

## CLONAL PROLIFERATIONS OF CELLS INFECTED WITH EPSTEIN-BARR VIRUS IN PREINVASIVE LESIONS RELATED TO NASOPHARYNGEAL CARCINOMA

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**Abstract** *Background.* The Epstein-Barr virus (EBV) is consistently detected in patients with nasopharyngeal carcinoma. To determine whether EBV infection is an early, initiating event in the development of this malignant tumor, we screened nasopharyngeal-biopsy samples, most of which were archival, for preinvasive lesions, including dysplasia and carcinoma in situ. Preinvasive lesions were found in 11 samples, which were tested for the presence of EBV.

*Methods.* EBV infection was detected with *in situ* hybridization for EBV-encoded RNAs (EBERs) and by immunohistochemical staining for latent membrane protein 1 (LMP-1). The larger samples were also tested for the EBV genome with the use of Southern blotting. The expression of specific EBV RNAs was determined by the amplification of complementary DNA with the polymerase chain reaction.

*Results.* Evidence of EBV infection was detected in all 11 tissue samples with dysplasia or carcinoma in situ. EBERs were identified in all eight samples tested, and

LMP-1 was detected in all six of the tested samples. Six of the seven samples tested for the EBV termini contained clonal EBV DNA. Transcription of the latent EBV gene products, EBV nuclear antigen 1, LMP-1, LMP-2A, and the BamHI-A fragment, was detected in most of the samples. Viral proteins characteristic of lytic lesions were not detected.

*Conclusions.* Preinvasive lesions of the nasopharynx are infected with EBV. The EBV DNA is clonal, indicating that the lesions represent a focal cellular growth that arose from a single EBV-infected cell and that EBV infection is an early, possibly initiating event in the development of nasopharyngeal carcinoma. Preinvasive lesions contain EBV RNAs that are characteristic of latent infection but not the viral proteins that are characteristic of lytic infection. The detection of the EBV-transforming gene, LMP-1, in all the neoplastic cells suggests that its expression is essential for preinvasive epithelial proliferations associated with nasopharyngeal carcinoma. (*N Engl J Med* 1995;333:693-8.)

THE Epstein-Barr virus (EBV) is an important factor in the development of nasopharyngeal carcinoma, an epithelial cancer.<sup>1,2</sup> Nasopharyngeal carcinoma is rare in North American and European whites, with an age-adjusted incidence of 1 case per 100,000 population. In contrast, it is among the most common cancers in southern China and parts of southeast Asia. An age-adjusted incidence of 26 cases per 100,000 has been reported among males in Hong Kong. Genetic and environmental factors appear to contribute to the elevated risk of nasopharyngeal carcinoma among the Chinese. Patients with nasopharyngeal carcinoma have elevated titers of IgA antibodies to EBV replicative antigens, including the viral capsid antigen. These antibodies, which frequently precede the appearance of the tumor, serve as a prognostic indicator of remission and relapse.<sup>1</sup>

Regardless of whether a patient with nasopharyngeal carcinoma lives in an area of endemic or sporadic incidence, all the tumor cells contain EBV DNA.<sup>3,4</sup> The EBV infection is latent, and the viral DNA is a clonal episome. The clonality of EBV DNA in nasopharyngeal carcinoma arises from the clonal expansion of a single EBV-infected cell.<sup>5</sup> If EBV infected a fully formed neoplastic lesion, one would expect to detect an occasional cell that lacked EBV or a polyclonal EBV infection.

Premalignant and preinvasive neoplastic lesions of the nasopharynx in patients in whom nasopharyngeal

carcinoma eventually develops are poorly documented and rare.<sup>6-9</sup> To determine whether EBV infection is an early, possibly etiologic event in the evolution of nasopharyngeal carcinoma, we screened a large number of nasopharyngeal-biopsy samples for early malignant changes, including dysplasia and carcinoma in situ. Eleven samples were identified and analyzed for the presence of EBV.

### METHODS

#### Tissue Samples

Most of the biopsy samples we analyzed were from an archive of nasopharyngeal-biopsy samples at the University of Malaya in Kuala Lumpur, Malaysia. Samples were examined microscopically for the presence of dysplastic or preinvasive nasopharyngeal mucosal lesions. Carcinoma in situ, as distinguished from dysplasia, was documented if the normal epithelial cells in the topmost layers of the tissue were absent.

A total of 5326 biopsy samples were screened. Invasive carcinoma, nasopharyngeal carcinoma, and various grades of dysplasia or carcinoma in situ were detected in 1811 samples, 56 (3 percent) of which contained preinvasive mucosal lesions with adjacent invasive cancer. In 11 samples (0.6 percent) there was dysplasia or carcinoma in situ without adjacent invasive carcinoma. Tissue was available from 8 of these 11 samples for studies of EBV. Cancer was diagnosed at follow-up examinations within 12 months after the tissue samples had been obtained in five of the eight patients whose samples were evaluated for the presence of EBV.

