# Second Field Tumors
## in head and neck cancer patients

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door

**Maarten Pieter Tabor**

geboren te Angerlo

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| | dr. R.H. Brakenhoff |

To my parents
To Lieke and Martijn

## Contents

# 1

# General introduction

# 1 Head and neck squamous cell carcinoma

## 1.1 Epidemiology

Head and neck squamous cell carcinoma (HNSCC) develops in the mucosal membranes of the upper aerodigestive tract. HNSCC comprises about 5% of all newly diagnosed cancer cases in the northern and western European countries and the United States (1). Annually there are approximately 500,000 new cases of HNSCC worldwide (2). In parts of Southeast Asia, HNSCC is the most common malignancy accounting for up to 50% of malignant tumors (3). In the year 1998, 2,300 Dutch individuals developed HNSCC and approximately 800 died from it (4). The incidence increases with age, with most patients over the age of 45. The average male to female ratio of patiens with HNSCC in the western developed countries is 3:1, but differs for the various anatomical locations (3). Over the last decade the incidence of HNSCC is increasing in females, presumably related to increased tobacco-smoking.

## 1.2 Treatment and prognosis

In general, one-third of the HNSCC patients presents with early stage (I and II) disease, while two-third presents with advanced disease (stage III and IV) (5). Early stage HNSCC can be cured with surgery or radiotherapy alone in the great majority of cases. Patients with advanced stages are mostly treated with a combination of surgery and radiotherapy.

Despite significant advances in HNSCC treatment modalities, long-term survival of HNSCC patients has only moderately improved during the last 20 years (2). An important reason for this lack of progress is the relatively high tumor recurrence rates observed for these patients. Local recurrences occur in about 10 to 30% of the cases with advanced tumors, even with histopathologically tumor-free surgical margins (6). Moreover, 10 to 20% of the patients develop regional recurrences and 15 to 25% distant metastases (7). Another reason for this lack of progress is the development of second primary tumors (SPT) in the upper aerodigestive tract (8). These SPT develop on average with a constant rate of 2 to 3% per year (9).

## 1.3 Histopathological examination HNSCC

After surgical excision of a primary HNSCC the resection margins are examined by a pathologist to screen for residual cancer as well as to assess the presence and grade of preneoplastic lesions (i.e., dysplasia). Presence of residual tumor in the resection margins, if left untreated, is associated with development of local recurrences and therefore indicates additional therapy such as re-excision or post-operative radiotherapy (10). The presence of epithelial dysplasia in the mucosal margins also needs to be considered when

deciding on post-operative clinical management. Squamous epithelial dysplasia is a histopathological premalignant condition morphologically characterized by aberrant differentiation, atypia and mitotic activity in more superficial epithelial layers (11). The presence of squamous epithelial dysplasia is scored to the standard criteria of the World Health Organization international histological classification of tumors as: a) normal mucosa, b) mild dysplasia, c) moderate dysplasia, d) severe dysplasia or carcinoma *in situ* and e) squamous cell carcinoma (12). Patients with high-grade preinvasive lesions (severe dysplasia and carcinoma *in situ*) in the surgical margins are believed to have a high risk for new tumor development, and therefore often receive additional treatment. In contrast, the presence of mild and moderate dysplasia is generally not considered an indication for further therapy. However, low-grade preinvasive lesions (mild and moderate dysplasia) can also develop into squamous cell carcinomas (13, 14). Notwithstanding the fact that the histological classification is standardized, the grading of dysplasia is at present not suitable for cancer risk assessment. It has been shown to be subjective and associated with a low predictive power (13-15). For these reasons histological grading is of limited value in patient management (16-18).

### 1.4 Local Recurrences

The relatively high incidence of local recurrences in patients with histopathologically tumor-free surgical margins strongly suggests that malignant and/or premalignant cells have remained in the patient. A local recurrence is defined according to clinical criteria, as occurring at a distance less than 1.5 cm or 2 cm away from that tumor and within three years after the (index) tumor (19-22). A generally accepted explanation for the development of local recurrences is that after treatment of the primary tumor, residual tumor cells have been left behind in the patients, which were not detected by routine histological examination of the resection margins, a phenomenon defined as minimal residual cancer (23). Another explanation for local tumor recurrence, based on the above clinical criteria, may be that preneoplastic cells have remained in the patient after resection of the primary tumor, which undergo subsequent malignant transformation resulting in the development of a new tumor.

### 1.5 Second primary tumors

Besides the clinical problems related to the index tumor, HNSCC patients are at high risk to develop second primary tumors (SPTs), often located in the same or adjacent anatomical region. These SPTs occur at a constant rate of 2 to 3% per year for more than 10 years (9). SPTs have in general a poor prognosis, because they arise at well-known unfavourable sites, such as the lungs or esophagus, or in previously irradiated or operated areas. Warren and Gates (1932) provided classification criteria for SPTs, which are still

used at present (19). These criteria are generally used to distinguish a SPT at the same anatomical site from a local recurrence, and at other sites from distant metastasis. These criteria are: 1) each of the tumors must present a definite picture of malignancy, 2) each must be distinct, and 3) the probability of one being a metastasis of the other must be excluded. Histopathology will often solve the issue whether the tumor is malignant, but the other two criteria are a matter of debate and confusion. Some investigators take 1.5 cm (20), others at least 2 cm (22) as distance criterion to distinguish a SPT from a local recurrence at the same or adjacent anatomical site. Finally, also the time interval between the occurrence of the primary and secondary carcinoma is considered by some investigators as a criterion. Tumors that arise after three years are usually considered SPTs (21).

Second primary tumors can be divided into two groups: synchronous SPTs, if they develop simultaneously or within six months after the index tumor, and metachronous SPTs, if they develop more than six months after the index tumor. Most SPTs are metachronous and develop during follow-up of HNSCC patients after curative treatment of their first tumor.

Many concepts have been presented to explain the development of SPTs in HNSCC patients. Initially, it was thought that all SPTs develop independently after extended epithelial exposure to carcinogens (24). This theory of independent origin was supported by comparison of specific genetic alterations (TP53 mutations and microsatellite alterations) between primary tumors and corresponding SPTs (25, 26). In contrast, several other reports using similar techniques suggested that at least a proportion of SPT in HNSCC patients have arisen from one clonal cell population (20, 27-30).

## 2 Field Cancerization and SPT

### 2.1 Field cancerization: Slaughter's concept

Given its relation to the development of local recurrences and SPTs, it is necessary to discuss the process of field cancerization. This concept was already proposed in 1953 by Slaughter *et al*. (24). The authors did not provide a clear definition, but based their theory on histopathological observations in a group of 783 patients with oral cancer. They described some features that seemed to be covered by the term field cancerization: 1) the surgical margins of surgically excised oral squamous carcinomas contained areas of morphologically abnormal mucosa, 2) multiple independent lesions were observed within the same resection specimen, 3) the incidence of second primary tumors found in the patient population was high, 4) the pattern of distribution of the SPT was remarkable since in half of these patients the two separate tumors occurred in the same anatomical area, and

5) the local recurrence rate in the patient population was high. From these observations they concluded that oral carcinomas (including local recurrences and SPT) originate by a process of "field cancerization", in which in a large area of epithelium has been preconditioned for cancer by long-term exposure to carcinogens.

### 2.2 Field cancerization: Polyclonal origin of SPT

As stated earlier, the process of field cancerization is not well defined. The most common interpretation of the work of Slaughter *et al*. is that a large area of the aerodigestive mucosa has been affected by long-term exposure to carcinogens. Multiple lesions exist in the mucosa of the aerodigestive tract that have independently developed. In this preconditioned epithelium, multiple carcinomas can develop, and these would thus not be genetically related. Molecular proof for this independent relation has been provided by comparing genetic alterations in primary tumors and corresponding SPT (25, 26). In addition, Waridel *et al*. were able to show the presence of multiple clones with different genetic aberrations in the oral mucosa (31). Patients with an SPT had more aberrant clones in the normal mucosa than patients without an SPT. Important questions with regard to this latter study are still open: what is the number and size of these clones and can these sensitive techniques also detect aberrant clones in normal control persons (e.g. smokers)?

### 2.3 Field cancerization: Monoclonal origin of SPT

In contrast to the independent origin of SPT, several other reports suggested that at least a proportion of SPT in HNSCC patients have arisen from one clonal cell population (20, 27-30). Various mechanisms have been proposed to explain this common clonal origin of SPT. The theory of micrometastatic spread by Bedi *et al*. (28), hypothesized that some SPT derive from migrated premalignant or malignant cells and hence have the same clonal origin as the primary tumor. At some point of transformation of the primary index tumor, some cells break away and migrate to another site. These cells may gradually replace normal mucosa in some fashion, migrate, or be shed into the saliva and settle down in an area where there is a small mucosal erosion. However, these preliminary results require further confirmative studies. The mechanism behind this common clonal origin needs to be further elucidated.

### 2.4 Field cancerization: tumor adjacent tissue

Moreover, field cancerization can be detected in mucosal tissue surrounding the primary tumor (24). The field cancerization concept predicts the presence of premalignant lesions with genetic alterations adjacent to a malignancy. However, there are conflicting reports as to whether these lesions are clonally related to the primary malignancy.

Califano *et al*. showed that morphologically abnormal mucosal cells surrounding preinvasive and microinvasive lesions shared common genetic alterations with these lesions and it was proposed to have arisen from a single progenitor clone (32). Another study supported this observation and was reason to assume that the field cancerization process precedes the development of cancer (33). It was stated that the study of tumor-adjacent field might reveal clues to the natural history of the primary tumor. In contrast, Lydiatt *et al*. showed in 6 of 13 patients discordant genetic alterations between tumors and their adjacent dysplastic lesions, suggesting in part a multifocal and independent origin (34). They questioned the utilization of surrounding dysplasia as a model for precursor lesions to the adjacent invasive carcinoma. These conflicting results may be explained by the selection of small patients groups and different panels of genetic markers. Ultimately, this issue should be solved by studies using more samples and more extensive panels of genetic markers.

## 3 Genetic Alterations in HNSCC

### 3.1 Tumorigenesis

To date it is widely accepted that an accumulation of genetic and epigenetic alterations in oncogenes and tumor suppressor genes forms the basis for the progression from a normal cell to a cancer cell, referred to as multistep carcinogenesis (32, 35). Some of the alterations specifically occur in genes that play a crucial role in the normal behavior of the cell, but often these changes appear in less important sequences and may be considered a consequence rather than a cause in carcinogenesis (35). In recent years considerable progress has been made in the genetic analysis of HNSCC development. Three types of genetic changes have widely been used to compare first and second primary tumors as well as to detect field cancerization; microsatellite instability (MSI), patterns of allelic loss and TP53 mutations. These changes occur frequently and are considered to be clonally preserved.

