Quantitative Epstein-Barr Virus DNA Analysis and Detection of Gene Promoter Hypermethylation in Nasopharyngeal (NP) Brushing Samples from Patients with NP Carcinoma[1]

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ABSTRACT

Purpose: **Nasopharyngeal carcinoma (NPC) is highly prevalent in southern China and characterized by a strong association with EBV. We aimed to detect EBV DNA and cancer-related gene promoter hypermethylation in nasopharyngeal (NP) brushing samples and provide a novel noninvasive approach for NPC detection.**

Experimental Design: **Twenty-eight NPC cases and 26 noncancerous subjects were prospectively recruited. NP brushing samples were subjected to quantitative real-time PCR analysis of EBV DNA and methylation-specific PCR analysis of the *DAP-kinase*, *RASSF1A*, and *p16* genes.**

Results: **EBV DNA quantity in NP brushing samples from NPC patients (median, 8.94 copies/actin) was significantly higher than that of controls (median, 0 copies/actin; $P < 0.0001$). Twenty-seven of 28 NPC patients had detectable EBV DNA in NP brushes, whereas 25 of 26 controls had undetectable or very low levels of EBV DNA. Elevated EBV DNA level in brushing samples as a tumor marker had a sensitivity of 96.4% and a specificity of 96.2% for NPC detection. Moreover, T_1 disease had a significantly lower EBV DNA level as compared with locally more advanced disease ($P = 0.037$). In brushing samples of NPC patients, the frequencies of *DAP-kinase*, *RASSF1A*, and *p16* promoter hypermethylation were 50.0%, 39.3%, and 46.4%, respectively. Seventy-eight percent of cases showed methylation of at least one gene. No aberrant hypermethylation was detected in control samples.**

Conclusions: **Our study demonstrated the feasibility of detecting multiple molecular tumor markers in NP brushing samples with a high sensitivity and specificity for NPC detection. It offers a powerful yet noninvasive approach for the diagnosis of NPC in high-risk populations.**

INTRODUCTION

NPC[3] is highly prevalent in southern China, including Hong Kong. The incidence of NPC in Hong Kong is 29.8/100,000, with a male:female ratio of 3:1. It is a major cause of cancer morbidity and mortality in this endemic region (1). The prognosis of the patient depends greatly on the staging of the disease. Early diagnosis is crucial to improve patient survival (2). However, NPC often presents with minimal or nonspecific local symptoms, and the nasopharynx is relatively inaccessible to routine examination (3). All of these things represent challenges in making an early diagnosis. An effective screening or diagnostic test is needed in high-risk populations. Given the premise of a strong association of NPC with EBV, a serological test for EBV-associated antibodies was advocated to be a screening test (4). Recently, quantitative EBV DNA analysis has also been shown to be a sensitive molecular marker for NPC (5). An alternative approach is to detect tumor markers in samples collected directly from the NP region via a noninvasive procedure, *e.g.*, NP brushing or swab. This approach will allow us to localize the disease process in the nasopharynx. Our previous study has demonstrated that exfoliative cytological specimens could be collected from the nasopharynx by a simple and noninvasive NP brushing procedure (6). Recent studies also showed that detection of EBV DNA from NP brushing/swab samples was feasible and appeared to be a useful tumor marker for NPC (7, 8). The observations supported the postulation that normal nasopharynx was not the reservoir of EBV and suggested that EBV DNA originated from EBV harbored in NPC cells. However, in a high-risk population, EBV DNA was frequently detected in exfoliated NP cells in normal subjects according to Hording's study (9). In our local high-risk population, the potential value of EBV DNA in NP brush/swab samples for NPC detection has yet to be investigated. Nevertheless, these studies suggested that tumor DNA can be collected from NP brushing/swab samples. Apart from EBV DNA,

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[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; NP, nasopharyngeal; ENT, ear, nose, and throat; COBRA, combined bisulfite restriction analysis; MSP, methylation-specific PCR; AJCC, American Joint Committee on Cancer; VCA, viral capsid antigen; EA, early antigen.

Table 1 Demographic data, EBV IgA titers, staging, EBV DNA quantity in brushing and plasma samples, and multiple gene methylation status in NPC cases

