Effect of Epstein–Barr virus infection on predisposition and postradiotherapeutic prognostic value among Libyan patients with nasopharyngeal cancer

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Received 04 December 2013
Accepted 08 May 2014

The Egyptian Journal of Otolaryngology
2014, 30:201–207

Background and objective
Libya is one of the North African countries with an endemic of nasopharyngeal carcinoma, particularly in the northern part of Libya as compared with its southern part. The clinical and histopathological presentation reveals no uniquely specific pattern of appearance; however, there is high deficiency in the data that confirm the possible predisposing factors that may play a role in the development of this common variety of head and neck cancer in this country, and whether these factors affect the patients' response to treatment. This study was conducted to determine the role of the Epstein–Barr virus (EBV) in the risk for nasopharyngeal cancer in the Libyan population; the results were correlated with radiotherapy response and improvement in the post-therapeutic prognostic value.

Patients and methods
Sixteen patients aged 9–80 years presented at the ENT Department, Althowra Central Teaching Hospital, Albyda, Libya, from September 2005 to January 2014 with variable patterns of clinical presentation suggestive of nasopharyngeal carcinoma. All patients were evaluated radiologically and endoscopically. The diagnosis was confirmed by biopsy and further histopathological assessment. For all patients, serological elucidation for IgG and IgM anti-EBV antibodies was carried out, and the results were correlated with age at incidence, sex of the patient, pattern of clinical presentation, pattern of histopathological presentation, response to radiotherapy, rate of recurrence, and 5-year survival.

Results
In all, 88% of patients showed a significant increase in serum IgG against viral capsid antigen of EBV; 83% of the cases presented with cervical masses and unilateral otitis media with effusion; 86% of cases showed lymphoepithelioma as histopathological pattern. All patients with lymphoepithelioma showed significant response to concomitant radiochemotherapy (100%) with high survival rate exceeding 5 years.

Conclusion
The EBV infection can be considered one of the main predisposing factors to nasopharyngeal carcinoma in the Libyan population. It was noted from this study that the induction of cancer by EBV is mainly by chronic infection rather than by acute infection; this was confirmed by significant elevation in serum IgG rather than IgM. In addition, it was postulated that the EBV infection is most likely associated with the lymphoepithelioma variety of histopathology rather than with the well-differentiated type, and as it was elucidated through this study that the association of the cancer with EBV infection increased the sensitivity of the tumor to concomitant radiochemotherpy, thus improving the 5-year survival after treatment.

Keywords:
Epstein–Barr virus, nasopharyngeal carcinoma, nasophargeal carcinoma at Libya

Egypt J Otolaryngol 30:201–207

1012-5574

Introduction

Nasopharyngeal carcinoma (NPC) is a tumor arising from the epithelial cells that cover the surface and line the nasopharynx. NPC was first described as a separate entity by Regaud and Schmincke in 1921 [1,2]. NPC is an uncommon disease; it is endemic in southern China, South-East Asia, and northern Africa [3]. In Malaysia, it is the second most common cancer in the male population. Also Chinese men in the fifth decade were at the highest risk. The incidence of the disease in southern China was 15 : 100 000 and in Hong Kong was 30 : 100 000. In contrast, the annual incidence of NPC in the UK is 0.25/million (age standardized) at age 0–14 years, 0.1/million at age 0–9 years, and 0.8/million at age 10–14 years. It seems reasonable to assume, on the basis of England and Wales's cancer registry data, that at least 80% of NPCs at age 15–19 years are carcinomas. This suggests an incidence of 1–2/million for NPC at age 15–19 years [4].

In northern Africa, NPC occurs with a higher incidence in Libya and Tunisia. The eastern part of Libya showed higher incidence of NPC as compared with the western part [5]. Some Libyan studies found that the highest

incidence was in the age group 40–49 years (32%) and the least in the age group 10–19 years (14%) [6]. It is well known that there is a pathogenesis triad that comprises the main three predisposing factors to NPC – namely, genetic, environmental, and viral infection. Regarding viral infection, it is well established that Epstein–Barr virus (EBV) is considered one of the most significant and strongly associated predisposing etiological factors to the pathogenesis of NPC [7]. EBV infection not only plays a significant role in the pathogenesis of NPC but also acts as one of the most highly valuable determinants of the histopathological pattern of this cancer as well as its response to radiotherapy [8].

