

Disparity of Sensitivities in Detection of Radiation-Naïve and Postirradiation Recurrent Nasopharyngeal Carcinoma of the Undifferentiated Type by Quantitative Analysis of Circulating Epstein-Barr Virus DNA^{1,2}

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ABSTRACT

Purpose: The purpose of this research was to compare the sensitivities of plasma EBV DNA in detection of postirradiation locally recurrent nasopharyngeal carcinoma (NPC), postirradiation distant metastatic NPC, and radiation-naïve NPC.

Experimental Design: Twenty-four patients with postirradiation local recurrence of NPC were assessed for plasma EBV DNA levels by a real-time quantitative PCR system. The results were compared with those of a cohort of 140 patients with newly diagnosed NPC and with those of 25 patients with distant metastatic relapse. EBV-encoded RNA positivity was also assessed in locally recurrent tumors and newly diagnosed tumors with undetectable plasma EBV DNA levels.

Results: Postirradiation locally recurrent tumors were associated with a significantly lower rate of detectable plasma EBV DNA compared with radiation-naïve tumors of comparable stage [stage I-II tumors: 5 of 12 (42%) versus 47 of 51 (92%), $P = 0.0002$; stage III-IV tumors: 10 of 12 (83%) versus 88 of 89 (99%), $P = 0.01$; Fisher's exact test], and compared with distant metastatic recurrences [15 of 24 (63%) versus 24 of 25 (96%), $P < 0.02$; Fisher's exact test]. The median EBV DNA level in patients with detectable EBV

DNA was also significantly lower in locally recurrent tumors than in radiation-naïve tumors. All of the tissue samples of tumors associated with undetectable EBV DNA levels, where available, were EBV-encoded RNA positive.

Conclusions: The sensitivity of EBV DNA in the detection of tumors regrowing from an irradiated site is much lower than that from a radiation-naïve site. Although plasma EBV DNA is very effective in detecting distant metastatic relapse of NPC, it cannot be relied on as the sole surveillance tool for detection of local relapse.

INTRODUCTION

Plasma/serum EBV DNA is a recently developed marker for NPC⁴ (1). It had been shown to have a high sensitivity for detection of NPC when measured by a quantitative PCR system (2) and was undetectable in most patients in remission from the cancer (3–5). Previous reports had also indicated that patients with recurrent NPC had elevation of plasma EBV DNA levels, suggesting a potential role of the marker in surveillance for postradiation therapy recurrence (4, 5). However, the number of patients with recurrent cancer studied was very limited. In the present study, the hypothesis that EBV DNA has a similar sensitivity in detection of recurrent NPC and radiation-naïve NPC was tested by comparing the sensitivities of the marker in three groups of patients: patients with local recurrences, patients with distant metastatic recurrences, and patients with radiation-naïve tumors.

MATERIALS AND METHODS

Study Design and Patient Groups. This is a case-control study involving three patient groups. Twenty-four patients with postradiation therapy local recurrence of NPC and another 25 patients with distant metastatic relapse of NPC were recruited over a 2.5-year period for quantitative assessment of plasma EBV DNA level at the time of diagnosis of the recurrent tumor. The 24 locally recurrent tumors were diagnosed at 1.1–15.5 years (median, 4.5 years) after the initial radiation therapy; they were not associated with any evidence of distant metastases. The 25 distant metastatic recurrences were diagnosed at 1 month to 2.7 years after radiation therapy; they were not associated with any evidence of local recurrence. Blood sampling was performed soon after confirmation of diagnosis of recurrent disease and before any oncological treatment for the recurrent disease was undertaken. Because the opportunity to recruit

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⁴ The abbreviations used are: NPC, nasopharyngeal carcinoma; EBV, EBV-encoded RNA.

Table 1 Plasma EBV DNA positivity rate according to tumor stage (6) in radiation-naïve tumor group and postirradiation locally recurrent tumor group

