Background: The presence or absence of metastatic disease in cervical lymph nodes is the single most important determinant of therapy and prognosis for patients with head and neck squamous cell carcinoma (HNSCC). However, histologic examination fails to detect metastatic disease in a subset of neck dissection specimens. The accuracy of neck staging may be improved by the use of molecular techniques. Cytokeratins 5, 14, and 20 may be appropriate markers for HNSCC because they are expressed in HNSCC but not in lymphatic tissue.

Design: To test the sensitivity of detection of cytokeratin 5, 14, and 20 messenger RNA by quantitative reverse transcription polymerase chain reaction (RT-PCR), full-length coding DNA sequences were cloned and transcribed. The expression of cytokeratin 5, 14, and 20 messenger RNA was quantified in 4 HNSCC cell lines and 11 tumors. A cell culture lymph node model was created.

Results: As few as 32 molecules of cytokeratin 14 could be detected using quantitative RT-PCR. Cytokeratins 5 and 14 were easily detected in all 4 HNSCC cell lines and almost all tumors. Cytokeratin 20 was not a useful marker, as expression was absent or significantly reduced in cell lines and tumors. In the lymph node model, cytokeratin 14 quantitative RT-PCR was able to detect 1 cancer cell in a background of 10 million lymphatic cells.

Conclusions: Quantitative RT-PCR detection of cytokeratin 5 or 14 is a sensitive new molecular technique that may be used for detection of cervical micrometastases in head and neck cancer.
tissue. Several lines of evidence suggest that cytokeratin proteins may be ideal candidates. Epithelial cells express cytokeratins in pairs (type I [large, basic] and type II [small, acidic]) that together make up the 8-nm intermediate filament of the cytoskeleton. The cytokeratin 5 and 14 pair is expressed specifically in mucosa and tumors of the oral cavity, oropharynx, hypopharynx, and larynx as well as mitotically active basal cells of stratified squamous epithelium. Malignant transformation may decrease but does not appear to eliminate cytokeratin 5 and 14 expression. The exact distribution of cytokeratin 20 is still under active investigation. Preliminary studies, using IHC to define expression of cytokeratin 20, suggested that it is restricted to intestinal epithelium and uroepithelium. However, follow-up studies using nonquantitative reverse transcription polymerase chain reaction (RT-PCR) confirm the presence of messenger RNA (mRNA) transcript in normal oral tissue, HNSCC tumors, and blood samples of patients with oral cancer. Because the presence of cytokeratin 20 mRNA transcripts in patients’ blood correlates with the presence of oral cancer, cytokeratin 20 has been proposed as a hematogenous marker of oral cancer recurrence.

Detection of cytokeratin proteins or mRNAs by means of molecular techniques accurately predicts micrometastatic disease in patients with lung, esophageal, gastric, breast, and colorectal carcinoma. Furthermore, the presence of molecule-detectable micrometastases correlates with a decreased disease-free interval for affected patients. Cytokeratin expression, as detected by RT-PCR or IHC, has been proposed as a marker of HNSCC, and early results have been promising. In this study, cytokeratins 5, 14, and 20 were chosen as potential markers because of the known expression patterns of cytokeratins 5 and 14 and because of promising cytokeratin 20 preliminary data.

Immunohistochemistry is the most common method used to detect cytokeratin-containing HNSCC cells in lymph nodes. This technique offers the advantage of preserving cellular and tissue morphological features. However, with HNSCC metastases, pathological identification of cell type may not add any diagnostic information, and tissue morphologic features are not necessary for staging, diagnosis, or treatment decisions. In addition, IHC is time- and labor-intensive as well as expensive. Reverse transcriptase polymerase chain reaction, which is more sensitive and less expensive than IHC, may be a better diagnostic test. Reverse transcriptase polymerase chain reaction has been used successfully to detect cancer cells in lymph nodes, most successfully with malignant melanoma, allowing for the detection of 1 cancer cell in a pool of 10 million noncancer cells, by assaying for the presence of tyrosinase transcript. Furthermore, the sensitivity of RT-PCR is more than twice that of serial sectioning for detecting metastatic melanoma in lymph nodes of patients with 1-mm-thick primary melanomas. The exquisite sensitivity of RT-PCR also constitutes its greatest disadvantage. In HNSCC, nonquantitative RT-PCR may lead to false-positive results secondary to contamination of surgical specimens with skin cells or the presence of benign epithelial rests in lymph nodes (found in 1.6% of cervical nodes). With the use of quantitative RT-PCR, a threshold level of cytokeratin expression can be defined that is consistent with an empirically determined “positive” result, distinguishing patients with nodal metastases from patients without neck disease. Although detection of cytokeratin transcript by RT-PCR has been described, previous studies in HNSCC have not taken advantage of the quantitative analysis aspect of quantitative RT-PCR.

