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Undifferentiated, Nonkeratinizing, and Squamous Cell Carcinoma of the Nasopharynx

Variants of Epstein-Barr Virus-Infected Neoplasia

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Nasopharyngeal carcinoma (NPC) samples of distinct histological types, including squamous cell carcinoma (WHO type 1), nonkeratinizing carcinoma (WHO type 2), and undifferentiated carcinoma (WHO type 3), were analyzed for Epstein-Barr virus (EBV) infection and gene expression by using in situ and biochemical techniques. The EBV-encoded RNAs (EBER) were detected in situ in most tumor cells of all three WHO types of NPC. In foci of squamous differentiation and keratinization within less differentiated NPC and throughout the expanse of well differentiated squamous cell carcinoma, EBER expression was less abundant. Latent membrane protein, an EBV-encoded membrane protein, was detected in 72% (36/50) of all NPC and 67% (6/9) of the cases of squamous cell carcinoma. The EBV genomes were present as clonal episomal forms, without detectable linear viral DNA, in all cases of squamous cell carcinoma analyzed. Polymerase chain reaction amplification of cDNA detected EBV transcription for Epstein-Barr nuclear antigen 1, latent membrane proteins 1 and 2, and BamHI A in all samples, indicating that all forms of NPC express the same EBV genes. These results reveal that EBER expression is significantly decreased in areas with squamous differentiation and confirm that all types of NPC, regardless of histological type or differentiation contain clonal episomal EBV genomes, express specific EBV genes, and are a clonal expansion of EBV-infected cells. (Am J Pathol 1995, 146:1355–1367)

Nasopharyngeal carcinoma (NPC) is an epithelial tumor that occurs worldwide but is characterized by marked geographical and population differences in incidence.[1] It is rare among Europeans and North American Caucasians, with an age-adjusted incidence rate of less than 1/100,000. It develops with high incidence in southern China and southeast Asia, where it may represent 25% of all cancers. In southern China, the age-adjusted incidence rate is approximately 25/100,000 for males in Hong Kong.[2] The tumor also occurs with increased incidence in other Chinese populations and in Alaskan Eskimos and with intermediate incidence in Mediterranean Africans.[3]

NPC has been classified into three types by the World Health Organization (WHO): squamous cell carcinoma (SCC, WHO type 1), nonkeratinizing carcinoma (NKC, WHO type 2), and undifferentiated carcinoma (UC, WHO type 3).[4] The classification is entirely on the basis of light microscopic appearances of the tumor cells. SCCs are tumors in which evidence of keratinization is unequivocally present as evidenced by the presence of intercellular bridges and the production of keratin, either intracellular or extracellular. In common with SCCs that occur elsewhere in the body, SCC of nasopharyngeal origin may be graded as well, moderately, or poorly differentiated cancers. A desmoplastic reaction to invading tumor cells is common, and the intense infiltrating lymphocytes, so conspicuous in the other histological types of NPC, are often lacking in SCC. NKC and UC are distinguished from SCC by the failure to demonstrate

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evidence of squamous differentiation on light microscopy. Tumor cells in NKC generally exhibit a pavemented or stratified arrangement, and individual tumor cells are separated by clearly defined cell margins. In UC, cell borders are indistinct and tumor masses appear as syncytia in sheet-like masses. A characteristic and striking feature is vesicularity of the nuclei and the presence of prominent nucleoli in UC cells. Additionally, in NKC and UC, a substantial lymphoid infiltrate is frequently present. NKC and UC of the nasopharynx, but not SCC, have been traditionally associated with Epstein-Barr virus (EBV) infection, as patients have elevated immunoglobulin (Ig)G and IgA titers to the viral capsid antigen and to the diffuse component of the early antigen.[5,6] In contrast, patients with SCC have serological profiles similar to those of control populations. In some studies, EBV DNA and EBV nuclear antigen (EBNA) have been detected only in UC and NKC but not in SCC. However, EBV DNA was found in two of four tumors with regions of differentiation.[7,8] EBV DNA was detected in low copy number on Southern blots in five of five cases of well differentiated SCC.[9] The DNA from one of the samples was cloned, and a recombinant fragment representing the fused viral termini was obtained, indicating the presence of covalently closed episomal viral DNA in the tumor.

This study was undertaken to further characterize the rare SCC arising in the nasopharynx and to ascertain its association with EBV. *In situ* hybridization (ISH) for the EBV-encoded RNAs (EBERs) and immunohistochemical detection of the EBV latent membrane protein (LMP-1) were utilized to identify EBV-infected cells and to determine the relationship between differentiation and the presence of EBV. Larger samples were also analyzed to determine the viral DNA structure and to identify the EBV genes expressed in these tumors. The data indicate that all forms of NPC are uniformly infected by EBV and contain homogeneous, clonal EBV genomes. In all forms of NPC, the same set of specific EBV genes were expressed with an apparent decrease in EBER abundance in areas of differentiation. The demonstration of clonal EBV and the consistent expression of specific EBV genes suggest that regardless of the state of differentiation, NPC is a clonal expansion of EBV-infected cells.

Materials and Methods

Tissue Samples

A total of 5326 nasopharyngeal biopsies were screened for examples of NPC, from which 1800 samples of histologically confirmed NPC were identified. The patients were predominantly Chinese with a small number of Malay (172) and Indian (20) patients. All samples of SCC analyzed in this study were from Chinese patients. The nasopharyngeal biopsies were examined histologically and classified according to established WHO criteria. In all, 320 cases (17%) of SCC were identified, of which 4 were examples of well differentiated SCC. The other cases were either moderately (174 cases) or poorly (142 cases) differentiated. Of these, 31 cases were selected for further study by both biochemical and *in situ* analyses. Included were all 4 cases of well differentiated SCC, 20 cases of moderately differentiated SCC, and 7 cases of poorly differentiated SCC. In addition, a prominent squamous or keratinizing component was identified in 360 biopsies of NPC of mixed histological type. Several types of analyses were performed on the collected tissues, although the types of analyses that could be performed on a given sample were limited by the sample size. Small specimens were studied by *in situ* analyses. There were 4 samples of SCC that were large enough for biochemical studies, including 2 examples of well differentiated SCC (samples 156 and 200) and two examples of moderately differentiated SCC (samples 159 and 453).

