1 Introduction

DNA methylation, a covalent chemical modification resulting in addition of a methyl group at the carbon 5 position of the cytosine ring in CpG dinucleotides, is one of the most consistent epigenetic changes occurring in human cancers (Jones and Baylin 2002; Jones and Baylin 2007; Esteller 2008). DNA methyltransferases (DNMTs) transfer methyl groups from S-adenosylmethionine to cytosines. DNA methylation normally promotes a highly condensed heterochromatin structure associated with deacetylation of histones H3 and H4, loss of histone H3, lysine 4 (H3K4) methylation, and gain of H3K9 and H3K27 methylation. DNA methylation is a stable modification inherited throughout successive cell divisions, and is essential for X-chromosome inactivation, genome imprinting, silencing of transposons and other parasitic elements, and proper expression of genes (Cedar and Bergman 2009; Delcuve et al. 2009).

Human cancer cells show a drastic change in DNA methylation status, i.e., overall DNA hypomethylation and regional DNA hypermethylation. DNA hypomethylation induces chromosomal instability through decondensation of heterochromatin and enhancement of chromosomal recombination during carcinogenesis. DNA hypermethylation of CpG islands around the promoter regions silences tumor-supressor genes. Translational epigenetics have come of age (Issa and Kantarjian 2009), and empirical analysis of DNA methylation status in clinical tissue samples in connection with the clinicopathological diversity of human cancers is assuming increasing importance for the diagnosis, prevention, and therapy of cancers (Kanai and Hirohashi 2007; Kanai 2008, 2009).

2 DNA Methylation Alterations During Multistage Hepatocarcinogenesis

Although in the 1990s various genetic alterations were revealed using classical analytical techniques such as Southern blotting, especially in hepatocellular carcinomas (HCCs) that were poorly differentiated, large in size, and associated with metastasis, only a few of the molecular events occurring in the earlier stage of hepatocarcinogenesis were known. Since DNA methylation alterations may be correlated with chromosomal instability, we examined DNA methylation status on chromosome 16, which is known to be a hot spot for loss of heterozygosity (LOH) in HCCs, using Southern blotting with a DNA methylation-sensitive restriction enzyme. DNA methylation alterations at multiple loci on chromosome 16, in comparison with normal liver tissue samples obtained from patients without HCCs, were frequently revealed even in samples of non-cancerous liver tissue showing chronic hepatitis or liver cirrhosis, which are widely considered to be precancerous conditions, indicating that DNA methylation alterations are a very early event during multistage

hepatocarcinogenesis. This was one of the earliest reports of DNA methylation alterations at the precancerous stage (Kanai et al. 1996).

Since the molecular weight of DNA fragments digested using a DNA methylation-sensitive restriction enzyme was higher in HCCs than in precancerous conditions, and the intensity of larger-sized bands appeared to be enhanced in the HCCs, the numbers of methylated CpG dinucleotides and cells showing DNA hypermethylation may increase progressively as precancerous conditions develop into HCCs. The incidence of DNA hypermethylation on chromosome 16 was significantly correlated with higher histological grade, portal vein involvement, and intrahepatic metastasis of HCCs. The presence of DNA methylation alterations in both precancerous conditions and progressed HCCs suggests that DNA methylation alterations in the precancerous stage may rapidly generate more malignant cancers (Kanai et al. 1996).

The E-cadherin tumor-suppressor gene is located on 16q22.1 near the hot spots for both DNA hypermethylation and LOH in HCCs. E-cadherin acts as a Ca^{2+}-dependent cell–cell adhesion molecule in the adherens junctions of epithelial cells (Hirohashi and Kanai 2003). Significant correlations between reduced expression of E-cadherin and poor prognosis have been reported in patients with cancers. We have demonstrated that the promoter region of the E-cadherin gene shows DNA methylation in human cancer cell lines lacking E-cadherin expression, and that E-cadherin expression is induced after treatment with the DNMT inhibitor 5-azacytidine in such cell lines (Yoshiura et al. 1995). At that time, only two genes, RB and VHL, were known to be tumor-suppressor genes silenced by DNA methylation. On the basis of our data, the E-cadherin gene became chronologically the third example of a tumor-suppressor gene silenced by DNA methylation.

DNA hypermethylation around the promoter region of the E-cadherin gene has been detected even in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis. Heterogeneous E-cadherin expression in such non-cancerous liver tissue, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity, might be due, at least partly, to DNA hypermethylation (Kanai et al. 1997). In HCCs, we found a significant correlation between DNA hypermethylation around the promoter region and reduced expression of E-cadherin (Kanai et al. 1997). This was the first demonstration of a significant correlation between DNA hypermethylation and reduced expression in a cohort of clinical tissue samples. DNA hypermethylation around the promoter region may participate in hepatocarcinogenesis through reduction of E-cadherin expression, resulting in loss of intercellular adhesiveness and destruction of tissue morphology.

The HIC (hypermethylated-in-cancer)-1 gene at the D17S5 locus (17q13.3) was the first tumor-suppressor gene to be identified in commonly methylated chromosomal loci in human cancers. We showed that DNA hypermethylation at the D17S5 locus was frequently detectable in non-cancerous liver tissue showing chronic hepatitis or cirrhosis (Kanai et al. 1999). The incidence and degree of DNA methylation at the D17S5 locus increased progressively as precancerous conditions developed

into HCCs. The expression level of HIC-1 mRNA in non-cancerous liver tissue showing chronic hepatitis or cirrhosis was significantly lower than that in normal liver tissue, and was further decreased in HCCs (Kanai et al. 1999).

The list of tumor-related genes whose expression levels are altered due to DNA hypo- or hypermethylation during hepatocarcinogenesis has recently been increasing. Silencing of cell cycle regulators such as p16 (Matsuda et al. 1999) and p15 (Wong et al. 2000), proapoptotic proteins such as TMS1/ASC (Kubo et al. 2004), matrix metalloproteinase inhibitor TIMP3 (Yu et al. 2002) and DNA repair protein MGMT (Matsukura et al. 2003), and multifunctional tumor-suppressor proteins such as RASSF1A (Schagdarsurengin et al. 2003) and 14-3-3-σ(Iwata et al. 2000), due to DNA hypermethylation has been reported in HCCs. DNA methylation of the cytokine mediator gene SOCS-1 (Okochi et al. 2003) has attracted attention because it may activate the JAK/STAT signaling pathway and mediate the molecular linkage between inflammation and hepatocarcinogenesis.

Microdissection techniques and PCR using microsatellite markers have been developed for detecting LOH in small numbers of cells from paraffin-embedded tissue samples. LOH has been reported even in microdissected specimens from tiny precancerous lesions in several organs. In order to re-examine whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis, we examined 308 microdissected specimens obtained from lobules, pseudo-lobules, and regenerative nodules in non-cancerous liver tissue from patients with HCCs, and the HCCs themselves, for LOH and microsatellite instability (MSI) using 39 microsatellite markers. In addition, using methylation-specific PCR and combined bisulfite restriction enzyme analysis, we also studied the DNA methylation status of C-type CpG islands of the p16, THBS-1, and human hMLH1 genes, and MINT 1, 2, 12, 25, and 31 clones, which are known to be methylated in a cancer-specific, but not age-dependent manner (Kondo et al. 2000). The low incidence of microsatellite instability in HCCs was compatible with absence of silencing of the hMLH1 gene by DNA hypermethylation during hepatocarcinogenesis (Kondo et al. 1999, 2000). In non-cancerous liver tissue showing chronic hepatitis, LOH for at least one marker was found in 20% of informative microdissected specimens. In non-cancerous liver tissue showing cirrhosis, LOH for at least one marker was found in 15% of informative microdissected specimens. LOH was never detected in normal liver tissue from patients without HCCs or in non-cancerous liver tissue showing no remarkable histological findings from patients with HCCs. Although no degree of DNA methylation of any of the examined CpG islands was ever detected in normal liver tissue from patients without HCCs, DNA hypermethylation was found on at least one CpG island in 58% of microdissected specimens of non-cancerous liver tissue showing no remarkable histological features obtained from patients with HCCs in which LOH was never detected (Kondo et al. 2000). The incidence of DNA hypermethylation on CpG islands overwhelmed that of LOH at all stages of chronic hepatitis, liver cirrhosis, and HCC. Thus, aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis, even when examined using PCR–LOH analysis and microdissection techniques.

