ORIGINAL ARTICLE

Epstein-Barr virus (EBV) subtype in EBV related oral squamous cell carcinoma in Okinawa, a subtropical island in southern Japan, compared with Kitakyushu and Kumamoto in mainland Japan

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Aim: In Okinawa, a subtropical island located between the East China Sea and Pacific Ocean, 2000 km south of mainland Japan, the incidence of oral squamous cell carcinoma is 1.5 times higher than that seen in mainland Japan, and a large number of these patients have been reported to be infected with the Epstein-Barr virus (EBV). This study aimed to gain a better understanding of the pathogenesis of this malignancy in this area by carrying out genomic analysis of EBV.

Methods: Fifty four patients with oral squamous cell carcinoma reported from 1997 to 1999 in Okinawa were compared with 21 and 20 patients from Kitakyushu and Kumamoto in Kyushu, mainland Japan, respectively. Diagnosis was confirmed by conventional histological examination of paraffin wax sections. EBV was detected by non-isotopic in situ hybridisation (NISH) and the polymerase chain reaction (PCR) (Bam HI-F, EBV nuclear antigen 2 (EBNA2), and latent membrane protein 1 (LMP-1) regions). Sequence analysis of the PCR products was also carried out.

Results: In Okinawa, 25 patients were found to be infected with EBV type A by analysing the 3′ sequence divergence of the EBNA2 genes. Six patients were positive for EBV type B, and eight for both type A and B. Therefore, type A virus infection was demonstrated in 33 of 54 patients, and type B in 14 of 54. In total, 39 of 54 patients were infected with EBV. However, the "f" variant was shown in only one patient, who was also infected with type A virus. In contrast, 97.0% of EBV type A infected patients showed a 30 bp deletion of the LMP-1 gene, but those infected with EBV type B did not. Sequence analysis of the type A virus EBNA2 gene revealed slight variations of the sequence (mutations)—^{48991}G→T and ^{48998}C→A—in 18 of 33 cases compared with the B95-8 strain, and in 14 cases, in addition to these, a further mutation of ^{48917}T→C was demonstrated; in the single remaining case, only one mutation at ^{49137}A→G was detected. The mutations at 48991 (G→T), and 49137 (A→G) are associated with amino acid changes Arg→Met and Thr→Ala, respectively. In contrast, no mutation was seen in the EBNA2 DNA from the 14 cases of type B virus when compared with that of the Jijoye strain. In Kitakyushu and Kumamoto, only 10 of 41 patients (six in Kitakyushu and four in Kumamoto) were infected with EBV. Among them, nine patients were infected with type A virus, and only one patient from Kitakyushu was infected with type B virus. The ^{48991}G→T and ^{48998}C→A mutations of the EBNA2 region were demonstrated in type A virus, but the ^{48917}T→C and ^{49137}A→G mutations were not when compared with the B95-8 strain. In the case of type B virus no mutation was noted. A 30 bp deletion was found in these nine cases of type A, but not in type B. The sequence analysis of EBV type A in Okinawa, Kitakyushu, and Kumamoto showed slight variations when compared with B95–8, but EBV type B LMP-1 did not when compared with the Jijoye strains.

Conclusion: In Okinawa, EBV infection was frequently demonstrated in oral squamous cell carcinoma ($p < 0.001$). However, in mainland Japan there was no significant correlation between EBV and oral squamous cell carcinoma. In Okinawa, EBV type B infection is approximately 10 times more common than in the mainland. However, in these areas—Okinawa, Kitakyushu, and Kumamoto—the frequency of the "f " variant was very low, whereas a high incidence of a 30 bp deletion of LMP-1 was noted. The number of EBV (including type A and/or B) infected oral squamous cell carcinomas in Okinawa was about three times higher than that seen in the mainland, although the frequency of oral squamous carcinoma was only 1.5 times higher than that seen in the mainland. A high prevalence of type B virus infection and slight differences in the EBNA2 gene sequence in the type A virus might influence the frequency of this carcinoma in Okinawa.

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Epstein-Barr virus (EBV) causes widespread infection; it is known to be the causative agent of infectious mononucleosis, and is associated with Burkitt's lymphoma, nasopharyngeal carcinoma, peripheral T cell lymphoma, and other cancers.[1–4] Recently, 7–10% of gastric carcinomas were also reported to be infected with EBV.[5–7] Furthermore, there are two subtypes of EBV, A (type 1) and B (type 2), which differ in their ability to transform B cells (a process known as

Abbreviations: EBV nuclear antigen 2, EBER1, EBV encoded RNA 1; EBNA2, EBV nuclear antigen 2; EBV, Epstein-Barr virus; HPV, human papillomavirus; LMP-1, latent membrane protein 1; NISH, non-isotopic in situ hybridisation; PCR, polymerase chain reaction; RBPJκ, recombination signal binding protein Jκ

Table 1 List of patients with oral squamous cell carcinoma in Okinawa, histological differentiation, and detection of Epstein-Barr virus (EBV)

