Serum Epstein Barr Virus as a Biomarker in Nasopharyngeal Carcinoma

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Abstract: Objectives: To assess the role of polymerase chain reaction (PCR) quantitative estimation of plasma Epstein-Barr viral (EPV) DNA load as a non-invasive diagnostic and/or prognostic modality for Egyptian nasopharyngeal carcinoma (NPC) patients. **Patients & Methods**: All patients underwent fiberoptic direct nasopharyngoscopy for nasopharyngeal inspection and to obtained tissue biopsy of a suspicious growth for pathological examination and grading according to WHO pathological grading. Primary tumor extent was evaluated by both MRI and CT scanning. Two venous blood samples were collected; one prior to initiation of therapy and at 4-weeks after the last session of radiotherapy for quantitative PCR determination of EBV plasma DNA loads. For primary treatment: external radiotherapy and brachytherapy boost for patients with lesions staged T1/T2 N0 M0; external radiotherapy + concomitant chemotherapy +adjuvant chemotherapy and uni- or bilateral neck block dissection for persistent nodal disease at 3 months for patients with nodal lesions. Follow-up included nasopharyngoscopy three-monthly for 2 years. **Results:** The study included 45 patients; 23 patients (51.1%) had nodal involvement. According to TNM staging; 3 patients had carcinoma in situ, 6 had stage-I, 12 had stage-II, 15 had stage-III and 9 patients had stage-IV lesions; 22 lesions were WHO grade I, 14 were grade II and 9 lesions were WHO grade III. There was a positive significant correlation between WHO pathological and TNM staging. MRI detected local invasion in 12 patients (26.7%). Qualitative PCR detected EBV viral DNA in all blood samples. Using Quantitative PCR technique, mean EBV DNA plasma load was 2188±642 copies/ml. There was a positive significant correlation between mean plasma viral load and TNM stage and WHO grade. Post-treatment quantitative PCR detected EBV DNA only in 10 (22.2%) patients with a mean plasma level of 61.5±33.7 copies/ml that was significantly lower than pre-treatment levels. **Conclusion:** PCR quantitative estimation of plasma Epstein-Barr viral DNA load is a valuable diagnostic test that showed a positive significant correlation with both TNM staging and WHO pathological grading of patients with NPC and could be used to assess the response to applied therapeutic modalities. Considering Egypt as a non-endemic area for NPC, quantitative estimation of EBV plasma load could be used as screening test for patients presenting by symptoms suspicious of NPC.
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1. Introduction

Nasopharyngeal carcinoma (NPC) has remarkable epidemiological features, including regional, racial, and familial aggregations. Moreover, it seems that there are some significant geographical and ethnic variables within the country, which predispose people for high incidence of NPC. The distinctive racial/ethnic and geographic distribution of NPC worldwide suggests that both environmental factors and genetic traits contribute to its development. It has been reported to be prevalent in three widely different populations; namely, Chinese in South East Asia, Arabs in North Africa and Eskimos in the Arctic **(Baizig *et al.*, 2011; Cao *et al.*, 2011; Kimura *et al.*, 2011)**.

At least three etiological factors; namely, ubiquitous Epstein Barr virus (EBV) infection, genetically determined susceptibility, and associated environmental factors are possibly contributing for the high incidence of NPC in various Chinese populations. However, environmental factors are numerous and appear to have a secondary role, mainly in the promotion of the neoplastic process **(Chang & Adami, 2006)**.

Epstein-Barr virus is a ubiquitous human gamma-1 lymphotrophic virus infecting over 90% of the adult population worldwide. Primary EBV infection usually occurs early in life and is followed by life long virus persistence, which again is asymptomatic in most cases. EBV is etiologically linked to NPC, endemic to Southern China, and Burkitt lymphoma endemic to equatorial Africa, both of which are rare elsewhere in the world **(Busson *et al.*, 2004; Chang *et al.*, 2009)**.

