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Original Article

Detecting plasma Epstein-Barr virus DNA to diagnose postradiation nasopharyngeal skull base lesions in nasopharyngeal carcinoma patients: a prospective study

Fa-Ya Liang[1], Wei Sun[1], Ping Han[1], Xing Lu[1], Ying-Ni Lian[2] and Xiao-Ming Huang[1]

Abstract

The diagnosis of postradiation nasopharyngeal skull base lesions in petients with nasopharyngeal carcinoma (NPC) is still a tough problem in clinical practice. An early and accurate diagnosis is important for subsequent management. We prospectively evaluated the diagnostic value of plasma Epstein-Barr virus (EBV) DNA in detecting postradiation nasopharyngeal skull base lesions in NPC patients. From July 2006 to September 2010, 90 patients with postradiation NPC (34 women and 56 men; median age: 42 years) met the selection criteria and were recruited in this study. All postradiation nasopharyngeal skull base lesions were found in the latest magnetic resonance imaging (MRI) examinations before endoscopic surgery, and the nasopharyngeal cavity was normal under flexible nasopharyngoscopy. Plasma EBV DNA detection was performed within 2 weeks before endoscopic surgery. A total of 90 endoscopic operations were successfully performed without any postoperative complications. Recurrences confirmed by postoperative pathology were found in 30 patients. The specificity, positive and negative predictive values of plasma EBV DNA detection were better than those of MRI. In addition, combining plasma EBV DNA detection with MRI improved the specificity and positive predictive values of MRI. Plasma EBV DNA detection followed by MRI would help to diagnose recurrence whereas MRI was unable. These results indicate that plasma EBV DNA is an effective and feasible biomarker for detecting postradiation nasopharyngeal skull base lesions in NPC patients.

Key words Nasopharyngeal carcinoma, plasma EBV DNA, diagnostic test, endoscopic surgery, postradiation lesions

Nasopharyngeal carcinoma (NPC) is an endemic Epstein-Barr virus (EBV)-associated carcinoma in southern China, where approximately 50 to 80 cases per 100 000 people are reported per year[1]. Although NPC is sensitive to radiotherapy, 20% –30% local recurrence has been reported[2]. In postradiation NPC patients, recurrence mainly occurs in the nasopharynx, skull base, parapharyngeal space, and paranasal sinus[3]. It is necessary to diagnose the recurrent lesions in these deep and complex anatomic regions, so that prompt therapy can be offered to improve prognosis[2].

For postradiation NPC patients, biopsies on nasopharyngeal skull base regions, such as the petrous apex, great wing of the sphenoid bone, base of sphenoid bone, cavernous sinus, and occipital clivus, are difficult to perform[4,5]. Radiotherapy can cause a variety of changes in radiation fields, including edema, necrosis, loss of tissue planes, fibrosis, and scarring. These changes may obscure the detection of tumor recurrence by computed tomography (CT) scans and magnetic resonance imaging (MRI)[6,7]. For uncertain CT/MRI findings in NPC patients after radiotherapy, positron emission tomography (PET) using 18-fluoro-2-deoxy-

Authors' Affiliations: [1]Department of Head and Neck Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510120, P. R. China; [2]Department of Oncology, the First People´s Hospital of Zhaoqing, Zhaoqing, Guangdong 526021, P. R. China.

Corresponding Author: Xiao-Ming Huang, Department of Head and Neck Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510120, P. R. China. Tel/Fax: +86-20-81332655; Email: xiaomingh@hotmail.com

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glucose (^{18}F-FDG) may provide an alternative diagnostic option for detecting possible recurrence. However, some false-positive results (e.g., inflammatory tissues) have also been found through FDG-PET examination[8-10]. Thus, imaging-based detection of postradiation nasopharyngeal skull base lesions in NPC patients is still limited[2,10,11]. Depending on the imaging examination, misdiagnosis may sometimes occur, but most nasopharyngeal skull base lesions lack histopathologic diagnoses[5,10]. Therefore, it is necessary to explore other effective methods for improving the diagnostic accuracy of postradiation lesions in nasopharyngeal skull base regions.

