Identification of Tyrosine Kinases Overexpressed in Head and Neck Cancer

Ho-Sheng Lin, MD; Gerald J. Berry, MD; Willard E. Fee, Jr, MD; David J. Terris, MD; Zijie Sun, PhD

Objective: To identify protein-tyrosine kinases (PTKs) that may be involved in the development and progression of head and neck squamous cell carcinoma (HNSCC).

Design: Messenger RNA from 7 HNSCC specimens was reverse transcribed to complementary DNA, and selective amplification of PTK complementary DNA was achieved using polymerase chain reaction (PCR) with degenerate PTK primers. The resulting PTK PCR products from these 7 HNSCC specimens were then cloned and randomly selected for sequencing. The PTKs that were represented multiple times in these randomly selected clones were selected as candidate PTKs that may be overexpressed in HNSCC. Antibodies against these candidate PTKs were then used for immunohistochemical studies on 8 other HNSCC specimens not used in the original selection of the candidate PTKs.

Results: Three known (EphA1, Brk, and Ron) and 2 novel (KIAA0728 and KIAA0279) PTKs were found to be highly expressed in the 7 HNSCC samples studied, based on the technique of reverse transcriptase–PCR with degenerate primers. Immunohistochemical studies with antibodies against the 3 known PTKs in 8 other HNSCC specimens not used in the previous reverse transcriptase–PCR reaction demonstrated overexpression of EphA1, Brk, and Ron in 12.5%, 37.5%, and 75% of these specimens.

Conclusions: In this study, we identified 5 PTKs that were overexpressed in HNSCC using a reverse transcriptase–PCR technique and confirmed the overexpression of 3 known PTKs in some of the 8 archival HNSCC specimens studied. Our finding suggests that the signaling pathways mediated through EphA1, Brk, and Ron may be involved in the development and progression of HNSCC.

Arch Otolaryngol Head Neck Surg. 2004;130:311-316

HEAD AND NECK CANCER ACCOUNTS FOR MORE THAN 4% OF ALL NEW CANCER CASES AND IS RESPONSIBLE FOR 2% OF ALL CANCER DEATHS IN THE UNITED STATES ANNUALLY.® Squamous cell carcinoma is the histopathologic type that constitutes more than 90% of cancer in this region.2 Although the development of head and neck squamous cell carcinoma (HNSCC) has long been associated with tobacco and alcohol use, the molecular mechanisms leading to carcinogenesis remain elusive. Protein-tyrosine kinases (PTKs) are regulatory proteins that play a significant role in cell growth, proliferation, differentiation, and apoptosis and include many growth factor receptors, cell cycle regulators, and cell signal transducers. They thus represent a major class of proto-oncogenes and have been implicated in pathogenesis of many human malignancies.3,4 Several PTKs have recently been identified to be overexpressed in HNSCC and show promise as useful diagnostic and prognostic markers.5 These include the vascular endothelial growth factor receptor,6 c-Met,7,11 and the epidermal growth factor receptor.12-15 Some of these markers also hold promise for aiding in the selection of the most effective cancer therapy16 and even for development of potential novel therapeutic strategies.17-19

Because all PTKs share a catalytic domain with 11 highly conserved subdomains (Figure 1),20-22 degenerate oligonucleotide primers can be used to selectively amplify the sequences that encode PTKs. This feature has led to the identification of many novel putative protein kinases. More than 100 distinct PTKs have now been identified. In the present study, we used an approach based on reverse transcriptase–polymerase chain reaction (RT-PCR) with degenerate primers24,25 to identify PTKs that are overexpressed in HNSCC specimens. The differential overexpression of these PTKs in HNSCC was then further confirmed using immunohistochemical studies.

From the Department of Otolaryngology/Head and Neck Surgery, Wayne State University, Detroit, Mich (Dr Lin); the Department of Pathology (Dr Berry) and the Division of Otolaryngology (Drs Fee and Sun), Stanford University Medical Center, Stanford, Calif; and the Department of Otolaryngology, Medical College of Georgia, Augusta (Dr Terris). The authors have no relevant financial interest in this article.
IHRDLAARN SDVWSFG R RPS DFG

Figure 1. Catalytic domains of the protein-tyrosine kinase family. The conserved residues are labeled I through XI and are shown within rectangular boxes.

