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Original

CpG Island Methylator Phenotype in Primary Gastric Carcinoma

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Abstract: Gastric cancers (GC) with methylation of multiple CpG islands have a CpG island methylator phenotype (CIMP) and they can have different biological features. The aim of this study was to investigate the DNA methylation status of GCs and its association with their clinicopathological features. We evaluated the methylation status of four genes (MINT1, MINT2, MINT25 and MINT31) in 105 primary GCs using bisulfite-pyrosequencing analysis. We classified tumors as CIMP-high (CIMP-H), CIMP-low (CIMP-L) or CIMP-negative (CIMP-N) based on the methylation of MINT1, MINT2, MINT25, and MINT31. Overall, the prevalence of CIMP-H, CIMP-L and CIMP-N was 22% (23/105), 52% (55/105) and 26% (27/105), respectively. We observed a significant difference in tumor stage (stages I–II vs. stages III–IV) between CIMP-H and CIMP-N tumors ($P = 0.0435$). No significant differences were observed in clinicopathological characteristics (gender, age, location and tumor differentiation) among the CIMP phenotypes. The prognoses of patients with a CIMP-H tumor is likely to be better than those with CIMP-L or CIMP-N tumors, but these differences are not statistically significant ($P = 0.074$ and $P = 0.200$). Our results suggest that CIMP may define a subgroup of GCs with distinct biological features.

Key words: gastric carcinoma, DNA methylation, CpG island methylator phenotype

Introduction

Gastric cancer (GC) arises from native gastric or metaplastic mucosa and is one of the most common malignancies worldwide[1)]. Both genetic and epigenetic alterations in a variety of tumor suppressor and tumor-related genes have functional roles during carcinogenesis. Changes in DNA methylation status are epigenetic events and represent the most common molecular alteration in human neoplasia[2)]. These changes in DNA methylation in cancer are classified as either genome-wide hypomethylation or gene promoter hypermethylation.

Promoter hypermethylation that leads to epigenetic silencing of multiple genes is an important mechanism in gastrointestinal carcinogenesis. Methylation of CpG dinucleotide-rich areas, termed CpG islands, occurs within the promoters of approximately 60% of human genes[3)]. These CpG

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islands are usually associated with long-term, irreversible, epigenetic silencing of X-linked and imprinted genes as well as tumor-related genes[2]. The concordant hypermethylation of multiple genes is termed the CpG island methylator phenotype (CIMP), and has been described in various cancers[4–7]. Recent studies have demonstrated that GCs can be classified as CIMP-high (CIMP-H), CIMP-low (CIMP-L) and CIMP-negative (CIMP-N) and that GCs with CIMP-H are associated with proximal tumor location, Epstein-Bar virus infection status and longer patient survival time[8].

The aim of this study was to evaluate the status of CIMP in primary GCs and to characterize the relationships between methylation status and clinicopathological features.

Materials and Methods

Patients and specimens

We collected a total of 105 primary GCs and 74 normal gastric mucosal tissues from 105 patients. All tissue specimens were obtained by endoscopic biopsy before treatment at Showa University Hospital. Corresponding adjacent normal-mucosa tissue specimens were also obtained from each patient case. Tumors were selected solely on the basis of availability. The ethics committee of Showa University School of Medicine approved the collection of tissue specimens.

Tissue specimens and DNA preparation

We examined 179 frozen tissue specimens (105 cancers and 74 adjacent normal-mucosa tissues) from 105 GC patients. Frozen tissue specimens were harvested by endoscopic biopsy and stored at −80°C until use. DNA was isolated from the frozen tissue specimens using standard proteinase K-phenol-chloroform extraction[9] or a QIAamp DNA mini kit (QIAGEN, Inc., Germantown, MD, U.S.A.).

Methylation-related genes and definition of CIMP

We studied the methylation status of four clones: MINT1, MINT2, MINT25 and MINT31. Firstly, we treated DNA methylation as a continuous variable. To define CIMP, however, we converted the continuous values into categorical variables (positive/negative), as defined by a methylation density greater than 15%. We classified GCs into three groups (CIMP-H, CIMP-L and CIMP-N), based on their methylation status of the promoters in CpG islands of MINT1, MINT2, MINT25, and MINT31. Tumors were classified as CIMP-H if three or more of these marker gene promoters were methylated; as CIMP-L if one or two marker gene promoters were methylated; and all other tumors were defined as CIMP-N.