EBER ISH

To detect expression of the small nuclear RNAs, the EBERs, ISH with fluorescein-conjugated oligonucleotide probes was used. The probes were obtained commercially and consisted of a mixture of both EBER-1 and EBER-2 (Dako Corp., Carpinteria, CA). Pretreatment of the slides included soaking in ethanol acetone (1:1 v/v), baking at 100 C, incubating in 3× SSC (sodium-buffered citrate), 1× Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin) for 3 hours at 65 C, dipping in 1% poly-L-lysine or 2% organosilane in acetone for 5 minutes, and storage at −70 C. The tissue sections, 4 to 5 μ thick, were placed on the slides, rehydrated, and air dried before they were treated with proteinase K in a solution of Tris-buffered saline (TBS) and washed and air dried again. The tissue sections were hybridized for 2 hours at 37 C in a 15 to 30-μl volume and washed in TBS (0.05 mol/L Tris/HCl, 0.15 mol/L NaCl, pH 7.6). Detection of hybridization utilized alkaline phosphatase-conjugated rabbit anti-fluorescein isothiocyanate antisera and was visualized by the colorimetric reaction of the enzyme conjugate with the substrate 5-bromo-4-chloro-3-

indoyl phosphate and the concomitant reduction of nitroblue tetrazolium.

LMP-1 Immunostaining

LMP-1 was identified with a pool of four anti-LMP-1 monoclonal antibodies (CS1–4) that were obtained commercially (Dako Corp.). The antibodies were detected with a secondary antibody that was tagged with immunoperoxidase. The formalin-fixed paraffin-embedded tissue sections were deparaffinized and rehydrated. A solution of 3% hydrogen peroxide was used to quench endogenous peroxidase activity, and the sections were rinsed in TBS. The sections were reacted with the primary antibody at a 1:50 dilution for 30 minutes, washed twice in TBS, reacted with biotinylated goat anti-mouse Ig for 30 minutes, and washed in TBS. The complex was detected with a streptavidin/horseradish peroxidase complex and incubated with diaminobenzidine tetrahydrochloride, which produces a dark brown precipitate.

Assessment of EBV Clonality

Tissue samples were processed as previously described, with purification of DNA and RNA through a CsCl step gradient.[10] To determine the EBV genome copy number and to identify the EBV termini, Southern blots were prepared with dilutions of Raji DNA representing 1, 5, and 50 copies of episomal DNA and sample DNA digested with *Bam*HI. The blots were hybridized with the *Xho*Ia probe, representing unique DNA adjacent to the terminal repeats as described previously.[11]

PCR Analysis of EBV Transcription

To analyze EBV expression, 2 μg of sample RNA was used to prepare cDNA. The RNA was annealed to 0.5 μg of a 20-mer oligodeoxythymidine primer by heating to 70 C for 10 minutes, followed by incubation on ice. Reverse transcription was performed in a 20-μl reaction volume at 45 C with Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Equivalent aliquots of RNA were processed in parallel without addition of enzyme to serve as a negative control. The cDNA was amplified by polymerase chain reaction (PCR) with oligonucleotide primers that span splices that are unique and distinguish each of the EBV mRNAs, as previously described (Table 1).[12] Negative controls containing all PCR reagents and water instead of template were included for all reactions. The PCR products were resolved by electrophoresis through 1% agarose gels with DNA molecular weight standards and transferred to nitrocellulose. Hybridizations were performed at 45 C with approximately 10^6 cpm/ml ^{32}P-end-labeled oligonucleotides and washed at 45 C in 1× SSC and 0.1% sodium dodecyl sulfate.

Results

Histology and in Situ *Identification of EBER RNAs*

An example of moderately differentiated SCC (sample 453) exhibited discrete islands of tumor cells with definite light microscopic evidence of squamous

Table 1. *Sequences of the Oligonucleotide Primers*

Specificity	B95–8 coordinates	Primer sequence
EBNA-1	67544–67563	5′-AGAGAGTAGTCTCAGGGCAT-3′
	108051–108032	5′-TCTTCTTTGAGGTCCACTGC-3′
EBNA-1 probe	107994–107975	5′-TCCTAGGCCATTTCCAGGTC-3′
EBNA-2	47940–47959	5′-GCGCCAATCTGTCTACATAG-3′
	48534–48515	5′-CCCCATGTAACGCAAGATAG-3′
EBNA-2 probe	48473–48454	5′-CGGGTGCTTAGAAGGTTGTT-3′
LMP-1	168666–168686	5′-AGTTAGAGTCAGATTCATGGC-3′
	169305–169285	5′-CCTTTGCTCTCATGCTTATAA-3′
LMP-1 nested	168888–168908	5′-GGAAGAAGGCCAAAAGCTGCC-3′
	169263–169243	5′-TCTTCAGAAGAGACCTTCTCT-3′
LMP-1 probe	168888–168908	5′-GGAAGAAGGCCAAAAGCTGCC-3′
LMP-2A	166820–166840	5′-GATTGCAACACGACGGGAATG-3′
LMP-2A	271–251	5′-AAGTGACAACCGCAGTAAGCA-3′
LMP-2 probe	229–249	5′-AAACTGCTGACACCGGTGACA-3′
BamHI A	157313–157332	5′-GTTTTGCGCCTGGAAGTTGT-3′
*Bam*HI A	160387–169370	5′-GGCTGACTCACCTGTTTG-3′
*Bam*HI A probe	159190–159171	5′-AGCTTTCCTTTCCGAGTCTG-3′
ZEBRA	102248–102267	5′-TCCGGGGGATAATGGAGTCA-3′
ZEBRA	102685–102666	5′-ACGCACACGGAAACCACAAC-3′
ZEBRA probe	102531–102518	5′-GCTGGAGGAATGCG