**METHODS**

**PATIENTS AND TISSUE SAMPLES**

Prior approval from the institutional review board of Stanford University, Stanford, Calif, and informed consent from the patients whose tissue specimens were used in this study were obtained. The cancer specimens were procured at the time of surgical extirpation at a site away from the margin of resection to avoid compromising the ability of the pathologists to assess the adequacy of margin. The tumor samples were immediately frozen in liquid nitrogen and then stored in a freezer at −80°C.

**RNA PURIFICATION FROM SURGICAL SPECIMENS**

A total of 7 cancer specimens were used for RNA extraction and isolation of tyrosine kinases. These specimens were confirmed to consist mostly of squamous cell carcinoma (>75%) on frozen sections. Two specimens were from the oral cavity (palate and buccal region), 1 from the oropharynx (tonsil), 1 from the hypopharynx, 2 from the supraglottic larynx, and 1 from the glottic larynx. Total RNA was isolated from approximately 1 g of tissue from each of the 7 cancer samples by means of RNAzol B (TM Cinna Scientific, Friendswood, Tex) per the manufacturer’s recommendation. After cell lysis, the lysate was mixed with 200 µL of chloroform and centrifuged at 14,000 g for 15 minutes. The top aqueous phase was then recovered, and the RNA pellet was precipitated using isopropanol. After dissolving in 50 µL of ribonuclease-free water, the purity and concentration of the total RNA were determined at the absorbance measurements of 260 nm and 280 nm with the use of a spectrophotometer (MBA 2000; PerkinElmer, Inc, Shelton, Conn).

**RT-PCR PROFILING OF TYROSINE KINASES**

Reverse transcription was performed using 5 µg of total RNA with oligo(dT) primer (Promega Corp, Madison, Wis) and avian myeloblastosis virus RT (Life Sciences, Inc, St Petersburg, Fla). The complementary DNA (cDNA) was then PCR amplified using Taq DNA polymerase and a series of degenerate sense primers from subdomains VI and antisense primers from subdomains VIII and IX (Figure 1 and Table 1). Primer sequences were chosen to amplify tyrosine kinases preferentially rather than serine/threonine kinases. In the first reaction, tyrosine kinases were amplified with degenerate primers from subdomain VI and antisense primers from subdomain VIII (Table 1). The resulting tyrosine kinase PCR fragments were directly subcloned into the vector (PGEM-T Easy Vector, Promega Corp) per the manufacturer’s instruction. Positive clones were identified by means of ampicillin and β-galactosidase selection. About 20 white colonies were randomly picked from each of the 7 cancer samples and sequenced. The sequence was then checked against the GenBank database using the Basic Local Alignment Search Tool (BLAST) program accessed through the National Center for Biotechnology Information (available at: http://www.ncbi.nlm.nih.gov).

**CLONING AND SEQUENCING**

The resulting tyrosine kinase PCR fragments were directly subcloned into the vector (PGEM-T Easy Vector, Promega Corp) per the manufacturer’s instruction. Positive clones were identified by means of ampicillin and β-galactosidase selection. About 20 white colonies were randomly picked from each of the 7 cancer samples and sequenced. The sequence was then checked against the GenBank database using the Basic Local Alignment Search Tool (BLAST) program accessed through the National Center for Biotechnology Information (available at: http://www.ncbi.nlm.nih.gov).

**IMMUNOHISTOCHEMICAL STUDIES ON PARAFFIN-EMBEDDED SPECIMENS**

Primary rabbit polyclonal antibodies against the 3 known human tyrosine kinases identified in this study were purchased (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). These include antibodies against Brk (C-17), EphA1 (G-18), and Ron (C-20). Eight surgical specimens not used in the previous RT-PCR reaction to select the candidate PTKs were selected from various head and neck subsites (Table 2) for immunohistochemical studies. These specimens were taken from patients who were initially treated with surgical excision and had never been exposed to radiation therapy or chemotherapy. Tissue blocks from the surgical margin were selected to include both the healthy and cancer tissues in the same slide. Tissue blocks were sliced at 5-µm thickness to prepare the slides.