Tumor stage	Newly-diagnosed NPC		Locally recurrent NPC	
	Total no. of patients	No. with detectable EBV DNA	Total no. of patients	No. with detectable EBV DNA
Stage I	14	12 (86%)	8	3 (38%)
Stage II	37	35 (95%)	4	2 (50%)
Stage III	40	40 (100%)	7	5 (71%)
Stage IV	49	48 (98%)	5	5 (100%)
Stage I-IV	140	135 (96%)	24	15 (63%)

patients with recurrent tumors was much less than for patients with radiation-naïve tumors, a larger sample of 140 patients with radiation-naïve tumors, which was about five times the sample size of the recurrent tumor groups, was used to obtain a length of $\leq 20\%$ on one side of a 95% confidence interval for any difference in sensitivities of EBV DNA between different patient groups. There were 2 patients in common in the locally recurrent tumor group and the radiation-naïve tumor group, but otherwise these two groups of patients did not overlap. Tumor stage definition for both the newly diagnosed tumors and the locally recurrent tumors was according to the Unio Internationale Contra Cancrum 1997 stage classification (6). Staging work-up for newly diagnosed tumors included in all of the cases clinical examination and computed tomography scan and/or magnetic resonance imaging of the nasopharynx/skull base/neck. Staging for locally recurrent tumors was based on imaging performed at the time of diagnosis of local recurrence. Histological proof was available in 22 of the 24 cases of locally recurrent tumors; in the other 2 patients whose recurrent tumors were located deep at the skull base, the diagnosis of local recurrence was based on unequivocal imaging findings. For the cases with zero copy per ml plasma EBV DNA levels, the tumor tissue blocks, if available (7 of 9 cases of locally recurrent tumors and 2 of 5 cases of radiation-naïve tumors), were retrieved for staining for EBER by *in situ* hybridization, as an indicator of the presence or otherwise of the EBV genome in the tumor cells. This tissue marker was chosen because of its consistent expression in EBV-bearing NPC cells (7).

Plasma EBV DNA Assay. Plasma EBV-DNA was measured by a quantitative PCR system by the method as described previously (2). Plasma samples were subjected to DNA extraction using a QIAamp Blood kit (Qiagen, Hilden, Germany) using the "blood and body fluid protocol" as recommended by the manufacturer. A total of 400–800 μl of the plasma samples were used for DNA extraction per column. The exact amount was documented for the calculation of the target DNA concentration. A final elution volume of 50 μl was used to elute the DNA from the extraction column. Circulating EBV DNA concentrations were measured using a real-time quantitative PCR system that amplified a DNA segment in the *Bam*HI-W fragment region of the EBV genome. The principles of real-time quantitative PCR and reaction set-up procedures were as described previously (2). Data were collected using an ABI Prism 7700 Sequence Detector and analyzed using the Sequence Detection System software (version 1.6.3). Results were expressed as copies of EBV genomes per ml of plasma. All

of the plasma DNA samples were also subjected to real-time PCR analysis for the β -globin gene, which gave a positive signal in all of the tested samples, thus demonstrating the quality of the extracted DNA. β -Globin results were expressed as genome equivalents of DNA per ml plasma. Multiple negative water blanks were included in every analysis.

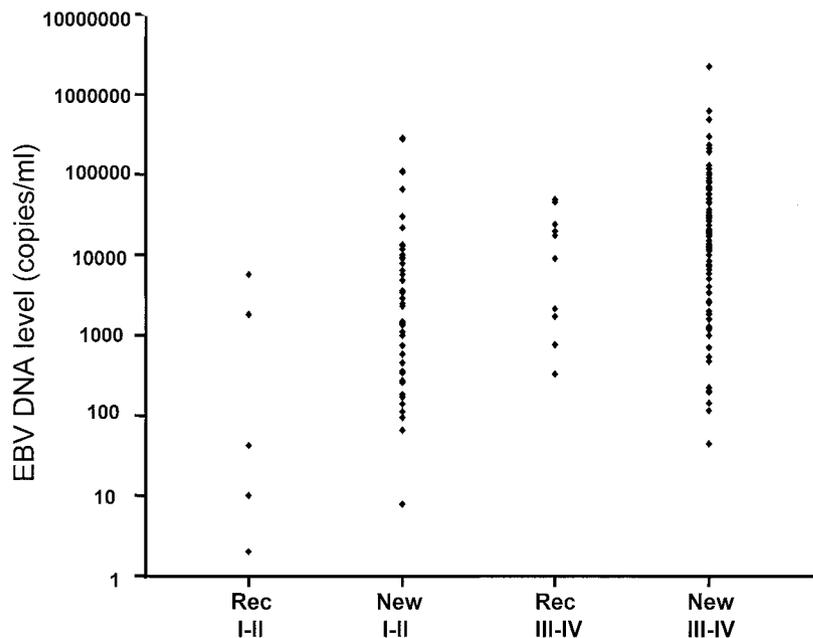
The study was approved by the affiliated institutions of the authors, and written informed consent was obtained from all of the patients studied.

