Circ J 2004; **68:** 1146–1151

Effects of Repeated Sauna Treatment on Ventricular Arrhythmias in Patients With Chronic Heart Failure

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Background The aim of the present study was to determine whether repeated 60°C sauna treatment improves cardiac arrhythmias in chronic heart failure (CHF) patients, because ventricular arrhythmias are an important therapeutic target in CHF.

Methods and Results Thirty patients (59±3 years) with New York Heart Association functional class II or III CHF and at least 200 premature ventricular contractions (PVCs)/24h assessed by 24-h Holter recordings were studied. They were randomized into sauna-treated (n=20) or non-treated (n=10) groups. The sauna-treated group underwent a 2-week program of a daily 60°C far infrared-ray dry sauna for 15 min, followed by 30 min bed rest with blankets, for 5 days per week. Patients in the non-treated group had bed rest in a temperature-controlled room (24°C) for 45 min. The total numbers of PVCs/24h in the sauna-treated group decreased compared with the non-treated group [848±415 vs 3,097±1,033/24h, p<0.01]. Heart rate variability (SDNN, standard deviation of normal-to-normal beat interval) increased [142±10 (n=16) vs 112±11 ms (n=8), p<0.05] and plasma brain natriuretic peptide concentrations decreased [229±54 vs 419±110 pg/ml, p<0.05] in the sauna-treated group compared with the non-treated group.

Conclusion Repeated sauna treatment improves ventricular arrhythmias in patients with CHF. (*Circ J* 2004; **68:** 1146–1151)

Key Words: Heart failure; Heart rate variability; Premature ventricular contractions; Sauna

Patients with chronic heart failure (CHF) have a high prevalence of potentially serious arrhythmias and consequently, a high incidence of sudden cardiac death[1–4] The presence of ventricular arrhythmias defines a higher-risk patient group with either ischemic or non-ischemic cardiomyopathy[5–9] Antiarrhythmic medications, such as class I drugs, have been tested in myocardial infarction survivors with depressed ventricular function and in atrial fibrillation patients with a history of congestive heart failure, and most were found not to be helpful and may even increase the occurrence of arrhythmias and cardiac mortality[10–12] Some studies have shown that amiodarone improves ventricular arrhythmias and sudden cardiac death mortality in patients with CHF, yet the improvement in total mortality remains controversial[13–15] Previous studies have demonstrated that vasodilators, such as angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, improve the prognosis and ventricular arrhythmias in patients with CHF[16–18] therefore arrhythmia is an important target for therapy in patients with CHF.

We have used thermal therapy with a 60°C dry sauna in patients with CHF, and found that it improves hemodynamic parameters, endothelial function, and clinical symptoms in many patients[19–21] Furthermore, we have demonstrated that repeated sauna treatment improves the prognosis in hamsters with CHF[22] It is well recognized that alterations in the neural control of the heart, characterized by decreased vagal activity and relative sympathetic predominance, play a key role in the occurrence of cardiac arrhythmias in patients with CHF[23] Several studies have shown that reduced heart rate variability (HRV), determined from 24-h ambulatory electrocardiographic (ECG) recordings, is associated with a greater risk for ventricular fibrillation and poor prognosis in patients with CHF[24–27] Therefore, we prospectively investigated the effects of thermal therapy on cardiac arrhythmias and HRV in patients with CHF.

(Received April 5, 2004; revised manuscript received September 21, 2004; accepted September 28, 2004)

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Methods

Study Population

We studied 30 patients with CHF, aged 28–80 years (mean age: 59±3 years): 24 patients (16 men, 8 women) had idiopathic dilated cardiomyopathy and 6 (5 men, 1 woman) had ischemic cardiomyopathy. Inclusion criteria included the presence of symptomatic CHF, left ventricular ejection fraction (LVEF) <50% by echocardiography, New York Heart Association (NYHA) functional class II–III, and >200 premature ventricular contractions (PVCs) per day on 24-h Holter monitoring. Seven patients were in NYHA functional class II, and the other 23 were in class III. They were randomized into a sauna-treated group (n=20) or a non-treated group (n=10). The mean number of PVCs/24h was 3,123±819; the mean cardiothoracic ratio (CTR) on chest radiography was 58.5±1.0% (range: 49–75%); and the mean LVEF on echocardiography was 29±2% (range:

Table 1 Baseline Clinical Characteristics of the 2 Groups

	Sauna-treated group (n=20)	*Non-treated group (n=10)*	*p value*
Age	*59±3*	*59±4*	*NS*
M/F	*14/6*	*7/3*	*NS*
DCM/ICM	*16/4*	*8/2*	*NS*
Atrial fibrillation (n)	*5*	*2*	*NS*
NYHA (I/II/III)	*0/5/15*	*0/2/8*	*NS*
Body weight (kg)	*57±3*	*53±3*	*NS*
Heart rate (beats/min)	*73±3*	*73±4*	*NS*
SBP (mmHg)	*107±4*	*108±5*	*NS*
DBP (mmHg)	*65±3*	*67±3*	*NS*
Drug therapy (%)			
Digoxin	*65*	*60*	*NS*
ACE inhibitors	*95*	*90*	*NS*
β-blockers	*55*	*40*	*NS*
Diuretics	*95*	*100*	*NS*
Nitrates	*30*	*30*	*NS*
Antiarrhythmic drugs (%)			
Mexiletine	*50*	*50*	*NS*

DCM, idiopathic dilated cardiomyopathy; ICM, ischemic cardiomyopathy; NYHA, New York Heart Association; SBP, systolic blood pressure; DBP, diastolic blood pressure; ACE, angiotensin-converting enzyme; NS, not significant.
All values are given as the mean±SE.

10–48%). All patients were receiving maintenance doses of medications for heart failure and arrhythmias, including angiotensin-converting enzyme inhibitors, diuretics, β-blockers, digitalis and antiarrhythmic drugs (mexiletine), and they were in a stable clinical condition for 1 month before entering the study. They also did not have symptomatic arrhythmias. Their medications were unchanged for at least 1 month before or during this study. Written informed consent was obtained from all patients prior to participation, and the protocol was approved by the Ethics Committee of the Faculty of Medicine, Kagoshima University.

Sauna Treatment

Thermal therapy with a far infrared-ray 60°C dry sauna was performed as previously reported.[19] Patients remained supine on a bed during the sauna for 15 min, followed by 30 min of bed rest with a blanket to keep them warm. Patients were weighed before and after the sauna treatment. Oral hydration with water was used to compensate for lost weight. Patients in the non-treated group remained supine on a bed in a temperature-controlled room (24°C) for 45 min.

Assessment of Clinical Symptoms

Clinical symptoms, such as dyspnea, fatigue, sleeplessness, edema, appetite-loss and constipation, were evaluated by a self-assessment quality of life (QOL) questionnaire.[20] Each item had 4 grades: remarkably improved, improved, no change, or worsened. Patients were classified into 3 groups based on the results of the questionnaire. Patients who answered 'improved' to more than 3 items were defined as the improved group, those who answered 'worsened' for at least 1 item were defined as the worsened group, and the others were defined as the unchanged group.

Laboratory Examination

A fasting blood sample was obtained in the morning to measure plasma concentrations of neurohormonal factors, including catecholamines, atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP). Plasma catecholamine (norepinephrine, epinephrine, and dopamine) concentrations were measured with high-performance liquid chomatography, and both plasma ANP and BNP concentrations were measured by radioimmunoassay. Chest radiography (CTR) and echocardiography (LVEDD, left ventricular end diastolic dimension; LAD, left atrial dimension; LVEF) also were performed.

