Micrometastatic Tumor Detection in Patients With Head and Neck Cancer

A Preliminary Report

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Objective: To apply a new immunocytochemistry (ICC) assay to peripheral blood samples for micrometastatic circulating tumor cell detection in patients with head and neck squamous cell cancer (HNSCC).

Design: The ICC assay uses established monoclonal antibodies that bind to tumor-associated antigens combined with an enrichment system that uses positive selection with anti-human epithelial antigen (EpCAM antibody) to detect circulating tumor cells.

Subjects: Eighteen consecutive patients newly diagnosed as having HNSCC are described.

Results: Of the 18 patients, 8 (44%) demonstrated circulating tumor cells using the ICC assay. The numbers of patients positive for circulating tumor cells per stage are as follows: stage I, 1 of 1; stage II, 0 of 2; stage III, 2 of 5; stage IV, 5 of 6; and unknown stage, 0 of 4. The numbers of patients positive for circulating tumor cells per location are as follows: oral cavity, 1 of 2; oropharynx, 3 of 4; glottic area, 3 of 5; supraglottic area, 1 of 3; and unknown primary 0 of 4.

Conclusions: Circulating tumor cells were identified in almost half of the patients using the ICC assay. In a literature review, we were not able to identify previous reports of circulating tumor cell detection in patients with HNSCC from peripheral blood samples using ICC or identify any study that has attempted to quantify circulating tumor cell levels. Although the clinical implications of circulating tumor cells in micrometastatic tumor detection in patients with HNSCC are still unknown, they may be significant. Long-term follow-up may help elucidate the patients in whom conventional treatment may fail and, thus, those who may benefit from different treatment; it may also assist with the detection of recurrence with a simple blood collection.


THERE ARE 42,000 new cases of head and neck cancer and 12,000 associated deaths in the United States each year. Worldwide, there are close to three quarters of a million new cases each year, with incidence rates varying on the distribution of risk factors, notably, tobacco chewing, smoking, and alcohol consumption. Of these cases, 95% are identified as squamous cell carcinoma. The 5-year survival averages 50%, depending on the origin and the extent of the tumor. Despite advances in technology and technique, morbidity and mortality rates have changed little during the past 30 years. Treatment failures are determined by the development of local and/or regional recurrence, distant metastasis, or a second primary tumor.

Why have morbidity and mortality rates not decreased significantly during the past 30 years? Local recurrence rates are as high as 50% to 60% for stage III and stage IV cancers, and 15% to 25% of those patients will develop distant metastases. Furthermore, almost one third of the patients with stage III and stage IV cancers who have tumor-free resection margins will develop a local recurrence. Is there a gap in the TNM staging system? Perhaps micrometastasis or minimal residual cancer has not been detected or represented by the current assessment standards. The presence of minimal residual cancer may correlate with a poor clinical outcome.

This study applies a new immunocytochemistry (ICC) assay, developed by IMPATH/BIS Laboratories, to peripheral blood samples for micrometastatic circulating tumor cell detection in patients with head and neck squamous cell cancer (HNSCC). The information generated by this study may help identify those patients in whom conventional treatment may fail and, thus, those who may benefit from different treatment; it may also...
MATERIALS AND METHODS

Institutional review board approval for the study was obtained through the Henry Ford Hospital, Detroit, Mich, while informed consent was also obtained from patients and/or guardians before initiating the study. Eighteen consecutive patients newly diagnosed as having HNSCC have been enrolled into this study to date. Patients were identified through Henry Ford Hospital’s interdisciplinary head and neck tumor board. The study is approved to examine 75 patients. Profiles of the 18 patients enrolled in the study are listed in Table 1.

Approximately 30 mL of blood was drawn from each patient during the head and neck tumor board examination or on the morning of surgery. The blood was collected using standard phlebotomy techniques and placed into 3 green-top tubes (a preservative, heparin sodium, was added to the vacucontainer). The green-top tubes were sent via Federal Express overnight to IMPATH/BIS Laboratories for ICC analysis.