Code	Sex	Age (yrs)	IgA VCA	IgA EA	AJCC T stage	AJCC N stage	M stage	AJCC stage	Brush EBV DNA copies/actin	Plasma EBV DNA copies/ml	Methylation status (biopsy/brush)		
											RASSF1A	*p16*	*DAP-kinase*
3	M	71	<5	<5	3	1	0	3	1.00	997.78	U/U[a]	M/M	M/M
9	M	55	20	20	2b	1	0	2b	19.88	1534.00	M/M	U/U	M/M
10	M	44	320	80	2b	2	0	3	5.08	6106.67	M/U	M/U	M/M
11	M	37	320	80	3	2	0	3	1.11	277.67	U/U	M/M	M/M
12	M	52	320	80	3	2	0	3	13.25	671.47	NA/M	NA/M	NA/U
13	F	63	320	80	4	2	0	4a	8.93	41692.31	NA/U	NA/U	NA/U
14	F	38	<5	<5	2a	1	0	2b	659.32	1235.39	NA/M	NA/M	NA/M
15	F	56	5	<5	2b	0	0	2b	2535.37	1563.64	NA/M	NA/M	NA/M
17	F	74	20	20	2a	1	0	2b	12953.93	1308.00	NA/M	NA/M	NA/U
18	M	44	20	5	4	3	0	4b	72.72	12920.00	NA/M	NA/M	NA/U
20	M	36	10	NA	2b	2	0	3	3384.96	2540.00	NA/M	NA/U	NA/U
21	M	45	320	80	2a	2	0	3	80.33	45955.56	NA/U	NA/U	NA/U
22	F	40	80	<5	1	3	0	4b	3.07	14920.00	NA/U	NA/U	NA/M
23	F	40	80	10	2a	2	0	3	137.24	11628.57	NA/M	NA/U	NA/U
24	M	49	80	5	1	2	0	3	0.11	2670.00	NA/U	NA/U	NA/U
25	M	48	40	5	4	1	0	4a	36.72	42.00	NA/M	NA/U	NA/U
26	M	69	320	>80	1	2	0	3	399.17	8166.67	M/M	M/M	M/M
33	M	43	80	10	4	3	0	4b	7.94	24900.00	M/U	M/U	U/U
34	M	68	<5	NA	1	0	0	1	0.20	545.56	U/U	M/M	M/M
40	M	61	320	5	1	0	0	1	8.95	938.00	U/U	M/U	M/M
51	M	57	20	<5	1	0	0	1	7.33	201.25	U/U	U/U	U/U
57	F	69	40	5	2a	0	0	2a	2.07	39.40	M/U	U/U	M/M
58	F	47	20	10	1	0	0	1	0.00	0.00	M/U	M/M	U/U
59	F	50	80	10	2b	0	0	2b	1.87	602.00	U/U	M/M	U/U
62	F	54	20	20	2b	0	0	2b	371.76	NA	U/U	U/U	U/U
63	M	47	80	10	4	1	0	4a	8.05	1696.00	M/U	M/U	M/M
65	M	54	80	20	1	2	0	3	46.75	81000.00	M/M	M/M	M/M
68	M	53	80	20	1	2	0	3	0.18	386.00	U/U	M/M	M/M

[a] U, unmethylated; NA, not available; M, methylated.

detection of other molecular tumor markers may also be applied. Promoter methylation has been increasingly recognized as an alternative mechanism leading to silencing of several cancer-related genes (10, 11). Detection of gene promoter hypermethylation in various types of body fluids has been shown to be a potential tumor marker (11–13). We have investigated the hypermethylation frequencies of multiple genes in NPC tumor tissues previously (14). We hypothesized that such epigenetic changes might also be detected in NP brushing samples.

We adopted a simple, noninvasive, and effective procedure, NP brushing, to obtain samples directly from the nasopharynx to detect EBV DNA quantity and tumor-specific epigenetic changes. We aimed to assess whether quantitative EBV DNA analysis and multiple gene hypermethylation might serve as useful diagnostic tumor markers in our high-risk population.

MATERIALS AND METHODS

Patients. Twenty-eight new NPC cases and 26 noncancerous controls seen in the ENT clinic between 1999 and 2001 at the Prince of Wales Hospital in Hong Kong were prospectively recruited with their consent. The case subjects were newly diagnosed NPC patients ($n = 28$; mean age, 52.3 years; range, 36–74 years; male:female ratio, 1.8). All tumors were histologically confirmed to be undifferentiated NPC (type III) according to the WHO classification (15). Staging was determined according to the AJCC with 4 stage I cases, 7 stage IIa/IIb cases, 11 stage III cases, and 6 stage IVa/Ivb cases (16). The control group ($n = 26$; mean age, 47.4 years; range, 20–80 years; male:female ratio, 0.6) consisted of family members of NPC patients with elevated EBV IgA VCA and/or EA titer or other noncancerous individuals with minor ENT complaints (Tables 1 and 2).

NP Brushing and DNA Extraction. The brushing procedure has been described previously (6). In brief, the nasal cavity was first examined with a headlight and a speculum to rule out any lesions that might cause difficulties in performing brushings. Five percent cocaine was then sprayed into the nasal cavities as a local anesthetic and vasoconstrictor. The brush used was Uterobrush (Medscand Medical, Malm, Sweden), a 24-cm long flexible wire shaft with a soft nylon brush at one end. A small plastic knob was attached to the distal end of the brush, which covered the wire tip and prevented possible trauma. The brush was covered with a sliding plastic sheath that protected the brush from contamination during introduction and withdrawal from the nasal cavities.