Ambulatory ECG Recording

Ambulatory ECG monitoring was by 2-lead 24-h Holter monitoring (DMC-4502, Nihon Koden, Tokyo, Japan). The Holter tape recordings were analyzed on a full disclosure unit that printed out each individual QRS complex for subsequent visual examination. Complete determination of PVC frequency with a description and quantification of complex forms (multiform PVCs, couplets, and ventricular tachycardia) was undertaken by manual analysis of the full disclosure data. For the purpose of this study, PVCs were defined as any beat of ventricular origin faster than the sinus rate, including the premature beats in couplets and ventricular tachycardia. Ventricular tachycardia was defined as ≥3 consecutive premature beats at a rate of ≥100 beats/min. There was an excellent correlation between the 2 observers with respect to determining the total number of PVCs ($r=0.99$), and the number of episodes of ventricular tachycardia ($r=0.99$). The technician and physician were unaware of the clinical information associated with the recording. Reproducibilities of the results of 24-h Holter monitoring performed twice were assessed in 13 patients with CHF: total beats, $r=0.99$, $p<0.0001$; PVCs, $r=0.91$, $p<0.0001$; couplets, $r=0.95$, $p<0.0001$; ventricular tachycardia, $r=0.95$, $p<0.0001$.

Analysis of HRV

Time-domain parameters of HRV were analyzed on a MARS8000 analysis system (GE Medical Systems Information Technologies, Milwaukee, WI, USA) from 2-lead 24-h Holter recordings. All tapes were manually edited for exclusion of artifacts and premature beats. A minimum of 18 h of analyzable data and a minimum of 85% successive RR intervals were required for a tape to be accepted as valid. The time interval between 2 consecutive QRS complexes was calculated as the normal-to-normal (NN) interval. Abnormal QRS complexes and RR intervals

Table 2 Frequency of Ventricular Arrhythmias and Heart Rate Variability at Baseline and After 2 Weeks in the 2 Groups

	Sauna-treated group		*Non-treated group*		*Comparison with both groups*	
	Baseline	*After 2 weeks*	*Baseline*	*After 2 weeks*	*At baseline*	*After 2 weeks*
PVCs/24 h (beats/24 h)	*3,161±1,104*	*848±415***	*3,048±914*	*3,097±1,033*	*NS*	*<0.0001*
Couplets (episodes/24 h)	*71±33*	*15±11***	*69±45*	*87±46*	*NS*	*<0.005*
VT (episodes/24 h)	*20±9*	*4±3***	*21±18*	*24±20*	*NS*	*<0.005*
Mean RR interval (ms)	*807±28*	*831±42*	*858±63*	*872±46*	*NS*	*NS*
SDNN (ms)	*113±8*	*142±10***	*111±10*	*112±11*	*NS*	*<0.005*

*PVCs, premature ventricular contractions; VT, ventricular tachycardia; SDNN, standard deviation of NN interval; NS, not significant. All values are given as the mean±SE; **p<0.01 vs baseline.*

Table 3 Various Parameters at Baseline and After 2 Weeks in the 2 Groups

	Sauna-treated group		*Non-treated group*		*Comparison with both groups*	
	Baseline	*After 2 weeks*	*Baseline*	*After 2 weeks*	*At baseline*	*After 2 weeks*
NYHA (I/II/III)	*0/5/15*	*0/15/5***	*0/2/8*	*0/2/8*	*NS*	*<0.005*
Body weight (kg)	*57±3*	*56±3*	*53±3*	*54±3*	*NS*	*<0.05*
SBP (mmHg)	*107±4*	*100±3*	*108±5*	*108±4*	*NS*	*NS*
DBP (mmHg)	*65±3*	*62±2*	*67±3*	*67±2*	*NS*	*NS*
CTR (%)	*59±1*	*56±2***	*58±1*	*58±1*	*NS*	*<0.05*
LVEDD (mm)	*64±2*	*61±2**	*64±3*	*64±3*	*NS*	*NS*
LAD (mm)	*46±2*	*44±2*	*47±2*	*46±2*	*NS*	*NS*
LVEF (%)	*29±2*	*33±2**	*29±3*	*31±3*	*NS*	*NS*
NE (pg/ml)	*431±56*	*415±76*	*414±42*	*455±84*	*NS*	*NS*
EP (pg/ml)	*25.3±4.1*	*25.0±3.4*	*24.9±4.3*	*28.3±6.0*	*NS*	*NS*
DOPA (pg/ml)	*13.7±3.1*	*13.7±3.0*	*14.2±4.2*	*14.2±3.2*	*NS*	*NS*
ANP (pg/ml)	*121±23*	*81±19***	*126±32*	*130±37*	*NS*	*NS*
BNP (pg/ml)	*425±102*	*229±54***	*415±98*	*419±110*	*NS*	*<0.01*

