Table I. List of probes and primers used in the present study.

	Sequences	Author/Ref.
EBNA-3C		Sample *et al*, (15)
Primers		
Sense	5'-AGAAGGGGAGCGTGTGTTGT-3'	
Antisense	5'-GGCTCGTTTTTGACGTCGGC-3'	
Probe		
Subtype A	5'-GAAGATTCATCGTCAGTGTC-3'	
Subtype B	5'-CCGTGATTTCTACCGGGAGT-3'	
BamHI-F		Sidagis *et al*, (16)
Primers		
Sense	5'-CACATTCTAGGTCCTGCATC-3'	
Antisense	5'-GGCAATGGGACGTCTTGTAA-3'	
Probe	5'-AAGGCTACCGTGCTAATTACCTCC-3'	
BamHI-I		Lung *et al*, (18)
Primers		
Sense	5'-ACCTGCTACTCTTCGGAAAC-3'	
Antisense	5'-TCTGTCACAACCTCACTGTC-3'	
XhoI site in LMP1		Chen *et al*, (19)
Primers		
Sense	5'-AGAAACACGCGTTACTCT-3'	
Antisense	5'-ACAATGCCTGTCCGTGCA-3'	

mucinous carcinoma (muc). The tumor location, defined as the predominant location of the tumor, was divided into the following three locations: cardia or upper third part, middle part of the stomach, and antrum or lower third part according to the guidelines of the Japanese Research Society for Gastric Cancer (8). Tumor location and histological classification of colorectal cancer were according to the guidelines of the Japanese Society for Cancer of the Colon and Rectum (9,10).

In situ hybridization. We examined the EBER-1 expression in the paraffin-embedded tissue obtained from the main tumor by *in situ* hybridization assay as described before (11). A case was considered EBER-1 positive on the basis of positive signals in carcinoma cells under microscopy. Paraffin sections from a known EBER-1-positive gastric cancer case were used as positive control, and a sense probe for EBER-1 was used as negative control in every assay.

Preparation of DNA. The formalin-fixed and paraffin-embedded specimen was cut into 10 μm thick slices, and DNA sample was prepared following the method reported (12). Deparaffinized sample was treated with proteinase K (200 μg/ml) at 37˚C overnight, followed by phenol/chloroform extraction and ethanol precipitation. Finally, the extracted DNA sample was dissolved in 50 μl of TE buffer.

Genotype-specific primer sets and probes. Four different regions, EBNA-3C, BamHI-F, BamHI-I, and XhoI site in LMP1, were used to determine viral genotypes. Table I shows the list of primer sets and probes used in the present study. Types A and B can be determined using EBNA-2, 3A, 3B or 3C gene (13-15). In the present study, we chose EBNA 3C for genotyping because of the higher detection rate of the primer set than those of EBNA-2 region in previous studies (16,17). Types A and B, identified by PCR amplification of EBNA-3C region, corresponded to 153 bp band and 246 bp band, respectively, and were confirmed by Southern blot hybridization with type-specific internal probes (15). The wild-type F and the f variant were identified by the presence of a 186-bp fragment in amplification of BamHI F region. After BamHI cleavage, the 186-bp fragment in the case of wild-type F and the 127-bp fragment in the case of the f variant. The wild-type F and the f variants were confirmed by Southern blot hybridization with the internal probe as described before (16). For the BamHI-I region, a 205-bp fragment was amplified using a primer set described previously (18), and types C and D were distinguished after cleavage by BamHI restriction enzyme. Types C had a 205-bp fragment and type D, cleaved fragments with 130 bp and 75 bp length. Type C and D were also confirmed by Southern blot hybridization with a cloned BamHI-I DNA fragment probe. To detect the XhoI polymorphism in exon 1 of LMP1 gene, a 497-bp DNA fragment was amplified with a primer set as described before (19). When two fragments, 340 bp and 157 bp were observed after XhoI digestion of the PCR product, the case was considered to contain the XhoI cleavage site. The 497-bp fragment of PCR product of B95-8 cell line was used as the probe to confirm XhoI cleavage site of LMP1 by Southern blot hybridization (20).

PCR and Southern blot hybridization. The template of PCR was mixed with the appropriate primer pair (1 μM each),

Table II. Sex and age distribution by histology in oral and esophageal cancer cases.[a]

	Male			Female		
	Number	Age		Number	Age	
	(No.)[b]	Mean ± SD[c]	Range	(No.)[b]	Mean ± SD[c]	Range
Oral cancer						
Well differentiated	35 (6)	52.7±14.0	21-80	21 (7)	48.6±12.1	28-70
Moderately differentiated	5 (1)	49.0±15.1	38-70	5 (1)	48.3±6.2	40-55
Total	40 (7)	52.2±14.0	21-80	26 (8)	48.6±10.9	28-70
Esophageal cancer						
Well differentiated	7	55.1±11.3	36-70	9 (1)	54.4±12.3	40-79
Moderately differentiated	12	54.0±7.9	40-62	5	51.2±17.3	27-65
Poorly differentiated	6 (1)	58.6±9.7	50-72	1	39	39
Total	25 (1)	55.3±9.1	36-72	15 (1)	52.1±13.8	27-79

[a]All cases were squamous cell carcinoma; [b]The figures in parentheses are the numbers of subjects without information on age; [c]The standard deviation of age was not calculated when there were less than 3 cases with information on age.

Table III. Sex and age distribution by tumor location or histology in stomach adenocarcinoma.

	Male			Female		
	Number	Age		Number	Age	
	(No.)[a]	Mean ± SD[b]	Range	(No.)[a]	Mean ± SD[b]	Range
Tumor location						
CEJ	14 (5)	53.2±6.6	41-60	4	52.3±6.4	46-60
Cardia	8	49.8±9.7	35-65	1	56	56
Middle	10 (3)	54.3±9.3	40-65	3	45.7±14.0	32-60
Antrum	32 (10)	53.0±14.0	29-75	24 (1)	53.3±10.8	25-66
Unknown	26 (5)	51.0±8.8	30-63	28 (2)	52.6±10.0	30-68
Total	90 (23)	52.2±10.5	29-75	60 (3)	52.5±10.1	25-68
Histology of Japanese classification						
tub1	7 (1)	56.0±10.2	40-70	7	55.7±7.9	45-65
tub2	35 (10)	52.4±10.4	30-75	18 (2)	51.9±10.0	30-64
muc	4 (1)	55.0±5.0	50-60	1	56	56
por1	22 (7)	56.3±9.3	40-70	13	53.5±9.4	35-68
por2	20 (4)	47.5±10.5	35-65	21 (1)	51.2±11.8	25-66
sig	2	39.5	29-50	0		
Total	90 (23)	52.2±10.5	29-75	60 (3)	52.5±10.1	25-68

[a]The figures in parentheses are the numbers of subjects without information on age; [b]The standard deviation of age was not calculated when there were less than 3 cases with information on age.

deoxyriboncleotide triphosphates (200 μM each) and Taq polymerase (Takara Shuzo, Kyoto, Japan) in a total amount of 100 μl PCR buffer. PCR products or PCR products digested with BamHI and XhoI were confirmed by electrophoresis in a 2% agarose gel and by staining with 0.5 μg/ml of ethidium bromide. Then, the electrophoretic pattern was photographed under UV-light.

The electrophoretic DNA was transferred onto a Hybond N+ nylon membrane (Amersham, Pharmacia Biotech, UK) by capillary blotting using 0.4 N NaOH solution. Membranes

Table IV. Sex and age distribution by tumor location or histology in colorectal adenocarcinoma.

	Male			Female		
	Number	Age		Number	Age	
	(No.)[a]	Mean ± SD[b]	Range	(No.)[a]	Mean ± SD[b]	Range
Tumor location						
Caecum	2 (1)	50	50	2	52.5	45-60
Ascending	6 (2)	33.8±6.9	24-40	3	43.0±18.7	23-60
Transvers	4	45.8±14.1	30-60	4 (2)	40.5	25-56
Descending	4 (1)	49.0±11.5	37-60	3 (1)	42.5	40-45
Recto-sigmoid	7 (2)	50.1±13.8	25-67	1	45	45
Unknown	5 (1)	52.8±10.2	40-64	3 (1)	40.0	40-40
Total	30 (7)	46.8±12.4	24-67	16 (4)	44.1±11.3	23-60
Histology						
Well differentiated	10 (3)	54.1±10.1	38-67	7 (2)	47.2±7.5	40-60
Moderately differentiated	17 (4)	45.6±12.3	24-64	4 (2)	41.5	23-60
Poorly differentiated	1	25	25	3	36.7±10.4	25-45
muc	2	40	40-40	1	56	56
sig	0			1	45	45
Total	30 (7)	46.8±12.4	24-67	16 (4)	43.8±11.7	23-60

[a]The figures in parentheses are the numbers of subjects without information on age; [b]The standard deviation of age was not calculated when there were less than 3 cases with information on age.

were prehybridized with hybridization buffer for 0.5-1 h at 42°C. After adding the probe, hybridization was carried out overnight at 42°C temperatures. Probes of types A and B, and BamHI F were labeled with Dig oligonucleotide 3'-end labeling kit and detected by Dig luminescent detection kit (Boehringer Mannheim, Germany). For detecting the BamHI-I fragment and XhoI polymorphism in LMP1, hybridization was carried out using the ECL direct labeling and detection kit (Amersham, Pharmacia Biotech, UK) according to the manufacturer's instructions.

