ORIGINAL ARTICLE

Role of Epstein-Barr virus DNA measurement in plasma in the clinical management of nasopharyngeal carcinoma in a low risk area

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Objective: To evaluate the role of quantitative measurement of Epstein-Barr virus (EBV) DNA in the clinical management of nasopharyngeal carcinoma (NPC) in a low tumour risk area (western Europe).

Methods: 22 consecutive Dutch NPC patients (11 europid) were studied. EBV DNA load in pretreatment and post-treatment plasma samples was determined. Three patients were also sampled at frequent intervals during treatment. RNA in situ hybridisation for the detection of EBV encoded RNAs (EBERs) was carried out on tumour biopsies of all cases.

Results: All patients with EBER positive NPC (20/22) showed a positive EBV DNA load in plasma at the time of diagnosis (median EBV DNA level, 4.1 log₁₀ copies/ml). Patients with EBER negative NPC had no detectable EBV DNA in plasma. After treatment, complete remission was achieved in all cases and concurrently EBV DNA in plasma became undetectable in all patients. In the three longitudinally evaluated cases, EBV DNA load gradually declined towards undetectable levels within three weeks after start of treatment. Two patients developed a distant metastasis with concomitant increases in EBV viral load. In addition, one EBER positive patient developed an EBER negative metastasis in the neck during follow up and in this case EBV DNA load remained undetectable at the time of recurrence.

Conclusions: Plasma EBV DNA load measurement appears to be useful in a low tumour risk area. However, development of local recurrences may not always coincide with raised levels of EBV DNA.

Nasopharyngeal carcinoma (NPC) is a disease with a remarkable geographical and racial distribution. It is highly prevalent in southern China, South-East Asia, and North Africa, but occurs relatively infrequently in Western areas, where it comprises merely 0.1% of all malignancies.^{1–4} NPC have been classified by the World Health Organisation (WHO) into three histological types: squamous cell carcinoma (type 1), non-keratinising carcinoma (type 2), and undifferentiated carcinoma (type 3). The prevalence of these three types of tumour is different in low incidence and high tumour risk areas.⁵ Furthermore, type 2 and 3 NPC are clearly associated with Epstein-Barr virus (EBV), whereas virus involvement in type 1 NPC is more controversial, especially in low tumour risk areas.⁶ Hence, both the prevalence and the association with EBV of the three histological types of nasopharyngeal carcinoma show different patterns in low tumour risk and high tumour risk populations.

Plasma or serum EBV DNA load is a recently developed marker for NPC in high tumour risk (Asian) populations. It was shown that the quantification of EBV DNA in plasma or serum, using real time quantitative polymerase chain reaction (PCR) technology, represents a promising tumour marker in patients from these areas.^{7–12} With respect to patients in these high tumour risk regions, plasma EBV DNA quantification can be a valuable tool in monitoring response to current treatments as well as in assessing the efficacy of new forms of therapy involving neo-adjuvant chemotherapy¹³ or salvage surgical treatment.¹⁴

However, little is known about the clinical use of EBV DNA load in plasma or serum from NPC patients in Western countries, where the tumour has a low incidence. The clinical relevance of this tumour marker in these low incidence areas may be different from that in regions where this tumour is

highly prevalent, as the association of NPC with EBV also differs between these areas.

The present study was conducted in a non-endemic NPC setting, to assess the clinical use of EBV DNA load measurements in plasma from NPC patients referred to a western European (Dutch) regional centre for head and neck cancer. The EBV status of the NPC from these patients was assessed using RNA in situ hybridisation (ISH) for the detection of EBV encoded RNAs (EBERs) in the tumour specimens.

METHODS

Patients

Patients with histologically confirmed NPC referred to the department of otorhinolaryngology of the Leiden University Medical Centre (Netherlands) between January 1998 and January 2005, from whom plasma samples at time of diagnosis were available, were included after informed consent. The patients' characteristics with respect to age, sex, and ethnic origin were recorded. All patients were investigated uniformly according to a routine diagnostic work up, comprising detailed clinical examination of the head and neck, nasopharyngoscopy, histological or cytological examination of tumour tissue, and radiological imaging techniques (computed tomography, magnetic resonance imaging, and ultrasonography). The tumours were subsequently staged according to the UICC staging criteria. Treatment consisted of radiotherapy or a combination of radiotherapy and neoadjuvant chemotherapy according to the stage. Additional blood samples were obtained during routine follow up after the

