STUDY ON CHANGES OF PLASMA CELL-FREE DNA OF EPSTEIN-BARR VIRUS DURING CHEMORADIOTHERAPY OF NASOPHARYNGEAL CARCINOMA PATIENTS

Pham Quynh Huong[1]; Vu Nguyen Quynh Anh[1]; Nguyen Dinh Ung[1]; Bui Tien Sy[2]
Ngo Thanh Tung[3]; Le Minh Ky[4]; Duong Thuy Linh[5]; Nguyen Van Ba[5]
Do Tram Anh[5]; Ho Anh Son[1]; Hoang Van Luong[1]; Nghiem Duc Thuan[5]; Ho Huu Tho[1]

SUMMARY

Objectives: To study fluctuation of plasma cell-free Epstein Barr virus DNA at pre-, mid- and post-radiotherapy of nasopharyngeal carcinoma patients. Methods: 21 nasopharyngeal carcinoma patients of stages I - IVB were followed up and the sensitive realtime PCR assay with detection limit of 25 copies/mL was performed for quantification of plasma cell-free Epstein Barr virus DNA. Results: Before radiotherapy, there were 71.43% of nasopharyngeal carcinoma patients with cell-free Epstein Barr virus DNA ≥ 300 copies/mL and 23.81% of nasopharyngeal carcinoma patients with detectable cell-free Epstein Barr virus DNA of less than 300 copies/ml. After radiotherapy, these numbers were 0% and 23.81%. Patients with undetectable cell-free Epstein Barr virus DNA after radiotherapy accounted for 76.19%, which was statistically different from before radiotherapy. Data of nasopharyngeal carcinoma patients from the middle of therapy was collected after 15 - 20 fractions. Conclusion: Changes of plasma cell-free Epstein Barr virus DNA, detected by our sensitive realtime PCR assay, represent a valuable biomarker for monitoring the response of nasopharyngeal carcinoma patients to radiotherapy.

** Keywords: Nasopharyngeal carcinoma; Cell-free Epstein Barr virus; DNA; Chemoradiotherapy.*

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is malignant tumors that arise from epithelium cells of the nasopharynx - the upper part of the throat that is situated behind the nasal cavity and near the base of skull. This cancer represents a significant disease burden in Southern China and also has an intermediate incident in Southeast Asia and a low incident in most parts of the world [1, 2].

In Vietnam, according to statistics from Vietnam National Cancer Hospital (1998), NPC was the fifth most common cancer after lung, uterus, ovary, breast and liver cancer. Recently, according to the databases from the International Agency for Research on Cancer GLOBOCAN 2012 (IARC),

1. Vietnam Military Medical University
2. Military Central Hospital,
3. Vietnam National K Hospital
4. Vietnam National ENT Hospital
5. 103 Military Hospital
Corresponding author: Ho Huu Tho (huuthottncydqs@yahoo.com
Date received: 20/10/2018
Date accepted: 02/12/2018

NPC ranks as the eighth most common cause of cancer-related death for both sexes, the fifth in male, with the age - standardized rate (ASR) of 7.7/100,000 population; and for female, the rank is tenth, with the ASR of 3.4/100,000 population.

The World Health Organization (WHO) classified NPC into three different histological types, and type III (undifferentiated carcinoma) are Epstein-Barr virus (EBV)-associated and commonly seen in the endemic areas, including Vietnam. The strong association between NPC and EBV has been reported for decades in many literatures.

The quantification of cell-free EBV DNA (*cf*-EBV DNA) was demonstrated as a useful tool for early detection of NPC. Moreover, the concentration of *cf*-EBV DNA in peripheral blood of NPC patients at diagnosis have been significantly correlated with tumor sizes, response to treatment [3], tumor clearance, and tumor recurrence. Therefore, levels of plasma *cf*-EBV DNA have been used as a reliable biomarker to follow-up the status of NPC patients at the beginning, in the middle and at the end of the radiotherapy regimen. Consequently, we aim to: *Evaluate the fluctuation of cf-EBV DNA levels among NPC patients during radiotherapy, with regards to improve management and prognosis of NPC patients.*

SUBJECTS AND METHODS

1. Clinical samples.

Peripheral blood was collected from 21 patients with histologically confirmed NPC, staged I to IVB disease who were treated at Vietnam National Cancer Hospital from May 2017 to May 2018 after informed consent was obtained. Histological subtypes and tumor stages were classified according to recent WHO classification and the American Joint Committee on Cancer Staging (AJCC) on cancer tumor-node-metastasis (TNM) staging system (2010), respectively. Within 6 hours of collection, all blood samples were transported to the research laboratory and were centrifuged at 120 g for 20 min. After that the plasma was carefully removed from the EDTA tubes and transferred into 1.5 mL eppendorf. The blood-cell fractions and the plasma samples were stored at -80°C for further processing.