Patient	Age (years) and sex		Location of the tumour	Stage and differentiation		EBV type	NISH	LMP	EBNA	30 bp
1	42	M	O	IV	W	–	–	–	–	
2	42	M	M	I	W	–	–	–	–	
3	48	M	M	III	W	A+B	+	+	+	+
4	70	M	M	IV	W	A+B	+	+	+	+
5	58	M	F	III	W	A+B	+	+	+	–
6	69	M	T	IV	W	A+B	+	+	+	+
7	57	M	T	I	W	A	+	+	+	+
8	67	M	O	IV	W	A	+	+	+	+
9	60	M	T	IV	W	–	–	–	–	
10	59	M	M	I	W	–	–	–	–	
11	77	M	O	III	W	A	+	+	+	+
12	43	M	T	III	W	A	+	+	+	–
13	75	M	T	IV	W	A	+	+	+	+
14	66	M	F	II	W	A	+	+	+	+
15	53	M	B	I	W	A	+	+	+	+
16	80	F	T	I	W	A	+	+	+	+
17	39	M	L	II	W	–	–	–	–	
18	70	M	T	II	W	A	+	+	+	+
19	47	F	T	II	W	A	+	+	+	+
20	60	M	T	III	W	A	+	+	+	+
21	78	F	M	III	W	A+B	+	+	+	+
22	70	M	T	I	W	B	+	+	+	–
23	57	M	T	II	W	–	–	–	–	
24	79	M	T	II	W	A	+	+	+	+
25	78	M	T	III	W	–	–	–	–	
26	65	M	O	III	W	A	+	+	+	+
27	80	M	F	II	W	B	+	+	+	–
28	64	M	T	III	W	A	+	+	+	+
29	86	M	M	IV	M	A	+	+	+	+
30	70	M	T	O	M	A	+	+	+	+
31	69	M	T	I	M	–	–	–	–	
32	55	M	B	I	M	A	+	+	+	+
33	60	M	L	IV	M	A	+	+	+	+
34	71	M	B	II	M	A	+	+	+	+
35	59	M	T	II	M	A	+	+	+	+
36	50	M	T	II	M	A	+	+	+	+
37	39	M	B	I	M	–	–	–	–	
38	70	M	T	I	M	–	–	–	–	
39	78	M	T	IV	M	–	–	–	–	
40	73	M	F	III	M	A	+	+	+	+
41	60	M	O	II	M	B	+	+	+	–
42	80	M	O	I	M	B	+	+	+	–
43	68	M	L	II	M	B	+	+	+	–
44	65	M	F	III	M	A+B	+	+	+	+
45	60	M	O	III	M	A+B	+	+	+	+
46	77	M	F	IV	M	A+B	+	+	+	+
47	47	F	B	II	M	–	–	–	–	
48	67	M	F	III	M	B	+	+	+	–
49	67	M	O	IV	M	–	–	–	–	
50	48	M	L	II	M	–	–	–	–	
51	80	M	F	III	P	A	+	+	+	+
52	57	M	O	III	P	A	+	+	+	+
53	63	M	O	II	P	A	+	+	+	+
54	61	M	L	IV	P	–	–	–	–	

Location: B, buccal mucosa; F, mouth floor; L, lower gum; M, maxilla; O, oropharynx; T, tongue.
Differentiation, degree of the histological differentiation of the tumour: M, moderately differentiated; P, poorly differentiated; W, well differentiated. NISH, non-isotopic in situ hybridisation of EBV encoded RNA 1; LMP, immunohistochemical demonstration of latent membrane protein 1 antigen; EBNA, immunohistochemical demonstration of the EBV nuclear antigen 2; 30 bp, 30 bp deletion of LMP-1.

immortalisation).[8 9] EBV nuclear antigen 2 (EBNA2) function is associated with this phenomenon.[10–14] Rickinson *et al* reported that the type B virus is much less efficient at immortalisation of B cells than the type A virus.[15] This may be one reason why the type B virus had long remained undiscovered. EBV type A virus is predominant in developed countries.[16] The type B virus was recently discovered in patients with Burkitt's lymphoma in central Africa and New Guinea, and in one study was found in 40% of patients with Burkitt's lymphoma.[17] Type B virus differs in the BamHI YH region encoding EBNA2,[17 18] and because of its restricted geographical distribution a close association with Burkitt's lymphoma was suggested. However, recent investigations have revealed that the type B virus is also frequently found in normal populations in the USA, and not only in the endemic area of Burkitt's lymphoma.[19] Sixbey *et al* reported type B virus infection in 40% of healthy individuals in the USA,[20] and Scully *et al* reported that the occurrence of B type virus infection in human immunodeficiency virus positive subjects was higher than that seen in the general community.[21]

In mainland Japan, type A virus has been reported to be predominant. Kunimoto *et al* reported that 37 of 79 cases of tonsillitis contained type A virus, and only one type B.[22] Tomita and colleagues[23] reported that type B virus was detected in one case in 37 cases of malignant lymphoma of the sinonasal region, compared with 22 of 37 cases with type A virus infection. Furthermore, Sidagis *et al* reported 73 cases of EBV

Table 2 List of patients with oral squamous cell carcinoma in Kitakyushu, histological differentiation and detection of Epstein-Barr virus (EBV)

Patient	Age (years) and sex		Location of the tumour	Diff	EBV type	NISH	LMP	EBNA	30 bp
1	52	M	T	W	A	+	+	+	+
2	73	M	M	W	A	+	+	+	+
3	57	M	T	W	–	–	–	–	–
4	48	M	T	W	–	–	–	–	–
5	52	F	B	W	–	–	–	–	–
6	58	F	T	W	–	–	–	–	–
7	62	M	F	M	–	–	–	–	–
8	54	F	M	M	A	+	+	+	+
9	52	M	T	M	A	+	+	+	+
10	55	M	T	M	–	–	–	–	–
11	73	F	T	M	A	+	+	+	+
12	83	M	M	M	–	–	–	–	–
13	62	M	T	M	B	+	+	+	–
14	52	M	F	M	–	–	–	–	–
15	74	M	T	M	–	–	–	–	–
16	90	F	U	M	–	–	–	–	–
17	83	M	M	M	–	–	–	–	–
18	43	M	T	M	–	–	–	–	–
19	53	M	F	M	–	–	–	–	–
20	70	M	B	M	–	–	–	–	–
21	45	M	F	P	–	–	–	–	–

Location: B, buccal mucosa; F, mouth floor; L, lower gum; M, maxilla; O, oropharynx; T, tongue. Diff, degree of the histological differentiation of the tumour: M, moderately differentiated; P, poorly differentiated; W, well differentiated. NISH, non-isotopic in situ hybridisation of EBV encoded RNA 1; LMP, immunohistochemical demonstration of latent membrane protein 1 antigen; EBNA, immunohistochemical demonstration of the EBV nuclear antigen 2; 30 bp, 30 bp deletion of LMP-1.

