J Gastroenterol 2006; 41:733–739
DOI 10.1007/s00535-006-1841-y

Journal of Gastroenterology

Phenotype analysis by MUC2, MUC5AC, MUC6, and CD10 expression in Epstein-Barr virus-associated gastric carcinoma

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Background. Gastric marker mucins (MUC5AC and MUC6) and intestinal marker molecules (MUC2 and CD10) have been used to determine the cell lineage of epithelial cell of gastric carcinoma (GC). ***Methods.*** To clarify the characteristics of Epstein-Barr virus (EBV)-associated GC, 18 cases were immunohistochemically evaluated along with 56 cases of EBV-negative GC. ***Results.*** MUC2 expression was lower in EBV-associated GC: immunostaining grades 0, 1, 2, 3, and 4 were observed in 10, 6, 1, 1, and 0 cases of EBV-associated GC, respectively, and in 18, 11, 15, 6, and 6 cases of EBV-negative GC, respectively ($P = 0.013$). CD10 positivity (grades 2–4) in EBV-associated GC was 6%, significantly lower than in EBV-negative GC (34%) ($P = 0.030$). When phenotypes of GC were categorized by the combined positivities of gastric markers (either MUC5AC or MUC6) and intestinal markers (either MUC2 or CD10), EBV-associated GC included primarily null (44%) and gastric (39%) types, but EBV-negative GC comprised null (7%), gastric (30%), intestinal (27%), and mixed (36%) types. The age of patients with gastric types was significantly younger for both EBV-associated GC and EBV-negative GC cases. ***Conclusions.*** Neoplastic epithelial cells of EBV-associated GC did not express MUC2 or CD10, and most of them were categorized as null or gastric types. EBV infection may occur in the epithelial cells of null or gastric phenotypes, which may be devoid of transdifferentiation potential toward intestinal phenotypes.

Key words: Epstein-Barr virus, gastric carcinoma, immunohistochemistry, MUC, CD10

Introduction

Epstein-Barr virus (EBV)-associated gastric carcinoma (GC) is a distinct subset of GC, accounting for 10% or less of total GC.[1] It occurs predominantly in the fundic gland mucosa of the gastric corpus, and its histology is characteristic, moderately differentiated tubular, or poorly differentiated solid-type histology admixed with lymphocytic infiltration of various degree. EBV-infected neoplastic gastric epithelia also show unique cell biological features, such as production of interleukin-1β and insulin-like growth factor (IGF)-1, and abnormal or decreased expression of CD44-variant and cytokeratin molecules.[2–5] However, few studies have investigated the cell lineage of neoplastic cells of EBV-associated GC.[6]

Mucins are high molecular weight glycoproteins, which are major components of the mucus layer covering the surface of epithelial tissues, and they consist of a mucin core protein (apomucin) and *O*-linked oligosaccharides.[7] Apomucins are encoded by 14 different mucin genes (*MUC1, 2, 3, 4, 5AC, 5B, 6, 7, 8, 9, 11, 12, 13*, and *16*). In the gastric mucosa, predominant mucins are MUC1, MUC5AC, and MUC6; MUC5AC is highly expressed in the foveolar epithelium, and MUC6 is expressed in the mucous neck cells of fundic glands and in the pyloric glands.[8] On the other hand, MUC2, similar to the CD10 molecule, is not present in the normal gastric mucosa, but is expressed in intestinal metaplasia.[9] These cell-specific molecules have been used to trace the cell lineage of neoplastic epithelial cells of the stomach.[7,10–14] Thus, in the present study, we investigated the phenotypic characteristics of EBV-associated GC to clarify its origin and the target of EBV-infection in the stomach.

Received: November 6, 2006 / Accepted: April 15, 2006
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Materials and methods

Patients and samples

The specimens used in the study were derived from stomachs resected for the treatment of gastric cancer at Jichi Medical School Hospital between 1997 and 1999. Surgically resected specimens were fixed with 10% formalin and embedded in paraffin. Histological and pathological data were evaluated according to the Japanese Classification of Gastric Carcinoma,[15] but the Lauren classification[16] was adopted for the histological classification. The presence of EBV in carcinoma tissue was determined by applying *EBER1* in situ hybridization to the formalin-fixed, paraffin-embedded sections, as has been reported previously.[17]

Eighteen cases of EBV-associated GC and 56 cases of EBV-negative GC were retrieved from the archives. The series included relatively many cases of EBV-associated GC, with the aim of finding the difference between GC with and without associated EBV. Otherwise, EBV-negative carcinomas were selected without any bias. The clinicopathological data of the tumors are summarized in Table 1.