3 Abnormalities of DNMTs During Hepatocarcinogenesis

3.1 Overexpression of DNMT1

With respect to the molecular backgrounds of DNA methylation alterations, we focused on abnormalities of DNMTs during hepatocarcinogenesis. The major DNMT, DNMT1, shows a preference for hemimethylated over unmethylated substrates in vitro, and targets replication foci by binding to proliferating cell nuclear antigen (PCNA) (Hermann et al. 2004). Thus, DNMT1 has been recognized as a "maintenance" DNMT that allows copying of the DNA methylation pattern on the parental strand to the newly synthesized daughter DNA strand. Mutational inactivation of the DNMT1 gene that can potentially cause genome-wide alterations of DNA methylation was never detected in HCCs (Kanai et al. 2003).

On the other hand, levels of DNMT1 mRNA expression are significantly higher in samples of non-cancerous liver tissue showing chronic hepatitis or cirrhosis than in normal liver tissue, and are even higher in HCCs (Sun et al. 1997; Saito et al. 2001). The incidence of DNMT1 overexpression in HCCs is significantly correlated with poorer tumor differentiation and portal vein involvement (Saito et al. 2003). Moreover, the recurrence-free and overall survival rates of patients with HCCs showing DNMT1 overexpression are significantly lower than those of patients with HCCs that do not (Saito et al. 2003).

3.2 Splicing Alteration of DNMT3b and DNA Hypomethylation in Pericentromeric Satellite Regions

Dnmt3b is required for DNA methylation of pericentromeric satellite regions in early mouse embryos (Okano et al. 1999). DNA hypomethylation in pericentromeric satellite regions is known to result in centromeric decondensation and enhanced chromosome recombination. Germline mutations of the DNMT3b gene have been reported in patients with immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, a rare recessive autosomal disorder characterized by DNA hypomethylation of pericentromeric satellite regions (Hansen et al. 1999). In HCCs, DNA hypomethylation of these regions is correlated with copy number alterations on chromosome 1, where satellite regions are rich (Wong et al. 2001).

The major splice variant of DNMT3b in normal liver tissue samples is DNMT3b3, which possesses the conserved catalytic domains. DNMT3b4, on the other hand, lacks the conserved catalytic domains, although it retains the N-terminal domain required for targeting to heterochromatin sites. Samples of normal liver tissue show only a trace level of DNMT3b4 expression. The levels of DNMT3b4 mRNA expression and the ratio of DNMT3b4 mRNA to DNMT3b3 in samples of non-cancerous liver tissue obtained from patients with HCCs, and in HCCs themselves, were significantly correlated with the degree of DNA hypomethylation in

pericentromeric satellite regions (Saito et al. 2002). DNA demethylation on satellite 2 was observed in DNMT3b4-transfected human epithelial 293 cells (Saito et al. 2002). Since DNMT3b4 lacking DNMT activity competes with DNMT3b3 for targeting to pericentromeric satellite regions, DNMT3b4 overexpression may lead to chromosomal instability through induction of DNA hypomethylation in such regions.

Furthermore, the growth rate of DNMT3b4 transfectants was approximately double that of mock-transfectants soon after the introduction of DNMT3b4, when chromosomal instability may not yet have accumulated. STAT 1, which acts as an effector of interferon signaling, and the genes implicated in interferon signaling, were upregulated in DNMT3b4 transfectants relative to mock-transfectants (Kanai et al. 2004). It had been reported previously that inhibition of DNA methylation in cultured human cancer cells by 5-aza-2′-deoxycytidine induces a set of genes implicated in interferon signaling, primarily via overexpression of STAT1, 2, and 3 (Karpf et al. 1999). In cancer cells, DNMT3b may act to maintain the DNA methylation status of not only pericentromeric satellite regions, but also specific genes, probably in cooperation with DNMT1, and this may explain why inhibition of DNMT3b activity by induction of DNMT3b4 produced a similar result to the general inhibition of DNA methylation obtained with 5-aza-2′-deoxycytidine. Overexpression of DNMT3b4 plays a role in multistage carcinogenesis not only by inducing chromosomal instability, but also by affecting the expression of specific genes.

4 Altered Expression of Methyl-CpG Binding Proteins (MBDs)

MBDs, such as MeCP2, MBD1, MBD2, and MBD3, bind to methylated CpG dinucleotides, and their transcriptional repression domain recruits a transcriptional co-repressor complex containing histone deacetylases (Esteller 2008). Although many researchers have focused on cross-talk between DNA methylation and histone modification, abnormalities of MBDs in human cancers do not seem to have attracted much attention. The expression level of MeCP2 mRNA in HCCs with portal vein involvement is significantly lower than that in HCCs without such involvement, suggesting that reduced expression of MeCP2 may be associated with malignant progression of HCCs (Saito et al. 2001). Reduced expression of MBD2 mRNA has been observed in HCCs, suggesting that this may be associated with a particular step in human carcinogenesis (Saito et al. 2001). Unlike other MBDs recruiting histone deacetylase complexes, MBD4 is thought to act as a thymine DNA glycosylase, repairing G:T or G:U mismatches at CpG sites. The expression level of MBD4 mRNA in HCCs is significantly lower than that in the corresponding non-cancerous liver tissue and is significantly correlated with poorer tumor differentiation and portal vein involvement (Saito et al. 2001). Reduced MBD4 expression may result in frequent C-T transitions in tumor-suppressor genes.

5 Genome-Wide DNA Methylation Analysis

5.1 DNA Methylation Alterations During Multistage Hepatocarcinogenesis Occur in a Genome-Wide Manner

Recently, we have employed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) (Inazawa et al. 2004) for DNA methylation analysis on a genomic-wide scale. Many researchers in this field use promoter arrays to identify genes that are methylated in cancer cells. However, the promoter regions of specific genes are not the only target of DNA methylation alterations in human cancers. Genomic regions in which DNA hypomethylation affects chromosomal instability may not be contained in promoter arrays. Moreover, aberrant DNA methylation of large chromosome regions, which are regulated in a coordinated manner in human cancers due to a process of long-range epigenetic silencing, has recently attracted attention (Frigola et al. 2006). Therefore, we used a BAC array that may be suitable for overviewing the DNA methylation status of individual large regions among all chromosomes. In fact, using BAMCA, we have shown successfully that particular DNA methylation profiles in the kidney at the precancerous stage are inherited by clear cell renal cell carcinomas developing in individual patients, and that these determine the aggressiveness of the tumors and patient outcome (Arai et al. 2009a). Diagnostic indicators for urothelial carcinomas have also been established using BAMCA (Nishiyama et al. 2009).

