DNA Methylation and Genetic Aberrations in Gastric Cancer

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Abstract

Background: Gastric cancer (GC) is one of the leading causes of cancer-related deaths worldwide. GC is a pathologically and molecularly heterogeneous disease. DNA hypermethylation in promoter CpG islands causes silencing of tumor-suppressor genes and thus contributes to gastric carcinogenesis. In addition, various molecular aberrations, including aberrant chromatin structures, gene mutations, structural variants, and somatic copy number alterations, are involved in gastric carcinogenesis. **Summary:** Comprehensive DNA methylation analyses revealed multiple DNA methylation patterns in GCs and classified GC into distinct molecular subgroups: extremely high-methylation epigenotype uniquely observed in GC associated with Epstein-Barr virus (EBV), high-methylation epigenotype associated with microsatellite instability (MSI), and low-methylation epigenotype. In The Cancer Genome Atlas classification, EBV and MSI are extracted as independent subgroups of GC, whereas the remaining GCs are categorized into genomically stable

(GS) and chromosomal instability (CIN) subgroups. EBV-positive GC, exhibiting the most extreme DNA hypermethylation in the whole human malignancies, frequently shows *CDKN2A* silencing, *PIK3CA* mutations, *PD-L1/2* overexpression, and lack of *TP53* mutations. MSI, exhibiting high DNA methylation, often has *MLH1* silencing and abundant gene mutations. GS is generally a diffuse-type GC and frequently shows *CDH1/RHOA* mutations or *CLDN18-ARHGAP* fusion. CIN is generally an intestinal-type GC and frequently has *TP53* mutations and genomic amplification of receptor tyrosine kinases. **Key Messages:** The frequency and targets of genetic aberrations vary depending on the epigenotype. Aberrations in the genome and epigenome are expected to synergistically interact and contribute to gastric carcinogenesis and comprehensive analyses of those in GCs may help elucidate the mechanism of carcinogenesis.

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Introduction

Gastric cancer (GC) is the third leading cause of cancer death worldwide [1]. GC is a histologically heterogeneous cancer. Most GCs are adenocarcinomas and are classified

into 2 main histological types: intestinal type and diffuse type, according to the Lauren classification [2]. These subtypes show biological differences in regional incidence, clinical features, and prognosis [3]. In the World Health Organization classification, GCs are classified into some subtypes: tubular, papillary, mucinous, poorly cohesive including signet ring cells and other subtypes, and mixed adenocarcinomas [4]. Furthermore, immunohistochemistry has already shown that GC includes adenocarcinoma with a gastric phenotype and also identified new subtypes of GC, including adenocarcinoma with differentiation of the fundic gland [5].

A variety of environmental factors are involved in developing GC, including smoking, alcohol, diet, and *Helicobacter pylori* (*H. pylori*) infection [3]. *H. pylori* is an important risk factor for GC, and a previous prospective study revealed that subjects infected with *H. pylori* had a high incidence of GC [6]. Most *H. pylori*-related GCs occur in the distal stomach and usually demonstrate intestinal-type histology [4]. Epstein-Barr virus (EBV), the well-known human herpes virus usually acquired during childhood, also contributes to gastric carcinogenesis [7]. EBV(+) GCs account for 7–15% of GCs and show characteristic clinical features such as proximal location of the stomach and relatively better prognosis [7]. EBV(+) GCs histologically demonstrate irregular sheets, trabecula, ill-defined tubes or syncytia of polygonal cells with dense infiltration of lymphocytes and are referred to as gastric carcinoma with lymphoid stroma [4].

Various molecular aberrations are involved in gastric carcinogenesis. Epigenetic aberrations, such as DNA methylation in the promoter CpG island, are involved in the development of GC [8, 9]. Aberrant methylation of tumor-suppressor genes causes transcriptional silencing, leading to carcinogenesis. Previous studies revealed that GCs were classified into at least 2 DNA hypermethylation subtypes with a high or low frequency of aberrant methylation at the CpG islands in the genome or in the 5' regions of genes [10, 11]. Additional studies reported that the frequency of DNA methylation in the promoter region was higher in EBV(+) GCs than other GCs, although the number of analyzed cancer-associated genes was limited [7, 12]. A comprehensive analysis with Illumina's Infinium BeadArray identified distinct DNA methylation patterns in GCs and classified GCs into 3 epigenotypes: extremely high-methylation epigenotype (E-HME), high-methylation epigenotype (HME), and low-methylation epigenotype (LME) [13]. E-HME is the unique phenotype of EBV(+) GCs, exhibiting aberrant hypermethylation of hundreds of genes specifically observed in this

subgroup, such as *CDKN2A* (*p16INK4A*). HME shows aberrant methylation of genes more frequently than LME; *MLH1* promoter hypermethylation is frequently observed in HME.

