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].

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.

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.

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

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.

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 KB_N from Cluster KA_N, also discriminated Cluster KB_T from Cluster KA_T without exception. When we examined each of the representative BAC clones characterizing both Clusters KB_N and KB_T, 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].

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).

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

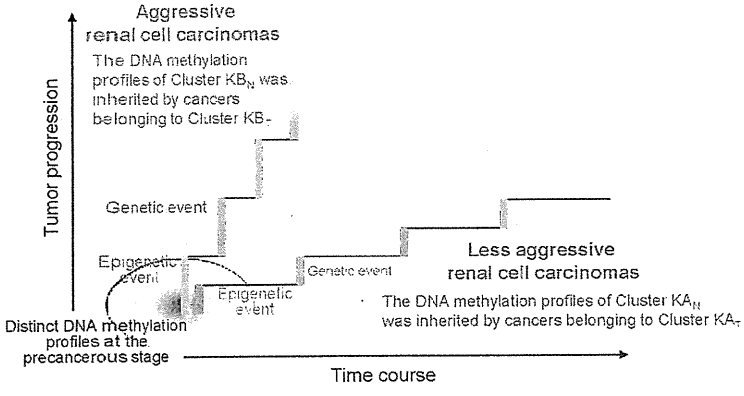

FIGURE 3.1
DNA methylation profiles in precancerous conditions and renal cell carcinomas (RCCs). Two-dimensional unsupervised hierarchical clustering analysis based on BAC array-based methylated CpG island amplification (BAMCA) data for non-cancerous renal tissue samples clustered patients with clear cell RCCs into two subclasses, Clusters KA_N and KB_N [98]. On the basis of the DNA methylation profiles of clear cell RCCs themselves, the patients with clear cell RCCs were divided into Clusters KA_T and KB_T [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.

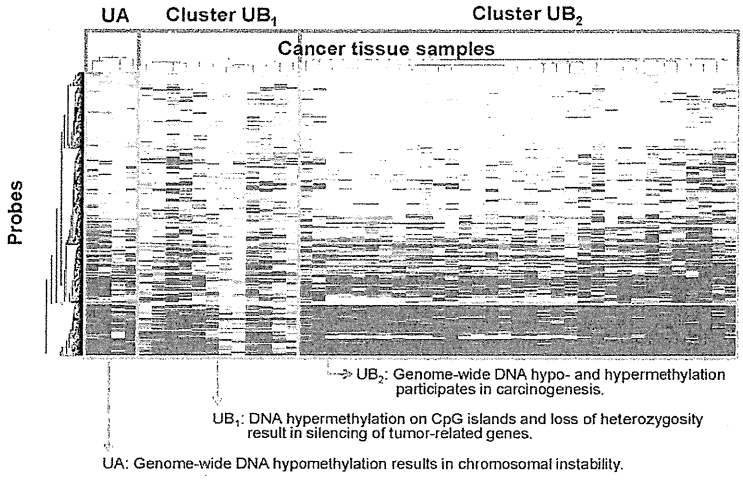

FIGURE 3.2
Hierarchical clustering analysis of urothelial carcinomas (UCs) based on array comparative genomic hybridization (CGH) data. In Cluster UA, copy number alterations, especially chromosomal gains, revealed by array CGH analysis, and DNA hypomethylation revealed by BAMCA were both accumulated in a genome-wide manner [109]. Cluster UB_1 showed accumulation of regional DNA hypermethylation on C-type CpG islands [109]. In Cluster B_2, 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 UB_1 [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 UB_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).

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

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].

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

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.

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.

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).

