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EBV SPECIFIC ANTIBODY-BASED AND DNA-BASED ASSAYS IN SEROLOGIC DIAGNOSIS OF NASOPHARYNGEAL CARCINOMA

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We assessed 5 EBV specific assays for their capacity to effect serologic diagnosis of suspected NPC. The assays were the immunofluorescent assays, VCA IgA and EA IgA, the enzyme-linked immunosorbent assays specific for EBNA 1 IgA or zta IgG and an EBV DNA assay. Serum samples were taken from 218 symptomatic NPC patients presenting consecutively at a public hospital in Hong Kong, 51 of whom were subsequently diagnosed as having NPC; 4 had EBV-associated lung cancer with similar serology as NPC. The remaining patients included 23 who had other cancers and 140 who had other diseases. Objectives of serodiagnosis under such clinical settings, therefore, are to both exclude and predict a diagnosis of NPC. None of the assays individually can meet both requirements adequately, however. The difficulty was best overcome by combining EBNA 1 IgA and zta IgG. It was shown that 68.3% of the patients gave a confirmed test results, negative or positive, by both tests. A confirmed negative result was associated with a negative predictive value of 99.1%, providing a clear indication to exclude a diagnosis of NPC; a confirmed positive result was associated with a positive predictive value of 86.8%, providing a clear indication to proceed with diagnostic work-up of NPC. The remaining patients gave equivocal test results, being positive for one or the other test, which were associated with a positive predictive value of 43.3% and 24.2%, respectively.

Key words: *NPC; EBV antibody; diagnosis*

Nasopharyngeal carcinoma (NPC) is a common cancer in China and Southeast Asia and is closely associated with Epstein-Barr Virus (EBV).[1] Patients with this tumor have high levels of a broad spectrum of EBV antibodies.[2,3] This feature does not appear to be directly induced by the tumor, as it persists in long-term survivors after successful treatment of the cancer and is also present in some apparently healthy individuals who have increased risk of the cancer.[4,5,6] EBV-specific T-cell immunity of NPC patients is also impaired,[7] and the impairment is associated with active disease and appears to be induced by the tumor.[8] It was shown that the level of circulating EBV-specific cytotoxic T-cell precursors (CTLp) was inversely related to plasma EBV DNA level in these patients, and viral burden was decreased concomitantly with increase in EBV CTLp level, after adoptive immune transfer of EBV-specific T cells.[8] Based on these findings, impaired T-cell immunity would appear to contribute, at least partly, to an increase in plasma EBV burden, as reported by different investigators.[9–13] The contention was consistent with immune T cells being the principal host surveillance of EBV replication.[14]

Early symptoms of NPC are innocuous, especially at early stages of the disease, and serologic diagnosis using EBV-specific antibody-based assays had become an integral part in diagnostic work-ups of NPC in most centers. The objectives are twofold: the first is to exclude and the second to predict a diagnosis of the cancer. The traditional immunofluorescent assays specific for VCA IgA[15] and EA IgA[2] are commonly used for determination of EBV antibodies. These subjective assays are being increasingly replaced by objective enzyme-linked immunosorbent assays (ELISA) produced with purified recombinant EBV antigens.[16–20] Because NPC patients sustain high levels of serum EBV DNA, it has been suggested that DNA-based assays may also be useful for serologic diagnosis of the cancer.[10] There have been few studies to evaluate these methods under defined clinical settings, however. Consequently, serology had not impacted on diagnostic work-ups of NPC to the extent it deserves. To make up for this deficiency, we evaluated performance of 5 antibody-based tests and a DNA-based test. These included the traditional IF assays, VCA IgA and EA IgA, the ELISA specific for EBNA 1 IgA,[21] zta IgG[22] and a PCR assay for the determination of EBV DNA. To minimize sampling bias, serum samples were taken from symptomatic NPC patients consecutively presenting in a public hospital in Hong Kong, and the city has a high incidence of NPC. The results show that only a combination of the IF tests or ELISA can meet both requirements of serologic diagnosis of NPC, that is, to exclude, as well as predict, a diagnosis of the cancer.