*NYHA, New York Heart Association; SBP, systolic blood pressure; DBP, diastolic blood pressure; CTR, cardiothoracic ratio; LVEDD, left ventricular end diastolic dimension; LAD, left atrial dimension; LVEF, left ventricular ejection fraction; NE, norepinephrine; EP, epinephrine; DOPA, dopamine; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; NS, not significant. All values are given as the mean±SE; *p<0.05 vs baseline, **p<0.01 vs baseline.*

were replaced by a linear interpolation algorithm. The standard deviation (SD) of all normal beat intervals and the mean length of the NN intervals (SDNN) were used for time-domain measures from the entire recording period. We analyzed 24 patients; 6 patients with atrial fibrillation were excluded.

Study Protocol

Sauna treatment was performed daily for 5 days each week, for a total of 2 weeks. All examinations were performed before the first treatment and on the day after the last treatment.

Statistical Analysis

All data are expressed as the mean±SEM. Differences in baseline characteristics were evaluated by the chi-square test and unpaired t-test. Within-group changes between baseline and after 2 weeks were evaluated by paired t-test or Wilcoxon signed rank test for variables that were not normally distributed. Between-group comparisons were evaluated by Mann-Whitney's U test using differences between baseline and after 2 weeks. A value of $p<0.05$ was considered statistically significant.

Results

Baseline Clinical Characteristics and Assessment of Clinical Symptoms

Baseline clinical characteristics are summarized in Table 1. There were no differences in age, gender, NYHA functional class, mean heart rate, blood pressure or use of drugs, such as digoxin, angiotensin-converting enzyme inhibitor, β-blockers, diuretics, nitrates, and antiarrhythmic drugs, at baseline between the 2 groups. All patients enrolled completed the study. In the sauna-treated group, no patient experienced dyspnea, angina pectoris or palpitations. Clinical symptoms related to dyspnea, fatigue, edema, appetite-loss, constipation and insomnia were improved in 17 of 20 patients and unchanged in 3 patients after the 2-week sauna treatment. However, no patients had worsening of clinical symptoms. In the non-treated group, clinical symptoms did not change after 2 weeks.

Cardiac Arrhythmias

At baseline, the total number of PVCs, couplets and episodes of ventricular tachycardia per day were similar between the 2 groups (Table 2). In the sauna-treated group, the total number of PVCs decreased in all patients 2 weeks after treatment. The total number of PVCs in the sauna-treated group was significantly decreased compared with the non-treated group after 2 weeks ($p<0.01$, Table 2). The total number of couplets and episodes of ventricular tachycardia per day also decreased significantly in the sauna-treated group compared with the non-treated group (Table 2). The prevalence of couplets and ventricular tachycardia in the sauna-treated group compared with the non-treated group was 45% vs 90%, $p<0.05$, and 20% vs 80%, $p<0.01$, respectively. The total number of PACs did not significantly change between the 2 groups after 2 weeks (170±102 vs 617±375, $p=0.07$).

HRV

There was no difference in SDNN at the baseline between the 2 groups, but after 2 weeks, SDNN was sig-

nificantly greater in the sauna-treated group compared with the non-treated group (Table 2).