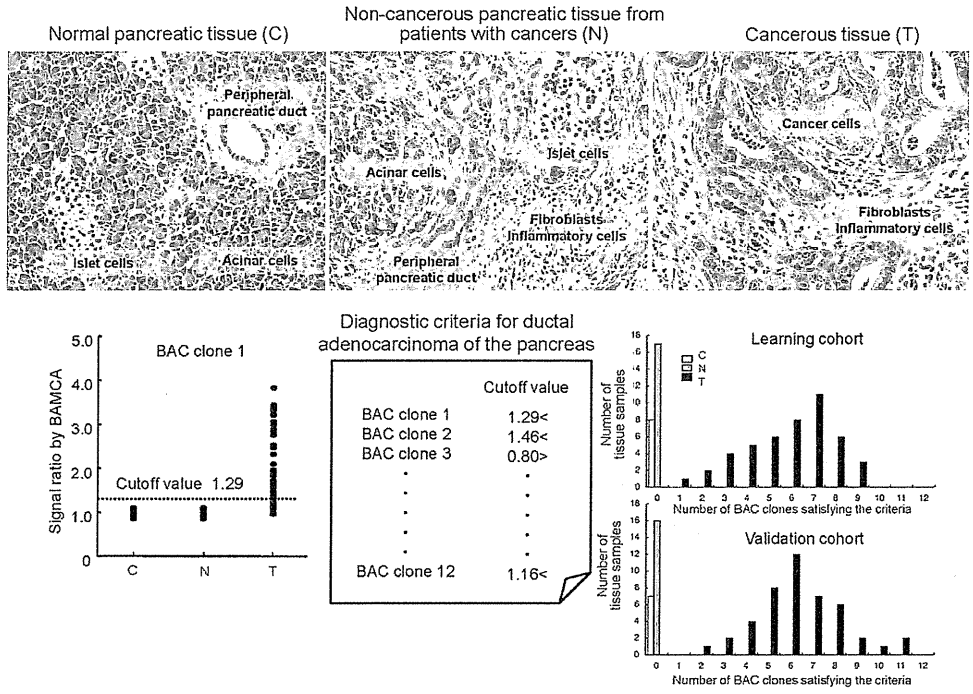

FIGURE 3.3

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.

3.10 CARCINOGENETIC RISK ESTIMATION BASED ON DNA METHYLATION PROFILES

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.

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-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.

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.

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.

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 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.

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

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.

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].

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

TABLE 3.1 DNA methylation alterations in human cancers that are correlated with the outcome of patients and can be used as prognostic indicators

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; 91: 1652–67
	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 stromal tumor	REC8	DNA hypermethylation	Gut 2012; 61: 392–401
	PAX3	DNA hypermethylation	Gut 2012; 61: 392–401
	p16	DNA hypermethylation	Gut 2012; 61: 392–401
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
Renal cell carcinoma	HOXA5	DNA hypermethylation	Pathol Int 2010; 60: 661–6
	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

Continued

TABLE 3.1 —continued

Tumor	Gene	DNA Methylation Status	References
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

human cancers that are correlated with patient outcome and can be used as prognostic indicators are summarized in Table 3.1.

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.

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), a proapoptotic gene, confers chemoresistance to melanoma and leukemia cells by mediating resistance to cytochrome c-dependent apoptosis [114]. These findings demonstrate the potential clinical utility of DNA methylation markers for individualized therapy of cancer patients.

3.12 NEW TECHNOLOGIES FOR DNA METHYLATION ANALYSIS AND FUTURE DIRECTIONS

Currently available forms of screening technology, such as single-base-pair resolution whole-genome DNA methylation analysis using second-generation sequencers, and international efforts aimed at determining reference epigenome profiles, are now opening new avenues of epigenome therapy for cancer patients. Although broad DNA methylation profiling was initially performed on the basis of two-dimensional gel electrophoresis, adaptation of microarray hybridization techniques used in gene expression and genome studies to the profiling of DNA methylation patterns opened the door to the era of the epigenome. Enzyme-based and affinity enrichment-based DNA methylation analysis techniques have been proved suitable for examination of human tissue samples using hybridization arrays [115]. Currently available high-throughput DNA sequencing technologies using second-generation sequencers are now capable of single-base-pair resolution for whole-genome DNA methylation analysis. Although projects involving analysis of large numbers of human tissue samples will still rely on array-based approaches for several more years, the trend will be towards bisulfite shotgun sequencing [94]. Nanopore sequencing provides single-molecule detection and avoids any bias introduced by differential amplification of methylation-derived states [116]. Moreover, third-generation sequencers for real-time sequencing can directly detect 5-methylcytosine without bisulfite conversion [117]. In addition, genome-wide analysis of histone modification and non-coding RNA is also being robustly performed. Thus, high-throughput mapping of the epigenome, i.e. an overview of DNA methylation, histone modification, non-coding RNA, and chromatin accessibility in normal, precursor and cancer cells, is now highly reproducible and standardized.

