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Molecular Markers and Changes of Computed Tomography Appearance in Lung Adenocarcinoma with Ground-glass Opacity

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Background: High-resolution computed tomography (HRCT) of lung adenocarcinoma at early stage shows pure ground-glass opacity (GGO) and most cases of pure GGO remain stable during follow-up. There is no consensus on the strategy for follow-up. Identification of the molecular mechanisms that are associated with the natural history of lung adenocarcinoma should provide useful information.
Methods: Twenty-three lung adenocarcinomas that were followed-up for more than 6 months pre-operatively by HRCT were included in this study. Patterns of radiological changes during the follow-up period were classified into three groups; type 1, pure GGO without consolidation; type 2, appearance or increase in consolidation within pure GGO; type 3, consolidation without pure GGO. Mutational analysis of the epidermal growth factor receptor (EGFR) and K-ras genes and immunohistochemical staining of p53 protein were performed.
Results: EGFR mutations were found in 17 cases (74%), and there was no K-ras mutation. Positive staining of p53 was found in 8 cases (35%). As for radiological findings during the follow-up period, the frequencies of EGFR mutations and positive p53 staining were 67 and 0% in type 1 ($n = 9$), 89 and 44% in type 2 ($n = 9$) and 60 and 80% in type 3 ($n = 5$).
Conclusions: EGFR mutations were frequently found in lung adenocarcinoma with GGO on HRCT in this study. Inactivation of p53 may be associated with the appearance of central consolidation within pure GGO on HRCT which reflects invasive features and may be useful as a molecular marker during the follow-up of pure GGO.

Key words: lung neoplasms – tomography – spiral computed receptor – epidermal growth factor – tumor suppressor protein p53 – adenocarcinoma – bronchioloalveolar

INTRODUCTION

Due to recent advances in computed tomography (CT) imaging and the prevalence of lung cancer screening with the use of helical CT, the frequency of small and early lung cancers which are invisible on chest X-ray is increasing in Japan (1). Most pure ground-glass opacity (GGO) lesions detected by helical CT are stable in size during the follow-up period and are pathologically atypical adenomatous hyperplasia (AAH) or bronchioloalveolar carcinoma (BAC), which shows lepidic growth without invasion (2). Although the prognosis after surgical resection is excellent (2), some lesions with pure GGO progress rapidly (3). Although intensive and careful follow-up is required for pure GGO, it remains unknown how long and how often these should be followed.

A hypothesis of multistage carcinogenesis of lung adenocarcinoma was proposed, but it is still unclear how the lesion progresses over time in terms of radiological, pathological and molecular characteristics. Since it is technically and ethically difficult to obtain tissue samples from these lesions, most studies that have sought to reveal the natural history of lung adenocarcinoma were based on radiological findings during the pre-operative follow-up period (3–6). None of them examined the molecular

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markers that are associated with the natural history of lung adenocarcinoma.

Recently, somatic mutations of the epidermal growth factor receptor (EGFR) gene were reported in lung adenocarcinoma (7,8). They have also been found in AAH and BAC (9,10). Our previous study revealed that EGFR mutations occur in the early stage of lung adenocarcinoma, such as AAH and BAC, suggesting that they play an important role in disease progression, whereas AAH with K-ras mutations stays indolent (10). Consequently, we hypothesized that these two mutually exclusive mutations might determine the natural history of pure GGO lesions.

Identification of the molecular mechanisms that affect the biological behavior of GGO lesions may offer useful information for determining the appropriate follow-up strategy for pure GGO lesions. We examined the radiological changes and genetic aberrations for lesions that were followed-up preoperatively to clarify the molecular markers that are associated with the natural history of pure GGO.

PATIENTS AND METHODS

This is a retrospective cohort study. This study was approved by the institutional review board (date of IRB approval: 30 September 2005). First, we selected patients with more than 6 months interval between their first visit to the hospital and the date of the operation by using the National Cancer Center Hospital Thoracic Surgery Division Database, which is an electronic prospective database for surgical records. All charts of listed patients were reviewed to obtain further clinical and pathological information and to select appropriate cases that fulfilled all the following criteria; surgical cases from January 2000 to December 2004, pathological diagnosis of primary lung adenocarcinoma and cases that were followed-up for >6 months before the operation using high-resolution CT (HRCT). The aim of this study is to clarify the natural history of lung adenocarcinoma in view of radiological, pathological and molecular findings, so we excluded other potential etiologies for GGO such as AAH, infection, respiratory bronchiolitis which had been followed up for >6 months using HRCT and then surgically resected. We also excluded mucinous BAC and mucin-producing adenocarcinoma of the lung. Twenty-three cases were included in the study. Ten patients were followed up because of pure GGO lesion with its size of 15 mm or less, to which surgical resection would not be indicated until radiological changes such as increase in size or attenuation, appearance of consolidation were observed. Other reasons were diagnosis of inflammation on HRCT at the initial presentation ($n = 4$), the previous history of lung surgery in the contralateral side ($n = 4$), other malignancy under treatment ($n = 2$) and patient's request for further follow-up ($n = 3$). The median follow-up interval between the initial and last HRCT scan before the operation was 18 (6–62) months.

Radiological Diagnosis

CT was performed on helical or multidetector scanners (X-Vigor, TCT-900S units or Aquilion V-detector; Toshiba Medical Systems, Tokyo, Japan) as described previously (11,12). The helical technique in 14 examinations consisted of 10.0-mm collimation for individual scans of the entire lung [120 kV (peak), 150 mA] and reconstruction using a standard algorithm. Additional thin-section CT images at the level of the lesion were obtained using 2.0-mm collimation, a 20-cm field of view, 120 kVp and 200 mA per rotation, 1.0-s gantry rotation and a high spatial frequency reconstruction algorithm. The remaining 32 examinations were evaluated on a multidetector CT scanner using axial 2.0-mm × 4 modes (four images per gantry rotation), 120 kVp, 200 mA, and 0.5-s scanning time. Thin section CT images were obtained using 2.0-mm sections reconstructed at 2.0-mm intervals using a high spatial frequency algorithm and were retrospectively retargeted to each lung with a 20-cm field of view. In 17 examinations, nonionic iodinated contrast material was administered intravenously. The scans were viewed on standard mediastinal window setting (window level, 70 H; window width, 400 H) and lung window setting (window level, −600 H; window width, 1500–2000 H).