Using the Biogenex EnVision (BioGenex, San Ramon, Calif), deparaffinization and microwave antigen retrieval were performed on the sections. This was followed by immunostaining using the Dako Autostainer (DakoCytomation, Carpinteria, Calif). Briefly, the slides were incubated in 0.3% hydrogen peroxide and blocked using a nonserum-based blocking agent. The slides were then incubated with primary antibodies to Ron (C-20), EphA1 (G-18, dilution 1:100), and Brk (C-17, dilution 1:50) (all antibodies from Santa Cruz Biotechnology, Inc). After the primary incubation, the slides were incubated with biotinylated secondary antibody (goat anti–rabbit antibody) for 10 minutes, followed by streptavidin-peroxidase reagent for 10 minutes. Finally, the slides were visualized using 3,3′-diaminobenzidine and counterstained with Mayer hematoxylineosin.

Analysis of immunohistochemical results was performed by a board-certified pathologist (G.J.B.). Staining intensities were graded semiquantitatively as 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. In addition, the percentage of cancer cells with positive findings for staining was noted.

**RESULTS**

**TYROSINE KINASE PROFILE OF HNSCC**

A total of 142 colonies (about 20 colonies per cancer sample) were picked at random from the plates for sequencing. Approximately 92% of the clones sequenced contained tyrosine kinase fragments. The other 8% of the
clones sequenced carried non–tyrosine kinase inserts such as proteasome, transforming growth factor β/H9252, and /H9252-actin. The sequences for PTKs EphA1 and Brk were isolated from all 7 cancer specimens, and they represent the most frequently isolated sequence in this study (Table 2). Ron, another known tyrosine kinase, was isolated in 3 cancer samples. Transcripts from tumor samples 3, 4, 5, and 6 were found to match uncharacterized human gene KIAA0728. In addition, transcripts from tumor samples 1 and 2 matched with another uncharacterized human gene KIAA0279 (Table 2).

### IMMUNOHISTOCHEMICAL ANALYSIS

The results of the immunohistochemical staining on 8 archival HNSCC specimens (not used in the previous RT-PCR to select candidate PTKs) using primary rabbit polyclonal antibodies against EphA1, Brk, and Ron are presented in Table 3 and Figure 2. Overall, we found a general increase in the intensity of staining in cancer cells compared with normal cells. Variability in the intensity of staining and the percentage of cells stained is observed across these 8 different tumor samples. A cancer sample was considered to have positive overexpression of a particular PTK when more than 50% (shown in boldface type in Table 3) of the cells stained positive with antibodies against EphA1, Brk, or Ron. In the 8 HNSCC samples examined, 1 sample (12%) overexpressed EphA1, 3 samples (38%) overexpressed Brk, and 6 samples (75%) overexpressed Ron. The expression of EphA1 and Ron was slightly elevated in the basal layer of epidermis in several adjacent noncancerous tissues. However, no expression of these PTKs was seen outside the basal layer in these normal tissues. The staining pattern for the HNSCC sample from the larynx (sample 1) is shown in Figure 2. Figure 2 A shows the hematoxylin-eosin stain of an infiltrating, moderately differentiated squamous cell carcinoma of the larynx. In Figure 2 B, 95% of the cancer cells have overexpression of Ron, as evidenced by the moderate intensity staining (2+) against antibody to Ron on the cell surface. In Figure 2 C, weak staining (1+) for EphA1 is seen in only about 20% of these cancer cells. Finally, about 40% of these laryngeal cancer cells overexpressed the nonreceptor tyrosine kinase Brk, as shown by the moderate amount of intracellular staining (Figure 2D).

###COMMENT

In this study, we applied the RT-PCR–based approach using degenerate primers and identified 3 known tyrosine kinases (EphA1, Brk, and Ron) that are overexpressed in HNSCC. An extensive review of the literature revealed that the overexpression of these 3 tyrosine kinases in HNSCC has never been reported. EphA1 (isolated from erythropoietin-producing hepatocellular carcinoma) is a receptor PTK that belongs to the Eph subfamily. This class of tyrosine kinase plays a critical role in cell-to-cell interactions and establishes tissue organi-
zation through signaling pathways that control axonal projection, cell migration, and the maintenance of cellular boundaries.27 A number of Eph receptors have been shown to be overexpressed in various tumors. Specifically, EphA1 has been shown to be transcribed at high levels in tumors of epithelial origin such as those from the breast, liver, lung, and colon.28,29 Thus, our finding that EphA1 is overexpressed in some of the carcinoma involving the head and neck region is consistent with its overexpression in other epithelial carcinomas. Breast tumor kinase (Brk) or PTK 6 (PTK6) is another novel PTK identified in this study. It is a nonreceptor tyrosine kinase and is normally expressed at a low level intracellularly in differentiating epithelial cells in skin and mu-

