S; *arrowhead*) by genomic PCR. *1,* HT; *2,* HTOA; *3,* HUOA; *4,* KF28; *5,* MH; *6,* OVKATE; *7,* OVSAHO; *8,* KFr13; *9,* HMKOA; *10,* MCAS; *11,* RMUG-L; *12,* RMUG-S; *13,* KK; *14,* OVISE; *15,* OVMANA; *16,* OVTOKO; *17,* RMG-I; *18,* RMG-II; *19,* ES-2; *20,* W3UF; *21,* HIOAnu; *22,* HMOA; *23,* HNOA; *24,* HTBOA; PLC, peripheral leukocytes. *Bottom,* mRNA expression of *CTGF, ENPP1, ENPP3, CRSP3, AKAP7, EPB41L2,* and *KIAA1913* in ovarian cancer cell lines and the normal ovary (*Ova*) and normal ovarian epithelial cell–derived cell line OSE-2a (*OSE*), detected by RT-PCR analysis. *Arrowhead,* the cell line with the homozygous deletion indicated in the genomic PCR analysis. Expression of *CRSP3, AKAP7,* and *EPB41L2* mRNAs was observed to some degree in most ovarian cancer cell lines, whereas *ENPP1, ENPP3, CTGF,* and *KIAA1913* showed frequent silencing. Notably, 12 of the 23 cell lines (50%) without a homozygous deletion of *CTGF* showed decreased expression. *D,* results of RT-PCR to reveal restored *CTGF* expression in HNOA and RMG-I cells after treatment with 5-aza-dCyd (5 μmol/L) for 5 d with or without TSA (100 ng/mL) for 12 h.

ovarian cancer lines (HTOA, HUOA, RMUG-L, RMG-I, HNOA, and KF28) but hypomethylated in the *CTGF*-expressing ovarian cancer lines (KK, OVISE, and HTBOA) and OSE-2a cells. Consequently, methylation of region 2A was likely to be inversely correlated with the expression status of *CTGF,* suggesting that region 2A may be crucial to regulate the basal transcription level of *CTGF.*

To compare the methylation and expression status of *CTGF* in a larger number of ovarian cancer lines, we did COBRA. Consistent with the results of bisulfite sequencing, no methylated allele was detected in region 1 among most of the lines tested regardless of the *CTGF* expression status (Fig. 2*B*). On the other hand, most of the ovarian cancer cells lacking *CTGF* expression, except OVMANA and OVTOKO, had a methylated allele without an unmethylated allele in region 2, whereas most of the ovarian cancer cell lines and OSE-2a cells expressing *CTGF* had an unmethylated allele with or without methylated allele. Notably, OVISE cells expressing *CTGF* were found to have only an allele methylated in region 2 by COBRA. In this cell line, bisulfite sequencing showed that region 2B containing two *Bst*UI sites was highly methylated but region 2A was hypomethylated (Fig. 2*A*), indicating that region 2A is a critical target site for epigenetic events affecting *CTGF* expression. However, mechanisms other than DNA methylation, including

histone modification, epigenetic silencing of transcription factors or upstream components of signaling pathway activating CTGF expression, and microRNAs (30), may also contribute to the direct or indirect silencing of *CTGF*. Indeed, restoration of *CTGF* expression by TSA and/or 5-aza-dCyd was also observed in OVMANA cell line only having unmethylated allele and OVTOKO cell line having unmethylated allele and partially methylated alleles (Fig. 2*B*; Supplementary Fig. S2*B*).

Promoter activity of the sequence around the *CTGF* CpG island. Because the sequence around the *CTGF* CpG island seems to be a target for methylation and closely related to gene silencing, we tested sequences around the CpG island for promoter activity, using three fragments covering this region (fragments 1–3 in Fig. 2*A*) and three types of ovarian cancer cell lines: RMUG-S with a homozygous deletion of *CTGF*, KK expressing *CTGF*, and KF28 lacking *CTGF* expression. Because region 1 is unlikely to be a critical target for methylation, we excluded it from the analysis. Fragments 1 and 3 containing region 2A showed a remarkable increase in transcriptional activity, whereas fragment 2 not containing region 2A showed very weak activity in all types of cell lines (Fig. 2*C*), suggesting that region 2A may contain critical sequence(s) for gene silencing.