Epstein and his group discovered novel viral particles within cultured cells from patients with Burkitt's lymphoma in 1964, 15 and 4 years later; this new virus was named EBV. EBV is a relatively large gamma herpes virus, and its DNA is double stranded and ~172 kb in length. Approximately 90% of the adult population throughout the world is EBV+ by serology. Elevated titers of IgA antibody to EBV viral capsid antigen (VCA) are usually found in patients with NPC. Therefore, this method of measuring patients' EBV-specific IgA antibodies is useful in screening for early detection of NPC [1–8]. Epstein–Barr virus nuclear antigen 1 (EBNA1) and Epstein–Barr virus-encoded small RNAs (EBERs) are expressed in all EBV+ cases of NPC, and latent membrane protein 1 (LMP1) is present in up to ~65% of cases. In almost all cases of EBV infection the oropharynx is the primary site of infection, as well as the site of viral replication [1–8]. The EBV is known to target primarily B lymphocytes. In-vitro studies demonstrate that EBV infects and potentially activates B cells by binding to the type-2 complement receptor, or to CD21, the putative EBV receptor. Hence, EBV appears to home to the oropharynx, and more specifically to the B cells within the oropharynx. The strain B95-8 can be found in EBV+ cell lines such as Raji, Namalwa, and CA46. These cell lines are all of B-lymphocyte lineage. This strain has been used as a benchmark to check for EBV positivity in NPC. The EBV latent proteins include the six nuclear antigens (EBNAs 1, 2, 3A, 3B, and 3C, and LP) and the three LMPs (1, 2A, 2B). EBNA-LP is transcribed from variable numbers of repetitive exons. LMP2A and LMP2B are composed of multiple exons located on either side of the terminal repeats region, which is formed during the circularization of the linear DNA to produce the viral episome. EBER1 and EBER2 are highly transcribed nonpolyadenylated RNAs and their transcription is a consistent feature of latent EBV infection [1–8].

It was in 1966 that Old and colleagues first discovered the relationship between EBV and NPC using in-situ hybridization and the anticomplement immunofluorescent assay. Subsequent studies by others demonstrated the expression of EBV latent genes – EBNA, LMP1, LMP2, and EBERs – in NPC cells confirming the infection of tumor cells by EBV [1–10]. Intriguingly, expression of EBV early antigen (EA) is positively correlated with the consumption of salted and preserved food, suggesting that development of EBV+ NPC could be related to dietary habits [21], providing another link to epidemiological studies on NPC. Future studies should consider the effects of dietary risk factors on the risk for specific histologic subsets of NPC and not assume that the disease is etiologically homogeneous [1–8,11–14]. EBV infection indeed precedes clonal expansion of malignant cells and is thought to contribute, at least in part, to the overall pathogenesis of NPC. Many studies have shown that undifferentiated NPCs are invariably EBV+, regardless of geographical origin. The process of EBV entry into keratinocytes and NPC cells is more complex, as both keratinocytes and NPC cells express only low levels of type-2 complement receptor. In addition, the relevance of serological tests for EBV infection in predicting the occurrence of NPC is presently still unclear. Specifically, a positive serological test for EBV EA may be found in more than 80% of patients with NPC in Singapore, yet many normal individuals also express a positive EBV EA serological test and never develop NPC. The procedures that researchers have carried out, such as PCR, revealed expression of the LMP2A and LMP2B genes and of latent transcripts running through the *Bam* HI A region of the EBV genome in the opposite direction to the conventional lytic cycle mRNAs transcribed over this region [1–8].

