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

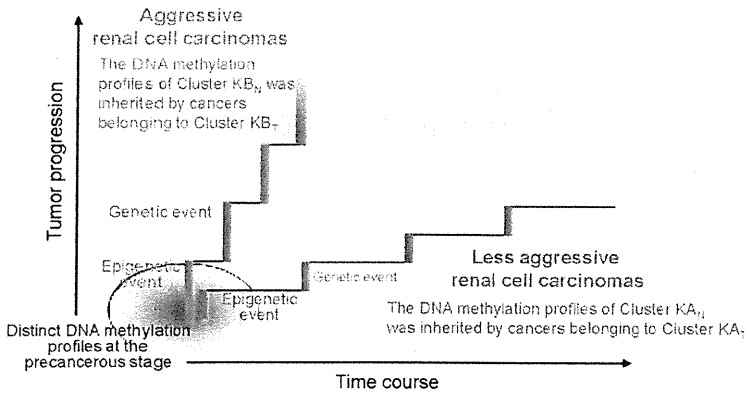

FIGURE 3.1
DNA methylation profiles in precancerous conditions and renal cell carcinomas (RCCs). Two-dimensional unsupervised hierarchical clustering analysis based on BAC array-based methylated CpG island amplification (BAMCA) data for non-cancerous renal tissue samples clustered patients with clear 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.

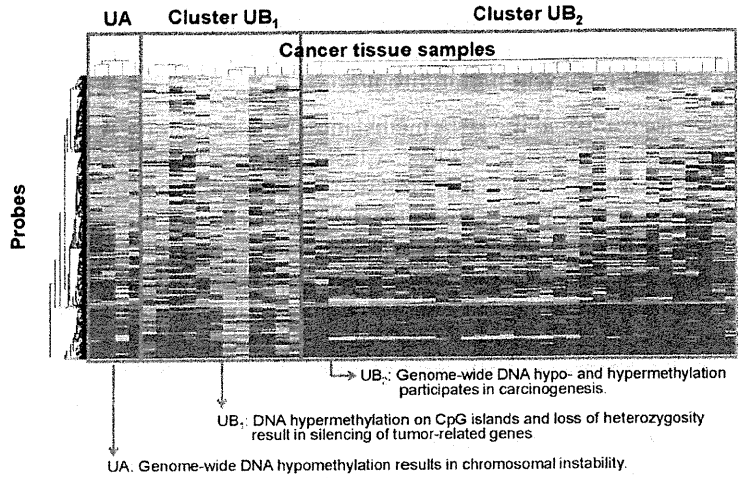

FIGURE 3.2
Hierarchical clustering analysis of urothelial carcinomas (UCs) based on array comparative genomic hybridization (CGH) data. In Cluster UA, copy number alterations, especially chromosomal gains, revealed by array CGH analysis, and DNA hypomethylation revealed by BAMCA were both accumulated 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).