Table 3 List of patients with oral squamous cell carcinoma in Kumamoto, histological differentiation and detection of Epstein-Barr virus (EBV)

Patient	Age (years) and sex		Diff	EBV type	NISH	LMP	EBNA	30 bp
1	72	M	W	–	–	–	–	–
2	66	F	W	–	–	–	–	–
3	70	M	W	–	–	–	–	–
4	74	F	W	–	–	–	–	–
5	60	M	W	A	+	+	+	+
6	56	M	W	A	+	+	+	+
7	87	F	W	A	+	+	+	+
8	68	M	W	–	–	–	–	–
9	78	F	W	–	–	–	–	–
10	71	M	M	–	–	–	–	–
11	74	F	M	–	–	–	–	–
12	79	F	M	–	–	–	–	–
13	77	M	M	–	–	–	–	–
14	72	M	M	–	–	–	–	–
15	56	M	M	–	–	–	–	–
16	50	M	M	–	–	–	–	–
17	84	F	M	–	–	–	–	–
18	59	M	M	–	–	–	–	–
19	63	M	M	–	–	–	–	–
20	55	F	P	A	+	+	+	+

Diff, degree of the histological differentiation of the tumour: M, moderately differentiated; P, poorly differentiated; W, well differentiated. NISH, non-isotopic in situ hybridisation of EBV encoded RNA 1; LMP, immunohistochemical demonstration of latent membrane protein 1 antigen; EBNA, immunohistochemical demonstration of the EBV nuclear antigen 2; 30 bp, 30 bp deletion of LMP-1.

related gastric carcinoma in mainland Japan, of which 51 cases were infected with type A virus, and only four with type B.[24]

"In mainland Japan, type A virus has been reported to be predominant"

In Okinawa, a subtropical island located between the East China Sea and Pacific Ocean, 2000 km south of mainland Japan, the incidence of oral squamous cell carcinoma is 1.5 times higher than that seen in mainland Japan.[25] We recently reported that 46 of 60 cases of oral squamous cell carcinoma were positive for EBV by the polymerase chain reaction (PCR).[26] In that paper we reported our molecular epidemiological analysis of EBV subtypes in oral squamous cell carcinoma by determining the 3′ sequence divergence of the EBV EBNA2 gene by PCR. The 30 bp deletion of the cytoplasmic C-terminal domain of the latent membrane protein 1 (LMP-1) gene and the BamHI "f" variant were also analysed.

MATERIALS AND METHODS

Tissue samples and cell lines

Fresh samples of oral squamous cell carcinoma (n = 54) from Okinawa (table 1) were obtained from the department of oral surgery, Ryukyu University Hospital. These were all patients with squamous cell carcinoma coming to surgery from April

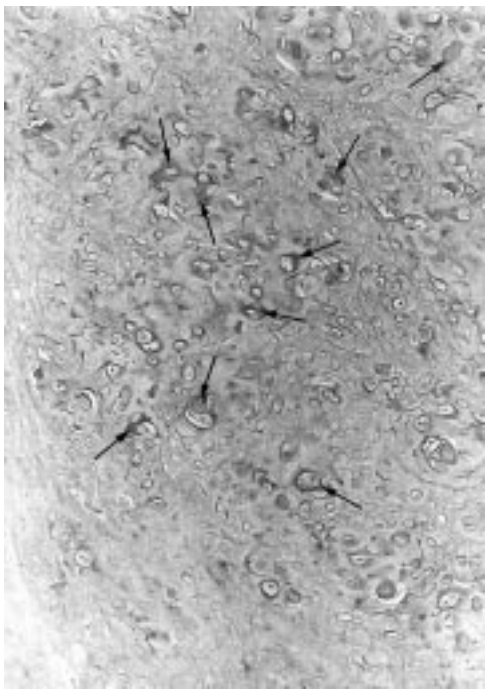

Figure 1 Immunohistochemical demonstration of the latent membrane protein 1 antigen. A large number of cancer cells were positive (arrows). Original magnification, ×200.

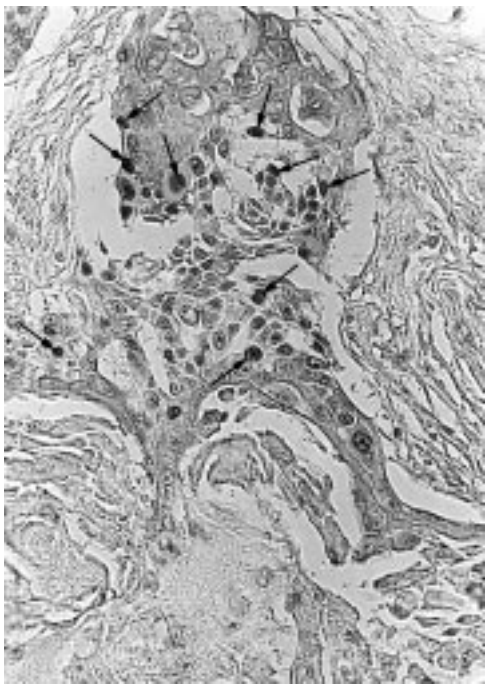

Figure 2 Demonstration of Epstein-Barr virus encoded RNA 1 in squamous cell carcinoma cells by non-isotopic in situ hybridisation. Numerous positive signals were seen (arrows). Original magnification, ×200.

1997 to March 1999. The tumours were grouped into well, moderately, and poorly differentiated types according to the World Health Organisation classification.[27] Most of the patients in our study were farmers, fishermen, or government employees. There were no miners or heavy industrial workers. Forty two patients were heavy smokers and eight were heavy drinkers. We compared these patients with those from Kitakyushu city and Kumamoto city in Kyushu in mainland Japan. The Okinawa prefecture (population, 1.27 million) is in southern Japan and is subtropical. Kitakyushu city (population, 1.01 million) and Kumamoto city (population, 0.64 million) are located in Kyushu in mainland Japan. Samples of oral squamous cell carcinoma from the department of oral surgery, University of Occupational and Environmental Health in Kitakyushu (n = 21) and Kumamoto University in Kumamoto (n = 20) in the same period from 1997 to 1999 were examined (tables 2,3). The cell lines B95-8 and Raji served as positive controls for type A, and the cell line Jijoye was used for type B.

Morphological investigation

Samples were fixed in 10% phosphate buffered formalin, routinely processed in paraffin wax, and sectioned at 4 μm. Haematoxylin and eosin staining were performed on 4 μm dewaxed sections. For the immunohistochemical staining, dewaxed sections were pretreated with 3% H_2O_2 for 20 minutes, washed, and blocked with a non-immune goat serum for 30 minutes. Antibodies to EBV EBNA2 (clone PE2) and EBV LMP-1 (clone CS1-4) were obtained from Dako (Dako A/S, Glostrup, Denmark). Slides were then incubated with diluted (1/100) primary antibodies for 25 minutes, washed three times, and incubated with biotinylated second antibody with avidin and biotinylated horseradish peroxidase complex (Dako, Kyoto, Japan). DAB (3′,3-diaminobenzidine; Dako) was used as a chromogen.

