Clinical Observations

SERO-EPIDEMIOLOGICAL STUDIES OF EPSTEIN-BARR VIRUS (EBV) INFECTION BY TESTING ANTIBODIES AGAINST EBV SPECIFIC DNASE (EDAb) AS A METHOD FOR EARLY DETECTION OF NASOPHARYNGEAL CARCINOMA (NPC)[*]

Huang Di 黄迪   Chen Haifeng 陈海峰   Cheng Luling 成昌玲
Fang Siming 方思明   Ma Jianshan 马建山   Zhang Jinming 张锦明
Chen Lizhen 陈丽珍   Fan Shunfa 范顺发   Li Jinglue 李经略

Sun Yet-Sen University of Medical Sciences, Cancer Center, Guangzhou 510060

In 1987, a mass survey of EDAb was carried out in Sihui county of Gunagdong Province (the highest incidence area of NPC in China) and the city of Guangzhou using the methods previously established by our lab. In order to study the correlation between EDAb level and NPC, the titre of DEAb and their distribution in sera from 430 patients with NPC and 5030 normal persons were detected. It was found that the AER (level of EDAb was represented by the anti-enzyme rate) of NPC patients gave a negative skew distribution and the Md=65.3% while the natural population (30–59 years old) gave a serious positive skew distribution and the Md is 7.9%. According to the pattern of EDAb distribution curve of 430 NPC patients and 2060 natural population. AER≥30% was defined as a cut-off point between EDAb positive and negative. Using this value we got a rate of 90.7% (390/430) positive diagnosis in NPC patients, while in natural population the positive rate was 3.3% (68/2060), and in the IgA/VCA positive (1:5) normal person the positive rate was 6.0% (41/681). As to the other tumors including head and neck tumors the positive rate was 3.4% (7/204), similar to the natural population. After a period of 62 months of follow-up surveillance, 15 of 224 EDAb positive normal persons found in the mass survey were diagnosed as NPC by histopathological examination. While in 4806 EDAb negative normal person only one case NPC patient was found in this period. 60% of these patients were diagnosed in their early stage of NPC. From these patients it was found that EDAb could appear in the sera of the patients as early as 62 months before NPC was definitely diagnosed. 2 of these patients were shown IgA/VCA negative and EDAb positive in mass survey. This suggested that in some of the precancerous patients EDAb could appear earlier than IgA/VCA. The signifi-cance of the sera EDAb positivity of normal person was discussed.

Key words: Nasopharyngeal carcinoma, Epstein-barr virus, DNase, Antibodies, Sero-epidemiology.

EBV is a common human herpesvirus, which is known to cause infectious mononucleosis (IM) and associate closely with NPC and Burkitts lymphoma (BL). In 1980, Cheng et al. reported that the frequency and levels of EDAb are elevated in patients with NPC.[1] The studies in the past decade have shown that the EDAb was a new serological tumor marker for

detecting NPC with high sensitivity and specificity.[2-6] However, further studies of EDAb in the natural population and its application to early detection of NPC were restricted due to various causes especially the incidence of NPC were high in the south China, while being otherwise quite rare in other parts of the world, hence NPC clinical and seroepidemiological data is very limited.

In 1987, sera were collected respectively from the Cancer Prevention Clinics of our Tumor Hospital in Guangzhou city and the countryside of Guangdong Province with high incidence of NPC.

The titers of EDAb we determined with the method developed in our laboratory.[7,8] The result of sero-epidemiological studies of EBV infection by testing EDAb of patients with NPC and healthy individuals were reported as follows.

MATERIALS AND METHODS

Sources of Sera

In the Cancer Prevention Clinics of Tumor Hospital, 1987 sera were collected from patients aged from 10 to 79 years, among them 430 cases were diagnosed pathologically NPC.

In addition, 50 sera from patients with head and neck tumors, 78 sera from patients with Esophageal Carcinoma and 76 sera from patients with other tumors were also collected as control groups.

In the region of Guangzhou, 2189 sera from VCA-IgA positive (>1:5) healthy individuals aged from 20 to 69 years.

In the Sihui county, 681 sera from VCA-IgA positive (>1:5) healthy individuals. 2060 sera from the natural population were collected, among the natural population, 190 cases were VCA-IgA positive and 68 cases were EDAb positive individuals were found, in which 12 persons were EDAb and VCA-IgA both positive.

