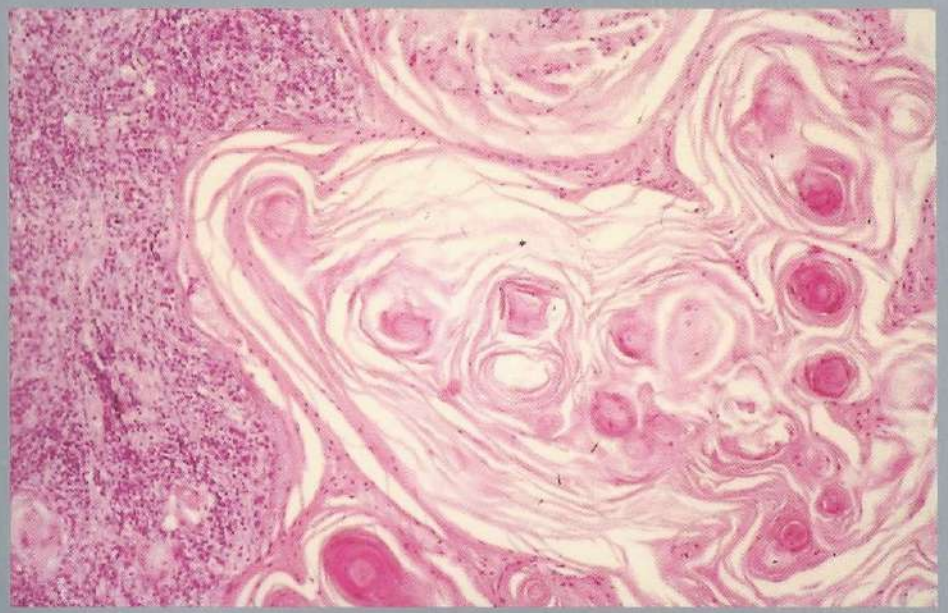
Staging of the N0 neck in oral cancer patients by USgFNAC Eline J.C. Nieuwenhuis 2003

Staging of the N0 neck in oral cancer patients by ultrasound guided fine needle aspiration cytology:

Is there a role for sentinel node identification and molecular diagnosis?



Eline J.C. Nieuwenhuis

Staging of the N0 neck in oral cancer patients by ultrasound guided fine needle aspiration cytology: is there a role for sentinel node identification and molecular diagnosis?

Eline J.C. Nieuwenhuis

The research described in this thesis was performed at the section Tumor Biology of the Department of Otolaryngology-Head and Neck Surgery, *Vrije Universiteit* Medical Center, Amsterdam, The Netherlands.
The research was financially supported by the Dutch Cancer Society (grant: VU 97-1967).

Thesis *Vrije Universiteit*, Amsterdam

ISBN 90-9017207-6

Printed by Budde Elinkwijk BV, Nieuwengein

ejcnieuwenhuis@hotmail.com

VRIJE UNIVERSITEIT

Staging of the N0 neck in oral cancer patients by ultrasound guided fine needle aspiration cytology:

Is there a role for sentinel node identification and molecular diagnosis?

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. T. Sminia,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op vrijdag 26 september 2003 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Eline Jeanette Catherina Nieuwenhuis

geboren te Sassenheim

promotoren: prof.dr. G.B. Snow
prof.dr. J.A. Castelijns

copromotoren: dr. R.H. Brakenhoff
dr. R. Pijpers

To my parents

To Menno

CONTENTS

1

General introduction

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) originating from the mucosal linings of the upper aerodigestive tract compromises about 5% of all newly diagnosed cancer cases in the Northern and Western European countries and the United States.[1] The most important route in the spread of HNSCC is through lymphatic pathways towards regional lymph nodes, rather than hematogenously to distant organs. The presence of lymph node metastases is the most important prognosticator for HNSCC, particularly as it is strongly associated with the risk of developing distant metastases. Distant metastases occur only in 7% of the patients without nodal metastases in the neck, while they occur in 50% of patients with > 3 lymph node metastases in the neck.[2] Apart from the presence of lymph node metastases, the number of metastases, the level(s) involved in the neck relative to the primary tumor, as well as the presence of extracapsular spread are important prognosticators.[3-7] In general, if lymph node metastases are diagnosed at presentation or develop during follow-up the survival rates are halved.

Table 1. UICC and AJCC staging of the neck

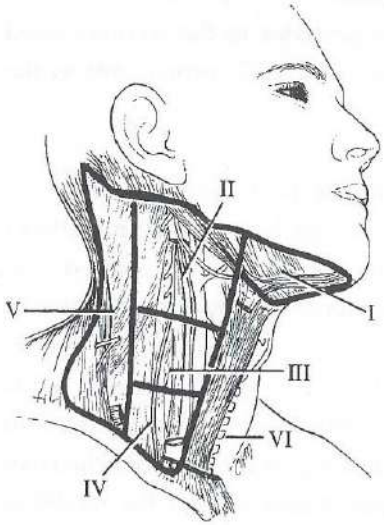
Nx: the neck cannot be assessed
N0: no regional lymph node metastases
N1: one ipsilateral metastasis ≤ 3 cm
N2a: one ipsilateral metastasis > 3 cm and ≤ 6 cm
N2b: multiple ipsilateral metastases ≤ 6 cm
N2c: contralateral or bilateral metastases ≤ 6 cm
N3: regional metastases > 6 cm

Tumor involvement of neck lymph nodes is categorized according to the classification of the UICC and AJCC (Table 1). The validity of any classification depends on the staging procedure employed. In this regard, the UICC and AJCC classification does not insist on additional staging

procedures to be used for the neck. Futhermore, this classification system does not incorporate the level(s) involved, which has been proven to be prognostically relevant.[3-7] Currently, most centers use the classification system of the American Academy of Otolaryngology/Head and Neck Surgery for the regional categorization of the lymph nodes in the neck (Figure 1).[8] In this classification system, level I corresponds to the submandibular and submental regions. Levels II, III and IV correspond to the jugulair chain nodes around the internal jugular vein (high-, mid-, and low-jugular). Level V harbour the lymph nodes posterior to the sternocleidoid muscle and the supraclavicular lymph nodes. Level VI corresponds to the juxtavisceral lymph nodes.

The presence of lymph node metastases in the neck does by no means always result in an incurable tumor proces. When lymph node metastases are diagnosed, the management is usually by surgery alone (in case of one lymph node metastasis without extracapsular spread) or by a combination of surgery followed by postoperative radiotherapy (in case of multiple lymph node metastases and/or the presence of extracapsular spread), while radiotherapy alone is appropriate in tumors which are primarily suited to treatment with radiotherapy, such as nasopharyngeal tumors. The choice of surgery lies between the classic radical neck dissection and the modified radical neck dissection, which involves the preservation of one or more non-lymphatic structures (accessory nerve, internal jugular vene and sternomastoid muscle), while others advocate selective neck dissection for N1 disease, which involves dissection of only those levels that are most likely to contain metastases depending upon the location of the primary tumor site with preservation of the non-lymphatic structures.[9-12] In general, the regional control rate after therapeutic neck dissection +/- postoperative radiotherapy in patients with oral or oropharyngeal squamous cell carcinoma varies from 88% to 94% when patients with recurrence at the primary site are excluded.[3,5,12,13] The number of tumor-positive lymph nodes and the presence of extracapsular spread are the most important prognosticators as to the risk of recurrence in the neck, as well as to the incidence of distant metastases. Failure in the neck after therapeutic neck dissection is almost invariably fatal.

Figure 1. Lymph node levels in the neck according to the classification system of the American Academy of Otolaryngology/Head and Neck Surgery



Level I: submandibular and submental lymph nodes
level II: subdigastric lymph nodes
level III: midjugular lymph nodes
level IV: low jugular lymph nodes
level V: lymph nodes located in the posterior triangle
level VI: juxtavisceral lymph nodes

MANAGEMENT OF THE CLINICALLY N0 NECK: A CONTROVERSIAL ISSUE

The management of the clinically N0 neck continues to be a controversial issue. There is general agreement that elective treatment of the neck is indicated when there is a high likelihood of occult lymph node metastases *and* when the neck needs to be entered for surgical treatment of the primary tumor and reconstruction, or the status of the neck cannot be adequately assessed, or the patient will be unavailable for regular follow-up. When there is *merely* a high likelihood of occult lymph node metastases, the choice is between elective treatment and watchful waiting. This question mainly arises in the smaller (T1 and T2) carcinomas of the oral cavity and oropharynx, because these usually can be excised adequately by the transoral route.

The rationale for elective treatment is based on the following premises. Firstly, occult metastases will inevitably develop into clinically manifest disease. Secondly, despite regular follow-up some patients will develop inoperable disease in the neck with a wait-and-see policy. Finally, untreated disease in the neck may give rise to distant metastases, while the lymph node metastasis is growing to clinically detectable size. The arguments against elective treatment of the neck are as follows. Firstly, a large proportion of patients are subjected to treatment that they do not require. Secondly, such treatment may remove or destroy a barrier to cancer spread in case of local recurrence or second primary tumor. Finally, elective treatment of the neck is associated with morbidity, although this is low with the modified types of neck dissection currently in use.[14-17]

Only few randomized trials have been performed to compare cure rates of patients who underwent elective neck treatment with those of patients subjected to a primary tumor excision only and a wait-and-see policy for the neck with delayed neck treatment once lymph node metastases become manifest during follow-up.[18-20] Although one of these studies showed a slight benefit in survival in favour of the patients treated with elective neck

dissection, none of these studies demonstrated a significant difference in survival between these two treatment policies. The drawback of these studies is the small number of patients included. However, a proper randomized trial on T1-T2 oral cancer patients will never be carried out because several thousands of patients are needed to allow for conclusions.

In most institutions around the world a frequency of occult metastases exceeding 20% is considered to be sufficient to justify elective treatment of the neck nodes in patients with squamous cell carcinoma of the upper aerodigestive tract.[21] When the assessment of the status of the neck nodes is carried out by means of *palpation alone*, most sites and stages of squamous cell carcinoma, including T1 and T2 tumors of the oral cavity and oropharynx, qualify for elective treatment on this basis (Table 2).[18-20, 22-28]

If the risk of undetected lymph node metastases can be reduced to below 20%, most head and neck surgeons would opt for watchful waiting. The question arises whether imaging techniques like Computer Tomography (CT), Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET), Ultrasound (US) or US guided Fine Needle Aspiration Cytology (USgFNAC) can achieve this aim.

Table 2. Percentage occult lymph node metastases in oral cancer patients with a clinically N0 neck treated with transoral excision and a wait-and-see policy for the neck or treated with combined primary tumor excision and elective neck dissection.

Author	Treatment	No. of patients	Occult metastases
Vandenbrouck *et al.*[18]	W&S	36	19/36 (47%)
	END	39	19/36 (49%)
Fakih *et al.*[19]	W&S	40	23/40 (57%)
	END	30	10/30 (33%)
Kligerman *et al.*[20]	W&S	33	11/33 (33%)
	END	34	7/34 (21%)
Khafif *et al.*[22]	W&S	137	95/137 (69%)
	END	99	42/99 (42%)
McGuirt *et al.*[23]	W&S	103	37/103 (36%)
	END	26	6/26 (23%)
Shah *et al.*[24]	END	192	65/192 (34%)
Cunningham *et al.*[25]	W&S	43	18/43 (42%)
Ho *et al.*[26]	W&S	28	13/28 (46%)
Byers *et al.*[27]	END	58	26/58 (45%)
Stoeckli *et al.*[28]	END	19	6/19 (32%)

In column 2 the initial treatment for the neck is indicated. Column 4 shows the number of patients who developed lymph node metastases during follow-up or the number of patients with lymph node metastases in their neck dissection specimen. W&S, wait-and-see policy for the neck; END, elective neck dissection.

STAGING OF THE N0 NECK

In many institutions throughout the world the neck is mainly staged by palpation. Although palpation has the advantage of being both easy to perform and inexpensive, it is generally accepted to be unreliable.[29-33] Therefore, much effort has been devoted to implement more accurate pre-operative staging techniques like CT, MRI, PET, US and USgFNAC.

CT and MRI

Some authors have discussed the issue of accuracy of CT and/or MR for the assessment of the N0 neck.[34-42] However, the sensitivities and specificities reported in these studies vary considerably. In general, between 40% and 60% of all non-palpable metastases are found using either one of these imaging techniques, but at the cost of a 25% to 35% false-positive rate. In most studies that compared accuracy of CT with MRI for the assessment of the neck, no significant differences between these modalities were found.[31,34,38] Several radiological criteria have been formulated to distinguish on CT or MRI normal or reactively enlarged lymph nodes from enlarged tumor-containing lymph nodes. These criteria include central necrosis and size, such as increased minimal axial diameter.[43] Central necrosis is the most reliable radiological criterium and can be recognized relatively easily. Unfortunately, necrosis is rarely found in small metastatic nodes. Although the search for more accurate and reliable criteria is progressing with exploration of other characteristics, such as those based on the use of superparamagnetic contrast agents [44] and dynamic-contrast-enhanced MRI,[45] the accuracy of CT and MRI to differentiate between reactively enlarged lymph nodes from those containing a metastatis in the N0-neck is still largely restricted by size criteria, which are apparently not accurate enough.

PET

More recently, other techniques like PET scanning were shown to allow detection of lymph node metastases in patients with a clinically N0 neck, and this new imaging technique shows promise for the future.[46-49] However, the published studies show conflicting results, and the value of this technique in patients with an N0 neck has yet to be proven.

US and USgFNAC

Besides CT , MRI and PET scanning also ultrasound imaging is widely used for neck staging, and it also has been reported to be superior to palpation in detecting lymph node metastases.[29,32] It is a relatively quick and safe technique, and suitable for repeated use during follow-up. Because many authors have found that lymph node metastases, can not be reliably diagnosed by using US alone, US combined with Fine Needle Aspiration Cytology has gained popularity, especially in Europe. In USgFNAC the lymph nodes at risk of harbouring metastases are being aspirated. From the aspirates cytological slides are prepared that are screened by microscopic examination by the pathologist for the presence of malignant cells. Particularly, selection of the most suspicious lymph nodes for aspiration is crucial, and is based on clinical information of the primary tumor, knowledge about the patterns of metastases from tumors at specific anatomical locations, and size criteria. Lymph nodes with a minimal axial diameter above 3 mm in level I and above 4 mm in the other levels are aspirated. This technique combines the relatively high sensitivity of US with the high specificity of fine needle aspiration cytology. Regarding the N0-neck the specificity has been reported to be 100%, and the sensitivity has been reported to vary between 42% and 73%.[29,31,35,50-53] The accuracy of USgFNAC very much depends on the experience of the ultrasonographer.

Since 1992, the management policy in our Institution has changed in patients with T1 or T2 oral and oropharyngeal carcinomas treatable by transoral excision of the primary tumor. Before 1992, elective treatment of the neck was included in the initial management in the majority of these patients. Since 1992, these patients are treated by transoral excision of the primary tumor only and a wait-and-see policy for the neck, when lymph nodes do not meet the size criteria to be aspirated or in case of tumor-negative cytology. The patients are frequently followed with intervals of 6 weeks, including USgFNAC examinations every 3 months in the first year after tumor excision, and afterwards when indicated. In an initial retrospective study of 77 patients treated according to this policy, it was shown that 14 (18%) patients developed lymph node metastases during follow-up.[54] Of these 14 patients 10 (71%) could be salvaged by delayed neck dissection +/- postoperative radiotherapy. Despite these encouraging clinical results, a further decrease of the false-negative rate is desirable. Possible causes of false-negative USgFNAC cases could be that 1) tumor containing lymph nodes do not meet US size criteria and are not aspirated, 2) aspiration is performed in a tumor-free part of a lymph node harboring a small metastasis (sampling error) or 3) the cytologic aspirate could not be evaluated or few tumor cells were missed. Sensitivity of USgFNAC might therefore be improved by a more adequate selection of the lymph node(s) at highest risk of harboring occult metastases, i.e. most notably the sentinel node. Secondly, molecular analysis of aspirated material might result in an improvement of routine cytology and a decrease of the false-negative USgFNAC rate.

THE SENTINEL NODE CONCEPT

Sentinel lymph node (SN) biopsy has been proposed as a potential alternative for elective lymph node dissection. The SN concept is fundamentally based on the theory of orderly spread of tumor cells within the lymphatic system. The first lymph node in a regional lymphatic basin that receives lymphatic flow from a tumour is considered to be the SN. The SN concept assumes that lymphatic metastases, if present, can always be found at least in the SN. A tumor-negative SN would preclude the presence of lymphatic malignant involvement.

In 1959 Gould presented a paper entitled: "Observations on a sentinel node in cancer of the parotid."[55] He suggested that an intraoperative frozen section of the SN could guide the surgeon in his decision concerning the necessity for a radical neck dissection. Despite this publication not much attention was paid to the implications of this procedure. Almost 20 years later Cabanas demonstrated the existence of "the so-called sentinel lymph node", by performing lymphangiograms via the dorsal lymphatics of the penis.[56] Guided by anatomical landmarks he determined which node was the SN. Again, the method was not widely accepted, probably due to the relatively crude localization technique. In 1992 the principle of the SN biopsy was reintroduced by Morton *et al.* in melanoma patients.[57-59] The authors used a blue dye to visualize the lymphatic channels and followed the lymphatic flow to the first blue appearing lymph node: the SN. Soon radio-labeled colloids and gamma-probe detection were introduced to identify the SN.

At present, many investigators have been assessing the feasibility and reliability of SN identification and biopsy. SN biopsy has been rapidly gaining popularity as a diagnostic procedure for several solid tumours, in particular for melanoma and breast cancer. In large series of patients with breast cancer and melanoma, preoperative SN identification by lymphoscintigraphy and subsequent SN biopsy has been shown to be an accurate staging technique. Histopathological analysis of the SN correctly reflects regional lymph node status in >98% of the cases.[60-63]

In the staging of head and neck cancer patients SN identification was introduced much later. At the time that the research presented in this thesis was conducted only few studies had been published and the results reported were not consistent. Particularly the large number of lymph nodes in the neck could hamper the accurate detection of the SN, and the relatively mild morbidity of modified radical or selective neck dissection in comparison to axillary dissection in for instance breast cancer did not lead to a great interest in the development of the methodology for this patient group. Moreover, SN identification with a radioactive tracer requires that the primary tumor is readily accessible for peritumoral injection, which is often not the case in head and neck cancer patients. Therefore, our study as well as the studies of others focus mainly on patients presenting with tumors in the oral cavity and to a lesser extent with tumors in the oropharynx.

MOLECULAR DIAGNOSIS

Recently, molecular techniques have been applied in a number of studies in head and neck cancer patients to detect the presence of micrometastases in cervical lymph nodes that are missed at routine histology.[64-66] In other types of cancer, including breast cancer and melanoma, the molecular detection of micrometastases in lymph nodes of patients with histologically tumor-negative lymph nodes has been shown to be prognostically important.[67-69] These studies emphasize the importance of molecular assessment of micrometastases in histopathologically tumor-negative lymph nodes, which may improve tumor staging.

Different types of molecular markers can be exploited for the detection of cancer cells in clinical samples, depending on the tissue compartment studied. These markers should be specific for tumor cells such that they correctly distinguish between normal cells and tumor cells. Furthermore, these markers should be present in a large part of a study population, in order to be broadly applicable. Tissue-specific markers (RNA/protein) like differentiation antigens such as keratins can be exploited for tumor cell detection in blood and bone marrow, while they cannot be used for detection of cancer cells in tissue samples such as resection margins where the normal counterparts (keratinocytes) are also present. In these cases tumor-specific genetic alterations (DNA markers), such as p53 mutations, can be exploited, and these have been used widely for molecular diagnosis in HNSCC and other tumor types.[70-75]

E48 antigen as molecular marker

At our laboratory, we are focusing on the exploitation of the tissue-specific marker antigen E48 (Ly-6D) as a potential marker for the detection of SCC. This antigen is a small membrane protein (15-22 kD) exclusively expressed on the outer surface of squamous carcinoma cells and their normal counterparts: the cells of transitional epithelia and the keratinocytes of stratified squamous epithelia.[76] In vitro experiments provided evidence that

the antigen is involved in signaling and (desmosomal) cell-cell adhesion of keratinocytes.[77] In 1995, the cDNA encoding the E48 antigen was cloned, and the gene structure was elucidated.[78,79] Sequence analysis revealed that the E48 antigen is a glycosylphosphatidylinositol-anchored membrane protein that shares 72% sequence homology with the mouse ThB antigen, a member of the murine Ly-6 family. Monoclonal antibody E48 that recognizes the antigen has been tested extensively in radioimmunoscintigraphy and biodistribution studies in HNSCC patients, demonstrating the in vivo tissue-specificity of the antigen.[80-82] 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.[83] However, the detection method used was a qualitative reverse transcriptase polymerase chain reaction (RT-PCR), whereas for the molecular detection of tumor cells in lymph node aspirates more precise quantification is preferred.

In initial experiments it was shown that lymph nodes of non-cancer controls showed a minor but consistent background signal, which hampers deciding whether a sample is positive for squamous tumor cells or negative. For these considerations it was decided to set-up a quantitative RT-PCR assay.

Real-time RT-PCR for squamous tumor cell detection using E48 as marker

In recent years, polymerase chain reaction (PCR) has proven to be a powerful tool for qualitative and semi-quantitative nucleic acid analysis. PCR and reverse transcriptase (RT)-PCR have permitted the analysis of minimal starting quantities of nucleic acid (as little as one cell equivalent). Although PCR has provided a powerful tool, it was a drawback that it could not be used quantitatively.[84] Several methods have been evaluated to perform quantitative (RT-)PCR analysis, but all these methods used endpoint measurement of the amount of amplimer formed at 30 or 40 cycles, while it is well known that the amount of amplimer at endpoint is hardly related to the amount of template at the start of the first cycle.[85-87] Moreover, these methods require various post-PCR manipulations that hamper a high sample throughput and might cause carry-over contamination.

Recently, a quantitative PCR system has been developed based on continuously monitoring fluorescence of dyes attached to an oligonucleotide that hybridizes as probe to an internal specific sequence between the PCR amplification primers.[88] The assay is based on the use of the 5'-3` exonuclease activity of *Taq* polymerase to cleave this nonextendible dual-labeled fluorogenic probe during the extension phase of PCR.[89,90] One fluorescent dye serves as a reporter and its emission spectra is quenched by the second fluorescent dye. Exonuclease degradation of the hybridization probe by the *Taq* polymerase causes separation of the quenching and reporter dye, resulting in an increase in peak fluorescent emission at 518 nm, which is measured by a light detector. By real time monitoring the logarithmic phase of amplification can be followed, and the cycles that the amplification passes a certain treshold during the logarithmic phase (the Ct value) is strongly correlated to the amount of template in the initial reaction mixture.

We decided to set up a quantitative real-time RT-PCR assay (Q-RT-PCR) using E48 as squamous cell specific molecular marker. For internal control of the RNA quality, and to determine the number of cells in the aspirate, we used mRNA transcribed from the gene encoding porphobilinogen deaminase (PBGD), a consistently expressed housekeeping gene.[91] Quantification of mRNA expression levels by real-time RT-PCR (Q-RT-PCR) is a sensitive and simple method that enables analysis of gene expression using very small amounts of mRNA while it allows at the same time analysis of a large number of samples and many different genes in the same experiment.

OUTLINE OF THIS THESIS

In our Department patients with T1-T2 N0 oral and oropharyngeal carcinomas undergo USgFNAC for initial staging of the neck. In case of negative USgFNAC at initial staging, these patients are treated by local excision of the primary tumor only and are spared an elective lymph node dissection. These patients are subsequently followed by palpation and USgFNAC at regular intervals. The retrospective study described above demonstrated a false-negative USgFNAC rate of approximately 20%.[42] These clinical data indicate that USgFNAC does not enable detection of all metastases. Possible causes of false negative results are: 1) tumor containing lymph nodes do not meet US size criteria and are not aspirated, 2) aspiration is performed in a tumor-free part of a lymph node harboring a small metastasis (sampling error), and 3) the aspirate was not evaluable by routine cytology or few tumor cells were missed. The aims of this thesis were to investigate whether identification and aspiration of the SN could improve the sensitivity of USgFNAC, and whether molecular diagnosis of the aspirates could improve routine cytology.

In **Chapter 2** the initial experience with the combined use of lymphoscintigraphic SN detection and (SN)-USgFNAC in 12 patients is described to investigate the feasibility of a SN procedure in patients with oral and oropharyngeal carcinoma.

In **Chapter 3** the lymphoscintigraphic findings in 82 patients explaining variables affecting SN visualization at lymphoscintigraphy is described. The number and localization of SNs are documented and recommendations concerning the imaging protocol are described.

To determine whether the SN concept holds true for HNSCC, and could be exploited for SN biopsy, preoperative SN identification using lymphoscintigraphy was carried out in a series of patients with cancer of the oral cavity or oropharynx, who were planned to undergo combined primary tumor excision and neck dissection. Both SNs and all other lymph nodes in the neck dissection specimens were thoroughly investigated by the pathologist, including serial sectioning at 250 µm intervals and immunohistochemistry. The results are described in **Chapter 4.**

In **Chapter 5** the additional value of SN identification and subsequent USgFNAC of the SN was investigated on the outcome of patients staged as N0 by USgFNAC who underwent transoral tumor excision and a wait-and-see policy for the neck.

In **Chapter 6** the real-time quantitative E48 RT-PCR assay for the detection of micrometastases in lymph node aspirate residues of HNSCC patients is reported. E48 Q-RT-PCR results of the lymph nodes aspirate residues were compared to cytology and clinical outcome.

In **Chapter 7** we describe the results of E48 Q-RT-PCR for the detection of disseminated tumor cells and micrometastases in histologically tumor-negative lymph nodes of patients without lymph node metastases and patients with one or two tumor-positive lymph nodes in their neck dissection specimens. The results were compared to histopathology and clinical outcome.

REFERENCES

1. Muir C, Weiland L. Upper aerodigestive tract cancers. *Cancer* 75:147-153, 1995.

2. Leemans CR, Tiwari R, Nauta JJP, Van der Waal I, Snow GB. Regional lymph node involvement and its significance in the development of distant metastases in head and neck cancer patients. *Cancer* 71:452-456, 1993.

3. Snow GB, Annyas AA, van Slooten EA, Bartelink H, Hart AA. Prognostic factors of neck node metastasis. *Clin Otolaryngol* 7:185-192, 1982.

4. Shah JP. Cervical lymph node metastases--diagnostic, therapeutic, and prognostic implications. *Oncology* 4:61-69, 1990.

5. Leemans CR, Tiwari R, van der Waal I, Karim AB, Nauta JJ, Snow GB. The efficacy of comprehensive neck dissection with or without postoperative radiotherapy in nodal metastases of squamous cell carcinoma of the upper respiratory and digestive tracts. *Laryngoscope* 100:1194-1198, 1990.

6. Stell PM, Morton RP, Singh SD. Cervical lymph node metastases: the significance of the level of the lymph node. *Clin Oncol* 9:101-107, 1983.

7. Jones AS, Roland NJ, Field JK, Phillips DE. The level of cervical lymph node metastases: their prognostic relevance and relationship with head and neck squamous carcinoma primary sites. *Clin Otolaryngol* 19:63-69, 1994.

8. Shah JP, Strong E, Spiro RH, Vikram B. Neck dissection: current status and future possibilities. *Clin Bulletin* 11:25-33, 1981.

9. Byers RM, Wolf PF, Ballantyne AJ. Rationale for elective modified radical neck dissection. *Head Neck Surg*10:160-167, 1988.

10. Majoufre C, Faucher A, Laroche C, De Bonfils C, Siberchicot F, Renaud-Salis JL, Pinsolle J. Supraomohyoid neck dissection in cancer of the oral cavity. *Am J Surg* 178:73-77, 1999.

11. Kolli VR, Datta RV, Orner JB, Hicks WL Jr, Loree TR. The role of supraomohyoid neck dissection in patients with positive nodes. *Arch Otolaryngol Head Neck Surg* 126:413-416, 2000.

12. Andersen PE, Warren F, Spiro J, Burningham A, Wong R, Wax M,K, Shah JP, Cohen JI. Results of selective neck dissection in management of the node-positive neck. *Arch Otolaryngol Head Neck Surg* 128:1180-1184, 2002.

13. Andersen PE, Shah JP, Cambronero E, Spiro RH. The role of comprehensive neck dissection with preservation of the spinal accessory nerve in the clinically positive neck. *Am J Surg* 168:499-502, 1994.

14. Kuntz AL, Weymuller EA, Jr. Impact of neck dissection on quality of life. *Laryngoscope* 109:1334-1338, 1999.

15. Magrin J, Kowalski L. Bilateral radical neck dissection: results in 193 cases. *J Surg Oncol* 75:232-240, 2000.

16. Shah S, Har-El G, Rosenfeld RM. Short-term and long-term quality of life after neck dissection. *Head Neck* 23:954-961, 2001.

17. El Ghani F, Van Den Brekel MW, De Goede CJ, Kuik J, Leemans CR, Smeele LE. Shoulder function and patient well-being after various types of neck dissections. *Clin Otolaryngol* 27:403-408, 2002.

18. Vandenbrouck C, Snacho-Garnier H, Chassagne D, Saravane D, Cachin Y, Micheau C. Elective versus therapeutic radical neck dissection in epidermoid carcinoma of the oral cavity-results of a randomized clinical trial. *Cancer* 46:386-390, 1980.

19. Fakih AR, Rao RS, Borges AM, Patel AR. Elective versus therapeutic neck dissection in early carcinoma of the oral tongue. *Am J Surg* 158:309-313, 1989.

20. Kligerman J, Lima RA, Soares JR, Prado L, Dias FL, Freitas EQ, Olivatto LO. Supraomohyoid neck dissection in the treatment of T1/T2 squamous cell carcinoma of oral cavity. *Am J Surg* 168:391-394, 1994.

21. Weiss MH, Harrison LB, Isaacs RS. Use of decision analysis in planning a management strategy for the stage N0 neck. *Arch Otolaryngol Head Neck Surg* 120:699-702, 1994.

22. Khafif RA, Gelbfish GA, Tepper P, Attie JN. Elective radical neck dissection in epidermoid cancer of the head and neck. A retrospective analysis of 853 cases of mouth, pharynx, and larynx cancer. *Cancer* 67:67-71, 1991.

23. McGuirt WF, Johnson JT, Myers EN, Rothfield R, Wagner R. Floor of mouth carcinoma. The management of the clinically negative neck. *Arch Otolaryngol Head Neck Surg* 121:278-282, 1995.

24. Shah JP, Candela FC, Poddar AK. The patterns of cervical lymph node metastases from squamous carcinoma of the oral cavity. *Cancer* 66:109-113, 1990.

25. Cunningham MJ, Johnson JT, Myers EN, Schramm VL, Thearle PB. Cervical lymph node metastasis after local excision of early squamous cell carcinoma of the oral cavity. *Am J Surg* 152:361-366, 1986.

26. Ho CM, Lam KH, Wei WI, Lau SK, Lam LK. Occult lymph node metastasis in small oral tongue cancers. *Head Neck* 14:359-363, 1992.

27. Byers RM, Clayman GL, McGill D, Andrews T, Kare RP, Roberts DB, Goepfert HG. Selective neck dissections for squamous cell carcinoma of the upper aerodigestive tract: patterns of regional failure. *Head Neck* 21:499-505, 1999.