Non-isotopic in situ hybridisation (NISH) for EBV EBER1

NISH for EBV was performed using the Dako DNA ISH detection kit for EBV encoded RNA 1 (EBER1) on dewaxed 4 μm sections. NISH was carried out according to the manufacturer's instructions.

Sample DNA preparation

DNA was extracted from fresh specimens of oral squamous cell carcinoma after treatment with proteinase K (1 μg/ml) freshly prepared in 10μM Tris/HCl (pH 8.0), 10μM EDTA, and 1% sodium dodecyl sulfate at 37°C overnight. The DNA was then purified by repeated extraction using phenol/chloroform/isoamyl alcohol (49/49/2), followed by ethanol precipitation.

The 110 bp β globin gene was detected in all samples according to the method of Saiki *et al* using their primers (PCO_3 and PCO_4).[28]

Analysis of the EBV subtype

Analysis of the EBV subtype was carried out by determining the 3′sequence divergence of the EBNA2 gene by means of PCR, as described by Borisch *et al*.[1] The EBNA2 region was amplified according to the methods of Borisch and colleagues[1] and Wu *et al*,[29] using primers gen 1 and gen 2 (table 4). A 1 μg sample of genomic DNA was used for the PCR. The reaction mixture contained 10mM Tris/HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.4mM of each dNTP, 200μM of each primer, and 2.4 units of Taq DNA polymerase (Cetus-Takara, Otsu, Japan). After the first denaturation at 99°C for five minutes, PCR was carried out for a total of 35 cycles: denaturation at 96°C for 30 seconds, annealing at 55°C for one minute, and extension at 72°C for one minute. After the PCR reaction, the PCR product was mixed with EBNA2A and EBNA2B specific primers for nested PCR to separate the subtypes A and B.

Sequencing of the EBNA2 type A and B genes

The PCR products of EBNA2, type A and B, were extracted from the agarose gel. The extracted DNA was cloned into a T-vector that was prepared from Bluescript (Stratagene, La Jolla, California, USA) according to the method of Marchuk *et al*.[31] The Bluescript plasmid is digested with EcoRV, and incubated with Taq (1 unit/μg plasmid/20 μl volume) using standard buffer conditions (10mM Tris/HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, and 200 μg/ml bovine serum albumin) in the presence of 2 dTTP for two hours at 70°C. The sequence analysis was carried out using a Hitachi SQ 5500 DNA sequencer (Hitachi, Tokyo, Japan).

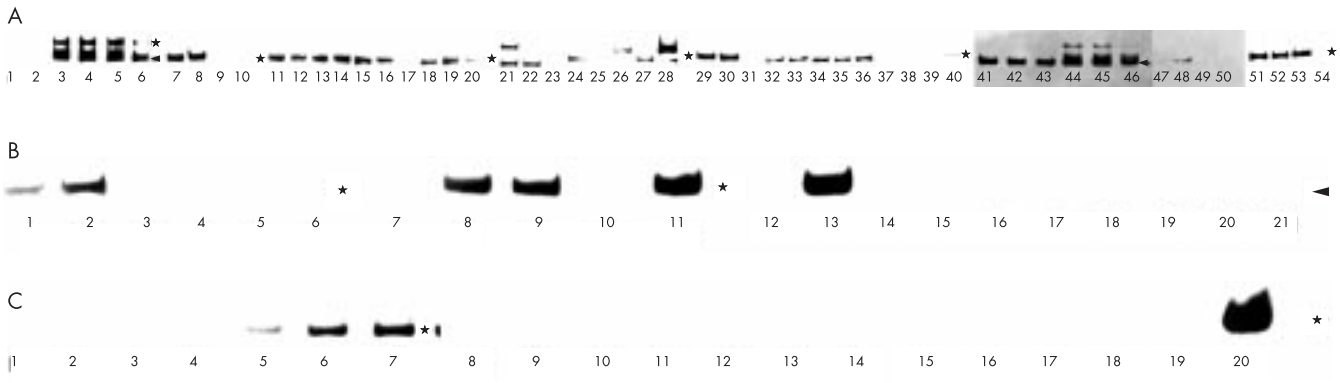

Figure 3 Demonstration of PCR products of Epstein-Barr virus (EBV) type A and B EBV nuclear antigen 2. Bands of 496 bp from EBV type A (*) and 150 bp from EBV type B (arrowhead) were demonstrated in patients from (A) Okinawa, (B) Kitakyushu, and (C) Kumamoto.

Table 4 List of primers and probes[29]

Primers and probes	Nucleotide sequence positions
EBNA2	
gen 1: 5′ AGGGATGCCTGGACACAA3′	44410–48827
gen 2: 5′ GTGCTGGTGCTGCTGGTGG3′	49410–49391
EBNA2 subtype 1	
A1: 5′ TCTTGATAGGGATCCGCTAGGATA 3′	48839–48862
A2: 5′ ACCGTGGTTCTGGACTATCTGGATC 3′	49335–49311
Probe: 5′ CTCTGTCACAACCGAGGCTTACC 3′	49048–49070
EBNA2 subtype 2[1]	
B1: 5′ CATGGTAGCCTTAGGACATA 3′	
B2: 5′ AGACTTAGTTGATGCCCTAG 3′	
Probe: 5′ AGGCCTACTCTTCCTCAACCCAG 3′	
BamHI-F	
F1: 5′ GGAACTGAGCCAGTAGGATA 3′	149–173
F2: 5′ AATGTTCTGCAGGGTAACGG 3′	980–960
LMP-1[30]	
F1: 5′ GTGGGGGTCGTCATCATCTC 3′	168190–168209
R2: 5′ CGGAAGAGGTTGAAAACAAA 3′	168350–168331
Probe: 5′ GGCGGGCCCTGGTCACCTCC 3′	168311–168330

EBNA2, Epstein-Barr virus nuclear antigen 2; LMP-1, latent membrane protein 1.