Bisulfite PCR and pyrosequencing analysis of DNA methylation

Bisulfite treatment was carried out as previously described[10]. Aliquots of 2 or 3 μL of bisulfite-treated DNA were used as the template for the bisulfite polymerase chain reaction (PCR). The sequences of primers and the PCR conditions used to amplify specific DNA fragments of various target genes have been reported previously[11, 12]. The protocol for pyrosequencing was

Table 1. Clinicopathological characteristics of gastric carcinomas with CpG island methylator phenotype

		CIMP-H (N = 23)	CIMP-L / N (N = 82)	*P* value
Gender	male	19 (82%)	50 (61%)	0.0534
	female	4 (18%)	32 (39%)	
Age (yr)	mean	67.8	66.8	0.2452
	(range)	(36–86)	(28–93)	
Tumor location	upper third	8 (34%)	31 (39%)	0.9651
	middle third	8 (34%)	31 (39%)	
	lower third	7 (32%)	24 (28%)	
Macroscopic type	type 0/2	12 (52%)	25 (31%)	0.278
	type 3/4	11 (48%)	57 (69%)	
Histology*	tub/pap	10 (45%)	36 (44%)	0.9326
	por/sig/muc	12 (55%)	45 (56%)	
	missing	1	1	
Clinical Stage**	stage I–II	9 (39%)	15 (18%)	0.0355
	stage III–IV	14 (61%)	67 (82%)	
Therapy	operation	6 (26%)	18 (22%)	0.4379
	chemotherapy	8 (35%)	32 (39%)	
	SR + CT	7 (30%)	26 (32%)	
	BSC	2 (9%)	6 (7%)	

* tub, tubular adenocarcinoma ; pap, papillary adenocarcinoma ; por, poorly differentiated adenocarcinoma; sig, signet-cell carcinoma; muc, mucinous adenocarcinoma.
** Stage was classified according to the International Union against Cancer TNM classification system. SR, surgical resection ; CT, chemotherapy ; BSC, best supportive care.

previously described in detail[13]. Pyrosequencing measures the methylation status of several CpG sites in DNA. Usually, these different sites show highly concordant methylation. Therefore, we averaged the methylation percentage of all the CpG sites that we measured for each gene.

Data analysis and statistics

Continuous variables (*e.g.*, age) were analyzed using the Mann-Whitney test. Categorical variables were compared between tumor groups using the χ^2 test or Fisher's exact test when testing small sample numbers. Survival was assessed using the Kaplan-Meier method. Survival curves were compared using the long-rank test. All tests were two sided, and $P < 0.05$ was considered statistically significant.

Results

CIMP status and clinicopathological characteristics

The prevalence of CIMP-H, CIMP-L and CIMP-N among the 105 GCs was 22% (23/105), 52% (55/105) and 26% (27/105), respectively. Table 1 shows the relationship between CIMP status and clinicopathological characteristics of the patients with GC in this study. We observed a significant difference in stage (early *vs.* late stage) between CIMP-H and CIMP-N GC patients ($P = 0.0435$), whereas no significant differences were observed in any other clinicopathological

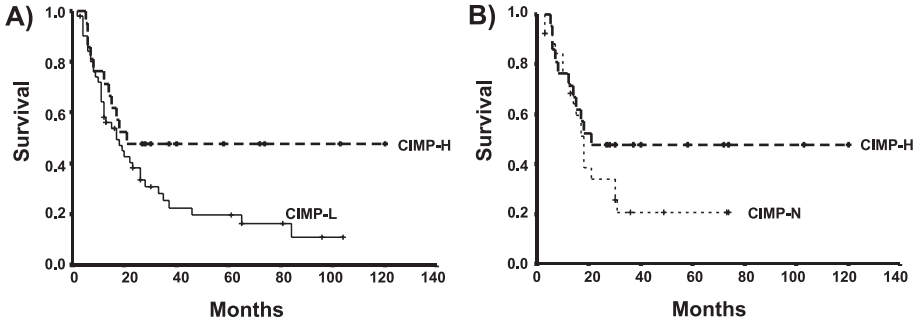

Fig. 1. Survival was compared among patients who had GCs with CIMP-H, CIMP-L and CIMP-N as assessed using the Kaplan-Meier method. Survival curves were compared using the log-rank test, but no significant differences were detected (CIMP-H *vs.* CIMP-L, $P = 0.070$; CIMP-H *vs.* CIMP-N, $P = 0.200$).

characteristics between patients with CIMP-H and CIMP-L/N GCs.

