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CHAPTER 3

c0003

DNA Methylation Alterations in Human Cancers

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CHAPTER OUTLINE

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s0010

3.1 INTRODUCTION: BIOLOGICAL ROLES OF DNA METHYLATION

p0015 Epigenetic processes, i.e. alterations to biological information without changes in the DNA sequences that are mitotically and/or meiotically heritable, go beyond DNA-stored information and are essential for packaging and interpretation of the genome [1]. The modulation of epigenetic profiles contributes significantly to embryonic development, differentiation, and transition from a stem cell to a lineage-committed cell, and underlies responses to environmental signals such as hormones, nutrients and inflammation [2]. DNA methylation is a key element of epigenetic mechanisms that include histone-modification, positioning of histone variants, nucleosome remodeling, and non-coding RNA. DNA methylation is a covalent

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chemical modification resulting in addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring of CpG dinucleotides. CpG sites are concentrated either in repetitive sequences or CpG islands in promoter regions.

p0020

The C-terminal catalytic domain of DNA (cytosine-5-)-methyltransferases (DNMTs) transfers methyl groups from S-adenosyl-L-methionine (AdoMet) to cytosines. Dietary folate, vitamins B6 and B12, methionine and choline can critically affect the synthesis of AdoMet [3]. The C-terminal catalytic domain of DNMTs is composed of five conserved amino acid motifs, namely I, IV, VI, IX and X [4,5]. Motifs I and X are filed together to form 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 forms the target recognition domain. The N-terminal regulatory domain of DNMT1 contains a PCNA (proliferating cell nuclear antigen)-binding domain, a cysteine-rich ATRX (alpha thalassemia/mental retardation syndrome X-linked) zinc finger DNA-binding motif, and a polybromo homology domain targeting DNMT1 to the replication foci. The preference of DNMT1 for hemimethylated over unmethylated substrates in vitro 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. Thus, DNMT1 has been recognized as the "maintenance" DNMT, whereas DNMT3A and DNMT3B show de novo DNA methylation activity in vitro [6]. However, since de novo methylation of CpG islands has actually been observed in human fibroblasts overexpressing DNMT1, DNMT1 is capable of de novo DNA methylation activity in vivo as well as having a maintenance function [3]. DNA methylation profiles in vivo may be determined on the basis of cooperation between DNMT1 and the DNMT3 family. DNMT3L lacks conserved motifs of the catalytic domain and cooperates with the DNMT3 family to establish an imprinting pattern [7].

 p0025

DNA methylation plays critical roles in the maintenance of chromatin integrity and regulation of gene expression [8]: (a) repetitive and parasitic sequences, such as retrotransposons and endogenous retroviral elements, are usually repressed due to DNA methylation and (b) methylation of CpGs islands can directly impede the binding of transcription factors to their target sites, thus prohibiting the transcription of specific genes. Moreover, methylation of CpG islands normally promotes a highly condensed heterochromatin structure, where active transcription does not occur. Weber et al. reported that approximately 70% of human genes are linked to promoter CpG islands and about 4% of CpG island promoters are methylated in somatic cells [9]. Methylation of CpG islands naturally takes place during X chromosome inactivation and imprinting, though the majority of CpG islands remain unmethylated during development and differentiation. Extensive changes in DNA methylation during the processes of differentiation are known to take place at CpG island shores, regions of comparatively low CpG density close to CpG islands [10].

p0030

On the other hand, DNA demethylation is a process involving removal of a methyl group from a nucleotide in DNA. Although passive demethylation occurs in the absence of methylation of newly synthesized DNA strands by "maintenance" DNMT during replication rounds, active removal of cytosine methylation has long remained a mystery. Recently, it has been proved that 5-methylcytosine can be converted to 5-hydroxymethylcytosine, an intermediate form potentially involved in active demethylation, by the 2-oxoglutarate and Fe (II)-dependent oxygenases TET1, TET2 and TET3 [11,12]. MLL (myeloid/lymphoid or mixed-lineage leukemia)—TET1 translocations have been found in patients with acute lymphoblastic leukemia [13] and deletions or mutations at the TET2 locus have been reported in myelodysplastic syndrome and acute myeloid leukemia [14], indicating that impairment of the conversion of 5-methylcytosine to 5-hydroxymethylcytosine may also participate in tumorigenesis [15]. Further investigation will be needed to elucidate the significance of conversion to 5-hydroxymethylcytosine.

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s0015
3.2 DNA METHYLATION ALTERATIONS IN HUMAN CANCERS

p0035 Heterozygosity of the *Dnmt1* gene, in conjunction with treatment using the DNMT inhibitor 5-aza-deoxycytidine, reduces the average number of intestinal adenomas in ApcMin mice [16]. On the other hand, genomic hypomethylation in p53+/− mice due to the introduction of a hypomorphic allele of Dnmt 1 induces sarcomas at an earlier age in comparison with littermates possessing normal levels of DNMT1 activity [17,18]. Increased loss of heterozygosity (LOH) accompanied by activation of endogenous retroviral elements has been observed in Dnmt1 hypomorphic mice [19]. These observations of genetically engineered animals clearly demonstrate a causal relationship between alterations of DNA methylation and human cancers.

p0040 In fact, human cancer cells obtained from clinical tissue specimens frequently show genome-wide DNA hypomethylation and region-specific DNA hypermethylation [20]. DNA hypomethylation induces a higher probability of translocation of parasitic sequences to other genomic regions, and chromosomal rearrangement resulting in chromosomal instability [21]. Furthermore, aberrant DNA hypomethylation can also induce activation of oncogenes and loss of imprinting. However, a more widely recognized epigenetic change in human cancers is DNA hypermethylation at the CpG islands of promoters that silences specific genes, including tumor-suppressor genes [20] such as CDKN2A (cyclin-dependent kinase inhibitor 2A), *CDKN2B* (cyclin-dependent kinase inhibitor 2B), *TP73* (tumor protein p73), *MLH1* (mutL homolog 1), *Apc* (adenomatosis polyposis coli), *BRCA1* (breast cancer 1), *MGMT* (O-6-methylguanine-DNA methyltransferase), *VHL* (von Hippel-Lindau tumor-suppressor), *GSTP1* (glutathione S-transferase pi 1), *CDH1* (cadherin 1) and *DAPK1* (death-associated protein kinase 1). DNA hypermethylation of tumor-suppressor genes frequently becomes the second hit for driver events in accordance with the two-hit theory [22]. Moreover, some tumor-suppressor genes, such as *TIMP3* (tissue inhibitor of metalloproteinase 3), *SFRP1* (secreted frizzled-related protein 1), *SFRP2*, *SFRP4*, *SFRP5* and *RASSF1* (Ras association (RalGDS/AF-6) domain family member 1), are seldom mutated, or their mutations have never been reported in human cancers [23]. Therefore, intensive screening of genes that are methylated in human cancers may be a strategy for identification of tumor-related genes that have potential as therapeutic targets. In some instances, genes can be silenced simultaneously due to a process of long-range epigenetic silencing, and the spreading of silencing seems to affect neighboring unmethylated genes through repressive chromatin [24].

p0045 MiRNAs are the best-known class of short non-coding RNAs, which are typically around 21 nucleotides in length, imperfectly aligned with the 3′UTR of target mRNAs, and induce their translational repression. Observations of silencing due to DNA hypermethylation have expanded to tumor-suppressive microRNAs (25), such as miR-34a and 34b/c, miR-124, miR-137, miR-152, miR-193a, miR-200, miR-203, miR-205, miR-218 and miR-345. In addition to their tumor-suppressor function, miRNAs can also serve as oncogenes to promote cancer growth. B-cell integration cluster (BIC)/miR-155 is the first miRNA shown to have such tumor-promoting activity. miR-10b is another oncogene highly associated with cancer metastasis. Transcription of miR-10b is regulated by the transcription factor Twist, and the downstream targets of miR-10b include homeobox D10. Other miRNAs with oncogene function include miR-17 clusters, miR-21, and miR-373 and miR-520c as metastasis-promoting miRNAs [26].

