Reduced Expression and Promoter Methylation of p16 Gene in Epstein-Barr Virus-associated Gastric Carcinoma

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Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) is a unique type of gastric carcinoma (GC), which is considered to develop in a different pathway from EBV-negative GC. To evaluate a possible role of p16, an inhibitor of G1/S transition of the cell cycle, in the carcinogenesis of EBVaGC, p16-immunohistochemistry and methylation-specific PCR analysis (MSP) were applied to surgically resected gastric carcinomas. When the percentage of p16-positive cells in more than 1000 carcinoma cells was expressed as p16 labeling index (p16-LI), it ranged from 2.5 to 88.1 (mean 42.9±24.4) in 70 gastric carcinomas. EBVaGC showed significantly lower values (n=15, 26.1±22.1) than EBV-negative GC (n=55, 47.5±23.2) (P=0.0036). Fresh frozen tissues of 55 gastric carcinomas (16 EBVaGC and 39 EBV-negative GC) were further subjected to MSP, to evaluate abnormal methylation of the promoter region of the p16 gene. The frequency of methylation was significantly higher in EBVaGC (14/16) than in EBV-negative GC (9/39) (<0.0001). The methylation-positive carcinomas showed significantly lower p16-LI (35.9±21.6) than the unmethylated ones (55.2±22.7) (P=0.0014). Thus, a marked decrease of p16 expression, caused by the aberrant methylation of the p16 gene promoter, is closely associated with the development of EBVaGC.

Key words: Epstein-Barr virus — Gastric carcinoma — p16 — Methylation

Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) accounts for 10% or less of gastric carcinoma (GC), and is distributed worldwide without any regional accumulation.1, 2 EBV is closely related to the genesis of EBVaGC: EBV-encoded small RNA (EBER) is present in nearly all of the carcinoma cells in the intra-mucosal stage. EBV in EBVaGC is monoclonal by Southern blot hybridization analysis with probes adjacent to the unique terminal repeat of EBV DNA.3 EBVaGC also has some characteristic clinicopathological features, such as male preference, predominant involvement of the proximal stomach, frequent accompaniment of atrophic gastritis, and moderately differentiated tubular or poorly differentiated solid type of histology.1, 2, 4) There are several specific molecules expressed in EBVaGC; variant forms of CD44, a cell-surface glycoprotein that acts as an adhesion molecule,5 and interleukin (IL)-1β,6 which may exert an autocrine growth effect in EBV-infected carcinoma cells. The carcinogenic process of EBVaGC is considered to be quite different from that of EBV-negative GC.

When we consider the carcinogenesis of a virus-associated neoplasm, both aspects, the virus itself and the infected cells, should be taken into consideration. Gene expression of EBV in EBVaGC is restricted to Latency-I genes (EBNA1, EBER1, BARF0, and LMP2A), as in Burkitt lymphoma. EBNA2 and LMP1, capable of immortalization of human lymphocytes or transformation of rodent fibroblasts, respectively, are not expressed in these neoplasms.7) As for the genetic changes in the infected cells, alteration of chromosomes 5q and 17p and microsatellite instability are both infrequent in EBVaGC.8) Recently, Schneider et al. reported that decreased expression of p16, an inhibitor of G1/S transition of cell cycle, was frequently observed in EBVaGC, although the fact may reflect the predilection of EBVaGC for the proximal stomach.9) In the present paper, we have also investigated the abnormality of p16 expression in gastric carcinoma with and without association of EBV, as well as the aberrant methylation of the promoter region of p16 gene, which might be responsible for the abnormality.10–15

MATERIALS AND METHODS

The material consisted of 70 cases of gastric carcinomas, which were surgically resected at Jichi Medical School or Tokyo Metropolitan Komagome Hospital from 1991 to 1998. Fresh tissue of gastric cancer was also obtained from 45 of 70 cases described above and from 10 additional cases without any selection. The tissues were frozen in liquid nitrogen immediately after surgical resec-
tion and stored at −80°C, until they were used for the methylation-specific PCR (MSP). Pathology of the resected stomachs was evaluated according to the Japanese Classification of Gastric Carcinoma.16) We also adopted Lauren’s classification of gastric carcinoma, intestinal and diffuse.17) To determine the presence or absence of EBV, EBER-1-in situ hybridization was performed as reported previously.3)