### 3.2 MSI and Patterns of Allelic Loss

Cancer cells are genetically unstable and display extensive chromosomal changes, including amplifications, deletions, and translocations. Some of these changes can be determined by allele-specific markers such as microsatellites because these markers allow distinction of maternal and paternal alleles. Microsatellites are small repetitive sequences that often are highly polymorphic in the population. By PCR amplification and subsequent electrophoretic separation, the maternal and paternal alleles can be distinguished, when the number of repeats differs between the two alleles (the marker is then called “informative”). These markers were originally exploited for genetic linkage studies and

later applied for genetic analysis of tumor DNA. The loss of a given marker in tumor DNA is generally considered as a hallmark of the loss of a specific tumor suppressor gene, located in proximity of that marker. Loss of the locus is thought to be one of the two inactivation steps in the complete inactivation of a tumor suppressor gene, one allele inactivated by mutation and one allele inactivated by loss (36).

In practice, tumor DNA is compared with normal DNA (usually isolated from blood lymphocytes) for multiple microsatellite markers. After PCR amplification and electrophoretic separation, two bands representing the two alleles are seen in the normal tissue DNA. When only one band is seen in the tumor DNA then the other allele has been lost, referred to as "loss of heterozygosity" (LOH). Because the method can theoretically not discriminate loss of one allele over the gain of another, the loss of a particular allele in a clinical sample can also be the result from amplification of the other allele. The term "allelic imbalance" seems therefore more correct. LOH analysis is generally performed in microdissected tumor tissue to ensure minimal contamination with normal cells. The presence of wild type DNA from the normal cells will overshadow the loss of an allele.

The genetic instability of tumors can also be reflected in changes in the length of the microsatellite (shifts), indicative of microsatellite instability (MSI). MSI is characterized as a tumor-specific change of length in a microsatellite, as a result of either insertion or deletion of repeating units, when compared with matched normal DNA (37). MSI has been described in numerous human neoplasms, but it is particularly common in most hereditary nonpolyposis colorectal carcinomas and in a proportion of nonhereditary colorectal tumors (38, 39). This seems to be an infrequent event in HNSCC, but the probability to detect MSI increases when AAAG nucleotides repeats are used, and the number of loci is extended (40).

### 3.3 TP53 Mutations

Alterations of the tumor suppressor gene TP53 through allelic deletion or mutation, or both, are the most frequently identified common genetic events in human tumors (41-43). The TP53 tumor suppressor gene encodes a stress-induced transcription factor, a phosphoprotein that steers cell cycle regulation and apoptosis. The TP53 gene maps on chromosome 17p13.1 and contains 3 functional domains, a transcription activation domain, a DNA-binding domain, and an oligomerization domain.

TP53 mutations are common in HNSCC. The incidence reported varies from 43% to 96%, which is most likely a reflection of differences in the mutation detection techniques (44, 45). Mutations can also be found in dysplastic lesions (46), showing that the gene is often mutated before the invasive cancer develops (47).

### 3.4 Genetic progression model HNSCC

Based on a genetic analysis of mucosal lesions with an apparently progressive histopathological appearance, Califano *et al*. (1996) presented a genetic progression model for HNSCC [Fig. 1] (32). In this study eighty-seven lesions of the head and neck, including preinvasive lesions and benign lesions associated with carcinogen exposure, were tested using microsatellite analysis for allelic loss at 10 major chromosomal loci. They showed that the spectrum and frequency of chromosomal loss progressively increased at each histopathological step from benign hyperplasia to dysplasia to carcinoma in situ to invasive cancer.

As the authors noted, this model is certainly not complete, and the temporal sequence of two closely occurring events in progression may in fact be arbitrary. Furthermore, the difficulty remains that not all of the genetic mutations involved in cancer development are known. Moreover, mucosa does not necessarily need to undergo histologic change to possess genetic alterations (34). In general, this model supports the multistep theory of progression by an increasing accumulation of genetic events, but is far from complete.

Using microsatellite analysis several chromosomal areas have been identified which are likely to harbor tumor suppressor genes for HNSCC (48-51). According to this model: chromosomal loss at 9p, 3p and 17p are supposed to be early transforming events, mutations in the p53 gene as well as chromosomal loss at 13q, 18q and 8p are supposed to be late transforming events in HNSCC carcinogenesis (32).

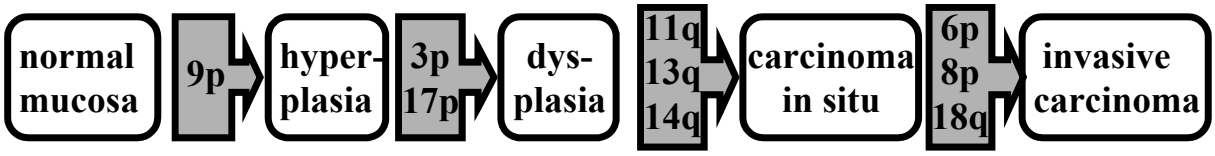


***Figure 1.*** *Genetic progression model for HNSCC according to Califano et al. (1996). Genetic alterations in HNSCC carcinogenesis are shown within the arrows. The numbers indicate chromosomal arms that show allelic losses, often indicative for loss of tumor suppressor genes.*

## 4 Aim of the study and outline of this thesis

As outlined above it is critical to establish new strategies to identify patients at risk and to optimize their management in order to prevent further cancer development. Therefore it is of uttermost importance to learn more about the molecular mechanisms behind local recurrences and SPTs.

Local recurrences are often thought to result from minimal residual cancer. Originally, it was thought that all SPT develop independently after widespread epithelial exposure to carcinogens. Recently, indications have been found for a common origin of index tumor and corresponding SPTs. Various mechanisms have been proposed to explain this proposed common clonal origin of SPT such as shedding of premalignant and/or malignant cells into the saliva and implantation at other sites or lateral migration of malignant cells.

Further improvements in the long-term survival of HNSCC patients can only be achieved if the factors responsible for development of SPTs and local recurrences are elucidated. Different mechanisms of development of secondary cancers may require different therapeutic strategies. When the pathobiological basis of SPTs and local recurrences are explained, it may become possible to identify the patients groups that are at high risk for new cancers at the time when the first tumor is diagnosed and treated.

The aim of the work described in this thesis was to investigate comprehensively the field cancerization concept and its relation to the development of local recurrences and SPTs after curative therapy of the primary tumor.

In **Chapter 2** we describe the reliability of microsatellite alterations, patterns of allelic loss and TP53 point mutations as clonal markers for the diagnosis of HNSCC.
In **Chapter 3** the genetic alterations in HNSCC and the accompanying macroscopically normal mucosa are studied by LOH and TP53-mutation analysis. Furthermore genetic differences between tumor and field(s) are described. In addition, the clinical significance of genetically altered mucosa remaining after treatment is discussed with respect to the development of SPT and/or local recurrences.
In **Chapter 4** the relations between molecular alterations, morphological grading and the number of proliferating cells in preneoplastic proliferating fields are described.
In **Chapter 5** we investigate whether first and second primary tumors are clonally related and originate from a single genetically altered field
In **Chapter 6** a pathobiological study of locally recurrent HNSCC in patients with histologically tumor-free resection margins is described.
In **Chapter 7** a summary, general discussion and future perspectives of this thesis are presented.

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# 2

# Discordance of Genetic Alterations Between Primary Head and Neck Tumors and Corresponding Metastases Associated With Mutational Status of the *TP53* Gene

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## Abstract

Ample molecular data are available on the progression from normal mucosa to invasive head and neck squamous cell carcinoma (HNSCC), but information on further genetic progression to metastatic disease is scarce. To obtain insight in the metastatic process we compared 23 primary HNSCC with 25 corresponding lymph node metastases (LNM) and 10 corresponding distant metastases (DM) with respect to *TP53* mutations and patterns of loss of heterozygosity (LOH) based on 26 microsatellite markers on six chromosome arms (3p, 9p, 17p, 13q, 8p and 18q). In 18 of the 23 patients, a *TP53* mutation was detected in the primary tumor, and in all cases the same *TP53* mutation was present in the corresponding LNM or DM. In nine of 20 patients with LNM and three of seven patients with DM, the LOH-pattern of the metastasis differed from that in the corresponding primary tumor at least one marker. Microsatellite markers located on chromosome arms 13q, 8p and 18q were most frequently discordant, providing evidence that alterations at these chromosomes occur late in HNSCC carcinogenesis. Moreover, evidence was found that DM had developed directly from the primary tumor and not from LNM. Remarkably, we observed that the mutational status of the *TP53* gene is associated significantly with the degree of genetic differences between primary HNSCC and corresponding metastases. All patients with *TP53* wild-type primary tumors showed significantly more discordant LOH-patterns in their corresponding LNM and DM, than patients with *TP53*-mutated tumors. The percentages were 100% versus 27% (LNM) and 100% versus 0% (DM), respectively ($P = 0.008$ and $P = 0.029$; two-sided Fisher exact test). This finding suggests that *TP53*-mutated tumors need less additional genetic alterations to develop metastases as compared to *TP53* wild-type primary tumors.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) makes up about 5% of all newly diagnosed cancer cases in the northern and western European countries and the United States (1). Despite advances in therapy, long-term survival of HNSCC patients has not improved significantly during the past 20 years (2). Important reasons for this lack of progress are the relatively high regional failure rates and the development of distant metastasis (DM) in patients at advanced stages of disease (3).The number of lymph node metastases (LNM) in the neck is important in terms of a patient's prognosis. For patients without LNM, there is a risk of only 7% of DM, whereas for patients with more than three positive lymph nodes this risk is almost 50%. Not only the number of LNM but also the level in the neck and the presence of extranodal spread are important prognosticators (3).

Recent discoveries in the area of cancer genetics have revolutionized the understanding of the process of primary tumor development, including HNSCC. HNSCC arises as the result of mutations and epigenetic alterations in tumor suppressor genes and oncogenes, reflected by loss of heterozygosity (LOH) at chromosome arms 9p, 3p, 17p, 4q, 18q, 13q, and 8p (4). Dysplastic lesions often show changes at chromosome arms 9p, 3p, and 17p, and therefore these alterations are referred to as "early" events (4, 5). The later changes that are associated with invasive growth and metastatic behavior are less well defined but, in a number of tumors, may encompass LOH at 18q and 8p (4, 6, 7, 8). Mutations in the *TP53* tumor suppressor gene are present in the majority of head and neck cancers. It is well known that loss of *TP53* function is associated with increased genetic instability, as evidenced in cell lines and *TP53*-deficient mice (9, 10). *TP53* status therefore might have a role in the genetic pathway to metastasis.

