Assessment of Regional Metastasis in Head and Neck Cancer

Robert P. Takes

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PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 20 juni 2000
te klokke 14.15 uur

door

Robert Paul Takes
geboren te Nieuwer-Amstel in 1963

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Contents

CHAPTER 1

General Introduction

INTRODUCTION

Regional metastasis is one of the most important factors in prognosis and treatment of patients with Head and Neck Squamous Cell Cancer (HNSCC). The presence of nodal metastasis will significantly affect the survival of the patient (1-3). Moreover, since lymphatic metastasis is the most common route of spread of HNSCC, a decision whether or not to treat the lymph nodes of the neck has to be made. It is therefore clinically relevant to assess as reliably as possible whether a patient has or will develop regional lymph node metastases.

The clinically negative (N0) neck

One of the basic issues in the management of patients with HNSCC is the treatment of the N0 neck. The question is whether or not to treat the neck electively in these cases. A high incidence of occult metastasis, the effectiveness of elective neck treatment and the failure of diagnostic tests for assessment of the neck are in favour of elective neck treatment. On the other hand, no study has conclusively demonstrated higher cure rates for patients who underwent elective neck treatment compared to patients subjected to a "watchful waiting" or "wait-and-see" policy. However, in the latter group, much will depend on the delay of treatment once the nodal metastases become manifest and therefore on the intensity of the follow-up of these patients. Morbidity, mortality and putative immunological consequences are some other arguments against elective neck treatment. Many, predominantly retrospective, studies have been performed on this subject with conflicting results (4-10). Of the few prospective studies, none showed a beneficial effect of elective neck treatment compared to a wait-and-see policy (11-13). However, up to date no general change of policy has been made. Due to the low sensitivity of most ways of examination with high false-negative rates, many patients still undergo elective neck treatment. Most head and neck oncologists will treat the neck electively if the incidence of occult metastasis is more than 15%. In a recent decision analysis, a risk of occult metastases higher than 20% was found indicative for elective neck treatment (14). As a consequence, most oral, oropharyngeal, hypopharyngeal and supra- and subglottic laryngeal carcinomas will be treated electively for the neck (15). So, even when no metastases can be detected the neck will be treated in the majority of the patients. As a result, many patients will receive an unnecessary treatment for their neck with possible morbidity (16-18). Shoulder dysfunction in particular can be a disabling consequence of neck dissections, even when the spinal accessory nerve is preserved (17-20).

If diagnostic means to assess the clinically negative neck improve, clinically occult metastases will be detected in more patients. These patients will be treated therapeutically instead of electively. The chance of the presence of regional metastasis in the remaining patients is reduced. To what extent this chance is reduced, or the posterior probability, is determined by the sensitivity and specificity of the diagnostic technique and by the incidence, or prior probability, of occult metastasis known from the literature. Quantification of this reduction in probability can be done by Bayes' theorem (15). This theorem is used in clinical decision analysis to calculate the probability of disease after interpretation of new diagnostic

information. If, by the use of improved diagnostic techniques, the probability of occult regional metastasis is reduced, the number of elective treatments can be decreased.

The clinically positive (N+) neck

Improved assessment of nodal metastasis will have other implications as well. A higher specificity, with lower numbers of false positive cases, will reduce the number of unnecessary treatments. So, better diagnostic techniques to detect or predict nodal metastasis will lead to a significant improvement in treatment strategies and, presumably, outcome.

IMAGING TECHNIQUES FOR THE NECK

Traditionally the neck of patients with HNSCC is examined by palpation. This method is, however, not very reliable (21-23). With the use of imaging techniques like Computed Tomography (CT), Magnetic Resonance Imaging (MRI) and Ultrasound (US) better accuracy, and sensitivity in particular, could be achieved (24-28).

Like CT and MRI the accuracy of US is limited if only radiological or morphological criteria for malignancy are used. Differentiation between nodes with and without metastases based on radiological characteristics only, has a relatively low specificity (22;27;29-35). Several radiological criteria to distinguish pathological, metastatically invaded, lymph nodes from normal or reactively enlarged nodes are described in the literature. The only criterion that is consistently found to be relevant is an irregular staining pattern of contrast. Necrosis in particular is considered to be the most reliable criterion for metastatic involvement. However, in a population of patients without palpable masses in the neck, the metastases will be small and therefore necrosis will not frequently be found. Reported size criteria for malignancy are not consistently the same in the different studies. In most studies an axial diameter in the range of 8 mm to 15 mm is found to be distinctive (25;36;37).

Due to the limited specificity in the range of 70 to 85% (23;31;33) of techniques like Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) patients may receive unnecessary neck treatment based on false-positive findings. More recently other techniques have been explored like Radio Immuno Scintigraphy (RIS) (38;39) and Positron Emission Tomography (PET) (40;41) but these techniques still have to prove their value in detecting nodal disease in clinical practice.

In recent years, Ultrasound (US) of the neck combined with Ultrasound Guided Fine Needle Aspiration Biopsy (UGFNAB) was demonstrated to be very accurate in the evaluation of regional metastatic disease (31;42;43). It combines the high sensitivity of US with the excellent specificity of fine needle aspiration biopsy (44-47). A sensitivity of as high as 98% and a specificity of 95% have been reported (43). In view of the test characteristics of US/UGFNAB, this diagnostic procedure may have a significant impact on clinical management of patients with HNSCC (15). Critics have put forward, however, that the results of both US and UGFNAB are very much

determined by the expertise of those who perform the investigation. Therefore, a need was felt to conduct a prospective multicenter study on the value of US/UGFNAB to verify the acclaimed accuracy of US/UGFNAB in the diagnosis of metastatic neck disease in patients with HNSCC and to investigate whether major differences in the accuracy of the combined procedure occur when the tests are performed by different investigators. The results of this study will be discussed in chapters 2 and 3.

Despite the improvement of diagnostic imaging techniques, the concept of elective neck treatment has not been abandoned. All imaging techniques have the fundamental limitation that a minimal size of metastases is required. Therefore, small metastatic deposits will still be undetected, and uncertainty about the true lymph node status of the neck will remain. Even Ultrasound (US) with Ultrasound Guided Fine Needle Aspiration Biopsy (UGFNAB), the most accurate technique to detect lymph node metastases to date, identifies clinically occult metastases with a sensitivity of no more than 76% according to the literature (48).

TUMOR BIOLOGY

The development of tumors is due to alterations in normal cellular processes like proliferation, differentiation, cell adhesion and programmed cell death or apoptosis. These alterations will result in phenotypic changes such as uncontrolled growth, invasive potential or ability to metastasize. The process of metastasis in particular is very complex. To metastasize, cells proliferate, lose contact with neighbouring cells, migrate through the interstitial matrix, invade blood and lymph vessels and grow out again in lymph nodes or distant organs. The metastatic cells, therefore, have to possess several (changes in) properties to be able to perform all these actions (49). These properties will be based on alterations in genes and their products. Based on the assumption that metastasis is mainly determined by properties of the primary tumor and its interaction with the surrounding structures it seems worthwhile to explore the possibility to predict the presence of metastases based on features of the primary tumor. In that case it would be possible to obtain additional information on the chance of metastasis, irrespective of the size of the metastases, by studying genetic alterations or other features of the primary tumors itself.

In the process of tumorigenesis and metastasis, many factors are involved. Several markers have been identified as relevant factors in these processes, but the exact genetic and cellular mechanisms are not yet fully unveiled. However, since several markers in different steps of carcinogenesis are already known, it seems worthwhile to investigate if useful clinical correlations can be established. If markers are to be used for clinical purposes, the techniques to study them should preferably be readily available and easy to perform. Histological features of the tumor (e.g. differentiation, growth pattern) and host response (e.g. tumor associated eosinophilic infiltration, inflammatory reaction surrounding the tumor) can be studied by simple light microscopy. Protein expression can be studied by immunohistochemistry using

antibodies directed at the proteins of interest. The techniques are relatively easy to perform and inexpensive. Studying gene amplifications using Southern blot techniques is more time consuming. A more recent technique, Fluorescence In Situ Hybridization (FISH), can make the study of amplifications much easier. Other techniques like the Polymerase Chain Reaction (PCR) have also made the assessment of other genetical alterations relatively simple.

Since the process of metastasis is very complex, it is unlikely that a single marker can predict metastasis reliably. It seemed therefore interesting to investigate wether a combination of relevant markers is able to predict metastasis. Our selection of markers was based on the relevance of the marker in the process of tumor progression and metastasis as described in the literature. Because metastasis is supposed to be a late event in tumor progression we selected markers playing roles in different phases of tumor development. Since the number of relevant markers is large and increasing, we limited ourselves to those we had experience with in our institute.

INVESTIGATED MARKERS

In the following part the investigated markers are introduced.

Cyclin D1/EMS1 (11q13)

Amplification of the 11q13 region has been found to be involved in a variety of human tumors. Amplification of this chromosome region is seen in a significant portion of breast cancers (50), squamous cell carcinomas of the oesophagus (51-53) and HNSCC (54-59). It appears to be correlated with several clinicopathological parameters including lymph node metastasis (reviewed by Schuuring (59)). Initially, indications of a relation with stage and prognosis were found in squamous cell carcinomas of the upper aerodigestive tract in relatively small series by some authors (51-54;57). Recently, however, a correlation of 11q13 amplification with the presence of lymph node metastasis (56;58;60-62) and prognosis (63) was found in several studies and on larger populations.

Of the genes located on the 11q13 region to date only 2 genes, cyclin D1 and EMS1, seem to be expressed. Cyclin D1 was first described as a candidate oncogene in 1991 by Motokura et al. as PRAD-1 (64) and plays an important role in cell-cycle regulation. It is acting as a regulator of the G1-S phase transition. Overexpression of cyclin D1 may cause deregulation of the cell cycle and may thus lead to tumorigenesis. Cyclin D 1 expression in HNSCC has been found in 44-49% of the cases (65-67). EMS 1 encodes an 80/85 kd cytoskeletal associated protein, cortactin. Since cortactin is associated with the cytoskeleton and cell contact sites, its overexpression may influence cell adhesion or migration properties (68).

A relation between expression of cyclin D1 and survival (63;69) and lymph node metastasis (61;70) has been described in HNSCC. Michalides et al., however, found no correlation of cyclin D1 expression with N-stage (65). So, although amplification

of the 11q13 genes seems to be correlated with the presence of metastasis, this correlation has not conclusively been confirmed for the expression of these genes.

Myc
The c-myc oncogene, located on 8q24, encodes the nuclear regulatory protein c-myc that is associated with tumorigenesis. The expression is related to the cell cycle and is supposed to determine, in a growth factor dependent way,proliferation or apoptosis. Expression of myc has not been studied extensively in HNSCC (71;72) but elevated expression has been associated with poor survival (72).

EGFR and neu
The Epidermal Growth Factor Receptor (EGFR) is a transmembrane glycoprotein which regulates cell growth in response to binding of Epidermal Growth Factor (EGF) or Transforming Growth Factor alfa (TGF alfa). Neu is a similar transmembrane protein to EGFR, but distinct from it.
In contrast to expression of neu, expression of EGFR is found in a considerable number of cases of HNSCC (73-76). A correlation between expression of EGFR and nodal metastasis has been described by some authors (76) but in general most studies failed to find a relation with metastasis of EGFR or neu expression (77-80).

Rb
Rb is a tumor suppressor gene and loss of the functioning protein may play a role in the progression of malignant tumors. Indeed, mutations of Rb were described to have prognostic value in some tumors (81-83). The role of Rb in HNSCC has not been studied extensively. In HNSCC Rb has predominantly been studied on the DNA level studying loss of heterozygosity (84-87) but more recently the expression of Rb has been studied too (88). A correlation of loss of expression with poorer survival (88) and with the presence of metastasis (89) has been reported.

P53
One of the most frequently studied markers in HNSCC in recent years is the tumor suppressor gene p53 (65;90-96). The normal function of the protein is to lead abnormal cells to apoptosis. Point mutations of p53 are one of the most frequent genetic alterations in HNSCC, leading to nuclear accumulation of the protein. Clinicopathological studies of alterations in p53 in HNSCC show varying results as mentioned in a review of Raybaud-Diogene (97). Some authors did not find any correlation of p53 expression with clinical parameters (98), metastasis (99) or survival (100;101). Others did find a correlation between nuclear p53 accumulation and survival although reports are contradictory: some found a correlation with worse survival rates (102;103), others with better (104). It has been suggested that the mutation of the p53 gene may be a more relevant parameter than its expression studied by immunohistochemistry: in some studies mutations did correlate with clinical parameters whereas the protein expression did not (98;105).

Bcl-2
One of the major apoptosis-regulatory gene families is represented by bcl-2 and its homologues. These proteins suppress apoptosis or programmed cell death and deficiencies in apoptosis contribute to carcinogenesis by allowing survival of cells with genetic instability and accumulation of gene mutations. Few studies on expression of bcl-2 in HNSCC have been published. In studies of bcl-2 expression, positive results were found in 17-32% of the cases (106-108). A correlation between bcl-2 expression and improved disease specific survival has been described (108;109) although others, in contrast, found a correlation with poor outcome in early HNSCC (110).
Although the prognostic value of bcl-2 expression in HNSCC has been studied more often, reports on its correlation with metastasis are rare. Spafford et al. failed to find a correlation (111). Theoretically, a correlation between bcl-2 expression and lymph node metastasis may be explained by the fact that inhibiting apoptosis may promote metastasis since many metastasis promoting genetic alterations may occur without cell death by apoptosis.

E-cadherin
E-cadherin is an important molecule in cell-cell adhesion. It has been demonstrated that down regulation of the E-cadherin gene is associated with poor differentiation, invasion and metastasis in several tumors (112-115). A relation of loss of expression of E-cadherin and the presence of metastases in different types of cancer has been described in several studies as reviewed by Jiang et al. (116). In some studies concerning HNSCC an indication (117;118) or correlation (119) was found between loss of expression of E-cadherin in the primary tumor and the development of nodal metastases. However other studies concerning HNSCC failed to find a statistically significant relation (120;121).

Ep-CAM
The monoclonal antibody 323/A3 recognizes a 40-kD surface antigen (122). Recently it was demonstrated that this surface antigen is an epithelium-specific intercellular adhesion molecule. Reflecting the function of the molecule it was named Ep-CAM (123;124).
Increased expression of Ep-CAM appears to result in decreased cadherin-mediated cell-cell adhesion and may lead to segregation of Ep-CAM positive cells from the parental cell population in vitro (125). This phenomenon may lead to the development of metastases in vivo. In contrast, other in vitro and animal studies of colorectal carcinomas suggest that higher expression would reduce the metastastic potential (126). Ep-CAM has not been studied extensively in HNSCC.

Desmoplakin
Desmosomes are disc like intercellular junctions mediating intercellular adhesion and providing membrane binding domains for intermediate filaments. Desmoplakins are located between the dense plaque of the desmosomes and the region of attachment of intermediate filaments. Changes in the presence of desmosomes or their components may influence cellular behaviour leading to invasion and

metastasis (127). A correlation between metastasis and desmoplakin has been described (128).

Nm23
The nm23 gene is a putative metastasis suppressor gene. Reduced expression of nm23 was found to be related to the presence of metastasis in several tumors (129-131) and recently this relation has also been described in oral carcinomas (132). In the few other studies on nm23 in HNSCC, no such relation was found (87;133).

DNA ploidy
DNA ploidy reflects the amount of genetic material in the nucleus of cells. In normal tissue most cells are in the resting (G0), diploid phase. In tumor cells there usually is genetic instability and therefore the DNA content can vary significantly from the normal diploid state. In recent reports on the DNA ploidy status in HNSCC correlations have been studied with clinical parameters like stage or metastasis. In studies of HNSCC of different sites, correlations with stage or nodal metastasis were found in some studies (134;135) but in other studies this correlation could not be established (136;137). A correlation with lymph node metastasis in cancer of the oral cavity was reported by several authors (138-142) although others only found a weak relation (143). A higher rate of lymph node metastases in aneuploid laryngeal carcinomas has also been reported (144).

Inflammatory reaction
Several histological features of tumors and their surrounding tissue have been studied for clinicopathological correlations. The degree of inflammatory reaction surrounding the tumor is suggested to reflect a host response to the tumor. It may therefore influence the progression of tumor growth and metastasis. A correlation between the presence of an inflammatory reaction and the absence of lymph node metastasis has been described in the literature (145;146).

Eosinophilic infiltration
In some studies a relation of eosinophilic infiltration surrounding the tumor and favourable prognosis has been described but a relation with lymph node metastasis was not found frequently (147-150). Others did not find any significant clinicopathological correlations (151).

Differentiation
A poor differentiation of tumors is supposed to be related to more aggressive tumor behaviour which may be reflected in a higher metastatic potential. A relation between lymph node metastasis and grade of differentiation (145;152;153) has been described by some authors. In many of these studies different types of, sometimes very elaborated, grading systems are used, often hampering comparison of the results of these studies.

Growth pattern
The growth pattern of tumors is also associated with tumor behaviour. A more invasive growth pattern is often thought to be indicating a more aggressive behaviour associated with recurrences and metastasis as has been reported by several authors (145;154;155).

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OUTLINE OF THIS THESIS

In chapter 2 a multi-center study on the value of Ultrasound with Ultrasound Guided Fine Needle Aspiration Biopsy (US/UGFNAB) in the assessment of the neck in patients with HNSCC is described.

In chapter 3 a study is discussed in which the value of US/UGFNAB was studied in a multicenter population of patients with clinically negative necks and compared to the results of Computed Tomography (CT) in that same population.

In chapter 4 a pilot study in laryngeal cancer is described. The aim of the study was to explore the possibility to predict nodal metastasis by studying features of the primary tumor. For this purpose histological features, protein expression using immunohistochemistry and DNA amplification using Southern blotting were investigated and correlated to the presence or absence of nodal metastases.

In chapter 5 the expression of genetic markers is studied in nodal metastases and their matched primary tumors. Differences in expression in the primary tumors and their metastases may suggest relevance in the process of regional metastasis.

In chapter 6 a study on the expression of a selection of previously studied markers on the material of the multi-center US/UGFNAB population is described. The aim of the study was to investigate if the correlations found in the pilot study could be reproduced in a larger number of cases.

In chapter 7 the expression of several genetic markers, studied by immunohistochemistry, was compared between the 3 major subsites of the head and neck. The head and neck region is often considered as one entity but since the biological behaviour of tumors arising in subsites of the head and neck varies, differences in intrinsic tumor factors can be expected.

In chapter 8 a study is described in which the expression of several genetic markers, studied by immunohistochemistry, was compared between biopsy material and the primary tumors they were taken from. If nodal metastases are to be predicted based on features of the primary tumors, only biopsy material will be available and due to sampling errors it is uncertain if this material is representative for the entire tumor.

In chapter 9 the value of the DNA ploidy status of the primary tumor in predicting the development of nodal metastasis is studied and discussed.

In chapter 10, the General Discussion, the findings in the studies described in the preceding chapters are summarized and the possibilities and limitations of the use of markers for clinical purposes are discussed.

CHAPTER 2

Regional Metastasis in Head and Neck Squamous Cell Carcinoma: Revised Value of US with UGFNAB

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ABSTRACT

Purpose
To verify the acclaimed accuracy of Ultrasound (US) combined with Ultrasound-Guided Fine-Needle Aspiration Biopsy (UGFNAB) in the detection of lymph node metastasis in the neck and to evaluate the interobserver variability.

Materials and methods
In a prospective, multicenter study of 185 patients with Head and Neck Squamous Cell Carcinoma (HNSCC), US (n=238 neck sides) with UGFNAB (n=178 neck sides) was used for evaluation of the lymph node status of the neck. Findings were correlated with those of histopathologic examination in 238 neck sides

Results
US/UGFNAB had a sensitivity of 77% and a specificity of 100%. Nineteen of 178 aspirations were nondiagnostic. There were no significant differences between the four participating hospitals or the individual sonologists ($p>0.05$).

Conclusion
Sensitivity of US/UGFNAB in this study was slightly lower compared with previous reports. Specificity was similar to previous reports. Interobserver variability appeared to be low. The validity of US/UGFNAB is high and warrants widespread use of the procedure for evaluation of the neck.

INTRODUCTION

The status of the lymph nodes in the neck is crucial to the treatment and prognosis of patients with Head and Neck Squamous Cell Carcinoma (HNSCC).
The prognosis is mainly determined on the basis of nodal disease: the presence of a single cervical lymph node metastasis in the ipsilateral side of the neck decreases the expected survival by approximately 50%. A contralateral affected node also reduces the expected survival by half (1).
In general, a patient presenting with HNSCC and regional metastasis will be treated with irradiation of the neck, surgery or both. Even when no nodes are detected, most head and neck oncologists will treat the neck electively when (clinically undetected) regional metastasis is likely. In most hospitals elective neck treatment will be performed if the incidence of occult metastasis is more than 15%. In clinical practice this means that patients with oral, oropharyngeal, hypopharyngeal and supra- and subglottic laryngeal carcinomas will be treated electively for the neck (2). If the probability of regional metastasis is reduced, the number of elective treatments will be decreased (2).

Until recently, accurate assessment of the neck of patients with HNSCC was not possible. Palpation and lymphangiography are not reliable (3-5). MRI and CT are useful (6-10), however, these are expensive and not always available. In addition, differentiation between nodes with and without metastases based on radiological characteristics only has a relatively low specificity (4,9,11-17). Immunologic assays may prove to be useful in the future, but are still under investigation (18).
Recently, Ultrasound (US) of the neck combined with Ultrasound Guided Fine Needle Aspiration Biopsy (UGFNAB) was demonstrated to be very accurate in the evaluation of regional metastatic disease (13,19,20). This technique combines the high sensitivity of US with the excellent specificity of fine needle aspiration biopsy (FNAB) (21-24). A sensitivity of 98% and a specificity of 95% have been reported (20).

In view of the test characteristics of US/UGFNAB, this diagnostic procedure may have a substantial impact on clinical management of patients with HNSCC (2). Opponents have suggested, however, that the results of both US and UGFNAB are very much determined by the expertise of those who perform the investigation. Therefore, a prospective, multicenter study on the value of US/UGFNAB was undertaken.
The object of our study was to verify the acclaimed accuracy of US/UGFNAB in the diagnosis of metastatic neck disease in patients with HNSCC. A second objective was to investigate whether major differences in the accuracy of the combined procedure occur when the tests are performed by different investigators. Furthermore, we studied other factors that could possibly influence the results of US/UGFNAB (eg, primary tumor site and node level).

MATERIALS AND METHODS

The multicenter study was performed at four hospitals in the Netherlands by 39 sonologists between March 1992 and September 1993. All patients with HNSCC (non-irradiated and irradiated) who underwent neck dissection(s) as part of their treatment were eligible for this study.
The neck of each patient was examined by an experienced head and neck oncologist (P.K., J.J.M., C.A.M., H.A.M.M., H.A.A.S., M.F.d.B., R.J.B.d.J.). The findings were recorded, together with other relevant clinical information. At this stage, cytological examination was not performed. Subsequently, the neck was examined by one of the sonologists (J.A.v.O., J.S.L., R.H.K., F.B.M.J.). All clinical information was provided. The findings of the sonologist(s) were recorded in a worksheet. Subsequently, UGFNAB of nodes that were depicted, was performed in 178 cases. In case of multiplicity, UGFNAB was performed of the largest node, nodes showing central hypo-echogeneity, or the most cranial and caudal nodes in the areas at highest risk for metastasis. The US examinations and UGFNABs were performed with the following scanners: a model 620/650CL (Aloka, Tokyo, Japan), with a 7.5 MHz linear-array probe, a model SSA 250A (Toshiba Europe, Zoetermeer, The Netherlands), with a 7.5 MHz mechanical sector type probe with a built-in water path, and a model 128 XP (Acuson, Mountain View, California), with a 7MHz linear array probe. The procedure was performed as described previously (20,25) (Figure 1).

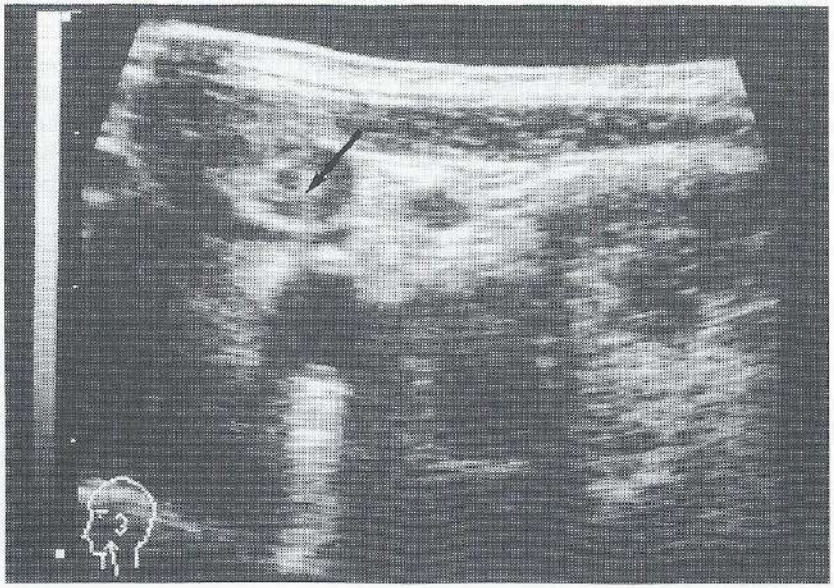

Figure 1. Ultrasound image of a lymph node metastasis during ultrasound-guided fine needle aspiration. The tip of the needle is depicted as a hyperechogenic spot marked by the arrow. For this image a 10 MHz sector-type probe with a built-in water path was used to obtain an optimal graphical representation.

Cytological examination was performed by experienced cytopathologists. Nondiagnostic aspirations had to be repeated. Because the trauma associated with rigid endoscopy may cause an increase in the number and size of lymph nodes, both palpation and ultrasound were preferably performed before endoscopy.
Neck dissection had to be performed within three weeks. The specimen was labeled by the surgeon as level I-V, according to the Memorial Sloan-Kettering classification (26). Subsequently the neck dissection specimen was histologically examined according to a standardized protocol and the findings of the pathologist (J.H.J.M.v.K., F.T.B., S.C.H.L., J.M.W.v.Y.) were recorded per level. The results of palpation and US/UGFNAB were compared with the results of the histopathological examination. We considered the results per neck side since the treatment of metastatic disease is for a neck rather than for a single node: the detection of a single metastasis results in the treatment of the whole neck side. Moreover, it is practically impossible to match an aspirated or palpable node with the same node in the neck dissection specimen.