GGO and consolidation were defined based on our previous study (13). A GGO appearance on HRCT corresponds to lepidic growth of cancer cells along alveolar walls (BAC features), whereas the proportion of consolidation is a predictor of pathological invasiveness (13). Patterns of radiological changes during the follow-up period were classified into three types (Fig. 1): type 1, pure GGO without consolidation during the follow-up period; type 2, appearance or increase in consolidation within pure GGO during the follow-up period; type 3, consolidation without pure GGO during the follow-up period. A board-certified general thoracic surgeon who was unaware of clinical and experimental information (K.S.) diagnosed the findings.

A board-certified clinician who was unaware of clinical and experimental information (H.K.) encircled the lesion using the segmented line selection tool in Image J software and measured the largest diameter and perpendicular size of

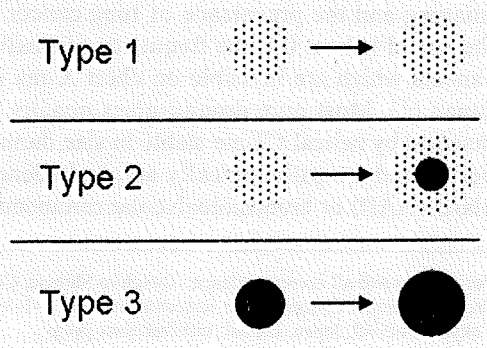

Figure 1. Schema of patterns of radiological changes during the follow-up period in this study.

the lesion (14). Corresponding slices in the initial and last HRCT examination were used. Tumor doubling time (TDT) was calculated using the formula proposed by Schwarts (15).

Mutational Analysis

Seven methanol-fixed and 16 formalin-fixed archives were used for mutational analysis of exons 18, 19 and 21 of the EGFR gene and exon 2 of the K-ras gene. Tumor DNA was purified by laser-captured microdissection (16). Information on primer sequences is available on request. Polymerase chain reactions and direct sequencing were performed as described previously (10). Peripheral lung tissue without cancer cells was used as a reference.

Pathological Diagnosis and Immunohistochemical Study

The pathological diagnosis of lung adenocarcinoma was categorized into four types according to the proposal by Ebright et al. (17): adenocarcinoma without BAC features, adenocarcinoma with BAC features (AwBF), BAC with focal invasion (BwFI) and pure BAC (PBAC).

Immunohistochemical staining of p53 was performed using a mouse primary antibody (1:100 dilution; clone DO7, Dako A/S, Glostrup, Denmark) and the avidin biotin complex method as described previously (18). A board-certified pathologist (K.T.) who was unaware of clinical and experimental information evaluated staining according to our previous criteria; +, when the proportion of tumor cells with definitely brownish nuclear staining was >20%; ±, when stained tumor cells were scattered, representing <20% of the tumor cells; −, when p53-positive cells were completely absent or seen only occasionally (19).

Clinical Information

Patient charts were reviewed to obtain clinical information. Patients who had quit smoking at least 1 year before the operation were defined as former smokers (20). Multiple lung cancers were discriminated from pulmonary metastases by applying the criteria proposed by Martini and Melamed (21). The TNM staging system revised in 1997 was adopted (22). All cases were cT1N0M0 and pT1N0M0. Surgical procedures were lobectomy in 11 cases, segmentectomy in 4 cases and wide wedge resection in 8 cases. The median follow-up period after the operation was 21 (9–65) months. Recurrence in the mediastinum was observed 12 months after the operation in one case with metachronous multiple lung cancers. The other 22 cases were alive without recurrence.

RESULTS

The 23 lesions are summarized in Table 1, and representative cases are shown in Figs. 2–5. Increase in size of 2 mm or more during follow-up period was observed in 10 cases (1 case was type 1, 5 were type 2 and 4 were type 3) and decrease in size of 2 mm or more was observed in one case of type 2 radiological classifications. The 8 men and 15 women had a median age of 66 years (37–77). Three were current smokers, 6 were former smokers and 14 were never smokers. There were seven cases of multiple lung cancers (metachronous in two cases and synchronous in five cases) and two cases were type 1, four were type 2 and 1one was type 3. Patients with type 3 radiological classification tended to have shorter interval between the initial and last HRCT examination and TDT of less than 24 months (Table 1).

EGFR mutations were found in 17 cases (74%), and all were somatic mutations (Table 2). There was no K-ras mutation in any of the 23 cases. Immunohistochemical staining of p53 was positive in eight cases (35%). Staining patterns were diffuse in all cases. Positive staining was

Table 1. Summary of clinical, pathological and radiological results

	Radiological findings		
	Type 1	Type 2	Type 3
Age, years			
Median	59	69	68
Range	40–75	53–77	37–74
Sex			
Female	7	6	2
Male	2	3	3
Smoking history			
Never	6	4	4
Former/current	3	5	1
Histological diagnosis			
PBAC	3	2	0
BwFI	6	7	3
AwBF	0	0	2
Size*, cm			
Median	1.4	1.6	1.6
Range	0.6–1.8	0.8–2.8	1.5–2.3
Interval**, months			
Median	20	16	12
Range	6–32	8–62	6–28
TDT			
0 < TDT < 24	3	3	4
24 < TDT	2	4	1
TDT < 0***	4	2	0

PBAC, pure bronchiolo-alveolar carcinoma; BwFI, BAC with focal invasion; AwBF, adenocarcinoma with BAC features; TDT, tumor doubling time; HRCT, high-resolution computed tomography.
*Size at the last HRCT examination.
**Interval between the initial and last HRCT examination.
***Reduction in volume during the follow-up period.