Importantly, changes in the epigenome are potentially reversible by drug treatments. This has significant implications for the prevention and therapy of human cancers. Indeed, several inhibitors of chromatin-modifying enzymes, including DNMT inhibitors, as well as HDAC inhibitors, have been approved by the US Food and Drug Administration and the EU, and are now being used in clinical practice [118,119]. However, to maximize the potential of such therapeutic approaches, a more comprehensive characterization of the epigenome changes that occur during normal development and adult cell renewal should be accomplished by international consortia.

Scientists and representatives of major funding agencies have decided to launch the International Human Epigenome Consortium (IHEC) [120]. Just as the Human Genome Project provided a reference "normal" sequence for studying human disease, high-resolution reference epigenome maps consisting of the various epigenetic layers of detailed DNA methylation as well as histone modification, nucleosome occupancy and corresponding coding, and non-coding RNA expression in different normal cell types will be provided by IHEC. Such a reference human epigenome will be available to the worldwide research community. Information on the methods utilized by IHEC members will be useful for producing large epigenomic datasets related to health and diseases in humans. It will become possible to compare profiles of different human populations, thereby helping to evaluate the impact of environment and nutrition on the epigenome. Epigenome reference maps will have an immediate impact on our understanding of cancers as well as diabetes, cardiopulmonary diseases, neuropsychiatric disorders, imprinting disorders, inflammation, and autoimmune diseases, and will hopefully lead to breakthroughs in the prevention, diagnosis, and therapy of human cancers.

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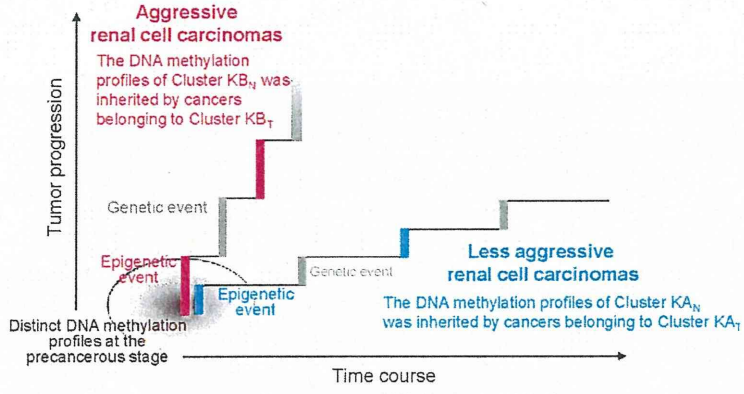


FIGURE 3.1
DNA methylation profiles in precancerous conditions and renal cell carcinomas (RCCs). See p. 39 for details.

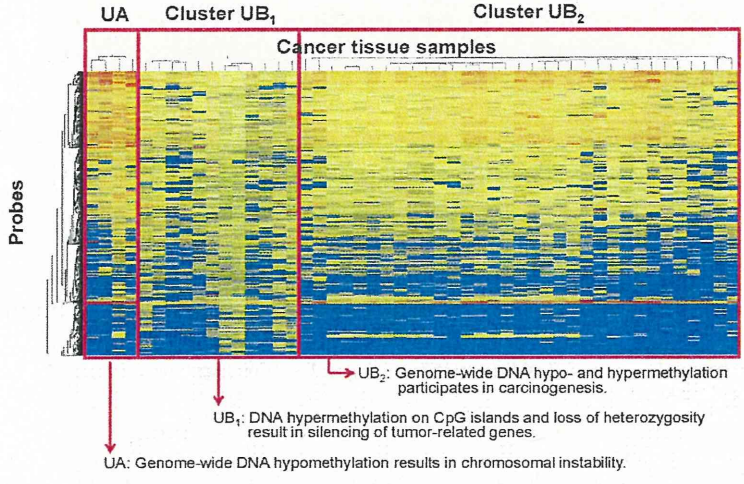


FIGURE 3.2
Hierarchical clustering analysis of urothelial carcinomas (UCs) based on array comparative genomic hybridization (CGH) data. See p. 40 for details.

