

# Quantitative Molecular Detection of Minimal Residual Head and Neck Cancer in Lymph Node Aspirates<sup>1</sup>

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## ABSTRACT

**Purpose:** Staging of the clinically N<sub>0</sub> neck in patients with head and neck squamous cell carcinoma (HNSCC) using ultrasound-guided, fine needle aspiration cytology (USgFNAC) has a false-negative rate of ~20% that might be caused by inaccurate cytology. Molecular analysis of aspirate residues might reduce the false-negative rate, and we therefore set up a quantitative reverse transcription-PCR (Q-RT-PCR) assay based on TaqMan technology using the squamous cell-specific antigen E48 (Ly-6D) as molecular marker.

**Experimental design:** The detection limit of the assay was determined in reconstruction experiments. The sensitivity of the assay was tested on cytological tumor-positive aspirate residues and the specificity on lymph node aspirate residues of noncancer controls. Subsequently, 235 lymph node aspirate residues of 64 HNSCC patients staged with USgFNAC were examined for the presence of E48 mRNA. E48 Q-RT-PCR results of the aspirated lymph nodes were compared with cytology and clinical outcome.

**Results:** The detection limit of E48 Q-RT-PCR was a single tumor cell in a background of 10<sup>6</sup> peripheral blood mononuclear cells. From the 41 aspirates that were not evaluable at cytology, 24 (59%) could be diagnosed with E48 Q-RT-PCR. In the 191 aspirates that were tumor negative or not evaluable at cytology, 8 samples from 6 patients were E48 positive. These results were confirmed by histology or clinical outcome in 3 of 6 patients. E48 Q-RT-PCR showed an increase in sensitivity from 56 to 67% and an increase in

frequency of reached diagnosis from 97 to 100% compared with cytology. The specificity decreased from 100 to 92%.

**Conclusions:** Real-time E48 Q-RT-PCR is an accurate technique for squamous cell detection in lymph node aspirates of HNSCC patients. The assay shows an increase in sensitivity and frequency of reached diagnosis compared with cytology. The test could be implemented routinely in USgFNAC to diagnose cases for which cytological examination is not conclusive.

## INTRODUCTION

SCC<sup>3</sup> represents 90% of the head and neck cancers and has a worldwide incidence of ~500,000 cases/year (1). The presence of lymph node metastases in the neck is the most important prognostic factor and determines clinical management of HNSCC patients (2). Therefore, accurate assessment of the regional lymph nodes for metastases is of crucial importance. Because sensitivity and specificity of palpation are limited (~60–70%; Ref. 3), a neck without palpable lymph nodes (N<sub>0</sub>) is at risk of harboring occult metastases.

To improve staging accuracy of the clinically N<sub>0</sub> neck, USgFNAC has gained popularity. The neck is examined by US, and suspicious (enlarged) lymph nodes are aspirated under US guidance and cytologically examined (4). Since 1992, we gradually changed our policy toward treatment of the neck in patients with T1–2 SCC of the oral cavity or oropharynx based on USgFNAC findings. Instead of routine elective neck dissection, we switched to a conservative wait-and-see policy. When USgFNAC of the neck is negative, suitable tumors are treated by transoral excision, and a wait-and-see policy for the neck is adopted, including USgFNAC at regular intervals. A recent evaluation of this policy at our department showed that ~20% of patients with initial negative USgFNAC findings developed lymph node metastases during follow-up, of whom 79% could be salvaged by delayed therapeutic neck dissection and postoperative radiotherapy (5).