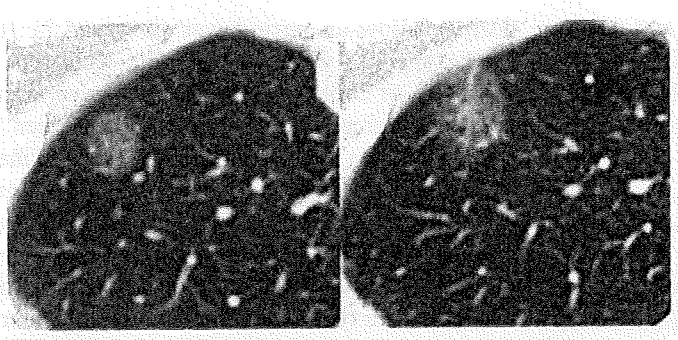

Figure 2. (Left: initial high-resolution computed tomography (HRCT); right: last HRCT; type 1 radiological finding) 66-year-old female, never smoker, 28-month interval; pure bronchioloalveolar carcinoma with del L747-T751.

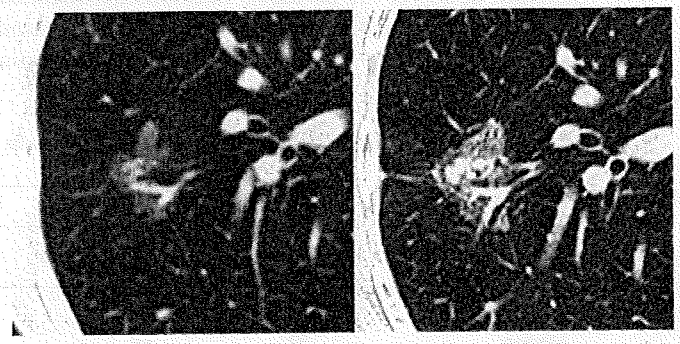

Figure 3. (Left: initial HRCT, right: last HRCT; type 2 radiological finding) 73-year-old female, never smoker, 11-month interval; BFWI with L858R.

observed both in the periphery of the lesion where cancer cells are lining along the alveolar walls and central areas where cancer cells are invading into the stroma.

The relationships between radiological findings, EGFR mutations and p53 staining are shown in Table 3. EGFR mutations were found in 67% of Type 1, 89% of Type 2 and 60% of Type 3. There was no trend between EGFR mutations and patterns of radiological changes during the follow-up period. The frequency of EGFR mutations was above 60% in all groups. The frequency of p53-positive was 44% in type 2 and 80% in type 3, in contrast to 0% in type 1. There was a trend between positive p53 immunohistochemistry and patterns of radiological changes during the follow-up period.

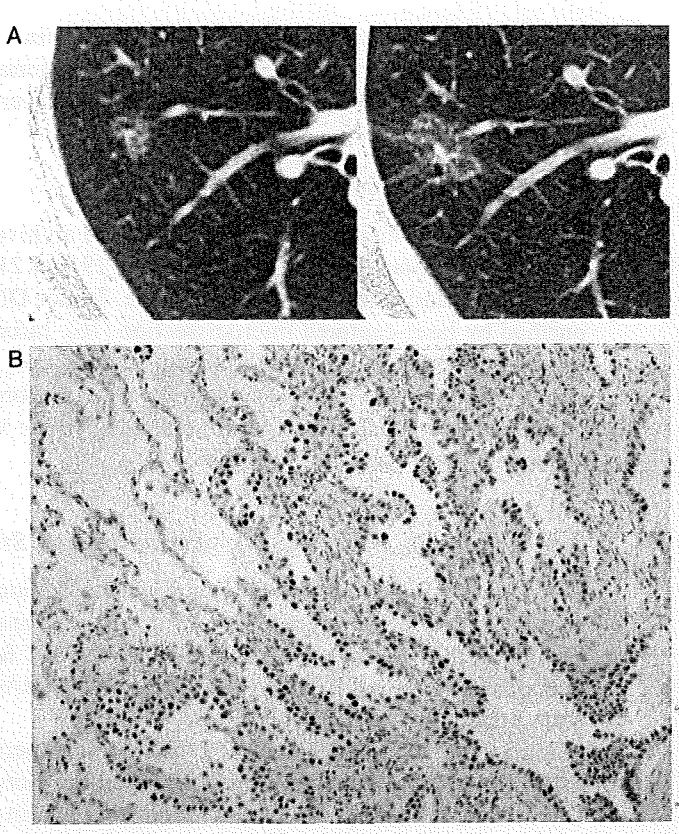

Figure 4. (A) (left: initial HRCT; right: last HRCT; type 2 radiological finding) 59-year-old male, former smoker, 62-month interval; BAC with focal invasion with del E746-A 750. (B) Immunohistochemical staining of p53 in the same case as in (A). p53 immunohistochemical staining showed reactivity in most tumor nuclei, but not in normal alveolar cells (hematoxylin and eosin staining, original magnification ×100).