Immunohistochemistry

Immunohistochemical staining was carried out with the following monoclonal antibodies: MUC5AC (clone, CLH2; optimal dilution, 1:50); MUC6 (CLH5; 1:50); MUC2 (Ccp58; 1:100); and CD10 (56C6; 1:100) (Novocastra, Newcastle, UK).

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections by the ABC method. Briefly, each section was deparaffinized with xylene and rehydrated in alcohol. For antigen retrieval, the sections were autoclaved in 0.01 M citrate-phosphate buffer, pH 6.0, for monoclonal antibodies against MUC2, MUC5AC, and MUC6, and in the same buffer, pH 7.0, for CD10. The sections were incubated with monoclonal antibodies at room temperature for 1 h. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol for 10 min, a standard avidin-biotin immunoperoxidase technique was used for visualization of the reactive product. The sections were incubated with avidin-biotin complex (Vectorstain ABC kit, Vector Laboratories, Burlingame, CA, USA), and the reaction product was visualized by incubation with 3′-3′-diaminobenzadine. Then, the slides were counterstained with Mayer's hematoxylin for 5 min and dehydrated in alcohol prior to mounting. Negative controls were made by replacing the primary antibodies with nonimmune mouse serum.

Table 1. Summary of the clinicopathological data of the examined gastric carcinomas

EBV-associated GC/EBV-negative GC	18/56
Sex (male/female)	42/32
Age	61.9 ± 13.5[a]
Tumor size (cm)	5.1 ± 3.7[a]
Location (upper/lower)	50/24
Depth (m/sm/mp/ss/se/si)	16/20/5/14/13/6
Histology (pap/tub1/tub2/por1/por2/sig)	3/24/15/10/11/11
Lauren's classification (intestinal/diffuse)	42/32
Lymphatic infiltration	49
Vessel infiltration	37
Lymph node metastasis	24

EBV, Epstein-Barr virus; GC, gastric carcinoma
[a] Mean ± SD
The author needs to explain the meaning of the symbols within parentheses in the Depth and Histology rows.

Evaluation of immunostaining

Scoring of the immunohistochemical results was as follows: grade 0, no positive cells; grade 1, some positive cells (<25%); grade 2, well-defined area of positive cells (25%–50%); grade 3, significant areas of positive cells (50%–75%); and grade 4, extensive areas of positive cells (>75%).[13,18] In the statistical analysis, immunostaining grades 2–4 were regarded as positive.

Statistical analysis

The relationships between gastric carcinoma with or without EBV and clinicopathological factors were evaluated by Fisher's exact probability test, Student *t* test, or unpaired Wilcoxon test. Differences were considered to be significant at $P < 0.05$.

Results

The immunohistochemical results for gastric-type mucin (MUC5AC and MUC6) and intestinal-type molecules (MUC2 and CD10) are presented in Table 2 and Fig. 1. Expression of MUC5AC and MUC6 were observed less frequently in EBV-associated GC than in EBV-negative GC, but the differences were not statistically significant. On the other hand, MUC2 expression was lower in cases of EBV-associated GC: immunostaining grades 0, 1, 2, 3 and 4 were observed in 10, 6, 1, 1, and 0 cases, respectively, of EBV-associated GC, and in 18, 11, 15, 6, and 6 cases, respectively, of EBV-negative GC. CD10 positivity (grades 2–4) in EBV-associated GC was 6%, which was significantly less frequent than in EBV-negative GC (34%).

Table 2. Expression of Mucin/CD10 molecules in EBV-associated GC and EBV-negative GC.