Three additional samples of dysplastic nasopharyngeal mucosa without concomitant carcinoma (samples 3, 6, and 9) were obtained at the Tumor Institute of Sun Yat Sen University in Guangzhou, China; DNA and RNA were extracted from these samples and analyzed with molecular probes. Thus, 11 samples of dysplasia or carcinoma in situ — 8 from Malaysia and 3 from China — were analyzed for the presence of EBV.

Because of heightened awareness of nasopharyngeal carcinoma among Malaysian Chinese people, nasopharyngeal biopsies are performed in many possibly symptomatic patients. From a collection of tissue samples obtained from such patients, 60 samples of normal nasopharyngeal tissue, identified in the screening, served as controls.

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These samples of normal epithelium were obtained from patients in the ear, nose, and throat clinic who had been followed for two years without evidence of cancer.

### In Situ Hybridization of EBV-Encoded RNA

Formalin-fixed, paraffin-embedded tissue was placed on slides pretreated with 1× Denhardt's solution (0.02 percent Ficoll, 0.02 percent polyvinylpyrrolidone, and 0.02 percent bovine serum albumin) and 1 percent poly-L-lysine or 2 percent organosilane. The tissue sections were deparaffinized, rehydrated, and hybridized to a fluorescein-conjugated oligonucleotide probe, obtained commercially, which consists of a mixture of the two EBV-encoded RNAs, EBER-1 and EBER-2 (Dako, Carpinteria, Calif.). Hybridization was detected by incubation with rabbit anti-fluorescein antibody tagged with alkaline phosphatase, followed by reaction with the substrate 5-bromo-4-chloro-3-indoyl phosphate and the colorimetric indicator nitroblue tetrazolium.

### Immunohistochemical Identification of Latent Membrane Protein 1

Latent membrane protein 1 (LMP-1) was identified with the use of a pool of commercially available mouse monoclonal antibodies (GS1 through CS4) and a standard immunoperoxidase staining procedure (Dako). The C15 tumor, an EBV-positive nasopharyngeal carcinoma passaged in nude mice, and previously identified, unambiguous examples of nasopharyngeal carcinoma were used as positive controls.<sup>10</sup> The specificity of the staining was confirmed by the use of EBV-negative squamous-cell carcinomas of the skin and the EBV-negative cell line HeLa as negative controls and by the use of staining without the anti-LMP-1 antibody.

### Assessment of EBV Clonality

Sufficient tissue was available for molecular analysis of EBV in four samples from the University of Malaya and three from Sun Yat Sen University. DNA from the tumors and dilutions of DNA from the Raji lymphoid cell line, representing 1, 5, and 50 copies of episomal DNA, were digested with *Bam*HI and analyzed by Southern blotting. The blots were hybridized with probes representing the left end (*Eco*RI-I) or the right end (*Xho*I-a) of the EBV genome, as described previously.<sup>5</sup>

### Analysis of EBV Transcription by the Polymerase Chain Reaction

To test for the expression of EBV genes, RNA obtained from the seven larger tissue samples was used to prepare complementary DNA (cDNA). Equal aliquots of RNA were processed with reverse transcriptase or, as a negative control, without reverse transcriptase. The individual EBV messenger RNAs (mRNAs) were identified by the amplification of cDNA with 20-base oligonucleotide primers that span a splice specific for each mRNA, as previously described.<sup>11</sup> Con-

trols containing all the polymerase-chain-reaction (PCR) reagents and water instead of template were included for all reactions. The PCR products were resolved by agarose-gel electrophoresis and transferred to nitrocellulose, followed by hybridization to <sup>32</sup>P-end-labeled oligonucleotide probes.

## RESULTS

### In Situ Detection of EBV

The preinvasive lesions were analyzed for transcripts of EBV genes and for EBV proteins by means of in situ hybridization and immunohistochemical staining. The small nuclear EBERs are the most abundant RNAs in latently infected cells; approximately 1 million copies per cell are consistently detected in tissue samples from patients with nasopharyngeal carcinoma.<sup>12,13</sup> The EBERs are not expressed in oral hairy leukoplakia, a permissive EBV infection, and are thus excellent diagnostic markers of a latent EBV infection.<sup>14,15</sup> We detected EBERs in all eight tested samples with dysplasia or carcinoma in situ (Table 1). Of the 56 samples that contained concomitant preinvasive and invasive lesions, 22 were analyzed for the expression of EBERs. The EBERs were readily detected throughout the invasive and preinvasive areas of the samples (data not shown). The results of EBER hybridization were negative in the 60 samples of normal nasopharyngeal tissue, confirming the results of a recent study.<sup>16</sup>

A tissue sample with carcinoma in situ (sample 7) is shown in Figure 1A. The immature atypical cells were characterized by an increased nuclear size, prominent nucleoli with occasional mitotic figures, and an absence of terminal differentiation in the upper layers toward the luminal surface. The basement membrane was intact, and lymphoid stroma was absent from the underlying fibrovascular stroma. EBERs were present in all the immature cells in the lesion but not in the underlying stroma (Fig. 1B). EBERs were also found in a tissue sample (sample 10) with severe dysplasia of the nasopharyngeal mucosa. The dysplasia showed evidence of differentiation in the uppermost layers of the epithelium (Fig. 1C); EBERs were detected in most cells, including some of the differentiated cells (Fig. 1D).