In samples of non-cancerous liver tissue obtained from patients with HCCs, many BAC clones showed DNA hypo- or hypermethylation (Panel N of Fig. 8.1a) in comparison with normal liver tissue from patients without HCCs (Panel C of Fig. 8.1a). Patients showing DNA hypo- or hypermethylation on more BAC clones in their non-cancerous liver tissue samples frequently developed metachronous or recurrent HCCs after hepatectomy, whereas patients showing DNA hypo- or hypermethylation on fewer BAC clones in their non-cancerous liver tissue samples rarely did so, suggesting that DNA methylation alterations at the precancerous stage may not occur randomly but are prone to development of more malignant HCCs potentially through the induction of chromosomal instability and silencing of tumor-suppressor genes (Arai et al. 2009b). In HCCs themselves, more BAC clones showed DNA hypo- or hypermethylation, the degree of which was further increased (Panel T of Fig. 8.1a) in comparison with non-cancerous liver tissue obtained from the same patients (Arai et al. 2009b).

There were no significant differences in the number of BAC clones showing DNA hypo- or hypermethylation in samples of normal liver tissue from male and female patients without HCCs, and in non-cancerous and cancerous liver tissue from male and female patients with HCCs, respectively. Wilcoxon test identified BAC clones in which DNA methylation status differed significantly between hepatitis B virus (HBV)- and hepatitis C virus (HCV)-positive patients with HCCs in both cancerous

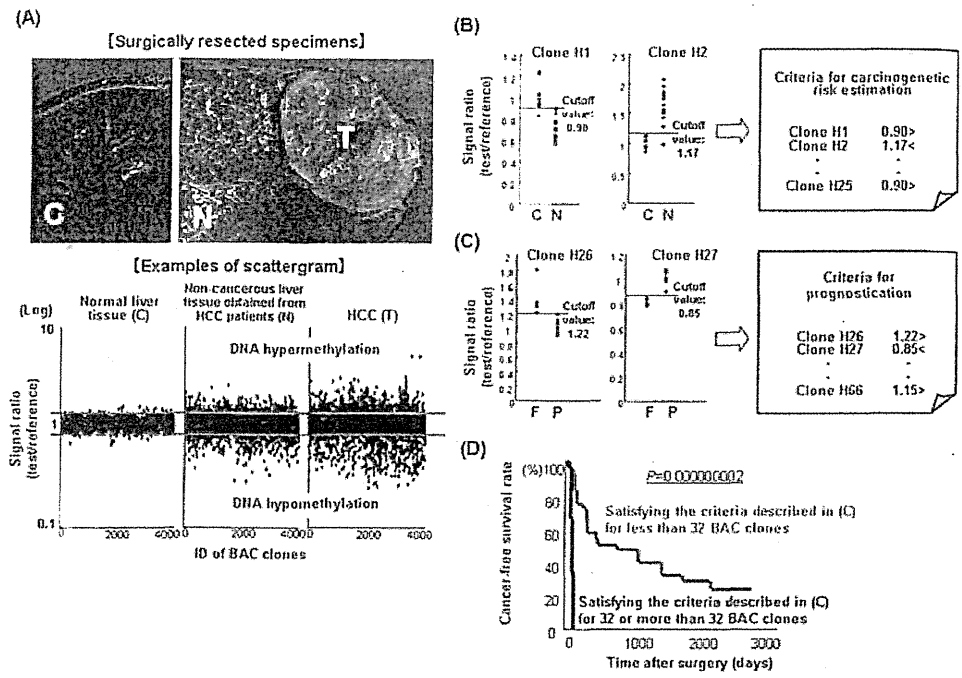

Fig. 8.1 Carcinogenetic risk estimation and prognostication based on genome-wide DNA methylation profiling. (a) Results of bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA). In non-cancerous liver tissue obtained from patients with HCCs (N), many BAC clones showed DNA hypo- or hypermethylation in comparison with normal liver tissue from patients without HCCs (C). In HCCs themselves (T), more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation was further increased. (b) Cutoff values for each of the 25 BAC clones selected using a bioinformatics approach were set to discriminate non-cancerous liver tissue obtained from patients with HCCs (N) from normal liver tissue (C), and the criteria for carcinogenetic risk estimation were established using the 25 BAC clones. (c) Cutoff values for each of the 41 BAC clones selected using a bioinformatics approach were set to discriminate the poorer outcome group (P) from the favorable-outcome group (F), and the criteria for prognostication were established using the 41 BAC clones. (d) The cancer-free survival rate of patients with HCCs in the validation cohort satisfying the criteria for 32 or more BAC clones was significantly lower than that of patients with HCCs satisfying the criteria for less than 32 BAC clones

and non-cancerous liver tissue. The DNA methylation status of such BAC clones may reflect the HBV- and HCV-specific molecular mechanisms inducing DNA methylation alterations.

5.2 *Carcinogenetic Risk Estimation Based on DNA Methylation Profiles*

The effectiveness of surgical resection for HCC is limited, unless the disease is diagnosed early at the asymptomatic stage. Therefore, surveillance at the precancerous stage will become a priority. To reveal the baseline liver histology, microscopic

examination of liver biopsy specimens is performed in patients with HBV or HCV infection prior to interferon therapy. Therefore, carcinogenetic risk estimation using such liver biopsy specimens would be advantageous for close follow-up of patients who are at high-risk of HCC development. 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.

To estimate the degree of carcinogenetic risk based on DNA methylation profiles, we omitted potentially insignificant BAC clones associated only with inflammation and/or fibrosis and focused on BAC clones for which DNA methylation status was inherited by HCCs from the precancerous stage, i.e., BAC clones in Groups I, II, III, and IV. Group I: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue and the average signal ratio of HCCs was even higher than that of non-cancerous liver tissue obtained from patients with HCCs. Group II: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue, and the average signal ratio of HCCs did not differ from that of non-cancerous liver tissue obtained from patients with HCCs. Group III: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue, and the average signal ratio of HCCs was even lower than that of non-cancerous liver tissue obtained from patients with HCCs. Group IV: BAC clones in which the average signal ratio of non-cancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue, and the average signal ratio of HCCs did not differ from that of non-cancerous liver tissue obtained from patients with HCCs. From the BAC clones of Groups I, II, III, and IV, in which the DNA methylation status was inherited by HCCs from non-cancerous liver tissue, the top 25 BAC clones for which DNA methylation status was able to discriminate non-cancerous liver tissue from patients with HCCs in the learning cohort from normal liver tissue with sufficient sensitivity and specificity were identified using a bioinformatics approach (Arai et al. 2009b). By 2D hierarchical clustering analysis using these 25 BAC clones, normal liver tissue and non-cancerous liver tissue obtained from patients with HCCs in the learning cohort were successfully subclassified into different subclasses without any error. We set the cutoff values for each of the 25 BAC clones to discriminate non-cancerous liver tissue obtained from patients with HCCs in the learning cohort from normal liver tissue, and established the criteria for carcinogenetic risk estimation using the 25 BAC clones (Fig. 8.1b). Based on these criteria, both the sensitivity and specificity for diagnosis of non-cancerous liver tissue samples obtained from patients with HCCs in the learning cohort, as being at high-risk of carcinogenesis, were 100% (Arai et al. 2009b). Our criteria enabled diagnosis of additional non-cancerous liver tissue samples obtained from patients with HCCs in the validation cohort as being at high-risk of carcinogenesis with a sensitivity and specificity of 96% (Arai et al. 2009b).