Despite these encouraging clinical results, an additional decrease of the false-negative rate is desirable. Possible causes of false-negative USgFNAC cases could be: (a) tumors containing lymph nodes do not meet US size criteria and are not aspirated, while all enlarged and aspirated nodes are tumor free; (b) aspiration is performed in a tumor-free part of a lymph node harboring a metastasis (sampling error); or (c) the aspirate was not evaluable by routine cytology, or few aspirated tumor cells

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<sup>3</sup> The abbreviations used are: SCC, squamous cell carcinoma; US, ultrasound; HNSCC, head and neck squamous cell carcinoma; USgFNAC, ultrasound-guided, fine needle aspiration cytology; PBMC, peripheral blood mononuclear cell; PBGD, porphobilinogen deaminase; dNTP, deoxynucleotide triphosphate; RT-PCR, reverse transcription-PCR; Q-RT-PCR, quantitative reverse transcription-PCR; C<sub>T</sub>, threshold cycle.

in the smear were missed. A previous study of our group showed that is not the case, as functional identification of the lymph node at highest risk to harbor a metastasis (the sentinel node) could not decrease the false-negative USgFNAC rate (5). These data indicated that the false-negative rate should be explained by sampling error or inappropriate cytological screening.

The routine cytological screening does meet limitations as 20% of the smears are not evaluable. This is in part caused by incorrect preparation of the smears that hampers morphological examination. Second, when low numbers of squamous cells in a smear are present, it is sometimes difficult to discriminate between a lymph node metastasis or a benign cyst. Finally, sometimes only few lymphoid cells are aspirated, and the diagnosis is considered inconclusive. Molecular analysis of residual aspirated material might overcome most of these problems, resulting in an improvement of routine cytology and a decrease of the false-negative USgFNAC rate.

Recent developments in molecular diagnosis enable detection of low numbers of cancer cells that are missed at routine histology (6–9). At our laboratory, we are focusing on the exploitation of SCC-associated antigen E48 (Ly-6D) as a potential marker for the detection of SCC. This antigen is specifically expressed in squamous cells of both normal, malignant, and transitional epithelia (10). In a previous study, we demonstrated that E48 transcripts can serve as a highly sensitive and specific molecular marker for squamous cell detection in blood and bone marrow (11). However, the suitability of this qualitative assay for molecular detection of residual tumor cells in lymph node aspirates meets limitations: (a) when we evaluated E48 RT-PCR on intact lymph nodes from noncancer controls, we noticed a slightly positive signal, suggesting that lymphatic tissues sometimes express E48 at a low level in contrast to blood and bone marrow (7); and (b) cytological screening of aspirates does not only assess the presence of squamous cells but also gives an indication on the number of cells aspirated and the quality of the preparation. Aspirates that contain insufficient material or show a poor quality are considered as nondiagnostic, and these patients are therefore not staged properly. On the basis of these considerations, we decided to set up a quantitative real-time RT-PCR assay using E48 as a squamous cell-specific molecular marker and the gene encoding PBGD as a marker for the number of cells in the aspirate. Recently, a technique has been described for real-time detection and precise quantification of mRNA expression levels in minute numbers of cells, using a dual-labeled fluorogenic probe (12, 13).

Here, we report a quantitative E48 RT-PCR assay for the detection of micrometastases in lymph node aspirates of HNSCC patients, and we determined the additional value of this approach for cytological diagnosis.

## PATIENTS AND METHODS

**Cell Lines and Tissue Samples.** The human HNSCC cell lines UM-SCC-22A and UM-SCC-22B used for reconstruction experiments were grown in DMEM as indicated previously (14). The confluence of the cultures before harvest was always 50–70%.

In total, 235 lymph nodes aspirates were obtained from 64 previously untreated patients with T1-T4 histopathologically

proven SCC of the oral cavity or oropharynx and a clinically negative neck at palpation ( $N_0$ ). US examinations of levels 1–5 of both sides of the neck were performed, and aspirates were taken from enlarged lymph nodes (>3 mm in level I and >4 mm in other levels). After preparation of two cytological smears per aspiration, the needle was washed in PBS to obtain aspirate residues for quantitative E48 RT-PCR. Of these 64 patients, 40 patients were planned for transoral tumor excision only and a wait-and-see policy for the neck. The remaining 24 patients were scheduled for combined tumor excision and elective neck dissection based on T-stage and/or to get adequate access to the tumor.