LMP-1 is the only EBV protein with transforming

Table 1. Data on EBV Infection in 11 Samples of Preinvasive Lesions from Patients with Nasopharyngeal Carcinoma.\*

SAMPLE NO.	HISTOLOGIC FINDING	EBV DNA CLONALITY	REVERSE-TRANSCRIPTASE PCR TESTS						EBER	LMP-1
			EBNA-1	LMP-1	LMP-2	<i>Bam</i> HI-A FRAGMENT				
						EBNA-2	ZEBRA			
1	Carcinoma in situ	ND	ND	ND	ND	ND	ND	ND	Yes	ND
2	Severe dysplasia	ND	ND	ND	ND	ND	ND	ND	Yes	ND
3	Mild dysplasia	Yes	No	Yes	Yes	No	No	No	ND	ND
4	Severe dysplasia	No	Yes	Yes	Yes	ND	ND	ND	Yes	Yes
5	Carcinoma in situ	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes
6	Mild dysplasia	Yes	Yes	Yes	Yes	Yes	No	No	ND	ND
7	Carcinoma in situ	ND	ND	ND	ND	ND	ND	ND	Yes	Yes
8	Carcinoma in situ	ND	ND	ND	ND	ND	ND	ND	Yes	Yes
9	Moderate dysplasia	Yes	Yes	Yes	No	No	No	No	ND	ND
10	Severe dysplasia	Yes	Yes	Yes	Yes	ND	ND	ND	Yes	Yes
11	Carcinoma in situ	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes

\*PCR denotes the polymerase chain reaction, EBER EBV-encoded RNA, LMP-1 latent membrane protein 1, EBNA-1 EBV nuclear antigen 1, LMP-2 latent membrane protein 2, EBNA-2 EBV nuclear antigen 2, ZEBRA the EBV replication activator *Bam*HI-Z, and ND test not done.

properties in rodent cell lines and is essential for immortalization of B lymphocytes *in vitro*.<sup>17,18</sup> This protein has been detected in approximately 60 percent of tissue samples from patients with nasopharyngeal carcinoma.<sup>19,20</sup> With immunohistochemical staining, we found LMP-1 in all cells in all six of the tested samples (Table 1).

In one tissue sample with carcinoma *in situ* and in one with dysplasia, coexpression of EBERS and LMP-1 was found in most of the cells in the abnormal epithelium. In a low-power microscopical view of the sample with carcinoma *in situ* (sample 5), the positive EBV hybridization was confined to the abnormal mucosa; occasional macrophages in the subepithelial lymphoid stroma stained weakly because of endogenous peroxidase activity (Fig. 2A). In the same section, strong staining for LMP-1 was found only in the epithelial cells (Fig. 2B).

In a sample with severe dysplasia (sample 4), EBERS

were detected in the majority of cells, with some of the abnormal cells apparently negative for the expression of EBV (Fig. 2C). A similar result has been reported in tissue from patients with nasopharyngeal carcinoma.<sup>21</sup> However, all the cells within this focus stained positively for LMP-1 (Fig. 2D). The degree of staining was particularly intense in the superficial layers of the epithelium. In contrast, the infiltrating lymphocytes, marked by clear cytoplasm and dark, condensed nuclei, did not express EBERS (Fig. 2D). Our studies revealed LMP-1 in all the samples with preinvasive neoplasia and in all the dysplastic cells, even when EBERS were not detected.

#### Clonality of EBV in Preinvasive Neoplasia

In cells latently infected by EBV, the viral genome is an episome formed by circularization of the linear EBV genome that is present in viral particles. Restriction-enzyme fragments of the termini of the circular episome

