

2016 Gastric Cancer: Global view

Genomic and epigenomic heterogeneity in molecular subtypes of gastric cancer

Byungho Lim, Jong-Hwan Kim, Mirang Kim, Seon-Young Kim

Byungho Lim, Jong-Hwan Kim, Seon-Young Kim, Genome Structure Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, South Korea

Jong-Hwan Kim, Mirang Kim, Seon-Young Kim, Department of Functional Genomics, University of Science and Technology, Daejeon 305-350, South Korea

Mirang Kim, Epigenome Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, South Korea

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Correspondence to: Seon-Young Kim, PhD, Genome Structure Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, South Korea. kimsy@kribb.re.kr
Telephone: +82-42-8798116

Fax: +82-42-8798119

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Abstract

Gastric cancer is a complex disease that is affected by multiple genetic and environmental factors. For the precise diagnosis and effective treatment of gastric cancer, the heterogeneity of the disease must be simplified; one way to achieve this is by dividing the disease into subgroups. Toward this effort, recent advances in high-throughput sequencing technology have revealed four molecular subtypes of gastric cancer, which are classified as Epstein-Barr virus-positive, microsatellite instability, genomically stable, and chromosomal instability subtypes. We anticipate that this molecular subtyping will help to extend our knowledge for basic research purposes and will be valuable for clinical use. Here, we review the genomic and epigenomic heterogeneity of the four molecular subtypes of gastric cancer. We also describe a mutational meta-analysis and a reanalysis of DNA methylation that were performed using previously reported gastric cancer datasets.

Key words: DNA methylation; Gastric cancer; Molecular subtype; Mutation; Next-generation sequencing

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Core tip: For the effective diagnosis and treatment of

gastric cancer, a recent sequencing study classified gastric cancer into four molecular subtypes, which include Epstein-Barr virus-positive, microsatellite instability, genomically stable, and chromosomal instability subtypes. This molecular subtyping will extend our knowledge for basic research and will be valuable for clinical uses. We herein discuss the genomic and epigenomic heterogeneity of the four molecular subtypes of gastric cancer. We also describe a meta-analysis result that was performed using previously reported sequencing datasets.

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INTRODUCTION

Gastric cancer (GC) is a heterogeneous disease that is affected by various genetic and environmental factors. Traditionally, GC has been divided into two histological subtypes, intestinal- and diffuse-type, on the basis of Lauren's classification^[1]. Intestinal-type GC is derived from gastric mucosa cells, characterized by well-differentiated glandular structures, and develops through well-characterized sequential pathological stages, such as chronic gastritis, atrophy, intestinal metaplasia, and dysplasia^[2]. Diffuse-type GC is characterized by poorly differentiated infiltrative growth with no definitive premalignant stage and is associated with aggressive behavior and poor prognosis^[3]. In addition to the histological subtypes, the clinicopathological characteristics of GCs vary from case to case, making it difficult to identify detailed subtypes and to choose a subtype-optimized therapeutic approach^[4].

Over the past decade, advances in sequencing technology and high-throughput analysis have delivered new insights into the genetic and epigenetic heterogeneity that underlies the distinct molecular subtypes of GC^[5-14]. Recently, The Cancer Genome Atlas (TCGA) network performed both sequencing-based and array-based approaches to investigate exome sequences, copy-number alterations, gene expression, DNA methylation, and protein activities in GCs, and GC was classified into four subtypes: Epstein-Barr virus (EBV)-positive, microsatellite instability (MSI), genomically stable, and chromosomal instability subtypes^[15]. This classification potentially has important biological and clinical implications for basic research, disease diagnosis, and drug treatment.

In this review, we summarize the genomic and epigenomic heterogeneity of the four molecular subtypes of GC. We describe a meta-analysis result that was conducted using the combined data of

eight previously reported exome sequencing studies. We also explain a CpG methylation result that was analyzed using TCGA DNA methylation profile data.

SUBTYPE-SPECIFIC GENOMIC ALTERATIONS IN GC

Genomic alterations in EBV-positive subtype

EBV, a gamma-herpes virus containing a 184-kb-long double-stranded DNA genome, was the first virus identified in human malignant cells (Burkitt's lymphoma)^[16]. It was also found in GC epithelial cells in 1990^[17]. EBV infection was found in approximately 8.7% of GCs and exhibited a distinct sex and anatomical prevalence^[18]: males were predominantly infected, and the proximal stomach such as the gastric cardia and fundus is the major infection site.

There are three latency programs of EBV (Latency I, II, and III) that are defined on the basis of EBV-derived latent gene expression. EBV-positive GC belongs to Latency I or II, which express EBV nuclear antigen I (EBNA1), EBV-encoded small RNA (EBER), BamHI-A rightward transcripts (BARTs), and latent membrane protein 2A (LMP2A)^[19]. Latency I neoplasms, including EBV-positive GC, do not express the representative EBV viral oncoproteins, EBNA2 and LMP1, suggesting that EBV contributes to GC development through other mechanisms. As opposed to EBNA2 and LMP1, viral LMP2A expression is one of candidate mechanisms involved in EBV-positive GCs. A previous study demonstrated that two GC cell lines (MKN1 and MKN7), when infected with recombinant LMP2A, recapitulated promoter hypermethylation and the repression of the *P TEN* tumor suppressor^[20], a phenomenon which has been previously observed in EBV-positive GCs.

