Pre-treatment serum inflammatory cytokines as survival predictors of patients with nasopharyngeal carcinoma receiving chemoradiotherapy

ADEL F. AL-KHOLY[1], OMMINEA A. ABDULLAH[1], MAMDOUH Z. ABADIER[1], MANAL M. HASSAAN[2], MOHAMED F. SHINDY[3], DALIA M. NOR EL-DIEN[4] and ALI HASANEEN[5]

[1]Department of Medical Biochemistry, Faculty of Medicine, Benda University, Benha 13511; [2]Department of Medical Biochemistry, Faculty of Applied Medical Sciences, Cairo 11811; Departments of [3]Otorhinolaryngology, [4]Clinical Pathology and [5]Internal Medicine, Faculty of Medicine, Benha University, Benha 13511, Egypt

Received May 31, 2016; Accepted August 10, 2016

DOI: 10.3892/mco.2016.1041

Abstract. The present study aimed to examine the predictability of pre-treatment serum levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α for determining the outcome of patients with nasopharyngeal carcinoma (NPC) assigned for chemoradiotherapy. A total of 35 patients with NPC were subjected to clinical examination and evaluation of performance status using Karnofsky scoring. Nasopharyngoscopy was performed for evaluation and to obtain a biopsy. Blood samples were obtained pre- and post-treatment for polymerase chain reaction quantitative estimation of Epstein-Barr virus (EBV) DNA plasma load and enzyme-linked immunosorbent assay for estimation of serum cytokines. All patients received chemoradiotherapy and were followed-up for 2 years. Cervical lymphadenopathy and recurrent attacks of epistaxis are the most common presenting symptoms. Treatment significantly decreased pre-treatment plasma EBV DNA load and serum levels of IL-6 and TNF-α, and increased serum IL-1β levels. Clinical staging and EBV DNA plasma load revealed positively significant correlation with pre-treatment serum levels of both IL-6 and TNF-α, while revealed negative significant correlation with serum IL-1β levels. The 2-year survival rate was negatively significantly correlated with pre-treatment levels of IL-6 and TNF-α, and EBV DNA viral load, while it was positively significantly correlated with pre-treatment performance scores and serum IL-1β levels. Statistical analyses defined high pre-treatment serum IL-6 levels as a significant specific predictor for high mortality rate. It was demonstrated that NPC was associated with high pre-treatment plasma EBV DNA load and serum cytokines, and chemoradiotherapy significantly reduced these high levels. High pre-treatment serum IL-6 level was a significant specific predictor for high mortality rate. Increased post-treatment serum levels of IL-1β indicated good therapeutic response and most probably a high survival rate.

Correspondence to: Dr Adel F. Al-Kholy, Department of Medical Biochemistry, Faculty of Medicine, Benda University, Farid Nada Street, Benha 13511, Egypt
E-mail: adel.alkholy@fmed.bu.edu.eg

Abbreviations: NPC, nasopharyngeal carcinoma; IL, interleukin; EBV, Epstein-Barr virus; TNF-α, tumor necrosis factor-α

Key words: nasopharyngeal carcinoma, inflammatory cytokines, plasma EB viral load, survival rate, chemoradiotherapy

Introduction

Nasopharyngeal carcinoma (NPC) is a group of malignant epithelial tumors with different etiopathogeneses and a broad range of histopathological appearances (1). Populations with elevated rates include the natives of Southeast Asia, the natives of the Artic region, and the Arabs of North Africa and parts of the Middle East (2). The well-known excess risk for NPC in North Africa is confirmed, with rates reaching the level of 5.4 in men and 1.9 in women, which are 10-times higher compared with that in Europe (3).

Epstein-Barr virus (EBV) is a ubiquitous, gamma-1 lymphotropic virus linked to NPC (4). Decoy receptor (DcR) 3 was overexpressed in NPC and its higher expression scores were observed in metastatic NPC; suggesting that DcR3 may enhance cell metastatic potential (5).

EBV-infected cells secrete EBV-encoded small RNAs, leading to the induction of type-I interferon and inflammatory cytokines, and subsequent immune activation (6). EBV latent membrane protein (LMP) 1 is an oncogenic protein (7) capable of upregulating IL-1α and IL-1β secretions from epithelial cells and is positively modulated by TNF-α (8). LMP1-expressing cells exhibited increased rates of haptotactic migration. Extracellular-regulated and mitogen-activated protein kinases (MAPK) may contribute to the oncogenicity of LMP1 through its ability to promote cell motility and enhance the invasive properties of epithelial cells (7).