Hardly any information is available on whether specific genetic alterations are related to metastatic behavior via either the lymphogenic or hematogenic route. Clinical experience indicates that lymphogenic metastases precede hematogenic metastases, suggesting that hematogenic metastases develop from lymphogenic metastases. There are some recent data, however, that hematogenic spread might occur at a relatively early stage (11, 12).

Comparing genetic abnormalities in primary head and neck tumors and corresponding metastases can give insight into the role of specific metastasis-suppressing or metastasis-promoting genes. Allelic losses in primary HNSCC and corresponding LNM have been compared in two studies, both of which showed small differences in LOH-pattern (13, 14). Also, with respect to *TP53* mutational status, comparisons between primary HNSCC and LNM have been reported, but with conflicting results. Some studies reported complete concordance of *TP53* mutations (15, 16), whereas other studies showed

different *TP53* mutations in primary HNSCC and corresponding LNM (17, 18). The comparison between primary HNSCC and corresponding distant metastases has not been studied. Furthermore, no studies have been published in which primary HNSCC and both LNM and DM from the same patient were analyzed. This approach may clarify the biological relationship between the processes of lymphogenic and hematogenic metastastic spread in HNSCC.

To study the genetic alterations involved in HNSCC metastasis, we analyzed both *TP53* mutations and LOH-patterns in corresponding specimens of primary tumors and LNM or DM from 23 HNSCC patients.

## Materials and methods

### Patients and tumor specimens

In total, 57 tissue samples of the primary tumor, LNM, or DM were collected from 23 HNSCC patients. From 16 patients samples of the primary tumor and corresponding LNM were obtained, from three patients samples of the primary tumor and corresponding DM were collected, and from four patients samples of the primary tumor and of both corresponding LNM and DM were acquired. Forty-two tissue samples were obtained as freshly frozen material and 15 tissue samples as archival paraffin-embedded material. Patients' characteristics are summarized in Table 1. Tumor stages (pTN) were determined according to the International Union Against Cancer (UICC) criteria (19). The DM were sampled from the skin (lower back in patient 19 and abdomen in patient 23), the lung (several lesions in both lungs from patients 20, 21, and 22), the iliac bone (patient 17), the spleen (patient 18), the kidney (patient 18), the heart (patient 18), and the liver (patient 22). The time period between resection of primary tumor and biopsy of the DM (in months) is cited in Table 1. All LNM were resected simultaneously with the primary tumor.

***TABLE 1.*** *TP53 Mutations in 23 Primary Head and Neck Tumors*

| Patient | Age | Sex | Site[a] | pTN | Time (mo)[b] | *TP53* mutation[c] | Exon | Codon | Aa change[d] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 48 | M | Vallecula | T2N2b | | G → A | 6 | 216 | Val → Met |
| 2 | 65 | M | FOM | T3N2b | | T → A | 5 | 163 | Tyr → Asn |
| 3 | 54 | M | Piriform sinus | T4N2b | | G → T | 8 | 298 | Glu → Stop |
| 4 | 55 | M | Vallecula | T4N2c | | 17 bp del | 7 | 247-252 | Frameshift |
| 5 | 67 | F | Tongue | T2N1 | | Wt | | | |
| 6 | 47 | M | Tonsil | T3N2a | | G → A | 8 | 273 | Arg → His |
| 7 | 60 | M | Piriform sinus | T4N2b | | 1 bp del | 6 | 219 | Frameshift |
| 8 | 55 | M | Epiglottis | T3N2c | | G → T | 7 | 248 | Arg → Leu |
| 9 | 54 | M | Piriform sinus | T4N2b | | 6 bp ins | 7 | 235 | Ins Tyr, Asn |
| 10 | 68 | M | Tongue | T3N2b | | 8 bp del | 5 | 126 | Splice site |
| 11 | 52 | M | Supraglottis | T4N3 | | G → T | 8 | 275 | Cys → Phe |
| 12 | 74 | M | FOM | T4N2b | | Wt | | | |
| 13 | 64 | F | Gingiva | T4N2b | | T → C | 5 | 127 | Thr → Asn |
| 14 | 72 | M | Tongue | T3N2b | | 1 bp del | 5 | 151 | Frameshift |
| 15 | 57 | M | Supraglottis | T3N2c | | C → G | 7 | 236 | Tyr → Stop |
| 16 | 62 | F | Base of tongue | T2N1 | | G → A | 8 | 273 | Arg → His |
| 17 | 53 | F | FOM | T3N2c | 4 | Wt | | | |
| 18 | 67 | F | FOM | T3N2c | 4 | T → A | 5 | 130 | Leu → His |
| 19 | 59 | M | FOM | T2N1 | 30 | 2 bp del | 9 | 307 | Frameshift |
| 20 | 52 | F | Tonsil | T3N2b | 21 | Wt | | | |
| 21 | 61 | F | Tonsil | T3N1 | 17 | Wt | | | |
| 22 | 59 | F | Transglottis | T4N2b | 11 | 1 bp del | 5 | 151 | Frameshift |
| 23 | 69 | M | Supraglottis | T2N0 | 13 | G → T | 7 | 248 | Arg → Leu |

*a FOM = floor of mouth*
*b Time period between resection of primary tumor and biopsy of the DM (in months)*
*c del, deletion; ins, insertion; wt, wild type*
*d Aa change = amino acid change*

**Microdissection and DNA extraction**

Freshly frozen tumor samples were cut on a cryomicrotome, and tissue sections (10 μm) were mounted on microscopic glass slides. From the paraffin-embedded tumor samples, 10 μm sections were obtained, placed on microscopic glass slides, and subsequently deparaffinized in xylene. In all cases, the first and last tissue sections were stained with hematoxylin-eosin for histological analysis and to guide microdissection. The other tissue sections were stained with 1% toluidine blue and 0.2% methylene blue and manually microdissected under a stereomicroscope. All microdissected samples were made up > 80 % of tumor cells. Dissected tissues were treated with 1 mg/ml proteinase K for 24 h at 52 ºC in 100 μl of buffer containing 100 mM TRIS (pH 9.0), 10 mM NaCl, 1% SDS, 5 mM EDTA. The DNA was purified by phenol-chloroform extraction and collected by ethanol precipitation using 2 μg of glycogen as carrier. The DNA was redissolved in LoTE-buffer (3 mM TRIS and 0.2 mM EDTA at pH 7.5). Normal DNA was isolated from blood samples obtained at the time of surgery or from connective tissue or muscle microdissected from the sections. The DNA concentration was measured by microfluorometry with the Hoefer Dynaquant (Amersham/Pharmacia Benelux NV, Roosendaal, the Netherlands).

**Selection of chromosomal loci for microsatellite analysis**

To evaluate LOH, we used 26 microsatellite markers located at chromosome arms 3p, 9p, 17p, 13q, 18q and 8p. These markers were selected because they frequently demonstrate LOH in HNSCC (4, 20-23). The following markers were used: *D3S1284* (3p12), *D3S1274* (3p12), *D3S1217* (3p13), *D3S1766* (3p14), *D3S1029* (3p21), *D3S1293* (3p24), *D9S171* (9p21), *D9S1748* (9p21), *D9S1751* (9p21), *IFNA* (9p21), *D9S162* (9p22), *D9S157* (9p22), *CHRNB1* (17p11-12), *TP53* (17p13.1), *D17S1866* (17p 13.3), *D13S294* (13q14.3), *D13S168* (13q14.3), *D13S170* (13q31), *D13S158* (13q32), *D18S34* (18q12), *D18S57* (18q12), *D18S35* (18q21), *D8S136* (8p21), *GZ-14/15* (8p22), *D8S261* (8p22) and *D8S1130* (8p23). Primer sequences were obtained from the Genome Database for all of these markers (http://gdbwww.gdb.org/).

**Microsatellite analysis**

Microsatellite analysis was carried out on an automated ABI PRISM sequencer (310 Genetic Analyzer; Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). One primer (Isogen Bioscience, Maarssen, the Netherlands) of each marker was end-labeled with one of the fluorescent dyes FAM, HEX or NED (Applied Biosystems). DNA (10 ng) was amplified by multiplex polymerase chain reaction (PCR) (involving two or three markers) in a total volume of 10 μl containing 2 pmol of each labeled and unlabeled

primer. Details of the multiplex PCR are available on request. The PCR buffer included 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, and 0.5 U of *Taq* DNA polymerase (AmpliTaq, Perkin Elmer, Gouda, the Netherlands). PCR amplifications for each primer set were performed for 35 cycles, consisting of denaturation at 94 ºC for 1 minute, annealing at a temperature between 55 and 65 ºC (depending on the primer set) for 1 minute, and extension at 72 ºC for 2 minutes. The amplified product was diluted in sterilized water, usually five times. For analysis, 12 µl of deionized formamide was combined with 0.5 µl of Genescan-350 [ROX] size standard (Applied Biosystems) and 1 µl of diluted PCR product in a Genetic Analyzer sample tube. The samples were loaded on the automated sequencer and run following the supplier's protocol. The data were analyzed with GeneScan Analysis software (version 1.2; Applied Biosystems). LOH was scored if one allele was decreased by more than 50% in the tumor sample when compared with the same allele in normal control DNA. Stutter correction was used as previously described for microsatellites that demonstrated a significant degree of polymerase slippage (24).

***TP53* sequencing**

The primary tumors of the 23 HNSCC patients were sequenced for exons 5-9 of the *TP53* gene. Sequencing was performed as described by Sidransky et al. (25). Briefly, an 1.8-kb fragment of the *TP53* gene, encompassing the exons 5-9, was amplified from DNA of microdissected frozen tumor specimens. For paraffin-embedded material the exons were amplified separately. Purified PCR products were sequenced directly by exon-specific primers using the radioactive dideoxynucleotide method (AmpliCycle Sequencing Kit; Applied Biosystems) (25). Primer sequences and reaction conditions are available on request. When a *TP53* mutation was detected in the DNA of the primary tumor, the mutation was confirmed for that particular exon in DNA isolated from the corresponding LNM and/or DM. When *TP53* mutations could not be detected in DNA of primary tumors, the DNA of the corresponding LNM and/or DM were sequenced for mutations in exons 5-9 of the *TP53* gene.