s0020
3.3 ABERRANT DNA METHYLATION IN PRECANCEROUS CONDITIONS ASSOCIATED WITH CHRONIC INFLAMMATION, PERSISTENT VIRAL INFECTION AND SMOKING

p0050 DNA methylation alterations are frequently observed even in precancerous conditions and early-stage cancers, suggesting that epigenetic alterations may precede the classical transforming events, such as mutations of tumor-suppressor genes, amplification of oncogenes and

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chromosomal instability. Environmental factors influence health, and epigenetic profiles are known to be responses to environmental signals. Thus, aberrant DNA methylation participates especially in precancerous conditions associated with chronic inflammation, persistent viral infection and smoking [27,28]. For example, in the 1990s, although LOH on chromosome 16 was frequently detected by classical Southern blotting in hepatocellular carcinomas (HCCs) associated with metastasis, the molecular events occurring in non-cancerous liver tissue showing chronic hepatitis or liver cirrhosis, which are widely considered to be precancerous conditions, were unknown. When we examined the DNA methylation status on chromosome 16 using Southern blotting with a DNA methylation-sensitive restriction enzyme, DNA methylation alterations at multiple loci were frequently revealed even in chronic hepatitis or liver cirrhosis, compared with normal liver tissue, indicating that DNA methylation alterations are a very early event during multistage hepatocarcinogenesis [29]. This was one of the earliest reports of DNA methylation alterations at the precancerous stage.

p0055 We then examined whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis. Bisulfite modification, which converts unmethylated cytosine residues to uracil, leaving methylated cytosine residues unchanged, was applied to micro-dissected specimens obtained from lobules, pseudo-lobules or regenerative nodules in non-cancerous liver tissue from patients with HCCs. Although no degree of DNA methylation of any of the examined C-type CpG islands, which are generally methylated in a cancer-specific but not age-dependent manner, was ever detected in normal liver tissue from patients without HCCs, DNA hypermethylation of such islands was frequently found even in microdissected specimens of non-cancerous liver tissue showing no remarkable histological changes obtained from patients with HCCs in which LOH was never detected by PCR using multiple micro-satellite markers. Thus it was directly confirmed that aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis [30].

p0060 DNA hypermethylation around the promoter region of the CDH1 gene at 16q22.1 [31], which encodes a Ca^{2+}-dependent cell–cell adhesion molecule [32], has been detected even in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis [33]. Heterogeneous E-cadherin expression in such non-cancerous liver tissue, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity, might be due, at least partly, to DNA hypermethylation [33]. Reduction of E-cadherin expression due to DNA hypermethylation around the promoter region may participate even in the very early stage of hepatocarcinogenesis through loss of intercellular adhesiveness and destruction of tissue morphology.

p0065 In addition to the chronic hepatitis and liver cirrhosis stages resulting from infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) [30,34,35], DNA methylation alterations are frequently found at the precancerous stage in various organs, especially in association with chronic inflammation and/or persistent infection with viruses. Epstein-Barr virus (EBV) infection in stomach cancers is significantly associated with marked accumulation of DNA hypermethylation of C-type CpG islands [36,37], and viral latent membrane protein 2A up-regulates DNMT1 in cultured cancer cells [38]. *Helicobacter pylori* infection, another etiologic factor that is believed to be involved in stomach carcinogenesis, has also been reported to strongly promote regional DNA hypermethylation [39]. Cervical intraepithelial neoplasia (CIN) is a precursor lesion for squamous cell carcinoma of the uterine cervix closely associated with human papillomavirus (HPV) infection. DNMT1 protein expression is increased even in low-grade CINs relative to normal squamous epithelia, and is further increased in higher-grade CINs and squamous cell carcinomas of the uterine cervix [40]. HPV-16 E7 protein has been reported to associate directly with DNMT1 and stimulate the methyltransferase activity of DNMT1 in vitro [41].

p0070 DNA hypermethylation at the HIC1 (hypermethylated in cancer 1) locus has been observed in non-cancerous lung tissues, which may contain progenitor cells for cancers, obtained from

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patients with non-small-cell lung cancers, and in the corresponding non-small-cell lung cancers [42]. HIC1 is a growth-regulatory and tumor-repressor gene [43] that was first identified in the commonly methylated chromosomal region in human cancer cells [44]. The incidence of DNA hypermethylation at this locus was significantly associated with poorer differentiation of lung adenocarcinomas. The incidence of DNA hypermethylation in samples of both non-cancerous lung tissue and non-small-cell lung cancer from patients who were current smokers was significantly higher than in patients who had never smoked [42]. The incidence of DNA hypermethylation in non-cancerous lung tissue from patients with non-small-cell lung cancers was significantly correlated with the extent of pulmonary anthracosis, as an index of the cumulative effects of smoking [27]. Cigarette smoking seems to be another background factor associated with alterations of DNA methylation during multistage carcinogenesis.

s0025 3.4 ABNORMAL EXPRESSION OF DNMTS IN HUMAN CANCERS

p0075 At least a proportion of DNA methylation alterations in human cancers may be attributable to abnormalities of DNMTs. In fact, altered expression of DNMTs has been reported in human cancers. For example, the levels of DNMT1 mRNA expression are significantly higher in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis than in normal liver tissue, and are even higher in HCCs [45,46]. The incidence of DNMT1 overexpression in HCCs is significantly correlated with poorer tumor differentiation and portal vein tumor involvement. Moreover, DNMT1 overexpression in tumors is inversely correlated with the recurrence-free and overall survival rates of patients with HCCs [47].

p0080 Ductal adenocarcinomas of the pancreas frequently develop after chronic damage due to pancreatitis. At least a proportion of peripheral pancreatic ductal epithelia with an inflammatory background may be at the precancerous stage. The incidence of DNMT1 protein expression increases with progression from peripheral pancreatic ductal epithelia with an inflammatory background, to another precancerous lesion, pancreatic intraductal neoplasia (PanIN), to well-differentiated ductal adenocarcinoma, and finally to poorly differentiated ductal adenocarcinoma, in comparison with normal peripheral pancreatic duct epithelia [48]. DNMT1 overexpression in ductal adenocarcinomas of the pancreas is significantly correlated with the extent of invasion to surrounding tissue, an advanced stage, and poorer patient outcome [48]. The average number of methylated CpG islands of examined tumor-suppressor genes in microdissected specimens of peripheral pancreatic ductal epithelia with an inflammatory background, PanIN and ductal adenocarcinoma was significantly correlated with the level of DNMT1 protein expression demonstrated immunohistochemically in precisely microdissected areas [49].

p0085 When the human DNMT3A and DNMT3B genes were first cloned, the expression levels of DNMT1, 3A and 3B were reported in ten paired samples of normal and cancerous tissue obtained from various organs. Robertson et al. observed ≥2-fold overexpression of DNMT3A in five of ten samples, DNMT1 in six of ten samples, and DNMT3B in eight of ten samples, and DNMT3B clearly showing the largest fold increases among the three enzymes [50]. On the other hand, the cancer phenotype associated with accumulation of DNA methylation on C-type CpG islands is defined as the CpG-island methylator phenotype (CIMP) [51], and such accumulation is generally associated with frequent silencing of tumor-related genes due to DNA hypermethylation only, or a two-hit mechanism involving DNA hypermethylation and LOH in human cancers of various organs [52]. Expression levels of DNMT1 mRNA and protein are significantly correlated with poorer differentiation and CIMP in stomach cancers, but no such association has been observed for the expression of DNMT2, DNMT3A or DNMT3B [53]. EBV infection in stomach cancers is significantly associated with marked accumulation of DNA methylation on C-type CpG islands and overexpression of DNMT1 protein, although

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Helicobacter pylori infection, another etiologic factor strongly promoting regional DNA hypermethylation, was not correlated with DNMT1 expression levels.

p0090 Urothelial carcinomas (UCs) of the urinary bladder are clinically remarkable because of their multicentricity and tendency to recur due to a "field effect". Even non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs can be considered precancerous, because they may have been exposed to carcinogens in the urine. Our immunohistochemical examinations have clearly revealed that the incidence of nuclear DNMT1 immunoreactivity is already higher in non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs, where the PCNA labeling index had not yet increased, compared to that in normal urothelia from patients without UCs, indicating that DNMT1 overexpression was not a secondary result of increased cell proliferative activity, but in fact preceded such activity [54]. The incidence of nuclear DNMT1 immunoreactivity showed a progressive increase in dysplastic urothelia, and during transition to UCs, being significantly correlated with accumulation of DNA methylation on C-type CpG islands [55].