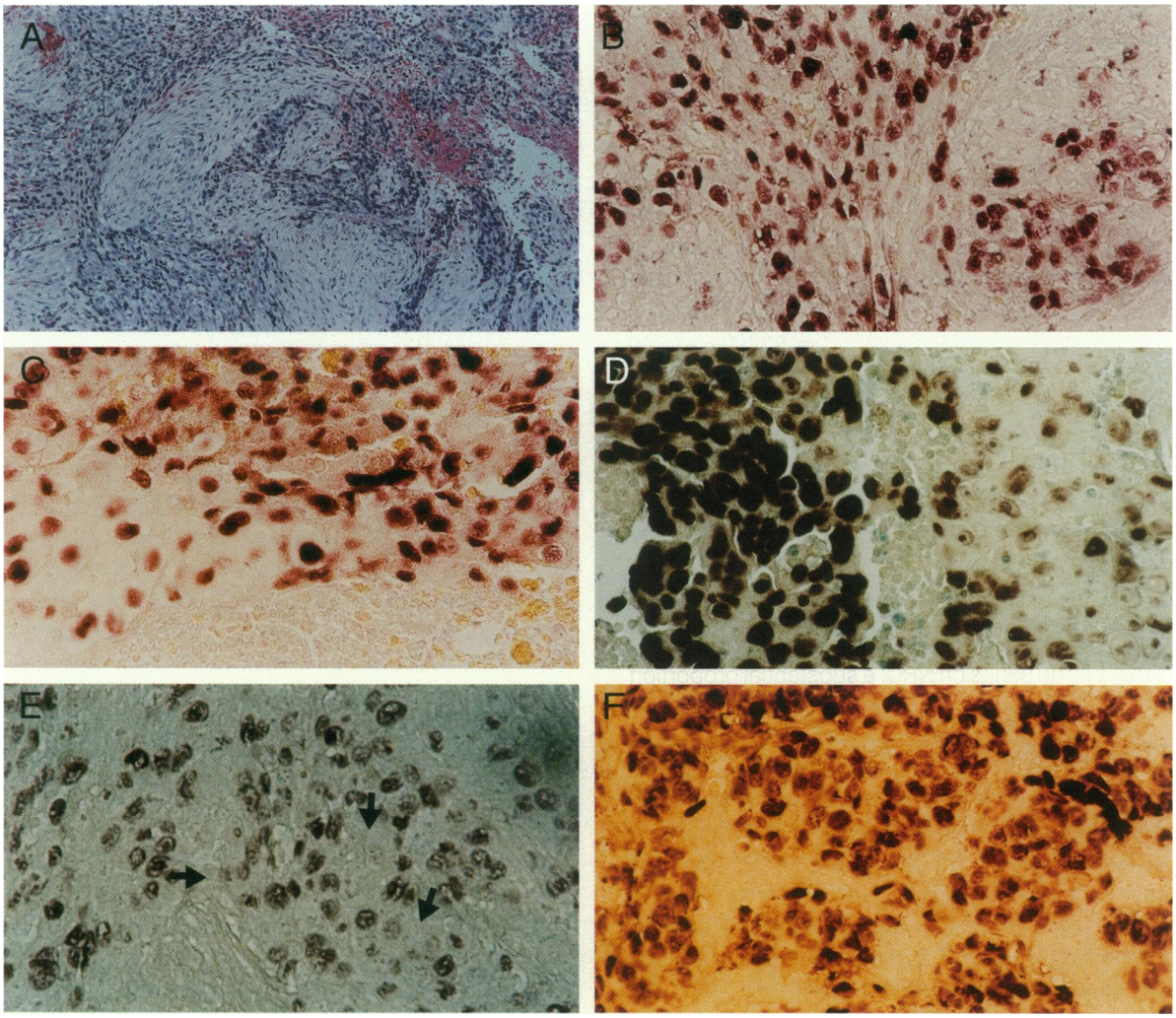

Figure 1. *Histology and EBER ISH of NPC.* **A:** *SCC of the nasopharynx (sample 453) NPC WHO type 1, moderately differentiated (hematoxylin and eosin; magnification, ×200). The tumor cells are present as ill-formed squamous islands with many cells with spindle cell morphology. A desmoplastic stromal reaction is apparent, and occasional scattered lymphocytes are present in the stroma.* **B:** *EBER ISH of SCC 453 (×400). Most of the spindle-shaped tumor cells have dark nuclear positivity of variable intensity. In a more differentiated focus, in the lower left of the field, EBERs were detected in some cells.* **C:** *EBER ISH in moderately differentiated WHO type 1 NPC (×400). In the periphery of the tumor nest, there is evidence of differentiation to maturation evidenced by the increase in size of the tumor cells and the increased clear cytoplasm. Most of the tumor cells within the keratinizing focus in the lower left had weakly detectable EBER signals. In contrast, most of the smaller, less differentiated tumor cells in the center of the tumor had strong EBER positivity. The stroma was negative for hybridization.* **D:** *EBER ISH to NPC with mixed histology (×400). The intensely EBER-positive tumor cells on the left consist of predominantly undifferentiated cells typical of NPC WHO type 3. A differentiated focus of keratinizing tumor cells is present in the right half of the section and has a striking reduction in the intensity of EBER hybridization, with EBER hybridization present in some cells and others staining weakly or not at all. Erythrocytes and lymphocytes in the tumor periphery and at the interface of the two types of NPC did not hybridize to the probe.* **E:** *EBER ISH of well differentiated SCC 156 (×400). Keratin whorl formation is evident in this section. The EBER signals are weak throughout the section and are faint or absent within the keratinizing centers of the tumor whorls (arrows).* **F:** *EBER ISH of NKC, WHO type 2 (×400). The tumor cells are present in confluent cords of cells, forming ill-defined masses with most cells intensely positive for EBER hybridization. The intervening clear areas are fibrous septae that did not hybridize to the EBER probe. The dark bar present in the panels is the image of the camera databack. The differences in background color are caused by differences in color balancing in different microscopes. All EBER ISH were photographed without counterstaining to emphasize the absence of nonspecific hybridization.*

differentiation. The majority of tumor cells were predominantly of the spindle cell type (Figure 1A). A prominent desmoplastic stromal reaction was evident, and occasional scattered lymphocytes were detected in the stroma.

The small nuclear EBERs are the most abundant RNA in latently infected cells and may be present at 10^6 copies per cell.[13,14] They are readily detected by EBER ISH. The EBERs are not expressed in the virus-producing EBV infection, oral hairy leukoplakia, and are consistently detected in NPC.[15,16] A parallel section of the tumor tissue presented above, SCC 453, was hybridized to single-stranded oligonucleotide probes, conjugated with FITC, homologous to the EBER1 and EBER2 RNAs. Within the tumor tissue, strong EBER positivity was localized to the nucleus in

the majority of the tumor cells. Hybridization was not detected to the intervening fibrous stroma (Figure 1B).