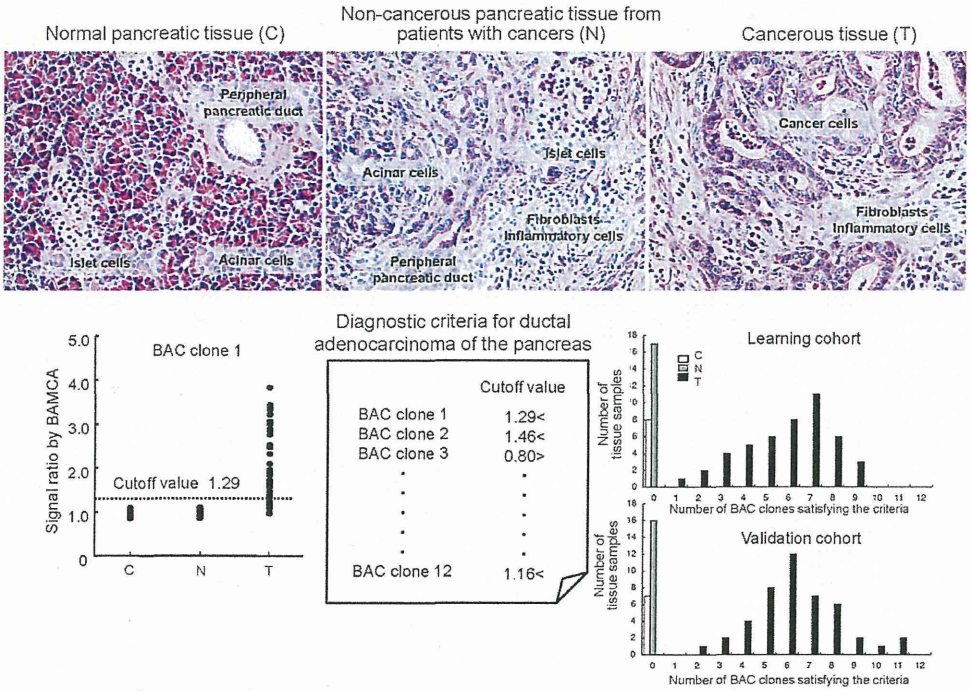
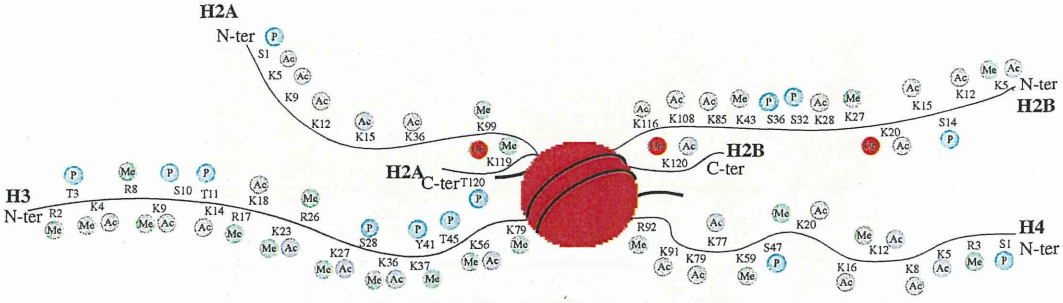


FIGURE 3.3
Diagnostic criteria based on DNA methylation profiles for ductal adenocarcinomas of the pancreas. See p. 42 for details.