Peripheral blood was shipped at room temperature to IMPATH/BIS Laboratories for enriched ICC analysis. Mononuclear cell fractions were isolated by gradient separation at 1500 rpm for 20 minutes (Ficoll-Hypaque; Pharmacia, Uppsala, Sweden), and washed twice in a medium of assorted salts, amino acids, vitamins, and D-galactose (Leibovitz L-15; GIBCO/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO/BRL). The mononuclear cell fraction was isolated using gradient separation (Ficoll-Hypaque). The mononuclear cells were then placed in phosphate-buffered saline containing 0.2% sodium citrate and washed twice at 1000 rpm for 10 minutes each. Following washing, up to $5 \times 10^5$ cells were incubated with blocking reagent and anti–cancer-conjugated microbeads (anti-human epithelial antigen; Miltenyi Biotech, Inc, Auburn, Calif) at 4°C for 30 minutes. The cells were washed twice with phosphate-buffered saline and 0.2% sodium citrate to remove unbound beads and placed in a separation column along with a magnet for 2 minutes at room temperature to bind cells. Bound cells were removed by removing the column from the magnet followed by gently flushing the column into a fresh tube with 1 mL of buffer using a plunger. All bead-cell conjugates recovered from the magnet were used in the cytopreparations and immunostained.

About 2 to 4 cytopreparations were typically made and stored for immunostaining at 4°C. Figure 1 represents an abridged schematic of this procedure.

Cytopreparations were fixed in 4% paraformaldehyde fixative, washed thoroughly in Dulbecco-modified phosphate-buffered saline (GIBCO/BRL) with detergent (1% Triton X; Sigma-Aldrich Co, St Louis, Mo), and placed on an automated immunostainer (TechMate; Ventana Medical Systems, Inc, Tucson, Ariz). Alkaline phosphatase immunostaining was performed, per protocol, as previously described. Briefly, slides were incubated in blocking solution and anticancer monoclonal antibody cocktail (anticytokeratin types 8 and 18, TFS-2 [a surface glycoprotein derived from breast cancer cells], and antikeratin). This step was followed by staining with alkaline phosphatase, chromogen, and finally hematoxylin. Buffer washes were performed between each step. All slides were then reviewed by a board-certified pathologist for tumor cell identification. Figure 2 demonstrates a hematoxylin-stained slide identifying tumor cells (arrow) along with several mononuclear cells that bypassed the magnetic separation process.

The number of tumor cells stained by the anticancer antibodies was recorded. The tumor concentration was determined by dividing the number of cancer cells detected by the number of cells enriched (the denominator being 1000000). The final recorded value was the number of tumor cells detected per 1000000 cells enriched. The sensitivity is 1 per 1000000, and the specificity is greater than 99%.4

