Nasopharyngeal Brush Biopsies and Detection of Nasopharyngeal Cancer in a High-Risk Population

Cathryn E. Tune, Per-Gunnar Liavaag, Jeremy L. Freeman, Michiel W. M. van den Brekel, Thomas Shpitzer, Jeroen D. F. Kerrebijn, David Payne, Jonathan C. Irish, Raymond Ng, Roy K. Cheung, Hans-Michael Dosch

**Background:** Nasopharyngeal carcinoma (NPC) is an important tumor in many countries. Ethnic and regional factors strongly influence disease risk. NPC is usually diagnosed late in disease development, and 10-year survival rates are as low as 10%. Epstein-Barr virus (EBV), a possibly causative agent, is present in all cells of essentially all undifferentiated NPCs. We wished to determine the following: 1) whether an ambulatory nasopharyngeal brush biopsy could provide sufficient tumor cell DNA for the detection of EBV and 2) whether the detection of EBV in this locale reflects the presence of tumor cells or simply EBV carrier status.

**Methods:** We collected nasopharyngeal tissue via ambulatory brush biopsies from 21 patients with newly diagnosed NPC and from 157 subjects with other otolaryngologic complaints. The majority of study subjects were from high-risk populations. Sample DNA was analyzed for the presence of EBV genomic sequences by use of the polymerase chain reaction (PCR).

**Results:** Ninety-six percent of samples yielded sufficient DNA for PCR amplification. Nineteen of 21 patients with NPC brushed positive for EBV DNA, while all but two (1.3%) of 149 informative control subjects were negative for EBV (two-sided \(P<.0001\)). One of the EBV-positive control subjects had an EBV-positive inverted sinonasal papilloma; the other EBV-positive control subject exhibited no overt clinical disease.

**Conclusion:** Demonstration of EBV DNA in nasopharyngeal brush biopsy specimens detects NPC with a sensitivity of at least 90% (95% confidence interval = 89.63%–91.32%) and a specificity of approximately 99% (95% confidence interval = 98.64%–98.68%). This technique merits further testing as a possible ambulatory screening strategy in high-risk populations. [J Natl Cancer Inst 1999;91:796–800]

Nasopharyngeal carcinoma (NPC) is a relatively rare tumor in Caucasians, but it occurs at high frequency in Pacific and Mediterranean rim countries, among the Inuit, and in coastal regions of Africa. NPC has strong genetic and environmental roots (1); e.g., 1%–8% of Southern Chinese are at risk of developing NPC (2). In Chinese immigrants to North America, NPC risk declines rapidly, but it remains 40-fold to 50-fold higher than that of resident Caucasians (3). More than 90% of NPC patients in North America are of Asian, Mediterranean, or African descent. In a worldwide perspective, NPC is a major cause of morbidity and mortality.

With often minimal, nonspecific local symptoms (4), more than two thirds of patients are diagnosed only late in the disease process by endoscopy and/or biopsies of metastatic lymph nodes (5). Primary radiotherapy of NPC usually induces remission, but relapse rates are high and are associated with increasing resistance to therapy (6). Important sites of treatment failure are the nasopharynx and distant metastases; 10-year survival rates are as low as 10% (5). Earlier detection would almost certainly improve overall survival (7).

Epstein-Barr virus (EBV) is a large herpesvirus carried by nearly all human adults; homologous viruses are carried by nonhuman primates (8). EBV is usually latent, infrequently producing infectious progeny (9). Cells maintain from few to
more than 50 virus copies as nonintegrated, double-stranded DNA episomes (10). B lymphocytes constitute the likely sole EBV reservoir, while persistent infection of epithelial cells is characteristic of malignant tumors such as NPC (11).

EBV is closely and perhaps causally linked to NPC; essentially all tumors and preinvasive lesions of the nasopharynx (12) carry clonal EBV genomes, and viral infection precedes malignant transformation (13–15). Accordingly, detection of EBV genomic DNA in nasopharyngeal biopsy specimens may predict the presence of NPC (16). However, the practical utility of diagnostic EBV detection is uncertain, since most human adults carry the virus. The high prevalence of EBV-specific serologies in carriers reduces its utility for prediction of NPC risk: Population screening through serology and endoscopy has not been satisfactory (17,18). We thus sought to test in a clinical setting an ambulatory brush biopsy procedure with polymerase chain reaction (PCR)-based EBV detection for identification of subjects who are likely to harbor NPC.