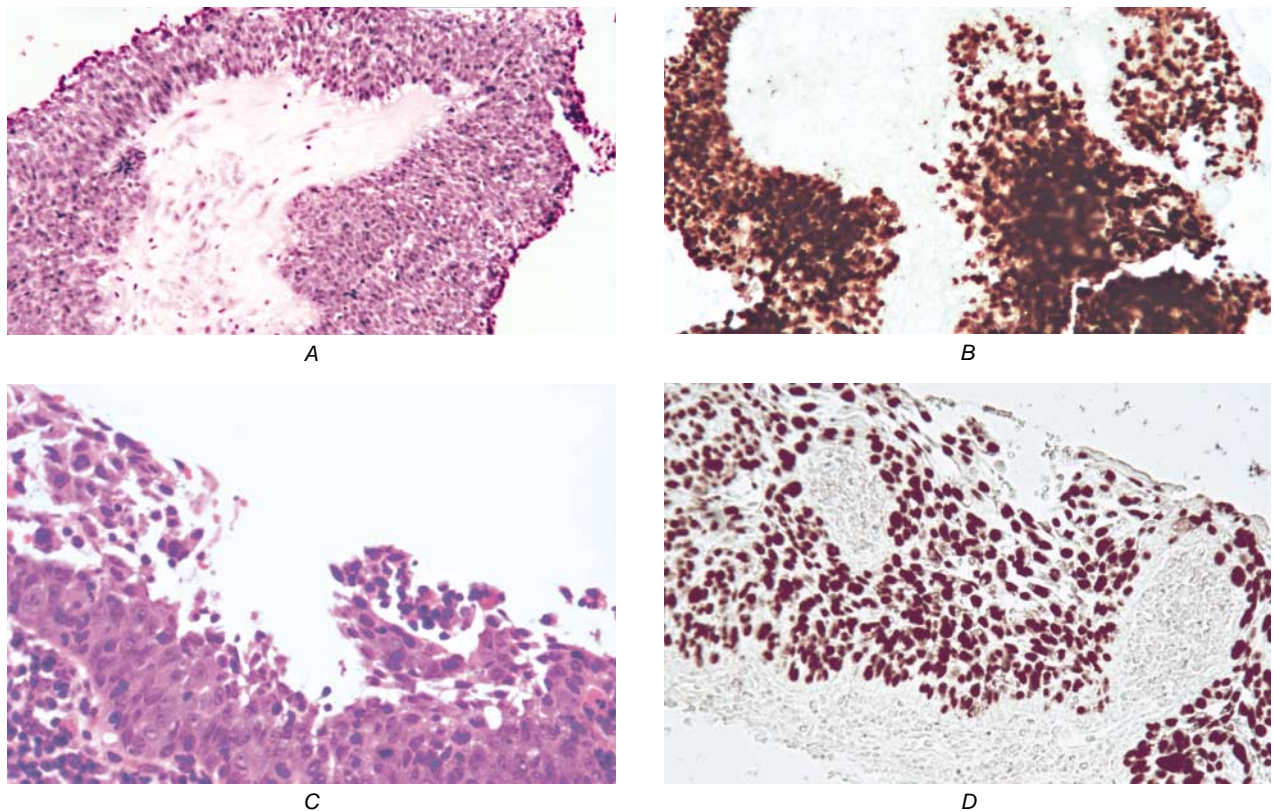


Figure 1. Histologic Features of Preinvasive Neoplasia of the Nasopharynx and Detection of EBV-Encoded RNA (EBER).

Panel A shows a polypoid mucosal lesion of the nasopharynx with carcinoma *in situ* (sample 7). The normal respiratory tract mucosa has been completely replaced by abnormal cells marked by a lack of differentiation, large nuclei with the loss of nuclear polarity, an increased ratio of cellular nuclei to cytoplasm, and prominent nucleoli. The basement membrane is intact, and the underlying stroma is composed of loose fibrovascular tissue and occasional inflammatory cells. There is no evidence of tumor invasion. (Hematoxylin and eosin,  $\times 630$ .) Panel B shows the same lesion with dark-brown nuclear staining, indicating the abundant expression of EBV within all nuclei of all cells throughout the full thickness of the epithelial layer ( $\times 630$ ). Panel C shows a mucosal lesion with severe dysplasia (sample 10), characterized by hypercellularity and an increased thickness of the mucosal epithelium, with the preexisting epithelial cells replaced by abnormal cells throughout most of the epithelium. There is some squamous maturation of proliferating cells in the topmost layers. The fragmentation of the epithelial layers is an effect of tissue processing. (Hematoxylin and eosin,  $\times 1260$ .) Panel D shows *in situ* hybridization of EBV throughout this severely dysplastic lesion. EBVs were also detected in most of the superficial, maturing epithelial cells but not in the underlying lymphoid cells ( $\times 1260$ ).