Neuro-Hormonal Factors

At baseline, there were no differences in the plasma concentrations of ANP, BNP, or catecholamine between the 2 groups. After 2 weeks, there were no differences in the plasma concentrations of ANP or catecholamine between the 2 groups, but the plasma concentration of BNP in the sauna-treated group was significantly lower than in the non-treated group (229±54 pg/ml vs 419±110 pg/ml, $p<0.05$; Table 3).

NYHA Functional Class, Chest Radiography, Echocardiography and Laboratory Parameters

At baseline, there were no differences in NYHA functional class, CTR or LVEDD between the 2 groups, but after 2 weeks, there was a significant difference in NYHA functional class, body weight, and CTR in the sauna-treated group; LVEDD did not change between the 2 groups. Laboratory parameters, including liver function tests (aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ-glutamyl transpeptidase etc), creatinine, electrolytes (Na, Cl, K) and hematocrit, did not change after 2 weeks in either group (data not shown).

Discussion

In the present study, we found that repeated 60°C sauna treatment improved ventricular arrhythmias. Furthermore, we observed that thermal therapy increased HRV and reduced the plasma concentration of BNP in patients with CHF.

The incidence of ventricular arrhythmias is extremely high in patients with CHF: approximately 80% or more of CHF patients have frequent ventricular premature beats and approximately 50% of them have runs of nonsustained ventricular tachycardia.[3,28–30] Sudden death because of ventricular arrhythmias accounts for approximately half of all deaths in patients with CHF.[4,31–33] Several studies have shown an association between ventricular arrhythmias and mortality in patients with CHF,[5–9,34–36] but unfortunately, current antiarrhythmic medications, such as class I drugs, have only limited efficacy in these patients and may even be associated with worsening ectopic activity and hemodynamic deterioration.[10–12] In large randomized trials with amiodarone, a potent antiarrhythmic drug with additional sympatholytic and minor negative inotropic effects, the Group for the Study of Survival in Heart Failure in Argentina (GESICA) demonstrated that low doses reduced ventricular arrhythmias and mortality in patients with CHF;[13] however, the Survival Trial of Antiarrhythmic Therapy in Congestive Heart Failure had conflicting results concerning mortality.[14] Previous studies have demonstrated that β-blockers, which also have antiarrhythmic effects, reduce mortality and the risk of sudden cardiac death, as well as ventricular arrhythmias, in patients with CHF[37] and other studies have shown that ventricular arrhythmias in patients with CHF are improved by treatment with non-antiarrhythmic drugs, such as angiotensin-converting enzyme inhibitors[16–18] and spironolactone.[38] Our present results demonstrated that thermal therapy reduced the total number of PVCs, couplets, and episodes of ventricular tachycardia in patients with CHF and we have already shown that thermal therapy reduced mortality in hamsters with CHF.[22] We suggest that improvement of ventricular arrhythmias may be one of the mechanisms by which repeated thermal therapy improves the prognosis in patients with CHF.

Although the mechanisms of ventricular arrhythmias occurring in patients with CHF are still unclear, experimental evidence suggests that the development of delayed and early afterdepolarization-induced triggered activity and automaticity, in addition to conditions favoring reentry, are related to arrhythmias in the setting of heart failure. Modulating factors, such as sympathetic activation, electrolyte disturbances and chronic left ventricular stretch, are also present in the setting of heart failure.[39,40] It is well-established that the sympathetic nervous system is activated in patients with CHF[41–43] and analysis of HRV provides important information about sympathetic nervous activity in these patients.[25,44] Data from the recent United Kingdom-Heart failure Evaluation and Assessment of Risk Trial (UK-HEART) suggest that reduced HRV, analyzed by a traditional time-domain method (including SDNN), is related to the risk of ventricular arrhythmias and sudden death in patients with CHF,[24] and we suggest that one of the mechanisms by which repeated sauna treatment significantly improves ventricular arrhythmias is by increasing HRV, although we have not clarified the underlying mechanisms of that effect of thermal therapy. On the other hand, the self-assessment QOL questionnaire revealed 17 of 20 patients who answered 'improved' to more than 3 of 6 clinical symptoms that comprised dyspnea, fatigue, sleeplessness, edema, appetite-loss and constipation, and furthermore, none of the patient answered 'worsened' for any symptom. Therefore, the improvement may be related to better mood as a result of repeated sauna treatment. Further study is needed.

