Epstein-Barr Virus, Human Papillomavirus, and Flow Cytometric Cell Cycle Kinetics in Nasopharyngeal Carcinoma and Inverted Papilloma among Egyptian Patients

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ABSTRACT: It is widely accepted that the Epstein-Barr virus is etiologically associated with the development of nasopharyngeal carcinoma. The human papillomavirus is also associated with inverted papilloma. We used the polymerase chain reaction technique to detect both viruses in both types of tumors. Flow cytometry was also used to study the DNA pattern and proliferative behavior of the tumors in relation to the viruses. EBV was detected in 13/20 (65%) of NPC specimens, and in none of IP (n = 10) or control specimens (n = 10). This indicates the contribution of EBV as an etiologic factor in NPC. Five cases of NPC (25%) were positive for HPV 16, two of them were EBV positive. Four HPV 16 positive cases were found among cases with inverted papilloma, but none among the control cases. Flow cytometry revealed that all NPC, IP, and control samples were diploid except one aneuploid NPC sample. Proliferative capacity (PC) of primary tumors was predictive of tumor recurrence in NPC. Using 13.6% as a cut-off point for PC, we were able to discriminate between high risk and low risk groups with 100% sensitivity and 86% specificity. PC can be

used as a baseline prognostic parameter in NPC, making it possible to modify courses of treatment in an attempt to inhibit tumor recurrence.

INTRODUCTION

The epithelial cells lining the nasopharynx are composed of stratified squamous cells, pseudo-stratified columnar ciliated cells, and patches of cells of an intermediate type. Nasopharyngeal carcinoma (NPC) is a malignant tumor which arises in the surface epithelium of the posterior nasopharynx from any of these three types of epithelial cells [18]. NPC is a rare disease among Caucasians but is one of the commonly occurring tumors in individuals in the southern region of China and South-East Asia. In these countries, it occurs about 10 to 50 times more often than in other countries [4]. The etiology of the disease seems to be multifactorial while evidence suggests that genetic, viral, and other environmental factors are involved [12]. The Epstein-Barr virus (EBV), a member of the human herpes virus family, is the primary cause of infectious mononucleosis, and has been linked to a number of malignancies, including Burkitt's lymphoma and NPC [16,19]. The virus infects through the oropharynx, invading B-lymphocytes that have EBV receptors in common with receptors for complement components [25]. It has been proved that EBV receptors are present in epithelial cells of the oropharynx and that the

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virus is able to infect nasopharyngeal epithelial cells *in vivo* [29]. Nasal inverted papilloma (IP) is a rare benign tumor that involves the mucous membranes of the nasal cavity and paranasal sinuses. The etiology of IP remains unknown. The human papillomavirus type 16 (HPV 16) has been suggested to be the etiological factor for this benign tumor [2]. The human papillomavirus (HPV) is an epitheliotrophic oncogenic virus that has been detected in a variety of head and neck tumors [2]. It is also strongly implicated in the genesis of human cervical carcinoma, epidermal squamous cell carcinoma, and squamous cell carcinoma of the upper respiratory tract [13]. In this study, we employed the polymerase chain reaction (PCR) technique to detect EBV and human papillomavirus (HPV 16) in fresh frozen and paraffin-embedded tissues from Egyptian patients with nasopharyngeal carcinoma and inverted papilloma. PCR provides a specific, rapid and sensitive means for the detection of viral genomes and may be used in routine diagnosis [7]. The technique is independent of the immune status of the patient and is able to detect very small amounts of viral copies in clinical samples. DNA content and proliferative rate in NPC and IP were also studied using flow cytometric analysis in order to correlate these parameters with prognoses for both tumors.

PATIENTS, MATERIALS AND METHODS

Patients

The study included twenty patients suffering from nasopharyngeal carcinoma, ten suffering from inverted papilloma, five control cases for NPC diagnosed as nonspecific inflammation of the nasopharynx, and five control cases for inverted papilloma diagnosed as inflammatory polyps of the nose. All patients presented to the Department of Otolaryngology of Ain Shams University hospitals in the period between May 1993 and July 1997. All the surgical materials were excised before any course of treatment was applied. Biopsies and archival paraffin blocks from all patients including those for control cases were examined histopathologically. NPC was typed according to WHO classification [21] and TNM staging was done according to the Ho staging system [5]. After radiotherapy all NPC carcinoma patients were clinically followed up every 3 months. Biopsies from the nasopharynx were taken and examined whenever recurrence was suspected.

