Clinical Evaluation of a New Molecular Method for Detection of Micrometastases in Head and Neck Squamous Cell Carcinoma

Carol G. Shores, MD, PhD; Xiaoying Yin, MD; William Funkhouser, MD; Wendell Yarbrough, MD

**Objective:** To better detect occult cervical metastases.

**Design:** RNA from 153 cervical lymph nodes was analyzed for the presence of squamous cell carcinoma using quantitative cytokeratin (CK) 14 real-time reverse transcription polymerase chain reaction (RT-PCR). Detection of CK RNA in pathologically negative nodes was further analyzed by semi-step sectioning and CK immunohistochemistry.

**Subjects:** Thirteen consecutive patients with head and neck squamous cell carcinoma (HNSCC) presenting to the Department of Otolaryngology/Head and Neck Surgery of the University of North Carolina at Chapel Hill for neck dissection.

**Results:** Cytokeratin detection was deemed nonspecific if expressed at fewer than 50 molecules of CK 14 RNA per nanogram total RNA. Of 35 HNSCCs, 33 expressed CK 14 RNA, and 15 lymph nodes with routine pathologically positive metastasis were also positive for CK 14 RNA. Four lymph nodes that were pathologically negative nodes were positive for CK 14 RT-PCR, with 2 containing metastases detected by semi-step sectioning.

**Conclusions:** Cytokeratin 14 RT-PCR is very sensitive for detecting micrometastasis in lymph nodes that are negative by routine pathological examination, with a relatively high false-positive rate. Quantitative CK 14 RT-PCR could be used to identify nodes negative for tumor by standard pathological analysis that should be examined by step sectioning and CK immunohistochemistry. Identification of micrometastases in patients with HNSCC will allow for appropriate and aggressive treatment of patients with metastatic disease.

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The presence or absence of metastatic disease in cervical lymph nodes is the single most important determinant of the therapy and prognosis for patients with head and neck squamous cell carcinoma (HNSCC). Given the import of nodal status, accurate staging of cervical lymph nodes is critical. However, routine analyses for metastases within lymph nodes from neck dissection specimens is typically limited to histological examination of 1 or only a few sections of each node stained by hematoxylin-eosin (H&E). Two lines of evidence demonstrate that routine light microscopic examination of neck dissection specimens fails to detect a portion of nodal metastases. First, regional recurrence rates of approximately 10% are reported in patients who had histopathologically negative neck dissection specimens, suggesting that metastases were present but not detected in the resected nodes. Second, retrospective studies using the more sensitive yet laborious technique of complete sectioning and cytokeratin (CK) immunohistochemistry (IHC) of the lymph node have found that 5% to 20% of patients with HNSCC have nodal metastases that are not identified by routine histological examination. Rhee et al found that 50% of patients with clinically N0 necks with negative findings by routine pathological examination had nodes that contained micrometastasis found by step sectioning and IHC.

Treatment is always indicated for clinically N-positive disease, but clinically N0 disease presents a diagnostic dilemma, as more than 30% of patients will have occult metastases. Occult metastasis is defined as a metastatic deposit undetected by clinical or radiographic examination. Occult metastases can be divided into 3 types: macrometastasis (>2-3 mm in largest dimension), micrometastases (<2-3 mm in largest dimension), and isolated tumor cells (individual or small clusters of tumor cells within the lymph node sinuses). The size and type of metastasis may influence the prognostic value of nodal metastases.
Clinical and Pathologic Staging of Neck Dissections

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Abbreviations: B neck, bilateral neck dissection; p, persistent neck disease after chemoradiation with complete response at primary site; r, recurrent disease after complete response to chemoradiation; resection 1*, primary tumor resected without treatment to the neck.

Simple observation of a neck with occult disease results in a relatively high rate of failure in the neck, frequently with ominous pathologic findings such as high-stage disease and extracapsular spread. However, the clinical significance of micrometastasis or isolated tumor cells in the neck has not been determined.

Cytokeratins are intermediate filaments that are part of the cytoskeleton of epithelial cells. Expression of these keratinlike proteins is specific for cells of epithelial origin and conserved during cell transformation or tumor development. In gastric, colon, esophageal, and lung cancer, detection of CK protein or messenger RNA (mRNA) using molecular techniques can detect micrometastatic disease in histologically negative nodes. Further, the presence of molecularly detected micrometastases correlates with a decreased disease-free interval for affected patients.