The chronic stretch of cardiac myocytes contributes to shortening of the action potential duration and mild decreases in the action potential amplitude and resting membrane potential.[45] These changes may be arrhythmogenic by increasing reentry and abnormal automaticity.[46] In patients with CHF, the ventricular wall is chronically stretched because of increases in ventricular volume and/or pressure overload. It is well-established that BNP is secreted predominantly by the ventricle in response to ventricular wall stretch.[47] On the basis of our findings, including previous data,[20] which showed significantly decreased plasma concentrations of BNP after 2 weeks of sauna treatment, we speculate that another mechanism responsible for decreased ventricular arrhythmias may be reduction of ventricular wall stretch.

Electrolyte disturbances, such as hypokalemia and hypomagnesemia, are prevalent in patients treated with diuretics and are implicated as a cause of ventricular arrhythmias associated with CHF. However, we did not observe significant changes in the electrolyte concentrations after 2 weeks (data not shown).

We have treated many CHF patients with sauna therapy and so far none of the in-hospital patients has shown any deterioration in their condition. However, thermal therapy does not appear to be indicated for CHF patients with aortic stenosis or obstructive hypertrophic cardiomyopathy because the pressure gradient is increased. In the present study, only CHF patients with NYHA functional class II or III underwent sauna treatment. It is well-known that the more severe the CHF, the more prevalent are ventricular arrhythmias. We evaluated the effects of sauna therapy on

ventricular arrhythmias at 2 weeks, but further studies of the long-term effects and benefit in CHF patients with NYHA functional class IV are needed.

In conclusion, repeated 60°C sauna treatment decreased ventricular arrhythmias in CHF patients with NYHA functional class II or III.

Acknowledgment

This study was supported in part by a Grant-in-Aid from the Japan Heart Foundation/Pfizer Grant for Cardiovascular Disease Research.

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ONCOLOGY REPORTS 12: 1093-1098, 2004

Epstein-Barr virus-associated gastric carcinoma in Papua New Guinea

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Received March 16, 2004; Accepted June 29, 2004

Abstract. Using *in situ* hybridization assay, we examined Epstein-Barr virus (EBV) encoded RNA (EBER) expression in 66 cases of oral cancer, 40 esophageal cancer cases, 150 stomach cancer cases, and 46 colorectal cancer cases diagnosed in the Pathology Department of Port Moresby General Hospital, University of Papua New Guinea during the period between 1986-2002. There were no malignancies with positive EBER expression except for the following two male stomach cancer cases: a male case with a gastric carcinoma in pylorus whose age was unknown; and a male case aged 55 years without information on location of tumor. Both cases were histologically classified as non-solid poorly differentiated adenocarcinoma of the Japanese histological classification. The frequency of EBV-associated gastric carcinomas was 1.3% (2/150), and was the lowest ever reported in the world. 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 genotype of the first case 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. The second case had EBV whose genotypes were type A, wild-type F at BamHI-F region, and the kept type of the XhoI cleavage site in LMP1. The BamHI-I region of this case could not be analyzed.

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Key words: Epstein-Barr virus, gastric carcinoma, Papua New Guinea

Introduction

In 1992, Shibata and Weiss (1) reported the presence of Epstein-Barr virus (EBV) genome in 16% of gastric adenocarcinomas in a small North American series, using *in situ* hybridization technique to detect EBV-encoded small RNA (EBER) genome in gastric tissue. A large-scale study in Japan, published in 1993, also showed the presence of EBER in 7% of gastric carcinomas (2). Subsequent studies revealed that the proportion of EBV-associated gastric carcinoma (EBV-GCs) was different from country to country, and ranged from 2 to 17% (3). In the present study, we examined the prevalence of EBV-GCs in Papua New Guinea (PNG).