Peripheral blood samples used for reconstruction experiments were obtained from healthy volunteers by venipuncture and collected in  $2 \times 7$  ml heparin vacutubes. PBMCs were prepared by Ficoll Hypaque density centrifugation according to the supplier. From ten noncancer patients, residues of ten aspirated lymph nodes in the neck were obtained for use as negative control. These patients, not suspected for cancer, presented with enlarged lymph nodes that after subsequent aspiration and cytology appeared to be reactive. All studies involving human subjects were approved by the Institutional Review Board, and informed consent was obtained from all subjects.

**Preparation and RNA Isolation of Lymph Node Aspirate Residues.** Residues of lymph node aspirates were collected in PBS. After centrifugation of the aspirate residues at  $220 \times g$  for 5 min, the needles were removed, and the pellet was resuspended in 1 ml of PBS and transferred to a 1.5-ml microcentrifuge vial. The cell pellets were collected by centrifugation at  $12,000 \times g$  for 5 min at  $4^\circ\text{C}$ . After removing the supernatant, the pellet was resuspended in 0.5 ml of RNazol B, and the RNA was isolated as described previously (11). Glycogen (20  $\mu\text{g}$ ) was used as a carrier for RNA precipitation. The amount of RNA was calculated from the absorbance at 260 nm.

**Preparation of Tumor Samples.** Biopsy specimens of the primary tumors of 47 of the 64 patients could be collected and snap frozen in liquid nitrogen. From these biopsies, 10- $\mu\text{m}$  cryosections were prepared. The first and last sections were stained by H&E. The other sections were stained with 1% toluidin blue and 0.2% methylene blue, and the neoplastic regions were manually microdissected and dissolved in 100  $\mu\text{l}$  of RNazol B. In total, two series of four sections were used as duplicates. Tissue was kept cold to avoid RNA degradation. Subsequently, RNA isolation was performed as described above.

**cDNA Synthesis and Real-time PCR Amplification using TaqMan Assay.** The sequences of the intron-spanning amplification primers E48–2s and E48 + 95 $\alpha\text{s}$ , as well as the sequence of the fluorescent probe E48 + 56sFAM, are listed in Table 1. In all experiments, a fluorogenic probe was used. These primers were selected with ABI/Prism Primer Express (Perkin-Elmer/Applied Biosystems, Warrington, United Kingdom). For internal calibration of the samples, we used mRNA transcribed from the gene encoding PBGD, a consistently expressed housekeeping gene (12). The sequences of the primers and probe for PBGD are listed in Table 1. Total RNA was reverse transcribed for 2 h at  $42^\circ\text{C}$ . One reverse transcription reaction (20  $\mu\text{l}$ ) consisted of 5  $\mu\text{l}$  of total RNA (maximum of 1  $\mu\text{g}$ ), 2  $\mu\text{l}$  of  $10 \times$  reverse transcription buffer (containing 600 mM KCl, 30 mM

Table 1 Primers and probes of E48 and PBGD used for RT-PCR amplification

Primers	Sequence 5'-3'
E48 product size: 116 bp	
E48 forward	AGATGAGGACAGCATTGCTGC
E48 reverse	GCAGACCACAGAATGCTTGC
E48 TaqMan	FAM-TTACCCTGCGCTGCCACGTGTG-TAMRA
PBGD product size: 135 bp	
PBGD forward	GGCAATGCGGCTGCAA
PBGD reverse	GGGTACCCACGCGAATCAC
PBGD TaqMan	FAM-CATCTTTGGGCTGTTTTCTCCGCC-TAMRA

MgCl<sub>2</sub>, and 500 mM Tris), 2 µl of 10 mM DTT, 2 µl of 10 mM dNTPs (2.5 mM for each dNTP), 1 µl of reverse primer E48 + 95αs or HuPBGD.R (25 pmol/µl), 7.85 µl of sterile H<sub>2</sub>O (Baxter), 0.05 µl of RNasin (40 units/µl), and 0.1 µl of avian myeloblastosis virus reverse transcriptase (10 units/µl). RNA isolated from microdissected tumor specimens and lymph node aspirates was analyzed in duplicate by Q-RT-PCR.

