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Serum Antibody Response to Lytic and Latent Epstein-Barr Virus Antigens in Undifferentiated Nasopharyngeal Carcinoma Patients from an Area of Nonendemicity▽

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Epstein-Barr virus (EBV)-associated *u*ndifferentiated *c*arcinoma of the *n*asopharyngeal *t*ype (UCNT) is highly prevalent in southeast China, where immunoglobulin A (IgA) antibodies to viral capsid antigen and early antigen (EA) represent important markers, routinely used to assist in diagnosing this malignancy. Our study aimed at determining the EBV serological profiles of 78 UCNT patients from Italy, an area of nonendemicity for this tumor, using different assays specific for both lytic and latent EBV antigens. Serum IgA against both EA and EBNA1 and IgG and IgA to the latent membrane protein 1 (LMP1), to EA, and to the EBV transactivator ZEBRA protein were assessed. These serological responses were then evaluated according to the clinicopathologic parameters at diagnosis. The sensitivities of the IgG assays were 37.7% for LMP1, 73.6% for EA, and 61.0% for ZEBRA. EA/EBNA1 IgA reactivity was 84.4%, and a high association (odds ratio [OR], 2.6; 95% confidence interval [CI], 1.7 to 4.0) with UCNT was observed. When EBV serological reactivities were analyzed according to the tumor, node, and metastasis staging system (TNM), a statistically significant association was found between N stage and IgG antibody rates for EA (OR, 3.6; 95% CI, 1.2 to 10.9) and ZEBRA (OR, 2.6; 95% CI, 1.2 to 5.5) and between M stage and IgG antibody rates for ZEBRA (OR, 7.1; 95% CI, 3.2 to 16.0) and LMP1 (OR, 14.0; 95% CI, 1.8 to 110.9). Our results show that no single serological marker allows the detection of all UCNT cases. EA/EBNA1 IgA represents a reliable marker for diagnosis, with a high predictive value also in areas where UCNT is not endemic, such as Italy. The analysis of serological results according to TNM classification is consistent with a progressive impairment of humoral immune response to EBV as the disease advances and may be used to improve the accuracy of diagnosis.

The *u*ndifferentiated *c*arcinoma of the *n*asopharyngheal *t*ype (UCNT) is the most frequent histological entity of nasopharyngeal carcinoma (NPC), and tumor cells from this type of cancer regularly harbor Epstein-Barr virus (EBV). Although viral protein expression in UCNT cells is largely restricted to EBNA1, latent membrane protein 1 (LMP1), and LMP2A (16), transcription of a large variety of viral genes, including those associated with EBV replication, was also detected (5, 43, 48). The clinical onset of this malignancy is associated with the occurrence of high titers of immunoglobulin A (IgA) antibodies, mostly to proteins involved in the productive cycle of EBV (3, 7, 11). Several lines of evidence indicate that the detection of humoral responses against various EBV antigens may be useful for both diagnostic and prognostic purposes and also for screening of NPC in geographic areas where this tumor is endemic (30, 47). However, the EBV antibody profile of UCNT patients from Western countries, where the tumor incidence is low and the genetic background different, has been poorly investigated (11). Furthermore, molecular definition of UCNT-specific markers for IgG and IgA responses is important for an accurate serodiagnosis of UCNT in areas of both endemicity and nonendemicity. In recent years, a number of candidate molecules have been proposed, which can be obtained from natural EBV-producer cell lines or recombinant sources or made by peptide synthesis (6, 9, 13, 14, 19, 22, 24, 26, 27, 41, 42, 45).

IgA EA/EBNA1 is a new-generation enzyme-linked immunosorbent assay (ELISA) based on the combined use of recombinant lytic and latent viral antigens, which showed a good analytical performance of sensitivity and specificity in NPC patients from areas of endemicity (8).

LMP1, a 63-kDa transmembrane protein considered the most important EBV-transforming protein, is one of the few EBV antigens expressed in UCNT and constitutes a target for both humoral and cellular immune responses (21, 31). Antibody reactivity to LMP1 has been described in patients with different EBV-related disorders by using a variety of techniques (4, 29, 32, 37), but only a few studies have investigated the anti-LMP1 antibody response in UCNT patients (26, 44).