BamHI "f" variant analysis

The "f" variant analysis was based on the presence of an extra enzyme site at the BamHI-F fragment region of EBV DNA. According to the method of Wu *et al*,[29] a PCR technique was used to detect the EBV BamHI "f" variant. PCR was carried out using a primer pair designed from the BamHI-F region (BamHI-F1 and BamHI-F2; table 4). The PCR conditions were the same as those described in the above section on the analysis of EBV subtype. After the PCR, 10 µl aliquots of the PCR products were digested by BamHI (10 U/µl) restriction enzyme (BRL, Grand Island, New York, USA) at 37°C for two hours. The products were analysed from the extra BamHI-F site by electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

Analysis of the C-terminal region of LMP-1 by PCR

The C-terminal cytoplasmic regions of the EBV type A and B LMP-1 genes were amplified using the primer pair F1/R2 (table 4).[30 32–34] The PCR amplification was carried out using a 1 µg sample of genomic DNA. The reaction mixture contained 200µM of primer pair, 10mM Tris/HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 0.4mM of each dNTP, and 2.4 units of Taq DNA polymerase. The reaction mixture was heated to 94°C for five minutes to denature the DNA–DNA hybrid. Each amplification cycle comprised one minute of denaturation at 94°C, one minute of annealing at 61°C, and one minute of extension at 72°C. The amplification was run for 50 cycles. The PCR products were subjected to 2% agarose gel electrophoresis. The DNA was extracted from the agarose gel and cloned into the T-vector. Sequence analysis was carried out using the Hitachi SQ5500 DNA sequencer (Hitachi, Tokyo, Japan).

Negative controls for PCR analysis

To prevent false positive results, the sample preparation and the PCR were carried out by three authors independently (MH, KT, JM). Furthermore, distilled water was used as a negative control and no positive reaction was obtained.

Statistical analysis

Statistical analysis was performed using the Mantel-Haentzel χ^2 method (Statistical Analysis System; SAS Institute Inc, Cary, North Carolina, USA). Significance was set at $p < 0.05$.

RESULTS

Morphological investigations, immunohistochemistry, and NISH

Twenty eight of the 54 Okinawan cases were well differentiated carcinomas. Twenty two and four of the 54 cases were moderately and poorly differentiated types, respectively (table 1). The mean (SD) age of the patients was 59 (2.8) years; 50 were men and four were women. One case was stage 0, 11 were stage I, and 42 were stage II–IV. Immunohistochemically, 36 of the 54 cases were positive for the LMP-1 (fig 1) and EBNA2 antigens. By means of NISH, EBV encoded RNA 1 (EBER1) was demonstrated in cancer cells (fig 2) from 36 of the 54 patients, all of whom were also positive for the LMP-1 and EBNA2 antigens immunohistochemically (table 1). There was no significant correlation between EBV infection and the histological differentiation of the carcinoma.

In Kitakyushu and Kumamoto, six of 21 and nine of 20 cases were well differentiated carcinomas, respectively (tables 2,3). The mean (SD) age of the patients in Kitakyushu and

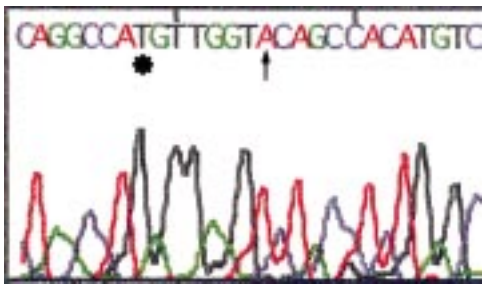

Figure 4 Sequence analysis of the PCR product (Epstein-Barr virus (EBV) type A nuclear antigen 2). Changes of T→G at position 48991 (*) and A→C at position 48998 (↑) were demonstrated in 18 of the patients when compared with the B95-8 strain.

Table 5 Numbers of Epstein-Barr virus (EBV) subtypes and "f" variants in Okinawa

EBV positive cases	EBV subtypes		"f" variant	
39	A	25	F	38
	B	6	f	1
	A+B	8		

Kumamoto was 61.5 (13.4) and 68.6 (10.2), respectively. All cases were stage I or II. Five and eight patients in Kitakyushu and Kumamoto, respectively, were women. Immunohistochemically, six patients in Kitakyushu and four in Kumamoto were positive for the LMP-1 and EBNA2 antigens, and these 10 patients were also positive for EBER1 by means of the NISH assay.

Detection of EBV EBNA2 DNA and EBV subtype analysis

Thirty nine of the 54 Okinawan patients were positive for EBV EBNA2 DNA using the PCR method (fig 3A; table 1). The analysis of EBV subtype was performed by determining the 3′ sequence divergence of EBNA2 using the designated PCR primers. The presence of a 496 bp product represents the type A strain, and a 150 bp the type B strain. EBV DNA samples from the B95-8 and Jijoye cell lines were used as controls for type A and B, respectively. Twenty five patients were positive for the type A strain alone, and six for type B. Eight patients were positive for both types A and B (table 1). Thus, in total 39 of the 54 patients were infected with EBV, of which 33 and 14 patients were positive for the type A and B viruses, respectively. EBV infection in the oral squamous cell carcinoma was frequently demonstrated ($p < 0.001$). However, no significant correlation was found between EBV type B infection and oral squamous cell carcinoma.

In the patients from Kitakyushu (n = 21) and Kumamoto (n = 20), 10 (six from Kitakyushu and four from Kumamoto) were positive for EBV EBNA2 DNA by PCR (fig 3B,C; tables 2,3). Nine patients were infected with type A, and only one patient (from Kitakyushu) was infected with type B, as assessed by analysis of the EBNA2 region.

In the mainland, there was no significant correlation between the detection of EBV DNA and oral squamous cell carcinoma.

Sequence analysis of EBNA2 DNA

The 496 bp products of the 33 cases of type A virus EBNA2 DNA and the 150 bp products of the 14 cases of type B virus EBNA2 DNA in the 41 EBV infected patients from Okinawa were analysed by a DNA sequencer. EBV DNA from the B95-8,[35 36] Raji, and Jijoye[18] cell lines was used as control DNA. In 18 of the 33 type A virus positive cases, two mutations (sequence variations) were found (^{48991}G→T and ^{48998}C→A) when compared with B95-8 DNA (fig 4). In 14 patients, an additional, third mutation (^{48917}T→C) was demonstrated (fig 5). In the remaining one type A positive patient, only one mutation (^{49137}A→G) was found. The mutations at 48991 (G→T) and at 49137 (A→G) were associated with amino acid changes—Arg→Met and Thr→Ala—respectively, but the mutations at 48917 and 48998 were not associated with amino acid changes (base exchanges only). The ^{48991}G→T and ^{48998}C→A mutations were also found in the Raji EBNA2 region. Furthermore, in Raji EBNA2, another mutation at 49140 was also seen when compared with the B95-8 strain, and this mutation was associated with the amino acid change Val→Leu (fig 6).