Results

We examined 66 cases of oral cancer, 40 esophageal cancer cases, 150 stomach cancer cases, and 46 colorectal cancer cases. All oral and esophageal cancers were squamous cell carcinomas, and histological distributions of oral cancer and esophageal cancer are shown in Table II. The distributions of tumor location and histology for adenocarcinomas of the stomach and colorectum are shown in Tables III and IV, respectively.

There were no malignancies with positive EBER expression except for two male stomach adenocarcinoma cases with Japanese histological classification of non-solid poorly differentiated adenocarcinoma, or por2, which is the diffuse type of Lauren classification (Table V). One case (case #1) with unknown age was gastric carcinoma in pylorus. The other (case #2) is 55 years old without information on location of tumor. There was no EBER-positive case in tumors with intestinal type of Lauren's classification.

Table V. Summary of EBER *in situ* hybridization assay of stomach adenocarcinoma.

Histology classification		
Lauren	Japanese	EBER-positive/total no.
Intestinal type		0/72
	tub1	0/14
	tub2	0/53
	muc	0/5
Diffuse type		2/78
	por1	0/35
	por2	2/41
	sig	0/2
Total		2/150

We examined genotypes of two EBV strains detected from gastric carcinomas. Four different regions of EBV genome were examined by PCR-RFLP, coupled with Southern blot hybridization. The EBV genotypes of case #1 were type A, wild-type F at BamHI-F region, type D of BamHI-I region and the kept type of the XhoI cleavage site in LMP1. Case #2 had EBV whose genotypes were type A, wild-type F at BamHI-F region, and the lost type of the XhoI cleavage site in LMP1. BamHI-I region could not be amplified in this case.

Discussion

EBV is a lymphotrophic virus, and more than 90% of adults in the world have evidence of past infection with EBV (21). Papua New Guinea (PNG) is not an exception, and average age at first infection was reported to be even earlier than that in other areas (22). PNG is a high-incidence area for Burkitt lymphoma, but Hodgkin disease is rare. Note here that both disorders are related to EBV infection. In the present study, we found only two EBER-positive cases with histological type of non-solid poorly differentiated adenocarcinoma (por2) among 150 gastric adenocarcinoma cases. The proportion of EBV-GCs observed in the present study was 1.3% (2/150), the lowest proportion found in the literature. According to the studies of Japanese series, EBER-positive case is more frequently observed in lymphoepithelioma-like carcinoma or adeno-carcinoma with histological type of moderately differentiated tubular adenocarcinoma (tub2), or solid poorly differentiated adenocarcinoma (por1) (2). However, neither case with lymphoepithelioma-like carcinoma nor EBER-positive case with histological type of tub2 and por1 was observed in the present study.

Kijima *et al*, examining epithelial carcinomas of the lung, breast, esophagus, colon, pancreas, thyroid and stomach, reported that EBER could be detected only in cancers of the stomach cancer (23). We examined EBER expressions in cancers of the digestive-tract organs other than stomach cancer as well. EBER expression could be detected only in cancer of the stomach and could not be detected in cancers of the oral cavity, esophagus, or colorectum. The cancer of the oral cavity seems worth mentioning. Recently, Higa *et al* reported that EBV could be detected in 72% of oral squamous cell carcinomas (24). The present study did not replicate their findings on oral squamous cell carcinomas in PNG.

Two EBV strains detected from gastric carcinomas had the same genotypes as far as type A/B and Bam HI-F region were concerned. Note here that type A and prototype F at BamHI-F region are predominant EBV genotype in Asian countries (16,25,26). On the other hand, the two detected strains had different genotypes in XhoI cleavage site in LMP1. It has been known that type C of BamHI-I region without the XhoI cleavage site in LMP1 is mainly Asian origin (16,27) and type D keeping the XhoI recognition site is common in Western countries (28,29). It is necessary to examine the genotype distribution of EBV among healthy subjects in order to evaluate the etiological significance of those observations.

Martin *et al* analyzed malignant tumors registered with the Tumor Registry of PNG from 1958-1988. Cancer incidence was generally low in PNG. During this period, carcinoma of oral cavity, cervix, breast, and skin, hepatoma, and lymphoma were the most common types of malignant lesions detected. The incidence of carcinoma of the oral cavity increased during the observation period (30). On the other hand, the incidence of squamous carcinoma of skin declined probably due to improved control of tropical ulcers. The incidence of stomach cancer is falling, and is not common.

Although it was once hypothesized that the areas having low incidence of gastric cancer experience relatively high proportions of EBV-GCs (31), it is now clear that there are many reports that cannot be explained by this hypothesis. For example, Chile and Colombia, where the proportion of EBV-GCs was reported to be as high as 17% (32) and 13% (33), respectively, whereas their gastric cancer risk is one of the highest in the world. Other examples are the UK and India, which have low proportions of EBV-GCs although their gastric cancer incidence rates are low (3,34). The present study adds another such example having low EBV-GC proportion in the country where the incidence of gastric cancer is relatively low.

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Loss of p16/CDKN2A Protein in Epstein-Barr Virus-Associated Gastric Carcinoma

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Key Words
p16^{INK4A} · Epstein-Barr virus · Gastric carcinoma

Abstract
We examined the expression of p16, the CDKN2A gene product, in EBV-associated gastric carcinomas (EBV-GCs). EBV-GCs were identified by detecting EBV-encoded small RNA (EBER) using an in situ hybridization assay of paraffin-embedded tissue. Two non-EBV-GC cases for each EBV-GC case were selected, matched for age, sex, tumor location, and depth of invasion. After excluding cases without sufficient tissue samples for immunohistochemical analysis, 54 EBV-GC and 117 non-EBV-GC cases were available for the present study. The loss of p16 expression was more frequently observed in EBV-GCs (89%) than non-EBV-GC cases (32%; $p < 0.001$). Among non-EBV-GC cases, the loss of p16 expression was more frequent in female cases (57%) than male cases (29%) ($p = 0.042$). Expression of p16 was not related to the location of tumor, clinical stage of tumor, age, or prognosis of the patients. In conclusion, the present study suggests that the loss of p16-related cell cycle regulation may be associated with the development of EBV-GC.

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Introduction

The cyclin-dependent kinase inhibitor, CDKN2A gene product, p16^{INK4A} (p16), is a tumor suppressor that binds to the complex of cyclin D1 and cyclin-dependent kinase 4 to repress its ability to phosphorylate the retinoblastoma protein, and, consequently, blocks G_1 cell cycle progression [1, 2]. Inactivation of the p16^{CDKN2A} gene, which has been shown in many different types of human carcinomas [3–5], including gastric cancer [6, 7], is considered to be mainly accomplished through methylation rather than gene mutations or homozygous deletion [8, 9].

In the early 1990s, in situ hybridization (ISH) of Epstein-Barr virus-encoded small RNA (EBER) became available, and revealed that about 10% of gastric carcinomas had an involvement of Epstein-Barr virus (EBV) [10, 11]. The expression pattern of latency-associated EBV gene products in EBV-associated gastric carcinomas (EBV-GCs) is similar to that of Burkitt's lymphoma, where the latent EBV gene products expressed are only EBERs, BARF-0, and EBNA-1 [12, 13]. Among latent membrane proteins (LMPs), only LMP-2A is occasionally expressed. Although LMP-1 is a strongly suspected candidate for an EBV oncogene, this protein is not ex-

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pressed in EBV-GCs. Interestingly, a recent report by zur Hausen et al. [14] showed that EBV-GC expressed BARF1, which had been reported to cause transformation of epithelial cells [15, 16].

Although the mechanism of developing EBV-GC has yet to be elucidated, Schneider et al. [17] recently reported a possible association between the loss of p16 expression and EBV-GC arising in the body of the stomach. The present study, trying to confirm their findings, examined p16 expression in EBV-GCs and non-EBV-GCs.

Materials and Methods

Subjects

Most of the subjects enrolled in the present study had participated in a study conducted by Tokunaga et al. [18]. We examined the EBER expression of all the gastric carcinomas diagnosed at the Kagoshima City Hospital during the period of 1976–1992, and identified 74 cases of EBV-GC. After excluding 10 cases without clinical information necessary for the present study, there were 64 EBV-GC cases. Two non-EBV-GC cases for each EBV-GC case were selected matching the EBV-GC case with respect to age, sex, tumor location, and depth of invasion. Ten EBV-GC cases and 11 non-EBV-GC cases did not have gastric tissue samples sufficient for immunohistochemical analysis. Thus, 54 EBV-GC and 117 non-EBV-GC cases were available for the present study.