We report optimization of quantitative RT-PCR for the detection of cytokeratin mRNA in HNSCC. In addition, we determined the sensitivity of this technique for detecting HNSCC in a background of lymphocytic cells. Cytokeratin 5 and 14 expression was confirmed in HNSCC tumors from multiple subsites within the head and neck, and, with the use of our experimental conditions, we could detect as few as 32 molecules of cytokeratin 14 RNA. Adequate testing and development of this technique may alter the current methods of detecting cervical micrometastases in HNSCC.
Full-length cDNA encoding cytokeratin 20 was PCR-amplified from SKBR-3 cells (human breast cancer cell line) with the conditions described above, except for a 5% dimethylsulfoxide concentration. After isolation and purification by electrophoresis through a 1% agarose gel, the PCR product was digested with EcoRI and BamHI and ligated (T4 ligase; Roche Diagnostics Corp, Indianapolis, Ind) into digested pBlueScript KS+ (Stratagene) cloning vector. After transfection into TG-1 cells, and colony selection on LB-Amp medium (isopropyl-β-D-1-thiogalactopyranoside [IPTG] and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal]), incorporation of the correct insert was confirmed by sequencing.

**DESIGN OF PRIMERS AND PROBE FOR QUANTITATIVE RT-PCR**

Oligonucleotide primers and probes specific for cytokeratins 5, 14, and 20 quantitative RT-PCR were designed (Primer Express software; Perkin Elmer Biosystems, Boston, Mass). Primers were synthesized at the Lineberger Comprehensive Cancer Center Nucleic Acids Core Facility (University of North Carolina, Chapel Hill), and custom FAM- and TAMRA-labeled fluorescence probes were purchased (Integrated DNA Technologies, Coralville, Iowa). Primer create amplicons of less than 150 base pairs that span intron-exon borders, and probes have a melting temperature 10°C higher than that of the primers:

**Cytokeratin 5**
- Detection primers
  - CK 5-01 5'-TGGATGAGCCGATGATCCA 3'
  - CK 5-02 5'-AGGCGTTACCGCAAGAC 3'
- Probe
  - CK 5-probe 5'-FAM-TGACAATGTCAAGAAACAGTGCGCCA-TAMRA 3'

**Cytokeratin 14**
- Detection primers
  - CK 14-01 5'-TCTGGGCAGCGATGACT 3'
  - CK 14-02 5'-CCATTGATGTGCTTCATCCA 3'
- Probe
  - CK 14-probe 5'-FAM-ACTCATGCGCAGGTTCAACTCTGTCTCAT-TAMRA 3'

**Cytokeratin 20**
- Detection primers
  - CK 20-07 5'-AAAAGAGGATACAGAGGAGAAAGTC 3'
  - CK 20-08 5'-AGACGATCAACCTCATTCTAA 3'
- Probe
  - CK 20-probe 5'-FAM-ATGGCCTACACAAGCATCTGGGCAAC-TAMRA 3'