Chromatin Modification	Residues modified	Function regulated
Acetylation	Lysine	Transcription, DNA repair, replication and condensation
Methylation (Lysine)	Lysine me1, me2, me3	Transcription, DNA repair
Methylation (Arginine)	Arginine-me1, Arginine-me2a Arginine-me2s	Transcription
Phosphorylation	Serine, Threonine, Tyrosine	Transcription, DNA repair and condensation
Ubiqutination	Lysine	Transcription, DNA repair
Sumoylation	Lysine	Transcription
ADP ribosylation	Glutamic	Transcription
Deimination	Arginine	Transcription
Proline isomerization	P-cis, P-trans	Transcription

Acetylation
Methylation
Phosphorylation
Ubiqutination

Different classes of histone modifications

FIGURE 4.1
Mammalian core histone modifications. See p. 55 for details.

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Chapter 10

Field Cancerization in Gastric Cancer

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Abstract

Frequent occurrence of multiple gastric cancers became clear as their endoscopic resection became popular. As an important mechanism, accumulation of aberrant DNA methylation of various genes, both driver and passenger genes, in "normal-appearing" gastric mucosae has been revealed.

Aberrant DNA methylation is induced by inflammation caused by *Helicobacter pylori* infection, a major causative agent of gastric cancers, and consists of permanent and temporary components that will remain and disappear, respectively, after discontinuance of *Helicobacter pylori* infection. Permanent methylation is almost absent in gastric mucosae of healthy individuals, and is present at low levels in gastric mucosae of patients with a single gastric cancer and at high levels in those of patients with multiple gastric cancers.

The presence of microsatellite instability in gastric cancers, mostly due to *MLH1* methylation, is also known to be associated with multiple gastric cancers. Accumulation of aberrant DNA methylation in gastric mucosae constitutes the major mechanism of field defect for gastric cancers.

10.1. Introduction

Gastric cancer is a major cause of cancer deaths world-wide [1]. Its incidence has markedly declined in the last century in the US and Europe [2], as shown by its crude mortality rate in Caucasian males at 33/100,000 in the early 20th century and at 5 in the late 20th century. However, gastric cancer incidence is still high in many Asian countries and Russia [1]. Histologically, gastric cancers are classified into intestinal and diffuse types, established by Lauren, in Western countries, and these two types largely correspond to differentiated and undifferentiated types in Japanese classification [3].

Early gastric cancers used to be treated by gastrectomy (total and partial gastrectomy), but now early intestinal-type gastric cancers are treated by endoscopic resection (ER),

including endoscopic mucosal resection and endoscopic submucosal dissection [4,5]. ER conserves a much larger part of gastric mucosae than partial gastrectomy, and brought a dramatic improvement of quality of life after treatment. At the same time, it became clear that metachronous gastric cancers occur in 8.5-14.0 % of patients after ER [6,7], which was much higher than the incidence after partial gastrectomy (1.8 – 2.4 %) [8,9]. Also, patients with multiple gastric cancers are known to have a higher risk of developing another gastric cancer than patients with a single gastric cancer [10,11]. The very high incidence of metachronous gastric cancers and high risk of gastric cancer patients with multiple gastric cancers strongly indicate that "field cancerization" or "field defect" is involved in gastric carcinogenesis. As its molecular basis, accumulation of aberrant DNA methylation in "normal-appearing" gastric mucosae is now recognized to be deeply involved [12,13]. The aberrant DNA methylation is induced by *H. pylori* infection, a major etiologic factor for gastric cancers [14], mainly through inflammation [12,15]. In this chapter, we will describe both epigenetic and genetic field defects, placing emphasis on the epigenetic field defect.

10.2. Conventional Changes Indicative of the Presence of Field Defect

The presence of individuals with high risk of gastric cancers has been known for a long time, and suggested that a field defect for gastric cancers is present. Conventionally, the presence of gastric atrophy and/or intestinal metaplasia in the stomach of gastric cancer patients has been well known [16]. Recently, much effort has been made to develop serum and other molecular markers to detect individuals with high risk of gastric cancers.