After collection. All sera were kept at -30 ℃ freezer before use.

Sihui county is one of the counties of Guangdong Province, 80 kilometers away from Guangzhou city all the residents in this county are native, most of them are farmers, of whom the crude incidence rate of NPC was 18.43, standardized-18.11 (Average, 1971– 1980). While in Guangzhou region, a fraction of the residents are not natives, all of them are city residents of whom the crude incidence rate was 10.98 standardized-10.55 (Average, 1970–1979).

Preparation of ^{14}C-DNA from E. Coli

[14]C-DNA was prepared from 15T (555–7) strain of E.Coli according to the method of Marmur, ^{14}C-thymidine was purchased from Atomic Energe Institute of Shanghai, Academia Sinica.

Preparation of EBV Specific DNase and Method of Determination

Preparation of EBV-DNase was previously described[8] and the titer of EDAb was measured as reported.[7] An anti-enzyme rate (AER) equal or greater than 30% was recorded as EDAb positive. An AER less than 30% was recorded as EDAb negative.

EBV Negative Serum and Raji Cell

EBV negative serum and Raji cell were presented by Dr. Nancy Raat-Traut of Liuberger Cancer Center, the University of North Carolina at Chapell Hill, USA.

Assays for VCA-IgA and EA-IgA

Performed using enzyme immunoassay method.

RESULTS

The Titer of EDAb and Their Distribution in Sera from Patients with NPC and the Natural Population

After the EDAb data of 2060 cases of natural population (Sihui county), 2189 cases of VCA-IgA positive healthy donors, 430 cases of NPC patients and 204 cases of other tumor patient were analysed. We found that the distribution of the levels of EDAb (AER) between NPC patients and natural population were quite different (Figure 1). The AER of NPC patients gave the negative skewness distribution Md=65.3 and AER of natural population appeared the serious positive skewness distribution. The Md of natural population in Sihui county is 7.9. The Md of VCA-IgA positive healthy donors in Guangzhou region is 9.0. Difference in Md of these two groups

has no any statistical significance ($P>0.05$), but there are some differences in the distribution of EDAb level, this indicated that there are some correlations between EDAb and VCA-IgA. When further analysis was performed, it was also shown that the EDAb distribution of VCA-IgA positive healthy donor and VCA-IgA negative healthy donors were different (Table 1).
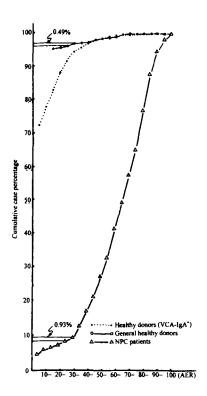

Fig. 1. Distribution of EDAb titers in sera from pts with NPC (430 cases), general healthy donors (2060 cases) and VCA-IgA* healthy donors (2189 cases)

The case number of high EDAb titer (AER) in VCA-IgA positive healthy donor is more than that of VCA-IgA negative healthy donor, which implied that there are some relationships between VCA-IgA and EDAb titre ($x^2=342.6$, $P<0.005$) and the Pearson Coefficient also showed that these was a low degree correlation between them (C=0.344).

The cut-off point between EDAb positive and negative was important for the clinical references. The result of EDAb determinations of 430 case NPC patients, 2060 case natural population and 2189 case

VCA-IgA positive healthy individuals, as indicated in Figure 1. In the EDAb titre region the percentage accumulated number of NPC patient increased slowly as the EDAb titre increase. While the AER reached a value of 30%, the curve showed a sharp turning and the percentage of accumulated number burst into a rapid increase. In the control group, this curve raises very slow and evenly. Authors found that it is a suitable to take AER=30% as a cut-off point and AER <30% as normal (negative) and AER≥30% as abnormal (positive).

Using this criterion, we can get the 90.7% diagnosis rate in NPC patients. The false negative rate is 9.3%. While in natural population the positive rate is 3.30%, as to the VCA-IgA positive healthy individual the positive rate is 5.12%–6.02.

