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Satoru Kondo,[1] Toshiyuki Horikawa,[1] Hajime Takeshita,[1] Chiharu Kanegane,[2] Yoshihito Kasahara,[2] Tzung-Shiahn Sheen,[3] Hiroshi Sato,[4] Mitsuru Furukawa[1] and Tomokazu Yoshizaki[1, 5]

Divisions of [1]Otolaryngology and [2]Pediatrics, Graduate School of Medicine and [4]Department of Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Ishikawa, Kanazawa 920-8640; and [3]Department of Otolaryngology, School of Medicine, National Taiwan University, 7, Chung-shan South Rd., Taipei, Taiwan

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We compared the amount of serum Epstein-Barr virus DNA (EBV-DNA) detected in patients with nasopharyngeal carcinoma (NPC) in a high-incidence area, represented by Taiwan, and a low-incidence area, represented by Japan, using real-time quantitative PCR. The median serum EBV-DNA value in 41 Japanese NPC cases was 5450 copies/ml, and that in in 23 Taiwanese cases was 2125 copies/ml. The median serum EBV-DNA value in all 64 NPC cases was significantly higher than in control groups. Using receiver-operating-characteristic (ROC) curves, the sensitivity and specificity of EBV-DNA quantification were determined (cut-off point, 6.87 copies/ml; sensitivity, 0.855; specificity, 0.885) and compared with those of EBV-viral-capsid-antigen (VCA) titers; the results showed that EBV-DNA was a more sensitive and specific parameter than EBV-VCA titer. Then, we analyzed 19 NPC patients in whom recurrence developed (11 Japanese and 8 Taiwanese), and 26 NPC patients in continuous remission. Although there was no significant difference in EBV-DNA values between Japanese and Taiwanese patients, the value was significantly higher in the 19 patients with recurrence than in those in remission. ROC analysis again revealed a higher diagnostic value of EBV-DNA than EBV-VCA. These results suggest EBV-DNA is a more reliable tumor marker than EBV-VCA in both high-incidence and low-incidence areas of NPC. (Cancer Sci 2004; 95: 508–513)

The restricted geographic and ethnic distribution of endemic nasopharyngeal carcinoma (NPC) is one of its most striking epidemiologic characteristics. In Southern China, where it is the third most common form of malignancy amongst males, incidence rates are between 15 and 50 per 100,000.[1)] In Taiwan, which is also considered a high-incidence area, the incidence rate was reported to be 8.3/100,000 (male) and 3.5/100,000 (female).[2, 3)] In Japan, the incidence rate was reported to be between 0.5 and 1 per 100,000, similar to those in North China and North America, which are both non-endemic or low-incidence areas.[4–6)]

Recently, cell-free Epstein-Barr virus DNA (EBV-DNA) has been detected in the plasma and serum of patients with NPC with the use of real-time quantitative PCR (RQ-PCR).[7–11)] Plasma/serum cell-free EBV-DNA has been shown to be measurable in the majority of NPC patients at the time of diagnosis, and appears to have prognostic significance. To our knowledge, these studies were conducted in an endemic area, represented by Hong Kong, while no study in a non-endemic area has been reported. In the present study, we compared by RQ-PCR the amount of serum EBV-DNA of NPC patients in a high-incidence area, represented by Taiwan, and a low-incidence area, represented by Japan. There have been some reports on the diagnostic value of serum EBV-DNA compared with EBV-VCA/EA Ig titers.[12)] However, these studies used an arbitrary cut-off and therefore were not statistically meaningful. For this report we used a different approach made possible by use of receiver-operating-characteristic (ROC) curve analysis[15, 16)] for thorough comparison of the sensitivity and specificity of serum EBV-DNA with EBV antibody titers. We also compared the results in patients with recurrence or in remission in both Japan and Taiwan.

Materials and Methods

Patients. Forty-one Japanese patients with histologically confirmed NPC under the care of the Department of Otolaryngology at Kanazawa University and the Central Hospital of Toyama Prefecture and 23 Taiwanese NPC patients at the National Taiwan University were recruited prior to initial therapy. Patients' characteristics are shown in Table 1A. All patients were investigated with endoscopy and computed tomography of the nasopharynx and neck. No patients had clinical evidence of distant metastasis on presentation. Staging of all patients was done according to the American Joint Committee on Cancer/International Union Against Cancer Stage Classification.[13)]

All patients had radiotherapy to the local and loco-regional lesion with or without cisplatin-based chemotherapy. After initial therapy, the patients were assessed by endoscopic examination of the nasopharynx and clinical examination every 4–12 weeks. If patients developed symptoms indicative of local recurrence or metastasis, this development was confirmed by nasopharyngeal biopsy and imaging.

