Research Article

Genomically stable gastric cancer characterized by hypomethylation in Wnt signal cascade.

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Short Title: Genomically stable gastric cancer

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Abstract

Introduction

Gastric cancer is divided into four subtypes by their molecular features linked with genetic alterations, e.g., Epstein-Barr virus (EBV), microsatellite instability-high (MSI-high), chromosomal instability (CIN), and genomically stable (GS), called as TCGA classification. In this study, we tried to clarify the epigenetic features of the four GC subtypes according to aberrant methylation status in 23 loci.

Methods

A total of 98 gastric cancers and their normal gastric mucosa samples were included in this study. We divided gastric cancers into TCGA subtypes which were determined in line with MSI-high, EBV, CIN, to GS by their molecular features. The 13 loci of polymorphic microsatellite sequences were used to determine loss of heterogeneity (LOH) for the detection of CIN. The MSI status was determined by three mononucleotide repeat markers. Infection of EBV was determined by recovering EBV *BNRF1* sequence from genomic DNA collected from gastric cancers. Methylation status of 23 loci was investigated by the combined bisulfite restriction analysis (COBRA). Status of other findings, e.g., *KRAS* mutations, HER2 expression status and infection of helicobacter pylori were confirmed.

Results

Gastric cancers were divided into MSI (13%), EBV (7%), CIN (53%), and GS (27%). By histological classification, poorly differentiated adenocarcinoma (por) was more in tumors categorized in MSI-high, and GS and signet-ring cell carcinoma (sig) was more in GS. Among the 23 loci investigated their methylation status, 18 loci were significantly hypermethylated in caner tissues. A unsupervised clustering divided gastric cancers into two clusters, and revealed that most GS tumors clustered together in a cluster that exhibited lower methylation levels, distinct from the other subtypes. The inter-variable clustering revealed that a cluster contained the three loci (*SFRP2*-region 1/2 and APC) belonging to the Wnt signal cascade (Wnt-associated loci). The mean methylation score of Wnt-associated loci was the lowest in GS tumors (MSI-high: 2.7 [95% confidence interval (CI), 2.3-2.9]; EBV:2.1[1.2-3.1]; CIN: 2.4 [2.2-2.7]; GS: 1.3 [0.8-0.7]). In contrast, the mean methylation score of the other 15 loci was significantly higher in MSI-high, while that in GS was as same as that in EBV or CIN (MSI- high: 10.4 [8.3-12.4]; EBV:5.7 [1.7-9.7]; CIN: 4.4 [3.6-5.1]; GS: 3.4 [2.2-4.6]). Additionally, the lower methylation score of Wnt-associated loci was observed only in sig tumors.

Conclusions

GS subtype tumors have the potential to possess distinct signatures in DNA hypomethylation profiles in Wnt signaling pathway, especially in signet-ring cell carcinoma.

Introduction

Gastric cancer is the fifth most common cancer and the third leading cause of cancer-related death worldwide (1). Currently, the generalized method for the classification of gastric cancer is based on its histologic subtype. Lauren's criteria, the most widely used histological classification of gastric cancer, separate the tumors into intestinal and diffuse types (2). Diffuse-type was renamed as poorly cohesive carcinoma in the 2010 World Health Organization classification and was further classified into two histologic subtypes: signet-ring cell carcinoma, characterized by globoid cytoplasmic mucin eccentrically displaced nuclei, and non- signet-ring cell carcinoma, referred to as poorly cohesive carcinoma not otherwise specified (3). However, those classifications have not led to the development of histological subtype-specific therapies. The Cancer Genome Atlas (TCGA) recently established classification systems for gastric cancer via comprehensive molecular analysis: Epstein-Barr virus (EBV)-positive tumors, microsatellite instability (MSI)-high tumors, somatic copy-number aberrations high cluster (chromosomal instability [CIN] tumors), and tumors without any of these features, called genomically stable (GS) tumors (4).