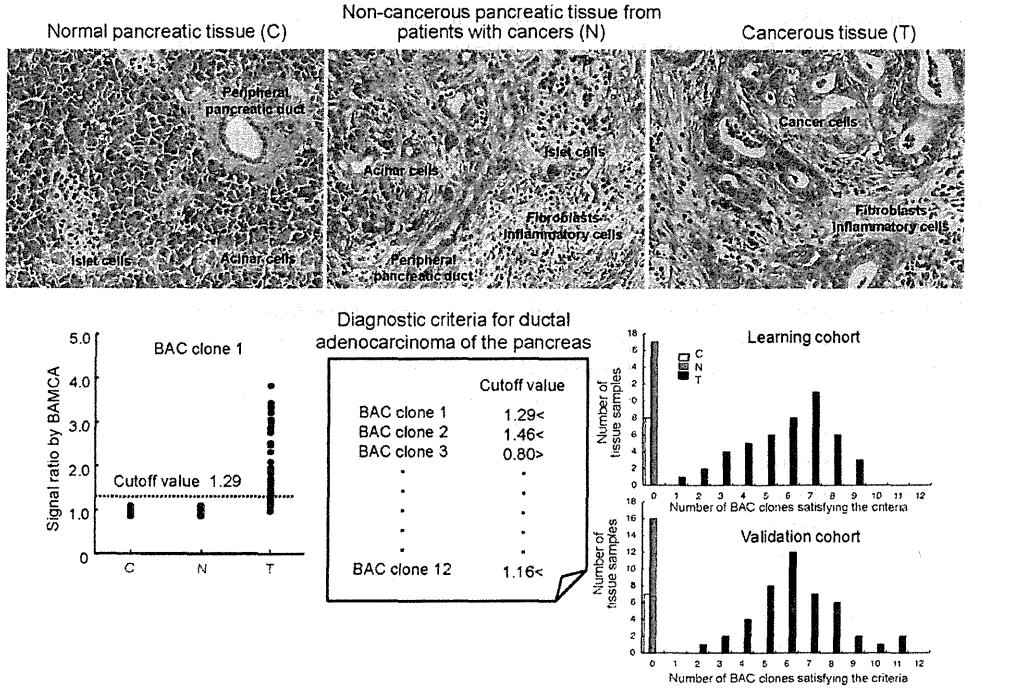

FIGURE 3.3

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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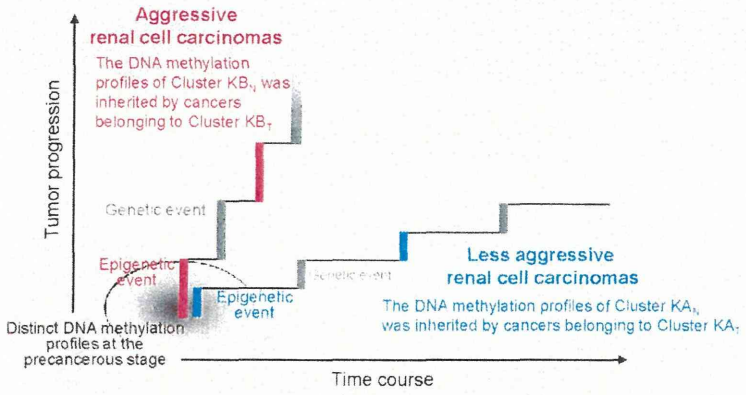


FIGURE 3.1
DNA methylation profiles in precancerous conditions and renal cell carcinomas (RCCs). See p. 39 for details.

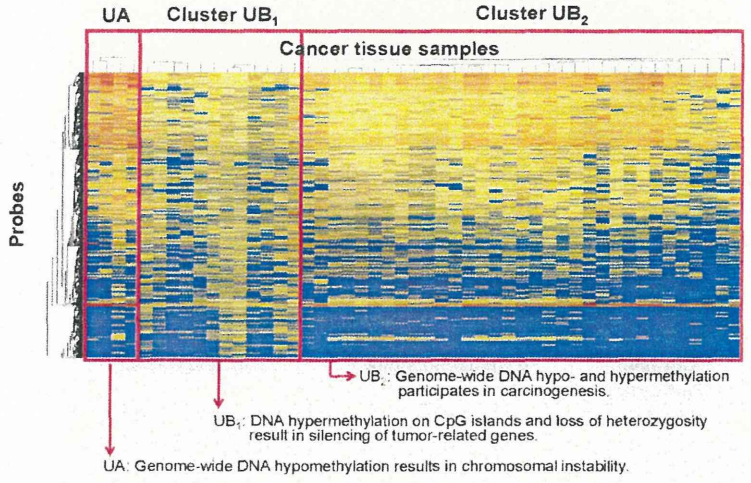


FIGURE 3.2
Hierarchical clustering analysis of urothelial carcinomas (UCs) based on array comparative genomic hybridization (CGH) data. See p. 40 for details.