For controls, sera from 12 patients with non-Hodgkin's lymphoma (NHL), 33 patients with head and neck squamous cell carcinoma (HNSCC), and 20 healthy individuals were investigated.

In addition, sera from 19 NPC patients who had an initial disease-free period, but subsequent tumor recurrence, were assayed for EBV-DNA; 11 were Japanese and 8 were Taiwanese. Patients' characteristics are shown in Table 1B. As controls, 26 NPC patients who had remained in continuous clinical remission, all of whom were Japanese, were recruited. Blood samples were taken immediately prior to therapy and at multiple occasions after initial therapy. Serum Ig titers were measured routinely. The EBV-DNA was measured from the serum obtained for the routine diagnostic examination. For the recent samples, written permission in accordance with the ethical standards of the Ethical Committee of Kanazawa University was obtained. However, older samples stocked in our laboratory were used without such permission, because we were examining only viral, not human, DNA.

[5]To whom correspondence should be addressed.
E-mail: tomoy@orl.m.kanazawa-u.ac.jp

Abbreviations: NPC, nasopharyngeal carcinoma; EBV, Epstein-Barr virus; ROC, receiver-operating-characteristics; VCA, viral capsid antigen; RQ-PCR, real-time quantitative PCR; NHL, non-Hodgkin's lymphoma; HNSCC, head and neck squamous cell carcinoma.

DNA extraction from serum samples. The serum samples were stored at −80°C until further processing. DNA was extracted using a QIAamp Blood kit (Qiagen, Hilden, Germany) using the manufacturer's protocol. Serum samples (100–200 μl) were used for DNA extraction after addition of 10 μg of poly(A) (Roche Diagnostics, Basel, Switzerland) as a carrier RNA. A volume of 50 μl of the final eluate was used.

Real-time quantitative EBV-DNA PCR. The serum EBV-DNA concentration was measured by means of RQ-PCR of the *BALF5* gene, which encodes the viral DNA polymerase, as reported previously,[14] with some modifications. Ten microliters of extracted serum DNA was used for this assay. As a positive control, a plasmid that contained the *BALF5* gene was constructed from pGEM-T vector (Promega, Madison, WI) and termed pGEM-BALF5. A standard graph of the C_T values obtained from serially diluted pGEM-BALF5 (from 10 to 10^6) was constructed. Each sample was tested in duplicate, and every analysis was tested with negative water blanks and carrier RNA.

Immunofluorescence assay. Indirect and anticomplement immunofluorescence (IF) assays were carried out with Nippon Kayaku VCA slides and kits (Nippon Kayaku, Tokyo) for the detection of IgG and IgA antibodies to VCA according to the manufacturer's instructions. The serum samples were serially diluted (1:2), with final dilutions ranging from 1:10 to 1:10,240. The concentration of the antibodies was expressed as a titer, with the endpoint corresponding to the last dilution that clearly showed fluorescence. If serum diluted 1:10 showed no fluorescence, that titer was expressed as 0.

Statistical analysis. Dr. SPSS II J for Windows statistical software (SPSS, Inc., Chicago, IL) was used for the data analysis. The Mann-Whitney test, the Kruskal-Wallis test, or the Mann-Whitney *U* test with Bonferroni adjustment was used for comparison of the copy numbers of EBV DNA and patient characteristics in each group. Sensitivity and specificity of the data were assessed by means of the ROC curve method. The differences between the two means were analyzed according to the method previously established.[15, 16] *P* values of <0.05 were considered to be statistically significant.