After the brush with covering sheath engaged the nasopharynx, the sheath was retracted, and the brush was turned for at least three complete rotations while brushing across the nasopharynx. The sheath was then pushed forward to cover the brush, followed by the withdrawal of the brush from the nasopharynx. To avoid contamination, the sheath and the terminal plastic knob at the end of the brush were wiped with 70%

Table 2 Demographic data, clinical status, EBV IgA titers, EBV DNA quantity in brushing and plasma samples, and multiple gene methylation status in control cases

Code	Sex	Age (yrs)	Status	IgA VCA	IgA EA	Brush EBV DNA copies/actin	Plasma EBV DNA copies/ml	Methylation status (biopsy/brush)			
								RASSF1A	*p16*	*DAP-kinase*	
19	F	41	Normal	<5	NA[a]	3	0.000739	0	U/U	U/U	U/U
28	F	32	Inc IgA	320	<5	0	0	U/U	U/U	U/U	
29	M	21	Normal	<5	NA	0	0	U/U	U/U	U/U	
30	M	55	Normal	<5	NA	0.000719	0	U/U	U/U	U/U	
31	F	39	FH	<5	NA	0	0	U/U	U/U	U/U	
32	F	62	Inc IgA, papilloma	80	<5	0.011142	100	U/U	U/U	U/U	
35	F	62	Normal	<5	NA	0	NA	ND	ND	ND	
36	F	75	Inc IgA	20	80	0.00166	NA	U/U	U/U	U/U	
37	F	64	Normal	<5	<5	0	0	ND	ND	ND	
38	F	43	Normal	<5	<5	0	0	ND	ND	ND	
39	M	49	FH, Inc IgA	20	<5	0.005354	38	U/U	U/U	U/U	
41	M	20	FH	<5	NA	0	0	ND	ND	ND	
42	F	60	Normal	<5	NA	0	0	ND	ND	ND	
43	F	33	Inc IgA	80	<5	0.010211	0	ND	ND	ND	
45	F	39	Normal	<5	NA	0	0	ND	ND	ND	
46	F	59	Inc IgA	20	NA	0	0	ND	ND	ND	
49	F	49	Normal	<5	NA	0	0	ND	ND	ND	
50	M	30	Inc IgA	40	NA	0	0	ND	ND	ND	
52	M	31	Inc IgA	160	<5	0.003693	0	ND	ND	ND	
53	F	49	Normal	<5	NA	0	0	ND	ND	ND	
55	F	34	FH	<5	<5	0.006892	0	U/U	U/U	U/U	
56	M	21	FH, Inc IgA	20	<5	0	0	ND	ND	ND	
60	M	51	Inc IgA	320	<5	0.838248	0	U/U	U/U	U/U	
61	M	80	Inc IgA	20	5	0.014796	0	U/U	U/U	U/U	
66	F	56	Normal	<5	NA	0	0	ND	ND	ND	
67	M	66	Inc IgA	160	10	0.0167	88	U/U	U/U	U/U	

[a] NA, not available; FH, family history of NPC; Inc IgA, increased EBV IgA titer; M, methylated; U, unmethylated; ND, not done.

alcohol pads. The terminal plastic knob was then cut away with a wire cutter. The brush was then cut and soaked in PBS and promptly sent to the laboratory. Bilateral brushing was performed for each case, and the samples were processed separately. After centrifugation, the supernatant of the brushing samples was discarded. The cell pellet was collected for standard phenol-chloroform extraction.

DNA Extraction from Formalin-fixed Paraffin-embedded Tissue Samples and Plasma Samples. Paraffin-embedded tumor tissues from 16 NPC patients were available for molecular analysis. The primary biopsies of the remaining 12 NPC cases were not available because they were referral cases. Ten 10-μm sections were cut and subjected to standard phenol-chloroform extraction. For tumor content < 80%, manual microdissection was performed to enrich the tumor content as described previously (17). For plasma, 400 μl were purified for DNA extraction using High Pure PCR Template Preparation Kit (Boehringer Mannheim) according to the manufacturer's protocol.

Detection of EBV DNA by Quantitative Real-time PCR. EBV DNA quantities in NP brushing samples were measured by real-time quantitative PCR analysis. All corresponding plasma samples, except those from one NPC patient (case 62) and two control subjects (cases 35 and 36), were available for EBV DNA quantitative analysis. The Taqman assay was based on DNA amplification of a 75-bp fragment of EBV *Bam*HI-W region as described previously (5). The PCR primers were custom-synthesized by Invitrogen Life Technologies, Inc. (Hong Kong). The nonextendible DNA probe with a fluorescent dye (FAM) attached to the 5′-end and a second fluorescent dye (TAMRA) linked to the 3′-end was synthesized by Applied Biosystems (Foster City, CA). Commercial reagents and PLATINUM Quantitative PCR SUPERMIX-UDG kit (Life Technologies, Inc.) were used according to the manufacturer's protocol. In brief, the PCR was set up in a reaction volume of 50 μl, including 5 μl of DNA template, 25 μl of 2× PLATINUM Quantitative PCR SUPERMIX-UDG, 300 nM of each of the amplification primers, and 50 nM of the fluorescent probe. The reactions were performed and analyzed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). A standard curve was run in parallel with each analysis using DNA extracted from an EBV-positive cell line, Namalwa (CRL-1432, American Type Culture Collection). Namalwa is a diploid cell line that has been previously reported to contain two integrated viral genomes/cell. Serial dilutions of Namalwa DNA were made. To express the DNA quantity in genome equivalents for the unknown samples, a conversion factor of 6.6 pg DNA/diploid cell was used. To adjust for the variation in the number of cells in different samples, we performed quantification of a fragment of the β-actin nuclear gene using a commercially available kit from Applied Biosystems. Each sample was analyzed in duplicate. Water blanks were included as controls in every analysis.