The BamHI-Z-encoded EBV replication activator (ZEBRA) is a key mediator of the switch from latency to productive cycle. Antibodies against ZEBRA are a marker of EBV reactivation and are regularly found in UCNT patients (65 to 87%), but only rarely among healthy people (19, 20, 25, 39).

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TABLE 1. Characteristics of UCNT study patients[a]

Clinicopathologic feature	Classification	No. (%) of patients
Gender	Male	58 (74.4)
	Female	20 (25.6)
Age (yr)	<50	38 (48.7)
	>50	40 (51.3)
Tumor size	T1	13 (16.7)
	T2a	8 (10.2)
	T2b	27 (34.6)
	T2x	2 (2.6)
	T3	15 (19.2)
	T4	13 (16.7)
Nodal stage[b]	N0	19 (24.4)
	N1	25 (32.0)
	N2	15 (19.2)
	N3a	3 (3.8)
	N3b	3 (3.8)
	N3x	12 (15.4)
Metastasis[b]	M0	67 (85.9)
	M1	10 (12.8)
Overall stage	I	5 (6.4)
	II	22 (28.2)
	III	19 (24.4)
	IVa	9 (11.5)
	IVb	14 (18.0)
	IVc	9 (11.5)

[a] Tumor, node, and metastasis and overall stage classifications were based on the International Union Against Cancer (UICC) classification system.
[b] The sum does not add up to the total because of a few missing values.

Our study was aimed mainly at evaluating the IgA EA/EBNA1 ELISA, IgG and IgA LMP1 Western blotting, and the IgA and IgG ZEBRA ELISA as new serological assays of possible clinical value for the diagnosis and monitoring of UCNT in patients from Italy, an area of nonendemicity. To this end, we have assessed the analytical performance of these three different serological assays and investigated whether the types and extents of serological responses against the different EBV markers correlated with the clinical stage at diagnosis.

MATERIALS AND METHODS

Patients and samples. In this study, 78 consecutive patients (native and resident in Italy) with histologically confirmed UCNT were enrolled between 1994 and 2005, prior to initial therapy. All patients were evaluated for their extent of disease by physical examination and fiberoptic nasopharyngoscopy; complete blood cell count; blood chemistry; computed tomography scan and/or magnetic resonance imaging scan of the nasopharynx, base of the skull, and neck; chest radiography; computed tomography scan of the abdomen; and bone scan. The medical records were reviewed, and all patients were restaged according to the 2002 edition of the Union International Contre le Cancer (UICC)/tumor, node, and metastasis (TNM) classification system for malignant tumors. Patient- and tumor-related characteristics are reported in Table 1. As a control group, 54 healthy donors matched by sex and age were selected from our blood bank and evaluated. Blood samples from healthy donors and UCNT patients were collected. Serum samples, together with plasma and peripheral blood mononuclear cell samples, were stored at −80°C for further laboratory analyses. The study was approved by our institution and informed consent was obtained from all study participants. EBV-specific antibody responses and EBV DNA viral loads were routinely assessed in serum samples before the commencement of oncologic therapy. The titers of IgG to the EBV EA and EBNA1 and titers of IgM to the viral capsid antigens (VCA) were measured with recombinant EA IgG, EBNA1 IgG, and VCA IgM ELISA kits (Biotest, Frankfurt, Germany), respectively, following the manufacturer's instructions and always using the appropriate internal controls. Serum EBV DNA was measured by real-time TaqMan PCR as previously described (1). All the other serological analyses described below were performed on aliquots of the stored frozen serum samples thawed before each experiment.

EA/EBNA1 IgA ELISA. The Bio-check EBV EA+EBNA-1 ELISA kit (Meditech Biotechnology Co., Ltd., Taipei, Taiwan) is an ELISA intended for the quantitative detection of human serum IgA antibodies. The measurement procedure was performed according to the recommendations of the manufacturer. This kit is a new-generation product, based on recombinant lytic and latent viral antigens, with reported sensitivity and specificity greater than 95% as calculated among assays of NPC patients (8). The IgA antibody titer was quantified by drawing a curve with the three included standards (8, 32, and 128 EBV units [EU]/ml) and expressed as EU/ml.

All the sample aliquots from patients and controls were thawed and run on the same day, and each plate, identically handled, included standards and controls from the kit.