After completion of the reverse transcription reaction, 5 µl of cDNA were used for PCR amplification in 60 cycles in a 45-µl reaction mixture, containing 5 µl of 10 × TaqMan buffer A (Perkin-Elmer/Applied Biosystems), 10 µl of 25 mM MgCl<sub>2</sub>, 0.5 µl of 100 mM dNTP, 0.6 µl of sense and antisense primers of E48 or PBGD (Table 1; 25 pmol/µl each), 1.5 µl of fluorescent probe (5 pmol/µl), 26.55 µl of sterile H<sub>2</sub>O (Baxter), and 0.25 µl of AmpliTaq Gold DNA-polymerase (5 units/µl; Perkin-Elmer/Applied Biosystems).

Real-time PCR was carried out on the ABI/Prism 7700 Sequence Detector System (TaqMan-PCR; Perkin-Elmer/Applied Biosystems) using a pre-PCR step of 10 min at 95°C, followed by 60 cycles of 15 s at 95°C and 60 s at 60°C. In each amplification, a serial dilution of UM-SCC-22A RNA ranging from 500,000 to 5 pg was run in parallel as standard curve for both E48 and PBGD. Subsequently, the C<sub>T</sub> was determined, *i.e.*, the cycle number at which the amount of amplified target crossed a fixed threshold. The original amount of E48 and PBGD mRNA in each sample was calculated from the standard curve relative to the cell line UM-SCC-22A, using the C<sub>T</sub> value. Preparations without RNA template were used as negative control.

## RESULTS

**Quantitative E48 RT-PCR.** Fig. 1A demonstrates the 10-fold serial dilution of UM-SCC-22A RNA for E48 ranging from 500,000 to 5 pg. Amplification was carried out in duplicate. The standard curve of these dilutions is shown in Fig. 1B. On the basis of these experiments, the dynamic range of quantitation was 5 log with a correlation coefficient of >0.99. The calculated PCR efficiencies were >95%. E48 transcripts can be detected reproducibly in 5 pg of UM-SCC-22A RNA, equivalent to approximately a single cell. The same amount of UM-SCC-22A RNA could be detected in a background of 2 µg of PBMC RNA corresponding to 10<sup>6</sup> cells (data not shown). In the range of 0.5–5 pg of UM-SCC-22A RNA, the duplicates are not always consistently positive, particularly <1.5 pg (7). The cutoff level to call a sample positive or negative was therefore set arbitrarily on 2 pg. This was also the lowest level of detection that duplicates were always within 20% variation. Moreover, all