Histology

Histologically, all cases were classified as intestinal or diffuse-type gastric carcinomas according to Lauren [19], and the location of a tumor, defined as the predominant location of the tumor, was divided into the following three sites: cardia or upper third part, middle part and antrum or lower third part according to the guidelines of the Japanese Research Society for Gastric Cancer [20]. The depth of invasion was classified as mucosal, submucosal, muscularis propria and subserosal involvement. A tumor invading beyond the submucosa is considered to be advanced cancer [20].

ISH Assay to Detect EBER

The presence of EBV was identified by the expression of EBER-1, the most abundant viral product in latently infected cells [21, 22]. An ISH assay of paraffin-embedded tissue samples obtained from the main tumor was conducted using a digoxigenin (DIG)-labeled EBER-1 oligonucleotide probe as described before [18, 23–25]. In brief, the tissue sections were deparaffinized, hydrated and predigested with pronase. After that, the tissue sections were hybridized overnight at 37 °C with a concentration of 500 ng/ml of DIG-labeled antisense EBER-1 probe (5′-agacaccgtcctcaccacccgggacttgta-3′). The hybridization signal was detected using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Germany) according to the instructions of the manufacturer. A case was considered to be EBER-1-positive based on a positive signal under microscopy. A lymph node section from a patient with infectious mononucleosis was used as positive control, and a sense probe for EBER-1 was used as a negative control in every assay [18]. In the present study, the case with EBER-1-positive tumor cells but not in the surrounding normal epithelial cells was determined as EBV-GC, and we defined the case with EBER-1-negative tumor cells as non-EBV-GC. The case with EBER-1-negative tumor cells but EBER-1-positive lymphocytes around the carcinoma was also determined as non-EBV-GC [18]. All EBV-GCs had the uniform presence of EBER-1 in tumor cells.

Immunostaining for p16

The presence of p16 expression was examined in gastric tissue sections as described previously [17]. Paraffin sections were dewaxed with xylene and rehydrated with an alcohol series. After the inactivation of endogenous peroxidase activity with H_2O_2, sections were heated for 30 min at 100 °C in 0.01 mol/l citrate buffer (pH 6.0) to retrieve antigen. As a primary antibody against p16, the monoclonal antibody G175-405 (PharMingen, San Diego, Calif., USA) at 2 μg/ml was applied to the sections and incubated overnight at 4 °C. The avidin-biotin-peroxidase complex method was used for immunohistochemical staining (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, Calif., USA). Positive internal controls were mature plasma cells present in every sample or adjacent gastric epithelium with intestinal metaplasia. Without knowledge of the EBV status, the expression of p16 protein in nuclear was graded as follows: (1) 0%, (2) 1–9%, (3) 10–49%, (4) 50–90%, and (5) more than 90% according to the proportion of p16-positive cells in a case. A case expressing more p16 than 0% of carcinoma cells was considered as p16 positive according to another report [17].

Statistical Analysis

Logistic regression analysis was conducted to examine the association of the EBER status (positive or negative) with p16 expression (positive or negative) using age, sex, tumor location (cardia, middle, or antrum) and depth of invasion as covariates. Maximum likelihood estimates of ORs and corresponding 95% CIs were calculated. The associations between the loss of p16 expression and clinicopathological factors were examined with univariate logistic regression models, and the p value was calculated by the likelihood ratio test. p value for trend of age was calculated using age as a continuous variable in a logistic model. All the p values presented were two-sided.

The multivariate survival analysis was conducted using the Cox proportional hazard model adjusting for the effects of tumor location and clinical stage. Maximum likelihood parameter estimates and likelihood ratio statistics in the Cox proportional hazard models were obtained with the use of a statistical package, STATA (Stata, USA).

Results

We examined the p16 expression in 54 EBV-GC cases and 117 non-EBV-GC cases (table 1). A loss of p16 expression was observed in 48 (89%) EBV-GC cases, and 6 (11%) EBV-GCs showed p16 expression in only 1–9% of carcinoma cells. However, p16 expression was not observed in 38 (32%) of 117 non-EBV-GC cases. The p16 staining pattern of 10 non-EBV-GC cases was a diffuse

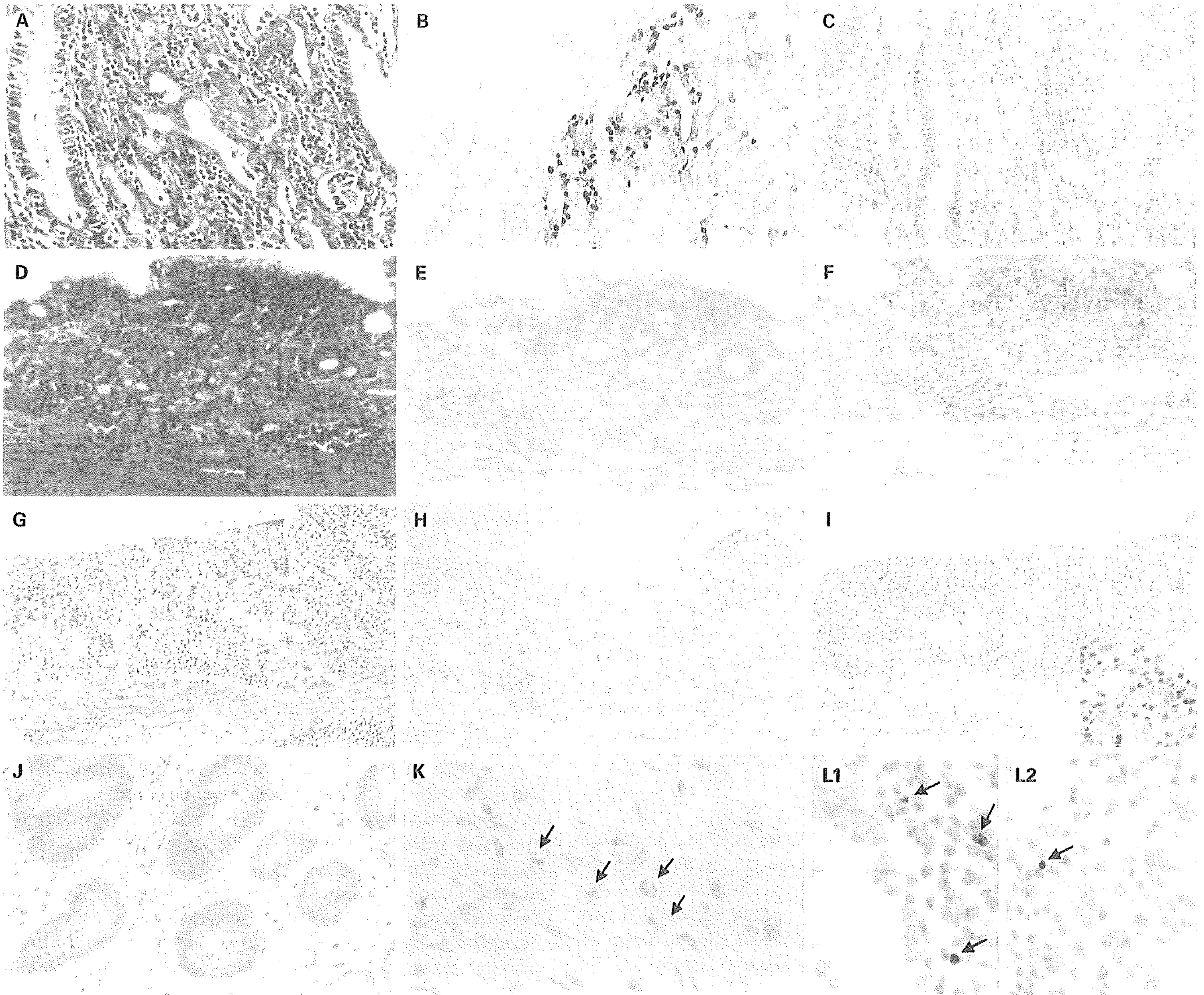

Fig. 1. A–C EBER-1-positive and p16-negative case. p16 is positive in plasma cells but not in carcinoma cells (**C**). **D–F** EBER-1-negative and p16-negative cases. **G–I** EBER-1-negative and p16-positive case. p16 is expressed in nuclei of carcinoma cells (**I** inset). **J** p16 expression in the nuclei of intestinal metaplastic cells. **K** p16 expression in plasma cells (arrows) and nuclei of carcinoma cells. **L** Non-EBV-GC case. EBER-1 was positive in part of the lymphocytes (arrows) but not in the adjusting normal epithelium (**L1**) and carcinoma cells (**L2**). **A**, **D**, **G** Hematoxylin and eosin staining. **B**, **E**, **H**, **L** ISH for EBER-1. **C**, **F**, **I**, **J**, **K** Immunohistochemical staining for p16.

pattern (proportion of p16-positive tumor cells ≥90%). The remaining p16-positive cases in both EBV-GCs and non-EBV-GCs showed a mosaic pattern of p16 staining (proportion of p16-positive tumor cells <90%). In an adjacent epithelium with intestinal metaplasia, the nuclei and cytoplasm of 20–30% of epithelial cells were p16 positive. Typical cases of EBV-GC and non-EBV-GC with or without p16 expression are shown in figure 1.