The EBV-associated gastric cancers are reported to be about 10%, more prevalent for males and younger patients and proximal gastric regions (5), and closely associated with CpG island methylator phenotype (CIMP) (6). In addition, EBV-associated gastric cancers had a high rate of *PIK3CA* and *ARID1A* mutations but rare *TP53* mutations (4). Other essential characteristics of EBV-associated gastric cancers for therapeutic objects were overexpression of programmed death-ligand (PD-L)1/2 combined with increased immune cell signaling signatures. The immune signature in EBV-associated gastric cancers is known to have a prominent lymphoid infiltration of the stroma and a high density of tumor-infiltrating lymphocytes (6), a promising candidate for anti-PD-1/PD-L1 therapy.

Gastric cancers with MSI-high signatures present a typical lack of function of the mismatch repair (MMR) genes, *MLH1*, *MSH2*, *MSH6*, or *PMS2* (and, rarely, in the non-MMR gene *EPCAM*, in which deletions induce epigenetic silencing of MSH2) (7). The deficiency of MMR complexes (dMMR) causes somatic accumulation of small insertion or deletion events at microsatellites in the genome. Additionally, as these mutations generate shifts in the reading frames of many of these genes, the resulting mutant alleles often encode novel amino acid sequences, sometimes termed "frameshift peptides," which may function as potent tumor-specific antigens (8). Indeed, MSI tumors show a markedly more fabulous presence of tumor-infiltrating lymphocytes (9, 10), and the dMMR tumor microenvironment strongly expressed several immune checkpoint ligands, including PD-1, PD-L1,

CTLA-4, LAG-3, and IDO, indicating that the active tumor microenvironment is counterbalanced by immune inhibitory signals that resist tumor elimination (11). MSI tumors consist of two clinical significances; sporadic tumors result from the hypermethylation of the MLH 1 gene promoter, and hereditary tumors, called Lynch syndrome, is caused by germline mutations in MMR genes (12).

CIN comprises about 50% of gastric cancer, characterized by highly variable chromosomal copy numbers without high mutation rates (4). Gastric cancer with a CIN signature is frequent at the gastroesophageal junction, correlates with Lauren histology's intestinal type, and indicates marked aneuploidy (4). For CIN characterized by copy number changes in chromosomes, Deng et al. used high-resolution genomic analysis to profile somatic copy number alterations in a panel of 233 gastric cancers (13). Regarding broad chromosomal regions, the most frequently amplified region included chromosomes 1q, 3q, 5p, 6p, 7pq, 8q, 12pq, 13q, 18pq, 19p, 20pq and 21p, and the most frequently deleted regions included chromosomes 3p, 4pq, 5q, 6q, 8p, 9p, 9q, 11q, 12p, 14q, 16q, 17p, 18p, 18q, 19p, 21q, and 21q (13). Frequently deleted chromosomal regions are usually characterized by loss of heterozygosity (LOH) and suggest the presence of tumor suppressor genes. CIN tumors often show receptor tyrosine kinase (RTK)–RAS activation by the focal amplifications of RTKs and cell cycle mediators (14). The genomic amplification of CIN tumors suggests the potential for therapeutic inhibition. Recurrent amplification of the gene encoding ligand VEGFA was notable given the GC activity of the VEGFR2 targeting antibody ramucirumab (15, 16).

GS subtype is classified according to the flowchart from the TCGA: first, molecular subtypes of EBV-positive and MSI tumors are assigned, and then the remaining tumors are further divided as GS or CIN: chromosomally unstable based on their degrees of aneuploidy(4). The GS subtype has low mutation rates and copy number alterations and has an enrichment of diffuse-type by Lauren histology(4). GS tumors are associated with the worst prognosis and are resistant to adjuvant chemotherapy among the four TCGA subtypes (17).

Because of the methylation microarray analyzing 1,315 CpG sites, gastric cancer was divided into four clusters with methylation degree; EBV, gastric CIMP, and clusters 3 and 4 (4). Although a population of CIN and GS subtypes was in cluster 4, characterized by lower methylation dense in 1,315 CpG sites both in cancer and normal stomach mucosa, features of DNA methylation between CIN and GS subtypes are still obscure (4). In this study, we focused on clarifying epigenetic features in the TCGA subtypes by analyzing the methylation status of the 23 loci.