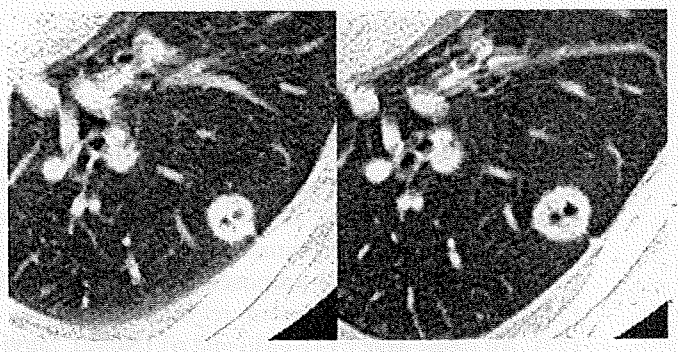

Figure 5. (Left: initial HRCT; right: last HRCT; type 3 radiological finding) 37-year-old male, never smoker, 6-month interval; adenocarcinoma with BAC features with wild type.

DISCUSSION

Several studies have been conducted to reveal the natural history of lung adenocarcinoma based on radiological findings during the pre-operative follow-up period (3–6). Most of them were based on the screening with low-dose CT, conventional CT with a 10 mm interval, which was not appropriate for a detailed and precise analysis of radiological findings. No study has ever examined molecular markers. To clarify the natural history of lung adenocarcinoma in view of radiological, pathological and molecular findings, we examined selected cases of patients who were followed-up for at least 6 months using HRCT pre-operatively and investigated the molecular markers that may be associated with the pattern of radiological changes during the follow-up period.

Biological behaviors of pure GGO may be determined by either initial aberrations of multistage carcinogenesis or additional aberrations during its progression. Since we

Table 2. EGFR mutations detected in the present study

Exons	Amino acids	Nucleotides	No. of patients
Exon 19	del E746-A750	del 2235-2249	1
	del E746-A750	del 2236-2250	6
	del E746-T751insS	del2236-2251 + insT	1
	del L747-T751	del 2239-2253	3
Exon 21	L858R	Substitution of G for T at nucleotide 2573	6

EGFR, epidermal growth factor receptor.

Table 3. EGFR mutations, immunohistochemical staining of p53 and patterns of radiological changes during the follow-up period

	Number	EGFR mutation	Deletions in Exon 19	L858R	Positive p53 IHC
Type 1	9	67%	4	2	0%
Type 2	9	89%	5	3	44%
Type 3	5	60%	2	1	80%

IHC, immunohistochemistry.

previously demonstrated that both EGFR and K-ras mutations were early events in lung adenocarcinoma (10), we examined whether these mutations were associated with the natural history of pure GGO.

We detected EGFR mutations in 17 cases (74%). Such a high incidence of EGFR mutations was probably due to the fact that all of the lesions in our study were AwBF, which frequently harbor EGFR mutations (20). We consistently observed EGFR mutations in most of the lesions with a GGO appearance (78% in types 1 and 2).

We classified lung adenocarcinoma in terms of the pattern of radiological changes during the follow-up period into three types and investigated differences between them from a molecular perspective. In our study, we should consider that the patterns of radiological changes during the follow-up period may not be distinct from each other, but rather they might be a transient finding in multistage carcinogenesis of lung adenocarcinoma. Lesions with a type 1 radiological classification might progress to type 2 and eventually type 3 during a long follow-up. The frequent EGFR mutations in type 1 (67%) suggested that they play a role in initiation (Table 3). However, EGFR mutations failed to distinguish the radiological classifications from each other, in contrast to our hypothesis, and this suggested that such mutations have little association with the progressive behavior of pure GGO.

There was no case of positive p53 staining in type 1 in our study, in contrast to type 2 (44%) and type 3 (80%). Our result suggests that the inactivation of p53 might be associated with the appearance of consolidation within a pure GGO lesion on HRCT which reflects invasive features of the lesion. Therefore, p53 should be a useful biomarker for determining the follow-up strategy for pure GGO lesions. The detection of serum p53 auto-antibodies, e.g. might complement a radiological follow-up, prevent the unnecessary exposure to X-rays and even precede HRCT findings to determine the appropriate timing for surgical intervention without delay (23,24). Previous study by others also demonstrated that among lung AwBF, the frequency of positive p53 staining was low in PBAC, whereas it increased in accordance with invasive features and they concluded that p53 played an important role in the progression of lung adenocarcinoma, although their study did not take into account periodic changes (19).

Since lung nodules that are stable for 2 years are considered to be benign, we defined a TDT cut-off value of 2 years in Table 1 (25). However, we have to be careful when applying TDT to lesions with GGO, in which cancer cells grow in a lepidic fashion without filling alveolar spaces. In addition, some lung adenocarcinomas retract the surrounding structures and shrink in size during their progression, which makes it difficult to interpret TDT (3).

Our study has several limitations, such as the small number of patients, the short follow-up between the initial and last HRCT examination and possible inter-observer variability of HRCT findings. Furthermore, the results may have been biased since we only considered surgically resected cases and we have no data on genetic aberrations for pure GGO that is stable and followed-up without surgical resection. Although K-ras mutations are frequently found in mucinous BAC or mucin-producing adenocarcinoma of the lung (26), we found no K-ras mutation in this study. The progression of multistage carcinogenesis in lung adenocarcinoma with K-ras mutations remains unclear.

It is important that we understand multistage carcinogenesis of lung adenocarcinoma to identify molecular biomarkers which can discriminate pure GGO lesions which progress from those which stay indolent. We found that the inactivation of p53 might be associated with the appearance of central consolidation within pure GGO on HRCT in this study. These markers should offer useful information for determining the appropriate strategy regarding the interval and duration of follow-up for pure GGO lesions detected by helical CT.

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Conflict of interest statement

None declared.

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help to decrease the high percentage of missing values. Second, studies should ensure that standard methods and antibodies to assess CA 19-9 concentration are used and should report survival according to different baseline CA 19-9 concentrations. These measures will allow appropriate comparisons of different studies and practical conclusions to be drawn to enable appropriate patient stratification in future phase III trials.