Why EBV is associated with different malignancies in different geographic regions remains puzzling and may be related to EBV genotypic variability through specific disease and geographic associations. Evaluation of the role of genetic

polymorphisms in NPC development reported a consistent evidence for associations of NPC and a handful of genes, including immune-related human leukocyte antigen (HLA) Class I genes, DNA repair gene RAD51L1, cell cycle control genes MDM2 and TP53, and cell adhesion/migration gene MMP2 **(Hildesheim & Wang, 2012)**.

Considering Egypt is a part of Upper Africa, whoever, it is non-endemic area for NPC; it is of prime interest to know if the plasma viral DNA load is elevated in Egyptian NPC cases. Thus, the present study aimed to assess the role of PCR quantitative estimation of plasma Epstein-Barr viral DNA load as a non-invasive diagnostic and/or prognostic modality for Egyptian NPC patients.

2. Patients and Methods

This prospective study was conducted at Departments of Otorhinolaryngology and Medical Biochemistry, Faculty of Medicine, Benha & Menufea Universities and Department of Radiation Oncology & Nuclear Medicine Departments, Faculty of Medicine, Menufea University.

The study comprised patients with suspected NPC. All patients underwent complete history taking and full general examination. Local NP examination was conducted using fiberoptic direct nasopharyngoscopy to allow inspection of the inside of the NP for abnormal growths, bleeding, or other signs of disease. If a suspicious growth was found, a true-cut biopsy was obtained for histopathological examination and graded according to WHO pathological grading: WHO-1 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 **(Neel *et al.*, 1993)**.

Fine needle aspiration biopsy of cervical lymph nodes was performed in patients presented with enlarged lymph nodes in the neck area especially if the primary lesion was not accessible. The primary tumor extent was evaluated by both MRI and CT scanning especially if MRI demonstrated erosion into the base of the skull by virtue of the change in signal of fatty bone marrow; CT scan was performed for assurance of presence of bone erosion. Metastatic work-up included chest X-ray, thoracic and abdominal CT scan and bone scintigraphy.

Epstein - Barr virus DNA Identification

Sampling:

Two venous blood samples were collected; one prior to initiation of therapy and another at 4-weeks after the last session of radiotherapy. Blood samples were collected in EDTA containing tubes, and then plasma was separated immediately and stored at -80ºC until use.

Quantitative EBV plasma DNA viral load determination

1. DNA was extracted from samples using the QIAamp® DNA minikit (Qiagen, Hamburg GmBH, Germany) according to the manufacturer's instructions.
2. 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:
   - Forward primer:
   - 5'-AGGAGTGGTGGCTCATGTCT-3'
   - Reverse primer:
   - 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) were reported by **Lo et al., (1999)** and were custom-made (Applied Biosystems, Foster City, CA, USA).
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 consisting of:
   - 4mM $MgCl_2$
   - 200 µM each of dATP, dCTP and dGTP
   - 400 µM of dUTP
   - 0.5 U AmpErase uracil N-glycosylase (UNG)
   - 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA).
6. Amplification reaction for each sample and standard was performed in duplicate.
7. 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.
8. To assess the inter-assay reproducibility of RTQ-PCR assay, the Namalwa DNA standards in five logs of concentration as mentioned were amplified in triplicates on four separate days. Amplification cycling was initiated by incubation at 50ºC for two minutes for UNG activation and initial denaturation at 95ºC for eight minutes, followed by 40 cycles of 95ºC for 30 seconds and 56ºC for one minute.
9. A standard curve was only accepted if the correlation coefficient is 0.996 or higher and its

slope ranged between -3.74 and -3.32, which correlated to amplification efficiency of between 85 and 100%, respectively.

10. The fluorescence detection threshold value was set at 10× the mean standard deviation of fluorescence in all reactions.
11. Viral load, expressed as viral copy number per ml of plasma, was determined as follows: 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)
VP: volume of plasma used for DNA extraction (200 µl).

Treatment Strategy included

a. For primary treatment: external radiotherapy and brachytherapy boost for patients with lesions staged T1/T2 N0 M0; external radiotherapy + concomitant chemotherapy +adjuvant chemotherapy and uni- or bilateral neck block dissection for persistent nodal disease at 3 months for patients with lesions staged T1- T2, ≥N1, M0/ T3-T4, any N, M0.
b. Salvage treatment for recurrent disease included external radiotherapy, salvage surgery and chemotherapy for patients with recurrent T1 N0/ T2 N0 lesions; neck dissection for patients with T≥0, recurrent N1, M0/ T2 N0 lesions and chemotherapy for patients with recurrent T3/recurrent T4 , any recurrent N lesions
c. Treatment for NPC with distant metastasis included chemotherapy, external radiotherapy and supportive care.

