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Clinical Research Paper

Combined determination of Epstein-Barr virus-related antibodiesand antigens for diagnosis of nasopharyngeal carcinoma

Yao-Ling Luo,[1,2,*] Guo-Ping Ou,[1,3] Pei-Dong Chi,[1,2] Yong-Neng Liang,[1,2] Yu-Hui Liu[1,2] and Ma-Yan Huang[1,4]

[1]State Key Laboratory of Oncology in South China; Guangzhou; Guangdong P.R. China; [2]Department of Laboratory; [3]Department of Nasopharyngeal Carcinoma; [4]Department of Pathology; Cancer Center; Sun Yat-sen University, Guangzhou, Guangdong P.R. China

Key words: nasopharyngeal neoplasm, diagnosis, EBV-DNase antibody, Epstein-Barr virus DNA, real-time fluorescence quantitative PCR

Background and Objective: With the development of molecular biology in recent years, many indexes for detecting Epstein-Barr virus (EBV) have been developed. This study was to evaluate the diagnostic value of combined determination of EBV-related antibodies and antigens, including VCA-IgA, EA-IgA, EBV-DNase antibody and EBV-DNA, in diagnosing nasopharyngeal carcinoma (NPC). Methods: Serum and plasma samples from 160 untreated NPC patients and 76 healthy donors were collected. VCA-IgA and EA-IgA in the serum samples were detected by immunoenzyme staining method. Raji cells were stimulated by ortho-butanoic acid and croton oil to detect EBV-DNase antibody. The content of EBV-DNA in the plasma samples was detected by real-time fluorescence quantitative polymerase chain reaction (RQ-PCR). The diagnostic values of the indexes for NPC were evaluated. Results: The sensitivity and specificity for diagnosing NPC were 90.0% and 89.5% for VCA-IgA, 75.0% and 94.7% for EA-IgA, 76.3% and 90.8% for EBV-DNase antibody, 68.8% and 88.2% for EBV-DNA, and 98.8% and 84.2% for combined determination. The positive rates of VCA-IgA and EA-IgA had no relationship with clinical stage of NPC ($p > 0.05$); nevertheless, the positive rates of EBV-DNase antibody and EBV-DNA were related with clinical stage ($p < 0.05$). Conclusions: The sensitivity of VCA-IgA and the specificity of EA-IgA are the highest while detecting solely. Combined determination could improve the diagnostic sensitivity and accuracy for NPC. EBV-DNase antibody and EBV-DNA could be helpful to evaluate the course of disease and classify the clinical stage of NPC.

The development of nasopharyngeal carcinoma (NPC) is closely associated with Epstein-Barr virus (EBV) infection.[1] Studies showed that anti-EBV antibodies and EBV-DNA often appear in the serum and plasma of most patients before clinical diagnosis of the disease.[2-5] With the development of molecular biology in recent years, especially the application of real-time fluorescence quantitative polymerase chain reaction (RQ-PCR) technology, more indexes for detecting EBV-related antibodies and antigens are available to be used as very important non-invasive detection markers for exploring the diagnosis and treatment of NPC. Single index detection has been reported widely,[3-9] however, both the sensitivity and accuracy in diagnosing NPC are unsatisfactory. Moreover, due to hidden lesions of NPC, missed diagnosis and misdiagnosis are often caused, and it is difficult to early diagnose this disease, hence, researchers are seeking an effective diagnostic approach to this disease. Few studies on the combined determination of EBV-related antibodies and antigens, including VCA-IgA, EA-IgA, EBV-DNase antibody and EBV-DNA, are seen. In the present study, combined determination of these four indexes was conducted to evaluate its value in the diagnosis, disease course assessment and clinical staging of NPC.