The first mutation frequently identified from EBV-positive GCs was the *ARID1A* (AT rich interactive domain 1A) mutation^[5,10]. The TCGA project revealed that *ARID1A* mutations occur in approximately 55% of EBV-positive GCs^[15]. Notably, the majority of *ARID1A* mutations are nonsense mutations that introduce premature stop codons. This result indicates that a loss-of-function of *ARID1A* may be involved in the tumorigenesis of EBV-positive GCs. Supporting the tumor suppressive role of *ARID1A* in GC, a loss of *ARID1A* expression has also been associated with lymphatic invasion, lymph node metastasis, mismatch repair deficiency, and poor prognosis^[21,22]. Given that *ARID1A* is a subunit of the SWI/SNF chromatin remodeling complex^[12], it would be interesting to investigate whether *ARID1A* mutations lead to the extreme hypermethylation phenotype of EBV-positive subtype, referred to as EBV-CIMP (CpG island methylator phenotype, see the section 'DNA methylation of EBV-positive subtype').

In addition to *ARID1A*, EBV-positive GCs have frequent mutations in *PIK3CA* and *BCOR* (BCL6 core-

pressor). In a TCGA cohort, approximately 80% of EBV-positive GCs acquired *PIK3CA* mutations and 23% had *BCOR* mutations^[15]. Interestingly, all of the identified *BCOR* mutations were nonsense or frameshift mutations, indicating that the inactivation of *BCOR* is associated with EBV-positive GC. Recurrent mutations of *BCOR* have also been found in other cancers, including medulloblastoma^[23], acute myeloid leukemia^[24], and rhabdomyosarcoma^[8]. Furthermore, fusion transcripts *BCOR-CCNB3* (Cyclin B3) and *BCOR-RARA* (Retinoic acid receptor alpha) were found in sarcoma^[25] and acute myeloid leukemia^[26], respectively, suggesting the importance of *BCOR* in the development of multiple types of tumors. *BCOR* acts as a transcriptional repressor, and a BCOR complex exhibits ubiquitylation and demethylation activities by recruiting a Polycomb group E3 ubiquitin ligase to histone H2A, a demethylase to histone H3K36, and an SCF E3 ubiquitin ligase^[27]. Thus, it is necessary to examine whether *BCOR* mutations participate in epigenetic chromatin remodeling and the establishment of EBV-CIMP.

Several copy-number alterations, including frequent 18q loss and 9p24.1 gain, were found in EBV-positive GCs^[10]. In particular, 9p24.1 amplification correlated with elevated expression levels of *JAK2* (Janus kinase 2), *CD274*, and *PDCD1LG2* (Programmed cell death 1 ligand 2)^[15]. The elevated expression of PD-L1 and PD-L2, which are encoded by *CD274* and *PDCD1LG2*, was known to mediate tumor evasion from host immune responses^[28]. Importantly, antibody-mediated blockade of PD-L1/2 recovers immune function and enhances antitumor activity^[29]. Moreover, EBV-positive GCs exhibited dysregulation in immune cell signaling, including IL-12 signaling^[15]. Hence, it is required to test the efficacy of therapeutic agents that are used to control immune cell signaling for the treatment of EBV-positive GCs.

Genomic alterations in MSI-high subtype

In the TCGA cohort, MSI-high GCs accounted for approximately 24% of GC patients. MSI-high GCs tended to be diagnosed at relatively older ages and contained a high proportion of intestinal-type GCs. This subtype is characterized by an extensive hypermethylation phenotype referred to as MSI-CIMP that is different from that of EBV-CIMP. A main criterion distinguishing MSI-CIMP from EBV-CIMP is the presence of *MLH1* (mutL homolog 1) silencing by promoter hypermethylation^[30].

MSI is associated with an absence of DNA mismatch repair activity. DNA mismatch repair genes, including *MLH1*, *MLH3* (mutL homolog 3), *PMS1* (PMS1 homolog 1), *PMS2*, *MSH2* (mutS homolog 2), *MSH3*, and *MSH6*, maintain genomic integrity by correcting errors (base-base mismatches and insertion/deletions) that are generated during DNA replication and recombination^[31]. Due to silencing of DNA mismatch repair genes by promoter hypermethylation, MSI-

high GCs exhibit hypermutation; tumors with mutation rates higher than 11-12 mutations per megabase were designated as hypermutated^[15,32]. The distinctly high mutational load of MSI-high GCs indicates that this GC subtype may have a unique mutational signature that is different from the other subtypes. Indeed, MSI-high GCs showed a high percentage of a C-to-T substitution signature, whereas the other GC molecular subtypes exhibited the enrichment of an A-to-C substitution signature^[33].

In addition to a distinct mutational signature, MSI-high GCs have a different repertoire of mutations compared to non-hypermutated GCs. Liu *et al.*^[33] revealed that MSI-high GCs acquired frequent mutations in *TP53* (Tumor protein p53), *ACVR2A* (Activin A receptor, type II A), *PTEN* (Phosphatase and tensin homolog), *PIK3CA*, *KRAS* (Kirsten rat sarcoma viral oncogene homolog), *ERBB2* (Erb-b2 receptor tyrosine kinase 2), *ZBTB1* (Zinc finger and BTB domain containing 1), *TRAPPC2L* (Trafficking protein particle complex 2-like), *GPR39* (G protein-coupled receptor 39), *GPR85*, and *CHRM3* (Cholinergic receptor, muscarinic 3). The TCGA project identified frequent mutations in *PIK3CA*, *ERBB3*, *RNF43* (Ring finger protein 43), *PTEN*, *TP53*, *KRAS*, *ARID1A*, *HLA-B* (Major histocompatibility complex, class I, B), *B2M* (Beta-2-microglobulin), and *NF1* (Neurofibromin 1), in hypermutated GCs^[15]. *TP53*, *PIK3CA*, and *PTEN* are the only genes that overlap between the two studies. Hypermutation in this subtype may cause numerous passenger mutations and hinder the detection of driver genes. Thus, rather than therapeutic approaches targeting mutated genes, therapeutic regimens targeting the MSI-CIMP may provide better options for the treatment of MSI-high GCs.