Materials and Methods

Patients

We collected tissue specimens of 98 primary gastric cancer and their matched normal gastric mucosa from gastric cancer patients who had undergone surgery at the Okayama University Hospital (Okayama, Japan), as described previously (18). All normal gastric mucosa tissues were obtained from sites adjacent to the tumor but at least 5 cm away from the tumor site. All patients provided written informed consent, and the ethical committee approved the study at the Okayama University Hospital. All patients also gave informed consent to use their data for future analyses. The pathological stage and the histological diagnosis were made according to the Japanese classification of gastric carcinoma (3rd English edition), with subclassification of malignant epithelial tumor: papillary adenocarcinoma (pap), tubular adenocarcinoma (tub), mucinous adenocarcinoma (muc), poorly differentiated adenocarcinoma (por), signet-ring cell carcinoma (sig), and special type (19). In this study, diffuse-type in this study has consisted of por and sig and categorization of sig-type tumors was according to the WHO classification (> 90% of signet ring cells) (3).

Detection of Loss of heterogeneity (LOH) and definition of chromosomal instability (CIN) phenotype

The 13 loci of polymorphic microsatellite sequences that are tightly linked to known tumor suppressor genes and DNA MMR genes, including the *MYCL* locus on 1p34 (MYCL), the *MSH2* locus on 2p16 (D2S123), the *UNC5C* locus on 4q23 (D4S1559, D4S2381, and D4S470), the *APC* locus on 5q21 (D5S346, D5S107), the *UNC5D* locus on 8p12 (D8S87), and the *p53* locus on 17p13 (D17S250, TP53), and the *DCC* locus on 18q21 (D18S35, D18S58, and D18S69) were used to determine LOH, as described previously (18). PCR amplifications were performed on genomic DNA templates from tumor and normal mucosa tissues using fluorescently labeled primers. The amplified PCR products were electrophoresed on an ABI 310R Genetic analyzer and analyzed by GeneMapper fragment analysis software (Thermo Fisher Scientific, Waltham, MA, USA). When comparing the signal intensities of the individual markers in the tumor DNA with that of the corresponding normal DNA, a reduction of at least 40% of the signal intensity was considered indicative of LOH. All 98 gastric cancer patients displayed at least three markers informative for the LOH status. CIN phenotype was categorized by calculating a LOH ratio of the informative markers of the 13 polymorphic microsatellite sequences. When a tumor showed a LOH ratio over 0, the tumor was categorized as

CIN-positive.

MSI analysis and definition of MSI phenotype

The MSI status was analyzed in all 98 gastric cancer patients by using 3 mononucleotide repeat markers (BAT26, NR21, and NR27) as described previously (18, 20). When at least one or more mononucleotide repeat markers displayed microsatellite instability, tumors were defined to MSI-high and the tumors without MSI in the three mononucleotide repeat markers were defined to non-MSI-high.

***KRAS* mutation analysis**

KRAS mutation status were analyzed in 98 gastric cancer samples as described previously (18).

Detection of *Helicobacter pylori (H. pylori)* and *Epstein-Barr* (EB) virus

To determine *H. pylori* infection status, we recovered the *EPIYA* repeat sequence in the *cagA* protein, which binds to the Src homology 2 domain-containing protein tyrosine phosphatase, SHP-2, on gastric epithelial cells. The *cagA* was recovered by PCR amplifications performed on genomic DNA templates from tumor tissues, as described previously (18). Infection of EBV was determined by recovering EBV *BNRF1* sequence from genomic DNA collected from gastric cancers, as described previously (10).

Immunohistochemical (IHC) analyses

IHC analysis for HER2 expression of samples was performed using the HercepTest kit (Dako, Carpinteria, CA, USA) and expression was evaluated by a HER2 scoring system (21). All staining was carried out manually with formalin-fixed paraffin-embedded tissues. Thin (4 μm) sections of representative blocks were deparaffinized and dehydrated using gradient solvents.