The OR of p16 expression comparing EBV-GCs with non EBV-GCs was 0.05 (95% CI: 0.02–0.1) after controlling for potential confounders such as age, sex, tumor location (cardia, middle, or antrum) and depth of invasion using a logistic regression model.

The frequencies of p16 expression by clinicopathological variables in EBV-GC and non-EBV-GC cases are shown separately in table 2. In non-EBV-GC cases, the

Table 1. Proportion of cases with p16 expression in EBV-GCs and non-EBV-GCs

p16 expression[a]	EBV-GC (n = 54)	Non-EBV-GC (n = 117)
0%	48 (89%)	38 (32%)
1–9%	6 (11%)	12 (10%)
10–49%	0	25 (21%)
50–89%	0	32 (27%)
90%–	0	10 (9%

[a] Percentage of carcinoma cells with p16 expression in a section.

loss of p16 expression was more frequent in female cases (57%) than male cases (29%) although EBV-GC cases did not show such a difference. The expression of p16 neither showed a significant association with age, tumor location, histology, depth of invasion nor clinical stage in both EBV-GC and non-EBV-GC cases.

The prognosis of gastric cancer cases was examined by survival analyses using Cox proportional hazard models. The average follow-up periods were 70.9 months (SD: 61.1) in EBV-GCs and 63.8 months (SD: 59.7) in non-EBV-GC cases. There was no significant association between p16 expression and the prognosis of the patients

Table 2. Proportion of cases with p16 expression by clinicopathological variables

		p16-negative	p16-positive	p value
EBV-GC cases				
Total (n = 54)		48 (89%)	6 (11%)	
Gender	Female	4 (67%)	2 (33%)	0.114
	Male	44 (92%)	4 (8%)	
Age, years	< 50	16 (100%)	0	0.550[a]
	50–59	4 (80%)	1 (20%)	
	60–69	19 (86%)	3 (14%)	
	70–	9 (82%)	2 (18%)	
Site	Antrum	12 (80%)	3 (20%)	0.406
	Middle	29 (94%)	2 (6%)	
	Cardia	7 (88%)	1 (13%)	
Histology	Intestinal type	24 (86%)	4 (14%)	0.437
	Diffuse type	24 (92%)	2 (8%)	
Clinical stage[b]	Early	11 (85%)	2 (15%)	0.586
	Advanced	37 (90%)	4 (10%)	
Non-EBV-GC cases				
Total (n = 117)		38 (32%)	79 (68%)	
Gender	Female	8 (57%)	6 (43%)	0.042
	Male	30 (29%)	73 (71%)	
Age, years	< 50	8 (25%)	24 (75%)	0.723[a]
	50–59	7 (44%)	9 (56%)	
	60–69	11 (28%)	28 (72%)	
	70–	12 (40%)	18 (60%)	
Site	Antrum	10 (30%)	23 (70%)	0.890
	Middle	22 (34%)	42 (66%)	
	Cardia	6 (30%)	14 (70%)	
Histology	Intestinal type	24 (31%)	54 (69%)	0.578
	Diffuse type	14 (36%)	25 (64%)	
Clinical stage[b]	Early	7 (25%)	21 (75%)	0.325
	Advanced	31 (35%)	58 (65%)	

[a] p value for trend.
[b] Depth of invasion in early cancer is mucosal or submucosal. Depth of invasion in advanced cancer is propia, muscularis, or subserosal involvement.

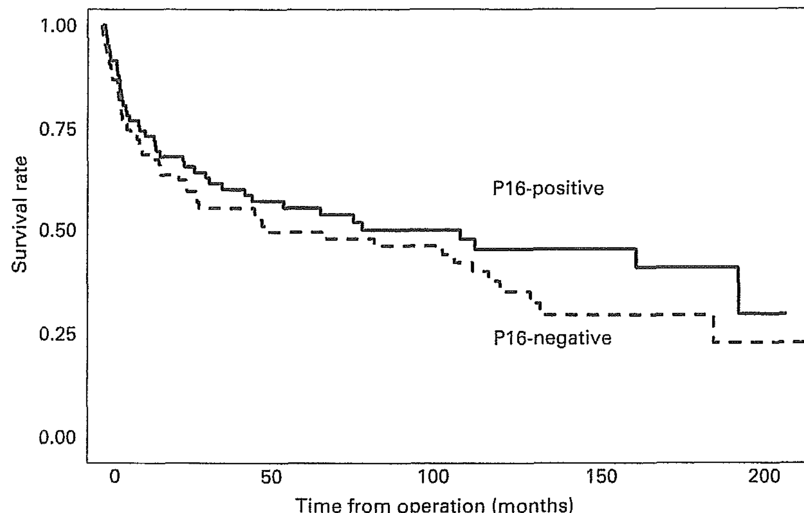

Fig. 2. Overall survival of 171 patients with gastric carcinoma according to p16 overexpression in tumor cells. —— = p16-positive cases (n = 85); ----- = p16-negative cases (n = 86). There was no significant difference between p16 expression and prognosis of patients (p = 0.42).

(hazard ratio: 0.8, 95% CI: 0.6–1.3) (fig. 2). EBER status did not modify the association between p16 expression and the prognosis of the patients (p = 0.32).

Discussion

The present study showed that p16 expression was more frequently lost in EBV-GCs than in non-EBV-GCs. This observation is consistent with the results reported by Schneider et al. [17]. However, the proportions of p16-negative cases in the present study (89 and 32% in EBV-GC and non-EBV-GC cases, respectively) were somewhat higher than those in their study (62 and 22% in EBER-positive and EBER-negative cases, respectively). Those differences indicate that sensitivity in the present study might be lower than that of Schneider et al. although the same monoclonal anti-p16 antibody was used in the two studies (PharMingen, clone G175-405). It should be noted here that both studies used the same cutoff point, 0%, for p16 positivity. Since the immunohistochemistry assay and grading of p16 expression were performed without knowledge of EBER status, it is unlikely that there was a bias in grading p16 expression.

Our finding that a loss of p16 expression was significantly associated with female GC patients is at variance with other studies, which reported an absence of gender difference in the loss of p16 expression [17, 26]. Interestingly, however, a study of colorectal cancer reported by Wiencke et al. [27] showed that the frequency of p16 methylation in female patients was about 9 times higher than in male patients. The association between aberrant methylation of p16 and gender should be clarified in further studies.

Hypermethylation of CpG islands in promoter region has been reported as a common way to silence the p16 gene in a wide variety of human primary tumors including gastric carcinoma [8, 9] although frequent somatic mutations of the p16 gene were also described in other tumors such as melanoma, and carcinomas of the pancreas and esophagus [9]. Recently, Kang et al. [28] reported that the methylation frequency of p16 and other genes in the EBV-GCs was more than 3 times higher than in the non-EBV-GCs and that there was an inverse correlation between the p16 immunostaining and its aberrant methylation. Significant reduction of p16 expression and promoter methylation of the p16 gene in EBV-GC was also reported from other ethnic populations [26, 29]. The results in these studies and the findings in the present study strongly suggest that aberrant methylation may be an important mechanism of EBV-related gastric carcinogenesis.

A loss of p16 expression is also reported in EBV-associated undifferentiated nasopharyngeal carcinoma [30], which has the EBV latency type different from that in EBV-GC (type I). The mechanisms responsible for inactivation of p16, a cell cycle inhibitor, are strongly suspected to be through deletion [31] and methylation of CDKN2A gene [32]. However, the mechanism of EBV involvement in aberrant p16 methylation of nasopharyn-

geal carcinoma has yet to be elucidated. That is also true for EBV-GCs; a recent report has suggested that methylation in EBV-GC does not depend on DNA methyltransferases [33]. According to the report by Resmus et al. [34], insertion of adenovirus DNA into the host genome can alter the cellular patterns of DNA methylation, possibly through the changes in the local DNA structure. Although episomal forms are commonest, there is a possibility of EBV DNA integration into the cellular DNA in Burkitt's lymphoma [35], and most of the EBV genome undergoes progressive methylation in the latent infection [36]. These observations suggest that the EBV infection causes the cellular methylation not only in the integrated viral DNA but also in the adjacent host DNA.

In the present study, patients with non-EBV-GC with a loss of p16 expression had a slightly worse prognosis although the association was not statistically significant. In addition, neither the depth of invasion nor the clinical stage was associated with p16 expression (data not shown). These findings are consistent with the results reported by Schneider et al. [17]. However, the aberrant p16 expression and deletion of CDKN2A gene were significantly associated with poor prognosis in other types of tumors [37–39]. The involvement of p16 in the cancer development stage may be different among tumors.

In conclusion, the present results suggest that a loss of p16-related cell cycle regulation may be associated with the development of EBV-GC. Further studies are required to elucidate the mechanism of p16 inactivation in EBV-GC.