2. Methods.

** Clinical diagnosis:*

NPC patients were diagnosed following the pathological examination of biopsied tissues and classified by TNM staging system (AJCC - 2010) [4]. Pre-treatment evaluation and treatment strategies were performed according to National Comprehensive Cancer Network (NCCN) guidelines.

** Quantification of plasma cf-EBV DNA:*

Cf-EBV DNA was quantified using the sensitive realtime PCR assay with a detection limit of 25 copies/mL that was described in detail in our recent publication [5]. Amplification process was performed on the Rotor Gene Q realtime PCR System (Qiagen, Germany).

** Evaluation of cf-EBV DNA levels at various time points:*

The plasma *cf*-EBV DNA levels were quantified at different time points as

following: Before, after, and in the middle of the treatment (between the fraction number 15 and 20, or about the fifth week from the start of the radiotherapy).

The changes in the plasma *cf*-EBV DNA level were classified into three categories during treatment-time points: (i) undetectable of *cf*-EBV DNA; (ii) detectable of *cf*-EBV DNA lower than 300 copies/mL; and (iii) detectable of *cf*-EBV DNA higher than 300 copies/mL.

** Statistical analysis:* Data was analyzed using the GraphPad Prism 6.0 and SPSS 20.0.

RESULTS

1. Patient and tumor characteristics.

From January 2017 to January 2018, three were 21 patients that achieved the approving criteria. The average age was 50 years (range, 29 - 65 years) and male accounted for 76.19%. The demographic and clinical characteristics of patients were shown in table 1.

Table 1: Patient demographic and clinical characteristics.

Age		
Median	50	-
Range	29 - 65	-
Gender		
Female	-	5 (23.81%)
Male	-	16 (76.19 %)
Histology (WHO classification)		
Type I (keratinizing squamous cell carcinoma)	-	0 (0%)
Type II (non-keratinizing squamous cell carcinoma)	-	1 (4.76%)
Type III (undifferentiated carcinoma)	-	20 (95.24%)
Tumor stage (AJCC staging system)		
I	-	3 (14.29%)
II	-	6 (28.57%)
III	-	8 (38.10%)
IVA - B	-	4 (19.05%)

Almost the patients included in this study were classified to undifferentiated carcinoma - type III (95.24%). Only 1 out of 21 patients (4.76%) belonged to type II - non-keratinizing squamous cell carcinoma. No patient of type I (keratinizing squamous cell carcinoma) was included in this study.

NPC patients were staged according to the AJCC (2010) classification system. The distribution of the 21 NPC patients according to the tumor stages as follows: 3 patients in stage I, 6 patients in stage II, 8 patients in stage III and 4 patients in stage IV. Greater number of patients in the present research was diagnosed at late stage (III - IVB) that accounted for 57.14%. Besides that, substantial portion of patients were detected at early stage I and II (14.29% and 28.57%, respectively). This research involved different treatment regimen, including treatment with radiotherapy, in combination with or

without chemotherapy and then plasma *cf*-EBV DNA was evaluated before, during and after treatment.

2. Alterations of plasma cf-EBV DNA at different time points: before, in the middle of and after treatment.

Table 2: Concentration of plasma *cf*-EBV DNA at before/middle/after treatment.

Undetectable	1 (4.76%)	11 (52.38%)	16 (76.19%)
Detectable < 300 copies/mL	5 (23.81%)	8 (38.10%)	5 (23.81%)
Detectable ≥ 300 copies/mL	15 (71.43%)	2 (9.52%)	0 (0%)
Total (∑n)	21 (100%)	21 (100%)	21 (100%)
Mean (copies/mL)	8,983	55	16
Standard error of mean (SEM) (copies/mL)	5,385	27	10

The comparison of plasma *cf*-EBV DNA concentration was assessed in 21 patients at three time points: Before, in the middle of and after treatment. Before radiation, *cf*-EBV DNA was detected in 20/21 NPC patients (95.24%) using our sensitive realtime PCR assay, with the concentrations mainly higher than 300 copies/mL (71.43%). However, a considerable number of samples with *cf*-EBV DNA were detected at lower than 300 copies/mL (23.81%). On the contrary, patients with undetected *cf*-EBV DNA in the middle of and after treatment were predominant, 52.38% (11/21 patients) and 76.19% (16/21 patients), respectively. These changes corresponded well to the effectiveness of radiotherapy among NPC patients.