DNA extraction, bisulfite modification and combined bisulfite restriction analysis

DNA was extracted from fresh-frozen tissue specimens of 98 gastric cancers and matched normal gastric mucosae using QIAamp DNA mini kits (Qiagen, Valencia, CA, USA). Bisulfite modification of genomic DNA clinical specimens was performed as described previously (18). The methylation status of the 23 loci (*APC* promoter 1A [*APC*], *CACNA1G*, *CHFR*, *CDKN2A*, *COX2*, *DAPK*, *DCC*, *HPP1*, *MGMT*-Mp region, *MGMT*-Eh region, *MINT1*, *MINT2*, *MINT31*, *MLH1*-A region, *MLH1*-D region, *p14ARF*, *RASSF2*-region 1, *RASSF2*-region 2, *RUNX3*, *SFRP2*-region 1, *SFRP2*-region 2,

UNC5C, and *3OST2*) in gastric tissues was analyzed by the combined bisulfite restriction analysis (COBRA), carried out in a 24.0-μL PCR reaction containing 12.0 μL of HotStarTaq Master Mix kit (Qiagen, Valencia, CA, USA) and 0.4 μM of each primer, as described previously (12, 18, 22). *MGMT*-Mp and -Eh region were the regions defined by Nagasaka T et al (23). *MLH1*-A and -D region were by Deng G et al (24). The region 1 and 2 of *SFRP2* and *RASSF2* were by by Nagasaka T et al (25) .

Statistical analyses

All statistical analyses were performed using JMP Genomics software (version 10.2; SAS Institute, Inc., Cary, NC, USA). Methylation status in the 23 loci were evaluated as both continuous and categorical variables (methylated: methylation level $\geq$ cut-off [%] of each locus; unmethylated: methylation level < cut-off [%] in **Table 2**). The cluster analysis was performed by the binary distance metric for clustering and Ward's method for linkage as implemented in the JMP Genomics software. Categorical variables were compared using the chi-squared test. The pair-wise comparisons for each of the subgroups were performed using a nonparametric multiple comparison method by the Dunn's Test, which computes ranks for all the data, not just the pair being compared. Thus, the reported P value by the Dunn's Test reflects a Bonferroni adjustment. All reported *P* values were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Features of methylation status in gastric cancer

We investigated the methylation status of 23 CpG island-related loci in 98 gastric cancers and their corresponding gastric normal mucosa specimens. The difference between gastric cancer tissues and normal mucosa was initially evaluated in continuous methylation level (%). Among the loci, 18 loci demonstrated hypermethylation significantly in tumor tissues compared with gastric normal mucosa (cancer-associated loci). We evaluated methylation status by a binary rank (methylated or unmethylated) according to each cut-off value estimated by the range of methylation levels observed in normal counterpart mucosa (**Table 1**). By those cut-off values, in all 18 cancer-associated loci, the frequency of methylated samples was significantly higher in tumor samples (**Supplementary Table 1)**, and the frequency of methylated samples in each locus was summarized in **Supplementary Table 2**.

Characteristics of gastric cancers divided into four TCGA subtypes

In line with a report from The Cancer Genome Atlas Research Network (TCGA), we tried to classify gastric cancers corresponding to their molecular features: first, molecular subtypes of MSI-high, then EBV-positive tumors, and finally, the remaining tumors were further divided as being CIN or GS based on their LOH ratio. 13 gastric cancers (13 %) were categorized as MSI-high by tumor genetic analyses. Of the remaining 85 gastric cancers, seven tumors (7%) were found EBV *cag A*-positive (EBV), and then 52 (53%) displayed their LOH ratio over 0 (CIN). The remaining 26 gastric cancers (27%) were classified as GS (**Fig. 1A**). Clinicopathological characteristics of each subtype are shown in **Table 2**. There were no significant differences in clinical characteristics in each group. In histological classification, poorly differentiated adenocarcinoma (por) was more in tumors categorized in MSI-high, and GS subtype (6 of 13 [46%] MSI-high and 13 o26 [50%] GS subtype, respectively) and signet-ring cell carcinoma (sig) was more in GS subtype (3 of 26 [12%] GS subtype), but not significant. *H. pylori* infection rate was no different among the groups. *KRAS* mutations were evaluated by direct sequence. Mutations were detected in the *KRAS* codon 12 (5 %, n = 5) and codon

13 (1 %, n = 1). *KRAS* codon 12 mutations consisted of p.G12D (c.35G> A, n = 4) and p.G12R (c.34G>C, n = 1), and codon 13 mutations included p.G13D (c38G>A, n = 1). Interestingly, one tumor with MSI-high displayed both *KRAS* codon 12 (p.G12D) and 13 mutations (p.G13D), and additionally, this tumor possessed EBV infection.