The number of BAC clones satisfying the criteria in non-cancerous liver tissue samples showing chronic hepatitis obtained from patients with HCCs was not significantly different from that in non-cancerous liver tissue samples showing cirrhosis obtained from patients with HCCs. In addition, the average number of BAC clones satisfying the criteria was significantly lower in samples of liver tissue obtained from patients who were infected with HBV or HCV, but who had never developed HCCs, than that in non-cancerous liver tissue samples obtained from patients with HCCs. Our criteria not only discriminated non-cancerous liver tissue obtained from patients with HCCs from normal liver tissue, but may also be applicable for classifying liver tissue obtained from patients who are followed up because of HBV or HCV infection, chronic hepatitis, or cirrhosis into that which may generate HCCs and that which will not (Arai et al. 2009b). We intend to validate the reliability of such risk estimation prospectively using liver biopsy specimens obtained prior to interferon therapy from a large cohort of patients.

5.3 Prognostication of Patients with HCCs Based on DNA Methylation Profiles

To establish criteria for prognostication of patients with HCCs, in the learning cohort, HCC samples obtained from patients who had survived more than 4 years after hepatectomy and HCC samples obtained from patients who had suffered recurrence within 6 months and died within a year after hepatectomy were defined as a favorable-outcome group and a poor-outcome group, respectively. Wilcoxon test revealed that the signal ratios of 41 BAC clones differed significantly between the two groups (Arai et al. 2009b). By 2D hierarchical clustering analysis using the 41 BAC clones, HCCs in two groups were subclassified into different subclasses without any error. We set cutoff values for each of the 41 BAC clones to discriminate the poor-outcome group in the learning cohort from the favorable-outcome group, and established criteria for prognostication using the 41 BAC clones (Fig. 8.1c). Multivariate analysis revealed that satisfying the criteria for 32 or more BAC clones was a predictor of recurrence, and was independent of parameters that are already known to have prognostic impact, such as histological differentiation, portal vein tumor thrombi, intrahepatic metastasis, and multicentricity (Arai et al. 2009b).

To confirm these criteria, additional HCC samples were analyzed by BAMCA as a validation study. The cancer-free (Fig. 8.1d) and overall survival rates of patients with HCCs satisfying the criteria for 32 or more BAC clones were significantly lower than those of patients with HCCs satisfying the criteria for less than 32 BAC clones. Such prognostication using liver biopsy specimens obtained before transarterial embolization and radiofrequency ablation may be advantageous even for patients who undergo such therapies. The reliability of such prognostication needs to be validated again prospectively using surgically resected specimens or biopsy specimens.

6 Perspective

DNA methylation alterations associated with DNA methyltransferase abnormalities, such as overexpression of DNMT1 and splicing alterations of DNMT3b, may participate in multistage hepatocarcinogenesis from the precancerous stage to the malignant progression stage. DNA methylation alterations at the precancerous stage may rapidly generate more malignant HCCs. Genome-wide DNA methylation profiling can provide optimal indicators for carcinogenetic risk estimation and prognostication using surgically resected specimens or liver biopsy specimens. Elucidation of the molecular backgrounds of DNA methylation alterations in chronic liver disease may provide clues for epigenetic prevention and therapy of HCCs.

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ORIGINAL ARTICLE

Integrated analysis of cancer-related pathways affected by genetic and epigenetic alterations in gastric cancer

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Abstract

Background The profiles of genetic and epigenetic alterations in cancer-related pathways are considered to be useful for selection of patients likely to respond to specific drugs, including molecular-targeted and epigenetic drugs. In this study, we aimed to characterize such profiles in gastric cancers (GCs).

Methods Genetic alterations of 55 cancer-related genes were analyzed by a benchtop next-generation sequencer. DNA methylation statuses were analyzed by a bead array with 485,512 probes.

Results The WNT pathway was activated by mutations of *CTNNB1* in 2 GCs and potentially by aberrant methylation of its negative regulators, such as *DKK3*, *NKD1*, and *SFRP1*, in 49 GCs. The AKT/mTOR pathway was activated by mutations of *PIK3CA* and *PTPN11* in 4 GCs. The MAPK pathway was activated by mutations and gene amplifications of *ERBB2*, *FLT3*, and *KRAS* in 11 GCs. Cell-cycle regulation was affected by aberrant methylation of *CDKN2A* and *CHFR* in 13 GCs. Mismatch repair was affected by a mutation of *MLH1* in 1 GC and by aberrant methylation of *MLH1* in 2 GCs. The p53 pathway was inactivated by mutations of *TP53* in 19 GCs and potentially by aberrant methylation of its downstream genes in 38 GCs. Cell adhesion was affected by mutations of *CDH1* in 2 GCs.

Conclusions Genes involved in cancer-related pathways were more frequently affected by epigenetic alterations than by genetic alterations. The profiles of genetic and epigenetic alterations are expected to be useful for selection of the patients who are likely to benefit from specific drugs.

Keywords Epigenetics · DNA methylation · Genetic alterations · Gastric cancer · Cancer-related pathway

Abbreviations

GC	Gastric cancer
CGI	CpG island
PGM	Personal Genome Machine
TSS	Transcription start site
CIMP	CpG island methylator phenotype

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Introduction

Genetic and epigenetic alterations are involved in gastric cancer (GC) development and progression by activating growth-promoting pathways and inactivating tumor-suppressive pathways. Genetic alterations consist of point mutations, small insertions and deletions, and chromosomal gains and losses, including gene amplifications. Among epigenetic alterations, aberrant DNA methylation of a promoter CpG island (CGI) is known to repress transcription of its downstream gene consistently, and a tumor suppressor gene can be permanently inactivated by this mechanism [1]. In gastric carcinogenesis, the contribution of aberrant methylation is known to be large because *Helicobacter pylori* (*H. pylori*) infection causes aberrant methylation [2].

Growth-promoting pathways activated in GCs include the WNT, AKT/mTOR, and mitogen-activated protein kinase (MAPK) pathways. These pathways can be activated not only by activating mutations of oncogenes but also by inactivation of their negative regulators. The WNT pathway can be activated by activating mutations of *CTNNB1* (β-catenin) and by inactivation of its negative regulators, such as *SFRP1* [3], *DKK3* [4], and *WIF1* [5]. The AKT/mTOR pathway can be activated by activating mutations of *PIK3CA* and by inactivation of its negative regulators, such as *PTEN* and *THEM4* [6]. The MAPK pathway can be activated by activating mutations and gene amplifications of *ERBB2* and *KRAS* and by inactivation of its negative regulators, such as *RASSF1A* [7].

Tumor-suppressive pathways inactivated in GCs include the RB/p16 pathway (cell-cycle regulation), mismatch repair, the p53 pathway, and cell adhesion. The RB/p16 pathway can be inactivated by mutations, losses, and aberrant DNA methylation of *RB* and *p16* [8], and by inactivation of a cell-cycle checkpoint gene, *CHFR* [9]. Mismatch repair can be affected by mutations, losses, and aberrant methylation of mismatch repair genes, such as *MLH1* and *MSH2* [10]. The p53 pathway can be inactivated by mutations and losses of *TP53* and potentially by inactivation of multiple members of its downstream genes, including *IGFBP7*, *MIR34b/c*, and *THBS1* [11]. Cell adhesion can be affected by mutations, losses, and aberrant methylation of *CDH1* and is known to be important for diffuse-type histology [12–14].