**Histopathological classification**

All hematoxylin-eosin slides were examined by a pathologist (J.A.K.) and scored according to the standard criteria of the World Health Organization international histological classification of tumors (26). Primary tumors and metastases were classified as follows: 1) well differentiated, 2) moderately differentiated, and 3) poorly differentiated. The pathologist had no information on molecular data before histopathological classification.

### Statistical Methods

The patients were stratified into two groups: patients with *TP53*-mutated primary tumors and patients with *TP53* wild-type primary tumors. Differences in frequency of discordant LOH and/or microsatellite instability (MSI) between primary tumors and corresponding metastases for the stratified groups were analyzed by the two-sided Fisher exact test. Differences in the total number of discordant LOH/MSI between the stratified groups were analyzed using the two-sided Mann-Whitney U test. When the *P* value was less than 0.05, the difference was considered significant.

## Results

### Comparison of *TP53* gene mutations in primary tumors and metastases

In 18 of the 23 patients (78%) a *TP53* mutation was detected in the primary tumor (Table 1). In five cases, a frameshift mutation was detected (three cases with a 1-bp deletion, one case with a 2-bp deletion, and one case with a 17-bp deletion). In one case, an insertion of 6 bp was found that resulted in the insertion of the amino acids tyrosine and asparagine, and in one case a splice site mutation was detected. Two (non-sense) mutations resulted in a stop-codon. The nine missense mutations included four transitions (G → A in three cases and T → C in one case) and five transversions (T → A in two cases and G → T in three cases). In all 18 patients, the same mutation was found in the corresponding LNM and/or DM. In the five cases with wild-type *TP53*, the corresponding LNM or DM also lacked a *TP53* mutation (Figure 1).

### Comparison of LOH/MSI patterns in primary tumors and metastases

In nine of 20 patients, different LOH patterns and microsatellite alterations were noted in primary tumors and LNM (Figure 1). In total, one discordant microsatellite alteration and 24 discordant allelic losses were seen among 402 informative allelic comparisons. In the three cases (patients 1, 12, and 20) with several LNM, the most discordant LNM was scored. Patient 1 had one discordant loss; patient 5 had one discordant microsatellite alteration; patients 2, 6, 17, and 19 had two discordant losses; patient 12 had four discordant losses; patient 20 had five discordant losses; and patient 21 had six discordant losses. Nine instances of discordance involved an allelic loss observed in the LNM but not in the primary tumor (patients 1, 12, 17, and 21). Fifteen instances of discordance involved an allelic loss in the primary tumor that was not evident in the corresponding LNM (patients 2, 6, 19, 20, and 21). In the last case, a microsatellite alteration was noted in the LNM but not in the primary tumor (patient 5).

In three of seven patients, different LOH patterns and microsatellite alterations were found in primary tumors and DM (Figure 1). In total, 18 discordant allelic losses and four discordant microsatellite alterations were noted among 141 informative allelic comparisons (Figure 1). Patient 21 had three discordant losses and two discordant microsatellite alterations, patient 20 had seven discordant losses, and patient 17 had eight discordant losses and two discordant microsatellite alterations (Figure 1). Eleven instances of discordance involved an allelic loss observed in the DM but not in the primary tumor (patients 17 and 20). Seven instances of discordance involved an allelic loss in the primary tumor that was not evident in the corresponding DM (patients 17, 20, and 21). In the other four cases, a microsatellite alteration was found in the DM but not in the primary tumor (patients 17 and 21).

The time period between resection of the primary tumor and the biopsy of the DM was not associated with increased differences in genetic alterations between primary and metastatic tumors. The average period between resection of the two tumors was 15 months for cases with identical LOH patterns and 14 months for cases with discordant LOH patterns.

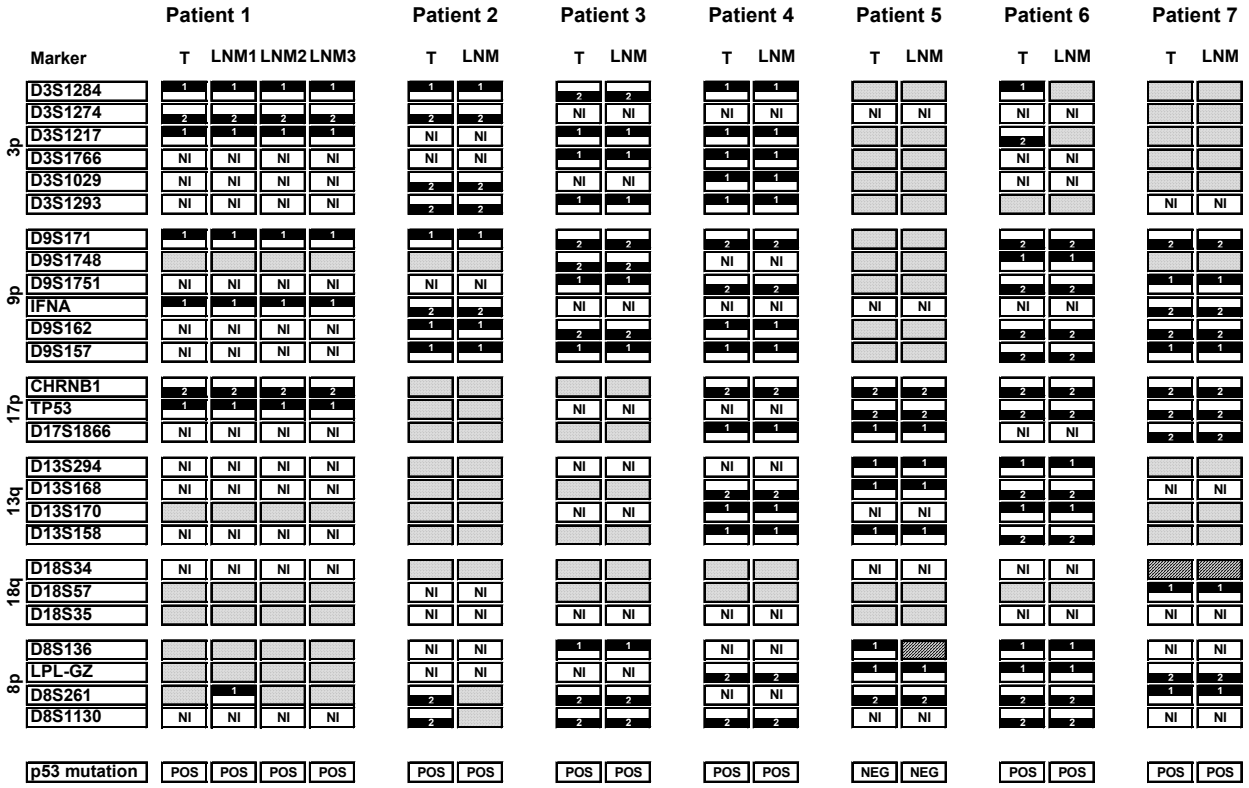
The frequency of discordant LOH/MSI between primary tumor and metastasis per microsatellite marker is depicted in Figure 2. Markers *D18S35* and *D8S1130* showed most discordances (30%), whereas markers *D3S1274*, *D9S171*, *D9S1751*, *D9S162* and *D9S157* showed no discordance at all (Figure 2).

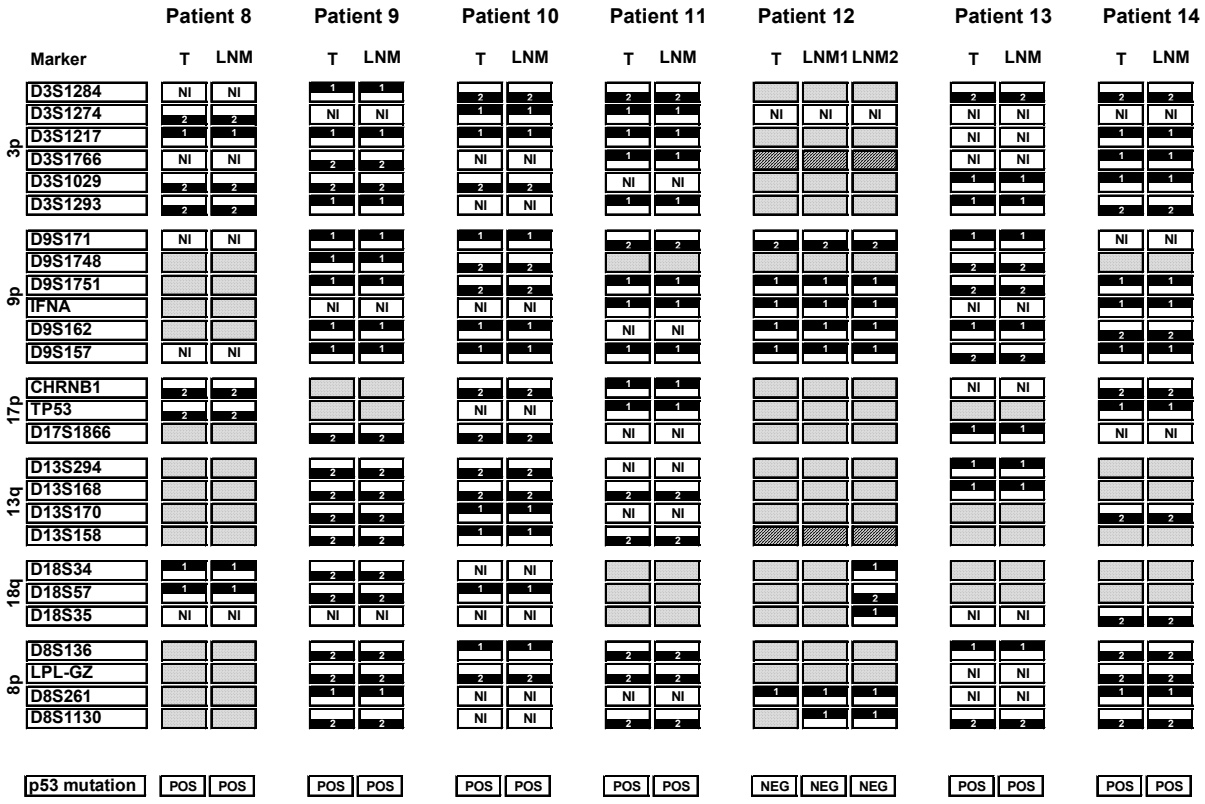
**Comparison between LNM and DM**

There were no statistically significant differences in the number of discordant allelic losses and microsatellite alterations between comparisons of primary tumor and corresponding LNM versus primary tumor and corresponding DM. Both LNM and DM could be obtained from four patients (17, 19, 20, and 21). Interestingly, in these four patients, the genetic alterations of the LNM differed from those in the DM, suggesting that these two types of metastasis arose from different subclones or acquired different alterations after separation from the primary tumor.