Genomic alterations in genomically stable subtype

Genomically stable GCs are classified according to guidelines from the TCGA network: first, molecular subtypes of EBV-positive and MSI-high tumors are assigned and then the remaining tumors are further divided as being genomically stable or chromosomally unstable based on their degrees of aneuploidy^[15]. Genomically stable GC is characterized by enrichment of diffuse-type GCs, a relatively younger patient age at diagnosis, and low mutation rates. Since the diffuse-type GC is an aggressive, invasive, and stem-like histological subtype, its rapid tumor progression may result in a diagnosis at an early age and may not provide enough time to accumulate mutations.

In contrast to intestinal-type GCs that are characterized by a corpus-dominated gastritis with gastric atrophy and intestinal metaplasia, diffuse-type GCs are characterized by gastritis throughout the stomach and a lack of atrophy^[34]. Diffuse-type GCs occur more uniformly throughout the world, whereas intestinal-type GCs are predominantly found in specific geographic areas (*i.e.*, Eastern Asia). The histological and genomic alterations of diffuse-type GCs are less

recognized compared to those of intestinal-type GCs, which develop through a sequence of events known as the Correa pathway^[34]. The highly infiltrative feature of diffuse-type GCs makes it difficult to obtain high purity tumor samples, thereby resulting in a low efficiency of mutation detection. Therefore, as the genomically stable property of diffuse-type GCs could be caused by low purity tumors, the genomic features of diffuse-type GCs should be interpreted with caution. Recent whole-genome or whole-exome sequencing followed by validation with deep sequencing may, in part, overcome this problem and may identify novel mutations of diffuse-type GCs.

Genomically stable GCs have frequent mutations in *ARID1A*, *CDH1* (Cadherin 1), and *RHOA* (Ras homolog family member A). In the TCGA cohort, mutations in *CDH1*, which encodes E-cadherin, were found in approximately 37% of genomically stable GCs. In addition to somatic mutations, germ-line mutations have been described as causative variants for hereditary diffuse-type GCs^[35-37]. A total of 90 out of 104 known germ-line *CDH1* mutations potentially cause a premature translation stop or lack of mRNA expression, thereby affecting the entire coding sequence and all functional domains of a protein^[38]. The inactivation of the cell adhesion molecule E-cadherin by mutations may, in part, explain a lack of cellular cohesion of diffuse-type GCs, which is the primarily histological feature.

Recently, three studies sequentially reported recurrent *RHOA* mutations in diffuse-type GCs^[10,14,15]. *RHOA* mutations were found in approximately 15%-23% of diffuse-type GCs. *RHOA* mutations occur in highly conserved hotspot sites, including R5W, G17E, L22R, Y34C, F39C/V, E40K/V, Y42C, L57V, and G62E. These *RHOA* mutations were clustered in two adjacent amino-terminal regions that are known to be functional domains associated with ROCK1 (Rho-associated, coiled-coil containing protein kinase 1) and other effector interaction or GTP binding^[39]. The most frequently mutated RhoA Y42 corresponds to HRAS Y40, which is required for the activation of mitogen-activated protein kinase^[40].

The functional effect of *RHOA* mutations is not conclusive whether it acts *via* loss-of-function or gain-of-function: both possibilities were revealed by two different studies. One study emphasized loss-of-function effect of *RHOA* mutations. Compared to wild-type RhoA, two different mutant RhoA proteins (Y42C and L57V) exhibited a decrease level of its active GTP-bound form. A subsequent functional study demonstrated that the overexpression of RhoA mutants Y42C or L57V in the intestinal organoid resulted in the evasion of cell detachment-induced apoptosis, termed anoikis^[10]. Given that lack of cellular cohesion, anchorage-independent growth, and resistance to anoikis may be prerequisites for the development of diffuse-type GCs^[13], the inhibition of anoikis by mutant *RHOA* may provide a selective

advantage with respect to tumorigenesis of diffuse-type GC.

Other study revealed a gain-of-function effect of *RHOA* mutations. siRNA-mediated *RHOA* knockdown largely decreased the growth rate of tumor cells harboring *RHOA* mutation; however, when expression was recovered using RhoA Y42C and G17E, tumor growth reinitiated. Conversely, wild type RhoA failed to rescue the growth inhibition affected by siRNA knockdown^[10]. In any case, drugs that modulate the RhoA signaling pathway may be valuable to treat diffuse-type GCs harboring *RHOA* mutations.

CLDN18-ARHGAP fusions were found in 15% of genomically stable GCs and were mutually exclusive from *RHOA* mutations^[15]. As a result, approximately 30% of genomically stable GCs have either *RHOA* or *CLDN18-ARHGAP* alterations. However, recent whole-genome sequencing studies revealed that there were no fusions in *CLDN18* or *ARHGAP6/26*^[10,11]. Instead, *RHOA* fusions were found, including *RHOA-COL7A1*, *RHOA-GPX1* by deletion, and *RHOA-RBM6* by inversion.