The test result of US/UGFNAB in fact consists of the combined results of two separate tests: US and UGFNAB (Table 1). US may be scored positive (lymph nodes depicted) or negative (no lymph nodes visualized). No morphological criteria were used. Merely the depiction of lymph nodes, therefore, was used for scoring the result of US. UGFNAB may be (1) not performed, (2) negative (reactive), (3) positive (metastatic) or (4) nondiagnostic. Therefore, 5 combinations of test results were distinguished (Table 1A). The cases with nondiagnostic aspirates (not repeated or repeatedly nondiagnostic) were excluded. So US/UGFNAB was considered negative if: no nodes could be visualized, nodes were too small to aspirate (< 5 mm) or nodes appeared to be reactive on cytologic examination. The test was considered positive if the aspirate contained tumor cells.
The reference standard (histopathologic examination) was considered negative when no evidence of metastases was found in neck dissection specimens and positive when one or more metastases were diagnosed.
Since the main objective of this study was to establish the value of US/UGFNAB in discriminating between *neck sides* with and without metastatic disease, the result of UGFNAB of one or more nodes was considered to be representative for the neck as a whole. In other words, when UGFNAB for example showed a reactive node which was confirmed by histopathologic examination but a metastasis was found in another lymph node which was not depicted by US, the test result was considered false-negative.
Differences in test-characteristics such as sensitivity and specificity were evaluated using the chi-squared test. Differences are considered statistically significant at $p<0.05$.

RESULTS

A total of 185 patients (133 men, 52 women; age range 25-85 years; mean age 59 years) participated in this study. Of these, 132 patients underwent unilateral and 53 underwent bilateral neck dissection, resulting in the inclusion of 238 neck sides. The primary tumor sites and tumor stages are listed in Table 2.

Palpation and US/UGFNAB

The results of US/UGFNAB are shown in Table 1. With palpation a sensitivity of 66% and a specificity of 92% were achieved (Table 3). For US/UGFNAB a sensitivity of 77% and a specificity of 100% were found (Table 3). Some nodes detected by palpation were not detected by using US/UGFNAB. Therefore, the results of palpation and US/UGFNAB are supplementary. When, as is done in clinical practice, the results of US/UGFNAB and palpation were combined, the sensitivity was 80% with a specificity of 92%.
A considerable proportion of the aspirates (19/178 cases) was nondiagnostic and UGFNAB was not repeated. In fact, in most of the initially nondiagnostic aspirates UGFNAB was not repeated. Histologic examination of the neck dissection specimens revealed that 12 of these specimens in fact contained metastases and 7 had reactive nodes. This might suggest that a nondiagnostic aspirate is more likely to be from a metastatic node. However, the proportion of neck sides containing metastatic nodes in this group (12 of 19 neck sides) reflects the prevalence of lymph node metastases in the entire population (155 of 238 neck sides). For the present study, the nondiagnostic aspirates were excluded.

Sonologists and Hospitals

To evaluate the inter-observer variability, we compared the results of 6 sonologists who examined at least 13 neck sides (varying from 13 to 53 neck sides) and the combined results of a group of 33 sonologists (78 neck sides) who performed the examination less frequently. No statistically significant differences were found between the characteristics of these (groups of) sonologists (Table 3) or between the participating hospitals (data not shown, p=0.14).

Primary Tumor Sites and Neck Levels

The results of US/UGFNAB for different primary tumor sites are summarized in table 3. Although there seem to be marked differences, note that the number of cases in some of the groups is fairly small. There were no statistically significant differences in sensitivity for the different primary sites (p=0.51).
To evaluate differences in detecting metastases in lymph nodes of the different neck levels (I-V) we investigated whether metastases were missed more often in particular regions. The number of lymph node metastases per level was listed and the fraction of metastases not detected by US/UGFNAB was calculated. No statistically significant differences were found between the various levels (p=0.52)(Table 4).

A: Separate Procedures

US	*UGFNAB*	*HIST -*	*HIST +*	*Total*
-	Not performed	34	17	51
+	Not performed	6	3	9
+	-	36	13	49
+	Non diagnostic	7	12	19
+	+	0	110	110
Total		83	155	238

B: Combined Procedure

US/UGFNAB	*HIST -*	*HIST +*	*Total*
-	76	33	109
+	0	110	110
Total	76	143	219

Table 1. Results of US/UGFNAB in 238 hemi-necks. Table A shows the results of US and UGFNAB separately. Table B contains the results of the combined procedure. The combination of US + and UGFNAB + is considered positive; no lymph nodes depicted by US, nodes too small for aspiration, and nodes reactive on cytologic examination are considered negative test results. In table B the 19 nondiagnostic aspirates are excluded. (HIST-= no lymph node metastases in neck dissection specimen, HIST+= lymph node metastases in neck dissection specimen).

Primary tumor site		
Larynx	:	60
Hypopharynx	:	15
Oropharynx	:	26
Floor of mouth	:	22
Oral tongue	:	36
Oral cavity others	:	15
Other sites	:	7
Unknown primary	:	4
Tumor stage		
T1	:	20
T2	:	40
T3	:	42
T4	:	48
Unknown primary	:	4
Recurrence	:	25
Unknown	:	6

Table 2. Primary tumor site and stage of tumor according to the TNM classification.

	N hemi-necks	PREV (%)	SENS (%)	SPEC (%)	PV+ (%)	PV- (%)	ACC (%)
Palpation and US/UGFNAB							
Palpation	238	65	66	92	94	59	75
US/UGFNAB	219	65	77	100	100	70	85
US/UGFNAB +Palpation	219	65	80	92	95	71	84
Breakdown per Sonologist							
A	13	85	82	100	100	50	85
B	13	71	90	100	100	75	92
C	15	60	67	100	100	67	80
D	48	58	68	100	100	69	81
E	20	60	75	100	100	73	85
F	37	54	80	100	100	81	89
Others	73	73	79	100	100	65	85
Breakdown per Primary Tumor Site							
Larynx	71	77	84	100	100	64	86
Hypopharynx	15	70	83	100	100	60	87
Oropharynx	28	93	73	100	100	22	82
FOM	38	37	57	100	100	80	84
Oral tongue	38	39	73	100	100	85	89
OC other	16	63	70	100	100	67	81
OC total	92	42	67	100	100	80	86

PREV= prevalence, SENS= sensitivity, SPEC= specificity, PV+= positive predictive value, PV-= negative predictive value, ACC= accuracy.
FOM = floor of mouth, OC = oral cavity.

Table 3. Diagnostic indices for palpation, US/UGFNAB, and US/UGFNAB with palpation. Next are the results of US/UGFNAB broken down according to sonologists and site of primary tumor.

Lymph node level	Number of metastases	Number not detected	Percentage not detected
I	44	10	23%
II	92	16	17%
III	65	9	14%
IV	31	3	10%
V	12	3	25%

Table 4. Percentage of undetected metastases per lymph node level with US/UGFNAB. The differences between the levels are not statistically significant (p=0.52).

DISCUSSION

To assess the status of lymph nodes in the neck in patients with HNSCC various (imaging) techniques have been explored. CT and MRI allow detection of small structures such as lymph nodes with high sensitivity. Although several radiological characteristics of metastatic nodes have been defined (size, shape, central necrosis, obliteration of fascial planes, contiguous nodes), several authors have criticized these criteria (4,12,15-17,27-29). In our opinion, differentiation between benign and metastatic nodes only on the basis of radiological characteristics remains difficult and unreliable.
US is characterized by a superior sensitivity rate for detection of lymph nodes (3,30). The detection of more lymph nodes, however, inevitably leads to a lower specificity: a considerable proportion of the lymph nodes which are detected by US will be benign. Like in CT and MRI, differentiation between reactive and metastatic nodes is based on morphological criteria (13,28,31). This leads to a relatively low specificity although some authors reported high specificity rates up to 91% with US alone (30).
With the introduction of the concept of Ultrasound Guided Fine Needle Aspiration Biopsy (UGFNAB) (19,20), the high sensitivity of US is combined with the high specificity of cytologic examination (20). Sensitivity and specificity have been reported as high as 98% and 95% respectively. In a subsequent similar study other authors reported an even higher specificity of 100% but at the expense of a lower sensitivity of 90% (13). Critics, however, doubted that these figures could be reproduced if the technique was performed by different sonologists in "daily practice".

In our study, only patients undergoing neck dissection as part of their treatment were included because histological examination was used as the standard of reference. Although this introduces an inevitable bias by increasing the number of cases with metastatic disease, all comparable studies of US/UGFNAB, CT and MRI are subject to this limitation.
The accuracy and sensitivity of US/UGFNAB in our study were not as high as found in previous studies. The specificity, however, was comparable. Compared with other diagnostic imaging techniques, the sensitivity of US/UGFNAB found in our study is in the range of that reported for CT (4,6,9,12,13,15,16) and MRI (5,9). The high specificity of up to 100% found in the present and previous studies (13,20) compares favorably with that of CT and MRI. Most studies concerning CT found a specificity of no more than 70 to 85% (4,9,12,13,15,16). Few studies found higher specificity rates (up to 94%) by using morphologic criteria (28).
In most studies concerning the value of CT and MRI for assessment of the neck, morphologic and/or size criteria are used. By choosing an optimal cut-off point, false-positive and false-negative results will be introduced. As a consequence, in these studies, a higher sensitivity results in a lower specificity and vice versa. For example, Stevens et al. (12), Close et al. (15), Hillsamer et al. (9) and Friedman et al. (11) found sensitivity rates of 97%, 86.5%, 84% and 95% paired to a much lower specificity of and 82%, 71%, 71% and 77% respectively. In contrast, Feinmesser et al. (4) found a relatively low sensitivity of 60% with a higher specificity of 85%.

Although US alone suffers from the same phenomenon, US/UGFNAB does not because US determines the sensitivity and UGFNAB the specificity.
Another factor influencing the results of these studies is the prevalence of metastasis. Studies with a high number of patients having metastasis or advanced stage disease will show higher sensitivity rates for the studied diagnostic techniques. For example, in studies with a relatively high number of clinically or histologically node positive cases higher sensitivity rates for CT were obtained up to 91% (6,12,15) whereas in studies with more node negative cases sensitivity rates for CT were lower, e.g. 60% (4). The only study, to our knowledge, in which the results of US/UGFNAB, CT and MRI were compared in the same study population showed superior results of US/UGFNAB (13).
Another advantage of US/UGFNAB over CT and MRI are the lower costs (in the Netherlands, the costs of CT are about 4 times as high as those of US/UGFNAB). Moreover, for US/UGFNAB patients do not have to lie down (for a prolonged period of time), which is more convenient in these predominantly elderly and/or dyspneic patients. In contrast with CT and especially MRI, US will not be problematic in patients inclined to be claustrofobic. Finally, in our opinion, FNAB is hardly a more invasive or risky procedure than the administration of intravenous contrast material in CT or MRI.

Unfortunately, in 19 cases of nondiagnostic aspirates, UGFNAB was not repeated. Repeating these aspirates, as was requested according to the protocol, would definitely have improved the test results. We cannot, however, prove this with our material because for most cases of nondiagnostic aspirates UGFNAB was not repeated. The rate of nondiagnostic UGFNABs (19 [11%] of 178 aspirates) is in the range found in previous studies (1-15%) (20,32,33).
Our data show that the results of US/UGFNAB are not as investigator dependent as often suggested. No major differences were found between experienced and less experienced sonologists.
Although the differences were not statistically significant, it appeared that the accuracy of US/UGFNAB was determined by the site of the primary tumor. There are two explanations for this finding. Firstly, this may be due to a difference in prevalence (table 3). This influences in particular the negative and positive predictive value. Secondly, different primary tumors metastasize to different neck levels and lymph node metastases in some levels are more difficult to detect by using US/UGFNAB than others. This phenomenon has been described in earlier studies (34,35). In our study, it seemed more difficult to detect lymph node metastases in levels I and V when compared to in levels II, III and IV, although the differences were not statistically significant. Therefore, the favorable sensitivity rates for laryngeal and pharyngeal carcinoma when compared to floor of mouth or oropharyngeal carcinoma, may be due to the fact that the former metastasize less frequently to level I.
The difficulty of detecting nodes in level I by US/UGFNAB may be caused by the mandible. However, nodes missed by US in this level may be detected by palpation: with bimanual palpation, examination of level I is relatively easy to perform. If the results of palpation are added to the results of US/UGFNAB in cancer of the floor of mouth, a primary tumor predominantly metastasizing to level I, sensitivity improves

from 57 to 79% at the expense of a specificity dropping from 100% to 83%. It seems justified, therefore, to use the combination of the results of both methods of examination in clinical practice (Table 3).

CONCLUSION

In this multicenter, prospective study the sensitivity of US/UGFNAB appeared to be slightly lower compared to previous studies but comparable with the sensitivity of CT and MRI. The specificity of US/UGFNAB found in our study is similar to that of previous studies and superior to the specificity of CT and MRI. Repeating UGFNAB for nondiagnostic aspirates may further improve the test-characteristics of US/UGFNAB.
Palpation remains an important tool for assessment of the lymph nodes of the neck. A combination of palpation and US/UGFNAB improves the sensitivity of the diagnostic procedure. In addition, in this study, the often suggested interobserver variability of US and UGFNAB could not be confirmed. The results of this study can be considered as a validation and recommendation of the use of US/UGFNAB for evaluation of the neck in patients with HNSCC.

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CHAPTER 3

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

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ABSTRACT

Purpose
Head and neck oncologists have not reached consensus regarding the role of contemporary imaging techniques in the evaluation of the clinically negative neck in patients with head and neck squamous cell carcinoma (HNSCC). The purpose of the present study was to compare the accuracy of ultrasound (US) with guided fine-needle aspiration biopsy (UGFNAB) and computed tomography (CT) in detecting lymph node metastasis in the clinically negative neck.

Methods and Materials
Sixty-four neck sides of patients with HNSCC were examined preoperatively by US/UGFNAB and CT at one of five participating tertiary care medical centers. The findings were correlated with the results of histopathologic examination of the neck specimen.

Results
UGFNAB was characterized by a sensitivity of 48%, specificity of 100%, and overall accuracy of 79%. Three cases had nondiagnostic aspirations using UGFNAB and were excluded. CT demonstrated a sensitivity of 54%, specificity of 92% and overall accuracy of 77%. UGFNAB detected two additional metastases not visualized on CT whereas CT did not detect any metastases not seen on UGFNAB. The results of UGFNAB were similar between the participating centers.

Conclusions
Approximately one-half of the clinically occult nodal metastases in our patient group were identified by both CT and UGFNAB. Overall, UGFNAB and CT demonstrated comparable accuracy. The sensitivity of CT was slightly better than UGFNAB but the latter remains characterized by a superior specificity.
The results of CT and UGFNAB did not appear to be supplementary. Choice of imaging modality for staging of the clinically negative neck depends on tumor site, T-stage, and the experience and preference of the head and neck oncologist. If CT is required for staging of the primary tumor, additional staging of the neck by UGFNAB does not provide significant additional value.

INTRODUCTION

Head and neck oncologists have not reached consensus regarding the role of contemporary imaging techniques in the evaluation of the clinically negative neck in patients with head and neck squamous cell carcinoma (HNSCC). While some head and neck oncologists frequently employ dissection of the clinically N_0 neck for staging purposes and potential therapeutic benefits,[1-9] others feel that the indications for elective neck dissection should be reconsidered in view of the reliability of contemporary imaging techniques in the assessment of cervical node status.[5-8] Studies which examine the value of imaging techniques in the N_0 neck are sparse and not strictly comparable.[8,9] The majority of reports concerned with imaging of the neck to evaluate nodal status include many patients with palpable nodes but few who are clinically N_0.[7,10-13] In addition, US examinations and CT scans are performed and reviewed by a dedicated radiologist in these studies whereas in clinical practice, US and CT scans are performed by a number of different radiologists as part of daily routine.
The purpose of the present study was to compare the accuracy of UGFNAB and CT in detecting lymph node metastasis in the clinically negative neck if performed in daily practice by sonologists and radiologists at different institutions.

Patients	: 50	
Hemi-necks	: 64	
Sex		
Male	: 40	(80 %)
Female	: 10	(20 %)
Primary tumor site		
Larynx	: 25	
Hypopharynx	: 2	
Oropharynx	: 6	
Oral Cavity	: 17	
Tumor stage		
T1	: 2	
T2	: 9	
T3	: 14	
T4	: 21	
Recurrence	: 3	
Unknown	: 1	

Table 1. Characteristics of the study population.

MATERIALS AND METHODS

This study was performed at the departments of Otolaryngology/Head and Neck Surgery, Radiology and Pathology (Indiana University Medical Center, University Hospital Rotterdam, Daniel den Hoed Cancer Center Rotterdam, University Hospital Nijmegen, University Hospital Leiden) and Maxillofacial Surgery (University Hospital Nijmegen). The study population of the Dutch hospitals was part of a multi-center study on UGFNAB[14] whereas the study population from Indiana University Medical Center represented a part of a single center study on UGFNAB[15] (Table 1). All patients with HNSCC staged N_0 clinically who subsequently underwent elective neck dissection(s) as part of their treatment were eligible for this study. A small number of patients (eight) received prior radiation to the neck and were staged N_0 by palpation in relation to a second primary tumor or local recurrence. Sixty-four neck sides of patients with HNSCC were examined by US/UGFNAB and CT for evaluation of lymph node status. The findings were correlated with the results of histopathologic examination of the neck dissection specimen.

Ultrasound examinations were performed in all participating centers by experienced sonologists who were aware of the primary tumor location and the clinical N_0 neck status of the patient. In centers in the Netherlands, all nodes greater than 5mm diameter depicted on US in high risk nodal drainage areas were considered "suspicious" and had UGFNAB performed. In some of these patients, no neck nodes were visualized on US. When multiple nodes were visualized, UGFNAB of the most suspicious nodes (largest node, nodes round rather than oval, nodes showing central hypoechogeneity, or the most cranial and caudal nodes in the areas at highest risk for metastasis) was done. The procedure was performed as described previously.[7] At Indiana University Medical Center (IUMC), lymph nodes visualized on US were aspirated if they met more than one of the following criteria: high risk nodal area, >7mm diameter, round rather than oval shape, clusters of enlarged nodes >7mm, central hypoechogeneity or loss of the normal fatty hilum. Cytological examination was performed by experienced cytopathologists. Nondiagnostic aspirations had to be repeated. At IUMC, cytological examination was performed on-site in the department of radiology. Nondiagnostic aspiration could be repeated during the same investigation.
The US examinations and UGFNABs were performed with an Aloka 620/650CL (Aloka Co Ltd, Tokyo, Japan), using a 7.5 MHz linear array probe, a Toshiba SSA 250A (Toshiba Europe, Zoetermeer, The Netherlands), using a 7.5 MHz mechanical sector-type probe with a built-in water path, an Acuson 128 XP (Acuson, Mountain View, California), using a 7MHz linear array probe, and an Advanced Technology Lab High Definition Machine (HDI 3000) with 10 MHz linear array probe (Bothell, Washington)(Figure 1).
The test result of UGFNAB in fact consists of the combined results of two separate tests: US and UGFNAB. US may be scored positive (suspicious lymph nodes depicted) or negative (no lymph nodes or no suspicious nodes visualized). Morphological criteria for US were not used in the Netherlands. Only minimum size criteria were employed. At IUMC, size and morphological criteria as outlined above were used to decide whether UGFNAB should be performed. Since lymph nodes in the size range of 4-8 mm were visualized in all patients evaluated at IUMC using the 10MHz HDI 3000 US machine, morphologic criteria were useful in deciding which nodes to aspirate. Cytological examination was positive, negative or nondiagnostic. Cases with nondiagnostic aspirates (not repeated or repeatedly nondiagnostic) were excluded. The Indiana University Medical Center had no nondiagnostic aspirates owing to the different set-up. Therefore, UGFNAB was considered negative if: 1) no nodes were visualized, 2) no suspicious nodes were visualized or 3) nodes appeared to be reactive on cytologic examination. The test was considered positive if the aspirate contained tumor cells (Table 2).

Table 2a.

US	UGFNAB	Hist -	Hist +	total
-	Not performed	27	8	35
+	Not performed	0	0	0
+	-	9	5	14
+	Non diagnostic	2	1	3
+	+	0	12	12
Total		38	26	64

Table 2b.

US/UGFNAB	Hist -	Hist +	total
-	36	13	49
+	0	12	12
Total	36	25	61

Table 2c.

CT	Hist -	Hist +	total
-	35	12	47
+	3	14	17
Total	38	26	64

Table 2. Results of US, UGFNAB and CT in 64 hemi-necks. Table 2a shows the results of US and UGFNAB separately. Table 2b contains the results of the combined procedure. The combination of US + and UGFNAB + is considered positive; no lymph nodes depicted or no suspicious lymph nodes depicted by US, and nodes reactive on cytologic examination are considered negative test results. In Table 2b the nondiagnostic aspirates are excluded. In Table 2c the results of CT are shown. (Hist - = no lymph node metastases in neck dissection specimen, Hist + = lymph node metastases in neck dissection specimen).

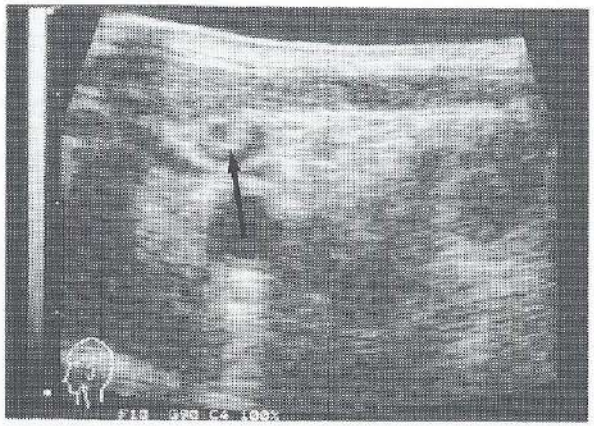

Figure 1. Ultrasound image of a lymph node metastasis during ultrasound-guided fine needle aspiration biopsy. The tip of the needle is depicted as a hyperechogenic spot marked by the arrow. For this image a 10 MHz sector-type probe with a built-in water path was used to obtain an optimal graphical representation.

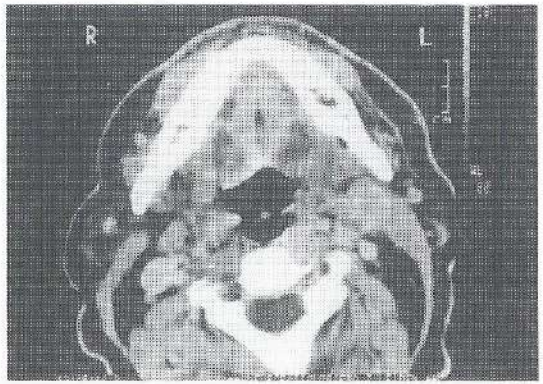

Figure 2. CT image of a clinically occult metastasis in the neck.

CT was performed with the use of IV contrast on an Elscint CT Twin scanner (Haifa, Israel), a Siemens Somaton plus, 4plus or 3 (Erlange, Germany), or a Philips Tomoscan 350 (Eindhoven, The Netherlands). Contiguous slices of 3-5 mm from the base of skull through the thoracic inlet were used. Interpretation of the CT was performed by an experienced radiologist at all participating centers. The radiologist was aware of the primary tumor location and the clinical N_0 status of the neck. The CT was considered positive for suspicious lymph nodes if nodes were identified with the following characteristics: size ≥ 1 cm, round shape, rim enhancement with contrast and central necrosis.[9,13,16-18] (Figure 2). The criterion standard, histopathologic examination of the neck specimen, was considered negative when no metastases were found and positive when one or more metastases were diagnosed.
Since endoscopy and biopsy of nodes under general anesthesia may cause an increase in the number and size of lymph nodes, both CT and ultrasound were

preferably performed prior to endoscopy. Neck dissection had to be performed within three weeks. The specimen was labelled by the surgeon (levels I-V, according to the Memorial Sloan Kettering classification).[19] Subsequently the neck dissection specimen was histologically examined per institutional protocol and the findings of the pathologist were recorded per nodal level. The results of CT and UGFNAB were compared with the results of the histopathological examination of the neck specimen. We considered the results per neck-side since the treatment of metastatic disease is for a neck rather than for a single node: the detection of a single metastasis results in the treatment of the whole neck side. Moreover, it is practically impossible to match a depicted or aspirated node with the same node in the neck dissection specimen.

RESULTS

The results of UGFNAB are shown in detail in Table 2. UGFNAB was characterized by a sensitivity of 48%, specificity of 100% and overall accuracy of 79%. Three cases using UGFNAB had nondiagnostic aspirations and were excluded. CT had a sensitivity of 54%, specificity of 92% and overall accuracy of 77% (Table 3). UGFNAB detected 2 out of 12 still occult metastases in the CT negative cases. CT did not detect any additional metastases in the US negative cases. There was only minor variation in the overall accuracy and sensitivity of UGFNAB between centers. The number of patients per center was too small, however, to permit proper statistical comparison between examiners. The number of nondiagnostic cases ranged from 3 out of 35 cases (9%) in the Dutch Hospitals (not more than 1 nondiagnostic case per Hospital) to 0 out of 29 cases (0%) at IUMC.

	US/UGFNAB	CT
No*	61	64
Sensitivity	48	54
Specificity	100	92
Predictive value +	100	82
Predictive value -	73	74
Accuracy	79	77
Prevalence	41	41
Nondiagnostic	3	0

* Of the UGFNAB examinations 3 of 64 were nondiagnostic. None of the 64 CT studies were nondiagnostic.

Table 3. Results of US/UGFNAB and CT.

DISCUSSION

The role of imaging techniques in the assessment of the clinically negative neck in patients with HNSCC continues to be debated in the literature. In part due to the difficulty of assessing the neck reliably on physical examination, the concept of elective neck treatment was introduced and is still common practice. Evaluation of the cervical lymph nodes for malignancy has improved significantly in recent years through the introduction of modern imaging techniques such as CT, magnetic resonance imaging (MRI), and UGFNAB. Accurate radiologic imaging could potentially allow for a more conservative approach regarding management of the clinically negative neck if the risk of occult metastatic disease could be reduced to less than 20%.[9,20-22]

The accuracy of CT and UGFNAB in evaluating the N_0 neck is difficult to assess from the literature. This may be explained by several factors. Differences in sensitivity and specificity rates between studies may be caused by widely varying criteria for differentiation between benign and metastatic nodes and differences between study populations. Most studies evaluating the role of CT and MRI for assessment of the neck employ morphological and/or size criteria. Selection of an optimal cut-off point for a specific criterion such as size shifts the false-positive and false-negative rates. As a consequence, in these studies, a higher sensitivity results in a lower specificity and vice versa. For example some authors report sensitivity rates of 84-97% paired to a much lower specificity of 71-82%.[10,16,18,23] In contrast others found a relatively low sensitivity of 60% with a higher specificity of 85%.[11] In our study, only minor variations existed between participating centers in terms of the accuracy and sensitivity of UGFNAB and the sensitivity and specificity of CT. This was probably due to the fact that UGFNAB methods and criteria for suspicious nodes were very similar although not exactly alike. Also, CT examinations are less operator-dependent than US and similarity of results between centers is not surprising.