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Histology-specific gender, age and tumor-location distributions of Epstein-Barr virus-associated gastric carcinoma in Japan

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Abstract. We examined 1,918 Japanese gastric cancer cases diagnosed during the period 1976-1995 to clarify histology-specific gender, age and tumor-location distributions of Epstein-Barr virus-associated gastric carcinoma (EBV-GC). EBV-GCs accounted for 4.5% and 6.1% of 1,088 intestinal-type and 830 diffuse-type gastric carcinomas, respectively. Both intestinal- and diffuse-type EBV-GCs showed male predominance, but the observed gender difference was statistically significant only in diffuse-type carcinomas ($P<0.001$). An age-dependent decrease of the EBV-GC proportion was observed in intestinal-type carcinomas ($P=0.002$), but not in diffuse-type carcinomas. In intestinal-type tumors, the estimated incidence of EBV-GCs reached its peak around age 70. Diffuse-type EBV-GCs appeared to have a much older peak incidence, if any. Both intestinal- and diffuse-type EBV-GCs were least prevalent in the stomach antrum. This study, examining the largest number of EBV-GCs in current literature, showed different patterns of age-dependence in intestinal- and diffuse-type EBV-GCs, suggesting that pathogenic pathways of EBV-GCs may be different in these 2 histological types.

Introduction

In the early 1990s, a small proportion of gastric carcinomas were demonstrated to be associated with Epstein-Barr virus (EBV), thanks to the *in situ* hybridization (ISH) technique to detect EBV-encoded small RNAs (EBERs) (1). EBV-associated gastric carcinomas (EBV-GCs) show uniform EBER expression in tumor cells but not in the surrounding normal epithelial cells (1-3). The major clinico-pathological features are male predominance and a predisposition to the upper two-thirds of the stomach (2). Although most of the studies reported the absence of age-dependence, several studies found an age-dependent decrease of the EBV-GC proportion among all gastric carcinomas (4-7). Histologically, EBV-GCs are more frequently observed in moderately differentiated tubular adenocarcinomas and solid poorly differentiated adenocarcinomas than in other histological types (5,7-11), according to the classification scheme of the Japanese Research Society for Gastric Cancer (12). When Lauren classification (13) was used, most of the studies conducted so far reported that diffuse-type EBV-GCs are slightly more common than intestinal-type EBV-GCs (3,5,7-9,14-21), even after excluding lymphoepithelioma-like carcinomas (LELCs), which almost always have EBER expression (22). There are several studies reporting the evident and statistically significant predominance of diffuse-type tumors among EBV-GCs (6,10,11,23-25).

The 2 histological types of Lauren classification are not only different in morphological features but also in etiological backgrounds. For example, the intestinal-type gastric carcinomas are predominant in high-risk countries whereas diffuse-type carcinomas are relatively frequent in low-risk countries. In Japan, intestinal-type carcinomas have markedly decreased over the years, whereas diffuse-type carcinomas show a relatively-stable time trend (26,27). The decreasing trend of intestinal-type tumors was also observed in the USA (28). According to Correa's hypothesis, intestinal-type gastric cancer is closely related to chronic inflammation leading to atrophy, intestinal metaplasia, and dysplasia (29). Although histology-specific analysis is important for understanding the etiological background of gastric carcinomas, histology-specific distributions of EBV-GCs according to gender, age and tumor location have yet to be examined in detail. This study, using by far the largest number of EBV-GC cases, examined the histology-specific distributions of EBV-GCs by these factors.

Materials and methods

Subjects. We examined 1,961 gastric carcinomas from Japanese patients ranging in age from 30 to 95 years. Twenty-five

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Key words: EBV, gastric carcinoma, EBER, histology, Japan

remnant cancer cases were excluded because of their unique pathologic features. Furthermore, 18 cases (12 male and 6 female cases) with LELC-type tumors were also excluded from the present analysis. Among the remaining 1,918 cases, 1,693 cases ascertained during the period 1976-1992 were those used in the previous study reported by Tokunaga *et al* (3). The remaining 225 new Japanese gastric cancer cases were collected from Kagoshima City Hospital during the period 1993-1995.

Histology. Gastric carcinomas were classified according to the classification scheme of the Japanese Research Society for Gastric Cancer (12). Histological patterns were considered as follows: well-differentiated tubular adenocarcinoma (tub1), moderately-differentiated tubular adenocarcinoma (tub2), solid poorly-differentiated adenocarcinoma (por1), non-solid poorly-differentiated adenocarcinoma (por2), signet-ring cell carcinoma (sig) and mucinous carcinoma (muc). According to Lauren classification, intestinal-type tumors included carcinomas with types tub1, tub2 and muc, and diffuse-type tumors included carcinomas with types por1, por2 and sig.

The location of a tumor, defined as the predominant location of the tumor, was divided into the following three categories: cardia or upper third part, middle part, and antrum or lower third part according to the guidelines of the Japanese Research Society for Gastric Cancer (12).

ISH assay to detect EBER. The ISH assay of paraffin-embedded tissue samples obtained from the main tumor was conducted using a digoxigenin-labeled EBER-1 oligonucleotide probe as previously described (30). A case was considered to be EBER positive based on an intensive nuclear dark purple signal under microscopy. In every ISH assay, lymph node section from a patient with infectious mononucleosis and a sense probe for EBER-1 were used as positive and negative controls, respectively.

Statistical analysis. We examined the proportion of EBV-GCs using logistic regression analysis. Gender, age and tumor subsite were included in logistic models as covariates. Maximum likelihood estimates of odds ratios (OR) and 95% confidence interval (CI) were calculated. The P-value for trend of age was calculated using age as a continuous variable in a logistic model. All the P-values presented were two-sided.

Table I. Results of logistic analysis, both intestinal and diffuse types.

Variables	EBER⁺/N	(%)	OR[a]	95% CI[a]
Gender				
Female	17/706	(2.4)	1	reference
Male	83/1212	(6.8)	3.2	1.9-5.6
		P<0.001		
Age				
≤49	17/296	(5.7)	1	reference
50-69	59/1052	(5.6)	1.1	0.6-1.9
≥70	24/570	(4.2)	0.8	0.4-1.6
		P for trend = 0.233		
Location				
Upper third (cardia)	19/229	(8.3)	1	reference
Middle	58/759	(7.6)	1.0	0.6-1.7
Lower third (antrum)	23/930	(2.5)	0.3	0.2-0.6
		P for heterogeneity <0.001		
Histology				
Diffuse	51/830	(6.1)	1	reference
Intestinal	49/1088	(4.5)	0.7	0.4-1.0
		P=0.065		

[a]ORs and 95% CIs were obtained from logistic regression analysis using gender, age, and tumor location as covariates.

Results

EBV-GCs accounted for 4.5% (n=49) and 6.1% (n=51) of 1,088 intestinal-type and 830 diffuse-type gastric carcinomas, respectively. When the gastric carcinomas were classified according to the classification scheme of the Japanese Research Society for Gastric Cancer, the percentage of EBV-GCs in tub1-, tub2-, por1-, por2-, sig- and muc-type tumors were 0.8%, 7.7%, 10.1%, 2.8%, 1.9% and 3.3%, respectively. EBV-GCs were most common in tub2- and por1-type carcinomas.

Table I shows the distribution of EBV-GCs according to gender, age, tumor location and histology. ORs and 95% CIs were obtained from logistic analysis using gender, age and tumor location as covariates. EBV-GC showed male predominance, and a relatively low frequency in the antrum, confirming clinicopathological features described by many previous studies. Diffuse-type tumors were slightly more common than intestinal-type carcinomas, but the difference between the 2 types was not statistically significant (P=0.065).

Table II summarizes the results obtained from logistic analyses conducted separately for the intestinal and diffuse types. Among intestinal-type tumors, EBV-GCs appeared to be predominant among the males but the gender difference was not statistically significant (P=0.143). Interestingly, age was inversely related to the ratio between EBER-positive and -negative tumors (P for trend = 0.002). The age-dependent decrease was observed regardless of tumor location (data not shown). Even when intestinal-type tumors were restricted to tub2-type tumors, which was most common among intestinal-type EBV-GCs, the age-dependent decrease of EBV-GC proportion was evident and statistically significant (P=0.004). Intestinal-type EBV-GCs were relatively less prevalent in the antrum. The proportion of intestinal-type EBV-GCs did not show evident dependence on calendar years (data not shown).

The results for diffuse-type carcinoma are also shown in Table II. In diffuse-type carcinomas, male predominance was evident and statistically significant (P<0.001). The magnitude of OR, comparing the ratio between EBER-positive and -negative tumors in 2 genders was larger in diffuse-type carcinomas (OR=5.1) than in intestinal-type carcinomas (OR=1.7). However, the 95% CIs of their ORs overlapped with each other; there was no statistically significant

Table II. Results of logistic analysis conducted separately for diffuse-type and intestinal-type gastric carcinomas.