28. Stoeckli SJ, Pfaltz M, Steinert H, Schmid S. Histopathological features of occult metastasis detected by sentinel lymph node biopsy in oral and oropharyngeal squamous cell carcinoma. *Laryngoscope* 112:111-115, 2002.

29. Baatenburg de Jong RJ, Rongen RJ, Lameris JS, Harthoorn M, Verwoerd CD, Knegt P. Metastatic neck disease: Palpation vs ultrasound examination. *Arch Otolaryngol Head Neck Surg* 115:689-690, 1989.

30. Van den Brekel MW, Castelijns JA, Croll GA, Stel HV, Valk J, van der Waal I, Golding RP, Meyer CJ, Snow GB. Magnetic resonance imaging vs palpation of cervical lymph node metastasis. *Arch Otolaryngol Head Neck Surg* 117:663-673, 1991.

31. Van den Brekel MWM, Castelijns JA, Stel HV, Golding RP, Meijer CJLM, Snow GB. Modern imaging techniques and ultrasound-guided aspiration cytology for the assessment of neck node metastases: a prospective comparative study. *Eur Arch Otorhinolaryngol* 250:11-17, 1993.

32. Atula TS, Varpula MJ, Kurki TJ, Klemi PJ, Grenman, R. Assessment of cervical lymph node status in head and neck cancer patients: palpation, computed tomography and low field magnetic resonance imaging compared with ultrasound-guided fine-needle aspiration cytology. *Eur J Radiol* 25:152-161, 1997.

33. Hao SP, Ng SH. Magnetic resonance imaging versus clinical palpation in evaluating cervical metastasis from head and neck cancer. *Otolaryngol Head Neck Surg* 123:324-327, 2000.

34. Friedman M, Mafee MF, Pacella BL Jr, Strorigl TL, Dew LL, Toriumi, DM. Rationale for elective neck dissection in 1990. *Laryngoscope* 100:54-59, 1990.

35. Righi PD, Kopecky KK, Caldemeyer KS, Ball VA, Weisberger EC, Radpour S. Comparison of ultrasound-fine needle aspiration and computed tomography in patients undergoing elective neck dissection. *Head Neck* 19:604-610, 1997.

36. Yucel T, Saatci I, Sennaroglu L, Cekirge S, Aydingoz U, Kaya, S. MR imaging in squamous cell carcinoma of the head and neck with no palpable lymph nodes. *Acta Radiol* 38:810-814, 1997.

37. Stern WB, Silver CE, Zeifer BA, Persky MS, Heller KS. Computed tomography of the clinically negative neck. *Head Neck* 12:109-113, 1990.

38. Hillsamer PJ, Schuller DE, McGhee RB, Chakeres D, Young DC. Improving diagnostic accuracy of cervical metastases with computed tomography and magnetic resonance imaging. *Arch Otolaryngol Head Neck Surg* 116:1297-1301, 1990.

39. Atula TS, Varpula MJ, Kurki TJI, Klemi PJ, Grenman R. Assessment of cervical lymph node status in head and neck cancer patients: palpation, computed tomography and low field magnetic resonance imaging compared withultrasound-guided fine needle aspiration cytology. *Eur J Radiol* 25:152-161, 1997.

40. Watkinson JC, Todd CE, Paskin L, Rankin S, Palmer T, Shaheen OH, Clarke SE. Metastatic carcinoma in the neck: A clinical/radiological/scintigraphic and pathological study. *Clin Otolaryngol* 16:187-192, 1991.

41. Stevens MH, Harnsberger R, Mancuso AA, Davis RK, Johnson LP, Parkin JL. Computed tomography of cervica lymph nodes; staging and management of head and neck cancer. *Arch Otolaryngol Head Neck Surg* 111:735-739, 1985.

42. Moreau P, Goffart Y, Collignon. Computed tomography of metastatic cervical lymph nodes. *Arch Otolaryngol Head Neck Surg* 116:1190-1193, 1990.

43. Van den Brekel MW, Stel HV, Castelijns JA, Nauta JJ, van der Waal I, Valk J, Meyer CJ, Snow GB. Cervical lymph node metastasis: assessment of radiologic criteria. Radiology 1990;177:379-384.

44. Anzai Y, Blackwell KE, Hirschowitz SL, Rogers JW, Sato Y, Yuh WT, Runge V, Morris MR, McLachlan SJ, Lufkin RB. Initial clinical experience with dextran-coated superparamagnetic iron-oxide for detection of lymph node metastases in patients with head and neck cancer. *Radiology* 192:617-618, 1994.

45. Noworolski SM, Fischbein NJ, Kaplan MJ, Lu Y, Nelson SJ, Carvajal L, Henry RG. Challenges in dynamic contrast-enhanced MR imaging of cervical lymph nodes to detect metastatic disease. *J Magn Reson Imaging* 17:455-462, 2003.

46. Stoeckli SJ, Steinert H, Pfaltz M, Schmid S. Is there a role for positron emission tomography with 18F- fluorodeoxyglucose in the initial staging of nodal negative oral and oropharyngeal squamous cell carcinoma. *Head Neck* 24:345-349, 2002.

47. Kresnik E, Mikosch P, Gallowitsch HJ, Kogler D, Wiesser S, Heinisch M, Unterweger O, Raunik W, Kumnig G, Gomez I, Grunbacher G, Lind P. Evaluation of head and neck cancer with 18F-FDG PET: a comparison with conventional methods. *Eur J Nucl Med* 28:816-821, 2001.

48. McGuirt WF, Williams DW 3rd, Keyes JW Jr, Greven KM, Watson NE Jr, Geisinger KR, Cappellari JO. A comparative diagnostic study of head and neck nodal metastases using positron emission tomography. *Laryngoscope* 105:373-375, 1995.

49. Adams S, Baum RP, Stuckensen T, Bitter K, Hor G. Prospective comparison of 18F-FDG PET with conventional imaging modalities (CT, MRI, US) in lymph node staging of head and neck cancer. *Eur J Nucl Med* 25:1255-1260, 1998.

50. Van den Brekel MW, Castelijns JA, Stel HV, Luth WJ, Valk J, Van der Waal I, Snow GB. Occult metastatic neck disease: detection with US and US-guided fine- needle aspiration cytology. *Radiology* 180:457-461, 1991.

51. Takes RP, Knegt P, Manni JJ, Meeuwis CA, Marres HAM, Spoelstra HAA, de Boer MF, Bruaset I, van Oostayen JA, Lameris JS, Kruyt RH, Joosten FBM, van Krieken HJM, Bosman FT, Henzen-Logmans SC, Wiersma-van Tilburg JM, Hermans J, Baatenburg de Jong RJ. Regional metastasis in head and neck squamous cell carcinoma: revised value of US with US-guided FNAB. *Radiology* 198:819-823, 1996.

52. Takes RP, Righi P, Meeuwis CA, Manni JJ, Knegt P, Marres HA, Spoelstra HA, de Boer MF, van der Mey AG, Bruaset I, Ball V, Weisberger E, Radpour S, Kruyt RH, Joosten FB, Lameris JS, van Oostayen JA, Kopecky K, Caldemeyer K, Henzen-Logmans SC, Wiersma-van Tilburg JM, Bosman FT, van Krieken JH, Hermans J, Baatenburg de Jong RJ. The value of ultrasound with ultrasound-guided fine-needle aspiration biopsy compared to computed tomography in the detection of regional metastases in the clinically negative neck. *Int J Radiat Oncol Biol Phys* 40:1027-1032, 1998.

53. Knappe M, Louw M, Gregor RT. Ultrasonography-guided fine-needle aspiration for the assessment of cervical metastases. *Arch Otolaryngol Head Neck Surg* 126:1091-1096, 2000.

54. Van den Brekel MW, Castelijns JA, Reitsma LC, Leemans CR, van der Waal I, Snow GB. Outcome of observing the N0 neck using ultrasonographic-guided cytology for follow-up. *Arch Otolaryngol Head Neck Surg* 125:153-156, 1999.

55. Gould EA, Winship T, Philbin PH, Hyland Kerr H. Observations on a "sentinel node" in cancer of the parotid. *Cancer* 13:77-78, 1960.

56. Cabanas, R. M. An approach for the treatment of penile carcinoma. *Cancer* 39:456-466, 1977.

57. Morton DL, Wen DR, Wong JH, Economou JS, Cagle LA, Storm FK, Foshag LJ, Cochran AJ. Technical details of intraoperative lymphatic mapping for early stage melanoma. *Arch Surg* 127:392-399, 1992.

58. Morton D, Wen D-R, Foshag L, Essner R, Cochran A. Intraoperative lymphatic mapping and selective cervical lymphadenectomy for early-stage melanomas of the head and neck. *J Clin Oncol* 11:1751-1756, 1993.

59. Cochran AJ, Wen DR, Morton DL. Management of the regional lymph nodes in patients with cutaneous malignant melanoma. *World J Surg* 16:214-221, 1992.

60. Pijpers R, Borgstein PJ, Meijer S, Hoekstra OS, van Hattum LH, Teule GJ. Sentinel node biopsy in melanoma patients: dynamic lymphoscintigraphy followed by intraoperative gamma probe and vital dye guidance. *World J Surg* 21:788-792, 1997.

61. Borgstein PJ, Pijpers R, Comans EF, van Diest PJ, Boom RP, Meijer S. Sentinel lymph node biopsy in breast cancer: guidelines and pitfalls of lymphoscintigraphy and gamma probe detection. *J Am Coll Surg* 186:275-283, 1998.

62. Morton DL, Thompson JF, Essner R, Elashoff R, Stern SL, Nieweg OE, Roses DF, Karakousis CP, Mozzillo N, Reintgen D, Wang HJ, Glass EC, Cochran AJ. Validation of the accuracy of intraoperative lymphatic mapping and sentinel lymphadenectomy for early-stage melanoma: a multicenter trial. Multicenter Selective Lymphadenectomy Trial Group. *Ann Surg* 230:453-463, 1999.

63. Muller MG, Borgstein PJ, Pijpers R et al. Reliability of the sentinel node procedure in melanoma patients: analysis of failures after long-term follow-up. *Ann Surg Oncol* 7:461-468, 2000.

64. McDonald LA, Walker DM, Gibbins JR. Cervical lymph node involvement in head and neck cancer detectable as expression of a spliced transcript of type II keratin K5. *Oral Oncology* 34:276-283, 1997.

65. Hamakawa H, Fukizumi M, Bao Y, Sumida T, Onishi A, Tanioka H, Sato H, Yumoto E. Genetic diagnosis of micrometastasis based on SCC antigen mRNA in cervical lymph nodes of head and neck cancer. *Clin Exp Metastasis* 17:593-599, 1999.

66. Cortesina G, Martone T, Galeazzi E, Olivero M, De Stefani A, Bussi M, Valente G, Comoglio PM, Di Renzo MF. Staging of head and neck squamous cell carcinoma using the MET oncogene product as marker of tumor cells in lymph node metastases. *Int J Cancer* 89:286-292, 2000.

67. McGuckin MA, Cummings MC, Walsh MD, Hohn BG, Bennett IC, Wright RG: Occult axillary node metastases in breast cancer: their detection and prognostic significance. *Br J Cancer* 73:88-95, 1996.

68. Cote RJ, Peterson HF, Chaiwun B, Gelber RD, Goldhirsch A, Castiglione-Gertsch M, Gusterson B, Neville AM: Role of immunohistochemical detection of lymph-node metastases in management of breast cancer. International Breast Cancer Study Group. *Lancet* 354:896-900, 1999.

69. Bostick PJ, Morton DL, Turner RR, Huynh KT, Wang HJ, Elashoff R, Essner R, Hoon DS: Prognostic significance of occult metastases detected by sentinel lymphadenectomy and reverse transcriptase-polymerase chain reaction in early-stage melanoma patients. *J Clin Oncol* 17:3238-3244, 1999.

70. Gath HJ, Brakenhoff RH: Minimal residual disease in head and neck cancer. *Cancer Metastasis Rev* 18:109-126, 1999.

71. Van Houten VM, Tabor MP, van den Brekel MW, Denkers F, Wishaupt RG, Kummer JA, Snow GB, Brakenhoff RH: Molecular assays for the diagnosis of minimal residual head-and-neck cancer: methods, reliability, pitfalls, and solutions. *Clin Cancer Res* 6:3803-3816, 2000.

72. Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, Goodman SN, Sidransky D: Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. *N Engl J Med* 332: 429-435, 1995.

73. Sidransky D, Von Eschenbach A, Tsai YC, Jones P, Summerhayes I, Marshall F, Paul M, Green P, Hamilton SR, Frost P. Identification of p53 gene mutations in bladder cancers and urine samples. *Science* 252:706-709, 1991.

74. Boyle JO, Mao L, Brennan JA, Koch WM, Eisele DW, Saunders JR, Sidransky D. Gene mutations in saliva as molecular markers for head and neck squamous cell carcinomas. *Am J Surg* 168:429-432, 1994.

75. Norberg T, Klaar S, Karf G, Nordgren H, Holmberg L, Bergh J. Increased p53 mutation frequency during tumor progression--results from a breast cancer cohort. *Cancer Res* 61:8317-8321, 2001.

76. Quak JJ, Balm AJ, van Dongen GA, Brakkee JG, Scheper RJ, Snow GB, Meijer CJ: A 22-kd surface antigen detected by monoclonal antibody E 48 is exclusively expressed in stratified squamous and transitional epithelia. *Am J Pathol* 136:191-197, 1990.

77. Schrijvers AH, Gerretsen M, Fritz JM, Van Walsum M, Quak JJ, Snow GB, Van Dongen GA. Evidence for a role of the monoclonal antibody E48 defined cell-cell adhesion in squamous epithelia and head and neck squamous cell carcinomas. *Exp Cell Res* 196:264-269, 1991.

78. Brakenhoff RH, Gerretsen M, Knippels EM, Van Dijk M, Van Essen H, Weghuis DO, Sinke RJ, Snow GB, Van Dongen, GA. The human E48 antigen, highly homologous to the murine Ly-6 antigen ThB, is a GPI-anchored molecule apparently involved in keratinocyte cell-cell adhesion. *J Cell Biol* 129:1677-1689, 1995.

79. Brakenhoff RH, Van Dijk M, Rood-Knippels EM, Snow GB. A gain of novel tissue specificity in the human Ly-6 gene E48. *J Immunol* 159:4879-4886, 1997.

80. De Bree R, Roos JC, Quak JJ, Den Hollander W, Van den Brekel MW, Van der Wal JE, Tobi H, Snow GB, Van Dongen GA. Clinical imaging of head and neck cancer with technetium-99m-labeled monoclonal antibody E48 IgG or F(ab')2. *J Nucl Med* 35:775-783, 1994.

81. De Bree R, Roos JC, Quak JJ, Den Hollander W, Wilhelm AJ, Van Lingen A, Snow GB, Dongen GA. Biodistribution of radiolabeled monoclonal antibody E48 IgG and F(ab')2 in patients with head and neck cancer. *Clin Cancer Res* 1:277-286, 1995.

82. De Bree R, Roos JC, Plaizier MA, Quak JJ, Van Kamp GJ, Den Hollander W, Snow GB, Van Dongen GA. Selection of monoclonal antibody E48 IgG or U36 IgG for adjuvant radioimmunotherapy in head and neck cancer patients. *Br J Cancer* 75:1049-1060, 1997.

83. Brakenhoff RH, Stroomer JG, Ten Brink C, De Bree R, Weima SM, Snow GB, Van Dongen GA. Sensitive detection of squamous cells in bone marrow and blood of head and neck cancer patients by E48 reverse transcriptase-polymerase chain reaction. *Clin Cancer Res* 5:725-732, 1999.

84. Raeymaekers LA. commentary on the practical applications of competitive PCR. *Genome Res* 5:91-94, 1995.

85. Fasco MJ, Treanor CP, Spivack S, Figge HL, Kaminsky LS. Quantitative RNA-polymerase chain reaction-DNA analysis by capillary electrophoresis and laser-induced fluorescence. *Anal Biochem* 224:140-147, 1995.

86. Williams SJ, Schwer C, Krishnarao AS, Heid C, Karger BL, Williams PM. Quantitative competitive polymerase chain reaction: analysis of amplified products of the HIV-1 gag gene by capillary electrophoresis with laser-induced fluorescence detection. *Anal Biochem* 236:146-152, 1996.

87. Mulder J, McKinney N, Christopherson C, Sninsky J, Greenfield L, Kwok S. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. *J Clin Microbiol* 32:292-300, 1994.

88. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 22:130-131, 134-138, 1997.

89. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A* 88:7276-7280, 1991.

90. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 6:986-994, 1996.

91. Mensink E, Van de Locht A, Schattenberg A, Linders E, Schaap N, Geurts van Kessel A, De Witte T. Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. *Br J Haematol* 102: 768-774, 1998.

2

Head and neck squamous cell carcinoma: US-guided fine-needle aspiration of sentinel lymph nodes for improved staging - initial experience

David R. Colnot, Eline J.C. Nieuwenhuis, Michiel W.M. van den Brekel, Rik Pijpers, Ruud H. Brakenhoff, Gordon B. Snow, Jonas A. Castelijns

Radiology 218:289-293, 2001

Abstract

Ultrasonography (US)-guided fine needle aspiration with cytologic examination was combined with lymphoscintigraphy for the identification of sentinel lymph nodes (SLNs) in 12 patients with a squamous cell carcinoma of the oral cavity or oropharynx. Dynamic lymphoscintigraphy and a hand-held gamma probe were used to depict the SLNs to be aspirated. Cytologic examination of the aspirated SLNs revealed neck lymph node status in patients who underwent neck dissection (n=6). In patients who underwent only transoral excision, one false-negative cytologic result was observed. This combined approach is expected to improve the detection of occult neck lymph node metastases.

Introduction

In patients with head and neck squamous cell carcinoma, a clinically negative neck (N0) is at risk of harboring occult metastases and is, therefore, treated electively with surgery or with radiotion therapy if this risk is estimated to be higher than 20%.[1] Elective neck dissection causes overtreatment for the majority of these patients, while no unequivocal advantage in survival has been demonstrated when it is compared with delayed neck dissection for patients with metastases in the neck.[2,3] Transoral excision of relatively small (T1 or T2) primary tumors of the oral cavity or oropharynx, followed by a wait-and-see policy with frequent ultrasonography (US) and US-guided fine-needle aspiration with cytologic examination, may be justified to prevent an elective neck dissection in these patients.[4]

Because cytologic criteria are more reliable than radiologic criteria, US-guided fine-needle aspiration with cytologic examination was found to be more accurate than US, computed tomography, or magnetic resonance imaging.[5] The sensitivity of US-guided fine-needle aspiration with cytologic examination for the clinically negative neck (N0) has been reported to be 44-73%, depending on, among other things, the experience of the ultrasonographer.[6-8] Since false-positive cytologic findings with lymph node aspirates are rare, specificity of US-guided fine-needle aspiration is often 100%. Inaccurate results at US-guided fine-needle aspiration and cytologic examination are thus attributed to an absence of enlarged lymph nodes, aspiration of the wrong lymph node, failure of cytological analysis due to the presence of few tumor cells, or the presence of micrometastases in parts of the lymph nodes not aspirated (sampling error). The selection of lymph node(s) to be aspirated is not easy and is based on known patterns of lymphatic spread[9] and on lymph node size and morphology as assessed with US.[8] However, assessment on the basis of drainage patterns and US morphology is prone to errors, and a functional technique to identify the lymph nodes at risk of harboring an occult metastasis could improve the accuracy and reliability of cancer staging in the neck in these patients.

The concept of the sentinel lymph node (SLN) is based on the orderly progression of tumor cells within the lymphatic system. Lymph flow mapping from the tumor site to the regional lymphatic drainage area can be used to identify the primary draining lymph node (ie, the SLN) that will be the first to receive metastatic tumor cells. Radiolabeled pharmaceuticals can be injected next to the tumor and a hand-held gamma probe is used to depict radioactive lymph nodes. Biopsy of the SLN has shown to be useful as an accurate staging technique in studies[10-12] with large series of patients with breast cancer and melanoma in which histopathologic analysis of the samples SLN was used to accurately predict the regional lymph node status in 97-99% of patients. Patients with a disease-free SLN may therefore be spared an elective regional lymph node dissection, which would reduce morbidity in a large number of patients. With head and neck cancer, there is limited experience in gamma probe-guided SLN biopsy.[13,14] To our knowledge, a combined SLN procedure with US-guided fine-needle aspiration with cytologic examination has not been performed. This approach is more attractive than biopsy since it is minimally invasive.

The purpose of our pilot study was to evaluate the feasibility of combining an SLN procedure with US-guided fine-needle aspiration and cytologic examination.

Materials and Methods

From June 1998 to December 1998, 12 consecutive patients (nine men, three women) with a clinically negative neck (N0) assessed at palpation, a histologically proved squamous cell carcinoma of the oral cavity or oropharynx, and scheduled transoral excision or combined tumor excision with unilateral neck dissection were included in this study. The protocol was approved by the institutional medical ethics committee, and informed consent was obtained from all patients after explanation of the procedure.

Depending on accessibility of the primary tumor, three or four submucosal peritumoral injections of technetium 99m-labeled colloidal albumin (Nanocoll, Sorin Biomedica, Sallugia, Italy; particle size, 3-80 nm; total content, 40-70 MBq) in aliquots of 0.2 mL of saline were administered by using a 25-gauge needle (Fig 1). Immediately after injection, lateral dynamic scintigraphy (20 60-second acquisitions in a 128 x 128 matrix) was performed by using a gamma camera with a large field of view equipped with low-energy parallel hole collimators (Dual Head Genesys Imaging System; ADAC Laboratories, Milpitas, Calif).

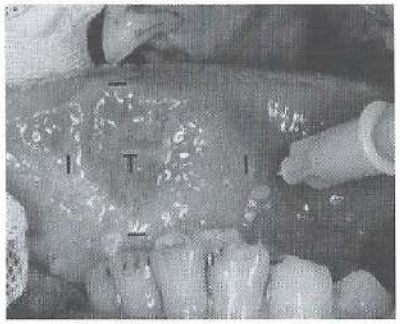


Figure 1. **Patient 11.** Peritumoral injection of ^{99m}Tc-labeled colloidal albumin in the submucosa immediately dorsal to a primry tumor (T) on left side of the tongue.

The dynamic studies were followed by static scintigraphy (120 seconds) in anterior and lateral projections to avoid superimposition caused by radioactive scatter originating from the injection site over radiolabeled lymph nodes in the neck. If no SLN was depicted, additional static scintigraphy was performed after 20 or 40 minutes. After the SLN was detected, its location was marked on the skin and confirmed by a nuclear medicine physician (R.P.) using a hand-held gamma probe (CTC 4; RMD, Watertown, Mass). This gamma probe with a 14-mm diameter uses a cadmium telluride semi-conductor and has a weight of 265 g, including a tungsten shield. The threshold was set between 120 and 160 KeV.

Within 1 hour, lymph nodes were identified at US (7.5-MHz linear-array transducer; Acuson, Moutain View, Callif) and fine-needle aspirates were obtained for cytologic examination of the SLN, additional radiolabeled lymph nodes, and other lymph nodes on the basis of US size criteria. US-guided fine-needle aspiration was performed by one radiologist (J.A.C.) using a syringe holder (Cameco, Taeby, Sweden) and a 0.6 x 25.0-mm needle. The location of the SLN was recorded per side and level according to the Memorial Sloan-Kettering Cancer Center classification (15) (Fig 2). After preparation of the cytologic smears, residues of the aspirates from the SLN, additional radiolabeled lymph nodes, and other lymph nodes were rinsed in phosphate-buffered saline and counted in a liquid scintillation counter (Wallac, Turku, Finland) to confirm correct aspiration.

Patients with a primary tumor staged T3 (three patients) or T4 (two patients) underwent planned unilateral elective neck dissection the next day, which enabled evaluation of the neck dissection specimen. The hand-held gamma probe was then used to retrieve all radioactive lymph nodes from the neck dissection specimen for separate histopathologic examination. A probe count less than 10% of the most radioactive lymph node was regarded as background. Histopathologic results of the identified SLN were compared with the cytologic results.

All seven patients with T1 or T2 staged tumors were initially scheduled for transoral excision of the primary tumor and a wait-and-see approach with strict follow-up of the neck, including palpation, US, and US-guided fine-needle aspiration with cytologic examination according to the policy of our department.[4] In these patients, verification from the neck dissection specimen could not be obtained, and, therefore, the outcome of the clinical follow-up was used to evaluate the SLN concept in these patients.

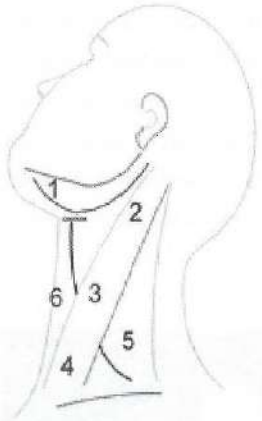
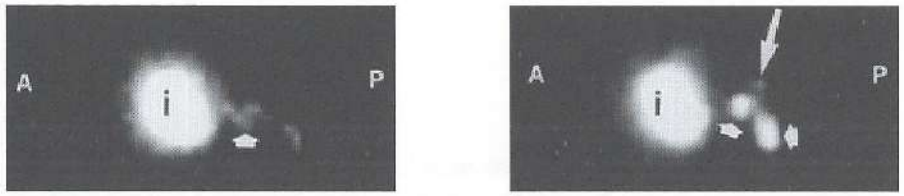


Figure 2. Drawing shows lymph node levels of the neck. Level 1 includes the submental and submandibular region. Levels 2, 3, and 4 correspond to the lymph nodes of the jugular chain (ie, high-, mid-, and low-jugular, respectively). Lymph nodes in the posterior triangle and supraclavicular region are located in level 5. Level 6 corresponds to the juxtavisceral lymph nodes.

Results

At dynamic lymphoscintigraphy (Figs 3, 4), at least one radiolabeled lymph node was identified after administration of ^{99m}Tc-labeled colloidal albumin in all but one patient (Table 1, patient 5). In this patient, no focal accumulation of radioactivity was visualized, but two level I lymph nodes that were aspirated on the basis of size criteria contained radioactivity and thus were identified afterward by means of liquid scintillation counting of the aspirates. Because of its location close to the injection site, scintigraphic visualization of the SLN in this patient was most likely disturbed by radioactive scatter originating from the injection site in the floor of mouth. In another patient, the SLN was located in level I (Table 1, patient 2), whereas in all other patients, scintigraphy showed the SLN in levels II and III. Subsequent independent localization with the hand-held gamma probe in a patient with a carcinoma in the floor of mouth was feasible if the SLN was located in level II or lower.



a. b.

Figure 3. **Patient 11. (a)** Left lateral dynamic lymphoscintigram obtained at 2 minutes after peritumoral injection of ^{99m}Tc-labeled colloidal albumin shows early transport of the tracer in a lymph vessel (arrow),without focal activity. **(b)** Left lateral static scintigram obtained at 20 minutes after injection shows two radiolabeled lymph nodes (short arrows) and a faint secondary focus (long arrow) above both lymph nodes, which represents spill of the radioactive tracer to a second-echelon lymph node. Fine-needle aspiration and cytologic findings of both radiolabeled lymph nodes were negative. In a and b, A = anterior, i = injection site, P = posterior.

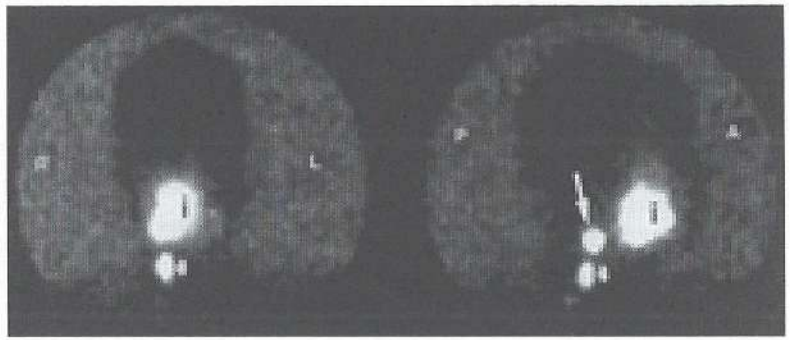


Figure 4. **Patient 10.** Anterior (left) and right lateral (right) projections of static scintigram. Right lateral projection shows the SLN (long arrow), which cannot be seen on the anterior projection because of superimposition, and the ballooning effect of the radioactive scatter originating from the injection site (i). A second radiolabeled lymph node is visible (short arrow). A = anterior, L = left, R = right.

Lymphoscintigraphy and/or counting of the aspirate was used to identify a total of 19 radiolabeled lymph nodes: in five patients, a single SLN was found, and in seven patients, additional radiolabeled lymph nodes were identified. Aspiration of a radiolabeled lymph node always revealed a positive result with the liquid scintillation counter. The mean radioactivity content of aspirate residues was 6,649 counts per minute (cpm) (range, 181-12,480 cpm). Counting of the aspirate residues of non-radioactive additional lymph nodes that were aspirated on the basis of US criteria resulted in background values (mean, 30 cpm). In one patient (Table 2, patient 9), two radiolabeled lymph nodes were considered too small for aspiration (<2 mm); therefore, a total of 17 radiolabeled lymph nodes were aspirated in 11 patients. In nine of these patients, cytologic findings were negative; in two patients, cytologic findings were positive.

Table 1. Characteristics of patients undergoing SLN and combined transoral excision with unilateral neck dissection.

Patient no/ age (y)	Tumor location	T-stage	Sizes of aspirated lymph nodes (mm)	Cytologic finding*	Histologic finding at neck dissection
1/67	Tonsil	T3	6†, 4‡, 4‡, 4‡	Negative	Negative
2/59	Tongue	T3	3†	Negative	Negative
3/57	Anterior pharyngeal arch	T3	5‡, 9†, 5†	Negative	Negative
4/72	Floor of mouth	T4	4‡, 5‡, 6‡, 6‡, 5†, 5†	Negative	Negative
5/64	Tongue	T4	3†, 5†, 5‡	Positive	Positive
6/46		T2	11†, 8‡, 6‡, 6‡, 5‡, 11‡, 6‡	Positive	Positive

*Cytologic findings in the aspirates of radiolabeled lymph nodes were used to correctly predict the regional lymph node status when combined tumor excision and neck dissection were performed.

† Radiolabeled lymph node.

‡ Nonradiolabeled lymph nodes aspirated because of US criteria.

Table 2. Characteristics of patients undergoing transoral excision with staging and strict follow-up with US-guided fine needle aspiration cytologic examination.

Patient no/ age (y)	Tumor location	T-stage	Sizes of aspirated lymph nodes (mm)	Cytologic finding	Follow-up (months)
7/59	Tongue	T2	6*	Negative	4†
8/44	Tongue	T1	5‡, 5*, 7‡	Negative	16
9/72	Tongue	T2	<2*, <2*	None	12
10/51	Tongue	T2	6‡, 5*, 4*	Negative	12
11/48	Tongue	T2	4*, 3*, 5‡, 4‡, 6‡, 7‡	Negative	9
12/59	Floor of mouth	T2	5*, 2*, 2‡	Negative	7

* Radiolabeled lymph node

† Lymph node metastasis was diagnosed at US-guided fine needle aspiration cytologic examination after 4 months follow-up.

