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Rapid Molecular Detection of Metastatic Head and Neck Squamous Cell Carcinoma as an Intraoperative Adjunct to Sentinel Lymph Node Biopsy

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Abstract

Objectives—Clinical staging of early head and neck squamous cell carcinoma (SCCHN) is often inaccurate, leading to elective neck dissection to detect the 30% of patients with micrometastatic disease. Sentinel node biopsy (SNB) accurately stages the regional lymphatics, but intraoperative pathology is only moderately sensitive and final pathology takes several days to complete. To facilitate immediate neck dissection where necessary, we have identified several promising marker genes of SCCHN metastasis and developed a rapid, accurate and automated quantitative real time PCR (qRT-PCR) assay for intraoperative use.

Design—Prospective tissue collection, retrospective pathologic correlation with qRT-PCR

Methods—From a 40-gene marker screen, we quantified expression of 11 potential tumor genes using a test set of primary tumors (n=32), metastatic (n=19), and benign (n=10) lymph nodes. Eight patients' paired primary tumor and metastatic nodes were included. A validation set of 442 grossly tumor-negative nodes was evaluated for expression of the most promising markers, comparing metastasis detection by qRT-PCR with pathologic analysis (H&E and immunohistochemistry). A novel multiplexed, automated, single-tube qRT-PCR assay was used to analyze over 100 lymph nodes using a two marker, 35-minute assay to determine its negative predictive value (NPV).

Results—Based on expression of 11 tumor-associated genes from the marker screen, the two most promising markers of SCCHN metastasis in the test set, p63 (p16) and tumor-associated calcium signal transducer 1 (*TACSTD1*), also known as epithelial cell adhesion molecule (*EpCAM*), were selected. Development of a multiplexed qRT-PCR assay for the detection of metastasis compared favorably with pathologic analysis in the additional 442 node

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set. A rapid, multiplexed assay using *PVA* and *TACSTD1* demonstrated excellent reproducibility, linearity, and accuracy (~96% NPV) for identifying positive (n=40) and negative (n=62) nodes in a validation subset.

Summary—Detection of metastatic SCCHN using multiplexed qRT-PCR can be rapid, accurate, and automated, and may enable SNB to be used for intraoperative decision-making. PCR amplification of tumor marker genes is an effective method of intraoperative molecular staging of SCCHN, and could more appropriately guide application of neck dissection in pN+ SCCHN patients, sparing 60–70% of pN0 patients from unnecessary neck dissection. This technique may also be used for identifying residual neck disease post-treatment, using outpatient fine needle aspiration (FNA) biopsy specimens.

Keywords

sentinel node biopsy; molecular staging; oral cancer; metastasis; RT-PCR

Introduction

Metastasis to cervical lymph nodes (LN) occurs in approximately 30% of patients with early squamous cell carcinoma of the head and neck (SCCHN) and is associated with regional recurrence and poor outcome^{1,2}. Physical examination, imaging, and histopathologic characteristics of the primary tumor are not accurate enough to determine TNM stage and to guide treatment reliably. Although close observation (i.e., watchful waiting) and elective neck irradiation remain options, most clinicians favor excision of the regional lymphatics at the time of resection of the primary cancer for accurate staging. However, 60–70% of the operated patients ultimately do not have nodal disease pathologically (pN0) and therefore are theoretically over-treated (Figure 1 schematic). More accurate, targeted, and less invasive approaches would prevent elective neck dissection (END) in many of the patients without metastatic disease.

Sentinel node biopsy (SNB) allows the surgeon to identify and excise targeted “first echelon” lymph nodes that reliably drain the site of a primary malignancy^{3,4}. This technique offers a less invasive means of staging lymphatic basins in patients with an identified primary cancer, and permits detailed histological, immunohistochemical (IHC) and molecular examination of clinically occult micro-metastases. The use of this technique might better direct the application of END in cN0 SCCHN patients, thereby reducing treatment-associated morbidity, which may even occur in a selective neck dissection (SND)⁵. The sequelae of SND include adhesive capsulitis of the shoulder, lower lip paresis, and changes in the contour of the neck. Recent studies have documented negligible morbidity or functional deficits with SNB, as compared with SND⁶.

A large multi-institutional pathologic validation trial has supported previous single institution studies, establishing that the sentinel node concept applies to SCCHN^{7–14}, as well as to cutaneous melanoma and breast cancer. The recently published American College of Surgeons Oncology Group (ACOSOG) prospective multi-center trial of 168 patients demonstrated that the pathologic status of the SLN correlated highly with the results of subsequent, immediate formal lymphadenectomy, yielding a negative predictive value (NPV) of 96% for accurately staging a pN0 neck using SNB¹⁵.

A major limitation of SNB currently remains the need for a reliable method of intraoperative detection of metastasis, so that a neck dissection could be performed at the same time when necessary. This would avoid a second surgery, with its associated costs, treatment delay, and the potential for complications. However, intraoperative frozen section and imprint cytology have insufficient sensitivity and reproducibility⁸, suggesting to the need for intraoperative

tools to permit accurate decision-making as to need for END (Figure 1). In addition, step sectioning and IHC, which are performed only on SLNs, typically add 1–2 days and may identify additional micrometastases¹⁵. A molecular technique, which can sample a larger portion of the SLN(s) and amplify microscopic tumor deposits, has the potential for greater accuracy and rapidity necessary for intraoperative decision making^{16,17}.