Another important factor influencing the results of radiologic examinations in these studies is the prevalence of cervical metastases in the study group. Inclusion of a high number of patients having metastasis or advanced stage disease will produce higher sensitivity rates for the studied diagnostic techniques. For example, in several studies with a relatively high number of clinically or histologically node positive cases, sensitivity rates for CT were obtained up to 91%[16,18,20] whereas in studies with more node negative cases, sensitivity rates for CT were lower, e.g. 60%.[11] In our study, all evaluated patients were staged clinically N_0 by an experienced head and neck oncologist.

Finally, results may be positively influenced by having the radiologic procedures performed by a confined number of investigators participating in the study.[7,8] Studies performed in a daily practice setting in which several individuals perform radiologic procedures may report considerably lower sensitivity rates.[14] In contrast to many other studies, the present study concerns the results of daily practice. Our results are therefore likely to be reproducible in other centers. Moreover, the direct

comparison of CT and UGFNAB in the same patient population in our study further enhances the reliability of the results.

In the present study, the overall accuracy of CT and UGFNAB in evaluating the N_0 neck in HNSCC was comparable. Our results showed a better sensitivity for CT and a superior specificity for UGFNAB. There seems to be no significant additional value, however, in combining CT and UGFNAB. Performing UGFNAB in the CT negative cases revealed 2 out of 12 (17%) still occult metastases. Although this may be considered of value, it is not a major improvement in results and is not statistically significant. Performance of CT in the US negative cases detected no additional metastases (0/8 cases). These results may be explained by the fact that both techniques require a minimum size threshold for a metastatic deposit before it can be detected. Very small tumor deposits not detected by one modality will likely not be detected by the other modality.[24, 25] If CT is performed for staging of the primary tumor, additional staging of the neck by UGFNAB does not seem to have significant additional value. While not tested, the same is probably true for MRI. In terms of staging of the primary tumor, CT and MRI are useful in evaluating skull base or intracranial involvement, prevertebral muscle invasion, carotid artery encasement and bone or cartilage invasion. Tumors that do not overly or invade bone and may not need a staging CT scan if tumor extent can be accurately assessed clinically and endoscopically. A subset of oral cavity and laryngeal lesions seem most appropriate in this respect. If imaging of the primary tumor by CT is not required, UGFNAB can be employed for evaluation of the neck. Patently, this assumes the availability of an experienced sonologist and cytopathologist who are involved in the care of a significant volume of HNSCC patients. In addition, it should be noted that retropharyngeal lymph nodes are better evaluated by CT or MRI. Tumors which frequently metastasize to this specific region usually require CT or MRI imaging of the primary tumor for staging. In these cases, additional UGFNAB staging of the neck would be unnecessary.

Interestingly, the sensitivity of both CT and UGFNAB in our study is somewhat lower than previously reported and is probably due to the patient population studied. As mentioned, both CT and UGFNAB have a detection threshold and metastases must achieve a minimum size to be detected. Our study population consisted entirely of patients without clinically palpable lymph node metastases. One would expect a higher proportion of patients with very small or microscopic lymph node metastases in our study group compared to studies including patients with palpable disease and therefore the lower sensitivity of UGFNAB and CT in our study is not surprising.

While UGFNAB produced no false positive results, the false negative results of UGFNAB can be divided into two categories (Table 2a): cases with metastases not detected by US and cases showing suspicious lymph nodes on US but with negative UGFNAB. The first group may be explained by the size detection threshold of US or by the location of the lymph nodes. Some nodal levels in the neck are more difficult to examine by US than others[14], e.g. the submandibular region. In the second group, a

sampling error may have occurred due to aspiration of an incorrect lymph node or aspiration of a non-involved part of a metastatic lymph node.

The impact of any imaging modality on the decision to electively treat the neck, depends on the ability to decrease the risk of occult neck disease to less than 20%. If evaluation by UGFNAB or CT decreases the risk of occult neck disease to below 20%, then perhaps elective neck treatment can be avoided. Both modalities detected approximately one-half of clinically occult neck metastases in our study. Therefore, if the risk of occult neck disease for a particular tumor is very high, even a negative UGFNAB or CT cannot drop the risk of occult disease below 20% and elective treatment of the neck should be undertaken. As technologies improve, a higher percentage of clinically occult nodes will likely be detected.

Finally, the differences in nondiagnostic UGFNAB results between the participating centers may be explained by the on-site cytological examination of the US-guided aspiration biopsies employed at IUMC. Onsite cytological examination allows for instant repetition of UGFNAB in the case of a nondiagnostic initial aspiration. Indeed, on-site cytological examinations yielded no nondiagnostic results of UGFNAB at IUMC. This method of FNA analysis may incur a higher cost however. the total number of nondiagnostic UGFNABs (3/64 or 5 %) is in the range found in previous studies (1-15%).[7,14,26,27]

CONCLUSIONS

Approximately one-half of the clinically occult nodes in our patient group were identified by both UGFNAB and CT. Overall, UGFNAB and CT demonstrated comparable accuracy for staging the N_0 neck in HNSCC. The sensitivity of CT was slightly better than UGFNAB but the latter remains characterized by a superior specificity. The results of CT and UGFNAB did not appear to be supplementary. Therefore, it seems most sensible to use either one of the modalities depending on tumor site, T-stage and the experience and preference of the head and neck oncologist. If CT is required for staging of the primary tumor, additional staging of the neck by UGFNAB does not provide significant additional value.

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CHAPTER 4

Expression of Genetic Markers in Lymph Node Metastases compared to their Primary Tumors in Head and Neck Squamous Cell Cancer

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ABSTRACT

Background
Regional metastasis is an important factor in the prognosis and treatment of Head and Neck Squamous Cell Carcinoma (HNSCC). The results of earlier studies suggested the possibility to predict nodal metastasis in HNSCC using biological markers. To identify which factors may be relevant in the metastatic behaviour of these tumors we studied the expression of several markers involved in tumor progression in both nodal metastases and their corresponding primary tumor.

Materials and Methods
Expression of p53, Rb, cyclin D1, myc, bcl-2, EGFR, neu, E-cadherin, Ep-CAM, desmoplakin1 and nm23 was studied in 54 primary tumors and their corresponding metastases of patients with HNSCC.

Results
Expression of most genes involved in tumorigenesis (p53, Rb, cyclin D1, myc, bcl-2, EGFR, neu, E-cadherin and desmoplakin1) was predominantly similar in primary tumors and metastases. The expression of nm23 and Ep-CAM was found to be reduced in metastases compared to their primary tumors.

Conclusion
Whereas most genetic alterations of primary tumors remain unchanged in the metastases, expression of cell adhesion molecule Ep-CAM and nm23 is lower in metastases compared to their primary tumor suggesting relevance in the process of metastasis. This also implies differences in regulation of markers involved in tumorigenesis and the process of metastasis.

INTRODUCTION

Many prognostic factors in Head and Neck Squamous Cell Carcinoma (HNSCC) have been studied and identified but regional metastasis remains one of the most important of these factors [1,2]. Moreover, since lymphatic metastasis is the most common route of spread of HNSCC, a decision whether or not to treat the lymph nodes of the neck has to be made. It is therefore clinically relevant to assess whether a patient has or will develop regional lymph node metastases.

The process of metastasis is very complex and supposedly a late event in tumorigenesis. Cells proliferate, lose contact with neighbouring cells, migrate through the interstitial matrix, invade blood and lymph vessels and grow out again in lymph nodes or distant organs. The metastatic cells, therefore, have to possess several properties to be able to perform all these actions [3]. These properties are based on several factors, including alterations in genes and their products and metastatic behaviour of a tumor will be based on overexpression of metastasis promoting factors and loss of expression of suppressing factors. Moreover, interaction of the tumor cells with surrounding structures and neighbouring cells is important.
Gene products involved in the process of tumorigenesis and metastasis are expected to play a role in the aforementioned processes like proliferation, migration and cell adhesion. Indeed many of such genes have been reported to be genetically altered in human invasive tumors. Some gene products are involved in cell cycle regulation (e.g. p53 and cyclin D1) and are predominantly associated with early steps in carcinogenesis. Others are involved in cell-cell or cell-matrix adhesion (e.g. E-cadherin and Ep-CAM) which are likely to affect invasive growth and metastasis. Altered expression of gene products, often the result of a genetic abnormality (e.g. amplification, loss of heterozygosity), can be evaluated directly by immunohistochemistry. Several studies have shown that changes in the expression of certain proteins are associated with metastastic behaviour and/or survival [4,5].
In a previous study [6] we investigated which histological parameters and expression of several markers using immunohistochemistry correlated with the presence of nodal metastasis. For this purpose, we compared a limited number of patients with laryngeal carcinoma with and without lymph node metastasis. We found that a set of parameters was able to predict metastasis in laryngeal carcinomas: the combination of inflammatory reaction, eosinophilic infiltration, and staining for Rb and Ep-CAM resulted in a high accuracy in assessing nodal metastasis. To evaluate wether the changed expression is relevant for the metastatic phenotype, expression of p53, Rb, cyclin D1, myc, bcl-2, EGFR, neu, E-cadherin, Ep-CAM, desmoplakin1 and nm23 was studied in 54 nodal metastases and their corresponding primary tumor.

MATERIALS AND METHODS

From the files of the department of pathology tissue blocks were retrieved of 54 resection specimens of laryngeal, pharyngeal and oral squamous cell carcinomas, which had been resected in the period of 1990-1995. All patients had undergone a uni- or bilateral cervical lymph node dissection. The primary tumor and corresponding metastatic tumor were studied. The population characteristics (age, sex, site and T stage) are summarized in table 1.

Age	mean:	61.4 years (38-87 years)
Sex	male:	35 (65%)
	female:	19 (35%)
Sites	larynx:	8 (15%)
	pharynx:	24 (44%)
	oral cavity:	22 (41%)
T stage	T1:	8 (10%)
	T2:	20 (26%)
	T3:	21 (27%)
	T4:	28 (36%)

Table 1. Population characteristics of 54 patients with HNSCC.

Protein expression was analysed using immunohistochemistry as previously described [6]. The antibodies used are summarized in Table 2. In brief, 5 µm sections of paraffin embedded tissue were de-waxed in xylol for 15 minutes and rehydrated through alcohol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase. Subsequently the sections were pretreated for antigen retrieval as follows: for p53, Rb, bcl-2, E-cadherin and desmoplakin1 the sections were first boiled in citrate buffer (pH 6.0) for 10 minutes and cooled down for at least 2 hours; for myc and Ep-CAM the sections were pre-treated with trypsin-solution (0,1% trypsin with 0,1% $CaCl_2$) pH 7.4 at 37 °C for 20 minutes. After washing with PBS, the primary antibody was applied for overnight incubation with 1% BSA in PBS. After washing with PBS the sections were incubated with the secondary antibody. For monoclonals, rabbit-anti-mouse IgG (RAM^{HRP}, Dako□P161) was applied for 45 minutes, then the sections were washed in PBS, and finally incubated with the tertiary antibody, swine-anti rabbit IgG ($SWAR^{HRP}$, Dako□P217) for 45 minutes. For polyclonals no tertiary antibody was used. For cyclin D1, E-cadherin and nm23 the avidin-biotin-peroxidase complex (ABC) staining method was applied.
After the final washing with PBS, staining was performed by means of 3-amino-9-ethylcarbazol (AEC) in dimethylformamide with H_2O_2 followed by counterstaining with Mayer-Haematoxylin for 30 seconds.

For each protein, all cases were stained in the same run. As positive control, tumor specimens were used that had shown positive results in former studies. As negative control the sections were processed without the primary antibody. Moreover, non-neoplastic cells in the section served as internal negative control.
Two observers (RPT; JHJMvK) evaluated the staining results. The primary tumors and metastases were scored in different sessions. Differences in scoring were discussed at a multi-headed microscope until agreement was reached. For each antibody the percentage of staining of tumor cells was estimated and cut-off points were made after a general viewing of all the cases. For convenience in reporting and before statistical analysis a dichotomy (positive vs. negative) was made for each antibody as previously described [7]. The cut-off points were made based on the distribution of staining results or analogous to other studies in literature since clear biological criteria are not available. For p53 and Rb the cut-off point was 0-15 % vs. >15%, for cyclin D1 and myc 0-5% vs. >5% and for bcl-2, E-Cadherin, EGFR, neu, Ep-CAM, desmoplakin1 and nm23, cases with no staining were compared with cases showing any staining. Some cases were not evaluable due to an insufficient amount of tumor in the specimens or non-evaluable staining results.

Antibody	Clone	Company	Dilution	Staining type
p53	p53-DO7	Novo-castra®	1:1000	N
Rb	1F8	Novo-castra®	1:100	N
cyclin D1	DSC-6	Novo-castra®	1:10,000	N
myc	9E11	Novo-castra®	1:200	N/C
bcl-2	100	Novo-castra®	1:50	C
EGFR	Ab-4	Oncogene®	1:20	M
neu	3B5	Pathology Leiden	1:20,000	M
E-Cadherin	HECD-1	Zymed®	1:250	M
Ep-CAM	Ab 323/A3	Centocor® Leiden	1:100	M
desmoplakin1	DP2.17	Progen®	1:20	C
nm-23	H1,H2	C.R.B. ®	1:20,000	C

Table 2. Panel of antibodies used
staining type: N=nuclear, M=membranous, C=cytoplasmatic.

Expression in the primary tumor was compared with that in the paired metastasis. Concordant (primary tumor and metastasis both negative or both positive) and discordant paires (one positive and the other negative) could be distinguished. We investigated in the discordant pairs whether one possibility occurred significantly more often than the other. This was evaluated by calculating the 95% confidence interval for the percentage of cases with positive staining results in the metastasis in the group of discordant cases. If this confidence interval does not cover 50%, it is an indication for a significant difference.

RESULTS

Most proteins showed predominantly similar rates of expression in primary tumor material and their metastases (table 3). Only few cases showed increased or decreased expression in the metastases. Moreover, for most proteins the number of cases with a higher expression in the metastases was balanced by a comparable number of cases with lower expression. Exceptions were nm23 and Ep-CAM, although even for these proteins still most cases showed similar expression.

For Ep-CAM 7 cases showed lower expression in the metastases compared to the primary tumor in contrast to no cases with higher expression in the metastases. This number of cases with lower expression in the metastases was significantly higher than with higher expression (0/7 gives 95% confidence interval from 0% to 41%). Positive staining results for Ep-CAM were found in 14/49 (29%) of the primary tumors and in 7/49 (14%) of the metastases (Table 3).

		concordance		discordance			
Primary:	n	-	+	-	+	% with positive	95% CI
Metastasis:		-	+	+	-	metastasis	
p53	47	19	25	3	0	3/3=100	29-100
Rb	47	8	34	3	2	3/5=60	15-95
CyclD1	48	21	20	3	4	3/7=43	10-82
Myc	48	41	1	2	4	2/6=33	4-78
bcl-2	44	29	5	6	4	6/10=60	26-88
EGFR	47	28	7	5	7	5/12=42	25-72
Neu	51	50	0	0	1	0/1=0	-
Ep-CAM	46	35	7	0	7	0/7=0	0-41
E-cadh	44	7	24	7	4	7/11=64	31-89
Desmopl	47	13	19	9	5	9/14=64	35-87
nm23	47	11	14	6	16	6/22=27	11-50

Table 3. Staining results of metastases compared to the primary tumors after dichotomizing the results for the 54 patients of the study population with nodal metastases. n = number of evaluable cases.
For nm23 and Ep-CAM, the combination of positive results in the primary tumor with loss of expression in the metastases ocurred significantly more than the combination of negative results in the primary tumor with expression in the metastases (95% confidence intervals of 11-50% and 0-41% respectively).

Expression of nm23 was seen in 30/47 (64%) of the primary tumors compared to 20/47 (43%) in the metastases group (Table 3). The combination of positive results in the primary tumor with loss of expression in the metastases (n=16) occurred

significantly more often than the combination of negative results in the primary tumor with expression in the metastases (n=6) (95% confidence interval of 11-50%).
Of the other markers the results of p53 were remarkably similar in primary tumor and metastasis. Only 3 cases showed discordant results: 3 primary tumors scored negative had metastases scored positive. Remarkably these 3 cases had very low expression in the primary tumor and a very high expression (>50%) in the metastases. Of the positively staining primary tumors all metastases were positive too (table 3). The fact that expression in the primary tumor and their metastasis is predominantly similar supports the idea that p53 alterations are early events in carcinogenesis.
Although some cases with discrepancies in expression between primary tumor and metastases were due to the fact that the expression was just on the negative or positive side of the cut-off point, others had very pronounced differences in expression. This was seen for all proteins.

DISCUSSION

In this study the expression of nm23 and Ep-CAM was found to be reduced in nodal metastases compared to the primary tumor suggesting a relevant role in the process of lymph node metastasis. A correlation of loss of expression in the primary tumor of these markers and the occurrence of nodal metastases could not be established though.

In our material the expression of nm23 and Ep-CAM in particular was reduced in metastases compared to their primary tumor. This phenomenon is not unexpected since nm23 is supposed to be a metastasis suppressor gene and Ep-CAM a cell adhesion molecule. Tumors or tumor subclones with loss of expression of nm23 therefore will tend to metastasize and loss of cell adhesion will facilitate metastasis as well. The change in expression of these proteins in the metastases compared to the primary tumor also implies differences in regulation of proteins involved in tumorigenesis (like p53 and cyclin D1) and those involved in the process of metastasis (e.g. Ep-CAM, nm23). Once changes in genes involved in tumorigenesis occur they seem to remain unchanged in tumor progression, whereas the expression of proteins involved in metastasis seem to be more dynamically regulated.

Reduced expression of nm-23 was found to be related to the presence of metastasis in several tumors [8-10] and recently this relation has also been described in oral carcinomas [11]. In the few other studies on nm23 in HNSCC, no such relation was found [6,12]. The only other study we found, studying expression of nm23 in primary tumors and their metastases was of Lee et al.. They found reduced expression of nm23 in 10 of 22 laryngeal carcinomas (46%) and in 4 of 5 lymph node metastases (80%) [13]. Although the number of cases of their study is too small for proper statistical analysis, these results are in line with our findings: loss of expression in 17/47 (36%) of the primary tumors and in 27/47 (57%) of the metastases. So, in our study, a trend of lower expression rate of nm23 was found in metastases compared to the primary tumors, suggesting involvement in the process of metastasis. This

finding is in concordance with the putative role of nm23 as a metastasis suppressor molecule.
Ep-CAM has not been studied extensively in HNSCC. In an earlier study we found a near significant relation between the loss of expression of Ep-CAM and the development of nodal metastasis [6]. Increased expression of Ep-CAM appears to result in decreased cadherin-mediated cell-cell adhesion and may lead to segregation of Ep-CAM positive cells from the parental cell population in vitro [14]. This phenomenon may lead to the development of metastases in vivo. In contrast, other in vitro and animal studies of colorectal carcinomas suggest that higher expression would reduce the metastastic potential [15].

The fact that we did not find a clearly reduced expression of E-cadherin in metastases compared to the primary tumors in our study may seem unexpected. A relation of loss of expression of E-cadherin and metastases has been described in several studies as reviewed by Jiang et al. [16] and therefore a reduced expression in nodal metastases compared to the primary tumors could be expected. However most studies concerning HNSCC failed to find a statistically significant relation between loss of expression of E-Cadherin in the primary tumor and nodal metastases [6,17-19]. In our study such a relation was not found either. Most studies, in contrast to ours, involved only few cases of paired primary tumors and metastases [17-20] and in most of them expression in primary tumors and nodal metastases was comparable and no correlation was found between E-cadherin expression and nodal metastasis [17;18;20]. Schipper et al., however, using a different antibody and different techniques, found complete loss of E-cadherin expression in 7/8 nodal metastases but they did not correlate the loss of expression in the primary tumor with the development of lymph node metastases [21], nor did they in a later study [22].
For amplification of the 11q13 region a correlation with nodal metastases in HNSCC has also been described [6;23;24]. Protein expression of the 11q13 genes has not been studied very frequently. We did not find a correlation between the expression of cyclin D1 and nodal metastasis and this is concordant with the results of others [25]. The expression found in primary the tumors was predominantly similar to that of their metastases. This is in concordance with the fact that cyclin D1 is probably an early event in carcinogenesis.

It may be clear from the discussion that the results of several studies are often varying or even contradictory. The discrepancies in results between different studies may be due to a number of factors related to immunohistochemistry. Differences in antibodies and staining techniques may of course affect the results. But in particular differences in scoring categories may influence results significantly. In absence of clear biological criteria, scoring categories are usually chosen rather arbitrarily. Most tumors show heterogeneity when expression of markers or chromosomal abberations are studied. Apparently, chromosomal abberations and protein expression are different in some parts of the tumor compared to others. This is usually thought to be a result of clonal evolution. In tumor progression some cells of a clone may acquire additional or different chromosomal alterations, resulting in a subclone with different properties. Therefore some parts of a tumor may develop properties that lead to more invasive growth and metastases, while other parts do not. In this

scenario, it would also be expected that the metastatic tumor deposits show features or aberrations similar to those of the subclones of the primary tumor that metastasized. As already mentioned earlier in the discussion, if this subclone is a relatively small part of the primary tumor this will not be reflected in the assessment and scoring of the marker in that primary tumor as a whole. However, this subpopulation of tumor cells may still be responsible for the metastasis. This fact makes the choice of (biologically relevant) cut-off points rather arbitrary. It may also hamper good comparison of results between studies. Moreover, the choice of cut-off points can also determine the presence or absence of correlations with clinical parameters. It may be that if a cut-off point is chosen differently the correlation with other parameters changes. More uniformity in methods is therefore necessary to obtain more consistent results and to obtain useable markers for useful clinical correlations.

CONCLUSIONS

The expression of nm23 and Ep-CAM is more often reduced than it is increased in metastases compared to the primary tumors, suggesting a relevant role of these markers in the process of metastases. This observation is in agreement with the hypothesis that downregulation of these genes is a late event in tumorigenesis. However, most markers show comparable rates of expression in primary tumors and their metastases.

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CHAPTER 5

Markers for Assessment of Nodal Metastases in Laryngeal Carcinoma

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ABSTRACT

Background
Regional metastasis is an important factor in the treatment and prognosis of patients with Head and Neck Squamous Cell Carcinoma (HNSCC). Although in recent years, imaging techniques have improved, it is still impossible to detect small metastatic deposits. Metastasis is mainly determined by properties of the primary tumor and its interaction with surrounding structures

Objective
To identify markers predicting the presence of metastases based on the features of the primary tumor.

Design
Correlation of the results of histological, immunohistochemical and molecular biological analysis with clinical and histopathological data.

Materials and Methods
Several histological features and biological markers were examined in 31 laryngeal carcinomas. The following markers markers were selected on their putative role in the process of metastasis and were studied using immunohistochemical and/or Southern blot techniques: proliferating cell nuclear antigen (*PCNA*), *p53*, retinoblastoma tumor-suppressor gen (*Rb*), *myc*, *bcl-2* (inhibitor of apoptosis), epidermal growth factor (*EGF*), *EGF*-receptor (EGFR), *neu*, *nm23* (also known as NME1, putative metastasis suppressor), *desmoplakin*, neuron cell-adhesion molecule (*N-CAM*), epithelial cell-adhesion molecule (*Ep-CAM*), *E-cadherin*, cyclin D1 (*CCND1*) and *EMS1*.

Results
The absence of an inflammatory reaction surrounding the tumor ($p=0.07$) or eosinophilic infiltration ($p=0.16$), positive immunostaining for *Rb* ($p=0.02$), negative immunostaining for *Ep-CAM* ($p=0.13$) and amplification of *CCND1* and *EMS1* ($p=0.05$) correlated with nodal metastasis. The combination of inflammatory reaction, eosinophilic infiltration, and staining for *Rb* and *Ep-CAM* resulted in a superior accuracy in assessing nodal metastasis.

Conclusions
These results indicate that it is possible to predict and exclude lymph node metastasis by studying features of the primary tumor only. When these results are confirmed in a larger series, biological markers may be powerful diagnostic tools with great impact on clinical decision making.

INTRODUCTION

The status of the neck determines treatment and prognosis of patients with head and neck squamous cell carcinoma (HNSCC). The presence of a single cervical lymph node metastasis in the ipsilateral neck decreases the expected survival by approximately 50% (1).
For detection of metastases by palpation or imaging techniques a minimal size of these metastases is required. Therefore, microscopic metastatic deposits will continue to evade recognition, and uncertainty about the true lymph node status of the neck will remain. Even Ultrasound (US) with Ultrasound Guided Fine Needle Aspiration Biopsy (UGFNAB), the most accurate technique to detect lymph node metastases to date, identifies clinically occult metastases with a sensitivity of no more than 76% (2). False-negative rates cause most head and neck oncologists to treat the neck of patients with tumors with a high propensity of metastasis electively: if the chance of metastases is estimated to be higher than 15% for a particular tumor, the neck will be treated even when no metastases can be revealed. Consequently, many patients will receive an unnecessary treatment for their neck with concomitant mortality and morbidity rates (3-5). Moreover the specificity in the range of 70 to 85% (6-8) of techniques such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) is a problem, and patients may receive needless neck treatment based on false-positive findings.
Based on the assumption that metastasis is mainly determined by properties of the primary tumor and its interaction with the surrounding structures, a study was designed to identify marker(s) predicting the presence of metastases based on features of the primary tumor. Metastasis is caused by a series of events and numerous possible factors have been identified (9-12). A number of markers playing putative roles in the different stages of tumor development and/or metastasis were selected. In most studies, a limited panel of markers was investigated; however, we used a wide range of markers (Table 1 and 2), and this allowed us to compare and combine several markers in the same study population. Moreover, we studied a circumscribed population of laryngeal carcinomas with a balanced and realistic number of node-positive and node-negative cases.
If biological markers prove to be reliable diagnostic tools, they may reduce the need for both elective and therapeutic neck treatment (13).

MATERIALS AND METHODS

Thirty-one patients with laryngeal carcinoma were evaluated (Table 3). Material of the primary (or recurrent) tumor was used. However, sufficient frozen material was not available in all cases and therefore DNA analysis was not performed in every case.
Patients in the node positive group had histologically proven lymph node metastases. The others were clinically and/or histologically node-negative and did not develop nodal metastases in the follow-up period (at least 2 years; one patient was unavailable for follow-up and one patient died of intercurrent disease after 4 months). Manifest metastasis would have developed in most patients with occult

metastasis within this period. Some of the patients received prior irradiation therapy (ie, 11 of 21 patients in the node negative group and 3 of 10 patients in the node positive group). Prior irradiation means that the material used was either taken of recurrent tumors after initial irradiation or resection material after the first course of radiotherapy in sandwich therapy. Patients in the node-negative group who were initially irradiated might have had (occult) metastases at presentation. These metastases might have been adequately eliminated with the use of radiotherapy. Thus, in these patients some uncertainty remains about the true lymph node status. However, this is not likely to influence the results significantly since if some of these cases would, in fact, have had (micro) metastases the correlation with metastases could have been influenced positively or negatively by this fact.