p0095 With respect to the mechanisms regulating the expression levels of DNMTs [56], the members of the miR-29 family, including miR-29a, miR-29b and mir-29c, have been shown to directly target DNMT3A and DNMT3B [57]. Enforced expression of miR-29s in lung cancer cell lines restores the normal patterns of DNA methylation, induces re-expression of methylation-silenced tumor-suppressor genes, and inhibits tumorigenicity in vitro and in vivo [57]. Enforced expression of miR-29b in acute myeloid leukemia cells resulted in markedly reduced expression of DNMT1, DNMT3A, and DNMT3B at both the RNA and protein levels [58]. Although down-regulation of DNMT3A and DNMT3B was the result of direct interaction of miR-29b with the 3′UTRs of these genes, miR-29b down-regulates DNMT1 indirectly by targeting Sp1, a transactivator of the DNMT1 gene [58]. miR-148 has been observed to bind to the coding region, outside the usual 3′UTR, of DNMT3B and to induce splicing alteration of DNMT3B in human cancer cells [59]. DNMT1 may also be directly regulated by miR-148 [60] and miR-126 [61]. Down-regulated miR-152 induces aberrant DNA methylation in HCC cells by targeting DNMT1 [62]. In addition to miRNAs, Hu-antigen R (HuR) proteins bind to target mRNAs and modify their levels of expression by altering their stability. HuR proteins target the 3′UTR of DNMT3B in human colon cancer cells, resulting in DNA hypermethylation of its target genes [63].

s0030

3.5 MUTATIONS, POLYMORPHISM AND SPLICING ALTERATIONS OF DNMTS AND HUMAN CANCERS

p0100 Even though our previous screening indicated that mutations of DNMT1 are not the major event during carcinogenesis in the liver and stomach [64], recent massively parallel DNA sequencing has identified somatic mutations including missense mutations, frameshifts, splice-site mutations and large deletions, which were predicted to affect DNMT3A translation in acute myeloid leukemia cells [65]. The overall survival of patients showing DNMT3A mutations was significantly shorter than that of patients without such mutations. Mutations of the DNMT3A gene, which reduce its enzymatic activity and alter the DNA methylation profiles, have also been reported in acute monocytic leukemia [66]. These observations add to the evidence for participation of aberrant DNMT activity in the pathogenesis of malignancies.

p0105 DNMT3A gene polymorphism can affect transcriptional levels of DNMT3A and susceptibility to cancers. The effect of a single nucleotide polymorphism, A/G, in the DNMT3A promoter region on transcriptional activity has been evaluated using a luciferase assay. Carriage of the A allele conferred significantly higher promoter activity in comparison with the G allele, and AA homozygotes had a six-fold increased risk of gastric cancer [67]. Similarly, a marked association between DNMT3B6 promoter C/T polymorphism and overall survival of patients

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with head and neck squamous cell carcinoma has been reported [68]: the homozygotes (CC-genotype and TT-genotype) survived significantly longer than the heterozygotes (CT-type). Such polymorphism may affect the gene expression profiles through distinct DNA methylation patterns.

p0110 Pericentromeric satellite regions are considered to be one of the specific targets of DNMT3B, since Dnmt3B−/− mice lack DNA methylation in such regions and die in utero [6]. DNA hypomethylation in pericentromeric satellite regions is known to result in centromeric decondensation and enhanced chromosome recombination. In fact, 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 of pericentromeric satellite regions [69]. In HCCs [70] and UCs [71], DNA hypomethylation of these regions is correlated with copy number alterations on chromosomes 1 and 9, respectively, where satellite regions are rich. The major splice variant of DNMT3B in normal liver tissue samples is DNMT3B3, which possesses the conserved catalytic domains. DNMT activity of human DNMT3B3 has been confirmed in vitro [72]. On the other hand, DNMT3B4 lacks the conserved catalytic domains, although it retains the N-terminal domain required for targeting to heterochromatin sites. Samples of normal liver tissue show only a trace level of DNMT3B4 expression. The levels of DNMT3B4 mRNA expression and the ratio of DNMT3B4 mRNA to DNMT3B3 in samples of non-cancerous liver tissue obtained from patients with HCCs, and in HCCs themselves, are significantly correlated with the degree of DNA hypomethylation in pericentromeric satellite regions [73]. DNA demethylation on satellite 2 has been observed in DNMT3B4-transfected human epithelial 293 cells [73]. Since DNMT3B4 lacking DNMT activity competes with DNMT3B3 for targeting to pericentromeric satellite regions, DNMT3B4 overexpression may lead to chromosomal instability through induction of DNA hypomethylation in such regions.

p0115 As another molecular mechanism involved in site-specific DNA methylation alterations, interaction between DNMT3A and c-myc has been reported. This interaction promotes the site-specific methylation of CpG dinucleotides localized in c-myc boxes in the promoter regions of the CDKN2a, CCND1 and TIMP2 genes [74]. The invalidation of c-myc reveals that c-myc allows recruitment of ~~DNM3A~~ on the c-myc box of c-myc-regulated genes. Monitoring transcription factor arrays have identified transcription factors interacting with DNMT3A and DNMT3B (such as CREB and FOS), those interacting with ~~DNM3A~~ (such as AP2alpha and p53) and those interacting with DNMT 3B (such as SP1 and SP4) [74]. Thus, direct interaction between DNMT 3A and/or DNMT 3B and transcription factors provides a rational molecular explanation for the mechanism of targeted DNA methylation.

s0035 3.6 SIGNAL PATHWAYS AFFECTING DNA METHYLATION STATUS DURING TUMORIGENESIS

p0120 Molecular links between the major signaling pathways involved in tumorigenesis and epigenetic events have been reported [75]. For example, correlations between the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and epigenetic events in tumorigenesis and progression have been attracting attention. It has been reported that PTEN methylation becomes progressively higher from benign thyroid adenoma to follicular thyroid cancer and to aggressive anaplastic thyroid cancer, which harbors activating genetic alterations in the PI3K/AKT pathway that correspond to a progressively higher prevalence [76]. An association of PTEN methylation with PIK3CA alterations and ras mutations has been reported in thyroid tumors [76]. Aberrant methylation and hence silencing of the PTEN gene, which coexists with activating genetic alterations of the PI3K/AKT pathway, may enhance the signaling of this pathway and contribute to tumor progression.

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p0125 With regard to BRAF-MEK signaling, BRAF is highly expressed in neurons. Expression of MAP2, a neuron-specific microtubule-associated protein that binds and stabilizes dendritic microtubules, is expressed in cutaneous primary melanomas and inversely associated with melanoma progression. Ectopic expression of MAP2 in metastatic melanoma cells inhibits cell growth by inducing mitotic spindle defects and apoptosis [77]. Levels of MAP2 promoter activity in melanoma cell lines are correlated with activating mutation in BRAF: hyperactivation of BRAF-MEK signaling activates MAP2 expression in melanoma cells through promoter demethylation or down-regulation of the neuronal transcription repressor HES1 [77]. Thus, BRAF oncogene levels can regulate the neuronal differentiation and tumor progression of melanoma. Genome-wide DNA methylation analysis after shRNA knockdown of BRAF V600E in thyroid cancer cells has revealed numerous methylation targets including hyper- or hypo-methylated genes with metabolic and cellular functions [78]. Among such genes, the HMGB2 gene plays a role in thyroid cancer cell proliferation, and the FDG1 gene in cell invasion [78]. A prominent epigenetic mechanism through which BRAF V600E can promote tumorigenesis is alteration of the expression of numerous important genes through DNA methylation alterations.