In a second example of a moderately differentiated SCC, multiple keratinizing foci were detected throughout the expanse of the tumor. In an example of one such keratinizing focus, the cells within the areas of differentiation are larger, with clearly defined cell borders and increased cytoplasmic volume (Figure 1C). The nuclei of these maturing cells are small and central, with regular margins. Hybridization with the EBER probe revealed variable intensity with decreased signal in the keratinizing focus (Figure 1C). This decrease in intensity was particularly evident in the periphery of the tumor nest where the tumor cells appeared more differentiated. The remaining moderately differentiated tumor cells within the center of the tumor were smaller with increased nuclear/cytoplasmic ratios and exhibited strong EBER signals. Hybridization was not detected in the adjacent fibrous stroma.

In any large series on NPC, a substantial proportion of tumors demonstrate mixed histological types of NPC.[17,18] In an example of a hybrid histological type of coexistent UC and SCC, EBER ISH revealed strong hybridization within the nuclei of the UC cells. In contrast, the cells of the adjacent SCC had a weaker but clearly identifiable hybridization signal within the tumor nuclei (Figure 1D). This result indicated a decreased amount of EBER expression in areas of differentiation.

Well differentiated SCC of the nasopharynx is extremely rare but is easily recognized and resembles well differentiated SCC elsewhere in the head and neck region.[17–19] Abundant keratin production, keratin whorl formation, and easily identifiable intracytoplasmic keratin within the tumor cells are typical features. An example of well differentiated NPC (sample 156) exhibited the characteristic abundant cytoplasm and keratin whorls. Positive hybridization with the EBER probe was detectable with variable intensity throughout the section. Within the well differentiated whorls, identified by arrows, most cells appeared EBER negative, again suggesting the down-regulation of EBER expression in areas of differentiation (Figure 1E). The low level of expression within this well differentiated tumor sample was strikingly distinct from the intense EBER staining that is characteristic of both NKC (WHO type 2) and UC (WHO type 3) types of NPC (Figure 1F).

In summary, the EBER ISH indicated that all examples of WHO 1 tumors, regardless of the degree of differentiation, were positive for EBER expression as were all examples of NKC and UC (Table 2). These data indicate that all forms of NPC are infected with EBV and confirm the previous studies that identified EBV in SCC.[9] The readily apparent difference in EBER intensity may account for the inability to detect EBV or EBER expression in some of the previous studies.[20]

Detection of EBV Latent Membrane Protein in Situ

The EBV latent membrane protein LMP-1 is the only EBV gene with transforming properties in cell lines.[21,22] LMP-1 expression has been detected in approximately 65% of NPC biopsy tissues.[23,24] In this study, staining with a pool of monoclonal antibodies to LMP-1 detected LMP-1 expression in 50% (2/4) of the well differentiated SCC samples and in 75% (3/4) of the moderately differentiated SCC samples. LMP-1 was also detected in 73% (30/41) of the NKC and UC samples (WHO types 2 and 3; Table 2).

Detection of LMP-1 was evident in most cells of all types of NPC (Figure 2). In an example of moderately differentiated NPC (sample 159), LMP-1 expression was clearly evident with coarse granular staining of the cytoplasm of most of the tumor cells. The scattered mononuclear cells and the adjacent stroma were negative (Figure 2A). Most cells within a second example of moderately differentiated SCC (sample 453) were also positive for LMP-1 with granular staining throughout the abundant cytoplasm. The infiltrating lymphocytes were negative (Figure 2B). These data indicated that EBV is present within the malignant epithelial cells and not in the infiltrating lymphocytes.

Table 2. *Analysis of NPC by EBER ISH and LMP-1 Immunostaining*

WHO type	EBER		LMP-1	
	Number	%	Number	%
1 (well differentiated)	4/4	100	2/4	50
1 (moderately differentiated)	20/20	100	3/4	75
1 (poorly differentiated)	7/7	100	1/1	100
1 (total)	31/31	100	6/9	67
2 and 3	89/89	100	30/41	73
Total	120/120	100	36/50	72

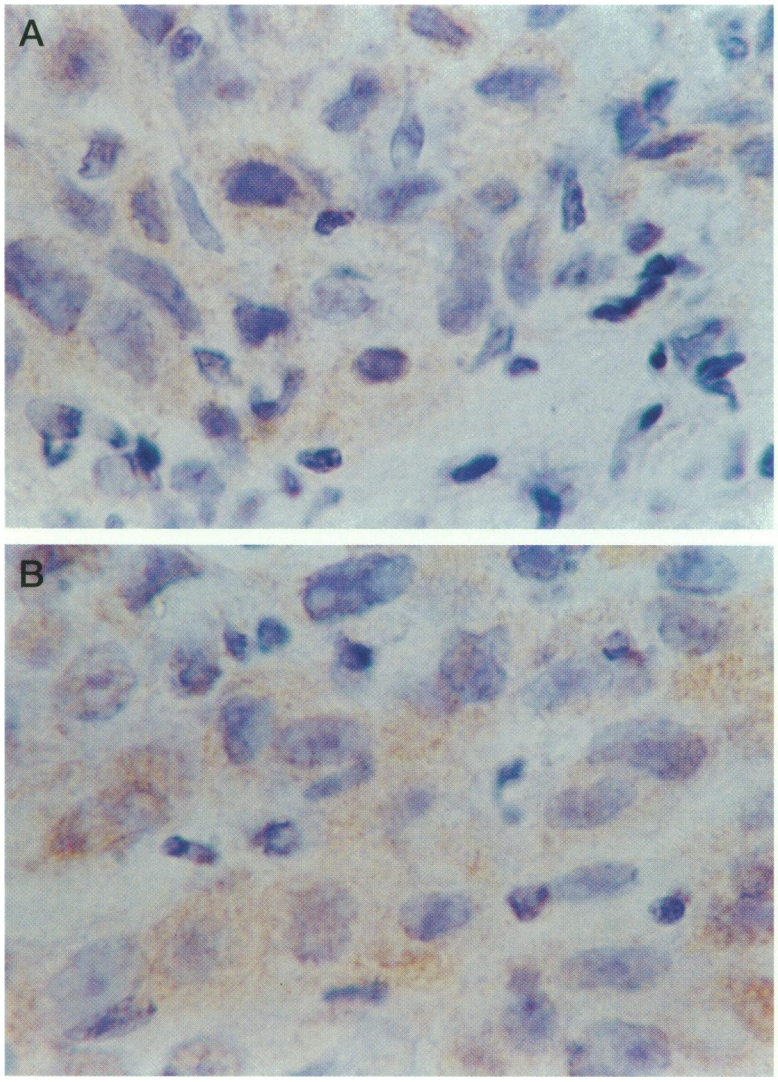

Figure 2. *LMP-1 immunostaining in NPC WHO type 1.* **A:** *Sample 159 (×675). LMP-1 was detected in most tumor cells as evidenced by the presence of brown granular staining of the cellular cytoplasm. The adjacent fibrous stroma and scattered mononuclear cells did not stain.* **B:** *Sample 453 (×675). The cytoplasm of the tumor cells was uniformly positive. In contrast, occasional infiltrating lymphocytes are clearly negative.*