Histological findings

All histopathological specimens were reviewed, without clinical data, by two authors (JHJMvK and RPT). The histological differentiation, growth pattern of the tumor margin, degree of inflammatory reaction that surrounded the tumor, and the presence of eosinophilic granulocytes were evaluated.

Histological differentiation was graded as "poorly", "moderately" or "well" differentiated. The growth pattern of the tumor margin was described as "infiltrating", "mixed" or "pushing". The degree of inflammatory reaction and eosinophilic infiltration were graded as "high", "intermediate" or "low".

Immunohistochemical studies

Sections of paraffin embedded tissue were de-waxed in xylene for 15 minutes, rehydrated through alcohol and then immersed in 1% hydrogen peroxidase for twenty minutes at room temperature. For *p53, ki-67, bcl-2, E-Cadherin, Collagen IV, Rb* and *desmoplakin* the sections were first boiled in citrate buffer (pH 6.0) for 25 minutes and cooled down for at least 2 hours. For *myc* and *Ep-CAM* the sections were pretreated with trypsin-solution (0,1% trypsin with 0,1% $CaCl_2$) pH 7.4 at 37 °C for 20 minutes. Then all sections were washed with PBS and normal goat serum was applied. The primary antibody was then applied and incubated overnight with 1% bovine serum albumine (BSA) in PBSS.

The sections were again washed with PBSS before they were incubated with the secondary antibody. For monoclonal antibodies, rabbit-anti-mouse IgG (RAM^{HRP}, Dako®P161, Dako Corp, subsidiary of Dakopatts, Carpinteria, Calif) was applied for 45 minutes, then washed in PBSS, and finally incubated with the tertiary antibody, swine-anti rabbit IgG ($SWAR^{HRP}$, Dako®P217) for 45 minutes. For polyclonal antibodies no tertiary antibody was used. For *neu* the avidin-biotin-peroxidase complex (ABC) staining method was applied.

After the final washing with PBSS, staining was performed by use of 3-amino-9-ethylcarbazol (AEC) in dimethylformamide with hydrogen peroxide, followed by counterstaining with Mayer haematoxylin for 30 seconds. The section was blued in tapwater and mounted with glycerinated gelatine.

The markers were scored according to their characteristic staining pattern (nuclear, cytoplasmatic, membrane) (Table 4). The results were scored on a semi-quantitative scale (+, ± and -). Staining results were considered positive (+) if the majority of tumor cells showed staining (> 50%). If staining was absent or confined to a few cells

(0-5%) the result was considered negative (-). Cases with partial staining were scored ± (5-50%). In the final analysis, partial (±) and negative (-) staining were clustered and set against positive staining (+). This clustering was done since staining of less than 50% will probably result in higher sampling errors in these tumors if the immunohistochemical studies had been performed on the biopsy material of the primary tumor. For *PCNA*, the percentage of positive staining tumor cells was counted.

DNA analysis

Southern blot analysis was performed as described previously (14). The DNA amplification of the following oncogenes was determined: *EMS1* (probe U21C8) and cyclin D1 (*CCND1*, probe U21B31A) both on the chromosome 11q13 area, *myc* (probe exon 2-3), and *EGFR* (probe 64.1)

An oncogene was considered amplified (+) when the intensity of the signal was increased at least 2-fold (in three different digests) relative to both the thyroglobulin (on chromosome 8) and SEA (on chromosome 11) signal, which served as an internal control for DNA loading and for polyploidy as we have described previously (14). Digested DNA from placenta and cell line UMSCC22b were used on each gel as a control for normal and 8 extra copies of the 11q13 region, respectively. In the final analysis, samples that showed no amplification and had a percentage of tumor cells of 20% or less were excluded. A possible amplification in these samples may not be detected because of the intermixture of normal cells.

Statistical methods

Marker outcome was related to nodal status in 2x2 contingency tables. The chi-square test with Yates correction was used. Correlations with a $p<0.05$ were considered statistically significant. However, correlations with a p between 0.05 and 0.20 were considered borderline significant.

To investigate whether a combination of markers was more predictive for the nodal status than a single marker, stepwise linear discriminant analysis was applied, using markers showing (borderline) significant correlation with metastasis. To characterize the parameters and markers evaluated in this study we also calculated sensitivity, specificity, positive and negative predictive values, and accuracy.

RESULTS

Histological parameters, immunohistochemical staining results, and gene amplifications were tested for correlation with the presence or absence of lymph node metastases. The over-all results are summarized in Table 1 and 2.

Histological parameters

A high inflammatory response and eosinophilic infiltration correlated with decreased incidence of lymph node metastasis (p=0.07 and 0.16 respectively). No relation between differentiation and growth pattern and lymph node metastasis was found.

Histological Feature	Nodal status Negative (n=21)	Positive (n=10)	Total (n=31)	p
Differentiation				1.00
-poor or moderately	17	8	25	
-well	4	2	6	
Growth pattern				0.82
-invasive or mixed	15	6	21	
-pushing	6	4	10	
Inflammatory reaction				0.07
-low or intermediate	13	10	23	
-high	8	0	8	
Eosinophilic infiltration				0.16
-low or intermediate	15	10	25	
-high	6	0	6	

Table 1. Relation between histological features and nodal status.

Immunohistochemical parameters

The expression of *Rb* showed a correlation with lymph node metastases. In the node-negative group 5% (1/19) of the tumors showed positive staining whereas 50% (5/10) of the node-positive cases showed positive staining (p=0.02). An example of staining for *Rb* is shown in Figure 1.

The 323/A3 antibody staining for *Ep-CAM* showed an inverse correlation with the presence of lymph node metastases (fig. 1). A negative staining result was related to the presence of metastases (p=0.13). An example of positive staining for *Ep-CAM* is shown in Figure 2.

No correlation was found between *p53, E-cadherin, EGF, nm23, desmoplakin* or *N-CAM* staining and the presence of lymph node metastasis.

Although 10% of the cases showed some positive staining for *myc* and 10% (3/29) showed moderately to strong staining for *bcl-2*, this percentage is too small for proper statistical analysis. Although we did find a faint staining for *EGFR* in some tumors

we did not consider this significant since the intensity and pattern were not characteristic. Staining for the *neu* product was negative in all of our cases.

The percentage of tumor cells staining with *PCNA* ranged from 0 to 90%. No significant correlation between the percentage of tumor cells staining with *PCNA* and the incidence of metastases was found (p=0.71). Neither was a correlation apparent if the cases were divided in two groups: one with greater than 50% positive staining of tumor cells and the other with less than 50% positive staining for *PCNA* (p=1.00).

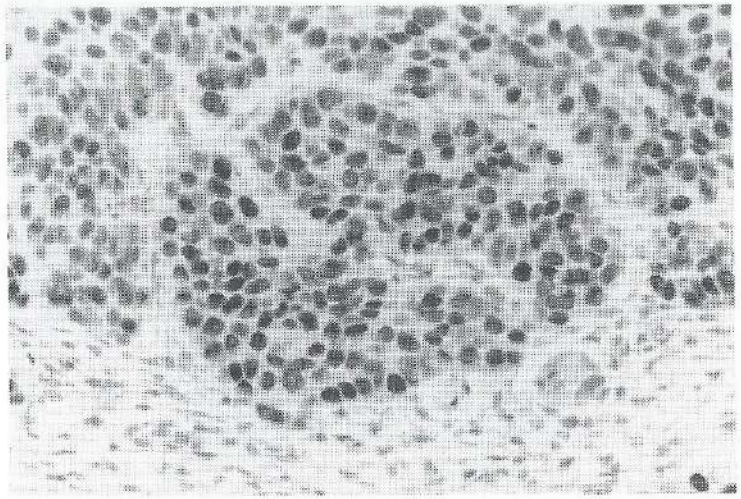

Figure 1. An example of immunostaining for Rb showing a nuclear staining pattern. Original magnification x400.

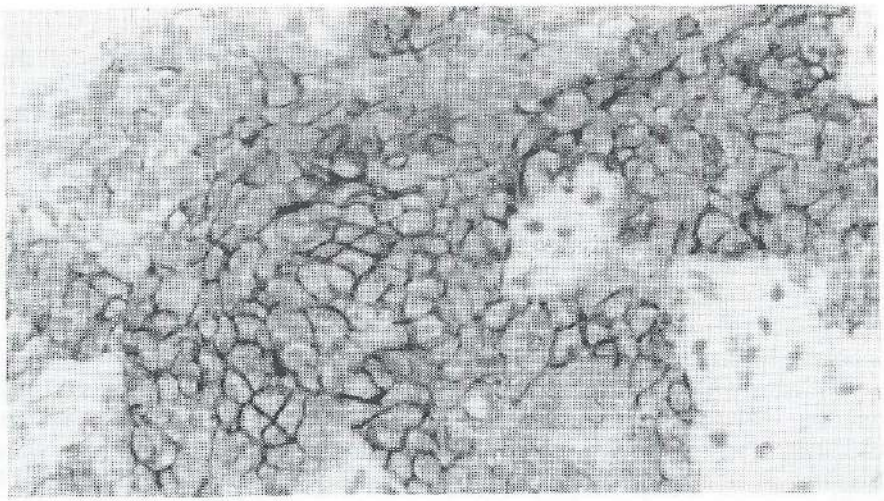

Figure 2. An example of positive immunostaining for Ep-CAM showing a membranous staining pattern. Original magnification x400.

score:	Node-negative Pos	Neg	Node-positive Pos	Neg	p
Immunohistochemical					
P53	3	16	3	7	0.68
Rb	1	18	5	5	0.02
MYC	1	18	0	10	1.00
bcl-2	2	17	0	10	1.00
neu	0	19	0	10	1.00
EGF	3	16	0	10	0.49
EGFR	0	19	0	10	1.00
nm23	5	14	2	8	1.00
N-CAM	8	8	4	4	1.00
Ep-CAM	6	13	0	10	0.13
E-cadh	11	8	7	3	0.52
desmopl	3	16	2	8	1.00
PCNA	12	7	6	4	1.00
DNA					
11q13	1	13	4	3	0.05
EGFR	0	16	1	6	1.00
MYC	2	14	0	7	1.00

Table 2. Relation between immunohistochemical and DNA markers and nodal status. For all markers pos = + and neg = - and ±, except PCNA pos = > 50% positive staining and neg = ≤ 50% positive staining. For CCND1/EMS1 (11q13) pos = amplification and neg = no amplification.

DNA analysis

Amplification of the 11q13 genes *CCDN1* and *EMS1* was found in 24% (5/21) of our specimens. In the node negative group only 7% (1/14) of the tumors showed amplification. In the node positive group 57% (4/7) amplifications were found. This difference was statistically significant (p=0.05).
Amplification of the *EGFR* gene was detected in only one of the 21 evaluable cases. *myc* amplification was seen in 2 of the 21 cases.

In summary, the parameters showing correlation with the presence of lymph node metastasis are: the absence of pronounced inflammatory reaction surrounding the tumor (p=0.07), positive staining for *Rb* (p=0.02), and amplification of *CCND1* and *EMS1* (p=0.05). To a lesser degree, a negative staining for *Ep-CAM* (p=0.13) and eosinophilic infiltration (p=0.16) showed a correlation with metastasis.
To investigate whether a combination of markers/parameters exhibits a stronger correlation with lymph node status than each marker individually, a stepwise logistic regression analysis was performed using only those markers showing (borderline) significant relation with lymph node metastasis (Table 5 and Table 6). The *Rb* expression showed the best correlation. Addition of subsequently inflammatory reaction, staining for *Ep-CAM* and eosinophilic infiltration further improved the correlation. By combining *Rb, inflammatory reaction, Ep-CAM* and

eosinophilic infiltration an accuracy of 86% was achieved with a sensitivity 100% and a specificity of 79% (Table 5). Although significant as single marker, amplification of the 11q13 genes *CCND1* and *EMS 1* was not picked up in this analysis since it apparently did not improve the results.
The exact combinations of staining results of the individual markers that correlate with the presence or absence of metastasis are presented in relation with nodal metastasis in Table 6.

Sex		
male		: 26
female		: 5
T stage		
T1		: 7
T2		: 7
T3		: 7
T4		: 10
Nodal status		
Positive ($N_{1,2,3}$)		: 10
Negative (N_0)		: 21
Prior irradiation		
Yes	-recurrence after initial RT (60/66/70 Gy)	: 11
	-"sandwich therapy" (20 Gy preoperative)	: 3
No	- biopsy material	: 4
	- initial surgery	: 13

Table 3. Patient, treatment and tumor characteristics of 31 patients treated for laryngeal carcinoma.

Antibody	Company	Material	Dilution	Staining type
PCNA	Zymed®	P	1:200	N
p53	Novo-castra ®	P	1:500	N
MYC	Novo-castra ®	P	1:200	N/C
bcl-2	Oxford ®	P	1:50	C
EGF	Oncogene®	P	1:50	M
EGFR	Oncogene®	P	1:25	M
neu	Pathology Leiden	P	1:200	M
nm-23	C.R.B.®	P	1:100	C
desmoplakin I	Progen ®	P	1:10	C
N-cam (SCCL)	Organon Technica®	F	1:100	M
Ep-CAM	Centocor® Leiden	P	1:100	M
E-Cadherin	Zymed®	P	1:1000	M

Table 4. Panel of antibodies used
material: F= frozen, P=paraffin
staining type: N=nuclear, M=membranous, C=cytoplasmatic.

A. Inflammatory reaction/Rb/Ep-CAM

	N -	N +	total
Combination -	13	0	13
Combination +	6	10	16
Total	19	10	29

accuracy 79%

B. Inflammatory reaction/Rb/Ep-CAM/eosinophilic infiltration

	N -	N +	total
Combination -	15	0	15
Combination +	4	10	14
Total	19	10	29

accuracy 86%

Table 5. Multivariate analysis (step-wise linear discriminant analysis). The combination of 3 markers (inflammatory reaction combined with Rb and Ep-CAM) has an accuracy of 79 % (23/29) in predicting lymph node metastasis (A). The addition of eosinophilic infiltration further improves the result with the accuracy rising to 86%, sensitivity 100% (10/10) and specificity 79% (15/19) (B). Accuracy is defined as the number of true positive and true negative results divided by the total number of cases. Data are given as the number of cases.

A. Inflammatory reaction (IR)/Rb

	IR- Rb+	IR- Rb-	IR+ Rb+	IR+ Rb-	total
N -	0	11	1	7	19
N +	5	5	0	0	10
Total	5	16	1	7	29

Chi square p=0.003

B. Inflammatory reaction (IR)/Rb/Ep-CAM (EpC)

	IR- Rb+ EpC-	IR- Rb+ EpC+	IR- Rb- EpC-	IR- Rb- EpC+	IR+ Rb+ EpC-	IR+ Rb+ EpC+	IR+ Rb- EpC-	IR+ Rb- EpC+	total
N -	0	0	6	5	0	1	7	0	19
N +	5	0	5	0	0	0	0	0	10
Total	5	0	11	5	0	1	7	0	29

Chi square p=0.002

Table 6. Relation with the presence of lymph node metastases using a combination of 2 markers: inflammatory reaction combined with Rb (A) and a combination of 3 markers: inflammatory reaction combined with Rb and Ep-CAM (B).
With a combination of inflammatory reaction and Rb, a clear correlation with metastasis is found (p=0.003 by chi-squared analysis). In case of an inflammatory reaction all cases are node negative irrespective of the staining results for Rb. In case of absence of an inflammatory reaction and positive staining for Rb all cases are node-positive. Only in the case of an absent inflammatory reaction and negative staining for Rb was the predictive value less (A). The addition of Ep-CAM results in a further improvement of the correlation (p=0.002 by chi-squared analysis) (B). Data are given as the number of cases

DISCUSSION

Regional metastasis is of paramount importance in patients with HNSCC. Current imaging techniques for assessment of these nodal metastasis, however, are deficient due to relatively high false negative and false positive rates. These techniques are limited since they are intended to detect the late, macroscopic result of a series of biologic events on a cellular and molecular level. It is likely that the result of these events (i.e. metastasis) may be assessed more accurately by studying these events in the primary tumor itself. In other words, instead of trying to detect the clinical result of the genetic aberrations, the genetic aberrations and altered protein expression themselves are studied. This may enable us to predict (or "detect") metastases

irrespective of their size, or to exclude metastasis based on features of the primary tumor. These features can be studied on biopsy material of the primary tumor which is relatively easily accessible in most HNSCCs.

Several relevant prognostic markers in HNSCC have been described (see the reviews of Clark (10), Issing (11), and others). However, they have not found a place in routine diagnostic strategies to date. To our opinion this is due to a number of factors.
First, histological markers (eg, the tumor associated inflammatory reaction, eosinophilic infiltration, grade of differentiation and growth pattern of the tumor) are supposed to be unreliable due to large inter- and intra-observer variability. However, when well-defined criteria are used, this problem can be minimized. In our study, correlation between the presence of an inflammatory reaction and the absence of lymph node metastasis was established. This is consistent with the results of other studies (15,16). In some studies a relation of eosinophilic infiltration surrounding the tumor and favourable prognosis has been described but a relation with lymph node metastasis was not found frequently (17-20). Others did not find any significant clinicopathological correlations (21). We found a relation between eosinophilic infiltration and the absence of metastasis, although our findings were not statistically significant (p=0.16).
A relation between lymph node metastasis and grade of differentiation (15,22,23) or growth pattern (15,24,25) has been described by some authors but could not be confirmed in our study.

Another factor that prevents widespread use of biological markers to predict tumor behaviour may lie in the fact that certain techniques, and more particular molecular biological techniques, are expensive, time-consuming, and, therefore, less suitable for daily clinical practice. However, certain chromosomal aberrations have been shown to have prognostic value or to have a correlation with metastasis and may therefore be clinically useful markers. Relevant markers in this respect are amplification and overexpression of the 11q13 genes *EMS1* and *CCND1*. The amplification of the 11q13 region has been described to be involved in a variety of human tumors. Amplification of this chromosome region is especially seen in a significant portion of breast cancers (26), squamous cell carcinoma of the oesophagus (27-29) and HNSCC (30-34). It appears to correlate with several clinicopathological parameters including lymph node metastasis (as reviewed by Schuuring (35)). Indications of a relation with stage and prognosis were found in squamous cell carcinoma of the upper aerodigestive tract in relatively small series by some authors (27-30,33) although others failed to detect such relations (31). Recently, however, a correlation of 11q13 amplification with the presence of lymph node metastasis was found in a series of 178 HNSCCs by Muller et al. (34) and in a series of 46 patients with HNSCC by Jares et al. (32). In our study, this relationship of 11q13 amplification with metastasis was confirmed. The amplification of 11q13 genes seems promising for assessment of metastasis, in particular if it would be possible to study these markers by immunohistochemistry. Recently, antibodies directed against the products of the relevant 11q13 genes, *EMS1* and *CCND1*, have been developed, allowing study of these markers by immunohistochemical stainings.

In contrast with molecular biological techniques, immunohistochemical stainings are relatively easy to perform and already play a significant role in daily practice of (differential) diagnostic histopathological procedures. However for reliable results, experience with newly developed antibodies is required: staining techniques have to be optimized and the specific staining patterns have to be recognized. Thus, there probably is a third factor with regard to why (immunohistochemical) markers are not used on a large scale yet. It may be expected that with growing experience, the reliability of staining techniques and their interpretation will increase, resulting in less variability of results.
Of the markers investigated using immunohistochemical stains in our study, *Rb* was the single nuclear factor which showed a positive correlation with nodal metastasis. Of the others (ie, *PCNA*, *p53*, and *MYC*) no correlation with metastasis was found. *Rb* is a tumor suppressor gene and loss of the functioning protein may play a role in the progression of malignant tumors. Indeed, mutations of *Rb* were described to have prognostic value in some tumors (36-38). The role of *Rb* in HNSCC has not been studied extensively, making it difficult to compare our findings with those of others. The finding that expression of *Rb* rather than loss of expression correlated with the presence of metastasis may seem unexpected. However, the same correlation has been described for breast cancer (39). Moreover the exact function of *Rb* and its interaction with other factors is not completely known yet, and its supressor effect in growth control does not necessarily mean a direct negative effect on the process of metastasis.
Concerning the growth factor receptors: clear positive staining for *EGFR*, or the related *neu* protein, was not found. According to other reports a relation with metastasis (40-43) is not to be expected.
Of the cell adhesion molecules studied, *Ep-CAM* showed a relation with lymph node metastasis. The cell-cell interaction is an important phenomenon in retaining integrity of epithelia. In carcinomas, the cell-cell interaction is often disturbed, leading to uncontrolled growth and metastasis. The monoclonal antibody 323/A3 recognizes a 40-kD surface antigen (44). Recently, it was demonstrated that this surface antigen is an epithelium-specific intercellular adhesion molecule. Reflecting the function of the molecule it was named *Ep-CAM* (45,46). To our knowledge, this is the first study that ha investigated the *Ep-CAM* expression in HNSCC. In our material the expression of *Ep-CAM* showed an inverse correlation with the presence of lymph node metastases.
It has been demonstrated that down-regulation of the *E-cadherin* gene is associated with poor differentiation, invasion and metastasis in several tumors including HNSCC (47-49) (reviewed by Birchmeier and collegues (50,51) and Takeichi (52)). In our study, however, *E-cadherin* did not prove to be relevant in predicting lymph node metastasis. Similarly, *desmoplakin* and *N-CAM* did not show a correlation with metastasis either.
Of the other markers that were studied, reduced expression of *nm-23* was found by others to be related to metastasis in several tumors (53-55), but not in HNSCC to our knowledge (56). We did not find a relation either.

Another reason why the use of markers has not found its place in the diagnostic work-up of lymph node metastasis may be the fact that most studies focus on single

markers only. These individual markers, however, did not show a relation with metastasis strong enough to be useful in clinical practice as a tool to predict or exclude metastasis. Since metastasis is a multistep process it is not very likely that a single marker will be able to predict metastatic behaviour. Therefore, it is sensible to study and combine several markers that all play (putative) roles in this process. Indeed through multivariate analysis we found that the combination of relevant markers in our study resulted in a high correlation with and predictive value for metastasis.

Finally, the exact role in the process of tumor progression and metastasis of many histological features or markers (including the markers used in this study) is not completely known. The correlation with certain clinical features may indicate which markers are the most relevant for clinical practice and warrant more (fundamental) investigation.
It seems that if some of the above mentioned reservations can be overcome, it may be possible in the near future to actually use markers in clinical practice. It may be possible to evaluate the nodal status of patients more reliably by studying the biopsy material of the primary tumor and using the relatively convenient and cost-effective immunohistochemical stainings.

CONCLUSIONS

In this study of patients with laryngeal carcinoma, absence of inflammatory reaction and absence of eosinophilic infiltration surrounding the tumor, the expression of *Rb* and the amplification of the chromosome 11q13 genes *CCND1* and *EMS1* showed a relation with the presence of metastasis. The expression of *Ep-CAM* showed an inverse relation with nodal metastasis. The combination of the individual results resulted in an even stronger correlation reflecting the several mechanisms underlying the process of tumor progression and metastasis.
The use of immunohistochemical satining is an easy, quick and cost-effective way to study the markers used in this study.
If these results can be reproduced in larger series, the use of a combination some of the investigated parameters could prove to be a powerful tool to predict or exclude lymph node metastases in individual patients with HNSCC, allowing more effective treatment strategies.

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CHAPTER 6

Expression of oncogenes, cell adhesion molecules and histological parameters as markers for nodal metastasis in Head and Neck Squamous Cell Cancer

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ABSTRACT

Background
To identify markers relevant as predictors of lymph node metastasis in Head and Neck Squamous Cell Cancer (HNSCC).

Methods
Expression of p53, Rb, cyclin D1, E-cadherin and Ep-CAM and traditional histological parameters like differentiation grade, growth pattern, tumor associated eosinophilic infiltration and inflammatory reaction, were studied in 121 primary tumors of patients with HNSCC. The correlation of these markers with the histologically verified presence of regional metastases was studied.

Results
Loss of expression of Rb (p=0.04) and marginally of E-cadherin (p=0.06 ns) correlated with the presence of lymph node metastasis. If the results are broken down to subsites, loss of E-cadherin expression in oral cancer (p=0.04) and absence of eosinophilic infiltration in laryngeal cancer (p=0.003) correlated with nodal metastasis. None of the other markers did. A combination of relevant parameters did not result in a much stronger correlation.

Conclusion
The expression of the investigated genetic markers and histopathological features of primary tumors can supply limited information on the metastatic behaviour of tumors.
Although the use of markers for regional metastasis would be a welcome additional tool, these results do not yet warrant the use of these parameters for clinical decision making concerning the treatment of the neck in patients with HNSCC.

INTRODUCTION

Regional metastasis is an important factor in the prognosis and choice of treatment of patients with Head and Neck Squamous cell Cancer (HNSCC). The presence of nodal metastasis will significantly affect the survival of the patient (1). Moreover, in most patients with HNSCC a decision whether or not to treat the lymph nodes of the neck has to be made. Diagnostic means to assess the lymph node status of the neck are not very reliable. Due to high false-negative rates, many patients with HNSCC will undergo elective neck treatment. For a considerable number of these patients this means an unnecessary treatment for their neck with significant morbidity (2-4).

The techniques to assess the nodal status of the neck have improved in recent years. However, even Ultrasound (US) with Ultrasound Guided Fine Needle Aspiration Biopsy (UGFNAB), the most accurate technique to detect lymph node metastases to date, identifies clinically occult metastases with a sensitivity of no more than 48 to 76% (5;6). Moreover, due to the limited specificity in the range of 70 to 85% (7-9) of techniques like Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) patients may receive unnecessary neck treatment based on false-positive findings. The limitation of all these techniques is that by palpation or imaging techniques small metastatic deposits will still be undetected, and uncertainty about the true lymph node status of the neck will remain.

