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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

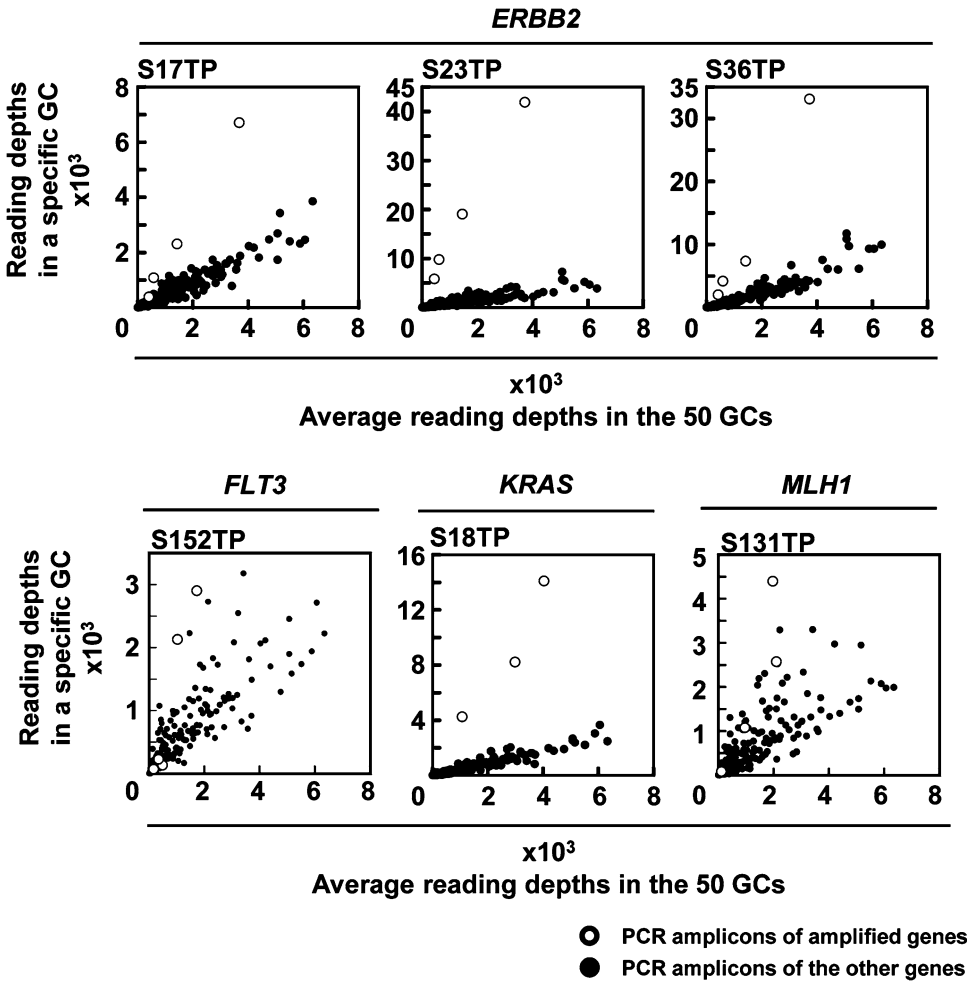

Fig. 1 Gene amplification of *ERBB2*, *FLT3*, *KRAS*, and *MLH1*. Reading depths of the PCR amplicons in a specific gastric cancer (GC) were plotted against the mean reading depths of the PCR amplicons in the 50 GCs. *ERBB2* was amplified in 3 GCs (S17TP, 3.6-fold; S23TP, 10.5-fold; and S36TP, 5.4-fold). *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. 2). 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