Table 1. Characteristics of Japanese and Taiwanese NPC

Characteristics	Japanese NPC	Taiwanese NPC	*P* value
A. Prior to therapy.			
Total patients	41	22	
Male/Female	33/8	15/7	NS
Age (mean yr; range)	58 (20–90)	50 (33–79)	NS
Histological type (WHO type)			NS
I	9	2	
II	22	17	
III	10	4	
Tumor classification			NS
T1	8	4	
T2	7	10	
T3	10	6	
T4	16	3	
Node classification			NS
N0	9	4	
N1	6	11	
N2	14	4	
N3	24	4	
Overall stage			NS
1	3	1	
2	1	6	
3	16	9	
4	21	7	
B. Recurrent cases.			
Total patients	11	8	
Male/Female	7/4	6/2	NS
Age (mean yr; range)	52 (20–68)	55 (29–65)	NS
Histological type (WHO type)			NS
I	1	2	
II	6	5	
III	4	1	
Overall stage (prior to initial therapy)			NS
I	0	0	
II	1	3	
III	3	1	
IV	7	4	
Recurrent stage			
rT1	7	0	
rT2	0	0	
rT3	1	1	
rT4	0	0	
rN1	1	3	
rN2	3	2	
rN3	0	0	

The values in this table show the number of patients. *P* values are from comparisons between the two groups. Tumor classification, node classification, and overall stage were based on the International Union Against Cancer (UICC) classification. No patients had distant metastasis detected. Histological types were classified according to WHO criteria.

Results

Serum EBV-DNA levels of Japanese and Taiwanese NPC cases. The serum EBV-DNA levels of both Japanese and Taiwanese NPC cases were quantitated by the RQ-PCR method. There was no significant difference between Japanese and Taiwanese cases in terms of distribution with respect to gender, age, TNM classification, stage, and histology (WHO type), as shown in Table 1A. Moreover, there was no significant difference between the EBV-DNA levels in Japanese NPC cases (median, 5450 copies/ml; range, 0–2,347,650 copies/ml) and Taiwanese NPC cases (median, 2125 copies/ml; range, 0–6,485,925 copies/ml), as shown Fig. 1A.

Quantitative analysis of serum from NPC and other diseases. To evaluate the significance of EBV-DNA elevation in patients with NPC, EBV-DNA was also quantitated in 20 healthy individuals, 12 NHL patients, and 33 HNSCC patients. The median EBV-DNA concentration in all 64 NPC patients (41 Japanese and 23 Taiwanese NPC patients) was 3625 copies/ml (range 0–6,485,925 copies/ml), which was significantly higher than that of healthy individuals (0 copies/ml, range 0–372 copies/ml), NHL patients (0 copies/ml, range 0–26,453 copies/ml), and HNSCC patients (0 copies/ml, range 0–4786 copies/ml) ($P<0.001$) (Fig. 1B). These results suggest that the EBV-DNA concentration was significantly increased in the serum of patients with NPC as compared with healthy individuals, and patients with NHL and HNSCC.

IgG and IgA VCA titers in NPC and other diseases. Elevation of serum IgG/VCA and IgA/VCA antibodies is a common feature in patients with NPC. Thus, serum IgG/VCA and IgA/VCA titers were also examined in healthy individuals, and patients with NHL and HNSCC. The values of median IgG/VCA titer were 1:640 (range 0–1:10,240) in NPC, 1:160 (range 1:40–1:320) in healthy individuals, 1:160 (range 1:40–1:10,240) in NHL, and 1:160 (range 1:40–1:5120) in HNSCC. The IgG/VCA titer in NPC was significantly higher than in healthy individuals and in patients with HNSCC ($P=0.005$, $P=0.01$, respectively). However, there was no significant difference between NPC and NHL (Fig. 1C).

The values of median IgA/VCA titer were 1:20 (range 0–

1:640) in NPC, 0 (range 0–1:20) in healthy individuals, 1:10 (range 0–1:80) in NHL, and 0 (range 0–1:320) in HNSCC. The IgA/VCA titer in NPC was significantly higher than in healthy individuals or HNSCC (P=0.017, P=0.044, respectively). However, no significant difference was found between NPC and NHL (Fig. 1D).

