The Diagnostic Accuracy of Reverse Transcription-PCR Quantification of Cytokeratin mRNA in the Detection of Sentinel Lymph Node Invasion in Oral and Oropharyngeal Squamous Cell Carcinoma: A Comparison with Immunohistochemistry

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Abstract  
Purpose: The main goal of sentinel lymph node (SLN) detection in head and neck squamous cell carcinomas is to limit neck dissections to pN+ cases only. However, intraoperative diagnosis cannot be routinely done using the current gold standard, serial step sectioning with immunohistochemistry. Real-time quantitative reverse transcription-PCR (RT-PCR) is potentially compatible with intraoperative use, proving highly sensitive in detecting molecular markers. This study postoperatively assessed the accuracy of quantitative RT-PCR in staging patients from their SLN.

Experimental Design: A combined analysis on the same SLN by serial step sectioning with immunohistochemistry and quantitative RT-PCR targeting cytokeratins 5, 14, and 17 was done in 18 consecutive patients with oral or oropharyngeal squamous cell carcinoma and 10 control subjects.

Results: From 71 lymph nodes examined, mRNA levels (KRT) were linked to metastasis size for the three cytokeratins studied (Pearson correlation coefficient, r = 0.89, 0.73, and 0.77 for KRT 5, 14, and 17 respectively; P < 0.05). Histopathology-positive SLNs (macro- and micrometastases) showed higher mRNA values than negative SLNs for KRT 17 (P < 10⁻⁴) and KRT 14 (P < 10⁻²). KRT 5 showed nonsignificant results. KRT 17 seemed to be the most accurate marker for the diagnosis of micrometastases of a size >450 μm. Smaller micrometastases and isolated tumor cells did not provide results above the background level. Receiver operating characteristic curve analysis for KRT 17 identified a cutoff value where patient staging reached 100% specificity and sensitivity for macro- and micrometastases.

Conclusion: Quantitative RT-PCR for SLN staging in cN₀ patients with oral and oropharyngeal squamous cell carcinoma seems to be a promising approach.

The extent of lymph node involvement in clinically and computed tomography scan N₀ (cN₀) oral and oropharyngeal squamous cell carcinomas is ~30% to 40% and is one of the main prognostic factors (1). Until recently, a neck dissection was advocated routinely both to assess nodal involvement and to remove occult minimal residual cancer (2). A more recent approach consists of limiting lymph node surgery to a staging procedure by taking only the sentinel lymph nodes (SLN) which are representative of the whole neck node system (3). Such a strategy aims to permit more thorough analysis of only a few lymph nodes to enhance the sensitivity and the specificity of the diagnosis of lymph node invasion and thus select only pN+ patients on whom to perform a neck dissection. Intraoperative diagnosis must therefore have a predictive negative value close to 100%. At present, many methods of SLN analysis are used. The reference method for pathologic analysis is histopathologic examination by serial step sectioning with immunohistochemistry, staining with anticytokeratin antibodies in cases of squamous cell carcinoma (4, 5). Such analysis is able to diagnose three levels of nodal invasion (6): isolated tumor cells <0.2 mm, micrometastases ≤2 mm, and macrometastases >2 mm. However, this technique is too time-consuming to be useful in routine intraoperative diagnosis (5), where frozen section analysis is most widely used. A single H&E-frozen section analysis in experienced hands leads to a sensitivity ranging from 50% to 85% (5). Intraoperative fine-sectioned frozen section can reach a sensitivity of 93% (7) but is time-consuming, and the inevitable loss of material can lead to some micrometastases being missed (5). Imprint...
cytology has also been described as a useful technique for intraoperative staging (8). However, the results reported for imprint cytology and frozen section are partly due to a high degree of attention and skill being given by pathologists during clinical studies; such results cannot usually be achieved during routine work (5). At present, the accuracy of the intraoperative diagnosis of neck involvement is the missing link in the SLN sampling strategy. An alternative to morphologic analysis is a molecular biology approach using reverse transcription-PCR (RT-PCR), which has the advantage of not being operator dependent and, with some adaptation, can be automated (9–11). This technique seems to be a valuable tool in the detection of lymph node invasion for other cancers such as breast adenocarcinomas (12) and skin melanomas (13). Recent publications have reported the usefulness of quantitative RT-PCR in the detection of lymph node invasion in head and neck squamous cell carcinomas using cytokeratin mRNA (10, 14–16). This technique seems to be a valuable tool in the detection of lymph node invasion for other cancers such as breast adenocarcinomas (12) and skin melanomas (13).