In order to decrease the false negative of NPC, we suggested to set up a warning value which was assigned as AER 25%. Using this criterion the false negative of NPC reduced to 8.37%, showing 0.93% decrease. For natural population, the positive rate increased 3.79, showing a 0.49% increased (Figure 1)

The Correlation of Sexes and Ages to the EDAb Titers

Four hundred and twenty-three cases of patient with NPC aged from 30 to 50 were analysed. The age groups of 10–19 and 70–79 were not included because there were only 7 cases in these two groups. In the natural population 2060 cases from the Sihui county were also analysed (Figure 2). The result showed that the Median titer of EDAb in sera from NPC patients was significantly higher than that from natural population. However, there was no significant difference between sexes both in NPC patients and natural population. The EDAb titers seemed not necessary to increase with the age. The result of statistical analysis showed that the rank correlation coefficient of EDAb titers to the ages in NPC patients was $\gamma_s=0.0199$ ($P>0.05$), indicating no correlation between EDAb titer and age. Thus the statistical analysis was not applied to the healthy donors.

The Difference of Other Data and Characters Related to EDAb in NPC Patients, EDAb Positive Healthy Individuals and Natural Population

All EDAb positive healthy donors found in mass screen accumulated to 224 cases (112 from Sihui

county, 112 cases from Guangzhou region) compared with EDAb positive NPC patients and 2060 natural population in various characters and indexes related to EDAb.
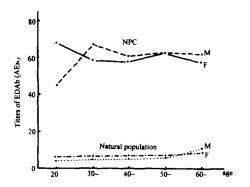

Fig. 2. The correlation of EDAb to sexes and ages male NPC; female NPC; male healthy donors; female healthy donors

The Difference in Sex Ratio

It is common to find that in the NPC patients male are more than females. The sex ratio of NPC may be 2–10:1 (male to female). In our paper, the sex ratio of NPC patients was 2.3:1 (301:129), while in EDAb positive healthy donors was 0.9:1 (106:118). The sex ratio of test EE in Sihui county is 0.8:1, while in Guangzhou region is 1.6:1.

The Difference of the Case Percentages Which Were Both EDAb and VCA-IgA Positive in NPC Patients and Healthy Donors

In NPC patients with both EDAb and VCA-IgA titres positive was 94.34% (350/371), in EDAb positive healthy individual was 17.65% (12/68), while in natural population this case percentage was merely

0.58% (Sihui county, 12/2060) and 0.69% (Guangzhou region, 119/17236). The significance of this difference was not clear, but it demonstrated these two indexes were quite disparate. Whether there are any relationships between these indexes in the process of the NPC development or not is still a question.

The Different Distributions of EDAb Titre in the Serum from EDAb Positive NPC Patients, EDAb Positive Healthy Donors and Natural Population

The EDAb titre distribution in EDAb positive NPC patients gave a negative skewness distribution. As to the natural population group manifested a serious positive skewness distribution type. The case percentage of the high titre level (AER≥50%) in EDAb positive NPC group was 80.3% (313/390), in EDAb positive healthy individual 35.3% (74/224), while in natural population group was only 0.9% (2/224). The differences were very significant (Figure 1, Table 2). These phenomena suggested that EDAb positive in these three groups might possess quietly different biological significance.

The Sensitivity and Specificity of EDAb in Detection of NPC

The results from Table 3 showed 90.7% (390/430) NPC patients were found to be EDAb positive, only 3.30% (68/2060) of the natural population were detected EDAb positive.

The specificity of this NPC auxiliary diagnosis was 0.9670 from the data of 2060 cases natural population and the sensitivity was 0.9070 from the 430 cases of NPC patient.

In this paper, we also analysed the data of patients with NPC who were both negative to VCA-IgA and EA-IgA and found that the 20 among 27 cases were EDAb positive (74%). In some of them, the EDAb titer were as high as to AER 89% (Figure 3, Table 4).