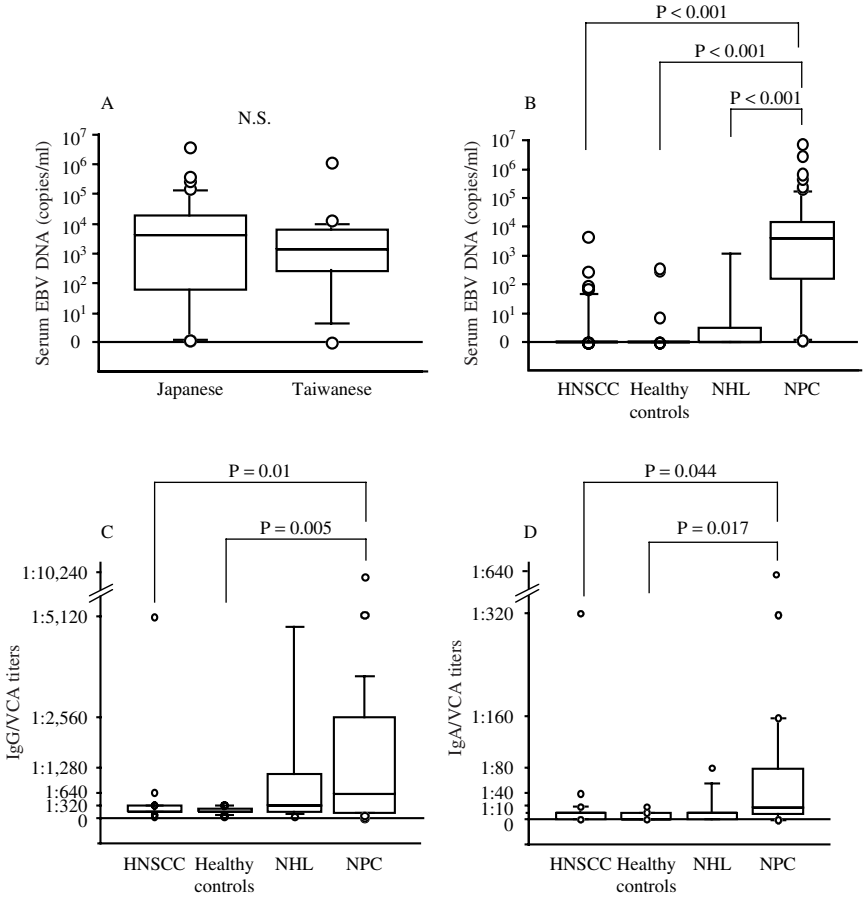

Fig. 1. A. Comparison of serum cell-free EBV-DNA in Japanese and Taiwanese NPC patients. The categories are on the *X*-axis. The *Y*-axis denotes the concentration of cell-free EBV-DNA (copies of EBV-DNA/ml of serum, common logarithmic scale) detected by the PCR system directed at the BALF5 region. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The open circles mark the data points outside the 10th and 90th percentiles. NS: not significant. B. Comparison of serum cell-free EBV-DNA in NPC, NHL, HNSCC, and healthy controls. The categories are on the *X*-axis. The *Y*-axis denotes the concentration of cell-free EBV-DNA. C. Comparison of titers of IgG antibodies to VCA in NPC, NHL, HNSCC, and healthy controls. The categories are on the *X*-axis. The *Y*-axis denotes the IgG antibody to VCA titer. D. Comparison of titers of IgA antibodies to VCA in NPC, NHL, HNSCC, and healthy controls. The categories are on the *X*-axis. The *Y*-axis denotes the IgG antibody to VCA titer.