### Materials and Methods

#### Patients

Between March 2003 and December 2004, every patient seen at the Head and Neck department of Montpellier Teaching Hospital for an untreated oral or oropharyngeal squamous cell carcinoma (cN0M0) was asked for consent to be involved in the study. The study received ethical approval from the clinical research board of the hospital. Eighteen consecutive patients were included and none refused to participate. The clinical, demographic, and histopathologic characteristics of the population are reported in Table 1.

#### Inclusion criteria

Inclusion criteria included men or women, ≥18 years old, without an upper age limit; patients suffering from primary squamous cell carcinoma of the oral cavity or of the oropharynx proven by recent biopsy; tumor site accessible for a peritumoral injection; operable tumor with regard to tumor-node-metastasis stage and its position; no history of previous treatment for any cancer of the upper aerodigestive tract; pretherapeutic N0 stage [i.e., (a) no palpable lymph nodes, (b) computed tomography scan with iodine injection: non-SLN were analyzed using a standard H&E technique. Histopathologic examination of the SLN by serial step sectioning and immunohistochemistry according to the classification of Hermanek (6). Non-SLN were analyzed using a standard H&E technique. Histopathologic examination of the SLN by serial step sectioning and immunohistochemistry according to the classification of Hermanek (6). Non-SLN were analyzed using a standard H&E technique.

#### Final pN stage

The presence of isolated tumor cells was not taken into account in determining the final pN stage.

### Table 1. Main demographic, clinical and pathologic characteristics of the population

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<tr>
<th>Patient no.</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Tumor location</th>
<th>cTNM</th>
<th>No. SLN</th>
<th>SLN location</th>
<th>Histopathology of SLN</th>
<th>Bilateral neck dissection</th>
<th>No. non-SLN (neck dissection)</th>
<th>Histopathology of non-SLN</th>
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<tr>
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<td>4</td>
<td>II, III</td>
<td>MI</td>
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</tr>
<tr>
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<td>34</td>
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<td>MI</td>
<td>No</td>
<td>18</td>
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<td>pN0</td>
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</table>

m = 52.8, SR = 0.77, T = 66, T = 622

Abbreviations: FOM, floor of mouth; OT, oral tongue; OP, oropharynx; ITC, isolated tumor cells; MI, micrometastases; MA, macrometastases; SR, sex ratio; m, mean; T, total; cTNM, clinical tumor-node-metastasis stage.

* Pathologic examination of the SLN by serial step sectioning and immunohistochemistry according to the classification of Hermanek (6).

† Non-SLN were analyzed using a standard H&E technique.

‡ pN stage was established according to the SLN and non-SLN status. The presence of isolated tumor cells was not taken into account in determining the final pN stage.
no sign of lymph node invasion—size <1 cm (1.5 cm for level IIA), ovoid, homogeneous, without iodine enhancement, and without any sign of perinodal invasion (high density of fat, vascular invasion, etc.) and the absence of lymph node groups (>3)); panendoscopic examination excluding another synchronous tumor and confirming the T stage.

**Exclusion criteria.** Exclusion criteria included one missing inclusion criterion; another tumor present; and contraindications to lymphoscintigraphy (e.g., allergy to Technetium-99 and pregnancy). Study exit criteria were failure in one of the phases of the protocol (i.e., Technetium injection, lymphoscintigraphy, surgery, or any technical or methodologic error reported by one of the investigators).

**Control population.** During a 1-month period, 10 consecutive patients planned for surgery for removal of a thyroid goiter were asked for consent to undergo a selective lymph node biopsy during the surgical procedure to constitute a control (noncancerous) population.