Follow-up included nasopharyngeal fiberoptic endoscopy and neck palpation every 3 months for 2 years. Dental examination was conducted every 6 months. Imaging studies included MRI and/or CT every 2-3 months after end of external radiotherapy for all T lesions and MRI and/or CT every 6 months for 2 years for T2 lesions.

Statistical analysis

Obtained data were presented as mean±SD, ranges and ratios. Results were analyzed using Wilxocon Ranked (Z-test) test for unrelated data. Possible correlations were studied using Pearson's correlation coefficient. 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 45 patients; 33 males and 12 females with a mean age of 59.4±10.4; range: 33-78 years. Female patients were significantly (*P*<0.05) older than males. Irrespective of gender distribution only 3 patients (6.6%) were younger than 40 years old and 5 patients (11.2%) older than 70 years while the remaining patients were in range of 40 to 70 years. Mean BMI of studied patients was 30.5±2.5; range: 24.8-35.4 Kg/m^2 and females had significantly (P<0.05) higher BMI compared to male patients. There were 31 smokers (68.9%); 28 males and 3 females; smokers were significantly (*P*<0.05) younger than non-smokers (Table 1).

Table (1): Patients' data

Gender; M:F		33:12	
Age (years)	Total		59.4±10.4 (33-78)
	<40 years	3 (6.7%)	35.3±2.5
	40-50 years	6 (13.3%)	47.3±1.9
	50-60 years	13 (28.9%)	56.2±2.7
	60-70 years	18 (40%)	65.3±3
	>70 years	5 (11.1%)	74±2.7
BMI (Kg/m^2)			30.5±2.5 (24.8-35.4)
Smoking	Smokers: non- smokers	31:14	
	Smokers; M:F	28:3	

Data are presented as mean±SD, numbers and ratios; ranges and percentage are in parenthesis

MRI detected local invasion, (Figs. 1 & 2) in 12 patients (26.7%), while the other 33 patients were free of local invasion. However, no distant metastasis were detected an all patients were considered M0.

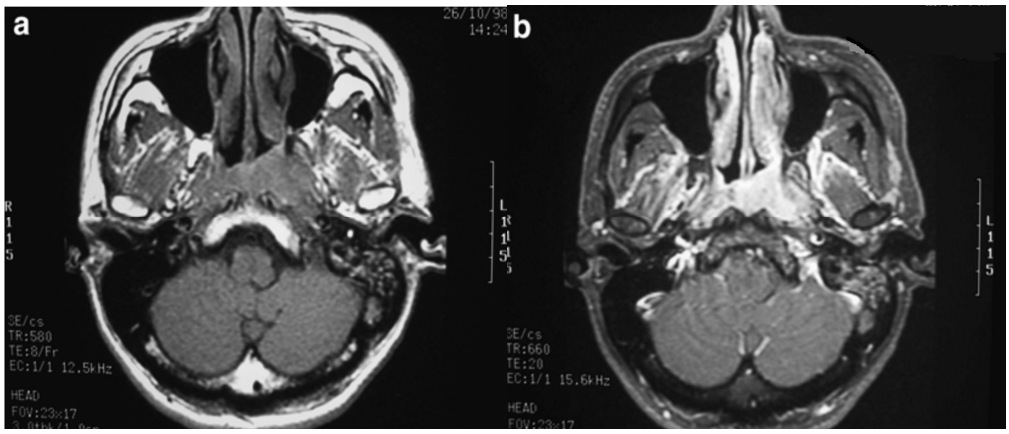

Fig. (1): Non-contrast enhanced Axial T1 (a) and contrast enhanced fat suppression axial T1 (b) showing a tumor mass across the roof of the nasopharynx with involvement at the left medial pterygoid plate that was more conspicuous (a). Also, the left medial pterygoid muscle was involved (b).