**Immunohistochemistry of p16** Four micrometer-thick sections were cut from the formalin-fixed and paraffin-embedded specimens. The sections were deparafinized in xylene, hydrated in alcohol, and treated with 0.3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. For antigen retrieval, the sections were autoclaved in 0.01 M citrate-phosphate buffer (pH 6.0) at 121°C for 10 min. They were blocked with normal goat serum at 37°C for 30 min, then immunohistochemistry was performed using a mouse monoclonal antibody specific for human p16 (clone F-12, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h. The sections were then stained with the Vectorstain ABC kit (Vector Laboratories, Burlingame, CA), and counterstained for 5 min with Mayer’s hematoxylin. As a negative control, the primary antibodies were omitted.

As for the interpretation of the immunohistochemical results, stromal cells served as an internal positive control, and nuclear staining for p16, but not cytoplasmic staining, was regarded as positive. Since the frequency of p16-positive carcinoma cells varied considerably within the whole carcinoma tissue, we counted the positive ratio rather than classifying the carcinoma into positive or negative. In each section, ten high power fields were selected at random, and at least 1000 tumor cells were evaluated to obtain the percentage of positively stained cells (p16-labeling index, p16-LI). All immunostained sections were coded and evaluated without prior knowledge of EBER-1-in situ hybridization or the outcome of MSP.

**MSP** Genomic DNA was extracted by a standard phenol/chloroform procedure. Bisulfite modification for MSP was

| Table I. Clinicopathological Characteristics and p16-expression in Gastric Carcinoma | No. of cases | Labeling index (mean±SD) | P value |
|---------------------------------------------|-------------|-------------------------|--------|
| Sex                                         |             |                         |        |
| Male                                        | 52          | 44.8±25.4               | 0.2317 |
| Female                                      | 18          | 37.5±21.3               |        |
| Location                                    |             |                         |        |
| Upper                                       | 16          | 40.4±26.8               | 0.8553 |
| Middle                                      | 37          | 43.2±24.3               |        |
| Lower                                       | 17          | 44.6±23.8               |        |
| Histologic type                             |             |                         |        |
| Intestinal                                  | 45          | 38.9±21.2               | 0.0896 |
| Diffuse                                     | 25          | 50.1±28.5               |        |
| Depth                                       |             |                         |        |
| Early                                       | 34          | 38.3±23.1               | 0.1403 |
| Advanced                                    | 36          | 47.3±25.2               |        |
| Lymphatic invasion                          |             |                         |        |
| Positive                                    | 48          | 45.6±25.6               | 0.1718 |
| Negative                                    | 22          | 37.1±22.5               |        |
| Vascular invasion                           |             |                         |        |
| Positive                                    | 46          | 46.0±25.5               | 0.1443 |
| Negative                                    | 24          | 36.9±21.6               |        |
| Lymph node metastasis                       |             |                         |        |
| Positive                                    | 26          | 48.6±23.9               | 0.1302 |
| Negative                                    | 44          | 39.6±24.4               |        |
| EBER-1                                      |             |                         |        |
| Positive                                    | 15          | 26.8±22.1               | 0.0036 |
| Negative                                    | 55          | 47.5±23.2               |        |
| MSP                                         |             |                         |        |
| Positive                                    | 18          | 33.7±21.3               | 0.0014 |
| Negative                                    | 27          | 57.1±20.6               |        |

MSP: methylation-specific PCR.