**SLN detection.** A previous preliminary protocol, evaluated in 20 patients (17), was adapted and modified as follows: isotopic lymphography was done after a peritumoral injection of colloid sulfur 99-Technetium (particle size ranging from 20 to 80 nm; Nanocis, CIS BIO International, France). The transoral injection of radiotracer was done on the operative day, after local anesthesia with 1% xylocaine infiltration, by a single otolaryngologist (R.G.). Three to five points of submucosal injection were used, surrounding the tumor area. The total dose was 33 MBq in 0.9 mL. Patients were asked to use a mouthwash to avoid contamination of the oral cavity and the oropharynx. Lymphatic mapping was achieved by static acquisition through both anterior and lateral views with patients placed in the Rose position. The images were taken with a Picker AXIS Gamma camera (variable-angle dual-head camera with a matrix of acquisition of 256 × 256; Cleveland, OH) 45 minutes after the injection for a period of 240 seconds. When hotspots were not seen, a subsequent acquisition was done (120 minutes after the injection) and additional oblique views were done. Hotspots were located externally with a mark on the skin. The method described was consistent with the recommendations of the first consensus conference on SLNs of head and neck squamous cell carcinoma (18). Surgery was done 3 to 6 hours after the lymphoscintigraphy. In 14 of 18 cases, the primary tumor was removed before the neck surgery. The primary tumor site was shielded by a malleable sterilized lead plate to reduce its radioactive signal. Elective SLN dissection (avoiding capsular disruption) was done through a short incision in the usual line used for a classic neck dissection. Detection was then done by a portable gamma probe (X-PROBE Clerad-ARIES France). After excision, the gamma probe confirmed ex vivo the radioactivity of the SLN and the absence of radioactivity in the neck. A modified radical neck dissection was then done at levels I, II, III, and IV depending on the tumor location. The neck dissection specimen and the tumor resection were analyzed by standard histopathologic techniques.

**Results of SLN detection.** One or more hotspots were located at lymphoscintigraphy from the 18 patients included (mean, 3; SD, 1.49) except in one case. Overall, 66 SLNs were collected. The average number of SLNs per patient was 3.67 (range, 0-7) and the average number of SLNs per neck side was 2.35 (28 neck dissections). This average was greater than the average number of hotspots because some hotspots involved more than one SLN. In one case (T1 of the anterior floor of mouth), a SLN could not be located either with preoperative lymphoscintigraphy or with perioperative gamma probe detection. This case was excluded from the study.

**Reference test: histopathologic analysis.** Because there is a risk of degradation of RNA with formol fixation and paraffin embedding, the SLNs were frozen. After splitting and printing, each half node was embedded in optimum cutting temperature compound, snap-frozen in precooled isopentane, and stored at −80°C. Each lymph node was sampled in its entirety. Two 5-μm-thick frozen slices were taken every 250 μm in RNase-free conditions (one slide for H&E stain and another for immunohistochemistry). For example, a 1-cm node would be sectioned 2 × 40 times. The rest of the lymph node tissue was blended and sent for quantitative RT-PCR analysis. Generally, the histopathologic examination used pancytokeratin antibodies (AE1/AE3). In the present study, the assumption was made that a three-marker analysis would be as accurate as a pancytokeratin analysis. For this purpose, we had to choose the proteins expressed strongly and most specifically in head and neck squamous cell carcinoma. The cytokeratins (CK) 5 and 14 were chosen because first, the basal layer of the oral stratified squamous epithelium mainly expresses these two CKs; second, because they are usually lymphocentric in squamous cell carcinoma (19); and third, recent research has reported the use of CK 5 and 14 in quantitative RT-PCR analysis with interesting results in head and neck squamous cell carcinoma (15, 16). CK 17 was chosen because it is typically expressed on the basal cells of complex epithelia and in hyperproliferative squamous epithelia and its usefulness in the histopathologic diagnosis of squamous cell carcinoma is well documented (20, 21). Finally, none of these CKs is normally expressed in the lymph node vessels (22).

Immunohistochemistry used primary antibodies anticytokeratin 5, 14, and 17 (Novocastra Laboratories Ltd., Newcastle, United Kingdom). The immunohistochemistry technique was done using the Ventana 3,3'-diaminobenzidine detection kit (streptavidin-biotin-peroxidase) with the NEXES Ventana Medical Systems automate (Ilkirch, France). All sections were carefully examined by a single pathologist (V.C.). Results were classified according to the Hermanek classification: isolated tumor cell, micrometastasis, and macrometastasis (6). The 10 control lymph nodes were sectioned following the same procedure.