	Intestinal type		Diffuse type	
Variables	EBER+/N (%)	OR (95% CI)[a]	EBER+/N (%)	OR (95% CI)[a]
Gender				
Female	9/316 (2.8)	1 (reference)	8/390 (2.1)	1(reference)
Male	40/772 (5.2)	1.7 (0.8-3.6)	43/440 (9.8)	5.1 (2.3-11.1)
	P=0.143		P<0.001	
Age				
≤49	10/95 (10.5)	1 (reference)	7/201 (3.5)	1 (reference)
50-69	30/611 (4.9)	0.4 (0.2-0.9)	29/441 (6.6)	2.1 (0.9-5.0)
≥70	9/382 (2.4)	0.2 (0.1-0.5)	15/188 (8.0)	2.3 (0.9-5.8)
	P for trend = 0.002		P for trend = 0.429	
Location				
Upper third	8/141 (5.7)	1 (reference)	11/88 (12.5)	1 (reference)
Middle	23/364 (6.3)	1.4 (0.6-3.3)	30/395 (7.6)	0.8 (0.4-1.6)
Lower third	13/583 (2.2)	0.4 (0.2-1.0)	10/347 (2.9)	0.2 (0.1-0.6)
	P for heterogeneity <0.001		P for heterogeneity = 0.001	

[a]ORs and 95% CIs were obtained from logistic regression analysis using gender, age and tumor location as covariates.

difference in the magnitude of the 2 ORs. The pattern of age-dependence in diffuse-type EBV-GCs was different from that of intestinal-type EBV-GCs. The proportion of EBV-GCs in diffuse-type carcinomas showed an increase in those ≥50 years of age. The trend of this increase was not statistically significant. The difference in the age-dependent patterns between the 2 histological types of Lauren classification was statistically significant in logistic analysis (P=0.002). As was the case with the intestinal-type tumors, the proportion of EBV-GCs among diffuse-type tumors was the lowest in cancer of the antrum. The proportion of diffuse-type EBV-GCs did not show an evident time trend (data not shown).

Discussion

The present study showed that the proportion of EBV-GCs in intestinal-type carcinomas decreased with age. On the other hand, age was not related to the proportion of EBV-GCs in diffuse-type tumors. The relatively low proportion of diffuse-type EBV-GCs in those aged <50 may be reflecting the presence of diffuse-type tumors with hereditary backgrounds in this relatively young age group (31). The results obtained here are similar to those reported by Hao *et al*, where only intestinal-type EBV-GCs showed an age-dependent decrease (6). Similar results were also obtained in a Chilean study [unpublished data, collected by the study conducted by Corvalan *et al* (11)]. Histology-specific age distribution of EBV-GCs was also examined by Hsieh *et al* (9). This Taiwanese study showed an age-dependent increase of intestinal-type EBV-GCs (statistical tests were not conducted). On the other hand, a study conducted in Colombia showed an age-dependent decrease both in intestinal and diffuse types (7).

Using the proportions of EBV-GCs specific for age and histology observed in the present study, as well as gastric cancer incidence in Japan in 1985 (32), we estimated age-specific incidence of intestinal- and diffuse-type EBV-GCs among men (Fig. 1). Incidence of EBV-GC among women was not estimated because the number of cases was too small to obtain reliable estimates. However, in statistical analysis, there was no evidence suggesting a significant gender difference in the EBV-GC incidence. The estimated incidence of intestinal-type EBV-GCs reaches its peak around 70 years of age (Fig. 1A). On the other hand, that of diffuse-type EBV-GCs does not have a peak in its age-specific incidence curve (Fig. 1B), suggesting a much older age peak, if any. Nasopharyngeal carcinoma (NPC), which is one of the most frequently observed carcinomas in southern China, and strongly suspected to be related to EBV, has its incidence peak at 50-60 years of age (33), which is much younger than that observed in intestinal-type EBV-GCs. The difference in the peak age between NPC and EBV-GC incidence curves suggests that age at exposure to etiologically important factors differs in NPC and EBV-GC patients. In most of the countries around the world, including southern China and Japan, the EBV infection takes place in early childhood (34). Therefore, it is unlikely for the ages at primary EBV infection to explain the different age distributions of these 2 cancers. Another possibility is the difference of ages at exposure to cofactors. In the case of NPC, salted fish intake in early childhood is an important cofactor in its etiology (35). Although no cofactors of EBV-GC are known, the male predominance of EBV-GCs strongly suggests the involvement of lifestyle-related factors in its etiology. The results obtained from the present study suggest that the age at exposure to those cofactors may be much older in the case of intestinal-type EBV-GCs, and even older in the case of diffuse-type EBV-GCs, when compared to NPC. As reviewed in the previous paragraph, the patterns of age-dependence observed in EBV-GCs are different from country to country. This fact may also explain the different age at exposure to cofactors from country to country.

Almost all the studies so far showed male predominance of EBV-GC. Among the highest gender ratios were 7.0

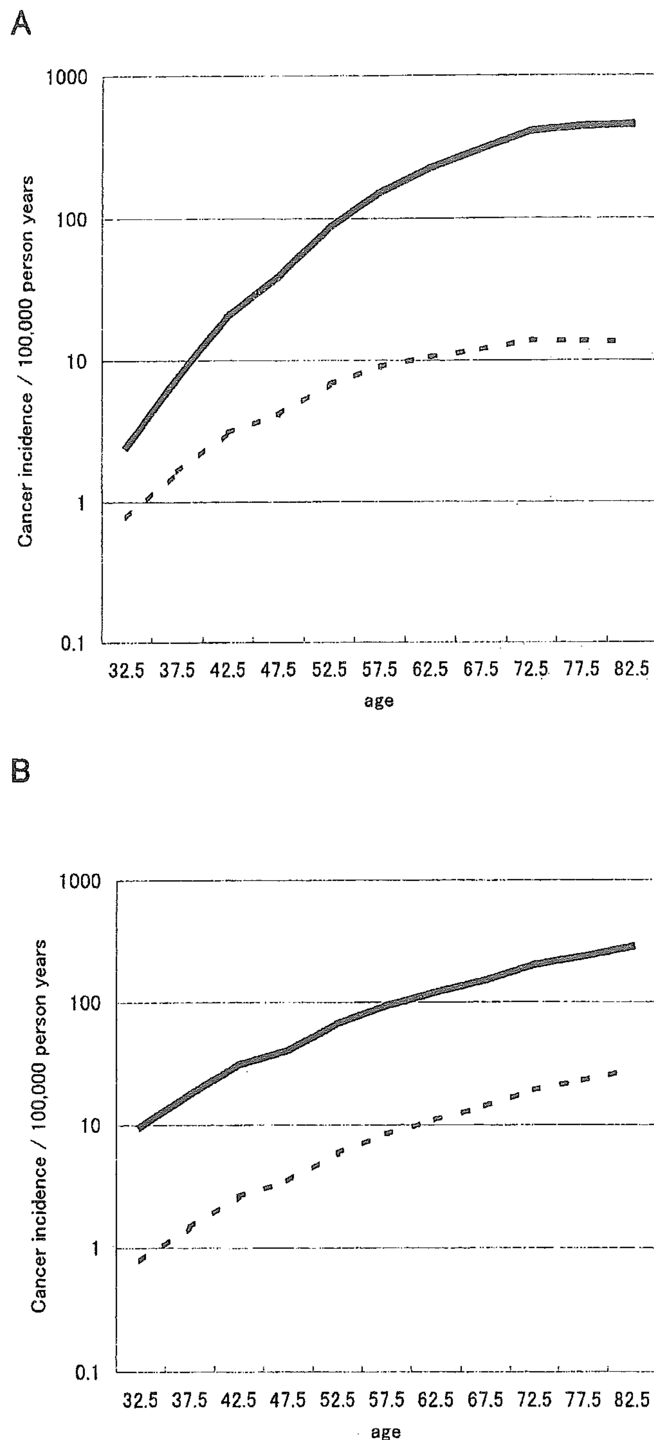

Figure 1. Estimated intestinal- and diffuse-type gastric cancer incidence in Japanese men. (A), Solid line, gastric cancer incidence of all intestinal-type tumors in Japanese men; dotted line, incidence of the intestinal-type EBV-GC in Japanese men. (B), Solid line, gastric cancer incidence of all diffuse-type tumors in Japanese men; dotted line, incidence of the diffuse-type EBV-GC in Japanese men.

observed in Caucasians living in Los Angeles (1), and 6.2 in Russians (9). Even among LELCs, which are known to be almost always EBER positive (22), the present study showed male predominance (LELC turned out to be EBER positive in all of the 12 male cases, and 4/6 female cases). To date, an exception is the Mexican study, reporting the gender ratio of only 1.2 (10). A study conducted in an area near Shanghai also reported a similar result (4). The present study showed the difference in magnitude of male predominance in intestinal- and diffuse-type tumors, suggesting that the varying gender ratio from country to country may be explained to some extent by the different distributions of various histological types. Although the underlying mechanism of male predominance in EBV-GC is not known, possible factors are the lifestyles more commonly observed in males than in females. Undifferentiated NPC, whose morphology is almost identical to that of gastric LELC, is known to have incidence about 2-fold higher in males than in females (33). The factors responsible for this male predominance have yet to be elucidated.

There are several studies reporting the predominance of diffuse-type tumors among EBV-GCs, as stated in the Introduction. This study did not confirm this notion. As shown in Table II, the ratio between intestinal- and diffuse-type EBV-GCs is affected by gender, age and tumor location. The conflicting results in the literature may be explained by the different distribution of those factors in various studies.