In contrast, in the 14 cases of type B virus, no mutation was demonstrated in the 150 bp PCR products when compared with Jijoye EBNA2 DNA (data not shown).

In nine cases of type A virus from Kitakyushu and Kumamoto, the ^{48991}G→T and ^{48917}C→A mutations were also

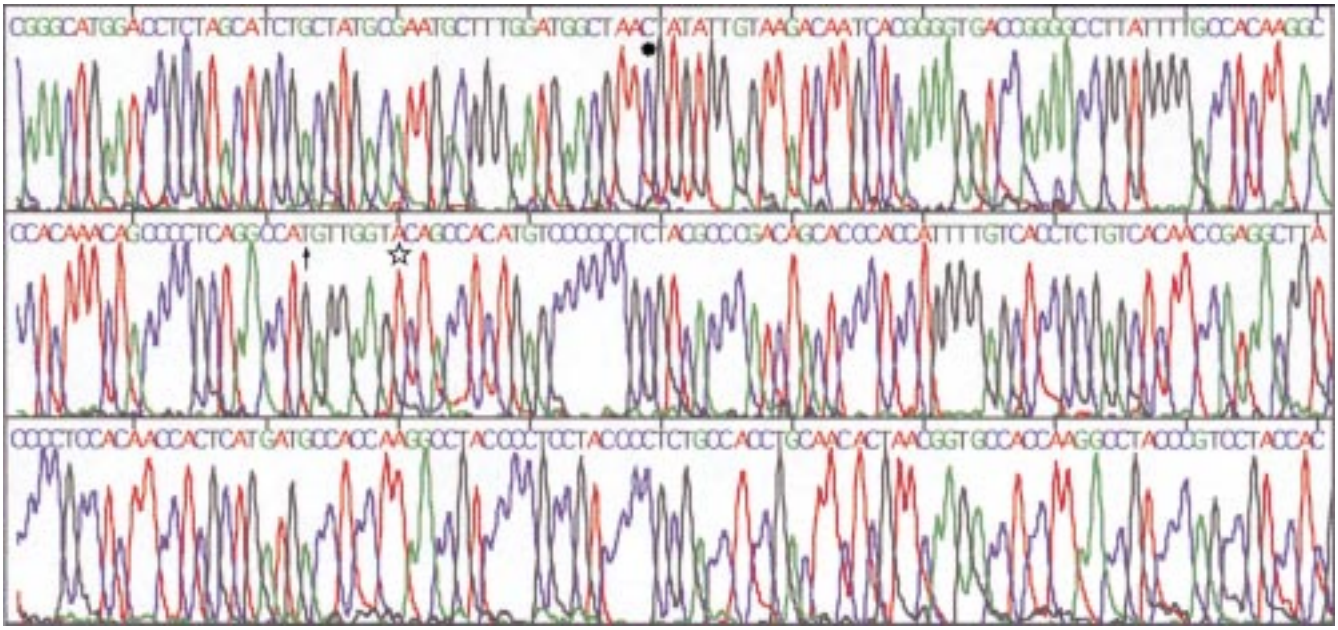

Figure 5 Sequence analysis of the PCR product (Epstein-Barr virus (EBV) type A nuclear antigen 2). There were three mutations: at 48917 (T→C; *), at 48991 (G→T; ↑), and at 48998 (C→A;) when compared with the B95-8 strain.

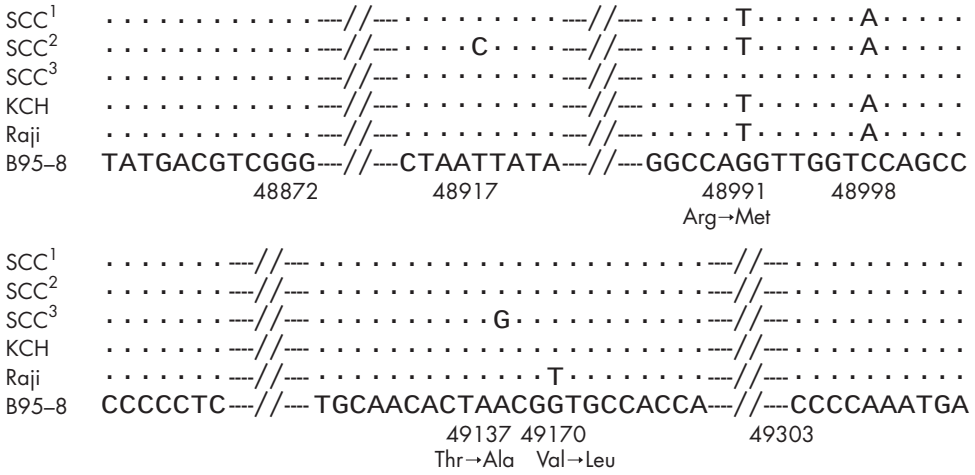

Figure 6 Schematic diagram of the Epstein-Barr virus (EBV) nuclear region 2 (EBNA2) of EBV type A virus. SCC[1]: the sequence of the type A virus EBNA2 region seen in 18 Okinawan patients. SCC[2]: the sequence of the type A virus EBNA2 region seen in 14 Okinawan patients. SCC[3]: the sequence of the type A virus EBNA2 region seen in the remaining Okinawan patient. KCH: the sequence of the type A virus EBNA2 region of the Kumamoto and Kitakyushu patients. Raji: the sequence of the EBNA2 region of the Raji strain. B95-8: the sequence of EBNA2 region of the B95-8 strain.