LMP1-based Western blotting. LMP1 was expressed in the EBV-genome-negative human B-cell line BJA-B, derived from Burkitt's lymphoma (28), and the transfected cells were a gift from J. Menezes (44). The selected plasmid pIgLMP1-carrying cells and, in parallel, the cells carrying the control plasmid (pIgNeo), obtained from pIgLMP1 after deletion of the LMP1 sequences, were maintained in the selection medium, RPMI 1640 containing 10% fetal calf serum (GIBCO, Invitrogen, Carlsbad, California) and 1 mg/ml of Geneticin (Sigma-Aldrich, St. Louis, MO). The cell lysates were then used to detect anti-LMP1 serum antibodies. For Western blotting, 5×10^6 cells were lysed in 50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, and sonicated three times for 10 to 15 s. The lysates were then clarified by centrifugation (14,000 × *g* for 30 min at 4°C) and their protein concentrations were determined with a commercial kit (Bio-Rad Laboratories, Hercules, CA). The protein samples (50 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and thereafter electrophoretically transferred to nitrocellulose sheets (Schleicher & Shuell, Keene, NH). After the transfer, the nitrocellulose sheets were cut into strips and blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) (Milk-PBS), for 1 h at room temperature. Human sera (diluted 1:50 in 2% Milk-PBS) were then incubated with the strips overnight at 4°C with shaking. The strips were washed five times with 5% Milk-PBS and finally incubated for 30 min with goat anti-human IgG horseradish peroxidase (HRP) or IgA HRP conjugate (Dako, Glostrup, Denmark) diluted 1/1,000 in 2% Milk-PBS. After three washes with 2% Milk-PBS and several washes with PBS–0.5% Tween 20, bound enzyme-labeled antibodies were detected using enhanced chemiluminescence Western blotting detection reagent (Amersham, Piscataway, NJ) according to the manufacturer's instructions.

In each batch analyzed, serum samples of the patients were run together with one negative control (serum from a person negative for EBV) and one positive control (pool of five serum samples from five LMP1-positive UCNT patients), as references for either the LMP1 location or the intensity of the reaction. For each patient, serum IgG and IgA were both evaluated. In Fig. 1, an example of the LMP1 Western blot is shown.

ZEBRA peptide-based ELISA. Half-area ELISA plates were coated with ZEBRA peptide corresponding to the major serologically reactive epitope in the ZEBRA protein (39) at 20 μg/ml, in 0.1 M Tris HCl, pH 8.8 (Costar, Cambridge, MA) overnight at room temperature. Sera were tested by an indirect two-step ELISA with a mouse monoclonal antibody against human IgG or IgA (1:5,000; Sigma Aldrich, St. Louis, MO) and an HRP-conjugated goat antibody to mouse IgG (1:2,000; Southern Biotechnology, Birmingham, AL), as previously described in detail (39). The cutoff level for the determination of positivity was calculated as the mean plus four standard deviations of the reactivity of 10 EBV VCA-negative serum samples. All the samples from patients and controls were thawed and run on the same day, and each plate included internal positive and negative EBV ZEBRA controls.

Statistical analysis. The Spearman correlation coefficient was used to analyze the correlation between IgA serum reactivity and EBV DNA viral load. Odds ratios (OR) and their corresponding 95% confidence intervals (CI) were obtained by unconditional multiple logistic regression models (2). The dependent variables were T stage (1, 2a versus 2b, 3, 4), N stage (0, 1 versus 2, 3) and M stage (0 versus 1). EA IgG, ZEBRA IgG, LMP1 IgG, and IgA were entered in the model, separated into two levels (negative or positive values). EBNA1/EA IgA and EBV DNA were entered as continuous variables, with their units set to 5 EU/ml and 500 copies/ml, respectively. The statistical calculations were performed using the SAS language program (reference version 9.13; SAS Institute Inc., Cary, NC). To verify the sample

