Epstein-Barr Virus Detection in Neck Metastases by In Situ Hybridization in Fine-Needle Aspiration Cytology: An Aid for Differentiating the Primary Site

Abstract
BACKGROUND: Nasopharyngeal carcinoma (NPC) is strongly associated with Epstein-Barr virus (EBV). The metastasis to cervical lymph nodes represents a frequent initial manifestation of NPC. The utility of EBV detection by polymerase chain reaction (PCR) in the diagnosis of occult NPC with cervical metastasis has been reported. Our previous study showed that EBER1 in situ hybridization was somewhat more sensitive and specific than PCR in detecting EBV in the evaluation of specimens from a population at high risk for NPC.

METHODS: Fine-needle aspiration cytologic specimens of neck masses from 30 patients were investigated, including 10 NPC primary, 19 squamous cell carcinomas from other site of head and neck (9 oral cavity, 2 paranasal sinuses, 2 oropharynx, 3 larynx, and 3 hypopharynx), and 1 diffuse large cell lymphoma. EBER1 in situ hybridization was performed on direct smears made of aspirations.

RESULTS: EBER1 signals were detected in all neck metastases from the nasopharynx, but none of the specimens from other primary sites. This study suggests EBER1 in situ hybridization can be used as a supplemental tool for differential diagnosis whenever fine-needle aspiration cytology confronts a neck metastasis without knowing the primary site.
Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignancies in Taiwan. The diagnosis of NPC is often difficult, because metastasis to a cervical lymph node is often the initial manifestation and the primary lesions tend to infiltrate submucosally and, eluding visual inspection or random biopsy. Both the primary site and neck metastases of NPC are radiosensitive. In addition, the differential diagnosis of neck mass is broad. Therefore, recognition of the primary nasopharyngeal origin in neck metastases is crucial to optimal therapeutic management.

NPC is strongly associated with Epstein-Barr virus (EBV). In our previous studies, the absolute relationship between the presence of EBV in neck metastatic squamous cell carcinoma and primary NPC is evident by using EBER1 in situ hybridization and polymerase chain reaction (PCR).

Fine-needle aspiration (FNA) cytology has been applied as the first-line investigation for head and neck masses. Although cytological features of metastatic NPC have been described, making a definite diagnosis by conventional cytological analysis may be difficult. In this study, we attempted to detect the presence of EBV by EBER1 in situ hybridization on direct smears obtained by fine-needle aspiration cytology in neck metastases. The aim of our work is to obtain a reliable, specific, rapid, and reproducible procedure with which to identify the nasopharyngeal histogenesis of metastases of unknown primaries.
MATERIAL AND METHODS

Case and Preparation of Samples

FNA cytology was performed with a 10 ml disposable syringe and 23-gauge needle. Direct smears made of the aspirated material were air dried by Riu's stain (a modified Wright's stain) and fixed in 95% ethanol by Papanicolau stain for cytological diagnosis. Additional air dried smear on silanated glass slides was for in situ procedures. These silanated slides were prepared by immersion in a 2% solution of 3-aminopropyltriethoxysilane in acetone for 5 minutes, followed by rinsing for 1 minute in water.

FNA specimens of neck masses from 30 patients were investigated, including 10 NPC primary, 19 squamous cell carcinomas from other site of head and neck (9 oral cavity, 2 paranasal sinuses, 2 oropharynx, 3 larynx, and 3 hypopharynx), and 1 diffuse large cell lymphoma. Histopathological correlation was available in all cases in biopsies or surgical specimens preceded or followed the FNA cytology.

Synthesis of Polymerase Chain Reaction-Derived, Digoxigenin-Labelled DNA Probe

The preparation of the probe was performed as in our previous report. Polymerase chain reaction (PCR) was performed using 2.5 units Taq polymerase (Perkin Elmer, Oak Brook, IL) in a 50 µL reaction containing 500 ng B95-8 genomic DNA, 50 pmol each oligonucleotide primer (sense primer, 5'-AGGTTTTGCTAGGGAGGA, antisense primer 5'-ACTTGACCGAAGACGGCA), 4 µL 10× dNTP labeling mixture: 1mM dATP; 1 mM dCTP; 1 mM cGTP; 0.65mM dTTP; and 0.35mM DIG-dUTP 9ph7.5) (Boehringer Mannheim, Germany, Cat No 1277065), 10 mM Tris-HCL (pH8.3), 50 mM KCl, and 1.5 mM MgCl2. An initial denaturation was performed at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, and extension at 72 °C for 60 seconds. Samples were overlaid with 1 drop of mineral oil and subjected to 35 cycles of amplification. PCR product was stored at −20 °C and was ready to be used in EBER in situ hybridization.

EBER1 in situ hybridization

After being dipped in phosphate-buffer saline for 2 minutes, the smears were immersed in 20 µg/mL proteinase K (Sigma), 0.3% triton-X in 100 mM Tris (pH 8.0), and 50 mM ethylene diamine tetraacetic acid (EDTA) for 8 min at 37°C. After proteinase K treatment, the slides were dipped in phosphate-buffered saline for 5 minutes to halt proteinase K activity. The hybridization cocktail consisted of 50% formamide, 5×SSPE (1×SSPE: 150 mM NaCl and 32 mM Na2H2PO4.H2O, pH 7.4), 10% dextran sulfate, 1% polyvinylpyrrolidone, 5×Denhart's solution, 2% sodium dodecyl sulfated, and 100 µg/mL salmon sperm DNA. PCR-derive DNA
probe was prepared at 1:20 dilution in a hybridization cocktail, denatured by boiling for 5 min, then quenched on ice. Samples were overlaid with 20 µl of hybridization mix, covered with paraffin coverslip and incubated at 55°C overnight.