**Histological comparison between primary tumors and metastasis**

Two primary tumors were scored as well differentiated, 10 as moderately differentiated, and 12 as poorly differentiated HNSCC. All metastases except the DM of patients 20 and 21 showed the same degree of differentiation as did the corresponding primary tumors.


Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
Patient 7
Marker
T
LNM1
LNM2
LNM3
LNM
3p
D3S1284
D3S1274
D3S1217
D3S1766
D3S1029
D3S1293
9p
D9S171
D9S1748
D9S1751
IFNA
D9S162
D9S157
17p
CHRNB1
TP53
D17S1866
13q
D13S294
D13S168
D13S170
D13S158
18q
D18S34
D18S57
D18S35
8p
D8S136
LPL-GZ
D8S261
D8S1130
p53 mutation
POS
NEG
NI


Patient 8
Patient 9
Patient 10
Patient 11
Patient 12
Patient 13
Patient 14
Marker
T
LNM
LNM1
LNM2
3p
D3S1284
D3S1274
D3S1217
D3S1766
D3S1029
D3S1293
9p
D9S171
D9S1748
D9S1751
IFNA
D9S162
D9S157
17p
CHRNB1
TP53
D17S1866
13q
D13S294
D13S168
D13S170
D13S158
18q
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p53 mutation
POS
NEG
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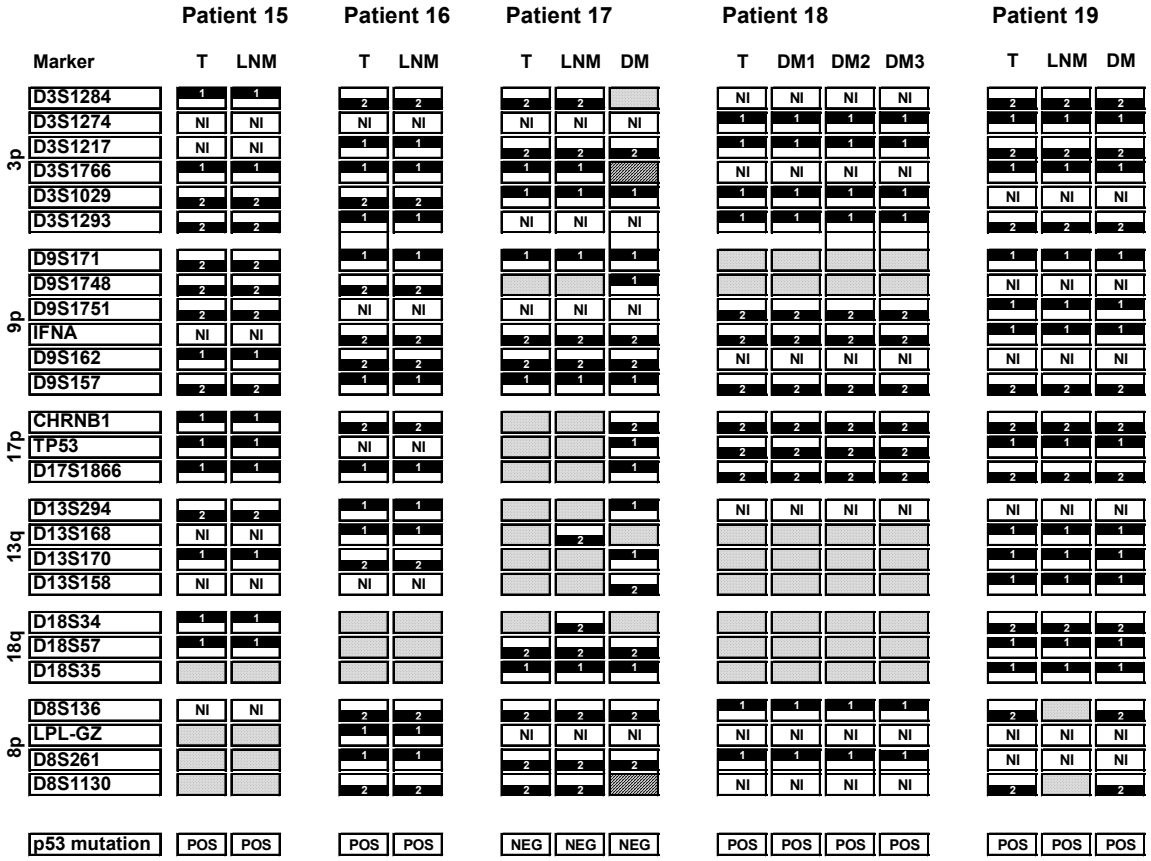
Patient 15
Patient 16
Patient 17
Patient 18
Patient 19
Marker
T
LNM
DM
DM1
DM2
DM3
3p
9p
17p
13q
18q
8p
D3S1284
D3S1274
D3S1217
D3S1766
D3S1029
D3S1293
D9S171
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D9S1751
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D9S162
D9S157
CHRNB1
TP53
D17S1866
D13S294
D13S168
D13S170
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p53 mutation
POS
NEG
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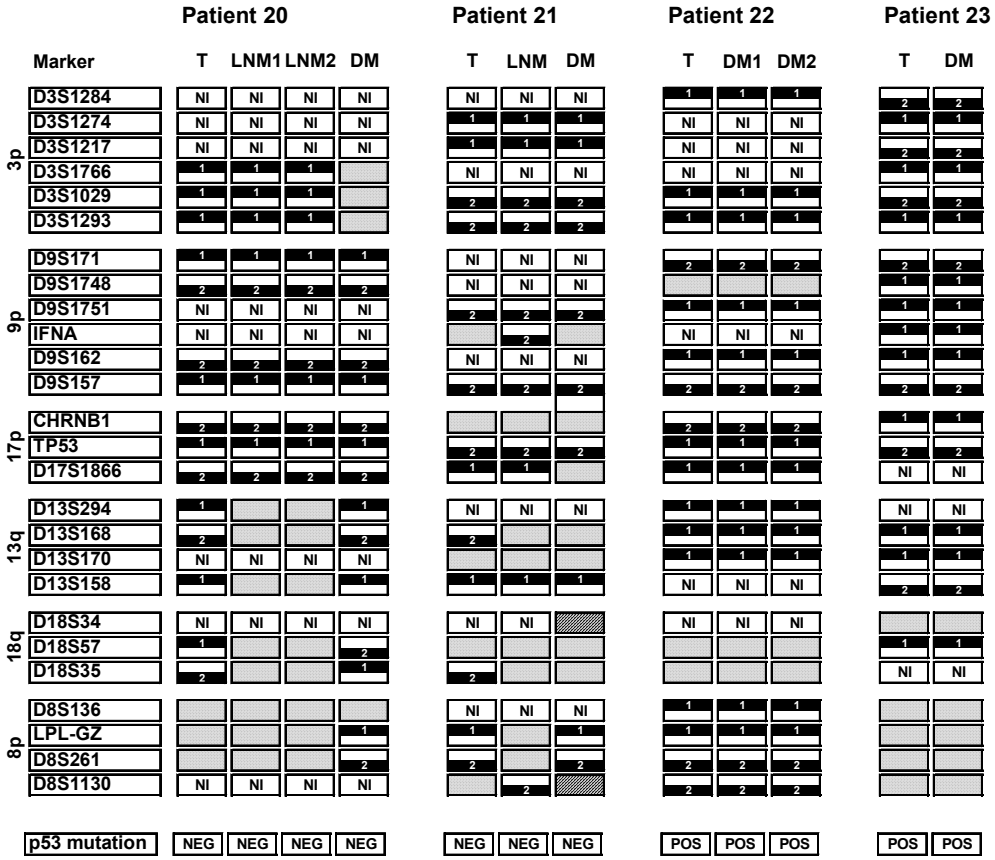
Patient 20
Patient 21
Patient 22
Patient 23
Marker
T
LNM1
LNM2
LNM
DM
DM1
DM2
3p
9p
17p
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18q
8p
D3S1284
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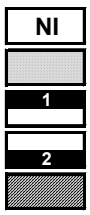
D3S1766
D3S1029
D3S1293
D9S171
D9S1748
D9S1751
IFNA
D9S162
D9S157
CHRNB1
TP53
D17S1866
D13S294
D13S168
D13S170
D13S158
D18S34
D18S57
D18S35
D8S136
LPL-GZ
D8S261
D8S1130
p53 mutation
NEG
POS
NI

***Figure 1.*** *Results of the 23 primary HNSCC and corresponding LNM and DM. The TP53 status are indicated as neg (wild type) and pos (mutated). The codon and type of mutation are listed in Table 1. T = tumor, LNM = lymph node metastasis, and DM = distant metastasis.*




*Not informative*
*No LOH*
*Larger allele is lost*
*Smaller allele is lost*
*Microsatellite instability*

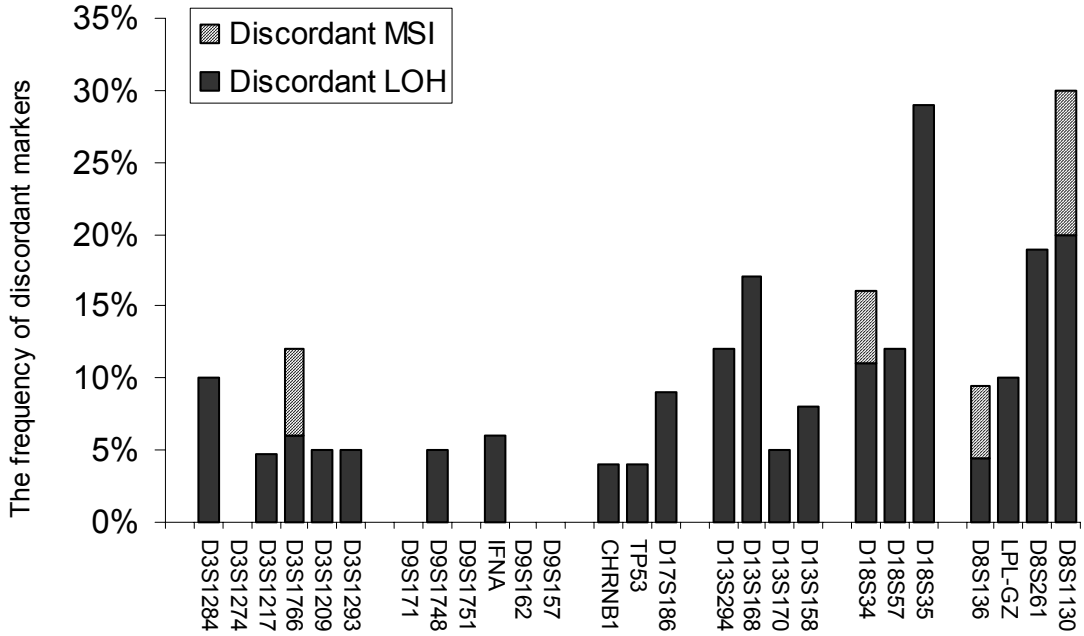



***Figure 2.*** *The rate of discordant LOH/MSI between primary tumor and metastasis (LNM and DM) is shown per microsatellite marker. Only informative markers were scored. When two or three LNM/DM were investigated, the LNM/DM that was the most discordant was scored. From patients with both LNM and DM, both metastases were scored separately. Notice the higher frequency of discordance in microsatellite markers located on chromosome arms 13q, 8p and 18q (proposed to be late markers in HNSCC carcinogenesis) compared with microsatellite markers located on chromosome arms 3p, 9p and 17p (proposed to be early markers in HNSCC carcinogenesis).*

**Comparison between tumors with and without a *TP53* mutation**

We noted that patients without a *TP53* mutation in the primary tumor showed higher rates of discordant LOH/MSI patterns in the corresponding LNM/DM than did patients with a *TP53* mutation in the primary tumor. The relationship between the mutational status of the *TP53* gene and the rates of discordance is shown in Table 2.