Materials

Propidium iodide stain (PI; from the Coulter DNA-Prep Reagent Kit, containing 50 μg/ml PI, 4 KU/ml bovine pancreas type III RNAse, 0.1% NaN$_3$, saline, and stabilizers) was obtained from Coulter Corporation (Miami, USA). Taq polymerase, dNTPs, and DNA markers were purchased from Promega (Madison, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

DNA extraction for PCR

From all patients, epithelial tissues recognized by macro- and histopathology were taken from fresh specimens and/or paraffin-embedded sections of 10 μm thickness. The DNA was first extracted from the fresh and paraffin-embedded tissue samples by the proteinase K digestion method slightly modified from Jackson et al., 1991 [15]. Briefly, for each fresh frozen tissue sample, 100 mg were cut into small slices and put into an Eppindorf tube. Three tissue sections of 10 μm thickness were cut from paraffin-embedded blocks, added directly to a microfuge tube without dewaxing. To all tubes, 500 μl of digestion buffer (100 mM NaCl, 10 mM tris-Cl, 25 mM EDTA, 0.5% SDS pH 8, 0.1 mg/ml freshly added proteinase K) was added. Tubes were then incubated in a tumbler (COBAS EIA incubator, Switzerland) for 24–48 hours at 37 ºC. An equal volume of Tris-saturated phenol/ chloroform/isoamyl alcohol (25:24:1) was then added. The tubes were mixed, phases were separated by centrifugation. The upper aqueous phase was collected using a wide-bore pipette and this step was repeated by adding an equal

volume of chloroform/isoamyl alcohol (24:1), mixed well, and microfuged. The upper aqueous phase was collected. Two volumes of cold absolute ethanol and one-tenth volume of 3 M sodium acetate (pH 5.2) were added. DNA was allowed to precipitate at −20 ℃ for at least one hour. Pelleting of DNA was done at 13,800 xg for 10 minutes. Ethanol was poured off. DNA was then washed twice using 80% ethanol and resuspended in 100 μl distilled water. DNA content and purity was estimated using UV at 260 and 280 nm.

PCR for EBV and HPV 16

In a 100 μl total reaction volume, crude DNA extract (about 1000 ng), or 50 ng control DNA were incubated in reaction mixture that contained: 10 mM Tris-hydrogen chloride (pH 8.3), 1.5 mM Mg chloride, 0.2 mM of each deoxynucleotide triphosphate, 50 pmol of oligonucleotide primers, and 0.25 units of thermostable Taq polymerase. DNA positive control for EBV was obtained from Raji cell line, derived from an EBV-infected African Burkitt's lymphoma [27]. Positive control for HPV 16 was derived from a cervical carcinoma specimen known to harbor the virus [11]. Placental tissue was used as negative control for EBV, and inflammatory nasal polyp as a negative control for HPV 16. Table 1 shows the oligonucleotide primers of EBV and HPV 16 used for PCR.

The reactions were overlaid with mineral oil and subjected to 30 cycles of amplification. For both EBV and HPV 16, the amplification cycle was 1 minute denaturation at 94 ℃, 2 minutes annealing of primers at 46 ℃, and 3 minutes for synthetic extension of the primers at 72 ℃

Table 1
Oligonucleotide primers for PCR of EBV and HPV 16

Oligonucleotide No.	Sequence
EBV	
Primers: 1041-1060	5` GTTCGCGTTGCTAGGCCACC 3`
1131-1150	5` AGGACCACTTTATACCAGGG 3`
HPV 16	
Primers: 320-339	5` ATTAGTGAGTATAGACATTA 3`
410-429	5` GGCTTTTGACAGTTAATACA 3`

[11,27]. The amplification products of PCR were allowed to diffuse in 2% agarose gel with 80 ng/ml ethidium bromide.

Sample Preparation for FCM

Fresh surgical biopsy specimens were immediately put in ice cold RPMI medium. From each sample a single cell suspension was made from recognized epithelial tissue within 30 minutes from sampling after removal of fat, blood accumulations, necrotic tissue, and normal-looking tissue. Mechanical (scissors and scalpel) disaggregation was performed, and cell clumps were removed by filtration through a 50 μm nylon mesh. After centrifugation, the yield of cells was calculated (microscopic examination) and split into two parts: one for flow cytometry and one for cytologic examination. Preparations with high content of lymphocytes were discarded.