To confirm that the primers amplify cytokeratins 5, 14, and 20, total RNA was isolated from UNC-7 cells by means of an RNA isolation kit (RNeasy Midiprep; Qiagen) and reverse transcribed (10 minutes at 25°C, then 30 minutes at 48°C, then 5 minutes at 95°C) by means of random hexamer primers and reverse transcriptase (Multiscribe; Applied Biosystems Inc) (reaction condition: 200mM each dNTP, 2.5mM random hexamers, 2.5mM magnesium chloride, 0.4-U/µL RNase inhibitor, 1.25-U/µL reverse transcriptase). The resulting cDNA was then subjected to 40-cycle PCR (15 seconds at 95°C, then 1 minute at 60°C for 40 cycles in 5.5mM magnesium chloride, 200mM each dNTP, 200mM each forward and reverse primer, 1 µL of DNA template, 0.025-U/µL Taq polymerase). Amplicons of the expected size were visualized by ethidium bromide staining followed by electrophoresis through a 3.5% agarose gel.

**ISOLATION OF TISSUE CULTURE CELL AND TISSUE RNA**

The UNC-7 and UNC-10 cells were previously derived from the lymph nodes of patients with primary squamous cell carcinoma of the lip and buccal mucosa, respectively. JHU-012 and JHU-022 were isolated from primary squamous cell tumors of the head and neck (sites not specified).27

**Characteristics of Head and Neck Squamous Cell Carcinomas**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Subsite</th>
<th>Primary Tumor Stage</th>
<th>Degree of Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Epiglottis</td>
<td>T3</td>
<td>Moderate</td>
</tr>
<tr>
<td>2</td>
<td>Supraglottis</td>
<td>T2</td>
<td>Well</td>
</tr>
<tr>
<td>3</td>
<td>Floor of mouth</td>
<td>T2</td>
<td>Moderate</td>
</tr>
<tr>
<td>4</td>
<td>Floor of mouth</td>
<td>T2</td>
<td>Moderate</td>
</tr>
<tr>
<td>5</td>
<td>Larynx</td>
<td>T4</td>
<td>Moderate</td>
</tr>
<tr>
<td>6</td>
<td>Tongue</td>
<td>T2</td>
<td>Moderate</td>
</tr>
<tr>
<td>7</td>
<td>Supraglottis</td>
<td>T2</td>
<td>Moderate</td>
</tr>
<tr>
<td>8</td>
<td>Larynx</td>
<td>T4</td>
<td>Moderate</td>
</tr>
<tr>
<td>9</td>
<td>Tongue</td>
<td>T3</td>
<td>Moderate</td>
</tr>
<tr>
<td>10</td>
<td>Tonsil</td>
<td>T4</td>
<td>Moderate</td>
</tr>
<tr>
<td>11</td>
<td>Maxillary sinus</td>
<td>T4</td>
<td>Well</td>
</tr>
</tbody>
</table>

Total RNA was isolated from the cultured cells with 1 of 2 methods (RNeasy Midiprep protocol; Qiagen; or RNAqueous-4PCR kit; Ambion, Inc, Austin, Tex). The RNA was quantified by spectrophotometry and a quantitation kit was used (Ribogreen RNA; Molecular Probes, Inc, Eugene, Ore). Fifty nanograms of total RNA from the HNSCC cell lines was used for quantitative RT-PCR.

Anonymous samples of human HNSCC tumors were obtained from our institution’s tissue procurement facility after approval by the institutional review board and after informed consent was obtained from patients undergoing surgical resection. Characteristics of the tumors are listed in the Table. Samples were placed immediately into liquid nitrogen and stored in liquid nitrogen until RNA isolation. The tumors were lysed with a motorized rotor-stator homogenizer, and total RNA was isolated (RNeasy Midiprep protocol, Qiagen) and quantified by spectrophotometry followed by quantitation (Ribogreen; Molecular Probes Inc). Ten nanograms of total tumor RNA was subjected to quantitative RT-PCR analysis for cytokeratins as described below.

To determine the sensitivity of cytokeratin quantitative RT-PCR, UNC-7 cells were serially diluted in Jurkat cells, in concentrations ranging from 1:100 to 1:10 million cells (UNC-7 cells to Jurkat cells), and total RNA was isolated from the cell mixtures. Five hundred nanograms of total RNA was subjected to quantitative RT-PCR analysis for cytokeratins as described in the next section.