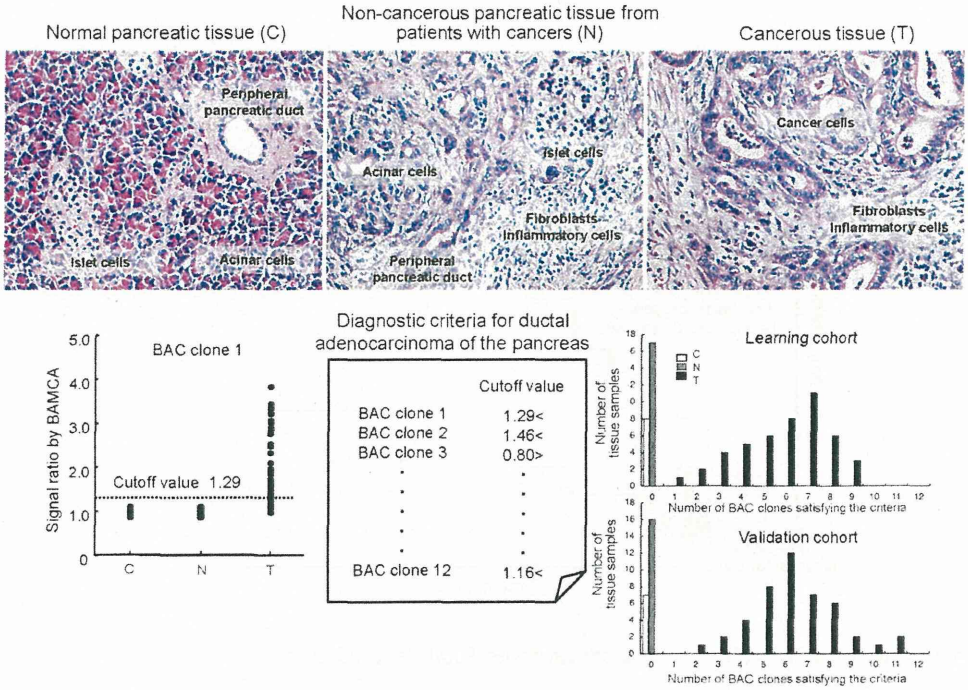
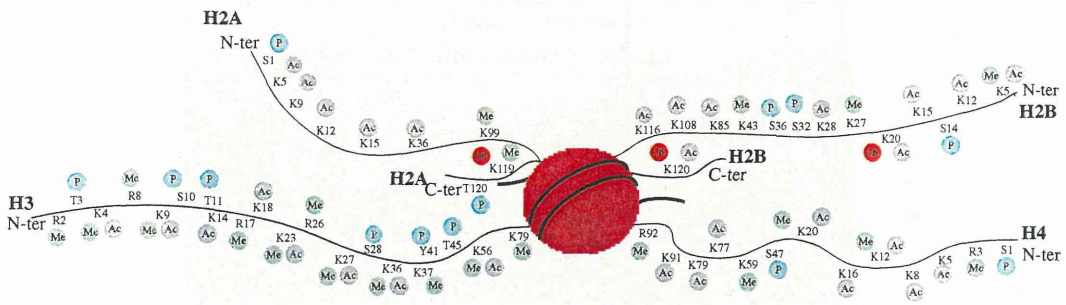


FIGURE 3.3
Diagnostic criteria based on DNA methylation profiles for ductal adenocarcinomas of the pancreas. See p. 42 for details.



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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SHORT COMMUNICATION

ANGPTL4 is a secreted tumor suppressor that inhibits angiogenesis

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Tumor suppressors with extracellular function are likely to have advantages as targets for cancer therapy, but few are known. Here, we focused on angiopoietin-like 4 (ANGPTL4), which is a secreted glycoprotein involved in lipoprotein metabolism and angiogenesis, is methylation-silenced in human cancers, but has unclear roles in cancer development and progression. We found a deletion mutation in its coiled-coil domain at its N-terminal in human gastric cancers, in addition to hypermethylation of the *ANGPTL4* promoter CpG islands. Forced expression of wild-type *ANGPTL4*, but not *ANGPTL4* with the deletion, at physiological levels markedly suppressed *in vivo* tumorigenicity and tumor angiogenesis, indicating that the latter caused the former. Tumor-derived ANGPTL4 suppressed *in vitro* vascular tube formation and proliferation of human umbilical vascular endothelial cells, partly due to suppression of ERK signaling. These showed that ANGPTL4 is a genetically and epigenetically inactivated secreted tumor suppressor that inhibits tumor angiogenesis.

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Keywords: epigenetics; angiogenesis; tumor suppressor; gastric cancer; DNA methylation

INTRODUCTION

Tumor-suppressor genes (TSGs) are somatically inactivated by genetic and/or epigenetic mechanisms.[1,2] Targeting TSGs for molecular target therapy has been attempted mainly for *p53*.[3,4] However, the attempts have not been easy, partly due to the fact that the *p53* gene product is neither a cell surface protein nor a typical enzyme.[5] Considering efficient delivery to targets, TSGs whose products function extracellularly as secreted proteins are likely to have advantages. So far, secreted frizzled-related proteins are known as secreted tumor suppressors,[6,7] but few others are known.

As a candidate, we previously identified that angiopoietin-like 4 (*ANGPTL4*), a member of the angiopoietin-like family, was silenced by aberrant DNA methylation of promoter CpG islands (CGIs) (methylation-silenced) in human cancers.[8,9] ANGPTL4 is a secreted glycoprotein, and is involved in lipoprotein metabolism through inhibition of lipoprotein lipase.[10] In contrast, the role of ANGPTL4 in angiogenesis remains controversial.[11–15] Likewise, its role in tumor formation also remains controversial—some reports suggesting its tumor-suppressive function[12,16,17] and others its oncogenic function.[18–20]

Here, we aimed to clarify the role of ANGPTL4 in cancer development and progression and also in tumor angiogenesis.