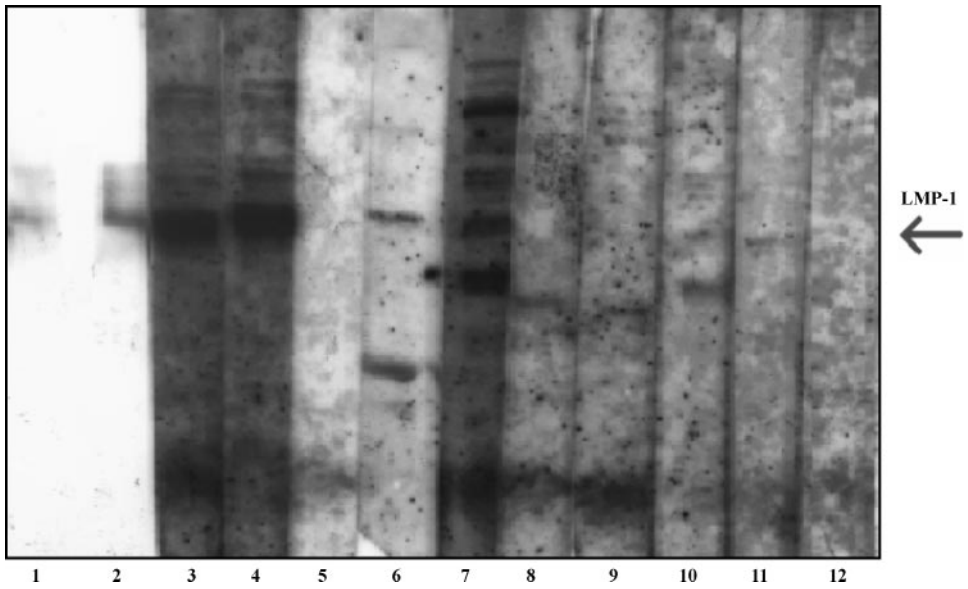

FIG. 1. Western blot analysis for detection of anti-LMP1 antibodies. The sera used in developing these strips (as detailed in Materials and Methods) were: five positive controls (serum pool from patients previously selected as LPM1 positive) (lanes 3, 4, and 7); one negative control (serum from healthy EBV seronegative person) (lane 5); and six sera from UCNT patients—three positive (lanes 6, 10, and 11) and three negative (lanes 8, 9, and 12) for LMP1 antibodies. Lanes 1 and 2 show positive controls in which monoclonal antibody s12 was used; the arrow shows the location of LMP1.

quality throughout the time frame of collection (1994 to 2005), all the serological data were statistically reanalyzed after our samples were divided into two categories: those collected before and after the year 2000.

RESULTS

IgA against recombinant EA/EBNA1 antigens. Sixty-eight out of 77 patients and 16/54 healthy donors were EA/EBNA1 IgA reactive according to the cutoff established by the manufacturer, with a sensitivity and specificity of 88.3% and 70.4%, respectively. However, in order to limit possible biases due to the heterogeneity of our patient population and to obtain the best discrimination between healthy carriers and UCNT patients, we calculated the optimal cutoff value by using the *r*eceiver *o*perating *c*haracteristic (ROC)