Abbreviations: EBER, Epstein-Barr virus encoded RNA; EBV, Epstein-Barr virus; ISH, in situ hybridisation; NPC, nasopharyngeal carcinoma; UICC, International Union Against Cancer

Table 1 Characteristics of the patients and the tumours

Patient	Age (y)	Sex	Ethnicity	Histological type	EBER-ISH	Tumour classification			Stage	EBV DNA load (log ₁₀ copies/ml)	
						T	N	M		Pretreatment	Post-treatment
1	38	F	Europid	UC	Neg	4	3	0	4b	UD	UD
2	59	M	Europid	UC	Neg	4	1	0	4a	UD	UD
3	49	M	Europid	UC	Pos	3	1	0	3	4.8	UD
4	52	M	Europid	UC	Pos	1	1	0	2b	4.1	UD
5	34	M	Indonesian	UC	Pos	3	0	0	3	4.2	UD
6	47	M	Europid	UC	Pos	1	3	0	4b	4.7	UD
7	60	M	Europid	UC	Pos	2	0	0	2	3.9	UD
8	35	M	African	UC	Pos	1	1	0	2b	4.8	UD
9	42	M	African	UC	Pos	2	0	0	2a	3.2	UD
10	36	M	Indonesian	UC	Pos	4	2	0	4a	5.1	UD
11	49	M	Europid	UC	Pos	3	2	1	4c	4.2	UD
12	39	F	African	UC	Pos	1	2	0	3	2.6	UD
13	36	M	African	UC	Pos	1	2	0	3	4.0	UD
14	47	M	Indonesian	UC	Pos	2	2	0	3	4.6	UD
15	39	M	Europid	UC	Pos	1	2	0	3	4.0	UD
16	47	M	Europid	UC	Pos	1	3	0	4b	4.3	UD
17	37	M	Europid	UC	Pos	4	2	0	4a	3.9	UD
18	60	M	Indonesian	UC	Pos	4	2	0	4a	3.4	UD
19	67	F	Indonesian	UC	Pos	1	0	0	1	3.3	UD
20	39	F	African	UC	Pos	2	1	0	2b	2.4	UD
21	45	M	African	UC	Pos	4	3	0	4b	4.2	UD
22	66	M	Europid	SCC	Pos	4	1	0	4a	2.9	UD
23	69	F	Europid	OC	NR	NR	NR	NR	NR	UD	NR
24	63	M	Europid	OC	NR	NR	NR	NR	NR	UD	NR

EBER-ISH, Epstein-Barr virus encoded RNA-in situ hybridisation; F, female; M, male; Neg, negative; NR, not relevant; OC, oropharyngeal carcinoma; Pos, positive; SCC, squamous cell carcinoma; UC, non-keratinising undifferentiated nasopharyngeal carcinoma; UD, undetectable; y, years.

completion of therapy. From three patients among the most recently referred cases, blood samples were also obtained longitudinally during treatment.

Extraction of EBV DNA and real time quantitative EBV DNA PCR

EDTA plasma samples were collected and stored at -80°C until further processing. Nucleic acids were extracted from 0.2 ml of the plasma or serum samples with the automated purification procedure of the MagnaPure LC (Roche Molecular Systems, Almere, Netherlands), using the Total Nucleic Acid isolation kit. Purified DNA was eluted in a final volume of 100 μl buffer. Real time quantitative PCR was carried out on these samples using the same primers and probes as previously described,¹⁵ but amplification was done in Qiagen Hostar Master mix (Qiagen, Hilden, Germany). The cycling protocol consisted of an activation step of 15 minutes at 95°C , and subsequently 50 cycles of 30 seconds at 95°C , 30 seconds at 55°C , and 30 seconds at 72°C for PCR amplification. The real time PCR was carried out on an iCycler iQ Multi-Colour Real Time PCR Detection System (BioRad, Veenendaal, Netherlands). Quantification has been carried out using a purified EBV standard (Advanced Biotechnologies, Columbia, Maryland, USA). To monitor the efficiency of the DNA extraction and PCR inhibition, clinical samples were spiked with a fixed amount of phocine herpes virus (PhHV) as an internal control. This target was co-amplified in the reaction and detected by a Cy5 labelled probe as described before.¹⁶

EBER in situ hybridisation

Histological specimens were fixed in 40 g/l formaldehyde formalin and routinely processed for paraffin embedment. EBV was identified by the expression of EBV encoded small RNA-1. Histological sections (4 μm) were subjected to ISH for EBV encoded small RNA-1. ISH for EBER was carried out using the Dako EBER probe and RNA ISH detection kit (Dako, Glostrup, Denmark). The slides were counterstained for 20 seconds in nuclear fast red, mounted, and examined using a standard microscope.