p0130 The Ras signaling pathway also regulates DNA methylation status. Forced expression of a cDNA encoding human GAP120 (hGAP), a down-modulator of Ras activity, or delta 9-Jun, a transdominant negative mutant of Jun, in adrenocortical tumor Y1 cells causes transformed cells to revert to their original morphology, resulting in a reduced level of DNA methylation through a reduction of both mRNA expression and the enzymatic activity of DNMTs [79]. Introduction of oncogenic Ha-ras into GAP transfectants has been found to increase the levels of DNA methylation and DNMT activity. Moreover, transient transfection CAT assays have demonstrated that the DNMT promoter in Y1 cells is activated by AP-1 and inhibited by down-regulators of Ras signaling [79]. In addition to Y1 cells, it has been reported that over-expression of unmutated Ha-ras in human T cells causes an increase in DNMT expression, and that DNMT is decreased by inhibitory signaling via the ras-MAPK pathway [80].

p0135 The apoptosis-promoting protein Par-4 has been shown to be down-regulated in Ras-transformed NIH 3T3 fibroblasts through the Raf/MEK/ERK MAPK pathway. The par-4 promoter is methylated in Ras-transformed cells through a MEK-dependent pathway, and treatment with a DNMT inhibitor restores the levels of both the Par-4 mRNA transcript and protein, suggesting that the Ras-mediated down-regulation of Par-4 occurs through promoter methylation [81]. In fact, it has been revealed that Ras transformation is associated with up-regulation of DNMT1 and DNMT3 expression [81].

s0040

3.7 DNA METHYLATION AND HISTONE MODIFICATIONS

p0140 DNA methylation determines chromatin configuration and regulates the expression levels of genes in cooperation with histone modifications [82,83]. Covalent histone modifications mark active promoters (methylation of lysine 4 of histone H3 [H3K4] and acetylation of histone H3 lysine 27 [H3K27]), active enhancers (H3K4 methylation, H3K27 acetylation), actively transcribed genes (H3K36 methylation), or heterochromatin regions (H3K9 methylation, H3K27 methylation) [82,83]. When methyl-CpG-binding proteins, such as MeCP2 and MBD2, bind to methylated CpG dinucleotide, their transcriptional repression domain recruits a co-repressor complex containing histone deacetylases (HDACs) [84]. On the other hand, histone methyltransferases, such as G9A and SUV39H1, are required to recruit DNMTs [85].

p0145 Transcriptionally repressive chromatin modifications within the promoters of tumor-suppressor genes silenced by DNA methylation are known to resemble the chromatin modifications of these genes in normal embryonic stem cells, e.g. polycomb (PcG) complex binding and H3K27 methylation. These genes also have an active marker, H3K4 methylation, in normal stem cells, and this bivalent state is converted to a primary active or repressive

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chromatin conformation after differentiation cues have been received [86]. During carcinogenesis, such modifications may render the genes vulnerable to errors, resulting in aberrant DNA methylation. These PcG complexes have been shown to directly interact with DNMTs, and possibly to promote cancer-specific gene silencing. EZH2, the PcG proteins in the polycomb repressive complex 2/3 (PRC2/3) that catalyzes the trimethylation of H3K27, may be a key player [87]. Overexpression of EZH2 is correlated with tumor progression and poorer prognosis in various cancers [88,89]. Depletion of EZH2 in cancer cells leads to growth arrest [90]. CBX7, another PcG protein, is a constituent of PRC1, and has also been shown to read the repressive histone marks, H3K9me3 and H3K27me3 [91]. Similarly to EZH2, CBX7 is able to recruit DNA methylation machinery to gene promoters and facilitate gene silencing during the development of cancers.

p0150 It has long been known that individual cancers each consist of heterogeneous cell populations. The recently proposed cancer stem cell hypothesis has emphasized that only certain subpopulations, known as cancer stem cells, cancer-initiating cells or tumor-propagating cells, have tumorigenic potential. These cancer-initiating cells are usually resistant to chemotherapy and radiotherapy, leading to treatment failure. Moreover, they may be capable of forming metastatic foci in distant organs. Despite the existence of such subpopulations, the cancer stem cell hypothesis continues to generate controversy. Since the PcG complex targets similar sets of genes in embryonic stem cells and cancer cells, much effort should be focused on how epigenetic mechanisms participate in the generation of cancer-initiating cells [20,23].

s0045 3.8 SUBCLASSIFICATION OF HUMAN CANCERS BASED ON DNA METHYLATION PROFILING

p0155 Almost all cancers are heterogeneous diseases composed of distinct clinicopathological subtypes. DNA methylation profiles may, at least partly, represent the molecular basis of each subtype [92,93]. Recently, analysis on a genome-wide scale has become possible using DNA methylation-sensitive restriction enzyme-based or anti-methyl-cytosine antibody affinity techniques that enrich the methylated and unmethylated fractions of genomic DNA [94]. These fractions can then be hybridized to DNA microarrays. Such DNA methylation profiling may provide new insight into disease entities and help to provide more accurate classifications of human cancers [23]. Such subclassification may yield clues for clarification of distinct mechanisms of carcinogenesis in various organs, and identify possible target molecules for prevention and therapy in patients belonging to specific clusters.

p0160 For example, progressive accumulation of genetic and epigenetic abnormalities has been best described in colon cancers. Clustering analyses based on either epigenetic (DNA methylation of multiple CpG island promoter regions) profiling or a combination of genetic (mutations of BRAF, KRAS, and p53 and microsatellite instability [MSI]) and epigenetic profiling have revealed distinct molecular signatures. Colon cancers were clustered into CIMP1, CIMP2, and CIMP-negative groups based on DNA methylation data [95]. CIMP1 is characterized by MSI and BRAF mutations and rare KRAS and p53 mutations. CIMP2 is associated with KRAS mutations and rare MSI, BRAF, or p53 mutations. CIMP-negative cases have a high rate of p53 mutation and lower rates of MSI or mutation of BRAF or KRAS. Together, the data show that colon cancers can be grouped into three molecularly distinct disease subclasses [95]. These three groups also differ clinically: CIMP1 and CIMP2 are more often proximal, CIMP1 has a good prognosis because it consists mostly of MSI-high cancers, and CIMP2 has a poor prognosis. Moreover, these groups may have distinct precancerous lesions that can be diagnosed endoscopically, such as serrated adenomas for CIMP1, and villous adenomas for CIMP2.

p0165 We focused on renal carcinogenesis and examined the DNA methylation status of C-type CpG islands of multiple tumor-related genes using bisulfite conversion. Even in non-cancerous

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renal tissue showing no remarkable histological changes obtained from patients with conventional-type clear cell renal cell carcinomas (RCCs), the average number of methylated CpG islands was significantly higher than in normal renal tissue obtained from patients without any primary renal tumor, regardless of patient age [96]. Stepwise accumulation of DNA methylation on CpG islands has been clearly shown to progress from normal renal tissue, to non-cancerous renal tissue showing no remarkable histological changes obtained from patients with RCCs, and to RCCs. Since it has not been possible to observe any histological change in non-cancerous renal tissue obtained from patients with RCCs, and RCCs usually develop from backgrounds without chronic inflammation or persistent viral infection, precancerous conditions in the kidney have been rarely described. However, from the viewpoint of altered DNA methylation, we have shown that it is possible to recognize the presence of precancerous conditions even in the kidney [96]. In other words, regional DNA methylation alterations may participate in the early and precancerous stage of multistage renal carcinogenesis. Surprisingly, the average number of methylated CpG islands in non-cancerous renal tissues obtained from patients with RCCs showing higher histological grades was significantly higher than that in equivalent tissue obtained from patients with low-grade RCCs, suggesting that precancerous conditions showing regional DNA hypermethylation may generate more malignant RCCs [96].

p0170

In order to further clarify the significance of DNA methylation alterations during renal carcinogenesis, we performed genome-wide DNA methylation analysis using BAC array-based methylated CpG island amplification (BAMCA), which may be suitable, not for focusing on specific promoter regions or individual CpG sites, but for overviewing the DNA methylation tendency of individual large regions among all chromosomes [92,93], in tissue samples. The average numbers of BAC clones showing DNA hypo- or hypermethylation in non-tumorous renal tissue obtained from patients with chromophobe RCCs and oncocytomas were significantly lower than the average number in non-tumorous renal tissue obtained from patients with clear cell RCCs [97]. In non-tumorous renal tissue from all examined patients with renal tumors (clear cell RCCs, papillary RCCs, chromophobe RCCs and oncocytomas), biphasic accumulation of DNA methylation alterations was evident. Among such patients, the recurrence-free survival rate of patients showing DNA hypo- or hypermethylation on more BAC clones in their non-tumorous renal tissue was significantly lower than that of patients showing DNA hypo- or hypermethylation on fewer BAC clones [97]. Significant DNA methylation profiles determining the histological subtype (chromophobe RCCs and oncocytomas vs clear cell RCCs) of future developing renal tumors and/or patient outcome (favorable outcome vs poorer outcome) may already be established at the precancerous stage.