Table 1. Patient Profiles

<table>
<thead>
<tr>
<th>Patient No./Sex/Age, y</th>
<th>Cancer Location</th>
<th>TNM Classification (Stage)</th>
<th>Tobacco Use, Pack-years</th>
<th>Alcohol Consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/75</td>
<td>Oropharynx</td>
<td>T3 N2 M0 (IV)</td>
<td>30</td>
<td>Heavy</td>
</tr>
<tr>
<td>2/F/23</td>
<td>Oral cavity</td>
<td>T2 N1 M0 (III)</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>3/F/68</td>
<td>Glottic area</td>
<td>T3 N0 M0 (III)</td>
<td>50</td>
<td>Moderate</td>
</tr>
<tr>
<td>4/M/72</td>
<td>Supraglottic area</td>
<td>T3 N0 M0 (III)</td>
<td>100</td>
<td>Heavy</td>
</tr>
<tr>
<td>5/M/78</td>
<td>Glottic area</td>
<td>T1 N0 M0 (I)</td>
<td>0</td>
<td>Occasional</td>
</tr>
<tr>
<td>6/F/61</td>
<td>Oropharynx</td>
<td>T3 N0 M0 (III)</td>
<td>60</td>
<td>Heavy</td>
</tr>
<tr>
<td>7/M/60</td>
<td>Oropharynx</td>
<td>T4 N3 M0 (IV)</td>
<td>85</td>
<td>Moderate</td>
</tr>
<tr>
<td>8/M/81</td>
<td>Oropharynx</td>
<td>T2 N0 M0 (II)</td>
<td>30</td>
<td>None</td>
</tr>
<tr>
<td>9/M/40</td>
<td>Unknown</td>
<td>T2 N0 M0 (II)</td>
<td>33</td>
<td>Heavy</td>
</tr>
<tr>
<td>10/F/31</td>
<td>Glottic area</td>
<td>T2 N0 M0 (II)</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>11/M/77</td>
<td>Supraglottic area</td>
<td>T3 N2 M0 (IV)</td>
<td>60</td>
<td>Moderate</td>
</tr>
<tr>
<td>12/M/52</td>
<td>Unknown</td>
<td>. . .</td>
<td>25</td>
<td>None</td>
</tr>
<tr>
<td>13/M/64</td>
<td>Glottic area</td>
<td>T4 N0 M0 (IV)</td>
<td>20</td>
<td>Occasional</td>
</tr>
<tr>
<td>14/F/63</td>
<td>Supraglottic area</td>
<td>T2 N0 M0 (IV)</td>
<td>45</td>
<td>Occasional</td>
</tr>
<tr>
<td>15/M/38</td>
<td>Unknown</td>
<td>T2 N0 M0 (IV)</td>
<td>45</td>
<td>Heavy</td>
</tr>
<tr>
<td>16/M/52</td>
<td>Oral cavity</td>
<td>T2 N0 M0 (IV)</td>
<td>45</td>
<td>Heavy</td>
</tr>
<tr>
<td>17/M/53</td>
<td>Unknown</td>
<td>. . .</td>
<td>30</td>
<td>Heavy</td>
</tr>
<tr>
<td>18/M/74</td>
<td>Glottic area</td>
<td>T3 N0 M0 (III)</td>
<td>45</td>
<td>None</td>
</tr>
</tbody>
</table>

assist with the detection of recurrence with a simple blood collection. By comparing and levels of circulating tumor cells with those of other malignancies, such as breast, colon, or lung cancer, insight may be gained into why head and neck cancer tends to be locally and/or regionally aggressive.
RESULTS

The number of circulating tumor cells identified per patient is listed in Table 2. Specifically, the number of cells enriched per 10,000,000, the positive fraction, and the positive fraction concentration are displayed. Only the positive fraction results are displayed, as there were no cells detected in any of the negative fractions collected.

To date, 8 (44%) of the 18 patients demonstrated circulating tumor cells using the ICC assay. The percentage of patients positive for circulating tumor cells per stage is illustrated in Figure 3, and the corresponding numbers are as follows: stage I, 1 of 1; stage II, 0 of 2; stage III, 2 of 5; stage IV, 5 of 6; and unknown stage, 0 of 4.

The percentage of patients positive for circulating tumor cells per location is illustrated in Figure 4, and the corresponding numbers are as follows: oral cavity, 1 of 2; oropharynx, 3 of 4; glottic area, 3 of 5; supraglottic area, 1 of 3; and unknown area, 0 of 4.