A recently conducted study suggested that diffuse-type GCs could potentially be further divided into two subgroups. One is diffuse-type GC with tubular cell morphology, which presents a mutational signature of NpTpT > NpGpT^[11]. The other is genetically quiet diffuse-type GC, which shows infrequent genetic changes and low clonality irrespective of the presence of a TpT dinucleotide mutational signature^[11].

Recent studies have revealed several genetic variants in diffuse-type GCs, despite their low tumor purity. However, a high-depth sequencing with high purity tumor samples would increase the likelihood of identifying more significantly mutated driver genes in the genomically stable subtype.

Genomic alterations in chromosomally unstable subtype

According to the TCGA project, chromosomally unstable GCs, which are classified based on degree of aneuploidy, account for approximately 50% of GC patients. Most patients in this subtype are histologically classified as intestinal-type GC^[15]. This subtype is characterized as having highly variable chromosomal copy numbers, although it does not exhibit a high mutation rate. This phenomenon may indicate that copy-number alterations and mutations occur through distinct oncogenic processes in different subsets of tumors. Supporting this assertion, a hierarchical classification of 3299 TCGA tumors from 12 cancer types revealed two main pan-cancer classes that are dominated by either mutations or copy-number alterations^[41].

Chromosomally unstable GC is primarily characterized by the enrichment of *TP53* mutations and recurrent chromosomal amplifications and deletions. This is consistent with the fact that *TP53* mutations cause chromosomal instability^[9,41,42]. Given that a majority of chromosomally unstable GCs are intestinal-type, it is

Table 1 Significantly mutated genes identified using a combined exome sequencing data from 629 gastric cancer patients

Gene	Nonsilent mutations from 629 GCs	P value	Q value
<i>TP53</i>	276	0	0
<i>KRAS</i>	41	0	0
<i>RHOA</i>	34	0	0
<i>PCDHGA6</i>	34	0	0
<i>DDI1</i>	27	0	0
<i>KRT73</i>	24	0	0
<i>OR10J3</i>	21	0	0
<i>GHSR</i>	20	0	0
<i>DHFR</i>	4	0	0
<i>CDH1</i>	86	6.00×10^{-15}	1.13×10^{-11}
<i>PREX2</i>	94	2.23×10^{-10}	3.82×10^{-7}
<i>PIK3CA</i>	100	1.75×10^{-9}	2.75×10^{-6}
<i>GLI3</i>	81	8.46×10^{-9}	1.23×10^{-5}
<i>KIF2B</i>	46	2.62×10^{-8}	3.53×10^{-5}
<i>GRM8</i>	47	1.73×10^{-7}	2.18×10^{-4}
<i>RPL22</i>	16	2.92×10^{-7}	3.44×10^{-4}
<i>DNAH5</i>	132	3.92×10^{-7}	4.20×10^{-4}
<i>EPB41L3</i>	48	4.07×10^{-7}	4.20×10^{-4}
<i>DCAF12L1</i>	29	4.23×10^{-7}	4.20×10^{-4}
<i>PLCL1</i>	38	4.79×10^{-7}	4.51×10^{-4}

GC: Gastric cancer.

reasonable to observe increased clonality and ploidy in intestinal-type GCs^[11].

Genomic amplification of receptor tyrosine kinases (RTKs) is the most apparent signature of chromosomally unstable GCs. Frequent amplification was found in the genomic regions of RTK-RAS, encompassing *EGFR* (epidermal growth factor receptor), *ERBB2*, *ERBB3*, *FGFR2* (fibroblast growth factor receptor 2), *MET* (MET proto-oncogene), *VEGFA* (vascular endothelial growth factor A), and *KRAS*^[43-45]. Because of this observation, administering trastuzumab, an anti-HER2 monoclonal antibody^[46,47], may be an therapeutic option for GCs harboring *ERBB2* amplification^[48]. The amplification of RTK-RAS had a mutually exclusive pattern within chromosomally unstable GCs^[49].

Other amplified genes are oncogenic transcription factors such as *MYC* (v-myc avian myelocytomatosis viral oncogene homolog), *GATA4* (GATA binding protein 4), and *GATA6*, and cell cycle regulators including *CCNE1* (cyclin E1), *CCND1*, and *CDK6* (Cyclin-dependent kinase 6). Meanwhile, chromosomal deletions have been found in genomic regions containing *FHIT* (Fragile histidine triad), *WWOX* (WW domain containing oxidoreductase), *STK3* (Serine/threonine kinase 3), *CDH1*, *CTNNA1* (Catenin alpha 1), *PARD3* (Par-3 family cell polarity regulator), and *RB1* (Retinoblastoma 1).

META-ANALYSIS OF PUBLISHED GC DATASETS

We conducted a meta-analysis to identify significantly

mutated genes that have not previously been recognized in GCs. We applied a MutSigCV algorithm to a combined exome data set of 629 GC patients from eight published studies^[5,7,11,14,15,33,50,51]. This analysis revealed 20 significantly mutated genes (Q -value < 0.001), including previously identified GC genes such as *TP53*, *RHOA*, *KRAS*, *CDH1*, *GLI3* (GLI family zinc finger 3) and *PIK3CA* (Table 1 and Figure 1). Additionally, this analysis identified previously unrecognized genes in GC, including *DDI1* (DNA-damage inducible 1 homolog 1), *DHFR* (Dihydrofolate reductase), *GHSR* (Growth hormone secretagogue receptor), *KRT73* (Keratin 73), *OR10J3*, *PCDHGA6* (Protocadherin gamma subfamily A, 6), *PREX2* (Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2), *KIF2B* (Kinesin family member 2B), *GRM8* (Glutamate receptor, metabotropic 8), *RPL22* (Ribosomal protein L22), *DNAH5* (Dynein, axonemal, heavy chain 5), *EPB41L3* (Erythrocyte membrane protein band 4.1-like 3), *DCAF12L1* (DDB1 and CUL4 associated factor 12-like 1), and *PLCL1* (Phospholipase C-like 1) (Table 1 and Figure 1).