MATERIAL AND METHODS

A total of 251 consecutive serum samples were received at the Department of Microbiology, Queen Mary Hospital, Hong Kong, for serologic diagnosis of suspected NPC between January and August 1999. Of these, 218 were the first samples taken on presentation; the remaining 33 were excluded, being repeated samples from the same patients. Fifty-one patients were subsequently diagnosed as having histologically confirmed NPC, 4 had lymphoepithelioma like cancer (LELC) of the lung, 23 had other non-LELC cancers and 140 did not have a tumor. Serum EBV antibody levels were determined by the IF assays, VCA IgA and EA IgA, and by ELISA specific for EBNA 1 IgA or for zta IgG (Sinoclone Co., Hong Kong, China). The ELISA were produced with purified recombinant peptides specified by EBV BKRF1 (EBNA 1) or BZLF1 (zta). Serum EBV DNA was determined by quantitative PCR (Q-PCR) in a LightCycler (Roche Diagnostics GmbH, Germany).

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Levels of VCA IgA and EA IgA were determined by titration. Cutoff values were set at 1:40 for VCA IgA and 1:10 for EA IgA. These values were set against panels of control sera from NPC patients and healthy subjects to optimally separate the 2 groups of control subjects. A positive reaction was indicated when antibody titer was equal to or exceeded the corresponding cutoff titers. Levels of EBNA 1 IgA and zta IgG were determined according to manufacturer instructions. A positive reaction is indicated when the OD value of test samples was equal to or exceeded the optical density (OD) value obtained concurrently with a reference sera. The reference sera had been calibrated against panels of sera from NPC patients and healthy donors to yield OD values that optimally separate the 2 groups of test subjects according to previously described method.[21]

DNA was extracted from 400 μl serum (V_s) using QIAamp blood kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was resuspended in 50 μl of elution buffer (V_{DNA}). Of the sample DNA extract (V_{ext}), 3 μl was added to 20 μl of LC-PCR master mix containing 1X FastStart DNA Master Hybridization Probes (Roche Diagnostics GmbH, Germany); 3.5 mM $MgCl_2$ and 0.5 μM of each of the EBV primers, forward (5′ TTCTGCTAAGCCCAACACTC 3′) and reverse (5′ CTGAAGGTGAACCGCTTA 3′) primer, were used for amplification. Cycling conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 55°C for 10 sec and 72°C for 10 sec, with ramp rates of 20°C/sec. Amplification product was monitored in real time using a pair of fluorescence labeled probes, EBV probe 1 (5′ CCAAGAACCCAGACGAGTCCGTA- FLUOR) and EBV probe 2 (5′ LC-Red 640-AAGGGTCCTCGTCCAGCAAGAAG-phosphate 3′). A cloned Bam HI W fragment of the EBV genome[23] was used as a reference standard, serially diluted to contain 10^1 to 10^5 copies and included in each test run. The limit of detection was 10 copies of the cloned EBV gene. Limiting concentration required for detection was calculated according to $[10 \times (V_{DNA} / V_s \times V_{ext})]$ and was estimated to be 416 genome copies per milliliter.

Sensitivity, specificity, positive predictive value and negative predictive value were calculated as follows: Positive prediction = [TP / (TP + FP)] × 100%; negative prediction = [TN / (TN + FN)] × 100%, sensitivity = [TP / (TP + FN)] × 100%; and, specificity = [TN / (TN + FP)] × 100%, where TP and FN are number of NPC patients giving a positive and negative reaction, respectively. FP and TN represent the number of non-NPC patients giving a positive and negative reaction, respectively.

RESULTS

Table I compares performance of EBV-specific antibody-based and DNA-based assays in serologic diagnosis of NPC. Serum samples were taken from 218 patients presenting consecutively with symptomatic NPC. Patients were divided into 2 groups, the NPC group comprised 51 NPC patients and 4 patients with EBV-associated LELC of the lung. The non-NPC group comprised 23 patients with other cancers; 140 patients with other diseases constituted the control non-NPC group. Serum antibody levels were determined by the IF assays specific for VCA IgA and EA IgA and ELISA's specific for EBNA 1 IgA and zta IgG. Serum EBV DNA level was determined by LightCycler Q-PCR. Limiting concentration required for detection at 95% confidence level by this method was previously determined to be approximately 400 genomes per milliliter. Apart from specificity and sensitivity, performance of the assays was assessed by their capacity to predict and exclude a diagnosis of the cancer. VCA IgA was the most sensitive of the assays and a negative test result was associated with a high negative predictive value of 96.1%. However, the assay was the least specific, and a positive test result by this assay was associated with a low positive predictive value of 44%. On the other hand, EBV DNA assay was the most specific of the assays, and a positive test result was associated with a positive predictive value of 91.2%. EA IgA has comparable specificity and was similarly predictive of the cancer as the DNA-based assay. However, neither assay was sufficiently sensitive for them to be also useful as screening tests. Sensitivity of the ELISA was variously lower than VCA IgA, and specificity of these assays was lower than the DNA-based assay and EA IgA.