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Overexpression of HDACs: a prognostic marker for gastric cancer identified by tissue microarray

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Acetylation and deacetylation of histones have important roles in transcriptional regulation in eukaryotic cells.[1] The acetylation status of histones is decided by the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs acetylate the ε-amino group of lysine residues on histones, thereby neutralising their positive charge and diminishing their ability to bind negatively charged DNA. An open chromatin configuration provides accessibility for transcription factors. Cross-talk between histone acetylation or methylation and DNA methylation has profound implications for gene expression. HDACs remove the acetyl groups, thereby allowing compacted chromatin to reform. So far, 18 HDAC isoforms have been identified and classified based on homology with yeast HDACs. HDACs also have many non-histone protein substrates, such as transcription factors and signal transduction mediators.

Histone acetylation is, along with DNA methylation, one of the most consistent epigenetic mechanisms of multistage human carcinogenesis.[2] Histone hypoacetylation affects the expression of genes involved in uncontrolled cell growth, differentiation, and apoptosis. The p21 gene is one of the best-studied targets of histone hypoacetylation in human cancers. In gastric cancer, overexpression at the mRNA and protein levels of HDAC isoform 1[3] has been reported in 25 samples of gastric-cancer tissue and in 71 samples of gastric-cancer tissue for HDAC isoform 2.[4] However, to my knowledge, there have been no published systematic reports about the expression levels of HDACs in large series of patients with gastric cancer.

In this issue of *The Lancet Oncology*, Weichert and colleagues[5] report an immunohistochemical study of the expression levels of HDAC isoforms 1, 2, and 3 in two cohorts of patients with gastric cancer (143 patients in a training group and 150 patients in a validation group). Initially, nuclear staining of HDACs in cancer cells was scored according to staining intensity and percentage of immunoreactive cells. After several grouping algorithms were tested, the simplest cut-off values were chosen for reproducibility, since clinicopathological and survival correlations were fairly robust. This system showed that concurrent overexpression of HDACs and overexpression of HDAC2 were significantly correlated with lymph-node metastasis and shorter survival. Immunohistochemical assessment of HDACs might therefore be of substantial prognostic value for gastric cancer, although the present system should be validated in appropriate prospective studies.

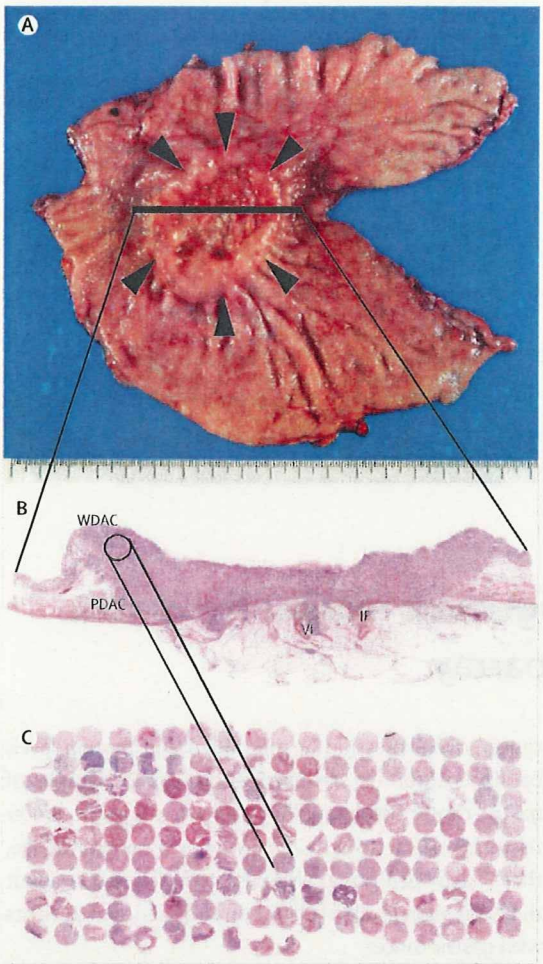

Figure: **Tissue microarray block including a cylindrical core from a gastric cancer**
(A) Macroscopic view of an advanced gastric cancer (arrowheads) in a distal gastrectomy specimen. (B) Histological heterogeneity is evident in a routine histological slide. WDAC=well-differentiated adenocarcinoma component. PDAC=poorly differentiated adenocarcinoma component. IF=invading front. VI=venous involvement. Haematoxylin-eosin staining. (C) A cylindrical core represents only some of the characteristics of this tumour. Haematoxylin-eosin staining. Construction of tissue microarray was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan. Photos courtesy of Yukihiro Nakanishi, Pathology Division, National Cancer Center Research Institute, Tokyo, Japan.

HDAC inhibitors induce the expression of p21 and other cell-cycle regulators, pro-apoptotic proteins such as Bax and Bad, and repress the expression of metastasis-promoting proteins, such as matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9), and pro-angiogenic factors such as vascular endothelial growth factor.[6] Healthy cells are relatively more resistant to HDAC-inhibitor-induced cell death. Therefore, HDAC inhibitors might be promising new anticancer drugs, and many are currently being tested in phase I or II clinical trials.[7] After appropriate validation, the present data obtained by Weichert and co-workers might help to predict therapeutic responses to HDAC inhibitors in patients with gastric cancer.

The immunohistochemical assessment system was established by Weichert's group with the use of tissue microarray. This technique allows rapid visualisation of molecular targets in thousands of tissue specimens at a time, and at the DNA, RNA, or protein level. Unlike methods that use conventional time-consuming molecular pathology, high-throughput molecular profiling of cancers by use of tissue microarray might be advantageous for screening diagnostic and treatment targets.[8,9] However, each cylindrical core constitutes only a very small portion of a tumour (figure), and thus carries only a small amount of molecular information. Weichert and colleagues took multiple cores from each tumour, and the cut-off values obtained from tissue microarray were validated in a large set of routine histological slides. This type of careful approach is strongly recommended when screening molecular targets using tissue microarray.