Recently, plasma EBV DNA has been reported as a useful marker for detecting, monitoring, and predicting the prognosis of patients with non-disseminated NPC who were treated with radiotherapy[12-16]. However, its diagnostic value in detecting postradiation nasopharyngeal skull base lesions of NPC has not been reported. In this prospective study of postradiation nasopharyngeal skull base lesions in NPC patients, the diagnostic value of plasma EBV DNA was evaluated by comparing its detection results with histopathologic data.

Materials and Methods

Selection criteria

Patients were included in this study according to the following criteria: (1) NPC patients had accepted one or two courses of radiotherapy. All patients achieved complete response, and were followed for at least 6 months after radiotherapy. (2) In the latest MRI before endoscopic surgery, abnormal signals were found in nasopharyngeal skull base regions, and the nasopharynx was normal (Figure 1A and 1B). (3) Flexible nasopharyngoscopy showed no signs of recurrence in the nasopharyngeal cavity, and the histopathologic results were negative (Figure 1C). (4) No distant metastasis was observed. (5) Karnofsky performance status scales were $\geqslant$60.

Patient characteristics

From July 2006 to September 2010, 34 women and 56 men with NPC (median age, 42 years; interquartile age range, 36–58 years) were recruited according to the selection criteria and diagnosed with postradiation nasopharyngeal skull base lesions after endoscopic surgery at the Sun Yat-sen Memorial Hospital. Plasma EBV DNA detection was performed within 2 weeks before endoscopic surgery. This study was approved by the Institutional Review Board of Sun Yat-sen Memorial Hospital.

Patients underwent radiotherapy at several locations, including the Sun Yat-sen University Cancer Center (78 patients), the Sun Yat-sen Memorial Hospital (7 patients), and other hospitals (5 patients). Of the 72 patients who underwent one course of radiotherapy, 35 underwent conventional radiotherapy (CRT) (total dose of the nasopharynx: 60–80 Gy), 32 underwent intensity-modulated radiotherapy (IMRT, 60–68 Gy), and 5 underwent CRT (60–66 Gy) followed by intracavitary brachytherapy (9–15 Gy). Among 18 patients who underwent 2 courses of radiotherapy, 7 underwent 2 courses of CRT (120–140 Gy), 5 underwent 2 courses of IMRT (136 Gy), 5 underwent 1 course of CRT (60–72 Gy) followed by 1 course of IMRT (60–68 Gy), and 1 underwent 1 course of CRT (72 Gy) followed by 1 course of X-knife (48 Gy).

Magnetic resonance imaging evaluation

All patients underwent MRI on a 1.5T MR whole-body system unit with either a Philips Gyroscan (Netherlands) or a GE Signa (USA) at the Sun Yat-sen Memorial Hospital or the Sun Yat-sen University Cancer Center. The protocols used for this study included pre-contrast coronal T1-weighted images, axial T2-weighted images, axial proton density images, sagittal T1-weighted images, post-contrast coronal T1-weighted images with fat saturation, and axial T1-weighted images without fat saturation. The upper limit covered the cavernous sinuses, and the lower limit reached the hypopharynx. MRI images were primarily evaluated by visual interpretation, requiring the independent agreement of at least two of three experienced radiologists.

The residual/recurrent tumors observed on MRI was graded using a five-point scoring system (0 = no lesion, 1 = definitely benign, 2 = probably benign, 3 = probably malignant, 4 = definitely malignant). Grades 3 and 4 were both considered positive[17].

DNA extraction from plasma samples

The preoperative plasma EBV DNA level was checked the day before surgery. Venous blood (5 mL) was collected from each patient according to a standard operating procedure and centrifuged at 2500 r/min at 4℃ for 10 min. Plasma samples from each patient were then collected and stored at −80℃. DNA from the plasma samples was extracted with the QIAamp Blood Kit (Qiagen, Germany) using the blood and body fluid protocol, as recommended by the manufacturer. In total, 500–1000 μL of each plasma sample was used for DNA extraction per column, with a final elution volume of 50 μL from the extraction column. The exact amounts were documented for the calculation of the target DNA

concentration.