**QUANTITATIVE DETECTION OF CYTOKERATIN 5, 14, AND 20 EXPRESSION**

Plasmids containing cytokeratin cDNA were linearized by digestion with EcoRI endonuclease (New England Biolabs, Inc); extracted with phenol, isoamyl alcohol, and chloroform; precipitated with isopropanol; and dried. Cytokeratin 5, 14, and 20 mRNA was then transcribed by means of an in vitro cell-free lysate (TNT In Vitro Transcription; Promega Corp, Madison, Wis). Purity and concentration of resulting mRNA was determined by electrophoresis, spectrophotometry, and the quantitation kit (Ribogreen RNA; Molecular Probes Inc). This mRNA was aliquoted and stored at −80°C. The RNA was diluted fresh for each experiment and used to generate standard concentration curves.

Initial experiments were carried out with a sequence detection system (ABI Prism 5700; Applied Biosystems Inc) with 40-µL reaction mixes, and latter experiments with a more sensitive system (ABI Prism 7900; Applied Biosystems Inc) with a 20-µL reaction mix. Sample RNA was always freshly diluted from concentrated, aliquoted stocks stored at −80°C. Optimal
probe and primer concentrations were empirically determined to be 200nM for cytokeratin 14 and 20 probes, 400nM for cytokeratin 5 probe, and 300nM for each primer. Thermocycler conditions were as follows: 10 minutes at 25°C (RT activation); 30 to 60 minutes at 48°C (RT inactivation); then 1 minute at 60°C alternating with 15 seconds at 95°C for 40 cycles (PCR). The thermocycler (ABI Sequence Detector; Applied Biosystems Inc) reported, for each sample, the number of PCR cycles necessary for PCR product to be detected above background (cycles to threshold).

Total RNA samples from cell lines were submitted for a parallel assay of ribosomal RNA (rRNA), to control for RNA yield and quality, with the use of commercially available rRNA-specific probes and primers (Applied Biosystems Inc). Total RNA from UNC-7 cells was used to generate a standard curve for rRNA assays, and the total RNA of other cell lines and tissues was compared with that of UNC-7 cells; in this way, the rRNA control was not absolutely quantitative, but relatively so. Because the purpose of this control was to evaluate the quality of total RNA isolate, this relative information was sufficient. Results from rRNA assays of cell lines and tissues were very similar, except in one isolate, which was excluded on the basis of these results (not shown).

**RESULTS**

**SENSITIVITY OF CYTOKERATIN DETECTION BY QUANTITATIVE RT-PCR**

Quantitative RT-PCRs for cytokeratins 5, 14, and 20 were optimized for the concentration of probe, primer, magnesium chloride, and dNTPs (data not shown). In vitro transcribed cytokeratin 5, 14, and 20 RNA was used to determine the limits of detection of the assays. As few as 32 molecules of standard cytokeratin 14 RNA and 160 molecules of cytokeratin 20 RNA were detected, while the cytokeratin 5 assay was less sensitive, detecting as few as 4000 molecules of specific RNA (Figure 1). Plotting cycle threshold vs number of cytokeratin mRNA molecules showed that the assays were linear over at least 5 orders of magnitude.

**CYTOKERATIN 5, 14, AND 20 EXPRESSION IN HEAD AND NECK CANCER CELL LINES**

Quantitative RT-PCR amplification of cytokeratins 5, 14, and 20 RNA from HNSCC cell lines (UNC-7, UNC-10, JHU-012, and JHU-022) showed that cytokeratin 5 and 14 expression is abundant in all cell lines tested (Figure 2A). Similar amounts of cytokeratins 5 and 14 were detected in 3 of the 4 cell lines tested (UNC-7, JHU-012, and JHU-022), whereas expression of cytokeratin 5 mRNA was markedly reduced in UNC-10 relative to the other cell lines. Cytokeratin 20 was detected only in UNC-7 cells, and at such low levels of expression that it approached the assay’s detection limit (2000 to 20000 times less than cytokeratin 5 or 14). No cytokeratin 20 expression was detected in the UNC-10, JHU-012, or JHU-022 cell line.