Materials and methods

Subjects. The present study examined cancer cases of digestive organs diagnosed in Pathology Department of Port Moresby General Hospital, the major teaching center of School of Medicine and Health Sciences, University of Papua New Guinea. Paraffin-embedded formalin-fixed tissues of the following cancers were examined: 66 cases of cancer of the oral cavity diagnosed in 2001; 40 cases of esophageal cancer for the period 1994-2002; 150 stomach adenocarcinoma cases for the period 1986-2002, and 46 colorectal cancer cases for the period 1989-2002.

Histological classification. Histological classifications of oral and esophageal cancers were made following the guidelines of Japan Society for Head and Neck Cancer (4), and Japanese Society for Esophageal Diseases, respectively (5). The gastric carcinomas were classified as the intestinal- and diffuse-type of Lauren classification (6), and subclassified according to the Japanese Classification of Gastric Carcinoma of Japanese Research Society for Gastric Cancer (7). Briefly, histological patterns were classified 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

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.

Acknowledgements

We thank Ms. Yoshie Minakami for her technical support in *in situ* hybridization assay. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Culture, Sports, Science, and Technology of Japan (12218231).

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Laboratory/Clinical Translational Research

Oncology

Oncology 2004;67:81–87
DOI: 10.1159/000080290

Received: April 14, 2003
Accepted after revision: February 2, 2004

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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0030–2414/04/0671–0081$21.00/0

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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′-agacaccgtcctcaccaccc-gggacttgta-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