In patient 9, both SLNs were considered too small for US-guided fine needle aspiration.

‡ Nonradiolabeled lymph node.

In all patients undergoing combined tumor excision with unilateral neck dissection (n=6), the cytologic findings in the SLN were in agreement with the histopathologic findings in the neck dissection specimen (Table 1). On the basis of the cytologic results in the aspirated SLN, clinical treatment was changed in one patient. This patient had a tongue carcinoma initially staged T2N0 (Table 1, patient 6) and was scheduled to undergo only a transoral excision, but cytologic findings were tumor positive. The aspirate of an 11-mm SLN located in level II was positive, as well as that of two other lymph nodes (level I and II) that were aspirated because of US size criteria. A combined transoral excision and neck dissection was performed. Histopathologic examination of the neck dissection specimen revealed six lymph node metastases, three in level I and three in level II.

The six patients undergoing transoral excision with a wait-and-see approach underwent follow-up with US-guided fine-needle aspiration with cytologic examination, with a mean follow-up of 11 months (range, 7-16 months) (Table 2). In one of these patients (Table 2, patient 7), a lymph node metastasis was diagnosed at US-guided fine-needle aspiration with cytologic examination after 4 months of follow-up after an initial negative aspiration and cytologic result of one SLN located in level II. Neck dissection was performed, and histopathologic examination of the specimen revealed a large lymph node metastasis with extranodal spread located from level II to IV. Therefore, this case should be considered a false-negative finding with US-guided fine-needle aspiration with cytologic examination of an SLN.

Discussion

The findings of this pilot study show that lymphoscintigraphy and fine-needle aspiration with lymphoscintigraphy by using a hand-held gamma probe and US guidance is a feasible approach in the identification and characterization lymph nodes in head and neck cancer. Moreover, aspiration of the visualized SLN can be confirmed by means of scintillation counting of the aspirate residue. However, difficulties in localization at scintigraphy or by using a hand-held gamma probe were encountered if an SLN was located in the submandibular region (level I) close to the injection site, that is, in case of a carcinoma of the floor of mouth. In this case, the presence of radioactive scatter originating from the injection site made separate localization of radiolabeled lymph nodes by using scintigraphy or the hand-held gamma probe impossible. As a consequence, correct aspiration can then be confirmed only by means of scintillation counting of the aspirate residue.

In their study with gamma probe-directed SLN biopsy in oral carcinomas, Koch *et al.*[13] also described this problem for one case. In our experience, when an SLN cannot be identified by using scintigraphy or the hand-held gamma probe, the ultrasonographer should focus on the submandibular and submental region. Findings in one of our cases (Table 1, patient 6) showed that even if an SLN is detected in level II, metastases in level I cannot be excluded. We are currently evaluating whether the use of shields at the injection site, digital subtraction techniques, and dynamic lymphoscintigraphy can reduce the risk of a missed radiolabeled lymph node close to the injection site.

An advantage of dynamic lymphoscintigraphy is its ability to discriminate between a true SLN and a spill to second echelon lymph nodes[16] (Figure 3). The risk of superimposition of radiolabeled lymph nodes by the injection site can be reduced by using anterior and lateral imaging (Fig 4).

Although we have validated our findings in only a small series of patients, the cytologic findings in the SLN aspirateswere used to correctly predict the

regional lymph node status when combined tumor excision and neck dissection were later performed (Table 1). Of all patients treated with transoral excision for the primary tumor and a wait-and-see approach with strict follow-up of the neck, one patient developed a large lymph node metastasis 4 months after surgical treatment of the primary tumor (Table 2). Most likely, this lesion was already present at the time of primary surgery, and it was therefore considered a false negative SLN aspiration. Since most recurrences occur within 2 years after primary treatment, a longer follow-up for this group of patients is mandatory.

As illustrated in Tables 1 and 2, the largest lymph node is not always visualized at lymphoscintigraphy. In four patients (Table 1, patient 4; Table 2, patient 8, 10, 11) the largest lymph node identified at US did not contain radiolabeled colloidal albumin. Lymphoscintigraphy thus resulted in the identification of smaller lymph nodes that would probably not have been selected for aspiration by using US-criteria. This observation had no consequence for patient care, since cytologic findings were negative for all aspirated lymph nodes in these patients. In one patient (Table 2, patient 9) two small radiolabeled lymph nodes (<2 mm) were identified by using the SLN procedure but were considered too small for fine-needle aspiration. Threfore, it could be concluded that the radioactivity attributed to these lymph nodes was determined only by the exclusion of the presence of other lymph nodes at the mark. Although the cytologic result of the aspirated SLN altered the surgical treatment in one patient (Table 1, patient 6), it seems likely that this SLN (11 mm) would also have been selected for aspiration because of US criteria.

It has been reported[12] with breast cancer that, ironically, extensive tumor infiltration of the true SLN can lead to lymph fluid rerouting, and, thus, the identification of alternative lymph nodes as being "sentinel", which can lead to a false-negative SLN. The combined use of lymphoscintigraphy and US might allow us to avoid this pitfall, since US will reveal (enlarged) lymph nodes with extensive tumor infiltration.

SLN biopsy is a routine procedure in breast and melanoma studies, but this seems unattractive for squamous cell carcinoma of the head and neck. An open biopsy, which requires an additional operation, is an invasive technique in which other structures, including lymph nodes, might have to be dissected to reach the SLN; thi procedure thereby complicates secondary surgical treatment of the neck in case of a positive SLN. Furthermore, an open biopsy is, in general, considered an indication for postoperative radiation therapy, which could have been avoided in some patients. In contrast to axillary lymph node dissection, selective neck dissection has a relatively low morbidity and is widely used as a staging procedure. Although debatable, some authors[17] even consider a selective neck dissection as a curative procedure in cases of limited metastatic disease. Therefore, the role of a SLN procedure in the head and neck might, in our opinion, be more attractive if combined with a minimally invasive technique such as US-guided fine-needle aspiration and cytologic examination.

In conclusion, these preliminary data show that US-guided fine-needle aspiration of SLNs is feasible. Whether this approach has the potential to increase the sensitivity of US-guided fine-needle aspiration with cytologic examination for staging in patients with a clinically negative neck (N0) must be determined in a larger series of patients.

References

1. Weiss MH, Harrison LB, Isaacs RS. Use of decision analysis in planning a management strategy for the stage N0 neck. *Arch Otolaryngol Head Neck Surg* 120:699-702,1994.

2. Hughes CJ, Gallo O, Spiro RH, Shah JP. Management of occult neck metastases in oral cavity squamous carcinoma. *Am J Surg* 166:380-383, 1993.

3. Vandenbrouck C, Sancho-Garnier H, Chassagne D, Saravane D, Cachin Y, Micheau C. Elective versus therapeutic radical neck dissection in epidermoid carcinoma of the oral cavity-results of a randomized clinical trial. *Cancer* 46:386-390, 1980.

4. Van den Brekel MWM, Reitsma LC, Quak, JJ, et al. Sonographically guided aspiration cytology of neck nodes for selection of treatment and follow-up in patients with N0 head and neck cancer. *Am J Neuroradiol* 20:1727-1731, 1999.

5. Van den Brekel MWM, Castelijns JA, Stel HV, Golding RP, Meijer CJLM, Snow GB. Computed tomography, magnetic resonance, ultrasound and ultrasound guided aspiration cytology for the assessment of the neck; a comparative study. *Eur Arch Otorhinolaryngol* 250:11-17, 1993.

6. Takes RP, Righi PD, Meeuwis CA, et al. The value of ultrasound with ultrasound-guided fine-needle aspiration biopsy compared to computed tomography in the detection of regional metastases in the clinically negative neck. *Int J Rad Oncol Biol Phys* 40:1027-1032, 1998.

7. Righi PD, Kopecky KK, Caldemeyer KS, Ball VA, Weisberger EC, Radpour S. Comparison of ultrasound-fine needle aspiration computed tomography in patients undergoing neck dissection. *Head Neck* 19:604-610, 1997.

8. Van den Brekel MWM, Castelijns JA, Stel HV, et al. Occult metastatic neck disease: detection with US and US-guided fine-needle aspiration cytology. *Radiology* 180:457-461, 1991.

9. Shah JP, Candela FC, Poddar AK. The patterns of cervical lymph node metastases from squamous carcinoma of the oral cavity. *Cancer* 66:109-113, 1990.

10. Morton D, Wen DR, Wong JH, et al. Technical details of intraoperative lymphatic for early stage melanoma. *Arch Surg* 127:392-399, 1992.

11. Veronesi U, Paganelli G, Galimberti V, et al. Sentinel-node biopsy to avoid axillary dissection in breast cancer with clinically negative lymph-nodes. *Lancet* 349:1864-1867, 1997.

12. Borgstein PJ, Pijpers R, Comans EFI, van Diest PJ, Boom RPA, Meijer S. Sentinel lymph node biopsy in breast cancer: guidelines and pitfalls of lymphoscintigraphy and gamma probe detection. *J Am Coll Surg* 186:275-283, 1998.

13. Koch WM, Choti MA, Civelek AC, Eisele DW, Saunders JR. Gamma probe-directed biopsy of the sentinel node in oral squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 124:455-459, 1998.

14. Shoaib T, Soutar DS, Prosser JE, et al. A suggested method for sentinel node biopsy in squamous cell carcinoma of the head and neck. *Head Neck* 21:728-733, 1999.

15. Shah JP, Strong E, Spiro RH, Vikram B. Neck dissection: current status and future possibilities. *Clin Bulletin* 11:25-33, 1981.

16. Pijpers R, Collet GJ, Meijer S, Hoekstra OS. The impact of dynamic lymphoscintigraphy and gamma probe guidance on sentinel node biopsy in melanoma. *Eur J Nucl Med* 22:1238-1241, 1995.

17. Clayman GL, Frank DK. Selective neck dissection of anatomically appropriate levels is as efficacious as modified radical neck dissection for elective treatment of the clinically negative neck in patients with squmaous cell carcinoma of the upper respiratory and digestive tract. *Arch Otolaryngol Head Neck Surg* 124:348-352, 1998.

3

Lymphoscintigraphy details of sentinel lymph node detection in 82 patients with squamous cell carcinoma of the oral cavity and oropharynx

Eline J.C. Nieuwenhuis, Rik Pijpers, Jonas A. Castelijns, Gordon B. Snow

Nuclear Medicine Communications 24:651-656, 2003

Abstract

Lymphoscintigraphy for sentinel node detection has been studied extensively in melanoma and breast cancer. In head and neck squamous cell carcinoma (HNSCC), however, experience in this field is relatively meagre. The purpose of this study was to document and evaluate lymphoscintigraphic findings in HNSCC patients. Eighty-two patients with clinical T1-T4 N0 SCC of the oral cavity or oropharynx received peritumoral injections of 25-75 MBq ^{99m}Tc-Colloidal Albumin (CA). Dynamic lymphoscintigraphy was performed in lateral projection during 20 min, followed by 2 min static imaging in anterior projection. In 26 patients, additional static images were obtained 2-6 hours after injection of the tracer. In four of 82 patients, both early and late imaging revealed no tracer transport. In 78 of 82 patients, one (60), two (14) or three (4) SNs could be visualized either by dynamic scintigraphy (73) or delayed static imaging (5). In 48 of 78 (62%) patients the SN was visualised within the first minute of dynamic imaging. In particular, SNs of tumors of the mobile tongue were visualised within the first minute. No effect of T-stage or ^{99m}Tc-CA dose on transport time of the tracer towards the SN was seen. The distribution of the SNs in the various levels of the neck relative to the primary tumor sites within the oral cavity was in concordance with the patterns of lymph node metastases as reported traditionally for patients with SCC in the oral cavity. This study demonstrates the different variables affecting SN identification with lymphoscintigraphy using ^{99m}Tc-CA in HNSCC patients.

Introduction

Squamous cell carcinoma represents roughly 90% of all cancers in the head and neck region, with a global incidence of 500,000 new cases per annum.[1] One of the most important factors in prognosis and treatment of patients with head and neck squamous cell carcinoma (HNSCC) is the presence of lymph node metastases in the neck. Only 7% of patients without lymph node metastases develop distant metastases, whereas this occurs in 50% of patients with more than three tumor-positive lymph nodes.[2] In addition, as the lymphatic system is the most common route of spread of HNSCC[3], a decision on whether or not to treat the lymph nodes of the neck electively has to be made.

The sentinel node (SN) concept signifies an important recent development in the continuing evolution of ideas concerning lymphatic metastases. The SN is the first node that receives lymphatic drainage from a malignant tumor and is therefore the first node to contain metastasis if lymphatic dissemination occurs.[4] A tumor-negative SN precludes the presence of lymphatic malignant involvement. Lymphoscintigraphy is oriented to identify the SN and, in melanoma and breast cancer, there is consensus on major points about guidelines for optimal image acquisition. For example, in most studies on SN localization in breast cancer, peritumoral injection of the tracer, followed by late imaging, is considered to be sufficient. In contrast, in case of cutaneous melanoma, early dynamic imaging is essential.[5-8] The technique for SN visualization in HNSCC patients has been the subject in only a few studies with relatively small patient numbers and, moreover, with highly discordant results.[9-13]

In contrast to the relatively orderly and predictable lymph flow pattern from the breast, the lymphatic system in the neck is the most extensive and variable lymphatic system of the entire body. These patterns of lymphatic spread are not predictable in the individual HNSCC patient, and therefore lymphoscintigraphy might be helpful to identify at-risk lymph nodes. After a pilot study of SN identification using lymphoscintigraphy in 12 HNSCC

patients[14], we decided to investigate further the efficacy and accuracy of lymphoscintigraphy for SN identification in a larger group of patients. So far, a number of areas remain unexplored regarding lymphoscintigraphy in HNSCC, such as the dose of colloid required and the use of either static or dynamic imaging.

In the present study, we describe the findings in 82 patients (including the 12 patients in our pilot study), explaining variables affecting SN visualization at lymphoscintigraphy. The aims of the study were 1) to document lymphoscintigraphic findings in HNSCC patients with respect to number and localization of SNs; and 2) to evaluate lymphoscintigraphy in HNSCC patients, including the optimization of the imaging protocol.

Patients and methods

Patient selection

From September 1998 to July 2001, 82 previously untreated patients with histologically proven squamous cell carcinoma of the oral cavity or oropharynx were enrolled in the study. All patients had a clinically N0 neck as assessed by palpation. The patient and tumor characteristics are shown in Table 1. The study protocol was approved by the Institutional Review Board and informed consent was obtained from all patients.

Table 1. Patient and Tumor Characteristics

No. of patients	82
Male/Female	45/37
Age (y)	
Median	61
Range	38-84
T-stage primary tumor	
T1	26
T2	33
T3	19
T4	4
Localization	
Mobile Tongue	36
Floor of mouth	21
Inferior Alveolar Process	7
Buccal Mucosa	5
Tonsil	7
Base of Tongue	3
Soft Palate	3

Lymphoscintigraphy

After local anesthesia with 10% lidocaine spray, 25 or 75 MBq ^{99m}Tc-labeled Colloidal Albumin (CA, Nanocoll®, Sorin Biomedica, Sallugia, Italy; particle size 3-80 nm) suspended in 0.4 ml saline, was administered by 2-4 submucosal peritumoral injections, depending on the size and accessibility of the primary tumor. In 66 of 82 patients, 25 MBq ^{99m}Tc-labeled CA was injected. In the final 16 patients we decided to increase the tracer dose to 75 MBq, as we had the impression that, in some cases, the low injected dose resulted in non-visualisation of lymphatic channels. All administrations were injected by one operator. Immediately following injection, the patients were asked to perform a mouthwash to prevent retention of tracer into the pharynx or oesophagus. Directly following the injections, dynamic lymphoscintigraphic images were obtained (20 x 60 s, 128 x 128 matrix, low energy high resolution collimator). The patient was in supine position with the tumor side facing the camera. After this, a flood-field-source image was obtained. Finally, a static image of 120 s was made in anterior projection to exclude superimposition of the injection site and SN and to check for contralateral drainage. After visualization of the SN(s), the position was marked on the overlying skin with the use of a ^{57}Co point-source-marker, and confirmed with a 14 mm diameter handheld gamma probe (CTC 4, RMD, Watertown, MA, USA). The SN was classified into one of the six different lymph node levels in the neck according to the classification system of the Memorial Sloan-Kettering Cancer Centre (Fig. 1). In 26 patients, additional static images were performed 2-6 h later. SNs were defined as nodes with afferent lymphatic channels or nodes clearly localized in different basins, visible within 20 min of dynamic scintigraphy or, if no transport was seen during dynamic and early static imaging, nodes visualised at later static images. In case of doubt, all visualized foci were considered to be SNs.

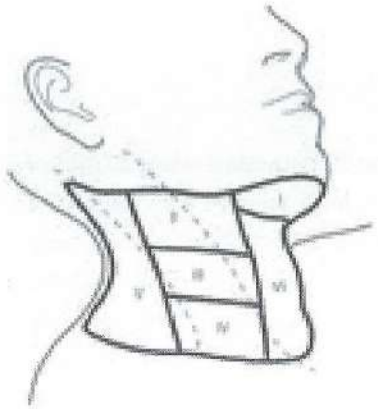


Figure 1. Anatomic localization of the six different lymph node levels in the neck according to the classification system of the Memorial Sloan-Kettering Cancer Centre.

Results

Imaging

At least one SN was visualized in 73 (89%) of the 82 patients during 20 min of dynamic scintigraphy (Fig. 2). In five patients at least one SN was visualized by late additional static images. In the remaining four patients, both early and late imaging revealed no tracer transport: two patients received 25 MBq and two patients received 75 MBq. One SN was visible in 60 (73%) patients, two SNs in 14 (17%) patients, and three SNs in four patients (5%). Of these four patients showing three SNs, three of 16 (19%) patients received a 75 MBq colloid dose and one of 66 (2%) received a 25 MBq tracer dose. In 25 of 78 (32%) patients, the focal accumulations appeared to be in conjunction with visible afferent lymphatic channels. With regard to the moment of SN visualization, in 48 of 78 (62%) patients, the SN was visualized within the first minute of dynamic imaging, in 25 of 78 (32%) patients during later dynamic imaging, and in five of 78 (6%) patients only at late static imaging. No effect of tracer dose (25 Mbq or 75 MBq) or T-stage on the delay until visualization of the SN was identified.

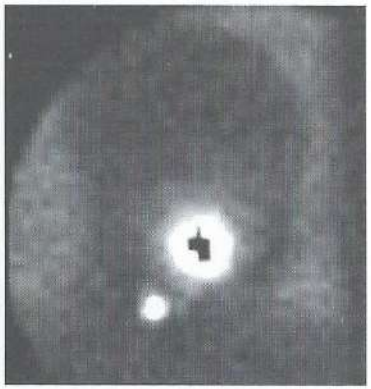


Figure 2. Lymphoscintigraphy reveals transport of the tracer from the injection site (large spot) along a faint lymphatic channel towards one sentinel node (small spot) in level II at early dynamic lateral imaging in a patient with a T2N0 tongue carcinoma located on the right side.

In Table 2, the time of appearance of SNs in relation to the tumor site is shown. These data demonstrate that, in most cases, SNs from tumors of the mobile tongue were visualized within 1 min, whereas, for example, SNs from the floor of mouth (FOM) appeared later during dynamic imaging. Overflow to non-sentinel lymph nodes occurred in 31 of 78 (40%) patients during dynamic lymphoscintigraphy.

Table 2. The timing of appearance of SNs in relation to tumor site.

Primary tumor site	Time to visualization of SNs			
	< 1 min	< 20 min	late imaging	no SN at early and late imaging
Floor of mouth (21)	8	10	0	3
Mobile tongue (36)	30	5	0	1
Inferior alveolar process (7)	2	3	2	0
Base of tongue (3)	2	1	0	0
Buccal mucosa (5)	2	2	1	0
Tonsil (7)	3	3	1	0
Soft palate (3)	1	1	1	0
Total (82)	48	25	5	4

In the first 21 patients, late imaging revealed 16 additional lymph nodes in 11 patients. These were clearly no SNs, but spill to second echelon nodes. Therefore, we decided to perform late imaging only in cases of negative dynamic scintigraphy. No SNs disappeared compared to the early images.

In six patients, bilateral drainage was seen. Two of these patients had tumors located in the midline of the floor of mouth (FOM) (1x T1, 1x T3), whereas four patients had tumors of the lateral tongue (2x T1, 1x T2, 1x T3). With regard to the timing of SN visualization in these patients, the ipsilateral SNs did not appear earlier than the contralateral SNs. Three additional patients

had contralateral drainage only. They presented with a tumor of the tongue located close to the midline, a tumor of the soft palate and a tumor of the tonsil, respectively.

Overall, the 82 lymphoscintigraphic studies revealed 99 foci to be considered as SNs, of which 41 were located in level I, 41 in level II, 14 in level III, one in level IV, one in level V, and one in the retropharyngeal region. Confirmation of the SN position with the handheld gamma probe proved to be reliable if the SN was located in levels II-V. Detailed analysis of drainage patterns based on the primary tumor site is given in Table 3. As can be seen in this table, tumors located in the FOM have dominant level I drainage, whereas tumors located on the mobile tongue drain to level I and II more or less equally. Tumors located more posterior have mainly level II and partly level III drainage. The prevalence of SNs in level IV and V was exceptional.

Table 3. Distribution of sentinel nodes visualised lymphoscintigraphy based on primary tumor site.

	Level					
Primary tumor site	I	II	III	IV	V	retropharyngeal
Floor of mouth (21)	12	5	3	-	1	-
Mobile tongue (36)	17	21	6	-	-	-
Inferior alveolar process (7)	4	4	3	1	-	-
Base of tongue (3)	1	2	-	-	-	-
Buccal mucosa (5)	4	1	-	-	-	-
Tonsil (7)	2	6	1	-	-	-
Soft palate (3)	1	2	1	-	-	1

Discussion

With respect to SN procedures in HNSCC, recently published studies have shown discordant results. Using radiolabeled colloid alone, lymphoscintigraphy identified SNs in only two out of five patients with SCC in the oral cavity, as described by Koch *et al.*.[9] In a group of 16 patients, Pitman *et al.*[15] were unable to find any SN in patients injected with blue dye alone. More encouraging results were published by Alex *et al.*[16], who reported successful SN identification in eight of eight HNSCC patients, using radiolabeled colloid and intraoperative localization with a handheld gamma probe, without preoperative lymphoscintigraphy. In our pilot study[14], using ^{99m}Tc-CA alone, lymphoscintigraphy identified SNs in 12 of 12 HNSCC patients, and ultrasound guided fine needle aspiration cytology (USgFNAC) of the SN correctly predicted the lymph node status. Another study by Shoaib *et al.*[11], who used both radiolabeled colloid and blue dye, could identify SNs in 36 of 40 HNSCC patients, and correctly reflected tumor positivity of the neck in 16 of 17 cases. Although the first four studies described relatively small patient numbers, they clearly indicate that the techniques for SN identification in HNSCC are highly variable and less well established compared with those for SN localization in breast cancer and melanoma.[17]

For this study, we used ^{99m}Tc-CA. This tracer shows rapid transport to the SN, clearly illustrated by the fact that, in 62% of the patients, the SN was visualized within the first minute of imaging. It is therefore important to start dynamic imaging as quickly as possible because it allows the visualization of lymphatic channels from the injection site to the lymph nodes, and provides the evidence required to consider them as true SNs. In this series, in 25 of 82 (30%) patients, one (22 patients) or two (three patients) lymphatic channels were demonstrated during dynamic lymphoscintigraphy. Lymphatic channels could not be followed up to an SN in 70%, mostly due to the proximity of the SNs to the injection site. In some cases, the relatively low injected dose may also have negatively influenced visualisation. For this reason, we decided to increase the tracer dose in the final16 patients.

However, with regard to identification rate, number of SNs and time to visualization of the SNs, no clear advantage was detected for either colloid dose. Although some degree of spill to non-sentinel lymph nodes was seen in 38% of the patients, these second echelon nodes could usually be easily distinguished from the true SNs at scintigraphy.

The findings of this study show that SN detection in HNSCC is a feasible approach, as in 95% of the patients at least one SN was identified. However, difficulties with the identification of SNs may be encountered during lymphoscintigraphy. The main difficulty in the localization of SNs at scintigraphy or by using a gamma probe arises from the proximity of SNs to the injection site, as in the case of a FOM carcinoma with a SN in level I.[9,11] In this study, no SN could be visualized in four patients, probably due to radioactive scatter originating from the primary tumor, as these patients presented with tumors located in the anterior oral cavity (FOM, n=3; mobile tongue, n=1). Lead shielding of the injection site might be helpful for identifying level I SNs theoretically. However, in case of a FOM SCC or an ulcerating and painful tumor that hampers movement of the tongue, this cannot be applied.

The existing pattern of lymph drainage from the injection depots determines which lymph node will be the first to receive the tracer. Our data are in concordance with the patterns of lymph node metastases as reported for the various primary tumor sites within the oral cavity[18,19], suggesting that the SN concept is also applicable in HNSCC patients. Shoaib *et al.*[11] have also found strong indications for the validity of the SN concept in HNSCC. The patterns of lymphatic drainage might be affected negatively by factors such as the tumor size, which hamper representative injections. Moreover, in patients with large tumors, one might expect lymphatic drainage to more SNs due to crossing borders between different lymphatic basins. However, we did not observe this in our patient group. For example, of the 36 patients with mobile tongue tumors, 29 patients were staged T1/2 and seven patients were staged T3/4. In 11 of 29 (38%) patients with T1/2 tongue tumors, more than one SN was identified, whereas, in two of seven (29%) patients with T3/4 tongue

tumors, more than one SN was identified. Furthermore, there is a risk of obstruction of lymphatic flow due to tumor growth in lymphatic channels or lymph nodes, which may result in deviation of the lymphatic flow to "false SNs".[20] In these cases, the tumor load may be large enough to be seen with other imaging techniques, including USgFNAC. After all, the SN concept is primarily aiming at excluding lymphatic metastases or demonstrating micrometastases.

In conclusion, this study demonstrates the variables affecting SN identification at lymphoscintigraphy using ^{99m}Tc-CA in HNSCC patients. Similar as in lymphoscintigraphy for cutaneous melanoma, SN visualization occurs in the majority of patients within 20 min after tracer administration. With regard to optimal time and type of imaging, early dynamic imaging directly after injection of the tracer, is superior to late static imaging. It seems reasonable to obtain late images only in case of negative dynamic scintigraphy. As a higher tracer dose might increase the visualization rate of lymphatic channels from the injection site to the lymph nodes, we recommend a 75 MBq ^{99m}Tc-CA dose.

References

1. Muir C, Weiland L. Upper aerodigestive tract cancers. *Cancer* 75: 147-153, 1995.

2. Leemans CR, Tiwari R, Nauta JJ, van der Waal I, Snow GB. Regional lymph node involvement and its significance in the development of distant metastases in head and neck carcinoma. *Cancer* 71:452-456, 1993.

3. Shah JP, Andersen PE. The impact of patterns of nodal metastasis on modifications of neck dissection. *Ann Surg Oncol* 6:521-532, 1994.

4. Morton D, Wen DR, Wong JH, et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. *Arch Surg* 127:392-399, 1992.

5. Borgstein PJ, Pijpers R, Comans EF, van Diest PJ, Boom RP, Meijer S. Sentinel lymph node biopsy in breast cancer: guidelines and pitfalls of lymphoscintigraphy and gamma probe detection. *J Am Coll Surg* 186:275-283, 1998.

6. Kapteijn BA, Nieweg OE, Valdes Olmos RA, et al. Reproducibility of lymphoscintigraphy for lymphatic mapping in cutaneous melanoma. *J Nucl Med* 37:972-975, 1996.

7. Birdwell RL, Smith KL, Betts BJ, Ikeda DM, Strauss HW, Jeffrey SS. Breast cancer: variables affecting sentinel lymph node visualization at preoperative lymphoscintigraphy. *Radiology* 220:47-53, 2001.

8. Valdes Olmos RA, Hoefnagel CA, Nieweg OE, et al. Lymphoscintigraphy in oncology: a rediscovered challenge. *Eur J Nucl Med* 26(Suppl):S2-S10, 1999.

9. Koch WM, Choti MA, Civelek AC, Eisele DW, Saunders JR. Gamma probe-directed biopsy of the sentinel node in oral squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 124:455-459, 1998.

10. Taylor RJ, Wahl RL, Sharma PK, et al. Sentinel node localization in oral cavity and oropharynx squamous cell cancer. *Arch Otolaryngol Head Neck Surg* 127:970-974, 2001.

11. Shoaib T, Soutar DS, MacDonald DG, et al. The accuracy of head and neck carcinoma sentinel lymph node biopsy in the clinically N0 neck. *Cancer* 91:2077-2083, 2001.

12. Klutmann S, Bohuslavizki KH, Brenner W, et al. Lymphoscintigraphy in tumors of the head and neck using double tracer technique. *J Nucl Med* 1999; 40:776-782.

13. Werner JA, Dunne AA, Ramaswamy A, et al. Sentinel node detection in N0 cancer of the pharynx and larynx. *Br J Cancer* 87:711-715, 2002.

14. Colnot DR, Nieuwenhuis EJ, Castelijns JA et al. Ultrasound guided aspiration of sentinel nodes for improved staging of head and neck cancer patients-initial experience. *Radiology* 218:289-293, 2001.

15. Pitman KT, Johnson JT, Edington H, et al. Lymphatic mapping with isosulfan blue dye in squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg* 124:790-793, 1998.

16. Alex JC, Sasaki CT, Krag DN, Wenig B, Pyle PB. Sentinel lymph node radiolocalization in head and neck squamous cell carcinoma. *Laryngoscope* 110:198-203, 2000.

17. Bass SS, Cox CE, Ku NN, Berman C, Reintgen SR. The role of sentinel lymph node biopsy in breast cancer. *J Am Coll Surg* 189:183-194, 1999.

18. Shah JP, Candela FC, Poddar AK. The patterns of cervical lymph node metastases from squamous carcinoma of the oral cavity. *Cancer* 66:109-113, 1990.

19. Werner JA, Dunne AA, Ramaswamy A, et al. Number and location of radiolabeled, intraoperatively identified sentinel nodes in 48 head and neck cancer patients with clinically staged N0 and N1 neck. *Eur Arch Otorhinolaryngol* 259:91-96, 2002.

20. Rahusen FD, Pijpers R, Van Diest PJ, Bleichrodt RP, Torrenga H, Meijer S. The implementation of the sentinel node biopsy as a routine procedure for patients with breast cancer. *Surgery* 128:6-12, 2000.

4

Histopathological validation of the sentinel node concept in squamous cell carcinoma of the head and neck

Eline J.C. Nieuwenhuis, Isaac van der Waal, C. René Leemans, Alain Kummer, Rik Pijpers, Jonas A. Castelijns, Ruud H. Brakenhoff, Gordon B. Snow

Submitted

Abstract

In patients with head and neck squamous cell carcinoma (HNSCC) the presence of lymph node metastases is the most important prognosticator. Sentinel node (SN) biopsy has been shown to be an accurate staging technique for patients with breast cancer and melanoma, and might be suited for HNSCC patients as well. This study was undertaken to determine whether the SN concept holds true for HNSCC, and could be exploited for SN biopsy.