However, in spite of such careful validation, the success of screening with the use of tissue microarray might be restricted if immunoreactivity varies substantially in a tumour. Cancers frequently show histological heterogeneity—eg, well-differentiated and poorly differentiated components might be present simultaneously in a single tumour. During microscopic assessment of routine histological slides, some candidate target molecules show differences in immunohistochemical staining intensity or subcellular localisation between the well-differentiated and poorly differentiated components, or between the intraepithelial region and the invading front, even in a single tumour.[10,11] Such heterogeneity in staining patterns, which can be easily overlooked in tissue microarray studies, evokes speculation about the function of the candidate molecule and its participation in multistage carcinogenesis. Not only the efficiency of tissue microarray, but the abundant information obtained from routine histological slides will be of utmost importance in cancer research.

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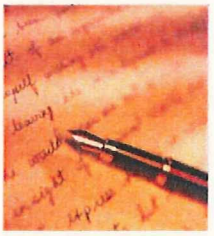

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Review Article

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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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

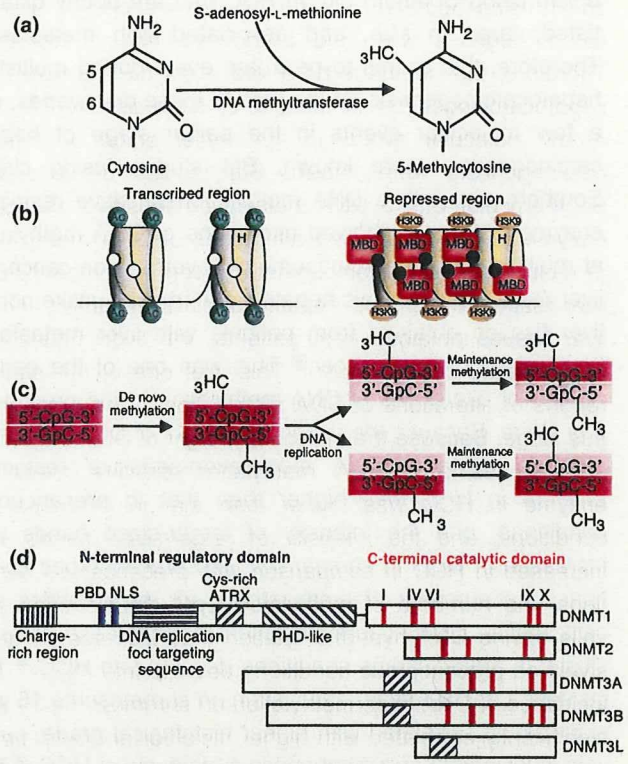

Figure 1 DNA methylation in mammals. (**a**) DNA methylation is a covalent chemical modification resulting in addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring in CpG dinucleotides. DNA methyltransferases (DNMT) transfer methyl groups from S-adenosyl-L-methionine to cytosines. (**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.

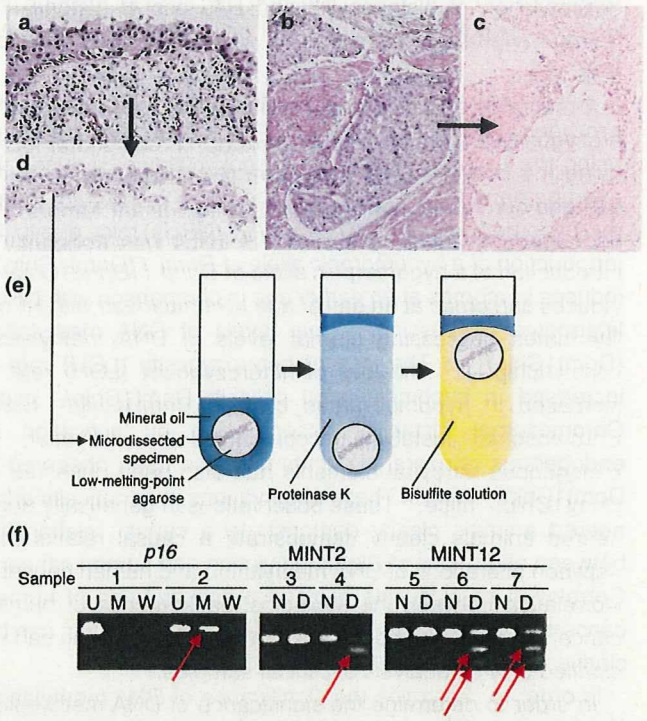

Figure 2 Analysis of DNA methylation status in tissue specimens. Tissue sample of carcinoma *in situ* (**a**) before and (**d**) during microdissection, and that of invasive carcinoma (**b**) before and (**c**) after microdissection. (**e**) Microdissected specimens were subjected to agarose bead-embedded 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.

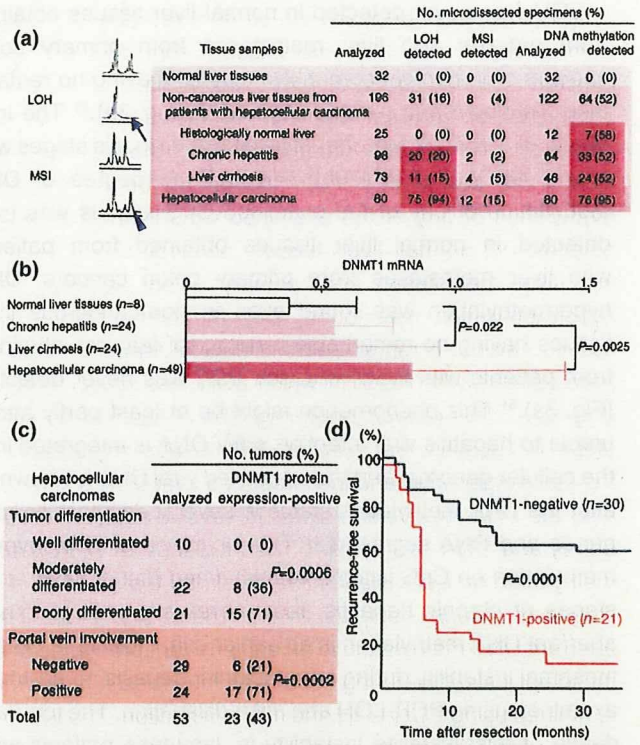

Figure 3 Alterations of DNA methylation during hepatocarcinogenesis. (**a**) Loss of heterozygosity (LOH; arrow) and microsatellite instability (MSI; arrowhead) were examined using 39 microsatellite markers; and DNA methylation status on 8 C-type CpG islands was examined on methylation-specific polymerase 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]

