Jpn. J. Cancer Res. **93**, 1195–1200, November 2002

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

Toshiya Osawa,[1,2] Ja-Mun Chong,[1] Makoto Sudo,[1,2] Kazuya Sakuma,[1,3] Hiroshi Uozaki,[1] Junji Shibahara,[1] Hideo Nagai,[3] Nobuaki Funata[4] and Masashi Fukayama[1,5]

[1]Department of Pathology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, [2]Department of Surgery, Yamanashi Medical University, Tamaho-cho, Nakakoma-gun, Yamanashi 409-3898, [3]Department of Surgery, Jichi Medical School, 3311-1 Yakushiji, Minami-Kawachi-machi, Kawachi-gun, Tochigi 329-0498 and [4]Department of Pathology, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-0021

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-

[5] To whom correspondence should be addressed.

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

Clinicopathologic factor	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[18] with the CpGenome DNA modification kit (Intergen, Purchase, NY). MSP was then performed to examine the methylation status of the promoter region of *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

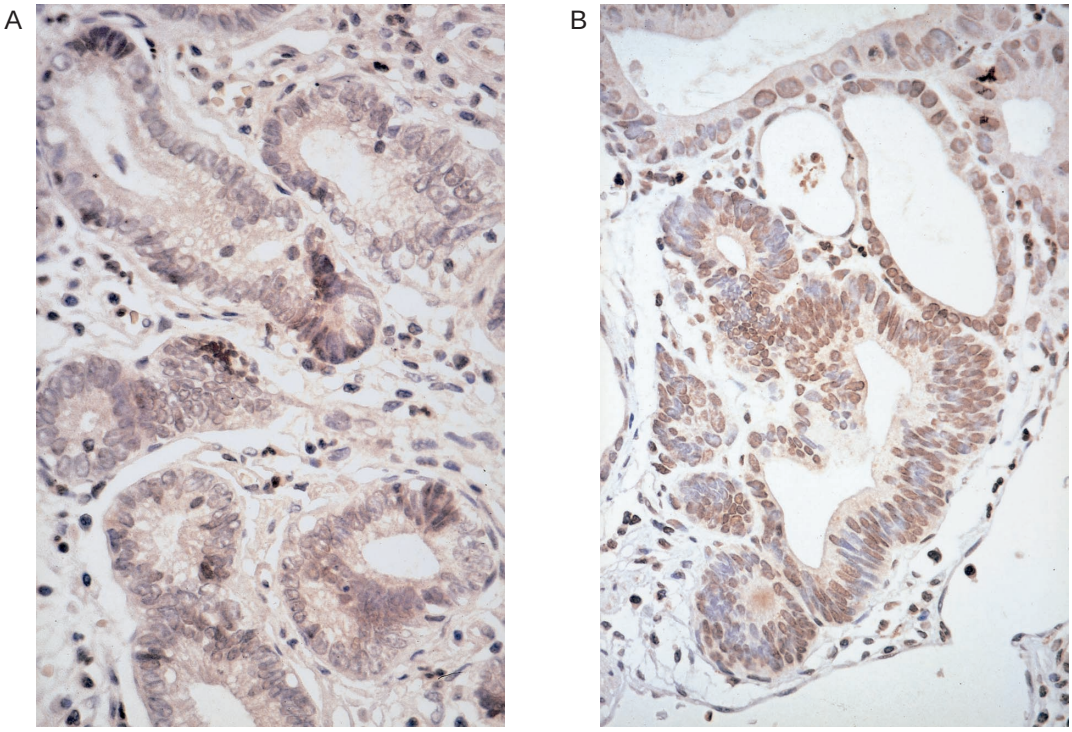

Fig. 1. Immunohistochemistry of p16 in gastric carcinoma with or without association of Epstein-Barr virus (EBV). p16-expression is markedly reduced in a case of EBV-associated gastric carcinoma (A), but there are still several cells showing positive staining. Since the neoplastic glands were situated 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).