The process of metastasis is a (late) result of changes in properties of cells and of interaction between tumor cells and surrounding cells and structures. To metastasize, cells proliferate, lose contact with neighbouring cells, migrate through the interstitial matrix, invade blood and lymph vessels and grow out again in lymph nodes or distant organs. The metastatic cells, therefore, have to possess several properties to perform all these actions (10). These properties of tumor cells will be based on changes in genes and their products. Based on the assumption that metastasis is mainly determined by properties of the primary tumor and its interaction with the surrounding structures (10) it is worthwhile to explore the possibility of predicting the presence of metastases based on features of the primary tumor. In that case it would be possible to obtain additional information on the chance on metastasis, irrespective of the size of the metastases, by studying features of the primary tumors themselves. In an earlier pilot-study it appeared to be feasible to assess the chance on metastasis in laryngeal cancers using a panel of relevant factors (11). If biological markers prove to be reliable diagnostic tools, they may reduce the need for both elective and therapeutic neck treatment (12). In contrast to most other studies we investigated both multiple traditional histological features as well as several markers. The expression of p53, Rb, cyclin D1, E-cadherin and Ep-CAM and histological parameters (differentiation grade, growth pattern, tumor associated eosinophilic infiltration and inflammatory reaction) were studied in 121 primary tumors of patients with HNSCC. The correlation between the expression of the markers and histological parameters and the presence of regional metastases and survival was studied.

MATERIALS AND METHODS

Expression of p53, Rb, cyclin D1, E-cadherin and Ep-CAM and histological parameters (differentiation grade, growth pattern, tumor associated eosinophilic infiltration and inflammatory reaction), were studied in 121 primary tumors of patients with HNSCC. All patients were previously untreated. The correlation of these markers and parameters with the histologically verified presence of regional metastases was studied.

From the files of the departments of pathology 121 tissue blocks were retrieved from resection specimens of laryngeal, pharyngeal and oral carcinomas, which were resected en bloc with the regional lymph nodes in the period of 1990-1995. The patients had been enrolled in a multicenter study on the value of US/UGFNAB in the assessment of the nodal status of the neck in patients with HNSCC (13). The population characteristics (age, sex, site, T, N and pN stage) are summarized in Table 1. The proteins were analysed using immunohistochemistry as previously described (11). The antibodies used are summarized in Table 2.

Age	mean:	59 years (34-85 years)
Sex	male:	85 (70%)
	female:	36 (30%)
Sites	larynx:	36 (30%)
	pharynx:	32 (26%)
	oral cavity:	53 (44%)
T	T1:	13 (11%)
	T2:	31 (26%)
	T3:	31 (26%)
	T4:	46 (38%)
N	N0	60 (50%)
	N+	61 (50%)
pN	pN0:	31 (26%)
	pN+:	90 (74%)

Table 1. Population characteristics of 121 patients with HNSCC.

Most proteins (except cyclin D1 and E-cadherin) were studied using the three step indirect method. In brief, 5 µm sections of paraffin embedded tissue were de-waxed in xylol for 15 minutes and rehydrated through alcohol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase. Subsequently the sections were pretreated for antigen retrieval as follows: for p53, Rb and E-cadherin the sections were first boiled in citrate buffer (pH 6.0) for 10 minutes and cooled down for at least 2 hours;

for Ep-CAM the sections were pre-treated with trypsin-solution (0,1% trypsin with 0,1% $CaCl_2$) pH 7.4 at 37 °C for 20 minutes. After washing with PBS, the primary antibody was applied for overnight incubation with 1% BSA in PBS. After washing with PBS the sections were incubated with the secondary antibody. For monoclonals, rabbit-anti-mouse IgG (RAM^{HRP}, Dako®P161) was applied for 45 minutes, then washed in PBS, and finally incubated with the tertiary antibody, swine-anti rabbit IgG ($SWAR^{HRP}$, Dako®P217) for 45 minutes. For polyclonals no tertiary antibody was used. For cyclin D1 and E-cadherin the avidin-biotin-peroxidase complex (ABC) staining method was applied.
After the final washing with PBS, staining was performed by means of 3-amino-9-ethylcarbazol (AEC) in dimethylformamide with H_2O_2 followed by counterstaining with Mayer-Haematoxylin for 30 seconds. The sections were blued in tapwater and mounted with glycerine gelatine.
All cases were stained simultaneously for each protein with appropriate specimens as positive and negative control. As positive control, tumor specimens were used that showed positive results in former studies. As negative control the sections were processed without the primary antibody. Moreover, non-neoplastic cells in the section served as internal negative control.

Antibody	Clone	Company	Dilution	Staining type
p53	p53-DO7	Novo-castra®	1:1000	N
Rb	1F8	Novo-castra®	1:100	N
Cyclin D1	DSC-6	Novo-castra®	1:10,000	N
E-Cadherin	HECD-1	Zymed®	1:250	M
Ep-CAM Ab	323/A3	Centocor® Leiden	1:100	M

Table 2. Panel of antibodies used
staining type: N=nuclear, M=membranous, C=cytoplasmatic.

Two observers (RPT; JHJMvK) evaluated the staining results. Differences in scoring were discussed at a multi-headed microscope until agreement was reached. For each antibody scoring categories were made. For convenience in reporting and before statistical analysis a dichotomy (positive vs. negative) was made as previously described (14). The cut-off points were made based on the distribution of staining results in the different scoring categories. For p53 and Rb the cut-off point was 0-15 % vs. >15 %, for cyclin D1 0-5% vs. >5% and for E-Cadherin and Ep-CAM cases with no staining were compared with cases showing any staining. Some cases were not evaluable due to the absence of sufficient tumor in the specimens or non-evaluable staining results. Traditional histological parameters were scored as described before (11).

The correlation of the investigated parameters with the histologically verified presence of lymph node metastasis was tested using the chi-squared test or Fisher's Exact test. For all analysis $p<0.05$ was considered significant.

RESULTS

The markers and histological parameters were studied in 121 cases of HNSCC and related to the development of nodal metastasis. The expression rates of the investigated markers are summarized in Table 3.

	pN0		pN+		
Score:	Pos/n	%	Pos/n	%	p value
Differentiation	5/31	16	17/90	19	1.00
Growth pattern	6/31	19	15/85	18	0.79
Inflammatory reaction	15/30	50	29/85	34	0.13
Eosinophilic infiltration	7/30	23	14/85	16	0.42
P53 (>15%)	16/30	53	45/87	52	1.00
Rb (>15%)	25/30	83	54/86	63	**0.04**
Cyclin D1 (>5%)	18/30	60	45/87	52	0.52
E-cadherin (>0%)	27/29	93	68/89	76	**0.06**
Ep-CAM (>0%)	10/30	33	33/86	38	0.67

Table 3.Relation of investigated parameters with lymph node metastasis (Pearson chi square test). n = number of investigated cases.
Differentiation: poorly/moderately = neg, well = pos; growth pattern: infiltrating = neg, bold = pos; inflammatory reaction: low/intermediate = neg, high = pos; eosinophilic infiltration: low/intermediate = neg, high = pos. The cut-off points for considering the staining result positive for each marker is noted between brackets.

A correlation between loss of expression of Rb ($p=0.04$) and marginally of E-cadherin ($p=0.06$) with lymph node metastases was found. None of the other markers or histological features showed a correlation with nodal metastasis (Table 3). T-stage ($p=0.26$, data not shown) and T1 and T2 vs. T3 and T4 ($p=0.83$, data not shown) did not correlate with metastasis either.

To see if the choice of cutt-off points in the scoring categories would influence the results, several cut-off points for considering results positive or negative were studied for each marker. Most of the cut-off points used in an earlier study (14) also resulted in the most significant correlations (15 % for Rb and 0% vs >0% for E-cadherin). For expression of p53 and cyclin D1 no correlation with lymph node

metastasis was found using any of the alternative cut-off points. For p53 the best alternative cut-off point of 50% did not result in a significant correlation ($p=0.67$) and neither did the cut-off point of 75% for cyclin D1 ($p=0.48$). For Ep-CAM only 2 scoring categories were made so no alternative cut-off points could be explored.

	pN0		pN+		
score:	Pos/n	%	Pos/n	%	p value
Larynx (n=36)					
Differentiation	0/6	0	4/30	13	1.00
Growth pattern	3/6	50	4/28	14	0.09
Inflammatory reaction	3/6	50	10/28	36	0.65
Eosinophilic infiltration	3/6	50	0/28	0	**0.003**
P53 (>15%)	3/6	50	12/29	41	1.00
Rb (>15%)	5/6	83	15/28	54	0.36
Cyclin D1 (>5%)	3/6	50	11/29	38	0.66
E-cadherin (>0%)	4/6	67	21/30	70	1.00
Ep-CAM (>0%)	2/6	33	12/27	44	1.00
Pharynx (n=32)					
Differentiation	1/6	17	4/26	15	1.00
Growth pattern	0/6	0	7/25	28	0.29
Inflammatory reaction	4/5	80	8/25	32	0.13
Eosinophilic infiltration	0/5	0	6/25	24	0.55
P53 (>15%)	5/5	100	14/25	56	0.13
Rb (>15%)	3/5	60	16/25	64	1.00
Cyclin D1 (>5%)	3/5	60	16/25	64	1.00
E-cadherin (>0%)	5/5	100	22/26	85	1.00
Ep-CAM (>0%)	2/5	40	10/25	40	1.00
Oral cavity (n=53)					
Differentiation	4/19	21	9/34	26	0.75
Growth pattern	3/19	16	4/32	13	1.00
Inflammatory reaction	8/19	42	11/32	34	0.77
Eosinophilic infiltration	4/19	21	8/32	25	1.00
P53 (>15%)	8/19	42	19/33	58	0.39
Rb (>15%)	17/19	89	23/33	70	0.17
Cyclin D1 (>5%)	12/19	63	18/33	55	0.58
E-cadherin (>0%)	18/18	100	25/33	76	**0.04**
Ep-CAM (>0%)	6/19	32	11/34	32	1.00

Table 4. Relation of investigated parameters with lymph node metastasis for each of the subsites of the head and neck: larynx, pharynx and oral cavity (Fisher's Exact Test). n = number of investigated cases.
Differentiation: poorly/moderately = neg, well = pos; growth pattern: infiltrating = neg, bold = pos; inflammatory reaction: low/intermediate = neg, high = pos; eosinophilic infiltration: low/intermediate = neg, high = pos. The cut-off points for considering the staining result positive for each marker is noted between brackets.

To investigate if a combination of parameters would be more informative, the results of the markers that showed some relation with nodal metastasis were combined. The combination of Rb and E-cadherin did not result in a better correlation with nodal metastasis (p=0.15, data not shown) and the addition of inflammatory reaction did not either (p=0.37, data not shown).

If the correlation of the investigated parameters with nodal metastasis is studied for the 3 subsites of the head and neck (larynx, pharynx, oral cavity) the results are different compared to the entire population (Table 4). For the larynx absence of eosinophilic infiltration correlated with the presence of nodal metastasis (p=0.003). For pharyngeal cancers no significant correlations were found and for oral cancers E-cadherin expression correlated with nodal metastasis (p=0.04). All tumors with loss of expression of E-cadherin had lymph node metastases.

Markers for assessment of nodal metastasis are particularly useful in the N0 neck. In Table 5 the results are summarized of the analysis performed in the subpopulation of 60 patients with no palpable masses in the neck. In this population marginally loss of expression of Rb (p=0.06 ns) correlated with the presence of nodal metastases. No relation between the other investigated parameters and nodal metastasis was found. In the subpopulation of patients with no detectable metastasis on examination with UGFNAB (n=49), no correlations between marker expression and the presence of nodal metastasis could be established (Table 6).

	pN0		pN+		
Score:	Pos/n	%	Pos/n	%	p value
Differentiation	5/27	19	7/33	21	1.00
Growth pattern	6/27	22	5/31	16	0.74
Inflammatory reaction	14/26	54	13/31	42	0.43
Eosinophilic infiltration	7/26	27	10/31	32	0.77
P53 (>15%)	14/26	54	14/33	42	0.44
Rb (>15%)	23/26	88	21/32	66	0.06
Cyclin D1 (>5%)	17/26	65	22/33	67	1.00
E-cadherin (>0%)	23/25	92	28/33	85	0.69
Ep-CAM (>0%)	9/26	35	12/32	38	1.00

Table 5. Relation of investigated parameters with lymph node metastasis in the clinically N0 group (Fisher's Exact Test). n = number of investigated cases.
Differentiation: poorly/moderately = neg, well = pos; growth pattern: infiltrating = neg, bold = pos; inflammatory reaction: low/intermediate = neg, high = pos; eosinophilic infiltration: low/intermediate = neg, high = pos. The cut-off points for considering the staining result positive for each marker is noted between brackets.

	pN0		pN+		
Score:	Pos/n	%	Pos/n	%	p value
Differentiation	4/26	15	6/23	26	0.48
Growth pattern	6/26	23	4/21	19	1.00
Inflammatory reaction	13/25	52	10/21	48	1.00
Eosinophilic infiltration	7/25	28	9/21	43	0.36
P53 (>15%)	14/25	56	10/22	45	0.56
Rb (>15%)	22/25	88	16/22	73	0.27
Cyclin D1 (>5%)	15/25	60	13/22	59	1.00
E-cadherin (>0%)	22/24	92	18/22	82	0.40
Ep-CAM (>0%)	8/25	32	7/23	30	1.00

Table 6. Relation of investigated parameters with lymph node metastasis in the group with no detectable lymph node metastases using Ultrasound Guided Fine Needle Aspiration Biopsy UGFNAB (Fisher's Exact Test). n = number of investigated cases.
Differentiation: poorly/moderately = neg, well = pos; growth pattern: infiltrating = neg, bold = pos; inflammatory reaction: low/intermediate = neg, high = pos; eosinophilic infiltration: low/intermediate = neg, high = pos. The cut-off points for considering the staining result positive for each marker is noted between brackets.

DISCUSSION

In recent years an increasing number of studies focus on finding parameters to assess the lymph node status of the neck in patients with HNSCC. Diagnostic imaging techniques improve continuously but have the fundamental limitation that the metastases need to have a minimal size of at least several milimeters to be detected. Moreover they suffer of a low specificity if the imaging is not combined with fine needle aspiration biopsy (FNAB) (7-9). More recently, histological features and changes in gene expression of tumors are explored to find predictors of nodal metastasis. In earlier studies the relation of individual markers with metastasis has only been studied in the context of detecting clinicopathological correlations in general. Now, studies are performed focussing on the issue of the management of the N0 neck. The feasibility is explored of assessing the chance on metastasis more reliably using a set of tumor related parameters (11;15).

In this study we found a correlation with nodal metastasis for the loss of expression of Rb and, nearly significant, E-cadherin. Combining the results of these two markers did not result in a better correlation with metastasis. If the correlation of the investigated parameters with nodal metastasis is studied for the 3 subsites of the head and neck (larynx, pharynx, oral cavity) the results are different compared to the entire population. For the larynx and pharynx this may be due to the low number of cases without metastasis since in this group the number of elective neck dissections

was low. Moreover, the number of cases per group is lower resulting in less statistical significant relations. The difference in results between the subsites may also underline the possible difference in intrinsic biological properties between tumors arising in the several subsites of the head and neck (14). The results in the clinically N0 group do not essentially differ from those of the entire group, except for E-cadherin. Probably due to the lower number of cases, statistical significance is not obtained for all parameters showing a correlation with metastasis in the entire group.

Loss of Rb correlated positively with the presence of nodal metastasis. In an earlier study of laryngeal carcinomas, however, we found an inverse correlation (11). We do not have a good explanation for this difference. It may due to the smaller number of cases in our previous study or a difference in population characteristics (e.g. some of the patients in the earlier study received prior radiotherapy and in the current study more higher stage tumors were included). The expression of Rb has not frequently been studied in HNSCC (16;17). A relation with the nodal status has been described in one of these studies (17).

The other marker that correlated with the presence of nodal metastasis in our study was E-cadherin. For the entire population, this relation was nearly significant but in oral cancers, all tumors with loss of expression of E-cadherin had nodal metastases ($p=0.04$). E-cadherin is an important molecule in cell-cell adhesion and changes in its expression may, together with other factors, play a role in the process of metastasis (18). A relation of loss of expression of E-cadherin and the development of metastases has been described in several studies as reviewed by Jiang et al. (19). In HNSCC, relations between loss of E-cadherin expression and the presence of nodal metastasis have also been reported (20). Other studies, however, failed to find a statistically significant relation between loss of expression of E-cadherin in the primary tumor and the occurrence of nodal metastases (21-23).

For the other markers (p53, cyclin D1 and Ep-CAM) no correlation with the development of lymph node metastasis was found in the current study.
One of the most frequently studied markers in recent years is the tumor suppressor gene p53. Point mutations of p53, leading to nuclear accumulation of the protein, are among the most frequent genetic alterations in HNSCC. Clinicopathological studies of alterations in p53 in HNSCC show varying results as discussed in a review of Raybaud-Diogene (24). Some authors did not find any correlation of p53 expression with clinical parameters (25), metastasis (26) or survival (27;28). Others did find a correlation between nuclear p53 accumulation and survival although reports are contradictory: some found a correlation with worse survival rates (29;30), others with better (31).
Cyclin D1 is a potentially relevant prognostic marker and marker for the development of metastasis. Cyclin D1 was first described as a candidate oncogene in 1991 by Motokura et al. as PRAD-1 (32) and plays an important role in cell-cycle regulation. Overexpression of cyclin D1 may cause deregulation of the cell cycle and thus contribute to tumorigenesis. Studies of HNSCC concerning amplification of the chromosome 11q13 region, harbouring the cyclin D1 and EMS-1 genes, indicated a relation of this amplification with the development of nodal metastasis (11;33-37).

The relation between lymph node metastasis and the expression of cyclin D1 has not been studied frequently. Similar to the results of our study, Michalides et al. found no correlation of cyclin D1 expression with N-stage (38). Other authors however did (36;39). So, although amplification of 11q13 genes seems to be correlated with the presence of metastasis, this correlation has not definitively been confirmed for the expression of these genes.
The expression of Ep-CAM has not been studied very frequently in HNSCC. Increased expression of Ep-CAM appears to result in decreased cadherin-mediated cell-cell adhesion and may lead to segregation of Ep-CAM positive cells from the parental cell population in vitro (40). This phenomenon may facilitate the development of metastases in vivo. Other in vitro and animal studies, however, suggest that expression of the molecule reduces the metastatic potential (41). In an earlier study we indeed found a near significant relation between loss of expression and the development of nodal metastasis (11). In the current study of a larger series of HNSCC this relation could not be confirmed, however.

The studies on correlations of expression of markers with nodal metastasis or other clinical parameters often show conflicting results. This may be due to a number of factors. Besides differences in techniques and antibodies, many of these factors are related to the heterogeneity of tumors. This heterogeneity of tumors, supposedly the result of genetic instability, results in the situation that no tumor is exactly alike and that no tumor consists of a population of identical cells (42;43). This may contribute to a reduction of effectiveness of therapeutic strategies since the intrinsic biological properties of cells within and between tumors may differ. To adjust treatment strategies, like those mentioned in the introduction, more individually for a certain patient and tumor, markers reflecting the intrinsic properties of tumors may be useful. However, the study of markers is again hampered by the heterogeneity of the tumors. Which change in expression of a certain marker has biological or clinical significance is often not known. The cut-off points splitting the results in "positive" and "negative" categories are therefore often based on arbitrary criteria like the distribution of results in the several scoring categories. Another consequence of the heterogeneity of tumors is that biopsy material which is used to study may not represent the entire tumor (44).

In several studies histological features of the tumor were found to correlate with survival and/or the development of lymph node metastases. Correlations between the presence of an inflammatory reaction and the absence of lymph node metastasis have been described by several authors (11;45;46). In some studies a relation of eosinophilic infiltration surrounding the tumor and favourable prognosis has been described but a relation with lymph node metastasis was not found frequently (47-50). Others did not find any significant clinicopathological correlations (51). A relation between the development of lymph node metastasis and grade of differentiation (45;52;53) or growth pattern (45;54-58) has also been described. So, traditional histological parameters seem to be able to provide useful information.

Since the process of tumor development and metastasis is very complex, it is unlikely that a single parameter will be able to predict the metastatic behaviour of a tumor.

However, in our study the number of single parameters showing a correlation with nodal metastasis was low and a combination of the most relevant parameters was not more predictive for nodal metastasis. If more relevant parameters can be identified in the future, the combination of these may provide important information on the metastatic behaviour of tumors and may therefore have impact on the clinical decision making concerning the N0 neck.

CONCLUSIONS

Expression of markers and histopathological features of primary tumors may be able to supply information on the metastatic behaviour of tumors and may therefore influence clinical decision making concerning the treatment of the neck in patients with HNSCC. The investigated parameters in this study, however, did not show correlations with nodal metastasis strong enough to be useful in clinical practice. Moreover, the inconsistent results between studies in literature hamper the actual introduction of these markers for clinical purposes. Uniform standards are required to make the results of studies comparable.

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CHAPTER 7

Differences in Expression of Oncogenes and Tumor Suppressor Genes in Different Sites of Head and Neck Squamous Cell Cancer

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ABSTRACT

Background
In most studies concerning chromosomal changes or protein expression in head and neck squamous cell carcinomas (HNSCC) no distinction is made between the sites within this area. The behaviour of tumors arising in one site or the other, however, differs significantly, suggesting different intrinsic tumor properties.
In this study we compared the expression of several proteins (p53, Rb, cyclin D1, myc, bcl-2, EGFR, neu, E-cadherin, Ep-CAM, Desmoplakin1 and nm23) in the three major sites of HNSCC (larynx, pharynx, and oral cavity).

Materials and methods
Expression of the proteins was studied by immunohistochemistry in 33 laryngeal, 31 pharyngeal and 36 oral carcinomas.

Results
Cyclin D1 had a very high level of expression in the pharynx ($p=0.0004$) and EGFR very low in the larynx ($p<0.0001$) compared to the other sites. To a lesser degree p53 ($p=0.05$), Desmoplakin1 ($p=0.04$) and Ep-CAM ($p=0.02$) showed statistically nearly significant differences. For the other proteins no statistically significant difference in expression was found.

Conclusion
These results show that the expression of a number of proteins is not identical in the three major localisations of HNSCC and indicate a different tumorbiology of cancers originating from different sites in the Head and Neck. It is therefore not justified to consider the different localisations as one entity. Moreover these differences may have clinical and prognostic relevance.

INTRODUCTION

An increasing number of genes involved in tumor development and progression is identified and studied in several types of tumors. Most of the known genetic alterations and proteins have been studied in the major tumor types and sites. The results of these individual studies reveal that the occurrence of genetic alterations and the changes of gene expression have a different pattern in for example colorectal, cervical, breast, bladder and lung carcinomas. This is to be expected in tumors so different in their biological and clinical behaviour. In most studies squamous cell carcinomas of the head and neck are considered as one tumor type. Although a histopathological entity, Head and Neck Squamous Cell Carcinoma (HNSCC) may behave differently at the (sub)sites within the area. This is reflected in differences in growth pattern, clinical behaviour and prognosis. For example, the differentiation of cancers of the oral cavity is generally better than of oropharyngeal carcinomas [1]. In addition, oropharyngeal carcinomas have a higher tendency for metastasis compared to oral cavity and laryngeal carcinomas [1,2] although local anatomical circumstances may also play a role.
Cancer arises through a series of genetic changes reflected by altered protein expression. In HNSCC, several genes have been identified to be associated with tumor development and progression. The fact that the behaviour of tumors arising in one site or the other is not identical, suggests different intrinsic tumor properties. It may be expected that genetic alterations, responsible for these properties of the tumor, will reflect these differences. Modern immunohistochemical staining techniques allow us to study the products of these genetic alterations.
The present study was undertaken to investigate differences in gene expression of different sites involved in HNSCC. Proteins were selected on their putative relevance in Head and Neck cancer. The selection was made based on reports in the literature and a previous study we performed concerning markers that could be predictive for metastasis in laryngeal carcinoma [3]. The expression of a range of possibly relevant genes was compared. We studied gene products involved in cell cycle regulation (p53, retinoblastoma gene product (Rb), cyclin D1, myc) growth factor receptors (epidermal growth factor receptor (EGFR), neu) apoptosis (p53, bcl-2) and cell adhesion (E-cadherin, epithelial cell adhesion molecule (Ep-CAM), desmoplakin1) and another putative relevant protein, nm23. Using immunohistochemical stainings, these proteins were studied in the 3 major sites of HNSCC: larynx, pharynx, and oral cavity.

MATERIALS AND METHODS

From the files of the department of pathology tissue blocks were retrieved from resection specimens of laryngeal (33), pharyngeal (31) and oral carcinomas (36), which were resected en bloc with the regional lymph nodes in the period of 1990-1995. The population characteristics (age, sex, differentiation, TNM stage) are summarized in Table I. The proteins were analysed using immunohistochemistry as previously described [3,4]. The antibodies used are summarized in Table II.

T stage	Larynx	Pharynx	Oral cavity	total
T1	2	3	6	11
T2	8	7	14	29
T3	8	9	9	26
T4	15	11	7	33
Tx	0	1	0	1
N stage				
N0	13	6	13	32
N1-3	20	25	23	68
Differentiation				
Poorly	17	17	19	53
Moderately/well	16	14	17	47
Sex				
Male	26	20	22	68
Female	7	11	14	32
Age (years)				
Average	61.5	59.9	60.0	60.4
Range	44-74	38-84	34-87	34-87
Total	33	31	36	100

Table I. Population characteristics.

Most proteins (except cyclin D1, E-cadherin and nm23) were studied using the three step indirect method. In brief, 5 µm sections of paraffin embedded tissue were dewaxed in xylol for 15 minutes and rehydrated through alcohol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase. Subsequently the sections were pretreated for antigen retrieval as follows: for p53, Rb, bcl-2, E-cadherin and desmoplakin1 the sections were first boiled in citrate buffer (pH 6.0) for 10 minutes and cooled down for at least 2 hours; for myc and Ep-CAM the sections were pretreated with trypsin-solution (0,1% trypsin with 0,1% $CaCl_2$) pH 7.4 at 37 ^{0}C for 20 minutes. After washing with PBS, the primary antibody was applied for overnight incubation with 1% BSA in PBS. After washing with PBS the sections were incubated

Dako®P161) was applied for 45 minutes, then washed in PBS, and finally incubated with the tertiary antibody, swine-anti rabbit IgG (SWAR^{HRP}, Dako®P217) for 45 minutes. For polyclonals no tertiary antibody was used. For cyclin D1, E-cadherin and nm23 the avidin-biotin-peroxidase complex (ABC) staining method was applied. After the final washing with PBS, staining was performed by means of 3-amino-9-ethylcarbazol (AEC) in dimethylformamide with H_2O_2 followed by counterstaining with Mayer-Haematoxylin for 30 seconds. The sections were blued in tapwater and mounted with glycerine gelatine.

All 100 cases were stained simultaneously for each protein with appropriate specimens as positive and negative control. As positive control, tumor specimens were used that showed positive results in former studies. As negative control the sections were processed without the primary antibody.