Real-time quantitative EBV DNA PCR

The real-time quantitative PCR was conducted as previously described[13,16]. The real- time quantitative PCR system was developed for EBV DNA detection toward the BamHI-W region. The system consisted of the amplification primers W-44F (5′-AGTCTCTGCCTCCA GGCA-3′) and W-119R (5′-ACAGAGGGCCTGTCCAC CG-3′) and the dual-labeled fluorescent probe W-67T (5′-[FAM] CACTGTCTGTAAAGTCCAGCCTCC [TAMRA]-3′).

The EBV DNA plasma concentration (expressed in copies/mL) was calculated using the following equation: $C = Q \times V_{DNA}/V_{PCR} \times 1/V_{EXT}$, in which C represents the target concentration in plasma (copies/mL), Q represents the target quantity (copies) determined by a sequence detector in PCR, V_{DNA} represents the total volume of DNA obtained after extraction, V_{PCR} represents the volume of DNA solution used for PCR, and V_{EXT} represents the volume of plasma/serum extracted (typically 0.5-1.0 mL).

Nasal endoscopic surgery

Postradiation nasopharyngeal skull base lesions occur at deep anatomic sites, thus making them difficult to biopsy for diagnosis. Based on our previous experience[18], nasal endoscopic surgery is an alternative method with which to perform biopsies in nasopharyngeal skull base regions. All operations were performed under general anesthesia with endotracheal intubation or loco-regional anesthesia. The surgical approach depended on the location of the lesion as demonstrated by MRI. The trans-sphenoidal approach was used for operation on the upper clivus, and the transnasal/trans-nasopharyngeal and/or trans-sphenoidal approaches were used for operation on the middle and/or lower clivus (Figure 1D). The pterygopalatine fossa and the medial wall of the infratemporal fossa were operated on through the medial wall of the maxillary sinus or with the Caldwell-lu approach. Operations on the cavernous sinus were performed via the trans-sphenoidal approach. The final diagnosis was confirmed by pathologic examination.

Statistical analysis

Statistical analysis was conducted with SPSS 16.0. The relationship between plasma EBV DNA and postoperative pathologic results was assessed with the Chi-square test. All P values were two-sided and considered significant at $P < 0.05$.

Results

Outcomes of operation

All 90 endoscopic operations were performed successfully. Median operative time was 55 min (interquartile range, 35–80 min), and median operative blood loss was 30 mL (interquartile range, 20–70 mL).

Postoperative pathologic results

Recurrences confirmed by postoperative pathology were found in 30 (33.3%) patients: 23 patients had poorly differentiated squamous cell carcinoma, 4 had osteoradionecrosis (ORN) with poorly differentiated squamous cell carcinoma, and 3 had undifferentiated squamous cell carcinoma. In the 60 patients without recurrence, 35 had chronic inflammation with ORN, 14 had inflammation with radiation-induced fibrosis, 6 had ORN, and 5 showed granulation.

Postoperative follow-up

All patients underwent endoscopic examinations at the periodic follow-up visits. None showed postoperative complications (Figure 1E and 1F). Thirty patients with recurrence were followed for a median of 25 months (interquartile range, 15–28 months); 2 showed distant metastases 24 and 29 months after the operation, respectively. The 60 patients without recurrence were followed for a median of 37.5 months (interquartile range, 16–43 months); 42 were followed for at least 24 months. Of these 42 patients, 2 died of serious bleeding of the nasopharynx 12 and 18 months after the operation, respectively; 1 died of inspiratory pneumonia 15 months after the operation; the remaining 39 showed no sign of recurrence.

