Applicability of Serum Interleukin-6 as a Screening Tool for Nasopharyngeal Carcinoma

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Abstract: Objectives: To evaluate the applicability of estimated serum IL-6 as a screening marker for nasopharyngeal carcinoma (NPC). **Patients & Methods:** The study included 30 patients with biopsy confirmed NPC and 10 healthy volunteers as control group for serum IL-6. Patients were subjected to full history taking, clinical examination with respect to nasopharyngeal region, nasopharyngoscopy and CT and/or MRI. Patients were clinically categorized using TNM clinical staging according to the American Joint Committee for Cancer Staging and biopsy findings were graded according to The World Health Organization (WHO) classification of NPC. All patients were assigned to receive their appropriate chemo-radiotherapy and completed their follow-up through attending ENT outpatient clinic. Blood samples were collected from patients prior to and after completion of their chemo-radiotherapy course for quantitative estimation of Epstein Barr virus (EBV) DNA plasma load and estimation of serum IL-6. **Results:** Mean pre-treatment serum IL-6 was significantly higher than control group. Plasma viral load showed a positive significant correlation with clinical staging, but the correlation was non-significant with WHO pathological type of the lesion. Estimated serum IL-6 levels showed positive significant correlation with plasma viral load, TNM clinical staging and WHO pathological type of the lesion. Estimated pre-treatment serum IL-6 levels showed high sensitivity for detection of early cases of NPC, while both parameters showed high specificity for determination of high WHO pathological grade. Treatment induced significant decrease of both serum IL-6 level and plasma viral load compared to pretreatment level, however post-treatment mean serum level of IL-6 was still significantly higher compared to pre-treatment level. **Conclusion:** Estimation of serum IL-6 could be used as a screening test for suspicious population especially wherein NPC was non-endemic for its high sensitivity and could be coupled with estimation of EBV DNA plasma load wherever available for proper diagnosis
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Key wards: Nasopharyngeal carcinoma, Interleukin-6, Epstein-Barr virus, quantitative PCR

1. Introduction

Nasopharyngeal carcinoma (NPC) is a disease with an extraordinary geographic and racial distribution worldwide. Except for certain populations, NPC is a rare human malignancy with an incidence well under 1 per 100, 000 population per year, constituting less than 0.3% of all malignant tumors and only 2% of all head and neck cancer **(Yu and Yuan, 2002)**.

Etiology of NPC is complex, involving multiple factors including genetic susceptibility, infection with the EBV and exposure to chemical carcinogens. During development of the disease, viral infection and multiple somatic genetic and epigenetic changes synergistically disrupt normal cell function, thus contributing to NPC pathogenesis **(Tao and Chan, 2007)**.

Epstein-Barr virus is a ubiquitous human gamma-herpes virus that infects resting B cells, stimulates their proliferation, and induces the outgrowth of virus-transformed lymphoblastoid cell lines expressing the nuclear antigens and the latent membrane proteins LMP1, LMP2A, and LMP2B **(Chen *et al.*, 2006)**. The LMP1 is of particular interest because it induces the oncogenic transformation of rodent fibroblast cell lines, is also essential for EBV-mediated primary B cell transformation *in vitro*, **(Iwakiri *et al.*, 2006)** and is associated with a number of human malignancies such as Hodgkin's disease, undifferentiated nasopharyngeal carcinoma, and EBV-related lymphoproliferative disease **(Pattle *et al.*, 2006)**. EBV-encoded small RNAs are secreted from EBV-infected cells and are recognized by toll-like receptor leading to induction of type-I interferon and inflammatory cytokines, and subsequent immune activation **(Iwakiri & Takada, 2010)**.

Of particular interest is the reported ability of LMP1 to induce production of cytokines; the EBV-encoded deoxyuridine triphosphate nucleotidohydrolase (dUTPase) is able to induce immune dysregulation *in vitro* as demonstrated by the inhibition of the replication of stimulated peripheral

blood mononuclear cells (PBMCs) and the upregulation of several proinflammatory cytokines including TNF-α, IL-1β, IL-8, IL-6, and IL-10 produced by unstimulated PBMCs treated with purified EBV-encoded dUTPase. Depletion of CD14-positive cells (monocytes) eliminated the cytokine profile induced by EBV dUTPase treatment. These data support the hypothesis that at least one protein of the EBV early antigen complex can induce immune dysregulation and may be involved in the pathophysiology of EBV-associated disease, **(Morris *et al.*, 2009, Lai *et al.*, 2010, Huang *et al.*, 2010)**.