The present prospective study aimed to determine the predictability of pre-treatment serum levels of IL-1β, IL-6

and TNF-α for the outcome of patients with NPC assigned for chemoradiotherapy.

Patients and methods

Patients. The current multi-center study was performed between May 2011 and December 2015, including 2-year follow-ups. The study protocol was approved by the Local Ethics Committee of Benha University, October 6 University and Tanta University. All enrolled patients or their nearest relatives provided written informed consent agreeing to the methodology for investigations and modalities of therapy prior to enrolment. The study included 35 patients with biopsy confirmed NPC. A total of 10 healthy volunteers were selected from those attending Benha University Blood Bank for blood donation after passing examination protocol for blood donation for being serologically negative for HCV, HBsAg and HIV, and with no history of previous infectious mononucleosis, no otorhinolaryngology diseases, recent infection or surgery within the last 3 months as a control for results of laboratory investigations.

Otorhinolaryngological evaluation. Patients were subjected to an assessment of full history, clinical examination with respect to nasopharyngeal region, nasopharyngoscopy and computed tomography and/or magnetic resonance imaging (MRI) to determine the full extent of the local and nodal spread of the tumor. The patients were clinically categorized using tumor-node-metastasis staging, according to the American Joint Committee for Cancer (AJCC) staging (9).

Nasopharyngoscopy was performed under general anesthesia to allow proper visualization, lesion identification and biopsy taking. Using a rigid 0˚, 30˚ sinus endoscope cupped biopsy forceps, the biopsy specimen was obtained, including the marginal adjacent tissue and the tumor itself. Specimens were maintained in prepared preservative and sent for histopathological examination. Pathological findings were graded according to the World Health Organization, which has classified NPC into three categories: i) WHO-1, defined as well-to-moderately differentiated squamous or transitional cell carcinoma with keratin production; ii) WHO-2, which is non-keratinizing carcinoma; iii) WHO-3, which is undifferentiated carcinoma, including lymphoepithelioma (10).

Performance status evaluation. All patients were evaluated for performance status criteria using the Karnofsky performance scale (KPS) (11). Performance status evaluation was performed pre-treatment and 6-months post-treatment for 2 years. Inclusion criteria included pathology proven to be WHO type II-III NPC, stage III/IV according to the AJCC staging criteria, no distant metastasis, an expected lifespan of at least 6 months, KPS score ≥70, neutrophil count >$2x10^9$/l and platelet count >$100x10^9$/l prior to treatment, and bilirubin <1.5 mg/dl, AST/ALT <2 times the upper limits of normal, serum creatinine <1.5 mg/dl and creatinine clearance rate >50 ml/min prior to treatment. The exclusion criteria included previous radiotherapy to the head and neck region, previous surgery in the primary tumor site or neck (unless for diagnostic biopsy), history of malignant tumors or simultaneous multiple tumors, a positive pregnancy test result for women of reproductive age, impaired renal or hepatic functions, diabetes mellitus or cardiac diseases.

Laboratory assessments. Blood samples were collected from patients prior to and following completion of their chemoradiotherapy course. The collected blood samples were divided into two samples.

The first sample was collected in EDTA-containing tubes and the plasma was separated immediately and stored at -80˚C until use for polymerase chain reaction (PCR) qualitative identification of EBV DNA and quantitative estimation of EBV DNA plasma load, according to the manufacturer's protocol (12). Briefly, a 200 µl aliquot of plasma from each sample was used. The DNA was extracted from the samples using the QIAamp® DNA minikit (Qiagen, Hamburg, Germany). The extracted DNA was quantified and checked for purity using a spectrophotometer (Shimadzu, Kyoto, Japan). Quantification of EBV DNA copies in plasma-derived DNA was performed using the iCycler iQ™ Real Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The quality of purified DNA from plasma samples was confirmed by conventional PCR amplification of the human β-globin gene using the following gene-specific primers: Forward: 5'-AGGAGT GGTGGCTCATGTCT-3' and reverse: 5'-CTCAAGGGATCC TCCCATTT-3'. Primers flanking the *Bam*H1W region (EBV coordinate: 14,649-14,724) of the EBV genome and TaqMan® probe (Applied Biosystems, Foster City, CA, USA) directed within this flanked region (EBV coordinate: 14,672-14,698) were reported by Lo *et al* (12) and were custom-made (Applied Biosystems). An aliquot of 5 µl purified DNA isolated from the plasma was used for amplification in a total reaction volume of 50 µl, which contained the following components: 300 nM each primer, 25 nM TaqMan® probe and TaqMan® PCR reagents. The amplification reaction for each sample and standard was performed in duplicate. The standard curve correlating the viral DNA copy to threshold cycle was constructed by amplifying 5 µl aliquots of serially diluted DNA isolated from Namalwa cells that contained 45, 450, 45,000, 100,000 and 450,000 EBV DNA copies/ml. The fluorescence detection threshold value was set at 10x the mean standard deviation of fluorescence in all reactions. EBV DNA load, expressed as viral copy number/ml of plasma, was determined using the following equation: EBV DNA copy/ml = Q (VE / VA) x 1 / VP. Where Q is the DNA copy determined from standard curve, VE is the volume of DNA eluent (50 µl), VA is the volume of DNA template amplified (5 µl) and VP is the volume of plasma used for DNA extraction (200 µl).