RESULTS

Patient characteristics and clinical outcome

We studied 22 patients with histologically confirmed NPC presenting at the Leiden University Medical Centre. Table 1 lists the characteristics of the patients and tumours. Twenty one patients had histologically proven undifferentiated nasopharyngeal carcinomas (WHO type 3) and one had squamous cell carcinoma (WHO type 1). Two non-NPC patients (with oropharyngeal carcinoma) served as negative controls. The median age at diagnosis was 45 years (range 34 to 67). Eleven patients were europid Dutch. Of the remaining 11, five were of Southeast Asian origin (Indonesia) and six of North African origin; three were first generation immigrants (born and raised in the Netherlands), while eight were immigrants. Seven of the latter had lived in the Netherlands for more than 15 years, and one for eight years. Both non-NPC patients were europid Dutch.

On completion of treatment, all patients had complete clinical responses as defined by clinical examination. However, after a median follow up of 1.5 years (range 0.3 to 5.2), one NPC patient died as a result of recurrence of his disease (patient 2: death after 10.6 months) and four other NPC patients presented with metastases: three distant (patients 11, 14, and 16) and one regional (patient 13).

Plasma EBV DNA load by tumour cell EBER status before and after treatment

EBER-ISH of nasopharyngeal tumour biopsy was positive in 20 of the 22 NPC patients (table 1), including the squamous cell carcinoma (WHO type 1). Both EBER negative NPCs were of the undifferentiated type (WHO type 3) and occurred in europid Dutch patients.

In all patients with EBER positive NPC, EBV DNA was detected in plasma at the time of diagnosis, with a median EBV DNA load of 4.1 log₁₀ (range 2.4 log₁₀ to 5.1 log₁₀), whereas in both patients with EBER negative NPC, EBV DNA was undetectable at presentation (table 1). After completion of treatment, EBV DNA load in plasma became undetectable in all EBER positive NPC patients (table 1). No EBV DNA could be detected in either of the controls (table 1).

Figure 1 Change in plasma EBV DNA concentrations in three patients with metastases of EBER positive nasopharyngeal carcinomas. Key clinical events are indicated in the respective panels. The scale of the y axis has been optimised for the concentration range for each case. (A) Five months after chemo-radiotherapy patient 13 presented with a regional metastasis in the neck. However, EBV DNA remained undetectable during follow up and the metastatic tumour cells were found to be EBER negative. (B) After an initial decrease in the EBV DNA load from 4.5 log₁₀ copies/ml before treatment to an undetectable level after treatment, EBV DNA load had reached a level of 7.4 log₁₀ copies/ml plasma at the time patient 14 presented with pulmonary metastasis. (C) Longitudinal follow up of EBV DNA load in plasma from patient 16 with lumbar spine metastasis. EBER, Epstein-Barr virus encoded RNA; EBV, Epstein-Barr virus.

Longitudinal analysis of plasma EBV DNA load after treatment, and correlation with tumour recurrence

After a median follow up of 1.5 years (range 0.3 to 5.2), five of the 22 NPC patients developed recurrence of their disease (patients 2, 11, 13, 14, and 16). Patient 2 died 10.6 months after initiation of his treatment. This case concerned an EBER negative tumour, and therefore additional plasma sampling during follow up was considered irrelevant for the purpose of this study. After completion of treatment, patient 11 was lost to follow up as he presented elsewhere, and attempts to obtain blood samples were unsuccessful. The EBV DNA load in plasma was available in three EBER positive NPC patients with metastases: regional in patient 13 and distant in patients 14 and 16.

In patient 13, after the initial decrease to undetectable levels, plasma EBV DNA was found to remain negative when a metastasis in the neck was diagnosed, five months after treatment (fig 1). After surgical removal, EBER-ISH was carried out on tumour tissue from the metastasis and vital tumour cells were found to be EBER negative.