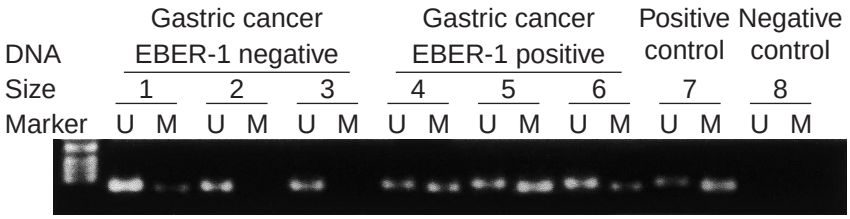

Fig. 2. Methylation-specific PCR (MSP) analysis of *p16* promoter in gastric carcinoma with or without association of Epstein-Barr virus (EBV). Bisulfite modification for MSP was followed by MSP to examine the methylation status of the promoter region of the *p16* gene. PCR products of unmethylated (U, 154 bp) and methylated (M, 145 bp) sequences are clearly identified on 2% agarose gel. Numbers 1–3, three cases of EBV-negative gastric cancer (GC); 4–6, three cases of EBV-associated GC; 7, positive control (universal methylated DNA); and 8, negative control (distilled water).

significantly 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.[19] 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.[11, 12, 15] 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.*[13] were also obtained by Tsujie *et al.*,[14] 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 variable expression fits better with recent findings of the regulation of p16-expression by promoter methylation. Bender *et al.*[20] 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.[21, 22] 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.[11] 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.*,[15] 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.[23] In viral infection, restriction to latent infection is induced by the methylation of the episomal DNA of EBV[24–26] or of the integrated virus genome, such as in human immunodeficiency virus type 1 (HIV-1)[27] and human T-cell leukemia virus type-1.[28, 29] 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.[7, 30] 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.[31] 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.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

(Received June 17, 2002/Revised August 27, 2002/Accepted September 5, 2002)

REFERENCES

1) Fukayama, M., Chong, J.-M. and Uozaki, H. Pathology and molecular pathology of Epstein-Barr virus-associated gastric carcinoma. *In* "Epstein-Barr Virus and Human Cancer," ed. K. Takada, Current Topics in Microbiology and Immunology No. 258, pp. 91–102 (2001). Springer, Berlin.
2) Fukayama, M., Chong, J.-M. and Kaizaki, Y. Epstein-Barr virus and gastric carcinoma. *Gastric Cancer*, **1**, 104–114 (1998).
3) Fukayama, M., Hayashi, Y., Iwasaki, Y., Chong, J.-M., Ooba, T., Takizawa, T., Koike, M., Mizutani, S., Miyaki, M. and Hirai, K. Epstein-Barr virus-associated gastric carcinoma and Epstein-Barr virus infection of the stomach. *Lab. Invest.*, **71**, 73–81 (1994).
4) Kaizaki, Y., Sakurai, S., Chong, J.-M. and Fukayama, M. Atrophic gastritis, Epstein-Barr virus infection, and Epstein-Barr virus-associated gastric carcinoma. *Gastric Cancer*, **2**, 101–108 (1999).
5) Chong, J.-M., Fukayama, M., Hayashi, Y., Funata, N., Takizawa, T., Koike, M., Muraoka, M., Kikuchi-Yanoshita, R., Miyaki, M. and Mizuno, S. Expression of CD44 variants in gastric carcinoma with or without Epstein-Barr virus. *Int. J. Cancer*, **74**, 450–454 (1997).
6) Chong, J.-M., Sakuma, K., Sudo, M., Osawa, T., Ohara, E., Uozaki, H., Shibahara, J., Kuroiwa, K., Tominaga, S., Hippo, Y., Aburatani, H., Funata, N. and Fukayama, M. Interleukin 1β expression in human gastric carcinoma with Epstein-Barr virus infection. *J. Virol.*, **76**, 6825–6831 (2002).
7) Imai, S., Koizumi, S., Sugiura, M., Tokunaga, M., Uemura, Y., Yamamoto, N., Tanaka, S., Sato, E. and Osato, T. Gastric carcinoma: monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein. *Proc. Natl. Acad. Sci. USA*, **91**, 9131–9135 (1994).
8) Chong, J.-M., Fukayama, M., Hayashi, Y., Takizawa, T., Koike, M., Konishi, M., Kikuchi-Yanoshita, R. and Miyaki, M. Microsatellite instability in the progression of gastric carcinoma. *Cancer Res.*, **54**, 4595–4597 (1994).
9) Schneider, B. G., Gulley, M. L., Eagan, P., Bravo, J. C., Mera, R. and Geradts, J. Loss of p16/CDKN2A tumor suppressor protein in gastric adenocarcinoma is associated with Epstein-Barr virus and anatomic location in the body of the stomach. *Hum. Pathol.*, **31**, 45–50 (2000).
10) Toyota, M., Ahuja, N., Suzuki, H., Itoh, F., Ohe-Toyota, M., Imai, K., Baylin, S. B. and Issa, J. P. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res.*, **59**, 5438–5442 (1999).
11) Shim, Y. H., Kang, G. H. and Ro, J. Y. Correlation of p16 hypermethylation with the p16 protein loss in sporadic gastric carcinomas. *Lab. Invest.*, **80**, 689–695 (2000).
12) Song, H. S., Jong, H. S., Choi, H. H., Kang, S. H., Ryu, M. H., Kim, N. K., Kim, W. H. and Bang, Y. J. Methylation of specific CpG sites in the promoter region could significantly down-regulate p16(INK4a) expression in gastric adenocarcinoma. *Int. J. Cancer*, **87**, 236–240 (2000).
13) Jang, T. J., Kim, D. I., Shin, Y. M., Chang, H. K. and Yang, C. H. $P16^{INA4A}$ promoter hypermethylation of non-tumorous tissue adjacent to gastric cancer is correlated with glandular atrophy and chronic inflammation. *Int. J. Cancer*, **93**, 629–634 (2001).
14) Tsujie, M., Yamamoto, H., Tomita, N., Sugita, Y., Ohue, M., Sakita, I., Tamaki, Y., Sekimoto, M., Doki, Y., Inoue, M., Matsuura, N., Monden, T., Shiozaki, H. and Monden, M. Expression of tumor suppressor gene p16(INK4) products in primary gastric cancer. *Oncology*, **58**, 126–136 (2000).
15) Kang, G. H., Lee, S., Kim, W. H., Lee, H. W., Kim, J. C., Rhyu, M.-G. and Ro, J. Y. Epstein-Barr virus-positive gastric carcinoma demonstrates frequent aberrant methylation of multiple genes and constitutes CpG island methylator phenotype-positive gastric carcinoma. *Am. J. Pathol.*, **160**, 787–794 (2002).
16) Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma—2nd English edition. *Gastric Cancer*, **1**, 10–24 (1998).
17) Lauren, R. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. *Acta Pathol. Microbiol. Scand.*, **64**, 31–49 (1965).
18) Hermans, J. G., Graff, J. R., Myöhänen, S., Nelkin, B. D. and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA*, **93**, 9821–9826 (1996).
19) Kamb, A. Cell-cycle regulators and cancer. *Trends Genet.*, **11**, 136–140 (1995).
20) Bender, C. M., Pao, M. M. and Jones, P. A. Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res.*, **58**, 95–101 (1998).
21) Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson,