***Table 2. Number of patients with discordant LOH/MSI pattern: Comparison of primary tumor and metastasis in TP53 (-) and TP53 (+) tumors***

| Type of metastasis | n [a] | Number of patients with discordant LOH/MSI pattern (%) | P-value |
|---|---|---|---|
| LNM[b] | | | |
| *TP53* (-) | 5 | 5/5 (100%) | 0.008 |
| *TP53* (+) | 15 | 4/15 (27%) | |
| Total | 20 | 9/20 (45%) | |
| DM[b] | | | |
| *TP53* (-) | 3 | 3/3 (100%) | 0.029 |
| *TP53* (+) | 4 | 0/4 (0%) | |
| Total | 7 | 3/7 (43%) | |

*a n, number of patients.*
*b LNM = lymph node metastasis; DM = distant metastasis*

Differences in the LOH/MSI patterns of the LNM compared with the primary tumor were significantly more common in *TP53* wild-type tumors than in *TP53*-mutated tumors (100% versus 27%; P=0.008 with the two-sided Fisher exact test). Differences in the LOH/MSI patterns of the DM compared with the primary tumor were also significantly more common in *TP53* wild-type tumors than in *TP53*-mutated tumors (100% versus 0%; P=0.029 with the two-sided Fisher exact test). The relative number of informative microsatellite markers that showed different results in primary tumor and the corresponding metastasis was calculated per patient. In cases with more than one LNM or DM, the mean number of differences was taken for calculation. The degree of discordance differed in the group of *TP53*-mutated tumors compared with the group of wild-type tumors for both LNM (P=0.006) and DM (P=0.019), using the Mann-Whitney U test. Interestingly, microsatellite alterations in the metastasis but not in the primary tumor were detected only in cases with wild-type *TP53*; once in a LNM (patient 5) and two times in a DM (patients 17 and 21; Figure 1).

## Discussion

In this study, we found complete concordance of *TP53* mutations in primary HNSCC and corresponding LNM and DM. The literature on this issue yields conflicting data. In a number of studies, the clonal stability of *TP53* mutations was confirmed (15, 16), whereas in other studies different *TP53* mutations in primary HNSCC and corresponding LNM have been reported (17, 18). Our findings support the idea that *TP53* mutations develop before metastasis (both LNM and DM) and are maintained during clonal outgrowth. These findings make *TP53* mutations very suitable as clonal markers for HNSCC.

The LNM and DM from HNSCC shared most of the allelic losses of the corresponding primary tumors but differed from them at a limited number of loci. Our observations confirm the data of the two studies that reported limited differences in LOH-patterns between HNSCC and corresponding LNM (13, 14). There were no significant differences in the rate of discordant genetic alterations between primary tumors and corresponding LNM and primary tumors and DM. Interestingly, analyses of LNM and DM obtained from the same patients showed that both types of metastasis had different patterns of genetic alterations. This genetic discordance between lymphogenic metastasis and hematogenic metastasis from the same patient is suggestive of subclone heterogeneity within the primary tumor at the time of the metastasis formation; alternatively, it points to further clonal evolution of metastatic cells after separation from the primary tumor. These findings also suggest that in a proportion of HNSCC patients hematogenic spread occurs at an early stage, and that DM do not develop from LNM. These results confirm the data in published reports (11, 12).

Microsatellite loci located at chromosome arms 13q, 8p and 18q scored the highest rate of discordance, providing additional evidence that alterations at these loci occur late in HNSCC carcinogenesis. LOH at these chromosome arms in primary tumors has been associated with tumor progression and poor prognosis in patients with HNSCC (6-8, 27). Microsatellite markers located at chromosome arms 3p, 9p and 17p are suitable as clonal markers in HNSCC, because the LOH patterns of these markers are relatively stable during tumor progression.

Discordant LOH patterns in metastases and primary tumors can be explained by low numbers of metastatic clones in the primary tumors. In support of this theory, the existence of intratumoral heterogeneity has been demonstrated in many tumor types, for example, melanomas (28) and ovarian tumors (29). Another explanation for the observed genetic differences between primary and metastatic tumors is that additional genetic alterations accumulate in the metastatic cell clone after separation from the parental tumor. If this is the case, time does not appear to be an important factor in this process,

since we did not see any influence of the time period between the resection of the primary and metastatic tumors on the number of discordant alterations.

The most remarkable finding of this study was that the status of the *TP53* gene was associated with the rate of discordant genetic alterations between primary tumor and the corresponding metastasis. The TP53 protein plays a central role in the cellular response to DNA damage. Increased levels and activity of TP53 result in blocking of the replication of damaged DNA and in the elimination of cells that have acquired DNA mutations (30). In the majority of HNSCC, *TP53* is inactivated as the result of a mutation in the gene. In the remaining HNSCC, *TP53* can be inactivated by binding to viral proteins (31), or as result of alterations in genes which products interact with TP53 (e.g., MDM2). Notwithstanding the limited number of investigated patients, we found that the presence of a *TP53* mutation in the primary tumor was associated significantly with the degree of genetic difference between the primary and corresponding metastatic tumor. A possible explanation is that tumor cell clones with a *TP53* mutation have a relatively high capacity to metastasize and do not need additional genetic hits, whereas tumor cell clones with wild-type *TP53* need additional genetic hits to metastasize. The implication of this explanation would be that head and neck tumors with a mutated *TP53* gene show a relatively poor prognosis. Conflicting data have been reported on this topic. Some studies showed no association between clinical outcome/disease progression and presence of *TP53* mutations (32, 33) whereas others did (34). In two additional studies, the effects of different types of mutations have been evaluated. One group showed that *TP53* DNA contact mutations resulted in an accelerated tumor progression and reduced survival (35), and the other group showed that *TP53* mutations that caused obvious changes in protein structure appeared to be an important prognostic factor in HNSCC (36).

The finding that the mutational status of the *TP53* gene is associated significantly with the degree of genetic difference between primary HNSCC and corresponding metastases might have implications for the interpretation of studies that were designed to predict the occurrence of metastases by genetic analysis of the primary tumor. In wild-type *TP53* tumors, the clone that makes up most of the tumor at the primary site differs genetically from the dominant clone in the metastasis. The genetic pattern of the primary tumor will therefore not represent the clone with metastatic capacity. To investigate whether specific genetic alterations are related to metastatic behavior, it seems more logical to focus on *TP53*-mutated HNSCC. The clone at the primary site in these cases is genetically identical to the clone with metastatic capacity. A comprehensive comparison of the genetic profiles of metastasizing and nonmetastasizing *TP53*-mutated tumors might uncover specific genetic alterations involved in the metastatic process.

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# 3

# Persistence of Genetically Altered Fields in Head and Neck Cancer Patients: Biological and Clinical Implications

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## Abstract

In 1953, Slaughter *et al.* [Slaughter *et al.*, Cancer, *6:* 963–968, 1953] proposed the concept of field cancerization in patients with squamous cell carcinoma of the head and neck (HNSCC) and discussed its clinical significance for the development of second primary tumors and local recurrences. To define the process of field cancerization and its putative clinical implications, we analyzed genetic aberrations in HNSCC and the accompanying macroscopically normal mucosa. In 28 HNSCC patients, loss of heterozygosity was determined in tumor and five non-contiguous mucosal biopsies using eight microsatellite markers at 9p, 3p, and 17p. For patients who showed loss of heterozygosity in their mucosal biopsies, all margins of the surgical specimen were subsequently analyzed to determine the extension of the field. In these cases, additional markers at 8p, 13q, and 18q as well as *p53* mutations were included to determine subclonal differences between field and tumor. Genetically altered fields were detected in 36% (10 of 28) of the HNSCC patients. The field varied in size between patients and consisted of genetically different subclones. In 7 of 10 cases, the field extended into the surgical margins. Genetic analysis strongly indicated a clonal relationship between tumor and accompanying genetically altered field. The picture emerges that these tumors have developed within a pre-existing preneoplastic field. One particular patient with a genetically altered field in a surgical margin developed a local recurrence after 28 months of follow-up. Microsatellite analysis showed that this recurrence had more molecular markers in common with the nonresected premalignant field than with the original tumor, suggesting that this persistent field has progressed further into a new malignancy. Our data show that genetically altered mucosa remains after treatment in a significant proportion of HNSCC patients, which may explain in part the high frequency of local recurrences and second primary tumors. Adequate identification and risk assessment of these genetically altered fields may have profound implications for future patient management.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) comprises about 5% of all newly diagnosed cancer cases in the Northern Western European countries and the United States (1). Despite advances in therapy, long-term survival of HNSCC patients has not significantly improved during the last 20 years (2). An important reason for this lack of progress is the relatively high recurrence rates observed in these patients. Local recurrences occur in about 10-30% of the cases with advanced tumors, even with histopathologically tumor-free surgical margins after resection (3). Another reason for this lack of progress is the development of second primary tumors (SPTs) in the upper aerodigestive tract (4). The reported incidence of these SPTs varies, but on average they develop with a constant rate of 2 to 3% new cases per year.

