Significance of Cell-Free Epstein-Barr Virus DNA in Monitoring Prognosis of Nasopharyngeal Carcinoma

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OBJECTIVE It has been reported that cell-free Epstein-Barr virus (EBV-DNA) in plasma was useful in diagnosing and monitoring nasopharyngeal carcinoma (NPC). The current study was designed to evaluate the significance of EBV-DNA in monitoring the prognosis of nasopharyngeal carcinoma and comparing its significance with that of plasma VCA/IgA and EA/IgA levels.

METHODS EBV-DNA, VCA/IgA, and EA/IgA levels in plasma were determined in NPC patients with different prognosis after radiotherapy, including 30 distant metastatic patients, 22 local recurrence patients and 24 individuals with remission who had been followed-up for more than 2 years after treatment. EBV-DNA was determined using a real-time quantitative PCR system, and levels of VCA/IgA and EA/IgA were measured using standard immunofluorescence. In a cohort study, the indexes were determined after different radiation periods for the 20 new cases of nasopharyngeal carcinoma.

RESULTS The median plasma EBV-DNA concentration was 135,100 copies/ml (interquartile range: 5,525-1,003 750) in metastatic group, 20,500 copies/ml (interquartile range: 0-58,500) in the local recurrence group and 0 copies/ml (interquartile range: 0-0) in the continuous remission group ($P<0.05$). The levels of VCA/IgA and EA/IgA showed no significant differences among the different groups. The high level of EBV-DNA concentration in the metastatic group was more than that in the local recurrence group. A level of 1,000,000 copies/ml of EBV DNA was an indication of distant metastasis of the NPC patients with a sensitivity of 27.3%. However, the sensitivity was 0 in the local recurrence group. For the 20 new patients, EBV-DNA concentration gradually decreased during the radiation period. Before radiation there were 32,050 copies/ml (interquartile range: 3,880-317,750), 0 copies/ml (interquartile range: 0-14 375) after a 40 Gy radiation dose and 0 copies/ml (interquartile range: 0-2940) after the radiation was finished ($P<0.05$). However, the levels of VCA/IgA and EA/IgA showed no significant difference.

CONCLUSION Determination of plasma cell-free EBV-DNA level is more valuable than evaluation of VCA/IgA and EA/IgA for monitoring the prognosis of NPC patients.

KEYWORDS: nasopharyngeal carcinoma (NPC), DNA, Epstein-Barr virus (EBV), polymerase chain reaction (PCR), prognosis.

With a high incidence in Southern China, nasopharyngeal carcinoma (NPC) is an important malignant tumor, for which

radiotherapy is the main treatment modality. Local recurrence and distant metastasis, however, are the most common causes of failure after primary radiotherapy in patients with NPC. [1-4] Therefore, periodic physical reexamination should be performed to detect small metastasis or local recurrence in order to improve survival.

The viewpoint that NPC is strongly associated with EBV has been well established. Detection of EBV-related antibody is a common tool for diagnosing and monitoring NPC patients. In recent years, due to application of real-time quantitative PCR, detection of cell-free EBV-DNA in plasma has attracted more and more attention. It has been published that cell-free EBV-DNA in serum was also useful in diagnosing and monitoring NPC. [5-7] We have previously published on the significance of EBV-DNA in plasma for NPC diagnosis and differential diagnosis. [8] This study was designed to evaluate the significance of EBV-DNA in monitoring the prognosis of NPC and to compare the rusults with monitoring VCA/IgA and EA/IgA plasma levels.

MATERIALS AND METHODS

The samples

During the period March, 2001 to June, 2002, 76 NPC patients were examined at the Cancer Center, Sun Yat-sen University after having been treated with radiotherapy. There were 30 patients with distant metastasis (including 10 bone, 8 liver, 6 lung, 3 multi-organ and 3 other), 22 with local recurrence and 24 in continuous remission who had been followed- up for over 2 years (range:2.0~4.6) after treatment. In addition, our study recruited 20 new NPC patients without distant metastasis who had pathological confirmation in the Department of nasopharyngeal carcinoma, Cancer Center of Sun Yat-sen University. The condition of the patients mentioned above was determined by clinical examination, imaging or pathological examination.