Two observers (RPT; JHJMvK) evaluated the staining results. Differences in scoring were discussed at a multi-headed microscope until agreement was reached. For each antibody the percentage of staining of tumor cells was estimated and scoring categories were made. For convenience in reporting and before statistical analysis a dichotomy (positive vs. negative) was made. The cut-off points were based on the distribution of staining results in the different scoring categories since clear biological criteria are not available. For p53 and Rb the cut-off point was 15 %, for cyclin D1 and myc 5% and for bcl-2, E-cadherin, EGFR, neu, Ep-CAM, desmoplakin1 and nm23 cases with no staining were compared with cases showing any positive staining.

Differences in expression between the three sites site were evaluated using the chi-squared test. Using Bonferoni correction to correct for multiple testing, we considered $p<0.005$ as significant.

Antibody	Clone	Company	Dilution	Staining type
p53	p53-DO7	Novo-castra®	1:1000	N
Rb	1F8	Novo-castra ®	1:100	N
cyclin D1	051	Novo-castra ®	1:5000	N
myc	9E11	Novo-castra ®	1:200	N/C
bcl-2	100	Novo-castra ®	1:50	C
EGFR	Ab-4	Oncogene®	1:20	M
neu	3B5	Pathology Leiden	1:20,000	M
E-cadherin	HECD-1	Zymed®	1:250	M
Ep-CAM	Ab 323/A3	Centocor® Leiden	1:100	M
desmoplakin1	DP2.17	Progen ®	1:20	C
nm-23	H1,H2	C.R.B.®	1:20,000	C

Table II. Panel of antibodies used
staining type: N=nuclear, M=membranous, C=cytoplasmatic.

RESULTS

In this series of 100 patients with HNSCC expression of p53 was seen in 50% of the cases, Rb in 79%, cyclin D1 in 47%, myc in 9%, bcl-2 in 11%, EGFR in 23%, neu in 2%, E-cadherin in 67%, Ep-CAM in 29%, desmoplakin1 in 55% and nm23 in 57% of the cases.

The results for each site are summarized in Table III. For each protein some of the cases could not be evaluated due to factors like insufficient material or poor technical staining results. Significant differences in expression between the 3 sites were found for cyclin D1 ($p=0.0004$) and EGFR ($p<0.0001$). The differences between the sites were nearly significant ($0.005<p<0.05$) for p53 ($p=0.05$), desmoplakin1 ($p=0.04$) and Ep-CAM ($p=0.02$).

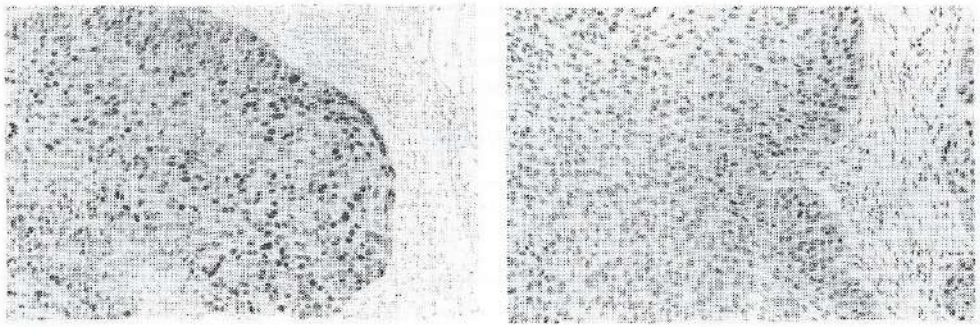
For cyclin D1 the results in pharyngeal carcinomas were distinct from the other sites. Positive results of cyclin D1 were seen in 76% of the pharyngeal carcinomas and in 36% of the laryngeal and 29% of the oral cavity carcinomas.

Expression of EGFR was not found in any of the laryngeal carcinomas and in only 14% (4/29) of the pharyngeal carcinomas in contrast to 51% (18/35) of the oral cavity carcinomas.

The expression rate of p53 was considerably higher in oral cavity carcinomas (63%) compared to laryngeal carcinomas (33%). Expression of desmoplakin1 was also higher in oral cavity tumors (71%) compared to laryngeal carcinomas (41%). For Ep-CAM more positive results were noticed in laryngeal carcinomas (45%) compared to pharyngeal (27%) and oral cavity (14%) tumors.

The differences were not related to a difference in T stage , N stage or differentiation (Table IV). The distribution of smaller (T1/T2) and larger (T3/T4) tumors ($p=0.07$), lymph node positive and negative cases ($p=0.18$), poorly and well or moderately differentiated tumors ($p=0.96$) was not significantly different between the 3 sites.

For the remaining proteins (Rb, myc, bcl-2, neu, E-cadherin, nm23) no statistically significant difference in expression was found between the three sites.

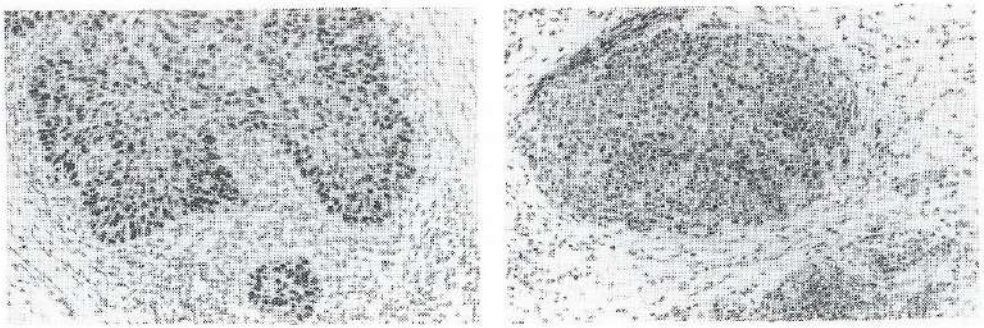


a b

Figure 1. Example of a positive (a) and negative (b) nuclear staining result for cyclin D1. In the tumor scored negative (b), only few nuclei show positive staining (original magnification x200).

	larynx	Pharynx	oral cavity	p	Total	Literature
p53	11/33 (33%)	16/30 (53%)	22/35 (63%)	0.05	49/98 (50%)	42 - 67 % [5-10]
Rb	24/33 (73%)	22/29 (76%)	30/34 (88%)	0.26	76/96 (79%)	74 - 88 % [13,14]
Cyclin D1	8/22 (36%)	22/29 (76%)	10/35 (29%)	**0.0004**	40/86 (47%)	44 - 49 % [7,15,16]
myc	5/33 (15%)	3/29 (10%)	1/36 (3%)	0.20	9/98 (9%)	No comparable studies [21], amplification 2-11% [19,22,23]
bcl-2	2/30 (7%)	6/27 (22%)	2/34 (6%)	0.08	10/91 (11%)	17 - 32 % [24-26]
EGFR	0/33 (0%)	4/29 (14%)	18/35 (51%)	**<0.001**	22/97 (23%)	44 - 90 % [27-29]
neu	0/33 (0%)	2/31 (6%)	0/36 (0%)	0.10	2/100 (2%)	0 - 47 % [10,30,32,33]
E-cadherin	23/33 (70%)	23/30 (77%)	20/35 (57%)	0.23	66/98 (67%)	22 - 58 % [34-36]
Ep-CAM	15/33 (45%)	8/30 (27%)	5/35 (14%)	0.02	28/98 (29%)	No references, 21% [3]
Desmopakin	13/32 (41%)	15/29 (52%)	25/35 (71%)	0.04	53/96 (55%)	No references, 17% [3]
Nm23	15/33 (45%)	17/29 (59%)	23/35 (66%)	0.23	55/97 (57%)	54 % [37]

Table III. The number and percentage of positive staining results per protein for each of the major sites of HNSCC and the range of positive staining results for all sites of HNSCC in other studies.



a b

Figure 2. Example of a positive (a) and negative (b) nuclear staining result for p53.
In the tumor scored negative (b), only few nuclei show positive staining (original magnification x200).

cyclin D1	Larynx	Pharynx	Oral cavity	Total	p
T1/T2	29%	67%	30%	39%	0.14
T3/T4	40%	79%	27%	51%	0.006
EGFR					
T1/T2	0%	11%	55%	31%	0.003
T3/T4	0%	16%	47%	18%	0.001
cyclin D1					
N0	39%	83%	31%	44%	0.09
N1-3	33%	74%	27%	48%	0.05
EGFR					
N0	0%	0%	46%	19%	0.005
N1-3	0%	17%	55%	25%	<0.001
cyclin D1					
poorly diff	54%	63%	28%	47%	0.11
well/mod diff	11%	92%	29%	46%	<0.001
EGFR					
poorly diff	0%	12%	44%	19%	0.002
well/mod diff	0%	17%	59%	27%	<0.001

Table IV. Percentage of positive staining results of the two proteins that differed most significantly in expression between the three sites (cyclin D1 and EGFR) stratified for T stage, N stage and differentiation in the three major sites. No major differences are found in the smaller (T1/T2) and larger (T3/T4) tumors, lymph node positive and negative cases, poorly and well or moderately differentiated tumors. In the final column the p values are shown for the differences in expression between the 3 sites, per subgroup. These p values are not completely comparable to those of the entire study population group since the number of cases in each subgroup is smaller than the entire study population, resulting in higher p values.

DISCUSSION

Since clinical behaviour of HNSCC is quite dependant on site it is questionable whether HNSCC may be considered as a single entity. Indeed in this study of 100 cases of HNSCC it is shown that there are clear differences in the expression of several genes known to be associated with tumor development and progression. This was found for cyclin D1 and EGFR and to a lesser degree for p53, desmoplakin1 and Ep-CAM. Although there are studies confined to gene expression in one specific site of HNSCC, only few studies compare this gene expression in several sites of HNSCC. As was found in our material, in these studies a difference of expression between the several sites has been noticed.
Combining the results of the 3 sites, the expression of most of the investigated proteins was similar to that found in other studies (Table 3).

The results of p53 expression were in the range found in other studies [5-10]. Nees et al. [11] studied expression of mutated p53. The highest percentage of p53 immunoreactivity was found in oropharyngeal and oral cavity tumors compared to laryngeal carcinomas. Lavieille et al. [9] also found highest expression in hypopharyngeal (67%) and oropharyngeal carcinomas compared to laryngeal cancers (12%). Similar figures are found in our material. However, Ahomadegbe et al., when studying p53 gene mutations, did not find major differences in the subsites (67% for oral cavity to 78% for laryngeal carcinomas) [12].
Rb has predominantly been studied on the DNA level and infrequently in HNSCC. In studies concerning the expression of Rb, the loss of expression of Rb was similar to our results [13,14].
Cyclin D-1 expression has been studied by others recently by immunohistochemistry and our finding of expression in 47% in the total series is close to the 44-49% found in those studies [7,15,16]. DNA amplification of the 11q13 region (harboring the cyclin D1 gene) has been studied more frequently and is found to be around 30% of HNSCC [17-19]. One of the rare studies in which a distinction and comparison between the sites has been made is a study of Muller et al. investigating 11q13 amplifications in HNSCC [18]. A difference of amplification rate was found between the several sites with a higher rate of amplification in pharyngeal carcinoma. Williams et al. found the highest number of amplifications in hypopharyngeal carcinomas [19]. These findings are consistent with our results: a higher rate of expression of cyclin D1 (gene on 11q13) was found in carcinomas arising in the pharynx. Amplification of 11q13 genes has been associated with a higher metastatic potential [18,20]. Therefore, the higher amplification rate of the cyclin D1 gene and the resulting overexpression in pharyngeal carcinomas may play a role in the fact that these tumors give rise to metastasis more often than cancers arising in other sites of the head and neck, although in our material this was not found (Table 4).

Expression of myc has not been studied extensively in HNSCC. In our study expression was 9%, which is lower than found by others although comparison is hampered due to the use of a different scoring system [21]. The expression found in our material is, however, compatible with the rate of amplification of the myc gene of 2-11% found in several studies [19,22,23].

Few studies on the expression of bcl-2 in HNSCC have been published. In studies of bcl-2 expression, positive results were found in 17-32% of the cases [24-25].
We do not have an explanation for the absence of any positive cases for EGFR in our series of laryngeal carcinomas. Others did find expression in a considerable number of cases of HNSCC [26,27]. Christensen et al. [28] found expression in 90 % of oral carcinomas, considering staining of more than 5% of the cells as positive staining result, compared to 51% in our material. One may speculate whether a technical factor may have played a role in our results for EGFR. Resection specimens of laryngeal carcinomas are usually decalcified and this might have resulted in the complete absence of positive staining results in these specimens. However, the difference in expression of EGFR in pharyngeal and oral cavity carcinomas is already significant. Since none of the other markers showed this complete absence of positive cases in laryngeal carcinomas, it is unlikely that the decalcification process may have affected other staining results.
We did not find neu expression in a significant number of cases in contrast to others [10,29,30]. Like in our series, Kearsley et al. [31] did not find any specific membrane staining and Field et al. [32] did not find any characteristic membranous staining either but merely a cytoplasmatic staining pattern.
E-cadherin expression found in our study was in the range found by others although often immunofluoresence instead of immunohistochemistry was used [33-36].
We did not find recent reports on Ep-CAM and desmoplakin1 expression in HNSCC except for our own recent study [3]. In one report on immunoreactivity of nm23 in laryngeal carcinoma a reduced expression was found in 46% of the cases, which is close to the 55% negative cases in laryngeal carcinoma in our series [37].

The staining results of most of the proteins in our study are comparable to the majority of the above mentioned studies. However, differences in techniques, antibodies and scoring systems used do not allow a reliable comparison between the studies. A more elaborate scoring, like our initial scoring system, can give more detailed insight in the pattern of expression of the individual markers. From these data other categories can be made to make these more comparable to other studies. As mentioned before, scoring systems and cut-off points are usually rather arbitrary since clear biological criteria are unavailable for most markers. Since most of our results are more or less similar to those of other studies, our cut-off points were apparently comparable.

The fact that important genes involved in tumor development are found to be differently expressed in the several sites of the head and neck may indicate a different path of tumor progression and behaviour in the subsites of HNSCC. This will probably be reflected in a different clinical behaviour of tumors arising in these sites and differences in prognosis. With increasing knowledge of the function of these proteins the clinical implications of these differences may become more explicit and treatment strategies may be adjusted for cancers arising in separate sites of the head and neck.

In conclusion, these results show that the expression of a series of genes is not identical in the several localisations of HNSCC and indicate a different tumor biology of

cancers originating from different sites in the Head and Neck. It is therefore not justified to consider the different localisations together in studies concerning molecular genetic changes and protein expression in HNSCC. Moreover the differences in gene expression may explain the differences in clinical behaviour of these tumors and may influence clinical decision making. To make protein expression suitable for clinical purposes the methods of staining and scoring should be standardized.

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CHAPTER 8

Protein Expression of Cancer Associated Genes: Biopsy Material Compared to Resection Material in Laryngeal Cancer

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ABSTRACT

Background
Changes in several (onco)genes and their protein expression play a role in the development of Head and Neck Squamous Cell Carcinoma (HNSCC). If protein expression is to be used for clinical purposes, the expression should preferably be evaluated during the initial diagnostic work-up when only biopsy material will be available. To investigate the correlation between assessment of expression in biopsy and resection material, protein expression in both was evaluated and compared.

Materials and Methods
In this study we compared the expression of P53, Rb protein, E-Cadherin, Ep-CAM, Desmoplakin1 and Cortactin by immunohistochemistry. Expression of the proteins was studied in biopsy and resection material of 26 laryngeal carcinomas.

Results
A variable rate of mismatches between the scoring on biopsy and resection material was found for the different proteins.

Conclusion
If protein expression is to be used in clinical practice and decision making it should be realised that a discrepancy exists between the expression in biopsy material and the complete resection material.

INTRODUCTION

An increasing number changes in relevant genes and gene products have been postulated to play a role in the development and progression of tumors. Furthermore, many studies have been performed to investigate the possible clinical relevance of these factors. Several examples of possible clinical applications of molecular changes in HNSCC in the diagnostic work-up phase have been published. Recently, a study performed in our institute demonstrated expression of certain proteins to be predictive for lymph node metastasis in laryngeal carcinoma [1]. In other studies the relation between expression of certain proteins and lymph node metastasis in HNSCC has been studied as well [2-6]. Others have investigated the relation of protein expression with the response to irradiation [7-10] or treatment failure in general [11,12].
To yield maximum results from the use of markers the results of the tests should be available during the diagnostic phase. However, in most studies clinical correlations of proteins expression are studied using tumor resection material, although most clinical decisions are made before resection of the primary tumor. For example whether or not to perform an elective neck dissection is usually decided before initial treatment since this elective neck dissection is preferably performed in one session with resection of the primary tumor. Therefore, if protein expression is to be used for correlation with lymph node metastasis, the expression should be studied in biopsy material instead of resection material. To estimate whether a tumor is radiosensitive or not also requires testing on biopsy material to facilitate the choice for adequate initial treatment. Some decisions can be made after initial treatment, eg adjuvant therapy.
In this study we compared the expression of several proteins in HNSCC by immunohistochemistry. Proteins involved in cell cycle regulation (p53, Rb) and cell adhesion (E-Cadherin, Desmoplakin, Ep-CAM) and Cortactin were studied in biopsy material as well as resection material from the same patients.

MATERIALS AND METHODS

Paraffin embedded tissue blocks were retrieved from the files of the department of pathology, and included specimens of both biopsy and resection material of 26 patients with laryngeal carcinoma to study the expression of the following proteins: P53, Rb, E-Cadherin, Desmoplakin, Ep-CAM and Cortactin. Half of the cases (13/26) were previously irradiated. Of these cases the biopsy material of the recurrent tumor was used for comparison with the resection material to exclude radiation induced differences between biopsy and resection material. The other cases were primary tumors without previous treatment and the initial biopsy material and subsequent resection material was used. Three cases were treated with irradiation prior to and after surgery ("sandwich therapy")
The population characteristics (age, sex, prior irradiation, TNM stage) are summarized in Table 1. The protein expression was analysed using immunohistochemistry as previously described [1,13]. The antibodies used were p53 (p53-DO7, Novo-castra®), Rb (clone 1F8, Novo-castra ®), E-Cadherin (clone HECD-1, Zymed®), desmoplakin1 (clone DP2.17, Progen ®), Ep-CAM (Ab 323/A3, Centocor® Leiden) and Cortactin (Pathology Leiden).

Sex	Female:	2	
	Male:	24	
T stage	T1:	0	
	T2:	1	
	T3:	6	
	T4:	9	
	Recurrence after irradiation:		10 (initially T1:3, T2:6, T3:1)
N stage	N0:	13	
	N1:	5	
	N2:	6	
	N3:	2	
Prior irradiation	13/26	- 10 recurrences (60,66, or 70Gy)	
		- 3 "sandwich" therapy (20Gy pre / 40 or 50Gy post)	

Table 1. Population characteristics

In brief, 5 µm sections of paraffin embedded tissue were de-waxed in xylol for 15 minutes and rehydrated through alcohol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase. Subsequently the sections were pretreated for antigen retrieval as follows: for P53, Rb, E-cadherin and desmoplakin the sections were first boiled in citrate buffer (pH 6.0) for 10 minutes and cooled down for at least 2 hours; for Ep-CAM and Cortactin the sections were pre-treated with trypsin-solution (0,1% trypsin with 0,1% $CaCl_2$) pH 7.4 at 37 °C for 20 minutes. After washing with PBS, the primary antibody was applied for overnight incubation with 1% BSA in PBS. After washing with PBS the sections were incubated with the secondary antibody. For monoclonals, rabbit-anti-mouse IgG (RAM^{HRP}, Dako®P161) was applied for 45 minutes, then washed in PBS, and finally incubated with the tertiary antibody, swine-anti rabbit IgG ($SWAR^{HRP}$, Dako®P217) for 45 minutes. For polyclonals no tertiary antibody was used. For E-cadherin the avidin-biotin-peroxidase complex (ABC) staining method was applied.
After the final washing with PBS, staining was performed by means of 3-amino-9-ethylcarbazol (AEC) in dimethylformamide with H_2O_2 followed by counterstaining with Mayer-Haematoxylin for 30 seconds. The sections were blued in tapwater and mounted with glycerine gelatine.

RESULTS

Two observers independently evaluated the sections (RPT; JHJMvK) according to the characteristic staining pattern of each antibody (nuclear, cytoplasmatic, membranous). Differences in scoring were discussed at a multi-headed microscope until agreement. The results were scored on a semi-quantitative scale (+, ± and -). Staining results were considered positive (+) if the majority of tumor cells showed staining (> 50%). If

staining was absent or confined to a few cells (0-5%) the result was considered negative (-). Cases with partial staining were scored ± (5-50%). For convenience of reporting, a dichotomy was made clustering the - and +/- categories versus + as was done in our previous study [1]. Biopsy material and resection material were scored at separate sessions by the same observers. To avoid bias, the results of the one session were not available at the other session.
Staining results of the biopsy and resection materials per protein are summarized in Table 2 and 3. In Table 4 the number of mismatches between biopsy and resection material is shown after dichotomizing the results. Discrepancy between the scoring results of biopsy material and resection material varied from 21% (4/24) for P53 to 52% (12/23) for E-cadherin (Table 5).
For diagnostic purposes, the biopsy material can be considered as a sample with a predictive value for the expression in the resection material. For the nuclear stainings (p53, Rb), a negative score in biopsy material shows good correlation with a negative score in resection material (13/14 and 6/7 respectively). For the cell adhesion molecules (E-cadherin, demoplakin, Ep-CAM) the negative predictive value was considerably lower (2/10, 5/9 and 8/14 respectively). For all proteins the positive predictive value was low.
Mismatches were found in almost all cases varying from 1 to 4 proteins (Table 6a). Prior irradiation was of no influence on the number of mismatches (Table 6a and 6b).

	resection +	biopsy +	resection -	biopsy -
p53	6 (23%)	8 (31%)	20 (77%)	16 (69%)
Rb	5 (19%)	10 (38%)	21 (81%)	13 (62%)
E-Cadherin	15 (58%)	3 (12%)	11 (42%)	20 (88%)
Desmoplakin	4 (15%)	6 (23%)	22 (85%)	16 (77%)
Ep-CAM	4 (15%)	3 (12%)	22 (85%)	21 (88%)
Cortactin	9 (35%)	7 (27%)	17 (65%)	16 (73%)

Table 2. Staining results of biopsy and resection specimens of 26 cases of laryngeal carcinoma.

p53	- resection	+/-	+	total
- biopsy	13	0	1	14
+/-	1	0	0	1
+	3	1	5	9
missing:2	17	1	6	24
Rb				
- biopsy	6	0	1	7
+/-	4	0	2	6
+	6	2	2	10
missing:3	16	2	5	23
E-Cadherin				
- biopsy	2	2	6	10
+/-	5	0	5	10
+	1	0	2	3
missing:3	8	2	13	23
Ep-CAM				
- biopsy	8	3	3	14
+/-	2	5	0	7
+	1	2	0	3
missing:2	11	10	3	24
Desmoplakin				
- biopsy	5	1	3	9
+/-	7	0	0	7
+	2	3	1	6
missing:4	14	4	4	22
Cortactin				
- biopsy	5	4	1	10
+/-	2	1	1.00	4
+	2	2	5.00	9
	9	7	7	23

missing:3

Table 3. Staining results of biopsy vs. resection material.

	correct		incorrect	
biopsy/resection	-/-	+/+	+/-	-/+
P53	14	5	4	1
Rb	10	2	8	3
E-Cadherin	9	2	1	11
Ep-CAM	18	0	3	3
Desmoplakin	13	1	5	3
Cortactin	12	5	4	2

Table 4. Number of matching scores of biopsy and resection material after dichotomy (- and +/- = - vs + = +).

Protein	match	mismatch	evaluable	total
p53	19 (79%)	5 (21%)	24	26
Rb	12 (52%)	11 (48%)	23	26
E-cadherin	11 (48%)	12 (52%)	23	26
Desmoplakin	14 (64%)	8 (36%)	22	26
Ep-CAM	18 (75%)	6 (25%)	24	26
Cortactin	17 (74%)	6 (26%)	23	26

Table 5. Discrepancy in staining results between biopsy and resection material.

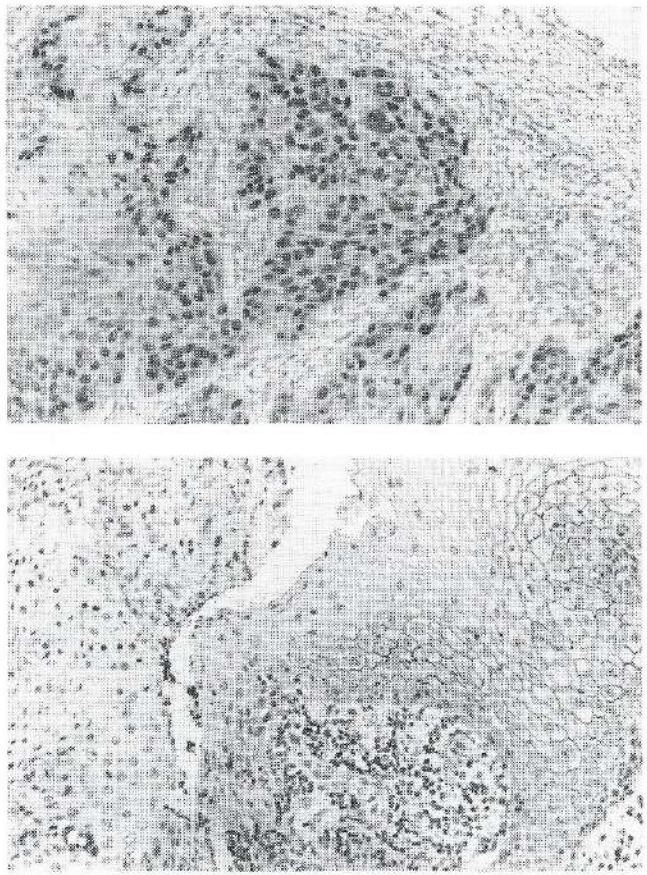
number of mismatches	number of cases irradiated (10)	non irradiated (13)	sandwich (3)
0	2	1	0
1	2	5	1
2	2	5	1
3	2	2	1
4	2	0	0

Table 6a. Number of mismatches between biopsy and resection material for the previously irradiated and non irradiated cases.

Table 6a. Number of mismatches between biopsy and resection material for the previously irradiated and non irradiated cases.

mismatches	p53	Rb	E-Cadherin	Desmoplakin1	Cortactin
prior RT	3/10	3/10	4/10	3/10	4/10
no prior RT	1/13	6/13	6/13	4/13	2/13
SW	0/3	2/3	2/3	1/3	0/3

Table 6b. Number of cases with mismatches between biopsy and resection material per marker for the previously irradiated and non irradiated cases.