Feature of LOH ratio in the TCGA subtypes

LOH status was evaluated by calculating the LOH ratio of informative markers of the 13 polymorphic microsatellite sequences. All 98 gastric cancers had at least three or more informative markers (**Supplementary Fig. 1**), and the mean LOH ratio of all tumors was 0.27 (95%CI; 0.21-0.33). In relation to the TCGA subtypes, the mean LOH ratio was larger in the order of CIN (0.45 [95%CI; 0.38-0.52]), EBV (0.26 [95%CI; 0.02-0.50]), and MSI (0.09 [95%CI; 0.02-0.16], **Fig. 1B**).

Hypomethylation in Wnt signaling pathway associated with GS tumors

On tumor samples, we performed unsupervised clustering by continuous methylation level in the 18 cancer-associated loci. The cluster analysis divided gastric cancers into two clusters (Cluster I and II in **Fig. 1C**), and revealed that most GS tumors clustered together in cluster I that exhibited lower methylation levels in the 18 cancer-associated loci, distinct from the other subtypes. Interestingly, an inter-variable clustering revealed that a cluster contained the three loci (*SFRP2*-region 1/2 and *APC*) belonging to the Wnt signaling pathway (**Fig. 1C**).

To clarify the association between the loci belonging to Wnt signaling pathway (Wnt-associated loci) and TCGA subtypes, each cancer or corresponding normal mucosa sample was given a numerical score to reflect the number of methylated loci. The methylation score was given by the number of loci methylated. The methylation score of Wnt-associated loci (*SFRP2*-region 1/2 and *APC*) and that of the other loci (the rest 15 cancer-associated loci) were calculated, respectively. In Wnt-associated loci, the mean methylation score was the lowest in GS tumors than the other TCGA subtypes (MSI-high: 2.7 [95% confidence interval (CI), 2.3-2.9]; EBV:2.1[1.2-3.1]; CIN: 2.4 [2.2-2.7]; GS: 1.3 [0.8-0.7]; **Fig. 2A**). In contrast, the mean methylation score in the other 15 loci was significantly higher in MSI-high, while that in GS was as same as that in EBV or CIN (MSI- high: 10.4 [8.3-12.4]; EBV:5.7

[1.7-9.7]; CIN: 4.4 [3.6-5.1]; GS: 3.4 [2.2-4.6]; **Fig. 2B**).

Concerning counterpart normal mucosa, the mean methylation score of Wnt-associated loci was significantly lower in normal mucosa obtained from GS cancers than that in normal mucosa from non-GS cancers (normal mucosa from GS cancers: 0.5 [0.2-0.7]; normal mucosa from non-GS cancers: 1.0 [0.8-1.2], *P*=0.0075, **Fig. 2C**). In contrast, there was no difference in the mean methylation score of the other loci between normal mucosa obtained from GS cancers and non-GS cancers (normal mucosa from GS cancers: 0.7 [0.3-1.0]; normal mucosa from non-GS cancers: 0.9 [0.6-1.1], *P*=0.3677, **Fig. 2D**).

Methylation signature in relation to histological subtype

GS gastric cancers are rink with diffuse-type gastric cancers in histology (4). As mucinous adenocarcinoma (muc) is contained both diffuse and intestinal features, in this study, diffuse-type gastric cancers consisted of two histological subtypes: poorly differentiated adenocarcinoma (por) and signet-ring cell carcinoma (sig). Although it became a small sample size in each subclassification, to further assess the feature of methylation signatures observed in GS tumors, we compared the methylation scores and status in line with subclassification of malignant epithelial tumors: pap, tub, muc, por, sig, and special type (**Fig. 3 and Supplementary Table 3**). Compare with counterpart normal mucosa, the methylation scores of both Wnt-associated loci and the other loci were significantly increased in por-type diffuse tumors, but those in sig-type diffuse tumors showed no difference with their counterpart normal mucosa. The lower methylation scores of Wnt-associated loci and the other loci in cancer were observed in sig-type diffuse tumors but there are no significant among six histopathological subtypes (**Supplementary Fig. 2**), as well as those scores of their counterpart normal mucosa (**Supplementary Fig. 3**). Of course, too small a sample size to conclude, not only between diffuse types but also among all histological subclassification, sig-type diffuse tumors have the potential to possess distinct signatures about DNA methylation profiles, especially in terms of hypomethylation.