p0175

We performed two-dimensional unsupervised hierarchical clustering analysis based on the genome-wide DNA methylation status (signal ratios obtained by BAMCA) of samples of non-cancerous renal tissue. On the basis of the DNA methylation profiles of these samples, the patients with clear cell RCCs were clustered into two subclasses, Clusters KA_N and KB_N [98]. The corresponding clear cell RCCs of patients in Cluster KB_N showed more frequent macroscopically evident multinodular growth, vascular involvement and renal vein tumor thrombi, and higher pathological tumor-node-metastasis (TNM) stages than those in Cluster KA_N. Our Clusters KA_N and KB_N in precancerous tissue can be considered clinicopathologically valid: the overall survival rate of patients in Cluster KB_N was significantly lower than that of patients in Cluster KA_N. DNA methylation alterations at the precancerous stage may even determine the outcome of patients with clear cell RCCs.

p0180

Two-dimensional unsupervised hierarchical clustering analysis based on BAMCA data for clear cell RCCs themselves was able to group patients into two subclasses, Clusters KA_T and KB_T [98]. Clear cell RCCs in Cluster KB_T showed more frequent vascular involvement and renal vein tumor thrombi, and also higher pathological TNM stages than those in Cluster KA_T. The overall survival rate of patients in Cluster KB_T was significantly lower than that of patients in

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Cluster KA_T. Multivariate analysis revealed that our clustering was a predictor of recurrence and was independent of histological grade, macroscopic configuration, vascular involvement or presence of renal vein tumor thrombi.

p0185 When we compared the DNA methylation profiles of non-cancerous renal tissue and those of the corresponding clear cell RCC, Cluster KB_N was completely included in Cluster KB_T. BAC clones, of which DNA methylation status significantly discriminated Cluster KBN from Cluster KAN, also discriminated Cluster KBT from Cluster KAT without exception. When we examined each of the representative BAC clones characterizing both Clusters KBN and KBT, the BAMCA signal ratio in the non-cancerous renal tissue was at almost the same level as that in the corresponding clear cell RCC developing in each individual patient [98]. Accordingly, we concluded that the genome-wide DNA methylation profiles of non-cancerous renal tissue are basically inherited by each corresponding clear cell RCC [99].

p0190 The average number of examined methylated C-type CpG islands was significantly higher in Cluster KB_T than in Cluster KA_T. The frequency of CIMP in Cluster KB_T was significantly higher than that in Cluster KA_T. Genome-wide DNA methylation alterations consisting of both hypo- and hypermethylation of DNA revealed by BAMCA in Cluster KB_T are associated with regional DNA hypermethylation on CpG islands. Moreover, a subclass of Cluster KB_N and KB_T based on BAMCA data showed particularly marked accumulation of copy number alterations [100]: specific DNA methylation profiles at the precancerous stage may be closely related to, or may be prone to, chromosomal instability. DNA methylation alterations in precancerous conditions, which do not occur randomly but are prone to further accumulation of epigenetic and genetic alterations, can generate more malignant cancers and even determine the outcome of individual patients [92] (Figure 3.1).

p0195 With respect to urothelial carcinogenesis, unsupervised hierarchical clustering of UCs based on array comparative genomic hybridization (CGH) data clustered UCs into three subclasses, Clusters UA, UB_1, and UB_2 [101] (Figure 3.2). In Cluster UA, copy number alterations, especially chromosomal gains, revealed by array CGH analysis, and DNA

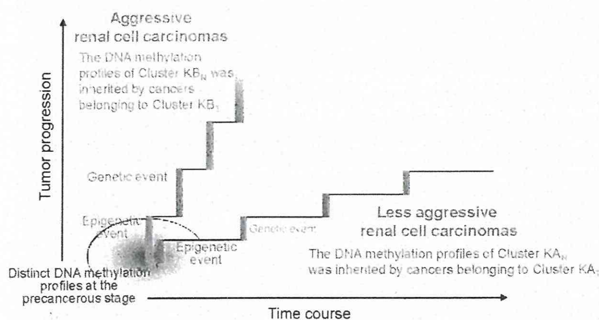

FIGURE 3.1
DNA methylation profiles in precancerous conditions and renal cell carcinomas (RCCs). Two-dimensional unsupervised hierarchical clustering analysis based on BAC array-based methylated CpG island amplification (BAMCA) data for non-cancerous renal tissue samples clustered patients with clear cell RCCs into two subclasses, Clusters KAN and KBN [98]. On the basis of the DNA methylation profiles of clear cell RCCs themselves, the patients with clear cell RCCs were divided into Clusters KAT and KBT [98]. Patients with more malignant RCCs and showing a poorer outcome were accumulated in Clusters KB_N and KB_T. The DNA methylation profile of Cluster KB_N was inherited by patients with RCCs belonging to Cluster KB_T. Regional DNA hypermethylation of C-type CpG islands and copy number alterations were accumulated in Cluster KB_T. DNA methylation alterations in precancerous conditions, such as the DNA methylation profile corresponding to Cluster KB_N, may be prone to further accumulation of epigenetic and genetic alterations, thus generating more malignant cancers, such as the RCCs in patients belonging to Cluster KB_T. This figure is reproduced in the color plate section.

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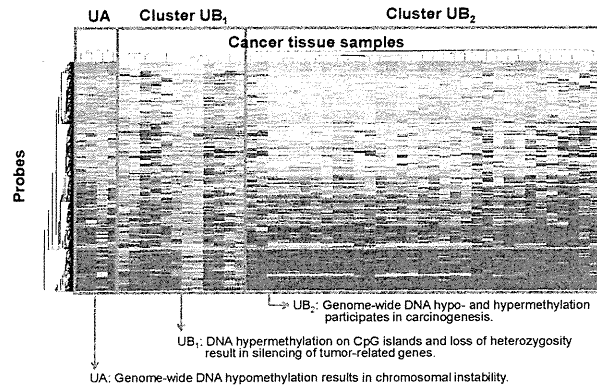

)015 **FIGURE 3.2**
Hierarchical clustering analysis of urothelial carcinomas (UCs) based on array comparative genomic hybridization (CGH) data. In Cluster UA, copy number alterations, especially chromosomal gains, revealed by array CGH analysis, and DNA hypomethylation revealed by BAMCA were both accumulated in a genome-wide manner [109]. Cluster UB1 showed accumulation of regional DNA hypermethylation on C-type CpG islands [109]. In Cluster B2, the number of BAC clones showing both DNA hypo- and hypermethylation by BAMCA was rather high, and the number of probes showing loss or gain by array CGH was rather low, in comparison to Cluster UB1 [109]. Genetic and epigenetic events appear to accumulate in a complex manner during the developmental stage of individual tumors. This figure is reproduced in the color plate section.

hypomethylation revealed by BAMCA, were both accumulated in a genome-wide manner, suggesting that DNA hypomethylation may result in chromosomal instability through changes in chromatin configuration and enhancement of chromosomal recombination [101]. Cluster UB1 showed accumulation of regional DNA hypermethylation on C-type CpG islands. Silencing of tumor-related genes due to DNA hypermethylation and chromosomal losses may be critical for the development of UCs belonging to Cluster UB1 [101]. In Cluster UB2, the number of BAC clones shown by BAMCA to have both DNA hypo- and hypermethylation was rather high, and the number of probes shown by array CGH to have loss or gain was rather low, in comparison to Cluster UB1 [101]. In addition to copy number alterations, genome-wide DNA methylation alterations may also participate in the development of UCs belonging to Cluster B_2. Taken together, the data suggest that genetic and epigenetic events accumulate in a complex manner during the developmental stage of individual UCs (Figure 3.2).