Two theories have been postulated to explain the high frequency of SPTs: (*a*) micrometastatic spread of (pre)malignant cells gives rise to genetically related SPTs; or (*b*) multiple transforming events give rise to genetically unrelated SPTs. The theory of micrometastatic spread was proposed by Bedi *et al.* (5). Other authors also suggested that at least a proportion of SPTs in HNSCC patients have arisen from one clonal population (6, 7).

The second theory is based on the concept of field cancerization. This concept was already proposed in 1953 by Slaughter *et al.* (8), who hypothesized that oral carcinomas (including SPTs) originate by a process of field cancerization, in which an area of epithelium has been preconditioned by long-term exposure to carcinogens. The authors based their theory on multiple histopathological observations in a group of 783 patients with oral cancer. They presented as supporting evidence that: (*a*) the surgical margins contained areas of abnormal mucosa; (*b*) multiple independent lesions were observed within the same resection specimen; (*c*) the incidence of SPTs found in their patient population was high; (*d*) the pattern of distribution of the SPTs was typical because in half of these patients the two separate tumors occurred in the same anatomical area; and (*e*) the local recurrence rate in oral cancer is high, which may be due to abnormal mucosa that was not excised during treatment of the primary tumor.

This concept of field cancerization can be interpreted in various ways to explain the phenomenon of SPTs. In the classical view, which is most commonly referred to, large areas of the aerodigestive tissue are affected by long-term exposure to carcinogens. In this preconditioned epithelium, multifocal carcinomas can develop as a result of independent mutations and thus would not be genetically related (9, 10). An alternative view of the field concept may also explain the phenomenon of SPTs. In this alternative model, a single cell is transformed and gives rise to one large extended premalignant field by clonal expansion and gradual replacement of normal mucosa. In this field of various subclones,

two separate tumors can develop after accumulation of additional genetic alterations. Both tumors have the same clonal origin and would thus share at least one early genetic event, which occurred before the initial clonal expansion. Intriguingly, this model of "expanding fields" could also explain the high rate of seemingly local recurrences. A premalignant field that extends beyond surgical margins and thus is not excised after surgical treatment of the index tumor could progress further and give rise to a "new" tumor at the same site.

It is now generally accepted that most solid tumors result from a multistep process involving the clonal evolution of abnormal cell populations that gain a selective growth advantage over normal cells by accumulating specific alterations in two group of genes, the proto-oncogenes and the tumor suppressor genes (11, 12). HNSCC is thought to progress through a series of well-defined histopathological stages that run parallel to specific genetic changes. Using microsatellite analysis, several chromosomal areas have been identified that are likely to harbor tumor suppressor genes for HNSCC (13–16). Chromosomal loss at 9p, 3p, and 17p is supposed to be an early transforming event in HNSCC and is therefore an ideal marker to study the concept of field cancerization (11). Mutations in the *p53* gene as well as chromosomal loss at 13q, 18q, and 8p are supposed to be late transforming events and are therefore more suitable to study subclonal differences between between field and tumor (11).

The aim of this study was to determine the frequency, extension, and persistence of the process of field cancerization in patients with HNSCC using extensive microsatellite analysis. Subclonal differences between tumor and field(s) were studied by microsatellite and *p53* mutation analysis. In addition, the clinical significance of genetically altered mucosa remaining after treatment was discussed with respect to the development of SPTs and/or local recurrences.

## Materials and Methods

### Patients and biopsies

The field cancerization study was approved by the Institutional Review Board Vrije Universiteit Medical Center, and written informed consent was obtained from all patients. In a pilot study, we enrolled 11 HNSCC patients to assess whether field cancerization could be detected at various sites by LOH. In total, two mucosal biopsies were analyzed from these patients, one < 0.5 cm (*F1*) and one > 0.5 cm (*F5*) from the edge of the tumor (Fig. 1). Genetically altered fields could be detected, but they appeared to be limited in size and distribution, and we decided to increase the number of biopsies for analysis to reduce the sampling error. In total, 28 patients who underwent surgical treatment for HNSCC in 1999 were subsequently included. Selection criteria used to include patients were tumor site (oral cavity and oropharynx) and histopathologically tumor-free margins. Selection on site was performed to enable sampling of exfoliated cells during clinical follow-up. Tumor stages (pTNM) were determined according to the criteria of the International Union Against Cancer (UICC) (17): T1N0 (seven patients), T2N0 (eight patients), T2N1 (three patients), T2N2b (six patients), T3N0 (one patient), T3N1 (one patient), and T3N2b (two patients). The distribution of the tumors by anatomical site was as follows: 20 tumors were located in the oral cavity (12 in the tongue, 6 in the floor of mouth, and 2 in the retromolar trigone); and 8 were located in the oropharynx (1 in the soft palate, 3 in the tonsil, 1 in the base of the tongue, and 3 in the anterior tonsillar pillar). Fourteen patients were treated postoperatively with radiotherapy. The age of the patients ranged from 38–77 years, with an average age of 57 years. In total, 13 patients were male, and 15 were female. Twenty patients were smokers, and 8 were never-smokers. Immediately after surgery, the surgical specimen was taken to the pathology department. In addition to the standard sampling, the specimens were analyzed according to another protocol: biopsies of the tumor and of macroscopically normal mucosa were taken from the surgical specimen, four of which (*F1–F4*; one per quadrant) were < 0.5 cm from the edge of the tumor, and one of which (*F5*) was > 0.5 cm from the edge of the tumor (Fig. 1). The distant mucosal biopsy (> 0.5 cm from the edge of the tumor) was taken only when the size of the surgical specimen was large enough (in 20 of 28 cases). All samples were directly snap-frozen in liquid nitrogen and stored at - 80°C until further processing. For patients who showed genetic evidence of field cancerization, the routinely paraffinembedded surgical margins (used for histopathological assessment of complete tumor excision) were also analyzed to study the extension of the field. Moreover, persistence of field cancerization was examined in exfoliated cells taken from the scar during clinical follow-up. Cells were collected using a cytobrush (Cytobrush Plus;

Medscand AB, Malmö, Sweden). The brushes were stirred in 4 ml of PBS, allowing the cells to be released.

The cells were washed once with PBS, and DNA was isolated from the pellets according to the protocol described for tissues (see below).

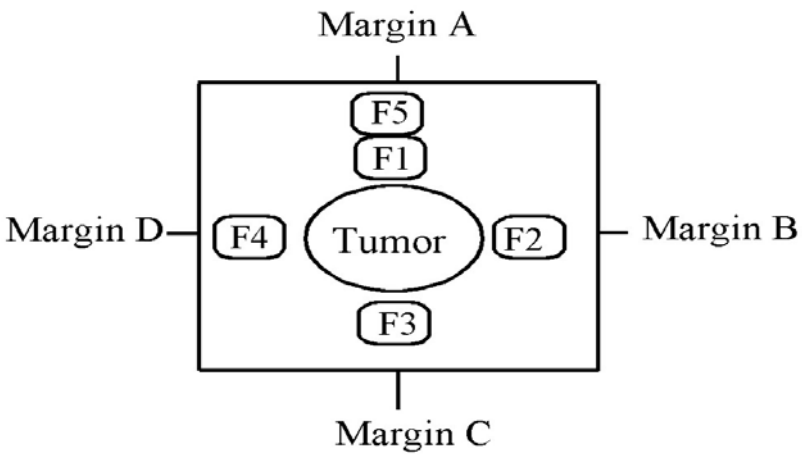



***Figure 1.*** *Schematic drawing of the surgical specimen. Biopsies of the tumor and macroscopically normal mucosa were taken from the surgical specimens; four biopsies (F1–F4; one per quadrant) were < 0.5 cm from the edge of the tumor, and one biopsy (F5) was > 0.5 cm from the edge of the tumor. The specimen was marked to enable reorientation after analysis of the biopsies. In patients who showed molecular evidence of field cancerization, the paraffin-embedded surgical margins (A–C or A–G, depending on the size of the specimen) were also analyzed to study the extension of the fields.*

**Microdissection and DNA extraction**

Fresh-frozen tumor and mucosa samples were cut on a cryomicrotome, and tissue sections (10 µm) were mounted on microscopical glass slides. From the paraffin-embedded surgical margins 10 µm sections were obtained, placed on microscopical glass slides and subsequently deparaffinized in xylene. In all cases the first and last tissue sections were stained with hematoxylin-eosin (HE) for histological assessment and to guide microdissection. After consultation of a pathologist (JAK), the other tissue sections were stained with 1% toluidine blue and 0.2% methylene blue and microdissected under a stereomicroscope. In the mucosal biopsies, histopathologically normal mucosal epithelium was separately dissected from histopathologically abnormal mucosal epithelium. All microdissected samples contained > 80 % of cells of interest (normal epithelium, dysplasia or tumor). Dissected tissues were treated with 1 mg/ml proteinase K for 24 h at

52 ºC in 100 µl buffer containing 100 mM TRIS (pH 9.0), 10 mM NaCl, 1% SDS, 5 mM EDTA. The DNA was purified by phenol-chloroform extraction and collected by ethanol precipitation using 2 µg glycogen as carrier. The DNA was redissolved in LoTE-buffer [3 mM TRIS; 0.2 mM EDTA (pH 7.5)]. Normal DNA was isolated from blood samples obtained at the time of surgery. The DNA concentration was measured by microfluorometry with the Hoefer Dynaquant (Amersham/Pharmacia Benelux NV, Roosendaal, the Netherlands).

**Histopathological classification**

All H&E-stained slides were examined by a pathologist (J. A. K.) and scored according to the standard criteria of the WHO international histological classification of tumors (18). Lesions were classified as: (*a*) normal mucosa; (*b*) mild dysplasia; (*c*) moderate dysplasia; (*d*) severe dysplasia or carcinoma *in situ*; and (*e*) squamous cell carcinoma. A representative selection of cases was independently screened by a second pathologist (Dr. P. J. van Diest). In the few cases for which there was a discrepancy in classification, a final consensus judgement was made. Neither pathologist had information on clinical and molecular data before screening.