It is well known that EBV contributes directly to tumorigenesis in NPC, primarily in the undifferentiated form of NPC, which is commonly found in South-East Asia. Unfortunately, research in NPC has been severely hampered by the lack of authentic EBV+ human NPC cell lines for study. Since 1975, there have been more than 20 reported NPC cell lines. However, many of these NPC-derived cell lines do not express EBV transcripts in long-term culture, and therefore that finding may dispute the fundamental theory of NPC carcinogenesis. In fact, only one EBV+ human NPC cell line (C-666) in long-term culture has been reported. Hence, most of the NPC cell lines may not be representative of the disease itself. To better understand and treat NPC, there is an urgent need to develop more EBV+ human NPC cell lines. Immunohistochemically, EBV acts to induce cell proliferation pathways in NPC through the activation of an antiapoptotic mechanism; that is, NPC is consistently associated with EBV infection. EBV-encoded LMP1, expressed

in most cases of NPC, has been suggested to have an important role in the pathogenesis and development of NPC, and its expression correlates with poor prognosis. LMP1 molecules aggregate in the cell membrane, and through two C-terminal activating regions (CTARs) they interact with tumor necrosis factor (TNF) receptor-associated factors and TNF receptor-associated death domain protein. *In vitro*, LMP1 expression in epithelia cells can upregulate the expression of intercellular adhesion molecule 1, CD40, and cytokines such as interleukin 6 and interleukin 8. LMP1 can also induce the expression of CD70 antigen, a member of the TNF family, in epithelial cells *in vitro*. LMP1 can induce a matrix metalloproteinase, MM-9, through CTAR-1 and CTAR-2, an effect blocked by overexpression of the inhibitor of nuclear factor κB (IκB). LMP1 encoded by EBV is a membrane protein that activates multiple signaling pathways and transcription factors, including nuclear factor κB (NF-κB). NF-κB activation is necessary for Hodgkin/Reed–Sternberg cells to proliferate and inhibit apoptosis. Furthermore, activation of NF-κB is essential for B-cell immortalization by EBV and LMP1-mediated transformation of fibroblasts. EBER-negative Hodgkin's lymphoma also displays constitutive NF-κB activation, indicating that without LMP1 other mechanisms exist to activate NF-κB. LMP1 has been shown to activate the mitogen-activated protein kinase pathways and Janus kinase (JAK) signal transducers and activators of transcription pathway in epithelial cells and B cells. LMP1 and 2A also activate the phosphatidylinositol-3-OH kinase (P13K)/Akt pathway, which is commonly activated inappropriately in malignancy [1–8,12–14]. A recent study showed that the P13K/Akt pathway is important in NPC pathogenesis. P13K/Akt pathway activation with subsequent phosphorylation and inactivation of GSK-3β and nuclear β-catenin accumulation was characteristic of primary NPC specimens. Thus, it is clear that NPC uses its viral proteins to activate numerous cellular pathways in the NPC tumor, which results in proliferation and prevents the transformed cells from dying [1,3,4,7].

There are a lot of difficulties in the performance and elucidation of proper research activity to confirm all these concepts and correlate them with clinical presentations, histopathological patterns, and radiotherapy response of NPC. These difficulties stem from the limited number of authentic EBV+ NPC cell lines in existence and from the inherent limitations to culturing NPC cells *in vitro*. Typically, nasopharyngeal biopsies provide only small tissue fragments. To increase the amount of available tissue, subcutaneous tumors may be first transferred to euthymic mice for initial in-vivo expansion before seeding into tissue culture flasks for in-vitro culture. Besides the potential introduction of murine cells, this method is likely to result in changes to the primary human biopsy tissue sample. In addition to these previously mentioned difficulties, in our area, which is considered one of the most important regions for endemic NPC, there was lack of sufficient data regarding nasopharyngeal carcinoma (NPC) as general and from pathogenesis point of view particular, for this reason this study was planned to achieve these specific aims:

1. To confirm whether there is any significant relationship between NPC and EBV infection in the Libyan population.
2. To postulate the possible correlation between EBV infection and clinical presentation of NPC in the Libyan population.
3. To elucidate the possible correlation between EBV infection and histopathological pattern of NPC in the Libyan population.
4. To demonstrate the possible correlation between EBV infection and the response of NPC to concomitant radiochemotherapy in the Libyan population.