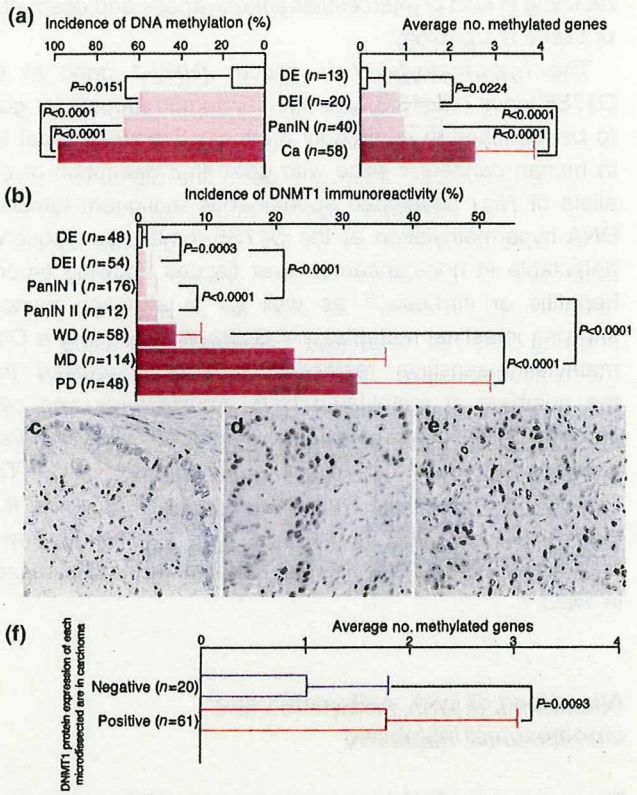

Figure 5 (**a**) Alterations of DNA methylation during pancreatic carcinogenesis. When DNA methylation status of the *p14*, *p15*, *p16*, *p73*, *APC*, *hMLH1*, *MGMT*, *BRCA1*, *GSTP1*, *TIMP-3*, *E-cadherin* and *DAPK-1* tumor-related genes was examined in microdissected specimens, the incidence of 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

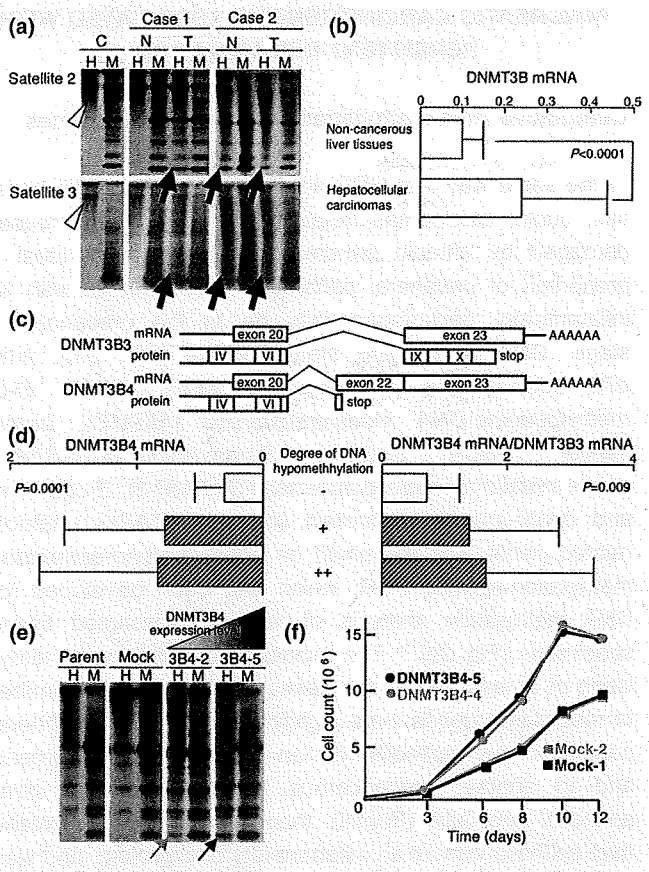

Figure 4 Overexpression of DNA methyltransferase 3B4 (DNMT3B4) associated with DNA hypomethylation in pericentromeric satellite regions during hepatocarcinogenesis. (**a**) Although satellites 2 and 3 were fully methylated in normal liver tissue obtained from a patient with liver metastasis from primary 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 intraepithelial 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-