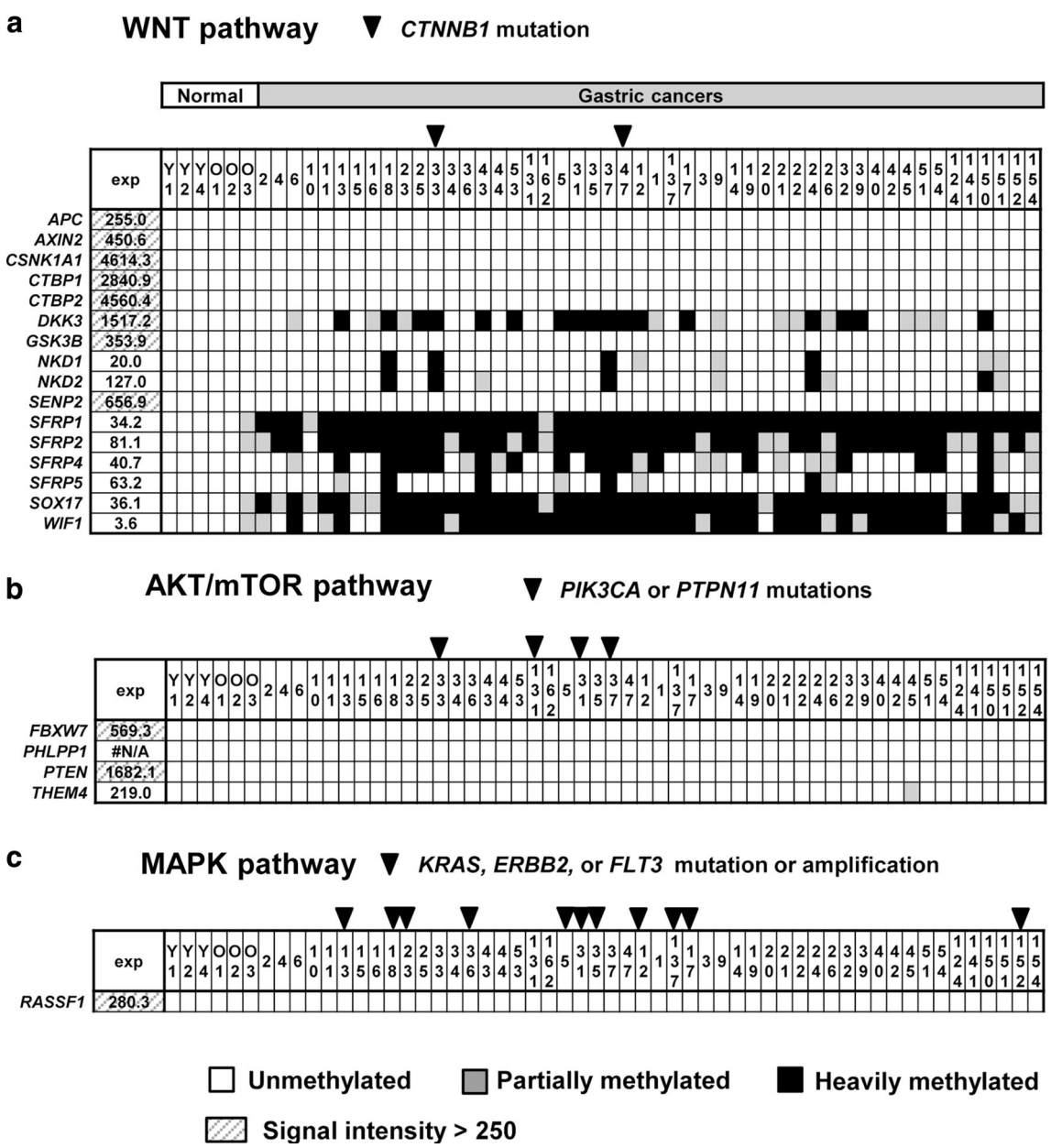

Fig. 2 Genetic and epigenetic alterations in three growth-promoting pathways. **a** In the WNT pathway, 2 GCs had point mutations of *CTNNB1* (*arrowheads*), and 49 GCs had heavy aberrant methylation of 1 or more of its 16 negative regulators. When limited to the 8 negative regulators with moderate or 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