The second sample was maintained in a plane container and allowed to clot. The serum was subsequently separated by centrifugation for 10 min at 1,000 x *g* using a refrigerated centrifuge. Serum was removed and stored in pyrogen-free Eppendorf tubes at -80˚C until assayed for estimation of serum levels of IL-1β (Quantikine ELISA kit; R&D Systems, Inc., Minneapolis, MN, USA) (13), IL-6 (Pelikine™ Inc., Concord, MA, USA) (14) and TNF-α (Pelikine™ Inc.) (15) using ELISA kits, according to the manufacturer's protocols.

Chemoradiotherapy procedure. All patients were assigned to receive the appropriate chemoradiotherapy at the Nuclear Medicine Department, Cancer Institute, Tanta University

(Tanta, Egypt). Patients received radiotherapy by the simplified intensity modulated radiotherapy technique to shorten the radiation time in each fraction, with prescription doses of 70, 66.5/68.25 and 61.25 Gy (in 35 fractions) for high-risk gross tumor volume of primary tumor and metastatic lymph nodes, and clinical target volume, respectively. Patients with low-risk clinical target volume, referred to levels IV and Vb without metastatic cervical lymph nodes, were radiated with 54 Gy/30 fractions in the anterior-posterior fields. All patients received neoadjuvant chemotherapy prior to radiotherapy with the TPF regimen (docetaxel 75 mg/m^2 and cisplatin 75 mg/m^2 on day 1, and 5-florouracil at 500 mg/m^2 on day 3) on continuous intravenous infusion for 120 h every 3 weeks for 3 cycles. Complete blood count, liver function and renal function were tested prior to each chemotherapy course, and only patients with a qualifying index were allowed to proceed with the next chemotherapy course. Concurrent chemoradiotherapy was administered following three courses of neoadjuvant chemotherapy. Concurrent chemotherapy consisted of weekly cisplatin (40 mg/m^2) during radiotherapy, with a maximum of seven courses (16).

Follow-up. Patients completed their follow-up by attending Otorhinolaryngology ENT and Internal Medicine outpatient clinics every 3 months for 2 years for nasopharyngoscopy and neck palpation and for MRI at 3 months after radiotherapy, then every 6 months. Performance status evaluation was performed 6-monthly following treatment for 2 years.

Statistical analysis. The sample size was calculated using the standard nomogram proposed by Kraemer and Thiemann (17). Considering the increasing frequency of NPC in North Africa (3), a sample size of >30 patients was determined to be sufficient to detect a difference at the 5% significance level and to give the trial 60% power (18). Sample size and power were recalculated and assured using Power and Sample Size Calculation Software program (version 3.1.2) provided by Department of Biostatistics, Vanderbilt University (Nashville, TN, USA). The obtained data were presented as the mean ± standard deviation, ranges, numbers and ratios. The results were analyzed using one-way analysis of variance, with post-hoc Tukey HSD test and χ^2. Sensitivity and specificity of estimated parameters as survival predictors were evaluated using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) compared with the null hypothesis that AUC=0.05. Possible associations were investigated using Spearman linear regression. Statistical analysis was performed using the SPSS (version 15) statistical package for Windows (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

The present study included 35 patients with NPC. Clinical presenting symptoms, clinical stage and pathological grading data are shown in Table I. All patients exhibited a gradual decrease of their performance scores throughout the follow-up period, but the extent of decrease was gradual with non-significant differences ($P>0.05$) between patient frequency among higher scoring items at 6 months post-treatment compared with pre-treatment frequency. Thereafter, at 12, 18 and 24 months post-treatment, the frequency of patients with high performance scores was significantly lower compared with the pre-treatment frequency. The mean of collective scoring throughout the follow-up period was significantly lower compared with the pre-treatment collective score (Fig. 1).