To this end, we have systematically investigated the identification of reliable tumor marker genes, expressed selectively in tumor⁺ nodes using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and developed a rapid, multiplexed qRT-PCR assay to provide immediate information regarding the status of the SLN, and have validated it for clinical application. This manuscript describes the identification and selection of the two most promising markers from a large screen of over 40 tumor-associated genes from a training set of SCCHN primary and metastatic tumors. These tumor markers, *PVA* and *TACSTD1*, were then validated in a test set of cervical lymph nodes (of which 62 of 442 were tumor⁺), comparing qRT-PCR with standard H&E and IHC analysis. After this validation was performed, the multiplexed, rapid (35 min) qRT-PCR assay was performed using a subset of 102 nodes, of which 40 were tumor⁺, to mimic the clinical picture of occult metastasis to cervical LN observed in early SCCHN. Our rapid, highly accurate qRT-PCR technique may facilitate more routine application of minimally invasive techniques to stage the neck, such as SLNB, and direct more appropriate use of neck dissection in SCCHN patients. Such a technique could be extended to the post-treatment setting for detection of residual disease, and to guide salvage therapy.

Materials and Methods

Patients and Lymph Node Collection

After receiving approval from the institutional review board (IRB), written informed consent was obtained from all patients donating specimens for this study. Primary tumors and histologically tumor-free or tumor-involved nodes were harvested at surgery and immediately snap-frozen at -80°C until RNA extraction was done. Of these specimens, eight sets of paired human head and neck primary tumors and tumor-containing metastatic lymph nodes (from the same 8 patients with SCCHN) were included in the initial tumor gene marker study¹⁶. Benign lymph nodes ($n = 21$) were obtained from patients undergoing surgery for nonmalignant esophageal disorders. All cervical lymph nodes were collected from patients undergoing neck dissections for HNSCC, with various primary sites including the oral cavity, oropharynx, larynx, and hypopharynx. A validation set of 442 lymph nodes from 92 patients were separately harvested, sliced in alternating 1mm sections, with half of the sections used for pathological diagnosis and the other half used for this study. In order to reduce discrepancy between the two halves, the research halves were embedded in OCT compound and one section was mounted on a slide, H&E stained, and read by a head and neck pathologist. The OCT embedded nodes were stored at -80°C prior to processing and RNA isolation.

Tissues and Pathologic Evaluation

Archived tissue from all patients in this study was reviewed by two individuals who specialize in head and neck pathology (RRS or SC), who were blinded as to the original diagnosis, which was reconfirmed histologically. Twenty 5- μm sections were cut from each optimal cutting temperature compound (OCT)-embedded tissue for RNA isolation. In addition, sections were cut and placed on slides for H&E and immunohistochemistry analysis at the beginning, middle (between the 10th and 11th sections for RNA), and end of the sections for RNA isolation (Figure 2). All three H&E slides from each specimen underwent pathologic review to confirm presence of tumor, percentage of tumor, and to

identify the presence of any contaminating tissues. All of the unstained slides were stored at -20° . Immunohistochemistry evaluation was done using the antibody pan keratin (AE1/AE3) mixture (DAKO, Carpinteria, CA), and Vector Elite ABC kit and Vector AEC Chromagen (Vector Laboratories, Burlingame, CA). Immunohistochemistry was used as needed to confirm the H&E histology.

Histologic analysis was performed for each lymph node, consisting of an H&E stained frozen section of alternating pieces for research, to be cut at a later date for both RNA isolation and histologic review by two pathologists, and consensus was reached using both H&E and IHC (“research pathology”), finally, alternate pieces of each node were fixed and embedded, and H&E stained sections were reviewed and entered into the medical record (permanent or “final” pathology). Finally, nodes were classified as negative, positive, as isolated tumor cells (ITC), or if tissues were considered inadequate, as non-diagnostic. From these data, each node was also given an “overall pathology” call based on all three evaluations, where any positive result trumped ITC and any ITC trumped negative results.

Screening Approach

Identification of Potential Tumor Markers—An extensive literature and public database survey was conducted to identify any potential markers. Resources for this survey included PubMed, OMIM, UniGene (<http://www.ncbi.nlm.nih.gov/>), GeneCards (<http://bioinfo.weizmann.ac.il/cards>), and CGAP (<http://cgap.nci.nih.gov>). Our survey goal was to identify genes with moderate to high expression in SCCHN and low expression in normal lymph nodes. In addition, genes reported to be upregulated in SCCHN, and genes with restricted tissue distribution were considered potentially useful. Finally, genes reported to be cancer specific, such as the cancer testis antigens and *hTERT*, were evaluated.

The screening was conducted in two phases. All potential markers entered the primary screening phase and expression was analyzed in 6 primary tumors and 10 benign lymph nodes obtained from patients without cancer (five RNA pools with 2 lymph node RNAs per pool). Markers that showed good characteristics for lymph node metastasis detection (consistent, high expression in tumors and very low expression in benign nodes) passed into the secondary screening phase. The secondary screen consisted of expression analysis on 26 primary tumors, 19 histologically positive lymph nodes, and 21 benign lymph nodes without cancer.

Validation cohort of lymph nodes—After the secondary marker screen, an independent cohort of 442 nodes from 92 different SCCHN patients was prospectively collected. This validation set was characterized pathologically using the same approach as in the primary and secondary screens for correlation with molecular results. Within this validation cohort, a set of 103 nodes (43 tumor⁺, 41.7%) were used to validate the rapid, multiplexed assay, which amplified two tumor marker genes, *PVA* and *TACSTD1*, and an internal control gene, *β -Glucuronidase (GUSB)*¹⁸.

RNA Isolation and cDNA Synthesis—RNA was isolated using the RNeasy minikit (Qiagen, Valencia, CA) essentially as described by the manufacturer. The only modification was that we doubled the volume of lysis reagent and loaded the column in two steps. This was found to provide better RNA yield and purity, probably because of diluting out the OCT in the tissue sections. Reverse transcription was done in 100- μ L reaction volumes with random hexamer priming and SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase. For the primary screen, two reverse transcription reactions were done, each with 1,000 ng of RNA. The cDNAs were combined and quantitative PCR was done using the equivalent of 20 ng RNA per reaction. For the secondary screen, the RNA input for

primary tumors and positive nodes was 1200 ng per reverse transcription reaction and 20 ng per quantitative PCR reaction, but this was increased to 2000 ng per reverse transcription reaction and 80 ng per quantitative PCR reaction for the benign nodes in order to improve sensitivity for detection of low background expression.