Among these genes, *GLI3*, a downstream component of the hedgehog pathway, was found to be a significantly mutated GC driver gene in two independent studies, our current meta-analysis and a previous whole-genome sequencing study by Wang *et al.*^[10]. Our results also identified *PREX2* as a significantly mutated gene. Supporting this, a recent whole-genome sequencing study identified *PREX2*, a negative regulator of PTEN, as a new candidate driver of melanoma^[52] and pancreatic cancer^[53].

GRM8 was identified as a new cancer driver gene in three studies, including our meta-analysis, a study conducted on 441 tumor samples encompassing breast, lung, ovarian, and prostate cancer^[54], and a whole-exome sequencing study of endometrial cancer^[55]. Thus, *GRM8* may be a promising therapeutic target for multiple types of cancer. Additionally, *RPL22* was found to be significantly mutated both in colorectal cancer with MSI^[56] and in GC. Other significantly mutated genes from our meta-analysis included *GHSR*, *KIF2B*, and *EPB41L3*, which have been shown to play crucial roles in tumorigenesis^[57-59]. Thus, further studies are required to evaluate the functional roles of these genes and their mutations during tumorigenesis. Moreover, our meta-analysis suggests that increasing the sample sizes still provides a chance to detect previously unrecognized significantly mutated genes.

SUBTYPE-SPECIFIC DNA METHYLATION IN GC

Thus far, over one hundred genes have been reported to be hypermethylated and downregulated in GC. To elucidate the subtype-specific methylation status of these reported genes, we analyzed CpG methylation levels of 86 genes and 14 microRNAs using the 295

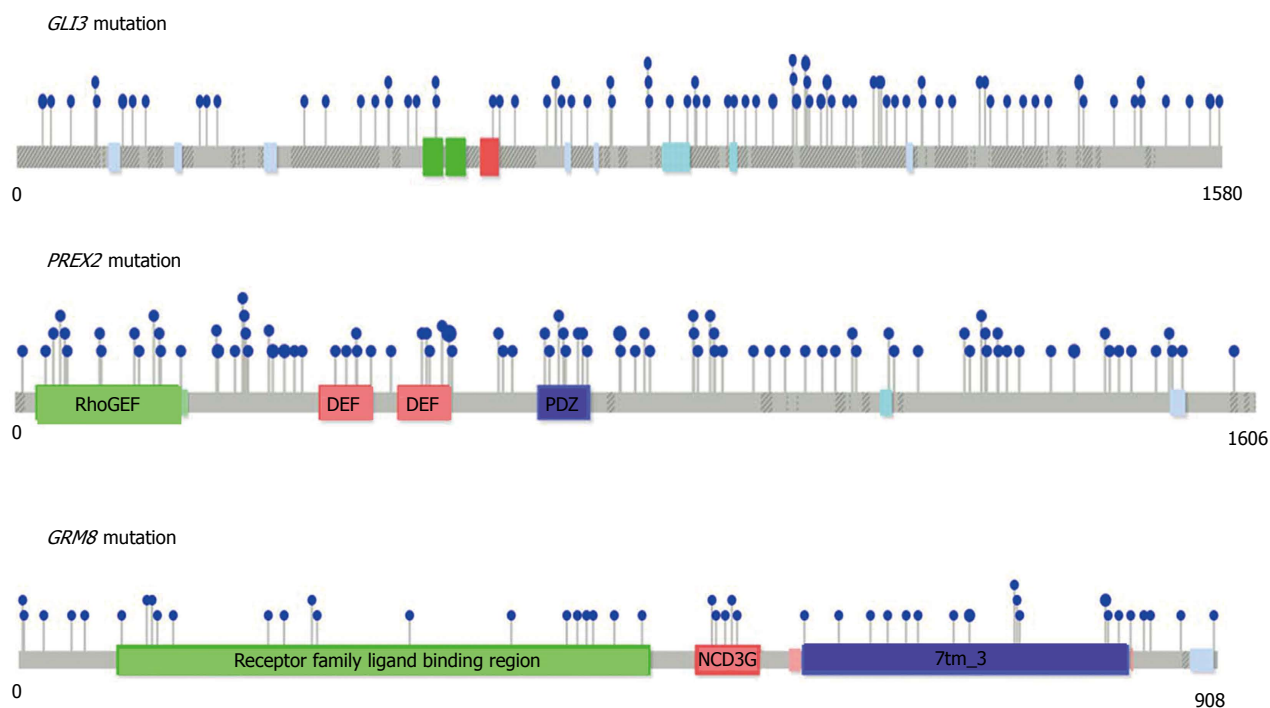


Figure 1 Mutational positions in three significantly mutated genes, *GLI3*, *PREX2*, and *GRM8*.

GC DNA methylation data that have been provided by TCGA^[15]. Supporting the extensiveness of EBV-CIMP, the majority of the hypermethylated genes were found in EBV-positive subtype (Figure 2). Using K-means clustering, we clustered the hypermethylated genes into three groups: hypermethylated in EBV-positive subtype (Figure 2A), hypermethylated in both EBV-positive and MSI-high subtypes (Figure 2B), and other hypermethylated genes (Figure 2C). In the remainder of this review, we will summarize the methylation patterns of these three groups.