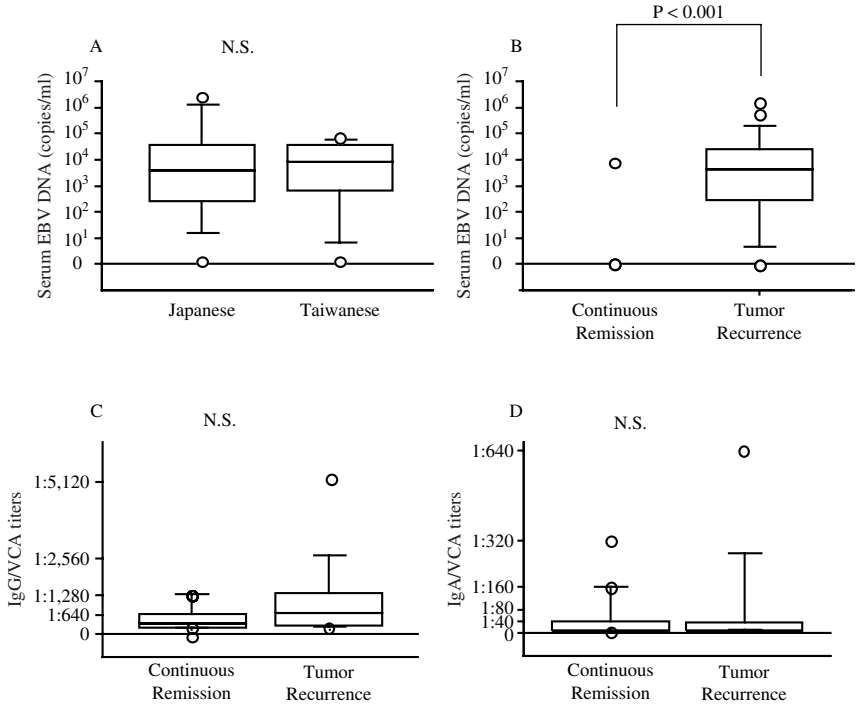

Fig. 2. A. Comparison of serum cell-free EBV-DNA in Japanese and Taiwanese NPC patients with recurrence. The categories are on the *X*-axis. The *Y*-axis denotes the concentration of cell-free EBV-DNA (copies of EBV-DNA/ml of serum, common logarithmic scale). B. Comparison of serum cell-free EBV-DNA in NPC patients who had recurrence and in those who remained in continuous clinical remission. The categories are on the *X*-axis. The *Y*-axis denotes the concentration of cell-free EBV-DNA (copies of EBV-DNA/ml of serum, common logarithmic scale). C. Comparison of IgG/VCA antibody titers in NPC patients who had recurrence and in those who remained in continuous clinical remission. NS: not significant. D. Comparison of IgA/VCA antibody titers in NPC patients who had recurrence and in those who remained in continuous clinical remission.

EBV-DNA and VCA titers in NPC patients with recurrence and in remission. To determine the diagnostic value of EBV-DNA, VCA/IgG, and VCA/IgA for tumor recurrence, these factors were again quantitated in 19 NPC patients in whom there was tumor recurrence (11 Japanese and 8 Taiwanese) and also in 26 in continuous clinical remission (all Japanese). As shown in Table 1B, there was no significant difference between Japanese and Taiwanese recurrent cases in terms of distribution with respect to gender, age, stage, and histology (WHO type). Moreover, there was no significant difference between the EBV-DNA levels in Japanese recurrent cases (median, 2754 copies/ml; range, 0–1,621,810 copies/ml) and Taiwanese recurrent cases (median, 6073 copies/ml; range, 0–47,533 copies/ml) as shown in Fig. 2A.

In the 19 recurrent cases, the median EBV-DNA concentration at recurrence was 2754 copies/ml (range 0–1,621,810 copies/ml), whereas that in the cases in remission was 0 copies/ml (range 0–7943 copies/ml). The elevation of the serum EBV-DNA concentration in the recurrence group was statistically significant ($P<0.001$, Fig. 2B). The median IgG/VCA titer was 1:640 (range 1:160–1:5120) in the recurrence group and 1:320 (range 1:20–1:2560) in the group in remission. The median IgA/VCA titer was 1:20 (range 1:10–1:640) in recurrence and 1:10 (range 0–1:320) in cases in remission. The elevations of both IgG/VCA and IgA/VCA titers in the recurrence group were not significant ($P=0.114$ and 0.142, respectively) (Fig. 2, C and D).

Diagnostic value of EBV-DNA, IgG/VCA, and IgA/VCA analyzed by ROC curves prior to therapy. To determine the diagnostic value of EBV-DNA levels versus IgG/VCA and IgA/VCA titers by ROC curve analysis, the data from 64 NPC patients prior to initial therapy were analyzed together with the data from 33 HNSCC patients and 20 healthy individuals as negative controls.

This analysis demonstrated the diagnostic values of the three assays compared with that of the eye examination, which is the "gold standard."[15, 16] The areas under the ROC curves were significantly larger than the area expected by chance alone for all three diagnostic tools ($P<0.001$, Fig. 3A, Table 2A). The diagnostic optimal cut-off point determined by this analysis was 6.87 copies/ml for EBV-DNA, 1:640 for IgG/VCA, and 1:20 for IgA/VCA. The sensitivity and specificity of EBV-DNA quantification obtained by this cut-off point were 0.855 and 0.885, respectively. The sensitivity and specificity of IgG/VCA with the optimal cut-off point were 0.590 and 0.897, and those of IgA/VCA were 0.597 and 0.897, respectively. Statistical analysis of the area under the ROC curves revealed that the area for serum EBV-DNA was significantly larger than the area for IgG/VCA or IgA/VCA ($P=0.008$, $P=0.011$, respectively). These results suggest that EBV-DNA quantification is a more sensitive and specific tumor marker for the diagnosis of NPC than IgG/VCA or IgA/VCA titers.