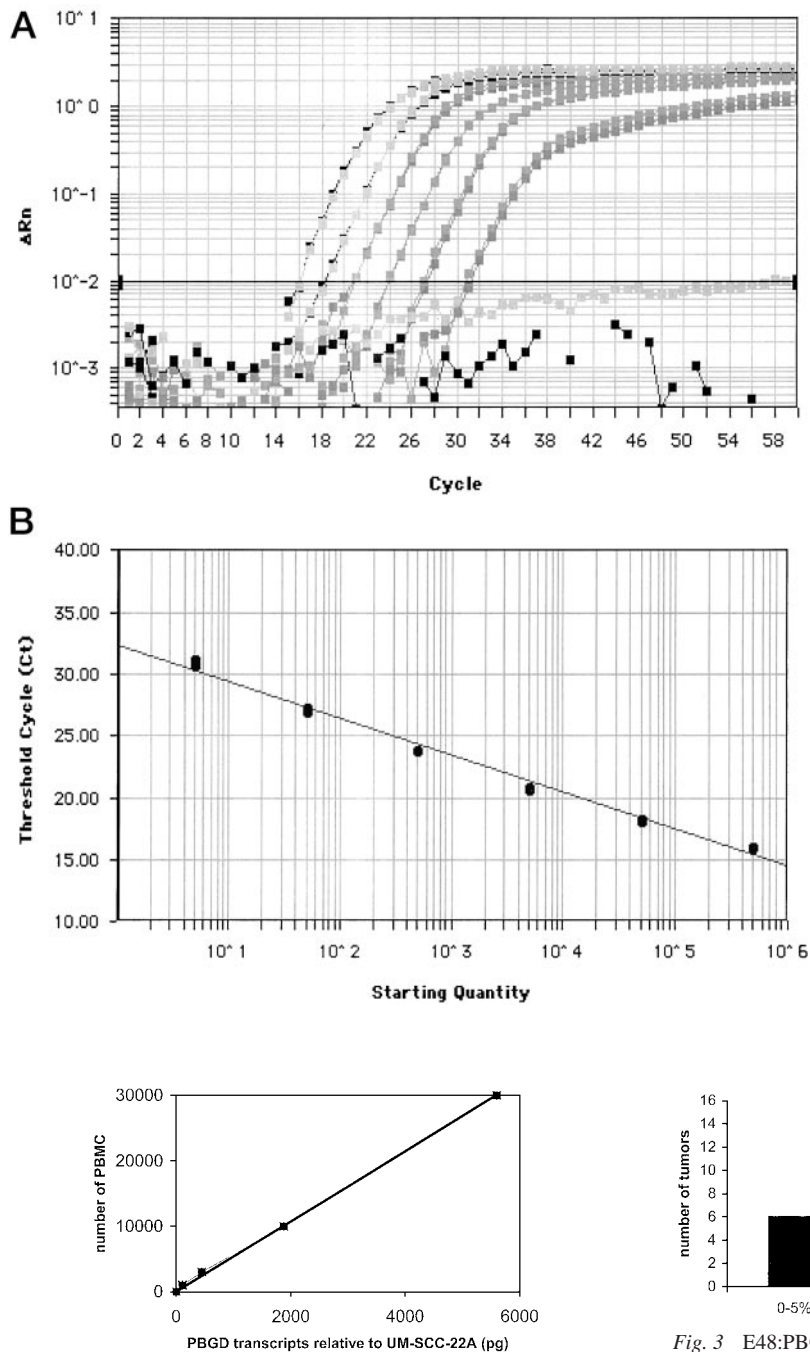
aspirates of noncancer controls were negative when using this cutoff level. Higher limits (5 or 10 pg) lead to a reduction in sensitivity (see below).

**Quantitation of Number of (Squamous) Cells in Lymph Node Aspirates.** Using PBGD as a marker for the number of cells in an aspirate, we established a calibration curve. After RNA isolation of PBMCs, the amount of RNA from 1,000; 3,000; 10,000; and 30,000 PBMCs was determined by PBGD Q-RT-PCR. The samples were tested in triplicate, and Fig. 2 shows the mean of all triplicates. On the basis of these results, an estimate of the number of cells in each aspirate can be deduced using the PBGD value. We arbitrarily defined an aspirate as representative when a minimum number of 1,000 PBMC cell equivalents was present.

**Limits Used for E48:PBGD Q-RT-PCR.** An aspirate was considered evaluable when the number of PBMCs ≥ 1000 cell equivalents based on PBGD Q-RT-PCR. An aspirate was considered positive when the E48 signal was >2 pg of UM-SCC-22A equivalents in duplicate.

**E48 Q-(RT)-PCR in HNSCC Tumors.** Approximately 70% of the HNSCC tumors are known to have a high (>50% cells intensively immunostained) E48 expression as assessed by immunohistochemical staining, indicating that in 30%, the expression is heterogeneous (15). From 47 of 64 patients, frozen biopsies were available, and the E48:PBGD ratios were determined. All Q-RT-PCR reactions on tumor biopsy specimens were performed in duplicate. The mean variation between the duplicates was in the range of 20%. The E48:PBGD ratios of all tumors were calculated relative to UM-SCC-22A. Subsequently, the E48:PBGD ratios of the tumors were subdivided into four classes, and in Fig. 3, the frequencies are depicted. As can be seen from the figure, 6 of 47 (13%) tumors had very low levels of E48 transcripts.