The mean pre- and post-treatment serum levels of the investigated cytokines were significantly higher compared with the control levels. The mean post-treatment plasma EBV DNA viral load and serum levels of IL-6 and TNF-α were

Table I. Enrolled patient data.

Patient information	Findings
Demographic data	
Age, years (range)	54.5±11.8 (37-71)
Gender (male:female)	2.18:1
Smoking history (%)	
Current	17 (48.6)
Ex-smoker	8 (22.8)
Non-smoker	10 (28.6)
Occupational exposure frequency	13 (37.1)
Presenting symptoms (%)	
Cervical lymphadenopathy	23 (65.7)
Recurrent epistaxis	16 (45.7)
Secretory otitis media	10 (28.6)
Headache	9 (25.7)
Otalgia	7 (20)
Frequency of symptoms (1:2:3:4:5)	17:9:7:1:1
Mean number (range)	1.86±1 (1-5)
Clinical stage (Stage II:III:IV)	13:13:9
Pathological grading[a] (Type 1:2:3)	15:12:8

[a]According to the World Health Organization. The data are presented as the mean ± standard deviation.

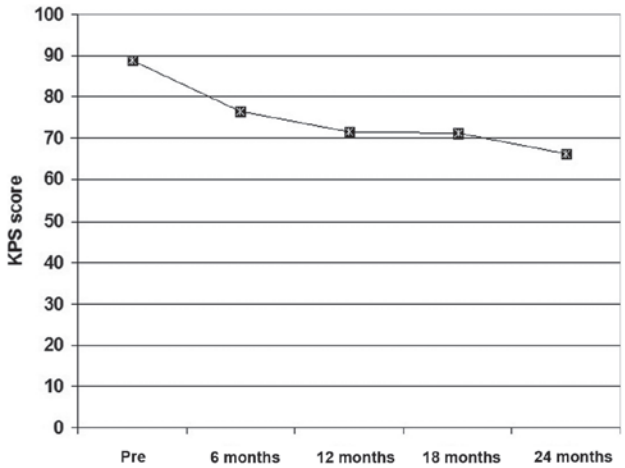

Figure 1. Mean collective KPS determined throughout the 2-year follow up compared with the pre-treatment score. KPS, Karnofsky performance score; Pre, pre-treatment.

Table II. Pre- and post-treatment laboratory findings of studied patients compared with control levels.

Cytokine level	Control	Patients		P-value[a]
		Pre-treatment	Post-treatment	
Serum IL-1β, ng/ml (P-value[b])	1.75±0.4	2.44±0.53 (0.012)	3.03±1.1 (0.035)	0.001
Serum TNF-α, ng/ml (P-value[b])	3.64±1.17	13.39±4.51 (0.0004)	11.06±2.71 (0.0004)	0.002
Serum IL-6, ng/ml (P-value[b])	13.53±2.84	189.89±43.36 (0.0001)	35.52±13.92 (0.0003)	0.0001
Plasma EBV DNA load, copies/ml	-	2,112.11±595.62	63.93±27.16	0.0001

[a]Comparing pre- and post-treatment levels; [b]Compared with control levels. The data are presented as mean ± standard deviation. IL, interleukin; TNF, tumor necrosis factor; EBV, Epstein-Barr virus.

significantly lower, but post-treatment serum levels of IL-1β were significantly higher compared with the pre-treatment levels (Table II).

Throughout the 2-years follow-up period, nine patients succumbed to mortality, giving a 2-year mortality rate of 25.7%. A total of 5 mortalities were associated with radiation-related injuries; 2 patients succumbed to secondary development of mucosal necrosis, 2 due to massive gastrointestinal hemorrhage and 1 secary to development of radiation encephalopathy. A total of 2 mortalities were secondary to local regional therapy failure and another 2 were secondary to distant metastasis.

At end of the 2-year follow-up, collective mortality rates were 44.5, 23.1 and 15.4% for patients with pre-treatment clinical stage IV, III and II, respectively. The 2-year survival rate demonstrated negative significant correlation with serum levels of IL-6 and TNF-α, and with plasma levels of EBV DNA viral load. It also demonstrated positive significant correlation with serum levels IL-1β and KPS; however, demonstrated positive non-significant correlation with pre-treatment clinical staging. Detailed correlations between the investigated parameters are shown in Table III.