Cytokeratin 14 protein is found in epithelial cells of the bladder, breast, lung, prostate, salivary gland, and oral mucosa. It is expressed in HNSCC and can be easily detected using quantitative reverse transcription polymerase chain reaction (RT-PCR). As few as 32 molecules of CK 14 RNA can be quantitated and 1 HNSCC cell can be detected in a pool of 10 million lymphocytes.

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Step sectioning with CK IHC is the gold standard for detection of occult metastasis but is expensive and time-consuming and therefore is not used for most general applications. Quantitative RT-PCR can be used to sample the entire node or a significant portion of the node for tumor-specific RNA expression and therefore will be less likely to miss a metastasis that occupies only a small portion of the node. We present herein the use of quantitative CK 14 RT-PCR to detect occult micrometastases.

METHODS

PATIENTS

Informed consent was obtained from all patients enrolled in this study, which was approved by the institutional review board of the University of North Carolina (UNC) at Chapel Hill. The management of patients with head and neck cancer was not altered for this prospective study. Thirteen consecutive patients with HNSCC presenting to the UNC Otolaryngology/Head and Neck Surgery Clinic and scheduled for a neck dissection were asked to participate; 3 patients underwent bilateral neck dissections. Clinical and pathological staging of these patients is listed in the Table. To evaluate sensitivity and specificity, patients with both clinically N0 and N-positive necks were enrolled. RNA was isolated from primary tumors from 4 of the neck dissection patients, and an additional 30 primary tumors were collected from other patients during surgical resection for HNSCC.

NODEnes

After the neck specimen was removed, lymph nodes were immediately dissected from each level of the neck. The lymph nodes were bivalved, then “breadloaved,” and every other piece submitted for standard paraffin embedding and H&E studies by the UNC Department of Pathology. The remaining portion of each node was subjected to CK 14 RT-PCR. Samples of the primary tumors were collected when present and large enough to sample without affecting analysis of the margins, as determined by the primary surgeon.

After standard pathological analysis, paraffin blocks of nodes deemed negative for metastatic squamous cell carcinoma (SCC) but exhibiting some level of detectable CK 14 RNA by RT-PCR were exhaustively sectioned. Four 5-µm-thick slides were retained every 200 µm throughout the block. At each level, adjacent slides were examined by H&E staining and CK IHC using the mixture of commercially available mouse monoclonal antibodies anti-CK AE1/AE3 (Biomedica, Foster City, Calif). Immunocomplexes were visualized using the avidin-biotinylated peroxidase complex technique (Vector Laboratories, Burlingame, Calif), and the slides were scored for CK expression by a blinded, single reviewer (W.F.).

The remaining node fragments and primary tumor samples were immediately fixed in RNA Later (Ambion, Austin, Tex) at 4°C for 18 hours, then stored at −70°C. The entire half of the node was lysed with a motorized rotor-stator homogenizer in a volume of lysis buffer that varied with the size of the node. After homogenization, total RNA was isolated from a portion of the lysate (RNAqueous-4PCR kit; Ambion) and quantified initially by spectrophotometry, then more precisely using Ribogreen staining (Molecular Probes Inc, Eugene, Ore). Ten nanograms of total tumor RNA was submitted to quantitative RT-PCR analysis for CK 14.
QUANTITATIVE CK 14 RT-PCR

Quantitative CK 14 RT-PCR was carried out as previously described. Briefly, oligonucleotide primers and probes specific for CK 14 quantitative RT-PCR were designed using Primer Express software (Perkin Elmer Biosystems, Boston, Mass). Primers were synthesized at the Lineberger Comprehensive Cancer Center Nucleic Acids Core Facility (UNC) and custom FAM- and TAMRA-labeled fluorescence probes were purchased (Integrated DNA Technologies, Coralville, Iowa). Primers 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:

Detection primers:
CK 14-01:
5’ TCTGGCAGGATGACT
CK 14-02:
5’ CACCTTGATGTCGGCTTCCA

Probe:
CK 14-probe:
5’ FAM-ACTCATGCGCAGGTTCAACTCTGTCTCAT-TAMRA

Quantitative RT-PCR experiments were carried out using the ABI Prism 7900 system (Applied Biosystems, Foster City, Calif) using a 20-µL reaction mix with 200-nmol/L probe and 300-nmol/L primer. Thermocycler conditions were 10 minutes at 25°C (RT activation); then 1 minute at 60°C alternating with 15 seconds at 95°C for 40 cycles (PCR). The ABI Sequence Detector thermocycler reported, for each sample, the number of PCR cycles necessary for PCR product to be detected above background (cycles to threshold [Ct]). Quantitation of CK 14 RNA is expressed as molecules of CK 14 RNA per nanogram total RNA.