A

B

Figure 1. Example of nuclear staining pattern for Rb (A) and membranous staining pattern for E-cadherin (B). In both examples a heterogeneity in the staining pattern is apparent, with parts of the tumor showing clear expression and parts showing absence of expression (original magnification 200x).

DISCUSSION

The results of this study show that in a significant number of the tumors assessment of protein expression in biopsy material is not identical to the expression found in the resection specimen. This may be due to a number of factors.
Probably the most important factor is that most tumors are heterogenous (Figure 1 A and B). Some parts of the tumor show expression of certain proteins while other parts do not. Since during biopsy only a small part of the tumor is removed, it is not necessarily representative of the entire tumor and a sampling error may be introduced. Some tumors may show more heterogeneity than others. But since almost all our cases showed mismatches of one or more proteins we are inclined to expect that the discongruency between biopsy and resection material may be encountered in any tumor. Radiotherapy did not seem to be an important factor in the number of mismatches and therefore heterogeneity.
In addition the pattern of expression may differ between the proteins. Although this phenomenon of heterogenous expression is probably seen for most proteins, it has not been reported and discussed in detail in most studies. For E-cadherin heterogeneous staining patterns have been described by some authors [4,14]. Schipper et al. [5,15] correlated this staining pattern to moderate differentiation of the tumor. Some proteins may show a more heterogenous staining pattern than others and this may account for the different rate of mismatches between the proteins.

Changes in expression due to tumor progression in the interval between biopsy and resection are unlikely, since this interval between diagnosis is usually short (several weeks at the most). Moreover, in our material expression of some oncogenes (mutated P53, loss of E-cadherin) was more unfavourable in biopsy material compared to the resection material making tumor progression an unlikely explanation for the discrepancy.
The 3 cases treated with "sandwich therapy" received irradiation in the interval between biopsy and resection. The number of mismatches, however, did not seem significantly different compared to the other cases. Although the number of cases is small this implies that irradiation does not affect the heterogeneity of the tumor.
The resection specimens of laryngeal carcinomas are usually decalcified in contrast to the biopsy material. One may speculate whether this influenced the staining results. However, since not intensity but percentage of positively staining results was scored, the effect of the different processing is probably neglectable. No differences in staining intensity in general between biopsy and resection material were noticed.

In conclusion, assessment of protein expression on biopsy material is not representative of the expression of the entire tumor. It is therefore doubtful if the results of immunohistochemistry performed on biopsy material in the diagnostic phase can be used in clinical decision making.

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CHAPTER 9

DNA Ploidy Status as a Prognostic Marker and Predictor of Lymph Node Metastases in Laryngeal Carcinoma

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ABSTRACT

Background
In patients with laryngeal carcinoma, nodal metastasis, response to radiotherapy and prognosis are important factors in clinical decision making. Parameters like tumor stage are considered insufficient for predicting these important items. The DNA ploidy status of the tumor may be a useful additional marker.

Materials and Methods
The DNA ploidy status of 38 laryngeal cancers was determined by flow cytometry. Correlations were studied with TNM stage, differentiation, survival, relapse-risk, response to radiotherapy and nodal metastasis.

Results
A positive correlation of DNA ploidy status with the development of lymph node metastases was found for diploid and peridiploid vs aneuploid tumors (p=0.007). No correlation was found between ploidy status and response to radiotherapy.
The overall survival (p=0.01) but not disease-specific survival and relapse-risk showed a correlation with the ploidy status.

Conclusions
The DNA ploidy status may be a useful marker for metastatic behaviour in HNSCC and may therefore be helpful in decision making concerning elective treatment of the neck.

INTRODUCTION

In patients with head and neck squamous cell carcinoma (HNSCC) metastatic behaviour and response to radiotherapy are important issues when making decisions on the treatment of these patients. But patients and their tumors, even of similar stage, are heterogenous. Tumors may show a wide range of characteristics concerning growth, metastatic behaviour, response to different treatment modalities and prognosis. If patients could be better selected for the appropriate treatments, the outcome might be improved. In patients with HNSCC, choices of treatment are currently mainly based on TNM stage. But TNM staging can not predict the behaviour of individual tumors and there are no tools to predict this behaviour more reliably. So a need is felt for additional parameters.

The potential clinical relevance of finding markers for lymph node metastasis is considerable. Regional metastasis is an important factor in the treatment and prognosis of patients with HNSCC but the accuracy of current diagnostic methods to detect these metastases is insufficient. For the detection of metastases by palpation or imaging techniques a minimal size of these metastases is required. Therefore, small and only microscopically detectable metastatic deposits will not be recognized, and uncertainty about the true lymph node status of the neck will remain. Even Ultrasound (US) with Ultrasound Guided Fine Needle Aspiration Biopsy (UGFNAB), the most accurate technique to detect lymph node metastases to date, identifies clinically occult metastases with a sensitivity of no more than 48-76% (1;2). Because of the high false-negative rates most head and neck oncologists will electively treat the neck of patients with tumors with a presumed high propensity to metastasize. Consequently, many patients will receive an unnecessary treatment for their neck with concomitant mortality and morbidity (3;4). If the probability of regional metastases can be reduced, the number of patients electively treated for their neck can also be diminished (5).

Some tumors respond favourably to radiotherapy and others do not. These latter patients should perhaps be treated with initial surgery, e.g. laser surgery, or should be monitored more carefully after irradiation. Although in the literature factors have been described that may supply information, there are no reliable criteria available to predict response to radiotherapy to date.

Chromosomal changes and histological features of tumors, can provide valuable information concerning the probability of nodal metastasis (6;7) or response to radiotherapy in HNSCC (8-12). However, the results of different studies are often conflicting and no marker in particular has yet been established as a marker for clinical use. The above mentioned and other applications of biomarkers, like cancer development risk and relapse risk assessment, were recently reviewed by Oh and Mao (13). Despite the fact that the molecular mechanisms of carcinogenesis are not yet fully understood, biomarkers may help to select patients for certain treatments, e.g. elective neck treatment (6;7). If, in addition to imaging techniques, biomarkers can further reduce the probability of nodal metastasis, it may be decided not to treat the neck electively.

The ploidy status of tumors reflects the DNA content of the tumor cells. Aneuploidy is the result of failing mitotic checkpoints (14). Based on the literature, the ploidy status of tumors may be a useful biomarker and helpful in the above mentioned clinical decision making in patients with HNSCC (15-24).

In the current study the correlation of ploidy status with the development of regional metastasis, the recurrence rates after radiotherapy and survival are studied in 38 patients with laryngeal carcinoma with a follow up of at least 10 years.

level		
	glottic:	27
	supraglottic:	8
	both/transglottic:	3
primary treatment		
	surgery and radiotherapy:	12
	radiotherapy alone:	26
T stage		
	1:	13
	2:	13
	3:	10
	4:	2
N status		
	positive:	9
	negative:	29
Sex		
	male:	33
	female:	5
Age		
	mean:	60.2 years
	range:	12 to 81 years

Table 1. Population characteristics of 38 patients with HNSCC.

MATERIALS AND METHODS

Paraffin embedded pre-treatment biopsy material of 38 patients who presented with laryngeal carcinoma in the period of 1973 to 1982 at the Leiden University Medical Center was used. The population characteristics are shown in Table 1. The patients were all treated by either initial radiotherapy or surgery (total laryngectomy) and radiotherapy. Follow-up was at least 10 years. The patients in the lymph node positive group had histologically proven lymph node metastases either initially or in the follow-up (regional recurrence). The patients in the lymph node negative group had no detectable metastases neither initially, nor in the follow-up period.

Cell preparation and staining procedures have been described elsewhere (25). The pepsin-digestion technique (0.5% pepsin in saline, pH 1.5 for 30 minutes) was used to release nuclei from 40-45 micrometer thick sections of paraffin embedded tissue specimens and they were stained with propidium iodide (PI). The DNA content was determined with a FACScan flow cytometer (Becton & Dickinson, Mountain View, CA, USA). On average, about 10,000 cells were measured in each sample.
When the DNA diagram showed two or more G0,1 populations, the left one was considered to represent the diploid, non-neoplastic cells in the specimen.

The degree of aneuploidy was expressed by the DNA index, i.e. the ratio between the modal channel number of the aneuploid G0,1 peak and that of the diploid G0,1 peak. Tumors with DI more than 1.00 were classified as aneuploid. To discriminate between diploid or peridiploid tumors and tumors with more severe aneuploidy that probably arose via polyploidization, a cut-off point of 1.4 was also made (25).

We investigated the correlation between DNA ploidy status, T stage and nodal status with overall and disease-specific survival and relapse-risk using Cox regression analysis. Actuarial survival plots were calculated according to the Kaplan-Meier method. The date of treatment was taken as starting point for this analysis. Overall survival was calculated by counting as death, all patients who died irrespective of the cause and disease specific survival by counting as death, all patients who died of their laryngeal cancer. The relation between DNA ploidy status and stage, differentiation, the response to radiotherapy and lymph node metastases was tested using the Fisher's Exact test. Correlations or differences with a $p<0.05$ were considered statistically significant.

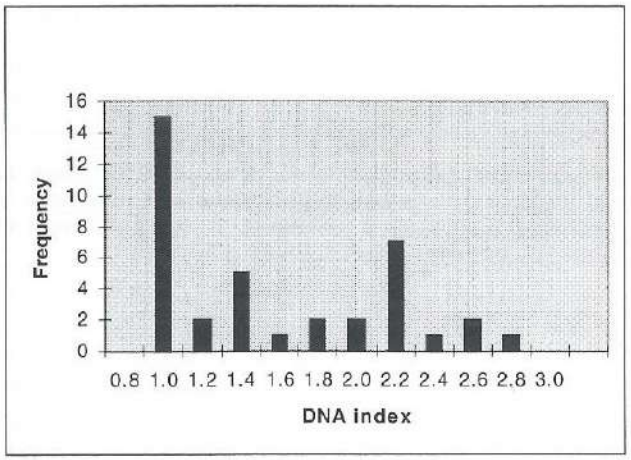

Figure 1. DNA index frequency distribution of 38 cases of laryngeal carcinoma.

RESULTS

The DNA index ranged from 1.0 to 2.78. The DNA index frequency distribution is shown in Figure 1. Of the 38 investigated cases 23 (61%) were aneuploid considering only tumors with a DNA index of 1.00 as diploid. Taking the diploid and peridiploid tumors together 18 (47%) of the cases were aneuploid.
With 1.4 as a cut-off point, a significant correlation of ploidy status with lymph node metastases was found (p=0.007). In the diploid-peridiploid group 1/20 (5%) patients had lymph node metastases whereas in the aneuploid group 10/18 (56%) of the patients had metastases. Using the cut-off point of 1.0 the relation was nearly significant (p=0.06) (Table 2).

	cut-off 1.0			cut-off 1.4		
	diploid	aneuploid	p	diploid	aneuploid	p
differentiation			0.16			0.03
poor	0	2		0	2	
moderate	7	15		9	13	
well	8	6		11	3	
N-	14	15	0.06	19	10	0.007
N+	1	8		1	8	
recurrence –	6	9	0.69	9	7	1.00
recurrence +	6	5		7	4	
T1	6	7	0.67	9	4	0.51
T2	5	8		6	7	
T3	4	6		4	6	
T4	0	2		1	1	
I	6	6	0.36	9	3	0.23
II	5	7		6	6	
III	4	6		4	6	
IV	0	4		1	3	

Table 2. Relation between ploidy status and differentiation, the development of lymph node metastases (N- = no lymph node metastasis, N+ = lymph node metastasis), recurrence after radiotherapy (recurrence- = no recurrence, recurrence+ = recurrence), T stage and overall stage grouping according to the UICC for the 2 cut-off points of the DNA index, 1.0 and 1.4.

UICC staging categories			
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IV	T4	N0/1	M0
	anyT	N2/3	M0
	anyT	anyN	M1

In the 26 patients treated with primary radiotherapy, no correlation was found between ploidy status and response to radiotherapy using either of the cut-off points (p=0.69 for 1.0 and p=1.00 for 1.4) (Table 2).
The DNA ploidy status of the tumors was not related to the T stage (p=0.67 for 1.0 and p=0.51 for 1.4) (Table 2) or overall UICC TNM stage grouping (p=0.36 for 1.0 and p=0.23 for 1.4) (Table 2). When diploid and peridiploid tumors were set against aneuploid tumors (cut-off point of 1.4) a correlation was found with the differentiation of the tumor (p=0.03) (table 2).

Table 3 summarizes the influence of aneuploidy (diploid/peridiploid vs. aneuploid), T stage and nodal status on survival, disease specific survival and relapse risk. As is shown by the Cox regression analysis, aneuploidy has a significant prognostic influence on overall survival (HR=3.47, p=0.01). In the aneuploid group survival was significantly worse than in the (peri)diploid group. The results with diploid vs. peridiploid and aneuploid tumors showed a similar difference in survival (HR=2.89, p=0.03). This correlation was independent of nodal status or T stage. T stage (T1 vs T2-T4) (p=0.20) did not correlate with overall survival and neither did the nodal status (p=0.79). For disease specific survival and relapse risk, none of the investigated variables was significant. For disease specific survival T stage came nearest to significance.
Figure 2 gives the Kaplan Meier plot for the influence of aneuploidy (diploid/peridiploid vs. aneuploid) on overall and disease specific survival.

		N	Overall survival (23 events)	Disease specific survival (9 events)	Relapse Risk (14 events)
			HR (95% CI)	HR (95% CI)	HR (95% CI)
Aneuploidy	<1.4	20	1.00	1.00	1.00
	≥1.4	18	3.47 (1.35-8.92) p=0.01	1.55 (0.35-6.86) p=0.57	0.93 (0.28-3.10) p=0.90
T	1	13	1.00	1.00	1.00
	2,3,4	25	1.87 (0.71-4.90) p=0.20	6.05 (0.74-49.5) p=0.09	1.14 (0.37-3.52) p=0.82
N	pN-	29	1.00	1.00	1.00
	pN+	9	0.87 (0.31-2.43) p=0.79	1.60 (0.32-7.92) p=0.56	1.80 (0.48-6.84) p=0.38

HR = Hazard Ratio
95% CI = 95% Confidence Interval

Table 3. Influence of aneuploidy (diploid/peridiploid vs. aneuploid), T stage and nodal status on survival, disease specific survival and relapse risk using Cox regression analysis.

Additional analysis of our data showed that in the diploid group of the 6 patients that died, 2 died of intercurrent disease. In the aneuploid group 12 of the 17 dead patients died of intercurrent disease. So in the aneuploid group more patients died of intercurrent diseases. Age correlated with ploidy status nearly significant (p=0.08, t-test). The average age in the diploid group was 55.7 compared to 63.1 years in the aneuploid group. For diploid and peridiploid vs. aneuploid tumors this difference was less pronounced (p=0.18, t-test). So there is a trend of more aneuploid tumors at higher age.

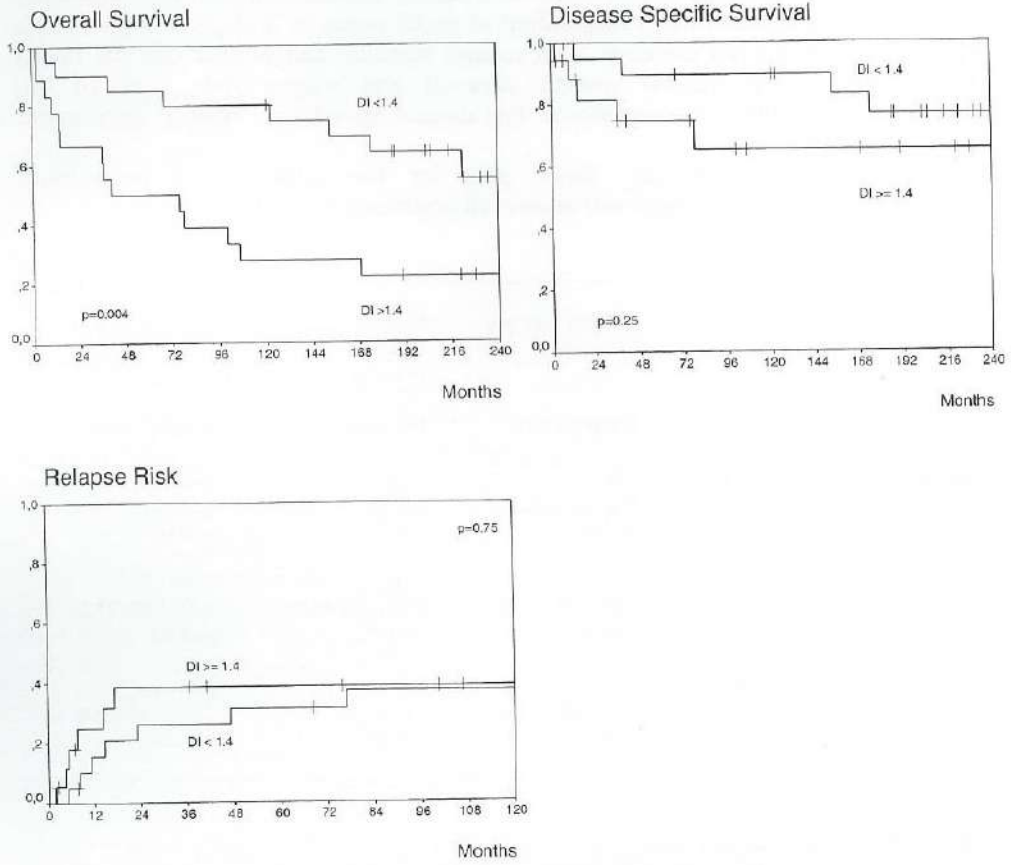

Figure 2. Kaplan Meier plots of overall survival, disease specific survival and relapse risk curves for ploidy status with 1.4 as cut-off point (Log Rank test).

DISCUSSION

In this study the ploidy status of laryngeal cancers correlated with the development of nodal metastasis but not with response to radiotherapy. Moreover, it showed a correlation with overall survival but not with disease-specific survival or relapse-risk.

In recent reports on DNA ploidy status in HNSCC correlations have been studied with clinical parameters like stage or metastasis. In studies of HNSCC of different sites, a correlation with stage or nodal metastasis was found in some studies (17;26) but in other studies this correlation could not be established (27;28). A correlation with lymph node metasasis in oral cavity carcinoma was found by several authors (16;18;20;21;23) although some only found a weak relation (7). Like in our material, Wolf et al. found a higher rate of lymph node metastases in aneuploid laryngeal carcinomas (19). So both in our study and in the literature DNA ploidy appears to correlate with nodal metastasis.

DNA ploidy may be a useful biomarker for the development of lymph node metastasis, as a single marker or in a combination with other relevant parameters and diagnostic techniques. Such a combination of relevant factors may obtain an even higher reliability in detecting or predicting lymph node metastases (6;7). In cases with no clinically or radiologically detectable nodal metastases, a further lowered probability of micro-metastases based on markers like DNA ploidy could support a decision not to perform elective neck treatment (5).

A relation of DNA aneuploidy with a higher risk of local recurrence after radiotherapy has been described in glottic carcinoma (22). We did not find a relation between ploidy status and recurrences after irradiation. This is consistent with the results of Pekkola-Heino et al. who did not find an association of ploidy status with radiosensitivity in HNSCC cell lines (29). Other markers, in particular p53 and bcl-2, have also been studied in this respect but with conflicting results (8;9;11;30-33). For example in some studies nuclear accumulation of p53 did not appear to be a significant predictor for control with radiotherapy in laryngeal carcinomas (10;31) or HNSCC in general (32). Others did find a relation of nuclear accumulation or mutation of p53 with response to radiotherapy in HNSCC patients (8;11;12;34;35) whereas mutations of p53 in oral cavity cell lines appear to render the tumor cells more radiosensitive (33). Expression of bcl-2 in HNSCC was demonstrated to give a better local control after accelerated RT (9). In conclusion, the results of studies of markers predicting response to radiotherapy are conflicting and it is therefore unlikely that they will play a role in clinical decision making in the near future.

Numerous markers have been studied for their prognostic significance. For the DNA ploidy status the results are again conflicting. Some authors did not find a statistically significant correlation with prognosis in HNSCC (9), squamous cell carcinoma of the oral tongue (36), supraglottic larynx (37) or pyriform sinus (38). Others did find a correlation with both relapse-free and/or overall survival in HNSCC (15;24;39). In laryngeal carcinomas a relation with relapse-free survival has been described too (19).

In our material we found a relation with overall survival but not with disease specific survival. This may seem difficult to explain. However, the distinction between the

two is not always clear in practice. Often factors related to for example the treatment of the disease, e.g. extensive surgery, may lead to mortality. Also if the condition of the patient deteriorates due to progression of the disease, the probability on death by intercurrent diseases may increase as well. We observed a trend towards a high frequency of aneuploid tumors at a higher age. The correlation between ploidy status and overall survival therefore may be influenced by the fact that aneuploidy is associated with higher age resulting in a higher chance to die of intercurrent diseases. So, it seems that the ploidy status of the tumor does not have a direct prognostic significance in our study.

CONCLUSIONS

The DNA ploidy status appears to be correlated with lymph node metastasis and overall survival, but not with disease-specific survival or relapse-risk, in laryngeal carcinomas. It may be useful in clinical decision making concerning the treatment of the neck. Although even as a single marker it shows a significant correlation with lymph node metastases, reliability to predict lymph node metastases may be improved by using it in addition to other relevant parameters and imaging techniques.

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CHAPTER 10

General Discussion

OUR RESULTS SUMMARIZED

In our multicenter study a sensitivity of 77% in the detection of nodal metastasis in the neck was found for US/UGFNAB. This is slightly lower compared to previous studies but comparable with the sensitivity of CT and MRI. The specificity of 100% of US/UGFNAB found in this study is similar to that of previous studies and superior to the specificity of CT and MRI (1-6). However, in the clinically N0 population, UGFNAB was characterized by a considerably lower sensitivity of 48%, while the specificity remained 100%. In this population CT demonstrated a sensitivity of 54% and a specificity of 92%, so UGFNAB and CT demonstrated comparable accuracy. The sensitivity of 48% of US/UGFNAB, the most accurate imaging technique to date, in detecting nodal metastases in the clinically N0 population appeared to be still quite poor and these results warranted the exploration of other means to estimate the chance on metastasis in patients with HNSCC.

Comparison of the expression of markers in metastasis and their primary tumor was performed to identify which markers may be relevant in the process of metastasis. The expression of nm23 and Ep-CAM in particular was more frequently reduced in metastasis compared to their primary tumors.
Studying several tumor related parameters in the pilot study of laryngeal cancers, relations with the presence of nodal metastasis were found for a limited inflammatory reaction and eosinophylic infiltration surrounding the tumor, expression of Rb and loss of expression of Ep-CAM and 11q13 amplification. A combination of some of these factors (Rb, Ep-CAM, inflammatory reaction and eosinophilic infiltration) resulted in a superior accuracy in assessing nodal metastasis. These results indicated that it may be possible to predict and exclude lymph node metastasis by studying features of the primary tumor.
In a larger series of cases of HNSCC, loss of expression of Rb and E-cadherin correlated with the presence of lymph node metastasis. In addition, we separately studied the value of the DNA ploidy status of primary laryngeal carcinomas for a correlation with clinical parameters. We established a correlation with the development of nodal metastasis and overall survival with the DNA ploidy status.

OUR RESULTS RELATED TO THE LITERATURE

Of the histological parameters, an inflammatory reaction surrounding the tumor and eosinophilic infiltration appeared to show some (although not statistically significant) relation with nodal metastasis. This was found in our pilot study of laryngeal carcinomas and in our multicenter study. A correlation between the presence of an inflammatory reaction and the absence of lymph node metastasis has also been described in the literature (7;8). Although in some studies a relation of eosinophilic infiltration surrounding the tumor and favourable prognosis has been described, a relation with nodal metastasis was not frequently reported (9-12).
Of the protein expressions, studied by immunohistochemical stainings, the expression of Rb, E-cadherin and Ep-CAM seem to be the most interesting concerning the relation with nodal metastasis. In our study of laryngeal carcinomas,

Rb and to a lesser extend, Ep-CAM showed a relation with nodal metastasis. Moreover, in the comparison of the expression of proteins in primary tumors and their paired metastases, Ep-CAM expression appeared to be more frequently decreased than increased in the metastases. No cases of increased expression in the metastases were found. The expression of Rb has not frequently been studied in HNSCC (13;14) but a relation with the nodal status has been reported in one study (14). No studies of Ep-CAM expression have been performed by others to our knowledge.
The relation of the expression of E-cadherin with nodal metastasis was not apparent in the pilot study on laryngeal carcinomas. In the multicenter study, however, this relation could be found and for oral cancer in particular. A relation of loss of expression of E-cadherin and the presence of metastases in different types of cancer has been described in several studies (15). In studies concerning HNSCC an indication (16;17) or correlation (18) between loss of expression of E-cadherin in the primary tumor and the development of nodal metastases has been reported. However, other studies concerning HNSCC failed to find a statistically significant relation (19;20).
Amplification of the 11q13 genes, cyclin D1 and EMS1 was found to be relevant in our study on laryngeal cancers. Studies of HNSCC concerning amplification of the chromosome 11q13 region, indicated a relation of this amplification with the development of nodal metastasis (21-26). The expression of cyclin D1 did not show a correlation with lymph node metastasis in our studies. The relation between lymph node metastasis and the expression of cyclin D1 has not been studied frequently and reports on the expression of the EMS1 product, cortactin, in HNSCC are even rarer. Studying expression of cyclin D1 with immunohistochemistry, Michalides et al. found no correlation of cyclin D1 expression with N-stage (27). Other authors however did report such a relation (25;28). So, although amplification of 11q13 genes seems to be correlated with the presence metastasis, this correlation has not definitively been confirmed for the expression of these genes.
In our study of laryngeal cancers a correlation between the ploidy status and the development of nodal metastasis was found. In studies concerning HNSCC, a correlation with stage or nodal metastasis was found in some studies (29;30) but in other studies this correlation could not be established (31;32). A correlation with nodal metasasis in oral cavity carcinoma was found by several authors (33-37) although some reported only a weak relation (38). Like in our material, Wolf et al. found a higher rate of lymph node metastases in aneuploid laryngeal carcinomas (39). So both in our study and in the literature DNA ploidy appears to show a correlation with nodal metastasis.

For the other investigated markers no correlation with the development of lymph node metastasis was found in the current study. Others than the investigated markers may be more relevant, like for example integrins, basement membrane components like collagens (40;41) and laminins (42), matrix metalloproteinases (MMP) (43;44), urokinase type plasminogen activators (uPA) and inhibitors (PAI) (45) and angiogenic factors or microvascular density (46;47). These have not been studied due to technical problems or lack of experience with these markers in our

laboratory. However, in the literature a correlation with the development of metastasis is not consistently found for these markers either.