Discussion/Conclusion

The comprehensive genome and proteome analyses of gastric cancer have uncovered molecular subtypes and identified dysregulated pathways and potential therapeutic targets (4). The TCGA project divided gastric cancers into four molecular subtypes: EBV subtype with extreme DNA hypermethylation, MSI subtype with elevated mutation rates and hypermethylation, CIN subtype with marked aneuploidy frequent focal amplification of receptor tyrosine kinases, and GS subtype with less specific genomic alterations. However, GS subtype demonstrated such less distinctive genomic alterations, including DNA methylation tightly associated with poor prognosis clinically and enrichment of diffuse-type pathological construction. We selected 23 promoter CpG loci associated with tumor suppressor genes in this study. The cluster analysis revealed that most GS tumors clustered together in lower methylation levels in the 18 cancer-associated loci of the 23 promoter CpG loci, similar to TCGA data. By inter-variable clustering, we found that three loci (*SFRP2*-region 1/2 and *APC*) belonging to Wnt signaling pathway made a significant cluster.

The Wnt signaling pathway regulates many cell functions, e.g., proliferation, migration, apoptosis, and differentiation, and is critical for embryonic development and in the homeostasis of several adult tissues, including the gastrointestinal tract (26). This pathway is deregulated in many cancers (27). Next-generation sequencing revealed that Wnt signaling is deregulated in gastric tumors at several pathways, including the ligand, receptors, and intracellular transduction components (26, 28). In addition to mutations, epigenetic changes are also observed in gastric tumors to Wnt inhibitors such as SFRPs (binds directly to Wnt ligands) (29, 30).

SFRPs and frizzled, a family of five secreted glycoproteins, are identified as possible negative modulators of the Wnt signal transduction pathway (31). The SFRPs are activated by binding Wnt proteins to the membrane-bound frizzled receptors, leading to inhibitor function to beta catenin (32, 33).

Among the four SFRPs (-1, -2, -4, and -5) with CpG islands in the promoter region, only SFRP2 was silenced and methylated in gastric cancer. Forced expression of SFRP2 in a gastric cancer cell line with hypermethylation in the *SFRP2* gene inhibits cell proliferation, induces cell apoptosis, and inhibits in vivo tumor growth (34). Consistent with these findings, Suzuki et al. demonstrated that overexpressed SFRPs reduced colony formation and induced apoptosis in colon cancer cells (35). These data suggest that SFRP2 acts as a functional tumor suppressor in gastric cancer, and its silencing may enhance tumor growth and expansion (34).

Other epigenetic studies have suggested that silencing of the genes by DNA hypermethylation at CpG islands tended to be accumulated in the multi-step pathway of gastric carcinogenesis (18, 36, 37). Promoter methylation of tumor suppressor genes has been detected in the early stages of gastric cancer development (37). Cheng YY et al. reported that methylation of *SFRP2* was detected in 73.3% of gastric cancer, 37.5% in intestinal metaplasia, and 20% in adjacent non-cancer tissues (34).

Similarly, our results demonstrated that methylation of *SFRP2*-region 1 and -2 was detected in 80% and 71% of gastric cancer, 32% and 16% in adjacent non-cancer tissues, respectively.