Table 1 shows the tumor-related characteristics of the patients. Primary carcinomas were staged according to the NPC '92 Staging System. [9]

Four ml of blood was drawn from each recruited subjects into a tube containing EDTA. After spinning the blood at 1,600 g in a micro-centrifuge, the plasma was isolated and stored at -20℃ for further analysis. Blood was drawn 3 times from the 20 new NPC cases, namely, at the time of pre-radiotherapy, after 40 Gy radiotherapy and post-radiotherapy. The total dose of radiotherapy averaged 70 Gy (range: 68~74 Gy). All 20 patients had complete tumor regression at the end of the radiotherapy.

DNA extraction

Fifty μl of plasma was utilized from each patient, and DNA from the plasma extracted using a QIAamp DNA mini blood kit (purchased from QIAGENG Inc, Catalog No. 51106) according to the instructions of the manufacturer. Ultraviolet absorbance of the extracted DNA was measured at 260 nm and 280 nm. The value

Table1. Tumor-related characteristics of the patients

	Metastasis number	Local recurrent number	Remissible number	Before treatment number	*P* value
Total patients	30	22	24	20	>0.05
Age($\bar{x}\pm s$)	47(47±10.96)	50(47±9.25)	48(48±10.95)	45(45±9.87)	
Gender					
Men	24	14	15	15	>0.05
Women	6	8	9	5	
Histopathologic type(WHO type)					
Squamous cell carcinoma	0	1	0	0	
Differentiated nonkeratinizing	4	2	1	2	>0.05
Undifferentiated carcinoma	26	19	23	18	
Clinical staging					
I	0	0	1	0	
II	2	5	7	3	<0.05
III	11	7	12	11	
IV	17	10	4	6	

of A_{260}/A_{280} was about 1.8.

Real-time quantitative PCR

Plasma EBV-DNA concentrations were measured using a real-time quantitative PCR toward the $BamH_1$-W fragment region of the EBV genome. The assay kit was provided by Da'an Gene Diagnostic Center, Sun Yat-sen University. The $BamH_1$-W system consisted of the amplification primers W-44F (5'-CCCAACACTCCACCA-CACC-3') and W-119R (5'-TCTTAGGAGCTGTCC-GAGGG-3'). A cloned fragment W of EBV was used as a positive control in each PCR reaction of different dilutions (10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies/μl). Multiple negative water blanks were included in every analysis. Reactions were performed using the PE 7700 Automatic Fluorescence Quantitative PCR System. The detailed reaction conditions and procedures were as described previously. [8]

Quantitative detection of plasma EBV-DNA

The plasma EBV-DNA concentration expressed in copies/ml was calculated according to the following equation: [5]

$$C=Q \times (V_{DNA}/V_{PCR})/V_{EXT}$$

Where C = target concentration in plasma (copies/ml), Q = target quantity (copies) determined by the sequence detector in a PCR, V_{DNA} = total volume of DNA obtained following extraction, V_{PCR} = volume of DNA solution used for PCR, V_{EXT} = volume of plasma extracted.

Detection of VCA/IgA and EA/IgA using immunofluorescence

Standard immunofluorescence was used to detect plasma VCA/IgA and EA/IgA. [10] Assay kits for both were supplied by Sun Yat-sen Genetic Engineering Company. The antibody titer standard was the highest dilution number of the positive plasma.

Statistical analysis

All statistical calculations were performed using the SPSS version 10.0. Analysis of variance and the rank sum test were used for quantitative data in accordance with a normal and non-normal distribution, respectively. Categorical data was analyzed by the χ^2 test, and in prospective study, the Friedman test was used for randomized block design. Sensitivity and specificity were computed according to the manual Medical Statistical and Computer Experiment. [11]

RESULTS

Plasma EBV-DNA copies in NPC patients with different prognosis after radiotherapy

For the plasma EBV-DNA concentration detected by real-time quantitative PCR, a significance difference existed among the metastatic group, local recurrence group and continuous remission group, namely, metastatic group > local recurrence group > continuous remission group. Selecting different cut-off points, sensitivity and specificity of the method, using plasma EBV-DNA to detect NPC recurrence and metastasis, were different. Under the same specificity, higher sensitivity was displayed in the metastatic group than in the local recurrence group. A level of 1,000,000 copies/ml of EBV-DNA indicated the presence of distant metastasis of NPC with a specificity of 100 % and a sensitivity of 27.3 %. However, the sensitivity was 0.0 % in the local recurrence group (Tables 2, 3).