Analysis of these genetic and epigenetic alterations is important for selection of patients who are likely to respond to specific molecular-targeted drugs, such as trastuzumab (*ERBB2* amplifications) [15] and everolimus (*PIK3CA* mutations) [16]. Also, the profiles of the alterations are expected to enable selection of patients who are likely to benefit from epigenetic drugs [17–20]. Nevertheless, until recently, these genetic and epigenetic alterations have been analyzed only individually because technologies for their comprehensive analysis have not been available at a reasonable cost. Now, point mutations and gene amplifications of a large number of target genes can be analyzed by benchtop next-generation sequencers [21], and a comprehensive DNA methylation profile can be analyzed using a bead array [22].

In this study, we aimed to establish an integrated profile of genetic and epigenetic alterations in GC-related pathways using these new technologies.

Materials and methods

Samples

Fifty GC and corresponding non-cancer samples were collected surgically (41 samples) or endoscopically (9 samples). Additionally, normal gastric mucosae of 6 healthy volunteers without current *H. pylori* infection were endoscopically collected. All the procedures were approved by the Institutional Review Boards and performed with informed consents. Among the 50 GC samples, 30 GC samples were used in our previous study [23]. The samples were stored in RNA*later* (Life Technologies, Carlsbad, CA, USA). Genomic DNA was extracted from the GC, non-cancer, and normal gastric mucosae samples by the phenol/chloroform method, and extracted DNA was quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan).

Analysis of somatic mutations

Sequence variations were obtained using the Ion Personal Genome Machine (PGM) sequencer (Life Technologies) as described previously [23]. Twenty GC samples were newly analyzed, and their reading depths are shown in Supplementary Table 1. The data were combined with the previously reported mutation data [23]. All the sequence variations identified by the Ion PGM sequencer were confirmed by dideoxy sequencing with primers listed in Supplementary Table 2. When a variation was absent in the corresponding non-cancer tissue, the variation was considered as a somatic mutation.

Analysis of gene amplifications

Gene amplifications of 33 genes with three or more polymerase chain reaction (PCR) amplicons were analyzed using the data of reading depths obtained by the Ion PGM

sequencer. Reading depths of the PCR amplicons in a specific GC sample were plotted against the mean reading depths of those in the 50 GC samples, and genes with PCR amplicons whose reading depths were larger (threefold or more) than those of the other genes were defined as amplified genes.

Selection of genes of cancer-related pathways

Genes involved in seven cancer-related pathways (the WNT pathway, the AKT/mTOR pathway, the MAPK pathway, cell-cycle regulation, mismatch repair, the p53 pathway, and cell adhesion) were selected from the Kyoto Encyclopedia of Genes and Genomes Pathway Database (http://www.genome.jp/kegg/). Regarding the signaling pathways activated in GCs, their negative regulators were selected. Regarding the pathways inactivated in GCs, their positive regulators and downstream effectors were selected. A total of 72 genes were selected as candidates for analysis of DNA methylation in this study.

Analysis of DNA methylation

DNA methylation levels of 485,512 probes (482,421 probes for CpG sites and 3,091 probes for non-CpG sites) were obtained using an Infinium HumanMethylation450 BeadChip array as described previously [24]. Twenty GC samples were newly analyzed, and the data were combined with the previously reported methylation data [23]. To adjust for probe design biases, intraarray normalization was performed using a peak-based correction method, Beta MIxture Quantile dilation [25]. The methylation level of each CpG site was represented by a β value that ranged from 0 (unmethylated) to 1 (fully methylated).

DNA methylation of a CGI in a promoter region, especially in the 200-bp upstream region from a transcription start site (TSS) (TSS200), is known to consistently silence its downstream gene, whereas that of downstream exons is weakly associated with increased expression [1, 26–28]. Therefore, we were careful to analyze DNA methylation of a CGI in a TSS200 as much as possible. To achieve this, probes for CpG sites were assembled into 296,494 genomic blocks smaller than 500 bp. Among the 296,494 genomic blocks, 59,757 were located in CGIs and 11,307 of them were located in TSS200s. Of the 72 genes selected for the cancer-related pathway analysis, 52 genes had genomic blocks in their promoter CGIs unmethylated in normal gastric mucosae. For *MLH1*, two genomic blocks in its two TSS200s were analyzed. For *CDKN2A* (*p16*), a genomic block immediately downstream of its TSS was analyzed because no genomic block was located in its TSS200, although it had a CGI spanning from its promoter region to exon 1. The positions of CpG sites of the 53 blocks are shown in Supplementary Table 3. The DNA methylation level of a genomic block was evaluated using the mean β value of all the probes within the genomic block, and the methylation status of the genomic block was classified into unmethylated (β value, 0–0.2), partially methylated (β value, 0.2–0.4), and heavily methylated (β value, 0.4–1.0).

Analysis of gene expression

The data of gene expression in normal gastric mucosae without *H. pylori* infection, analyzed by the GeneChip Human Genome U133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA, USA), were obtained from our previous study [23]. Genes with signal intensities of 250 or more were defined as expressed genes.

Survival curve and statistical analysis

The Kaplan–Meier survival curves were drawn using SPSS 13.0J (SPSS Japan, Tokyo, Japan) for overall survival (OS) of 41 patients whose prognostic information was obtained. The differences in the survival rates were evaluated using the Mantel–Cox test. Association between a pathway alteration and clinicopathological characteristics was evaluated by the Fisher exact test (gender, histological differentiation, depth of tumor, lymph node metastasis, and recurrence) and the Student's *t* test (age). *H. pylori* infection status was not evaluated because it is known that most GC patients had current or past infection of *H. pylori* [29].

Results

Point mutations and gene amplifications in GCs

Among the 50 GCs analyzed for mutations of the 55 cancer-related genes, 27 GCs had 35 somatic mutations, among which 32 and 3 were missense and nonsense mutations, respectively (Table 1). Five oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, *PIK3CA* and *PTPN11*, and four tumor suppressor genes, *CDH1*, *MLH1*, *SMARCB1*, and *TP53*, were mutated. *TP53* was most frequently mutated (19 of the 50 GCs), and *CDH1*, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA* were mutated in 2 or more GCs.

Gene amplification was analyzed for the 33 cancer-related genes in the 50 GCs (Fig. 1, Supplementary Table 4). *ERBB2* was amplified in 3 GCs (S17TP, 3.6-fold; S23TP, 10.5-fold; and S36TP, 5.4-fold; respectively). *FLT3* (S152TP, 3.7-fold), *KRAS* (S18TP, 5.8-fold), and *MLH1* (S131TP, 3.5-fold) were amplified in 1 GC. The combination of point mutations and gene amplifications showed that 58 % of GCs (29 of the 50 GCs) had at least one genetic alteration of the 55 cancer-related genes.