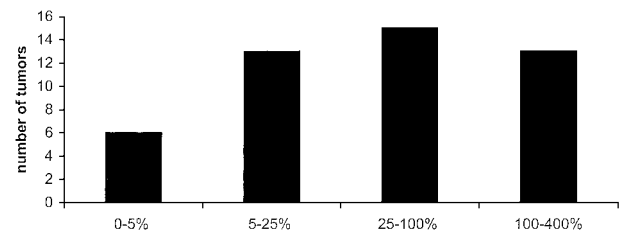
**Correction for Heterogeneity of E48 Expression.** The number of tumor cells in an aspirate residue might be related to the risk for developing metastases. We hypothesized that the number of squamous cells in a specific lymph node aspirate could be estimated, even when the E48 expression is slightly different between tumors. This was tested in a reconstruction experiment using a squamous cell line with a high E48 expression (UM-SCC-22A) and a squamous cell line with a low E48 expression (UM-SCC-22B). As a first step, we determined by E48:PBGD Q-RT-PCR that the E48:PBGD ratio of UM-SCC-22B is 10 ± 0.5-fold lower as compared with that of UM-SCC-22A. Subsequently, 100; 500; 1,000; and 5,000 cells of both cell lines were seeded in



**Fig. 2** Relation between the number of PBMCs from blood and the amount of UM-SCC-22A PBGD RNA equivalents. The amounts of 1,000; 3,000; 10,000; and 30,000 PBMCs correspond to 119; 444; 1,876; and 5,599 pg of UM-SCC-22A RNA equivalents, respectively (mean of triplicate measurements).

100,000 PBMCs, respectively. Using E48 Q-RT-PCR, and using correction for the level of E48 expression in the cell lines, the numbers of UM-SCC-22A cells determined (mean of duplicates) assays were 55; 325; 713; and 6,452 and for UM-SCC-22B was 52; 381; 729; and 3,489, respectively. The

**Fig. 1** A, serial dilution of UM-SCC-22A RNA ranging from 500,000 to 5 pg analyzed by E48 Q-RT-PCR.  $\Delta R_n$  on the Y axis indicates the fluorescent signal of the cleaved probe in the amplification reaction, measured real-time during cycling. The PCR cycles are indicated on the X axis. After a phase with exponential amplification (steep part of the curve), the amount of PCR product stabilizes by increasing shortage of reagents. The cycle at which the amplification plot crosses the  $\Delta R_n$  value of 0.01 was defined as  $C_T$ . The  $C_T$  value is an accurate measure of the amount of template (E48 or PBGD transcripts) in the sample. B, standard curve of E48 Q-RT-PCR in a serial dilution ranging from 500,000 to 5 pg of UM-SCC-22A RNA. The  $C_T$  value was indicated on the Y axis and the amount of RNA (in pg) on the X axis. Correlation coefficient was 0.991. Using this calibration curve of the standard cell line UM-SCC-22A, the amount of E48 RNA or PBGD RNA in a sample can be quantified accurately.



**Fig. 3** E48:PBGD ratios were calculated for 47 microdissected tumor biopsy specimens and compared with the E48:PBGD ratio of UM-SCC-22A. The tumor ratios were subdivided into four classes, and the frequencies are depicted in a bar graph.

experiment was performed twice with similar results. These data demonstrate that the quantitative use of E48 Q-RT-PCR permits estimation of the number of squamous cells in a lymph node aspirate, even when the tumor expresses E48 at a lower or higher level than cell line UM-SCC-22A. Obviously, E48 expression in the tumor should then be determined.