RESULTS

Locally Recurrent Tumors versus Radiation-Naïve Tumors: Sensitivity of EBV DNA. The sensitivity of EBV DNA in diagnosis of locally recurrent tumors is significantly lower than in diagnosis of radiation-naïve tumors [15 of 24 (63%) versus 135 of 140 (96%); $P < 0.0001$; Fisher's exact test]. It is observed that the locally recurrent tumor group contained a higher proportion of early stage recurrent tumors than the radiation-naïve tumor group (Table 1). Table 1 also shows that EBV DNA positivity is generally more frequent in advanced-stage tumors, in both the recurrent tumor group and the radiation-naïve tumor group. In order that comparisons can be made between patient groups with comparable extent of disease, additional comparisons were carried out separately for early stage (stage I-II) tumors and advanced-stage (stage III-IV) tumors. A significantly lower rate of plasma EBV DNA positivity was found in locally recurrent tumors compared with radiation-naïve tumors of a similar stage [5 of 12 (42%) versus 47 of 51 (92%) for stage I-II tumors; $P = 0.0002$; Fisher's exact test; 10 of 12 (83%) versus 88 of 89 (99%) for stage III-IV tumors; $P = 0.01$; Fisher's exact test]. Cochran-Mantel-Haenszel Statistics was also applied to compare EBV DNA positivity rates in recurrent tumors and radiation-naïve tumors to adjust for the effect of stage; the difference was also significant ($P < 0.0001$). The test for homogeneity of the effect among stages is $P = 0.4665$, indicating that the difference in EBV DNA sensitivities between recurrent tumors and radiation-naïve tumors is independent of the effect of tumor stage.

Locally Recurrent Tumors versus Radiation-Naïve Tumors: Quantitative EBV DNA Levels. Among tumors with detectable plasma EBV DNA, the median EBV DNA level of recurrent tumors was also significantly lower than that of radiation-naïve tumors (42 copies/ml versus 2492 copies/ml in stage I-II tumors; $P = 0.03$; Wilcoxon's rank-sum test). For the 2 patients who had EBV DNA assay at the time of first diagnosis

Fig. 1 Serum EBV DNA concentrations in patients with detectable EBV DNA levels. *Rec I-II*, locally recurrent stage I-II NPC; *New I-II*, new cases of stage I-II NPC; *Rec III-IV*, locally recurrent stage III-IV NPC; *New III-IV*, new cases of stage III-IV NPC. The Y axis is in natural logarithmic scale.



and also at the time of local recurrence (as mentioned in “Materials and Methods”), both patients had detectable EBV DNA associated with the radiation-naïve tumor; 1 patient had undetectable EBV DNA for the recurrent tumor and the other had a much lower level of detectable EBV DNA associated with the recurrent tumor. Fig. 1 shows the distribution of circulating EBV DNA concentrations in different patient groups.

Locally Recurrent Tumors versus Distant Metastatic Recurrence. The sensitivity of EBV DNA in the detection of locally recurrent tumors was significantly lower than for distant metastatic recurrence [15 of 24 (63%) versus 24 of 25 (96%); $P < 0.02$; Fisher’s exact test]. In fact, the sensitivity of EBV DNA for the diagnosis of distant metastatic recurrence (24 of 25 cases; 96%) was very similar to that of stage I-IV radiation-naïve tumors (135 of 140 cases; 95%).

Tissue EBER Status. All of the cases with available tissue samples, both postirradiation locally recurrent tumors ($n = 20$) and radiation-naïve tumors with zero copy/ml of EBV-DNA levels ($n = 2$), were EBER-positive.

Relation of Plasma EBV-DNA to Histological Type. Among the 22 histology-confirmed locally recurrent tumors, the proportion of WHO type I histology was similar for tumors with zero plasma EBV-DNA levels (1 of 8 cases) and tumors with elevated plasma EBV DNA levels (2 of 14 cases).

DISCUSSION

The major cause of morbidity and mortality in patients with NPC remains postradiation therapy disease recurrence, the frequency of which rises with increasing disease stage. Currently, diagnosis of relapse relies on the detection of macroscopic disease by direct inspection of the nasopharynx or by an imaging procedure followed, when practical, by histological confirmation.