Table 2. The plasma EBV-DNA copies in NPC patients with different prognosis

Group	EBV-DNA (copies/ml)		*P* value	
	M	Interquartile range	Local recurrent	Remissible
Metastasis	135,100	5,525-1,003,750	0.033	0.001
Local recurrent	20,500	0-58,500	-	0.001
Remissible	0	0-0	-	-

NPC: nasopharyngeal carcinoma EBV: Epstein-Bar virus M:median

Table 3. Sensitivity and specificity at different cut-off points of EBV-DNA in detecting distant metastasis and local recurrence of NPC patients

Cut-off points	Distant metastasis		Local recurrence	
(copies/ml)	Sensitivity(%)	Specificity (%)	Sensitivity(%)	Specificity (%)
0	86.3	87.5	66.7	87.5
10,000	72.7	91.7	58.3	91.7
100,000	50.0	95.8	16.7	95.8
1,000,000	27.3	100.0	0.0	100.0

VCA/IgA and EA/IgA levels in NPC patients with different prognosis after radiotherapy

Using immunofluorescence, VCA/IgA and EA/IgA levels were determined among the metastatic group, local recurrence group and continuous remission group. As shown in Table 4, VCA/IgA and EA/IgA concentrations among the 3 groups showed no significant difference ($P>0.05$).

The change of the plasma EBV-DNA level of NPC patients during radiotherapy

All 20 of the prospectively recruited NPC patients had complete tumor regression after complete radiotherapy. The plasma EBV-DNA , VCA/IgA and EA/IgA levels of these patients were determined at the time of pre-radiotherapy, after 40 Gy radiotherapy and post-radiotherapy. The results indicated that the plasma EBV-DNA gradually declined in the radiotherapy treatment period, while VCA/IgA and EA/IgA showed no significant changes (Table 5).

Table 4. VCA/IgA and EA/IgA levels in NPC patients with different prognosis after radiotherapy

	VCA/IgA		EA/IgA	
Group	M	Interquartile range	M	Interquartile range
Metastasis	320	160–640	40	10–80
Local recurrent	320	80–640	20	10–160
Remissible	240	160–640	40	0–80

DISCUSSION

Invasion and development of NPC are closely correlated with EBV, so there are various EBV-related antigens and antibodies in the peripheral blood of the patients of NPC. Since it is very convenient to get peripheral blood from patients, the EBV-related hematological index is commonly used to monitor prognosis of NPC patients. At present, VCA/IgA and EA/IgA are often determined to diagnose and monitor NPC. It is currently unknown, however, whether the levels of these antibodies are of prognostic value. According to Liu et al.,[12] the 10-year survival rate of patients with the high VCA/IgA antibody titer group was less than that of the low titer group ($P<0.05$), whereas other prospective study revealed that there was no relationship between antibody level and prognosis of NPC patients.[13,14]

Recently, it was reported that plasma EBV-DNA in NPC patients after radiotherapy was associated with tumor recurrence and metastasis, and thus, analysis of plasma EBV-DNA after treatment might be a valuable tool to monitor the response to treatment in NPC patients. Lo et al. [6,7] indicated that the median plasma EBV-DNA concentration in 10 patients with tumor recurrence was determined to be 32,350 copies/ml, while that in patients in continuous remission for a mean period of 2 years was 0 copy/ml. Furthermore, in a prospective study, patients with recurrence or metastasis within the first year after treatment had a higher median plasma EBV-DNA concentration than those without pathogenic changes (41,756 copies/ml versus 5,807 copies/ml). Peng et al. [15] investigated the correlation between the changes of plasma EBV-DNA levels and clinical responses during concomitant chemo-radiotherapy in locally advanced NPC patients. They found that EBV-DNA could not be detected at 2~5 weeks of the therapy in 15 patients who had complete tumor regression at the end of the radiotherapy, while 3 patients had a continuously high EBV-DNA, and following clinical examination after treatment found that they still had residual tumors. Further investigation, however, is needed to elucidate which EBV-related parameter might provide more information regarding the prognosis of NPC patients.