| Table II. Clinicopathological Characteristics and Methylation Status of p16 Gene Promoter in Gastric Carcinoma | p16 unmethylated group (n=32) | p16 methylated group (n=23) | P value |
|-------------------------------------------------|------------------------------|-----------------------------|--------|
| Age (year)                                      | 61.4±16.9                    | 60.4±13.8                   | 0.9105 |
| Sex                                             |                              |                             |        |
| Male                                            | 23                           | 19                          | 0.3554 |
| Female                                          | 9                             | 4                           |        |
| Location                                        |                              |                             |        |
| Upper                                           | 8                             | 7                           | 0.2052 |
| Middle                                          | 15                            | 14                          |        |
| Lower                                           | 9                             | 2                           |        |
| Histologic type                                 |                              |                             |        |
| Intestinal                                      | 13                            | 10                          | 0.8324 |
| Diffuse                                         | 19                            | 13                          |        |
| Depth                                           |                              |                             |        |
| Early                                           | 6                             | 7                           | 0.3144 |
| Advanced                                        | 26                            | 16                          |        |
| Lymphatic invasion                              |                              |                             |        |
| Positive                                        | 30                            | 18                          | 0.0891 |
| Negative                                        | 2                             | 5                           |        |
| Vascular invasion                               |                              |                             |        |
| Positive                                        | 28                            | 17                          | 0.1975 |
| Negative                                        | 4                             | 6                           |        |
| Lymph node metastasis                           |                              |                             |        |
| Positive                                        | 23                            | 14                          | 0.3909 |
| Negative                                        | 9                             | 9                           |        |
| EBER-1                                          |                              |                             |        |
| Positive                                        | 2                             | 14                          | <0.0001|
| Negative                                        | 30                            | 9                           |        |
performed with the CpGenome DNA modification kit (Intergen, Purchase, NY). MSP was then performed to examine the methylation status of the promoter region of the \( p16 \) gene with the CpG WIZ Amplification kit (Intergen) according to the manufacturer’s recommendation. The temperature profiles for the amplification were as follows: 95°C for 5 min, 35 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The amplification was always accompanied by a positive control (universal methylated DNA; Intergen) and a negative control (distilled water). PCR products were electrophoresed in 2% agarose gel, which was stained with ethidium bromide.

**Statistical analysis** Statistical analysis was carried out using the Mann-Whitney \( U \) test, and two-tailed Fisher’s exact test. Differences with \( P \) value <0.05 were considered as significant.

**RESULTS**

The results are presented in Tables I and II.

**p16-immunohistochemistry** Seventy cases (15 EBVaGC and 55 EBV-negative GC) were immunohistochemically evaluated. The frequency of p16-positive carcinoma cells varied considerably within the carcinoma tissue: p16-LI ranged from 2.5 to 88.1 (mean 42.9±24.4, Fig. 1). P16-LI was not different in regard to sex of the patients, or any pathological factor of the carcinoma, including location, histologic type, depth, lymphatic and vascular invasion and lymph node metastasis (Table I). On the other hand, EBVaGC showed significantly lower values (26.1±22.1) than EBV-negative GC (47.5±23.2) (\( P=0.0036 \)).

**Aberrant methylation of the promoter region of the \( p16 \) gene** We next evaluated the aberrant methylation of the promoter region of the \( p16 \) gene in gastric carcinomas, since this might be responsible for the down-regulation of the gene expression. Fifty-five carcinoma tissues (16 EBVaGC and 39 EBV-negative GC) were examined. In each sample, PCR products for both or either of unmethylated (154 bp) and methylated (145 bp) primers were clearly identified (Fig. 2), and there were 18 cases which showed MSP products (Table II). It is especially noteworthy that 14 of 16 EBVaGC (87.5%) showed aberrant methylation in the \( p16 \) gene promoter. The frequency is

Fig. 1. Immunohistochemistry of p16 in gastric carcinoma with or without association of Epstein-Barr virus (EBV). p16-expression is markedly reduced in a case of EBV-associated gastric carcinoma (A), but there are still several cells showing positive staining. Since the neoplastic glands were situated beyond the mucosal layer, the positive cells are not integrated normal epithelial cells. Also note the positive staining in the interstitial cells. On the other hand, there are many positive cells for p16 in a case of EBV-negative gastric carcinoma (B).
significant higher than that of EBV-negative GC (9/39, 23.1%) (<0.0001). Otherwise, the methylation status had no relationship with any clinicopathologic factors.