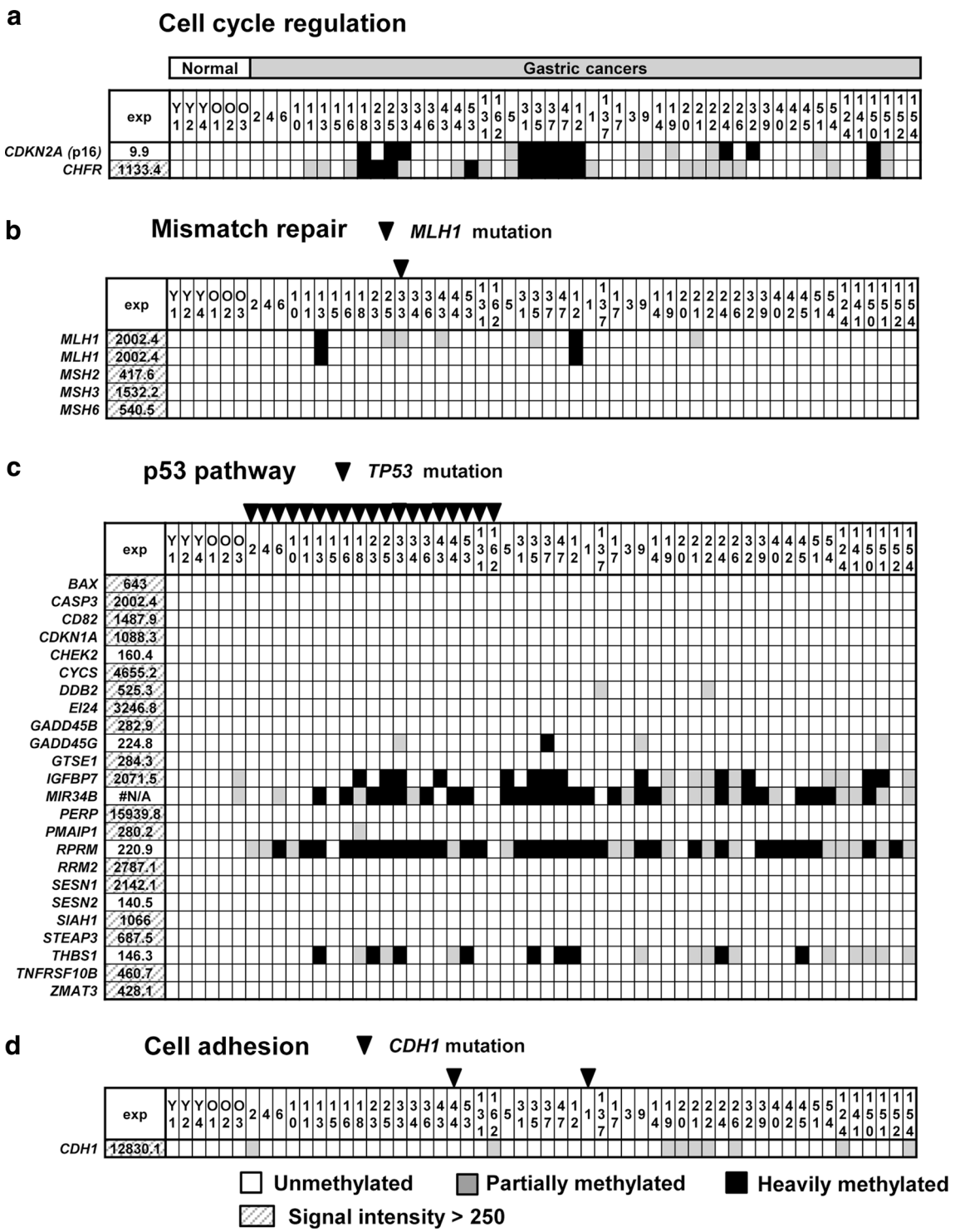

Fig. 3 Genetic and epigenetic alterations in four tumor suppressor pathways. **a** In cell-cycle regulation, none of the 50 GCs had point mutations of *CDKN2A*, whereas 13 GCs had heavy aberrant methylation of *CDKN2A* and/or *CHFR*. **b** In mismatch repair, 1 GC had a point mutation of *MLH1* (*arrowhead*), 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.