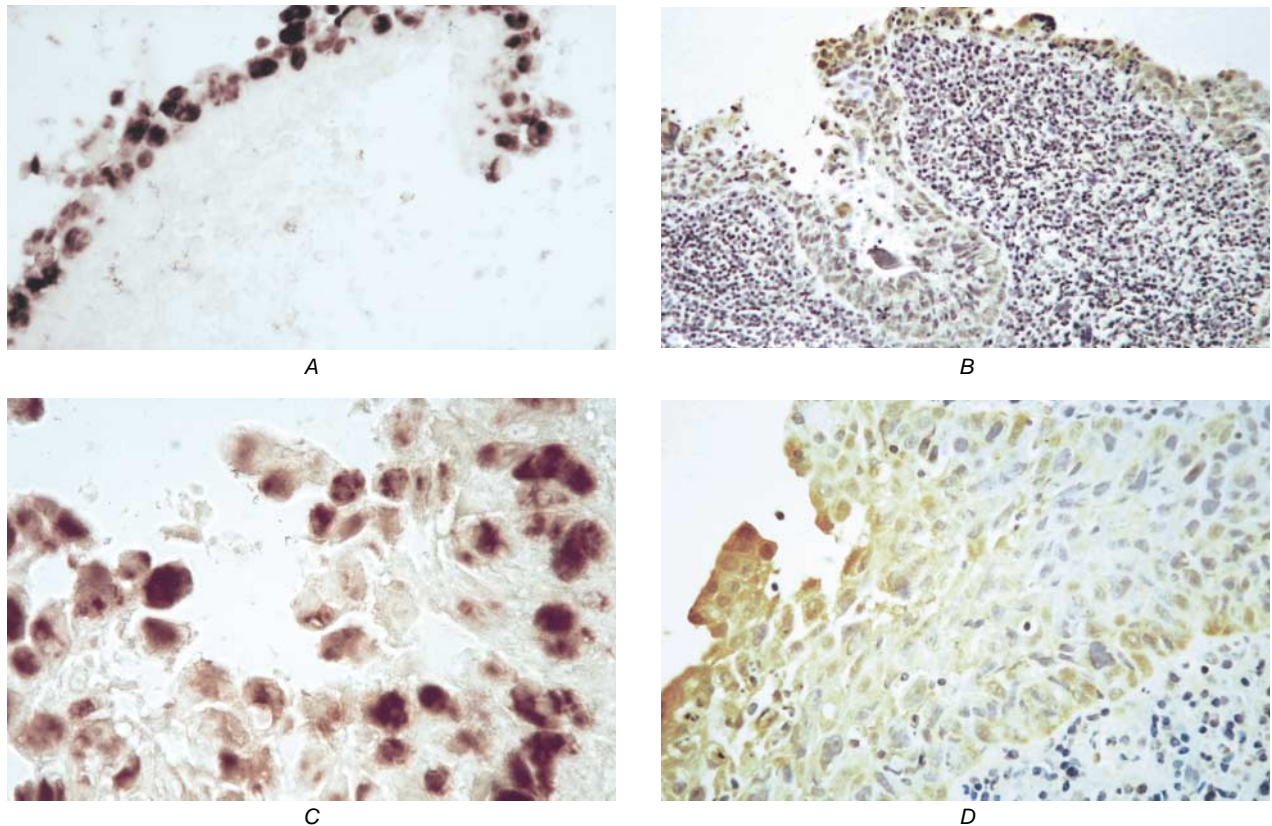


Figure 2. EBV-Encoded RNA (EBER) and Latent Membrane Protein 1 (LMP-1) in Tissue Samples from Patients with Preinvasive Nasopharyngeal Neoplasias.

In Panel A, which shows a tissue sample from a patient with carcinoma *in situ* (sample 5), the overlying atypical epithelial cells are positive for EBER. Much of the superficial mucosa is eroded. EBERs are not present in the underlying lymphoid stroma ( $\times 1260$ ). Panel B shows the same sample with brown immunostaining for LMP-1 in the overlying epithelium, which is intense in the superficial layers. LMP-1 is not present in the lymphoid cells of the submucosa, although there is occasional weak staining of macrophages because of endogenous peroxidase activity ( $\times 630$ ). Panel C shows a sample with severe dysplasia (sample 4). EBERs are present, in varying intensity, within the nuclei of most of the epithelial cells ( $\times 2500$ ). Panel D shows variably intense brown staining for LMP-1 in all the cells from sample 4. The intensity of the stain increases near the luminal surface. The occasional infiltrating small lymphocytes within the epithelial layer, with dark nuclei and clear cytoplasm, are negative for LMP-1 ( $\times 1260$ ).

mal DNA can be detected by probes from both ends of the linear genome.<sup>5</sup> In a monoclonal infection, the fused termini yield a single restriction-enzyme fragment, whereas multiple fused fragments suggest a polyclonal infection. Smaller fragments that do not hybridize to both probes represent linear genomes produced by viral replication.<sup>5</sup>

EBV clonality was evaluated in four samples of tissue with nasopharyngeal carcinoma from the University of Malaya and in three samples of tissue with dysplastic nasopharyngeal mucosa from Sun Yat Sen University. A single EBV terminal restriction-enzyme fragment was detected in three of the four samples from Malaysia, which were also positive for EBER and LMP-1 expression (Table 1 and Fig. 3), and in all three samples from China. In sample 6, smaller fragments that might have represented linear DNA were detected, in addition to a single fused terminal fragment (Fig. 3). The presence of linear EBV DNA in this sample was probably due to viral replication in a small percentage of the cells. The absence of detectable EBV DNA in one

(sample 4) that was positive for EBER and LMP-1 may have been due to an absence of abnormal cells in the portion of the sample that was processed for DNA analysis.