Results: Nine of 66 SLNs were positive for tumor (i.e., containing at least one micrometastasis (n = 6) or macrometastasis (n = 3). The number of metastases per SLN was one in six cases, two in two cases, and three in one case. Patients were staged by taking into account the maximum size of metastasis found in their SLN. Seven patients (41.18%) were therefore staged SLN+ due to the presence of a micrometastasis (n = 4) or a macrometastasis (n = 3) in at least one of their SLNs. None of the invaded SLNs showed extracapsular spread. Five other patients were staged as having isolated tumor cells only (29.41%). For patient staging, isolated tumor cells without micro- metastases or macrometastases were not considered pathologic given that the clinical relevance of such a status is unknown. No pathologic tissue was found in the 10 control nodes from patients without cancer. From the 622 non-SLNs obtained following systematic neck dissection, formal histopathology found two additional nodal metastases in patients 7 and 10. These patients also had comparable metastases diagnosed in their SLNs.

**Real-time RT-PCR.** Lymph node tissue remaining after tissue section for immunohistochemical analysis was used for real-time RT-PCR quantification of CK 5, 14, and 17 mRNA. For this, total RNA was extracted from frozen tissues using the RNeasy midi kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. RNA was quantified using spectrophotometry and RNA quality was checked by visualizing 185 and 285 RNA on an agarose gel after ethidium bromide staining. Reverse transcription was done using 1.5 μg of total RNA from patient samples or calibrator, random hexamers, and the Omniscript kit from Qiagen. Samples were incubated for 10 minutes at 65°C, cooled on ice for 5 minutes, and incubated with reverse transcriptase for 1 hour at 37°C. Reverse transcriptase was then inactivated by heating at 95°C for 5 minutes. The resulting cDNA was aliquoted and stored at −20°C until further use.

Real-time RT-PCR was done using a SYBR Green approach (Light Cycler Fast DNA Master™ SYBR green kit) and Light Cycler technology (Roche, Mannheim, Germany). Primers for mRNA CK 5, 14, and 17 (Table 2) were selected using the Lasergene Primer Select 5.07 software (DNASTAR, Inc., Madison, WI). To avoid false detection of genomic DNA amplification was done on spliced regions of the gene. Primers for hypoxanthine phosphoribosyltransferase (HPRT), chosen as a housekeeping gene, have been described elsewhere (23). Two
Table 2. Primers and PCR conditions for CK 5, 14, and 17

<table>
<thead>
<tr>
<th>Primers</th>
<th>Gene accession no.</th>
<th>Annealing temperature (°C)</th>
<th>PCR size product (nt)</th>
</tr>
</thead>
<tbody>
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<td>NM-000424</td>
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<tr>
<td></td>
<td>1895 R, 5'-CCGGGGAGAGGTGGTGAGAC-3</td>
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<td>NM-000526</td>
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<tr>
<td></td>
<td>1334 R, 5'-AGAAGCTGGAGAGGAAGTG-3'</td>
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<td></td>
<td>702 R, 5'-ATCTCCTCCTGTTGTCTCTTC-3'</td>
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</table>

nontemplate controls were included in each amplification run and all measurements were done in duplicate under blind conditions by a single technician (M.D.). For real-time PCR optimization, the specificity of all PCR products was determined by the presence of a single band of the expected size after migration on agarose gel, and PCR efficiency was assessed for each run by generating a calibration curve giving reproducible slopes of −3.9, −3.4, −3.5, and −3.6 for CK 5, 14, 17, and HPRT, respectively. For each variable, the intra-assay coefficient of variation of Ct values was <2% and the inter-assay coefficient of variation was <5% as calculated by the SE. Linearity for each target gene and housekeeping gene was >99%. mRNA levels for the 3 CKs (KRT 5, 14, and 17) in lymph nodes were determined using a calibration curve constructed with serial dilutions of a cDNA obtained after reverse transcription of total RNA extracted from a metastatic lymph node and expressed relative to the HPRT housekeeping gene. These results were then normalized to a control lymph node sample (calibrator) treated under the same conditions as the patient samples and included in each PCR run and finally expressed as arbitrary units. The approximate mean duration of the quantitative RT-PCR procedure was 180 minutes.

From the 66 SLNs harvested, 61 SLNs were analyzed by quantitative RT-PCR. The 10 control lymph nodes underwent the same procedure. The mean HPRT Ct value obtained using RNA extracted from the 61 SLN was 22.8 ± 2.1 (mean ± SD), indicating good quality RNA samples. Two SLNs were lost because of inadvertent formalin fixation following surgery (in patient 15). RNA extraction failed in three additional SLNs (4.7%), giving RNA of poor quality as judged by the high Ct level of the HPRT gene (>32) in patients 16 and 18. All of these five SLNs were free of disease by serial step sectioning with immunohistochemistry. Because other SLNs were available for these patients, none was excluded.