Three-dimensional chromatin organization is also involved in the development of GC. Aberrant chromatin structures are associated with the regulation of gene expression, and these chromatin structures are detected in cancer [14, 15]. Recent studies revealed long-range chromatin interaction in regions related to genomic rearrangements [16]. Genomic rearrangements are involved in “enhancer hijacking,” promoting the expression of *CCNE1* and *IGF2*. Our latest study of EBV(+) GC has shown that episomal viral genomes interact with human genome and rewire the human chromatin structure [17]. Silenced enhancers are aberrantly activated by the chromatin rewiring (named “enhancer infestation”) and can upregulate neighboring proto-oncogenes to promote gastric carcinogenesis.

Genetic aberrations also contribute to gastric carcinogenesis [18]. *TP53* gene mutations are the most frequent genetic aberrations in GCs [19]. *TP53* gene mutations are found in about half the cases of GCs. Other mutations are observed in several genes, including *CDH1*, *ARID1A*, *KRAS*, *PIK3CA*, *APC*, *CTNNB1*, and *SMAD4* [20]. In addition, *RHOA* mutations are detected in some cases of diffuse-type GCs [21, 22]. In 2014, The Cancer Genome Atlas (TCGA) proposed a molecular classification of GCs [23]. In this classification, EBV(+) GC exhibiting extremely high methylation uniquely, and microsatellite instability (MSI) involving high methylation, for example, *MLH1*, are extracted as independent subgroups of GC. The remaining GCs, thus mostly exhibiting LME, are categorized into 2 subtypes according to the somatic copy number alterations (sCNAs): chromosomal instability (CIN) and genomically stable (GS) subgroups. This molecular classification has now been widely known throughout the world. In this study, we will review the classification of GC into molecular subtypes, especially by DNA methylation and genetic aberrations.

Epigenetic Aberrant in Gastric Cancer

DNA Methylation and Gastric Carcinogenesis

DNA methylation is the common epigenetic aberration. The methylation of 5-position of cytosine bases at CpG dinucleotides such as 5-methylcytosine is well known [8, 9]. CpG islands in promoter regions are basically unmethylated, and expression of the gene is regu-

lated by histone modification. Aberrant methylation of promoter CpG islands causes the transcriptional silencing, leading to the expression of the downstream gene. The silencing of tumor-suppressor genes caused by methylation is one of the major mechanisms of carcinogenesis [8, 9, 18].

The level of DNA methylation is affected by environmental factors. Previous studies showed that aging and smoking are involved in the accumulation of aberrant methylation in CpG islands [24]. In addition, chronic inflammation is known to accelerate the accumulation of aberrant methylation in noncancerous tissues. For example, ulcerative colitis, chronic hepatitis, and reflux esophagitis cause the inflammation, leading to aberrant CpG island methylation [7]. This aberrant CpG island methylation of these noncancerous tissues is considered to associate with carcinogenesis. Indeed, the mouse colitis model induced by dextran sodium sulfate revealed that DNA methylation of CpG island was accumulated in colonic mucosa [25].

Chronic inflammation caused by *H. pylori* is associated with gastric carcinogenesis [7]. *H. pylori* infection causes aberrant promoter methylation, silencing the expression of tumor-suppressor genes such as *RUNX3*, *LOX*, and *CDH1*. Previous studies suggested that chronic inflammation caused by *H. pylori* infection may induce aberrant DNA methylation rather than any factor of *H. pylori* itself [26]. Another study using a Mongolian gerbil model supported that both *H. pylori* infection itself and aberrant methylation in gastric mucosa contribute to *H. pylori*-related gastric carcinogenesis [27].