It was concluded from the above that none of the tests can be used reliably to both exclude and predict a diagnosis of NPC. The difficulty was partly overcome by combining the 2 IF tests. Table II shows that 61.5% of the patients gave a confirmed test results of being either positive or negative by both tests. A negative test result by both tests had the same high negative predictive value as afforded by VCA, and a positive result by both tests had the same high positive prediction as afforded by EA IgA. Thus, by combining the features of the 2 tests, a confirmed test result provides a clear indication to proceed with diagnostic work-up of NPC or to exclude a diagnosis of the cancer. The remaining patients yielded equivocal results of being positive for VCA IgA only, and this was associated with a low positive predictive value of 14.7%.

The same can be achieved also by combining EBNA1 IgA with zta IgG (Table II) or zta IgA (not shown). 68.3% of the patients gave a confirmed test result, which was associated with either a negative predictive value of 99.1% or a positive predictive value of 86.8%. The remaining samples yielded equivocal results, being positive for EBNA 1 IgA or zta IgG associating with positive predictive values of 43.3% or 24.2%. The performance achieved by thus combining the 2 ELISA markedly surpassed that by the either of these tests individually.

Table III compares serologic diagnosis by the combination of the ELISA or the IF assays. Of a total of 155 confirmed test results obtained by the combination of ELISA, 100 (64.5%) also yielded the same confirmed test results by the combination of the IF tests,

TABLE I – SEROLOGICAL DIAGNOSIS OF NPC BY INDIVIDUAL ANTIBODY BASED AND DNA BASED

Assays	NPC[4] positive (sensitivity)[8]	NPC[4] negative	Non-NPC[5] negative (specificity)[9]	Non-NPC[5]positive	Positive prediction[6]	Negative prediction[7]
VCA lgA[1]	51 (92.7%)	4	98 (60.1%)	65	44.0%	96.1%
EA lgA[1]	40 (72.7%)	15	158 (96.9%)	5	88.9%	91.3%
EBNA1 lgA[2]	46 (83.6%)	9	141 (86.5%)	22	67.6%	94.0%
zta lgG[2]	41 (74.5%)	14	135 (82.8%)	28	59.4%	90.6%
EB DNA[3]	31 (56.4%)	24	160 (98.2%)	3	91.2%	87.0%

[1]Cutoff titers for VCA IgA = 1:40, and EA IgA = 1:10.–[2] ELISA's include reference sera had been calibrated to yield the respective optimal cutoff OD values and they were tested concurrently with test samples. A positive reaction is when antibody level of test sample exceeded that of the cutoff OD and negative reaction, when level of test sample is the same or less than the cut off values.–[3] EBV DNA was determined by Q-PCR system. A standard containing 10^1 to 10^5 copies of Bam HI W fragment of the EBV genome clone was included in each test run.–[4] NPC group included 51 patients having confirmed NPC and 4 with EBV related lung cancer.–[5] Non-NPC group included 23 patients with other cancers and 140 patients with other diseases symptomatically similar as NPC.–[6] Positive prediction = TP / (TP + FP) × 100%.–[7] Negative prediction = TN / (TN + FN) × 100%.– [8] Sensitivity = TP / (TP + FN) × 100%.–[9] Specificity = TN / (TN + FP) × 100%.– TP and FN refer to patients in the NPC group tested positive and negative, respectively.–TN and FP refer to patients in the non-NPC group tested to be negative and positive, respectively.