*Correspondence to: Yao-Ling Luo; State Key Laboratory of Oncology in South China; and Department of Laboratory; Cancer Center; Sun Yat-sen University, Guangzhou, Guangdong 510060 P.R. China; Tel.: 86.20.87343438; Fax: 86.20.87343198; Email: luoyaolingluo@126.com

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Materials and Methods

Serum (plasma) samples. A total of 160 patients with pathologically confirmed NPC, treated at the Sun Yat-sen University Cancer Center from May 2006 to December 2007, including 116 men and 44 women, were enrolled. The median age of the patients was 49 years (range, 20–78 years). Based on the WHO classification, 149 patients had undifferentiated non-keratinizing NPC, five had differentiated non-keratinizing NPC, and six had poorly differentiated squamous cell carcinoma. According to the '92 Fuzhou staging system for NPC, eight patients had stage I disease, 27 had stage II disease, 72 had stage III disease, and 53 had stage IV disease. Venous blood samples were taken from the 160 patients before treatment and 76 healthy adults (control) undergoing physical examination, and centrifuged to separate serum and plasma (EDTA-anticoagulated), which were then preserved in the refrigerator (at 2–8°C).

Major reagents and instruments. VCA-IgA and EA-IgA detection kits were purchased from Zhongshan Bio-tech Co., Ltd (Product No. TV021). DNA extraction kit was purchased from TIANGEN Biotech (Beijing) Co., Ltd (Catalog No. DP304). The primers and probes for EBV were purchased from Shanghai GeneCore Biotechnology Co., Ltd. Raji cell line was purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences of Chinese Academy of

Table 1 **Diagnostic values of the four Epstein-Barr virus (EBV) detection indexes for nasopharyngeal carcinoma (NPC)**

Index	Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)
VCA-IgA	90.0(144/160)	89.5(68/76)	89.8(212/236)	94.7(144/152)	81.0(68/ 84)
EA-IgA	75.0(120/160)	94.7(72/76)	81.4(192/236)	96.8(120/124)	64.3(72/112)
EBV-DNase antibody	76.3(122/160)	90.8(69/76)	80.9(191/236)	94.6(122/129)	64.5(69/107)
EBV-DNA	68.8(110/160)	88.2(67/76)	75.0(177/236)	92.4(110/119)	57.3(67/117)
Combination	98.8(158/160)	84.2(64/76)	94.1(222/236)	92.9(158/170)	97.0(64/ 66)

Table 2 **The positive rates of four EBV detection indexes and the median level of EBV-DNA in NPC patients at different clinical stages**

TNM stage	Cases	VCA-IgA (%)	EA-IgA (%)	EBV-DNase antibody (%)	EBV-DNA (%)	Median EBV-DNA level (copies/mL)
Ⅰ	8	87.5 (7/ 8)	50.0 (4/ 8)	12.5 (1/ 8)	25.0 (2/ 8)	1 460
Ⅱ	27	92.6(25/27)	66.7(18/27)	63.0(17/27)	48.1(13/27)	28 400
Ⅲ	72	87.5(63/72)	73.6(53/72)	79.2(57/72)	73.6(53/72)	79 350
Ⅳ	53	92.5(49/53)	84.9(45/53)	88.7(47/53)	79.2(42/53)	137 000

Sciences. The LS 6500 Liquid Scintillation Counter was purchased from Beckman Coulter Co., USA. The 7900 Real-Time PCR System and PCR product automatic analysis software SDS (version 2.0) was purchased from ABI Co., USA.

Detection methods. VCA-IgA and EA-IgA in serum samples were detected by immunoenzyme staining method according to the instructions. Serum samples were 2-fold diluted from 1:10 to 1:1280. When the percentage of positive cells reached over 15%, the dilution fold was regarded as positive antibody titer. VCA-IgA dilution fold of ≥ 1:40 and EA-IgA dilution fold of ≥ 1:10 were considered positive.

EBV-DNase antibody in serum samples was detected according to the method reported by Chen et al.[10] Raji cells were stimulated with ortho-butanoic acid and croton oil to obtain EBV-DNase antigen for detecting the level of EBV-DNase antibody, namely, antienzyme rate. The antienzyme rate was calculated using the following formula: antienzyme rate = (1 - CPM value of test serum sample) / CPM value of normal fresh serum sample) × 100%. An antienzyme rate of > 30% was considered positive.