Data collection. Data from the histopathologic analysis and from the quantitative RT-PCR analysis were prospectively collected in an Excel file (Microsoft Corporation, Redmond, WA). The only information available to the investigators was the number of the patient and the number of each SLN. Readers of the index test and the reference standard were blinded to the results of the other test.

Statistical method. The aim of the data analysis was first to assess the relationship between quantitative RT-PCR results and SLN invasion in comparison with immunohistochemistry and, second, to test the accuracy of quantitative RT-PCR in patient staging. Statistical analysis was done using the software SAS, V 8.2 (SAS Institute, Inc., Cary, NC).

Assessment of the relationship between the quantitative RT-PCR results and SLN invasion was achieved as follows. First, SLNs containing macrometastases and micrometastases were compared with other groups (i.e., isolated tumor cells, negative SLNs, and control nodes) using the Kruskal-Wallis test because nonparametric data distribution occurred. In case of a significant difference, the four groups were compared with one another, applying the Bonferroni’s correction to the multiple Mann-Whitney tests. The threshold for significance for each of these four tests was then 5%/4 = 1.25%. Second, quantitative RT-PCR values (KRT 5, 14, and 17) obtained for each SLN were correlated to the size of micrometastases and macrometastases as well as to the number of isolated tumor cells, calculating the Pearson correlation coefficient (r).

The accuracy of patient staging was assessed by forming three patient groups according to the histopathologic diagnosis of their SLN invasion: patients with at least one invaded SLN (i.e., macrometastasis or micrometastasis), “SLN+ patients”; patients without any SLN invasion, “SLN− patients”; and “control patients.” For the staging of patients with quantitative RT-PCR, the maximum value of mRNA expressed as log10 measured in all of their SLNs was taken into account. These values were compared among the groups using the Kruskal-Wallis test for each KRT factor. Thereafter, a receiver operating characteristic curve analysis was done between SLN+ and SLN− patients: areas under the curve (AUC) were computed with their 95% confidence intervals (95% CI; ref. 24) and then compared (25, 26). AUCs are known to represent the continuous test accuracy in the patient’s classification into negative or diseased subjects, without the threshold actually chosen. A comparison of AUCs might then show the best continuous test. Acturally, a cutoff value was chosen to reach a 100% negative predictive value and a 100% sensitivity. Specificity and positive predictive values were computed with a 95% CI.

Results

SLN analysis. Lymph nodes were grouped according to serial step sectioning with immunohistochemistry: control (n = 10), negative (n = 40), isolated tumor cell (n = 12), micrometastasis (n = 6), and macrometastasis (n = 3). The positive SLN group was made up of macrometastasis and micrometastasis. Interestingly, no difference was found in mRNA levels measured with real-time RT-PCR (KRT) between control and negative groups for the three CKs tested (P = 0.84, P = 0.21, and P = 0.68 for KRT 17, KRT 14, and KRT 5; Fig. 1A-C). The positive SLN group (macrometastasis + micrometastasis) showed higher KRT 17 values compared with the control node group (P < 10−4) and the negative node group (P < 10−6; Fig. 1A). Similar results were obtained for KRT 14 with a significant difference between the macrometastasis and micrometastasis groups and the negative node group (P < 10−2; Fig. 1B) but the difference was not significant between the positive SLN and isolated tumor cell groups (P = 0.049). KRT 5 was the least useful because no significant difference was observed between groups (P = 0.26; Fig. 1C). None of the KRTs allowed the isolated tumor cell node group to be distinguished from control or negative nodes (P > 0.03). With regard to individual KRT values, all SLNs with macrometastases (n = 3) showed higher values for the three KRTs than the maximum value obtained for isolated tumor cell nodes, control, and negative nodes. Four of six SLNs with micrometastasis showed a higher KRT 17 value than that of negative or control nodes.
The two SLN with values comparable to the negative and control nodes had micrometastases sized <450 μm. Examples of invaded SLNs are shown in Fig. 2 with corresponding KRT 17 values. With regard to KRT 14, only two of six SLNs with micrometastasis showed a higher value than that of negative or control nodes. The four lowest KRT 14 values were obtained for micrometastases sized <700 μm.