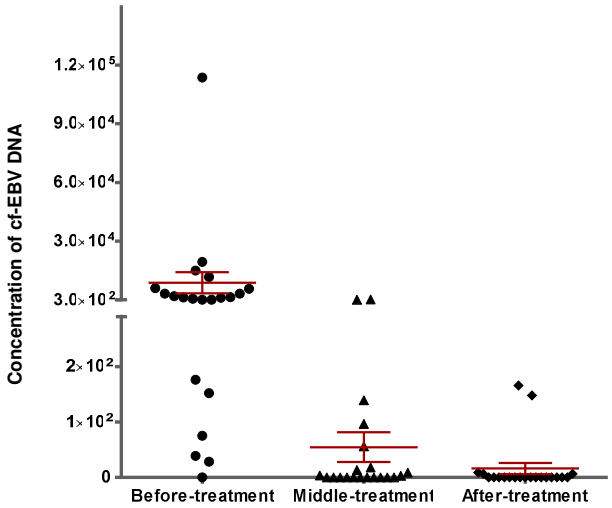

Figure 1: Comparison of mean cell-free EBV DNA concentration before, middle and after treatment of NPC patients.

(Plasma samples from NPC patients, before, middle and after treatment were analyzed for the cell-free EBV DNA concentration. Chart represents mean ± SEM. Differences in means are explained in the text)

Our data also showed a notable drop of plasma *cf*-EBV DNA concentrations at the middle of the treatment between the fractions 15 and 20. After that time point, the concentration of *cf*-EBV DNA gradually decreased, but with relatively lower rates. In particular, a mean *cf*-EBV DNA concentration of 8 983 ± 5 385 copies/mL (range, 0 - 114,000 copies/mL) was detected before treatment, then was significantly reduced to 55 ± 27 copies/mL (range, 0 - 472 copies/mL) in the middle of radiotherapy, and gradually reduced to 16 ± 10 copies/mL (range, 0 - 166 copies/mL) after treatment. Interestingly, only one case out of 21 NPC patients had undetectable *cf*-EBV DNA in plasma from the beginning till the end of the treatment, and no patient showed plasma *cf*-EBV DNA higher than 300 copies/mL after treatment. The notable decrease of the plasma *cf*-EBV DNA in the middle of the treatment may reflect the reduction of the tumor burden, which would mainly occur during the first half of the radiotherapy regimen.

3. Plasma *cf*-EBV DNA concentration and their clinical correlations.

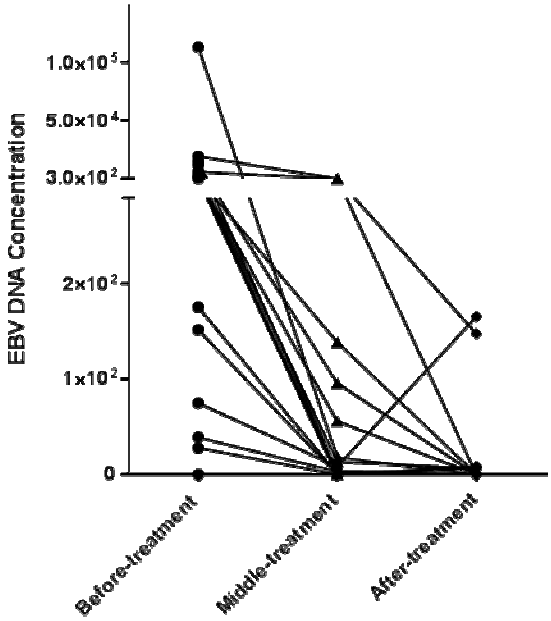

Figure 2: Changes of plasma cf-EBV DNA during treatment of 21 NPC patients**.**

Next, we examined the changes of the level of plasma *cf*-EBV DNA during radiotherapy. After 15 - 20 fractions of radiotherapy, with or without chemotherapy, depending on clinical manifestations, plasma *cf*-EBV DNA became undetectable in approximately 50% of NPC patients. At the end of the regimen, the number of cases with undetectable *cf*-EBV DNA further increased to 76.19%. On other hand, number of case with plasma *cf*-EBV DNA higher than 300 copies/mL dropped from 71.43% at beginning to 9.52% in the middle, then to zero case at the end of the radiotherapy. The difference of the plasma *cf*-EBV DNA between the two time points,

before and middle of the chemoradiotherapy is statistically significant with $p < 0.001$ (Wilcoxon test). On the other hands, the difference of the plasma *cf*-EBV DNA between the middle and the end point of the treatment is just borderline with $p = 0.099$.