Patients and methods

Sixteen patients aged 9–80 years presented at the ENT Department, Althowra Central Teaching Hospital, Albyda, Libya, from September 2005 to January 2014 with variable clinical presentations suggestive of nasopharyngeal growth were included in this study. The patients underwent further radiological and endoscopic evaluations and biopsies were taken for histopathological confirmation of NPC diagnosis.

All patients were assessed immunologically for EBV antibody VCA in the form of VCA–IgG and VCA–IgM. All patients were indicated for concomitant radiochemotherapy after confirmation of diagnosis and full staging. The immunological results were correlated with the clinical manifestations, histopathological patterns, and patients' response to the treatment.

Statistical analysis

Data were expressed using descriptive analysis as mean ± SEM and percentages. Test of significance was carried out using the χ^2-test and two-way analysis of variance. A probability value of less than 0.05 was considered significant; the degree of significance was determined using the level of SD test. Student's *t*-test was used for dependent variables, and the contingency coefficient was calculated as measurement of association between nominal variables.

Results

As shown in Tables 1 and 2 the NPC incidence was correlated with patients' demographic factors — namely, age and sex. The percentage of male patients was higher (57%) than that of female patients (43%). The highest percentage of patients (58%) was found in the age group 39–48 years, followed by age 29–38 years (14%). As illustrated in Table 3 there was a significant elevation in VCA–IgG in 88% of NPC cases as compared with VCA–IgM. As demonstrated in Table 4 the most common clinical presentation of NPC among Libyan patients at Albyda city was neck mass (81%), followed by halitosis (64%), weight loss and metabolic changes (56%), and unilateral otitis media with effusion (53%). As can be seen in Table 5 the most common radiological presentation of NPC among Libyan patients at Albyda city was obliteration of unilateral Rosenmullar fossae (64%), followed by obliteration of bilateral Rosenmullar fossae, localized bone destruction, base of skull invasion, parapharyngeal space invasion, and intracranial invasion (36, 18, 18, 9, and 9%, respectively). Table 6 shows the different varieties of histopathological presentations among NPC Libyan patients at Albyda city. Lymphoepithelioma (LE) constituted 86% of NPC cases that were presented at the ENT Department, Althowra Central Teaching Hospital, Albyda, Libya.

Table 7 demonstrates the relationship between the histopathological pattern of NPC on the one hand and response to concomitant radiochemotherapy with 5-year survival rate on the other. LE showed significantly (P<0.001) the highest response to treatment with the maximum 5-year survival rate and no evidence of recurrence throughout 7 years of follow-up.

Table 1 Sex distribution in relation to the incidence of nasopharyngeal carcinoma in the Libyan population at Albyda city

Types of sex	%
Male (n = 9)	57
Female (n = 7)	43
Total (n = 16)	100

P < 0.05.

Table 2 Age distribution in relation to the incidence of nasopharyngeal carcinoma in the Libyan population at Albyda city

Age intervals	%
9–18 (n = 1)	7
19–28 (n = 0)	0
29–38 (n = 2)	14
39–48 (n = 10)	58
49–58 (n = 1)	7
59–68 (n = 0)	0
69–78 (n = 1)	7
79–88 (n = 1)	7
Total (n = 16)	100

P < 0.05.

Table 3 The incidence of positive serological evaluation for Epstein–Barr virus among Libyan nasopharyngeal carcinoma patients at Albyda city

	Serological results					
	Negative		Weak positive		Positive	
	N	Titer (AU/ml) (mean ± SEM)	*N*	Titer (AU/ml) (mean ± SEM)	*N*	Titer (AU/ml) (mean ± SEM)
VCA–IgG	2	7.9	0	—	14	132.93 ± 6.7
VCA–IgM	16	4.6 ± 0.529	0	—	0	0

Biological reference intervals: for VCA–IgG: negative <10, weak positive 10–15, positive >15; for VCA–IgM: negative <16, weak positive 16–24, positive >24; VCA, viral capsid antigen; P<0.05.