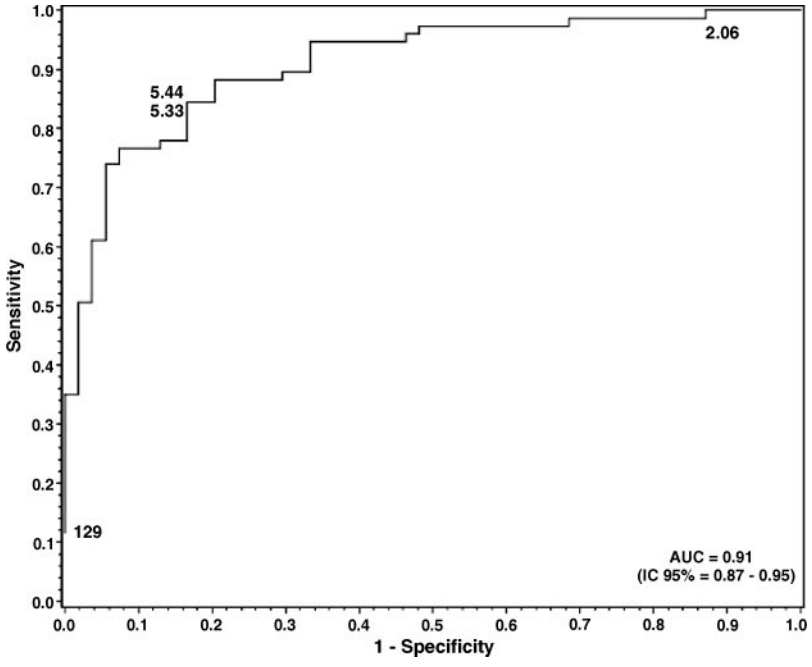

FIG. 2. ROC curve of IgA EA/EBNA1 ELISA results. The ROC curve plot was made by using the data from healthy donors ($n = 54$) and UCNT patients ($n = 78$). AUC, area under the ROC curve.

TABLE 2. Results of statistical analyses for UCNT patients according to EA/EBNA1 IgA serum titer

No. (%) of UCNT patients	No. (%) of healthy donors	EA/EBNA IgA serum titer (EU/ml)	OR (95% CI)[a]
18 (23.4)	50 (92.6)	<8	1[b]
30 (39.0)	3 (5.6)	8–31	28.6 (7.6–108.4)
29 (37.6)	1 (1.8)	≥32	78.8 (9.9–625.7)

[a] The estimates are from sex- and age-adjusted multiple logistic regression models. As the EBNA1/EA IgA titer increased (by 5-EU/ml increments), a statistically positive ($P = 0.001$) association with UCNT emerged (OR = 2.56; 95% CI, 1.65–3.95).
[b] Reference category.

curve, as shown in Fig. 2. The optimized cutoff was 5.4 EU/ml, with a sensitivity and specificity of 84.4% and 83.3%, respectively. The assay accuracy, as defined by the area under the ROC curve, was 0.91 (95% CI, 0.87 to 0.95).

Globally, UCNT patients of the present series showed high IgA antibody titers. We classified all the IgA data from patients and controls into three groups, according to the three different intervals defined by the standards (<8, 8 to 31, and ≥32 EU/ml): 23.4% of the patients had undetectable IgA (versus 92.6% of the healthy donors), 39.0% had IgA levels between 8 and 31 EU/ml (versus 5.6% of the healthy donors), and 37.6% were IgA reactive, with ≥32 EU/ml (versus 1.8% of the healthy donors). A statistically significant positive association emerged between increases of 5 EU/ml of the IgA titer and UCNT cases (OR, 2.6; 95% CI, 1.7 to 4.0) ($P < 0.001$) (Table 2).

Fifty-four out of 78 (69.2%) UCNT patients had detectable viral loads, with a median serum EBV DNA value of 5,307 copies/ml (ranging from 128 to 162,756 copies/ml). All the healthy donors had undetectable EBV viremia. No association between IgA serum reactivity and EBV DNA levels was found (Spearman rank correlation coefficient, 0.07; $P = 0.57$).

IgG and IgA against ZEBRA peptide. Forty-seven out of 77 (61.0%) patients and only 3 out of 54 (5.6%) healthy controls were IgG ZEBRA serum reactive. No detectable IgA reactivity to ZEBRA was observed in UCNT patients or controls.

IgG and IgA against LMP1. IgG and IgA serum reactivities against LMP1 were evaluated by Western blot analysis of the whole series of serum samples ($n = 78$) from UCNT patients and of 9 serum samples from the healthy controls. Twenty-nine out of 78 (37.7%) patients were found to be IgG seropositive and 25/78 (32.1%) were IgA seropositive. Ten (12.8%) patients showed both IgG and IgA LMP1 seroreactivity. No IgG or IgA serum reactivity specific for LMP1 was detected among the controls.

EBV seroreactivity and tumor stages. UCNT patients were classified according to the TNM stage of disease as detailed in Table 1. The OR and 95% CI were calculated to study the associations between serological reactivity against the different viral antigens and the clinical TNM (Table 3). When UCNT patients were classified according to the lymph node status (N0, 1 versus 2, 3), the EA and ZEBRA IgG reactivities were, respectively, 3.6-fold (95% CI, 1.2 to 10.9) and 2.6-fold (95% CI, 1.7 to 4.0) greater among patients at earlier stages of malignancy (N0, 1).