The EBV DNA level in brushing samples was expressed as the ratio of the copy number of the EBV genome:the copy number of the β-*actin* gene, whereas in the plasma sample, it was expressed as copies of EBV genome/ml plasma.

Bisulfite Modification and COBRA. The C promoter of EBV DNA within the virion is unmethylated. However, in latent

EBV from NPC cells, the C promoter is methylated (18). To investigate the nature of EBV DNA in brushing samples, we examined EBV C promoter methylation status in some of our samples. Five NP brushing samples and five primary NPC biopsy samples were investigated by COBRA. Blood and NP brushing samples from a patient with infectious mononucleosis were used as unmethylated controls. Genomic DNA from primary tumor and brushing samples was modified by bisulfite treatment and purified using the CpGenome DNA Modification Kit (Intergen, Purchase, NY) according to the manufacturer's instructions.

The COBRA protocol has been described previously (19). Bisulfite-modified DNA was amplified with specific primers for EBV C promoter. Primer sequences for bisulfite-treated DNA were 5′-CGTAGGATCCCTTAAACTCTCTTATTAACTATA-3′ (upstream) and 5′-TCGAAGATCTAATGTGTTTTAATTAGAAATTT-3′ (downstream; Ref. 20). PCR reactions were performed in a volume of 50 μl containing 1× PCR buffer, 4 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphate, 10 pmol of each primer, and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems). The PCR involved an initial denaturation at 95°C for 12 min; followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and polymerization at 72°C for 1 min; followed by a final extension at 72°C for 5 min. PCR products were then digested by restriction enzyme *Taq*I at 60°C overnight. Digested samples were analyzed on 10% polyacrylamide gels and stained by ethidium bromide.

MSP. NP brushing samples of 28 NPC cases and 16 corresponding primary tumor biopsies were studied. NP brushing samples from 12 noncancerous control cases were also included. Three cancer-related genes with a high frequency of methylation in NPC (*DAP-kinase*, *RASSF1A*, and *p16*) were included (14). The methylation statuses of these genes were detected by MSP analysis. The modified DNA was used as a template for PCR amplification using primers specific for either methylated or modified unmethylated DNA. The primer sequences and PCR conditions for *DAP-kinase* (21), *RASSF1A* (22), and *p16* (23) were described previously. Fifteen μl of PCR products were loaded onto a 10% nondenaturing polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

Statistical Analysis. Mann-Whitney test (two-tailed) was used to compare the nonparametric variables between groups. $P < 0.05$ was considered as statistical significance. The statistical analysis was performed using the statistical software SPSS Version 10.0.

RESULTS

Serum Antibody Titers against EBV IgA VCA/EA. The sera titers of EBV IgA VCA and EA for every patient enrolled in our study were determined by using an indirect immunofluorescence technique. The results are summarized in Tables 1 and 2. Raised EBV IgA VCA or EA titer (≥5) was seen in 12 of 26 (46.7%) control subjects and 25 of 28 (89.3%) NPC patients. The sensitivity for cancer detection was 89.3% and the specificity was 53.8% when anti-VCA IgA ≥ 5 and/or EA ≥ 5 were set as cutoff points. The positive predictive value was 67.5%, and the negative predictive value was 82.3%.

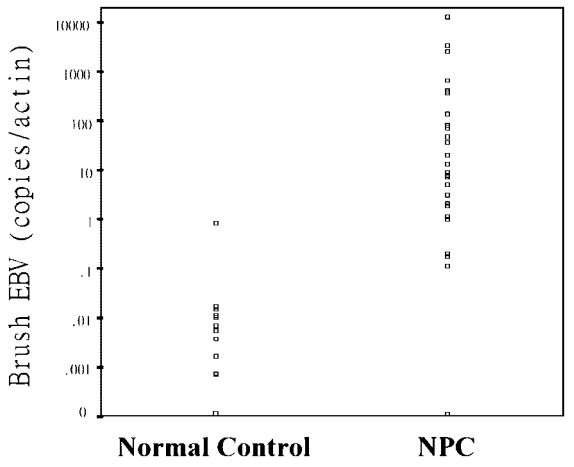

Fig. 1 EBV DNA quantity in NP brushing specimens from NPC patients and noncancerous control subjects. The quantity of EBV DNA was expressed as the ratio of copy number of EBV genome: copy number of the β-*actin* gene. The *Y* axis was expressed in a log scale. A statistically significant difference in EBV DNA level was demonstrated between the two groups (Mann-Whitney test, $P < 0.0001$).