ROC curve analysis defined high pre-treatment serum IL-6, serum TNF-α and plasma EBV DNA as sensitive predictors of mortality, with descending order of significance compared with the null hypothesis of AUC=0.5, while high serum IL-1β as a specific predictor for survival with significantly higher AUC compared with the null hypothesis (Table IV and Fig. 2). Regression analysis defined high pre-treatment serum IL-6 as a specific significant predictor for mortality (β=-0.529, t=3.584, P=0.001).

Discussion

Clinical presentation of studied cases of NPC was mosaic and non-specific. The major presenting symptoms were cervical lymphadenopathy, epistaxis of unexplained origin and varied otological symptoms and signs. Similarly, Adham *et al* (19) reported that the initial diagnosis of NPC is difficult to make since early signs and symptoms of NPC are not specific to the disease.

The estimated pre-treatment plasma EB viral DNA load were decreased significantly following the completion of the treatment protocol and demonstrated a positive significant correlation with clinical tumor aggressiveness. These findings indicated a close association between the presence of NPC and EBV viremia, and the probability of reliance on estimation of plasma EBV DNA load as a non-invasive diagnostic modality for NPC that provided a pre-treatment knowledge about disease stage and response to applied therapy.

In line with these data, Ji *et al* (20) reported that at the cutoff point of 0 copies/ml plasma EBV DNA had a sensitivity, positive and negative predictive values of 86.8, 30 and 99.3%, respectively, for NPC detected within the first year of follow-up. Additionally, it had a sensitivity of 81.5 and 100% for patients who had early and advanced NPC, respectively. Hutajulu *et al* (21) documented elevated viral DNA in the patient circulation, as well as nasopharyngeal site underline the role of EBV for NPC development.

In trials to explore the association between NPC and EBV infection, experimental studies using NPC lines infected *in vitro* with EBV showed that *in vitro* EBV infection resulted in the activation of signal transducer and activator of transcription (STAT)-3 and nuclear factor-κB signaling cascades in nasopharyngeal epithelial cells. This resulted in increased expression of their downstream targets. These findings suggest that EBV infection may manipulate multiple cellular signaling cascades to protect infected cells from immunological attack and to facilitate cancer development (22,23).

Estimated post-treatment levels of IL-6 and TNF-α were significantly decreased compared with pre-treatment levels that showed positive significant correlation with clinical staging and EBV DNA plasma load; a finding indicating a close association between NPC and high serum levels of both inflammatory cytokines. The detected association between clinical staging and pre-treatment plasma EBV DNA viral load on one side and pre-treatment serum cytokines was approved experimentally by Zhang *et al* (24) who reported that EBV-infected nasopharyngeal epithelial cells exhibited enhanced response to IL-6-induced STAT-3 activation through overexpression of the IL-6 receptor, thus promoting growth and invasive properties EBV-positive NPC cells. Ansari *et al* (25) observed that EBV facilitates its genome persistence and evasion of host immune responses through activation of caspase-1, which cleaves the pro-forms of inflammatory IL-1β, IL-18 and IL-33 cytokines. Song *et al* (26) suggested that TNF-α may be a promoter for NPC local spread and metastasis through the induction of inhibitor of apoptosis proteins, which contribute to both tumor

Table III. Spearman correlation between various factors.

	2-year survival		Plasma EBV DNA		Clinical staging	
Factor	Rho	P-value	Rho	P-value	Rho	P-value
Karnofsky performance score	0.379	0.025	-0.395	0.017	-0.716	0.0009
Clinical staging	-0.248	>0.05	0.499	0.002	-	-
Serum IL-1β (ng/ml)	0.415	0.013	-0.425	0.011	-0.555	0.001
Serum TNF-α (ng/ml)	-0.458	0.006	0.350	0.039	0.394	0.019
Serum IL-6 (ng/ml)	-0.541	0.001	0.504	0.002	0.806	0.0004
Plasma EBV DNA (copies/ml)	-0.401	0.017	-	-	-	-

IL, interleukin; TNF, tumor necrosis factor; EBV, Epstein-Barr virus.

Table IV. Receiver operating characteristic curve analysis of clinical staging and laboratory findings as predictors for 2-year survival determined by AUC.