#### Analysis of EBV Expression

Latently infected lymphocytes and nasopharyngeal carcinoma cells express a specific subgroup of EBV genes. Lymphoid cell lines express six EBV nuclear antigens, EBNA-1 through EBNA-6, and two integral membrane proteins, LMP-1 and LMP-2. In nasopharyngeal carcinoma, EBNA-1, LMP-1, LMP-2, and transcription from the *Bam*HI-A fragment of the EBV genome are detected.<sup>22-27</sup> We used PCR with complementary DNAs to detect the distinctive RNAs corresponding to the EBNA-1, EBNA-2, LMP-1, and LMP-2 transcripts; *Bam*HI-A transcription; and a replicative gene product, the EBV replication activator *Bam*HI-Z (ZEBRA).<sup>28</sup>

Table 1 summarizes the results of these studies. Most of the preinvasive neoplastic lesions contained the characteristic RNAs of nasopharyngeal carcinoma

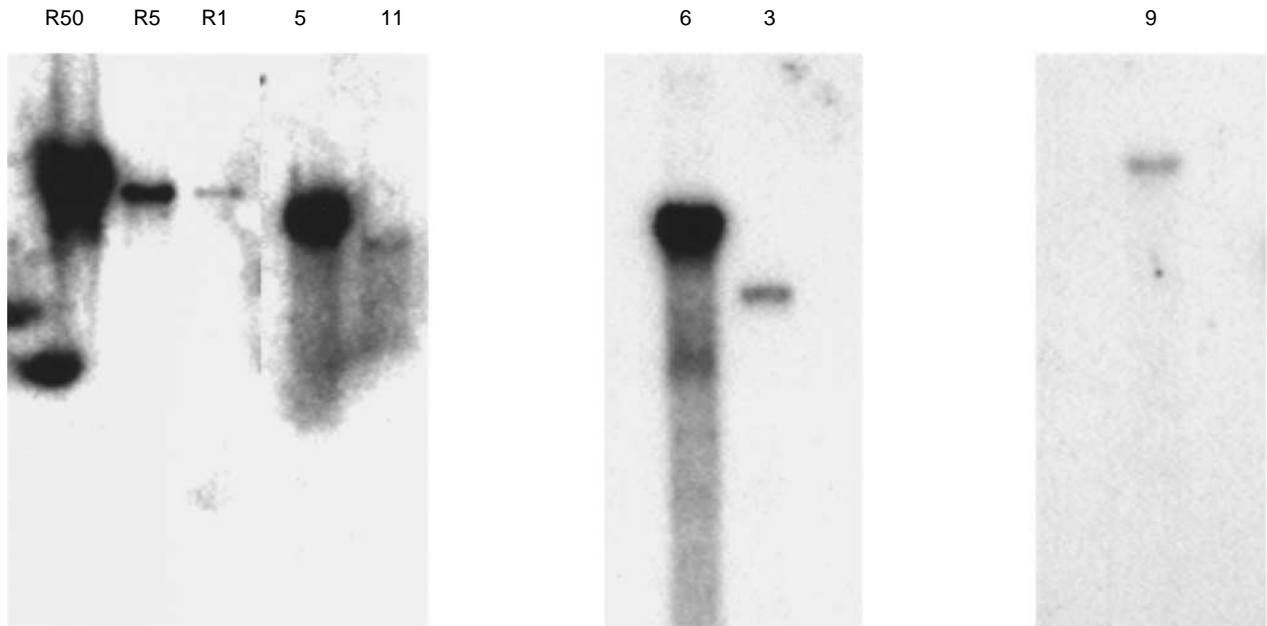


Figure 3. Detection of EBV DNA in Five Samples of Tissue with Preinvasive Neoplasia (Samples 3, 5, 6, 9, and 11).

Southern blots prepared with DNA from tissue with atypical hyperplasia, dysplasia, and carcinoma in situ, digested with *Bam*HI, and dilutions of Raji-cell DNA representing approximately 50 copies, 5 copies, and 1 copy of EBV per cell (R50, R5, and R1, respectively) were hybridized to a single-stranded RNA probe representing the *Xho*I-a fragment. A single restriction-enzyme fragment was detected in most samples, indicating the presence of clonal, episomal EBV DNA. Sample 6 had additional, smaller fragments that probably represented linear DNA.

— that is, EBNA-1, LMP-1, LMP-2, the *Bam*HI-A fragment, and the EBER RNAs (Table 1). Transcripts encoding EBNA-2, which is typically present with latent infection in lymphocytes, or ZEBRA, which distinguishes the earliest stage of lytic infection, were not detected in any of the samples assayed (Table 1). Therefore, the pattern of expression of viral genes in the preinvasive samples was identical to that in the samples with nasopharyngeal carcinoma.

#### DISCUSSION

The results of this study strengthen the evidence that EBV is critical to the pathogenesis of nasopharyngeal carcinoma. The data demonstrate latent EBV infection of all the cells in all 11 samples of preinvasive lesions. The presence of a single clonal form of EBV in these lesions suggests that nasopharyngeal dysplasia or carcinoma in situ is a focus of EBV-induced cellular proliferation and that EBV infection precedes the acquisition of invasiveness by these tumors.