s0050

3.9 DIAGNOSIS OF CANCERS IN BODY FLUIDS AND BIOPSY SPECIMENS BASED ON DNA METHYLATION PROFILES

p0200 The incidence of DNA methylation alterations is generally high in human cancers derived from various organs. Therefore, DNA methylation alterations are applicable as biomarkers for early diagnosis of patients with cancers [102]. Cancer diagnosis based on DNA methylation alterations was initially attempted using body fluids, such as urine, that can be collected non-invasively. For example, DNA hypermethylation of regulatory sequences at the GSTP1 gene locus is present in the majority of primary prostate carcinomas, but not in normal prostatic tissue or other normal tissues. Matched specimens of primary tumor, peripheral blood lymphocytes, and simple voided urine were collected from patients with prostate cancers at various clinical stages, and the DNA methylation status of GSTP1 was examined using methylation-specific PCR [102]. Decoding of the results indicated that urine from prostate cancer patients contained shed cancer cells or debris. Furthermore, there was no case where urine-sediment DNA harbored methylation when the corresponding tumor was negative, suggesting the feasibility of molecular diagnosis using DNA methylation status as an indicator of prostatic cancer cells in urine [103].

p0205 Quantitative analysis has been introduced for cancer diagnosis based on DNA methylation alterations. For example, quantitative fluorogenic real-time PCR assay has been used to

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examine primary tumor DNA and urine sediment DNA from patients with UCs of the urinary bladder for promoter hypermethylation of multiple genes in order to identify potential biomarkers for bladder cancer [104]. The promoter methylation pattern in urine generally matched that in the primary tumors. A selected gene panel including CDKN2A, MGMT, and GSTP1 was validated in urine-sediment DNA samples from an additional validation cohort of patients with UCs of various stages and grades, and from additional age-matched control subjects [104]. Testing of such a gene panel using quantitative methylation-specific PCR assay has been shown to be a powerful non-invasive approach for detection of cancers.

p0210 DNA methylation may become an alternative biomarker which can compensate for the demerits of conventional diagnostic techniques. Gastrointestinal endoscopy followed by pathological diagnosis of biopsy specimens is useful for diagnosis of stomach cancers. However, the diagnostic power depends on the technical skill of the endoscopist. Endoscopic biopsy is a topical procedure whereby only a small portion of the lesion is removed. Moreover, gastrointestinal endoscopy is neither comfortable nor risk-free for patients, and is associated with frequent morbidity. Therefore, a method for sensitive and specific detection of early gastric cancer has been established using DNA methylation analysis of gastric washes [105]. This revealed a close correlation between the DNA methylation level of the MINT 25 locus in tumor biopsy specimens and that in gastric washes. MINT25 methylation had high sensitivity, specificity, and area under the receiver operating characteristic curve for tumor cell detection in gastric washes [105]. In addition, even when compared with potential protein or mRNA biomarkers in gastric washes, DNA methylation in such samples may be optimal because of its stability and amplifiability.

p0215 In general, pancreatic biopsy yields only a small amount of tissue, and in specimens of pancreatic juice the cellular morphology is not well preserved due to degeneration. We applied the BAMCA method to normal pancreatic tissue obtained from patients without ductal adenocarcinomas, non-cancerous pancreatic tissue obtained from patients with ductal adenocarcinomas, and cancerous tissue. The results of BAMCA for normal pancreatic tissue samples reflected the DNA methylation profiles of normal peripheral pancreatic duct epithelia (the origin of ductal adenocarcinomas), acinar cells and islet cells. In samples of non-cancerous pancreatic tissue obtained from patients with ductal adenocarcinomas, BAMCA revealed DNA hypo- or hypermethylation on many BAC clones in comparison to normal pancreatic tissue samples. Microscopic observation of non-cancerous pancreatic tissue samples obtained from patients with ductal adenocarcinomas revealed lymphocytes and fibroblasts associated with various degrees of chronic pancreatitis, which is considered to be one of the precancerous conditions for ductal adenocarcinomas (Figure 3.3). Our previous studies using microdissection and immunohistochemistry revealed accumulation of DNA hypermethylation of tumor-related genes associated with DNMT1 overexpression, even in peripheral pancreatic duct epithelia at the precancerous stage [48,49]. Therefore, the results of BAMCA for samples of non-cancerous pancreatic tissue from patients with ductal adenocarcinomas may reflect the DNA methylation profiles of peripheral pancreatic duct epithelia at the precancerous stage, lymphocytes, fibroblasts, acinar cells, and islet cells. In order to diagnose ductal adenocarcinomas in tissue samples, cancer-specific DNA methylation profiles should be discriminated from those of normal or precancerous peripheral pancreatic duct epithelia, lymphocytes, fibroblasts, acinar cells, and islet cells. Therefore, we identified 12 BAC clones whose DNA methylation status was able to discriminate cancerous tissue samples from both normal pancreatic tissue and non-cancerous pancreatic tissue samples obtained from patients with ductal adenocarcinomas in the learning cohort with a specificity of 100%. Using the criteria that combined these 12 BAC clones, cancerous tissues were precisely diagnosed with 100% sensitivity and specificity in both the learning and validation cohorts [106]. Our diagnostic criteria may be advantageous for supporting the histological and cytological assessment of pancreatic cancers (Figure 3.3).

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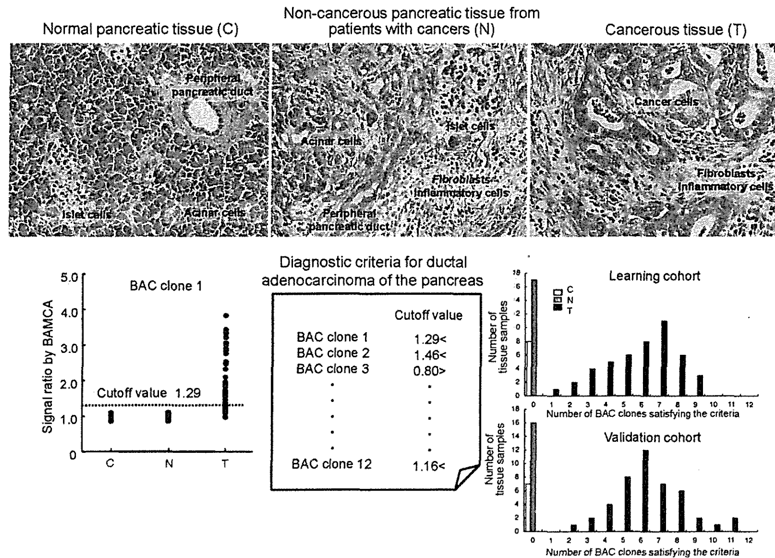

0020 **FIGURE 3.3**

Diagnostic criteria based on DNA methylation profiles for ductal adenocarcinomas of the pancreas. In order to diagnose ductal adenocarcinomas in tissue samples, cancer-specific DNA methylation profiles should be discriminated from those of normal and precancerous peripheral pancreatic duct epithelia, inflammatory cells, fibroblasts, acinar cells, and islet cells. Therefore, we identified 12 BAC clones for which the DNA methylation status was able to discriminate cancerous tissue (T) samples from both normal pancreatic tissue obtained from patients without pancreatic cancers (C) and non-cancerous pancreatic tissue from patients with cancers (N). Using criteria that combined these 12 BAC clones, cancerous tissue samples were precisely diagnosed with 100% sensitivity and specificity in both the learning and validation cohorts [106]. In general, pancreatic biopsy yields only a small amount of tissue, and in specimens of pancreatic juice the cellular morphology is not well preserved due to degeneration. Our diagnostic criteria may be advantageous for supporting the histological and cytological assessment of such specimens. This figure is reproduced in the color plate section.

s0055

3.10 CARCINOGENETIC RISK ESTIMATION BASED ON DNA METHYLATION PROFILES

p0220

DNA methylation alterations play a role even in the early and precancerous stage during multistage carcinogenesis. Since even subtle alterations of DNA methylation profiles at the precancerous stage are stably preserved on DNA double strands by covalent bonds, they may be better indicators for risk estimation than mRNA and protein expression profiles, which can be easily affected by the microenvironment of precursor cells. Personalized prevention by elimination of inflammatory conditions, viruses, and other microorganisms, together with prohibition of smoking, which causes DNA methylation alterations, may be applicable to patients with precancerous conditions.