With respect to the absence/presence of distant metastases at presentation, the ZEBRA and LMP1 IgG reactivities were 7-fold (95% CI, 3.5 to 16.0) and 14-fold (95% CI, 1.8 to 110.9) greater, respectively, for patients without distant metastasis (M0) than for patients from the M1 group.

TABLE 3. Results of statistical analyses among UCNT TNM stages according to serum reactivity against the different EBV antigens evaluated and the EBV DNA viral load

Antigen	OR (95% CI)[a]		
	T1 + T2a versus T2b + T3 + T4	N0 + N1 versus N2 + N3	M0 versus M1
EA IgG[b]	0.91 (0.28–3.01)	3.57 (1.17–10.91)	1.06 (0.25–4.47)
ZEBRA IgG[b]	1.59 (0.62–4.08)	2.60 (1.23–5.51)	7.09 (3.15–15.99)
LMP1 IgG[b]	0.73 (0.25–2.14)	1.11 (0.45–2.71)	14.00 (1.77–110.87)
LMP1 IgA[b]	0.40 (0.12–1.36)	0.61 (0.23–1.58)	0.51 (0.14–1.87)
EA/EBNA1 IgA[c]	1.05 (1.01–1.10)	1.09 (1.04–1.14)	1.11 (1.05–1.18)
EBV DNA[d]	0.96 (0.92–1.00)	0.99 (0.98–1.00)	1.00 (0.99–1.01)

[a] Estimates are from multiple logistic regression models, including terms for sex and age.
[b] Reference category, negative values.
[c] Increases are 5 EU/ml.
[d] Increases are 500 copies/ml.

Interestingly, the EA/EBNA1 IgA serum response, with the unit set to 5 EU/ml, could be significantly associated with all TNM stages (OR ranging from 1.05 to 1.11), although the association with a lower stage of malignancy was moderate.

In our UCNT patients, the serum EBV viral load was not significantly associated with the TNM stage.

Multivariate models, including adjustment for patients' sex and age, did not materially affect these results. No consistent heterogeneity was observed after the samples were divided into two time periods (1994 to 2000 and after 2000); thus, the age of the samples did not influence our results.

DISCUSSION

EBV-associated diseases are characterized by distinct antibody patterns to various EBV-determined antigen specificities (15). Patients with UCNT, in particular, usually have elevated titers against multiple viral antigens, particularly IgA antibodies against lytic antigens (i.e., EA and VCA), consistent with the tumor's origin from nasopharyngeal mucosa. A panel of serological tests focused on the use of defined recombinant EBV proteins, as well as different testing approaches, has been successfully used in high-risk populations, such as those of southern China, Hong Kong, and Taiwan, for screening purposes, to detect early relapse after therapy, and to better define prognosis. However, the serum EBV antibody profile of UCNT patients from areas of nonendemicity has only rarely been investigated (11, 44).

In this study, the serum reactivities to different EBV-encoded antigens expressed in different phases of virus infection (i.e., EBNA1 and LMP1 during latency, EA in the early phase, and ZEBRA in the immediate-early phase of virus replication) were examined in untreated Italian UCNT patients, followed at our institution.

In our series of UCNT patients, the new assay allowing the simultaneous detection of IgA to EA and EBNA1 antigens showed slightly lower sensitivity and specificity than the results reported in the Chinese study (8), although the predictive value for UCNT was also high in our patients. As for all

available EBV serologic tests, the use of this new assay for screening purposes in areas of nonendemicity, as opposed to high-risk populations, is probably not warranted, due to the relatively low incidence of UCNT and the lack of screening programs. On the other hand, this new assay may constitute a useful diagnostic support in patients with rhinopharyngeal lesions repeatedly showing aspecific inflammatory cellularity when biopsied. In these cases, the analysis of seroreactivity (particularly the IgA) to EBV antigens may help in the diagnostic process, avoiding unnecessary delays. Further studies are, however, required to firmly assess the role of these serological assays in the diagnostic work-up of UCNT patients.

Our analysis did not disclose any significant positive correlation of serum IgA antibody titers with serum EBV DNA loads, consistent with a recent study carried out with patients from Indonesia (36). These findings are, however, different from those observed during the follow-up of patients from regions of endemicity (34).