Table 1. The correlation between EDAb and VCA-IgA titers in sera

| VCA-IgA | No. of pts | EDAb titers (AER, %) | | | | | | | | | Total |
		15	15-	30-	40-	50-	60-	70-	80-	90-	
+	681	73.27	20.70	2.94	1.03	0.73	0.44	0.88	0	0	99.99
	1870	95.72	1.28	0.64	0.85	0.53	0.75	0.05	0.05	0.11	99.99

Groups	Case	EDAb titre (AER)							Total
		30-	40-	50-	60-	70-	80-	90-	
EDAb⁺ NPC pts	390	8.78*	11.02	15.90	17.69	21.28	19.74	5.64	100.00
EDAb⁺ healthy donors**	224	40.18	25.45	15.17	11.60	6.70	0.45	0.45	100.00
Natural population*** •	224	1.79	0.89	0.45	0.45	0.00	0.00	0.00	3.58

*Case percentage **Total case of EDAb positive healthy individual found in mass survey
***Sampling from 2060 natural population

Table 3. Sensitivity and specificity of EDAb in distinguishing patients with NPC
from those with other tumors and healthy individuals

Sources of sera	No. of pts tested	No. of pts with EDAb⁺	EDAb⁺ ratio (%)
NPC	430	390	90.70
Other head & neck tumors*	50	3	6.00
Esophageal carcinoma	78	4	5.13
Lung cancer	33	0	0
Other tumors**	43	0	0
Healthy individuals			
1. Natural population in Sihui county *	2060	68	3.30
1–1 VCA-IgA ⁻	1870	56	2.99
1–2 VCA-IgA⁺	190	12	6.32
2. VCA-IgA⁺ in region of Guangzhou	2180	112	5.12
3. VCA-IgA⁺ in Sihui county **	681	41	6.02
4.VCA-IgA ⁻ in Sihui county (control)**	100	3	3.00

*Laryngo carcinoma, glosso carcinoma and thyroma etc.   **Hepatocarcinoma and leukemia.
*Sera collected in April 1987.   **Sera collected in July 1987.

Table 4. EDAb titre of pats with whom were both
negative of VCA-IgA and EA-IgA

Item	EDAb titre (AER)		
	≥50%	30–50%	<30%
Case No.	10	10	7
%	37	37	25.93

carcinoma (VN). There were only 2 and 3 cases respectively in undifferentiated carcinoma (UC), adenocarcinoma (AC). The results in Table 5 showed that there was not significant difference between titers of PD and VN ($P>0.05$).

Table 5. Correlation of EDAb titers to
different pathological types

Types	No. of pats	EDAb (Md)
PD	381	64%
VN	40	71%
UC	2	78%, 91%
AC	3	39%, 38%, 33%

PVS:PD,   VN $P>0.05$

The Correlation of EDAb Titers to the Different Pathological Types and Different Clinical Stagings

In 430 cases of NPC patients, 381 of them were poorly differentiated squamous cell carcinoma (PD) and 40 cases of them were vesicular nucleus cell

Three hundred and thirty-eight cases of NPC patients indifferent stagings were analysed (Table 6). The result showed that there were not significant differences in EDAb titers among different stagings ($P>0.50$).

Table 6. correlation of EDAb titers to different clinical stagings

Stagings	No. of pats	EDAb (Md)
I	7	0%, 30%, 50%, 72%, 85%, 88%, 89%
II	81	63.2%
III	182	65.3%
IV	68	65.0%

P=0.81. The difference among II, III, IV stagings is not significant. Clinical stagings were divided according to Changsha's TNM staging system, 1977.

The Correlation of EDAb to VCA-IgA and EA-IgA

In 410 of 430 of NPC, serum EDAb VCA-IgA and were determine simultaneously to study their correlation.

The correlations of EDAb to VCA-IgA and EA-IgA were analysed by computer. Linear regression analysis showed that the coefficients of EDAb to 1 g (1+VCA-IgA) and 1 g (1+EA-IgA) were $\gamma_{VCA}=0.3369$ ($P<0.01$) and $\gamma_{EA}=0.4643$ ($P<0.01$) respectively (Figure 3). These showed that there was merely low correlation of EDAb to VCA-IgA and EA-IgA titers and the correlation was increased with the elevation of VCA-IgA and EA-IgA. This results were also agreeable with our previous report.[3]
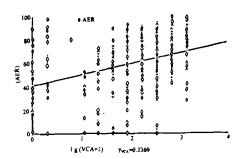
The incidence of EDAb, VCA-IgA and EA-IgA in sera from 410 patients with NPC were also analysed in Table 7. The results showed that most of the NPC patients (89.74%, 350/390) had both positive to EDAb and VCA-IgA. There were only a few of NPC patients (1.70%, 7/410) who had negative both to EDAb and VCA-IgA. This meant that the combination of EDAb and VCA-IgA testing would greatly increase the chance for detection of NPC.