Rare cases of nasopharyngeal mucosal dysplasia have been detected by nasopharyngoscopy during intensive screening programs in Guangdong province, China.<sup>9</sup> The lesions were marked by a thickening of the epithelial layer, with a loss of normal stratification, abnormal morphologic features, and pleomorphic nuclei.<sup>9</sup> However, these samples were not analyzed for EBV infection. In another study, EBV DNA was detected by in situ hybridization in only some cells in a few samples of tissue with carcinoma in situ.<sup>29</sup> This finding suggests that EBV infection occurred after the initial neoplastic

event. However, we found evidence of EBV infection in all samples of preinvasive lesions, with the use of several methods. In situ hybridization is considerably more sensitive for the detection of EBER than for the detection of EBV DNA, because, when expressed, EBERs are present at very high levels. In addition, LMP-1 was detected in all abnormal cells, even in some that were negative for EBER, indicating that the lesions were homogeneously infected.

Analysis of the EBV genomic termini and the expression of viral genes revealed that the preinvasive lesions contained predominantly latent infections and expressed the same EBV genes as those expressed in nasopharyngeal carcinoma. EBNA-2, which is essential for the in vitro transformation of lymphocytes and in those with leiomyomas.<sup>30,31</sup> The absence of the expression of EBNA-2 in the preinvasive neoplasias is consistent with its absence in nasopharyngeal carcinoma and indicates that EBNA-2 is not required for altered epithelial growth.<sup>19,20</sup> The absence of the expression of ZEBRA confirms the latent infection of these lesions by EBV.

Evidence of viral replication has been detected in oropharyngeal epithelial cells during primary or reactivated infection with EBV.<sup>32</sup> Sporadic reactivation is probably the result of reintroduction of the virus into epithelium from lymphocytes.<sup>33</sup> Normally, this type of reactivation would lead to viral replication and cytolysis. In nasopharyngeal carcinoma, however, the cascade of viral replication does not proceed. Instead, la-

tent EBV genes are expressed, which probably induce the infected cells to proliferate, thus forming a preinvasive lesion. The rare detection of preinvasive neoplasia (in 11 of the 1811 samples, or 0.6 percent) and preinvasive neoplasia with nasopharyngeal carcinoma (in 56 of the 1811 samples, or 3 percent) and the progression to nasopharyngeal carcinoma within one year in five cases suggest that EBV-induced clonal proliferation can progress rapidly to cancer.

The consistent expression of specific viral genes and the detection of LMP-1 in all cells in the preinvasive lesions suggest that certain EBV gene products contribute to the abnormal proliferation of the cells. The development of latent infection, rather than lytic infection, in oropharyngeal cells and critical virus-transforming functions may be the rare events that lead to the development of nasopharyngeal carcinoma. The development of latent infection in a nasopharyngeal epithelial cell may be influenced by the way in which the virus enters the cell or by the presence of a preexisting abnormality, perhaps caused by genetic or environmental factors.<sup>33,34</sup> Genetic changes such as mutations of the p53, retinoblastoma, or *ras* genes have not been detected in nasopharyngeal carcinoma,<sup>35,36</sup> but other genetic changes may develop while the tumor is growing and contribute to its progression and metastasis.