Table 1 List of somatic mutations identified in the 50 gastric cancers (GCs)

Sample name	Gene	Coverage	Variant frequencies	Nucleotide change	Amino acid change	References
S1TP	*CDH1*	399	10.3	c.1198G>A	p.Asp400Asn	[23]
S2TP	*TP53*	496	34.1	c.581T>G	p.Leu194Arg	[23]
S3TP	No mutation					This study
S4TP	*TP53*	438	74.2	c.581T>G	p.Leu194Arg	[23]
S5TP	*KRAS*	1626	54.4	c.38G>A	p.Gly13Asp	[23]
	SMARCB1	50	56	c.1130G>A	p.Arg377His	[23]
S6TP	*TP53*	2077	24.7	c.820G>C	p.Val274Leu	[23]
S9TP	No mutation					[23]
S10TP	*TP53*	2030	41.1	c.833C>A	p.Pro278His	This study
S11TP	*TP53*	10211	53.4	c.844C>T	p.Arg282Trp	[23]
S12TP	*ERBB2*	24516	63.8	c.2264T>C	p.Leu755Ser	[23]
S13TP	*TP53*	70	15.7	c.478A>G	p.Met160Val	[23]
	ERBB2	482	23.9	c.2264T>C	p.Leu755Ser	[23]
S14TP	No mutation					[23]
S15TP	*TP53*	534	40.3	c.743G>A	p.Arg248Gln	[23]
S16TP	*TP53*	453	36.2	c.660T>G	p.Tyr220Ter	[23]
S17TP	No mutation					[23]
S18TP	*TP53*	1946	26.5	c.537T>A	p.His179Gln	[23]
S19TP	No mutation					[23]
S20TP	No mutation					[23]
S21TP	No mutation					This study
S22TP	No mutation					[23]
S23TP	*TP53*	565	67.8	c.537T>A	p.His179Gln	[23]
S24TP	No mutation					[23]
S25TP	*TP53*	609	45.6	c.401T>G	p.Phe134Cys	This study
S26TP	No mutation					This study
S31TP	*KRAS*	1979	56.6	c.35G>T	p.Gly12Val	This study
	PTPN11	7391	56.8	c.182A>G	p.Asp61Gly	This study
S32TP	No mutation					[23]
S33TP	*MLH1*	4092	45.4	c.1744C>G	p.Leu582Val	[23]
	CTNNB1	11994	20.5	c.101G>A	p.Gly34Glu	[23]
	PIK3CA	276	49.3	c.1633G>A	p.Glu545Lys	[23]
	TP53	1142	34.9	c.524G>A	p.Arg175His	[23]
S34TP	*TP53*	551	28.3	c.641A>G	p.His214Arg	[23]
S35TP	*KRAS*	770	41.3	c.35G>T	p.Gly12Val	[23]
S36TP	*TP53*	1142	34.9	c.524G>A	p.Arg175His	[23]
S37TP	*PIK3CA*	59	15.3	c.1624G>A	p.Glu542Lys	[23]
S39TP	No mutation					This study
S40TP	No mutation					[23]
S42TP	No mutation					[23]
S43TP	*TP53*	239	74.9	c.1024C>T	p.Arg342Ter	[23]
S44TP	*CDH1*	368	10.3	c.119C>T	p.Thr40Met	This study
	TP53	1163	14.6	c.818G>A	p.Arg273His	This study
S45TP	No mutation					[23]
S47TP	*CTNNB1*	4591	33.7	c.121A>G	p.Thr41Ala	[23]
S51TP	No mutation					This study
S53TP	*TP53*	1467	20.2	c.844C>T	p.Arg282Trp	This study

Table 1 continued

Sample name	Gene	Coverage	Variant frequencies	Nucleotide change	Amino acid change	References
S54TP	No mutation					This study
S124TP	No mutation					This study
S131TP	*PIK3CA*	266	17.3	c.1633G>A	p.Glu545Lys	This study
	TP53	898	67.8	c.493C>T	p.Gln165Ter	This study
S137TP	*KRAS*	508	34.4	c.35G>A	p.Gly12Asp	This study
S141TP	No mutation					This study
S150TP	No mutation					This study
S151TP	No mutation					This study
S152TP	No mutation					This study
S154TP	No mutation					This study
S162TP	*TP53*	605	36.5	c.400T>G	p.Phe134Val	This study

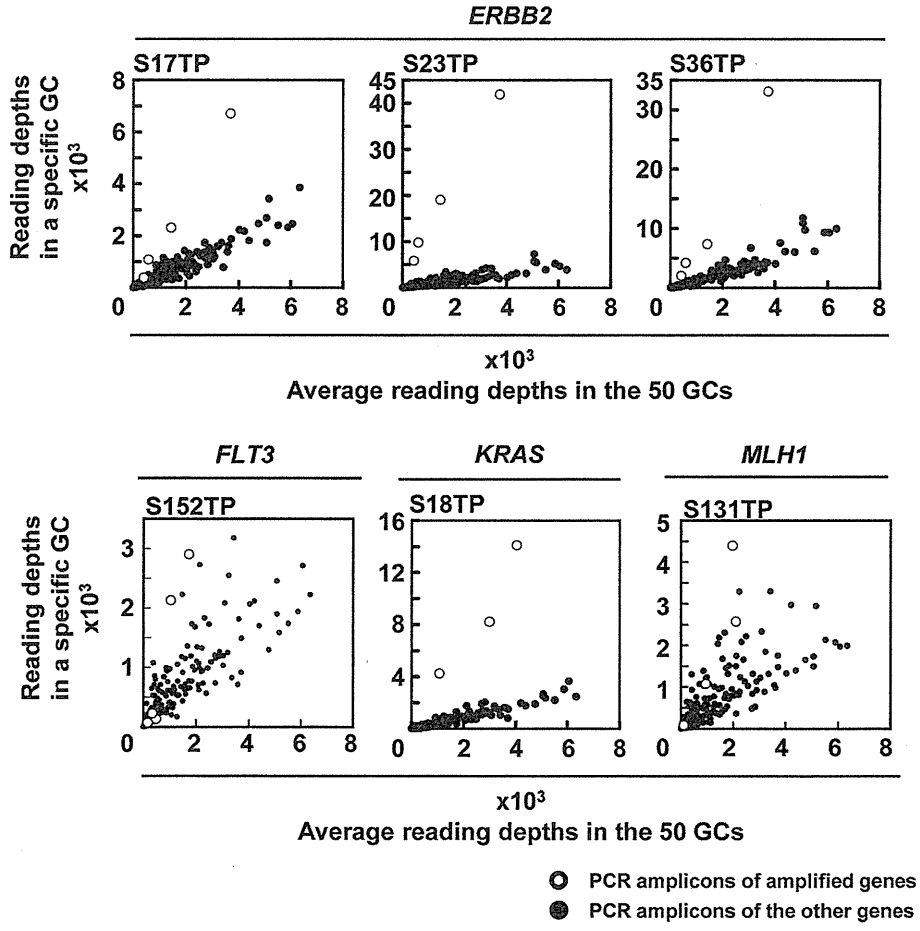

Fig. 1 Gene amplification of *ERBB2*, *FLT3*, *KRAS*, and *MLH1*. Reading depths of the PCR amplicons in a specific gastric cancer (GC) were plotted against the mean reading depths of the PCR amplicons in the 50 GCs. *ERBB2* was amplified in 3 GCs (S17TP, 3.6-fold; S23TP, 10.5-fold; and S36TP, 5.4-fold). *FLT3* (S152TP, 3.7-fold), *KRAS* (S18TP, 5.8-fold), and *MLH1* (S131TP, 3.5-fold), respectively, were amplified in 1 GC each. *Open circles* show the reading depths of PCR amplicons of the amplified genes

Growth-promoting pathways affected by epigenetic and genetic alterations

Aberrant DNA methylation of the 53 promoter CGIs of the 52 genes involved in the seven cancer-related pathways was combined with genetic alterations in the 50 GCs (Fig.). First, potential activation of growth-promoting pathways by aberrant methylation of their negative regulators, in addition to activating genetic alterations (point mutations and gene amplifications), were analyzed. Regarding the WNT