Quantitative PCR quantification with SYBR Green—All quantitative PCR for marker screening was done on the ABI Prism 7700 Sequence Detection Instrument (Applied Biosystems, Foster City, CA). Relative expression of the marker genes was calculated using the ΔCT methods previously described¹⁶ and with *GUSB* as the endogenous control gene. All assays were designed for use with 5′ nuclease hybridization probes although the primary screening was done using SYBR Green quantification to save cost. Assays were designed using the ABI Primer Express Version 2.0 software and, where possible, amplicons spanned exon junctions in order to provide cDNA specificity. All primer pairs were tested for amplification specificity (generation of a single band on gels) at 60°C, 62°C, and 64°C annealing temperature. In addition, PCR efficiency was estimated using SYBR Green quantification before use in the primary screen. Further optimization and more precise estimates of efficiency were done with 5′ nuclease probes for all assays used in the secondary screen. A mixture of the Universal Human Reference RNA (Stratagene, La Jolla, CA) and RNAs from human placenta, thyroid, heart, colon, PCI-13 cell line and the SKBR3 cell line served as a universal positive expression control for all the genes in the marker screening process. For SYBR Green I-based quantitative PCR, each 50-μL reaction contained 1× TaqMan buffer A (Applied Biosystems), 300 nmol/L each deoxynucleotide triphosphate, 3.5 mmol/L MgCl₂, 0.06 units/μL Amplitaq Gold (Applied Biosystems), 0.25× SYBR Green I (Molecular Probes, Eugene, OR), and 200 nmol/L each primer. The amplification program consisted of two stages with an initial 95°C Taq activation stage for 12 minutes followed by 40 cycles of 95°C denaturation for 15 seconds, 60°C, 62°C, or 64°C anneal/extend for 60 seconds, and a 10-second data collection step at a temperature 2°C to 4°C below the T_m of the specific PCR product being amplified. After amplification, a melting curve analysis was done by collecting fluorescence data while increasing the temperature from 60°C to 95°C over 20 minutes.

Tissue processing for RNA isolation and Research Pathology review—Five-micron serial sections were cut from each OCT embedded lymph node half, with the initial and final tissue sections were mounted on slides for histologic analysis with hematoxylin and eosin (H&E) staining (Figure 2). The next adjacent tissue section was mounted on slides and stained with pancytokeratin immunohistochemistry (IHC) for histopathology. Immunohistochemical evaluation was done using the antibody pan keratin (AE1/AE3) mixture (DAKO, Carpinteria, CA), and Vector Elite ABC kit and Vector AEC Chromagen (Vector Laboratories, Burlingame, CA). The intervening sections were distributed 4:1:4:1:4 etc., such that 4 sections were immediately placed in chaotropic lysis buffer for RNA isolation (total of 50–60 sections) and every fifth section was mounted on a slide for histologic review (every five sections are considered a level (Figure 2). All unstained slides from levels 2–9, were fixed in acetone and stored at –20 °C. All H&E and IHC slides from level 1 and 10 of the lymph nodes were reviewed by two specialized head and neck pathologists (R.S. and S.C.) to confirm presence of tumor, percentage of tumor, and identify the presence of contaminating tissues, as were slides from the GeneXpert™ (Cepheid, Sunnyvale, CA) validation set.

RNA Isolation and cDNA Synthesis—RNA was isolated using the Stratagene RNA isolation minikit (Stratagene, La Jolla, CA) using the manufacturers described protocol. Reverse transcription was done in 100-μL reaction volumes with random hexamer priming and SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase as per our previous

studies (33). All screening quantitative PCR was performed on the Stratagene MX300P cycler (Stratagene, La Jolla, CA). Relative expression of the marker genes (*PVA* and *TACSTD1*) was calculated using the ΔCT methods that were previously described¹⁶, and with β -glucuronidase (*GUSB*) as the endogenous control gene. *TACSTD1* is also known as epithelial cell adhesion molecule (*EpCAM*). Primer and probe sequences for each gene were as published previously¹⁶.

GeneXpert™ Multiplex Assay Development—Multiplex qRT-PCR assays can suffer from limited linear dynamic range due to common reagent utilization. The SmartCycler™ and the GeneXpert™ both employ a method called temperature controlled primer limiting to inhibit the assay that reaches threshold first in order to decrease competition between the simultaneous assays. The *PVA*, *TACSTD1*, *GUSB* (triplex) assay used in this study is very similar to our previously published GeneXpert™ assay for breast cancer¹⁹. Duplex and then triplex assays were first developed on the SmartCycler™ (2–3 hr machine) before transitioning to the GeneXpert™ (35 min machine). Briefly, PCR efficiency and dynamic range were assessed for duplex assays (*TACSTD1/GUS* and *PVA/GUS*) using serial dilutions of cDNA with high expression of the respective target genes (normal esophagus RNA for *PVA*, normal colon RNA for *TACSTD1*) in a background of total spleen cDNA. Linear dynamic range for the triplex assay was then tested to ensure that all three assays functioned well in the ranges needed for lymph node classification. At this point, the triplex qPCR assay was incorporated into the Cepheid GeneXpert™ system to facilitate automated RNA isolation, reverse transcription and qPCR. Reproducibility of the quantification of each marker gene was then tested on the GeneXpert™ using serial dilutions of total RNA known to have high expression of *PVA* and *TACSTD1*. RNAs were diluted in a background of lysate from lymph nodes negative for disease in order to mimic a true test scenario. Six serial dilutions were performed and each assay was repeated four times on the GeneXpert™ in order to assess reproducibility across a range of target gene expression. Primer and probe sequences used in the GeneXpert™ for each gene were previously published¹⁶.