In 22 patients with T2-4 N0 oral or oropharyngeal SCC who were planned to undergo combined primary tumor excision and elective unilateral (n=17) or bilateral (n=5) neck dissection, the day before surgery SN identification using lymphoscintigraphy after peritumoral injections of ^{99m}Tc-labeled colloidal albumin was carried out. After removal of the neck dissection specimens all SNs, all other radioactive lymph nodes and all non-radioactive lymph nodes were retrieved for histopathological analysis including serial sectioning (SS) at 250 μm intervals and immunohistochemistry (IHC).

In 3 patients (including 4 neck sides) no SN could be identified at lymphoscintigraphy, while in the other 19 patients a total of 25 SNs was visualized in 21/23 neck sides. In one patient the SN identified on scintigraphy could not be found in the neck dissection specimen. In 20 neck dissection specimens of 19 patients 23 SNs and 30 additional radioactive lymph nodes could be identified. Of the 7 necks in which SNs were not identified, 5 necks concerned 4 patients who underwent bilateral neck dissections. At routine histological examination at least one SN was tumor-positive in 8/19 patients. In one patient a metastasis was detected in a non-radioactive lymph node, while the SN was tumor-free, also at SS and IHC. In the remaining 10 patients all radioactive (n=26) and all non-radioactive (n=279) lymph nodes did not contain tumor at histopathological analysis, including SS and IHC. The sensitivity of the SN procedure for predicting lymph node metastases therefore was 89% (8/9 patients), when a SN was identified. The overall accuracy of the SN procedure for predicting the presence or absence of lymph node metastases in the neck was 95% (18/19 patients).

Our study seems to validate the SN hypothesis for oral (and oropharyngeal) cancer. The role of SN biopsy in the management of the N0-neck in such patients has yet to be established through prospective trials. SN identification (and thus biopsy) seems not reliable in patients with tumors located in or close to the midline.

Introduction

The lymph node status of the neck is the most important prognosticator for patients with head and neck squamous cell carcinoma (HNSCC).[1] If metastases in the neck are diagnosed, the neck should be treated. Furthermore, it is widely accepted to treat the neck electively when the risk of occult metastases is estimated to be higher than 20%.[2] If the status of the neck is assessed by palpation alone, most sites and stages of HNSCC qualify for elective treatment on this basis.[3] For the majority of patients, however, this policy results in overtreatment of the neck. Much effort has been directed to increase the accuracy of the assessment of the N0 neck. A prospective study comparing palpation, imaging techniques and Ultra Sound guided Fine Needle Aspiration Cytology (USgFNAC) has shown that USgFNAC is the most accurate technique to detect occult lymph node metastases in the neck.[4] In experienced hands the sensitivity for the N0 neck can reach 73% with a specificity of 100%,[5] although others reported sensitivities in the range of 42% to 50%.[6-8] In case of negative USgFNAC at initial staging, a wait-and-see policy for the N0 neck is considered justified in some centers in early stage oral and oropharyngeal cancer.[9]

Sentinel lymph node (SN) biopsy has been proposed as a potential alternative for staging of the N0 neck. The SN concept is based on the theory of orderly progression of tumor cells within the lymphatic system. This so called "sentinel node" will be the first lymph node to receive metastatic tumor cells. The SN concept assumes that lymphatic metastases, if present, will always be found at least in the SN. A tumor-negative SN would preclude the presence of other regional metastases. In large clinical studies of patients with breast cancer and malignant melanoma, preoperative SN identification by lymphoscintigraphy and subsequent SN biopsy has been shown to be an accurate staging technique.[10-13] Histological analysis of the SN correctly reflected regional lymph node status in >98% of the cases.

SN identification in HNSCC patients has been recently investigated by various groups, but the results are not consistent.[14-28] These studies particularly focus on the accuracy and feasibility of SN procedures in HNSCC, including the optimal technique for SN identification and biopsy. However, as yet not much attention has been paid to the histopathological examination of the SN. Analysis of the SN is in most studies limited to a single Hematoxylin-Eosin (H&E) stained section per lymph node. Clearly, the chance of detecting metastatic tumor cells in a lymph node depends on the amount and distribution of tumor. Clusters of tumor cells can easily be missed in just one random section of a lymph node. To increase the chance of finding small metastases, it is desirable to make step sections through the lymph node at regular intervals.[29] Step sections are obviously only necessary when the first section appears to be tumor-negative.

Clearly, first the principle of the SN concept that lymphatic spread is not a random event, but that it follows an orderly and predictable pattern must be validated in HNSCC. In this study we report on the histopathological validation, including serial sectioning (SS) and immunohistochemistry (IHC) of the sentinel node concept in a series of patients with oral and oropharyngeal cancer in whom the neck has been staged N0 by palpation.

Patients and methods

Patients

Between May 1998 and January 2002 22 patients with histologically proven squamous cell carcinoma of the oral cavity or oropharynx and a clinically N0 neck based on palpation were enrolled in the study (Table 1). The population consisted of 9 female and 13 male patients. The median age was 59 years (range 44-80 years). The majority of these patients presented with large (T3-4) primary tumors, because for patients with small (T1-2) primary tumors treatable by transoral excision we maintain a wait-and-see policy for the neck in case of negative USgFNAC findings at initial staging. All patients were planned for combined primary tumor excision and elective modified radical neck dissection. In one patient a supraomohyoid neck dissection was carried out on the contralateral neck side. The study protocol was approved by the Institutional Review Board and informed consent was obtained from all patients.

SN identification at lymphoscintigraphy

The day before surgery, the patients received 75-80 MBq ^{99m}Tc-labeled colloidal albumin (CA) suspended in 0.4 ml saline (Nanocoll®, Sorin Biomedica, Sallugia, Italy, particle size 3-80 nm) by three to four submucosal peritumoral injections. Before injection, the patients received local anesthesia with 10% lidocaine spray. Within two minutes after injection, lateral dynamic lymphoscintigraphic images were obtained (20 x 60 sec acquisitions) in a 128 x 128 x 16 matrix, the patient lying in supine position under a gamma camera with a low-energy high-resolution parallel-hole collimator. Subsequently, static scintigraphy was performed during 120 sec in anterior projection to reduce overprojection from the injection site over the SNs. After visualization of the SNs, the position was marked on the overlying skin with the use of a point-source ^{57}Co marker, and confirmed with a 14 mm diameter handheld gamma probe (CTC 4, RMD, Watertown, MA, USA). A focal accumulation was considered to be the SN if an afferent lymphatic channel could be seen. In case of multiple foci without visualization of lymphatics the most intense focus was considered to be the SN.

USgFNAC

In total, 21/22 patients underwent US examinations of levels I through V of both sides of the neck using a 7.5 MHz linear array transducer 7 (Acuson Company, Mountain View, CA, USA, and ATL HDI 3000, Bothell, WA, USA). One patient refused USgFNAC. The minimal axial diameter of every lymph node as depicted with US was measured on the screen, as this is the most relevant size criterion.[30] USgFNAC was performed of the visualized SNs as well as of enlarged lymph nodes (a minimal axial diameter of 3 mm in level I and 4 mm in other levels). The median number of aspirated lymph nodes was three per patient with a maximum of five.

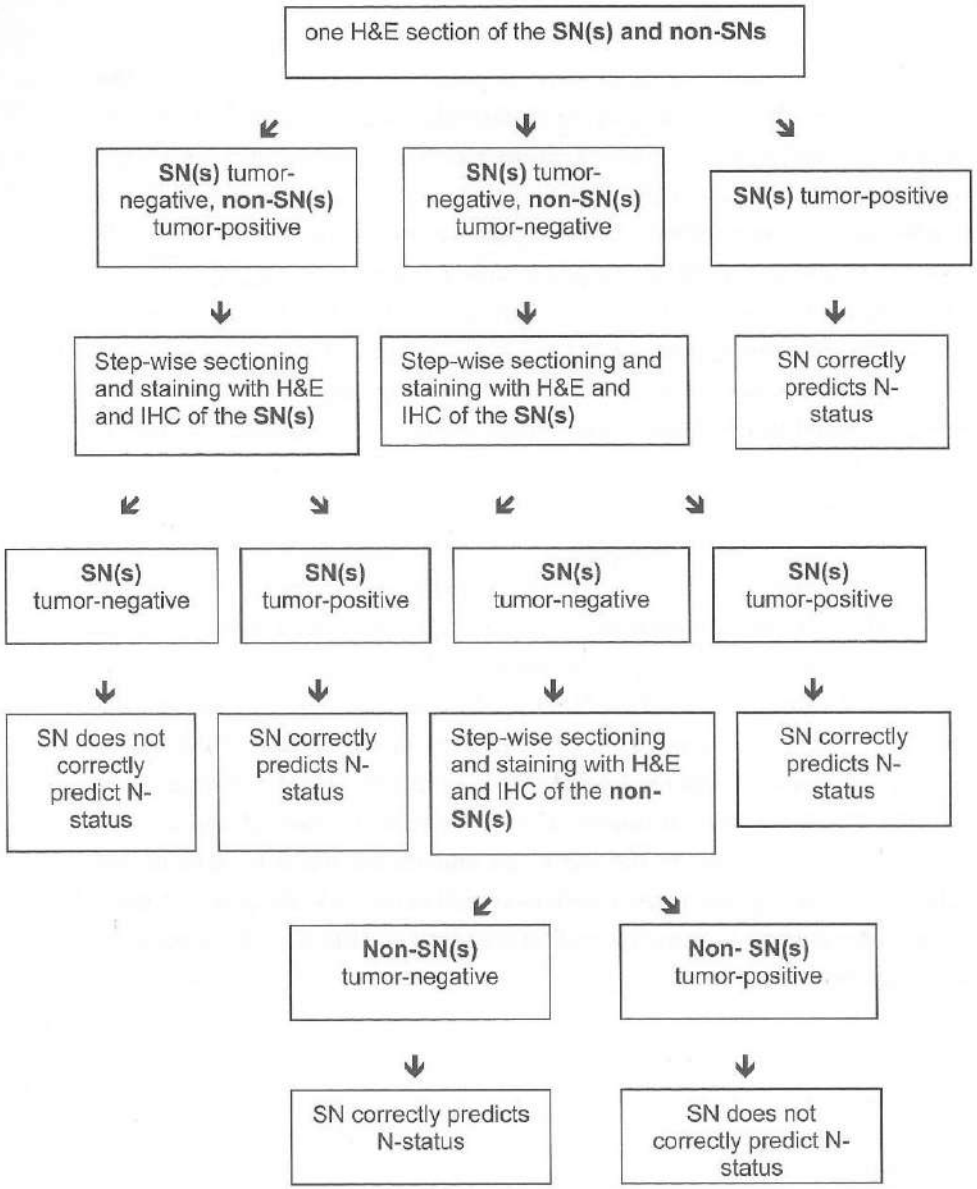
SN identification in neck dissection specimens

The next day, all patients underwent excision of the primary tumor and elective modified radical neck dissection. In five patients a bilateral neck dissection was carried out, as the primary tumor was located in or close to the midline. One of these five patients underwent a supraomohyoid neck dissection on the contralaterdal side and the other four patients underwent bilateral modified radical neck dissection. The remaining 17 patients underwent unilateral modified radical neck dissection. After removal of the neck dissection specimen, the entire specimen was taken to the Department of Nuclear Medicine, it was placed on a gamma camera and scintigraphy was performed to facilitate identification of the SNs. Subsequently, the handheld probe was used to retrieve the SNs a well as other radioactive lymph nodes from the neck dissection specimen for separate histological examination. A count rate of less than 10% of the most radioactive lymph node was considered to be background.

Histopathological analysis, including SS and IHC of SNs and other lymph nodes

The SNs and all other lymph nodes were fixed in formalin and embedded in paraffin. Lymph nodes smaller than 0.5 cm were processed intact, those between 0.5 cm and 1.0 cm were bisected, and those larger than 1.0 cm were cut in pieces of approximately 0.5 cm. Of all lymph nodes one H&E-stained section was made for routine analysis. The analysis scheme indicated in Figure 1 was used. If the SN contained tumor by a routine H&E-stained section, then the remaining lymph nodes were further examined by routine analysis only. If the SN was tumor-free as determined by a H&E-stained section, four additional steps were made through the tissue blocks at intervals of 250 μm. At each step two sections were prepared, one stained with H&E and the other with anticytokeratin antibody. These step section analyses are standard in our center for SN evaluation in different cancers.[29] A 1% AE1:AE3 solution (Boehringer Mannheim Biochemica, Mannheim, Germany) was used for immunostaining. This is a 1:20 mixture of mouse monoclonal antibodies AE1 and AE3, which recognizes almost all human epithelial keratins with minimal cross-reactivity to formalin fixed lymph node reticular cells. If the SN remained tumor-free after additional H&E-staining and immunostaining, then all other lymph nodes were examined similarly: four additional steps were made and stained as described.

Figure 1. Histopathological analysis of SNs and other lymph nodes (non-SNs).



Results

SN identification at lymphoscintigraphy

In Table 1 T-site and T-stage, N-stage at palpation and by USgFNAC, the number of SNs identified at lymphoscintigraphy and in the neck dissection specimen as well as the number of additional radioactive lymph nodes, the histopathological results of the SNs and non-radioactive lymph nodes, and the pN-stage are summarized. In three patients, including four necks (Table 1, cases 7, 14 and 18), no SN could be identified at lymphoscintigraphy, while in the other 19 patients a total of 25 SNs in 21/23 necks was visualized at dynamic lymphoscintigraphy or at later static imaging. Thus, in six necks of 22 patients no SN was identified at lymphoscintigraphy. Four of these six necks concerned three patients undergoing bilateral neck dissection (cases 4, 12, and 14), and two necks concerned two patients undergoing unilateral neck dissection (case 7 and 18).

In only 2/19 patients the focal accumulations appeared to be in conjunction with visible afferent lymphatic channels. In the remaining patients lymphatics to the SN were not visualized, and therefore the most intense focus was considered to be the SN. One SN was visible in 13 patients and two SNs in 6 patients. Three of these six patients showed bilateral SNs (cases 8, 21 and 22). Case 8 presented with a floor of mouth (FOM) carcinoma on the left side and underwent unilateral neck dissection. Cases 21 and 22 had FOM carcinomas located on the right side and on the left side close to the midline, respectively; these cases underwent bilateral neck dissection. A total of 23 additional focal accumulations that were not classified as SN were seen at lymphoscintigraphy.

USgFNAC

As described earlier, one patient (case 21) refused USgFNAC. In six of the remaining 21 patients USgFNAC demonstrated lymph node metastases, while in 15/21 patients no lymph node metastases were demonstrated. Four patients (cases 4, 7, 9 and 12) had one, one patient had two (case 8) and one patient (case 10) had three lymph node metastases, respectively. The minimal axial diameter of these metastatic lymph nodes varied from 3 mm to 12 mm.

SN identification in neck dissection specimens

In total, 27 neck specimens from 22 patients were explored for radioactive lymph nodes. Each neck was considered separately. In 20 of the 27 necks SNs as defined by lymphoscintigraphy were found in the neck dissection specimen. In seven necks no SNs were identified, including the forementioned six necks (cases 4, 7, 12, 14, and 18) in which no SNs were identified at lymphoscintigraphy, as well as one additional neck (case 21) in which the SN identified at lymphoscintigraphy could not be found in the neck dissection specimen. The SN concept was therefore evaluated in the remaining 20 necks of 19 patients.

In total, 23 SNs in 20 necks visualized at scintigraphy the day before surgery as well as 30 additional radioactive lymph nodes were identified in the neck dissection specimen. In four necks (cases 8, 9, 17 and 22) no additional radioactive lymph nodes were identified in the specimen, whereas in the remaining 16 necks one to four additional radioactive lymph nodes were identified. In 13 of these 16 necks the additional radioactive lymph nodes were found in other levels than the SNs at scintigraphy.

in the neck dissection specimen, the number of additional radioactive lymph nodes in the neck, the number of tumor-involved SNs and other lymph nodes, and the pN-stage per patient.

Case	Primary site	Location L/R/M	T-stage	N-stage using palpation only	N+ after USgFNAC (no of tumor-positve nodes)	No of SNs at scintigraphy	Type and site of neck dissection	No of SNs in specimen	No of additional RLNs	No of tumor-positive SNs in specimen	No f other lymph nodes with tumor in specimen	pN-stage (after steps)
1	Tongue	R	T3	N0		1	MR r	1	1	0	0	N0
2	Trig R	L	T3	N0		1	MR l	1	1	0	0	N0
3	Trig R	R	T4	N0		1	MR r	1	3	0	0	N0
4* R	FOM	M	T4	N0		0	MR r	0	0	NA	1	N2c
L					N+ (1)	1	MR l	1	1	1	2	
5	Trig R	R	T2	N0		2	MR r	2	3	0	0	N0
6	FOM	R	T2	N0		2	MR r	2	2	1	1	N2b
7	Tonsil	L	T2	N0	N+ (1)	0	MR l	0	(3)#	NA	1	N2b
8	FOM	L	T3	N0	N+ (2)	2‡	MR l	1	0	1	2	N2b
9	FOM	R	T3	N0	N+ (1)	1	MR r	1	0	1	1	N2b
10	Tonsil	R	T3	N0	N+ (3)	1	MR r	1	3	1	2	N2b
11	Tonsil	L	T3	N0		1	MR l	1	1	0	0	N0
12* R	Tongue	L	T3	N0		0	MR r	0	0	NA	1	N2c
L					N+ (1)	1	MR l	1	3	0	1	
13	Tongue	L	T3	N0		2	MR l	2	1	0	0	N0
14* R	FOM	M	T4	N0		0	MR r	0	0	NA	1	N1
L						0	MR l	0	0	NA	0	
15	Buccal M	L	T2	N0		1	MR l	1	4	1	1	N2b
16	FOM	L	T3	N0		1	MR l	1	2	1	0	N1
17	Tongue	R	T3	N0		1	MR r	1	0	0	0	N0
18	Tongue	L	T3	N0		0	MR l	0	0	NA	0	N0
19	FOM	R	T2	N0		1	MR r	1	1	0	0	N0
20	Tongue B	R	T3	N0		1	MR r	1	1	0	0	N0
21* R	FOM	R	T3	N0	†	1	MR r	0	0	NA	4	N2c
L						1	MR l	1	2	1	1	
22* R	FOM	L/M	T2	N0		1	SOH r	1	1	0	0	N0
L						1	MR l	1	0	0	0	
Total					**6N+ (9)**	**25**		**23**	**30**	**8**	**19**	**11N0, 11 N+**

*These 5 patients underwent bilateral neck dissection, the results are summarized per neck side; † This patient refused USgFNAC; ‡ Lymphoscintigraphy of this patient showed bilateral SNs; # In this patient the SN concept could not be validated, because no SN was identified at scintigraphy. Although in the specimen 3 RLNs were found, these are not included in the total of additional RLNs. Trig R, trigonum retromolare; FOM, floor of mouth; Buccal M, buccal mucosa; Tongue B, base of tongue; L, left; R, right; M, midline; MR, modified radical neck dissection right or left; SOH, supraomohyoid neck dissection; RLNs, radioactive lymph nodes, NA, not assessable, because SNs were not identified. In the final column the pN-stage per patient is outlined, instead of per neck side.

Histopathological analysis, including SS and IHC of SNs and other lymph nodes

A number of 53 (range 1-5 per neck) radioactive lymph nodes -23 SNs and 30 additional radioactive lymph nodes- and 533 other lymph nodes (range 17-46 per neck) were identified in the 20 neck dissection specimens of 19 patients. Routine H&E histological examination of the SNs showed metastases in 8/19 patients (42%), whereas all SNs and all other radioactive lymph nodes of the other 11 patients were tumor-free by one H&E stained section. One of these 11 patients with a T3 tongue carcinoma extending to the floor of mouth (case 12) who underwent bilateral neck dissection, had a metastasis in a non-radioactive ipsilateral lymph node in level I determined by routine analysis. Additional step-wise analysis of H&E and AE1/AE3 stained sections of the four radioactive lymph nodes of this patient did not reveal any metastases, and this case was therefore considered as false-negative. The radioactive lymph nodes and the non-radioactive lymph nodes of the remaining 10 patients were therefore examined using four additional H&E and IHC stained step sections.

All 26 radioactive lymph nodes -13 SNs and 13 additional radioactive lymph nodes- of the remaining 10 patients were analysed by four additional steps stained with AE1/AE3 IHC and routine H&E. As no tumor was detected, the 279 non-radioactive lymph nodes of these patients were examined similarly. All lymph nodes were tumor-free.

In the group of eight patients with tumor-positive SNs, in only one patient the SN was the only lymph node that harbored tumor (case 16) as determined by routine histopathological analysis. Thus, in seven patients other lymph nodes contained tumor in addition to the SNs. In four of these seven patients (cases 6, 9, 15, and 21) one additional tumor-positive lymph node was found, and in three other patients (cases 4, 8 and 10) two additional tumor-positive lymph nodes were found as determined by routine histopathological analysis.

When a SN was found in the neck dissection specimen, the sensitivity of the SN procedure for predicting the presence of tumor was 89% (8/9 patients) (Figure 2). The overall accuracy of the SN procedure for predicting the presence or absence of lymph node metastases in the neck was 95% (18/19 patients).

Figure 2. Relationship between the histopathological status of the SN and that of the neck in 19 patients in whom a SN was identified

	SN tumor-positive	SN tumor-negative	Total
Patients with a tumor-positive neck	8	1	9
Patients with a tumor-negative neck	X	10	10
Total	8	11	19

Ten of the 11 patients with histologically tumor-positive lymph nodes underwent USgFNAC preoperatively, which detected occult lymph node metastases in 6/10 (60%) patients. When each neck is considered separately, USgFNAC demonstrated lymph node metastases in 6/12 (50%) necks. As mentioned before, one patient (case 21) refused USgFNAC, who had histologically bilateral lymph node metastases.

Histological examination of the 7 necks in which no SN was identified

In one patient (case 14) SNs were not identified in both sides of the bilateral neck dissection and histological examination revealed one tumor-positive lymph node with extra nodal spread in level I. In three other patients (cases 4, 12 and 21) who underwent bilateral neck dissection SNs were not identified in either one of both neck sides. Histological examination of the three necks of these three patients revealed one (cases 4 and 12) and four (case 21) lymph node metastases. Two other patients (case 7 and 18) in which no SN was identified underwent unilateral neck dissection, and histological examination of the neck dissection specimen revealed two and no tumor-involved lymph nodes, respectively.

Discussion

This study was performed to determine the histopathological validation of the SN concept in patients with oral or oropharyngeal cancer and a clinically N0 neck staged by palpation. One of the conditions for performing SN biopsy is the identification of a SN at lymphoscintigraphy preoperatively. Our study demonstrated a SN identification rate of 86% (19/22 patients) by lymphoscintigraphy, which comes close to the rates demonstrated in other studies on SN identification in HNSCC. In nine patients with T2N0 oral or oropharyngeal cancer Taylor et al. reported a SN identification rate of 89% at lymphoscintigraphy.[20] Another study by Mozilla et al. showed SN visualization in 95% of 41 patients.[23] Pitman et al. demonstrated a 95% identification rate as well in 20 patients with oral cancer.[27] On the other hand, Koch et al. identified SNs at lymphoscintigraphy in only 2 out of 5 patients with SCC in the oral cavity using radiolabeled colloid alone.[14] Unfortunately, several studies describe the use of lymphoscintigraphy preoperatively, but the results on SN identification are not reported.[17,21,25]

In six necks no SN could be identified at lymphoscintigraphy. Two patients presenting with a FOM carcinoma might have had drainage to SNs in level I that was not visualized due to radioactive scatter originating from the primary tumor site. In several studies, proximity of SNs to the injection site has been reported to be the main difficulty in SN identification during lymphoscintigraphy.[14,17,21] Four of the six necks, in which no SN could be identified, concerned three patients undergoing bilateral neck dissection (cases 4, 12, and 14). This experience has not been described in other studies.[14,20,23] An explanation could be that the tracer volume (0.4 ml) used in this study was too low to allow for adequate distribution over both neck sides in the patients planned for bilateral neck dissection. This might be overcome using a double tracer volume for patients presenting with tumors close to the midline, who are planned for bilateral neck dissection. The two other necks concerned two patients undergoing unilateral neck dissection (case 7 and 18). One of these two patients had a T3 tongue carcinoma, and ulceration of the tongue tumor might have resulted in leakage of the tracer

out of the injection site into the oral cavity, preventing lymphatic uptake. This has also been described by Shoaib et al., in a study in which three patients with indurated and ulcerated tongue tumors showed blue dye leakage into the oral cavity.[21] Probably, the colourless injected colloid followed the same pathway.

One patient (case 8) with a well lateralized floor of mouth carcinoma on the left showed bilateral SN drainage at lymphoscintigraphy. The contralateral neck side of this patient remained tumor-free after follow-up of 38 months. Others have reported on bilateral drainage patterns in patients with unilateral primary tumors as well.[19,20] This is not surprising, as it is well known that lymphatic drainage pathways in the oral cavity may cross the midline.

In one patient (case 21) with a T3 FOM carcinoma the SN as identified on lymphoscintigraphy the day before surgery could not be found in the neck dissection specimen. We could not find any explanation for this observation.

Although 23 SNs as identified at lymphoscintigraphy were found in the neck dissection specimen, 30 additional radioactive lymph nodes were removed, ranging from one to four per patient. This range of radioactive lymph nodes in the neck dissection specimens is in agreement with other studies on SN identification in HNSCC, where the number of radioactive lymph nodes is reported to be as high as six per patient.[17-19,27] Although at preoperative lymphoscintigraphy additional focal accumulations were visualized, still, a difference was observed between the total number of additional focal accumulations at scintigraphy and the total number of additional radioactive lymph nodes found in the neck dissection specimen. One explanation might be that two SNs located very closely to each other appear as one focal accumulation at scintigraphy due to limitations in resolution of the gamma camera, which was the case in two patients in our study. Another explanation could be spill of the radioactive tracer to second echelon nodes, which might occur in the timespan between injection and sampling, which lasted in our study more than 24 hours. A possible solution might be

injection of the tracer several hours before surgery. Thereby, it is of great importance to start as quickly as possible with dynamic imaging because it might increase visualization of lymphatic channels from the injection site to the lymph nodes, which provides the evidence required to distinguish SNs from second echelon nodes. Another solution might be the use of a radioactive tracer with a larger particle size, for example ^{99m}Tc-sulphur colloid (^{99m}Tc-SC) (particle size 50-1000 nm), which might diminish the spread to second echelon nodes. A study by Pijpers et al. comparing ^{99m}Tc-CA and ^{99m}Tc-SC in melanoma patients showed that ^{99m}Tc-SC causes less spill to second echelon nodes, but at the expense of a less frequent visualization of lymphatic channels.[31] The main question is whether lymph node metastases can be found in these 30 additional radioactive lymph nodes without tumor-involvement of the "first" 23 SNs identified at scintigraphy. In this study the lymph node metastases, if any, were always found in the 23 SNs (except for the one false-negative case), whereas the 30 additional radioactive lymph nodes did not reveal tumor. Based on these results the surgeon performing the SN biopsy might consider to remove only the SNs first visualized at scintigraphy instead of taking out all radioactive lymph nodes. For this purpose, precise marking of only the "first echelon" SN on the overlying skin after scintigraphy might guide the surgeon in determining which lymph node to biopsy.

In the group of eight patients with tumor-positive SNs at routine histopathology, the SN was the only tumor-positive lymph node in only one patient, whereas in the remaining patients one or two additional tumor-positive lymph nodes were found. These findings are in sharp contrast to other studies, which report the SN being the only tumor-involved lymph node in more than 60% of the cases.[20,21,24,25] This discrepancy could be explained by the fact that the majority of our patients presented with large primary tumors, with a higher risk to develop lymph node metastases.

One false-negative case was observed in this study. At histological examination the tumor-positive (non-radioactive) lymph node (8 mm) was completely replaced by cancer cells. This lymph node metastasis was already

detected by USgFNAC preoperatively, and this patient would therefore not have been considered for SN biopsy. However, for the scope of this study, we included this patient for evaluation of the SN concept. The heavy tumor load in this lymph node might have caused lymph obstruction resulting in deviation of the lymphatic flow to "false SNs". This phenomenon is one of the most important pitfalls of the SN technique and has been described for SNs in other types of cancer, including breast cancer and melanoma.[10,11]

In the 10 patients without evidence of metastases by routine histological examination, we did not find lymph node metastases using four additional H&E stained and immunostained step sections of the radioactive lymph nodes and non-radioactive lymph nodes. So far, only two other studies reported the use of additional H&E stained and immunostained step sections for SN biopsy in oral cancer patients. Using additional SS and IHC, Ross et al. detected metastatic SNs in 2/28 patients, whose SNs were initially staged tumor-negative at routine histopathology,[25] while Civantos et al. upstaged 2/10 patients using additional SS and IHC on initially tumor-negative SNs.[28] It is, therefore, very likely that our up to now "negative" outcome of additional SS and IHC analysis is an accidental finding, related to the small number of patients investigated. The importance of thorough histological examination of the SNs, including SS and IHC, can not be overemphasized.

The sensitivity of USgFNAC in this study is 60%, which compares favourably to most other studies, where the sensitivity has been reported to vary from 42% to 50%,[6-8] although one study demonstrated a sensitivity of 73%.[5] SN identification and examination revealed lymph node metastases in four additional patients (case 6, 15, 16, and 21), including the patient who refused USgFNAC. This indicated that SN biopsy might be an adjunct diagnostic tool in case of negative USgFNAC findings at the initial neck staging.

When a SN was found in the neck dissection specimen, the sensitivity of the SN procedure for predicting the presence of tumor was 89% with an overall accuracy of 95%. These rates come close to the sensitivity and overall accuracy rates which has been quoted in the literature. In studies including

at least ten patients, the sensitivity has been reported to vary between 89% and 100%, while the overall accuracy has been reported to vary between 97% and 100%.[21,23-25,27] As mentioned before, our study population, including mainly patients with more advanced T-stage and a higher incidence of lymph node metastases, might have caused a lower sensitivity rate as compared to those reported in the literature.

It may be concluded from our study that the SN concept seems to be valid for patients with a squamous cell carcinoma of the oral cavity or oropharynx, although our study group was relatively small and consisted of patients with mainly advanced T-stages. Furthermore, it appears from our results that SN identification (and thus biopsy) in patients with tumors located in or close to the midline (and planned for bilateral neck dissection), seems not reliable. It is important to emphasize that the role of SN biopsy in patients with oral and oropharyngeal cancer need yet to be established. This should be investigated in patients presenting with mainly T1 and T2 tumors. Particularly in these patients elective neck dissection causes considerable overtreatment, and omission of neck dissection in case of tumor-negative SN biopsy will therefore reduce morbidity. From Canniesburn Hospital in Glasgow a multicenter trial is organized, in which the decision to perform a neck dissection is based on the histopathological examination of the SN after biopsy. If the outcome of such studies would demonstrate that SN biopsy is a reliable staging method for the N0 neck, we suggest performing SN biopsy only in patients with a N0 neck after USgFNAC, as USgFNAC can detect approximately 50% of occult metastases at initial staging, while this technique is less invasive and more patient friendly.

Acknowledgements

We would like to thank Thea Tadema en Willem W. de Jong (Department of Pathology, *Vrije Universiteit* Medical Center, Amsterdam) for their technical assistance. We would like thank Paul van Diest for assistance with the study design and critically reading the manuscript. Joop Kuik is acknowledged for the statistical analysis concerning the study design.