<table>
<thead>
<tr>
<th>Sites</th>
<th>EphA1</th>
<th>Brk</th>
<th>Ron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stain Intensity, Normal/Carcinoma Tissue</td>
<td>% of Cells Positive for Stain</td>
<td>Stain Intensity, Normal/Carcinoma Tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>0/1</td>
<td>20</td>
<td>0/2</td>
</tr>
<tr>
<td>Tonsil 1</td>
<td>0, 1 (Basal layer)/1</td>
<td>40</td>
<td>0/1</td>
</tr>
<tr>
<td>Supraglottic</td>
<td>0, 1 (Basal layer)/1</td>
<td>10</td>
<td>0/1</td>
</tr>
<tr>
<td>Retromolar trigone</td>
<td>0/2</td>
<td>95</td>
<td>0/1</td>
</tr>
<tr>
<td>Buccal</td>
<td>0/0</td>
<td>0</td>
<td>0/1</td>
</tr>
<tr>
<td>Oral tongue</td>
<td>0/1</td>
<td>20</td>
<td>0/1</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>0/1</td>
<td>10</td>
<td>0/1</td>
</tr>
<tr>
<td>Tonsil 2</td>
<td>0/1</td>
<td>10</td>
<td>0/1</td>
</tr>
</tbody>
</table>

*Staining intensity grades are described in the “Immunohistochemical Studies on Paraffin-Embedded Specimens” subsection of the “Methods” section. Boldface type indicates positive overexpression of a particular protein-tyrosine kinase.

Figure 2. Immunohistochemical staining for Ron, EphA1, and Brk in laryngeal cancer. A, Infiltrating, moderately differentiated squamous cell carcinoma of the larynx (hematoxylin-eosin, original magnification ×400). B, Immunostaining for Ron receptor shows moderate (2+) membranous staining (original magnification ×400). C, Weak immunostaining for EphA1 receptor in laryngeal carcinoma (original magnification ×400). D, Staining for Brk, which is a nonreceptor tyrosine kinase, shows moderate (2+) intracellular staining (original magnification ×600). Staining intensity grades are described in the “Immunohistochemical Studies on Paraffin-Embedded Specimens” subsection of the “Methods” section.
cosa of normal alimentary canal. Overexpression of Brk has been reported in breast cancer, melanoma, and colon cancer. Again, our finding of increased expression of Brk in some HNSCC is consistent with findings in other epithelial malignancies. This finding brings fresh insight regarding the role of Brk in cancers of epithelial origin. Finally, Ron is a receptor tyrosine kinase that belongs to the subfamily of hepatocyte growth factor receptor. It has structural similarity to c-Met, which is a well-known proto-oncprotein overexpressed in diverse human tumors including melanoma, rhabdomyosarcoma, breast cancer, colon cancer, ovarian cancer, and prostate cancer. A high level of expression of c-Met has been correlated with increased invasiveness and potential for metastasis. Although Ron has not been as well studied as c-Met, Ron has been reported to be overexpressed in breast cancer. We demonstrated herein that it is also overexpressed in some squamous cell carcinomas involving the head and neck region. Seventy-five percent of the HNSCCs used in this study demonstrated increased expression of Ron.

Our results indicated that in the 8 HNSCC samples examined, 12.5% of tumor cells overexpressed EphA1, 37.5% overexpressed Brk, and 75% overexpressed Ron. This variability in the overexpression of these PTKs among different HNSCC specimens is not surprising and may reflect the intrinsic biological differences among these different HNSCCs. Although histologically homogeneous, squamous cell carcinomas represent an extremely heterogeneous group of diseases with a wide spectrum of clinical behaviors. We now know that there are multiple different molecular pathways that can eventually lead a cell down the path of carcinogenesis. These different molecular pathways are likely responsible for the difference in clinical behavior (eg, growth rate, propensity for regional or distant metastasis, invasion into nerve or vessels, and response to chemotherapeutic agents and radiation therapy) of these cancers. The different molecular events that are involved in carcinogenesis may be responsible for the differential overexpression of PTKs among HNSCC. We also observed in this study the heterogeneity of cellular expression of the PTKs within the same tumor (Figure 2). Overexpression of a given PTK was observed in a certain percentage of the tumor cells and can range from 10% in some tumors to 100% in other tumors. There have been suggestions that this heterogeneity of expression may be due to microenvironmental factors that regulate receptor expression and heterogeneity of tumor cells within the same tumor tissue. However, more needs to be learned about the intratumor variation in PTK expression.