EBV DNA Quantity in NP Brushing and Plasma samples. Quantitative analysis of EBV DNA was performed on all brushing samples and corresponding plasma samples when available from 28 NPC patients and 26 control noncancerous subjects. Bilateral NP brushing was performed for each case, and the samples were processed separately. The one with higher EBV DNA quantity was used for data analysis. All NP brushing samples were positive for PCR analysis of the β-*actin* gene, suggesting sufficient yields of genomic DNA. EBV DNA was detectable in 27 of 28 (96.4%) brushing samples of NPC patients, and the median EBV DNA quantity was 8.94 copies/actin (range, 0–12,953 copies/actin; Table 1). For noncancerous subjects, EBV was not detectable or was detectable at an extremely low level in 25 of 26 cases (96%), and the median EBV DNA quantity was 0 copies/actin (range, 0–0.8 copies/actin; Table 2). A statistically significant difference in EBV DNA quantities in brushing samples between NPC and noncancerous subjects was demonstrated ($P < 0.0001$; Fig. 1). Although we set the threshold of a positive test as 0.05 EBV copy/actin, it resulted in one false positive (case 60) and one false negative case (case 58) for NPC detection. The test thus predicted NPC with a sensitivity of 96.4% and had a specificity of 96.2%. The positive predictive value was 96.4%, and the negative predictive value was 96.2%.

The EBV DNA quantity in NP brushing of control subjects was significantly lower than that in T_1 ($P < 0.0001$), N_0 ($P < 0.0001$), or stage I/II diseases ($P < 0.0001$; Table 3). T_1 disease had a significantly lower level of EBV DNA quantity in NP brushing samples as compared with locally more advanced disease (T_2–T_4; $P = 0.037$; Table 4). No statistically significant difference was demonstrated between node-negative and node-positive cases and between lower stage and higher stage diseases.

As a comparison, corresponding plasma samples from NPC patients and noncancerous control subjects were also analyzed for EBV DNA by real-time PCR. The median EBV DNA concentration for NPC patients was 1,534 copies/ml (range,

Table 3 The *P* values (Mann-Whitney test) of EBV DNA quantity in brushing samples/plasma samples of control subjects as compared with different T stages (T_1/T_2, T_3, T_4), N stages (N_0/N_1, N_2, N_3), and overall stages (stage I, II/III, IV) of NPC cases.

	T_1	$T_2/T_3/T_4$	N_0	$N_1/N_2/N_3$	Stage I/II	Stage III/IV
P for control brush EBV DNA (copies/actin)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
P for control plasma EBV DNA (copies/ml)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Table 4 Comparison of EBV DNA quantity in brushing samples/plasma samples in different T stages (T_1 *versus* $T_2/T_3/T_4$), N stages (N_0 *versus* $N_1/N_2/N_3$, and overall stages (stage I/II *versus* III/IV) of NPC cases. Mann-Whitney test was used.

NS, no statistical significance was demonstrated.

			Brush EBV DNA (copies/actin)			Plasma EBV DNA (copies/ml)		
		Case no.	Median	Range	*P*	Median	Range	*P*
T	T_1	9	3.07	0.00–399.17	0.037	938.00	0.00–81,000.00	NS
	$T_2/T_3/T_4$	19	19.88	1.00–12,953.93		1548.00	39.40–45,955.56	
N	N_0	8	4.70	0.00–2,535.37	NS	546.56	0.00–1,563.64	0.003
	$N_1/N_2/N_3$	20	16.57	0.11–12,953.93		2605.00	42.00–81,000.00	
Stage	I/II	11	8.95	0.00–12,953.93	NS	770.00	0.00–1,563.64	0.006
	III/IV	17	8.93	0.11–3,384.96		6106.67	42.00–81,000.00	

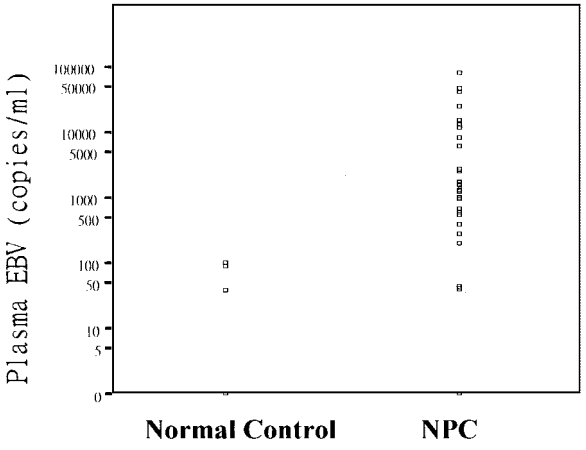

Fig. 2 EBV DNA quantity in plasma of NPC patients and noncancerous control subjects. The quantity of EBV DNA was expressed as EBV copy number/ml plasma. The *Y* axis was expressed in a log scale. The difference in EBV DNA level in these two groups was statistically significant (Mann-Whitney test, $P < 0.0001$).