Methylation of the *CTGF* promoter region in primary ovarian cancer tumors. To determine whether the aberrant methylation of *CTGF* also takes place in primary tumors, we did MSP with primer sets targeting the sequence around the most frequently methylated sites around region 2A in a panel of 66 primary ovarian cancer tumors (Fig. 2*D*). Specificity and sensitivity of MSP and the comparison of sensitivity between MSP and COBRA were shown in Supplementary Fig. S3. Consistent with the results of the bisulfite sequencing and COBRA (Fig. 2*A* and *B*), a representative cell line lacking *CTGF* expression (RMUG-L) was methylated, whereas the *CTGF*-expressing cell line (OSE-2a) was unmethylated, as expected. We detected *CTGF* hypermethylation in 39 of the 66 primary ovarian cancer tissues (59%; Fig. 2*D*; data not shown). To confirm the results of the MSP analysis quantitatively, we did bisulfite sequencing in some of representative cases. Aberrant hypermethylation was observed in ovarian cancer tissues, which showed a methylation pattern in the MSP analysis, whereas tumors with an unmethylated pattern in the MSP analysis showed hypomethylation (Fig. 2*D*). To confirm that the methylation of *CTGF* is associated with gene silencing in primary ovarian cancer, we then examined the expression status of *CTGF* mRNA using real-time RT-PCR with cDNA prepared from 43 primary ovarian cancer tumors except for mucinous type tumors, which contain a larger amount of noncancerous cell contamination compared with other types of ovarian cancer. We found that primary tumors showing methylation of the *CTGF* region 2A by MSP expressed the gene at a significantly lower level than tumor without methylation (P = 0.041, Mann-Whitney U test; Fig. 2*D*), suggesting that the methylation of *CTGF* promoter and the gene silencing through this mechanism were not an artifact arising during the passage of ovarian cancer cell lines *in vitro*, but rather may be a true cancer-related event during the pathogenesis of ovarian cancer. However, no clear association between the methylation status of *CTGF* region 2A and the clinicopathologic characteristics was observed (Supplementary Table S4).

Association between expression level of CTGF and clinicopathologic characteristics in primary cases. To further clarify the clinical significance of the *CTGF* gene in ovarian cancer, the expression level of CTGF protein in primary ovarian cancer tissues was evaluated by immunohistochemistry using a CTGF-specific antibody (Supplementary Fig. S1). The results of the immunohistologic staining were classified as level 0 (negative staining), level 1 (1–10% of tumor cells stained), level 2 (10–50% of tumor cells stained), and level 3 (>50% of tumor cells stained). A high level of immunoreactivity for CTGF (level 3) was detected in normal ovarian epithelium (Fig. 3*A*). The CTGF protein was predominantly found in the cytoplasm or the membrane of normal or tumor epithelial cells. Although some ovarian cancer specimens showed high levels of CTGF (Fig. 3*B*), no or very weak immunoreactivity (levels 0 and 1) for CTGF was frequently observed in other ovarian cancer specimens (Fig. 3*C*). A low (levels 0 and 1) and high expression levels (levels 2 and 3) of CTGF were found in 84 of 103 (82%) cases and 19 of 103 (18%) cases, respectively. The relationship between the expression level of CTGF protein and the clinicopathologic characteristics is summarized in Table 2. In contrast to the *CTGF* mRNA level,

Table 1. High-level amplifications (log 2 ratio, >2.0) and homozygous deletions (log 2 ratio, <−2.0) detected in 24 ovarian cancer cell lines by array-CGH analysis using MCG Cancer Array-800

Alteration	BAC	Locus*		Cell line (N = 24)		Known candidate target gene†
		Chromosome band	Position	n	Name	
High-level amplifications (log 2 ratio, >2.0)	RP11-438012	2q14.2	chr2: 120,629,082–120,846,427	1	OVISE	*GL12*
	RP11-300I6	11q13.3	chr11: 69,162,462–69,323,966	1	ES-2	*CCND1, FGF3*
	CTD-2234J21	11q13.3	chr11: 69,307,612–69,307,884	1	ES-2	*CCND1, FGF3*
Homozygous deletions (log 2 ratio, <−2.0)	RP11-7I16	3p24.1	chr3: 30,541,893–30,705,070	1	KFr13	*TGFBR2*
	RP11-69I8	6q23	chr6: 132,249,163–132,410,700	1	RMUG-S	None
	RP11-70L8	9p21.3	chr9: 21,732,608–21,901,258	1	HTBOA	*CDKN2A, MTAP*
	RP11-145E5	9p21.3	chr9: 21,792,634–22,022,985	1	HTBOA	*CDKN2A, MTAP*
	RP11-10I6	18q21.1	chr18: 46,348,632–46,493,352	1	RMUG-S	*SMAD4*

* Based on UCSC Genome Browser, May 2004 Assembly.
† Putative oncogenes or tumor suppressor genes located around BAC.