The ZEBRA protein, the master activator of EBV reactivation, was also employed for UCNT serological diagnosis by different authors (19, 20, 25), with sensitivities of 75 to 87% depending on the different assays used. Using a peptide-based ELISA, we found a high specificity (94.4%) and almost the same sensitivity (61%) observed in NPC sera from Chinese patients (63%) (39). In contrast, no IgA response could be detected, suggesting that IgG and IgA responses to this ZEBRA peptide are independent events.

With regard to EBV latent genes and considering, in particular, the relevance of LMP1 in the pathogenesis of EBV-associated tumors, we assessed IgG and IgA antibody responses against LMP1 by using a sensitive Western blot assay. Both IgG (37.7%) and IgA (32.1%) reactivities to LMP1 were observed. These results were similar to those previously reported with the same method by Xu et al. (44), although with slightly lower rates in our patients, and were different from the findings obtained by Meij et al. (26), who detected LMP1 reactivity in only a small fraction of NPC patients (7.5%) by using immunoblot and immunofluorescence assays based on recombinant protein.

To assess whether these EBV serological markers could be clinically informative in Italian UCNT patients, the IgG and IgA antibody responses were analyzed according to the TNM stage. IgG EA and ZEBRA reactivities were more prevalent in patients with earlier N stage (N0/N1) diagnoses, and IgG reactivities to ZEBRA and LMP1 were more frequently detected in patients without distant metastases at presentation (M0). These results are consistent with a progressive impairment of humoral immune response to EBV as the disease is diagnosed at an advanced stage. These alterations, however, are probably confined to the site of the tumor, since the numbers of circulating $CD4^+$ cells were not significantly different among UCNT patients with different TNM stages (data not shown). Moreover, we have previously reported that Italian UCNT patients had percentages and absolute numbers of $CD4^+$ memory ($CD45R0^+$), $CD4^+$ naive ($CD45RA^+/CD62L^+$), and activated $CD4^+$ ($HLA\text{-}DR^+$) lymphocytes similar to those of healthy donors (46). Intriguingly, the extent of the EA/EBNA1 IgA response seems to decrease as the tumor progresses; even low associations were computed. This could be due to the high local levels of cytokines, such as transforming growth factor β and interleukin-10, which suppress immune responses and concomitantly promote the isotypic switch to IgA in B cells present in the tumor microenvironment (18, 35). Other immunomodulatory cytokines and growth factors could also contribute to deregulating the immune response in UCNT patients, as recently emphasized (38). Elucidation of this issue will be relevant to understanding more thoroughly the complex pathogenesis of UCNT.

A limited correlation between the levels of EBV VCA IgA and the overall stage of the disease at presentation was reported (17), whereas high levels of serum anti-LMP1 IgA, detected by ELISA, correlated with more advanced stages of disease, with a clear trend of an increase in optical density values as the disease progressed (44). A recent study, based on the use of an immunoblot technique allowing side-by-side analysis of IgG and IgA reactivities against a wide spectrum of EBV proteins, reported that the antigen-recognition patterns of both IgG and IgA increased with the disease's stage, most significantly at stage 2 and later (11). Technical differences related to the serological assays employed, together with the different genetic backgrounds of the study populations and the different criteria for TNM classification, may at least partly explain these different results. In our serological evaluation, we chose to consider the T, N, and M stages separately, taking into account the prognostic correlates and the relapse pattern (33). This classification also considers separately three distinct sites that may have different microenvironments. The first group (T1) is an early stage of the disease; the second one (T2b to T4) includes tumors with an advanced T stage that may locally relapse; the N2 to N3 stages include later-stage tumors that can systematically relapse; and the M1 stage is characterized by low rates of survival.

In conclusion, the study of UCNT serum reactivity to multiple EBV antigens may be used to better define the clinical stage of the disease and improve the accuracy of diagnosis and prognosis, even in areas where UCNT is nonendemic, such as Italy. Our results provide additional support to available evidence indicating that no single serological marker allows the detection of all UCNT cases, the combined use of different markers being therefore essential for the early identification of the disease (10, 12, 23, 40). Our findings also show that EBV serum reactivities may correlate with clinicopathologic features of UCNT when the data are analyzed according to the disease stage. Further studies should be undertaken to assess the possible clinical value of these serological markers during the follow-up of UCNT patients from areas of nonendemicity.

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