The present study aimed to estimate pre-treatment serum levels of IL-6 in patients with biopsy confirmed NPC and to correlate it with clinical staging, pathological grading and EBV DNA plasma load estimated using quantitative so as to determine applicability of estimated serum IL-6 as a screening marker for NPC.

2. Patients & Methods

After obtaining a written fully informed patients consent; 30 patients with biopsy confirmed NPC were enrolled in the study. The study also included 10 healthy volunteers chosen 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 or recent infection or surgery within the last 3 months.

Patients were subjected to full history taking, clinical examination with respect to nasopharyngeal region, nasopharyngoscopy and CT and/or MRI to determine the full extent of the local and nodal spread of the tumor. Patients were clinically categorized using TNM staging according to the American Joint Committee for Cancer Staging **(AJCC, 2002)**.

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

All patients were assigned to receive their appropriate chemo-radiotherapy at Nuclear Medicine Department, Cancer Institute, Tanta University; patients with lesions staged T1N0M0/T2N0M0 received external radiotherapy and brachytherapy boost and patients with lesions staged T1- T2, ≥N1, M0 or T3-T4, any N, M0 received external radiotherapy plus concomitant chemotherapy. Radiotherapy was applied in daily fractionated dose of 2Gy/fraction for a total dose of 66Gy for cases with T1 and/or N1 lesions and 70 Gy for lesions of ≥T2 and/or ≥N2. Patients completed their follow-up through attending ENT outpatient clinic as follows: nasopharyngoscopy and neck palpation every 3 months for 2 years and MRI at 3-months after radiotherapy, then 6 monthly.

Laboratory investigations:

Blood samples were collected from patients prior to and after completion of their chemo-radiotherapy course for qualitative identification of EBV DNA and quantitative estimation of EBV DNA plasma load and estimation of serum IL-6.

Blood samples were collected from patients in EDTA-containing tubes and plasma was separated immediately and stored at -80ºC until use for DNA extraction according to the manufacturer's instructions, **(Lo *et al.*, 1999)**.

Another blood sample was obtained from both patients and controls; collected blood samples were kept in a plane container and allowed to clot then serum was separated by centrifugation at 3000 rpm for 10 min. Serum was removed, placed in pyrogen-free Eppendorf tubes and stored at -80°C until assayed (within one month) for estimation of serum levels of IL-6 with an ELISA kit from Pelikine™ Inc., Concord, USA. IL-6 values were read using a 96 well microplate ELISA reader (Dynatech. MR 7000), **(Gaines-Das *et al.*, 1993)**.

Detection of EBV infection by PCR technique on plasma samples

1. A 200 µL aliquot of plasma from each sample was used. DNA was extracted from samples using the QIAamp® DNA minikit (Qiagen, Hamburg GmBH, Germany).
2. 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, Hercules, CA, USA).
3. The quality of purified DNA from plasma samples was validated by conventional PCR amplification of the human β-globin gene using gene-specific primers:
   - βF: 5'-AGGAGTGGTGGCTCATGTCT-3'
   - βR: 5'-CTCAAGGGATCCTCCCATTT-3'.

4. Primers flanking the *Bam*H1W region (EBV coordinate: 14649-14724) of the EBV genome and TaqMan® probe (Applied Biosystems, Foster City, CA, USA) directed within this flanked region (EBV coordinate: 14672-14698) as reported by **Lo *et al.* (1999)**.
5. An aliquot of 5 µL of 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 of each primer, 25 nM of TaqMan® probe, TaqMan® PCR reagents.
6. 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 per ml. The fluorescence detection threshold value was set at 10× the mean standard deviation of fluorescence in all reactions.
7. EBV DNA load, expressed as viral copy number per ml of plasma, was determined using the following equation: EBV DNA copy/ml = Q (VE/VA)*1/VP. Where; Q: DNA copy determined from standard curve; VE: volume of DNA eluent (50 µL), VA: volume of DNA template amplified (5 µL) and VP: volume of plasma used for DNA extraction (200 µL).