GeneXpert™ Analysis: Twenty-four 5- μ m sections of OCT-embedded tissue were sectioned into 800 μ L of GeneXpert™ lysis buffer (Cepheid). The lysis buffer was filtered through a 0.22-mm syringe filter (Osmonics Inc, Westborough, MA), and loaded into a GeneXpert™ cartridge. The automated processes of RNA isolation, reverse transcription, and QRT-PCR on the GeneXpert are described elsewhere. Briefly, the filtered tissue lysate was placed into a reservoir on the GeneXpert cartridge (Supplementary Figures 2 and 3) and all necessary reagents for RNA isolation, reverse transcription and quantitative PCR were placed in additional reservoirs. The cartridge was placed in the GeneXpert™ and the remaining steps were fully automated. The tissue lysate was first passed over an RNA isolation resin, washed, and then RNA is eluted into a clean reservoir. Reverse transcription reagents are added and the mixture is pumped into the integrated PCR tube and heated for cDNA synthesis. The cDNA is then removed from the PCR tube and PCR reagents (primers, probes, etc.) are added. The mixture was returned to the PCR tube and thermal cycling is done (overall total time ~ 35 minutes). Probe fluorescence was monitored at each cycle and results are updated on the monitor in real time. For this study, the rapid PCR assay consisted of a multiplex qRT-PCR for *PVA*, *TACSTD1*, and the endogenous control gene, *GUSB*.

Analysis of Lymph Nodes using the GeneXpert™—One hundred and three (n=102) lymph nodes were analyzed on the GeneXpert™. Frozen tissue pieces, which were re-cut for RNA and tissue sections, were evaluated blindly by two fellowship trained head and neck pathologists (GeneXpert research Pathology). Twenty-four, 5- μ m sections of OCT-embedded tissue were sectioned into 800 μ L of GeneXpert lysis buffer (Cepheid) for each node, with two initial and final tissue sections mounted on slides for H&E and IHC

histopathology. The lysate was then filtered through a 0.22- μ M syringe filter (Osmonics Inc) and loaded into a GeneXpert™ cartridge. The exact details of the RNA isolation, reverse transcription, and QRT-PCR on the automated GeneXpert™ system are described (35).

Statistical Analysis—In the primary screen, data from the melting curve were analyzed using the ABI Prism 7700 Dissociation Curve Analysis 1.0 software (Applied Biosystems). The first derivative of the melting curve was used to determine the product T_m as well as to establish the presence of the specific product in each sample. In general, samples were analyzed in duplicate PCR reactions and the average cycle time (C_t) value was used in the expression analysis. However, in the secondary screen triplicate runs were done for each individual benign node and the lowest C_t value was used in the calculation of relative expression to obtain the highest value of background expression for the sample.

Generation of Prediction Rules: After the primary marker screen, six markers that passed the secondary screen were evaluated individually and in combination with other markers. The characteristics used to evaluate markers were sensitivity, specificity, classification, accuracy and the area under the receiver operating characteristic curve. For individual markers, a cutoff value was determined that maximized the classification accuracy (proportion of lymph nodes correctly classified). In cases wherein classification accuracy was 100%, the cutoff was set at the midpoint between the highest expressing negative node and the lowest expressing positive node. Markers were also combined into pairs for lymph node classification and a linear prediction rule was generated for each pair. The rule was equivalent to the linear predictor that equalized the fitted probabilities above and below the linear boundary. That is, points on the boundary line had a predicted probability midway between the numerical scores assigned to positive and negative nodes. For example, if positive nodes were assigned a score of 2 and benign nodes a score of 1, predicted scores >1.5 were classified as positive.

Internal Validation of Prediction Rules: Internal validation of prediction rules was conducted by nonparametric bootstrap resampling using Efron and Tibshirani's improved bootstrap method¹⁶, in which 500 bootstrap samples of lymph nodes are selected from the pool of all positive and negative nodes. The optimism in the original estimates of sensitivity, specificity, and classification accuracy are then calculated as the difference between the bootstrap classification statistic applied to the original data and applied to the bootstrap data. The average difference over all bootstrap samples is computed and reported as the bias in the values derived from the observed data and then subtracted from the original estimates to produce the bootstrap-validated estimates.

Diagnostic accuracy of standard qRT-PCR and the GeneXpert assays: The reference standard was established: 1) research pathology, a consensus of 2 academic pathologists applying frozen H&E and IHC. To explore the diagnostic potential of individual markers with both standard qRT-PCR and the GeneXpert™, ROC curves were constructed and the cutoff value of each marker that produced the highest overall classification accuracy was selected. The diagnostic parameters sensitivity, specificity, positive predictive value, negative predictive value and overall accuracy were calculated for each cutoff. In addition the area under the ROC curve was estimated. For the purpose of developing a diagnostic model, and identifying which marker or combination of markers are most important a different strategy was used. In this approach, the sample of 442 lymph nodes were collected from 92 patients and therefore were not considered to be independently distributed. For this reason we applied a generalized linear model with random effects. This model used a logit link to estimate the binomial variable, positive or negative by pathology, as a function of marker RNA expression level controlling for the random effect of patient.²⁰ The best fitting

model was selected based on the likelihood ratio test and the number of model parameters (Akaike's Information Criteria, ²¹). A 'GeneXpert' classification model was developed with conventional (fixed effects) logistic regression. The model was used to predict the probability that a node was positive and the range of predicted probabilities served as a list of potential cutoff values in an ROC analysis. In addition to logistic regression models, recursive partitioning and nearest neighbor models were fit but neither method compared favorably to logistic regression. All diagnostic operating characteristics were cross validated by either bootstrap resampling (200 reps) or leave-10-out cross-validation.