Our results seem promising but it is already apparent from these results that the correlation with nodal metastasis is not consistently found for the same parameters. In the larynx study and the larger series of HNSCC, different parameters were found to correlate with lymph node metastasis. Reviewing the literature these differences in results are even more pronounced. There may be several possible explanations for this fact

Differences between subsites of the Head and Neck
In our study we demonstrated a difference in expression of several genetic markers between tumors arising in the different subsites of the Head and Neck (chapter 7). This difference is probably reflected in the variation of the biological behaviour of tumors arising in different subsites of the head and neck. In our studies, this expression indeed appeared to be different in tumors arising in the larynx, pharynx or oral cavity. Many studies concerning HNSCC make no distinction between these sites but it seems questionable if results found in tumors of one site are applicable to tumors of other sites. Since some studies are restricted to certain subsites of the head and neck and other studies consider all these subsites together, the results of these studies are actually incomparable. This fact is not a limitation of the use of biomarkers but it leads to an inconsistancy of results and will therefore not promote the acceptance of this use of biomarkers for clinical purposes.

Scoring of markers and parameters
Standardization of scoring systems is necessary to obtain results which are comparable with those of others. Although there are some fundamental difficulties in the scoring of proteins, as will be discussed later in the context of tumor heterogeneity, more uniformity is required in the creation of scoring categories. For the assessment of histological features of the tumor or the surrounding tissues different scoring systems are used as well. The many scoring systems are sometimes very elaborate but are mostly not universally used by others. This lack of standardization also hampers the comparison of study results. Moreover, some of the investigated parameters, like depth of invasion or tumor thickness, can only be assessed on the resection specimen and not on biopsy material. They are therefore not useful in the process of decision making concerning the treatment of the neck.

Reference standard for nodal metastasis
Finally, in most studies, like in our study, the nodal status is based on (routine) histopathological examination of the neck dissection specimen. However, small metastatic deposits or micrometastases may still remain undetected in this way. If the lymph nodes are examined more meticulously, in a number of the cases small metastases may be detected, which were neither noticed preoperatively nor histopathologically (48;49). Some studies even use the clinical N stage to test correlations with certain tumor biological parameters. Which reference standard is used for the nodal status will influence the outcome of a study.

LIMITING FACTORS IN THE USE OF MARKERS FOR CLINICAL PURPOSES

Several factors other can play a role in the variability of study results and in the limitations and the acceptance of the use of potential markers for the prediction of metastasis or other clinical purposes. These factors may be related to the differences in techniques and antibodies that are used, but many of these factors are related to the heterogeneity of tumors.

Techniques and antibodies
The protein expression of genes is studied in different ways by different authors. Techniques like immunofluorescence, immunohistochemistry and Western blotting are used for this purpose making comparison of results of different studies difficult. Moreover, many different antibodies directed against the same protein of interest are available and this may also contribute to a variability in results.

Heterogeneity of tumors
This heterogeneity of tumors, supposedly the result of genetic instability, makes that no tumor is exactly alike and that no tumor consists of a population of identical cells (50;51). This may contribute to a reduction of effectiveness of therapeutic strategies since the intrinsic biological properties of cells within and between tumors may differ. To adjust treatment strategies more individually, markers reflecting these intrinsic properties of tumors may be useful. However, the study of markers is again hampered by this same heterogeneity of the tumors as will be discussed below.

Scoring of markers
Chromosomal aberrations and protein expression are different in some parts of the tumor compared to others. This is usually thought to be a result of clonal evolution. In tumor progression some cells of a clone may acquire additional or different chromosomal alterations, resulting in a subclone with different properties. If this subclone is a relatively small part of the primary tumor, the expression of a studied marker in this tumor will probably be scored negative. However, this subpopulation of tumor cells may still be responsible for a certain biological behaviour like metastasis. This fact makes the choice of biologically relevant cut-off points rather arbitrary. Which percentage of cells showing expression should be considered as a positive result and which percentage as a negative result? As a result, in different studies cut-off points are often not the same. Moreover, the choice of cut-off points can also determine the presence or absence of correlations with clinical parameters. It may be that if a cut-off point is chosen differently the correlation with other parameters changes. More uniformity in methods is therefore necessary to obtain more consistent results and making different studies comparable. This is essential to obtain usable markers for clinical correlations. In absence of biological criteria, an option to circumvent some of the above mentioned problems may be to create scoring categories of for example intervals of 10% and to make cut-off points based on ROC curves (52). In this way an optimal cut-off point can be detected if the marker expression is used as a test or predictor for a certain clinical event like metastasis.

Biopsy material
If markers are to be used in decision making on the treatment of the neck, the only available material to study before treatment will be the biopsy material. Another consequence of the heterogeneity of tumors is that this biopsy material may not represent the entire tumor. In our study the expression of markers in the biopsy material appeared not to be identical to the expression in the entire tumor (chapter8). It is therefore questionable if studies of marker expression on biopsy material can be used for prediction of clinical behaviour of the tumor.

Dynamics of tumor biology
A more fundamental weakness of any technique to study genetic changes to predict biological behaviour of tumors is that tumorigenesis, and the process of metastasis in particular, is a dynamic process. The moment material of a tumor is taken for study, the results will just reflect the current status of the tumor: one moment in time of a dynamic process. It is questionable if such a dynamic process can be studied at all in this way.

OUR CONCEPT OF THE USE OF MULTIPLE MARKERS

One of the most important reasons that markers are not yet used for the evaluation of the nodal status of patients may be that most studies focus on single markers only. Since the process of metastasis is very complex with many factors involved, it is not likely that a single marker is sufficient to predict the metastatic behaviour of a tumor. As was already mentioned in the introduction, this was the reason that, in contrast to most other studies at the time, we decided to study and combine several relevant markers on the same population to see if useful correlations and predictive values for nodal metastasis could be obtained. Recently, more studies combining several tumor related parameters to predict nodal metastasis are reported (38).
Although many genes or markers have been identified as relevant in tumor development and progression, the exact mechanisms of these processes have not yet been elucidated. The processes like cell cycle regulation and cell adhesion are very complicated and yet not fully understood. An increasing number of alterations in genes and their expression is identified that may play a role in the process of tumor progression and metastasis. A combination of some of the identified markers in our studies and markers that will be identified in the future may provide valuable information on parameters like metastasis.

PERSPECTIVES

All the above discussed matters influence the possibility to use biomarkers for clinical purposes unfavorably. This is also reflected in the results of our studies, although some of the results are promising. Probably, with the increasing knowledge of the processes of tumorigenesis and metastasis and the improvement of techniques to detect chromosomal aberrations and changes in expression, some of these factors will be overcome. It is therefore likely that the study of chromosomal aberrations and

changes in genes and their expression will play a role in clinical decision making in the future. To accomplish this, the most important conditions seem to be the following: multiple instead of single tumor related parameters should be studied in relation to the clinical parameter of interest (eg metastasis); a distinction between the subsites of the Head and Neck should be made; and more uniformity in scoring systems is required.
Currently, modern imaging techniques are the most reliable tools to detect lymph node metastasis in the neck. US/UGFNAB seems to be the most reliable technique of these as was confirmed by the results of our studies. Other promising imaging techniques, like PET, are under investigation but all imaging techniques are limited in their accuracy since metastasis must have a minimum diameter to be detected. Indeed, in a population of patients with clinically undetected nodal metastasis, the sensitivity drops significantly as was found in our study for US/UGFNAB.
Clinicians confronted with certain clinical problems are looking for new or additional tools helpful in clinical decision making. On the other hand fundamental researchers are interested in finding clinically relevant applications for the results of their work. As it seems, in the case of the prediction of nodal metastasis in HNSCC by the use of biomarkers there is still a considerable gap between laboratory and clinic.

CONCLUSION

Head and Neck Squamous Cell Carcinomas arising in different sites of the head and neck are different in their genetic changes reflecting their differences in biological behaviour.
In addition to imaging techniques, marker expression may be useful for the assessment of regional metastasis in HNSCC. But for a reliable prediction of nodal metastasis, allowing a change in the concept of elective neck treatment, the results of marker studies are still too variable due to a number of factors. Therefore, up to date the use of US with UGFNAB seems to be the most reliable technique to assess the nodal status of the neck in patients with HNSCC.

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Summary

In the introduction the importance of the detection of regional nodal metastasis in patients with HNSCC was discussed. Regional metastasis is one of the most important factors in the prognosis and treatment of patients with Head and Neck Squamous Cell Cancer (HNSCC). Imaging techniques are currently used to assess the nodal status of the neck. These techniques are however limited in their accuracy by the fact that the metastases need to have a minimal diameter to be detected. This warranted a search for new techniques to predict nodal metastasis more reliably. The biological behaviour of tumor cells is to a great extend determined by changes in genes and their expression in these cells and the interaction with surrounding structures and cells. The metastatic behaviour of tumors may therefore be assessed by studying these factors and this may enable us to predict nodal metastasis irrespective of their size.

In chapter 2 the value of Ultrasound with Ultrasound Guided Fine Needle Aspiration Biopsy (US/UGFNAB) in the assessment of the neck in patients with HNSCC was studied in a multi-center study. Recent studies concluded that US/UGFNAB is the most accurate technique to detect or exclude regional metastasis in patients with HNSCC. Critics however suggested that the good results found in these study were probably irreproducible in clinical practice since the technique is supposedly very investigator dependant. In our study no significant inter-observer variability was found. The sensitivity in the detection of nodal metastasis in the neck was 77%. This was slightly lower compared to previous studies but comparable with the sensitivity of CT and MRI. The specificity of 100% of US/UGFNAB found in this study was similar to that of previous studies and superior to the specificity of CT and MRI. These results can be considered as a validation and recommendation of the use of US/UGFNAB for the evaluation of the neck in patients with HNSCC.

In chapter 3 the value of US/UGFNAB in the assessment of the neck in patients with HNSCC was studied in a subpopulation of patients with clinically negative necks and compared to Computed Tomography (CT). In this study, UGFNAB was characterized by a sensitivity of 48% with a specificity of 100%. For the use of CT a sensitivity of 54% and specificity of 92% was found. The sensitivity of US/UGFNAB of 48% in detecting nodal metastases in the clinically N0 population, appeared to be considerably lower than in the previous part of the study considering the entire population of HNSCC patients. This is not unexpected considering the fact that the population consisted of patients with no palpabel nodes, implying the presence of only small metastases. However, it means that about half of the metastases is detected in this population. This warranted the exploration of other means to assess the chance on metastasis in patients with HNSCC.

In chapter 4 expression of genetic markers was studied in nodal metastases and their matched primary tumors since differences in expression in the primary tumors and their metastases may suggest relevance in the process of regional metastasis. The expression of nm23 and Ep-CAM in particular was reduced in metastasis compared to their primary tumors. Since nm23 is a metastasis suppressor gene and Ep-CAM a cell adhesion molecule, loss of expression of these molecules may facilitate metastasis.

In chapter 5 a pilot study in laryngeal cancer was described. The aim of the study was to explore the possibility to predict nodal metastasis by studying features of the primary tumor. For this purpose histological features, protein expression using immunohistochemistry and DNA amplification using Southern blotting were investigated and correlated to the presence or absence of nodal metastases. Relations with the presence of nodal metastasis were found for limited inflammatory reaction and eosinophylic infiltration surrounding the tumor, expression of Rb, loss of expression of Ep-CAM and 11q13 amplification. A combination of some of these factors resulted in a superior accuracy in assessing nodal metastasis. These results indicated that it may be possible to predict and exclude lymph node metastasis by studying features of the primary tumor only.

In chapter 6 the expression of a selection of previously studied markers was studied on the material of the multi-center US/UGFNAB population to investigate wether the correlations found in the pilot study could be reproduced in a larger number of cases. In this larger series, loss of expression of Rb and E-cadherin correlated with the presence of lymph node metastasis.

In chapter 7 the expression of several genetic markers studied by immunohistochemistry was compared between the 3 major subsites of the head and neck (larynx, pharynx, oral cavity). The head and neck region is often considered as one entity and tumors arising in this region are often studied without distinction between the subsites. However, the biological behaviour of tumors arising in subsites of the head and neck varies. Therefore differences in intrinsic tumor factors can be expected, reflected in differences in protein expression. The expression of several proteins indeed appeared to be different in tumors arising in the larynx, pharynx or oral cavity and it seems not justified to consider these sites as one entity.

In chapter 8 the expression of several genetic markers studied by immunohistochemistry was compared between biopsy material and the primary tumors they were taken from. If nodal metastases are to be predicted based on features of the primary tumors, only biopsy material will be available and it is uncertain if this material is representative for the entire tumor.
In our study the expression of markers in the biopsy material appeared not to be identical to the expression in the entire tumor. It is therefore questionable if studies of marker expression on biopsy material can be used for prediction of clinical behaviour of the tumor.

In chapter 9 the value of the DNA ploidy status of the primary tumor in predicting the development of nodal metastasis was studied. We found a correlation with the development of nodal metastasis and overall survival in this material.

Some of the above mentioned results seem promising. In addition to imaging techniques, marker expression may reduce the probability of metastasis. But for a reliable prediction of nodal metastasis, allowing a change in the concept of elective neck treatment, the results of marker studies are still too variable. Therefore up to

date the use of US with UGFNAB seems to be the most reliable technique to assess the nodal status of the neck in patients with HNSCC.

Samenvatting

In de introductie wordt het belang van de detectie van regionale metastasering bij patiënten met een plaveiselcelcarcinoom van het hoofd-halsgebied (HNSCC) besproken. Regionale metastasering is een van de belangrijkste factoren bij de behandeling en de prognose van patiënten met hoofd-halstumoren. Momenteel worden vooral beeldvormende technieken gebruikt voor beoordeling van de hals. Deze technieken hebben echter de beperking dat metastasen een minimale grootte moeten hebben om te worden gedetecteerd. Dit gegeven leidde tot het zoeken naar andere technieken om metastasen beter te kunnen voorspellen. Het biologisch gedrag van tumoren wordt voor een groot deel bepaald door veranderingen in genen en hun expressie en de interactie van de cellen met omliggende structuren en cellen. De hypothese is dat het metastaseringsgedrag van tumoren derhalve beoordeeld zou kunnen worden door deze factoren te bestuderen. Het zou dan mogelijk kunnen zijn metastasen onafhankelijk van hun grootte te voorspellen.

In hoofdstuk 2 werd de waarde van echografie met echogeleide cytologische punctie (US/UGFNAB) bij de beoordeling van de hals van patiënten met hoofd-halscarcinomen in een multicenter onderzoek bestudeerd. Recente studies concludeerden dat US/UGFNAB de meest betrouwbare techniek is om regionale halskliermetastasering aan te tonen of uit te sluiten. Critici suggereerden echter dat de goede resultaten van deze studies waarschijnlijk niet te reproduceren zouden zijn in de dagelijkse praktijk omdat het onderzoek verondersteld wordt onderzoekersafhankelijk te zijn. In onze studie werd geen significante variabiliteit tussen de verschillende onderzoekers gevonden. De sensitiviteit bij de detectie van metastasen was 77%. Dit was iets lager dan in voorgaande studies maar vergelijkbaar met de sensitiviteit van CT en MRI. De specificiteit van 100% was gelijk aan die van eerdere studies en superieur aan de specificiteit van CT en MRI. Deze resultaten kunnen worden beschouwd als een validatie van de techniek en een aanbeveling voor het gebruik van US/UGFNAB voor de beoordeling van de hals bij patiënten met een hoofd-halscarcinoom.

In hoofdstuk 3 bestudeerden wij de waarde van US/UGFNAB bij de beoordeling van de hals van patiënten met hoofd-halscarcinomen in een deelpopulatie zonder palpabele afwijkingen in de hals en vergeleken deze met CT. In deze studie werd US/UGFNAB gekarakteriseerd door een sensitiviteit van 48% en een specificiteit van 100%. Voor CT werd een sensitiviteit van 54% en een specificiteit van 92% gevonden. De gevonden sensitiviteit van US/UGFNAB van 48% bleek aanzienlijk lager dan die gevonden in de eerdere studie van de gehele populatie patiënten met hoofd-halscarcinomen. Dit is niet onverwacht aangezien de populatie van patiënten zonder palpabele klieren kleinere en dus moeilijker te detecteren metastasen zal hebben. Het betekent echter wel dat slechts de helft van de metastasen in deze populatie wordt gedetecteerd. Dit rechtvaardigde het onderzoeken van andere methoden om de kans op metastasen in te schatten bij patiënten met hoofd-halscarcinomen.

In hoofdstuk 4 werd de expressie van genetische markers bestudeerd in lymfkliermetastasen en hun gepaarde primaire tumoren. Verschillen in expressie in primaire tumoren en hun metastasen zijn suggestief voor relevantie in het proces van regionale metastasering. De expressie van nm23 en Ep-CAM was verminderd in de

metastasen in vergelijking met hun primaire tumoren. Aangezien nm23 een metastaseringssuppressorgen is en Ep-CAM een celadhesiemolecuul zal verlies van expressie van deze moleculen metastasering kunnen faciliteren.

In hoofdstuk 5 beschrijven wij een pilotstudie van larynxcarcinomen. Het doel van de studie was de mogelijkheid te onderzoeken om lymfkliermetastasen te voorspellen door het bestuderen van eigenschappen van de primaire tumor zelf. Voor dit doel werden histologische kenmerken, eiwitexpressie bestudeerd m.b.v. immunohistochemie en DNA amplificatie bestudeerd m.b.v. Southern Blotting onderzocht en gecorreleerd aan de aan- of afwezigheid van lymfkliermetastasen. Relaties met de aanwezigheid van metastasen werden gevonden voor verminderde aanwezigheid van ontstekingsinfiltraat en eosinofiel infiltraat rond de tumor, expressie van Rb, verlies van expressie van Ep-CAM en amplificatie van het chromosoomgebied 11q13. Een combinatie van een aantal van deze factoren resulteerde in een superieure accuratesse voor de beoordeling van halskliermetastasen. Deze resultaten wijzen erop dat het mogelijk lijkt lymfkliermetastasering te voorspellen door het bestuderen van kenmerken van de primaire tumor zelf.

In hoofdstuk 6 werd de expressie van een selectie van eerder bestudeerde markers bestudeerd op het materiaal van de populatie van de multicenter echografiestudie. Dit met het doel te zien of de correlaties die gevonden waren in de pilotstudie van larynxcarcinomen gereproduceerd konden worden in een grotere serie. In deze grotere serie correleerden verlies van expressie van Rb en E-cadherine, vaso-invasieve groei en perineurale groei met de aanwezigheid van lymfkliermetastasen.

In hoofdstuk 7 werd de expressie van diverse genetische markers, bestudeerd m.b.v. immunohistochemie, vergeleken tussen de 3 voornaamste gebieden binnen het hoofd-halsgebied (larynx, pharynx, mondholte). Het hoofd-halsgebied wordt vaak beschouwd als een eenheid en tumoren die in dit gebied ontstaan, worden vaak bestudeerd zonder onderscheid te maken tussen de verschillende lokalisaties binnen dit gebied. Het biologische gedrag van de tumoren die in de diverse lokalisaties ontstaan verschilt echter van elkaar. Derhalve kunnen ook verschillen in intrinsieke tumorfactoren worden verwacht. Dit zou tot uiting kunnen komen door verschillen in eiwitexpressie. Deze expressie van diverse markers was inderdaad verschillend in de tumoren ontstaan in de larynx, pharynx of mondholte en het lijkt dus niet terecht deze lokalisaties als een entiteit te beschouwen.

In hoofdstuk 8 werd de expressie van diverse genetische markers bestudeerd m.b.v. immunohistochemie vergeleken tussen biopsiemateriaal en de primaire tumoren waar ze van werden genomen. Als lymfkliermetastasen zouden moeten worden voorspeld, gebaseerd op eigenschappen van de primaire tumoren, zal immers alleen biopsiemateriaal beschikbaar zijn. Het is echter onzeker of dit materiaal representatief is voor de gehele primaire tumor. In onze studie bleek de expressie van markers in het biopsiemateriaal niet gelijk aan de expressie in de gehele tumor. Het is derhalve twijfelachtig of onderzoek van markerexpressie in biopsiemateriaal kan worden gebruikt voor het voorspellen van klinisch gedrag van de tumor.

In hoofdstuk 9 werd de waarde van de DNA ploidystatus van de primaire tumor bij het voorspellen van de ontwikkeling van lymfkliermetastasen bestudeerd. Wij vonden een correlatie met de ontwikkeling van lymfkliermetastasen en algehele overleving in dit materiaal.

Enkele van de bovenstaande resultaten lijken veelbelovend. Naast beeldvormende technieken zou markeronderzoek aanvullende informatie kunnen geven t.a.v. regionale metastasering van hoofd-halscarcinomen. Maar voor een betrouwbare voorspelling van lymfkliermetastasen, die een verandering in het concept van electieve halsbehandeling zou kunnen betekenen, zijn de resultaten van markerstudies nog te variabel. Derhalve is tot nu toe het gebruik van echogeleide cytologische punctie de meest betrouwbare techniek om de lymfklierstatus van de hals te bepalen in patiënten met een carcinoom van het Hoofd-Halsgebied.

Addendum

IMMUNOHISTOCHEMISTRY

Immunohistochemistry can be used to study expression of proteins by the use of antibodies against an antigene or protein of interest. The antibodies are coupled to a staining substance which can be visualised by light microscopy. In this way nuclear, cytoplasmatic and membranous staining patterns can be seen, depending on the function and localisation of the protein. The advantage of the technique is that it is relatively easy to perform and inexpensive.
The antibodies belong to a group of proteins called immunoglobulins (Ig). Each immunoglobulin is composed of two identical heavy chains and two identical light chains. Five classes of immunoglobulins can be distinguished: IgG, IgA, IgM, IgD and IgE. The most frequently used antibodies in immunohistochemistry are IgG and IgM. The antibodies can be polyclonal or monoclonal. Polyclonal antibodies are produced by different cells and are therefore immunochemically dissimilar. They react with different epitopes on the antigen they are raised against. The most frequently used animal for the production of these antibodies is the rabbit. Monoclonal antibodies are produced by clones of plasma cells, are immunochemically identical and react with a specific epitope on the antigen against which they are raised. The animal used for the production of these antibodies is almost exclusively the mouse. The advantages over polyclonal antibodies include high homogeneity and absence of nonspecific antibodies.
Different techniques can be used for immunoenzymatic stainings. The most commonly used are the indirect immuno peroxidase and Avidin-Biotin method. In the two-step indirect method the primary antibody binds to the antigen and a secondary antibody directed against the primary antibody is then applied, followed by the staining solution. Usually the primary antibody is raised in mice and therefore in the next step a rabbit-anti-mouse (RAM) antibody is used. In the three-step indirect method an additional antibody is applied, usually swine-anti-rabbit (SWAR). The use of secondary and tertiairy antibodies will amplify the staining signal with greater color intensity.
Another technique is the Avidin-Biotin Complex method (ABC). For this method a secondary antibody is biotinylated, a mild process whereby biotin is attached to the antibody. Avidin has a high affinity for biotin and has four binding sites for biotin. Avidin-biotin complexes bind to the biotin on the antibody. So after the primary antibody subsequently the biotinylated secondary antibody and preformed avidin-biotin complexes are applied.

SOUTHERN BLOTTING

Southern blotting is a technique suitable to study gene amplification. The procedure is quite labour intensive and time consuming and requires the use of radioactive labeling. Tumor DNA is isolated from the tumor cells and is cut in smaller fragments using restriction enzymes. These enzymes cut the DNA at defined sequences. The DNA fragments can be seperated by size using electrophoresis. The DNA fragments will migrate towards the positive electrode and the extend of migration will be determined by the size of the fragment. This gel is subsequently transferred or

blotted on a filter or blot. Hybridization of DNA is the formation of double-stranded DNA between two single-stranded DNA fragments. This is possible due to the complementary nature of double-stranded DNA. A probe consisting of a fragment of single-stranded DNA and with a complementary sequence to the gene or region of interest is labeled radioactively and is used for hybridization. The radioactive band can be visualized by exposing the blot to X-ray film.
To study gene amplification, the amount of radioactivity is compared to the amount of the control DNA of non malignant cells. In this way the number of copies of the gene can be determined.

Nawoord

"Er is geen moeilijkere taak dan goed te bedanken."

Gilles Ménage

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Mijn ouders die altijd de vrijheid en mogelijkheid gaven mijzelf te ontplooien en datgene te ondernemen wat ik wilde en mij hierbij altijd gesteund hebben met enthousiasme en belangstelling.

Curriculum Vitae

The author of this thesis was born on June 18, 1963 in Nieuwer-Amstel, the Netherlands. He graduated at the Keizer Karel College in Amstelveen in 1981 and subsequently studied at the Free University in Amsterdam where he received his medical degree in 1988. After serving as a medical officer in the Dutch Army, he worked in the Netherlands Cancer Institute in Amsterdam as a resident on the departments of Surgery, Internal Medicine and Head and Neck Surgery from 1990 until 1993. In his last year at this institute he worked on a radioimmunotargeting project for the detection of metastases in patients with colorectal, breast and head and neck cancer. In 1993 he started at the department of Otorhinolaryngology of the University Hospital Leiden working on a multicenter study on the value of ultrasound-guided fine needle aspiration biopsy and biomarkers for the assessment of nodal metastasis in head and neck cancer, the subject of this thesis. His training in Otorhinolaryngology started in 1995 and was finished in februari 2000. Since then, he is a staff-member at the department of Otorhinolaryngology of the Leiden University Medical Center.

De auteur van dit proefschrift werd geboren op 18 juni 1963 te Nieuwer-Amstel. Na het voltooien van het VWO aan het Keizer Karel College te Amstelveen studeerde hij van 1981 tot 1988 Geneeskunde aan de Vrije Universiteit te Amsterdam. Van 1989 tot 1990 werd de militaire dienstplicht vervult als officier-arts bij een geneeskundige eenheid. Van 1990 tot 1993 was hij werkzaam als arts-assistent in het Nederlands Kanker Instituut/Antoni van Leeuwenhoekhuis te Amsterdam op de afdelingen chirurgie, interne geneeskunde en hoofd-halschirurgie. Het laatste jaar van deze periode werkte hij als studiecoördinator van een immunotargetingproject met radioactief gelabelde monoclonale antilichamen en een project voor monitoring van de speekselklierfunctie bij radiotherapie van het hoofd-hals gebied. Vanaf 1993 is de auteur werkzaam op de afdeling Keel-, Neus- en Oorheelkunde van het Academisch Ziekenhuis Leiden: tot 1995 als onderzoeker waarbij aan het onderwerp van dit proefschrift werd begonnen en vanaf 1995 in opleiding tot Keel-, Neus- en Oorarts. Deze opleiding is in februari 2000 voltooid. Sinds die datum is hij als stafarts verbonden aan de afdeling Keel-, Neus- en Oorheelkunde van het Leids Universitair Medisch Centrum.

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