An interesting finding from this study was the isolation of 2 novel proteins, KIAA0728 and KIAA0279, which may represent putative kinases with unknown function. Further studies are ongoing to elucidate the function of these 2 novel proteins.

Finally, the technique of RT-PCR with degenerate primers appears to be an effective method for identification of differentially overexpressed PTKs in HNSCC. However, previous studies have shown that different types of protein kinases may be preferentially selected by using different combinations of degenerate primers. Multi-

COSA OF NORMAL ALIMENTARY CANAL. OVEREXPRESSION OF BRK HAS BEEN REPORTED IN BREAST CANCER, MELANOMA, AND COLON CANCER. AGAIN, OUR FINDING OF INCREASED EXPRESSION OF BRK IN SOME HNSCC IS CONSISTENT WITH FINDINGS IN OTHER EPITHELIAL MALIGNANCIES. THIS FINDING BRINGS FRESH INSIGHT REGARDING THE ROLE OF BRK IN CANCERS OF EPITHELIAL ORIGIN. FINALLY, RON IS A RECEPTOR TYROSINE KINASE THAT BELONGS TO THE SUBFAMILY OF HEPATOCYTE GROWTH FACTOR RECEPTOR. IT HAS STRUCTURAL SIMILARITY TO C-MET, WHICH IS A WELL-KNOWN PROTO-ONCOPROTEIN OVEREXPRESSED IN DIVERSE HUMAN TUMORS INCLUDING MELANOMA, Rhabdomyosarcoma, Breast Cancer, Colon Cancer, Ovarian Cancer, AND PROSTATE CANCER. A HIGH LEVEL OF EXPRESSION OF C-MET HAS BEEN CORRELATED WITH INCREASED INVASIVENESS AND POTENTIAL FOR METASTASIS. ALTHOUGH RON HAS NOT BEEN AS WELL STUDIED AS C-MET, RON HAS BEEN REPORTED TO BE OVEREXPRESSED IN BREAST CANCER. WE DEMONSTRATED HEREIN THAT IT IS ALSO OVEREXPRESSED IN SOME SQUAMOUS CELL CARCINOMAS INVOLVING THE HEAD AND NECK REGION. SEVENTY-FIVE PERCENT OF THE HNSCCS USED IN THIS STUDY DEMONSTRATED INCREASED EXPRESSION OF RON.

OUR RESULTS INDICATED THAT IN THE 8 HNSCC SAMPLES EXAMINED, 12.5% OF TUMOR CELLS OVEREXPRESSED EPHA1, 37.5% OVEREXPRESSED BRK, AND 75% OVEREXPRESSED RON. THIS VARIABILITY IN THE OVEREXPRESSION OF THESE PTKS AMONG DIFFERENT HNSCC SPECIMENS IS NOT SURPRISING AND MAY REFLECT THE INTRINSIC BIOLOGICAL DIFFERENCES AMONG THESE DIFFERENT HNSCCs. ALTHOUGH HISTOLOGICALLY HOMOGENEOUS, SQUAMOUS CELL CARCINOMAS REPRESENT AN EXTREMELY HETEROGENEOUS GROUP OF DISEASES WITH A WIDE SPECTRUM OF CLINICAL BEHAVIORS. WE NOW KNOW THAT THERE ARE MULTIPLE DIFFERENT MOLECULAR PATHWAYS THAT CAN EVENTUALLY LEAD A CELL DOWN THE PATH OF CARCINOGENESIS. THESE DIFFERENT MOLECULAR PATHWAYS ARE LIKELY RESPONSIBLE FOR THE DIFFERENCE IN CLINICAL BEHAVIOR (EG, GROWTH RATE, PROPENSITY FOR REGIONAL OR DISTANT METASTASIS, INVASION INTO NERVE OR VESSELS, AND RESPONSE TO CHEMOTHERAPEUTIC AGENTS AND RADIATION THERAPY) OF THESE CANCERS. THE DIFFERENT MOLECULAR EVENTS THAT ARE INVOLVED IN CARCINOGENESIS MAY BE RESPONSIBLE FOR THE DIFFERENTIAL OVEREXPRESSION OF PTKS AMONG HNSCC. WE ALSO OBSERVED IN THIS STUDY THE HETEROGENEITY OF CELLULAR EXPRESSION OF THE PTKs WITHIN THE SAME TUMOR (FIGURE 2). OVEREXPRESSION OF A GIVEN PTK WAS OBSERVED IN A CERTAIN PERCENTAGE OF THE TUMOR CELLS AND CAN RANGE FROM 10% IN SOME TUMORS TO 100% IN OTHER TUMORS. THERE HAVE BEEN SUGGESTIONS THAT THIS HETEROGENEITY OF EXPRESSION MAY BE DUE TO MICROENVIRONMENTAL FACTORS THAT REGULATE RECEPTOR EXPRESSION AND HETEROGENEITY OF TUMOR CELLS WITHIN THE SAME TUMOR TISSUE. HOWEVER, MORE NEEDS TO BE LEARNED ABOUT THE INTRATUMOR VARIATION IN PTK EXPRESSION.