**SUBJECTS AND METHODS**

**Patients**

Written informed consent to undergo nasopharyngeal brushing under an approved study protocol was given by 157 control subjects (mean age = 52.9 years; range = 6–94 years; male-to-female ratio = 1.26), 149 of whom attended otolaryngology clinics at the Toronto Mount Sinai Hospital, the Toronto Hospital–General Division, and the Scarborough Centenary Health Center and eight of whom were residents of a Toronto Chinese retirement home and were brushed at that locale. We employed a recruitment preference for middle-aged patients of Asian descent (55.4%), with the remainder random samples from the general, largely Caucasian population. Two groups of control subjects were used: subjects with minor otolaryngological complaints (n = 101, or 64.3%) and patients with head and neck cancers other than NPC (n = 56, or 35.7%). A random subset (n = 38) of these 157 brush biopsy donors were tested for EBV carrier state by serology and, as noted above, were EBV seropositive. In addition, 21 patients (18, or 85.7%, of Asian descent) with established or suspected NPC underwent nasopharyngeal brushing before therapy. NPC histopathology was established in all patients studied. Nine NPC patients were diagnosed and brushed in T1/T2 tumor stages and 12 in T3/T4 stages (5), a pattern consistent with previous medical center experience.

**Study Design**

Our study consisted of an initial unblinded and a subsequent blinded study (Fig. 1). The open study involved 29 of 157 total control subjects and nine of 21 NPC patients. This phase was used for technology proof. The blinded study enrolled 140 subjects (128 of 157 control subjects and 12 of 21 NPC patients). The sample sizes were sufficient to detect a 20% difference between EBV-positive brushings from control subjects and patients with a power of 0.95, a type 1 error of 0.02, and a type 2 error of 0.05. A secondary study question reflected an observation in the technology proof phase, where two NPC patients brushed EBV positive only in one nostril. Hence, one study branch employed unilateral brushing (n = 77 subjects), while a second employed bilateral brushing (n = 63 subjects) with a single cytology brush.

In a third, cross-sectional survey, 32 NPC patients were recruited in the course of their regular follow-up in radiation oncology clinics at the Toronto Princess Margaret Hospital, 2 weeks to 21 years after receiving high-dose radiation therapy. Of these patients, one patient maintained residual local and metastatic disease after radiotherapy, and eight (25%) demonstrated disease recurrence locally (n = 1) or regionally (n = 2) or had metastatic disease alone (n = 2) or together with local/regional recurrence (n = 3).

**Brush Biopsy**

For acquisition of nasopharyngeal tissue fragments, we modified a gynecologic cytology brush (Cytobrush; Medscord, Hollywood, FL), The 19-cm plastic stem carries a 1-cm bristled head for desquamation of tissue fragments. We developed a plastic sheath to prevent acquisition of tissue or potentially EBV-containing sputum during nasal cavity passage. Brush and sheath were gas sterilized and carried no EBV contamination on four random checks. The nasal cavity and nasopharynx were locally anesthetized with topical 3% cocaine or 4% lidocaine HCl (Xylocaine; Astra Pharma Inc., Mississauga, ON, Canada) with 1% phenylephrine HCl (Neo-Synephrine; Sanofi Winthrop, Markham, ON, Canada). Once positioned in the Rosenmüller recess, the sheath was retracted and the brush was rotated to dislodge tissue fragments. The sheath was then re-advanced before removal from the nose. The loaded brush was placed in a 50-mL sterile plastic tube containing 10 mL of phosphate-buffered saline to rinse off the cytologic material. The nasopharyngeal brushing procedure was tolerated by the patient with brief discomfort. No patient developed a serious nosedbleed, and no complications were recorded.