In patient 14, pulmonary metastases were detected five months after initiation of treatment. Concurrently plasma EBV DNA load had increased to a level of 7.4 log₁₀ copies/ml plasma after the initial decrease to undetectable levels (fig 1).

In patient 16, one month after the completion of treatment, plasma EBV DNA load progressively increased from an undetectable level to 3.3 log₁₀ copies/ml plasma (fig 1). Initially no recurrences were detected but eventually positron emission tomography indicated a lumbar metastasis; by then EBV DNA load in the plasma had reached a level of 4.0 log₁₀ copies/ml plasma (fig 1).

Of the 20 EBER positive NPC patients, 16 remained disease-free after treatment and concurrently their EBV DNA load in plasma remained undetectable during follow up.

Longitudinal analysis of plasma EBV DNA load during treatment

In three EBER positive NPC patients (patients 15, 16, and 17), the course of the EBV DNA load in plasma was assessed throughout treatment and during follow up after treatment. In all three patients EBV DNA load decreased to undetectable levels within three weeks after the start of chemo-radiotherapy. In patient 15, EBV DNA load initially increased after one week of chemo-radiotherapy before it reached undetectable levels in the second week after treatment (fig 2). In patient 16 the EBV DNA load in plasma fell rapidly and became undetectable in the second week after initiation of chemo-radiotherapy (fig 1). In patient 17, the EBV DNA load decreased from 3.9 log₁₀ copies/ml plasma before treatment to an undetectable level within three weeks after start of chemo-radiotherapy (fig 2).

Correlation between plasma EBV DNA load and clinical stage

Six of 20 EBER positive NPC patients presented with stage I/II and 14 with stage III/IV disease. The median EBV DNA load in patients with stage I/II and stage III/IV disease were 3.6 log₁₀ copies/ml plasma (range 2.4 log₁₀ to 4.8 log₁₀) and 4.2 log₁₀ copies/ml plasma (2.6 log₁₀ to 5.1 log₁₀), respectively (fig 3). This difference did not reach statistical significance, most probably because of the small numbers.

DISCUSSION

In NPC patients from high tumour risk regions (such as South-East Asia), the quantification of EBV DNA in plasma

Figure 2 Longitudinal follow up of plasma EBV DNA load during and after chemo-radiotherapy in patients with EBER positive nasopharyngeal carcinomas. Key clinical events are indicated in the respective panels. The scale of the y axis has been optimised for the concentration range for each case. EBER, Epstein-Barr virus encoded RNA; EBV, Epstein-Barr virus.

Figure 3 Plasma EBV DNA concentrations according to the stage of disease. The median load, the interquartile 50% range (box), and the range of values (whiskers) are given. Median values did not reach statistical significance.

or serum can be of great clinical value, as it represents a promising non-invasive tumour marker.⁸ Furthermore, in these patients where the association with NPC and EBV is very strong, plasma EBV DNA quantification is also useful to assess the efficacy of new treatments.^{13 14 17}

A recent report by the Dutch Cancer Institute indicated that the NPC patient population in the Netherlands comprises an increasing number of patients from Asia and North Africa.¹⁸ In the same study, 88% of the 109 evaluated NPC cases were undifferentiated carcinomas and only 6% were differentiated squamous cell carcinoma. No information regarding EBV association was included in this report, but as the undifferentiated type of NPC shows the most consistent association with EBV worldwide, and is EBV associated in nearly 100% of the cases,⁴ it could be expected that plasma EBV DNA load in NPC patients in the Dutch population might be clinically relevant as a tumour marker.

In the present study, the applicability of detecting plasma EBV DNA as a tool for disease management in NPC patients in a low tumour risk area was assessed. The association between NPC and EBV was established by the detection of EBERs in tumour cells using an ISH assay. As EBERs are expressed in all forms of EBV latency, the EBER sequence is usually detectable in the nucleus of latently EBV infected cells. Furthermore, its high level of expression facilitates detection by ISH.^{19–21} Therefore, EBER-ISH has served as a diagnostic gold standard of EBV presence in tissue samples.⁶

In the current study, 21 of 22 NPC (95.5%) were of the undifferentiated type and 90% (19/21) of these undifferentiated type NPC were EBV related, as they were EBER positive. Only one NPC was a histologically proven squamous cell carcinoma, which was found to be EBER positive as well. Although squamous cell NPCs from low incidence regions are not invariably EBV associated as they are in the high incidence areas, approximately one third of the cases from a low incidence regions have been reported to be EBV positive as detected by EBER-ISH.⁶

Eleven NPC patients (50%) were euroid Dutch and two of these had EBER negative nasopharyngeal carcinomas (both were of the undifferentiated type). Although undifferentiated NPCs are considered to be consistently associated with EBV, EBER negative type 2 and type 3 NPCs have been described previously.^{22 23} In an Italian study,²³ eight of 24 NPCs (types 2 and 3), and in a recent Israeli study,²² three of 40 patients with types 2 and 3 NPCs, were negative by EBER-ISH.