COMMENT

Micrometastatic tumor cell detection in patients with head and neck cancer is a relatively uncharted field of study when compared with other cancers, such as breast, prostate, and colorectal cancer and neuroblastoma. Previous efforts in patients with head and neck cancer have also focused primarily on bone marrow specimens. Wollenberg et al., using ICC directed against epithelial cell marker cytokeratin 19, demonstrated micrometastasis in bone marrow specimens in 41 of 108 patients with HNSCC. Bone marrow specimens positive for tumor cells also correlated with higher rates of local and distant recurrences and with shorter disease-free intervals. Gath et al., using monoclonal antibody A45-B/B3 directed at oral cavity tumors, demonstrated that 10 (32%) of 31 patients had tumor cells in bone marrow specimens. van Dongen et al., demonstrated the use of monoclonal antibodies E48 and U36 directed against HNSCC-associated antigens for the detection and treatment of minimal residual cancer. According to the study, polymerase chain reaction and ICC were used successfully in the detection of cells in bone marrow and peripheral blood; however, no specific data or patient profiles were presented for review. Most recently, Kawamata et al., using reverse transcription polymerase chain reaction, demonstrated the expression of cytokeratin 20 in the peripheral blood samples of 10 of 11 patients with oral squamous cell carcinoma. In summary, a literature review has not iden-
tified previous reports of circulating tumor cell detection in peripheral blood samples of patients with HNSCC using ICC and has not found any study that has attempted to quantify circulating tumor cell levels.

The uniqueness of this study is that it not only uses advanced ICC techniques to analyze peripheral blood but also seeks to quantify tumor load. The ICC assay used (IMPATH/BIS Laboratories) is capable of reliably detecting a single tumor cell among 1000000 mononuclear cells. This technique also has the added advantage of actually being able to see the tumor cell, while reverse transcription polymerase chain reaction techniques may be amplifying some other undesirable product, most notably skin cells. The timing for the blood collection for analysis was scheduled during the head and neck tumor board examination or on the morning of surgery. The presumption is that the number of circulating tumor cells will decrease after treatment; however, this was not addressed in this study. Documenting this reduction will be essential in future studies so that this assay might be used for tumor surveillance following definitive treatment.

Of all 18 patients studied thus far, 8 (44%) demonstrated circulating tumor cells using the ICC assay. If the 4 patients who had primary tumors of unknown origin were discarded (as all 4 were negative for circulating tumor cells), then 8 (57%) of 14 patients would have been positive for circulating tumor cells. Although lesions of the oropharynx had the highest percentage of positive results (3 [75%] of 4), there are too few patients enrolled in the study to statistically conclude a higher rate of micrometastasis. Similarly, although 100% (1/1) of the patients with stage I cancer and 0% (0/2) of the patients with stage II cancer were positive for circulating tumor cells, the number of patients for these 2 stages is too few to draw definitive conclusions. To draw statistically significant conclusions, a total of 75 patients, including all stages, will ultimately need to be examined.

What is the clinical significance of these findings? The presence of minimal residual disease in bone marrow specimens provides prognostic information, most notably in patients with neuroblastoma. More recently, however, the presence of circulating tumor cells in peripheral blood in patients with prostate cancer has also been shown to affect overall survival and disease-free days. Specifically, patients with circulating tumor cells had decreased disease-free and overall survival (49 and 122 days, respectively) when compared with patients with no circulating cells (251 and 347 days, respectively). There are also some preliminary data in patients with breast and colorectal cancer.

Our hope is that by demonstrating circulating tumor cells in the blood and by quantifying that tumor load, we will be able to stratify patients and, thus, tailor their treatment. Perhaps those patients who present with circulating tumor cells will need more aggressive or neoadjuvant therapy to effectively treat the tumor. It will also be interesting to compare circulating tumor cell loads between different types of cancers to better understand why certain tumors tend to be more locally and/or regionally aggressive vs those that tend to metastasize early. Finally, we hope to use this blood test in patient follow-up care for recurrence detection, as this is a simple and minimally invasive technique.

CONCLUSIONS

The presence of circulating tumor cells in peripheral blood samples was successfully demonstrated in 8 (44%) of the 18 patients studied with HNSCC. Although the clinical implications of circulating tumor cells in micrometastatic tumor detection in patients with HNSCC are still unknown, they may be significant. Long-term follow-up may help elucidate the patients in whom conventional treatment is more likely to fail and, thus, those who may benefit from different treatment; it may also assist with the detection of recurrence with a simple blood collection.

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Figure 4. Results are presented as the percentage of patients positive for circulating tumor cells per location.