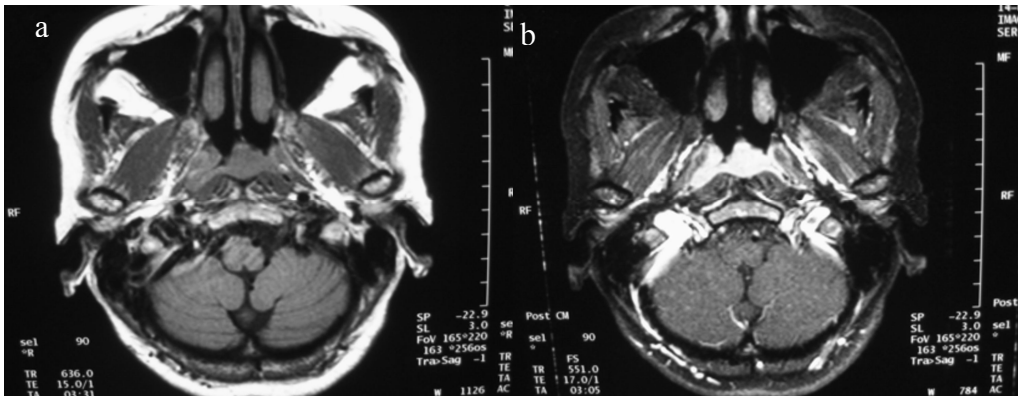

Fig. (2): Non-contrast enhanced Axial T1 (a) and contrast enhanced fat suppression axial T1 (b) showing tumor involvement of the right medial pterygoid muscle (a) as the tissue plane between the nasopharynx and the right medial pterygoid muscle was obscured, widened and full.

Nodal involvement was detected in 23 patients (51.1%); 10 patients (22.2%) had N1, 9 patients (20%) had N2 and 4 patients (8.9%) had N3 nodal involvement. Three patients (6.6%) had carcinoma in situ, 6 patients (13.4%) had stage-I lesion, 12 patients (26.7%) had stage-II lesions, 15 patients (33.3%) had stage-III lesions and 9 patients (20%) had stage-IV lesions, (Table 2).

Table (2): Patients' distribution according to TNM classification

Stage	Subgroup		Number (%)	Total
0 (T *in situ* N0 M0)			3 (6.6%)	3 (6.6%)
I (T1 N0 M0)			6 (13.3%)	6 (13.3%)
II	II-A (T2a N0 M0)		5 (11.1%)	12 (26.7%)
	II-B	T2b N0 M0	5 (11.1%)	
		T1 N1 M0	2 (4.4%)	
III	III-A	T3 N0 M0	3 (6.6%)	15 (33.3%)
		T3 N1 M0	5 (11.1%)	
	III-B	T1 N2 M0	4 (8.8%)	
		T2 N2 M0	2 (4.4%)	
		T3 N2 M0	1 (2.2%)	
IV	IV-A	T4 N1 M0	3 (6.6%)	9 (20%)
		T4 N2 M0	2 (4.4%)	
	IV-B	T3 N3 M0	4 (8.9%)	
	IV-C (any T, any N, M1)		0	

Data are presented as numbers; percentages are in parenthesis

Twenty-two patients (48.9%) had lesions of WHO pathological grade I and 14 patients (31.1%) had lesions of WHO pathological grade II lesions; while the other 9 patients (20%) had lesions of WHO pathological grade III, (Table 3). There was a positive significant correlation (r=0.699, P<0.001) between WHO pathological and TNM staging.