The survival of patients with CIMP-H, CIMP-L and CIMP-N GCs was characterized using Kaplan-Meier analysis and was compared using the long-rank test (Fig. 1). Patients with CIMP-H GCs tended to show longer survival time compared to those with CIMP-L or CIMP-N GCs (median survival: CIMP-H, 19 months; CIMP-L, 13 months; CIMP-N, 15 months, respectively). It should be noted that these differences were not statistically significant ($P = 0.074$ and $P = 0.200$, respectively).

Discussion

Toyota *et al*[14] proposed a definition of CIMP status in GCs that is based on the DNA methylation of the MINT markers, which were originally identified from colon cancer cells. GCs are classified as CIMP-L tumors and CIMP-H tumors, which are more likely to show distinct clinicopathological characteristics than CIMP-L tumors[8]. Several reports[8,14–17] have shown the prevalence of CIMP-H in GCs to range from 24% to 41% using MINT markers. The current study found CIMP-H in 22% of GCs using four MINT genes (MINT1, MINT2, MINT25 and MINT31). In other studies, CIMP was defined by the DNA methylation status of tumor-related genes, rather than MINT genes[18–20]. The prevalence of CIMP-H tumors in the present study is similar to previous results obtained using MINT markers. Although several sets of CIMP markers have been reported, CIMP-positive GCs are likely to define distinct clinicopathological features.

Kusano *et al*[8] reported that CIMP status is associated with the distinct clinicopathological features of GCs. Their study indicated that GCs with CIMP-H more frequently showed proximal tumor location, diffuse histology, and longer patient survival time, compared with CIMP-L or CIMP-N[8]. In the current study, CIMP-H was detected significantly more frequently in early (stages I–II) than late (stages III–IV) stage GCs. No significant differences were detected in any other clinicopathological characteristics, such as tumor location and differentiation, among the CIMP phenotypes. The higher frequency of CIMP-H in early stage GCs may account for the better prognosis for those patients. Chang *et al*[18] reported a lower frequency of lymph node metastasis in GCs with CIMP-H, which may reflect the lower malignant potential of GCs with

CIMP-H. On the other hand, Mitsuno *et al*[21] recently reported a strong association between CIMP-H and cancer-related genes. Patients with p16INK4a methylation were found to specifically benefit from 5-FU based adjuvant chemotherapy for GCs[21]. Several reports[22-24] have suggested that CHFR (Checkpoint with Forkhead-associated and RING finger domains) methylation might be a predictive marker for sensitivity of GCs to chemotherapy with microtubule inhibitors. Esteller *et al*[25] proposed that promoter hypermethylation of MGMT (O^6-methylguanine DNA-methyltransferase) was linked to the responsiveness of brain tumors to alkylating agents. These positive relationships between the DNA methylation of cancer-related genes and therapeutic efficacy might be implicated in the longer survival time of GC patients with CIMP-H. Our data shows that the survival time of GC patients with CIMP-H is likely to be longer than those with CIMP-L/CIMP-N GCs, but no statistical differences were observed in survival time among patients with different CIMP status. It should be noted that the proportion of patients at stages III–IV in the current study is higher than in the study by Kusano *et al* (77% *vs.* 56%, respectively)[8]. As shown in Figure 1, approximately half of the CIMP-H patients were terminated from the study for follow-up at 20 months. Nine patients died after chemotherapy or surgical resection followed by chemotherapy (stage IV, eight patients; stage IIIB, one patient), three patients (all stage II) moved to another hospital. These terminations from the study may have contributed to our failure to observe significant differences in survival among patients with different CIMP status. No significant difference was found in other parameters such as tumor location and histologic differentiation between GCs with CIMP-H *vs.* CIMP-L/CIMP-N. These observations could be explained by multiple factors, including: 1) about half of the samples in this study were stage IV GC, which more frequently show diffuse type cancer and shorter patient survival time than other types, independent of CIMP status[17]; 2) EB-virus status was not examined in this study because EBV-positive CIMP-H GCs are more likely to be diffuse carcinomas or to be located proximally in the stomach, compared with EBV-negative tumors[8]; 3) we used different CIMP-markers from previous studies.

In summary, our data indicates that CIMP-H represents a specific subgroup of GCs with distinct biological features. Clarification of the utility of CIMP status as a valuable prognostic marker for GCs awaits further study.

Conflict of interest

The authors have declared no conflict of interest.

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