	Number with expression grade 0, 1/2, 3, 4 Positivity %			
	Gastric mucin		Intestinal-type molecule	
	MUC5AC	MUC6	MUC2*	CD10**
EBV-associated GC (n = 18)	5, 5/1, 6, 1 44%	9, 5/2, 2, 0 22%	10, 6/1, 1, 0 11%	11, 6/1, 0, 0 6%
EBV-negative GC (n = 56)	12, 9/13, 15, 7 63%	24, 8/17, 6, 1 43%	18, 11/15, 6, 6 48%	31, 6/11, 7, 1 34%

*Significantly lower in EBV-associated GC; Fisher's exact test, $P = 0.005$, and unpaired Wilcoxon test, $P = 0.013$
**Significantly lower in EBV-associated GC; Fisher's exact test, $P = 0.030$

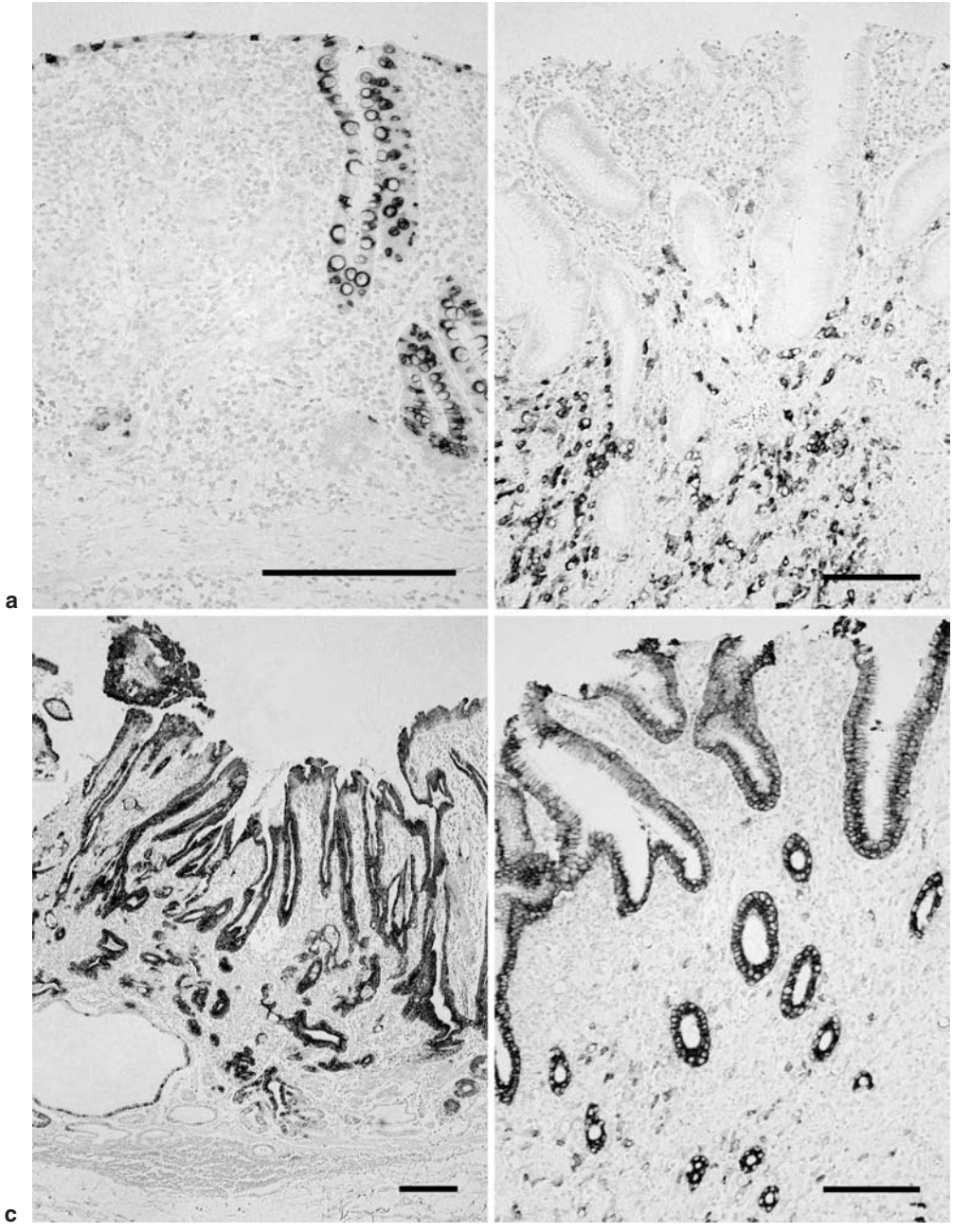

Fig. 1. a Immunohistochemical staining with anti-MUC2 antibody in a case of Epstein-Barr virus (EBV)-associated gastric carcinoma (GC). Cancer cells in the lamina propria mucosae were negative, whereas noncancerous epithelium showing intestinal metaplasia was positive. **b** Positivity for MUC2 in a case of EBV-negative GC: a poorly differentiated adenocarcinoma in the lamina propria mucosae. The noncancerous surface epithelium was negative. **c** Positivity of MUC5AC in a case of EBV-associated GC. **d** A case of EBV-negative GC (the same as shown in **b**) was negative for MUC5AC. The noncancerous surface epithelium was positive for MUC5AC

Relationship of cell-specific molecules with clinicopathological factors in GC

To obtain insight into the significance of these molecules in GC, their correlations with clinicopathological factors were statistically evaluated in EBV-associated GC and EBV-negative GC. No significant relationship was identified in cases of EBV-associated GC. In contrast, MUC2 expression was less frequent in advanced-stage EBV-negative GC than in early-stage cases ($P = 0.023$), and MUC5AC expression was more frequent in cases of EBV-negative GC in female patients than in cases in male patients ($P = 0.047$).

Phenotype classification of EBV-associated GC and EBV-negative GC

On the basis of the criteria of Tsukashita et al.,[11] four phenotypes of GC were recognized by the combination of the positivities of gastric markers (either MUC5AC or MUC6) and intestinal markers (either MUC2 or CD10): null (–/–), gastric (+/–), intestinal (–/+), and mixed (+/+) phenotypes (Fig. 2 and Table 3).