References

1. Leemans CR, Tiwari R, Nauta JJ, van der Waal I, Snow GB. Regional lymph node involvement and its significance in the development of distant metastases in head and neck carcinoma. *Cancer* 71:452-456, 1993.

2. Weiss MH, Harrison LB, Isaacs RS. Use of decision analysis in planning a management strategy for the stage N0 neck. *Arch Otolaryngol Head Neck Surg* 120:699-702, 1994.

3. Ali S, Tiwari RM, Snow GB. False positive and false negative neck nodes. *Head Neck Surg* 8:78-82, 1985.

4. Van den Brekel MWM, Castelijns JA, Stel HV, Golding RP, Meyer CJ, Snow, GB. Modern imaging techniques and ultrasound-guided aspiration cytology for the assessment of neck node metastases: a prospective comparative study. *Eur Arch Otorhinolaryngol* 250:11-17, 1993.

5. Van den Brekel MW, Castelijns JA, Stel HV et al. Occult metastatic neck disease: detection with US and US-guided fine- needle aspiration cytology. *Radiology* 180:457-461, 1991.

6. Righi PD, Kopecky KK, Caldemeyer KS, Ball VA, Weisberger EC, Radpour S. Comparison of ultrasound-fine needle aspiration computed tomography in patients undergoing neck dissection. *Head Neck* 19:604-610, 1997.

7. Takes RP, Righi P, Meeuwis CA et al. The value of ultrasound with ultrasound-guided fine-needle aspiration biopsy compared to computed tomography in the detection of regional metastases in the clinically negative neck. *Int J Radiat Oncol Biol Phys* 40:1027-1032, 1998.

8. Knappe M, Louw M, Gregor RT. Ultrasonography-guided fine-needle aspiration for the assessment of cervical metastases. *Arch Otolaryngol Head Neck Surg* 126:1091-1096, 2000.

9. Nieuwenhuis EJ, Castelijns JA, Pijpers R, et al. Wait-and-see policy for the N0 neck in early stage oral and oropharyngeal SCC using ultrasound guided cytology: is there a role for identification of the sentinel node? *Head Neck* 24:282-289, 2002.

10. Pijpers R, Borgstein PJ, Meijer S, Hoekstra OS, van Hattum LH, Teule GJ. Sentinel node biopsy in melanoma patients: dynamic lymphoscintigraphy followed by intraoperative gamma probe and vital dye guidance. *World J Surg* 21:788-792, 1997.

11. Borgstein PJ, Pijpers R, Comans EF, van Diest PJ, Boom RP, Meijer S. Sentinel lymph node biopsy in breast cancer: guidelines and pitfalls of lymphoscintigraphy and gamma probe detection. *J Am Coll Surg* 186:275-283, 1998.

12. Morton DL, Thompson JF, Essner R et al. Validation of the accuracy of intraoperative lymphatic mapping and sentinel lymphadenectomy for early-stage melanoma: a multicenter trial. Multicenter Selective Lymphadenectomy Trial Group. *Ann Surg* 230:453-463, 1999.

13. Muller MG, Borgstein PJ, Pijpers R et al. Reliability of the sentinel node procedure in melanoma patients: analysis of failures after long-term follow-up. *Ann Surg Oncol* 7:461-468, 2000.

14. Koch WM, Choti MA, Civelek AC, Eisele DW, Saunders JR. Gamma probe-directed biopsy of the sentinel node in oral squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 124:455-459, 1998.

15. Pitman KT, Johnson JT, Edington H, et al. Lymphatic mapping with isosulfan blue dye in squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg* 124:790-793, 1998.

16. Klutmann S, Bohuslavizki KH, Brenner W, et al. Lymphoscintigraphy in tumors of the head and neck using double tracer technique. *J Nucl Med* 40:776-782, 1999.

17. Shoaib T, Soutar D, Prosser JE, et al. A suggested method for sentinel node biopsy in squamous cell carcinoma of the head and neck. *Head Neck* 21:728-733, 1999.

18. Alex JC, Sasaki CT, Krag DN, Wenig B, Pyle PB. Sentinel lymph node radiolocalization in head and neck squamous cell carcinoma. *Laryngoscope* 110:198-203, 2000.

19. Chiesa F, Mauri S, Grana C, et al. Is there a role for sentinel node biopsy in early N0 tongue tumors? *Surgery* 128:16-21, 2000.

20. Taylor RJ, Wahl RL, Sharma PK, et al. Sentinel node localization in oral cavity and oropharynx squamous cell cancer. *Arch Otolaryngol Head Neck Surg* 127:970-974, 2001.

21. Shoaib T, Soutar DS, MacDonald DG, et al. The accuracy of head and neck carcinoma sentinel lymph node biopsy in the clinically N0 neck. *Cancer* 91:2077-2083, 2001.

22. Colnot DR, Nieuwenhuis EJ, Castelijns JA et al. Ultrasound guided aspiration of sentinel nodes for improved staging of head and neck cancer patients-initial experience. *Radiology* 218:289-293, 2001.

23. Mozzillo N, Chiesa F, Botti G, et al. Sentinel node biopsy in head and neck cancer. *Ann Surg Oncol* 8:103S-105S, 2001.

24. Werner JA, Dunne AA, Ramaswamy A, et al. Sentinel node detection in N0 cancer of the pharynx and larynx. *Br J Cancer* 87:711-715, 2002.

25. Ross G, Shoaib T, Soutar DS, et al. The use of sentinel node biopsy to upstage the clinically N0 neck in head and neck cancer. *Arch Otolaryngol Head Neck Surg* 128:1287-1291, 2002.

26. Werner JA, Dunne AA, Ramaswamy A, et al. Number and location of radiolabeled, intraoperatively identified sentinel nodes in 48 head and neck cancer patients with clinically staged N0 and N1 neck. *Eur Arch Otorhinolaryngol* 259:91-96, 2002.

27. Pitman KT, Johnson JT, Brown ML, Myers EN. THESIS: Sentinel Lymph Node Biopsy in Head and Neck Squamous Cell Carcinoma. *Laryngoscope* 112:2101-2113, 2002.

28. Civantos FJ, Gomez C, Duque C, et al. Sentinel node biopsy in oral cavity cancer: correlation with PET scan and immunohistochemistry. *Head Neck* 25:1-9, 2003.

29. Van Diest PJ, Peterse HL, Borgstein PJ, Hoekstra O, Meijer CJ. Pathological investigation of sentinel lymph nodes. *Eur J Nucl Med* 26:S43-49, 1999.

30. Van den Brekel MW, Stel HV, Castelijns JA, et al. Cervical lymph node metastasis: assessment of radiologic criteria. *Radiology* 177:379-384, 1990.

31. Pijpers R, Borgstein PJ, Meijer S, Krag DN, Hoekstra OS, Greuter HNJM, Teule GJJ. Transport and retention of colloidal tracers in regional lymphoscintigraphy in melanoma: influence on lymphatic mapping and sentinel node biopsy. *Melanoma Res* 8:413-418, 1998.

5

Wait-and-see policy for the N0 neck in early-stage oral and oropharyngeal squamous cell carcinoma using ultrasonography-guided cytology: is there a role for identification of the sentinel node?

Eline J.C. Nieuwenhuis, Jonas A. Castelijns, Rik Pijpers, Michiel W.M. van den Brekel, Ruud H. Brakenhoff, Isaac van der Waal, Gordon B. Snow, C. René Leemans

Head Neck 24: 282-289, 2002

Abstract

Management of the N0 neck in patients with head and neck squamous cell carcinoma (SCC) remains controversial. We describe the outcome of patients who underwent transoral tumor excision and a wait-and-see policy for the neck staged N0 by ultrasonography-guided cytology (USgFNAC). Because selection of lymph nodes for USgFNAC is currently based on size criteria, we investigated the additional value of sentinel node (SN) identification.

The outcome of 161 patients with T1-T2 oral/oropharyngeal SCC was determined. In a subgroup of 39 patients the SN was identified and aspirated in addition.

SN identification and aspiration was possible in 38 of 39 patients, but without decreasing the false-negative rate of USgFNAC. During follow-up (12-99 months) 34 of 161 (21%) patients developed lymph node metastases. After therapeutic neck dissection and postoperative radiotherapy 27 of 34 (79%) could be salvaged (88% regional control).

Wait-and-see seems justified in case of negative USgFNAC. Strict follow-up with USgFNAC is required. SN identification and aspiration is feasible, but did not improve lymph node selection.

Introduction

The lymph node status of the neck is the most important prognosticator for patients with head and neck squamous cell carcinoma (HNSCC). Not only presence, but also number of nodal metastases, level in the neck, size of the nodes, and presence of extranodal spread are important prognostic features.[1] If metastases in the neck are diagnosed, the neck should be treated. As both sensitivity and specificity of palpation are in the range of 60 to 70%, a neck without palpable lymph nodes (N0) is still at risk of harboring occult metastases. Consequently, much effort has been devoted to increase accuracy of assessment of the N0 neck. It is widely accepted to treat the neck electively when the risk of occult metastases is estimated to be higher than 20%.[2] For most patients, this policy results in overtreatment of the neck, although no real benefit in survival has been demonstrated compared with delayed neck dissection for lymph node metastases.[3]

Although both computed tomography (CT) and magnetic resonance imaging (MRI) of the neck have been found to be superior to palpation in detecting cervical metastases, these modalities still have a relatively low accuracy for the N0 neck. In contrast, US-guided fine- needle aspiration cytology (USgFNAC) has a higher sensitivity and specificity and is more cost-effective than CT and MRI. In experienced hands, the sensitivity for the N0 neck can reach 73% with a specificity of 100%,[4] although others reported sensitivities in the range of 42% to 50%.[5-7]

Since 1992, we gradually changed our policy towards the neck for patients with T1-T2 oral carcinomas who could be treated by transoral tumor excision. In case of negative USgFNAC at initial staging, these patients are treated by local excision of the primary tumor and are spared an elective lymph node dissection. These patients are subsequently followed by palpation and USgFNAC at regular intervals (wait-and-see policy). In a retrospective study, it was shown that approximately 20% of the patients with negative USgFNAC at the time of presentation developed a neck node metastasis during follow-up.[8] These clinical data indicate that USgFNAC

does not enable detection of all occult metastases. Possible causes of false-negative results may be aspiration of the wrong node, aspiration of the wrong part of a node containing a small metastasis (sampling error) and false interpretation of the cytologic slides. The selection of lymph nodes for aspiration is currently based on known patterns of lymphatic spread and particularly on size criteria.[4] However, the number of nodes in the neck is large, and the patterns of spread are not always consistent. Sensitivity of USgFNAC might be improved by a more functional selection of the lymph node(s) at highest risk of harboring occult metastases (i.e., most notably the sentinel node (SN)).

The sentinel node concept is based on the orderly progression of tumor cells within the lymphatic system. Metastasis to lymph nodes is not a random event and can be determined by identifying the lymph flow from the tumor site to the first draining lymph node. In theory, the SN will thus be the first node to contain metastases; examination of this node should predict accurately regional lymph node status. In a large series of patients with breast cancer and melanoma, preoperative SN identification by lymphoscintigraphy and subsequent SN biopsy has been shown to be an accurate staging technique. Histopathological analysis of the SN correctly reflected regional lymph node status in >98% of the cases.[9-12]

In a pilot study we have evaluated the feasibility of the combined use of SN lymphoscintigraphy and USgFNAC.[13] Our initial experience in 12 patients showed that lymphoscintigraphic identification and subsequent aspiration of SNs was a feasible technique in patients with a SCC of the oral cavity or oropharynx with a N0 neck. Based on these results, we decided to investigate whether the combined use of lymphoscintigraphy and SN-USgFNAC could decrease the false-negative rate in a larger group of patients, compared with our former USgFNAC policy of enlarged lymph nodes only. Therefore, all patients treated between 1993 and 2000 by transoral excision for a T1-T2 oral or oropharyngeal tumor with negative USgFNAC findings at initial staging were included. This group consisted of 122 patients staged with USgFNAC of enlarged lymph nodes only

(USgFNAC) and 39 patients staged with additional SN aspiration (SN-USgFNAC).

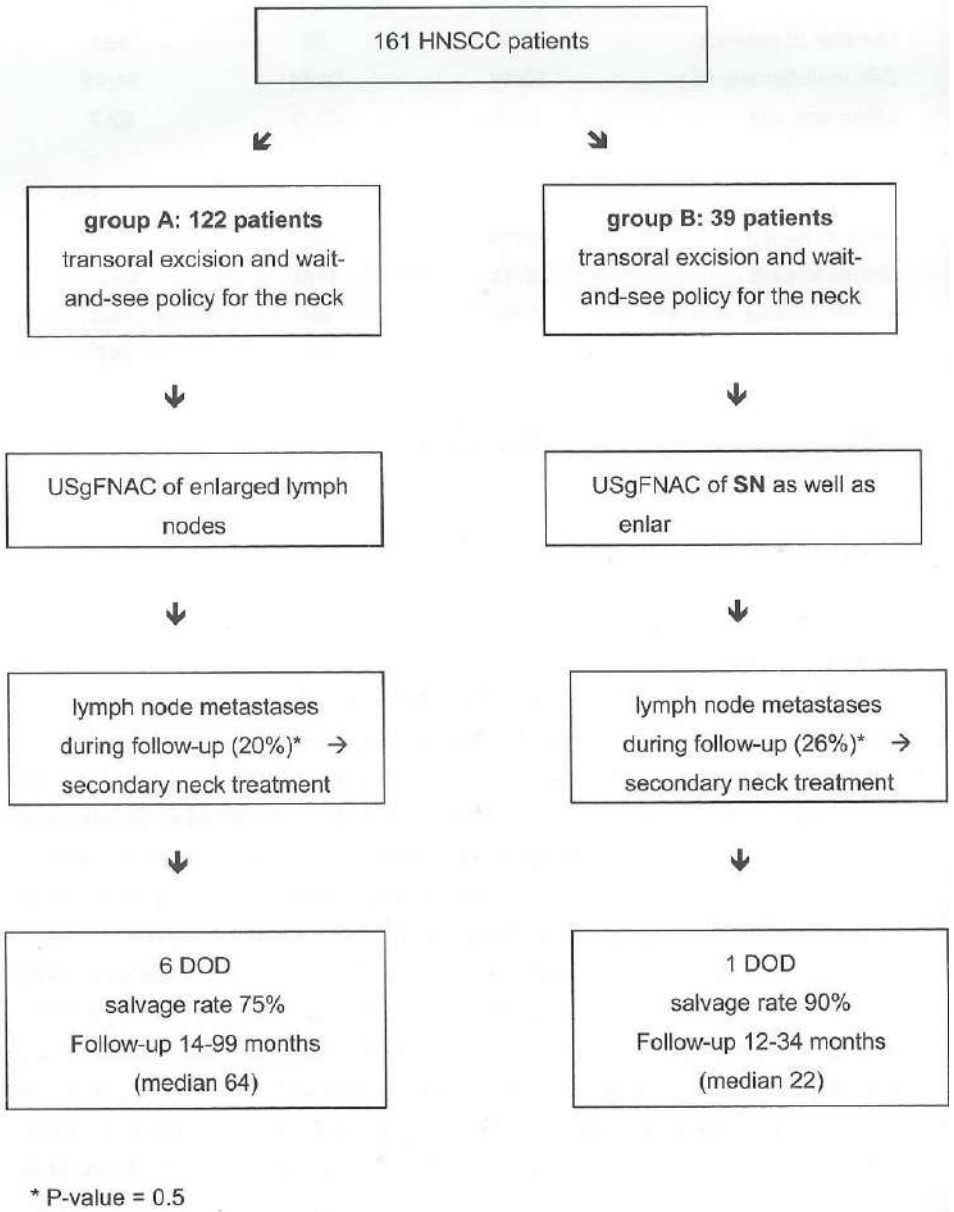
The aim of this study is to evaluate the clinical outcome of these patient groups in terms of neck failures and salvage rates after therapeutic neck treatment. Second, we want to investigate whether additional SN aspiration could decrease the false-negative USgFNAC rate.

Patients and methods

Patient selection

The study design is shown in Figure 1. The study group consisted of 161 previously untreated patients with a T1-T2 N0 histologically proven squamous cell carcinoma of the oral cavity or oropharynx. Patient characteristics are shown in Table 1. All patients were treated with transoral excision of the primary tumor on the basis of negative USgFNAC findings at initial staging. Patients received strict follow-up of the neck, including USgFNAC every 3 months for the first 2 years. A group of 122 patients (group A), treated between 1993 and 1998, prospectively entered the study. In this patient group USgFNAC of suspected lymph nodes was performed based on size criteria only. In a group of 39 patients (group B), treated between 1998 and half of 2000, USgFNAC of the SN was performed in addition to USgFNAC of enlarged lymph nodes. Characteristics of both patients groups staged by either USgFNAC only or SN-USgFNAC are shown in Table 1. With respect to gender, age, tumor stage, and site, both groups show a similar distribution. The SN protocol was approved by the Institutional Review Board, and informed consent was obtained from all patients.

Figure 1. study design and outcome.



* P-value = 0.5

Table 1. Patient characteristics

	USgFNAC	SN-USgFNAC	Total
Number of patients	122	39	161
Sex male/female (%)	52/48	49/51	51/49
Mean age (yr)	63.4	61.0	62.7
	T1 / T2	T1 / T2	T1 / T2
Floor of mouth	28/19	7/3	35/22
Mobile tongue	32/14	17/9	49/23
Inferior alveolar process	12/0	1/1	13/1
Buccal mucosa	3/6	0/1	3/7
Soft palate	5/3	0/0	5/3
Total	80/42	25/14	105/56

USgFNAC

US examinations of levels 1 through 5 of both sides of the neck were performed using a 7.5-MHz linear array transducer 7 (Acuson Company, Mountain View, CA, USA, and ATL HDI 3000, Bothell, WA, USA). The minimal diameter of every lymph node as depicted with US was measured on the screen, because this is the most relevant size criterion.[14] Lymph nodes with a minimal diameter of 3 mm in level I and 4 mm in other levels were selected for USgFNAC. In group A the number of aspirated lymph nodes in the ipsilateral neck side varied from 0 to 4 per patient and in the contralateral neck side from 0 to 2 per patient. In group B, these numbers varied from 0 to 5 for the ipsilateral side and from 0 to 2 for the contralateral side, respectively. Aspiration of the enlarged lymph nodes was performed using a syringe holder (Cameco, Taeby, Sweden) and a 0.6 x 25-mm needle. From each aspiration two cytological smears were prepared. One smear was fixed with 70% ethanol and stained with Papanicolaou and one smear was air-dried and stained with May-Grünwald-Giemsa. Follow-up ranged from 14 to

99 months (median, 64 months) in group A and 12 to 34 months (median, 22 months) in group B.

SN identification and aspiration

After local anesthesia with lidocaine 10% spray, 39 patients received 15-20 MBq ^{99m}Tc-labeled colloidal albumin (CA) suspended in 0.4 ml saline (Nanocoll®, Sorin Biomedica, Sallugia, Italy, particle size 3-80 nm), by 3 to 4 submucosal peritumoral injections. Within 2 minutes after injection, lateral dynamic lymphoscintigraphic images were obtained (20 x 60 sec acquisitions) with the patient in supine position under a gamma camera with a low-energy high-resolution parallel-hole collimator. Subsequently, static scintigraphy was performed during 120 sec in anterior projection to reduce superimposition caused by radioactive scatter originating from the injection site over the SNs. After visualization of the SN(s), the position was marked on the overlying skin with the use of a point-of-source ^{57}Co marker and confirmed with a 14 mm diameter handheld gamma probe (CTC 4, RMD, Watertown, MA, USA). From 9 patients additional static images were acquired 2 to 4 hours after injection. Subsequently, USgFNAC was performed of the visualized SNs and the enlarged lymph nodes. The numbers of aspirated lymph nodes for both ipsilateral and contralateral neck side in group B were described previously. After preparation of the cytological smears, the needle and syringe were washed in phosphate-buffered saline to obtain residues that were used for radioactivity counting in a gamma counter (Wallac, Turku, Finland).

Statistical analysis

Neck metastases-free survival was determined by the Kaplan-Meier method. Patients who developed local recurrences, second primary HNSCC or other tumors were censored in the neck analysis from the date of presentation. Differences in frequency distributions were analyzed by the χ^2-Fisher's exact test. P values <0.05 were considered as significant.

Results

Group A: USgFNAC only

Initial US did not show enlarged lymph nodes in 45 of the 122 (37%) patients, and, consequently, aspirations were not performed. In 40 (33%) patients only ipsilateral lymph nodes were aspirated, whereas from the remaining 37 (30%) patients bilateral enlarged lymph nodes were detected and aspirated. During follow-up 24 of the 122 patients (20%) developed lymph node metastases 1 to 21 months after transoral excision of the primary tumor. All 24 patients were treated by a therapeutic neck dissection. In total, 22 patients received postoperative radiotherapy based on histopathological analysis of the neck dissection specimen.

Group B: SN identification and SN-USgFNAC

In 35 out of 39 (90%) patients at least one SN was visualized by lymphoscintigraphy after peritumoral injection of ^{99m}Tc-CA. One SN was visible in 20 patients, 2 SNs in 14 patients, and one patient showed 3 SNs after 20-min dynamic imaging. All SNs were located ipsilateral. In total, 51 SNs were identified scintigraphically, which were located in level I in 20 cases, in level II in 22 cases, in level III in 8 cases, and in level IV in 1 case. Confirmation of the SN position with the handheld gamma probe proved to be reliable if the SN was located in levels II trough IV. In 4 patients with primary tumors in the anterior oral cavity, dynamic scintigraphy did not enable identification of a SN independent of the injection site, probably due to radioactive scatter originating from the primary tumor. However, in 3 out of these 4 patients the SN could be identified afterwards by measurement of radioactivity in at least one of the aspirated residues of enlarged lymph nodes. Hence, in only 1 out of 39 patients we did not succeed in identifying a SN. In 9 patients static images were acquired 2 to 4 hours post injection, which showed additional hot spots in 4 patients.

In 10 of the 38 patients 13 lymph nodes would not have been selected for aspiration on the basis of the size criteria. From this group, 11 lymph nodes of 9 patients were aspirated. The minimal diameters of these SNs varied from 1.6 mm to 3.8 mm. From 2 of these 11 SNs insufficient material was aspirated for a cytological diagnosis, both with a minimal diameter of 1.6 mm. From the other 9 SNs with minimal diameters ranging from 1.9 mm to 3.8 mm, diagnostic smears were obtained; these were all tumor cell-negative at cytologic diagnosis.

During follow-up, 10 of these 39 patients (26%) developed a lymph node metastasis in the neck within 1.5 to 8 months after treatment of the primary tumor. All but 2 patients developed metastases in levels of the neck, which corresponded to those of the SNs identified at the initial staging. One of these 2 patients developed a lymph node metastasis on the contralateral side. These 10 patients all underwent therapeutic neck dissection, and 9 also received postoperative radiotherapy based on histopathological findings.

Clinical outcome

In group A, 20% (24 of 122) developed lymph node metastases in the neck vs 26% (10 of 39) in group B (p=0.5). No further significant difference was demonstrated with respect to gender, age, stage, and site of the primary tumor. As described earlier, in group B, 11 additional lymph nodes were aspirated because of the SN procedure. Because these lymph nodes were all tumor-negative or contained insufficient material at cytologic examination, we could not demonstrate additional value of SN aspiration. Therefore, the results in terms of neck failures and the occurrence of second primary tumors and recurrences are presented for the total group of 161 patients (group A and B).

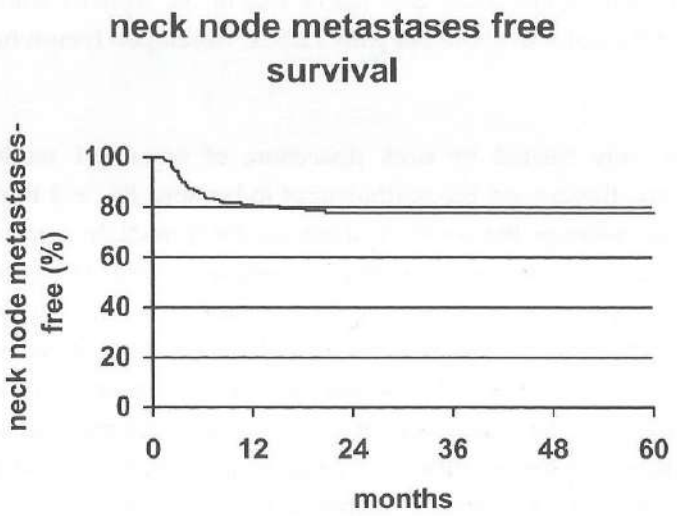


Figure 2. Proportion of patients free of neck metastases after transoral excision (78%).

Of the 161 patients, 34 (21%) developed lymph node metastases in the neck during follow-up without a local recurrence (Figure 2). However, it should be mentioned that in 17 of 161 patients, follow-up was between 12 and 24 months. In 10 patients neck metastases were detected by USgFNAC, whereas in 24 patients suspicious lymph nodes were found at palpation and cytologically confirmed by USgFNAC. These neck failures were located ipsilateral in 32 cases, bilateral in one case, and contralateral in one patient with a T1 SCC of the mobile tongue. The interval between treatment of the primary tumor and the occurrence of cervical metastases was less than 12 months in 30 cases and more than 12 months in 4 cases (Figure 2). Of the 34 patients, 23 patients had primary tumors staged T1 (mobile tongue n = 13, floor of mouth (FOM) n = 6, inferior alveolar process n = 3, buccal mucosa n = 1), whereas 11 patients had T2 tumors (mobile tongue n = 5, FOM n = 4, buccal mucosa n = 1, soft palate n = 1). This distribution corresponds with

the initial stage distribution. Thus, 21% (23 of 105) of the patients with T1 SCC and 19% of the patients (11 of 56) with T2 SCC developed lymph node metastases.

All 34 patients were treated by neck dissection, of whom 31 received postoperative radiotherapy on histopathological indications (ie, ≥ 2 tumor-containing lymph nodes or the presence of extranodal spread). In 2 patients there was no indication for postoperative radiotherapy, and one patient refused radiotherapy. Histopathological examination of the dissection specimen of the 34 patients revealed involved nodes in level I in 18 patients, level II in 25 patients, level III in 15 patients, level IV in 8 patients, and one patient had positive lymph nodes in level V. One positive lymph node was found in 10 patients, 2 positive nodes in 9 patients, 3 in 3 patients, and 4 or more involved nodes in 12 patients. Extranodal spread was found in 24 patients (71%).

Twenty-seven of 34 (79%) patients with neck metastases were salvaged after therapeutic neck dissection. Seven of the 34 patients (21%) died of disease: 4 patients had regional recurrences in the treated neck, 2 patients died of distant metastases, and one patient died of aggressive lymph node metastases in the contralateral neck side. Six of these seven failures died within 3 to 8 months after neck dissection, one patient died after 20 months. The proportion of patients free of neck recurrences in the treated neck side after neck failure is 88% (Figure 3). In total, 6 patients died of other causes 11 to 75 months after neck dissection, without evidence of recurrence of their index tumor or metastases thereof. The other 21 patients with neck failures are alive without evidence of disease with a follow-up ranging from 5 to 78 months after neck dissection.

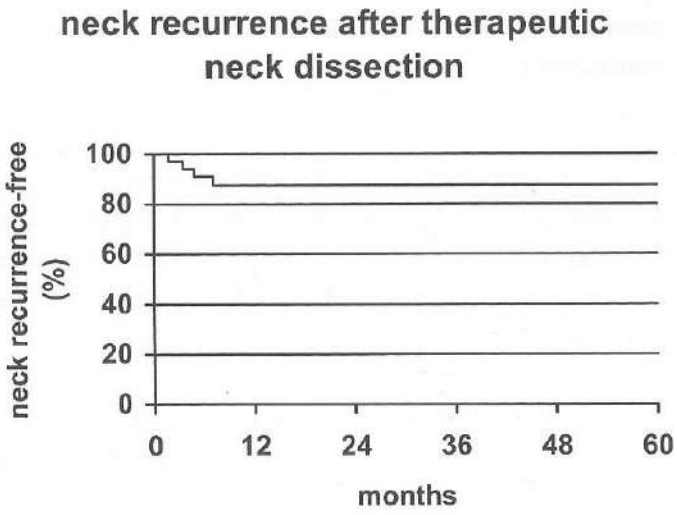


Figure 3. Proportion of patients free of neck recurrence after treatment for neck failures (88%).

The characteristics of the 27 patients with neck metastases who were salvaged after therapeutic neck dissection vs the 7 patients who died of disease after therapeutic neck dissection are shown in Table 2. From the table it can be seen that the number of tumor-containing lymph nodes and the presence of extranodal spread differed significantly between the two groups. Tumor stage of the primary tumor, size of the tumor-containing nodes at USgFNAC, and interval to the development of neck metastases did not differ significantly. USgFNAC detected lymph node metastases in 10 patients, of whom one patient (10%) died of disease. In contrast, 24 patients developed clinically suspicious metastases, of whom 6 patients (25%) died of disease. Thus, patients whose metastases were found by USgFNAC tend to have a better prognosis as compared to patients with palpable lymph nodes, although this observation is not statistically significant.

Table 2. Characteristics of 7 patients who died of their cancer and of 27 patients who were salvaged after treatment (neck dissection +/- postoperative radiotherapy) for neck failure.

		USgFNAC		Histopathology		
No. of cases (follow-up)	T1/T2	Interval in months (median)	Size in mm of pos. lymph nodes (median)	No of meta ≤ 3/ >3 (median)	% of lymph nodes with ENS	Follow-up in months (median)
7 (DOD)	6/1	3-18 (4)	6-20 (12)	2/5* (4)	94%† (33/35)	3-20 (6)
27 (NED/DOC)	17/10	1-21 (4)	5-30 (10)	23/4* (2)	53%† (37/70)	5-77 (19)

The interval between transoral resection and positive USgFNAC is indicated in column 3, whereas the next column specifies the size of positive lymph nodes detected at USgFNAC. Number of positive nodes and extranodular spread (ENS) found at histopathology, follow-up after therapeutic neck treatment. DOD, dead of disease; NED, no evidence of disease; DOC, dead of other cause.

* P-value = 0.007

† P-value < 0.0001

Discussion

Controversy exists about the proper management of the neck if no metastases are detected clinically. The key question in this debate is whether there is a difference in treatment outcome between elective neck treatment and observing the neck. Comparison between these two policies has been the topic in many studies[3,15-22], but only three of these studies were prospective.[3,15,16] All three described relatively small numbers of patient groups, and none used imaging initially or during follow-up. Remarkably, a statistically significant difference in overall survival between elective treatment or observing the neck could not be demonstrated. An important issue in selecting patients for either elective neck dissection or observation policy is the risk of occult metastases. USgFNAC can help to decrease the risk of occult metastases, but cannot reduce it to zero. For most T1-T2 oral and oropharyngeal carcinomas this risk is estimated to be in the range of 30% to 40%.[16-23] Because the reported sensitivity of USgFNAC in the palpably N0 neck is in the range of 44% to 73%, the risk of occult metastases can be diminished to approximately 15% to 25% using USgFNAC.[4-7]

This study demonstrates that SN lymphoscintigraphy and subsequent fine-needle aspiration is a feasible approach in HNSCC. Recently published studies on SN procedures in SCC and melanomas in the head and neck region showed discordant results.[24-26] The main difficulty is to visualize SNs located in proximity of the injection site, as in the case of a floor of mouth carcinoma with a SN in level I. In our study, lymphoscintigraphy did not reveal a SN in 4 of 39 patients; all had tumors located in the anterior oral cavity. Also with the handheld probe, these SN(s) could not be identified. However, residues of aspirated enlarged lymph nodes in level I from 3 of these patients contained radioactivity, and these nodes were therefore considered as SN(s). These data indicate that the ultrasonographer should focus on the submandibular and submental region, when a SN cannot be visualized by lymphoscintigraphy. Another problem is that the tracer travels quickly: in the majority of patients a SN was identified within one minute.