DNA methylation of EBV-positive subtype

As stated above, EBV has been identified in epithelial malignancies including GC, and nearly 9% of GCs are EBV-positive^[60]. Hypermethylation of tumor suppressor genes is a key abnormality in EBV-positive GCs^[61].

Unsupervised clustering of CpG methylation clearly revealed that EBV-positivity is the major GC molecular subtype^[15]. The most representative feature of EBV-positive GCs is an extensive hypermethylation phenotype EBV-CIMP, which includes *CDKN2A* promoter hypermethylation. EBV-positive GCs exhibit a global, non-random CpG island hypermethylation phenotype in promoter regions of many cancer-related genes, including p14ARF, p15, p16INK4A, p73, *TIMP3*, E-cadherin, *DAPK*, and *GSTP1*^[62]. This CpG island hypermethylation leads to downregulation of the expression level of many tumor suppressor genes that are responsible for GC tumorigenesis.

Interestingly, three DNA methyltransferases, *DNMT1*, *DNMT3A*, and *DNMT3B*, are overexpressed in EBV-positive GC compared to other subtypes (Figure

3). Although the precise molecular mechanism that leads to an increase in the expression of *DNMTs* during EBV infection is not fully understood, the expression of EBV genes such as *LMP2A* has been reported to activate *DNMT1* transcription by inducing the phosphorylation of STAT3 (Signal transducer and activator of transcription 3)^[20]. As *DNMT1* plays an important role in the establishment, maintenance, and regulation of tissue specific global methylation patterns, the upregulation of *DNMT1* by viral *LMP2A* might drive the extensive EBV-CIMP. Further studies are required to determine whether EBV viral proteins affect the expression level of other DNA methylation regulators, including *DNMTs*, *HDACs* (Histone deacetylases), and *TETs* (Tet methylcytosine dioxygenases).

Apoptosis-related genes such as *RASSF1* (Ras association domain family member 1), *DAPK1* (Death-associated protein kinase 1), and *GADD45G* (Growth arrest and DNA-damage-inducible gamma) exhibit EBV-positive subtype-specific methylation (Figure 2A). *RASSF1A* is hypermethylated and inactivated in lung, breast, ovarian, kidney, prostate, thyroid, and other cancers^[63]. *RASSF1A* possesses tumor suppressor function through its modulation of apoptosis *via* the Hippo and Bax pathways and by controlling the cell cycle^[64]. Figure 4A illustrates the high methylation level of *RASSF1A* in EBV-positive GCs of the TCGA cohort. *RASSF1A* hypermethylation has been detected in 43% of primary GCs and 60% of GC cell lines^[65], and significant *RASSF1A* silencing was found in advanced GC^[65]. Therefore, it is important to elucidate a relationship between EBV infection and progressive methylation of *RASSF1A* during tumorigenesis of GC.