E., Burger, P. C., Baylin, S. B. and Sidransky, D. 5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. *Nat. Med.*, **1**, 686–692 (1995).
22) Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M. and Issa, J. P. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv. Cancer Res.*, **72**, 141–196 (1998).
23) Yoder, J. A., Walsh, C. and Bestor, T. H. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.*, **13**, 335–340 (1997).
24) Allday, M. J., Kundu, D., Finerty, S. and Griffin, B. E. CpG methylation of viral DNA in EBV-associated tumours. *Int. J. Cancer*, **45**, 1125–1130 (1990).
25) Robertson, K. D. and Ambinder, R. F. Mapping promoter regions that are hypersensitive to methylation-mediated inhibition of transcription: application of the methylation cassette assay to the Epstein-Barr virus major latency promoter. *J. Virol.*, **71**, 6445–6454 (1997).
26) Salamon, D., Takacs, M., Rjvari, D., Uhlig, J., Wolf, H., Minarovits, J. and Niller, H. H. Protein-DNA binding and CpG methylation at nucleotide resolution of latency-associated promoters Qp, Cp, and LMP1p of Epstein-Barr virus. *J. Virol.*, **75**, 2584–2596 (2001).
27) Bednarik, D. P., Cook, J. A. and Pitha, P. M. Inactivation of HIV LTR by DNA CpG methylation. *EMBO J.*, **1**, 1157–1164 (1989).
28) Saggioro, D., Panozzo, M. and Chieco-Bianchi, L. Human T-lymphotropic virus 1 transcriptional regulation by methylation. *Cancer Res.*, **50**, 4968–4973 (1990).
29) Saggioro, D., Forino, M. and Chieco-Bianchi, L. Transcriptional block of HTLV-1 LTR by sequence-specific methylation. *Virology*, **182**, 68–75 (1991).
30) Gratama, J. W. and Ernberg, I. Molecular epidemiology of Epstein-Barr virus infection. *Adv. Cancer Res.*, **67**, 197–255 (1995).
31) Mikovits, J. A., Young, H. A., Vertino, P., Issa, J.-P. J., Pitha, P. M., Turcoski-Corrales, S., Taub, D. D., Petrow, C. L., Baylin, S. B. and Ruscetti, F. W. Infection with human immunodeficiency virus type 1 upregulates DNA methyltransferase, resulting in *de novo* methylation of the gamma interferon (IFN-γ) promoter and subsequent downregulation of IFN-γ production. *Mol. Cell. Biol.*, **18**, 5166–5177 (1998).