This stresses the importance of performing dynamic scintigraphy, in order to try to distinguish the true SN(s) from second echelon nodes.

Despite the theoretical advantage to identify lymph nodes at risk not only by a size criterion, but also by the functional criterion of the SN, the combination of USgFNAC with a SN procedure did not reveal any improvement with regard to USgFNAC only. On the one hand, it may be argued that the validity of the SN concept has not yet been uniformly established for HNSCC. However, in a control group of patients treated with combined primary tumor excision and elective neck dissection, there is some preliminary evidence that the SN concept is valid.[27] Another explanation could be that some patients had only micrometastases in the SN that were missed at aspiration (sampling error). In a previous study we have shown that 25% of all tumor-positive neck dissection specimens obtained from elective dissections contain exclusively metastases smaller than 3 mm, whereas 16% contained exclusively metastases smaller than 1 mm.[28] In this group of 39 patients with negative SN-USgFNAC findings, 26% of the patients developed neck node metastases, which is in agreement with our earlier results (no significant difference). The failure rate for both groups together is 21%.

Because not all occult lymph node metastases are detected, the salvage rate in these patients is of crucial importance for the impact of a wait-and-see policy. Surgery and postoperative radiotherapy could salvage 79% (27 of 34) of the patients who developed neck failures during follow-up. However, it should be mentioned that in some patients follow-up is less than 2 years. In the literature, others reported salvage rates varying from 27% to 59% after development of cervical metastases,[15-21] and only one study reported a salvage rate of 82%.[3] These differences could be attributed to the fact that in none of the studies imaging was used initially or during follow-up. Furthermore, this study shows that the regional control of 88% after delayed therapeutic neck dissection is not disadvantageous compared to the regional control rate after elective neck dissection as reported in the literature.[29] In the neck dissection specimens of the 7 patients who died of tumor-related causes

a total of 35 positive lymph nodes were found at histopathology (2-10 per patient), of which 33 showed extranodal spread. This is in sharp contrast to the histopathologic examination of the neck dissection specimen of the 27 patients who remained disease-free after neck dissection. In these patients less than 3 involved nodes was reported in 23 patients. Furthermore, 7 of 27 patients did not have extracapsular spread in their specimen. These data indicate that the prognosis after neck failure is related to the metastatic extension and the presence of extranodal spread. Therefore, it is of significant importance to keep the interval between excision of the primary tumor and neck treatment short. We maintain a strict policy of follow-up visits to the outpatient clinic every 6 weeks, including USgFNAC every 3 months. Because 30 of the 34 neck failures were detected within 12 months after transoral excision of the primary tumor, this policy applies in particular for the first year, whereas afterwards USgFNAC can be performed when indicated.

In conclusion, USgFNAC at initial staging can reduce the risk of occult metastases for T1-T2 SCC of the oral cavity or oropharynx considerably, which justifies local excision of the tumor and a wait-and-see policy for the neck.[4-7] SN identification and aspiration did not decrease the false-negative rate in this study, and we have therefore abandoned its use in this setting. Using USgFNAC for follow-up enabled early detection of a significant percentage of neck failures and a high salvage rate with therapeutic neck dissection. However, it should be remembered that techniques such as USgFNAC are technically demanding, and results from centers experienced in this procedure cannot be generally applied. It must therefore be emphasized that each institution intending to use such a technique to help modify existing treatment strategy, would first need to verify the accuracy of the procedure in their own hands.

References

1. Leemans CR, Tiwari R, Nauta JJ, van der Waal I, Snow GB. Regional lymph node involvement and its significance in the development of distant metastases in head and neck carcinoma. *Cancer* 71:452-456, 1993.

2. Weiss MH, Harrison LB, Isaacs RS. Use of decision analysis in planning a management strategy for the stage N0 neck. *Arch Otolaryngol Head Neck Surg* 120:699-702, 1994.

3. Vandenbrouck C, Snacho-Garnier H, Chassagne D, Saravane D, Cachin Y, Micheau C. Elective versus therapeutic radical neck dissection in epidermoid carcinoma of the oral cavity-results of a randomized clinical trial. *Cancer* 46:386-390, 1980.

4. Van den Brekel MW, Castelijns JA, Stel HV et al. Occult metastatic neck disease: detection with US and US-guided fine- needle aspiration cytology. *Radiology* 180:457-461, 1991.

5. Righi PD, Kopecky KK, Caldemeyer KS, Ball VA, Weisberger EC, Radpour S. Comparison of ultrasound-fine needle aspiration computed tomography in patients undergoing neck dissection. *Head Neck* 19:604-610, 1997.

6. Takes RP, Righi P, Meeuwis CA et al. The value of ultrasound with ultrasound-guided fine-needle aspiration biopsy compared to computed tomography in the detection of regional metastases in the clinically negative neck. *Int J Radiat Oncol Biol Phys* 40:1027-1032, 1998.

7. Knappe M, Louw M, Gregor RT. Ultrasonography-guided fine-needle aspiration for the assessment of cervical metastases. *Arch Otolaryngol Head Neck Surg* 126:1091-1096, 2000.

8. Van den Brekel MW, Castelijns JA, Reitsma LC, Leemans CR, van der Waal I, Snow GB. Outcome of observing the N0 neck using ultrasonographic-guided cytology for follow-up. *Arch Otolaryngol Head Neck Surg* 125:153-156, 1999.

9. Pijpers R, Borgstein PJ, Meijer S, Hoekstra OS, van Hattum LH, Teule GJ. Sentinel node biopsy in melanoma patients: dynamic lymphoscintigraphy followed by intraoperative gamma probe and vital dye guidance. *World J Surg* 21:788-792, 1997.

10. Borgstein PJ, Pijpers R, Comans EF, van Diest PJ, Boom RP, Meijer S. Sentinel lymph node biopsy in breast cancer: guidelines and pitfalls of lymphoscintigraphy and gamma probe detection. *J Am Coll Surg* 186:275-283, 1998.

11. Morton DL, Thompson JF, Essner R et al. Validation of the accuracy of intraoperative lymphatic mapping and sentinel lymphadenectomy for early-stage melanoma: a multicenter trial. Multicenter Selective Lymphadenectomy Trial Group. *Ann Surg* 230:453-463, 1999.

12. Muller MG, Borgstein PJ, Pijpers R et al. Reliability of the sentinel node procedure in melanoma patients: analysis of failures after long-term follow-up. *Ann Surg Oncol* 7:461-468, 2000.

13. Colnot DR, Nieuwenhuis EJ, Castelijns JA et al. Ultrasound guided aspiration of sentinel nodes for improved staging of head and neck cancer patients-initial experience. *Radiology* 218:289-293, 2001.

14. Van den Brekel MW, Stel HV, Castelijns JA et al. Cervical lymph node metastasis: assessment of radiologic criteria. *Radiology* 177:379-384, 1990.

15. Fakih AR, Rao RS, Patel AR. Prophylactic neck dissection in squamous cell carcinoma of oral tongue: a prospective randomized study. *Semin Surg Oncol* 5:327-330, 1989.

16. Kligerman J, Lima RA, Soares JR et al. Supraomohyoid neck dissection in the treatment of T1/T2 squamous cell carcinoma of oral cavity. *Am J Surg* 168:391-394, 1994.

17. Khafif RA, Gelbfish, GA, Tepper P, Attie, JN. Elective radical neck dissection in epidermoid cancer of the head and neck. A retrospective analysis of 853 cases of mouth, pharynx, and larynx cancer. *Cancer* 67:67-71, 1991.

18. Ho CM, Lam KH, Wei WI, Lau SK, Lam LK. Occult lymph node metastasis in small oral tongue cancers. *Head Neck* 14:359-363, 1992.

19. Shingaki S, Kobayashi T, Suzuki I, Kohno M, Nakajima T. Surgical treatment of stage I and II oral squamous cell carcinomas: analysis of causes of failure. *Br J Oral Maxillofac Surg* 33:304-308, 1995.

20. Yuen AP, Wei WI, Wong YM, Tang KC. Elective neck dissection versus observation in the treatment of early oral tongue carcinoma. *Head Neck* 19:583-588, 1997.

21. Persky MS, Lagmay VM. Treatment of the clinically negative neck in oral squamous cell carcinoma. *Laryngoscope* 109:1160-1164, 1999.

22. Sessions, DG, Spector GJ, Lenox J et al. Analysis of treatment results for floor-of-mouth cancer. *Laryngoscope* 110:1764-7172, 2000.

23. Shah JP, Candela FC, Poddar AK. The patterns of cervical lymph node metastases from squamous carcinoma of the oral cavity. *Cancer* 66:109-113, 1990.

24. Shoaib T, Soutar DS, MacDonald DG et al. The accuracy of head and neck carcinoma sentinel lymph node biopsy in the clinically N0 neck. *Cancer* 11:2077-2083, 2001.

25. Jansen L, Koops HS, Nieweg OE et al. Sentinel node biopsy for melanoma in the head and neck region. *Head Neck* 22:27-33, 2000.

26. Koch WM, Choti MA, Civelek AC, Eisele DW, Saunders JR. Gamma probe-directed biopsy of the sentinel node in oral squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 124:455-459, 1998.

27. Nieuwenhuis EJC, Pijpers RH, Castelijns JA, Brakenhoff RH, Snow GB, van den Brekel MWM. New developments in the staging of head and neck cancer patients using ultrasound guided aspiration of sentinel lymph nodes. Presented at the EANM Annual Congress, Paris September 2-6, 2000. Abstract: *Eur J Nucl Med* suppl; 27(8):986, 2000.

28. Van den Brekel MW, van der Waal I, Meijer CJ, Freeman JL, Castelijns JA, Snow GB. The incidence of micrometastases in neck dissection specimens obtained from elective neck dissections. *Laryngoscope* 106:987-991, 1996.

29. Leemans CR, Tiwari R, van der Waal I, Karim AB, Nauta JJ, Snow GB. The efficacy of comprehensive neck dissection with or without postoperative radiotherapy in nodal metastases of squamous cell carcinoma of the upper respiratory and digestive tracts. *Laryngoscope* 100:1194-1198, 1990.

6

Quantitative molecular detection of minimal residual head and neck cancer in lymph node aspirates

Eline J.C. Nieuwenhuis, Lies H. Jaspars, Jonas A. Castelijns, Branco Bakker, Richard G.A. Wishaupt, Fedor Denkers, C. René Leemans, Gordon B. Snow, Ruud H. Brakenhoff

Clinical Cancer Research 9:755-761, 2003

Abstract

Staging of the clinically N0 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 trancription-PCR (Q-RT-PCR) assay based on TaqMan technology using the squamous cell-specific antigen E48 (Ly-6D) as molecular marker.

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.

The detection limit of E48 Q-RT-PCR was a single tumor cell in a background of 10^6 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%.

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

Squamous cell carcinoma (SCC) 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%)[3] a neck without palpable lymph nodes (N0) is at risk of harboring occult metastases.

To improve staging accuracy of the clinically N0 neck, ultrasound-guided fine needle aspiration cytology (USgFNAC) has gained popularity. The neck is examined by ultrasound (US) and suspicious (enlarged) lymph nodes are aspirated under US-guidance and cytologically examined.[4] Since 1992, we gradually changed our policy towards 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, a additional decrease of the false-negative rate is desirable. Possible causes of false-negative USgFNAC cases could be 1) tumor containing lymph nodes do not meet US size criteria and are not aspirated, or all enlarged and aspirated nodes are tumor-free, 2) aspiration is performed in a tumor-free part of a lymph node harboring a metastasis (sampling error) or 3) the aspirate was not evaluable by routine cytology, or few aspirated tumor cells in the smear were missed. A previous study of our group showed that this 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 could 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. First, 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] Second, 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 non-diagnostic, 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

squamous cell specific molecular marker, and the gene encoding porphobilinogen deaminase (PBGD) as 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 Dulbecco's modified essential medium (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 (N0). 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, > 4 mm in other levels). After preparation of two cytological smears per aspiration, the needle was washed in phosphate buffered saline (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 x 7 ml heparin vacutubes. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll Hypaque density centrifugation according to the supplier. From 10 noncancer patients residues of 10 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 x *g* for 5 min the needles were removed, the pellet was resuspended in 1 ml PBS and transferred to a 1.5 ml microcentrifuge vial. The cell pellets were collected by centrifugation at 12,000 *g* for 5 min at 4°C. After removing the supernatant the pellet was resuspended in 0.5 ml RNAzol™ B and the RNA isolated as described previously.[11] Glycogen (20 µg) was used as 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-µm cryosections were prepared. The first and last section were stained by hematoxilin and eosin. 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 µl 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+ 95as, 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, UK). For internal calibration of the samples, we used mRNA transcribed from the gene encoding porphobilinogen deaminase (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 hours at 42°C. One reverse

transcription (RT) reaction (20 µl) consisted of 5 µl total RNA (maximum of 1 µg), 2 µl 10x RT-buffer (containing 600 mM KCl, 30 mM $MgCl_2$, 500 mM TRIS), 2 µl 10 mM DTT, 2 µl 10 mM dNTPs (2.5 mM for each deoxynucleotide triphosphate), 1 µl of reverse primer E48+95as or HuPBGD.R (25 pmol/ µl), 7.85 µl sterile H_2O (Baxter), 0.05 µl RNasin (40 U/µl), and 0.1 µl AMV reverse transcriptase (10 U/µl). RNA isolated from microdissected tumor specimens and lymph node aspirates was analyzed in duplicate by Q-RT-PCR.

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*-CATCTTTGGGCTGTTTTCTTCCGCC-*TAMRA*

After completion of the RT reaction, 5 µl of cDNA was used for PCR amplification in 60 cycles in a 45 µl reaction mixture, containing 5 µl 10x TaqMan buffer A (Perkin-Elmer/Applied Biosystems, Warrington, UK) 10 µl 25 mM $MgCl_2$, 0.5 µl 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 sterile H_2O (Baxter), and 0.25 µl AmpliTaq ™Gold DNA-polymerase (5 U/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 sec at 95 °C and 60 sec at 60 °C. In each run a serial dilution of UM-SCC-22A RNA ranging from 500,000 pg to 5 pg was run in parallel as standard curve for both E48 and PBGD. Subsequently, the threshold cycle (C_T) 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_T 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 pg to 5 pg. Amplification was carried out in duplicate. The standard curve of these dilutions are shown in Fig. 1B. On the basis of these experiments, the dynamic range of quantitation was 5 log with a correlation coefficient > 0.99. The calculated PCR efficiencies were >95%. E48 transcripts can be detected reproducibly in 5 pg 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 PBMC RNA corresponding to 10^6 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 set on 2 pg. This was 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 (5-10 pg) lead to a reduction in sensitivity (see below).

Quantitation of Number of (Squamous) Cells in Lymph Node Aspirates

Using PBGD as 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 amount of cells in each aspirate can be deduced using PBGD. We arbitrarily defined an aspirate as representative when a minimum number of 1,000 PBMC cell equivalents was present.

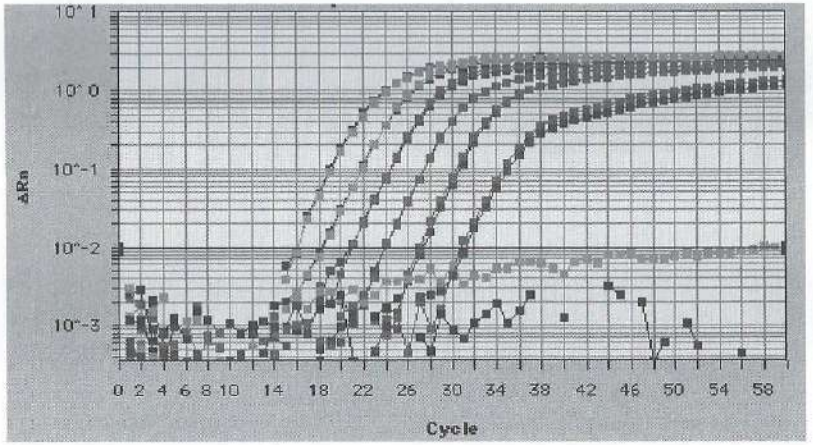


Figure 1A. Serial dilution of UM-SCC-22A RNA ranging from 500,000 to 5 pg analysed by E48 Q-RT-PCR. ΔRn on the Y axis indicates the fluorescent signal of the cleaved probe in the amplification reaction, measured real-time during cycling. The PCR cycles are indicated on the X axis. After a phase with 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 ΔRn 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.

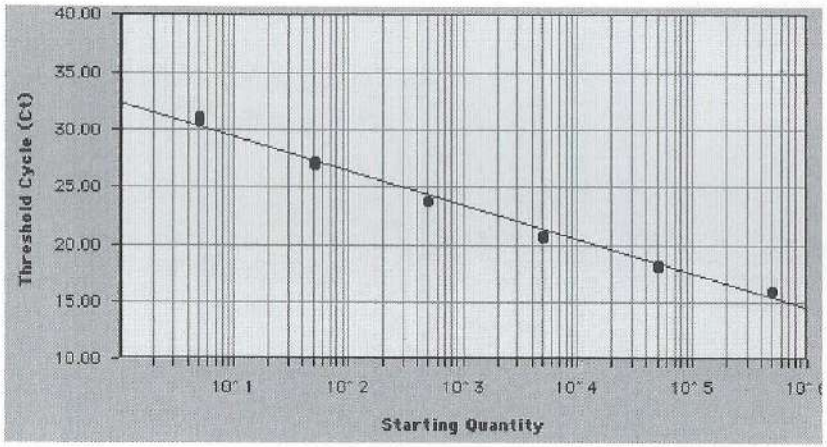


Figure 1B. Standard curve of E48 Q-RT-PCR in a serial dilution ranging from 500,000 pg to 5 pg of UM-SCC-22A RNA. The C_T value was indicated on the Y axis and the amount of RNA (in pg) on the X axis. Correlation coefficient was 0.991. Using this calibration curve of the standard cell line UM-SCC-22A, the amount of E48 RNA or PBGD RNA in a sample can be quantified accurately.

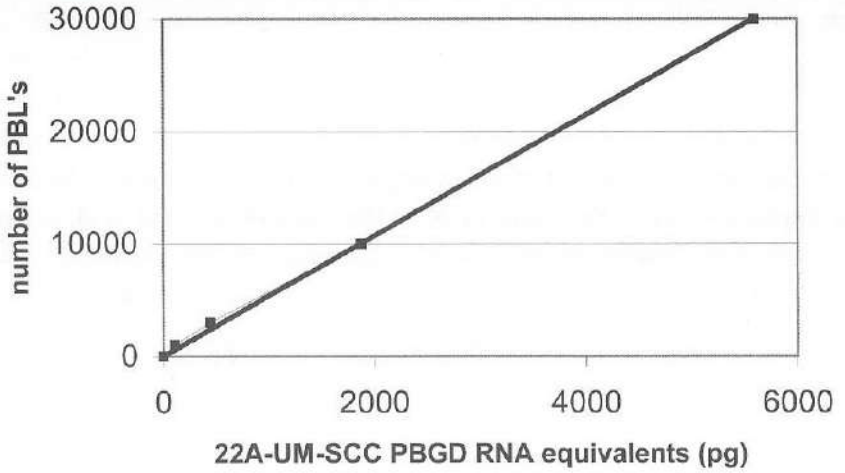


Figure 2. Relation between the number of PBMCs from blood and the amount of UM-SCC-22A PBGD RNA equivalents. The amounts of 1,000; 3,000; 10,000; and 30,000 PBMCs correspond to 119; 444; 1,876; and 5,599 pg of UM-SCC-22A RNA equivalents, respectively (mean of triplicate measurements).

Limits used for E48:PBGD Q-RT-PCR

An aspirate was considered evaluable when the number of PBMCs ≥ 1,000 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.

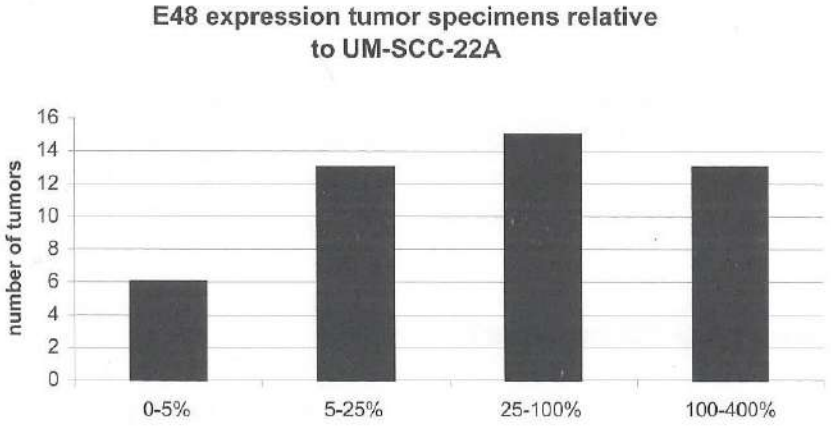


Figure 3. E48:PBGD ratios were calculated for 47 microdissected tumor biopsy specimens and compared to the E48:PBGD ratio of UM-SCC-22A. The tumor ratios were subdivided into four classes, and the frequencies are depicted in a bar graph.

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 ± 0.5 fold lower as compared to that of UM-SCC-22A. Subsequently, 100, 500, 1,000 and 5,000 cells of both cell lines were seeded in 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) were 55; 325; 713; 6,452 and for UM-SCC-22B 52; 381; 729; 3,489, respectively. The 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.

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 analysed with E48 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 to 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) 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 most likely because the corresponding tumors did not express E48. Of the remaining 191 lymph node aspirates that were tumor-negative or not evaluable at cytology 8 (4%) were E48 positive.

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

		PBGD > 1,000 cells		
		Evaluable	Not evaluable	Total
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		
		E48 positive	E48 negative	Total
cytology	Tumor-positive	18	2	20
	Tumor-negative	**8**	184	192
	Total	26	186	212

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 analysed 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 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, the 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.

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%)

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 Hematoxylin-Eosin section, and 10% (1 of 10) using immunohistochemical staining. In a second study, using p53 point mutations as 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. First, the technique permits precise quantification of minute amounts of mRNA transcripts over a 5-log range. Second, PCR is performed in a closed tube system and does not require post-PCR manipulations, thereby preventing possible contamination. Third, 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 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 varying from 0.01 to 1.94 pg, which were considered negative using the cutoff level. The fact that this ectopic E48 mRNA expression was not demonstrated in the analyzed noncancer controls might be due 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 then 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 to 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 metastases is left untreated.

In conclusion, real-time quantitative E48 RT-PCR appears to be an accurate technique for assessment of micrometastases 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 out 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 (212 of 235). 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.

References

1. Muir, C., Weiland, L. Upper aerodigestive tract cancers. *Cancer* 75: 147-153, 1995.

2. Leemans CR, Tiwari R, Nauta JJ, van der Waal I, Snow GB. Regional lymph node involvement and its significance in the development of distant metastases in head and neck carcinoma. *Cancer* 71: 452-456, 1993.

3. Ali S, Tiwari RM, Snow GB. False-positive and false-negative neck nodes. *Head Neck Surg* 8: 78-82, 1985.

4. Van den Brekel MW, Stel HV, Castelijns JA, Nauta JJ, Van der Waal I, Valk J, Meyer CJ, Snow GB. Cervical lymph node metastasis: assessment of radiologic criteria. *Radiology* 177: 379-384, 1990.

5. Nieuwenhuis EJ, Castelijns JA, Pijpers R, Van den Brekel MW, Brakenhoff RH, Van der Waal I, Snow GB, Leemans CR. Wait-and-see policy for the N0 neck in early stage oral and oropharyngeal SCC using ultrasound guided cytology: is there a role for identification of the sentinel node? *Head Neck* 24: 282-289, 2002.

6. Gath HJ, Brakenhoff RH. Minimal residual disease in head and neck cancer. *Cancer Metastasis Rev* 18:109-126, 1999.

7. Van Houten VM, Tabor MP, Van den Brekel MW, Denkers F, Wishaupt RG, Kummer JA, Snow GB, Brakenhoff RH. Molecular assays for the diagnosis of minimal residual head-and-neck cancer: methods, reliability, pitfalls, and solutions. *Clin Cancer Res* 6: 3803-3816, 2000.

8. Pantel K. Detection of minimal disease in patients with solid tumors. *J Hematother* 5: 359-367, 1996.

9. Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, Goodman SN, Sidransky D. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. *N Engl J Med* 332: 429-435, 1995.

10. Quak JJ, Balm AJ, Van Dongen GA, Brakkee JG, Scheper RJ, Snow GB, Meijer CJ. A 22-kd surface antigen detected by monoclonal antibody E 48 is exclusively expressed in stratified squamous and transitional epithelia. Am *J Pathol* 136: 191-197, 1990.

11. Brakenhoff RH, Stroomer JG, Ten Brink C, De Bree R, Weima SM, Snow GB, Van Dongen GA. Sensitive detection of squamous cells in bone marrow and blood of head and neck cancer patients by E48 reverse transcriptase-polymerase chain reaction. *Clin Cancer Res* 5: 725-732, 1999.

12. Mensink E, Van de Locht A, Schattenberg A, Linders E, Schaap N, Geurts van Kessel A, De Witte T. Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. *Br J Haematol* 102: 768-774, 1998.

13. Fink L, Seeger W, Ermert L, Hanze J, Stahl U, Grimminger F, Kummer W, Bohle RM. Real-time quantitative RT-PCR after laser-assisted cell picking. *Nat Med* 4: 1329-1333, 1998.

14. Brakenhoff RH, Gerretsen M, Knippels EM, Van Dijk M, Van Essen, H, Weghuis DO, Sinke RJ, Snow GB, Van Dongen GA. The human E48 antigen, highly homologous to the murine Ly-6 antigen ThB, is a GPI-anchored molecule apparently involved in keratinocyte cell-cell adhesion. *J Cell Biol* 129: 1677-1689, 1995.

15. De Bree R, Roos JC, Quak JJ, Den Hollander W, Snow GB, Van Dongen GA. Clinical screening of monoclonal antibodies 323/A3, cSF-25 and K928 for suitability of targetting tumours in the upper aerodigestive and respiratory tract. *Nucl Med Commun* 15: 613-627, 1994.

16. Van den Brekel MW, Van der Waal I, Meijer CJ, Freeman JL, Castelijns JA, Snow GB. The incidence of micrometastases in neck dissection specimens obtained from elective neck dissections. *Laryngoscope* 106: 987-991, 1996.

17. Partridge M, Li SR, Pateromichelakis S, Francis R, Phillips E, Huang XH, Tesfa-Selase F, Langdon JD. Detection of minimal residual cancer to investigate why oral tumors recur despite seemingly adequate treatment. *Clin Cancer Res* 6: 2718-2725, 2000.

18. Liefers GJ, Cleton-Jansen AM, Van de Velde CJ, Hermans J, Van Krieken JH, Cornelisse CJ, Tollenaar RA. Micrometastases and survival in stage II colorectal cancer. *N Engl J Med* 339: 223-228, 1998.

19. Wang AM, Doyle MV, Mark DF. Quantitation of mRNA by the polymerase chain reaction. *Proc Natl Acad Sci USA* 86: 9717-9721, 1998.

20. Becker-Andre M, Hahlbrock K. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Res* 17: 9437-9446, 1989.

7

Assessment and clinical significance of micrometastases in lymph nodes of head and neck cancer patients detected by E48 (Ly-6D) quantitative reverse transcription-polymerase chain reaction

Eline J.C. Nieuwenhuis, C. René Leemans, Alain Kummer, Fedor Denkers, Gordon B. Snow, Ruud H. Brakenhoff

Laboratory Investigations, in press

Abstract

The presence of lymph node metastases is the major determinant for prognosis in head and neck squamous cell carcinoma (HNSCC). It is at present unknown whether the same holds true for the presence of histologically undetectable micrometastases. We analysed 456 histologically tumor-negative lymph nodes of 23 HNSCC patients without (pN0) and 18 patients with one or two tumor-positive lymph nodes (pN+) in their neck dissection specimens at histopathology. To detect the presence of disseminated tumor cells and micrometastases in these lymph nodes real-time quantitative RT-PCR was exploited using E48 (Ly-6D) transcripts as squamous cell-specific molecular marker. The results were compared to histopathology and clinical outcome. E48 transcripts were detected in lymph nodes of 5 of 23 (22%) patients in the pN0 group, and in histologically negative lymph nodes of 10 of 18 (56%) patients in the pN+ group. In the pN0 group the presence of E48-positive lymph nodes was significantly associated with a distinctly poor cause-specific survival as compared to those with E48-negative lymph nodes. Our results indicate that E48 real-time quantitative RT-PCR is a suitable method for the detection of micrometastases in lymph nodes of HNSCC patients. Moreover, detection of micrometastases seems clinically relevant, but should be confirmed in a large multi-center trial.

Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for 5% of all newly diagnosed cancers in the Western world.[1] Dissemination to lymph nodes in the neck is one of the most important prognosticators for HNSCC. Only 7% of patients without lymph node metastases develop distant metastases, while this occurs in 50% of patients with more than three tumor-positive lymph nodes.[2] Moreover, when tumor is diagnosed in the regional lymph nodes, the neck should be treated. The clinical management is further determined by the number of tumor-containing lymph nodes, the involved level(s) in the neck, and the presence of extranodal spread. Therefore, accurate assessment of the regional lymph nodes for metastases is of crucial importance. However, classical diagnostic modalities, such as radiology and histopathology are of limited sensitivity to detect small numbers of tumor cells.

Recently, molecular techniques have been applied in a number of studies to detect the presence of micrometastases that are missed at routine histology for the early diagnosis of.[3-7] Also in other types of cancer, including breast cancer, esophageal cancer and melanoma, the molecular detection to detect micrometastases in lymph nodes of patients with histologically tumor-negative lymph nodes has been shown to be prognostically important.[8-10] It has been demonstrated for example, that CEA as a marker for tumor cells was successfully applied in patients with stage II (histologically negative lymph nodes) colorectal cancer. The presence of CEA transcripts in lymph nodes significantly correlated with a poor prognosis.[11] These studies emphasize the importance of molecular assessment of micrometastases in histopathologically tumor-negative lymph nodes, which may improve tumor staging.