Table (3): Patients' distribution according to WHO pathological stage of tumor and TNM staging

WHO grade		TNM stage	
Grading	Number	Stage	Number
I	22 (48.9%)	0	3 (6.6%)
		I	6 (13.3%)
		II	9 (20%)
		III	4 (8.9%)
II	14 (31.1%)	II	3 (6.6%)
		III	7 (15.7%)
		IV	4 (8.9%)
III	9 (20%)	III	4 (8.9%)
		IV	5 (11.1%)

Data are presented as numbers; percentages are in parenthesis

Qualitative PCR detected EBV viral DNA as shown in figure 3. Using Quantitative PCR technique, the mean EBV DNA plasma load was 2188±642; range: 1169-3954 copies/ml. Mean viral plasma load was significantly (p<0.05) higher in patients with TNM stage IV lesions compared to levels estimated in patients with other lesions' stages. Also, the mean viral plasma load was significantly (p<0.05) higher in patients with TNM stage III lesions compared to levels estimated in patients with lesions staged 0, I and II. Mean viral plasma load was significantly (p<0.05) higher in patients with TNM stage II and I lesions compared to levels estimated in patients with lesions staged 0 with a non-significantly higher levels in patients with TNM II lesions compared to those staged I, (Table 4, Fig. 4).

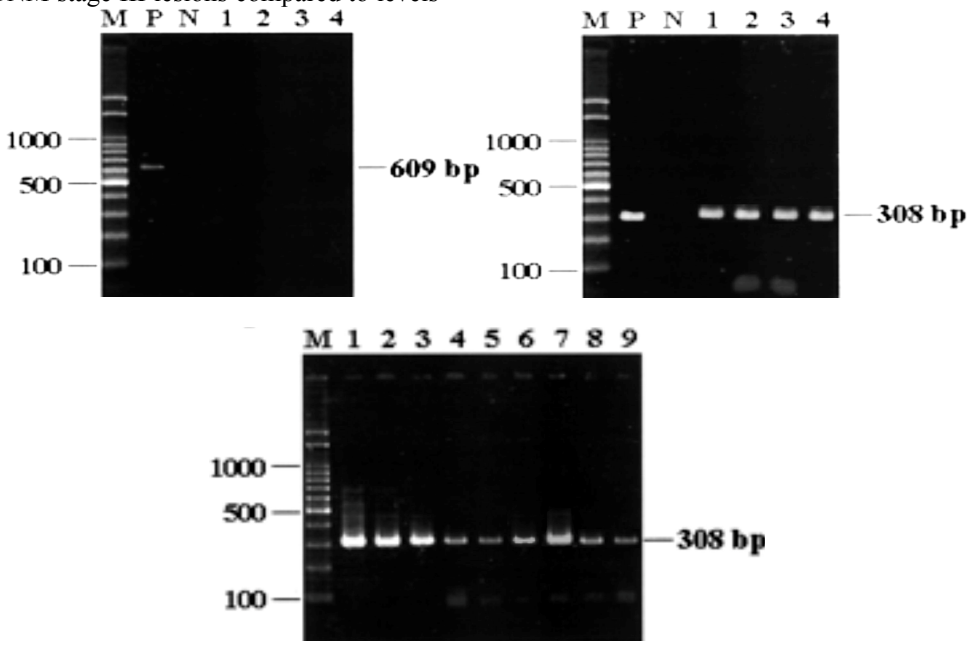

Fig. (3): Agarose gel electrophoresis of the nested PCR products for EBV DNA:

A. Results of the first PCR round using outer *EBNA-1* primers. M, 100-bp ladder DNA marker; P, positive control; N, negative control; lanes 1 to 4, patient samples.
B. Results of nested PCR using inner *EBNA-1* primers.
C. Sensitivity of nested PCR by serial dilution test. The detection limit was 10^{-4} ng DNA. Lanes 1 to 9, 10x dilutions from sample containing 1-ng DNA.

Table (4): Mean (±SD) plasma viral load in studied patients categorized according to TNM staging

TNM Stage	Plasma viral load (copies/ml)	Statistical analysis	
		t	*P*
Stage 0 (n=3)	1337±101		
Stage I (n=6)	1744±297	3.015	P_1<0.01
Stage II (n=12)	1854±385	4.103 0.150	P_1<0.01 P_2>0.05
Stage III (n=15)	2381±508	7.250 3.884 2.839	P_1<0.001 P_2<0.01 P_3<0.05
Stage IV (n=9)	2894±581	7.708 5.481 4.018 1.617	P_1<0.001 P_2=0.005 P_3=0.008 P_4>0.05