AN INTERESTING FINDING FROM THIS STUDY WAS THE ISOLATION OF 2 NOVEL PROTEINS, KIAA0728 AND KIAA0279, WHICH MAY REPRESENT PUTATIVE KINASES WITH UNKNOWN FUNCTION. FURTHER STUDIES ARE ONGOING TO ELUCIDATE THE FUNCTION OF THESE 2 NOVEL PROTEINS.

FINALLY, THE TECHNIQUE OF RT-PCR WITH DEGENERATE PRIMERS APPEARS TO BE AN EFFECTIVE METHOD FOR IDENTIFICATION OF DIFFERENTIALLY OVEREXPRESSED PTKS IN HNSCC. HOWEVER, PREVIOUS STUDIES HAVE SHOWN THAT DIFFERENT TYPES OF PROTEIN KINASES MAY BE PREFERENTIALLY SELECTED BY USING DIFFERENT COMBINATIONS OF DEGENERATE PRIMERS. MULTIPLE OPTIONS EXIST IN THE CONSTRUCTION OF DEGENERATE PRIMERS. THE PRIMERS CAN BE CHOSEN FROM DIFFERENT DOMAINS AND CAN BE CONSTRUCTED IN DIFFERENT LENGTH AND DEGENERACY (TABLE 1). THE PARTICULAR PRIMERS USED IN THIS STUDY OBVIOUSLY DID NOT SELECT FOR CERTAIN TYPES OF TYROSINE KINASES SUCH AS C-MET AND EPIDERMAL GROWTH FACTOR RECEPTOR, WHICH ARE WELL KNOWN TO BE OVEREXPRESSED IN MOST HNSCCs. THIS BIAS OF SELECTION OF PTKs BASED ON THE TYPE OF DEGENERATE PRIMERS USED CAN CERTAINLY BE A CRITICISM OF THIS STUDY. FURTHER STUDIES WILL BE NEEDED TO USE DIFFERENT COMBINATIONS OF DEGENERATE PRIMERS TO CAPTURE THE LARGEST NUMBER OF DIFFERENT TYROSINE KINASES POSSIBLE.

CONCLUSIONS

In this study, we identified 3 known tyrosine kinases (EphA1, Brk, and Ron) that are overexpressed in HNSCC by using an RT-PCR technique with degenerate primers. We confirmed their overexpression in some HNSCCs using immunohistochemical studies. Two possible novel kinases were also identified. Our finding suggests that the signaling pathways mediated through EphA1, Brk, and Ron may be involved in the development and progression of HNSCC.

Submitted for publication April 21, 2003; final revision received June 23, 2003; accepted June 26, 2003.

Corresponding author: Ho-Sheng Lin, MD, Department of Otolaryngology/Head and Neck Surgery, Wayne State University, 5 East University Health Center, 4201 St Antoine, Detroit, MI 48201 (e-mail: hlin@med.wayne.edu).

REFERENCES