Results

Primary screening for suitable tumor markers of nodal metastatic disease

Our literature and database surveys identified 40 genes overexpressed by aerodigestive tract malignancies for evaluation in the primary tumor marker screen ^{16,22}. All of these genes were analyzed for expression in 6 primary SCCHN and 10 benign lymph nodes, using qRT-PCR. Resulting data for the 11 genes with the highest median expression in tumors, and well-described biological function in SCCHN, are shown in Table 1. In addition, we calculated the ratio of relative expression between the lowest expressing tumor and the highest expressing benign node, and between the median expression in tumors and the highest expressing benign node. Some genes had no detectable expression in benign nodes and therefore ratios could not be calculated.

Using three parameters (median expression in the tumors, lowest tumor/highest benign node and median tumor/highest benign node ratios), two genes were selected with expression characteristics most suitable for detection of SCCHN lymph node metastases. These genes, *PVA* and *TACSTD1*, had median tumor/highest benign node ratios >500 (Table 1), and therefore had the potential to detect small foci of tumor, while still discriminating negative nodes. In addition, all genes except cytokeratin (*CK*)19 also had lowest tumor/highest benign node ratios >100 indicating that they were expressed at reasonably high levels in all six tumors tested in the primary screen. *MAGE-A3*²² and *CK7* were omitted from the secondary screen due to a combination of low median tumor/highest benign node expression ratios and relatively low median expression in tumors. Similarly, *CK18* was excluded based on a combination of a low expression ratio in primary tumors. *EGFR* was included in the secondary screen based on its frequent use as a prognostic and therapeutic marker in SCCHN ²³, but its differential expression between tumor positive and benign nodes was not sufficiently promising (data not shown).

Secondary tumor marker screen using the best 6 tumor markers identified in the primary screen

For the secondary marker screen, histologic evaluation of 26 primary tumor specimens was then performed for the best markers, specifically focused on *PVA* and *TACSTD1*. Similarly, we studied 19 histologically positive nodes, in which the median tumor percentage was 70% (range, 2–90%). Eight patients' paired primary tumors and metastatic nodes were included. The relative expression profiles of the 11 markers selected for the secondary screen are shown in Table 1. The data show that all markers are expressed in positive lymph nodes as well as in primary tumors indicating that metastatic tumor cells continue to express these genes. Figure 3A indicates that relative expression cutoff values can be established (dotted line) to provide the most accurate classification of histologically positive and benign nodes. These cutoff values were used to calculate classification characteristics (sensitivity, specificity, area under the receiver operating curve, ROC, and overall classification accuracy) for each marker, as well as using a standard time-frame (3–4 hr) multiplex assay

with simultaneous amplification of both markers. These results are presented in Figure 3B and Table 2.

Pathologic analysis of nodal disease

As an independent validation set, 442 lymph nodes from 92 patients (demographic data provided in Table 3) were analyzed through i) research frozen section (H&E plus IHC) and ii) final pathology of fixed sections. Overall (combined) results classified 380 nodes as negative [2 nodes were diagnosed as positive for isolated tumor cell (ITC)⁺ and treated as pN0] and 62 nodes as tumor-positive (Figure 3B). Comparison of two separate, blinded pathologists' reviews with the overall pathology result (excluding ITC, which were treated as benign) indicated that concordance was >99% in all cases. However, some discrepancy of sensitivity between research pathology and permanent pathology was observed (86.9% and 91.8% respectively). The difference between research and permanent pathology results with overall (consensus) pathology was presumably due to sampling error of slightly different sections of the nodes, which were processed as shown in Figure 2. We focused on research pathology since these sections were closer (adjacent) to those used for RNA extraction and PCR.

Validation of qRT-PCR markers and correlation with histologic detection of metastasis

After selection of *PVA* and *TACSTD1* as the most promising gene markers from the secondary screen, the 442 cervical lymph node validation set was analyzed by qRT-PCR for these markers, and molecular results were compared to research pathology (Tables 4–5 and Figure 3B). In general, qRT-PCR results were slightly more concordant with research pathology than with overall pathology (mean accuracy 96% vs 94%), probably reflecting the increased sampling error inherent in the comparison with overall, consensus pathology. Individually, all markers demonstrated high overall accuracy compared with both pathology endpoints but *PVA* clearly provided superior sensitivity (92% vs research pathology, see Table 4) and hence, superior negative predictive value. ROC curve analysis also identifies *PVA* as the best marker although the AUC confidence intervals for *PVA* and *TACSTD1* overlap. Paired combinations of markers were also evaluated (Table 2, 4 and 5) with *TACSTD1* and *PVA* providing the best correlation with pathology.

Automated GeneXpert™ rapid assay development and reproducibility assessment

For application of a rapid qRT-PCR assay suitable for use within an intraoperative time frame, a triplex assay was developed for *PVA*, *TACSTD1* and control gene, *GUSB*. PCR efficiencies for multiplex assays ranged from 96.3% to 98.8% (Figure 4) and the triplex assay showed good linearity in the range needed to discriminate positive from negative nodes. Replicate experiments on the GeneXpert™ resulted in an intra-class correlation coefficient of 0.90 for *PVA* and 0.97 for *TACSTD1*. The coefficient of variation was 18.2% for *PVA* and 3.9% for *TACSTD1*.