Another evident feature of EBV-GC observed in the present study is its low prevalence among carcinomas of the antrum regardless of histological type. Note here that fundic-gland mucosa exists in only a small part of the lower one-third of the stomach, whereas it covers the upper-third of the stomach except cardia. Thus, our observation suggests that EBV-GC tends to occur, but unlikely exclusively, in the fundic-gland epithelium, which is featured by oxyntic cells. Interestingly, as pointed out by Fukayama *et al*, EBV-GCs are more frequently found in the zone intermediate between fundic and pyloric gland mucosa (36). The intermediate zone moves in proximal direction as age advances (37). The age-dependent decrease of intestinal-type EBV-GCs observed in the present study may be explained by the shift of the zone, whose nature may be changed by the shift or the advancement of age (or both).

This study, examining by far the largest number of EBV-GCs in current literature, showed different patterns of age-dependence in intestinal- and diffuse-type EBV-GCs, suggesting that pathogenic pathways of EBV-GCs may be different in these 2 histological types.

Acknowledgements

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HTLV-1 provirus load in peripheral blood lymphocytes of HTLV-1 carriers is diminished by green tea drinking

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Human T-cell lymphotropic virus type 1 (HTLV-1) is causatively associated with adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Since a high level of HTLV-1 provirus load in circulating lymphocytes is thought to be a risk for ATL and HAM/TSP, diminution of HTLV-1 provirus load in the circulation may prevent these intractable diseases. Our previous study (*Jpn J Cancer Res* 2000; 91: 34–40) demonstrated that green tea polyphenols inhibit *in vitro* growth of ATL cells, as well as HTLV-1-infected T-cells. The present study aimed to investigate the *in vivo* effect of green tea polyphenols on HTLV-1 provirus load in peripheral blood lymphocytes on HTLV-1 carriers. We recruited 83 asymptomatic HTLV-1 carriers to examine HTLV-1 provirus DNA with or without administration of capsulated green tea extract powder. Thirty-seven subjects were followed up for 5 months by measuring HTLV-1 provirus load after daily intake of 9 capsules of green tea extract powder per day (equivalent to 10 cups of regular green tea), and 46 subjects lived *ad libitum* without intake of any green tea capsule. The real-time PCR quantification of HTLV-1 DNA revealed a wide range of variation of HTLV-1 provirus load among asymptomatic HTLV-1 carriers (0.2–200.2 copies of HTLV-1 provirus load per 1000 peripheral blood lymphocytes). Daily intake of the capsulated green tea for 5 months significantly diminished the HTLV-1 provirus load as compared with the controls (*P*=0.031). These results suggest that green tea drinking suppresses proliferation of HTLV-1-infected lymphocytes *in vivo*. (Cancer Sci 2004; 95: 596–601)

Human T-cell lymphotropic virus type 1 (HTLV-1) is a causative agent of adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–4] The major route of HTLV-1 infection is mother-to-child transmission via breast milk, and both bottle-feeding and short-term breast-feeding reduce the risk of neonatal infection with HTLV-1.[5,6] However, once HTLV-1 infects T lymphocytes and integrates its provirus into the host genome, the virus cannot be excluded from the body. A high level of HTLV-1 provirus load in circulating lymphocytes of HTLV-1 carriers is a risk factor for HTLV-1-related diseases.[7–10] It is thus likely that diminution of HTLV-1 provirus load in circulating lymphocytes may prevent HTLV-1 carriers from contracting ATL or HAM/TSP.

Green tea polyphenols have antioxidant and anti-mutagenic activities,[11–14] and induce apoptosis in a variety of tumor cells.[15,16] These anti-tumor activities of green tea have been supported by epidemiological findings that green tea drinking lowers the risk of stomach cancer.[17,18] Other Japanese studies showed that consumption of green tea amounting to more than 10 cups per day lowered the incidence of cancers of the stomach, lung and other sites.[19–21]

We previously reported that green tea polyphenols inhibit *in vitro* growth of ATL cells as well as HTLV-1-infected T-cells, by inducing apoptosis.[22] The present study was conducted to assess the *in vivo* effect of green tea to diminish HTLV-1 provirus load in peripheral blood lymphocytes of asymptomatic HTLV-1 carriers.

Materials and Methods

Study subjects and blood samples. Ninety-five subjects were recruited from asymptomatic HTLV-1 carriers living in Kagoshima prefecture, an HTLV-1 endemic area in southern Kyushu, Japan, all of whom had given informed consent to answer a questionnaire regarding their lifestyle (such as habitual green tea consumption, smoking and alcohol drinking habits), to make daily records of taking capsules of green tea extract powder, and to donate peripheral blood for monthly examination of HTLV-1 provirus load. The female subjects were recruited from those who had participated in the surveillance for mother-to-child transmission of HTLV-1,[6] and the male subjects were their husbands and relatives. The subjects were randomly assigned to two groups by the minimization method using age and gender as risk factors: 47 subjects who took the capsulated green tea extract powder, designated as the GT(+) group and 48 subjects who lived *ad libitum* without intake of any green tea capsules, designated as the GT(−) group. Both groups were followed up monthly for 5 months. We drew 5–7 ml of peripheral blood with citrate anticoagulant from each subject and collected the buffy coat to enrich peripheral blood lymphocytes by centrifugation at 2000*g* for 10 min at ambient temperature. The buffy coats were frozen at −30°C until used for examination of HTLV-1 provirus DNA. The study protocol was reviewed and approved by the Medical Ethical Committee of Kagoshima University.

Capsules of green tea extract powder. We used organic green tea leaves grown without the use of pesticides to prepare the capsules of green tea extract powder. In brief, the dry green tea leaves were infused into hot water (95°C) with ascorbic acid to stabilize green polyphenols, then the infusion was reduced to powder by a spray-dry method. The green tea extract powder was capsulated as "Nanchariki" (Satsuma Shuzo Co., Ltd., Kagoshima, Japan). Ingredients of the green tea extract powder were analyzed with a HPLC-UV detection system as depicted in Fig. 1. One capsule of the powder contained 27.3 mg of (−)-epigallocatechin-3-gallate (EGCg), which is the main constituent of green tea polyphenols. One cup of regular green tea, made by infusion of 2 g of dried leaves with 150 ml of hot wa-

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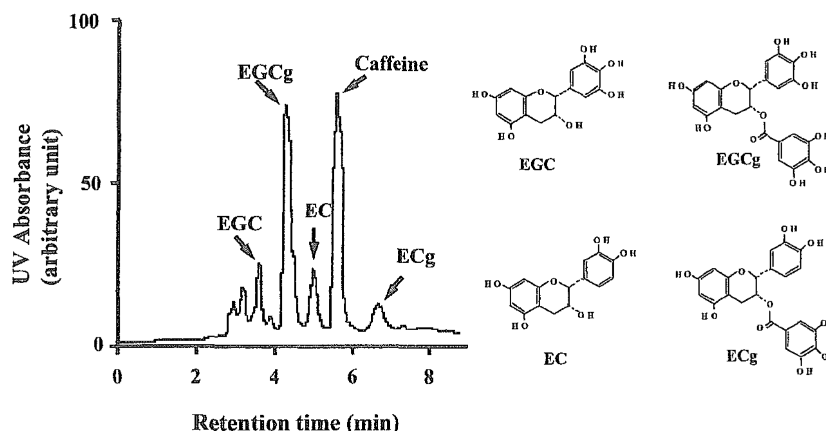

Fig. 1. HPLC profile of polyphenols and caffeine in green tea extract powder ("Nanchariki"). Ten milligrams of "Nanchariki" powder was dissolved in 10% methanol solution by sonication and vigorous vortexing. The mixture was passed through a 0.45 μm filter to get a sample solution of the green tea extract powder. An aliquot of the solution was subjected to HPLC with UV detection (mobile phase, methanol/water (35/65 containing 0.2% phosphoric acid); flow rate, 1.0 ml/min; column, C_{18} reversed-phase column (150×4.6 mm i.d., particle size 5 μm); column temperature, 40°C; UV detection, 280 nm). Polyphenol compounds were analyzed with reference to authentic (–)-epigallocatechin-3-gallate (EGCg), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECg), epicatechin (EC) and caffeine purchased from Mistui Norin Co., Ltd. (Shizuoka, Japan) and Nacalai Co., Ltd. (Tokyo), respectively. The chemical structures of EGC, EGCg, EC and ECg are shown.

ter, contains 24.0 mg of EGCg. Therefore, 9 capsules of "Nanchariki" contain 245.7 mg of EGCg, which is equivalent to 10 cups of regular green tea.