In the current study, 11 patients (50%) were non-euroid immigrants from South-East Asia (Indonesia) and North Africa, all presenting with EBER positive tumours. Most of the non-euroid patients were either long term Dutch residents or first generation immigrants. It has been reported previously that migrants from high risk NPC regions continue to show high rates of NPC irrespective of their country of

Take home messages

- Plasma or serum Epstein-Barr virus DNA load, developed as a marker for nasopharyngeal cancer in high tumour risk (Asian) populations, appears also to be useful in a low tumour risk area.
- The development of local recurrences may not always coincide with raised levels of EBV DNA.

migration, and this risk remains high for subsequent generations.⁴ Thus the non-euroid patients in the current study should be considered a high tumour risk population in a low tumour risk area. However, the patients described in this study reflect the population presently encountered in Dutch hospitals and as this study was intended to assess the clinical relevance of EBV DNA load measurements in a low risk (Dutch) area, they were included in this evaluation.

All patients (euroid and non-euroid) with EBER positive NPC (20 of 22) showed significant levels of EBV DNA in the plasma at time of diagnosis, and the EBV DNA load in plasma declined to undetectable levels after treatment with radiotherapy or chemo-radiotherapy. These results are consistent with those obtained from studies in high tumour risk areas⁸ and with results described from one previous low risk cohort.^{24 25} With regard to disease recurrence, it was found that an increased EBV DNA load in plasma correlated with the development of distant metastasis in one non-euroid patient with EBER positive NPC. However, in another non-euroid patient the EBV DNA load remained undetectable despite the occurrence of regional recurrent disease (patient 13). It has previously been reported that, in contrast to distant recurrences, local recurrences of NPC in patients from high tumour risk regions cannot always be detected by EBV DNA load measurements in plasma or serum.^{26 27} It is suggested that the small tumour mass present in these regional recurrences accounts for the lower detection rate compared with distant metastases,²⁷ or that radiation affects the release mechanism of EBV DNA without disrupting the reproductive capacity of the tumour cells.²⁷ Remarkably, in the case of patient 13, EBER-ISH on tumour cells from the regional metastasis was negative, suggesting that the recurrent tumour was no longer associated with EBV. To our knowledge, this is the first report of a regional recurrence of an EBV positive NPC manifesting itself without a renewed EBV association after chemo-radiotherapy. As a result, such a redifferentiation can impose limitations on the clinical applicability of the quantitative EBV DNA assay as well as on the employment of new therapeutic approaches such as adoptive immunotherapy.¹⁷ This unusual phenomenon is currently being further examined to determine its clinical relevance.

In conclusion, plasma EBV DNA concentrations can be used as a useful molecular marker for the detection and monitoring of NPC patients in low risk regions as well as in high risk regions. No differences in the characteristics of EBV DNA concentrations were noticed between low risk patients (euroid) and high risk patients (non-euroid) living in a low risk area. However, unlike in high NPC incidence areas, where the association of this tumour with EBV is nearly 100%, a smaller proportion of NPC are EBV positive in low tumour risk regions. As a result the application of plasma EBV DNA load as an NPC tumour marker in a low risk area is restricted to cases in which the association of the tumour with EBV is confirmed by EBER-ISH on tumour tissue. Thus this assay can only be used to sustain the diagnosis of NPC and not to replace routine diagnostic procedures in a low NPC

risk area. Once the association of the tumour with EBV is established, the EBV DNA load in the plasma can be used to monitor treatment and recurrences. In this respect, another limitation also described for high tumour risk regions should be taken into account, which is that certain recurrences cannot be detected by the EBV DNA load. Thus routine post-treatment follow up is essential and cannot be completely replaced by plasma EBV DNA testing.

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