Epigenetic Aberrant and Carcinogenesis in EBV(+) Gastric Cancer

EBV infection is associated with epigenetic aberrant and gastric carcinogenesis [28]. Comprehensive DNA methylation analyses showed that EBV infection could cause de novo DNA methylation in an LME GC cell, MKN7 [13]. Furthermore, EBV infection also induced aberrant DNA methylation within a month in an immortalized normal gastric epithelial cell line, GES1 [29]. The mechanism of epigenetic aberration and gastric carcinogenesis during EBV infection has not been completely resolved; however, repression of *TET2*, a resistance factor against DNA methylation, is involved in DNA methylation [30].

Moreover, not only DNA methylation but also other epigenetic alterations caused by EBV infection may contribute to carcinogenesis. EBV infection causes enhancer activation and repression, which may be associated with

gastric carcinogenesis [31]. A recent study suggested that enhancer activation through ATF3 might be involved in carcinogenesis [32]. Our latest study has revealed that interaction of episomal EBV genome may directly rewire the host chromatin structure to aberrantly activate proto-oncogenes and promote gastric carcinogenesis [17]. In the study, Hi-C analysis showed the specific interaction between viral and human genomes in EBV(+) GC cell lines and primary tissues of human EBV(+) GC. The human genomic regions interacting with the EBV genome were named as EBVIRs (EBV-interacting regions), and defined by high-resolution technique using 4C-seq. EBVIRs were found to be modified bivalently with active histone marks such as H3K4me1 and H3K27ac and repressive histone mark H3K9me3. This is because EBV genome preferentially interacts with H3K9me3(+) heterochromatins and converts the regions from repressive H3K9me3(+) state to active H3K4me1(+)/H3K27ac(+) state. Enhancers silenced in the heterochromatin are thus aberrantly activated in EBVIRs and can upregulate nearby GC-related genes such as *TGFB2* and *MZT1* [17].

Accumulation of DNA Methylation and Risk of Developing GC

Epigenomic aberrations accumulated in apparently normal tissues reportedly modify tumor risk and are thus suggested to be utilized as tumor risk markers [33, 34]. In noncancerous gastric mucosa with *H. pylori* infection, both driver genes and passenger genes have been methylated [35]. Some passenger genes are more highly methylated in gastric mucosa of subjects with GC than those without GC. This result suggests that the methylation level of particular passenger genes may help predict the risk of developing GC. A recent study also suggested that the methylation levels of gastric mucosal may be useful to predict the risk of developing GC [36]. Indeed, a prospective clinical study revealed that patients with high methylation of marker genes showed a higher incidence of metachronous GC [37, 38]. However, the marker gene that reflects the risk of primary GC has not yet been established.

It also remains unclear how promoter regions of each gene are methylated after *H. pylori* infection. A previous study identified DNA methylation-sensitive genes and DNA methylation-resistant genes [39]. DNA methylation-sensitive genes, including *CDKN2A*, are methylated in the whole promoter regions by EBV infection, whereas DNA methylation-resistant genes methylated only in the surrounding of promoter regions. Besides, a comprehensive DNA methylation analysis using GES1 with EBV in-

Fig. 1. Distribution of DNA hypermethylation of marker genes in epigenotypes. GC is classified into distinct molecular subgroups: E-HME, HME, and LME. In E-HME, all GCs are positive for EBER in situ hybridization. In HME, *MLH1* is frequently silenced. In LME, all GCs are negative for EBER in situ hybridization and lack *MLH1* silencing. The frequency of PRC-target genes is larger in high and common markers than in all genes. PRC-target genes in

embryonic stem cells are enriched in both high and common markers (*) but not significant in EBV markers and normally methylated markers (NS). E-HME, extremely high-methylation epigenotype; HME, high-methylation epigenotype; LME, low-methylation epigenotype; GC, gastric cancer; PRC, polycomb repressive complex; EBV, Epstein-Barr virus.

fection revealed that alternant DNA methylation was acquired from CpG peripheral regions to core regions [29]. Although the process of acquiring DNA methylation in cell lines has been reported, further studies are necessary to be conducted to clarify whether these dynamic changes in methylation status are also confirmed in clinical samples of the non-neoplastic gastric mucosa.