Paired X^2 analysis were performed according to the data of Table 7. It was found that the diagnosed effect of VCA-IgA was a little higher than EDAb, EDAb was higher than EA-IgA, and VCA-IgA was

higher than EA-IgA. Owing to the positive rate of VCA-IgA in normal individual may be as high as 17%[9] and the positive rate of EDAb in natural population of Sihui county (most high risk area in China) was merely 3.30%. It was apparent that the EDAb level assay possessed of higher sensitivity and specificity in early detection of NPC and distinguishing NPC from other cancers particularly from head and neck cancers.

Table 7. Incidence of EDAb, VCA-IgA and EA-IgA in sera from pats with NPC

EDAb	VCA-IgA	EA-IgA	No. of pats
+	+	+	260 (63.42%)
+	+	−	90 (21.95%)
+	−	+	1 (0.24%)
+	−	−	20 (4.88%)
−	+	+	8 (1.95%)
−	+	−	24 (5.85%)
−	−	+	0 (0%)
−	−	−	7 (1.71%)

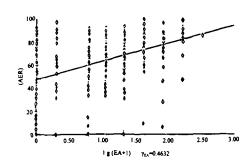+, − represented positive and negative results respectively

Fig. 3. Correlation of EDAb to VCA-IgA and EA-IgA titers in patients with NPC

Results of Follow-up Surveillance in 224 Persons with Positive EDAb

Of 224 people with EDAb positive serum, 15 developed NPC during the period of 62 months following the initial report of EDAb positive (from July 1987 to Sept. 1992). Among them, 5 persons were at NPC stage I, 4 at stage II, 5 at stage III and I at IV. That is to say, 60% of the those patients (9/15) were diagnosed in their early stage of NPC (stages I and II). In the EDAb negative cohert, on the other hand, only one person (1/4806) developed NPC (stage I) in the same period. It is worthy to mention, one of two people found positive in all three predictive assays for high risk for NPC (EDAb 52%, VCA-IgA 1:5, severe dysplasis of NP cytological smear). The one who failed to follow the advice of periodic check-up for NPC, was diagnosed with NPC when he showed up for enlarged lymph nodes and erosive lesions on both side of his neck. Unfortunately, the patient had already been at stage IV disease at the time of diagnosis and died two months thereafter due to NPC metastasis. Early diagnosis and the timely medical intervention of NPC could have saved his life, if he could have followed medical advice and came for periodic medical cheek-up.

DISCUSSION

Since the discovery of EBV specific DNase antibody in serum of patients with NPC, a number of reports showed that titration of EDAb was useful in early detection of NPC.[1-5] Due to the complexity of EDAb assay method and the low incidence in most of the world besides South China. It was difficult to collect enough amounts of NPC patients and the related natural population. Therefore the investigations of sero-epidemiology of EDAb and early diagnosis of NPC were restricted.

By analysis of the results of large sample of NPC patients, other tumors, especially non-NPC head and neck tumors (Table 3) and the natural population. It was found that it is suitable to take the AER≥30% as a cut-off point and AER<30% as normal (negative) and AER≥30% as abnormal (positive). Since vast majority of NPC patients (90.7%) tested were found positive with AER≥30% and only a very small proportion of natural population (3.3%) tested fell into this positive category, giving a sharp demarcation

between NPC and healthy donor as well as other tumors. After a period of 62 months (1987. 4–1992. 9) follow-up surveillance, 15 of 224 EDAb positive natural population found in the mass screening were diagnosed as NPC by histopathological examination. While in 4806 EDAb negative natural population, only one case of NPC was fond in this period suggesting this indicator to be specific and sensitive to NPC.

This criterion and methods have been successfully demonstrated in "double blind test"[5] at Cancer Center, Sun Yat-Sen University of Medical Sciences. Thus, the Cancer Center has adopted this method as a part of their standard diagnosis for early detection of NPC. To date (1997. 6), the center has applied this method to over 45000 cases and has found the result quite good.