APC, first identified as the gene responsible for familial adenomatous polyposis, is an essential negative regulator of Wnt signaling; as a component of the degradosome complex, APC promotes the proteasomal degradation of the Wnt effector molecule beta-catenin. APC inactivation by hypermethylation leads to the stabilization of beta-catenin in the cytoplasm due to dysregulation of the beta-catenin degradation (38, 39). Two promoters have been identified in *APC*, termed promoter 1A and promoter 1B (38). Through alternative splicing, promoter 1A produces transcript 1A, and promoter 1B produces three transcripts. The role of *APC* methylation in gastric cancer is more controversial than in other gastrointestinal tumors (38). Hosoya et al. proposed that methylation of *APC* promoter 1A was frequently observed and acted as a passenger in human gastric carcinogenesis, while promoter 1B was unmethylated (40). Therefore, in this study, we evaluated methylation status in *APC* promoter 1A. As several studies demonstrated that *APC* gene promoter methylation was obserbed in normal gastric mucosa, Cle´ment G et al. precisely investigated and reported that *APC* promoter 1A showed monoallelic methylation (around 50% methylation in a quantitative manner) in normal gastric mucosa, not due to imprinting but most likely due to allelic exclusion (41). In this study, the mean methylation ratio of *APC* promoter 1A was 14.6% (95%CI, 12.4-16.8%) in counterpart normal mucosa. Therefore, a methylated case was defined as 20% or methylation in *APC* promoter 1A. If we defined a methylated case as the case that observed methylation in *APC* promoter 1A as 5% or more, 76 out of 98 normal counterpart mucosa (78%) were categorized as methylated (data not shown). Although evidence of the monoallelic methylation is not as straightforward as that Cle´ment G et al. (41), at least a little less than 80% of normal mucosa shows methylation of *APC* promoter 1A.

In this study, we focused on clarifying epigenetic features in the TCGA subtypes by analyzing the methylation status of the 23 loci. By unsupervised clustering by continuous methylation level in the 18 cancer-associated loci, gastric cancers were divided into two clusters (Cluster I and II in this study), and revealed that most GS tumors clustered together in cluster I that exhibited lower methylation levels, distinct from the other subtypes. Additionally, an inter-variable clustering revealed that a cluster contained *SFRP2*-region 1/2 and *APC*.

In Wnt-associated loci, the mean methylation score was the lowest in GS tumors than the other TCGA subtypes, whereas the mean methylation score in the other 15 loci was significantly higher in MSI-high, while that in GS was as same as that in EBV or CIN. Similarly, in concerning counterpart normal mucosa, the mean methylation score of Wnt-associated loci was significantly lower in normal mucosa obtained from GS cancers than that in normal mucosa from non-GS cancers, whereas there was no difference in the mean methylation score of the other loci between normal mucosa obtained from GS cancers and non-GS cancers.

As GS cancers are rink with diffuse-type (muc and sig, in this study) in histology. The lower

methylation score of Wnt-associated loci was observed only in sig-type diffuse tumors compared with the other histological subtypes including muc-type. Additionally, the methylation score of the other loci was lower in sig-type diffuse tumors compared with por-type diffuse tumors. Pathologically, frequent coexistence of sig- and non-sig-type (muc-type in this study) is observed. As we diagnosed sig-type cancers according to the WHO classification (> 90% of signet ring cells) (3), thus, the differences in relation to methylation score between sig-type and non-sig-type might be observed.

Recently, Togasaki K et al. demonstrated that organoids generated by diffuse-type gastric cancer referred as poorly cohesive carcinoma not otherwise specified (por-type in this study) transformed into signet-ring cell carcinoma-like structures on removal of Wnt and R-spondin from the culture medium (42). Our results consistent with this finding. The lower methylation score of *SFRP2* and *APC* in Wnt signaling pathway, which observed in signet-ring cell carcinoma, would activate both SFRP2 and APC, leading to inhibitor function to beta-catenin (32, 34). Thus, the lower methylation in *SFRP2* and *APC* promoters may be equal to Wnt removal from the culture medium.

This study has limitations. The sample size is small to conclude. Especially, by histological subclassification, sig-type diffuse tumors were observed only five cases. Classification of TCGA subtype was not based on the same strategy. However, GS subtype in this study demonstrated such less distinctive genomic alterations, including DNA methylation and enrichment of diffuse-type pathological construction. Moreover, in clinical setting, to classify gastric cancer into TCGA subtype is still difficult, especially to distinguish between CIN and GS. Our result demonstrated, that we could predict GS tumors by analyzing methylation status of Wnt-associated loci in cancer and counterpart normal mucosa. To confirm our results, a future prospective analysis will be required.

In conclusion, the mean methylation score of Wnt-associated loci was significantly the lowest in GS tumors and their counterpart normal mucosa compared with the other TCGA subtypes. Among GS subtype characterized by diffuse histological type, sig tumors have the potential to possess distinct signatures in DNA hypomethylation profiles.