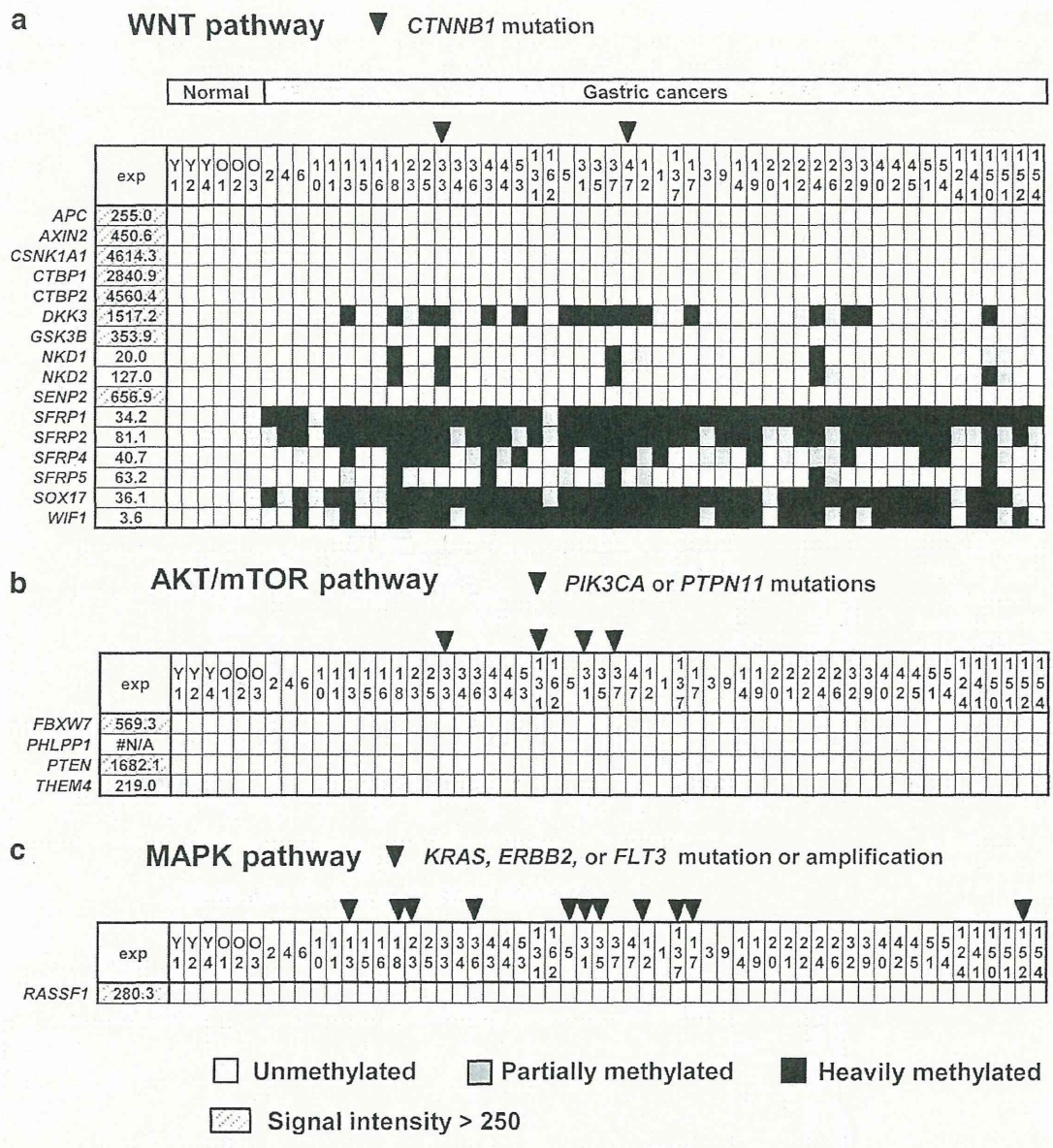

Fig. 2 Genetic and epigenetic alterations in three growth-promoting pathways. **a** In the WNT pathway, 2 GCs had point mutations of *CTNNB1* (*arrowheads*), and 49 GCs had heavy aberrant methylation of 1 or more of its 16 negative regulators. When limited to the 8 negative regulators with moderate or abundant expression in normal gastric mucosae (shown by *hatching*), 17 GCs had aberrant methylation of one or more of them. **b**, **c** In the AKT/mTOR pathway, 4 GCs had point mutations of *PIK3CA* or *PTPN11* (*arrowheads*). In the MAPK pathway, 11 GCs had genetic alterations of *ERBB2*, *FLT3*, or *KRAS* (*arrowheads*). In contrast, none of the 50 GCs had heavy aberrant methylation of negative regulators of the AKT/mTOR or MAPK pathway

pathway, 49 of the 50 GCs had heavy aberrant methylation of 1 or more of its 16 negative regulators, such as *DKK3*, *NKD1*, and *SFRP1* (Fig. 2a). To exclude a concern that we analyzed methylation of genes which had little expression in normal gastric mucosae and thus were susceptible to methylation [30], we confirmed that 8 of the 16 negative regulators were moderately or abundantly expressed (signal intensity >250) in normal gastric mucosae. When limited to these 8 genes, only *DKK3* was heavily methylated in 17 GCs. In contrast, only 2 GCs had point mutations of *CTNNB1*. Regarding the AKT/mTOR pathway, none of the 50 GCs had heavy aberrant methylation of its 4 negative