EBV-associated GC consisted basically of two types, the null type (44%) and the gastric type (39%), whereas EBV-negative GC consisted of null (7%), gastric (30%), intestinal (27%) and mixed (36%) types. The proportion of null types was significantly different between EBV-associated and EBV-negative GC. The difference persisted even after further classification into subgroups by tumor stage or tumor location (Fig. 2b–e).

Phenotypes showed an age-dependent distribution; the gastric type of EBV-associated GC occurred in significantly younger patients than the other types of EBV-associated or EBV-negative GC. The mixed type of EBV-negative GC occurred in older patients than the other types of EBV-negative GC.

Discussion

MUC5AC, MUC6, MUC2, and CD10 are used to determine the cell lineage of epithelial cells of the stomach.[7,10–14] In this context, it is interesting that MUC6 and MUC2 expression of EBV-negative GC was less frequent in advanced-stage than in early-stage disease. It is possible that some markers are lost as the carcinoma progresses, for example, MUC2 and MUC6 in this study. According to Kawachi et al.,[14] differentiated GCs at the minute stage within the mucosa usually develop at first without mucin expression, that is, the null type in our study, and they begin to produce mucin only as the tumor grows larger. In the present study, EBV-associated GC dominantly showed null or gastric phenotypes, suggesting that EBV-associated GC either retains the phenotype of an early stage of carcinogenesis or genuinely differentiates. Since both types of intestinal markers, MUC2 and CD10, were rarely expressed in EBV-associated GC even at an early stage, the absence of both markers was not due to loss of expression. Rather, their absence indicates that the neoplastic epithelial cells of EBV-associated GC are devoid of transdifferentiation potential toward the intestinal epithelium.

Phenotype analysis has been applied to GC, coupled with the recognition of four types: null, gastric, intestinal, and mixed types.[11,14] In EBV-associated GC, two phenotypes predominated, null and gastric types. The former type was rarely identified in EBV-negative GC, and thus indicates a specific cell lineage of EBV-associated GC. In our previous studies,[17] some *EBER*-positive cells were identified in surface epithelial cells in the nonneoplastic mucosa of the stomach. The target of EBV infection in the stomach may be the proliferating component of the neck zone, which shows the null or gastric phenotype.