The diagnostic value of plasma EBV DNA in detecting postradiation nasopharyngeal skull base lesions of NPC

Standard curve of plasma EBV DNA detection The standard concentration ladders were 106, 105, 104, 103, and 102 copies/mL. Concentrations of plasma EBV DNA are expressed as copies of EBV genome per mL of plasma. Figure 2A presents the results obtained using the real-time PCR system. The amplification curves shifted to the right as the input target quantity were reduced. Figure 2B shows the relationship between the threshold cycle of the EBV DNA PCR and the input target quantity. The linearity of the graph demonstrates the large, dynamic range and the accuracy of real-time

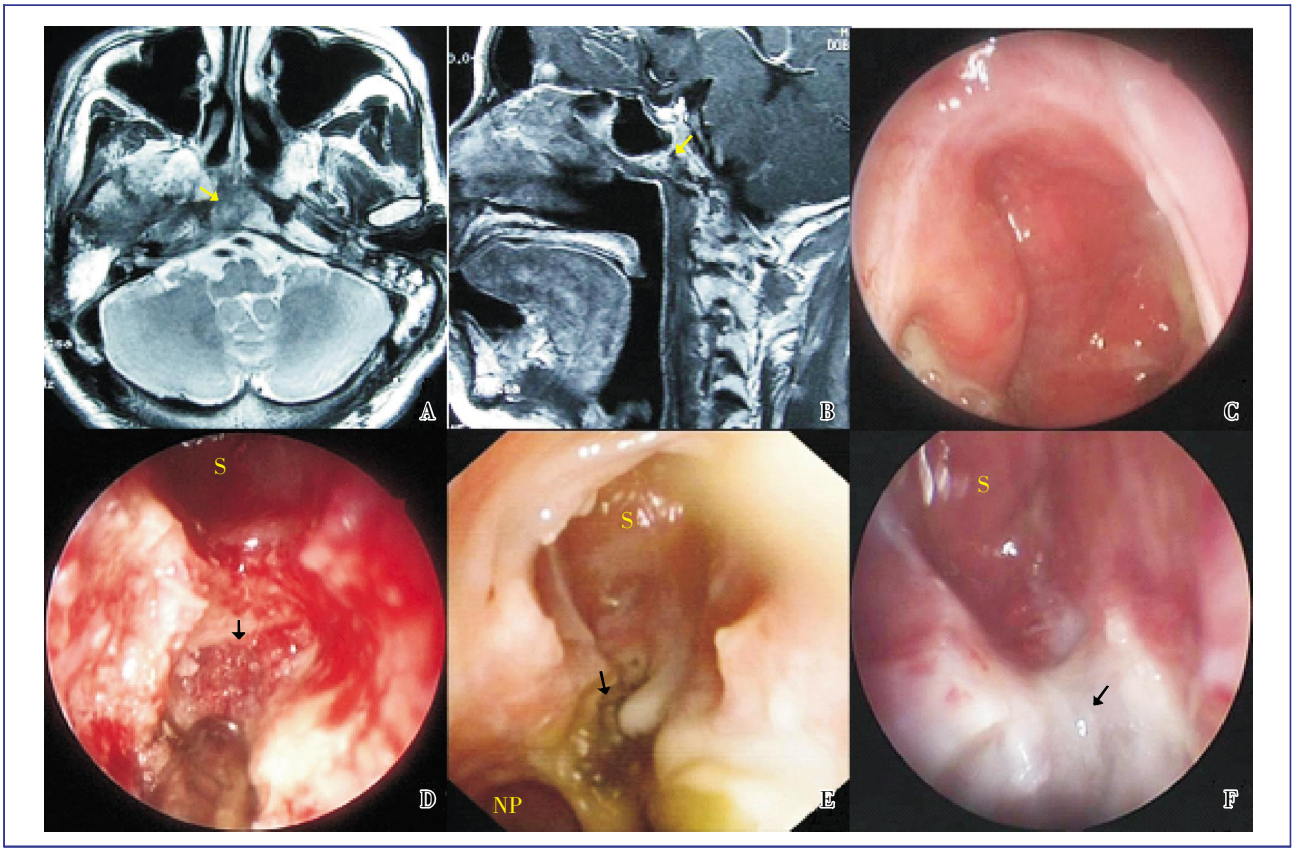

Figure 1. **Preoperative examination, operation, and postoperative follow-up in a nasopharyngeal carcinoma (NPC) patient with a postradiation nasopharyngeal skull base lesion.** This NPC patient was a 59-year-old man who underwent one course of radiotherapy. The 1-year follow-up MRI shows signals indicating probable malignancy. Postoperative pathology showed osteoradionecrosis. A, T2-weighted image on the axial plane shows normal marrow signal in the right clivus changed to heterogeneous slightly hypointense signal with a patchy, slightly hyperintense signal inside (yellow arrow). B, enhanced T1-weighted image on the sagittal plane shows heterogeneous enhancement in the right clivus lesion with patchy, non-enhanced areas inside (yellow arrow). C, the nasopharynx cavity is normal and there are no signs of recurrence under flexible nasopharyngoscope. D, the right clivus is exposed and abraded for biopsy via transnasal approach (black arrow). E, at the 2-month follow-up after surgery, the endoscopic