Statements

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Statement of Ethics

This study was approved by the ethics committee of Okayama University (Institutional Review Board [IRB] no.: 80) and Kawasaki Medical School (IRB no.: 3196-3). The patients provided written informed consent and all studies were conducted in accordance with the Declaration of Helsinki.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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Author Contributions

Toshiaki Toshima performed all genetic analyses and drafted the manuscript. Hiroaki Tanioka and Yoshiko Mori assisted with data interpretation. Takehiro Tanaka performed the immunohistochemical analysis and pathological diagnosis. Kazuya Yasui, Keisuke Kimura, Akihiro Nyuya, and Shuya Yano extracted DNA and analyzed genetic and epigenetic mutations. Yuzo Umeda and Toshiyoshi Fujiwara provided the patient samples and summarized the clinicopathological data. Takeshi Nagasaka designed the project, performed the epigenetic analyses, assisted with the interpretation of all data, secured funding, and drafted the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

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Figure legends

Fig. 1.

A, A flowchart outlines how tumors were classified into molecular subtypes.

B, Loss of heterogeneity (LOH) ratio of gastric tumors in the subtypes: microsatellite instability (MSI, blue), Epstein–Barr virus (EBV)-positive (red), chromosomal instability (CIN, purple) and genomically stable (GS, green). The green horizontal bar depicts the mean LOH ratio. *P* values were by the Dunn's test.

C, The heatmap represents unsupervised clustering of DNA methylation at 18 cancer-associated loci for 98 tumors into two clusters (I and II). Inter-variable clustering divided 18 cancer-associated loci into four clusters. The top of cluster in the Inter-variable clustering is consist of three Wnt-associated loci (*SFRP2*-region 1/2 and *APC* promoter 1A, red).

Fig. 2.

Mean methylation score of tumors calculated by the Wnt-associated loci (**A**) and the other 15 cancer-associated (**B**) in TCGA subgroups. Mean methylation score of counterpart normal mucosa calculated by the Wnt-associated loci (**C**) and the other 15 cancer-associated (**D**) in TCGA subgroups. In the box plot diagrams, the horizontal line within each box represents the median; the limits of each box are the interquartile ranges, the whiskers are the maximum and minimum values, and the green horizontal bar within each box depicts the mean value. The numbers over the green horizontal bar denote the mean methylation score. The *P* values were statistical differences among any 2 individual groups calculated by the Dunn's Test.

Fig. 3.

Mean methylation score of tumors and their counterpart normal mucosa calculated by the Wnt-associated loci (**A**) and the other 15 cancer-associated (**B**) in histological subgroups. In the box plot diagrams, the horizontal line within each box represents the median; the limits of each box are the interquartile ranges, the whiskers are the maximum and minimum values, and the green horizontal bar within each box depicts the mean value. The numbers over the green horizontal bar denote the mean methylation score. The *P* values were statistical differences among tumor and counterpart normal mucosa calculated by the Dunn's Test. T and N denote tumors and counterpart normal mucosa, respectively.

Supplementary Fig. 1

A, Number of the markers displaying LOH. The green horizontal bar depicts the mean number of the markers displaying LOH. Histogram was presented on the right panel.

B, Number of informative polymorphic microsatellite sequences. The green horizontal bar depicts the mean number of informative polymorphic microsatellite sequences.

Histograms were presented on the right panel, respectively.

Supplementary Fig. 2

Mean methylation score of tumors calculated by the Wnt-associated loci (A) and the other 15 cancer-associated (B) in histological subgroups. In the box plot diagrams, the horizontal line within each box represents the median; the limits of each box are the interquartile ranges, the whiskers are the maximum and minimum values, and the green horizontal bar within each box depicts the mean value. The numbers over the green horizontal bar denote the mean methylation score. None of pair-wise comparison for each of subgroups was not significant by by the Dunn's Test.

Supplementary Fig. 3

Mean methylation score of the counterpart normal mucosa calculated by the Wnt-associated loci (A) and the other 15 cancer-associated (B) in histological subgroups. In the box plot diagrams, the horizontal line within each box represents the median; the limits of each box are the interquartile ranges, the whiskers are the maximum and minimum values, and the green horizontal bar within each box depicts the mean value. The numbers over the green horizontal bar denote the mean methylation score. None of pair-wise comparison for each of subgroups was not significant by by the Dunn's Test.