The authors had also collected sera from 98 patients with various degrees of nasopharyngeal lesions, among then 44 cases were EDAb positive and 54 cases were EDAb negative. After repeated biopsies within two months, 34 cases (77.3%, 34/44) from EDAb positive patients were diagnosed as NPC pathologically, while in the EDAb negative group, only 5 cases (9.2%, 5/54) were confirmed with NPC. These results demonstrated that there was a positive correlation between NPC development and serum EDAb level. It was also showed that there was implication of the determination of EDAb in the early detection of NPC.[6]

As we have pointed out previously that the medical significance of the serum EDAb positive of NPC patients and healthy individuals may be quite different. Therefore, it was speculated that of the EDAb positive persons a small percentage might be in various pre-cancerous stages, but most (including those with AER≥50%) are merely showing an immunologic response to EBV infection. Of the 224 EDAb positive healthy donors studied over a 62-month follow-up period, only 15 cases actually developed full blown NPC (6.7%, 15/224) which is still a low percentage. Thus these results suggest that the probability of NPC occurrence in the EDAb positive individual is low. As to the small percentage of EDAb positive individuals might be in various pre-cancer stages, whether they will develop NPC or reverse to normal situation depends on both internal and extratrenal environments of each person.

In this mass screening and the later follow-up. We found for the first time two cases of NPC patients who had contact with HMPA (Hexamethyl phos-

phoramide, a strong carcinogen for the nasal tumor in rats) for a long term. This finding primarily revealed that the probability of human NPC may be induced by the chemicals.[10]

The data from this experiment revealed that there was no significant difference in the levels of EDAb between the patients with stage II, III and IV. It may be related to the criteria of the clinical staging of NPC. Recently CT scanning has been routinely used for the diagnosis of NPC, many involvements of primary tumor can be easily found by CT scanning such as the invasion to the parapharyngeal space, carotic sheath region and the bone of the skull base etc., that can not be diagnosed by conventional clinical examination. But in 1987 when our field survey was carried out. We could only grouped the patients with Changsha's TNM staging system (1979, Changsha, China) which was not based on clinical examination combined with CT scan. Now a new clinical staging system (1992, Fuzhou, China) is being adopted for clinical use. A more precise TNM staging may be helpful to make a thorough study to the relationship between the levels of EDAb and the clinical stage of NPC.

REFERENCES

1. Chang YC, Chen JY, Ronald G, et al. Frequence and levels of antibodies to Epstein-Barr virus specific DNase are elevated in the patients with nasopharyngeal carcinoma. Proc Natl Sci USA 1980; 77(10):6162.

2. 黄迪, 潘启超, 罗慧玲, 等. EB 病毒 DNA 诱导酶的检定应用于鼻咽癌早期发现的初步研究. 科学通报 1982; 7(6):378.

3. 黄迪, 潘启超, 焦晓蓉, 等. 鼻咽癌及其它癌症病人血清抗 EB 病毒特异性 DNA 酶活性的研究. 癌症 1982; 1(2):91.

4. 黄迪. 关于"抗 EBV-DNase"抗体(EDAb)直测 100 例实验经过及结果报告. 中山医科大学学报 1990; 11(4):封 2

5. Chen JY, Chen YC, Liu MY, et al. Antibodies to Epstein-Barr virus specific DNase as a marker for field survey of patients with nasopharyngeal carcinoma in Taiwan. J Med Virol 1989; 27(4):269.

6. 黄迪, 陈丽珍, 张锦明, 等. Epstein-Barr 病毒特异性 DNA 酶抗体检测在鼻咽癌早期发现中的提示性. 中华肿瘤杂志 1993; 15:289.

7. 陈海峰, 黄迪. 鼻咽癌患者血清中 Epstein-Barr 病毒特异性 DNA 酶抗体检测方法的探讨. 生物化学杂志 1989; 5(2):131.

8. 黄迪, 郑美莲, 罗慧玲, 等. 巴豆油正丁酸对 Raji 细胞的联合诱导作用. 生物化学杂志 1986; 2(3):21.

9. Epstein MA, Achong BG. Epstein-Barr Virus. 1st ed. Spring Verlag. Berlin: Heidelberg. New York. 1979; p432.

10. Huang D, Zhang JM, Liang XM. The finding of patients suffering from nasopharyngeal carcinoma (NPC) who contacted with the hexamethyl phosphoramide (HMPA) for a long time. Chin Journal Can Res 1995; 7(4):258.