Data are presented as mean±SD; *P*<0.05 = significant difference; *P* >0.05 = non-significant difference
P_1: significance versus stage 0; P_2: significance versus stage I; P_3: significance versus stage II
P_4: significance versus stage III

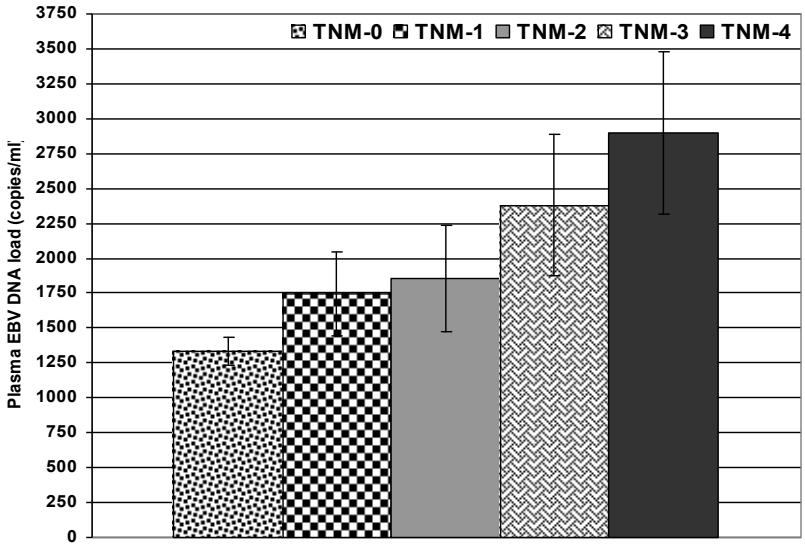

Fig. (4): Mean (±SD) EBV DNA plasma load estimated in studied patients categorized according to TNM classification

According to WHO grading; mean viral plasma load was significantly higher in patients had WHO grade III lesions compared to levels estimated in patients had lesions staged I and staged II and in patients had WHO grade II lesions compared to levels estimated in patients with lesions staged I, (Table 5, Fig. 5). There was a positive significant correlation between mean plasma viral load and TNM stage (r=0.540, *P*=0.025) and WHO grade (r=0.606, *P*=0.001), (Fig. 6).

Table (5): Mean (±SD) plasma viral load in studied patients categorized according to WHO grading

WHO Stage	Plasma viral load (copies/ml)	Statistical analysis	
		t	*P*
Stage I (n=22)	1822±483		
Stage II (n=14)	2324±524	2.670	P_1<0.05
Stage III (n=9)	2832±581	5.665 2.349	P_1<0.001 P_2<0.05

Data are presented as mean±SD *P* <0.05 = significant difference P_1: significance versus stage I P_2: significance versus stage II

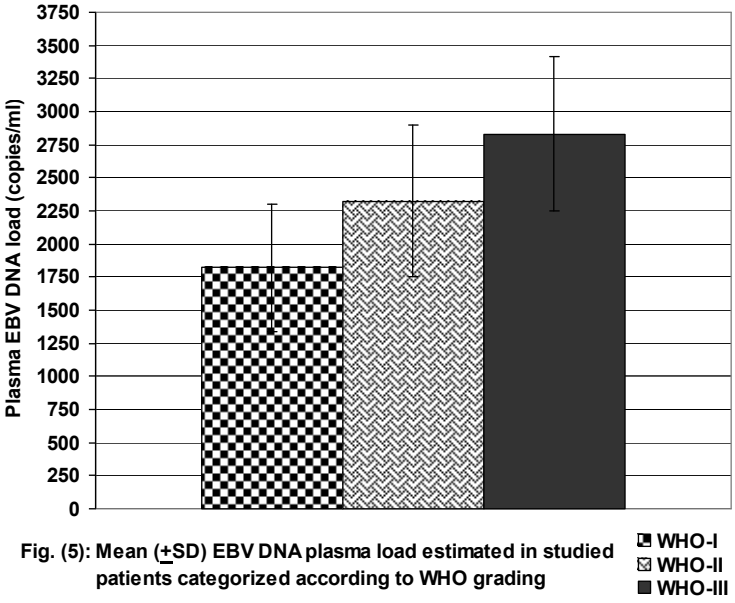

Fig. (5): Mean (±SD) EBV DNA plasma load estimated in studied patients categorized according to WHO grading