The number of specific RNA transcripts per cell was estimated by means of the calculated molecular weights of each in vitro transcribed control RNA (Figure 2B). The UNC-7, JHU-012, and JHU-022 cells contained about 59000 to 88000 cytokeratin 5 transcripts per cell, almost 1000-fold more than UNC-10 cells, which contained only about 100 transcripts. Of interest, expression of cytokeratins 5 and 14 was roughly equivalent in

![Figure 1](https://example.com/figure1.png)

![Figure 2](https://example.com/figure2.png)
UNC-7 and UNC-10 cells, whereas in the JHU-012 and JHU-022 cells, expression of cytokeratin 5 exceeded that of cytokeratin 14 approximately 5-fold. Similar levels of cytokeratin 5 and 14 RNA in each cell line are expected, as cytokeratin 5 and 14 heterodimerize in vivo. Cytokeratin 20 levels were estimated at 7 transcripts per cell in UNC-7 and were not detected in UNC-10, JHU-012, or JHU-022 cells.

DETECTION OF CYTOKERATIN 5 AND 14 mRNA IN HUMAN TUMORS

The RNA was isolated from 11 primary human HNSCCs of various subsites and stages (Table), and cytokeratin 5, 14, and 20 mRNA was quantitated by RT-PCR (Figure 3). Cytokeratin 14 RNA was easily detected in all tumors, with a variation in expression level of 2 orders of magnitude. Cytokeratin 5 mRNA was detected in all but 1 sample, tumor 6, a T2 lesion of the tongue. In tumor 7 (T2 of the supraglottis), cytokeratin 14 expression was 100 times lower than that of cytokeratin 5, but in all other tumors, the expression levels of cytokeratins 5 and 14 were similar. Although most of the tumors displayed moderate differentiation, the expression levels of cytokeratins 5 and 14 did not vary consistently with the 2 well-differentiated tumors, 2 and 11. Cytokeratin 20 RNA was not detected in any of the tumors. Cytokeratin 20 was not found consistently in HNSCC cell lines or tumors, and the cytokeratin 5 assay was not as sensitive as cytokeratin 14 RT-PCR. Therefore, cytokeratin 14 quantitative RT-PCR was used for detection of HNSCC cells in the lymph node model system.

DETECTION OF HNSCC CELLS IN AN IN VITRO LYMPH NODE MODEL

A possible use of quantitative cytokeratin RT-PCR is more sensitive detection of micrometastasis of HNSCC within lymph nodes. Micrometastasis is defined as a nest of tumor cells 3 mm or less in diameter in a lymph node that is 1 cm or less in diameter. Therefore, the assay must specifically detect a small amount of cytokeratin RNA within a large sample of nonspecific RNA. To determine whether an abundance of lymphocyte RNA altered the detection limits of the assay, we created a lymph node model that quantitatively varies the ratio of HNSCC cells to lymphocytes.

Total RNA was isolated from Jurkat cells, a human T-cell leukemia line, and no cytokeratin 5, 14, or 20 RNA was detected by quantitative RT-PCR in this cell line (data not shown). Total RNA was then isolated from dilutions of UNC-7 cells into Jurkat cells at ratios of 1:100 to 1:10 million cells (UNC-7 cells to Jurkat cells). Five hundred nanograms of total RNA from these mixtures was subjected to quantitative cytokeratin 14 RT-PCR, and the number of cytokeratin mRNA molecules in each sample was determined. Cytokeratin 14 RNA could be reliably and repeatedly detected in the mix of 1 UNC-7 cell in 10 million Jurkat cells. On the basis of previous studies determining the number of cytokeratin 14 messages in each UNC-7 cell, we expected 29 molecules of cytokeratin 14 RNA to be present in the 1:10 million cell mixture and detected 59 molecules (Figure 4). Excellent correlation between measured and expected cytokeratin 14 detection was observed at all dilutions of UNC-7 to Jurkat cells (Figure 4). The amount of cytokeratin 14 RNA detected in the 1:10 million cell mix is equivalent to less than 1% of the cytokeratin 14 RNA found in 1 UNC-7 cell.