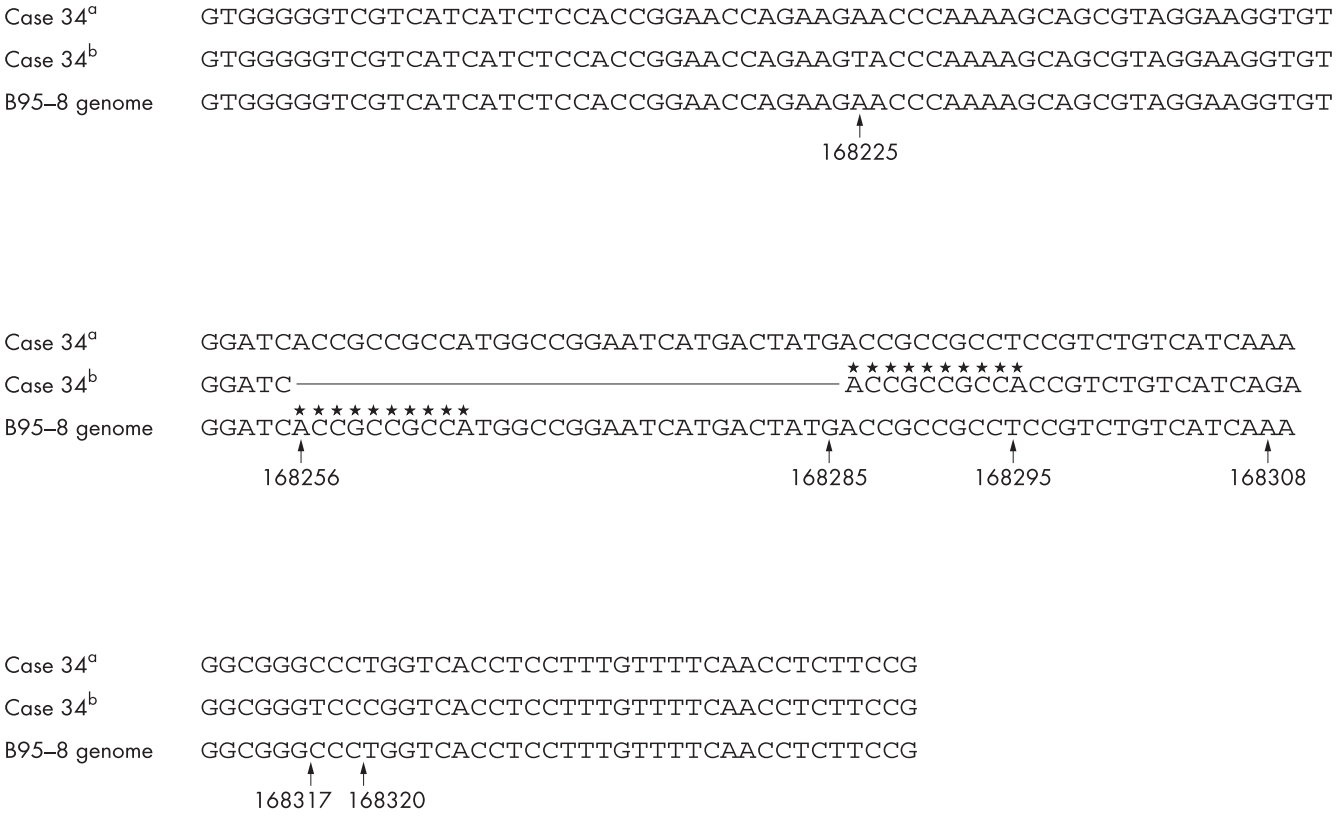

Figure 7 Schematic diagram of the latent membrane protein 1 (LMP-1) region of Epstein-Barr virus (EBV) type A. Case 34[a]: the sequence of the 161 bp product of case 34. Case 34[b]: the sequence of the 131 bp product of case 34. B95-8: the sequence of the B95-8 LMP-1 region. The straight line indicates the 30 bp deletion from position 168256 to 188285; the line of asterisks indicates the repeated sequence.

noted, but the [48137]A→G mutation was not. Furthermore, in the one type B virus case from Kitakyushu, no mutation was seen.

BamHI "f" variant analysis

The "f" variant analysis was based on the presence of an extra BamHI restriction enzyme site in the F fragment region of EBV DNA. The PCR amplified product of the prototype BamHI-F fragment is 831 bp in size. However, after cleavage with the BamHI restriction enzyme, two fragments of 702 bp and 129 bp in size were yielded for the "f" variant. Only one EBV type A infected Okinawan case (case 6 in table 5) was "f" variant. All other cases (Okinawa, Kitakyushu, and Kumamoto cases) belonged to the prototype BamHI-F type.

Analysis of the LMP-1 C-terminal region

The 30 bp deletion in the LMP-1 gene resulted in a 131 bp PCR (as opposed to 161 bp in the wild-type gene) in the EBV type A and B LMP-1 genes. In the 33 EBV type A positive Okinawan patients, 32 had the 30 bp deletion of the LMP-1 gene (position, 168256–85; table 1; fig 7), whereas the 30 bp deletion was not demonstrated in patients infected with type B virus. In the patients with the 30 bp deletion, the T at position 168295 was changed to A. Then the repeated sequence ACCGCCGCCA (at 168286–95), which was the same as the sequence at 168256–65, was noted and a 30 bp deletion at 168256–85 was demonstrated. Furthermore, additional mutations at 168225, 168308, 168317, and 168320 were seen when

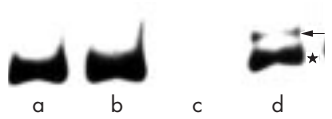

Figure 8 Southern blot hybridisation of PCR products after amplification of the latent membrane protein 1 (LMP-1) gene (case 34). Lane a (case 32), a 131 bp product was amplified; lane b (case 33), a 131 bp product was amplified; lane c, negative control (distilled water); lane d (case 34), both 161 (←) and 131 (*) bp products were amplified.

compared with the EBV DNA sequence from the B95-8 cell line. The mutations at 168225, 168317, and 168320 resulted in amino acid changes, Ser→Thr, Gly→Asp, and Glu→Arg, respectively. In contrast, the sequence of the 161 bp product was the same as that from the B95-8 cell line. Interestingly, both the 161 bp and the 131 bp products of the type A virus were demonstated in patients 34 from Okinawa (figs 7, 8). Although it is possible that this tumour favoured the different EBV type A infection, the virus could represent changes over a long period of time. In contrast, nine EBV type A positive patients from Kitakyushu and Kumamoto harboured the 30 bp deletion of the LMP-1 gene, although the one type B infected case did not (tables 2,3). However, the PCR products of both the type A and type B virus LMP-1 genes from Kitakyushu and Kumamoto showed no additional sequence variations when compared with the B95-8 (type A) and Jijoye (type B) strains.