Diagnostic value of EBV-DNA, IgG/VCA, and IgA/VCA analyzed by ROC curves at recurrence. By ROC curve analysis, we determined the diagnostic value of EBV-DNA levels and IgG/VCA and IgA/VCA titers at tumor recurrence. The 19 NPC patients who had tumor recurrence served as the positive subjects, and the 26 who were in continuous complete remission were used as the negative subjects.

The areas under the ROC curves were significantly larger than the area expected by chance alone for EBV-DNA quantification ($P<0.001$, Fig. 3B, Table 2A). For IgG/VCA and IgA/VCA titers, however, there were no significant differences from the area expected by chance alone, which means that their diagnostic value was poor. The diagnostic optimal cut-off point determined by this analysis was 60.3 copies/ml for EBV-DNA. The sensitivity and specificity of EBV-DNA quantification obtained by this cut-off point were 0.909 and 0.926, respectively. However, we were not able to determine the cut-off points for VCA-titers, because they did not have high diagnostic value. Statistical analysis of the area under the ROC curves revealed that the area for serum EBV-DNA was significantly larger than the area for either IgG/VCA or IgA/VCA ($P=0.004$; and $P<0.001$, respectively, Table 2B). These results suggest that EBV-DNA is a more sensitive and specific tumor marker for the diagnosis of NPC than IgG/VCA or IgA/VCA, even at tumor recurrence.

Discussion

The recent demonstration of circulating EBV-DNA in plasma or serum of NPC patients has opened up new possibilities for detecting and monitoring NPC in endemic areas such as South China, and also high-incidence area such as Taiwan and elsewhere in Southeast Asia.[7–11, 12, 17, 18] In the first part of this study, we explored serum EBV-DNA levels in NPC patients in a low-incidence area, Japan. The results showed that the amount of serum EBV-DNA in Japanese NPC patients was increased similarly to that in Taiwanese who showed a statistically similar distribution of clinical background. Moreover, there was no significant difference in the EBV-DNA concentration between the two areas, suggesting that quantification of serum EBV-DNA is useful as a screening method for NPC in low-incidence as well as high-incidence areas.

In addition to HNSCC and healthy individuals, the EBV-DNA concentration was significantly higher in NPC patients

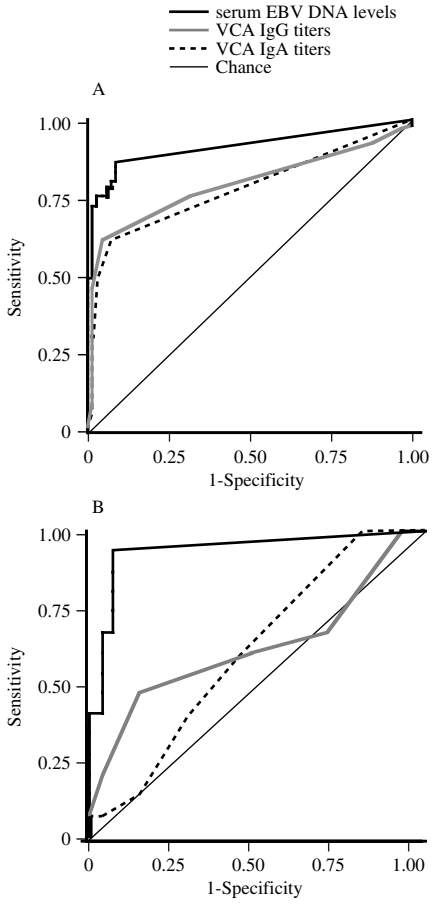

Fig. 3. A. ROC curves of serum EBV-DNA levels and IgG and IgA antibodies to VCA prior to initial therapy. B. ROC curves of serum EBV-DNA levels and IgG and IgA antibodies to VCA at recurrence.

Table 2.

A. Results of ROC curve analysis prior to initial therapy and at recurrence

Assays	% of area under ROC curve	Standard error	95% CI	Determined cut-off	Sensitivity	Specificity
Prior to therapy						
EBV-DNA quantification	90.7[1)]	0.029	0.849–0.964	6.87	0.855	0.885
IgG/VCA	77.7[1)]	0.044	0.690–0.864	1:640	0.590	0.897
IgA/VCA	77.2[1)]	0.044	0.687–0.858	1:20	0.597	0.897
At recurrence						
EBV-DNA quantification	93.5[1)]	0.045	0.846–1.023	60.3	0.909	0.926
IgG/VCA	62.0[2)]	0.099	0.426–0.813	ND	ND	ND
IgA/VCA	60.9[2)]	0.87	0.437–0.780	ND	ND	ND

