Background: Nasopharyngeal carcinoma is often associated with neck lymph node (LN) metastases, which in many cases is the only manifestation of this disease. The submucosal and infiltrative characteristics of nasopharyngeal carcinoma make this type of cancer difficult to diagnose. Nasopharyngeal carcinoma has also been reported to be strongly associated with the Epstein-Barr virus.

Methods: We examined 36 nasopharyngeal carcinomas (from 30 primary sites and from 6 metastasized LNs), 13 metastasized LNs of other head and neck cancers, and 12 primary unknown neck metastases using an in situ hybridization technique.

Results: In the nasopharyngeal carcinomas, in situ hybridization with an Epstein-Barr virus-encoded small RNA identified the Epstein-Barr virus in 20 (67%) of the 30 primary sites and in 3 (50%) of the 6 metastasized LNs. Epstein-Barr virus was not detected in metastasized LNs of other head and neck cancers, but was detected in 1 of the primary unknown neck metastases.

Conclusion: In situ hybridization using a digoxigenin-labeled Epstein-Barr virus-encoded small RNA probe is useful for the differential diagnosis of metastasized LNs when the primary site is unknown.


NASOPHARYNGEAL carcinoma (NPC) is one of the most common malignancies in southern China and Taiwan, accounting for up to 18% of all cancers. However, it is rare in Japan, North America, and northern Europe. Nasopharyngeal carcinoma affects women more than other head and neck malignancies, and also tends to affect younger age groups compared with most other cancers. Five-year survival rates for NPC range from 30% to 48%. Nasopharyngeal carcinoma is frequently accompanied by neck lymph node (LN) metastasis, which in many instances is the only manifestation of this disease. An endoscopic examination or a biopsy of the nasopharynx often fails to detect the primary site because of the submucosal and infiltrative characteristics of NPC. This difficulty in diagnosing the primary site may result in suboptimal therapy. Thus, development of a sensitive, specific, and reliable procedure to identify NPC in metastasized LNs will allow early diagnosis of NPC and optimal therapeutic treatment of this tumor.

The strong association of NPC with the Epstein-Barr virus (EBV) is well documented. The significant relationship between the presence of EBV in neck metastasis and primary NPC has been shown by in situ hybridization (ISH) and the polymerase chain reaction. Previous studies have detected EBV in metastatic LNs of NPC, but not in other primary sites. Encouraged by these reports, we attempted to detect the presence of EBV using ISH with an EBV-encoded small RNA (EBER) in the neck metastases of patients with primary unknown squamous cell carcinoma (SCC). This study tests the efficacy of this method for identifying the nasopharyngeal histogenesis of metastases of unknown primary tumors.

METHODS

MATERIALS

Paraffin-embedded specimens of NPC and primary unknown neck LN metastases were randomly selected from patients treated at the Department of Otolaryngology–Head and Neck Surgery, University of Tokyo Hospital, Tokyo, Japan, from January 1, 1987, to December 31, 1999. Thirty-six of the specimens were NPCs (from 30 primary tissues and 6 LNs) and 12 were neck metastases of primary unknown SCCs. Thirteen specimens of metastases from
other head and neck cancers were also used as negative controls. The pathological diagnoses of these specimens according to Union Internationale Contre le Cancer criteria are summarized in the Table.

### ISH TECHNIQUE

Serial sections (5 µm) were cut from formaldehyde-fixed paraffin-embedded specimens and mounted on silane-coated slides (Dako Japan, Tokyo). Paraffin was removed from the sections, and they were rehydrated by means of a xylene and alcohol series. In situ hybridization was performed using an ISH kit (Rembrandt; Kreatech, Amsterdam, the Netherlands) according to the manufacturer’s protocol. Briefly, sections were first pre-digested with pepsin solution at 37°C for 30 minutes. Sections were then hybridized with either EBV nuclear antigen 1 or an EBER oligonucleotide probe at 37°C for 2 hours to detect EBV DNA or messenger RNA (mRNA), respectively. Alkaline phosphatase–conjugated digoxigenin was applied for 30 minutes at 37°C for detection, and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt substrate was applied for 10 minutes at 37°C and used as a chromogen. Sections were counterstained with nuclear fast red.

### STATISTICAL ANALYSIS

The Fisher exact test was used to evaluate the statistical significance in the frequencies of EBV according to the pathological classifications.