GeneXpert™ rapid analysis of model lymph node cohort

A total of 102 lymph nodes were evaluated using the triplex assay on the GeneXpert. Results were compared to concomitant research pathology (examination of frozen sections taken adjacent to the tissue used for the rapid, multiplex qRT-PCR) and consensus pathology (a consensus based on all histologic analyses performed on the node). Of the 102 nodes, 40 (39.2%) were tumor positive by consensus pathology and 62 were tumor negative. These results are shown in Figure 5. Sampling slightly different nodal sections in the setting of micrometastatic deposits led to discrepancy in 7 nodes from research pathology to consensus results. Using the rapid, multiplex assay, negative predictive value compared with

research pathology, the most accurate comparison of adjacent tissue sections analyzed pathologically, was 95.5% (Table 4).

For each lymph node section tested in this validation set using the rapid assay, *TACSTD1* and *PVA* expression data from the triplex GeneXpert™ assay was converted to a probability of being positive using a logistic regression model, to conform to the clinical scenario in which approximately 30% of nodes are tumor⁺, instead of 39.2% (40/102) by final consensus pathology or 35% by research pathology in this data set. We used 35% since research pathology more accurately conformed to the section tested after RNA isolation. Using this approach, the overall accuracy of qRT-PCR compared with research pathology was 94.2% and compared with consensus pathology was 90.3%. The sensitivity and specificity compared with research pathology were 91.7% and 95.5% respectively and compared with consensus pathology were 86% and 93.3%. Ten-fold cross validation indicated that these accuracy levels are likely to be quite robust as the estimated accuracy only dropped by 4–5%.

Discussion

To facilitate selection of pN+ SNB patients who require END, we have developed a completely automated, multiplex qRT-PCR assay that can accurately detect lymph node metastasis in SCCHN. This assay can be performed in approximately 35 minutes and thus could be performed intraoperatively, in conjunction with frozen section to detect metastasis, even those with 3–5% nodal involvement. This study is the first of its kind in SCCHN to adapt the use of a rapid automated qRT-PCR assay that can be used in an intraoperative setting to detect lymph node metastasis.

Because many patients with SCCHN routinely undergo END, the ability to accurately and rapidly stage the cN0 neck has great clinical application, to avoid morbidity of open neck dissection. SLN biopsy is a promising, minimally invasive technique that has recently been validated to accurately stage the neck in early SCCHN¹⁵, with a negative predictive value (NPV) of 96%. However, widespread clinical application of SNB is severely hampered by the lack of an intraoperative method that is rapid and accurate, to detect metastatic disease.

To accomplish this goal, an extensive tumor gene marker screen was used, in which two distinct mRNA markers were identified, and then validated in both single and multiplex assays using a large independent set of over 440 grossly tumor-negative lymph nodes. Furthermore, after validation of these novel markers, we adapted the qRT-PCR assay to a rapid time-frame, and applied the triplex assay (2 tumor marker genes and one control gene) to a subset of 102 lymph nodes (of which 40 nodes were tumor⁺), to demonstrate the accuracy and clinical applicability of this approach.

Although intraoperative frozen section has been evaluated for SNB, its sensitivity is not very high (~60%, ref.⁸) and an inherent problem with intraoperative frozen section is sampling error, since even 1 or 2 5μm frozen sections of a 1mm lymph node only corresponds to 0.1%, which is visually analyzed and therefore may miss metastasis. This is particularly true if the metastatic deposit is located near the capsule, as opposed to the mid-portion of the node most likely to be bi-lobed into 2 sections. Frozen section histology is also subject to both technical difficulties in sample and section preparation, including interpretive challenges, all of which are heavily dependent on the skill and experience of the pathologist and support staff. Thus, even final assessment of step sectioned formalin-fixed, paraffin embedded (FFPE) sections, which samples a larger percent of the node and is the “gold standard,” may not find micrometastatic deposits (<2mm and >0.2 mm), not to mention isolated tumor cells (tumor clusters <0.2mm), as suggested by the fact that 7–10% of pN0

patients after END suffer recurrent disease in the neck ^{24,25}. Hence, a method of evaluating a large portion of SLN for metastasis that is both rapid and accurate would be of great benefit.

Others have tried to predict nodal metastasis using gene expression profiles from a primary tumor biopsy ^{26,27}. However, no reliable gene prediction profile has been developed to use the primary tumor mRNA profile in classifying presence of disease in cN0 patients. Thus, direct analysis of the draining SLN is necessary, a procedure which has been recently validated in a large prospective multi-center trial ¹⁵. The GeneXpert™ assay has several advantages over prior methods of molecular diagnosis, as well as conventional pathologic analysis, in detecting occult metastasis in SLNs of SCCHN. First of all, this is the first instrument that can do a fully automated RNA isolation and qRT-PCR in less than 35 minutes, which could easily fit into an intraoperative time frame. Closure of the primary tumor defect and/or neck incisions could be undertaken after SLN removal and during pathologic and qRT-PCR analysis. Second, the objective nature of the assay eliminates the uncertainty and discordance rates with frozen section pathology analysis, a clearly documented issue ⁸. Finally, with further work, this wet assay can become automated into a dry assay which will remove any doubt of potential human error with the sample processing (i.e. pipetting error). Further development of the PVA assay, such as was done for *TACSTD1* in breast cancer ¹⁹, should lead to higher PCR efficiency. Thus, we anticipate technical improvement as greater developmental effort is devoted to preparing rapid PVA amplification for clinical evaluation.

Our results show that the GeneXpert™ assay had a very high NPV (>96%), however 3 tumor⁺ nodes were ultimately “missed”, i.e. were below the qRT-PCR threshold. Histologic analysis of these 3 nodes showed < 5% tumor, which was absent on later sections that were likely placed in RNA lysis buffer and analyzed by qRT-PCR. The fact that PVA and *TACSTD1* did not reveal high expression in both cases could be an artifact of the tissue sectioning protocol. Moreover, the GeneXpert™ could detect < 3% tumor in a lymph node that two pathologists missed on H&E analysis, showing that the qRT-PCR assay reliably detects micrometastatic disease. Furthermore, optimization of these marker assays, such as inclusion of a larger number of alternating sections for PCR versus frozen section analysis, will be used to avoid sampling errors in micrometastatic disease.