As for the correlation between methylation and the expression of p16, the methylation-positive group showed significantly lower p16-LI (35.9±21.6) than the unmethylated group (55.2±22.7) (P=0.0014).

**DISCUSSION**

The p16 gene, located on chromosome 9p21, is a tumor suppressor gene, which inhibits G1/S transition of the cell cycle by binding to cyclin D/cyclin-dependent kinase 4 (CDK4) or CDK6. Loss of p16-expression has been observed in various neoplasms, such as leukemia, brain tumor, malignant melanoma, esophageal carcinoma and lung carcinoma. In the present study of p16-immunohistochemistry in gastric carcinoma, we observed marked decrease of p16-expression rather than total loss, as was reported in several studies. It is unlikely that the difference in the observations is due to the methods used, since the same results as ours and that of Jang et al. were also obtained by Tsujie et al. who used antibody derived from a different source. Rather, the difference may be due to the sensitivity of the detection system or the size of the examined tissue. We consider that our view of variation of expression fits better with recent findings of the regulation of p16-expression by promoter methylation. Bender et al. demonstrated that the expression of p16 is highly affected by the frequency, but not by the existence itself, of methylation of the p16 promoter in human bladder, colon cancers, and melanoma cell lines.

DNA methylation is a powerful modifying factor of genomic DNA. It is an epigenetic event, which occurs at cytosine located 5'- to guanosine in the CpG dinucleotide (CpG). Recently, such hypermethylation of CpG-islands of tumor suppressor genes has been regarded as one of the pathways of suppressor gene inactivation. In the present study, we observed a significant correlation between aberrant methylation by MSP and decreased p16-expression. This supports the idea that aberrant methylation of the promoter region may be responsible for the decreased expression of p16 in gastric carcinoma. However, it is still undetermined whether hypermethylation occurs in a uniform manner or in a sub-population of the carcinoma. Another possibility is that the maintenance of hypermethylation is considerably disturbed in the progression of the carcinoma, even if hypermethylation plays an important role in the early stage of the development of the carcinoma. This seems likely in EBVaGC, since methylation was observed in nearly all of the cases.

The decrease of p16-expression and the methylation of the p16 gene promoter were both intimately associated with EBV infection in gastric carcinomas. The latter result is consistent with the data of Kang et al., and our findings clearly demonstrated that EBV infection is the sole factor correlating with the methylation of p16 gene promoter among various clinicopathological factors. An important role of DNA methylation is as a genome defense mechanism against infectious agents. In viral infection, restriction to latent infection is induced by the methylation of the episomal DNA of EBV or of the integrated virus genome, such as in human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type-1. Hypermethylation of the promoter region of LMP1 of EBV genome results in the silencing of LMP1-expression, which has been observed in various EBV-associated tumors, including EBVaGC. Furthermore, methylation is not restricted to the viral DNA; acute infection of lymphoid cells by HIV-1 results in increased DNA methyltransferase expression, an overall increase in methylation of DNA in infected cells, and even the de novo methylation of a CpG dinucleotide in the interferon (IFN)-γ gene promoter. Thus, it is possible that exaggeration of virus-induced methylation leads to the aberrant methylation of the CpG-island of tumor suppressor genes in EBV-infected cells, resulting in the development of EBVaGC.
In conclusion, marked decrease of p16-expression, caused by the aberrant methylation of p16 gene promoter, is closely associated with the development of EBVaGC. Further studies are necessary, such as global profiling of the promoter methylation and the methyltransferase expression, to clarify the mechanism and significance of the methylation in this unique type of gastric carcinoma, EBVaGC.

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