Statistical analysis

Data were presented as mean±SD, ranges, numbers and ratios values. Results were analyzed using Wilcoxon' s test for unrelated data. Possible relationships were investigated using Pearson linear regression. Specificity and sensitivity of estimated serum IL-6 for diagnosis of NPC were evaluated individually against EBV DNA plasma load using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) and significance was adjusted against the null hypothesis that AUC=0.5. Statistical analysis was conducted using the SPSS (Version 15, 2006) for Windows statistical package. *P* value <0.05 was considered statistically significant.

3. Results

The study included 30 patients with NPC with mean age of 56.8±8; range: 38-71 years. There were 21 male and 9 female patients with male:female ratio of 2.33:1. Nineteen males (63.3%) were smokers, while the other 11 patients (36.7%); 4 males and 7 females were non-smokers. Seven patients (23.3%); 5 males and 2 females had past history of occupational exposure to dust and wood dust.

Clinical presentation was a composite picture of multiple symptoms, however, cervical lymphadenopathy was the main presenting symptom in 20 patients (66.7%), followed by recurrent attacks of epistaxis that had no medical cause in 16 patients (53.3%) and 7 patients (23.3%) had secretory otitis media. Headache was the main symptom in 3 patients (10%), and otalgia in 4 patients (13.3%).

All patients were free of distant metastasis (M_0). Ten patients were staged II; 5 were $T_2N_0M_0$, 3 were $T_1N_1M_0$ and 2 patients were staged $T_2N_1M_0$. Another 12 patients had stage III lesions; 5 were staged $T_3N_1M_0$, 4 were staged $T_1N_2M_0$ and 3 patients were staged $T_2N_2M_0$. Eight patients had stage IV lesions; 3 patients were staged $T_4N_1M_0$, another 3 were staged $T_2N_3M_0$ and only 2 patients had $T_3N_3M_0$ lesions. Histopathological examination of biopsies taken revealed the presence 14 specimens of WHO type 1, 10 specimens of WHO type 2 and 6 specimens WHO type 3, (Table 1).

Table (1): Clinical and histopathological categorization of cases of NPC

Clinical staging		Histopathological grading	
Stage II	10 (33.3%)	WHO type 1	14 (46.7%)
Stage III	12 (40%)	WHO type 2	10 (33.3%)
Stage IV	8 (26.7%)	WHO type 3	6 (20%)

Data are presented as numbers; percentages are in parenthesis

Qualitative PCR could detect EPV-DNA, (Fig. 1) in all blood samples. Mean pre-treatment EBV DNA viral load as estimated by quantitative PCR was 2126.2±665; range: 1098-3248 copies/ml. The mean pre-treatment serum IL-6 was 175.6±32.8; range: 128-235 ng/ml and was significantly higher than mean serum IL-6 level estimated in controls; 26.3±3.5; range: 19-29 ng/ml, (Fig. 2).

Plasma viral load showed a positive significant correlation with clinical staging of the lesion, (r=0.604, *p*=0.026), but the correlation was non-significant (r=0.344, *p* >0.05) with WHO pathological type. On contrary, estimated serum IL-6 levels showed positive significant correlation plasma viral load, (r=0.428, *p* =0.018), TNM clinical staging of the lesion, (r=0.432, *p* =0.017) and WHO pathological type, (r=0.513, *p* =0.004).

Both serum IL-6 levels and plasma viral load were significantly higher in patients staged IV compared to those staged II and III with non-significantly higher levels in patients staged III. Moreover, estimated serum IL-6 levels were significantly higher in patients had lesions type 3 compared to those had lesions type 1 and 2 with a non-significantly higher levels in patients with lesions type 2 compared to those with lesions of type 1. On

contrary, plasma viral load showed non-significant difference among patients categorized according to WHO histopathological type of lesion, (Table 2).