The *MUC2*, *MUC5AC*, and *MUC6* genes are located on chromosome 11p15, which is known as the aggregate

Table 3. Cell type classification in EBV-associated and EBV-negative GC

	EBV-associated GC			EBV-negative GC			
	Null	Gastric	Others	Null	Gastric	Intestinal	Mixed
Number	8	7	3	4	17	15	20
Age	63.5 ± 12.7	43.9 ± 13.9*	71.3 ± 14.6	59.0 ± 11.2	59.9 ± 12.7	62.0 ± 10.50	68.2 ± 11.6**
Female	2 (25%)	2 (29%)	1 (33%)	1 (25%)	12 (71%)	5 (33%)	9 (45%)
Advanced stage	4 (50%)	4 (57%)	0 (0%)	3 (75%)	12 (71%)	7 (47%)	8 (40%)
Upper location	5 (63%)	7 (100%)	3 (100%)	1 (25%)	12 (71%)	9 (60%)	13 (65%)
Lauren's intestinal type	2 (25%)	3 (43%)	0 (0%)	2 (50%)	10 (59%)	6 (40%)	9 (45%)
Nodal involvement	3 (38%)	3 (43%)	0 (0%)	2 (50%)	7 (41%)	2 (13%)	7 (35%)

* Significantly younger patients, $P = 0.0038$, compared with other types of EBV-associated GC, and $P = 0.00012$, compared with the remaining cases; unpaired Student *t* test

** Significantly older patients, $P = 0.023$, compared with other types of EBV-negative GC, and $P = 0.0013$, compared with the remaining cases