Methylation of Target Genes in Each Epigenotype

GCs are subdivided into subgroups according to aberrant DNA methylation: E-HME, HME, and LME (Fig. 1) [13]. E-HME is uniquely observed in GC associated with EBV(+) GCs. This epigenotype has specifically methylated genes, including *CDKN2*, which are unmethylated in other epigenotypes. HME is associated with MSI and has

frequent methylation of *MLH1* (46%). HME has methylated genes that are unmethylated in LME. Interestingly, these methylated genes are also methylated in E-HME, except for *MLH1* [13]. LME has methylated genes that are unmethylated in normal gastric mucosa. These methylated genes are also methylated in other epigenotypes.

A comprehensive analysis of DNA methylation in GC has proposed 3 subtypes of marker genes identifying each epigenotype [13]. EBV markers are specifically methylated only in E-HME. High markers are methylated in both E-HME and HME, but not methylated in LME. Common markers are methylated in all epigenotypes; however, are unmethylated in normal gastric mucosa. The frequency of polycomb repressive complex (PRC)-target genes is lower in EBV markers than in both high

Table 1. Methylation marker genes and enriched gene ontology terms

Enriched GO terms	Included methylated genes
<i>EBV markers</i>	
Chemotaxis	<i>BMP4</i> , <i>EFNA5</i> , and <i>TGFB2</i>
Regulation of cellular response to growth factor stimulus	<i>TGFB11I</i> , <i>PELO</i> , and <i>SEMA6A</i>
Cell surface receptor signaling pathway involved in cell-cell signaling	<i>BMP4</i> , <i>TGFB11I</i> , and <i>LGR6</i>
Developmental growth	<i>TGFB2</i> , <i>EFNA5</i> , and <i>FBLN5</i>
<i>High markers</i>	
Cell morphogenesis involved in differentiation	<i>FBLN1</i> , <i>NKX6-1</i> , and <i>THY1</i>
Synaptic signaling	<i>EGR2</i> , <i>PTPRD</i> , and <i>WNT7A</i>
Embryonic morphogenesis	<i>FOXL2</i> , <i>GATA3</i> , and <i>RET</i>
Synapse organization	<i>PTPRD</i> , <i>WNT7A</i> , and <i>TMEFF1</i>
<i>Common markers</i>	
Axon development	<i>PTPRM</i> , <i>SLIT3</i> , <i>UNC5C</i> , and <i>EPHA6</i>
Synapse organization	<i>ADGRB3</i> , <i>POU4F1</i> , and <i>TMEFF2</i>
Cell-adhesion molecules	<i>CDH4</i> , <i>NCAM1</i> , <i>NCAM2</i> , and <i>JAM2</i>
Regulation of neuron differentiation	<i>PTPRO</i> , <i>SFRP1</i> , <i>RGMA</i> , and <i>MIR137</i>

Three groups of methylation marker genes have been reported to determine methylation epigenotypes of GC [29]. The GO analysis using reported markers has identified significantly enriched GO terms in marker genes. Representative enriched GO terms are listed in the table. These GO terms include several methylated genes which are considered to function as tumor-suppressor genes. GO, gene ontology; EBV, Epstein-Barr virus; GC, gastric cancer.

and common markers. These differences in frequency of PRC-target genes suggest that the methylation mechanism in E-HME is different from other epigenotypes [13, 28]. Another comprehensive analysis of DNA methylation in both GES1/MKN7 and clinical GC samples also extracted 3 subtypes of marker genes that identify each epigenotype [29]. Even with these extracted markers, the frequency of PRC-target genes in the EBV markers differs from that in other markers.

The gene ontology (GO) terms enriched in the reported EBV markers [29] are summarized in Table 1: chemotaxis (e.g., *BMP4*, *EFNA5*, and *TGFB2*), regulation of cellular response to growth factor stimulus (e.g., *TGFB11I*, *PELO*, and *SEMA6A*), cell surface receptor signaling pathway involved in cell-cell signaling (e.g., *BMP4*, *TGFB11I*, and *LGR6*), and developmental growth (e.g., *TGFB2*, *EFNA5*, and *FBLN5*). High markers show enrichment of GO terms such as cell morphogenesis involved in differentiation (e.g., *FBLN1*, *NKX6-1*, and *THY1*), synaptic signaling (e.g., *EGR2*, *PTPRD*, and *WNT7A*), embryonic morphogenesis (e.g., *FOXL2*, *GATA3*, and *RET*), and synapse organization (e.g., *PTPRD*, *WNT7A*, and *TMEFF1*). Common markers show enrichment of GO terms such as axon development (e.g., *PTPRM*, *SLIT3*, *UNC5C*, and *EPHA6*), synapse organization (e.g., *ADGRB3*, *POU4F1*, and *TMEFF2*), cell-adhesion mole-