Our present study put the emphasis on comparing values of plasma EBV-DNA, VCA/IgA and EA/IgA in monitoring prognosis of NPC patients. We compared the 3 parameters in NPC patients with different prognosis after radiotherapy and concluded EBV-DNA was remarkably superior to VCA/IgA and EA/IgA in monitoring the treatment response. The plasma EBV-DNA concentration in the metastatic group and local recurrence group was higher than that in the continuous remission group. However, the levels of VCA/IgA and EA/IgA among the 3 groups showed no significant differences. In our prospective study for 20 new NPC patients, we found that the EBV-DNA concentration rapidly declined with the shrinkage of the tumors. When the dose of radiotherapy reached 40 Gy, median plasma EBV-DNA concentration had declined to 0 copy/ml, while VCA/IgA and EA/IgA

Table 5. The plasma EBV-DNA, VCA/IgA, and EA/IgA levels of NPC patients during radiotherapy

	Before radiotherapy		40 Gy radiation dose		Radiotherapy finished		
	M	Interquartile range	M	Interquartile range	M	Interquartile range	*P* value
EBV-DNA(copies/ml)	32,050	3,880–317,750	0	0–14,375	0	0–2,940	0.000
VCA/IgA	320	160–640	320	160–640	320	160–640	0.753
EA/IgA	40	10–80	40	20–80	40	20–80	0.990

levels showed no significant change during the treatment. We considered the reason as follows: the EBV antibody level is maintained in NPC patients for a relatively long period and it is not closely associated with the tumor burden, but the patient's immunity. Especially, in a short time, the antibody level has no significant relationship with the treatment response in NPC patients, while cell-free EBV-DNA can sensitively reflect the change of the tumor burden . Furthermore, the finding that plasma EBV-DNA concentrations rapidly varied with the change of the tumor size demonstrated that most of the EBV-DNA in peripheral blood is derived from the tumor.

This present study also indicated that the EBV-DNA concentration in the metastatic group was significantly higher than that in the local recurrence group. And cases with a high EBV-DNA level more likely came from the metastatic group than from the recurrence group. Hence, distant metastasis should be taken into consideration in NPC patients with a high plasma EBV-DNA concentration. This may be related to the high tumor load in NPC patients with distant metastasis, as well as the EBV from the metastasized tumor which more readily released EBV into the peripheral blood.

Results for changes in the plasma EBV-DNA during radiotherapy in our study were somewhat different from what Peng et al. reported. [15] In their study, for the patients who had complete tumor regression, EBV-DNA could not be detected at 2~5 weeks of the therapy, while we observed 2 cases with complete tumor regression at the end of radiotherapy and found that their plasma EBV-DNA was detectable but lower than that before the therapy. There may be two reasons leading to this finding: first, tiny residual or metastatic tumor existed in these patients, which, however, could not be detected using present techniques, while the EBV-DNA in the plasma was already positive. According to Lo et al., [6] significant elevations in plasma EBV-DNA, remained sometimes up to 6 months before detectable clinical deterioration was observed in the patients who subsequently developed tumor recurrence or metastasis; second, EBV-DNA possibly derived from non-tumor tissue. We will follow-up the two patients to determine other reasons for their positive plasma EBV-DNA.

In summary, we confirmed that the plasma cell-free EBV-DNA was a useful tool to monitor the response to treatment in NPC patients, and was significantly superior to determination of EBV-related antibodies. Nevertheless, further prospective investigation shall be developed to learn the relationship between plasma EBV-DNA and the time to relapse or metastasis, as well as whether EBV-DNA can serve as an independent prognostic indicator.

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