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## REFERENCES

- Henle G, Henle W. Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. *Int J Cancer* 1976;17:1-7.
- zur Hausen H, Schulte-Holthausen H, Klein G, et al. EBV DNA in biopsies of Burkitt tumors and anaplastic carcinoma of the nasopharynx. *Nature* 1970;228:1056-8.
- Raab-Traub N, Flynn K, Pearson G, et al. The differentiated form of nasopharyngeal carcinoma contains Epstein-Barr virus DNA. *Int J Cancer* 1987;39:25-9.
- Desgranges C, Wolf H, De-Thé G, et al. Nasopharyngeal carcinoma. X. Presence of Epstein-Barr genomes in separated epithelial cells of tumors in patients from Singapore, Tunisia and Kenya. *Int J Cancer* 1975;16:7-15.
- Raab-Traub N, Flynn K. The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. *Cell* 1986;47:883-9.
- Anderson MC. Premalignant and malignant diseases of the cervix. In: Fox H, ed. Haines and Taylor's obstetric and gynecological pathology. New York: Churchill Livingstone, 1987:225-77.
- Rosen PP, Braun DW Jr, Kinne DE. The clinical significance of pre-invasive breast carcinoma. *Cancer* 1980;46:Suppl:919-25.
- Crissman JD, Zarbo RJ. Dysplasia, in situ carcinoma and progression to invasive squamous cell carcinoma of the upper aerodigestive tract. *Am J Surg Pathol* 1989;13:Suppl 1:5-16.
- Li ZQ, Chen JJ, Li WJ. Early detection of nasopharyngeal carcinoma (NPC) and nasopharyngeal mucosal hyperplastic lesions (NPHL) with its relationship to carcinomatous change. In: Prasad U, Ablashi DV, Levine PH, eds. Nasopharyngeal carcinoma — current concepts. Kuala Lumpur, Malaysia: University of Malaya Press, 1983:17-23.
- Busson P, Ganem G, Flores P, et al. Establishment and characterization of three transplantable EBV-containing nasopharyngeal carcinomas. *Int J Cancer* 1988;42:599-606.
- Chen C-L, Sadler RH, Walling DM, Su Iu, Hsieh HC, Raab-Traub N. Epstein-Barr virus (EBV) gene expression in EBV-positive peripheral T-cell lymphomas. *J Virol* 1993;67:6303-8.
- Arrand JR, Rymo L. Characterization of the major Epstein-Barr virus-specific RNA in Burkitt lymphoma-derived cells. *J Virol* 1982;41:376-89.
- Howe JG, Steitz JA. Localization of Epstein-Barr virus-encoded small RNAs by in situ hybridization. *Proc Natl Acad Sci U S A* 1986;83:9006-10.
- Gilligan K, Rajadurai P, Resnick L, Raab-Traub N. Epstein-Barr virus small nuclear RNAs are not expressed in permissively infected cells in AIDS-associated leukoplakia. *Proc Natl Acad Sci U S A* 1990;87:8790-4.
- Randhawa PS, Jaffe R, Demetris AJ, et al. Expression of Epstein-Barr virus-encoded small RNA (by the EBER-1 gene) in liver specimens from transplant recipients with post-transplantation lymphoproliferative disease. *N Engl J Med* 1992;327:1710-4.
- Sam CK, Brooks LA, Niedobitek G, Young LS, Prasad U, Rickinson AB. Analysis of Epstein-Barr virus infection in nasopharyngeal biopsies from a group at high risk of nasopharyngeal carcinoma. *Int J Cancer* 1993;53:957-62.
- Wang D, Liebowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* 1985;43:831-40.
- Kaye KM, Izumi KM, Kieff E. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc Natl Acad Sci U S A* 1993;90:9150-4.
- Fahraeus RJ, Fu HL, Ernberg I, et al. Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. *Int J Cancer* 1988;42:329-38.
- Young LS, Dawson CW, Clark D, et al. Epstein-Barr virus gene expression in nasopharyngeal carcinoma. *J Gen Virol* 1988;69:1051-65.
- Wu TC, Mann RB, Epstein JI, et al. Abundant expression of EBER1 small nuclear RNA in nasopharyngeal carcinoma: a morphologically distinct target for the detection of Epstein-Barr virus in formalin-fixed paraffin-embedded carcinoma specimens. *Am J Pathol* 1991;138:1461-9.
- Kieff E, Liebowitz D. Epstein-Barr virus and its replication. In: Fields B, Knipe D, eds. *Virology*. New York: Raven Press, 1990:1889-910.
- Gilligan K, Sato H, Rajadurai P, et al. Novel transcription from the Epstein-Barr virus terminal *EcoRI* fragment, DJIhet, in a nasopharyngeal carcinoma. *J Virol* 1990;64:4948-56.
- Gilligan KJ, Rajadurai P, Lin J-C, et al. Expression of the Epstein-Barr virus *BamHI* A fragment in nasopharyngeal carcinoma: evidence for a viral protein expressed in vivo. *J Virol* 1991;65:6252-9.
- Brooks L, Yao QY, Rickinson AB, Young LS. Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *J Virol* 1992;66:2689-97.
- Busson P, McCoy R, Sadler R, Gilligan K, Tursz T, Raab-Traub N. Consistent transcription of the Epstein-Barr virus LMP2 gene in nasopharyngeal carcinoma. *J Virol* 1992;66:3257-62.
- Hitt MM, Allday MJ, Hara T, et al. EBV gene expression in an NPC-related tumour. *EMBO J* 1989;8:2639-51.
- Countryman J, Miller G. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. *Proc Natl Acad Sci U S A* 1985;82:4085-9.
- Yeung WM, Zong YS, Chiu CT, et al. Epstein-Barr virus carriage by nasopharyngeal carcinoma in situ. *Int J Cancer* 1993;53:746-50.
- Young L, Alfieri C, Hennessy K, et al. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *N Engl J Med* 1989;321:1080-5.
- Lee ES, Locker J, Nalesnik M, et al. The association of Epstein-Barr virus with smooth-muscle tumors occurring after organ transplantation. *N Engl J Med* 1995;332:19-25.
- Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS. Epstein-Barr virus replication in oropharyngeal epithelial cells. *N Engl J Med* 1984;310:1225-30.
- Sixbey JW, Yao QY. Immunoglobulin A-induced shift of Epstein-Barr virus tissue tropism. *Science* 1992;255:1578-80.
- de Thé G. Epidemiology of Epstein-Barr virus and associated diseases in man. In: Roizman B, ed. *The herpesviruses*. New York: Plenum Press, 1982: 25-104.
- Effert P, McCoy R, Abdel-Hamid M, et al. Alterations of the p53 gene in nasopharyngeal carcinoma. *J Virol* 1992;66:3768-75.
- Sun Y, Hegamyer G, Colburn NH. Nasopharyngeal carcinoma shows no detectable retinoblastoma susceptibility gene alteration. *Oncogene* 1993;8: 791-5.