**Detection of EBV Genomic DNA**

Cellular material was collected by centrifugation at 4400g for 10 minutes at 25°C and incubated overnight at 56°C in lysis solution containing 1× PCR buffer (1 mM Tris-hydrochloride [pH 8.3], 5 mM potassium chloride, 0.15 mM magnesium chloride, and 0.0001% [wt/vol] gelatin), 0.45% (vol/vol) Nonidet P-40, 0.45% (vol/vol) polyoxyethylene-sorbitan monolaurate, and 0.1 mg/mL Proteinase K (Sigma Chemical Co., St. Louis, MO). After boiling for 10 minutes, DNA was precipitated in 70% ethanol at −20°C overnight or isolated with the use of a commercial kit (QIAGEN Inc., Santa Clarita, CA) with similar results. We chose to amplify regions in the Epstein-Barr nuclear antigen (EBNA) 1 (5′-TGAATACCAACCAAGAAGGTG-3′ and 5′-GTTCAG-3′), EBNA 2 (5′-AGTTCCTTCGTGAGGATC-3′ and 5′-GTGAGGCGCATTTTTGCA-3′ and 5′-TTTCTAGTCTAGCCTAGG-3′) and the Epstein-Barr viral interleukin 10 (vIL-10) genes (5′-CGAAGGTTAGTGTGCACCTCT-3′ and 5′-TACTCTTGTCTCACAACC-3′) for identification of viral DNA (16,19). Amplification of a genomic region in the human islet cell antigen 69 (ICAP69) gene (20) (5′-GACCTGACTGCGTG-3′ and 5′-TACATGCTAGTGCAGAATT-3′) provided a marker for the presence of intact genomic DNA. To apply multiplex PCR (21) with these primers, we developed (22) a technique that used fluorescent-labeled oligonucleotides for amplification of the three test gene regions in a single tube and analysis on an automated DNA sequence (A.L.F.; Pharmacia, Montreal, PQ, Canada). Multiplex PCR products were also analyzed by Southern blotting and hybridization with radioactive reporter probes (EBNA 1: 5′-
TTTCTACGTGACTCCTAGCC-3' or 5'- TGAATACCCACAAAGGTGGT-3'; vIL-10: 5'-ACCTGGAGGAAGTCATGCCA-3'; ICAp69: 5'-CGCTGACCTCGACCCACTCTC-3') as previously described (16). DNA from paraffin-embedded tissue was analyzed as above, with the addition of a xylene immersion step to remove paraffin (16). Periodic switching of primer combinations provided a safeguard against laboratory cross-contaminations. Negative control samples containing water instead of DNA were always processed in parallel to patient samples. Pipette tips were plugged. DNA from 2D5, an EBV growth-transformed B-cell line (19), was used as an EBV-positive control.

Statistical Analysis

Fisher’s exact test was used for statistical comparisons; Katz’s approximation was used to calculate relative risks. All P values were two-sided and considered significant for $P<.05$.

RESULTS

Fig. 2, A, shows a typical Southern blot of PCR products following amplification of viral EBNA 1, vIL-10, and human ICAp69 genes, probed with radiolabeled, internal oligonucleotides. Fig. 2, B, shows results of a subset of samples analyzed on an automated DNA sequencer measuring laser-induced fluorescence (22). The multiplex PCR assay identifies samples with sufficient DNA and detects the presence of EBV genomes.

Table 1 combines observations from the open and blinded studies that are described in Fig. 1. There was insufficient DNA for the amplification of the ICAp69 (human, cellular) control gene (20) in eight (5%) of the control samples. As a result, they were eliminated from further analysis. Brushings of NPC patients turned out to have sufficient DNA for analysis, and 19 (90%) were positive for the presence of EBV genomic DNA. Two brushing samples, one each from patients with tumors of stage T1 or T2, remained negative upon re-analysis. EBV genomic DNA was present, however, in tissue obtained from the paraffin-embedded T2 tumor sample (Fig. 3). This, therefore, represents a true false-negative brushing result. Tissue from the T1 tumor was not available. In this cohort, there was no significant trend between stage of tumor and EBV detection ($P = .46$).

Two (1.3%) of the 149 informative control subjects brushed positive for the virus. One patient was diagnosed with EBV-positive inverted sinonasal papilloma, a rare condition associated with EBV in some, but not all, surveys (23,24). The second sample was obtained from one of eight residents of a Chinese retirement home, who were brushed at that locale. This 88-year-old Chinese female did not have overt clinical disease at the time of brushing, but she refused further investigation.

The detection of EBV genomic DNA in nasopharyngeal brush biopsy samples thus predicts NPC with a sensitivity of 90% (95% confidence interval [CI] = 89.63%–91.32%). When inverted sinonasal papilloma was included, the detection sensitivity for EBV-associated tumors was 91% (95% confidence interval [CI] = 90.14%–91.68%). The specificity for overt NPC was close to 99% overall (95% confidence interval [CI] = 98.64%–98.68%). There was a statistically significant association between an EBV-positive brush biopsy specimen and the presence of NPC ($P < .0001$; relative risk = 10.3; 95% CI = 2.7–38.7). This significance was unchanged when test subjects and patients in the blinded study only were analyzed.

To determine the utility of single versus bilateral brushing, we compared 77 subjects who were brushed through one nostril with 63 who were brushed bilaterally. This latter cohort was found after unblinding to include 10 patients with NPC. However, the remaining 77 subjects were found to include only two such patients, precluding meaningful comparisons.