cules (e.g., *CDH4*, *NCAM1*, *NCAM2*, and *JAM2*), and regulation of neuron differentiation (e.g., *PTPRO*, *SFRP1*, *RGMA*, and *MIR137*). Multiple tumor-suppressor genes are included in these categories, and their silencing by DNA methylation might be associated with gastric carcinogenesis of each epigenotype.

TCGA classification stratified LME into GS and CIN subgroups. Although GS and CIN are indistinguishable by DNA methylation, their histological and genetic findings are different and will be discussed in the next chapter.

Genetic Aberrations in Gastric Cancer

Genetic Mutations

Genetic mutation is one of the most common mechanisms of carcinogenesis [18]. HME has especially frequent mutations, including *TP53*, *PIK3CA*, *KRAS*, *ARID1A*, *PTEN*, *ERBB3*, and *HLA-B* [23]. In this subtype, histocompatibility complex class I genes, including *B2M* and *HLA-B*, are frequently mutated. These mutations may work better in hypermutated GCs by suppressing the immune response [23].

Some of these genes are also mutated in other epigenotypes, such as *TP53*, *ARID1A*, *KRAS*, and *PIK3CA* [23]. *TP53* mutation is the most frequent genetic aberration

Fig. 2. Molecular aberrations in epigenotypes. E-HME is observed in EBV-positive GC; frequently have *CDKN2A* methylation, *PIK3CA* mutations, and PD-L1/2 overexpression; and generally lack *TP53* mutations. Proto-oncogenes such as *MZT1* are activated by “enhancer infestation” [17]. HME often has *MLH1* methylation and abundant gene mutations, including *PIK3CA*, *ARID1A*, and RTK. LME contains GS and CIN subtypes. The GS subtype frequently has *CDH1/RHOA* mutations or *CLDN18-ARHGAP* fusion. The CIN subtype has genomic amplification of RTKs and frequently has *TP53* mutations. In each column, *open* and *closed circles* show unmethylated and hypermethylated regions, and the *strikethrough* shows gene mutation. E-HME, extremely high-methylation epigenotype; HME, high-methylation epigenotype; LME, low-methylation epigenotype; RTK, receptor tyrosine kinase; GS, genomically stable; CIN, chromosomal instability. MSI, microsatellite instability; EBV, Epstein-Barr virus; *H. pylori*, *Helicobacter pylori*.

Table 2. Characteristic histology and major molecular aberrations in each epigenotype

	Representative histology	DNA methylation	Gene mutations	Other genetic/epigenetic aberrations
E-HME	Gastric carcinoma with lymphoid stroma	<i>CDKN2A</i>	<i>PIK3CA</i> <i>ARID1A</i> <i>BCOR</i>	Latent EBV infection Enhancer infestation <i>PD-L1/2</i> overexpression
HME	None	<i>MLH1</i>	<i>PIK3CA</i> <i>ARID1A</i> RTK	
LME				
GS	Diffuse type		<i>CDH1</i> <i>RHOA</i>	<i>CLDN18-ARHGAP</i> fusion
CIN	Intestinal type		<i>TP53</i>	RTK-RAS activation

The histology is different among molecular subtypes [4]. In particular, E-HME demonstrates characteristic histology referring to as gastric carcinoma with lymphoid stroma. Gene mutations, structural variants, and sCNAs are also different among the subtypes [23]. E-HME, extremely high-methylation epigenotype; HME, high-methylation epigenotype; LME, low-methylation epigenotype; EBV, Epstein-Barr virus; RTK, receptor tyrosine kinase; GS, genomically stable; CIN, chromosomal instability; sCNAs, somatic copy number alterations.