10.2.1. Histological Changes Associated with Increased Risk

Gastric atrophy is characterized by loss of gastric glandular cells, and appearance of fibrous tissue. Intestinal metaplasia is characterized by the appearance of intestinal-type epithelia in the stomach, and is considered as an abnormal differentiation. Gastric atrophy and intestinal metaplasia are produced as a result of chronic inflammation due to *H. pylori* infection. A prospective study involving 5,373 subjects for more than 10 years revealed that subjects with moderate atrophy at the baseline had a hazard ratio of 2.22 to develop gastric cancers [17].

10.2.2. Serum and Molecular Markers for Increased Risk

Most serum markers to detect individuals at high risk for gastric cancers are related to atrophy of gastric mucosae [18]. Among these, the pepsinogen concentrations are most widely used. Pepsinogen I is produced in chief and mucous neck cells, and Pepsinogen II is produced in not only chief and mucous neck cells but also in cardiac, pyloric, and duodenal Brunner gland cells. Since gastric atrophy advances from the pyloric glands towards the cardiac glands, the level of pepsinogen I and the ratio of pepsinogen I/II decrease with the

advancement [19]. If individuals are classified according to *H. pylori* infection status and the presence of atrophy, gastric cancer risk increases in the order of Group A (*H. pylori*-negative, atrophy-negative), Group B (*H. pylori*-positive, atrophy-negative), Group C (*H. pylori*-positive, atrophy-positive), and then Group D (*H. pylori*-negative, atrophy-positive) [20]. Groups B, C, and D have hazard ratios of 3.0, 3.7, and 32 compared with Group A [20,21].

Molecular risk markers that can be assessed in gastric biopsy specimens are still analyzed for research purpose, and their clinical usefulness has not been established. For example, expression of brain-type glycogen phosphorylase in non-cancerous gastric mucosae has been reported to be useful to predict occurrence of another gastric cancer [22]. *CDX2* is known as a master regulator of the intestinal phenotype, and is expressed in intestinal metaplasia. However, its expression has been shown to progressively decrease from intestinal metaplasia, dysplasia, and then cancers [23].

10.3. Epigenetic Field for Cancerization

Epigenetic mechanisms are deeply involved in gastric cancers because tumor-suppressor genes that can be inactivated by genetic or epigenetic mechanisms are more frequently inactivated by epigenetic mechanisms in gastric cancers [24]. Now, the deep involvement is underlain by a mechanism, induction of aberrant DNA methylation by *H. pylori* infection in gastric mucosae and formation of "epigenetic field defect".

10.3.1. Epigenetic Alterations

Epigenetic modifications are characterized by their inheritance upon somatic cell division, and represented by DNA methylation and histone modifications. They control development, cellular differentiation, and reprogramming by establishing gene usage patterns. DNA methylation of a promoter CpG island (CGI) is known to cause silencing of its downstream gene (figure 10-1) [25].

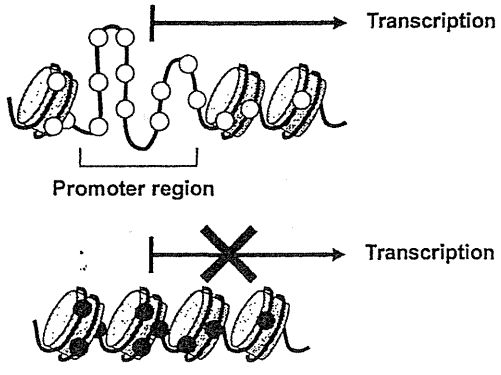

Figure 10.1. Silencing effect of DNA methylation of a CGI in promoter region. Most CGIs in gene promoter regions lack DNA methylation, and no nucleosome is formed. Therefore, transcription factors and RNA polymerase can have access to DNA, and the gene is transcribed. In contrast, if the CGI is methylated, a nucleosome is formed. Therefore, transcription machinery does not have access to DNA, and the gene is silenced.