Interestingly, two patients showed a persistence of plasma *cf*-EBV DNA during their treatment process and above 100 copies/mL after radiotherapy. In detail, the plasma *cf*-EBV DNA concentration gradually reduced through three time points (6 - 290 copies/mL, 336 copies/mL and 148 copies/mL, respectively) in one case; while in the other patient, the plasma *cf*-EBV DNA concentration decrease from 75 copies/mL before treatment, to 8.5 copies/mL in the middle of the treatment, then suddenly increased to 166 copies/mL. During follow-up, both of these two cases were then determined to have a relapse and bone metastasis, while the others have been reported to be recurrence-free.

DISCUSSION

Nasopharyngeal carcinoma is endemic in Southern Asia, and type III - undifferentiated carcinoma (WHO classification) is the most common type of NPC and that was also proven in our data with the amount of patients harboring undifferentiated carcinoma accounted for 95.24%. Clinical signs and symptoms of NPC are often nonspecific, so it is difficult to detect, particularly, in the early stage [6], leading to high risk of relapse or recurrent metastasis. Over the past 15 years, plasma *cf*-EBV DNA has been recognized as a valuable biomarker for early detection and prognosis of undifferentiated NPC [7], especially for monitoring treatment response of NPC patients. The highly sensitive assay of *cf*-EBV DNA quantification has been successfully established by our group, for the first time, in Vietnam for the detection of early NPC lesions with the sensitivity of 96.9%. This assay, which is a blood-based or “liquid biopsy” testing, is a noninvasive procedure [8]. In this study, our sensitive assay for quantification of *cf*-EBV DNA has been utilized for detection and monitoring of residual disease during treatment process of NPC patients.

As shown in figure 1, the association of *cf*-EBV DNA among three time points of radiotherapy (before, in the middle and after treatment) of NPC patients has been described. In detail, high plasma *cf*-EBV DNA level (≥ 300 copies/mL) was frequently detected in plasma of untreated NPC patients (71.43%) and a majority of the patients (19/21) experienced a significant decline in *cf*-EBV DNA following radiotherapy. The results of quantification of *cf*-EBV DNA higher than 300 copies/mL at two following time points of middle and after treatment were 9.52% and 0%, respectively, decreased significantly than before treatment. So, all of NPC patients after treatment in this study had detectable *cf*-EBV DNA below 300 copies/mL results. This data was reaffirmed the important role of using sensitive quantitative *cf*-EBV DNA assay for monitoring and assessing treatment outcome of NPC patients.

There was a significant difference between the level of *cf*-EBV DNA at the beginning and the middle NPC (*figure 2*). Our data was showed interesting association of post-treatment *cf*-EBV DNA levels with the treatment outcome. In particular, two

cases had *cf*-EBV DNA persistent during treatment, and its levels were still above 100 copies/mL at the end of the treatment. These patients were diagnosed skeletal recurrence metastasis recently. Other patients, who had undetectable or below-detection-limit levels of *cf*-EBV DNA, haven't shown any evidence of relapse or recurrent metastasis. These clinical observations underscore and confirm the prognostic value of plasma *cf*-EBV DNA for monitoring and guiding optimal strategies with each NPC patient.

CONCLUSION

This study showed the changes of plasma *cf*-EBV DNA concentrations during treatment of NPC patients using the high sensitive *cf*-EBV DNA quantification assay. The promptitude and high accuracy make this assay not only an excellent candidate for screening and early detection of NPC among high-risk populations, but a valuable tool for monitoring the response to radiotherapy of NPC patients.

REFERENCES

1. Yu M.C, J.M. Yuan. Epidemiology of nasopharyngeal carcinoma. Semin Cancer Biol. 2002, 12 (6), pp.421-429.

2. Razak A.R et al. Nasopharyngeal carcinoma: The next challenges. Eur J Cancer. 2010, 46 (11), pp.1967-1978.

3. Hsu C.L et al. Plasma Epstein-Barr virus DNA concentration and clearance rate as novel prognostic factors for metastatic nasopharyngeal carcinoma. Head Neck. 2012, 34 (8), pp.1064-1670.

*4. Edge S.B, C.C. Compton. T*he American Joint Committee on Cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol. 2010, 17 (6), pp.1471-1474.

5. Ho H.T et al. Establishment of ultrasensitive PCR assay targeting cell-free EBV DNA for early detection of nasopharyngeal carcinoma. 2017, 59 (3), p.7.

6. Tabuchi K et al. Early detection of nasopharyngeal carcinoma. J Otolaryngol. 2011, p. 638058.

7. Ambinder J.K.a.R. The Biology and Clinical Utility of EBV Monitoring in Blood.