A simple, reliable, and sensitive blood test for postradiation

therapy recurrence would clearly be valuable, being less resource intensive and permitting a “whole body” screen rather than relying on symptoms to point to the site of disease. Plasma/serum EBV DNA has been shown to be a potentially useful marker for such purpose. It had been shown to be undetectable in most patients who are in remission from disease post-therapy, using qualitative (3, 5) or quantitative (4) assay systems. It was rarely detectable in healthy subjects, as shown in a previous study where only 7 of 197 (3.6%) healthy controls had detectable circulating EBV DNA, using the same assay system of the present study (8). On the other hand it was found to be frequently elevated in patients with recurrent NPC (4, 5). However, the data on recurrent NPC is very limited, because the opportunity to encounter these tumors is much less than radiation-naïve tumors, and only a few patients had been evaluated in previous studies. Although the sensitivity of the test in newly diagnosed cancer was only 59–75% using qualitative assay (3, 5), the sensitivity by the present quantitative assay system is as high as 95%. This stands in contrast with an overall sensitivity of only 62% for detection of locally recurrent tumors by the same system.

We cannot explain the disparity of EBV DNA positivity between locally recurrent tumors and radiation-naïve tumors of a similar stage. Because all of the tissues tested were, in common with the vast majority of NPC cases in Southern China, EBER-positive, an EBV DNA-negative clone developing exclusively in the recurrent NPC without any EBV DNA-positive clone seems unlikely. Whereas it is known that the WHO type I differentiated tumors are associated with greater difficulty in demonstration of presence of EBV genome compared with WHO type II and III tumors (9, 10), WHO type I tumors were uncommon in our patient population. There were only 3 cases of locally recurrent tumors of WHO type I histology, and 2 of them were associated with elevated plasma EBV DNA levels. Among

the locally recurrent tumors, similar proportions of WHO type I tumors were found in patients with zero EBV DNA levels (1 of 8) and in patients with elevated EBV DNA levels (2 of 14). The second possibility is that there is a threshold of tumor mass that is required before plasma levels start to rise, and that the locally recurrent cases have a mass that falls below this figure. This hypothesis could explain the disparity in positivities between local recurrences and distant metastatic recurrences, which are expected to contain a much higher tumor burden. However, it could not explain the disparity in positivities between treatment-naïve cases and locally recurrent cases of similar staging and thereby presumably similar tumor loads. The third possibility is that radiation can switch off the EBV DNA production/release machinery without totally disrupting the reproductive capacity of tumor cells; this hypothesis implies that nondetection of plasma EBV DNA in NPC may just be a “chopmark” of prior exposure to high-dose radiation, which may not necessarily be associated with tumor eradication, thus explaining the appreciable proportion of postirradiation recurrent tumors with undetectable circulating EBV DNA. Perhaps the most likely scenario is that the postirradiation changes in the nasopharynx, which involved decrease in vascularity and stromal fibrosis (11), somehow interfered with the efflux of EBV DNA into plasma. This hypothesis is compatible with the presence of EBV genome as shown by EBER positivity in the locally recurrent tumor tissue.

The reason for the disparity of plasma EBV DNA positivity between postirradiation locally recurrent NPC and postirradiation distant metastatic NPC is not clear. It is logical to suggest that the higher tumor burden in distant metastatic tumor accounts for its much higher detection rate. This hypothesis is supported by the known association between tumor stage and quantitative plasma EBV DNA level in an earlier study (12). An alternative hypothesis, which may not be exclusive with the tumor burden hypothesis, is that disappearance of plasma EBV DNA is primarily a marker of prior radiation exposure, and the difference in positivity between locally recurrent tumors and distant metastatic tumors is because of the presence of prior radiation exposure in the former and absence of radiation exposure in the latter. This hypothesis is based on the generally held assumption that the postirradiation distant recurrences actually originate from subclinical micrometastases in the circulation, seeded from the radiation-naïve primary tumor before the commencement of radiation therapy. In other words, the distant metastatic population, despite their clinical manifestation being temporally postirradiation, were actually derived from the preirradiation primary tumor, unlike the locally recurrent tumor cells, which regrows from the irradiated nasopharyngeal site.

On the clinical management side, the practical message from the present study is clear. It confirms that EBV DNA is a very sensitive test for the diagnosis of radiation-naïve NPC at

presentation and distant metastatic recurrent disease. However, for the detection of local recurrence, especially early stage local recurrence, post-treatment follow-up examination of the nasopharynx is essential and cannot be completely replaced by EBV DNA testing, although a positive result from the latter should nonetheless lead to inspection of the nasopharynx and other potential metastatic sites.

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