EBER ISH and LMP-1 immunostaining were performed in histologically normal biopsies of the postnasal space of various categories of patients and were consistently negative (data not shown). The control cases included samples from patients suspected of having nasopharyngeal lesions but presenting with normal or reactive mucosa on biopsy, patients with high risk for developing NPC, and adjacent normal mucosa of patients with NPC.

Identification of the EBV Termini

EBV virion DNA is a double-stranded linear molecule with multiple, variable numbers of copies of a direct tandem repeat of approximately 500 bp at each terminus. When treated with restriction endonucleases, arrays of heterogeneous restriction fragments are produced that vary by increments of 500 bp.[25,26] The ends of the linear termini fuse after infection to form the covalently closed episomal intracellular DNA. Although these fragments are potentially very heterogeneous, single fused terminal restriction fragments were detected in NPC and in Burkitt's lymphoma.[11] The detection of a homogeneous fragment indicated that the viral episomes within a cell were clonal with regard to the number of terminal repeats and suggested that clonality of the EBV episome could be used as a predictor of cellular clonality. The correlation between viral and cellular clonality has been proven in studies of lymphoid cell lines.[27] Other malignancies associated with EBV, including Hodgkin's disease and T cell lymphomas, have also been shown to contain clonal EBV termini, and oligoclonal proliferations have been detected in immunosuppressed patients.[12,28]

Frozen tissue samples were available for biochemical analyses from four examples of well and moderately differentiated SCC. These samples were processed to obtain DNA and RNA. Southern blots prepared with sample DNA digested with *Bam*HI and dilutions of Raji DNA representing approximately 50, 5, and 1 copies of EBV per cell were hybridized to the *Xho*Ia probe that represents the unique DNA adjacent to the terminal repeats at the right end of the genome. A single EBV terminal restriction enzyme fragment was detected in all four samples of well and moderately differentiated SCC, which were also positive for EBER and LMP-1 expression (Table 3, Figure 3). Ladder arrays of smaller fragments that are indicative of viral replication were not detected in any of these samples. The detection of a single EBV terminal fragment suggests that these examples of SCC are clonal proliferations of a latently EBV-infected cell.

PCR Analysis of EBV Transcription

Multiple EBV genes are expressed in latently infected lymphocytes. These include six nuclear antigens and two integral membrane proteins.[29] The EBV latent mRNAs are intricately spliced with distinct exon patterns for each of the transcripts.[29] The six nuclear antigens, EBNAs, are encoded by a large primary transcript that is differentially spliced for each of the individual mRNAs. The PCR has been used to detect the specific splices of the EBV mRNAs, by using oligonucleotide primers that span the introns of the individual mRNAs.[12,30,31] PCR analysis of cDNAs was used to screen for the specific splices that distinguish the EBNA-1, EBNA-2, LMP-1, and LMP-2 mRNAs and transcription of *Bam*HI A, all of which are expressed in latently infected lymphoid cell lines.[28,30,32,33] PCR primers were also used to detect the mRNA encoding the EBV replication activator gene, ZEBRA.[34] For all PCR analyses, equal aliquots of RNA were used to prepare cDNA with and without reverse transcriptase to serve as a negative control.

The EBNA-1 protein binds to the latent origin of replication and is required for replication of the EBV episome.[35] EBNA-1 is expressed in all latently infected cells, including the epithelial cells of NPC. Sequence analysis of cDNAs indicated that all EBNA-1 mRNAs contain an exon within the *Bam*HI U fragment.[36,37] To specifically detect the EBNA-1 mRNA, oligonucleotide amplimers were chosen that span a 40-kb intron within the mRNA from *Bam*HI U to the coding sequences within *Bam*HI K. The EBNA-1 mRNA is present in low abundance but is readily detected by PCR amplification. Amplification of contaminating DNA or unprocessed and unspliced mRNA is prohibited by the large size of the intron. In the four samples of SCC, the 216-bp product representing the spliced EBNA-1 transcript was detected by hybridization with an EBNA-1-specific oligonucleotide probe (Table 3, Figure 4A).

EBNA2 is a transcriptional transactivating protein that is essential for B lymphocyte transformation in vitro.[38–40] The protein has not been detected in NPC or in EBV-positive T cell and Hodgkin's lymphomas but has been identified in post-transplant lymphomas.[12,28,41,42] The EBNA-2 mRNAs include exons from the *Bam*HI W, Y, and H fragments. The EBNA-2 mRNA splices from the Y2 exon to the EBNA-2 YH coding exon. Amplification with oligonucleotide primers for this splice and hybridization with an EBNA-2-specific oligonucleotide probe did not produce any detectable product (data not shown). Therefore, in contrast to EBNA-1, transcription of the EBNA-2 gene was not detected in the three examples of SCC that were tested. The lack of detectable EBNA-2 transcription in all types of NPC indicates that EBNA-2 expression is not required for maintenance or regulation of latent EBV infection in epithelial cells and apparently does not contribute to the altered growth properties of the epithelial cells.

The EBV genes expressed during latency have been primarily defined in lymphoblastoid cell lines.[29] However, an abundant family of transcripts, partially encoded by the *Bam*HI A fragment, was identified in NPC. These transcripts are present at much higher levels in NPC than in lymphoid cell lines.[32,33,43] The *Bam*HI A transcripts are intricately spliced, and multiple different RNA forms have been detected.[32,33] *Bam*HI A transcription has previously been detected

Table 3. *Results of Clonality Assay and Reverse Transcriptase-PCR Analysis of WHO Type 1 NPC*

Sample No.	EBER ISH	LMP-1 IS	Clonal termini	Reverse transcriptase-PCR					
				EBNA-1	LMP-1	LMP-2	BamHI A	EBNA-2	BZLF-1
156	+	+	+	+	+	+	+	–	–
200	+	+	+	+	+	+	ND	ND	ND
453	+	++	+	+	+	+	+	–	–
159	+	++	+	+	+	+	+	–	–

ND, not determined.