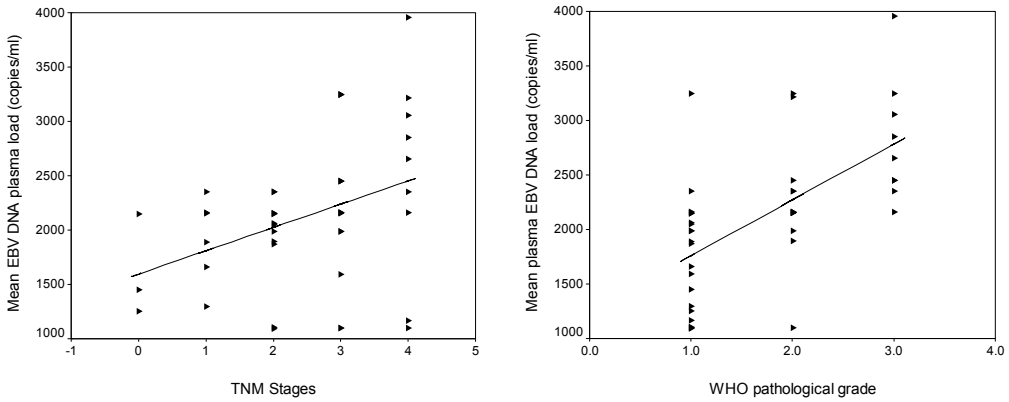

Fig. (6): Correlation between mean plasma viral load and TNM stages and WHO pathological stage

Post-treatment quantitative PCR detected EBV DNA only in 10 (22.2%) post-treatment samples, while the others were free of viral DNA. Mean EBV DNA plasma load estimated in these patients (n=10) after completion of treatment protocol was 61.5±33.7; range: 23-145 copies/ml with a significant decrease of mean plasma viral load after treatment compared to that estimated prior to treatment.

4. Discussion

The study included 45 patients with mean age of 59.4±10.4; 9 patients (20%) were younger than 50 years, 13 patients (28.9%) were aged in range of >50-60 years and 23 patients (51.1%) were older than 60 years. These data define a fact that the frequency of development of NPC in Egypt was maximal in patients older than 50 years (80% of studied patients) and go in hand with **Paulino & Grupp (2006)** who reported that NPC has a bimodal age distribution; a small peak is observed in late childhood, and a second peak occurs in people aged 50-60 years. Moreover, a male: female ratio of 2.75:1 was reported and agreed also with **Paulino & Grupp (2006)** who reported that among patients with NPC male preponderance exists with male-to-female ratio is approximately 2:1.

There were 31 smokers (68.9%); 28 males and 3 females, smokers were significantly younger than non-smokers and female smokers were significantly younger than non-smoker, while male smokers were non-significantly younger than non-smoker. These data point to an etiological relation between smoking and development of NPC that occurs earlier in smokers and agreed with various studies evaluated risk factors for acquisition of NPC; **Feng *et al.*, (2009)**, reported that tobacco, cannabis and domestic cooking fumes intake are risk factors for NPC in western North Africa. **Ekburanawat *et al.* (2010)**, suggested that cigarette smoking, past history of ear or nose disease and occupational exposure to wood dust may play a role in the development of NPC in the Thai population. **Cao *et***

***al.* (2011)**, provide the first molecular epidemiological evidence that methylenetetrahydrofolate reductase polymorphism was associated with the risk of NPC and this association is especially noteworthy in heavy smokers. **Polesel *et al.* (2011)**, reported that in western populations, NPC includes two separate entities: the differentiated NPC, associated with tobacco smoking and the undifferentiated NPC, upon which tobacco smoking has little or no influence. Moreover, **Ji *et al.* (2011)**, demonstrated that cigarette smoking and family history of cancer could serve independently and jointly as risk factors for etiology of NPC and might affect the risk of histology-specific NPC differently