RESULTS AND DISCUSSION

Inactivation of *ANGPTL4* by epigenetic and genetic mechanisms in human gastric cancers

ANGPTL4 methylation was detected in 10 of 91 human gastric cancers (11%) by quantitative real-time methylation-specific PCR (Figure 1a). The mRNA and protein expression levels of ANGPTL4 in cancers with *ANGPTL4* methylation were significantly lower than those in cancers without methylation (Supplementary Figure S2). Methylation status did not have any association with clinicopathological features, but had a significant association with Epstein-Barr virus infection status and the presence of the CGI methylator phenotype[21] (Supplementary Figure S1 and Supplementary Table S1). In non-cancerous gastric mucosae of 71 gastric cancer patients and gastric mucosae of 58 healthy volunteers, the methylation level was also quantified. It was significantly higher in cancer patients than in healthy volunteers and in individuals with *H. pylori* infection than in those without (Figure 1b). This suggested the potential involvement of *ANGPTL4* methylation in the formation of an epigenetic field for cancerization, a predisposed normal-appearing tissue.[22]

ANGPTL4 mutation was then analyzed in 89 of the 91 gastric cancers (due to sample availability), and a somatic 21-bp deletion in exon 1 was identified in one specimen (cancer #217T) without *ANGPTL4* methylation (Figures 1c and d). ANGPLT4 consists of an N-terminal coiled-coil domain (CCD) and a C-terminal fibrinogen-like domain,[23,24] and the 21-bp deletion was located in the CCD (Supplementary Figure S3). The CCD is reported to be critical for regulation of the anti-angiogenic activity of ANGPTL4,[13] and the deletion here involved one of the two cysteine residues (Cys76 and Cys80) essential for the activity regulation by oligomerization.[25,26]

Loss of heterozygosity (LOH), which suggests the presence of a TSG,[27] was detected in 4 of 16 samples (25%) informative for a C/T polymorphism at the second position of codon 266. The locus of

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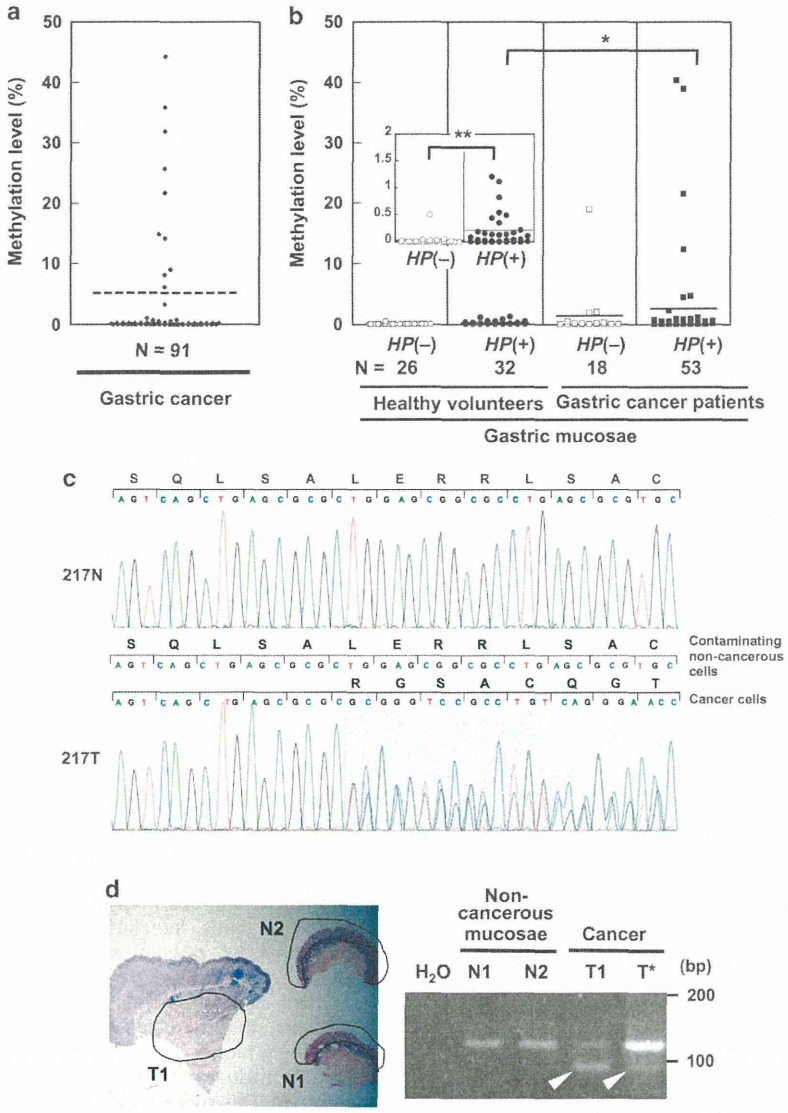