**Table 2A** Comparison of cytology and PBGD Q-RT-PCR results for 235 lymph node aspirates of 64 HNSCC patients

	PBGD >1000 cells		Total
	Evaluable	Not evaluable	
Cytology			
Evaluable	188	6	194
Not evaluable	24	17	41
Total	212	23	235

**Table 2B** Comparison of cytology and E48 Q-RT-PCR results of 212 lymph node aspirates of 64 HNSCC patients, containing >1000 PBMC cell equivalents

	E48 Q-RT-PCR		Total
	E48 positive	E48 negative	
Cytology			
Tumor positive	18	2	20
Tumor negative	8	184	192
Total	26	186	212

**E48 Q-(RT)-PCR of Lymph Node Aspirate Residues from HNSCC Patients and Noncancer Controls.** Ten lymph node aspirate residues of 10 noncancer controls were tested, and no positive E48 signal was detected in any of the cases, whereas the amount of cells was sufficient on the basis of PBGD Q-RT-PCR. Subsequently, 235 lymph node aspirate residues from 64 HNSCC patients were analyzed with E48 and PBGD Q-RT-PCR. Of these 235 aspirates, 41 had been reported as “not evaluable” by cytology, whereas representative PBGD signal was detected in 24 (59%) of the aspirate residues (Table 2A). From Table 2A, it can be seen that 212 of 235 aspirates were evaluable as judged by PBGD Q-RT-PCR. Subsequently, the E48 Q-RT-PCR results of the lymph node aspirates were compared with cytology (Table 2B). Of the 21 cytological tumor-positive aspirates, 18 showed E48 transcript-positive signals in duplicate, varying from 13 to 89,000 pg (median ~2,500 pg) of UM-SCC-22A equivalents. Of the three E48-negative aspirates, one did not contain sufficient material on the basis of PBGD Q-RT-PCR (<1,000 cell equivalents). The two other residues were negative because the corresponding tumors did not express E48. Of the remaining 191 lymph node aspirates that were tumor negative or not evaluable at cytology, eight (4%) were E48 positive.

**Clinical Outcome.** In eight lymph node aspirates of six HNSCC patients who were staged as tumor negative, E48 transcripts were detected by Q-RT-PCR. Two of these six patients received combined tumor excision and elective neck dissection, of which histopathology revealed lymph node metastases in levels of the neck corresponding to those of the positive aspirates found by RT-PCR. A third patient also received combined tumor excision and elective neck dissection with postoperative radiotherapy, but no metastases were found at routine histological examination. A metastasis could not be found in the neck as well when the node was analyzed by step-wise sectioning at five deeper levels. For the remaining three patients, a wait-and-see policy for the neck was adopted; thus far, one patient developed

**Table 3** Comparison of cytology and E48 RT-PCR on sensitivity, specificity, and frequency of reached diagnosis based on 64 patients

	Sensitivity	Specificity	Diagnosis reached
Cytology	15/27 (56%)	37/37 (100%)	62/64 (97%)
E48 RT-PCR	18/27 (67%)	34/37 (92%)	64/64 (100%)

a metastasis after 6 months. The other two patients have not developed clinically manifest lymph node metastases with a follow-up of 13 and 28 months, respectively.

In Table 3 the sensitivity, specificity, and frequency of reached diagnosis of E48 RT-PCR and routine cytology are shown of all patients irrespective of the E48 expression in the tumor. From the table, a clear benefit on the sensitivity is seen, the most important parameter for neck staging, as well as a benefit in the frequency of reached diagnosis. The specificity is decreased when E48 RT-PCR is applied.

## DISCUSSION

Until now, the presence of minimal disease in lymph nodes of HNSCC patients was investigated only in a few studies (9, 16, 17). In a previous study, we analyzed 76 neck dissection specimens from 64 HNSCC patients (16). The percentage of additional micrometastases detected in tumor-negative neck dissection specimens was 3% (2 of 62) using one additional H&E section, and 10% (1 of 10) using immunohistochemical staining. In a second study, using p53 point mutations as a marker, tumor cell DNA could be identified in 21% of histopathological negative lymph nodes (9). The reason that these micrometastases in lymph nodes are missed by routine histopathology is most likely sampling error, because usually only single sections are analyzed. Although step-wise sectioning and immunostaining will decrease the number of unidentified metastases, a certain percentage will still be missed. These data indicate that micrometastases occur frequently in the neck and that molecular techniques can improve sensitivity. We therefore decided to exploit a Q-RT-PCR method on the detection of micrometastases.