The present study relied on estimation of plasma EBV DNA viral load depending on the previous findings of **Zhang *et al.* (2004)** who used quantitative analysis of EBV DNA levels in plasma, peripheral blood cells (PBCs) and tumor tissue in NPC and found plasma EBV-DNA level was significantly increased in TNM stage, whereas there was no significant difference of PBCs EBV-DNA loads in difference stages of NPC and concluded that plasma EBV-DNA level is a more sensitive and reliable biomarker than PBCs EBV-DNA loads for reflection the tumor volumes in NPC patients. Also, **Kondo *et al.* (2004)** compared the amount of plasma EBV DNA detected in patients with NPC in a high- and low-incidence areas using quantitative PCR with EBV-viral-capsid-antigen (VCA) titers and found EBV-DNA is a more reliable tumor marker than EBV-VCA titers in both high- and low-incidence areas of NPC.

Through the present study qualitative PCR detected EBV DNA in all studied blood samples obtained of the 45 cases of NPC, irrespective of the stage or pathological grade. These data point to a fact that nasopharyngeal malignancy was found to be strongly associated with viral infection. **Liu *et al.* (2006)** attributed these findings to the fact that EBV, being a persistent virus, i.e. virus persists in some cells and does not disappear from all infected cells after subsidence of the acute attack, induces immuno-suppression, immortalization and malignant transformation of the inhabitant cells. In support of these data, **Ogino *et al.* (2007)** reported that NPC cells may utilize multiple immunoescape mechanisms, including dysfunction of HLA class I antigens and Fas/FasL apoptosis pathways. Furthermore, FasL expression appears to be associated with IL-10 upregulation in EBV positive NPC cells. **Li *et al.* (2009)** reported several associations between the frequency of HLA class I genes in certain populations and the risk of developing NPC and populations in geographical areas at higher risk of developing NPC display HLA distribution patterns different and sometimes opposite from areas of low incidence, whereas populations in areas with intermediate incidence display a totally independent pattern.

Mean EBV DNA plasma load was 2188±642; range: 1109-3954 copies/ml with a positive significant correlation between mean plasma viral load and both TNM stage and WHO grade. These findings indicated a close relationship between plasma viral load and tumor aggressiveness and differentiation. In hand with these results; **Adham *et al.* (2012)**, reported that early-age EBV infection combined with frequent exposure to environmental carcinogenic co-factors is suggested to cause NPC development and undifferentiated NPC (WHO-III) is the most frequent histological type and is closely associated with EBV.

Viral DNA was detected in only 10 patients (22.2%) after completion of treatment with a mean EBV DNA plasma load of 61.5±33.7copies/ml. There was a significant decrease of mean plasma viral load after treatment compared to pre-treatment load; a finding indicating the possibility of using pretreatment plasma EBV load as predictor for response to therapy. These data are in agreement with **Kalpoe *et al.* (2006)** and **Lacy *et al.* (2006)** who found plasma EBV DNA load measurement appears to be useful marker especially in a low tumor risk area and with **Lin *et al.* (2007)** who reported that EBV DNA was detected in the pretreatment plasma of 94.1% of patients in comparison to 20.4% after treatment and concluded that plasma EBV DNA is the most valuable prognostic factor for NPC and more chemotherapy should be considered for patients with persistently detectable EBV DNA after concurrent chemoradiotherapy.

Also, **An *et al.* (2011)**, who found plasma EBV DNA is of predictive value for prognosis in metastatic/recurrent NPC patients undergoing palliative chemotherapy and pre-treatment plasma EBV DNA level as well as the early decrease of plasma EBV DNA after chemotherapy enabled easy and early discrimination between patients who will and those who will not benefit from continued treatment. Moreover, **Hassan *et al.* (2011)**, suggested that the EBV DNA load quantification after treatment may be a useful predictor of disease progression and survival.

It could be concluded that PCR quantitative estimation of plasma Epstein-Barr viral DNA load is a valuable diagnostic test that showed a positive significant correlation with both TNM staging and WHO pathological grading of patients with NPC and could be used to assess the response to applied therapeutic modalities. Considering Egypt as a non-endemic area for NPC, quantitative estimation of EBV plasma load could be used as screening test for patients presenting by symptoms suspicious of NPC.

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