Figure 1. Aberrant methylation and a mutation of *ANGPTL4* in human gastric cancers, and methylation in non-cancerous gastric mucosae. (**a**) *ANGPTL4* methylation levels in gastric cancer specimens. Cancer samples were obtained from 91 gastric cancer patients undergoing gastrectomy with informed consents and approval by the institutional review boards. Some of the samples and methylation data were obtained from our previous study.[33] Quantitative real-time methylation-specific PCR was conducted with sodium bisulfite-treated DNA and primer sets specific to methylated and unmethylated sequences (Supplementary Table S2). Using a cutoff value of 6% (broken line), as in previous studies,[34–36] 10 cancer specimens were considered to have aberrant methylation. (**b**) *ANGPTL4* methylation levels in gastric mucosae of 58 healthy volunteers (30 male and 28 female; average age = 55 years) and 71 non-cancerous gastric mucosae of gastric cancer patients (50 male and 21 female; average age = 67 years) obtained by endoscopic biopsy of the antral region. *H. pylori* infection status was analyzed by a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan), rapid urease test (Otsuka, Tokushima, Japan) or culture test (Eiken, Tokyo, Japan). The methylation level in gastric mucosae was significantly higher in gastric cancer patients than in healthy volunteers. The mean methylation level is shown by a horizontal line. $^{*}P < 0.05$, $^{**}P < 0.01$ (the unpaired Welch's *t*-test, two-sided). (**c**) A deletion mutation in a human gastric cancer specimen. All the seven exons and splice donor/acceptor sites of *ANGPTL4* were amplified by PCR (Supplementary Table S2), and the PCR products were directly cycle sequenced. The sequences of a gastric cancer specimen (217T) and its corresponding non-cancerous tissue (217N) between nucleotides 385 and 423 are shown. A 21-bp deletion in exon 1 was detected (shown in the gray background). (**d**) Confirmation of the deletion mutation using DNA samples obtained from a single tissue section. A 117-bp region encompassing the deletion was amplified by PCR, and the deletion was detected as a PCR product with a shorter size (96 bp). DNA from the cancer (T1), but not that from non-cancerous areas (N1 and N2), yielded the shorter product (shown by arrows). T*, genomic DNA extracted from frozen tumor tissues. If LOH was present in T1, the band intensity ratio was expected to be 1:1 (wild type:deletion mutant) (fraction of cancer cells was pathologically assessed to be 61–67%). If LOH was not present, it was expected to be 2:1. The ratio observed was ~1:2, and LOH was considered to be present in T1.

ANGPTL4, 19p13.3, has been suggested to contain TSGs, due to frequent LOH of the region in several types of cancers, such as pancreatic and colon cancers.[28–30] In addition, two human gastric cancer cell lines (MKN28 and AGS) without *ANGPTL4* expression had methylation of its promoter, and their treatment with 5-aza-2′-deoxycytidine (5-aza-dC), a DNA methylation inhibitor,