Because of the close association between EBV and NPC, we expected that the virus would disappear after successful induction of remission by radiotherapy. In a small cross-sectional study, we obtained 30 informative brushings from 32 radiation-treated NPC patients (Table 1). Of 21 patients in remission, three brushed positive for EBV DNA and are being monitored at this time. Of nine patients with recurrent or residual disease, only two brushed positive for EBV. One patient was diagnosed with a local recurrence at the time of brushing, while a second had a regional neck recurrence treated 6 months earlier. All other patients with recurrences, including all patients with distant
DISCUSSION

The diagnosis of NPC is often difficult because of the nonspecific nature of the clinical symptoms and difficulty in visualizing the nasopharynx. Primary lesions can be submucosal and are easily missed by endoscopic examination. Many tumors are detected late or remain undiagnosed until they present as squamous cell metastases of the neck, often without overt pathology at the primary site (16). Early NPC detection should improve cure rate, morbidity, and incidence of metastasis (25).

Given the close association between EBV and NPC, the utility of EBV as a target for NPC diagnostics (16) critically depends on the virus’s tissue distribution in a carrier (26), in particular, the absence of virus in or on the nasopharyngeal mucosa. We (16,23,26,27) have tested and confirmed this premise over the past years in tissue samples from well over 200 donors without NPC. However, some PCR-based studies from regions where NPC is endemic have reported considerable, even large proportions of all nasopharyngeal tissue samples as positive for EBV (28,29). It is conceivable that the tissue distribution of EBV is different in NPC-endemic regions and in low-incidence areas such as Toronto. However, preliminary studies with collaborating research centers in New Zealand and Israel do not support this possibility. Studies from our laboratory (16,23,26,27) and other laboratories (30,31) failed to find a nasopharyngeal EBV reservoir. Contamination with infectious (i.e., linear) EBV derived from sputum may explain the large number of positive samples in studies of nasopharyngeal washings (29) and swabs (32). EBV, as one probable causative agent in NPC, provides a good tumor marker, and—as demonstrated here—a simple, ambulatory procedure that relies on the detection of viral DNA is effective and practical for tumor diagnosis. These data provide justification for a prospective study of this technique for detecting NPC in high-risk cohorts.

Since brush biopsies are performed without endoscopic guidance, the areas actually brushed may vary somewhat. We do not know the topographic distribution of EBV-positive tumor cells in the super-

ficial nasopharyngeal mucosa. However, the fact that nasopharyngeal brush biopsies were able to retrieve EBV-positive tissue from patients known to have NPC implies that tumor cells routinely gain access to the mucosal surface. Our additional question of whether unilateral, as opposed to bilateral, brushing of the nasopharynx is adequate for tumor detection remained unanswered by our blinded study because there was an unexpected disparity in the number of NPC case patients between the two test branches. However, since bilateral brushing was necessary for EBV (and, thus, tumor) detection in at least two patients with NPC from the open study, we would recommend bilateral brushing as a routine to avoid missing small, localized tumors.

It was unexpected that three of 21 treated patients who were in apparent remission exhibited sustained or re-acquired EBV positivity. Each of these patients is being followed clinically for signs of relapse. Relapsed tumors contain the same viral clone as the primary lesion (33), and our observation may indicate viral persistence in residual tumor cells that escaped radiotherapy in these patients. However, EBV detection was inefficient in patients with known recurrence. Scari-

fication after radiotherapy may reduce overall the ability of nasopharyngeal brushing to detect EBV positivity in patients with recurrent NPC. A long-term, prospective study of newly treated patients is required to test the value of nasopharyngeal brush biopsy in monitoring for residual disease and/or tumor recurrence.

Implementation of the Pap smear as routine for cervical cancer screening was associated with an almost 70% decline in mortality from the disease (34–36). The extent to which this cytologic examination has been adopted worldwide reflects its cost-effectiveness and ease of usage (37). Cervical carcinoma, although globally still one of the most common cancers in women (35), does not approach the high prevalence of NPC in the populous areas in which this cancer is endemic. A study is in development to examine whether nasopharyngeal brushing could serve as part of a screening program in these high-risk populations.

REFERENCES


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Notes

Supported by the Medical Research Council (MRC) of Canada, Temmy Latner-Dynacare, the Saul A. Silverman Family Foundation, and the Tauba and Solomon Spiro Family Foundation as an Isabel Silverman Canada-International Scientific Exchange Program (CISEPO) Project. C. E. Tune is a recipient of an MRC Doctoral Research Award. Manuscript received October 20, 1998; revised January 27, 1999; accepted March 8, 1999.