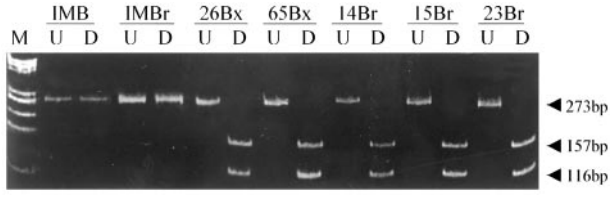

Fig. 3 COBRA assay. A 273-bp PCR fragment cleaved by *Taq*I when methylation is present in bisulfite-modified DNA. *M*, ΦX174 DNA marker (MBI Fermentas); *U*, undigested DNA; *D*, DNA digested with *Taq*I. A fully unmethylated pattern was seen in DNA samples from blood (*IMB*) and NP brushing (*IMBr*) of a patient with infectious mononucleosis. Fully methylated pattern was seen in NPC biopsies (*26Bx* and *65Bx*) and NP brushing (*14Br*, *15Br*, and *23Br*) samples.

0–81,000 copies/ml), whereas for noncancerous patients, it was 0 copies/ml (range, 0–100 copies/ml; Tables 1 and 2; Fig. 2). Three of 24 (12.5%) noncancerous control subjects showed elevated EBV DNA in plasma (case 32, 100 copies/ml; case 39, 38 copies/ml; and case 67, 88 copies/ml). One of these subjects had a history of sinonasal inverted papilloma (case 32). All of these patients had almost undetectable EBV DNA in brushing samples, and the NP biopsies were negative for malignancy. The one NPC patient with no detectable EBV DNA in plasma (case 58) also showed negative EBV DNA in NP brushing samples and has been mentioned earlier. Detectable plasma EBV DNA has a sensitivity of 96.3%, a specificity of 87.5%, a positive predictive value of 89.7%, and a negative predictive value of 95.5% for the detection of NPC.

Similar to NP brushing, the plasma EBV DNA quantity in control subjects was significantly lower, even when compared with those with T_1 disease ($P < 0.0001$), N_0 disease ($P < 0.0001$), or stage I/II disease ($P < 0.0001$; Table 3). The plasma level of EBV DNA was significantly higher in node-positive cases than in node-negative cases ($P = 0.003$) and higher stage cases (stage III and IV) than in lower stage cases (stage I and II; $P = 0.006$; Table 4), but no statistically significant difference was seen in T_1 *versus* T_2, T_3, and T_4 cases.

Methylation Status of EBV C Promoter as Determined by COBRA. To investigate the nature of EBV DNA, we examined the methylation status of the EBV C promoter from five NP brushing samples and five primary NPC biopsies by COBRA. All of these samples were completely methylated at the selected *Taq*I recognition site (Fig. 3). The absence of the unmethylated sequence of the EBV C promoter implied that only latent EBV was detected in the NP brushing samples.

Detection of Multiple Gene Methylation in NP Brushing Samples of NPC Patients. The frequencies of methylation of *DAP-kinase*, *RASSF1A*, and *p16* were determined by MSP. NP

brushing samples of 28 NPC cases and 16 corresponding primary tumor biopsy samples were investigated. NP brushing samples from 12 noncancerous control cases were also included. In brushing samples from NPC patients, the frequencies of gene promoter hypermethylation of *DAP-kinase*, *RASSF1A*, and *p16* were 50.0% (14 of 28), 39.3% (11 of 28), and 46.4% (13 of 28), respectively. The results are listed in Tables 1 and 2, and representative MSP results are shown in Fig. 4. In brushing samples of NPC, 78.6% (22 of 28) exhibited aberrant promoter hypermethylation in at least one of the three genes studied. Thirty-six percent (10 of 28) of the brushing samples from NPC patients had only one gene methylated, 29% (8 of 28) had two genes methylated, and 14% (4 of 28) had all three genes methylated. In NPC primary tumor tissues of the 16 available cases, identical methylation status was detected in both NP brushing samples and biopsy samples in 100% of cases (16 of 16) for *DAP-kinase*, 69% of cases (11 of 16) for *RASSF1A*, and 75% of cases (12 of 16) for *p16*. In 62.5% (10 of 16) of the cases, the MSP result of NP brushing exactly matched the corresponding tumor for all three genes. Notably, gene hypermethylation was not found in brushing samples when such alteration was not seen in the corresponding primary tumor. Moreover, promoter hypermethylation of any of the three genes was not detected in NP brushing samples of 12 noncancerous control cases. The methylation status of these genes did not correlate with the T, N, or overall staging of the diseases.