After hybridization, slides were washed in SSPE twice, 0.1% sodium dodecyl sulfate for 5 minutes at 55°C, and SSPE twice for 5 minutes. Alkaline phosphatase F'ab antibody fragments to digoxigenin (Boehringer Mannheim, Germany) were prepared at 1:500 dilution in 100 mM Tris (pH 7.5), 150 mM NaCl containing 1% sheep serum, and 0.3% triton-X. Slides were incubated for 1 hour at room temperature after adding 40 µL of this solution. Slides were washed in 100mM Tris (pH7.5) and 150mM NaCl for 5 minutes, and 100 mM Tris (pH 9.5), 100 mM NaCl, and 50 mM MgCl2 for 5 minutes at room temperature. Color development proceeded by incubating the slides in a solution of 45 µL nitroblue tetrazolium salt solution (75 ng/mL in dimethylformamide) and 35 µL 5-bromo-4cloro-3 indolyl phosphate toluidinium salt (50mg/mL in dimethylformamide) in 10mL of 100 mM Tris-HCL (pH9.5), 100 mM NaCl, and 50 mM MgCl2 at 37°C for 2 hours. The slides were rinsed in distilled water and counterstained with 0.5% eosin in 80% ethanol for 10 seconds. Counterstained slides were cleared in xylene for 30 seconds and mounted with coverslides.

RESULTS

FNA cytological smears in ten cases of metastatic neck mass from NPC showed malignant cells in a background of reactive lymphocytes (Fig. 1). The malignant cells were in loosely cohesive clusters or singly. The nuclei were round to oval and with mild to moderate pleomorphism. Nucleoli were prominent in most cases. EBER1 in situ hybridization signals were detected in these ten FNA specimens (Fig. 2). The staining was localized the nuclei of the majority of epithelial cells but not in adjacent lymphocytes.

FNA cytological smears in 19 cases of neck masses from other sites of head and neck tumor showed keratinizing or poorly differentiated squamous cell carcinoma. None of these 19 cases expressed EBER1.

FNA cytologic smear in the case of lymphoma showed dispersed malignant cells with large nuclei and coarse chromatin in a background of reactive lymphocytes. No EBER1 signal was detect in the malignant cells. Following excision biopsy revealed a lymphoma, diffuse B cell type.

DISCUSSION

The application of lymph node FNA is a widely accepted procedure to diagnose and provide hints as to the primary site of a malignancy. Particularly in NPC, the
metastasis to cervical lymph nodes represents a frequent initial manifestation. Sometimes, the primary lesion remains elusive since NPC has submucosal, infiltrative growth. Therefore, recognition of the primary nasopharyngeal origin in lymph node FNA cytology is crucial. However, making a definite diagnosis of metastatic NPC only by cytological morphology may be difficult. The differential diagnosis of cervical lymphadenopathy is broad. In addition, in WHO type III NPC dissociated cells with single eosinophilic nucleoli may be mistaken for high-grade non-Hodgkin's lymphoma or melanoma. This study was therefore performed to develop a supplemental tool for securing an accurate diagnosis of nasopharyngeal origin in lymph node FNA cytology.

EBV identification is a specific marker of NPC within the head and neck tumors. The utility of EBV detection by the PCR in FNA specimens from neck lymph node metastases to identify cases of NPC has been reported. However, amplified EBV genomes were demonstrated by PCR in metastatic carcinomas other than NPC. A major drawback of PCR is the sacrifice of tissue architecture and positive results may be obtained from the presence of latent EBV in lymphocytes. Our previous study detected EBER1 signal by in situ hybridization in the nuclei of metastatic malignant epithelial cells to lymph nodes but not in the host lymphocytes. In addition, the sensitivity and specificity of EBER1 in situ hybridization for NPC detection are optimal. suggest the method is the choice for qualifying cytological diagnosis.

Pacchioni et al described the detection of EBV by EBER1 in situ hybridization in neck metastases. In situ procedures were performed on sections of cell blocks made of the aspirated materials. EBER1 expressed in all metastases of NPC but in none of metastases of tumors of different types. Our study confirms and extends this report, as in situ procedure was used on direct smears but not section of cell blocks. The advantage of direct smears is more simple, rapid, and saving the cost.

In our present report, EBER1 was found in the nuclei of metastatic NPC to lymph nodes, but was not evident in the host lymphocytes in FNA cytological smears. None of the 20 nodes with other types of cancer showed positive EBER1 signal. These observations suggest EBER1 in situ hybridization can be used as a supplemental tool for differential diagnosis whenever fine-needle aspiration cytology confronts a neck metastasis without knowing the primary site. The presence of EBV in metastatic lesions would make the diagnosis of metastatic NPC in patients of occult primary. The absence of EBV would suggest other potential primary sites.

This present report is the first to demonstrate the utility of EBER1 in situ hybridization in detecting EBV in direct smears obtained by fine-needle aspiration in neck metastases. Diagnostic material can be made into direct smears and obtained by a rapid, safe, simple, cost-effective, and noninvasive procedure, such as FNA. This
assay would be a rapid, specific, sensitive, and nonradioisotopic method to confirm nasopharyngeal origin and should be routinely applied in neck metastasis of unknown primary. The application of this tool will resolve a diagnostic dilemma and will be useful in clinical management of occult primary NPC.
REFERENCES

1. Department of Health, the Executive Yuan: Cancer registry annual report in Taiwan Area. Taipei: Department of Health, the Executive Yuan, 1998.


LEGENDS

Figure 1. Fine-needle aspiration showing loosely clustered malignant cells in a background of lymphocytes (Papanicolaou’s stain).

Figure 2. EBER1 in situ hybridization on direct smear. Positive signal in the nuclei of tumor cells but not in adjacent lymphocytes.