DISCUSSION

It has been reported that the incidence of EBV infection varies geographically. Taiwan, the nearest neighbour of Okinawa, and southern China (which historically had close relations with Okinawa, including some migration) have been reported as high EBV infection areas. In Taiwan, Wu *et al* reported that the type A virus was detected in 25 of 30 cases of nasopharyngeal carcinoma, and the remaining five were infected with both the type A and type B viruses.[29] In southern China (Shanghai, People's Republic of China), Chen *et al* reported that only one of 16 nasopharyngeal carcinoma biopsies was infected with the type B virus, and the other 15 cases were infected with the type A virus.[37]

A high frequency of the BamHI "f" variant of EBV was reported from southern China,[38 39] and this "f" variant might be associated with the development or maintenance of nasopharyngeal carcinoma. However, in Okinawa, the incidence of type B virus infection was much higher than that reported from southern China; in our series, 14 of the total 39 EBV infected carcinomas from Okinawa were infected with the type B virus. In addition, the "f" variant form was found in only one case, so that the frequency of "f" variant positive cases also differs greatly from that seen in Taiwan[29] and southern China. Again, in contrast to Okinawa, in mainland Japan the incidence of type B virus infection (1.3–5%) was slightly lower than that of Taiwan and southern China.[22–24 40] In our present study, only one of 41 cases from Kitakyushu in the mainland had type B infection. The incidence of type B virus infection in Okinawa (34.1%) was nearer to that seen in Taiwan (16.7%) than to that seen in mainland Japan and southern China, but was still about twice as high as that seen in Taiwan. However, the incidence of the "f" variant in mainland Japan has been reported to be very low (1.4%),[24] and is similar to that seen in Okinawa. In our present study, the EBNA2 gene of the type A virus in Okinawa had non-silent mutations (sequence variations) at positions 48991 and 49173. In contrast, the type A virus EBNA2 gene in Kitakyushu and Kumamoto had only the 48991 mutation when compared with the B95-8 strain. The interaction of EBNA2 with C-promoter binding factor 1 or recombination signal binding protein Jκ (RBPJκ),[10] is thought to be associated with the tumorigenecity of the EBNA2 gene. These mutations, associated with the amino acid changes seen in the Okinawan strain type A virus EBNA2, might have an influence on the interaction between the EBNA2 protein and RBPJκ. However, Ling and colleagues[11] and Henkel and colleagues[10] reported that mutant EBNA2, having mutations at amino acids 323 and 324 of the EBNA2 transcript, failed to have such an influence. The present amino acid changes were located at positions 165 and 180, different from those reported by Ling *et al*,[11] and an influence on such an interaction was obscure. LMP-1 has also been reported to be a viral oncogene.[12] The transmembrane domains, together with the C-terminal cytoplasmic domain, are required for the maximal activation of NF-κB.[41 42] Furthermore, Hu *et al* reported a 30 bp deletion and point mutations in the C-terminal cytoplasmic region of the LMP-1 gene in Chinese nasopharyngeal carcinomas,[32] and this 30 bp deletion mutant transformed rodent cells more efficiently.[43] However, to date there are no in vivo data indicating that 30 bp deletion mutants show altered function when compared with EBV strains without the 30 bp deletion.[44–47] In mainland Japan, Mori *et al* also reported the 30 bp deletion of the LMP-1 gene in 91.7% of EBV related gastric carcinoma and 83.3% of throat washings from healthy adults.[48] In our present study, the LMP-1 genes of the type A virus from the mainland all showed the 30 bp deletion and in Okinawa the 30 bp deletion was seen in 97.0% of the EBV type A infected patients. The 30 bp deletion mutants are common in Okinawa, similar to mainland Japan. Furthermore, there were no sequence variations of the LMP-1 genes in the mainland EBV when compared with the B95-8 strain.

> "In Okinawa, the incidence of type B virus infection was much higher than that reported from southern China"

As reported previously, human papillomavirus (HPV) infection in oral squamous cell carcinoma, especially well differentiated cases, in Okinawa is higher than that seen in mainland Japan.[26] HPV might affect the histological differentiation of squamous cell carcinoma.[26] The number of well differentiated cases in Okinawa was higher than that seen in the mainland. However, recently the number of such cases has decreased greatly. In general, the prevalence of smoking in Okinawa has not been particularly high, although most of the patients in our series from Okinawa and the mainland were heavy smokers, and there is no clear correlation between smoking and EBV infection. Furthermore, only eight patients in our present series were heavy drinkers. However, no correlation between alcohol drinking and EBV infection has been demonstrated. Thus, in Okinawa, both EBV and HPV might be associated with the tumorigenecity of the oral mucosa. However, recently HPV infection has been decreasing,[49] and the role of EBV in tumorigenicity should be studied further. In Okinawa, the subtropical climate and hygiene conditions (water supply and sewage disposal, etc) might influence the incidence of EBV. The high incidence of type B infection may reflect in part the previous history of the region. More than 100 years ago Okinawa was an independent kingdom, and had close relations with China, Taiwan, and South East tropical Asian countries.

In conclusion, the rate of EBV infection in oral squamous cell carcinomas in Okinawa was about three times higher than that seen in mainland Japan, although the incidence of these carcinomas was only 1.5 times higher in Okinawa than in the mainland. High EBV type B infection and the slight differences in the EBNA2 gene might influence the incidence of oral squamous cell carcinoma in Okinawa.

Take home messages

- In Okinawa, Epstein-Barr virus (EBV) infection was associated with oral squamous cell carcinoma ($p < 0.001$), although this was not the case in mainland Japan
- In Okinawa, EBV type B infection is approximately 10 times more common than in the mainland
- In Okinawa and the mainland the frequency of the "f " variant was very low, whereas a high incidence of a 30 bp deletion of LMP-1 was noted
- The number of EBV infected oral squamous cell carcinomas in Okinawa was about three times higher than that seen in the mainland, although the frequency of oral squamous carcinoma was only 1.5 times higher
- A high prevalence of type B virus infection and slight differences in the EBV nuclear antigen 2 gene sequence in the type A virus might influence the frequency of this carcinoma in Okinawa

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