Table 4 Different varieties of clinical presentations among Libyan nasopharyngeal carcinoma patients at Albyda city

Types of clinical presentations	%
Neck mass	81
Unilateral otitis media with effusion	53
Unilateral recurrent epistaxis	37
Unilateral nasal obstruction	46
Headache	27
Facial pain	19
Trigeminal neuralgia	18
Abducent nerve palsy	9
Halitosis	64
Snoring and apnea attacks	46
Speech changes	46
Parapharyngeal space invasion	9
Dysphagia	9
Weight loss and metabolic changes	56
Jugular foramen syndrome	18

Table 5 Different varieties of radiological presentations among Libyan nasopharyngeal carcinoma patients at Albyda city

Types of clinical presentation	%
Obliteration of unilateral Rosenmullar fossae	64
Obliteration of bilateral Rosenmullar fossae	36
Localized bone destruction	18
Parapharyngeal space invasion	9
Base of skull invasion	18
Paranasal sinus invasion	0
Intracranial invasion	9

Table 6 Different varieties of histopathological presentations among Libyan nasopharyngeal carcinoma patients at Albyda city

Types of histopathological presentation	%
Well-differentiated squamous cell carcinoma	0
Nonkeratinizing carcinoma	14
Lymphoepithelioma	86
Clear cell carcinoma	0
Spindle cell carcinoma	0
Anaplastic carcinoma	0

Table 7 Response to the concomitant chemoradiotherapy and 5-year survival among nasopharyngeal carcinoma Libyan patients at Albyda city

Responses to treatment	Histopathological pattern		
	LE	NKC	Total
Yes			
> 5-year survival	13	0	13
Developed recurrence	1	0	1
No	0	2	2
Total	14	2	16

LE, lymphoepithelioma; NKC, nonkeratinizing carcinoma; $P < 0.001$.

Discussion

The incidence of NPC has remained high in endemic regions. Diagnosing the disease in the early stages requires a high index of clinical acumen and, although most cross-sectional imaging investigations show the tumors precisely, confirmation is dependent on histology. The EBV-encoded RNA signal is present in all NPC cells, and early diagnosis of the disease is possible through the detection of raised antibodies against EBV. The quantity of EBV DNA detected in blood indicates the stage and prognosis of the disease. Radiotherapy with concomitant chemotherapy has increased the probability of survival and led to the development of improved techniques (such as intensity-modulated radiotherapy), aiding in the early detection of recurrence. Further, application of appropriate surgical salvage procedures has contributed to improved therapeutic results. Screening of high-risk individuals in endemic regions together with developments in gene therapy and immunotherapy might further improve outcome [1–6].

NPC has a unique and complex etiology that is not completely understood. Although NPC is rare in most populations, it is a leading form of cancer in a few well-defined populations, including natives of southern China, South-East Asia, the Arctic, the Middle East, and North Africa. The distinctive racial/ethnic and geographic distribution of NPC worldwide suggests that both environmental factors and genetic traits contribute to its development [6,7,9–17].

Convincing evidence implicates dietary factors as the primary cause of NPC among the Chinese. A series of case–control studies conducted in various Chinese populations with distinct risk for NPC, ranging from the very high-risk Cantonese populations to the relatively low-risk northern Chinese, have suggested that ingestion of salted fish and other kinds of preserved foods constitutes the most important cause of NPC among these people. Preliminary data on Malays in South-East Asia, Eskimos in Alaska, and Arabs of North Africa also suggest that ingestion of preserved foods may be responsible for their raised incidence of NPC [6,7,9–17].

A recent study suggests that, among occupational hazards such as exposure to smoke, construction sites, metal, wood dust, motor fuel and oil, paint and varnishes, etc., only wood dust was statistically significant for the development of NPC. Therefore, the development of NPC disease is multifactorial, with genetics, diet, and environmental exposure all playing large roles [6,9,11].

The positive VCA–IgA and intake of salted fish were associated with a strong excess risk for NPC. The association persisted after adjustment for other factors. The combination of salted fish and EBV was strongly associated with NPC, and more so than EBV or salted fish *per se*. Multivariate analyses showed that VCA–IgA was the most important predictor of NPC, and salted fish the second most important. These results suggest that EBV has a strong effect on the development of NPC. The exclusion of EBV and genetic factors in earlier epidemiological studies may have resulted in an overestimation of salted fish as an important etiological factor causing NPC [18–27].