p0225

Since HCC usually develops in liver already affected by chronic hepatitis or liver cirrhosis associated with HBV and/or HCV infection, the prognosis of patients with HCC is deemed poor unless the cancer is diagnosed at an early stage. Therefore, surveillance at the precancerous stage becomes a priority. In clinical practice, especially intensive surveillance should be performed for patients at high risk of HCC development, even if the patients are asymptomatic. Therefore, we applied the BAMCA method to samples of liver tissue. Wilcoxon test showed that 25 BAC clones, whose DNA methylation status was inherited by HCCs from non-cancerous liver tissue in patients with HCCs, were able to discriminate such non-

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cancerous liver tissue from normal liver tissue obtained from patients without HCCs. The criteria for carcinogenetic risk estimation that combined the 25 BAC clones allowed diagnosis of non-cancerous liver tissue from patients with HCCs in the learning cohort as being at high risk of carcinogenesis with 100% sensitivity and specificity [107]. In the validation cohort, these criteria allowed such discrimination with 96% sensitivity and specificity [107]. In patients with HCCs, there were no significant differences in DNA methylation status in these 25 BAC clones between samples of non-cancerous liver tissue showing chronic hepatitis and those showing cirrhosis, indicating that the criteria we employed were not associated with inflammation or fibrosis. In addition, the average number of BAC clones satisfying these criteria was significantly lower in liver tissue from patients with HBV or HCV infection but without HCCs than in non-cancerous liver tissue from patients with HCCs. DNA methylation status in these 25 BAC clones does not simply depend on hepatitis virus infection but may actually reflect the risk of carcinogenesis itself. Therefore, our criteria not only discriminate non-cancerous liver tissue from patients with HCCs from normal liver tissues, but may be capable of discriminating patients who may or may not develop HCCs from among those who are being followed up for HBV or HCV infections, chronic hepatitis, or cirrhosis.

p0230 Next, to precisely identify the CpG sites having the largest diagnostic impact on each of the 25 BAC clones and to improve the sensitivity and specificity of carcinogenetic risk estimation, we quantitatively evaluated the DNA methylation status of 203 Sma I sites on these 25 BAC clones using highly quantitative pyrosequencing of tissue specimens. In order to overcome PCR bias, we optimized the PCR conditions for each pyrosequencing primer set. It was revealed that 30 regions including 45 CpG sites had the largest diagnostic impact. Using these 30 regions, we then established criteria revised on the basis of pyrosequencing for estimation of carcinogenetic risk [108]. The revised criteria allowed diagnosis of all samples of non-cancerous liver tissue obtained from HCC patients in the validation cohort as being at high risk of carcinogenesis, with improved sensitivity and specificity [108]. It is feasible that only one CpG site in the promoter region was included in the revised criteria, because DNA methylation status in genomic regions, which do not directly participate in gene silencing, may be altered at the precancerous stage before alterations in the promoter regions themselves occur. Many CpG sites with evident diagnostic impact are located within non-CpG islands, gene bodies, and non-coding regions that have been overlooked as DNA methylation biomarkers. Meticulous examination of such regions may be important for identifying optimal indicators of carcinogenetic risk.

p0235 During the surveillance period, in order to clarify the baseline liver histology, liver biopsy is performed in patients with HBV or HCV infection prior to interferon therapy. Therefore, carcinogenetic risk estimation using such liver biopsy specimens will be advantageous for close follow-up of patients who are at high risk of HCC development. We have confirmed that carcinogenetic risk estimation using pyrosequencing is applicable to routine formalin-fixed, paraffin-embedded liver biopsy specimens. Our next step is to validate the reliability of such risk estimation prospectively using liver biopsy specimens obtained prior to interferon therapy from a large cohort of patients with HBV or HCV infection.

p0240 As mentioned above, UC is clinically remarkable because of its multicentricity due to the "field effect". Even non-cancerous urothelia showing no remarkable histological changes obtained from patients with UCs can be considered to be at the precancerous stage, because they may have been exposed to carcinogens in the urine. In fact, principal component analysis based on BAMCA data have revealed progression of DNA methylation alterations from normal urothelia to non-cancerous urothelia obtained from patients with UCs, and to UCs themselves. Unsupervised hierarchical clustering analysis of patients with UCs based on the DNA methylation status of their non-cancerous urothelia showed that the DNA methylation profiles of non-cancerous urothelia were significantly correlated with the

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invasiveness of UCs developing in individual patients, suggesting that DNA methylation alterations at the precancerous stage may generate more malignant cancers [109]. The combination of DNA methylation status on 83 BAC clones was able to completely discriminate between non-cancerous urothelia from patients with UCs and normal urothelia, and allowed diagnosis of non-cancerous urothelia from patients with UCs as having a high risk of carcinogenesis, with 100% sensitivity and specificity [109]. Differences in DNA methylation profiles between muscle-invasive UCs and non-invasive UCs have also been extensively examined: frequent DNA hypermethylation of the HOXB2 [110] and RASSF1A [111] genes is known to be associated with invasiveness of UCs.

s0060

3.11 PERSONALIZED MEDICINE BASED ON DNA METHYLATION PROFILES: PROGNOSTICATION OF PATIENTS WITH CANCERS AND PREDICTION OF RESPONSE TO CHEMOTHERAPY

p0245

Since DNA methylation alterations frequently correlate with clinicopathological parameters of cancers, they can be used as prognostic indicators in patients with cancers. For example, based on BAMCA data, 41 BAC clones, whose DNA methylation status was able to discriminate HCC patients who survived more than 4 years after hepatectomy from patients who suffered recurrence within 6 months and died within a year after hepatectomy, have been identified [107]. The DNA methylation status of these 41 BAC clones was correlated with the cancer-free survival rate of HCC patients in the validation cohort. Prognostication based on our criteria may be promising for supportive use during follow-up after surgical resection, since multivariate analysis revealed that our criteria are able to predict overall patient outcome independently of parameters observed in hepatectomy specimens, such as the degree of histological differentiation, presence of portal vein tumor thrombi, intrahepatic metastasis and multicentricity, which are already known to have a prognostic impact. Such prognostication using liver biopsy specimens obtained before transarterial embolization, transarterial chemoembolization, and radiofrequency ablation may be advantageous even for patients who undergo such therapies.

p0250

Even when surgery is performed with curative intent for patients with pancreatic cancers, the rate of recurrence is very high. Although previous studies have suggested the efficacy of adjuvant chemotherapy, it needs to be carried out carefully, paying close attention to adverse reactions. In order to decide the indications for such adjuvant chemotherapy, prognostic criteria should be explored. We have identified 11 BAC clones whose DNA methylation status was able to discriminate patients showing early relapse from those without relapse in the learning cohort with 100% specificity, and this was correlated with the recurrence-free and overall survival rates in the validation cohort [106]. Multivariate analysis revealed that satisfying the prognostic criteria using these 11 BAC clones was a parameter independent of surgical margin positivity and lymph node metastasis at the time of surgery [106].

p0255

The quality of life of patients with urinary bladder cancers is generally poor after total cystectomy. In general, therefore, after therapeutic diagnosis of UC tumors obtained by transurethral resection, patients are followed-up by repeat cystoscopy examinations. In patients showing sudden prominent malignant progression, it is difficult to determine the appropriate timing of total cystectomy. Therefore, prognostic indicators need to be explored. The combination of DNA methylation status on 20 BAC clones selected by Wilcoxon test was able to completely discriminate patients who suffered recurrence after surgery from patients who did not [109]. DNA methylation profiling may thus provide optimal indicators for prognostication in patients with UCs. Other recently published DNA methylation alterations in human cancers that are correlated with patient outcome and can be used as prognostic indicators are summarized in Table 3.1.