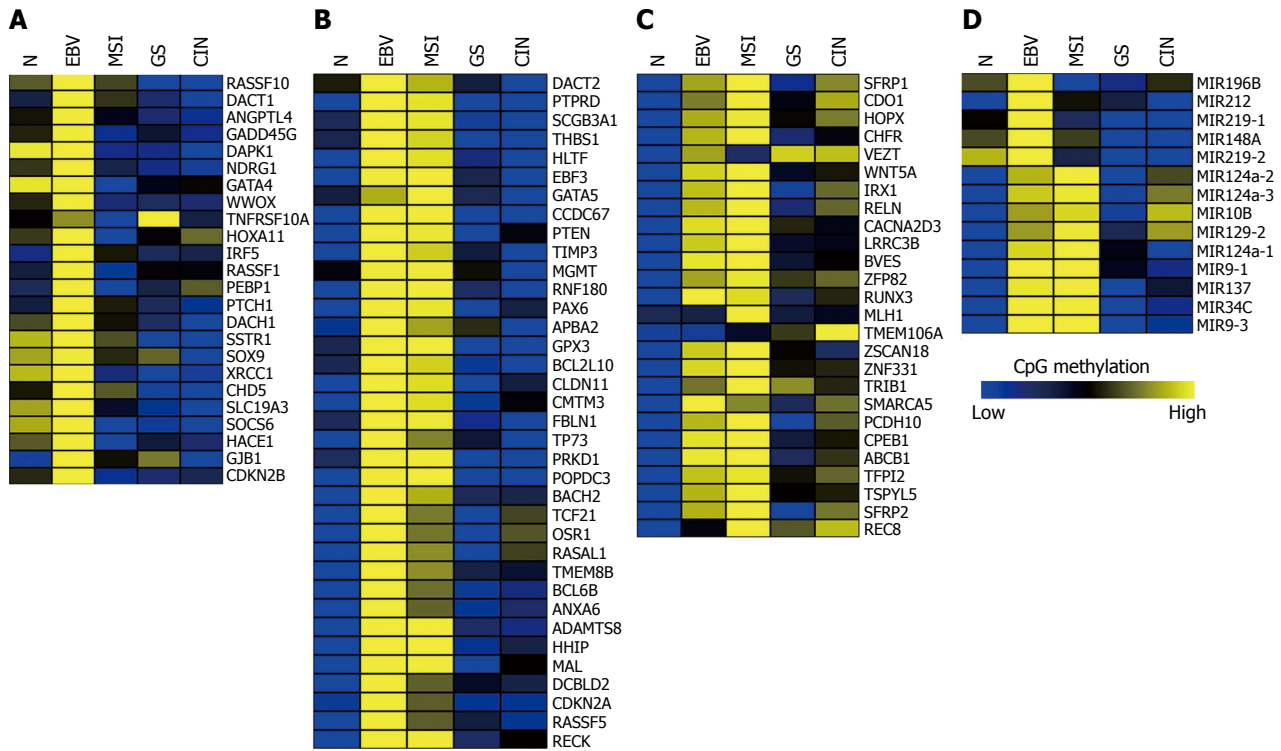


Figure 2 Methylation profiles of the previously reported 86 genes and 14 miRNAs analyzed using the Cancer Genome Atlas methylation data. A: Genes hypermethylated in Epstein-Barr virus (EBV)-positive gastric cancers (GCs); B: Genes hypermethylated in both EBV-positive and MSI-high GCs; C: Genes hypermethylated in more than two GC subtypes; D: Methylation profiles of miRNAs in four GC subtypes.

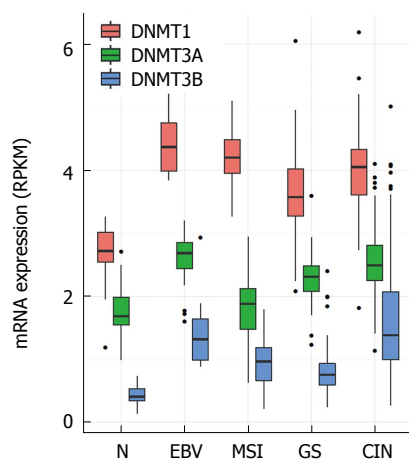


Figure 3 Expression levels of *DNMT1*, *DNMT3A*, and *DNMT3B* analyzed using the Cancer Genome Atlas RNA sequencing data. EBV: Epstein-Barr virus.

Interestingly, aberrant methylation of *RASSF1A* was detected in 67% of EBV-positive GCs but only in 4% of EBV-negative GCs^[66].

DNA methylation of MSI-high subtype

The most common feature of MSI-high subtype is the hypermethylation of *MLH1* promoter. In addition to *MLH1*, many other tumor suppressive genes are frequently hypermethylated in MSI-high subtype^[67], exhibiting MSI-CIMP. Interestingly, we found high mutation rates in DNA methylation regulators, *DNMTs* and *TETs*, in MSI-high GC (Supplementary Figure 1)^[68],

although we cannot rule out the possibility that these high mutation rates are caused by hypermutation in MSI-high GCs. Fifty-two percent (33 of 64) of MSI-high GCs exhibited truncating or missense mutation in *TETs* (Supplementary Figure 1).

As shown in Figure 4B, *MLH1* is hypermethylated only in MSI-high subtype, whereas numerous tumor suppressor genes exhibited hypermethylation patterns in both the EBV and MSI GC subtypes (Figure 2B). This group includes many development-related genes, such as *GATA5* (GATA binding protein 5), *HHIP* (Hedgehog interacting protein), *OSR1* (Odd-skipped related 1), *PAX6* (Paired box 6), and *POPDC3* (Figure 2B). GATA factors are zinc finger DNA binding proteins that control the development of diverse tissues, including the gastrointestinal tract^[69,70], and epigenetic inactivation of *GATA4* and *GATA5* has been reported in GC^[71,72]. Using TCGA data, we found that *GATA4* is hypermethylated in the EBV-positive subtype of GC, whereas *GATA5* is hypermethylated in both EBV and MSI subtypes (Figure 4C).

Hypermethylated genes across more than two subtypes

A subset of genes was found to be hypermethylated in more than two subtypes of GC (Figure 2C). Genes such as *SFRP1* (Secreted Frizzled-related protein 1), *BVES*, *IRX1* (Iroquois homeobox 1), *RUNX3* (Runt-related transcription factor 3), and *WNT5A* (Wingless-type MMTV integration site family member 5A) belong to this subset (Figure 4D). The Wnt signaling is important for cell proliferation during development