COMMENT

Quantitative cytokeratin RT-PCR offers many advantages as a technique for improving detection of metastatic HNSCC. It is more sensitive and faster than other molecular methods and has the potential to be performed intraoperatively. Cytokeratin levels can be quantified to determine a threshold, reducing the risk of false-positive results. The standardized technique can be taken from institution to institution and widely disseminated. In addition, quantitative RT-PCR samples an entire lymph node, in contrast to routine pathological methods, in which the limited sampling of each lymph node may be a major limitation to detecting micrometastases. It has
been calculated that a single section through a 0.5-cm lymph node, which samples only 1/1000th of the node, has only an 11% chance of detecting a 0.5-mm metastatic deposit and only a 4% chance of detecting a 0.2-mm one. However, small sampling of a node containing a micrometastasis may not be the only problem. In a study of nodal metastases in esophageal cancer, detection of tumor cells by IHC was compared with standard hematoxylin-eosin staining in adjacent sections. Despite the knowledge that an adjacent section was positive by IHC staining, metastatic cells were not identified by hematoxylin-eosin staining of sections from 67 nodes, highlighting the poor sensitivity of hematoxylin-eosin staining for small numbers of cancer cells. Cytokeratin RT-PCR not only allows whole-node sampling but is also specific for individual metastatic epithelial cells.

Micrometastases are defined as tumor deposits of less than 2 to 3 mm within a node. In a cohort of patients with cancer of the oral cavity, 21.9% were found to have micrometastases significantly predicts recurrence. With the use of IHC to detect occult metastases, 50% of patients with micrometastases died as frequently of HNSCC as patients with established metastases. Since the presence of metastatic cancer cells within the sentinel node will mandate therapy for the neck, the sentinel node must be thoroughly examined. Step sectioning and cytokeratin IHC are sensitive methods of detecting few metastatic HNSCC cells within a node, but the technology is expensive and labor intensive and takes several days for processing and pathological review. Quantitative RT-PCR is an emerging molecular technique that is relatively inexpensive and can be performed within 1 to 2 hours. Quantitative cytokeratin RT-PCR combined with sentinel node biopsy has the potential to boost both sensitivity and specificity of nodal staging, while reducing the number of neck dissections.

In the study presented, variations in cytokeratin expression between HNSCC cell lines and tumors were observed. Variations in cytokeratin expression in upper respiratory mucosa from different anatomic subsites have been reported, but our sample size is too small to conclude whether the variable cytokeratin RNA levels observed were due to anatomic site. We hope to use our method of quantitative RT-PCR to further define the anatomically based variation of cytokeratin expression in HNSCC.

Our results show that quantitative cytokeratin RT-PCR is a feasible method for sensitive detection of HNSCC using cytokeratin 14 mRNA as a molecular marker. Because cytokeratin 20 expression was undetectable in 11 of 11 primary HNSCC samples, our data do not support the use of cytokeratin 20 as a marker for HNSCC metastases. Likewise, with the use of our experimental conditions, cytokeratin 5 was not detected as sensitively as cytokeratin 14. Relative insensitivity of cytokeratin 5 quantitative RT-PCR may be due to small variations in the melting temperature of the probes and primers or decreased specific activity of the cytokeratin 5 probe. Using quantitative RT-PCR detection of cytokeratin 14 transcripts, we can detect 1 cancer cell in a background of 10 million lymphoid cells, far surpassing the sensitivity of other methods. In addition, quantitative cytokeratin RT-PCR is reproducible from assay to assay and can detect cytokeratins over 5 logs of expression (Figure 1). The ability to quantify cytokeratin expression by means of quantitative RT-PCR may decrease the possibility that a truly negative node will be labeled as containing a me-
mation in cervical lymph nodes should similarly allow de-
scription within cervical lymph nodes. In practice, an empirically es-
sentation of cytokeratin expression within the lymph node. In head and neck cancer, the use of quantitative RT-PCR to detect occult me-
metastases can be established. Indeed, in esophageal cancer, this study was presented at the annual meeting of the
American Head and Neck Society; May 4, 2003; Nashville, Tenn.

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