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t0010 **TABLE 3.1**

Tumor	Gene	DNA Methylation Status	References
Glioma	MGMT	DNA hypermethylation	PLoS One 2011; 6: e23332 J Neurooncol 2011; 102: 311–16
	LINE-1	DNA hypomethylation	PLoS One 2011; 6: e23332
	POTEH	DNA hypomethylation	Brain Res 2011; 1391: 125–31
Head and neck cancer	miRNA-137	DNA hypermethylation	Cancer 2011; 117: 1454–62
	ESR1	DNA hypermethylation	Clin Epigenetics 2010; 1: 61–69
	HIC1	DNA hypermethylation	Clin Epigenetics 2010; 1: 61–69
	LATS2	DNA hypomethylation	BMC Cancer 2010; 10: 538
	p16	DNA hypermethylation	Oral Oncol 2010; 46: 734–9
Salivary gland cancer	RUNX3	DNA hypermethylation	Cancer Sci 2011; 102: 492–7
Lung cancer	RASSF1A	DNA hypermethylation	Carcinogenesis 2011; 32: 411–16.
Esophageal cancer	p 14	DNA hypermethylation	J Clin Pathol 2011; 64: 246–51
	p 15	DNA hypermethylation	J Clin Pathol 2011; 64: 246–51
	p 16	DNA hypermethylation	J Clin Pathol 2011; 64: 246–51
	p 21	DNA hypermethylation	J Clin Pathol 2011; 64: 246–51
	p 27	DNA hypermethylation	J Clin Pathol 2011; 64: 246–51
	p 57	DNA hypermethylation	J Clin Pathol 2011; 64: 246–51
	p 73	DNA hypermethylation	J Clin Pathol 2011; 64: 246–51
	PAX6	DNA hypermethylation	Ann Surg Oncol 2011; 18: 1185–94
	ENST00000363328	DNA hypermethylation	Ann Surg Oncol 2011; 18: 1185–94
Stomach cancer	Claudin-4	DNA hypomethylation	Lab Invest 2011 ~~doi: 10.1038/ labinvest.2011.117.~~
	BNIP3	DNA hypermethylation	Oncol Rep 2011; 25: 513–18
	DAPK	DNA hypermethylation	Oncol Rep 2011; 25: 513–18
	S100A6	DNA hypomethylation	Am J Pathol 2010; 177: 586–97
	EphA1	DNA hypermethylation	Oncol Rep 2010; 24: 1577–84
Colorectal cancer	fibulin-3b	DNA hypermethylation	Neoplasma 2011; 58: 441–8
	p16	DNA hypermethylation	Anticancer Res 2011; 31: 1643–6 Oncol Rep 2011; 25: 789–94
	LINE-1	DNA hypomethylation	Cancer 2011; 117: 1847–54
	RASSF1A	DNA hypermethylation	J Cell Physiol 2011; 226: 1934–9
	SFRP2	DNA hypermethylation	Clin Invest Med 2011; 34: E88–95
	DSC3	DNA hypermethylation	Br J Cancer 2011; 104: 1013–19
	IGFBP3	DNA hypermethylation	Clin Cancer Res 2011; 17: 1535–45
	EVL	DNA hypermethylation	Clin Cancer Res 2011; 17: 1535–45
	hMLH1	DNA hypermethylation	Oncol Rep 2011; 25: 789–94
	PPARG	DNA hypermethylation	PLoS One 2010; 5: e14229
	MGMT	DNA hypermethylation	Cancer Causes Control 2011; 22: 301–9
	IGF2	DNA hypomethylation	Gastroenterology 2010; 139: 1855–64
	RARβ2	DNA hypermethylation	Tumour Biol 2010; 31: 503–11
Gastrointestinal ctromal tumor	REC8	DNA hypermethylation	Gut ~~2011 doi:10.1136/gut.2011.241034~~
	PAX3	DNA hypermethylation	Gut ~~2011 doi:10.1136/gut.2011.24103~~
	p16	DNA hypermethylation	Gut ~~2011 doi:10.1136/gut.2011.24103~~
Hepatocellular carcinoma	RASSF1A	DNA hypermethylation	Asian Pac J Cancer Prev 2010; 11: 1677–81
	CADM1	DNA hypermethylation	Oncol Rep 2011; 25: 1053–62
	WIF-1	DNA hypermethylation	Tumour Biol 2011; 32: 233–40
	RELN	DNA hypermethylation	Ann Surg Oncol 2011; 18: 572–9
	~~HOXA5~~	~~DNA hypermethylation~~	~~Pathol Int 2010; 60: 661–6~~

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TABLE 3.1 —continued

Tumor	Gene	DNA Methylation Status	References
Renal cell carcinoma			
	MSH2	DNA hypermethylation	Pathol Int 2010; 60: 661–6
	hsa-miR-9	DNA hypermethylation	Oncogene 2010; 29: 5724–8
Neuroblastoma	CASP8	DNA hypermethylation	Mol Carcinog 2011; 50: 153–62
	TMS1	DNA hypermethylation	Mol Carcinog 2011; 50: 153–62
	APAF1	DNA hypermethylation	Mol Carcinog 2011; 50: 153–62
Breast cancer	Endoglin	DNA hypermethylation	Oncogene 2011; 30: 1046–58
	RASSF1A	DNA hypermethylation	Breast Cancer Res Treat 2011; 129: 1–9
	CDO1	DNA hypermethylation	BMC Cancer 2010; 10: 247
Cervical cancer	APC1A	DNA hypermethylation	Int J Oncol 2011; 39: 683–8
Endometrioid cancer	CDH1	DNA hypermethylation	Cancer Invest 2011; 29: 86–92
Ovarian cancer	GREB1	DNA hypomethylation	Oncology 2011; 80: 12–20
	TGIF	DNA hypomethylation	Oncology 2011; 80: 12–20
	TOB1	DNA hypomethylation	Oncology 2011; 80: 12–20
	TMCO5	DNA hypermethylation	Oncology 2011; 80: 12–20
	PTPRN	DNA hypermethylation	Oncology 2011; 80: 12–20
	GUCY2C	DNA hypermethylation	Oncology 2011; 80: 12–20
	HERV-K	DNA hypomethylation	Int J Gynecol Cancer 2011; 21: 51–7
Trophoblastic tumor	ASPP1	DNA hypermethylation	Mod Pathol 2011; 24: 522–32
Melanoma	LINE-1	DNA hypomethylation	J Transl Med 2011; 9: 78
Acute myeloid leukemia	CEBPA	DNA hypermethylation	Leukemia 2011; 25: 32–40
Multiple myeloma	p16	DNA hypermethylation	Ann Hematol 2011; 90: 73–9

~~DNA methylation alterations in human cancers that are correlated with the outcome of patients and can be used as prognostic indicators~~

p0260

In addition, DNA methylation profiles may be predictive indicators of response to chemotherapy. One such example is silencing of the mitotic checkpoint gene CHFR (checkpoint with forkhead and ring finger domains) in gastric cancers. Mitotic checkpoints prevent errors in chromosome segregation that can lead to neoplasia, and it is notable that gastric cancers often show impaired checkpoint function. CHFR expression was silenced by DNA methylation of the 5′ region of the gene in tested gastric cancer cell lines and primary gastric cancers; expression was restored by treatment with 5-aza-2′-deoxycytidine. In addition, histones H3 and H4 were found to be deacetylated in cell lines showing aberrant methylation. Cells not expressing CHFR showed impaired checkpoint function, leading to nuclear localization of cyclin B1 after treatment with microtubule inhibitors such as docetaxel or paclitaxel. Absence of CHFR appears to be associated with the sensitivity of cells to mitotic stress caused by microtubule inhibition, and restoration of CHFR expression by 5-aza-2′-deoxycytidine or adenoviral gene transfer restores the checkpoint. By affecting mitotic checkpoint function, CHFR inactivation likely plays a key role in gastric cancer tumorigenesis [112]. Moreover, aberrant methylation of CHFR appears to be a good molecular marker with which to predict the sensitivity of gastric cancers to microtubule inhibitors.

p0265

Another example is MGMT, a DNA repair protein, which reverses the addition of alkyl groups to the guanine base of DNA. Silencing of MGMT due to DNA methylation in glioma is a useful predictor of response to alkylating agents such as carmustine or temozolomide [113]. Similarly, methylation of a mismatch repair gene, hMLH1, in ovarian and colon cancer cell lines confers chemoresistance to many chemotherapeutic agents. Treatment with a DNA demethylating agent, 5-aza-2′-deoxycytidine, can reactivate hMLH1 and reverse the chemoresistance. Likewise, silencing of APAF-1 (apoptotic peptidase activating factor-1),