As confirmed by a series of local studies conducted at different regions of Libya, NPC can remain dormant for a long time, causing few primary symptoms; hence, it is difficult to make an early diagnosis. It has an early tendency to cervical lymph node involvement regardless of the size of the primary tumor. Regional lymph node enlargement, headache, and nasal and ear symptoms were common presenting complaints. Cranial nerve palsies and trismus were found in the late stages [28].

Thus, on the basis of the correlation between all previously mentioned reviews and the results of this study we can note that male patients have a higher incidence of NPC compared with female patients and the peak age at incidence ranged from 39 to 48 years. These results are in agreement with those of many other national and international studies. These figures can be explained by the higher probability for exposure of the male population, particularly the middle-aged group, to predisposing environmental factors from industrial and occupational hazards compared with females [6,9,12–14].

The most commonly presenting clinical pattern of NPC was cervical metastatic lymph node enlargement. This can be discussed in relation to three pathoanatomical facts:

(a) The nasopharyngeal cavity is considered a wide cavity with sufficient space that makes the

small-sized tumors asymptomatic at early stage in relation to otorhinopharyngeal obstructive symptoms and signs;
(b) The nasopharynx is rich in lymphatic drainage with much decussated distributions resulting in early lymphatic spread to sentinel lymph node groups and subsequently to other draining lymph node station groups; and
(c) The lateral wall of the nasopharyngeal cavity is directly connected to the parapharyngeal space through the sinus of Morgagni, resulting in the facilitation of local and rapid invasion of tumor into this space.

In addition, the histopathological profile of the tumor may determine its clinical and radiological presentation; that is, nondifferentiated tumors of epithelial origin and those of mesenchymal origin, such as sarcomas, have higher tendency for more aggressive and rapidly invasive behavior [12–14,26,28].

In accordance, the detection of EBNA and viral DNA in NPC types II and III has revealed that EBV can infect epithelial cells and is associated with their transformation. It was illustrated from the results of this presenting study that EBV infection has a significant role in the predisposition for NPC, particularly LE (type III). The serum VCA–IgG was significantly elevated as compared with VCA–IgM. This may indicate the association of the risk for cancer with longer duration of the histoimmunological activity of the virus, resulting in the stimulation of production of IgG rather than IgM, because, immunologically, it is well known that a high titer of IgG is almost always associated with chronic and prolonged subclinical stage of infection rather than the acute and severe stage [1–8,11,14,16,18,21].

In contrast, it was shown through this presenting study that the most common histopathological pattern of NPC is LE, which is considered one of the undifferentiated categories of NPC (type III). This may determine the response to concomitant radiochemotherapy and further improvement in the 5-year survival rate, because it is well established that LE is classified as a highly sensitive variety of NPC to radiotherapy. In the same manner, LE is strongly associated with EBV infection as a significant etiological predisposing factor. In agreement with all previously mentioned concepts, our results postulated that LE, which is the most common pattern recognized among Libyan patients with NPC, had been confirmed with a high titer of VCA–IgG of EBV. Those patients showed sufficient response to concomitant radiochemotherapy, and thus a high rate of 5-year survival.

Therefore, from this original descriptive study we can conclude that NPC in the Libyan population commonly presents clinically with cervical metastatic lymph node enlargement, halitosis, and unilateral otitis media with effusion. From the histopathological point, the most common pattern is LE, which has a significant association with EBV infection. Thus, it has shown significant response to treatment and subsequent significant elevation of 5-year survival rate.

Libya is considered one of the North African countries with an endemic epidemiological presentation of NPC, encouraging a continuous and wider spectrum of research into this type of malignancy. Further studies are required for elucidation and estimation of antibodies against other more specific viral products at the cellular level, which may require more advanced immunohistochemical techniques.

Acknowledgements

Conflicts of interest

None declared.

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