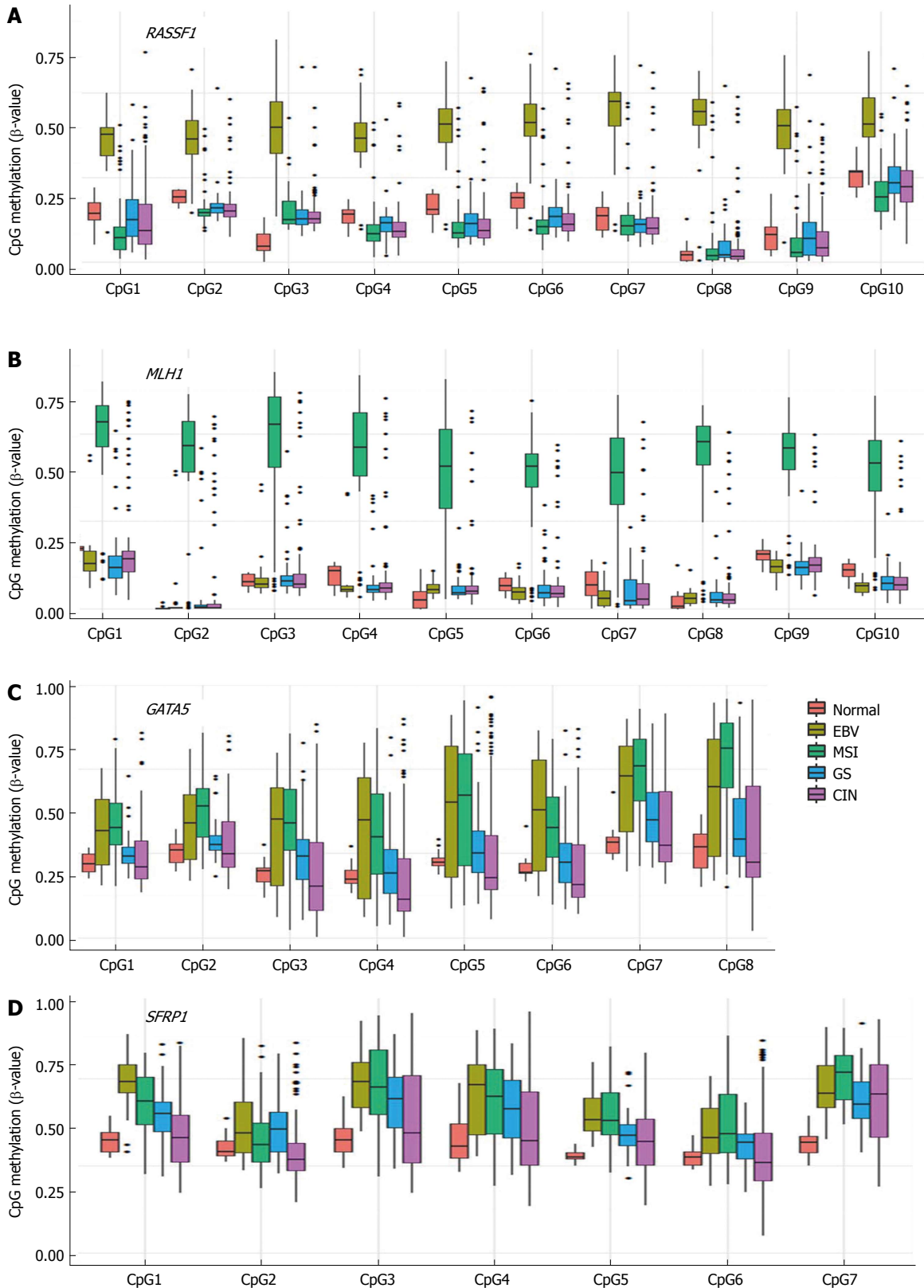


Figure 4 CpG methylation level in promoter regions of *RASSF1* (A), *MLH1* (B), *GATA5* (C), and *SFRP1* (D) analyzed using the Cancer Genome Atlas methylation data.

of the gut, and activation of the signaling pathway has been implicated in gastric carcinogenesis^[2]. SFRP proteins are secreted glycoproteins that inhibit the Wnt signaling either by competing with Wnt ligands to bind to Fz receptors or by binding directly to Fz^[73]. *SFRP1* hypermethylation has been detected in 91% of primary GCs and 100% of GC cell lines^[74]. This hypermethylation of *SFRP* was found to occur during an early stage of GC^[74]. Hypermethylation of *WNT5A*, a non-transforming WNT family member that antagonizes the Wnt signaling^[75], has also been frequently detected in early GC^[76]. Thus, the aberrant methylation patterns of these genes during early GC may serve as useful markers for the early detection of GC.

HYPERMETHYLATED MICRORNAS IN GC

miRNAs are single-stranded, non-coding, small RNAs (18-22 nucleotides in length), which are involved in various biological processes. The aberrant expression of miRNAs and their target genes has a critical role in cancer initiation, progression, and metastasis^[77]. The aberrant DNA methylation of miRNAs has frequently been reported in GC^[78]. The TCGA miRNA-seq data revealed a subtype-specific aberrant DNA methylation pattern of miRNAs (Figure 2D). *miR-196B*, *miR-212*, *miR-148A*, *miR-219-1*, and *miR-219-2* are hypermethylated in the EBV-positive subtype of GC, whereas *miR-9-1*, *miR-137*, *miR-34C*, and *miR-9-3* are hypermethylated in both the EBV and MSI subtypes. Finally, *miR-10B*, *miR-129-2*, *miR-124a-1*, *miR-124a-2*, and *miR-124a-3* are hypermethylated in all of the GC subtypes. Further studies on subtype-specific epigenetic regulation of miRNAs will enable to understand the regulation mechanism of miRNA-driven target genes for GC development.

CONCLUSION

We herein discussed four molecular subtypes of GCs. Each subtype has unique characteristics that facilitate the effective diagnosis and treatment of GCs. The EBV-positive subtype has EBV-driven extensive CpG hypermethylation. The MSI subtype has hypermutation and extreme CpG hypermethylation along with *MLH1* silencing. The genomically stable GC subtype exhibits diffuse-type histology harboring frequent *RHOA* or *CDH1* mutations. The chromosomally unstable subtype has the RTK-RAS activation caused by copy-number amplification. This classification simplifies and clarifies the heterogeneous characteristics of GC, thus serving as a foundation for future research, diagnosis, and treatment of GC. Nonetheless, there are many obstacles that must still be overcome. For instance, defining only four molecular subtypes of GC may oversimplify the complexity of the disease. Therefore, further classification of each molecular subtype may be required. Additionally, causative genetic variants that

drive the genomically stable GC subtype are still largely unknown. Finally, since a primary purpose of genomic studies is to discover therapeutic targets for GC treatment, the classification scheme may eventually be utilized to facilitate personalized medicine. In that sense, the druggable targets that underlie each GC subtype should be further investigated.

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