TABLE II – SEROLOGICAL DIAGNOSIS OF NPC BY COMBINATION OF IMMUNOFLUORESCENT ASSAYS OR ELISA

		NPC	Non-NPC	Total	Prediction
VCA IgA	EA IgA				
[+]	[+]	40	5	45	[+]88.9%
[−]	[−]	4	94	98	[−]95.9%
Subtotal				143 (61.5%)	
[+]	[−]	11	64	75	[+]14.7%
[−]	[+]	0	0	0	
Subtotal				75 (38.5%)	
Total				218	
EBNA 1 IgA	zta IgG				
[+]	[+]	33	5	38	[+]86.8%
[−]	[−]	1	116	117	[−]99.1%
Subtotal				155 (68.3%)	
[+]	[−]	13	17	30	[+]43.3%
[−]	[+]	8	25	33	[+]24.2%
Subtotal				63 (31.7%)	
Total				218	

Positive prediction [+]. – Negative prediction [−].

TABLE III – CORRELATION BETWEEN SEROLOGICAL DIAGNOSIS OF NPC BY ELISA'S AND IMMUNOFLUORESCENT ASSAYS

Diagnosis by IF	Definitive diagnosis by ELISA	Per cent
Concordant	100	64.5
Equivocal	51	32.9
Discordant	4	2.6
Total	155	

Definitive diagnosis = positive or negative by both tests.

32.9% gave an equivocal result, and less than 3% gave a discordant finding by the combined IF tests.

DISCUSSION

Apart from intrinsic sensitivity and specificity, we have determined predictive values of 4 EBV-specific antibody-based assays and a DNA-based assay in serologic diagnosis of suspected NPC. To minimize sampling bias, test samples were taken from suspected NPC patients consecutively presenting at a public hospital in Hong Kong, about 20% of whom were subsequently diagnosed as having confirmed NPC; the rest had other diseases with similar symptoms to NPC. Prevalence of the cancer is probably similar for suspected NPC patients presenting in other centers in China and Southeast Asia, where NPC is common. Objectives of serologic diagnosis under such clinical setting, therefore, are to provide a clear indication at an early stage in the diagnostic work-up as to whether to proceed with the diagnostic work-up for NPC or pursue other courses of investigation. The results showed that none of the tests individually could meet both requirements adequately. The traditional VCA IgA assay is the most sensitive of the assays and it is also the most suitable as a screening test to exclude a diagnosis of NPC, having a negative predictive value of 96.1%. The DNA-based test and EA IgA were the most specific, and hence the most predictive of the assays, whereby a positive test result by either test is a clear indication to proceed with diagnostic work-up of NPC. However, neither test was sufficiently sensitive to serve as a screening test also. The PCR method used by Lo *et al.*[10] appears to be more sensitive than those used by other investigators,[9,11–13] including the one used in the present study, but performance of the test in serodiagnosis of NPC has not been evaluated under defined clinical settings. EBNA 1 IgA is moderately predictive of NPC and it also affords a high negative predictive value second only to VCA IgA.

In routine practice, the inadequacy was overcome by using the 2 IF tests in tandem, in which VCA IgA is used as a screening test to exclude a diagnosis of NPC. A positive test result is then subject to confirmation by EA IgA. We confirmed that this practice combines the high negative predictive value afforded by VCA IgA and the high positive predictive value afforded by EA IgA to provide a reliable diagnosis for 61.5% of symptomatic NPC patients. We showed that a positive test result by both tests is a clear indication to proceed with diagnostic work-up for NPC and a negative test result by VCA IgA provides a reliable exclusion of the cancer. The remaining patients gave an equivocal test result, being positive for VCA IgA only, which was associated with a low positive predictive value that does not significantly impact on diagnostic work-up of the cancer. Reliable serodiagnosis was also achieved by combining EBNA1 IgA with zta IgA, and because antigenic specificity of the 2 ELISA is distinct,[5] the 2 tests were found to complement one another to achieve a performance that markedly exceeded that by either ELISA individually. We showed that 68.3% of the patients gave a confirmed test result that was associated with a positive predictive value of 86.8% or a negative predictive value of 99.1%. We further showed that there was a high degree of concordance between the 2 combined approaches using the IF assays or ELISA.

In conclusion, our results showed that none of the antibody-based or DNA-based assays individually could meet both requirements of serologic diagnosis, which are to exclude as well as predict a diagnosis of NPC. The inadequacy was partly overcome by using a combination of 2 IF tests or 2 ELISA. It was shown that, for about two-thirds of the patients, a negative test finding by both tests simultaneously is a clear indication to exclude a diagnosis of the cancer, and a positive finding by both tests is a clear indication to proceed with diagnostic work-up of the cancer. The combination of ELISA is the preferred approach, because of its objectivity and convenience.

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