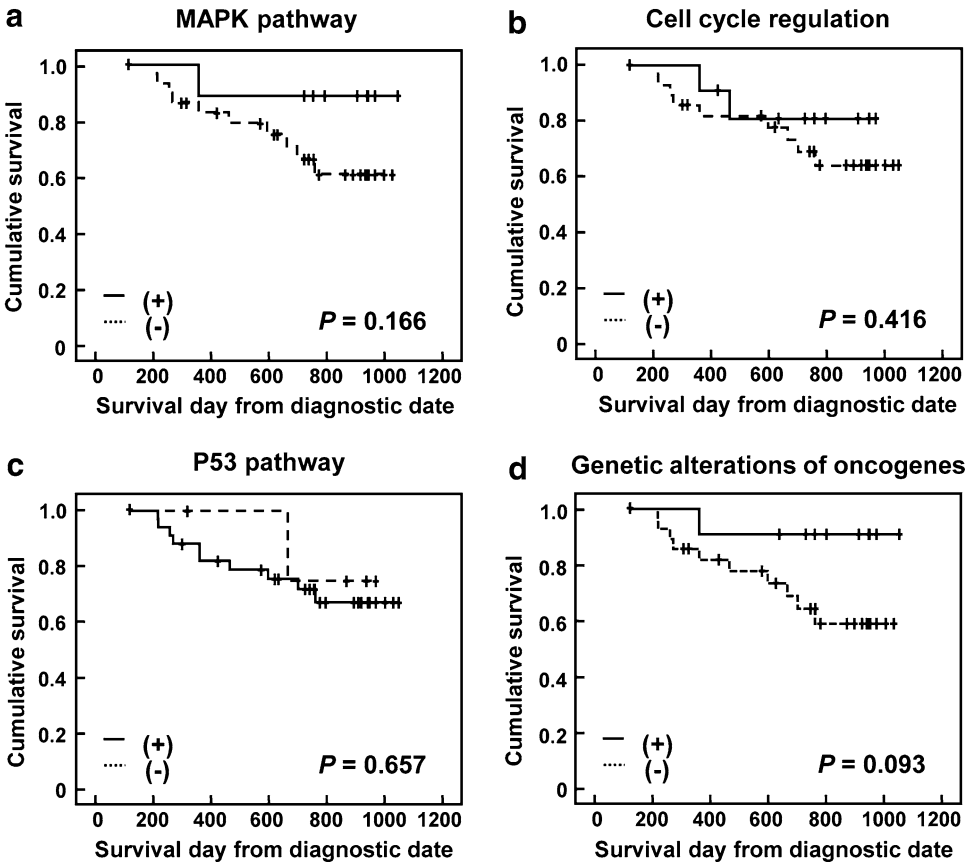

Fig. 4 Associations between a pathway alteration and patient prognosis. Kaplan–Meier curves were drawn using OS. **a** Patients with alterations of the MAPK pathway ($n = 11$) might have better prognosis than those without ($P = 0.116$). **b**, **c** The genetic or/and epigenetic alterations of cell-cycle regulation and the p53 pathway did not show any associations. **d** Patients with genetic alterations of oncogenes ($n = 12$) tended to have better prognosis than those without ($P = 0.093$)

These results showed that epigenetic alterations are deeply involved in gastric carcinogenesis. Aberrant DNA methylation can be restored by the DNA-demethylating drugs 5-azacytidine (azacitidine) and 5-aza-2′-deoxycytidine (decitabine), which are clinically used for patients with myelodysplastic syndromes [34]. Recently, clinical trials using DNA-demethylating drugs for solid tumors have been actively conducted [35], and efficacy was shown in recurrent metastatic non-small cell lung cancer [36]. There is a possibility that these epigenetic drugs are useful for the treatment of GCs.