Expression levels and metastasis size were significantly linked for each CK with the Pearson correlation coefficient, $r = 0.89$, 0.73, and 0.77 for KRT 5, 14, and 17, respectively, with $P < 0.05$ (Fig. 3); however, this was not the case for isolated tumor cell ($r < 0.1$).

**Patient staging.** Patients with serial step sectioning with immunohistochemistry were staged and formed into three groups: control patients ($n = 10$), negative neck patients (SLN−, $n = 10$), and positive neck patients (SLN+, $n = 7$). The maximal quantitative RT-PCR value showed significant differences between groups with $P < 0.012$ for KRT 5 and $P < 10^{-3}$ for KRT 14 and 17. Receiver operating characteristic curve analyses showed an AUC of 87.1% (95% CI, 68.1-100%) for KRT 5, 82.8% (95% CI, 61.3-100%) for KRT 14, and 100.0% for KRT 17 (Fig. 4). KRT 17 seemed to be the best marker (nonsignificant difference between AUC, $P > 0.1$). Receiver operating characteristic curve analysis of KRT 17 allowed determination of a cutoff value (2.3075) at which the specificity (95% CI, 69.2-100%) and the sensitivity (95% CI, 59.0-100%) were 100%. This cutoff allowed an accurate classification of the nodal status of patients with quantitative RT-PCR, as well as with serial step sectioning with immunohistochemistry (Fig. 5). For a SLN invasion prevalence of 41.18%, the positive predictive value was 100% (95% CI, 59.0-100%) and the negative predictive value was 100% (95% CI, 69.1-100%).

**Discussion**

This study supports the potential clinical value of quantitative RT-PCR for SLN staging in head and neck squamous cell carcinoma. With a 100% accuracy in the staging of neck disease from the SLN, CK 17 seems to be a useful marker. These preliminary results are encouraging and it is hoped that they will now be validated in a larger multicenter study.

In this study, the SLN detection rate of 94%, the average of 2.35 SLN per neck, and the prevalence of nodal invasion (41.18%) with macrometastases and micrometastases were consistent with the literature (27), suggesting that the population studied here was representative. The sampling methods used, which allow the same tissue to be sent for histopathology and for quantitative RT-PCR, were dramatically different from those previously reported (10, 11, 15, 16, 28) where lymph nodes were bisected. Because a nodal metastasis is generally solitary (refs. 29, 30; 6 of 9 in the present study), sampling of the entire SLN is required for diagnostic accuracy. The quantitative RT-PCR method used here showed good reproducibility as all lymph node samples were analyzed in duplicate in the quantitative RT-PCR runs with an intra-assay coefficient of variation <2%. In addition, KRT results were no different when comparing control and negative nodes.

Quantitative RT-PCR is known to be able to detect and quantify levels of expression of CKs or other markers in primary head and neck squamous cell carcinoma (11, 15). We have observed that CK mRNA levels correlate well with metastasis

![Fig. 1. RT-PCR quantification of CK mRNA (KRT) for each group of SLNs and control nodes as determined by serial step sectioning with immunohistochemistry.](image)
size, underlining the quantitative value of quantitative RT-PCR analysis. However, with SLN analysis, the clinical relevance of quantitative RT-PCR is dependent on its ability to detect small amounts of neoplastic tissue when diluted within a large amount of normal lymphatic tissue. Although the CKs tested were specific for head and neck squamous cell carcinoma, we did detect an underlying background level. This probably arises from infinitesimal natural CK expression in healthy lymphatic tissue, mainly from dendritic cells (30), leading to an overlap in KRT values between negative and positive nodes especially for KRT 14 (16). As a consequence, the discriminatory power of quantitative RT-PCR is dependent on the ratio between CK expression levels in normal and pathologic tissues. This varies depending both on the marker studied and on the quantity of neoplastic tissue in a SLN. Ferris et al. (11) screened 40 potential markers using primary tumor and gross metastatic deposits compared with benign nodes. An accuracy of 100% was obtained with pemphigus vulgaris antigen in detecting neoplastic tissue. Thereafter, a rapid quantitative RT-PCR method was tested on seven pathologic lymph nodes, including two with micrometastases of unreported size. However, this screening study was not designed to assess diagnostic accuracy in the detection of minute metastatic deposits in either lymph nodes or SLN. Furthermore, the lymphatic tissue was not sampled in its entirety and CK 17 was not assessed. A matched

Fig. 2. CK immunohistochemistry of SLN sections showing metastatic deposits. A, macrometastase (×10); B, two contiguous 420- and 650-µm micrometastases (×100); C, 260-µm micrometastase located near the subcapsular sinus (×200); D, isolated tumor cells in the subcapsular sinus (×400).