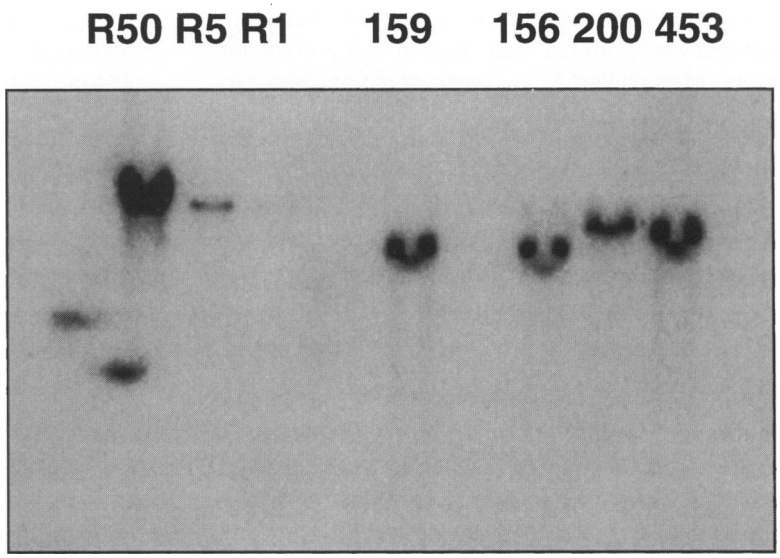

Figure 3. *Identification of EBV DNA in SCC. Histologically proven samples of SCC were processed to obtain DNA. Southern blots prepared with sample DNA digested with* Bam*HI were hybridized to a single-stranded RNA probe from the* Xho*Ia fragment in comparison with dilutions of Raji DNA representing 50, 5, and 1 copy of EBV DNA per cell. In each sample, a single restriction enzyme fragment was detected, suggesting clonal episomal forms of EBV DNA.*

in all NPC samples tested.[30,43] In this study, oligonucleotide primers that span one of the introns from the abundant 4.8-kb *Bam*HI A mRNA were used to detect expression. Hybridization with a *Bam*HI A oligonucleotide probe detected the 350-bp product representing processed *Bam*HI A transcription in the three samples of SCC that were tested (Table 3, Figure 4B).

LMP-1 is an integral membrane protein and is the only EBV gene product that has transforming properties *in vitro*.[21,22] LMP-1 is essential for EBV-induced lymphocyte transformation[44] and has been detected on immunoblots in approximately 65% of NPC samples[23,24] and in this study was detected in 72% of the samples by immunohistochemistry (Table 2). To detect LMP-1 mRNA, two sets of nested amplimers were chosen that span the two LMP-1 introns. The amplification product representing genomic LMP-1 DNA or unprocessed RNA is 375 bp whereas a 222-bp product is generated from the spliced LMP-1 mRNA. The 222-bp product was detected by hybridization in all four cDNA samples but was not detected in the control amplifications (Figure 5A, Table 3) prepared without reverse transcriptase. This result was consistent with the immunohistochemical detection of the LMP-1 protein. The consistent detection of LMP-1 in all forms of NPC suggests that its expression contributes to the altered growth properties of the neoplastic cells.

The LMP-2 proteins, LMP-2A and LMP-2B, are also integral membrane proteins that associate with LMP-1 in the plasma membrane.[45] In lymphocytes, LMP-2A binds to the *lyn* and *fyn* tyrosine protein kinases and blocks signaling through the Ig receptor. This inhibition of lymphocyte activation blocks induction of replicative gene functions and helps maintain a latent infection.[46] Transcription of LMP-2A is consistently detected in NPC.[30,31] The LMP-2 genes contain exons from both ends of the linear EBV genome and can only be transcribed across the fused termini of the episomal form.[47,48] Primer pairs were utilized that span the fused termini of EBV from exon 1A to exon 2. Hybridization with an exon 2-specific probe detected the 312-bp product, indicative of transcription of LMP-2A mRNA, in all four SCC samples (Figure 5B, Table 3). Normal epithelial cells are thought to be permissive for viral replication; therefore, the consistent expression of LMP-2A in NPC suggests that it may also contribute to maintenance of latent infection in epithelial cells.[49]

The EBV replication activator gene, ZEBRA, is the single viral gene responsible for activating the cascade of viral expression leading to permissive, replicative infection.[34] The protein is encoded by three exons; therefore, oligonucleotide primers were chosen that span the two introns (Table 1). Amplification and hybridization with the ZEBRA-specific oligonucleotides did not produce any detectable product (data not shown). It is thought that differentiation of epithelial cells may activate viral replication; however, the lack of ZEBRA expression in these examples of differentiated NPC indicated that these infections are predominantly latent, in agreement with the absence of linear forms of viral DNA in the termini analyses (Figure 3).

Discussion

In most large series of NPC samples, SCC or WHO type 1 NPC is the least common type of NPC and in