Factor	AUC	SE	P-value	95% CI
Clinical stage	0.346	0.109	>0.05	0.132-0.560
Serum IL-1β (ng/ml)	0.774	0.085	0.016	0.607-0.940
Serum TNF-α (ng/ml)	0.199	0.105	0.008	-0.007-0.405
Serum IL-6 (ng/ml)	0.143	0.076	0.002	-0.005-0.291
Plasma EBV DNA load (copies/ml)	0.235	0.106	0.019	0.028-0.442

AUC, area under curve; SE, standard error; CI, confidence interval; IL, interleukin; TNF, tumor necrosis factor; EBV, Epstein-Barr virus.

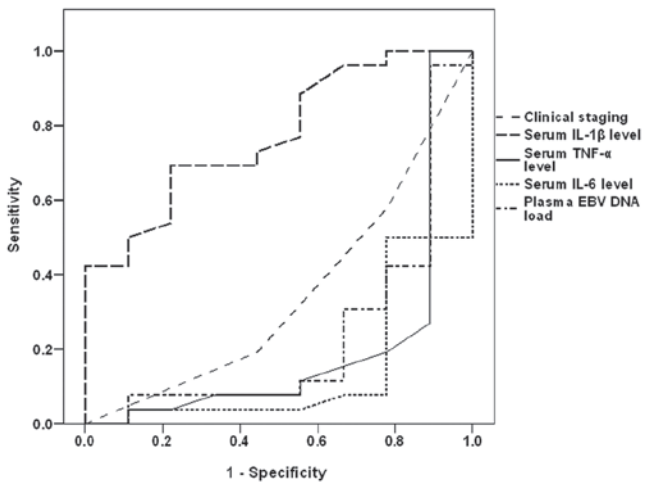

Figure 2. Receiver operating characteristic curve analysis of the clinical staging and pre-treatment laboratory data as predictors for the 2-year survival.

progression and tumor metastasis. Liao *et al* (27) used NPC cell lines to detect that IL-6 promoted NPC cell proliferation in a dose- and time-dependent manner, and this was accompanied by increasing cyclin D1 and Bcl-2 expression, STAT-3 activation, and inhibition of Bax and p21 expression. Liu *et al* (28) observed that in certain macrophages of the tumor stroma of NPC tissue, IL-6- and TNF-α-dependent expression of tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase led to suppressed proliferation of T cells and impaired cytotoxic activity of CD8(+) T cells, thus facilitating immune escape.

Notably, the estimated pre-treatment levels of IL-1β were significantly lower compared with post-treatment levels and had negative significant correlation with clinical staging and plasma EBV DNA viral load. These data may indicate an abnormal behavior of IL-1β, which despite being an inflammatory cytokine, appears to have anticancer action. In line with these findings, Chen *et al* (29) experimentally reported that tumor inflammasomes, which are critical for IL-1β production, serve a key role in tumor control by recruiting neutrophils, and their expression levels manifested by increased levels of IL-1β are favorable prognostic markers and promising therapeutic targets in patients.

The reported 2-year survival rate demonstrated negative significant correlation with pre-treatment serum levels of IL-6 and TNF-α, while showed positive significant correlation with pre-treatment serum level of IL-1β. Statistical analyses defined high pre-treatment serum IL-6 as a significant specific predictor for high mortality rate. These findings go in hand with Lu *et al* (30) who reported that pre-treatment serum levels of IL-2 and TNF-α were closely associated with the overall survival of patients with NPC, with >2-fold increase in the risk of mortality in patients with low IL-2 expression and/or high TNF-α expression compared with those with high IL-2 or low TNF-α levels. Visconti *et al* (31) demonstrated that elevated IL-6 and IL-10 levels appear to be independently associated with worse prognosis in terms of overall and disease-free survival in cancer patients. Reitter *et al* (32) reported that

elevated levels of IL-6, IL-8 and IL-11 were associated with worse survival of cancer patients. Cheng *et al* (33) revealed that NPC patients who had high IL-8 levels had significantly shorter overall survival and disease-free survival.

In conclusion, NPC is associated with high pre-treatment plasma EBV DNA load and serum cytokines, and chemo-radiotherapy significantly reduced these high levels. High pre-treatment serum IL-6 level is a significantly specific predictor for high mortality rate. Increased post-treatment serum levels of IL-1β indicated good therapeutic response and most probably high survival rate.

Acknowledgements

The authors would like to thank staff members, assistants, technicians and workers at Tanta Cancer Institute for providing assistance for researchers and patients during the chemoradiotherapy.

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