Table (2): Mean pre-treatment levels of IL-6 and EBV DNA load in studied patients categorized according to TNM clinical staging and WHO pathological grading

		IL-6 (ng/ml)	Statistical analysis		EBV DNA (copies/ml)	Statistical analysis	
			t	*p*		t	*p*
TNM stages	Stage II (n= 10)	154±25.1			1815.6±502		
	Stage III (n=12)	172.3±17.8	1.396	p_1>0.05	1988.4±532	0.437	p_1>0.05
	Stage IV (n=8)	212.3±26.7	3.866 3.761	p_1=0.006 p_2=0.007	2721.1±700	2.754 2.709	p_1=0.028 p_2=0.030
WHO types	Type 1 (n= 14)	167.3±22.8			1846.9±555		
	Type 2 (n= 10)	168.7±38	0.339	p_3>0.05	2253.4±630	2.194	p_3=0.056
	Type 3 (n= 6)	206.7±29.1	3.128 4.423	p_3=0.026 p_4=0.007	2565.8±756	2.573 1.458	p_3=0.050 p_4>0.05

p_1: significance versus TNM stage II
p_2: significance versus TNM stage II
p_3: significance versus WHO type 1
p_4: significance versus TNM WHO type 2

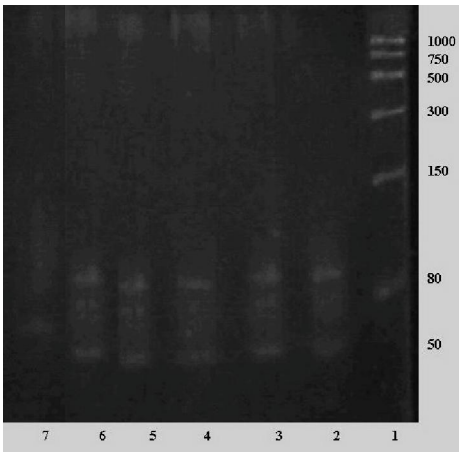

Fig. (1): Electrophoretic analysis of EBV amplification product. The 80 bp was obtained. Lane 2,7: positive & negative controls, respectively. Lanes 3,4,5,6: NPC specimens positive for EBV. Lane 1: DNA standard size marker.

Evaluation of predictability of estimated pre-treatment serum IL-6 level and EBV DNA plasma load for clinical staging and pathological grading of lesions using ROC curve analysis revealed that estimated pre-treatment serum IL-6 level showed high sensitivity with an AUC=0.183 that showed significant difference (p=0.004) compared to the null hypothesis that AUC=0.5, while pre-treatment plasma viral load showed an AUC=0.403 which showed non-significant (p >0.05) difference compared to the null hypothesis, (Fig. 3). On the other hand, both parameters showed high specificity for determination of high WHO pathological grade with AUC that showed significant difference compared to the null hypothesis; AUC=0.823, p =0.016 for serum IL-6 and AUC=0.771, p =0.043 for plasma viral load, (Fig. 4).

Treatment induced significant decrease of both serum IL-6 level (42.7±10; range: 23-58 ng/ml) and plasma viral load (52.1±25; range: 20-102 copies/ml)) compared to pretreatment level, however post-treatment mean serum level of IL-6 was still significantly higher compared to pre-treatment level, (Fig. 2 & 5).

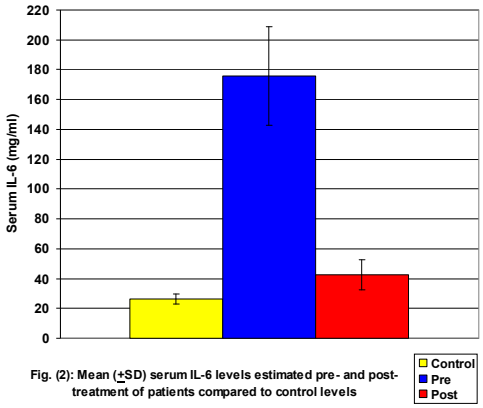

Fig. (2): Mean (±SD) serum IL-6 levels estimated pre- and post-treatment of patients compared to control levels

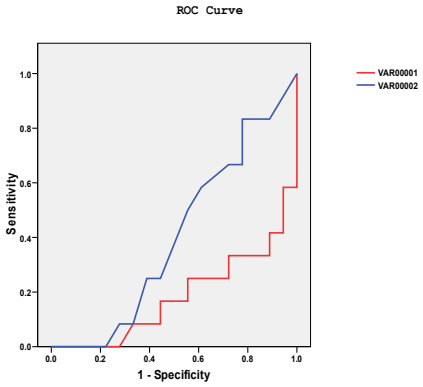

Fig. (3): ROC curve analysis of predictability of pre-treatment estimated levels of IL-6 and EBV DNA viral load for TNM staging of lesions.