examination shows the biopsied region covered by a scab (black arrow). F, at the 6-month follow-up after surgery, the endoscopic examination shows mucosal epithelialization and no signs of recurrence in the biopsied region (black arrow). S, sphenoid sinus; NP, nasopharynx.

quantitative PCR.

Comparisons between plasma EBV DNA detection and MRI Plasma EBV DNA was detected in 34 (37.7%) of the 90 patients. The median plasma EBV DNA levels were 4600 copies/mL (interquartile range, 910–40 025 copies/mL) in patients with recurrent NPC leisions and 0 copies/mL (interquartile range, 0–0 copies/mL) in patients without recurrent lesions (Figure 3). Plasma EBV DNA level was significantly different between the recurrent NPC group and the non-recurrent group ($P < 0.001$). The sensitivity and specificity of plasma EBV DNA-based detection of recurrent NPC were 90% and 88.3%, respectively; the positive and negative predictive values were 79.4% and 94.6%, respectively.

Of the 72 patients who underwent one course of radiotherapy, plasma EBV DNA level was significantly different between the recurrent NPC group and the non-recurrent group ($P < 0.001$). The sensitivity and specificity of plasma EBV DNA-based detection of recurrent NPC were 89.5% and 88.7%, respectively; the positive and negative predictive values were 73.9% and 95.9%, respectively. Of the 18 patients who underwent two courses of radiotherapy, plasma EBV DNA level was significantly different between the recurrent group and the non-recurrent group ($P = 0.002$). The sensitivity and specificity of plasma EBV DNA-based detection of recurrent NPC were 90.9% and 85.7%, respectively; the positive and negative predictive values were 90.9% and 85.7%, respectively.