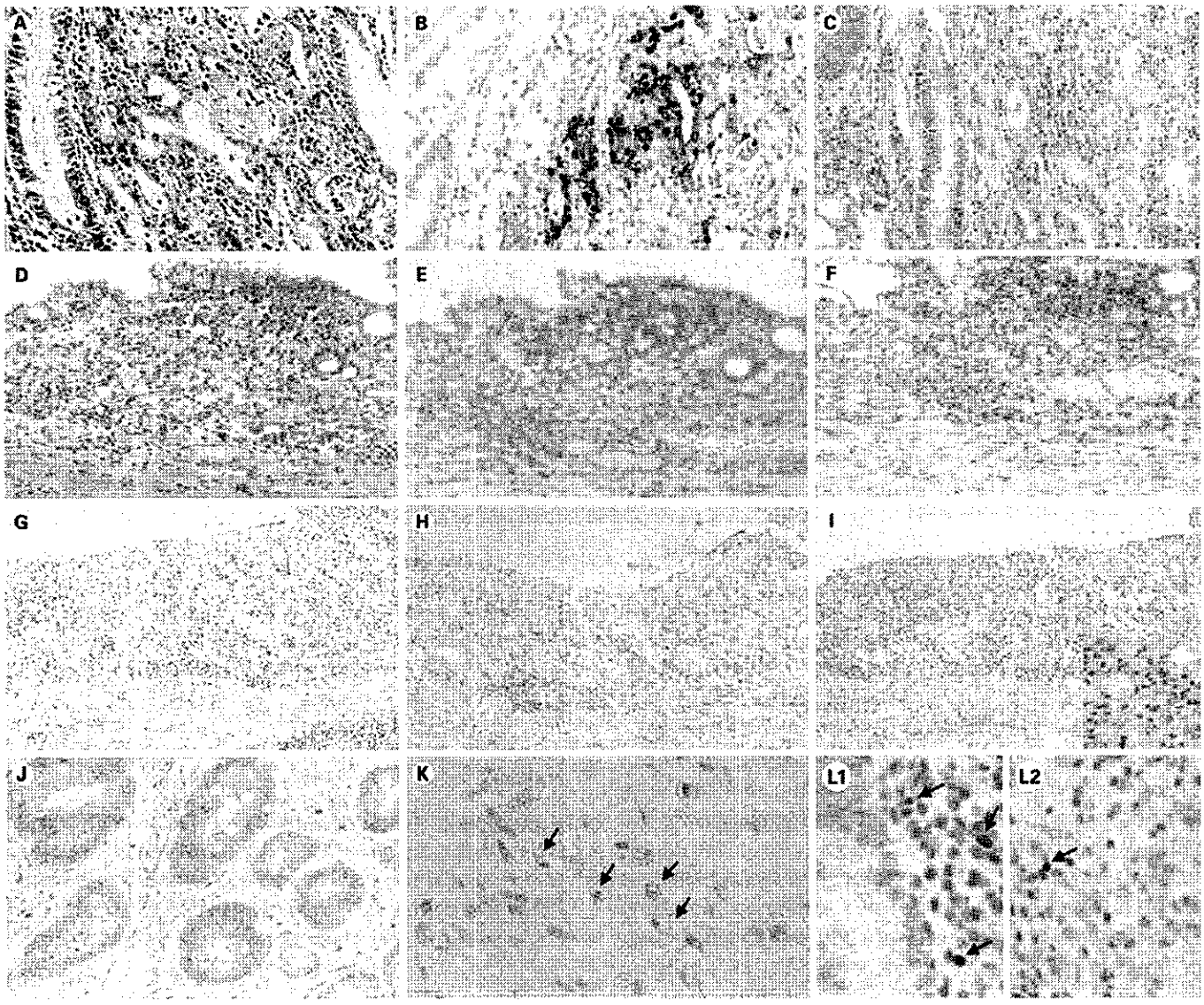

Fig. 1. A–C EBER-1-positive and p16-negative case. p16 is positive in plasma cells but not in carcinoma cells (**C**). **D–F** EBER-1-negative and p16-negative cases. **G–I** EBER-1-negative and p16-positive case. p16 is expressed in nuclei of carcinoma cells (**I** inset). **J** p16 expression in the 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.

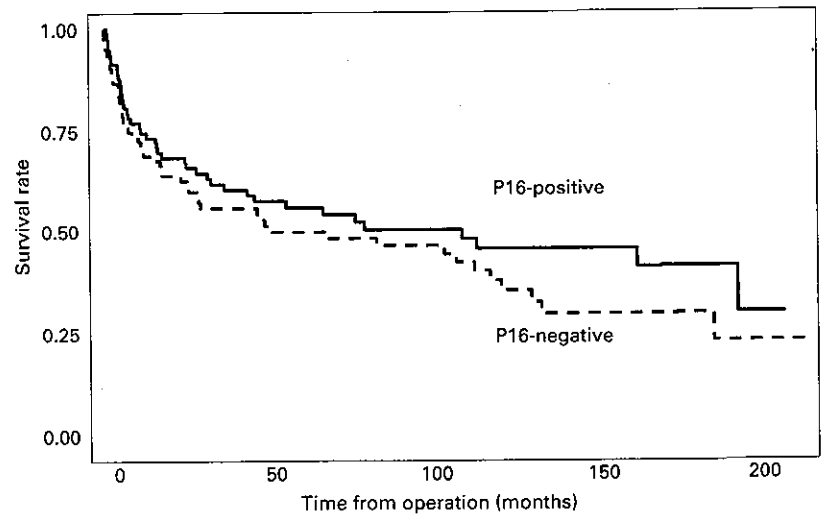

Fig. 2. Overall survival of 171 patients with gastric carcinoma according to p16 overexpression in tumor cells. —— = p16-positive cases (n = 85); ----- = p16-negative cases (n = 86). There was no significant difference between p16 expression and prognosis of patients (p = 0.42).

(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.

Acknowledgments

The authors appreciate the skillful assistance of Ms. Yoshiko Arimura with immunohistochemistry. This study was supported by Grants-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Culture, Sports, Science and Technology of Japan (12218231 and 13220016).

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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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Received December 31, 2003; Accepted March 19, 2004

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