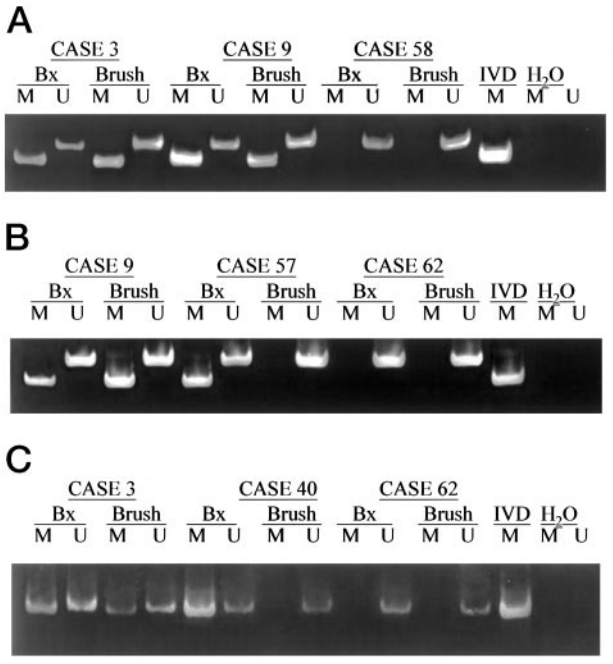

Fig. 4 MSP analysis of *DAP-kinase* (*A*), *RASSF1A* (*B*), and *p16* (*C*) in primary NPC and NP brushing specimens. *U*, reactions using primers specific for unmethylated CpG sites; *M*, reactions using primers specific for methylated CpG sites; *IVD*, *in vitro* methylated DNA.

DISCUSSION

NPC is prevalent in southern China. Both genetic and environmental factors have been implicated in its carcinogenesis (1). EBV has a strong association with NPC. In this endemic region, the majority, if not all, NPC cancer tissues contained latent EBV infection. Detection of EBV DNA in NP brushing samples as a molecular marker for NPC was also based on the premise that normal nasopharynx is not an EBV reservoir (24). Previous studies have demonstrated the absence of EBV in normal NP epithelium (25, 26). Initial attempts using NP brush or swab for EBV detection reported a high false positive rate, and this may be explained by contamination during the sampling procedure through the nasal passages (9, 27). Subsequent studies with attention to prevention of contamination advocated that detection of EBV DNA in brush/swab samples could be a sensitive and specific marker for NPC (7, 8). Because we are in an endemic region, we are in a good position to explore whether such approaches may be applicable to our local population.

Our results indicated that NP brushing was a simple and noninvasive procedure to collect NP tissue samples for molecular analysis. Quantitative EBV DNA analysis represented a sensitive (sensitivity, 96.4%) and specific (specificity, 96.2%) marker for NPC in this endemic region. One of the advantages of quantitative analysis is the allowance of a good cutoff between cases and controls. The only false negative case (case 58) had undetectable EBV DNA in both NP brushing and plasma samples. This patient was a 47-year-old female with a family history of NPC and was found to have elevated EBV IgA titers. NP biopsy was performed, and she proved to have early-stage ($T_1N_0M_0$, stage I) undifferentiated NPC. *In situ* hybridization for EBV-encoded mRNAs (EBER) was positive. The reason(s) accounting for the absence of detectable EBV DNA in brushing and plasma samples remained uncertain. The false positive case in a noncancerous control subject (case 60) showed slightly elevated EBV DNA (0.8 copy/actin) in brushing sample. The NP biopsy of this patient was negative for malignancy, and *in situ* hybridization for EBER was also negative. Contamination during sample collection might be one of the explanations. However, we cannot exclude the possibility of obscured malignancy. This patient has been followed-up regularly to monitor any clinical evidence of NPC.

In our study, EBV DNA quantity was correlated with T stages of disease (Table 4). It was logical to assume that more tumor cells (hence, a larger amount of EBV DNA) could be brushed out from a more locally advanced tumor. On the other hand, the lack of association between EBV DNA quantity in NP brushes and nodal status and staging was not unexpected.

Our results also implied that EBV DNA originated from latently infected NPC cells. The status of latent infection could also be demonstrated in the NP brushing samples in our study. In lytic replication with the production of new virions, the EBV C promoter is not methylated, whereas in latent EBV infection, the C promoter is inactivated and hypermethylated (18, 28). Five NP brushing samples and five primary tumor biopsy samples were studied using COBRA. All of these samples harbored the methylated sequence of the EBV C promoter only, indicating the latent status of EBV, and no lytic component was detected.