Of the 90 patients, 59 patients were MRI-positive

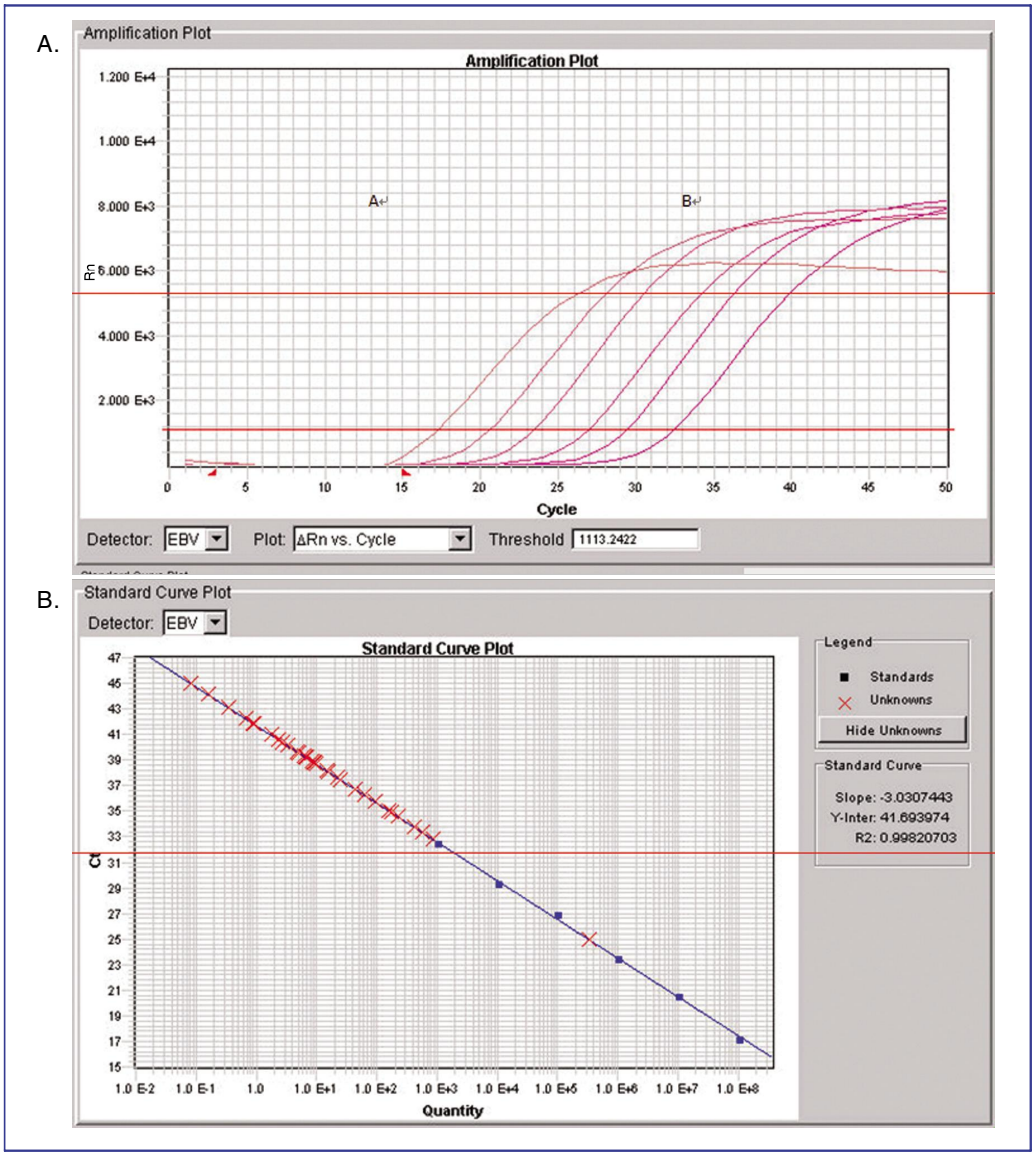

Figure 2. **Detection of plasma Epstein-Barr virus (EBV) DNA by real-time polymerase chain reaction (PCR).** A, amplification plot of fluorescence intensity against the PCR cycle. Each plot corresponds to a particular input target quantity marked by a corresponding symbol. The *X*-axis denotes the cycle number of a quantitative PCR reaction. The *Y*-axis denotes the Rn, which is the fluorescence intensity over the background. B, a plot of the threshold cycle against the input target quantity. The input target quantity is expressed as copies of EBV DNA.

and 31 patients were MRI-negative. The sensitivity and specificity of MRI-based detection of postradiation nasopharyngeal skull base lesions in NPC patients were 90% and 46.7% , respectively, and the positive and negative predictive values were 45.8% and 90.3% , respectively (Table 1).

We also combined plasma EBV DNA detection and MRI as a serial test. Results showing both positive plasma EBV DNA detection and positive MRI were considered serial test positive, whereas results showing neither plasma EBV DNA detection nor positive MRI signals were considered serial test negative. The sensitivity and specificity of serial test were 80% and 91.6%, respectively; the positive and negative predictive values were 82.7% and 90.1%, respectively (Table 1).

If the tests designed in order, the second test would proceed while the first test result was positive. Both the first and second tests positive were considered order test positive. The first test positive and the second test negative were considered order test negative. When MRI followed by plasma EBV DNA detection, the sensitivity and specificity of order test were 88.9% and 28.6%, respectively; the positive and negative predictive values were 82.8% and 40% , respectively. When plasma EBV DNA detection followed by MRI, the sensitivity and specificity of order test were 96% and 85.3%, respectively; the positive and negative predictive values were 82.6% and 96.7%, respectively (Table 1).