Full-length CK 14 complementary DNA containing plasmid was linearized by digestion with EcoRI endonuclease (New England Biolabs, Beverly, Mass); extracted with phenol, isoamyl alcohol, and chloroform; precipitated with isopropanol; and dried. Cytokeratin 14 mRNA was then transcribed using an in vitro cell-free lysate (TNT In Vitro Transcription; Promega, Madison Wis). Purity and concentration of resulting mRNA was determined by electrophoresis, spectrophotometry, and the Ribogreen RNA Quantitation Kit (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.

RESULTS

Quantitative RT-PCR was used to examine 34 primary tumors for CK 14 RNA expression (11 tumors previously reported16). Of the 34 primary tumors, 32 (94%) expressed detectable levels of CK 14 RNA. Expression in primary tumors ranged widely, from 57 to 213,400 CK 14 RNA molecules per nanograms of total RNA (Figure 1). These data suggest that quantitative CK 14 RT-PCR will be applicable to most HNSCCs.

Cytokeratin 14 is not normally expressed in lymph nodes. To verify this, 4 lymph nodes were harvested from 2 patients undergoing submandibular gland resection for chronic sialoadenitis and were found to contain no detectable CK 14 RNA. Cytokeratin 14 is expressed in normal submandibular gland tissue, which is included in a level 1 neck dissection. Six samples from 5 patients revealed levels ranging from 300 to 3800 molecules of CK 14 per nanogram of RNA.

A total of 153 lymph nodes were collected from 13 patients undergoing neck dissection for the treatment of CK 14 expressing HNSCC. Nodes were collected in the operating room, divided lengthwise, and sliced into several pieces. Alternating slices were fixed in formaldehyde for H&E staining or preserved in RNA Later (Ambion) for CK 14 RT-PCR. In 15 nodes, routine pathological examination detected metastatic SCC. All 15 nodes had detectable CK 14 RNA, ranging from 104 to 198,000 molecules of CK 14 RNA per nanograms of total RNA. There were 138 nodes that had no tumor detected by routine pathological examination, and in 90 of these nodes, no CK 14 RNA was detected (Ct >40). In the remaining 48 nodes, a level of CK 14 RNA above 0.00 molecules per nanogram of total RNA was found, ranging from 0.03 to 28,960 molecules of CK 14 RNA per nanogram of total RNA.

It is likely that the high sensitivity of RT-PCR could lead to false-positive detection of CK 14, particularly when extremely low levels are detected. We therefore sought to establish a cutoff point of CK 14 RNA detection, below which the test result would be considered negative for metastases. To determine if standard pathological examination...
tion had missed small nests of metastatic SCC, the remaining paraffin-embedded halves of the 48 nodes with detectable levels of CK 14 RNA were subjected to semi-step sectioning with subsequent H&E staining and CK IHC.

Semi-step sectioning and CK IHC revealed 4 nodes in 3 patients (2.3% of nodes) with metastatic disease undetected by routine pathological evaluation. Two nodes, with levels of CK 14 RNA of 587 and 28958 molecules CK 14 RNA per nanograms of total RNA, were found to contain CK IHC–positive cells. One node contained a metastatic deposit 3.3 mm across, and was therefore an occult metastases, not a micrometastasis (Figure 2A and B). The other node contained a 1-mm micrometastatic deposit (Figure 2C and D). Two nodes with low levels of CK 14 RNA (1.29 and 2.86 molecules of CK 14 RNA per nanogram of total RNA) had CK-positive cells detected by IHC. One node contained a few scattered CK-positive cells detected by IHC in 1 level (Figure 3A and B). The other node had a focus of CK IHC–positive cells seen on 3 consecutive sections taken 400 µm apart (Figure 3C and D). The low levels of CK 14 RNA detected by quantitative RT-PCR may be due to a geographic miss, with the micrometastasis present only in the portion of the nodes submitted for routine pathological examination and not present in the half analyzed by RT-PCR.

Using this information, an arbitrary threshold of 50 molecules of CK 14 RNA per nanogram of total RNA was established. When above this threshold, a node was considered likely to contain a metastatic focus. Sensitivity and specificity of quantitative CK 14 RT-PCR was determined by using this threshold. When compared with routine pathological examination, CK RT-PCR had a sensitivity of 1.00 and a specificity of 0.96, with 5 apparent false-positives (Figure 4). However, when the paraffin blocks of these 5 nodes were subjected to step sectioning with CK IHC, 2 were found to contain metastases that were undetected by routine pathological examination. The remaining 3 blocks had 1657, 1244, and 767 molecules of CK 14 RNA per nanogram of total RNA, but no SCC was detected in the half node submitted for step sectioning. Although these may be true false-positives, they also represent geographic misses, with a micrometastasis present only in the pieces of nodes analyzed by RT-PCR, but not present in the portion of the node examined by semi-step sectioning and CK IHC.