While other lymph node qRT-PCR studies report homogenizing whole portions of, or even complete lymph nodes for RNA isolation, we believe that lymph nodes can be processed in such a way to permit both qRT-PCR and routine pathologic evaluation in parallel on immediately adjacent tissue sections (41). In this way, we anticipate that the GeneXpert™ assay may be used in the common clinical scenario in which the pathologist suspects metastatic disease in the frozen section, but cannot confirm the diagnosis solely based on H&E analysis. Instead of running the risk of having the patient potentially return for surgery in the case that IHC staining picks up metastatic disease, one can run the GeneXpert™ assay in less than 35 minutes intraoperatively, providing over 95 % accuracy that the SLN is free of tumor. The high NPV is of greatest clinical importance ¹⁵, because it saves those patients free of disease from having a longer procedure (END) and inpatient hospital stay. The nodal analyses could be performed during an intraoperative sequence in which the primary tumor and/or neck incisions are being closed, with a high likelihood (70%) that no further surgery will be needed.

We and others have previously shown that RT-PCR can potentially be more sensitive than routine pathology for analysis of lymphnodes ^{19,28}. In SCCHN specifically, studies on molecular analysis of cervical lymph node metastasis have used a variety of techniques, including PCR amplification to detect *p53* mutations ²⁹, immunohistochemistry staining

(with or without serial sectioning) and histopathologic examination⁸ and slower, standard RT-PCR-based analysis of tumor marker gene expression³⁰. Nieuwenhuis et al. showed the prognostic value of qRT-PCR in pN0 SCCHN patients²⁸. This same group also showed the potential for molecular staging of cervical nodes by using tissue obtained via fine-needle aspiration³¹. Our technique could be readily adapted to this clinical scenario, in the setting of clinical or radiographic suspicion.

Our comprehensive marker screen to identify the best mRNA markers for detection of lymph node metastases in SCCHN can also provide biological insights as to mechanisms of metastasis. This marker screen has identified two (*PVA* and *TACSTD1*) extremely robust, tumor-related markers, any one of which might be used in a single-marker assay. *PVA*, also known as desmoglein 3 (*DSG-3*), is a 130-kDa surface glycoprotein that is the serologic target in the autoimmune skin disease pemphigus vulgaris. *PVA* is a member of the desmoglein subfamily of the desmosomal cadherins and the gene encoding this protein has been mapped to the long arm of chromosome 18 and is known to contain 15 exons. *TACSTD1*, also known as *EPCAM*, is a human cell surface antigen that has been used in studies to detect lymph node micrometastases in a variety of tumor types, including SCCHN³². To our knowledge, we were the first to identify *PVA* to detect lymph node metastases in SCCHN¹⁶.

This rapid, multiplex assay was incorporated into a completely automated RNA isolation and QRT-PCR instrument (the GeneXpertTM), which was developed by Cepheid, Inc. (Sunnyvale, CA) for molecular diagnostic testing and is currently in use for bioterrorism applications. The GeneXpertTM is an automated cartridge processor with integrated real-time PCR capability (Supplementary Figure 2). Each single-use GeneXpert cartridge consists of multiple reagent reservoirs, a syringe barrel, and a valve mechanism that allows transfer of reagents between reservoirs. In addition, the cartridges used in this study have a solid-phase matrix for nucleic acid purification and isolation. Finally, each cartridge has a PCR tube that can be accessed from specific reagent reservoirs to allow loading of reaction components into the PCR tube for reverse transcription and/or quantitative PCR. The GeneXpertTM instrument itself interfaces with the cartridge to control reagent movement and also houses heat plates and optical excite/detect blocks to facilitate real-time PCR. The details of RNA isolation and qRT-PCR using the GeneXpertTM have been published elsewhere¹⁹. Biotechnology companies will hopefully develop these and other instruments to facilitate further development of this field.

In conclusion, we have demonstrated that qRT-PCR can detect, with high sensitivity and specificity, metastatic disease in lymph nodes of patients with SCCHN. In addition, we have shown the feasibility of automated, intraoperative staging of cervical lymph nodes and the possibility that such an approach may eventually prove superior to conventional pathology. Staging of the cN0 neck is currently a topic of intense interest in the head and neck oncologic community, with the goal that therapeutic surgical and adjuvant treatment be administered to those most likely to benefit from it. Whereas the ACOSOG Z0360 trial effectively validated the multiple single-institution studies, supporting SNB for staging the cN0 neck in SCCHN, it is unlikely that SNB will be widely accepted without a rapid, accurate, and standardized method of staging the SLN(s). Our development of such an assay and identification of discriminatory marker genes provides crucial data necessary for the incorporation of qRT-PCR into future clinical studies applying SLN mapping to clinical practice for patients with this disease. Separate studies evaluating its use in the post-treatment setting may be warranted. Further efforts, such as the conversion to a dry assay on the GeneXpertTM system, as well as the implementation of this technology into a multicenter SLN trial, are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Current algorithm for intraoperative diagnosis of SLN status in SCCN

Low sensitivity of intraoperative frozen section or touch prep leads to the need for 12 of 30 patients with micrometastasis to undergo repeat surgery (completion neck dissection), after final pathologic review of the SNB. A rapid (intraoperative, accurate test for metastasis would potentially reduce the need for a second procedure, and serve as an adjunct to pathologic review for intraoperative decision-making.

Figure 2. Pathologic and molecular tissue handling and sectioning protocol

Nodes were harvested at surgery and subjected to the sectioning process shown, for either histologic (H&E or IHC) or qRT-PCR analysis. This allowed for comparison of qRT-PCR results with histologic assessment of metastatic disease by H&E and IHC analysis, for correlation of molecular data with the “gold standard” diagnostic test.