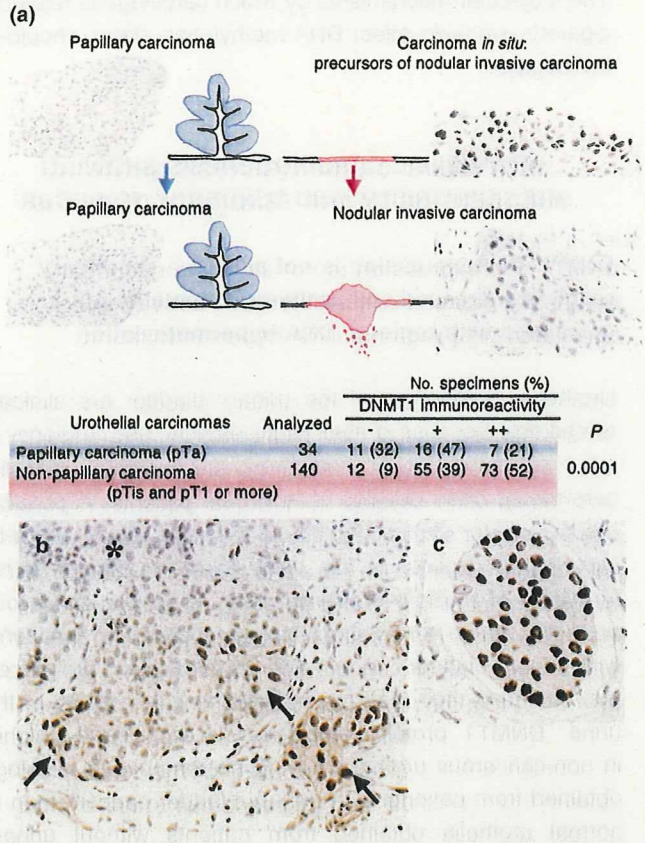

Figure 6 Immunohistochemistry of DNA methyltransferase 1 (DNMT1) in urothelial carcinomas. (**a**) The incidence and intensity of nuclear DNMT1 immunoreactivity were significantly higher in nodular invasive carcinomas with aggressive clinical courses and carcinomas *in situ*, which are considered to be precursor lesions of nodular invasive carcinoma, than in papillary carcinomas, which usually remain non-invasive.[76] +, >30% of the cells had the same nuclear staining intensity as the positive internal control lymphocytes; ++, >30% of the cells had stronger intensity. (**b**) In invasive carcinomas, cancer cells occasionally have particularly strong nuclear DNMT1 immunoreactivity at the invading front (arrows) or (**c**) in involved lymphatic vessels.[76] (*) Center of the cancer nest.

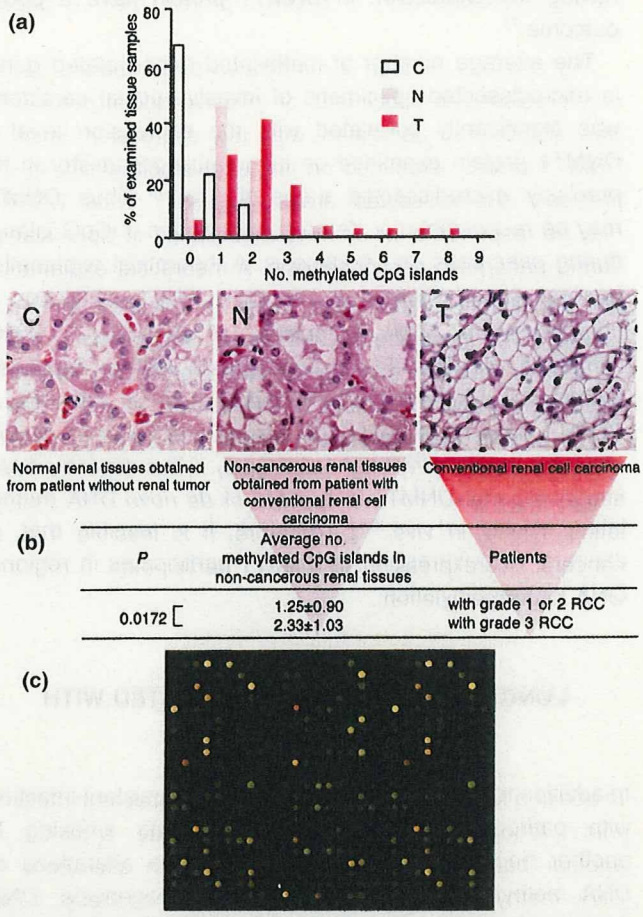

Figure 7 Precancerous conditions with alterations of DNA methylation generate more malignant renal cell carcinomas (RCC). (**a**) Even though non-cancerous renal tissues obtained from patients with renal cell carcinomas lacked remarkable histology (N), the average number of methylated CpG islands was significantly higher in N than in normal renal tissues obtained from patients without renal tumors (C).[80] The average number of methylated CpG islands was even higher in renal cell carcinomas (T).[80] (**b**) The average number of methylated CpG islands in N was significantly correlated with a higher histological grade of corresponding renal cell carcinomas developing in individual patients,[80] suggesting that regional DNA hypermethylation in precancerous conditions generates more malignant renal cell carcinomas. (**c**) Example of hybridization in the bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) method using MCG Whole Genome Array-4500 constructed by Dr Johji Inazawa, Tokyo Medical and Dental University, Tokyo, Japan and Dr Misao Ohki and Dr Fumie Hosoda, National Cancer Center Research Institute, Tokyo, Japan. This technique allows high resolution and genome-wide analysis of DNA methylation status, and suggests that the future development of more malignant RCC is determined by the genome-wide DNA methylation profile at the precancerous stage.

lia and urothelial carcinomas, concurrent DNA hypermethylation of three or more examined C-type CpG islands was significantly correlated with overexpression of DNMT1 protein.[77] Further examination is required to clarify the molecular mechanisms of recruitment of DNMT1 in a sequence-specific manner during carcinogenesis.

Alterations of DNA methylation participate particularly in the development of nodular invasive carcinomas via widely spreading carcinomas *in situ*

Although the incidence and intensity of nuclear DNMT1 immunoreactivity are correlated significantly with the histological grade of urothelial carcinomas, they appear not to be simply correlated with the depth of invasion.[76] Therefore, the morphological structures of urothelial carcinomas were examined (Fig. 6a). Urinary bladder cancers are classified as papillary or nodular according to their macroscopic