Real-time PCR quantification of HTLV-1 provirus load in peripheral blood lymphocytes. High-molecular DNA was isolated from the frozen buffy coat of blood samples pretreated with RCLB solution (Genome Science Laboratories, Fukushima, Japan) to remove red blood cell components and with SMI TEST EX-R&D (Genome Science Laboratories) to extract DNA. The standard HTLV-1 DNA was prepared from MT-2 cells.[23] The real-time PCR quantification of HTLV-1 DNA was performed in a LightCycler System (Roche Diagnostics, Mannheim, Germany) by intra-assay using a series of duplicate measurements of 12 test samples with standard DNA of 4 different dilutions for each assay. The duplicate intra-assay for HTLV-1 provirus load in peripheral blood lymphocytes was run by simultaneous measurements of β-globin DNA and HTLV-1 DNA using the standard DNAs, β-globin DNA from Roche Diagnostics, and HTLV-1 provirus DNA from MT-2 cells. One peripheral blood lymphocyte has 2 copies of *β-globin* gene (equivalent to 6 pg of β-globin DNA) and one MT-2 cell has 8 copies of HTLV-1 provirus DNA (equivalent to 6 pg of HTLV-1 DNA). The β-globin PCR primer and probe sets were commercial kits (Roche Diagnostics). The HTLV-1 primer set corresponded to the highly conserved HTLV-1 pX region, SK43 (CGGATACCCAGTCTACGTGT, nucleotide positions 7358–7377) and SK44 (GAGCCGATAACGCGTCCATCG, nucleotide positions 7516–7496).[24] The HTLV-1 pX probe set was newly designed by ourselves for the two adjacent parts of the pX region which were labeled with different fluorophores (SONPX1: 5′-TACATCGTCACGCCCTACTGGCCAC-fluorescein-3′, nucleotide positions 7438–7462 and SONPX2: 5′-LC-red640-TGTCCAGAGCATCAGATCACCTGGG-phosphate-3′, nucleotide positions 7464–7488) according to the manufacturer's instructions.

The PCR amplification was initiated with pre-incubation at 95°C for 10 min using FastStart polymerase (Roche Diagnostics). For β-globin amplification, 40 cycles of PCR were carried out: 95°C for 10 s, 50°C for 10 s and 72°C for 10 s. For HTLV-1 pX amplification, we performed 45 cycles of PCR at 95°C for 10 s, 50°C for 10 s and 72°C for 10 s. The final volume (20 μl) of the PCR amplification mixture for HTLV-1 pX, 3 m*M* $MgCl_2$, 0.5 μ*M* each of the primer set, 0.2 μ*M* each of the oligonucleotide probes, 2 μl of LightCycler FastStart DNA Master Mix (Roche Diagnostics) and 2 μl of template DNA solution diluted to 25 ng/μl, while those for β-globin were from commercially available kits (Roche Diagnostics). The HTLV-1 provirus load was expressed as number of copies per 1000 cells using the following formula: HTLV-1 provirus load=[(HTLV-1 pX copy number)/(β-globin copy number/2)]×1000. The detection limit of this method was 0.2 copies of HTLV-1 provirus/1000 cells.

Statistical analysis of difference in HTLV-1 provirus load. To examine the significance of the diminution of HTLV-1 provirus load after taking the green tea capsules, we calculated the difference of HTLV-1 provirus load between the baseline (0 month) and each month, and compared the difference between the GT(–) and GT(+) groups by time after taking the capsules, using a *t* test. We also performed subgroup analysis of subjects whose values of HTLV-1 provirus load at baseline were either below or above median value (lower or higher provirus load group). The trend of change in HTLV-1 provirus load values during the intervention period was compared between the GT(–) and GT(+) groups using a linear regression model with a group interaction term (GT(–)=0 and GT(+)=1) and time (month), after adjusting for the contribution of habitual green tea drinking (0, 1–3, 4–9, 10–14, ≥15 cups of regular green tea per day). Differences of demographic distribution and lifestyles at baseline between the GT(–) and GT(+) groups were examined by using the *t* test and χ^2 test. Statistical analysis was carried out using STATA ver.7 (Stata Corp., TX).

Results

Profile of study subjects. We recruited 95 subjects at the beginning of this study; however, 12 dropped out due to difficulty in monthly donation of blood samples and low compliance with daily intake of the capsules of green tea extract powder. Thus, we followed up 83 subjects for 5 months, 37 subjects in the GT(+) group and 46 subjects in the GT(–) group. The age distribution in the GT(+) group was slightly shifted to the younger side as compared with the GT(–) group, but the difference was not statistically significant. Lifestyle factors (green tea consumption, smoking and alcohol drinking habits) in the GT(+)

and GT(−) groups were similar at baseline, and there was no significant difference between the groups (Table 1). Most subjects tolerated the daily intake of "Nanchariki" without any complaint of gastrointestinal symptoms, except for one subject who developed an indisposition in stomach and bowel movement after 2 months of daily intake of "Nanchariki" capsules.

Properties of green tea extract powder and content of EGCg. Green tea contains antioxidants and other bioactive components.[12,14,22] HPLC-UV analysis revealed that the "Nanchariki" powder contained 4 polyphenols (EGC, EGCg, EC, ECg) and caffeine as major ingredients (Fig. 1). Amounts of EGC, EGCg, EC and ECg in the "Nanchariki" powder were estimated to be 84.7, 68.8, 32.5 and 10.7 mg/g, respectively. The amount of caffeine was 32.7 mg/g. Freshly prepared regular green tea showed the same profile of polyphenols and caffeine as that of "Nanchariki" powder (data not shown). Nine capsules of "Nanchariki" contained 245.7 mg of EGCg (equivalent to 10 cups of regular green tea). This is estimated to be as much as one-third of the maximum tolerated dose of green tea extract in humans, 4.2 g green tea extract (34 cups of regular green tea)/m^2/day.[25]

Real-time PCR quantification of HTLV-1 provirus load among HTLV-1 carriers. We used the real-time PCR system in LightCycler for quantification of HTLV-1 provirus load in peripheral blood lymphocyte DNA. Our duplicate intra-assay system reduced the error in PCR measurement, providing a coefficient of variation of less than 8% for test samples containing 18.0–200.2 copies of HTLV-1 provirus/1000 cells, and enabled accurate quantitative measurements of HTLV-1 provirus load in peripheral blood lymphocytes (Fig. 2). We found a wide range of individual variation of HTLV-1 provirus load, from 0.2–200.2 copies of HTLV-1/1000 cells, and the median value was 18.0 copies/1000 cells, giving a skewed distribution of HTLV-1 provirus load among HTLV-1 carriers (Fig. 3).

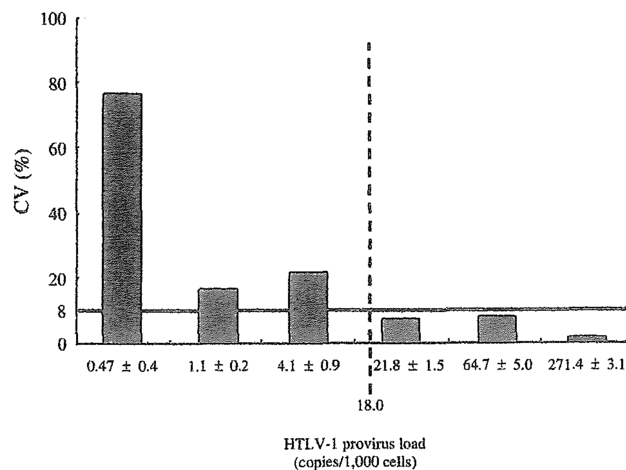

Fig. 2. Relationship between HTLV-1 provirus load and coefficient of variation. Coefficient of variation (CV) was determined by quadruplicate measurements of HTLV-1 provirus load using serially diluted DNA samples of HTLV-1 carrier's lymphocytes. CV (%) was calculated by means of the following formula: CV (%)=(SD of HTLV-1 provirus load in quadruplicate measurement/mean of HTLV-1 provirus load in quadruplicate measurement)×100. The axis shows the amount of HTLV-1 provirus load in each dilution of HTLV-1 DNA (copies/1000 cells). The ordinate shows CV (%). Subjects with more 18.0 copies of HTLV-1 provirus load/1000 cells had a CV of less than 8%.

Table 1. Characteristics of study subjects

	Number (%)		*P* value[3),4)]
	GT(−)[1)]	GT(+)[2)]	
Age			
25–39	23 (50)	22 (59)	0.692
40–59	22 (48)	14 (38)	
60–77	1 (2)	1 (3)	
Total	46 (100)	37 (100)	
Sex			
Male	2 (4)	1 (3)	0.690
Female	44 (96)	36 (97)	
Habitual consumption of green tea at baseline			
0 cup/day	12 (26)	14 (37)	0.188
1–3 cups/day	15 (32)	11 (30)	
4–9 cups/day	12 (26)	10 (27)	
10–14 cups/day	1 (2)	1 (3)	
15 or more cups/day	3 (7)	0 0	
Unknown	3 (7)	1 (3)	
Smoking habit at baseline			
Never or former	35 (76)	31 (84)	0.573
Current	8 (17)	5 (14)	
Unknown	3 (7)	1 (3)	
Alcohol drinking habit at baseline			
None	24 (52)	19 (51)	0.117
<1 day/week	7 (15)	9 (24)	
1–4 days/week	3 (7)	6 (16)	
5> day/week	7 (15)	1 (3)	
Unknown	5 (11)	2 (6)	

1) GT(−): Without administration of green tea capsules.
2) GT(+): With administration of green tea capsules (9 capsules per day).
3) Distributions of age between GT(−) and GT(+) were compared by *t* test.
4) Differences of sex and lifestyle between GT(−) and GT(+) were examined by χ^2 test.