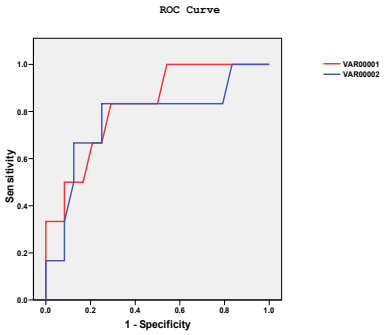

Fig. (4): ROC curve analysis of predictability of pre-treatment estimated levels of IL-6 and EBV DNA viral load for WHO grading of lesions.

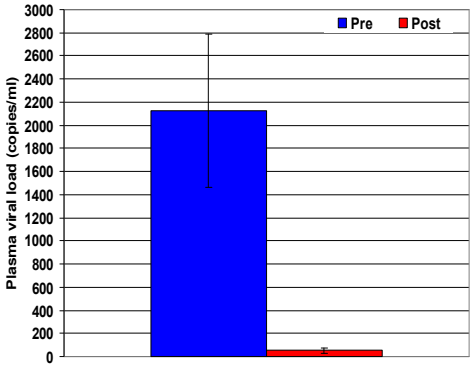

Fig. (5): Mean (±SD) pre- and post-treatment plasma EBV DNA level determined in patients and controls

Example of the studied cases (Case No. 7)

A male patient aged 70 years presented by recurrent epistaxis without evident systemic cause for epistaxis. CT examination (Fig. 6a) showed on coronal scan a nasopharyngeal mass in the vault of nasopharynx (*). Nasal endoscopy showed a fleshy mass in the vault of the nasopharynx (T) with multiple hemorrhagic spots and areas of necrosis. The mass bled easily on touch. The mass could be visualized through both right (Fig. 6b) and left (Fig. 6c) nostrils.

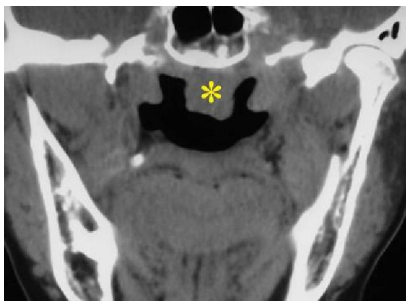

Fig. (6a): CT imaging (coronal view) showing a mass suspended from the vault of nasopharynx (*)

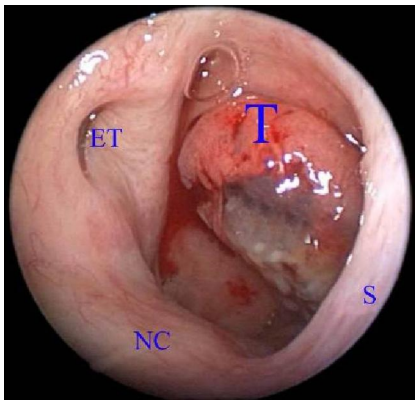

Fig. (6b): Nasal endoscopic view through right nostril showing a mass in the vault of nasopharynx (T) with hemorrhagic spots and areas of necrosis. The mass bled on touch. S: nasal septum, ET: Eustachian Tube: NC nasal cavity

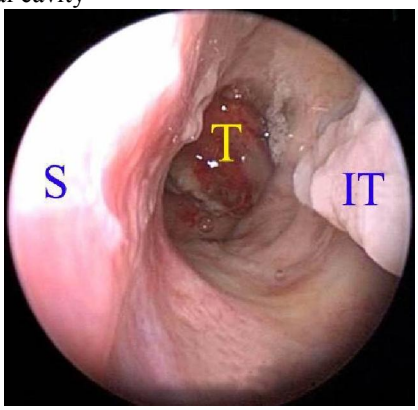

Fig. (6c): Nasal endoscopic view through left nostril showing the mass in the vault of nasopharynx (T) with hemorrhagic spots. S: nasal septum, IT: inferior turbinate

4.Discussion

The current study detected high pre-treatment plasma viral load in all studied blood samples that significantly decreased after completion of treatment protocol, a finding indicating a close relationship between presence of NPC and plasma viral load which may be causal or concomitant or result relationship, however, indicating intimate relation between EBV viremia and presence of NPC. In hand with this outcome, there was a positive significant correlation between plasma viral load and tumor spread as manifested clinically and non-significant with WHO pathological grading, so not related to the extent of cancer differentiation.