among several driver genes of GCs [19]. p53 contributes to DNA repair, cell cycle control, and programmed cell death [40]. *TP53* mutations have been identified in both intestinal type and diffuse type and are detected in 71% of the CIN subtype [23]. Almost all EBV(+) GCs lack *TP53*

mutation; however, *PIK3CA*, *ARID1A*, and *BCOR* mutations are frequently observed in EBV(+) GCs. *RHOA* mutations are frequently observed in diffuse type/GS subtype. Because patients with diffuse-type GCs have a poor prognosis, *RHOA* mutation can be a clinically significant

target for treatment. *CDH1* germline mutation is widely known to be associated with hereditary diffuse gastric cancer. *CDH1* somatic mutation is frequently detected in diffuse type/GS subtype. TCGA study reported that 37% of the GS subtype has the *CDH1* somatic mutation [23].

sCNAs and Structural Variants

sCNAs and structural rearrangements also play an important role in cancer development through the selective activation or inactivation of oncogenes and tumor-suppressor genes [23]. The most frequently amplified genes in GC are associated with receptor tyrosine kinase (RTK)/RAS/mitogen-activated protein kinase (MAPK) signaling such as *HER2*, *EGFR*, *MET*, *FGFR2*, and *RAS* [3, 23]. There may be RTK/RAS/MAPK-related amplifications in 30–40% of GCs. In these GCs, therapeutic effects targeting RTK/RAS/MAPK-related signal transduction are expected. Additionally, amplifications of *JAK2*, *CD274* (*PD-L1*), and *PDCD1LG2* (*PD-L2*) are frequently observed in EBV(+) GCs [23]. These aberrant amplifications can be a target for immunotherapy.

TCGA study reported the *CLDN18-ARHGAP26* fusions in diffuse type/GS subtype [23]. *ARHGAP26* is associated with the Rho-effector pathway. The *CLDN18-ARHGAP* fusions are enriched in GS subtype and exclusively exist with both *RHOA* and *CDH1* mutations. This exclusivity suggests that both *CDH1/RHOA* mutations and *CLDN18-ARHGAP* fusion contribute to carcinogenesis depending on cell adhesion-related Rho signaling in this subtype [3, 23].

Summary of Histology and Genetic Aberrations in Each Epigenotype

Various molecular aberrations, including DNA methylation and gene mutations, are involved in gastric carcinogenesis. Interestingly, the frequency of genetic aberrations differs among epigenotypes (Table 2) [23]. E-HME is uniquely observed in EBV(+) GCs. EBV(+) GCs histologically demonstrate poorly differentiated carcinoma with dense infiltration of lymphocytes, referred to as gastric carcinoma with lymphoid stroma [4]. EBV(+) GCs frequently have *CDKN2A* silencing by DNA methylation. *PIK3CA* mutations, *PD-L1/2* overexpression, and lack of *TP53* mutations are frequently observed in this epigenotype (Fig. 2) [3, 23]. MSI, which is categorized as HME, often has *MLH1* silencing by DNA methylation. Although the MSI subtype generally lacks targetable amplifications, MSI has abundant gene mutations, including *PIK3CA*, *ARID1A*, and RTK. MSI also frequently has a mutation of histocompatibility complex class I genes, including *B2M*

and *HLA-B*, which may work better in hypermutated GCs by suppressing the immune response [23]. LME contains GS and CIN subtypes. GS subtype is generally of diffuse type and frequently has *CLDN18-ARHGAP* fusion or *CDH1/RHOA* mutations [3]. The CIN subtype, which has genomic amplification of RTKs, is generally of intestinal type and frequently has *TP53* mutations [23].

Conclusion

DNA methylation in the CpG island is associated with gastric carcinogenesis. GCs are classified into 3 epigenotypes by DNA methylation. In addition to DNA methylation, variable genetic aberrations are involved in gastric carcinogenesis. Interestingly, the frequency and targets of genetic aberrations vary depending on the epigenotype. Aberrations in the genome and epigenome are expected to synergistically interact and contribute to gastric carcinogenesis, and comprehensive analyses of those in GCs may help elucidate the mechanism of carcinogenesis.

Conflict of Interest Statement

The authors have no conflict of interest to disclose.

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Author Contributions

G.U. and A.K. drafted the manuscript. K.M., Y.M., M.U., and S.F. revised the manuscript critically for important intellectual content. M.F., T.U., and A.K. supervised this review article.

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