At our laboratory, we are focusing on the exploitation of SCC-specific antigen E48 (Ly-6D) as a potential marker for the detection of HNSCC. This antigen is specifically expressed in squamous cells of both normal, malignant and transitional epithelia.[12] 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.[13] Subsequently, we described a quantitative real-time RT-PCR assay to assess the presence of squamous cells in lymph nodes aspirates. In this study 235 lymph node aspirate residues of 64 HNSCC patients staged with ultrasound-guided-fine-needle-aspiration-cytology (USgFNAC) were examined for the presence of E48 mRNA and compared with routine cytology. The gene encoding porphobilinogen deaminase (PBGD), a consistently expressed housekeeping gene, was used to determine the quality and quantity of isolated RNA. Our data demonstrated a clear benefit of molecular diagnosis on the sensitivity as well as a benefit in the frequency of diagnosis. 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.[14]

Minimal data exist on the presence and clinical significance of minimal residual disease in lymph nodes of HNSCC patients. In a previous study we analyzed 76 neck dissection specimens from 64 HNSCC patients.[15] The percentage of additional micrometastases detected in tumor-negative neck dissection specimens was 3% (2/62) when one additional section was analysed by routine histopathological examination, and 10% (1/10) when using immunohistochemical staining with anti-cytokeratin antibodies. In another study, using p53 point mutations as marker, the presence of tumor cell DNA was detected in 21% of histopathologically tumor-negative lymph nodes.[3] In none of these studies, however, the clinical significance could be determined.

Moreover, the sensitivity of these techniques is still not optimal as only limited amounts of tissues can be analyzed. Using quantitative E48 RT-PCR, however, we can specifically detect a single squamous cell in a background of 10^6 peripheral blood mononuclear cells.[14]

The aim of the present study is to investigate the presence and prognostic relevance of micrometastases in lymph nodes of HNSCC patients using a quantitative E48 RT-PCR assay.

Patients and methods

Subjects

Between August 1997 and July 2000 69 patients with histopathologically proven HNSCC entered the study. The protocol was approved by the Institutional Review Board, and informed consent was obtained from all patients. All patients were treated by radical primary tumor excision and uni- or bilateral neck dissection, depending on the extension of the tumor towards the midline. A uni-variate analysis of this patient group showed that the 41 patients with 0, 1 or 2 histologically tumor-positive lymph nodes in their neck dissection specimen had a relative good prognosis compared to the 28 patients with more than two tumor-involved lymph nodes ($P<0.001$, data not shown). Therefore, we proceeded with these 41 patients for further molecular detection of micrometastases. The cohort consisted of 30 men and 11 women, with an age ranging from 40 to 77 years (median, 57). In the neck dissection specimens the different levels were identified according to the classification system of the American Academy of Otolaryngology/Head and Neck Surgery, and from each level the two largest lymph nodes were removed. One half of the two largest lymph nodes and the remaining lymph nodes in the various levels were fixed in formalin and embedded in paraffin for routine histopathological examination. The other half of the two largest lymph nodes was snap-frozen in liquid nitrogen and stored at –80 °C until RNA isolation. Nodes larger than one cm in diameter were not halved, but were sliced and alternately embedded in paraffin or snap-frozen. Histopathological examination showed no tumor-positive lymph nodes in 23 patients (pN0 group), one tumor-positive lymph node in 11 patients (pN+ group) and two tumor-containing lymph nodes in 7 patients (pN+ group). Follow-up visits were performed every six weeks during the first year after treatment to check for recurrent disease, whereafter the interval gradually increased. From 3 non-cancer patients 15 lymph nodes from the neck were obtained and served as negative control. In total 22 histopathological tumor-positive lymph nodes from the pN+ patient group were used as positive control.

RNA Isolation

The lymph nodes were homogenized using a micro pestle in one ml RNAzol™ B (Campro Scientific bv., Veenendaal, Holland). After addition of 100 µl chloroform the solution was mixed vigorously for 15 sec and put on ice for 5 min. After centrifugation at 12,000 x *g* for 15 min at 4°C, the aqueous phase was transferred to another microcentrifuge vial, an equal volume of isopropanol was added for precipitation and the solution was incubated on ice for 15 min. Glycogen (20 µg) was used as carrier for RNA precipitation. The RNA precipitate was collected by centrifugation at 12,000 x *g* for 30 min at 4°C. The pellet was washed in 70% ethanol, and centrifuged at 12,000 x *g* for 5 min at 4°C. The pellet was dissolved in 25-100 µl of RNAse-free water, when necessary incubated at 65°C for 15 min. The amount of RNA was calculated from the absorbance at 260 nm. Subsequently, concentrations of 100 ng/µl total RNA were prepared.

cDNA Synthesis and Real-time PCR Amplification

The sequences of the intron-spanning amplification primers and probe for E48 were: AGATGAGGACAGCATTGCTGC, GCAGACCACAGAATGCTTGC, and *FAM*-TTACCCTGCGCTGCCACGTGTG-*TAMRA*, respectively. These primers were selected with ABI/Prism™ Primer Express™ (Applied Biosystems, Warrington, UK). For internal calibration of the samples we used mRNA transcribed from the gene encoding PBGD, a consistently expressed housekeeping gene. The sequences of the intron-spanning amplification primers and probe for PBGD were: GGCAATGCGGCTGCAA, GGGTACCCACGCGAATCAC, and *FAM*-CATCTTTGGGCTGTTTTCTTCCGCC-*TAMRA*. In total 5 µl RNA (500 ng) was heated to 65 °C for 5 min and cooled directly on ice. Subsequently, 15 µl of RT mixture was added. The RT mixture consisted of 2 µl 10x RT-buffer (600 mM KCl, 30 mM $MgCl_2$, 500 mM Tris), 2 µl 10 mM DTT, 2 µl 10 mM dNTPs (2.5 mM each deoxynucleotide triphosphate), 1 µl of reverse primer E48+95αs or HuPBGD.R (25 pmol/ µl), 7.85 µl sterile H_2O, 0.05 µl RNasin (40

U/µl), and 0.1 µl AMV-RT (10 U/µl). The reaction mixture was incubated for 2 hours at 42°C. All reactions were performed in duplicate.

After completion of the RT reaction, 5 µl of cDNA was used for PCR amplification in 60 cycles in a 50 µl reaction mixture, containing 5 µl 10x TaqMan buffer A (Applied Biosystems), 10 µl 25 mM $MgCl_2$, 0.5 µl 100 mM dNTP, 0.6 µl of sense and antisense primers of E48 or PBGD (25 pmol/µl each), 1.5 µl of fluorescent probe (5 pmol/µl), 26.55 µl sterile H_2O (Baxter), and 0.25 µl AmpliTaq ™Gold DNA-polymerase (5 U/µl, Applied Biosystems). Real-time PCR was carried out on the ABI/Prism™ 7700 Sequence Detector System (Applied Biosystems), using a pre-PCR step of 10 min at 95 °C, followed by 60 cycles of 15 sec at 95°C and 60 sec at 60°C. In each experiment a serial dilution of UM-SCC-22A RNA ranging from 500,000 pg to 50 pg was run in parallel as calibration curve for both E48 and PBGD. The original amount of E48 and PBGD mRNA in each sample was calculated from the standard curve relative to the human HNSCC cell line UM-SCC-22A. The range of duplicates was usually within 20% variation, and the mean of duplicates was used for further calculations.

Quality Assurance and Controls

The primers, probes and buffers were prepared in a laboratory that is isolated from PCR product analysis and sample preparation. RNA isolation, cDNA synthesis and preparation of PCR reactions were performed in a pre-PCR laboratory. To prevent amplimer carry-over contamination, all materials and reagents were transported in a one-way direction from the pre-PCR laboratories to the post-PCR laboratory. Sample to sample carry-over contamination was further avoided using different pipet sets and filtertips (Greiner Bio-One, Kremsmünster, Austria). Preparations without RNA template were used as negative RT-PCR control.

Statistical Analysis

Cause-specific and disease-free survival were determined by the Kaplan-Meier analysis, and differences between the curves were calculated by the log-rank test. Differences in frequency distributions were analyzed by Fisher's exact test. P values <0.05 were considered as significant.

Results

E48 RT-PCR of Lymph Nodes

The 10-fold serial dilution of UM-SCC-22A RNA for E48 amplification ranging from 500,000 pg to 5 pg is shown in Figure 1. The standard curve of these dilutions is depicted in Figure 2. Based on these experiments, the dynamic range of quantitation was 5-log with a correlation coefficient > 0.99. From previous dilution experiments using the cell line UM-SCC-22A the detection limit of this E48 RT-PCR assay was a single tumor cell in a background of 10^6 peripheral blood mononuclear cells (PBMCs).[14] The cut-off level to call a sample positive or negative was set on 2 pg relative to UM-SCC-22A. This was the lowest level that duplicates were always within 20% variation. Setting the limits higher caused a reduction in sensitivity.[14] We defined a lymph node sample as representative when a minimum number of 1,000 PBMCs was present, which corresponds to a PBGD RNA value of approximately 100 pg UM-SCC-22A equivalents.[14]

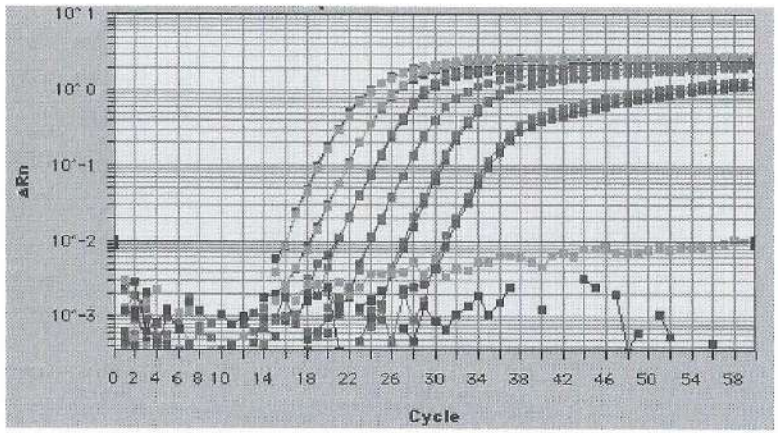


Figure 1. Serial dilution of UM-SCC-22A RNA ranging from 500,000 to 5 pg analyzed by E48 Q-RT-PCR. PCR cycles are shown on the X axis. ΔRn on the Y axis indicates the fluorescent signal of the cleaved probe. A ΔRn of 0.01 was chosen to define the treshold cycle (C_T).

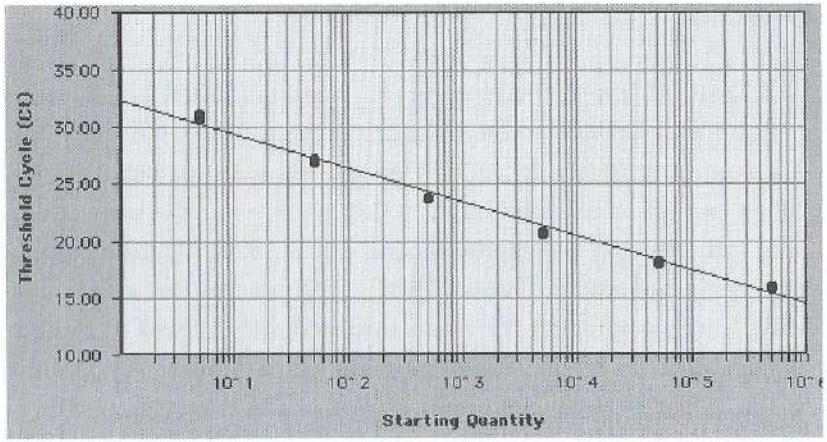


Figure 2. Standard curve of E48 Q-RT-PCR in a serial dilution ranging from 500,000 pg to 5 pg of UM-SCC-22A RNA. The C_T value on the Y axis was plotted to the log amount (in pg) of UM-SCC-22A RNA at the X axis. The correlation coefficient was 0.991. The amount of E48 (and PBGD) mRNA of each clinical sample was calculated relative to this standard curve of UM-SCC-22A RNA.

In total 15 lymph nodes of 3 non-cancers controls were collected and tested. In none of these lymph nodes a positive signal was detected. A total of 456 lymph nodes from 41 patients (range 7 to 20 per patient, median 10) were selected for E48 RT-PCR. Only 1/456 lymph nodes did not yield sufficient RNA as assessed by the control PCR from PBGD. All 22 histopathological tumor-positive lymph nodes showed E48 transcript-positive signals in duplicate. Of the remaining 433 lymph nodes that were tumor-negative at routine histopathology 24 (5.5%) revealed E48 positive signal. Overall, 15/41 (37%) patients showed E48 transcript-positive signals (in duplicate) in at least one lymph node.

Molecular N-Staging in Relation to Histopathology

Table 1 and 2 presents the data on patient characteristics, E48 RT-PCR results of the analyzed lymph nodes and the clinical outcome of the pN0 group and the pN+ group, respectively. In the pN0 group 5/23 (22%) patients showed E48 signal in one (4) or two (1) lymph nodes, whereas in the pN+ group 10/18 (56%) patients had E48 signal in one (7) or more (3) histologically tumor-negative lymph nodes. Overall, 15/41 (37%) patients had at least one E48-positive lymph node, and 7/15 were upstaged with regard to N-stage (N0 to N1 or N1/2a to N2b). T-stage and site did not differ significantly between the patients with and without E48-positive lymph nodes as determined by Fisher's exact test. In only one patient in this study the detection of the micrometastases would have influenced postoperative treatment (Table 2, case 8). The other patients had already received postoperative radiotherapy based on the clinical T-stage (T3 or T4) or the histopathological status of the neck (multiple tumor-positive lymph nodes and/or extranodal spread).

From Table 2 it can be seen that in 6 of 10 patients the E48-positive lymph nodes were found in similar levels as the metastases found at routine histopathology. However, in 4 out of 10 cases (case 2, 8, 9, and 16) additional lymph node metastases were found in levels not adjacent to the histopathological tumor-involved levels. In none of the lymph nodes micrometastases could be identified after histopathological review of the paraffin sections by the pathologist.

Molecular N-Staging and Clinical Outcome

When all patients were considered (pN0 and pN+) molecular N-staging was not related to a poor cause-specific survival. However, when the pN0 patients were considered separately, than there was significant correlation between molecular N-staging and cause-specific survival ($P = 0.002$, data not shown). It should be noted, however, that the number of patients and events is small.

Table 1. Tumor characteristics, molecular analyses and clinical outcome of 23 pN0 patients (pN0 group).

				Lymph nodes				
Case	Tumor site	T-stage	RT	No. analysed	No. E48 positive	Level(s) involved	Patient status	F-up (r
1	Tonsil	2	N	10	0	-	Alive	48.5
2	M Tongue	2	Y	7	0	-	Alive	50
3	Tonsil	3	Y	12	2	I	DOD	28
4	Larynx	3	Y	10	0	-	Alive	46.5
5	Inf Alv Process	4	N	10	0	-	Alive	48
6	Inf Alv Process	4	N	12	0	-	Alive	43
7	FOM	1	N	10	0	-	Alive	41.5
8	Inf Alv Process	3	Y	10	0	-	Alive	44
9	Tonsil	3	Y	10	0	-	Alive	46
10	Tonsil	3	Y	14	1	III	DOD	14
11	Inf Alv Process	2	N	10	0	-	DOC	19
12	Oropharynx	2	N	10	0	-	Alive	41
13	Inf Alv Process	4	Y	10	0		Alive	39
14	M Tongue	2	N	10	0	-	Alive	34
15	Inf Alv Process	4	Y	8	0	-	Alive	9
16	Tonsil	3	Y	10	0	-	DOC	21
17	FOM	4	Y	15	0	-	Alive	32.5
18	B Tongue	2	Y	10	0	-	Alive	31
19	M Tongue	3	Y	10	1	III	Alive	24
20	FOM	2	N	14	1	II	Alive	23
21	Inf Alv Process	4	Y	9	0	-	Alive	19
22	Inf Alv Process	4	Y	10	1	III	Alive	20
23	Buccal Mucosa	4	Y	10	0	-	Alive	20

M Tongue, mobile tongue; B Tongue, base of tongue; FOM, floor of mouth; Inf Alv Process, Inferior alveolar process; RT, postoperative radiotherapy; Y, yes; N, no; DOD, dead of disease; DOC, dead of other cause. Follow-up (F-up) is indicated in months.

Table 2. Tumor characteristics, molecular analyses and clinical outcome of 18 patients with one (case 1-11) or two (case 12-18) lymph node metastases found at routine histopathology (pN+ group).

				Lymph nodes					
Case	Tumor site	pTN-stage	RT	pos at PA level	No. analysed RT-PCR	No. E48-positive	level(s) involved	Patient status	F-up (m)
1	Piriform Sinus	T2N1	Y	II	11	0	-	Alive	53.5
2	FOM	T3N1	Y	III	10	3	II V	DOD	35.5
3	Tonsil	T3N1	Y	II	10	0	-	Alive	53
4	Tonsil	T3N1	Y	II	11	0	-	DOD	23
5	B Tongue	T2N1	Y	II	12	0	-	Alive	50.5
6	Tonsil	T3N1	Y	II	13	1	II	Alive	48
7	Tonsil	T3N1	Y	II	12	2	II	Alive	49
8	M Tongue	T2N1	N	III	13	1	I	Alive	47
9	Oropharynx	Rec*	N	III	10	2	II	Alive	35.5
10	Tonsil	T2N1	Y	II	10	6	I, II III,IV	Alive	30.5
11	FOM	T2N1	Y	II	11	0	-	Alive	31.5
12	FOM	T3N2b	Y	I/II	20	0	-	DOC	17
13	Oropharynx	T3N2b	Y	II	9	1	II	DOD	8
14	Piriform Sinus	T4N2b	Y	II/IV	14	1	IV	DOC	9
15	FOM	T2N2b	Y	I L I R	16	0	-	Alive	48
16	Oropharynx	T2N2b	Y	II	12	1	IV	Alive	46
17	Oropharynx	T2N2b	Y	III/IV	10	1	IV	DOD	22
18	M Tongue	T2N2b	Y	III	11	0	-	DOD	19

The pathological TN stage is indicated in column 3, and the levels of the tumor-positive lymph nodes found at routine histopathology in column 5. The number of additional metastases assessed by E48 RT-PCR is indicated in column 7, whereas the next column specifies the levels of these E48-positive lymph nodes. M Tongue, mobile tongue; B Tongue, base of tongue; Rec, recurrence; RT, postoperative radiotherapy; Y, yes; N, no; DOD, dead of disease; DOC, dead of other cause; FOM, floor of mouth. Follow-up (F-up) is indicated in months. *This patient developed a local recurrence after primary radiotherapy.

Discussion

The presence or absence of lymph node metastases is the strongest prognosticator in HNSCC. Not only the presence, but also the number of nodal metastases, the level(s) in the neck, the size of the nodes, and the presence of extranodal spread are important prognostic factors.[16-20] Although pre-operative node assessment using imaging techniques has improved over the years, histological assessment is the most reliable diagnostic modality, particularly for detecting smaller metastases, and therefore the gold standard. However, patients can harbor occult metastases that are missed by histology as well. These remain undetected mainly because of sampling error as only single paraffin sections are analyzed routinely.

Immunohistochemical staining using monoclonal antibodies have been shown to improve the detection of micrometastases. For example, Izbicki et al (Table 3) demonstrated in patients with esophageal carcinoma the presence of micrometastases by immunostaining with Ber-EP4 antibody in 17% of lymph nodes that were tumor-free on basis of routine histopathological analysis, which correlated with a significant worse prognosis.[21] However, their statistical analyses involved a comparison between patients with lymph node metastases versus patients without lymph node metastases with BerEP4 positive cells, regardless of histologically identifiable metastases. When patients with histologically overt lymph node metastases were excluded from the analysis, a comparison of patients with versus those without occult lymph node metastases yielded no significant effect on overall survival. Recently, the histologically pN0 patient group was enlarged and it was shown that the presence of Ber-EP4-positive cells in lymph nodes was significantly associated with a reduced relapse-free survival.[22] In another study Glickman *et al.* detected histologically occult lymph node metastases in 20/78 (26%) esophageal carcinoma patients using immunostaining with a pancytokeratin antibody, but these findings were not associated with patient outcome.[23]

Table 3. Micrometastases detection in pN0 and pN+ patients in esophageal cancer and HNSCC.

	Source year	No of patients	upstaged patients†	Marker	Method	No of molecular or IHC positive LN per patient
Esophageal carcinoma	Glickman et al 1999	78 pN0	26%	AE1/AE3	IHC	1-4
	Hosch et al 2000	54 pN0	56%	Ber-EP4	IHC	
		72 pN1	82%			
	Kano et al 2000	3 pN0	100%	SCC antigen	IHC	1-5
		11 pN1	91%			1-8
HNSCC	McDonald et al 1998	16 pN0	63%	CK5	RT-PCR	1-8
		8 pN+*	75%			1-8
	Cortesina et al 2000	5 pN0	100%	MET	Q-RT-PCR	2-5
		15 pN1-2c	33%			
	This study	23 pN0	22%	E48	Q-RT-PCR	1-2
		18 pN+	56%			1-6

†% of patients with molecular or immunostaining tumor-positive but histopathologically tumor-negative lymph nodes *N+: 1-3 histopathological tumor-positive lymph nodes

Kano *et al.* analyzed lymph nodes of three patients without histologically involved lymph nodes and lymph nodes of 11 patients with nodal involvement, and found in 13 patients upstaging of the lymph node status.[24] These studies demonstrated that step-wise sectioning and immunostaining decrease the number of unidentified metastases. However, the enormous workload and costs are major disadvantages for immunostaining of multiple sections and will always hamper implementation in routine practice. In contrast, RT-PCR is less laborious and might overcome, at least in part, the problem of sampling error, as by RT-PCR the entire lymph node can be assessed. This technique was used by McDonald *et al.*, who examined 196 lymph nodes of 24 HNSCC patients for the presence of cytokeratin 5 mRNA.[25] They demonstrated the presence for cytokeratin 5 transcripts in 40% of the lymph nodes that were histologically negative for tumor. However, the prognostic value of this molecular N-staging was not discussed.

Real-time quantitative RT-PCR offers several advantages over conventional RT-PCR. First, the technique permits precise quantitation of minute amounts of mRNA transcripts over a 5-log range. Second, PCR is performed in a closed tube system and does not require post-PCR manipulations, thereby preventing possible contamination. Third, because the lack of post-PCR processing time, sample throughput is increased. Cortesina *et al.* investigated the expression of the MET oncogene in 20 HNSCC patients using quantitative RT-PCR.[26] In 40% of the histologically negative lymph nodes the presence of MET mRNA was demonstrated. Again, the relationship between the presence of MET expression in lymph nodes and prognosis was not evaluated.

In this study we evaluated the prevalence and prognostic significance of histologically occult lymph node metastases in HNSCC patients exploiting the SCC-associated E48 antigen (Ly-6D), expressed on normal, malignant and transitional epithelia, as molecular marker. 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.[13]

Our results show that 22% of pN0 patients and 56% of pN+ patients had E48 transcript-positive signals in at least one of the resected histologically tumor-negative lymph nodes. Survival analysis revealed that patients in the pN0 group with one or more molecular-positive lymph nodes had a significantly worse cause-specific survival compared to patients with molecular-negative lymph nodes, although this conclusion is based on only small patient numbers and a small number of events.

In conclusion, real-time E48 Q-RT-PCR is an accurate technique for assessment of micrometastases in lymph nodes. Our study shows that the frequency of lymph node metastasis are underestimated. Moreover, our results demonstrate that micrometastases in HNSCC patients without histopathological positive lymph nodes contribute to a poor prognosis. It has been shown previously that only 7% of N0 patients develop distant metastases.[2] When sensitive molecular assays such as E48 Q-RT-PCR are exploited for N-staging the percentage could even be much lower, suggesting that in HNSCC hematogenic dissemination follows initial lymphatic spread. Large multicenter studies will be necessary to confirm these preliminary results in a large patient group. Although in this study only frozen material was used, which provides RNA of good quality, recent improvements in quantitative RT-PCR analyses on RNA isolated from archival, formalin-fixed and paraffin-embedded material will help to initiate studies in larger cohorts.[27] A direct application of E48 Q-RT-PCR could be the combination with sentinel node identification and biopsy. The putative additional value of this approach should be further investigated.

Acknowledgements

We thank Prof. I. van der Waal (Department of Oral and maxillofacial surgery/Oral Pathology, *Vrije Universiteit* Medical Center, Amsterdam) for histological review of all paraffin sections.

References

1. Muir C, Weiland L. Upper aerodigestive tract cancers. *Cancer* 75:147-153, 1995.

2. Leemans CR, Tiwari R, Nauta JJ, Van der Waal I, Snow GB. Regional lymph node involvement and its significance in the development of distant metastases in head and neck carcinoma. *Cancer* 71:452-456, 1993.

3. Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, Goodman SN, Sidransky D. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. *N Engl J Med* 332: 429-435, 1995.

4. Gath HJ, Brakenhoff RH. Minimal residual disease in head and neck cancer. *Cancer Metastasis Rev* 18:109-126, 1999.

5. Pantel K, Cote RJ, Fodstad R. Detection and clinical importance of micrometastatic disease. *J Natl Cancer Inst* 91:1113-1124, 1999.

6. Pantel K. Detection of minimal disease in patients with solid tumors. *J Hematother* 5:359-367, 1996.

7. Van Houten VMM, Tabor MP, Van den Brekel MWM, Denkers F, Wishaupt RG, Kummer JA, Snow GB, Brakenhoff RH. Molecular assays for the diagnosis of minimal residual head-and-neck cancer: methods, reliability, pitfalls, and solutions. *Clin Cancer Res* 6:3803-3816, 2000.

8. Bostick PJ, Morton DL, Turner RR, Huynh KT, Wang HJ, Elashoff R, Essner R, Hoon DS. Prognostic significance of occult metastases detected by sentinel lymphadenectomy and reverse transcriptase-polymerase chain reaction in early-stage melanoma patients. *J Clin Oncol* 17:3238-3244, 1999.

9. Cote RJ, Peterson HF, Chaiwun B, Gelber RD, Goldhirsch A, Castiglione-Gertsch M, Gusterson B, Neville AM. Role of immunohistochemical detection of lymph-node metastases in management of breast cancer. International Breast Cancer Study Group. *Lancet* 354:896-900, 1999.

10. McGuckin MA, Cummings MC, Walsh MD, Hohn BG, Bennett IC, Wright RG. Occult axillary node metastases in breast cancer: their detection and prognostic significance. *Br J Cancer* 73:88-95, 1996.

11. Liefers GJ, Cleton-Jansen AM, Van de Velde CJ, Hermans J, Van Krieken JH, Cornelisse CJ, Tollenaar RA. Micrometastases and survival in stage II colorectal cancer. *N Engl J Med* 339:223-228, 1998.

12. Quak JJ, Balm AJM, van Dongen GAMS, Brakkee JGP, Scheper RJ, Snow GB, Meijer CJLM. A 22-kd surface antigen detected by monoclonal antibody E48 is exclusively expressed in stratified squamous and transitional epithelia. *Am J Pathol* 136:191-197, 1990.

13. Brakenhoff RH, Stroomer JG, Ten Brink CBM, De Bree R, Weima SM, Snow GB, Van Dongen GAMS. Sensitive detection of squamous cells in bone marrow and blood of head and neck cancer patients by E48 reverse transcriptase-polymerase chain reaction. *Clin Cancer Res* 5:725-732, 1999.

14. Nieuwenhuis EJC, Jaspars LH, Castelijns JA, Bakker B, Wishaupt RGA, Denkers F, Leemans CR, Snow GB, Brakenhoff RH. Quantitative molecular detection of minimal residual head and neck cancer in lymph node aspirates. *Clin Cancer Res* 9:755-761, 2003.

15. Van den Brekel MWM, Van der Waal I, Meijer CJLM, Freeman JL, Castelijns JA, Snow GB. The incidence of micrometastases in neck dissection specimens obtained from elective neck dissections. *Laryngoscope* 106:987-991, 1996.

16. Snow GB, Annyas AA, Van Slooten EA, Bartelink H, Hart AA. Prognostic factors of neck node metastasis. *Clin Otolaryngol* 7:185-192, 1982.

17. Stell PM, Morton RP, and Singh SD. Cervical lymph node metastases: the significance of the level of the lymph node. *Clin Oncol* 9:101-107, 1983.

18. Leemans CR, Tiwari R, Van der Waal I, Karim AB, Nauta JJ, Snow GB. The efficacy of comprehensive neck dissection with or without postoperative radiotherapy in nodal metastases of squamous cell carcinoma of the upper respiratory and digestive tracts. *Laryngoscope* 100:1194-1198, 1990.

19. Shah JP. Cervical lymph node metastases-diagnostic, therapeutic, and prognostic implications. *Oncology* 4:61-69, 1990.

20. Jones AS, Roland NJ, Field JK, Phillips DE. The level of cervical lymph node metastases: their prognostic relevance and relationship with head and neck squamous carcinoma primary sites. *Clin Otolaryngol* 19:63-69, 1994.

21. Izbicki JR, Hosch SB, Pichlmeier U, Rehders A, Busch C, Niendorf A, Passlick B, Broelsch CE, Pantel K. Prognostic value of immunohistochemically identifiable tumor cells in lymph nodes of patients with completely resected esophageal cancer. *N Engl J Med* 337:1188-1194, 1997.

22. Hosch S, Kraus J, Scheunemann P, Izbicky JR, Schneider C, Schumacher U, Witter K, Speicher MR, Pantel K. Malignant potential and cytogenetic characteristics of occult disseminated tumor cells in esophageal cancer. *Cancer Res* 60:6836-6840, 2000.

23. Glickman JN, Torres C, Wang HH, Turner JR, Shahsafaei A, Richards WG, Sugarbaker DJ, Odze RD. The prognostic significance of lymph node micrometastasis in patients with esophageal carcinoma. *Cancer* 85:769-778, 1999.

24. Kano M, Shimada Y, Kaganoi J, Sakurai T, Li Z, Sato F, Watanabe G, Imamura M. Detection of lymph node metastasis of oesophageal cancer by RT-nested PCR for SCC antigen gene mRNA. *Br J Cancer* 82:429-435, 2000.

25. McDonald LA, Walker DM, Gibbins JR. Cervical lymph node involvement in head and neck cancer detectable as expression of a spliced transcript of type II keratin K5. *Oral Oncol* 34:276-283, 1998.

26. Cortesina G, Martone T, Galeazzi E, Olivero M, De Stefani A, Bussi M, Valente G, Comoglio PM, Di Renzo MF. Staging of head and neck squamous cell carcinoma using the MET oncogene product as marker of tumor cells in lymph node metastases. *Int J Cancer* 89:286-292, 2000.

27. Specht K, Richter T, Muller U, Walch A, Werner M, Hofler H. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tissue. *Am J Pathol* 158:419-429, 2001.

8

Summary and Conclusions

In the introduction (**Chapter 1**) it is outlined that in patients with head and neck squamous cell carcinoma (HNSCC) the management of the clinically N0 neck continues to be a controversial issue, particularly in patients with oral and oropharyngeal cancers which can be excised transorally. The choice is between elective treatment and watchful waiting. In most institutions around the world a frequency of occult metastases exceeding 20% is considered to be sufficient to justify elective treatment of the neck in patients with squamous cell carcinoma of the upper aerodigestive tract. When the assessment of the status of the neck nodes is carried out by means of *palpation alone*, most sites and stages of HNSCC, including T1 and T2 tumors of the oral cavity and oropharynx, qualify for elective treatment on this basis. At our center Ultrasound guided Fine Needle Aspiration Cytology (USgFNAC) is used for the staging of the N0 neck in patients with T1-T2 oral and oropharyngeal carcinomas. In case of negative USgFNAC at initial staging, these patients are treated by excision of the primary tumor only, followed by a wait-and-see policy for the neck with frequent USgFNAC examinations during follow-up. In a retrospective study it was shown that USgFNAC is capable of reducing the risk for developing delayed lymph node metastases considerably to approximately 20% in these patients. However, further improvement of the sensitivity rate of USgFNAC remains desirable. Possible causes of false-negative USgFNAC are: 1) tumor containing lymph nodes do not meet US size criteria and are not aspirated, 2) aspiration is performed in a tumor-free part of a lymph node harboring a small metastasis (sampling error), and 3) the aspirate was not evaluable by routine cytology or few aspirated tumor cells in the smear were missed.