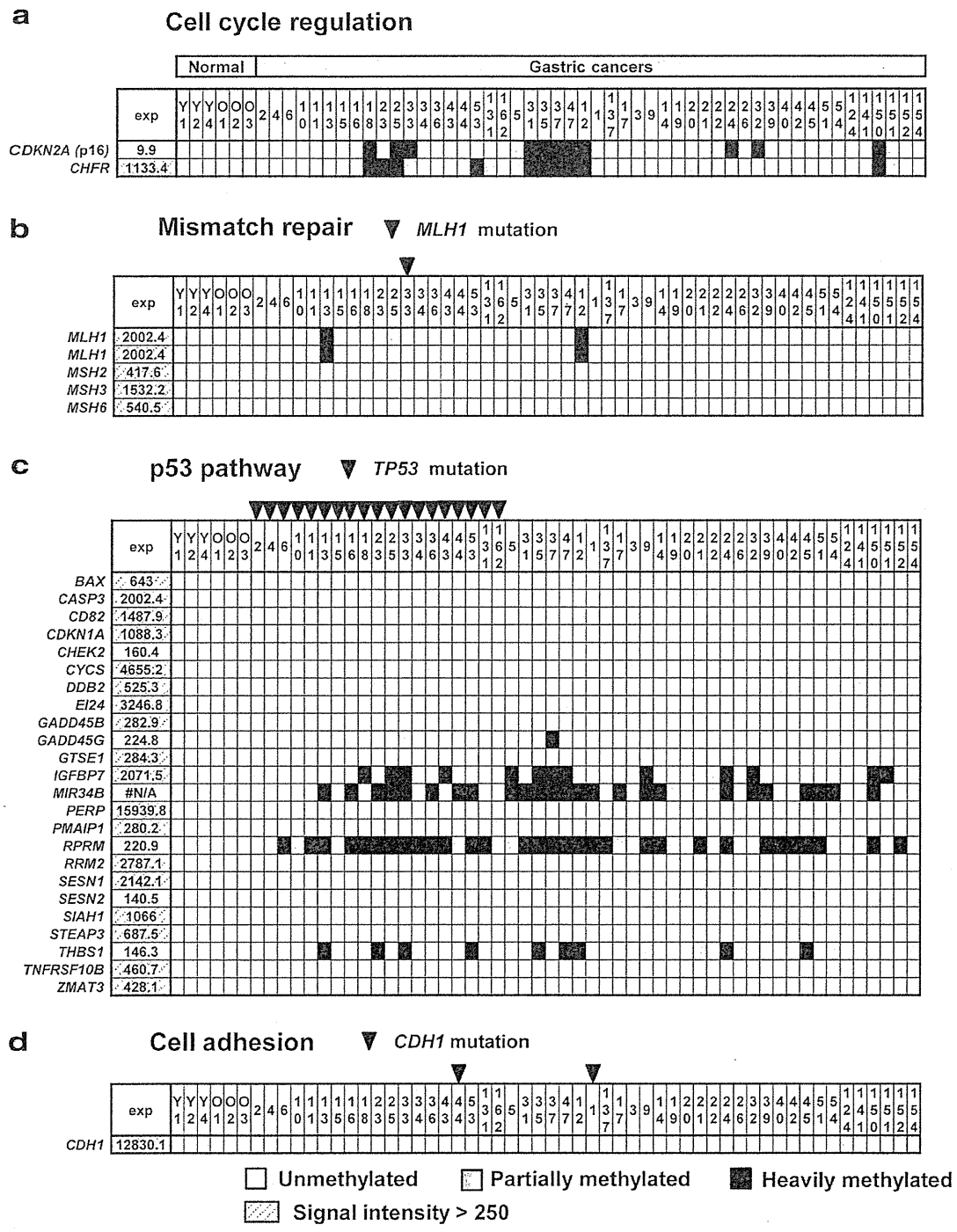

Fig. 3 Genetic and epigenetic alterations in four tumor suppressor pathways. **a** In cell-cycle regulation, none of the 50 GCs had point mutations of *CDKN2A*, whereas 13 GCs had heavy aberrant methylation of *CDKN2A* and/or *CHFR*. **b** In mismatch repair, 1 GC had a point mutation of *MLH1* (*arrowhead*), and 2 GCs had heavy aberrant methylation of *MLH1*. **c** In the p53 pathway, 19 GCs had point mutations of *TP53* (*arrowheads*), and 38 GCs had heavy aberrant methylation of 1 or more of its downstream genes. When limited to the genes with moderate or abundant expression in normal gastric mucosae (shown by *hatching*), 13 GCs had heavy aberrant methylation of *IGFBP7*. **d** In cell adhesion, 2 GCs had mutations of *CDH1* (*arrowheads*), and none of the 50 GCs had heavy aberrant methylation of *CDH1*

regulators, and 4 GCs had point mutations of *PIK3CA* or *PTPN11* (Fig. 2b). Regarding the MAPK pathway, none of the 50 GCs had aberrant methylation of its 1 negative regulator, and 11 GCs had genetic alterations of *ERBB2*, *FLT3*, or *KRAS* (Fig. 2c).

Tumor-suppressive pathways affected by epigenetic and genetic alterations

We then analyzed tumor-suppressive pathways inactivated in GCs. Regarding cell-cycle regulation, 13 of the 50 GCs had heavy aberrant methylation of *CDKN2A* and/or *CHFR*, whereas none of the 50 GCs had point mutations of *CDKN2A* (Fig. 3a). Regarding mismatch repair, 2 GCs had heavy aberrant methylation of *MLH1*, and 1 GC had a point mutation (Fig. 3b).

Regarding the p53 pathway, it is known that *TP53* itself cannot be methylation silenced because it does not have a CGI in its promoter region. However, its downstream genes with promoter CGIs could be methylation silenced. Twenty-four downstream genes had promoter CGIs and 38 GCs had heavy aberrant methylation of 1 or more of the 24 genes (Fig. 3c). Among the 24 genes, *IGFBP7* was abundantly expressed (signal intensity = 2,071.5) in normal gastric mucosae, and 13 GCs had its heavy aberrant methylation. Nineteen GCs had point mutations of *TP53*.

Regarding cell adhesion, none of the 50 GCs had heavy aberrant methylation of *CDH1*, and 9 GCs had partial aberrant methylation. At the same time, 2 GCs had its point mutations (Fig. 3d). Taken together, these results showed that genes in GC-related pathways were more frequently affected by epigenetic alterations than by genetic alterations.

Association between pathway alterations and clinicopathological characteristics

Associations between the pathway alterations and clinicopathological characteristics were analyzed using the data of 41 GCs with clinical information. First, the GCs were classified into two groups by the presence of genetic or/and epigenetic alterations of one of the seven cancer-related pathways (the WNT pathway, the AKT/mTOR pathway, the MAPK pathway, cell-cycle regulation, mismatch repair, the p53 pathway, or cell adhesion), and by that of genetic alterations of oncogenes. Then, from these classifications, those with reasonable statistical power (five or more in both groups) were selected for the clinicopathological analysis (namely, alterations of the MAPK pathway, cell-cycle regulation, and the p53 pathway, and genetic alterations of oncogenes).

As a clinicopathological factor, first, an association with prognosis was analyzed by drawing Kaplan–Meier curves using OS. The prognosis of patients with alterations of the MAPK pathway and genetic alterations of oncogenes tended to be better than that of patients without such alterations ($P = 0.166$ and 0.093, respectively; Fig. 4a,d). In contrast, alterations of cell-cycle regulation and the p53 pathway did not show any associations (Fig. 4b,c). Then, associations with other clinicopathological characteristics (gender, age, histological differentiation, depth of tumor, lymph node metastasis, and recurrence) were analyzed (Table 2). The presence of genetic alterations of oncogenes was associated with lymph node metastasis ($P = 0.021$). In contrast, alterations of the MAPK pathway, cell-cycle regulation, and the p53 pathway were not associated with any clinicopathological characteristics.

Discussion

In this study, we showed (i) that 15 and 21 of the 50 GCs had genetic alterations of oncogenes and tumor suppressor genes, respectively, and (ii) that genes in cancer-related pathways were more frequently affected by epigenetic alterations than by genetic alterations. When genetic and epigenetic alterations were combined, all the 50 GCs had alteration of cancer-related pathways. Although it is still necessary to confirm that activities of cancer-related pathways were indeed impaired by these genetic and epigenetic alterations, all the genes analyzed here were at least reported to be involved in the pathways. These pathways were considered to be potential targets for drugs.

Among the 50 GCs, some GCs had mutations and amplifications of target genes of molecular-targeted therapy. Three GCs had *ERBB2* amplifications and 4 other GCs had point mutations of genes involved in the AKT/mTOR pathway. The 3 GCs with *ERBB2* amplifications are expected to respond to trastuzumab, which was shown to improve survival of patients with *HER2* (*ERBB2*)-positive advanced GC in the ToGA trial [15]. The 4 GCs with point mutations of genes involved in the AKT/mTOR pathway might respond to everolimus, whose efficacy was shown for renal cell carcinoma [16] and breast cancer [31]. Clinical trials for GC are in progress [32, 33].

Tumor suppressor genes, such as *CDH1*, *CDKN2A*, and *MLH1*, were inactivated more frequently by epigenetic alterations than by genetic alterations. In addition, inactivation of negative regulators of the WNT pathway by epigenetic alterations was observed in all the 50 patients.