Real-time RT-PCR offers several advantages over conventional RT-PCR: (a) the technique permits precise quantification of minute amounts of mRNA transcripts over a 5-log range; (b) PCR is performed in a closed tube system and does not require post-PCR manipulations, thereby preventing possible contamination; and (c) because the lack of post-PCR processing time, sample throughput is increased.

Ectopic expression of small amounts of epithelial mRNA might be detected in lymph nodes, which can cause unwanted positive results. This phenomenon was also described in a previous study where the presence of micrometastases in lymph nodes of patients with colon cancer was assessed, using carcinoembryonic antigen as a marker (18). In our study, we set the cutoff level at 2 pg of UM-SCC-22A equivalents. At this level of detection, the analysis was reliable, and all aspirates of noncancer controls were negative. We noticed a low expression of E48 in 40 of 235 lymph node aspirates demonstrating UM-SCC-22A equivalents ranging from 0.01 to 1.94 pg, which were considered nega-

tive using the cutoff level. The fact that this ectopic E48 mRNA expression was not demonstrated in the analyzed noncancer controls might be attributable to the relatively low numbers of aspirates available (0 of 10) compared with those of HNSCC patients (40 of 235;  $P = 0.37$  Fisher's exact test). We consider the very low amounts of E48 expression in samples of HNSCC patients as illegitimate E48 expression of lymphoid tissue (background) rather than the presence of tumor cells in the aspirate, although this is arbitrarily for cases that are close to the cutoff level.

Using E48 Q-RT-PCR for the detection of squamous cells in lymph node aspirates, the methodological sensitivity in this study was 90%, because E48 RNA was found in 18 of 20 cytological tumor-positive aspirate residues (one case could not be evaluated). The methodological specificity was 100% (10 of 10 E48 negative). Using Q-RT-PCR, 24 of 41 (59%) aspirates could be analyzed that could not be assessed by cytology. Of these 24 cases, one aspirate was E48 positive, an observation that was clinically confirmed because this patient developed a lymph node metastasis during follow-up.

In six patients overall, E48 mRNA was detected in lymph node aspirates that were tumor negative at cytology. In three of these six patients, these findings were clinically confirmed, because tumor-infiltrated lymph nodes were found at histopathology of neck dissection specimens (two patients), and a lymph node metastasis developed during follow-up (one patient). In the remaining three patients, the E48-positive aspirates could not be confirmed clinically nor histologically. One explanation for the difference in clinical outcome for the patient of whom the neck was left untreated could be the relatively short follow-up period of 13 months. The difference in clinical outcome could not be explained by the number of tumor cells, because the tumor cell number of these patients did not differ considerably from the tumor load in the other three patients. Another explanation for these three cases could be that normal squamous cells of the skin were introduced in the lymph node by fine needle aspiration. The sensitivity of E48 Q-RT-PCR was 67%, a considerable improvement as compared with cytology (57%). The specificity, however, decreased from 100 to 97%. For exploitation of this assay, the sensitivity is of largest importance to prevent that the neck of patients with lymph node metastases cancer is left untreated.

In conclusion, real-time quantitative E48 RT-PCR appears to be an accurate technique for assessment of micro-metastases in lymph node aspirates. The assay shows an increase in sensitivity and frequency of reached diagnosis in relation to cytology, permits analyses of a large number of samples simultaneously, and is less labor intensive as compared with other quantitative PCR methods (19, 20). The upstaging of patients with E48 RT-PCR was clinically confirmed in three of six patients (50%). It should be noted that we analyzed only aspirate residues that were left after slide preparation. We might therefore have missed cases. E48 Q-RT-PCR on aspirate residues is almost always evaluable (188 of 194). The test could be implemented routinely in USgFNAC to diagnose cases for which cytological examination is not conclusive. Furthermore, it might be of interest in the future to use a series of molecular markers in parallel that

might lead to an increased sensitivity. Moreover, markers specific for normal keratinocytes or squamous tumor cells could be exploited to enhance the specificity, excluding the presence of normal keratinocytes from the skin that might have been introduced in the aspirate by the needle.

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