B. Difference under the ROC curve for each assay

Assays	EBV-DNA quantification vs. IgG/VCA	EBV-DNA quantification vs. IgA/VCA	IgG/VCA vs. IgA/VCA
Prior to therapy			
Z	2.66	2.57	0.12
P value	0.008*	0.011*	0.907
At recurrence			
Z	2.90	3.32	0.15
P value	0.004*	<0.001*	0.933

IgG/VCA: EBV-specific IgG antibody to viral capsid antigen. IgA/VCA: EBV-specific IgA antibody to viral capsid antigen. ROC curve: receiver-operating-characteristic curve. CI: confidence intervals.

1) The areas under the ROC curves were significantly larger than the area expected by chance alone.

2) The area under the ROC curve was not significantly larger than the area expected by chance alone.

ND: For IgG/VCA titer and IgA/VCA titers at recurrence, no cut-off point could be determined; those assays did not give efficient diagnostic values.

Z: critical ratio of normal distribution. * Statistically significant ($P<0.05$).

than in NHL patients, whereas neither IgG/VCA nor IgA/VCA showed a significant difference between the two groups. The present findings suggest that RQ-PCR analysis of serum EBV-DNA could be useful for differential diagnosis between NPC and NHL.

There has been no previous study regarding the optimal cut-off point using the ROC curve method for EBV-VCA antibody titers, or for EBV-DNA quantification. This strict statistical method gave us an optimal EBV-DNA cut-off point of 6.87 copies/ml prior to therapy, and 60.3 copies/ml at recurrence. In contrast, the cut-off point for the IgG/VCA titer was 1:640 prior to therapy, and that for the IgA/VCA titer was 1:20. Furthermore, the findings obtained from the ROC analysis clearly demonstrate that serum EBV-DNA is a more sensitive parameter than the VCA titers. Although comparison of VCA protein instead of VCA Ig titer with EBV-DNA might be better, we designed this experiment because VCA titers had been considered to be the "gold standard" tumor markers for NPC.

The cross-sectional study on the level of serum EBV-DNA in NPC patients with tumor recurrence and in those with complete remission revealed significantly higher levels of circulating cell-free EBV-DNA in patients with tumor recurrence than in patients with complete remission. Occasionally, antibody titers to VCA in NPC patients continue to show high levels after initial treatment. IgA/VCA titers decrease gradually to undetectable levels within 12 to 30 months even in patients in whom complete remission was maintained.[19–22)] Increases in IgA/VCA titer are a well-known predictor of tumor recurrence; however, it is also known that IgA/VCA sometimes fails to detect recurrence, probably for the above reason.

ROC analysis was again utilized at recurrence. The VCA titers were a relatively useful marker prior to initial therapy, but did not have high diagnostic value at recurrence. In addition, ROC analysis revealed clear superiority of serum EBV-DNA quantification over antibodies to VCA in terms of both sensitivity and specificity.

Our data for sensitivity of both IgG and IgA VCA antibodies seem lower than those in many other reports, most of which were from NPC-endemic areas. The results could be due to geographic differences between high and low NPC incidence areas. These data suggest that EBV-DNA quantification is a more valuable diagnostic tool in a low-incidence area for NPC than in a high-incidence area.

Levels of EBV-DNA in the present study were relatively low in comparison with the study by Lo *et al.*[7, 8)] The present RQ-PCR system was constructed to examine the *BALF5* gene; in contrast, in their PCR system the *Bam*HI-W region of the EBV genome or the *EBNA1* gene was utilized. Both of these regions of the genome contain multiple repeat units, but whether these affect the PCR results is unknown. In addition, we used subcloned plasmid DNA as a positive control, whereas Lo *et al.* used an EBV-positive cell line, Namalwa. In addition, they used plasma, but not serum samples. While we used 200 µl of serum, they used 800 µl of plasma. The differences in the systems may have led to the difference in the concentration of EBV-DNA detected. These factors suggest that EBV-DNA should be quantitated using a unified PCR system.

In conclusion, detection of cell-free EBV-DNA using RQ-PCR is suggested to have high fidelity and high diagnostic value for tumor recurrence in NPC patients. Moreover, the present results suggest that serum cell-free EBV-DNA quantification is a more reliable tumor marker than EBV serological titers in both high-incidence and low-incidence areas for NPC.

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