50 μm Sections from each paraffin block were deparaffinized according to Hedley's method, modified by McLemore et al. 1990 [20]. Then 1 ml of 0.5% prewarmed pepsin in 0.9% saline at pH 1.5 was added to each sample, followed by incubation for 45 to 60 minutes at 36 ℃, with vortexing at intervals of 10 minutes. All of the residual solid tissue was then removed, and the remaining nuclear suspension was filtered through 50 μm nylon mesh. The suspension was then centrifuged at 800 xg for 5 minutes, and the supernatant was decanted. The pellet was resuspended in 1 ml Hanks solution, centrifuged as before, and the supernatant was decanted. The wash step was repeated twice. The final concentration was adjusted to 10^6 nuclei/ml.

Following disaggregation, both fresh and paraffinized samples were stained with propidium iodide, as follows: a 100 μL aliquot of each sample was lysed and stained by the Coulter DNA-Prep, which sequentially dispenses and mixes 100 μL of Lysing Permeabilizing Reagent (LPR) and 1 ml of staining solution (containing 50 μg/ml propidium iodide and 4 KU/ml bovine pancreas type III RNAse) into each sample. Finally, the samples were incubated at room

temperature for 60 minutes, in darkness, prior to flow cytometric analysis [8].

Flow Cytometry

Flow cytometric analysis was performed with a Coulter EPICS® Profile II flow cytometer, configured with a 488 nm argon ion laser. Peripheral blood lymphocytes were used as external standard for fresh tissue material. For paraffin-embedded tissue, 50 μm sections from tonsil block were processed in parallel with each run and were used as an external standard. Twenty thousand events/samples were acquired. DNA aneuploidy was defined as any population with a distinct additional peak (s), or the presence of a tetraploid population greater than 15%. The CV was defined as the standard deviation as a percentage of the mean DNA value of the diploid peak. Samples were excluded when CV exceeded 5%.

Statistical Analysis

All analyses were performed using Statistical Package for the Social Sciences (SPSS) software.

RESULTS

The study included twenty patients with nasopharyngeal carcinoma, 10 patients with inverted papilloma, and 5 control patients for both types of tumors. The age of NPC patients had a wide range (7–75 years, mean ± SD 48 ± 15.7), 16 of them (80%) were in the age group between 35–64 years. The ratio between males and females was 2.3:1. The age of IP patients ranged from 37 to 64 years (49.1 ± 9.86). According to WHO classification, patients with type 3 NPC comprised 90% of the total number of patients, a minority were type 2 (10%). Most of the patients were classified according to the Ho staging system as stage III (45%), less common was stage II (30%) and the least was stage I (25%). According to our proposed staging system, IP patients were classified as
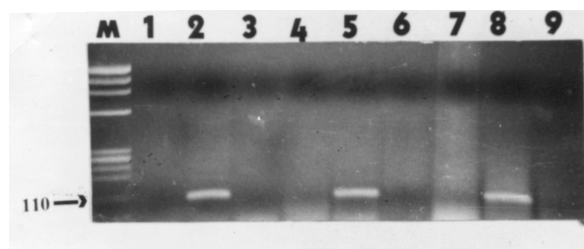

Fig. 1. Analytical gel electrophoresis showing 110 bp product of the PCR amplification reaction of the EBV. M represents φ × 174 DNA/Hae III marker. The arrow points to 110 band level. Lane 1: negative control, lane 2: positive control, lane 3: amplification control, lanes 5 and 8: positive samples, lanes 4, 6, 7 and 9: negative samples.
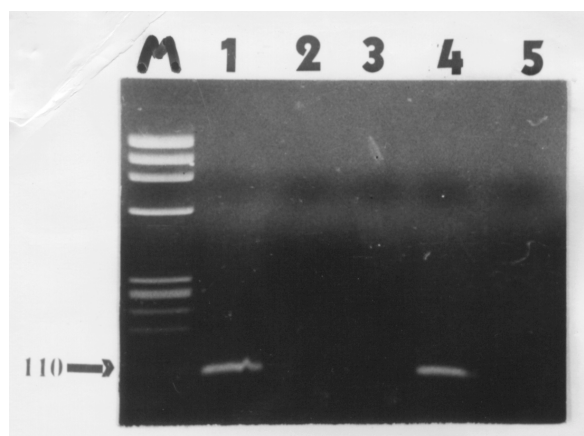

Fig. 2. Analytical gel electrophoresis showing 110 bp product of the PCR amplification reaction of the HPV 16. M represents φ × 174 DNA/Hae III marker. The arrow points to 110 band level. Lane 1: positive control, lane 2: negative control, lane 3: amplification control, lane 4: a positive sample, lane 5: a negative sample.

stage I (60%), stage II (20%), stage III 10%, and one patient (10%) as stage IV, as he had intracranial extension. All IP patients were males, 6 of them showed squamous metaplasia, 3 showed dysplasia. However, none of the patients showed malignant transformation in any part of the tumor tissue.