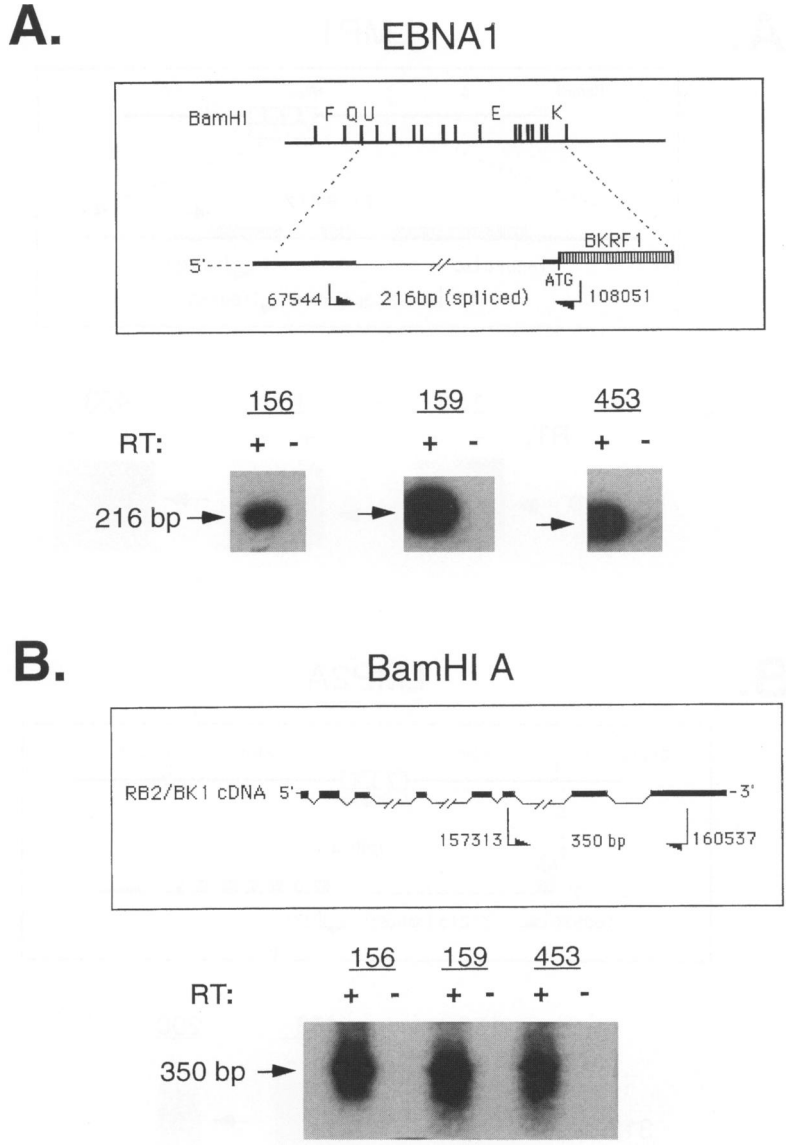

Figure 4. *Transcription of EBNA-1 and* Bam*HI A. Equal aliquots of RNA were used to prepare cDNA with (RT⁺) or without (RT⁻) reverse transcriptase.* **A:** *Detection of EBNA-1 mRNA. Oligonucleotide probes from the* Bam*HI U and* Bam*HI K fragments were used to detect the EBNA-1 mRNA. The EBV* Bam*HI fragments are denoted above. The hatched bar represents the EBNA-1 coding sequences. The spliced EBNA-1 mRNA is represented by a 216-bp PCR product that was detected in all four samples analyzed.* **B:** *Detection of* Bam*HI A transcription. The structure of the abundant 4.8-kb mRNA is shown with the specific splice characteristic of this mRNA. The spliced product representing the rightward* Bam*HI A transcript is 350 bp and was detected in all samples analyzed.*

this study represented 17% of the NPC samples analyzed. In contrast to SCC occurring in other regions of the head and neck region such as the tongue, hypopharynx, and larynx, well differentiated SCC of the nasopharynx is exceedingly uncommon. In a review of 1000 consecutive cases of NPC in Taiwan, not one case of well differentiated SCC were detected,[19] and in a separate study only 7 cases of well differentiated SCC were found, with the majority of the cases being either moderately or poorly differentiated.[50] The incidence of well differentiated SCC in this study was 0.2% (4/1800) of the total number of NPC cases.

WHO type 1 NPC has generated much controversy because of conflicting data concerning its association with EBV. Earlier studies demonstrated elevated IgG and IgA titers to viral capsid antigen and early antigen in patients with WHO types 2 and 3 NPC, whereas patients with WHO type 1 tumors tended to have serological profiles similar to that of control populations.[5,6] It has been suggested, on the basis of serological data and earlier EBV DNA hybridization studies, that SCCs and undifferentiated carcinomas are different diseases.[51] Although some studies have been unable to demonstrate the presence of EBV in SCCs of the nasopharynx,[20] others have demonstrated that this form of NPC is EBV associated.[9,52,53]

In the previous studies that did not detect EBV, ISH was used to demonstrate viral presence. In a sample of 87 NPCs, which included 8 cases of SCC, EBV DNA was not detected in any of the SCC samples, although an addendum reported that EBV DNA was detected by PCR amplification in 1 case of SCC.[20] However, in