### RESULTS

In this study, we first performed ISH using an EBV nuclear antigen 1 probe to identify EBV DNA on 22 primary specimens of NPCs (18 undifferentiated and 4 poorly differentiated carcinomas) as a pilot study. However, an EBV nuclear antigen 1 signal was identified in only 1 (5%) of the undifferentiated carcinomas (UNPs). Because this frequency was significantly lower than we expected, we instead performed EBER ISH. The EBER signals were identified in 20 of the 30 primary sites (Table). In the primary lesions, the association of EBV was significantly higher in the UNPs, 15 of 18, than in the poorly differentiated carcinomas, 3 of 8 \( (P = .06) \). As expected, an EBER signal was not detected in an adenoid cystic carcinoma. A similar tendency toward a higher EBV association with UNPs was observed in neck metastases of NPC. An EBER signal was detected in 3 of 4 UNPs, but not in a poorly differentiated SCC (Table). The EBER-ISH signal in metastatic LNs was localized in the nuclei of most of the tumor cells, but was not seen in adjacent lymphocytes (Figure, A).

As for the neck metastases of primary unknown SCCs, an EBER signal was detected in only 1 of 12 specimens (Figure, B). This patient presented to our clinic with a single metastasis on the left side of the neck. An excisional biopsy was performed, and the pathological diagnosis of the tumor was poorly differentiated SCC. There were no abnormal findings on an endoscopic examination, and a computed tomographic scan did not show any additional LN metastases. Because we were unable to detect the primary site, the patient was treated with radiotherapy. She has been followed up for 2 years, with no evidence of disease. No EBER signals were detected in any of the neck metastases of the other head and neck cancers.

### Table: In Situ Hybridization With an EBER in Patients With NPC and Primary Unknown Carcinoma

<table>
<thead>
<tr>
<th>Type of Ca</th>
<th>No. of Patients</th>
<th>EBER Signal Detected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC</td>
<td>30</td>
<td>20 (67)</td>
</tr>
<tr>
<td>Primary lesion</td>
<td>18</td>
<td>15 (83)</td>
</tr>
<tr>
<td>Undifferentiated Ca</td>
<td>3</td>
<td>2 (67)</td>
</tr>
<tr>
<td>keratinizing Ca</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Adenoid cystic Ca</td>
<td>6</td>
<td>3 (50)</td>
</tr>
<tr>
<td>LN</td>
<td>4</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Poorly differentiated SCC</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Primary unknown SCCs (LN)</td>
<td>12</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Oropharyngeal SCC (LN)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Poorly differentiated SCC of the tongue (LN)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cancer of the nasal cavity (LN)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Transitional cell Ca</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Olfactory neuroblastoma</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hodgkin disease (LN)</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: Ca, carcinoma; EBER, Epstein-Barr virus–encoded small RNA; LN, neck lymph node metastasis; NPC, nasopharyngeal carcinoma; SCC, squamous cell carcinoma.

*Data are given as number (percentage) of patients.
The strong association of NPC with EBV has been shown by various methods, including immunoblotting, nucleic acid hybridization, ISH, and polymerase chain reaction. Among them, polymerase chain reaction and ISH have been reported as sensitive methods for detecting EBV in NPCs. Of these 2 methods, ISH has the advantage of being able to precisely localize EBV in tumor cells. In our study, ISH signals were observed in the nuclei of tumor cells, but not in adjacent lymphocytes. Recently, the trend for using ISH to detect EBV has changed from EBV DNA to EBERs. The abundance and stability of these mRNAs provide a higher sensitivity than DNA ISH, as shown by the results of the present study. DNA ISH using an EBV nuclear antigen 1 probe detected EBV in only 1 of 22 specimens, whereas RNA ISH using an EBER probe detected 22 of 30 specimens in primary sites, suggesting the significantly higher sensitivity of RNA ISH.

The close association of nonkeratinizing carcinoma and UNP with EBV is widely accepted, but the association of keratinizing SCC with EBV is still controversial. In this study, we detected EBV in 2 of 3 keratinizing SCCs. On the other hand, a marginally significant difference (P = .06) was found in the frequency of EBV between nonkeratinizing carcinoma (3 [38%] of 8) and UNP (15 [83%] of 18). This variation of EBV association with NPC may reflect technical factors and real geographic variations. Further studies with a larger population are needed to examine this issue.

This study tested the efficacy of the EBER-ISH technique in identifying the nasopharyngeal histogenesis of neck metastases of unknown primary tumors. The EBER signals were found in the nuclei of LN metastases, but not in host lymphocytes, in accordance with previous reports. In addition, an EBER signal was detected in 1 of the metastatic SCCs of unknown origin, but in none of the 13 LNs with other types of cancer. These observations suggest that EBER ISH may be used as a supplemental tool for the differential diagnosis of a neck mass when the primary site is unknown. Recently, Lee et al succeeded in detecting EBV by the EBER-ISH technique in fine-needle aspiration cytologic specimens from neck metastases of NPCs. This assay is a specific, sensitive, and nonradioisotopic way to confirm nasopharyngeal origin and should be routinely performed on neck metastases with an unknown primary site.

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