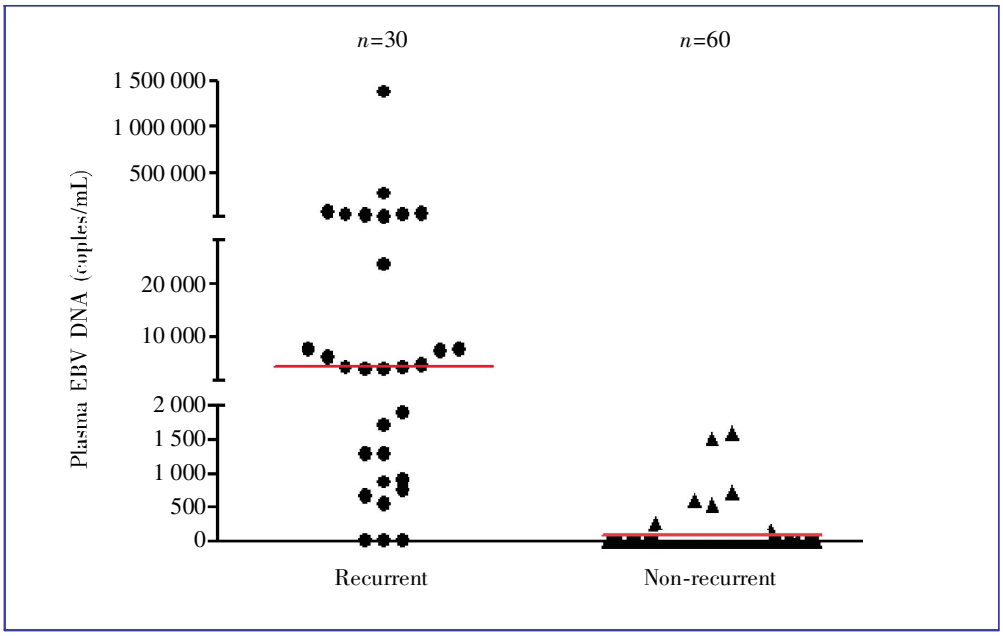

Figure 3. **Plasma EBV DNA levels in patients with and without recurrent NPC.** Scatter diagram shows that the preoperative plasma EBV DNA levels in the recurrent NPC group was significantly higher than that in the non-recurrent group (P = 0.03).

Table 1. Different diagnostic methods compared with pathologic results

Diagnostic method	Pathologic results (cases)		P
	Recurrent group	Non-recurrent group	
EBV DNA			<0.001
Positive	27	7	
Patients underwent 1 course of radiotherapy[a]	17	6	
Patients underwent 2 courses of radiotherapy[b]	10	1	
Negative	3	53	
Patients underwent 1 course of radiotherapy	2	47	
Patients underwent 2 courses of radiotherapy	1	6	
MRI			0.001
Positive	27	32	
Negative	3	28	
Serial test[c]			<0.001
Positive	24	5	
Negative	6	55	
MRI followed by EBV DNA detection			0.268
Positive	24	5	
Negative	3	2	
EBV DNA detection followed by MRI			<0.001
Positive	24	5	
Negative	1	29	

[a]P < 0.001, vs. EBV DNA-negative patients underwent 1 course of radiotherapy; [b]P = 0.002, vs. EBV DNA-negative patients underwent 2 courses of radiotherapy. [c]Combined plasma EBV DNA detection and MRI.

Discussion

The nasopharyngeal skull base is one of the recurrence regions in NPC patients[5]. Early and accurate diagnosis of recurrence is important for subsequent management. Because nasopharyngeal skull base lesions occur in deep anatomic sites, such as the petrous apex, great wing of the sphenoid bone, base of sphenoid bone, cavernous sinus, and occipital clivus [5], histopathologic examinations are sometimes difficult to perform in these regions. Hence, the diagnosis of postradiation nasopharyngeal skull base lesions in patients with NPC is still a tough problem in clinical practice[10].