Similarly, high pre-treatment serum levels of IL-6 showed significant decrease after completion of treatment protocol and showed a positive significant correlation with both clinical and pathological grading. These obtained data indicated interplay between these three heads of the triangle; NPC, IL-6 and EBV, which

heavily studied experimentally for establishment. **Stewart *et al.* (2004)** and **Lo *et al.*(2006)** established NPC lines stably infected in vitro with EBV and found these infected cells, similar to primary tumors of NPC, exhibited a type II EBV latency expression pattern and in vitro EBV infection resulted in the activation of signal transducer and activator of transcription (STAT)-3 and NF-κB signal cascades in nasopharyngeal epithelial cells with increased expression of their downstream targets, these findings suggest that EBV latent infection is able to manipulate multiple cellular signal cascades to protect infected cells from immunologic attack and to facilitate cancer development. Also, **Buettner *et al.* (2006)** stated that LMP-1 expression in epithelial cells may be regulated through a loop involving activated STAT3, LMP1, LMP1-mediated induction of IL-6 expression and STAT3 activation through the IL-6 receptor.

These early studies were further documented recently; **Chew *et al.*(2010)**, suggested that EBV LMP1 was able to confer resistance of apoptosis and increased MMP-9 production in NPC cells and when cultured cells expressing the EBV LMP1 were treated with IL-6 displayed increased MMP-9 production, up-regulation of bmi-1 oncogene expression and down-regulation of nasopharyngeal carcinoma-associated gene 6, a tumor suppressor gene, expression and concluded that these findings implicate the roles of EBV LMP1, laminin and IL-6 in the promotion of invasion and metastasis in NPC. **Zhao *et al.* (2012)** documented that LMP1 expression is positively associated with metastasis in NPC and **Yoshizaki *et al.*(2012)** reported that LMP1 upregulates each step of metastasis, and contribute to highly metastatic feature of NPC, a tumor suppressor gene p53 is mostly intact and overexpressed in NPC whereas expression of p16, a cyclin-dependent kinase inhibitory protein, is down-regulated in 2/3 of NPC. Moreover, in a feedback fashion, **Murata *et al.*(2012)**, reported that IL-6-modulated inducible nitric oxide synthase expression via STAT3 and epidermal growth factor receptor in EBV-associated NPC and documented that such epigenetic alteration may occur by controlling the DNA methylation through IL-6-mediated JAK/STAT3 pathways.

The reported elevated pre-treatment serum IL-6 levels was found to have significant predictability for the presence of NPC, so could be applied as a screening test for detection of suspicious cases especially where NPC is not endemic and in patients with ambiguous presentation. In hand with these assumptions; **St John *et al.* (2004)** detected significantly higher concentrations of IL-6 in serum of patients with oral cavity and oropharyngeal squameous cell carcinoma (OSCC) and confirmed these results at both the mRNA and the protein levels, and using statistical analysis these findings indicated that IL-6 in serum hold promise as biomarker for OSCC. De **Schutter *et al.* (2005)** found IL-6 pretreatment serum level above the median was the only independent predictor of local control, disease-free survival and overall survival of patients with head and neck squameous cell carcinoma (HNSCC) including NPC and **Riedel *et al.*(2005)** found the majority of the patients with HNSCC have significantly high serum IL-6 concentrations compared to healthy individuals with a significant correlation with tumor stage and a significant difference of IL-6 serum concentration of tumors with positive and negative lymph nodes and concluded that IL-6 serum determinations might serve as a biological marker and help to identify advanced HNSCC. **O *et al.*(2007)** found NPC screening in a high-risk, non-endemic population using serologic markers is effective and series testing is a statistically sound and economically feasible strategy and concluded the necessity of the development of a cost-effective NPC screening strategy in a high-risk, non-endemic population. **Chang *et al.* (2011)**, reported that the levels of IL-6, IL-8, interferon-inducible protein 10 and macrophage inflammatory protein-3α were significantly elevated in patients with advanced disease stages and correlated with EBV DNA and concluded that simultaneous, large-scale measurement of multiple cytokines may improve NPC detection and prognostic prediction

The obtained data and review of literature allow concluding that estimation of serum IL-6 could be used as a screening test for suspicious population especially wherein NPC was non-endemic for its high sensitivity and could be coupled with estimation of EBV DNA plasma load wherever available for proper diagnosis.

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