According to a genome-wide analysis of methylated genes, several hundred to 1,000 genes whose promoter CGIs are aberrantly methylated are accumulated in cancers [37]. Expression levels of most of these genes are absent or very low in normal cells [30]. Most of them are considered not as drivers of carcinogenesis but as passengers. Therefore, we separately analyzed TSS200 CGIs of genes expressed in normal gastric mucosae. These genes are known to frequently include driver genes in carcinogenesis [38]. *DKK3* involved in the WNT pathway and *IGFBP7* involved in the p53 pathway were expressed in normal gastric mucosae and frequently methylated in GCs. It is known that downregulation of *DKK3* is correlated with tumor progression [39], and that *IGFBP7* can inhibit cell growth and induce apoptosis [40]. These results supported that aberrant methylation of *DKK3* and *IGFBP7* was involved in gastric carcinogenesis.

Patients with genetic alterations of oncogenes had a significantly smaller number of lymph nodes with metastasis than those without, and their prognosis tended to be better than those without. Although detailed mechanisms are unknown, it is known that oncogene mutations are associated with the CpG island methylator phenotype (CIMP), and that the prognosis of the CIMP(+) patients tends to be better than that of the CIMP(−) patients in GCs [23].

In conclusion, an integrated profile of genetic and epigenetic alterations of GC-related pathways was obtained using a benchtop next-generation sequencer and a bead array. The profile is expected to be useful for selection of molecular-targeted and epigenetic drugs for individual patients.

Table 2 Associations between genetic/epigenetic alterations and clinicopathological findings

Variable	*N*	Alterations of MAPK pathway		*P* value	Alterations of cell cycle regulation		*P* value	Alterations of p53 pathway		*P* value	Genetic alterations of oncogenes		*P* value
		+	−		+	−		+	−		+	−	
Gender				1.000			0.398			0.567			1.000
Male	34	8	26		9	25		29	5		10	24	
Female	7	1	6		3	4		7	0		2	5	
Age				0.743			0.101			0.436			0.451
Mean ± SD (range)		67.3 ± 11.2 (54–88)	68.8 ± 12.0 (38–88)		73.2 ± 12.2 (54–88)	66.6 ± 11.2 (38–83)		69.0 ± 11.8 (38–88)	64.6 ± 11.6 (47–76)		70.7 ± 11.7 (54–88)	67.6 ± 11.8 (38–84)	
Histological differentiation				0.231			0.494			0.146			0.278
Differentiated	14	5	9		3	11		14	0		6	8	
Undifferentiated	27	4	23		9	18		22	5		6	21	
Depth of tumor				0.088			0.370			0.852			0.230
T1	1	0	1		1	0		1	0		1	0	
T2	9	4	5		2	7		9	0		4	5	
T3	14	4	10		3	11		12	2		4	10	
T4	17	1	16		6	11		14	3		3	14	
Lymph node metastasis				0.070			0.524			0.173			0.021
N0	6	3	3		3	3		6	0		4	2	
N1	7	3	4		1	6		6	1		3	4	
N2	10	1	9		2	8		7	3		0	10	
N3	18	2	16		6	12		17	1		5	13	
Recurrence				0.441			0.305			1.000			0.084
Negative	25	7	18		9	16		22	3		10	15	
Positive	16	2	14		3	13		14	2		2	14	

Depth of tumor and lymph node metastasis are based on the 7th edition tumor-node-metastasis classification of the International Union Against Cancer

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