In general, image examinations play an important role in identifying postradiation nasopharyngeal skull base lesions in patients with NPC. However, a variety of radiation-induced changes, such as edema, necrosis, loss of tissue planes, fibrosis, and scarring, may obscure the diagnosis or even lead to misdiagnosis [6,7]. Nevertheless, there are no prospective studies about histopathology and imaging examination on NPC patients with postradiation nasopharyngeal skull base lesions. In our study, MRI examination was compared with postoperative histopathology. The results showed that both the specificity and positive predictive value of MRI were low in detecting postradiation nasopharyngeal skull base lesions in NPC patients. This may be due to nonspecific signals caused by inflammation with ORN and radiation-induced fibrosis. An inflammatory change of the skull base region can occur months or years after radiotherapy[19,20] and lead to MRI signals similar to local recurrence/residual tumors [21]. In addition, differentiating ORN from recurrence is difficult when ORN has a variable signal on MRI, such as high signal intensity or mixed signal intensity on T2-weighted images [22]. Thus, radiation-induced abnormalities could be misdiagnosed according to MRI. For inconclusive MRI results, several researchers have suggested that ^{18}F-FDG PET may provide more evidence for differential diagnosis [4,23]. However, ORN and/or inflammation may also cause ^{18}F-FDG hypermetabolism and give rise to false-positive interpretations [8,9,24,25]. Based on previous reports [10,22] and our results, imaging examinations are limited in their ability to help identify and differentiate postradiation nasopharyngeal skull base lesions in NPC patients. Therefore, it is necessary to explore an effective, minimally invasive diagnosis method for postradiation nasopharyngeal skull base lesions in NPC patients.

Recently, EBV DNA, which has been detected in the serum/plasma of NPC patients, has became an effective biomarker for early diagnosis, monitoring recurrence, and predicting prognosis in NPC patients [13,14,16,26,27]. Also, plasma EBV DNA detection by real-time PCR is simple and minimally invasive[28]. For these reasons, we evaluated the effectiveness of plasma EBV DNA detection in distinguishing postradiation nasopharyngeal skull base lesions in NPC patients and found that its diagnostic value was superior to MRI. EBV is well known to be closely related to NPC. NPC cell proliferation and EBV replication result in increased plasma EBV DNA copy number [12]. However, after radiotherapy, plasma EBV DNA might be detected in several patients with NPC in clinical remission [16]. We also detected low level of plasma EBV DNA in 7 patients without recurrence. Although histopathology confirmed radiation-induced abnormalities, these patients should be followed up closely to monitor recurrence. Plasma EBV DNA is also related to tumor volume [12]. Plasma EBV DNA was not detected in 3 recurrent NPC patients with small tumor volume in our study, which led to false negative diagnosis. When plasma EBV DNA- based detection and MRI examination were combined, both the specificity and the positive predictive value were higher than those of MRI alone. When plasma EBV DNA detection was followed by MRI, the sensitivity, specificity, positive and negative predictive values were all higher than those of MRI. These results suggest that plasma EBV DNA detection followed by MRI would help to differentiate recurrence and non-recurrence. Our study indicated that plasma EBV DNA detection is useful for diagnosing recurrent NPC when postradiation nasopharyngeal skull base lesions were found by MRI in NPC patients. If both plasma EBV DNA and MRI results are positive, a biopsy is needed to avoid misdiagnosis of recurrent NPC. Nasal endoscopic biopsy is a feasible and alternative way to access in nasopharyngeal skull base regions. If the results of plasma EBV DNA detection are inconsistent with MRI results, tracking plasma EBV DNA level bimonthly or quarterly is recommended. Moreover, it is necessary to perform a surgical biopsy if plasma EBV DNA level is continuously high or increases gradually. Hence, these procedures will help improve the diagnostic accuracy and reduce the misdiagnosis rate for postradiation nasopharyngeal skull base lesions in NPC patients.

Conclusions

Plasma EBV DNA is an effective and feasible biomarker for detecting postradiation nasopharyngeal skull base lesions in NPC patients. Combining plasma EBV DNA detection and MRI would help to increase diagnostic accuracy and reduce the misdiagnosis rate, especially when nasal endoscopy is feasible in nasopharyngeal skull base regions.

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