The presence of nodal metastasis is a critical prognosticator in HNSCC and is used to direct treatment to the...
The data presented in this report support the use of CK 14 quantitative RT-PCR to increase the sensitivity of routine pathological examination of a clinically N0 neck. This technique may be used to examine all nodes removed during a neck dissection that are negative by standard pathological examination or could be coupled with sentinel node biopsy to more accurately predict neck metastasis in the nodes most likely to drain the tumor bed.

The prognostic significance of micrometastasis in HNSCC has not been established, although it has been suggested that the presence of micrometastasis increases the risk of regional recurrence. The prognosis of isolated tumor cells vs micrometastases (<2–3 mm) vs macrometastases (>3 mm) may vary, and the difference is not consistently reported. Micrometastases occur in 3% to 7% of nodes and 9% to 22% of patients with clinically N0 neck disease. Woolgar reported in 1999 that patients with only micrometastasis had outcomes similar to patients with N0 neck disease. In this study only nodes larger than 2.4 cm that appeared tumor free on initial assessment of 1 section were then step sectioned, so micrometastatic deposits in nodes smaller than 2.4 cm would have been missed, confounding the analyses. In a later study, Woolgar et al demonstrated a trend toward higher survival rates in patients with only micrometastasis compared with larger intranodal deposits in patients with clinically N0 neck disease. Enepekides et al also failed to find a difference in regional recurrence rates or survival in patients with neck disease that was staged N0 by initial H&E staining, but CK positive after step sectioning.

**Figure 3.** Hematoxylin-eosin (A and C) and cytokeratin immunohistochemistry of adjacent sections (B and D) of nodes without cytokeratin RNA detected by quantitative reverse transcriptase polymerase chain reaction but not by routine pathological examination. Bar is 250 µm for parts A and B and 100 µm for parts C and D.

**Figure 4.** Cytokeratin 14 quantitative reverse transcription polymerase chain reaction (RT-PCR) compared with routine pathological examination alone in the detection of nodal metastasis. The cutoff point of 50 molecules of cytokeratin 14 RNA per nanogram of total RNA was used to determine a positive or negative RT-PCR test result.
ever, not all of these authors divided positive nodes by the size of metastatic deposit, and they included single CK-positive cells, which may not portend a worse prognosis compared with established colonies of tumor cells. Larger studies are needed to establish the significance of the presence of single cells or nests of tumor cells in cervical lymph nodes.

Cytokeratin deposits, defined as a CK-positive particle lacking tumor nuclei, are thought to arise from dead or dying tumor cells and can be observed in primary tumors after neoadjuvant chemotherapy.19 Similar to micrometastases, they are frequently found in the subcapsular sinus of the node. In esophageal cancers, CK deposits were not correlated with survival, while the presence of micrometastases predicted a poorer prognosis when compared with patients with N0 disease.10 The use of RT-PCR may help to distinguish RNA-free CK deposits from living cells.

Gene expression in tumor cells varies with the degree of differentiation, and it is unlikely that a single genetic marker will identify all HNSCCs. In the present study, 94% of the tumors expressed CK 14. Other possible markers include SCC antigen, which has been used in a similar study to detect nodal micrometastases.20 These markers could be combined to use with multiplex quantitative RNA analysis.

Quantitative RT-PCR is more sensitive than routine pathological examination, since the entire node can be homogenized and then a portion sampled for the presence of HNSCC-specific markers. This corrects for the sampling error introduced by examining only a few sections of a fixed node. However, homogenization of the entire node leaves no tissue for microscopic verification of the presence of metastatic disease. In this study, half the node was reserved as paraffin-embedded tissue, while half the node was examined in its entirety for CK 14 RNA. If a small metastatic deposit is present in only half of the node, it will lead to a geographic miss, with nonconcordant CK 14 RT-PCR and step sectioning with CK IHC. It is not possible to verify if this is the cause of the false-positive and false-negative nodes found in this study.

Decreasing the sampling error intrinsic in routine pathological examination should increase the detection of clinically significant metastatic disease, as demonstrated in this study by the discovery of an otherwise undetected 3.3-mm metastatic deposit. Use of this technique should therefore more accurately identify patients who will benefit from postoperative radiation therapy.

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REFERENCES