Positive samples for the presence of EBV and HPV 16 genomes were judged from the presence of a 110-bp fragment after the electrophoretic analysis of the PCR products (Figures 1 and 2).

EBV was detected in 65% of specimens of NPC. It was not detected in any of the tissue specimens of IP or any of the control cases. No correlation was found between the EBV and either the age or pathologic type. Using Chi-square, a statistically significant correlation was found between the EBV and stages II and III as compared with stage I NPC (P < 0.05). Recurrence was reported in 6 cases (30%) of NPC patients during a follow-up period (1–4 years, mean 22 months). Most recurrences were nodal while a minority was systemic. EBV genome was detected in 5 (83%) of these recurrent cases. HPV 16 genome was detected by PCR in 5 cases (25%) of NPC patients, 2 of whom were harboring EBV too. The 2 cases who had a dual infection were NPC type 3; one of them was 42-year-old male in stage I of the disease, the other was a 45-year-old female patient who had NPC stage II. None of them had had recurrent disease after 4 years of follow-up. Positive HPV 16 infection was not correlated to age, sex, pathologic type or staging. Only one of the recurrent cases of NPC was HPV 16 positive. HPV 16 genome was detected in 40% of patients with IP, and in none of the control specimens. There was no significant correlation between the HPV 16 and the age or clinical staging of IP patients. However, a highly significant correlation was found between HPV 16 and dysplasia. All cases with dysplasia were HPV positive, while only one case without dysplasia was HPV positive. No significant correlation of the HPV 16 and metaplastic changes in inverted papilloma. It should be noted that 33% of cases with metaplasia were HPV positive.

By flow cytometry, 19/20 NPC cases were found to be diploid with a DNA index (DI) of 1. Only one specimen was found to be aneuploid with a DI of 1.142. This specimen belonged to a 75-year-old male patient with stage III, WHO type 2 NPC, and who was EBV positive. Proliferative capacity (PC = SPF + G2/M) was calculated. A cut-off value of PC, which maximized the sum of sensitivity and specificity and exhibited the maximum predictive values for recurrence in NPC, was estimated to be 13.6 (Figure 3). Above this value recurrence is highly
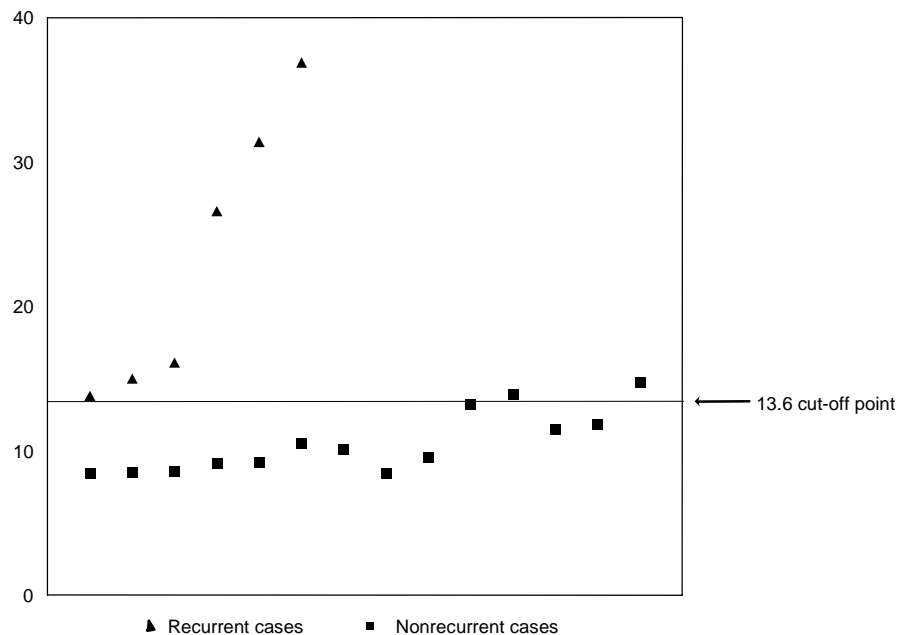

Fig. 3. A scatter diagram representing the distribution of proliferative capacity in the recurrent and nonrecurrent NPC patients.

probable. The mean value of PC was 15.64 ± 8.81. Using the Mann-Whitney test, the correlation between proliferative capacity (PC) and its recurrence was found to be significant (mean of PC in recurrent group was 23.3 versus 10.53 in non-recurrent group, $P < 0.05$). Neither EBV nor HPV 16 was found to have a significant correlation with PC values. All IP specimens were diploid, with a DI of 1. The mean of PC was 9.13. There was no significant correlation between the pathologic changes, whether dysplasia or metaplasia, and the value of PC. Also, no significant correlation was found between the HPV 16 and PC in cases of inverted papilloma.