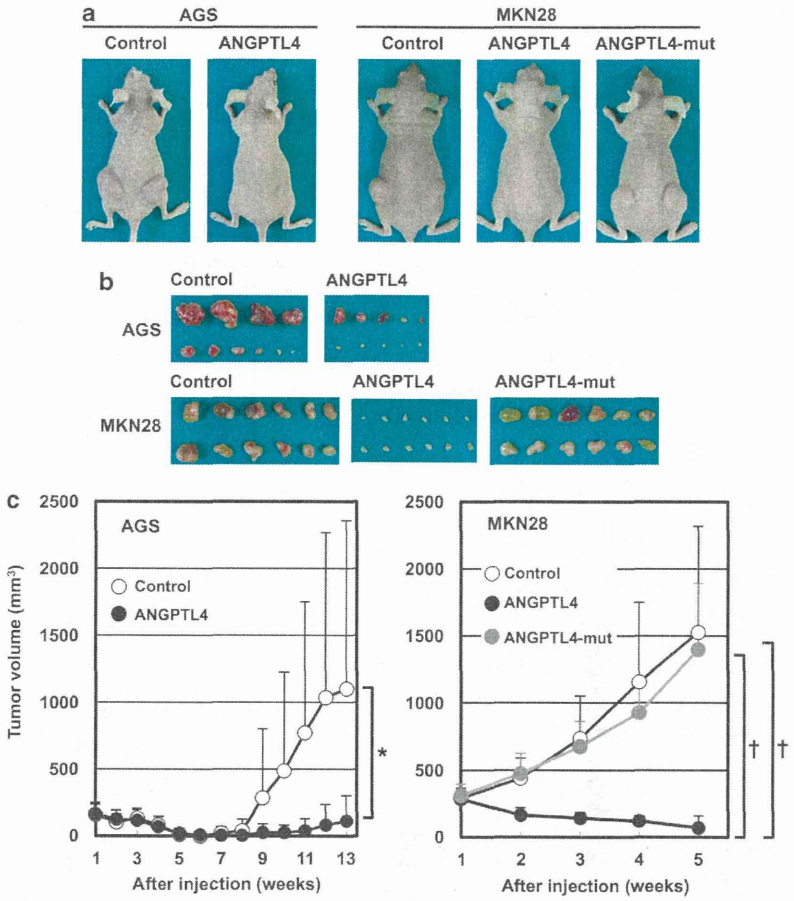

Figure 2. The effect of *ANGPTL4* and its mutant with the 21-bp deletion on tumor formation. The complementary DNA of wild-type *ANGPTL4*, its mutant with the deletion, and *EGFP* as a control were inserted into a mammalian expression vector pIRESpuro3 with the human cytomegalovirus immediate early promoter (Clontech, Mountain View, CA, USA). Individual vectors were transfected into MKN28 or AGS gastric cancer cell lines, and transfectants were selected with puromycin (0.3 μg/ml). Athymic nude mice (BALB/cAJc1-nu/nu, CLEA, Tokyo, Japan) were subcutaneously injected with cells (1×10^7 cells) mixed with an equal volume of Matrigel (BD Biosciences, San Diego, CA, USA). All the animal experiments were approved by the Committee for Ethics in Animal Experimentation, and conducted in accordance with the Guidelines for Animal Experiments of the National Cancer Center. (**a**) Representative photographs of transplanted tumors at 13 weeks (AGS) and 5 weeks (MKN28). *ANGPTL4* markedly suppressed tumor formation, while its mutant with the deletion lacked the activity. (**b**) Macroscopic views of the tumors resected at 13 weeks (AGS) and at 5 weeks (MKN28). Introduction of *ANGPTL4* markedly suppressed tumor sizes in both cell lines. The variable degree of suppression in AGS might have been due to the lower ANGPTL4 expression level (Supplementary Figure S5c). (**c**) Tumor growth curves after the injection. The volume of tumor (mm^3) was calculated by the formula: (length × $width^2$)/2. A tumor volume is shown as a mean ± s.d. ($N = 10$ in AGS and $N = 12$ in MKN28). $^*P < 0.05$, $^\dagger P < 0.001$ (Student's *t*-test).

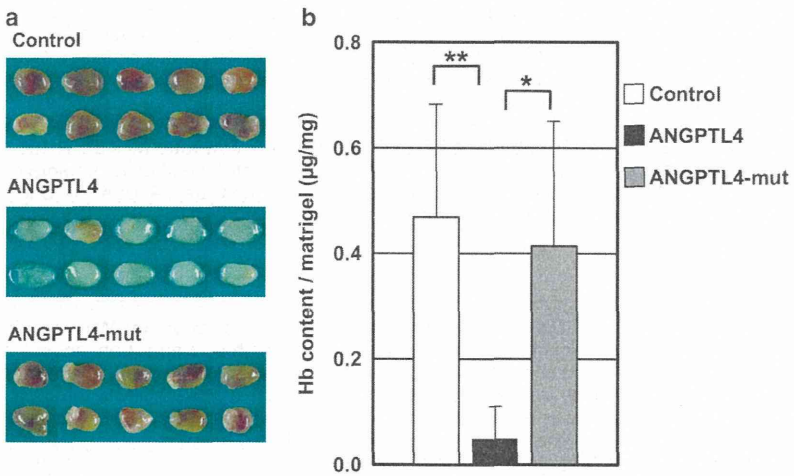

Figure 3. For caption see next page.