A **Figure 3.**

A. Distribution of relative expression (upper panel) of PVA and TACSTD1 in histologically positive and negative lymph nodes as determined by review of adjacent tissue sections (research pathology) The Y-axis shows relative expression as delta CT and the horizontal line indicates the most accurate cut-off determined from ROC curve analysis (lower panel). **B. Marker combinations and correlation with lymph node classification according to research pathology.** Green open circles = negative nodes, red plus signs = positive nodes, Axes show ΔCT .

Figure 4. Reproducibility and linearity tests of the rapid, 35 minute assay using the GeneXpert™
The rapid assay is performed in the linear assay arrange with high reproducibility.

Figure 5. Results of the triplex GeneXpert assay compared to research pathology results
 The first panel shows the risk score (probability of being positive calculated based on the *TACSTD1* and *PVA* expression) for each node. The second panel shows expression of *TACSTD1* and *PVA* in positive (+) and negative (o) nodes. The graph shows horizontal and vertical boxed lines to identify the most accurate cut-off for positive and negative nodes determined by ROC curve analysis. Sensitivity and specificity are listed numerically in the table (third panel).

Table 1

Relative expression for selected tumor markers analyzed in SCCHN tumors and benign lymph nodes in primary marker screen

Gene	Accession Number	Median Tumor	Median Benign Node	Highest Benign Node	Lowest Tumor/Highest Benign Node	Median Tumor/Highest Benign Node
PVA	NM_001944	67.476213	—*	0.021197	1770.6	3183.3
TACSTD1	NM_002354	56.962817	0.009753	0.049549	339.0	1149.6
CK19	NM_002276	22.922226	0.003691	0.018136	0.3	1263.9
SCCA1/2	NM_006919	13.372098	0.000014	0.015571	147.0	858.8
NTS	NM_006183	4.880276	0.532185	2.321408	0.003	2.1
CK18	NM_000224	3.890994	0.033609	0.073557	22.1	52.9
PTHrP	NM_002820	3.175056	0.001186	0.004425	117.8	717.5
Survivin	NM_001169	0.570393	0.022251	0.113834	1.2	5.0
MMP7	NM_002423	0.506164	0.009453	0.099098	2.5	5.1
CEA	NM_004363	0.467097	0.000175	0.000270	340.1	1730.3
MAGEA3	NM_005362	0.457354	0.000328	0.001271	0.1	359.9

Note: Data are ranked by median expression in tumors.

* Nondetectable level in 40-cycle quantitative PCR.

Single- and two-marker prediction characteristics on positive and benign nodes in the secondary screen

Table 2

Marker	Observed data			Parametric bootstrap estimate *				Classification bias †
	Sensitivity	Specificity	Classification accuracy	Sensitivity	Specificity	Classification accuracy	Classification accuracy	
PVA	1	1	1	1	1	1	1	0
TACSTD1	1	0.952	0.975	0.983	0.944	0.962		0.013
PVA + TACSTD1	1	1	1	0.993	1	0.997		0.003

* Five hundred bootstrap samples of lymph node expression levels were generated and a new decision rule based on the most accurate cutoff was formulated each time (total of 500 decision rules). The optimism for each bootstrap sample is calculated as the difference between the classification statistics applied to the original data and applied to the bootstrap data. The average over all bootstrap samples is computed and reported as the bias in the values derived from the observed data (Efron's enhanced bootstrap prediction error estimate)

† Bias is the enhanced bootstrap estimate of optimism, or the amount that classification accuracy is overestimated when tested on the original data.

Clinical features and risk factors in 92 previously untreated SCCHN patients, from whom validation set lymph nodes (n=442) were harvested

Table 3

Age	60 yr
Gender	79M/13F
Smoking history	78/92 (84.8%)
Alcohol history	58/92 (63.0%)
pT status	
T1	28/92 (30.4%)
T2	25/92 (27.2%)
T3	20/92 (21.7%)
T4	19/92 (20.7%)
pN status	
N0	50/92 (54.3%)
N1	12/92 (13.1%)
N2	30/92 (32.6%)
N3	0/92 (0%)
Site:	
Oral cavity	57/92 (62%)
Oropharynx	11/92 (12%)
Larynx	20/92 (21.7%)
Hypopharynx	2/92 (2.2%)
Other	2/92 (2.2%)

Table 4

GeneXpert™ vs Research Pathology results
10-Fold Cross-Validation of GeneXpert Logistic Model

Logistic Model Cross-classification (Re-substitution)			
Research Pathology	Predicted Node Status		Total
	Pos	Neg	
Pos	33	3	36
Neg	3	64	67

Logistic Model 10-fold CV			
Research Pathology	Predicted Node Status		Total
	Pos	Neg	
Pos	30	6	36
Neg	5	62	67

Method	Sensitivity	Specificity	PPV	NPV	Accuracy
Resubstitution	.917	.955	.917	0.955	0.942
10 fold CV	.833	.625	0.857	0.912	0.893

Table 5

GeneXpert™ vs Consensus Pathology Results
10-Fold Cross-Validation of GeneXpert Logistic Regression Model

Logistic Model Cross-classification (Re-substitution)			
Consensus Pathology	Predicted Node Status		Total
	Pos	Neg	
Pos	37	6	43
Neg	4	56	60

Logistic Model 10-fold CV			
Consensus Pathology	Predicted Node Status		Total
	Pos	Neg	
Pos	35	8	43
Neg	6	54	60

Method	Sensitivity	Specificity	PPV	NPV	Accuracy
Resubstitution	.860	.933	.902	0.903	0.903
10 fold CV	.814	.900	.854	0.871	0.864