Plasma EBV-DNA was determined by RQ-PCR. DNA was extracted using plasma DNA extraction kit and used as templates to amplify and detect the BamHI-W region (GenBank Accession No. V01555) of the EBV-DNA using the ABI7900 Real-Time PCR System. Positive templates were prepared by cloning the EBV BamHI-W fragment into a plasmid and serially diluted to 2.98×10^8, 2.98×10^7, 2.98×10^6, 2.98×10^5, 2.98×10^4, 2.98×10^3 and 2.98×10^2 copies/mL. The amplification of serially diluted positive templates was conducted simultaneously with that of samples to plot positive standard curve to quantify samples. Multiple negative controls and blank controls were run for each PCR amplification. PCR amplification results were automatically analyzed using the SDS software (version 2.0). Quantitative PCR results for plasma EBV-DNA were converted according to the following formula: $C = Q \times (V_{DNA} / V_{PCR}) \times (1 / V_{EXT})$, where C represents the concentration (copies/mL) of EBV-DNA in test plasma samples, Q represents the concentration of EBV-DNA obtained by PCR, V_{DNA} represents the final diluted volume of DNA extracted from plasma samples, V_{PCR} represents the volume of DNA added to the PCR reaction system, and V_{EXT} represents the volume of plasma used to extract DNA. DNA levels of $> 1 \times 10^3$ copies/mL were considered positive.

Statistical analysis. Statistical analysis was performed using the SPSS10.0 software package. The positive rates were compared using the Chi-square test while the means of multiple independent samples were compared using the Kruskal-Wallis test. $p < 0.05$ was considered as significance. The sensitivity and specificity were calculated according to the following formulas: sensitivity = positive cases in NPC group / total cases in NPC group × 100%; specificity = negative cases in control group / total cases in control group × 100%.

Results

Diagnostic values of four EBV-related indexes for NPC. In control group, VCA-IgA was detected in eight patients, EA-IgA in four patients, EBV-DNase antibody in seven patients, and EBV-DNA in nine patients; 12 patients had at least one index being detected. For single index determination, the sensitivity of VCA-IgA was the highest (90.0%) and the specificity of EA-IgA was the highest (94.7%) (Table 1). Combined determination raised the sensitivity and accuracy to 98.8% (158/160) and 94.1% (222/236), respectively, but had no significant impact on the specificity.

Comparison of combined determination of four EBV-related indexes in NPC patients at different clinical stages. As seen in Table 2, the differences in the positive rates of VCA-IgA and EA-IgA were not significant among the patients at different stages ($p > 0.05$); the positive rate of EBV-DNase antibody was significantly lower in the patients at stage I than in those at stages II, III and IV ($p < 0.05$), and lower in the patients at stage II than in those at stage IV ($p < 0.05$); the positive rate of EBV-DNA was significantly lower in the patients at stages I and II than in those at stages III and IV ($p < 0.01$). The more advanced the clinical stage was, the higher the EBV-DNA level (copies) was (Kruskal-Wallis test, $p = 0.005$).

Discussion

NPC is a malignant tumor commonly seen in southern Chinese population. Although the mechanism behind the development of this disease is still unclear, numerous studies indicate that EBV is closely associated with the development and progression of NPC. Observing

the antigen expression profile of activated EBV gene-harboring Raji cells, at least EA-D, EA-R, EBV-DNase, DNA polymerase and thymidine kinase are found. Since a variety of antibodies against the antigens of EBV appear in the serum of most NPC patients, serological diagnosis of NPC should be oriented to combined determination of multiple indexes.[2,3] In recent years, some studies [4,5] have shown that tumor-derived EBV-DNA is detectable in the plasma of NPC patients, and the content of EBV-DNA is associated with the severity, efficacy, recurrence and metastasis of NPC.