For comparison, corresponding plasma EBV DNA quantitative analysis was performed. Plasma EBV DNA quantity has been shown to be a good molecular marker for NPC (5). It was associated with disease staging and shown to be useful for

monitoring of disease progression (29, 30). Our current data also demonstrated that plasma EBV DNA quantity was a sensitive and specific maker for NPC detection. In addition, node-positive *versus* node-negative cases and higher stage *versus* lower stage cases contained a significantly larger quantity of plasma EBV DNA (Table 4). In contrast to NP brushing, no significant difference was demonstrated among T stages. It appeared that the EBV DNA quantity detected in the NP brushing samples reflected the local tumor load in the nasopharynx, whereas the plasma EBV DNA quantity reflected the tumor load in the whole body. In our series, three noncancerous control subjects had elevated plasma EBV DNA. One of the control cases had a history of sinonasal inverted papilloma. The possible association between sinonasal inverted papilloma and EBV may deserve further investigation (8). Nevertheless, various EBV-associated conditions may result in detectable plasma EBV DNA. These included infectious mononucleosis and EBV-associated malignancies, such as certain subsets of lymphoma and gastric carcinoma (31, 32). The causes of elevated plasma EBV DNA in the two other control subjects remained unclear. However, for these three subjects, significant elevation of EBV DNA in corresponding NP brushing samples was not detected. Detection of an elevated level of EBV DNA from NP brushing would help to localize the disease in the nasopharynx. The potential diagnostic value of quantitative EBV DNA analysis may be illustrated by one of our NPC cases. The patient was a 57-year-old man (case 51) who presented with minor ENT symptoms with a IgA VCA titer of 20. No obvious NP tumor mass was detected in endoscopic examination, and the initial NP biopsy was negative for malignancy. However, both NP brushing and plasma EBV DNA were elevated (Table 1). Repeated multiple random NP biopsies were then performed under general anesthesia. Only one of the biopsy specimens revealed undifferentiated carcinoma. An early-stage NPC ($T_1N_0M_0$, stage I) was diagnosed. Detection of NPC at an early stage is critical to improve patient prognosis. However, the nasopharynx remained a difficult area for routine examination. It is not unusual to have repeated biopsies before one can confirm the diagnosis in suspicious cases. Our data indicated that the EBV quantities in both NP brushing and plasma of control subjects were significantly lower than those of NPC cases. More importantly, the differences could be demonstrated even when comparing the controls with individuals with early-stage disease (T_1, N_0, or stage I/II; Table 3). Quantitative EBV DNA analysis in NP samples would help to localize the disease process in the nasopharynx and might contribute to early recognition of the disease.

We also explored the utility of other molecular markers for NPC detection in NP brushing samples. Gene promoter hypermethylation is an increasingly recognized mechanism for inactivation of cancer-related genes (10). Such epigenetic changes could also be detected in various types of body fluid, for example, serum, urine, saliva, and bronchial brushing specimens (11–13, 33–35). These observations suggested that detection of promoter hypermethylation of various tumor-specific genes might serve as a useful tumor marker (12). The multiple gene methylation profile in NPC has been demonstrated in our previous study (14). Simultaneous inactivation of several pathways by aberrant methylation in NPC has been documented with high frequencies of *DAP-kinase*, *RASSF1A*, and *p16* gene methylation (14). The current study demonstrated for the first time that aberrant promoter hypermethylation of *DAP-kinase*, *RASSF1A*, and *p16* could be detected in NP brushing samples from NPC patients. The frequencies of gene promoter hypermethylation of *DAP-kinase*, *RASSF1A*, and *p16* were 50.0% (14 of 28), 39.3% (11 of 28), and 46.4% (13 of 28), respectively. In 10 of 16 NPC cases for whom the primary tumor specimens were available, identical methylation status of three genes was identified in both brushing specimens and biopsy specimens (Table 1). Gene hypermethylation was not found in brushing samples when such alteration was not seen in the corresponding primary tumor. Moreover, only unmethylated alleles of the three genes were detected in 12 noncancerous control subjects, including the one with elevated EBV DNA quantity in NP brushing (case 60). These results demonstrated that the test was highly specific for cancer detection. Taking the presence of at least one gene showing methylation in NP brushing samples as a positive test, the sensitivity of detecting NPC was 78.6% with 100% specificity. Notably, hypermethylation of *p16* was detected in brushing samples as well as the primary tumor sample from NPC case 58 (Table 1). However, the brushing samples were not positive for *RASSF1A* as in the primary tumor tissue. As described earlier, this case was negative for EBV DNA in both brushing and plasma samples. Combining methylation and quantitative EBV DNA analysis in NP brushes would increase the sensitivity for NPC detection to 100%. These results further demonstrated the potential diagnostic value of multiple tumor marker detection in brushing samples. Recently, a real-time PCR-based assay (quantitative MSP) for the detection and quantification of CpG island methylation has been introduced. The potential advantages of quantitative MSP analysis of multiple tumor markers may deserve additional studies.

In conclusion, NP brushing provided sufficient materials for multiple tumor marker analysis. It offered a powerful yet noninvasive approach for the detection of NPC. Our data provide justification for a larger prospective study for this approach in detecting NPC in high-risk populations.

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Quantitative Epstein-Barr Virus DNA Analysis and Detection of Gene Promoter Hypermethylation in Nasopharyngeal (NP) Brushing Samples from Patients with NP Carcinoma

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