Fig. 3. The expression level of the CK as a function of the size of the SLN metastasis. Pearson correlation coefficient (r) shows a good correlation for the three KRT.

Fig. 4. Receiver operating characteristic curves for each KRT. The AUC was 100% for KRT 17 (nonsignificant differences between AUCs, P > 0.05).
Fig. 5. Patient staging: maximal expression level of CK 17 for each patient as a function of serial step sectioning with immunohistochemistry. The receiver operating characteristic curve analysis exhibits a cutoff value of 2.3075 achieving 100% sensitivity and specificity.

analysis using pemphigus vulgaris antigen and CK 17 would clearly be worth further investigation.

Although molecular markers are specific, false-positive results can occur in cases of cross-reactivity with normal epithelial tissue such as salivary gland (10), which naturally expresses CKs. Such cross-reactivity was observed in a preliminary study7 with expression levels comparable to those obtained for macrometastases. To avoid this problem, careful dissection of the lymph node must be done to remove any contaminating nonlymphatic tissue. There was no case here where quantitative RT-PCR was positive and histopathology was negative, as has been reported for melanoma (28). In our opinion, the reason for this finding in melanoma was that the histopathologic examination did not involve complete node sampling and serial sectioning. This probably resulted in inaccurate reporting of false-negative histopathology.

The method used in this study was not effective for isolated tumor cell detection as CK expression levels were no different between SLNs with isolated tumor cells and negative and control nodes, and the number of isolated tumor cells was not correlated with KRT levels. However, this study was not designed to detect such circulating tumor cells. For such detection, not only would a molecular marker with better discrimination be required but also thinner serial sectioning, permitting an accurate count being made of single tumor cells.

With regard to accurate neck staging, the determination of a cutoff value for KRT 17 leading to 100% sensitivity and specificity is very encouraging. In our staging approach, the maximal value of KRT for each patient was compared with the histopathologic SLN status. KRT 17 failed to diagnose two minute micrometastases sized <450 μm in two patients but this inaccuracy was negated by the presence of larger metastases in another SLN being quantitative RT-PCR positive in the same two patients. Therefore, all patients were correctly staged following complete analysis. However, the presence of a single micrometastasis sized <450 μm in only one SLN is possible (although not found in this study) and, therefore, formal postoperative histopathologic analysis is mandatory to avoid false-negative staging.

These results now need to be corroborated by future larger studies. A more powerful study may find a significant difference in areas under receiver operating characteristic curves, confirming the superiority of KRT 17, and could reduce the interval for the choice of the cutoff value for log (KRT 17), which was quite large [i.e., 2.1271 (maximum value of SLN− patients) and 2.3075 (minimum value of SLN+ patients)]. To run a multicenter study, standardization of technique and result reporting would be required. Assessment of the diagnostic accuracy of a rapid intraoperative quantitative RT-PCR technique (9–11) will be required before advocating it for intraoperative staging. Ultimately, a study of the prognostic significance of the quantitative RT-PCR results will be required.

Finally, it must be stressed that histopathologic examination at present remains irreplaceable in clinical practice, allowing the detection of negative nodes during surgery (by frozen section) as well as allowing us to validate the result of the quantitative RT-PCR analysis and to establish the definitive pN stage. Therefore, if quantitative RT-PCR analysis of SLNs is to be used in clinical practice, it should be included in a multi-modality diagnostic protocol similar to the one reported here.

By achieving a good predictive negative value, quantitative RT-PCR may help the surgeon to select patients with oral or oropharyngeal carcinomas that definitely require neck dissection. Such a high level of accuracy is mandatory for wider acceptance of the SLN sampling strategy in cN0 head and neck cancer.

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The Diagnostic Accuracy of Reverse Transcription-PCR Quantification of Cytokeratin mRNA in the Detection of Sentinel Lymph Node Invasion in Oral and Oropharyngeal Squamous Cell Carcinoma: A Comparison with Immunohistochemistry

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