In the present study, EBV-related antigens and antibodies were simultaneously detected to evaluate the diagnostic value of combined determination of VCA-IgA, EA-IgA, EBV-DNase antibody and EBV-DNA for NPC. We found that the sensitivity of single determination of VCA-IgA, EA-IgA, EBV-DNase antibody and EBV-DNA was 90.0%, 75.0%, 76.3% and 68.8%, respectively, which are consistent with those reported previously.[3,6,7] The determination of VCA-IgA and EA-IgA had the highest sensitivity and specificity, respectively, which are consistent with the results reported by Zhang et al.[3] Combined determination of these four indexes raised the sensitivity and accuracy to 98.8% and 94.1%, respectively, and had no significant impact on the specificity. We believe that antibodies are formed against EBV-encoded antigens. EBV in NPC tissues can enter the blood circulation. EBV-DNA is detectable in the blood circulation, indicating that EBV-encoded antigens also appear in the serum. Theoretically, combined determination of the above-mentioned four indexes can simultaneously reflect the levels of EBV antibodies and antigens in the body, thereby, significantly improve the value of serological examination in screening and diagnosis of NPC. In this study, only two samples were negative for all four indexes, whereas the remaining samples were positive for at least one index. This result indicates that combined determination has the advantage of complementarity, which can improve its diagnostic value and is conductive to early detection of NPC. More strong vigilance is required in patients who are negative for first biopsy but positive for EBV detection. If necessary, multiple biopsies should be conducted to avoid misdiagnosis and missed diagnosis.

Clinical stage is a major factor affecting the prognosis of NPC. Though VCA-IgA and EA-IgA have been used for the screening and auxiliary diagnosis of NPC, no evidence indicates that they are reliable indexes for evaluating the clinical stage, therapeutic efficacy and recurrence of this disease. The main reason is that these two antibodies have a long half-life in human body and may maintain high titers even after EBV has been eliminated from the body for a long term. Our results showed that the positive rate of VCA-IgA had no significant differences among NPC patients at different stages, while the positive rate of EA-IgA among these patients was different but with no significance, fully corroborating the above-mentioned view. We found that the median plasma level and positive rate of EBV-DNA ($P < 0.01$) and the positive rate of EBV-DNase antibody ($p < 0.05$) were higher in NPC patients at more advanced stages. These results indicate that free EBV-DNA level in the plasma is matched with the clinical stage of NPC and can well reflect the clinical features of NPC. The plasma content of free EBV-DNA is positively correlated with tumor burden in NPC patients. Plasma EBV-DNA may be released by tumor cells into peripheral blood. Thus, with the progression of the illness, the content of plasma EBV-DNA will increase accordingly. The presence of EBV-DNase suggests that the virus is multiplying. The level of EBV-DNase in infected patients can directly reflect the activity of viral replication. With the enlargement of lesions and the extension of infiltrative area, the level of EBV-DNase is enhanced accordingly. We think that EBV-DNA and EBV-DNase antibody levels are of great significance for the clinical staging of NPC since they can well reflect tumor growth changes and are helpful to evaluate the progression of disease. Thus, it is expected that these indexes will be used as molecular markers for the staging of NPC.[6, 8]

In summary, we believe that combined determination of multiple indexes has the advantage of complementarity and can thereby improve the efficiency and accuracy of the diagnosis of NPC and reduce the occurrence of misdiagnosis and missed diagnosis. Since EBV-DNA and EBV-DNase antibody are sensitive and reliable indexes that can reflect tumor growth changes, they may be used to diagnose NPC, evaluate the progression of the disease and assist to assess TNM staging. It has been reported[9,11,12] that dynamic detection of these two indexes before and after treatment are of great significance for evaluating the therapeutic efficacy, prognosis, recurrence and metastasis of NPC, and may help clarify the underlying mechanisms of the development and progression of NPC.

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