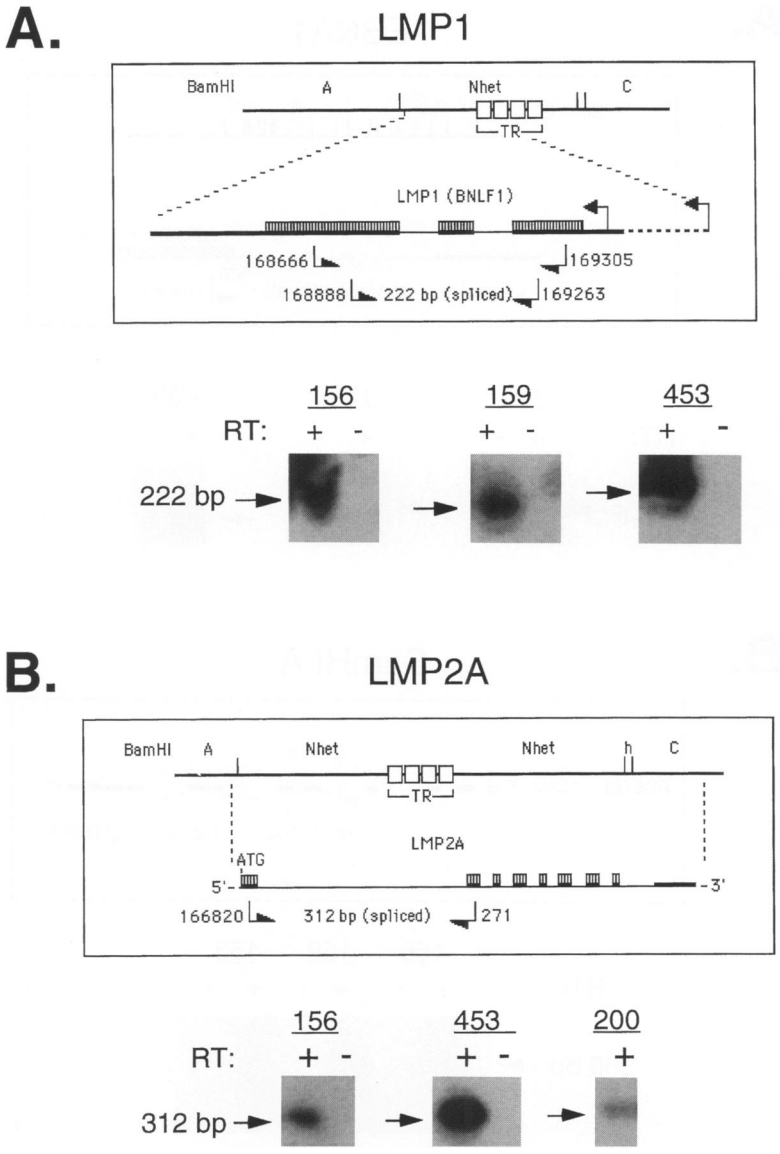

Figure 5. *Expression of LMP-1 and LMP-2A in SCC NPC.* **A:** *Detection of the LMP-1 mRNA. The location of the LMP-1 coding sequences near the right end of the linear genome and the structure of the mRNA with the exons that form the LMP-1 open reading frame are indicated. Amplification of the spliced LMP-1 mRNA or genomic DNA produces 222-bp or 375-bp products, respectively. The spliced mRNA was detected in all four samples analyzed.* **B:** *Detection of the LMP-2A mRNA. The LMP-2 genes are transcribed across the terminal repeats. Amplification from the first exon of the LMP-2A mRNA from the right end of the linear genome to the second exon at the left end of the linear genome generates a 312-bp PCR product that was detected in all samples analyzed.*

that study, EBV DNA was detected in only 68/77 cases of UC and in only 1 case of NKC, raising concerns about the sensitivity of detection in this study. This is supported by the fact that the authors were able to amplify by PCR DNA from 7 cases of UC, one of which had been negative for EBV DNA by ISH. In another study, EBV DNA was demonstrated in 8 cases of well differentiated squamous carcinoma of the nasopharynx by DNA slot blot hybridization (5/8 positive) and PCR analysis (6/8 positive).[54] In addition, two recent studies consistently detected EBV in all NPC cases tested, including 1 case of keratinizing carcinoma[52] and by ISH in 3 cases of well differentiated SCC of the nasopharynx.[53] DNA was detected in most tumor cells of NKC and UC but was demonstrable in only some of the WHO type 1 tumor cells.[52] It is possible that the failure to detect EBV DNA reflects the sensitivity of the ISH assay, which is not as high in tissue sections as in the cell lines used as controls.

It has been suggested that the biochemical detection of EBV DNA in SCC reflects the presence of EBV-infected lymphocytes in the mucosal lymphoid tissue of the nasopharynx. However, histological studies have shown that such lymphoid infiltrates are rare in SCC,[7,50] and the data presented here reveal that EBV is specifically localized to the epithelial cells and clearly absent from the infiltrating lymphocytes.

EBER ISH was used for analysis because this type of analysis permits visualization of morphology, allows simultaneous assessment of the integrity of the specimen, and localizes the positive signals to spe-

cific tissues, cells, or even cellular compartments. EBER ISH permits evaluation of gene expression in paraffin-embedded tissue sections.[16] The results of the present study demonstrate that EBERs are expressed in most cells of NPC including the well differentiated SCC. However, the abundance of EBERs in SCC was clearly lower than that observed in NKC and UC. These results suggest that there is a reduction in EBER detection in the maturing, differentiating cells, a phenomenon demonstrated dramatically in the case of mixed carcinoma (Figure 1D). This finding would suggest that EBER expression may be downregulated once tumor cells differentiate and produce keratin.

Parallels may be drawn with the molecular events that occur in SCC and in oral hairy leukoplakia, a permissive, virus-producing EBV infection of the lingual and buccal mucosa in human immunodeficiency virus-infected individuals. Oral hairy leukoplakia is a characterized by abundant keratin production with proliferation of the squamous epithelium. The viral infection is unique in EBV pathogenesis in that the infection is permissive with the production of linear viral DNA and expression of LMP-1 and replicative antigens in the absence of EBER expression.[15] A similar situation may occur in the SCC lesions in which differentiation could inhibit EBER expression.

Careful histological studies have indicated that the different histological types of NPC are variants of a homogeneous group of neoplasms.[55] Histogenetically, all NPC is squamous in origin, and this essential squamous nature of both NKC and UC has been confirmed by both electron microscopy and immunohistochemistry.[49,50] Dedifferentiation in tumors is known to occur in sarcomas.[56] This phenomenon is well recognized in NPC, and it is not unusual for lesions that recur, or tumors metastatic to lymph nodes, to demonstrate histological features that differ from those observed at the time of initial diagnosis. Shanmugaratnam et al[50] found that 8% of the UC and 26% of the NKC presented as SCC when the lesions recurred or metastasized. Similarly, 50% of the tumors initially diagnosed as SCC recurred as either NKC or UC. From the morphological point of view, the existence of mixed histological types of NPC and the phenomenon of dedifferentiation in the clinical course and biological evolution of NPC are persuasive arguments for the histogenetic commonality and relatedness of all three WHO types of NPC.

All four examples of well differentiated SCC analyzed here had clonal forms of the EBV episome without evidence of linear DNA. The IgA viral capsid antigen titers were not elevated in these patients. The combined results of the reverse transcriptase PCR analyses and the LMP-1 immunohistochemistry indicate that EBNA-1, LMP-1, LMP-2, and *Bam*HI A are consistently transcribed in SCC. EBV gene transcription in latently infected lymphoid and epithelial cells is complex, although there are clearly specific viral-epithelial cell interactions.[3] However, despite the apparent differences in the morphological appearances of the three types of NPC, the identity of their histogenetic origin and squamous nature, the frequent admixture of varying histological types of NPC within a single tumor, and the demonstration of dedifferentiation suggest that there are greater similarities than differences between the forms of NPC. The commonality of all three forms of NPC is further reinforced by the results presented here with the demonstration of clonal EBV in the cases of well differentiated SCC and the pattern of EBV expression identical to that which has been described for NKC and UC. The overall conclusion, on the basis of epidemiological, pathological, and molecular biological grounds, is that all types of NPC are variants of an EBV-associated malignancy.

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