Sentinel lymph node (SN) biopsy has been proposed as a potential alternative for elective lymph node dissection. The SN concept is fundamentally based on the theory of orderly spread of tumor cells within the lymphatic system. The first lymph node in a regional lymphatic basin that receives lymphatic flow from a tumour is considered to be the SN. The SN concept assumes that lymphatic metastases, if present, can always be found at least in the SN. A tumor-negative SN would preclude the presence of lymphatic malignant involvement. SN biopsy has been rapidly gaining

popularity as a diagnostic procedure for several solid tumours, in particular for melanoma and breast cancer. Histopathological analysis of the SN correctly reflects regional lymph node status in >98% of the patients with these tumor types. SN identification has been introduced much later in the staging of head and neck cancer patients and the results so far reported are not consistent.

It has recently been shown for a variety of human cancers, that molecular techniques are superior to histological and cytological analysis for the detection of small numbers of tumor cells in a background of overwhelming numbers of normal cells.

The aims of this thesis are: 1) to investigate whether identification and aspiration of the SN could improve the sensitivity of USgFNAC to detect occult lymph node metastases in the clinically N0 neck, and 2) whether molecular diagnosis of the aspirates could improve routine cytology.

In **Chapter 2** a pilot study is described, in which the feasibility of combining SN identification and USgFNAC of the SN is evaluated in 12 patients. It was found that SN identification at lymphoscintigraphy, subsequently gamma probe guided identification of the SN, and then USgFNAC of the SN was feasible in HNSCC patients with primary tumor sites accessible for injection of ^{99m}Tc-Coloidal Albumin and a clinically N0 neck. In the 6 patients who subsequently underwent a neck dissection, the earlier cytological findings in the SN aspirates correctly reflected the status of the lymph nodes in the neck (2 positive, 4 negative), while in the 6 patients treated with transoral excision of the primary tumor only and a wait-and-see policy for the neck one false-negative case was observed.

In **Chapter 3** the variables affecting SN visualization at lymphoscintigraphy are reported based on the lymphoscintigraphic findings in 82 HNSCC patients, receiving peritumoral injections of 25 or 75 MBq ^{99m}Tc-labeled Colloidal Albumin. In 95% of the patients at least one SN could be identified either by dynamic scintigraphy or delayed static scintigraphy. In 62% of the

patients the SN was visualised within the first minute of dynamic imaging. We recommend a 75 MBq ^{99m}Tc-CA injection dose directly followed by dynamic imaging in order to increase the visualization rate of lymphatic channels from the injection site to the lymph nodes, thereby facilitating differentation between SNs and second echelon nodes.

In **Chapter 4** a study is described to attempt to validate the principle of the SN in head and neck cancer patients. In 22 patients with T2-4 N0 oral or oropharyngeal SCC who were planned to undergo combined primary tumor excision and elective unilateral (n=17) or bilateral (n=5) neck dissection, SN identification using lymphoscintigraphy after peritumoral injections of ^{99m}Tc-labeled colloidal albumin was carried out the day before surgery. After removal of the neck dissection specimens all SNs, all other radioactive lymph nodes and all non-radioactive lymph nodes were retrieved for histopathological analysis including immunohistochemistry (IHC) and serial sectioning (SS) at 250 μm intervals. Preoperative USgFNAC detected occult metastases in 60% of the patients with histologically tumor-positive lymph nodes. When a SN was identified (19/22 patients), the sensitivity of the SN procedure for predicting the presence of lymph node metastases was 89% (8/9 patients), and the overall accuracy of the SN procedure to predict the status of the lymph nodes in the neck 95% (18/19 patients). It further appeared that SN identification is not reliable in patients with primary tumors located in or close to the midline.

In **Chapter 5** the results of a follow-up study of patients with T1-T2 oral or oropharyngeal carcinomas treated with transoral excision of the primary tumor only and a wait-and-see policy for the neck based on initial negative USgFNAC findings, are described. To investigate the putative role of SN identification and aspiration, patient group A (n = 122) was staged using USgFNAC of suspected lymph nodes based on size criteria only, whereas patient group B (n = 39) was staged using USgFNAC of the SN in addition to USgFNAC of enlarged lymph nodes according to the standard size criteria. A benefit of identification and aspiration of the SN could not be demonstrated.

Sampling error of USgFNAC of the SN is most probably the cause of this observation.

In **Chapter 6** the set-up is described of a quantitative real-time RT-PCR assay using E48 as squamous cell specific molecular marker and the gene encoding porphobilinogen deaminase as marker for the number of cells in a specific sample. The detection limit of the assay was shown to be a single squamous cell in 10^6 peripheral blood mononuclear cells. The sensitivity of E48 Q-RT-PCR was shown to be higher as compared to the sensitivity of routine cytological examination, but at the expense of a decrease in specificity. This E48 Q-RT-PCR assay seems particularly useful in case aspirates can not be diagnosed conclusively by routine cytology.

In **Chapter 7** a study is reported, in which the prognostic relevance of micrometastases in lymph nodes, as detected by E48 Q-RT-PCR, in HNSCC patients was investigated. In total 456 lymph nodes of 41 patients treated by radical primary tumor excision and neck dissection with 0, 1 or 2 histopathologically tumor-positive lymph nodes, were analyzed for squamous cells by E48 Q-RT-PCR. In 5/23 (22%) pN0 and in 10/18 (56%) pN+ patients E48 transcript-positive signals were detected in at least one of the resected histologically tumor-negative lymph nodes. The presence of micrometastases in the pN0 group was prognostically relevant.

Conclusions

In patients with a well lateralized squamous cell carcinoma in the oral cavity or oropharynx accessible for peritumoral injection of radioactive tracer and a clinically N0 neck, the SN procedure is a feasible and reliable approach with a high SN identification rate using dynamic and static scintigraphy and the handheld gamma probe. Our work supports the hypothesis of the SN concept in these patients. However, SN identification (and thus biopsy) in patients with primary tumors located in or close to the midline with potential spread to the contralateral neck side seems not reliable.

Addition of a functional criterion (SN identification) to the usual size criteria for USgFNAC did not decrease the false-negative rate of USgFNAC, probably due to sampling error when aspirating the SN. This problem can not be resolved by the current aspiration techniques used at USgFNAC. However, performing a *biopsy* of the SN with extensive histopathological examination, including step sectioning and staining by both H&E and IHC, as well as with molecular assessment using e.g. E48 Q-RT-PCR, might be a useful adjunct diagnostic tool in case of negative USgFNAC findings at initial staging of the N0 neck. The role of SN biopsy in patients with squamous cell carcinoma of the oral cavity or oropharynx, however, needs yet to be established in prospective trials, particularly in patients with T1-T2 tumors.

Real-time E48 Q-RT-PCR is an accurate technique for squamous cell detection in lymph node aspirates of HNSCC patients. The sensitivity of E48 Q-RT-PCR was shown to be higher as compared to the sensitivity of routine cytological examination, but at the expense of a decrease in specificity. This assay might be implemented routinely in USgFNAC in cases for which cytological examination is not conclusive.

9

Samenvatting en Conclusies

In circa 5% van alle kwaadaardige tumoren die jaarlijks worden gediagnosticeerd in Noord/West Europa en de Verenigde staten, betreft het een plaveiselcelcarcinoom uitgaande van het slijmvlies in het hoofd/hals gebied (HHPCC) (**Hoofdstuk 1**). Deze tumoren hebben meer neiging te metastaseren via de lymfebanen naar de regionale lymfeklieren in de hals dan uit te zaaien via het bloed naar organen op afstand. De status vande lymfeklieren in de hals is bij HHPCC de belangrijkste prognostische factor. Wanneer zich bij patiënten met een HHPCC halslymfekliermetastasering voordoet, verslechtert de prognose met een factor twee, dat wil zeggen de kans om vijf jaar te overleven wordt gehalveerd. Echter, de aanwezigheid van lymfekliermetastasen in de hals betekent geenszins dat het proces incurabel is. Halslymfekliermetastasen worden over het algemeen chirurgisch behandeld door middel van een zogenaamde nekdissectie. Wanneer bij pathologisch onderzoek van het nekdissectiepreparaat multipele lymfekliermetastasen en/of groei buiten de lymfeklieren worden vastgesteld, wordt postoperatieve radiotherapie gegeven.

De behandeling van de klinisch tumor-negatieve hals (N0 hals) is nog altijd een controversiële kwestie. In het algemeen is electieve behandeling van de hals geïndiceerd wanneer een grote kans op aanwezigheid van occulte lymfekliermetastasen bestaat *en* wanneer de hals geopend moet worden voor chirurgische excisie van de primaire tumor en reconstructie, of de hals niet adequaat gestadiëerd kan worden of de patiënt niet beschikbaar is voor regelmatige follow-up. Indien *alleen* een hoge kans bestaat op occulte metastasen bestaat, is de keuze tussen electieve behandeling van de hals of "watchful waiting". Dit probleem doet zich met name voor bij kleinere (T1-T2) tumoren in de mondholte of orofarynx, aangezien deze transoraal verwijderd kunnen worden.

De argumenten vóór electieve behandeling van de hals zijn gebaseerd op de volgende uitgangspunten. Ten eerste zullen occulte metastasen onvermijdelijk uitgroeien tot klinisch manifeste ziekte. Ten tweede ontwikkelen sommige patiënten bij een "watchful waiting" beleid, ondanks regelmatige follow-up controles, inoperabele metastasen in de hals. Tenslotte kunnen onbehandelde "occulte" metastasen in de hals aanleiding geven tot afstandsmetastasen, terwijl zij uitgroeien tot klinische detecteerbare metastasen. De argumenten tégen electieve behandeling van de hals zijn de volgende. Ten eerste krijgt een grote groep patiënten een behandeling die zij niet nodig hebben. Ten tweede neemt deze behandeling een barrière tegen verspreiding van kanker weg, hetgeen van belang is wanneer zich een locaal recidief of een tweede primaire tumor in het hoofdhalsgebied voordoet. Tenslotte gaat electieve behandeling van de hals gepaard met de nodige morbiditeit, al is die minder na de gemodificeerde vormen van nekdissectie die tegenwoordig worden verricht dan na de klassieke radicale nekdissectie.

In de meeste centra in de wereld wordt bij patiënten met HHPCC electieve behandeling van de hals gerechtvaardigd geacht wanneer het risico op occulte metastasen groter is dan 20%. Indien alléén palpatie wordt gebruikt voor de stadiëring van de hals, kwalificeren de meeste localisaties en stadia van HHPCC, inclusief T1-T2 tumoren in de mondholte en orofarynx, zich op basis van dit criterium voor electieve behandeling van de hals.

In ons centrum wordt, behalve palpatie, echo-geleide dunne-naald aspiratie cytologie (USgFNAC) gebruikt voor de stadiëring van de N0 hals bij patiënten met een T1-T2 mondholte- of orofarynxcarcinoom. Bij deze techniek worden lymfklieren geselecteerd voor aspiratie op basis van grootte criteria (minimale axiale diameter > 3 mm in level I, > 4 mm in de overige levels). Indien USgFNAC geen aanwijzingen voor halskliermetastasen laat zien, ondergaan deze patiënten alleen een transorale excisie van de primaire tumor en wordt de hals ongemoeid gelaten, echter, met een strict follow-up beleid ("watchful waiting"), inclusief frequente USgFNAC onderzoeken van de hals. Een retrospectieve studie, uitgevoerd in ons centrum, heeft aangetoond dat het risico op occulte metastasen bij deze patiënten m.b.v.

initiële USgFNAC aanzienlijk kan worden terug gedrongen van ruim 40% tot ongeveer 20%, terwijl m.b.v. USgFNAC tijdens follow-up lymfekliermetastasen vaker eerder ontdekt worden en daardoor vaker alsnog genezing wordt bereikt met nekdissectie (+/- postoperatieve bestraling). Een verdere verbetering van de sensitiviteit van USgFNAC om occulte halslymfekliermetastasen te detecteren, blijft echter wenselijk. De mogelijke oorzaken van fout-negatieve uitslagen van USgFNAC zijn: 1) tumor-positieve lymfeklieren voldoen niet aan de selectie criteria voor aspiratie en worden derhalve niet gepuncteerd, 2) een kleine metastase in een tumor-positieve lymfeklier wordt gemist bij aspiratie (sampling error) of 3) het aspiraat is niet evalueerbaar bij cytologie. De sensitiviteit van USgFNAC zou mogelijk verbeterd kunnen worden door een gerichtere selectie voor aspiratie van de lymfeklier(en) die het meeste risico heeft op metastasen, de zgn. schildwachtklier. Bovendien zou moleculaire analyse van de lymfklieraspiraten gevoeliger kunnen zijn dan de analyse door routine cytologie, resulterend in een verlaging van het fout-negatief USgFNAC percentage.

Het schildwachtklier (SWK) concept veronderstelt dat lymfogene metastasering van kanker niet een willekeurig proces is, maar op een ordelijke te voorspellen wijze plaatsvindt. Het SWK concept gaat ervan uit dat elke tumor in eerste instantie draineert op één of hooguit enkele lymfeklieren, de SWK(en). Wanneer rondom de primaire tumor een colloïdale radioactieve tracer wordt ingespoten, zal deze via de lymfebanen naar de SWK(en) getransporteerd worden. Met behulp van scintigrafie en een gammaprobe kan de SWK geïdentificeerd en vervolgens verwijderd worden. Een bij PA onderzoek tumor-negatieve SWK zou de aanwezigheid van lymfekliermetastasen elders in het regionale lymfeafvloed gebied van de primaire tumor uitsluiten en een electieve lymfeklierdissectie overbodig maken. Bij patiënten met een melanoom of mammacarcinoom is het SWK concept uitvoerig onderzocht; histopathologisch onderzoek van de SWK voorspelt in meer dan 98% van de gevallen de status van de overige lymfeklieren correct. SWK identificatie werd bij patiënten met HHPCC pas

veel later geïntroduceerd en de resultaten ervan zijn tot op heden inconsistent.

Recent is beschreven dat moleculaire technieken beter in staat zijn kleine aantallen tumorcellen in een achtergrond van grote aantallen normale cellen op te sporen dan cytologisch en histopathologisch onderzoek. Als moleculaire markers worden zowel weefsel-specifieke markers (RNA/eiwit) als tumor-specifieke markers (DNA) gebruikt. In ons laboratorium wordt de weefsel-specifieke marker E48 gebruikt voor de detectie van plaveiselcellen. Dit antigeen wordt uitsluitend tot expressie gebracht op het oppervlak van maligne en benigne plaveiselcellen.

Het doel van deze dissertatie was tweeledig: 1) onderzoeken of identificatie en aspiratie van de SWK de sensitiviteit van USgFNAC voor het opsporen van occulte metastasen in een N0 hals kan verbeteren bij patiënten met een mondholte of orofarynxcarcinoom, en 2) onderzoeken of routine cytologisch onderzoek van lymfklieraspiraten verbeterd kan worden door gebruik te maken van moleculaire technieken.

In **hoofdstuk 2** wordt de techniek en uitvoerbaarheid van SWK identificatie, gevolgd door USgFNAC van de SWK, besproken op basis van een pilot-studie met 12 HHPCC patiënten en een N0 hals. Na injectie van een colloïd met kleine deeltjesgrootte gelabeld met ^{99m}Technetium (^{99m}Tc-CA), werd een lymfoscintigram vervaardigd. De gevisualiseerde SWK(en) werd(en) gemarkeerd op de huid met behulp van een radioactief gelabelde pen en gecontroleerd met een gamma probe, waarna USgFNAC werd verricht van de gemarkeerde SWK en van andere lymfeklieren op basis van de gebruikelijke grootte criteria. SWK identificatie gevolgd door USgFNAC van de SWK bleek een uitvoerbare techniek bij patiënten met een primaire tumor toegankelijk voor injectie van het colloïd. In 6 van de 12 patiënten die een gecombineerde excisie van de primaire tumor en een electieve halsklierdissectie ondergingen, voorspelde de cytologie van de SWK de status van de halslymfeklieren correct (2 tumor-positief, 4 tumor-negatief). In de andere 6 patiënten waarbij alleen de primaire tumor transoraal werd

verwijderd en een afwachtend beleid ten aanzien van de hals werd gevoerd, was er één fout-negatieve SWK op basis van USgFNAC van de SWK.

In **hoofdstuk 3** worden de resultaten beschreven van lymfoscintigrafie in een groep van 82 HHPCC patiënten die elk een dosis van 25 of 75 MBq ^{99m}Tc-CA peritumoraal kregen geïnjecteerd. Direct na injectie werd gedurende 20 minuten dynamische scintigrafie verricht in laterale richting, gevolgd door een statische anterieure opname van 2 minuten. Indien bij deze opnames geen SWK werd gevisualiseerd, werd op een later tijdstip nog een opname verricht. In 95% van de patiënten werd tenminste één SWK gevisualiseerd op het lymfoscintigram direct tijdens dynamische opnames of bij de latere statische opnames. Opvallend was dat bij 62% van de patiënten de SWK werd geïdentificeerd binnen de eerste minuut tijdens dynamische scintigrafie. Bij 40% van de patiënten was er overloop van het radioactieve colloïd vanuit de SWK naar een volgend lymfeklierstation: zgn. tweede echelon lymfeklieren. Teneinde goed te kunnen differentiëren tussen een SWK en een tweede echelon klier, heeft een dosis van 75 MBq ^{99m}Tc-CA, direct gevolgd door dynamische scintigrafie de voorkeur omdat daarmee de kans op visualisatie van de lymfebaan naar de SWK wordt vergroot.

In **hoofdstuk 4** wordt een validatiestudie beschreven waarin de betrouwbaarheid van het SWK concept voor patiënten met een HHPCC wordt onderzocht. In 22 patiënten met een T2-T4 mondholte of orofarynxcarcinoom en een N0 hals bij palpatie die gepland waren voor een gecombineerde excisie van de primaire tumor en een electieve uni- of bilaterale halsklierdissectie, werd één dag pré-operatief de SWK geïdentificeerd met behulp van lymfoscintigrafie, gevolgd door USgFNAC van de SWK. Nadat de halsklierdissecties waren uitgevoerd, werden alle SWK-en, alle andere radioactieve lymfeklieren en alle niet-radioactieve lymfeklieren verwijderd uit het preparaat en histopathologisch onderzocht, inclusief immunohistochemie (IHC) en additionele Hematoxiline en Eosine (H&E) coupes. Pré-operatieve USgFNAC detecteerde occulte metastasen in 60% van de patiënten met histopathologisch tumor-positieve lymfeklieren. Indien een SWK werd geïdentificeerd (19 van de 22 patiënten), was de

sensitiviteit van de SWK procedure om lymfekliermetastasen aan te tonen 89% (8 van de 9 patiënten). De voorspellende waarde van de SWK procedure voor het aantonen cq uitsluiten van lymfekliermetastasen was 95% (18 van de 19 patiënten). De SWK procedure bleek niet betrouwbaar bij patiënten met een primaire tumor dichtbij of in de mediaanlijn.

In **hoofdstuk 5** worden de resultaten besproken van een follow-up studie van patiënten met een T1-T2 mondholte of orofarynxcarcinoom, behandeld met transorale excisie van de primaire tumor alléén en een afwachtend beleid ten aanzien van de N0 hals gebaseerd op negatieve USgFNAC bevindingen bij initiële stadiëring. Om de potentiële rol van SWK identificatie en aspiratie te onderzoeken, werden de uitkomsten in patiëntengroep A (n=122), gestadiëerd met USgFNAC van lymfeklieren geselecteerd op basis van de gebruikelijke grootte criteria alléén, vergeleken met die in patiëntengroep B (n=39), die werd gestadiëerd met USgFNAC van zowel de SWK als van lymfeklieren volgens de gebruikelijke grootte criteria. Een voordeel van SWK identificatie en aspiratie kon niet worden aangetoond. Sampling error van USgFNAC van de SWK is de meest waarschijnlijke oorzaak voor deze observatie.

In **hoofdstuk 6** is onderzocht in hoeverre routine cytologie van lymfeklieraspiraten verbeterd kan worden door gebruik te maken van moleculaire technieken. Hiertoe is een kwantitatieve real-time RT-PCR methode (Q-RT-PCR) opgezet, waarbij E48 wordt gebruikt als plaveiselcel-specifieke moleculaire marker en waarbij het coderende gen voor porfobilinogeen deaminase wordt gebruikt als maat voor het aantal cellen in een bepaald monster. De detectiegrens van deze methode was één plaveiselcel in een achtergrond van één miljoen perifere bloedlymfocyten. De sensitiviteit van E48 Q-RT-PCR bleek hoger te zijn dan de sensitiviteit van routine cytologisch onderzoek, maar ging ten koste van een daling van de specificiteit. De E48 Q-RT-PCR test bleek met name waardevol voor de analyse van lymfeklieraspiraten waarop bij routine cytologie geen diagnose kan worden gesteld.

In **hoofdstuk 7** wordt de prognostische betekenis onderzocht van de aanwezigheid van micrometastasen in lymfeklieren van HHPCC patiënten, gedetecteerd met de E48 Q-RT-PCR test. In totaal werden 456 lymfeklieren van 41 patiënten met deze moleculaire methode geanalyseerd op de aanwezigheid van plaveiselcellen. Alle patiënten hadden een radicale primaire tumor excisie met halsklierdissectie ondergaan, waarbij histopathologisch onderzoek 0 (pN0 groep), 1 of 2 (pN+ groep) tumor-positieve lymfeklieren aantoonde. In 5 van de 23 (22%) pN0 patiënten en in 10 van de 18 (56%) pN+ patiënten werd E48 expressie aangetoond in tenminste één van de histopathologisch tumor-negatieve lymfklieren. De aanwezigheid van micrometastasen bleek prognostisch relevant in de pN0 groep.

Conclusies

Bij patiënten met een gelateraliseerd primair plaveiselcelcarcinoom in mondholte of orofarynx, toegankelijk voor peritumorale injecties van radioactief colloïd, en een N0 hals, is de SWK procedure een goed uitvoerbare en betrouwbare techniek. Met lymfoscintigrafie en de gamma probe wordt een hoog SWK identificatie percentage gehaald. Het SWK concept lijkt voor deze groep HHPCC patiënten valide te zijn. Echter, SWK identificatie (en biopsie) lijkt niet betrouwbaar te zijn bij patiënten met een HHPCC gelocaliseerd in of dichtbij de mediaanlijn.

Het toevoegen van een functioneel criterium (SWK identificatie) aan de gebruikelijke grootte criteria voor USgFNAC heeft het fout-negatieve USgFNAC percentage niet verlaagd, waarschijnlijk als gevolg van sampling error bij de aspiratie van de SWK. Dit probleem kan niet worden opgelost met de huidige aspiratie technieken zoals die gebruikt worden bij USgFNAC. Een *biopsie* van de SWK met uitgebreid histopathologisch onderzoek, inclusief additionele H&E en IHC coupes, en moleculaire methoden zoals E48 Q-RT-PCR, lijkt van aanvullende diagnostische betekenis te kunnen zijn in geval van negatieve USgFNAC bevindingen bij initiële stadiëring van de hals. De rol van SWK biopsie bij patiënten met een plaveiselcelcarcinoom

van mondholte of orofarynx, in het bijzonder T1-T2 tumoren, en een N0 hals, zal echter alleen middels een prospectieve trial definitief kunnen worden vastgesteld.

Real-time E48 Q-RT-PCR is een nauwkeurige techniek voor het opsporen van plaveiselcellen in lymfeklieraspiraten van HHPCC patiënten. De sensitiviteit van E48 Q-RT-PCR blijkt hoger te zijn dan de sensitiviteit van routine cytologie, echter, ten koste van de specificiteit. De test kan routinematig worden toegepast bij USgFNAC voor de analyse van lymfeklieraspiraten waarop bij routine cytologie geen diagnose kan worden gesteld.

List of publications

J Cloos, **EJC Nieuwenhuis**, DI Boomsma, DJ Kuik, MLT van der Sterre, F Arwart, GB Snow, BJM Braakhuis.
Inherited Susceptibility to Bleomiycin-Induced Chromatid Breaks in Cultured Peripheral Blood Lymphocytes.
Journal of the National Cancer Institute 91:1125-1130, 1999.

EJC Nieuwenhuis, DR Colnot, R Pijpers, JA Castelijns, RH Brakenhoff, GB Snow, MWM van den Brekel. Lymphoscintigraphy and ultrasound guided fine needle aspiration cytology of sentinel lymph nodes in head and neck cancer patients.
Recent Results in Cancer Research 157:206-217, 2000.

DR Colnot, **EJC Nieuwenhuis**, JA Castelijns, R Pijpers, RH Brakenhoff, GB Snow, MWM van den Brekel.
Ultrasound guided aspiration of sentinel nodes for improved staging of head and neck cancer patients-initial experience.
Radiology 218:289-293, 2001.

EJC Nieuwenhuis, JA Castelijns, RH Brakenhoff, RH Pijpers, AJ Kummer, GB Snow, MMW van den Brekel.
New developments using non invasive techniques to assess the status of the neck in head and neck cancer patients.
Research Trends, Current Topics in Radiology 2:41-45, 2000.

S Meijer, MWM van den Brekel, **EJC Nieuwenhuis**.
Letter to the editor.
Head and Neck 22(7):733-735, 2000.

EJC Nieuwenhuis, JA Castelijns, R Pijpers, MMW van den Brekel, RH Brakenhoff, I van der Waal, GB Snow, CR Leemans.
Wait-and-see policy for the N0 neck in early stage oral and oropharyngeal SCC using ultrasound guided cytology: is there a role for identification of the sentinel node?
Head Neck, 24:282-289, 2002.

EJC Nieuwenhuis, R Pijpers, JA Castelijns, GB Snow.
Lymphoscintigraphic details of sentinel lymph node detection in 82 patients with squamous cell carcinoma of the oral cavity and oropharynx.
Nuclear Medicine Communications 24:651-656, 2003.

EJC Nieuwenhuis, LH Jaspers, JA Castelijns, B Bakker, RGA Wishaupt, F Denkers, CR Leemans, GB Snow, RH Brakenhoff.
Quantitative molecular detection of minimal residual head and neck cancer in lymph node aspirates.
Clinical Cancer Research 9:755-761, 2003.

EJC Nieuwenhuis, CR Leemans, A. Kummer, F. Denkers, GB Snow, RH Brakenhoff.
Asessment and clinical significance of micrometastases in lymph nodes of head and neck cancer patients using E48 real-time PCR.
Laboratory Investigations, in press.

AG de Nooij-van Dalen, GAMS van Dongen, SJ Smeets, **EJC Nieuwenhuis**, M Stigter-van Walsum, GB Snow, RH Brakenhoff.
Characterization of the human LY-6 antigens, the newly annotated member Ly-6K included, as molecular markers for head and neck squamous cell carcinoma.
International Journal of Cancer 103:768-774, 2003.

Chapters

EJC Nieuwenhuis, DR Colnot, R Pijpers, JA Castelijns, PJ van Diest, RH Brakenhoff, GB Snow, MWM van den Brekel.
Ultrasound guided fine needle aspiration cytology of sentinel lymph nodes in head and neck cancer patients.
Lymphologie gegen Ende des 20. Jahrhunderts. Eds. BM Lippert, IO Rathcke, JA Werner. Shaker Verlag Aachen 197-206, 1999.

MWM van den Brekel, **EJC Nieuwenhuis**, JA Castelijns.
Bildgebung und Behandlung von zervikalen Lymphknotenmetastasen.
Symposiumband Klinische Onkologie 2000/'01. Ed. S.L.Roth. Dressler & Schreiner GmbH Düsseldorf 11-15, 2000.

EJC Nieuwenhuis, JA Castelijns, MMW van den Brekel, GB Snow, CR Leemans.
Observing the N0 neck in T2 oral and oropharyngeal carinoma using ultrasound guided cytology.
Metastases in Head and Neck Cancer. Eds. BM Lippert, JA Werner, Marburg 257-261, 2001.

EJC Nieuwenhuis, MMW van den Brekel, RH Brakenhoff, R Pijpers, CR Leemans, GB Snow, JA Castelijns.
Assessment of the N0 neck using ultrasound guided cytology of sentinel lymph nodes in head and neck cancer patients.
Metastases in Head and Neck Cancer, Eds. BM Lippert, JA Werner, Marburg 185-189, 2001.

Acknowledgements

Many people have contributed to the work described in this thesis. In particular, I would like to gratefully acknowledge the following persons for their support, motivation, inspiration, time and energy:

Promotors	Prof. dr Gordon B. Snow Prof. dr Jonas A. Castelijns
Co-promotors	Dr Ruud H. Brakenhoff Dr Rik Pijpers
Scientific Board	Prof. dr P.J. van Diest Prof. dr J.S. Laméris Prof. dr C.R. Leemans Prof. dr K. Pantel Prof. dr J. Teule Prof. dr I. van der Waal
Vrije Universiteit Medical Center	Department of Otolaryngology/Head and Neck Surgery Department of Tumorbiology Department of Radiology Department of Nucleair Medicine Department of Pathology
	Dr. Michiel W.M. van den Brekel Dr. Jacqueline Cloos Dr. Alain Kummer Dr. Lies H. Jaspers
Analysts	Richard Wishaupt, Janny Dijkstra, Fedor Denkers, Willem de Jong, Branco Bakker
Statistical support	Joop Kuik
Hebbes chicks	Es, Maas, Mik, Wen, Ood
Paranymphs	Jolijn Brouwer, Marein van der Torn

Curriculum vitae auctoris

The author of this thesis, Eline Vriens-Nieuwenhuis, was born on May 7, 1971 in Sassenheim. She graduated from high school at the Rijnlands Lyceum Sassenheim in 1990. In the subsequent year, physics, mathematics and chemistry were added to her curriculum at the Boerhaave Scholengemeenschap in Leiden. During these years she worked as a nutrition nurse in the Diaconessenhuis in Leiden. In 1991 she started Medical School at the University of Leiden. Initially, she worked at the Bloodbank Leiden. From 1992 to 1998 she worked as an organ allocation coordinator for the Eurotransplant Foudation. From 1996 to 1997 she spend seven months at the Section Tumor Biology of the Department of Otolaryngology/Head and neck Surgery (Prof. dr G.B. Snow), Vrije Universiteit Medical Center in Amsterdam, participating in a study assessing the heritability of mutagen sensitivity. After graduation from Medical School in 1998 she returned to the Department of Otolaryngology/Head and neck Surgery (Prof. dr G.B. Snow), Vrije Universiteit Medical Center, to perform the studies described in this thesis (Prof. dr G.B. Snow and Prof. dr J.A. Castelijns). In 2002 she started her surgical training at the Jeroen Bos Ziekenhuis in Den Bosch (dr J. Wever). Currently, she is a resident in surgery at the University Medical Center Utrecht (Prof. dr I.H.M. Borel Rinkes).