DISCUSSION

It is widely accepted that EBV is etiologically associated with the development of nasopharyngeal carcinoma in which the tumor cells contain EBV episomes with clonal integration [24]. It affects about 90% of the world population before adulthood [14]. This suggests that other mechanisms of cellular interaction may be involved in malignant transformation. As with many virus-associated tumors, the first evidence for an association between EBV and NPC was derived from serological studies. Although serology might be a useful screening tool for NPC in high-incidence areas such as China, it lacks sensitivity and specificity. Typically, patients have elevation in IgG and IgA titres to the viral capsid antigen [23]. However, such antibodies are also found in the general population, especially in high-risk regions [10]. The polymerase chain reaction technique used in this study enabled us to detect EBV and HPV 16 DNA in fresh frozen and in paraffin-embedded tissues from Egyptian patients with NPC and IP. Since PCR uses only a small fragment of cellular DNA, formalin-fixation and paraffin-embedding have only a minor effect on the rate of detection of viral DNA. In addition, the technique is very rapid, sensitive and accurate. EBV genomes can also be demonstrated in NPC by in situ hybridization and

blotting analysis [9]. However, unlike PCR, these technologies cannot be applied to archival samples and are less sensitive and rapid.

In this study, EBV genome was detected in 65% of tissue specimens of NPC and in none of the control specimens. This percentage is lower than that reported in high-risk areas (90% or more) [1,6]. The difference may be due to different genetic or environmental factors. The viral genome was significantly correlated with stage II and III. It was also detected in 83% of the recurrent cases indicating virus association with more aggressive forms of the disease. Its presence may indicate bad prognosis. No association was found between the virus and IP.

The viral genome of human papillomaviruses contains coding regions referred to as open reading frames (ORFs), and a noncoding region referred to as NCR. ORFs that encode nonstructural proteins are known as early (E) genes, and those that encode structural proteins are known as late (L) genes. Papillomaviruses that infect humans have seven (E) and two (L) genes [28]. Integration of the virus disrupts the viral genome in the E1-E2 region, resulting in the failure of transcription of the late genes and possibly uncontrolled transcription of the E6 and E7 genes [26]. E7 protein of HPVs 6, 11, 16, and 18 bind to the gene product of the well-known tumor supressor gene retinoblastoma (RB) [17]. E6 protein of HPVs 16 and 18 but not 6, or 11 bind to the gene product of another tumor supressor gene, p53 [3]. HPV 16 was selected for study from the various types of HPVs because it has a high tropism to epithelial tissues. The virus causes metaplasia followed by dysplasia, and is associated with dysplastic changes in cases of cervical intraepithelial neoplasia [14]. In our study, HPV 16 was found in 5 cases with NPC (25%). Two of them were also harboring EBV. This indicates the minor role of HPV 16 as an etiological factor for NPC. On the other hand, we detected HPV 16 in 40% of patients with IP, but in none of the control cases. A significant correlation was found between the virus and dysplasia in IP. The recent study done by Caruana et al. 1997 [3] showed the same strong correlation. This association was proved earlier

in cervical carcinoma [14]. The unstable behavior of IP can be explained, in part, by the presence of the HPV 16 virus.

The proliferative activity of a tissue has been estimated in a variety of ways. Flow cytometry can provide measurements of proliferative rates of tumors by measuring the fraction of cells in S and G_2/M phases of the cycle. This study is the first to evaluate PC as a predictor for recurrence in NPC. Using 13.6 as a cut-off value, we were able to discriminate between high-risk and low-risk groups (100% sensitivity and 86% specificity). Other prognostic parameters as serologic markers for EBV (IgG and IgA antibodies against VCA, EA and EBNA) lack the prediction of recurrence at the time of diagnosis. They are only helpful in the follow-up of patients after treatment. The only exception to these is the ADCC (antibody-dependent cellular cytotoxicity) which can be used as a baseline prognostic parameter. However, unlike PC, serologic markers in general are affected by other factors such as the immune status of the patient [22]. In IP, all specimens were diploid with low PC as expected in benign tumors. The lack of correlation between HPV 16 and PC in IP suggests that the virus is not the sole factor implicated in the proliferative potential of IP although it is related to the presence of a premalignant pathologic change, namely dysplasia, within the tumor cells.

Acknowledgments

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