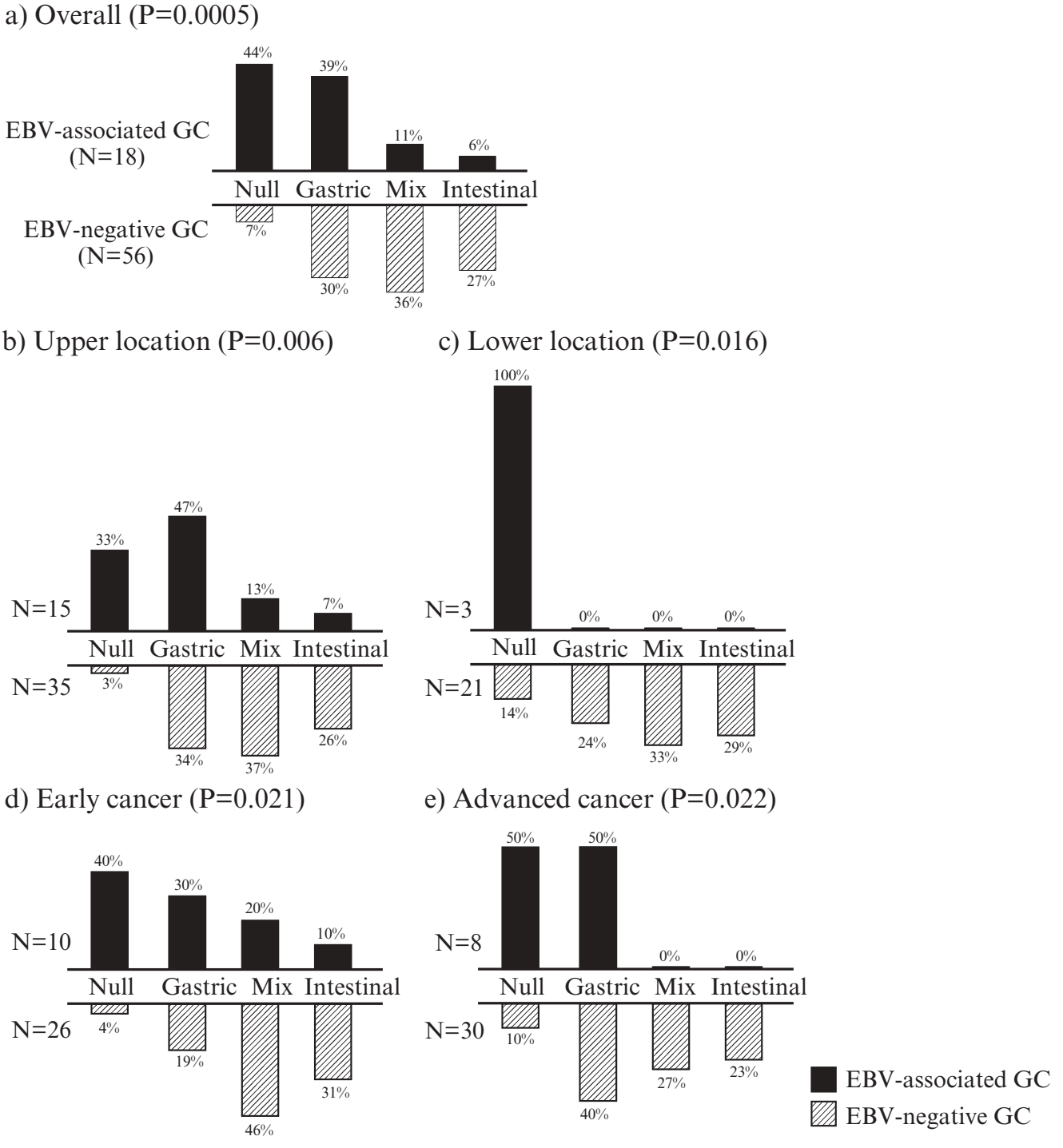

Fig. 2a–e. Distribution of mucin phenotypes in EBV-associated GC and EBV-negative GC. **a** Overall: EBV-associated GC expressed two predominant phenotypes: the null and gastric types; the intestinal type was very rare ($P = 0.0005$). EBV-negative GC primarily expressed three phenotypes: the gastric, intestinal, and mixed types. **b, c** Phenotype distribution among GCs with upper or lower locations. **d, e** Phenotype distribution among early and advanced cancer. Among these subgroups, the phenotypes of EBV-associated GC differed from those of EBV-negative GC

site of imprinting genes such as *IGF2* and *H19*.[19] Recently, some studies have reported that the expression of MUC2 or MUC5AC may be regulated by promoter methylation in pancreatic[20] and colon cancer cells.[21] On the other hand, global and nonrandom DNA methylation occurs in EBV-associated GC,[22,23] raising the possibility that epigenetic changes are responsible for the silencing of the genes *MUC2*, *MUC5AC*, and *MUC6*. However, our preliminary study of EBV-infection in gastric cancer cell lines showed consistency in the expression of these molecules (data not shown).

Among EBV-negative GC, advanced-stage tumors expressed more MUC2 than early-stage tumors. MUC2 might be related to less biological aggressiveness. Utsunomiya et al.[24] reported that MUC2 expression is a prognostic factor for a favorable outcome in GC patients. Our data are compatible with their report. There was a correlation between MUC5AC and male sex in our study, but no such relationship was reported by another study.[25]

It is interesting that the phenotypes of GC correlated with age in both EBV-associated and EBV-negative GC (Table 3). The gastric phenotype of GC was observed in relatively younger patients, suggesting, at least in EBV-associated GC, an age-dependent capacity of the stem cells of the stomach; the potential to differentiate toward the authentic gastric phenotype might be lost as a patients ages.

In EBV-negative GC, the mixed type occurred in a significantly older population than the other phenotypes. According to Sugai et al.,[26] each phenotype shows a distinct profile of genetic abnormalities in morphologically differentiated GC, such as 3p loss of heterozygosity (LOH) in the gastric type and 5q LOH in the intestinal type. The mixed phenotype of differentiated GC showed a different LOH pattern or microsatellite instability, suggesting that these types develop independently. It is thus further necessary to extrapolate these findings and to clarify the significance of phenotype classification in diffuse-type and advanced-stage carcinomas with respect to genetic abnormalities and biological behavior.[10,13]

In conclusion, EBV-associated GC showed a characteristic expression pattern of MUC5AC, MUC6, MUC2, and CD10, namely, null and gastric phenotypes. Therefore, EBV infection may occur in the epithelial cells of null or gastric phenotypes and be devoid of transdifferentiation potential to the intestinal epithelium, thus corresponding to the gastric-committed stem cells of the glands. Phenotype analysis may be also useful in EBV-negative GC to identify a specific subgroup, such as a mixed phenotype.

Acknowledgments. This work was supported by a Grant-in Aid for Scientific Research (A) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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