**Selection of chromosomal loci for microsatellite analysis**

To detect the molecular presence of field cancerization, we examined the status of chromosomes 9p, 3p, and 17p using eight polymorphic microsatellite markers. The specific markers used in this study were selected because they identify a minimal area of loss at putative tumor suppressor gene loci and because they are lost frequently and early during HNSCC tumorigenesis (11). The tumor and concordant microdissected mucosal biopsies were analyzed using the following eight microsatellite markers at 3p12 (D3S1284), 3p14 (D3S1766), 3p21 (D3S1029), 3p24 (D3S1293), 9p21 (D9S171), 9p22 (D9S157), 17p11–12 (CHRNB1), and 17p13 (TP53). From patients who showed LOH in one or more mucosal biopsies, microsatellite markers at other chromosomal loci were also examined to study the differences between tumor and genetically altered field in the mucosa. These markers are proposed to detect late events in the HNSCC carcinogenesis, based on the frequencies with which these alterations are found at different premalignant stages (11, 19). The additional microsatellite markers used were located at the following chromosomal regions: (*a*) 8p22 (D8S261); (*b*) 8p23 (D8S1130); (*c*) 13q14 (D13S294); (*d*) 13q31 (D13S170); (*e*) 18q12 (D18S34 and D18S57); and (*f*) 18q21 (D18S35). Moreover, in these patients, the routinely paraffin-embedded surgical margins were also analyzed (with both marker sets) to study the extension of the field. Primer sequences were obtained from the Genome Database for all of these markers (http://gdbwww.gdb.org/).

**Microsatellite Analysis.**

The analysis of microsatellite markers was performed with two different methods. The first experiments were performed by PCR amplification with radioactively labeled primers (Isogen Bioscience, Maarssen, the Netherlands), followed by electrophoretic gel separation. Before amplification, one primer (5 pmol) was end-labeled with [$\gamma$-$^{32}$P]ATP (0.74 MBq; Amersham, Hertogenbosch, the Netherlands) and T4 polynucleotide kinase (Roche, Almere, the Netherlands) in a total volume of 10 µl. The PCR reactions were carried out in a final reaction volume of 10 µl containing 10 ng of genomic DNA, 0.5 pmol of labeled primer, and, respectively, 1.5 or 2.0 pmol of each unlabeled primer. The PCR buffer included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, and 0.5 unit of Taq DNA polymerase (AmpliTaq; Perkin-Elmer, Gouda, the Netherlands). PCR amplification was performed for 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C to 65°C (depending on the primer set) for 1 min, and extension at 72°C for 2 min. One-third of the PCR product was separated electrophoretically on 6% urea-formamide-polyacrylamide gels and visualized by autoradiography. All PCR products were quantified by scanning densitometry and ImageQuant software (version 3.1; Molecular Dynamics). Allelic loss was defined when $(S^n/L^n)/(S^t/L^t)$ was <0.5 or >2.0. $S^n$ and $S^t$ are the densitometric signals from the small allele of the normal and tumor DNA, respectively, and $L^n$ and $L^t$ are the densitometric signals from the large allele of the normal and tumor DNA, respectively. When the alleles differed in size by only 2 or 4 bp, a stutter band from the large allele often comigrated with the full-length product amplified from the smaller allele. In these cases, stutter correction was used. For a particular marker, the relative contribution to the stutter bands is calculated from a noninformative sample and used to calculate the relative abundance of the second allele to the first stutter band of the first allele (20). Later experiments were carried out on an automated ABI PRISM sequencer (310 Genetic Analyzer; PE Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). One primer of each marker was end-labeled with one of the fluorescent dyes (FAM, HEX, or NED; PE Applied Biosystems). Microsatellites (10 ng) were amplified by multiplex PCR (involving two or three markers) in a total volume of 10 µl containing 2 pmol of each labeled and unlabeled primer. Details of the multiplex PCR are available on request. PCR buffer and PCR conditions were the same as those described above. The amplified product was diluted in sterilized water, usually five times. For analysis, 12 µl of deionized formamide were combined with 0.5 µl of Genescan-350 (ROX) size standard (PE Applied Biosystems) and 1 µl of diluted PCR product in a Genetic Analyzer sample tube. The samples were loaded on an ABI PRISM 310 Genetic Analyzer and run following the supplier's protocol. The data were analyzed with GeneScan Analysis software (version 1.2; PE Applied

Biosystems). LOH was scored using the formula described above when necessary after stutter correction.

**p53 Sequencing and Plaque Assay.**

For patients whose mucosal biopsies showed genetic evidence of field cancerization, tumor DNA was analyzed for *p53* mutations. When a mutation was detected, DNA of the genetically altered fields were subsequently sequenced. Sequencing was performed as described previously (21). In short, a 1.8-kb fragment of the *p53* gene encompassing the exons 5–9 was amplified from the DNA of microdissected tissue specimens. Purified PCR products were sequenced directly by exon-specific primers using the radioactive dideoxynucleotide method (AmpliCycle Sequencing Kit; Perkin-Elmer, Norwalk, CT; Ref. 21). Primer sequences and reaction conditions are available on request. Plaque assays were performed on *p53* exon fragments amplified from exfoliated cell DNA as described by Van Houten *et al.* (20). In short, the amplimers were digested with *Eco*RI, cloned in 56 lambda GT11 vector arms, packaged *in vitro*, and plated on *Escherichia coli* K12 LE392. Approximately 5000 plaques were hybridized differentially with either an end-labeled mutant or wild-type oligonucleotide as probe. After autoradiography, the number of mutant/wild-type plaques was calculated.

## Results

**Molecular Evidence for Field Cancerization.**

In the pilot study of 11 patients, we analyzed two mucosal biopsies, one < 0.5 cm (*F1*) and one > 0.5 cm (*F5*) from the edge of the tumor (Fig. 1). In 2 of 11 patients, genetic alterations were detected in both mucosal biopsies. In the first patient, the field also extended into the margins of the surgical specimen, whereas in the second patient the field appeared to be limited in size because it did not reach the resection margins. Based on these observations, we concluded that analysis of biopsies taken in only a single quadrant adjacent to the tumor could result in an underestimation of the number of patients with genetically altered fields. For the 28 patients subsequently enrolled, we therefore analyzed the tumor and four adjacent mucosal biopsies surrounding the tumor (one per quadrant; < 0.5 cm from the edge of the tumor) and, when available, one distant mucosal biopsy (> 0.5 cm from the edge of the tumor).

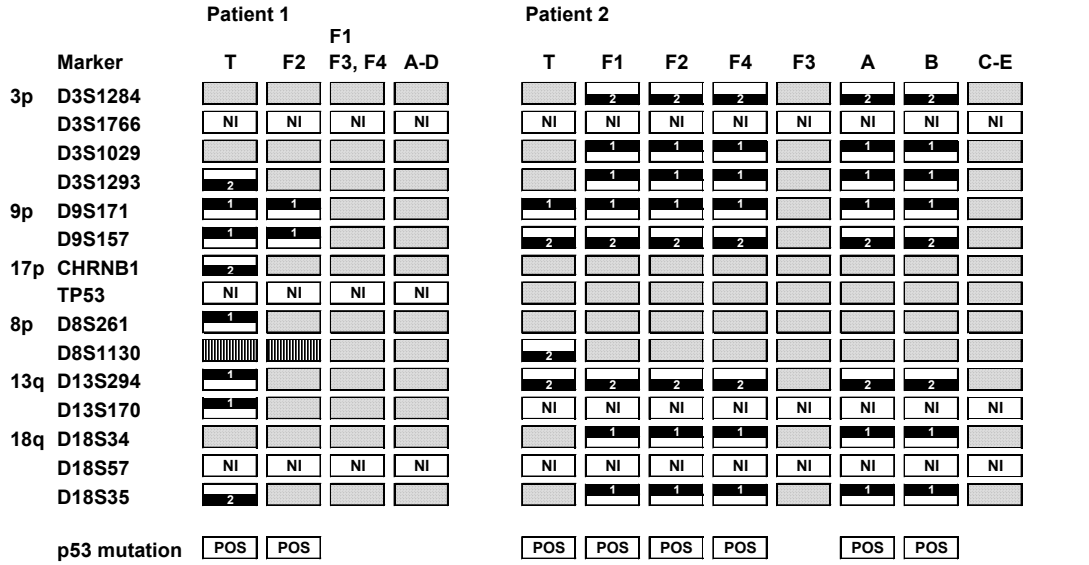
The tumors and the mucosal epithelium of the biopsies were microdissected and analyzed for LOH. When present, histologically abnormal mucosal epithelium was microdissected separately from the histologically normal mucosal epithelium. In the tumors of these 28 patients, LOH was scored in at least one chromosomal locus (Table 1).

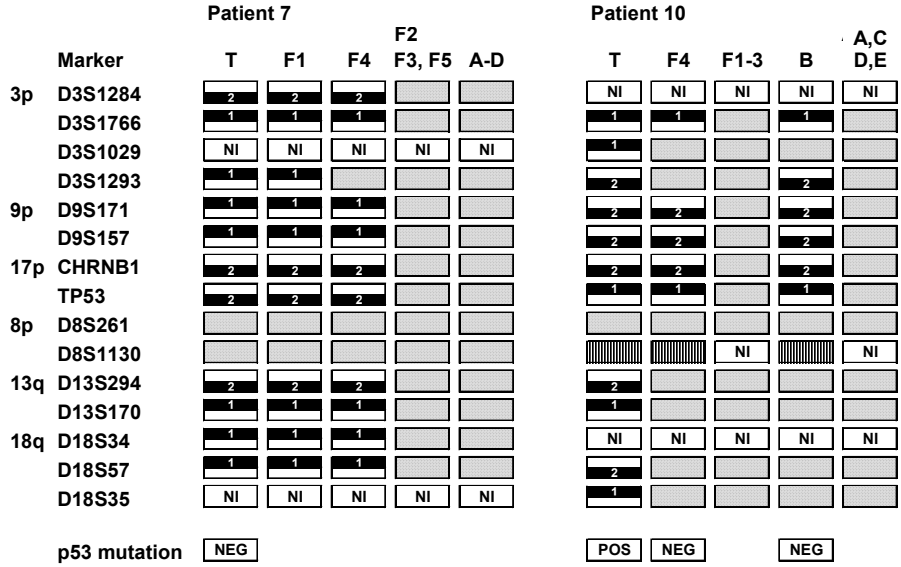
In 10 of 28 (36%) patients, at least one mucosal biopsy showed LOH in one or more microsatellite marker(s). Fig. 2 shows the summary of the microsatellite analysis of these 10 patients. The number of mucosal biopsies with genetic alterations varied from one to three per patient (Fig. 2). In the 10 patients who showed genetic evidence of field cancerization, the paraffin-embedded surgical margins were also analyzed to study the extension of the field. In 7 of 10 patients, the surgical margins showed LOH in one or more microsatellite marker(s) (Fig. 2). In all cases, the localization of the surgical margins that showed genetic alterations corresponded to that of the mucosal biopsy or biopsies with LOH, strongly suggesting that these genetically altered fields were connected. The number of surgical margins that showed genetic alterations varied from one to two per patient (Fig. 2). Hence, the size of the field varies considerably between patients and can extend beyond the surgical margins.

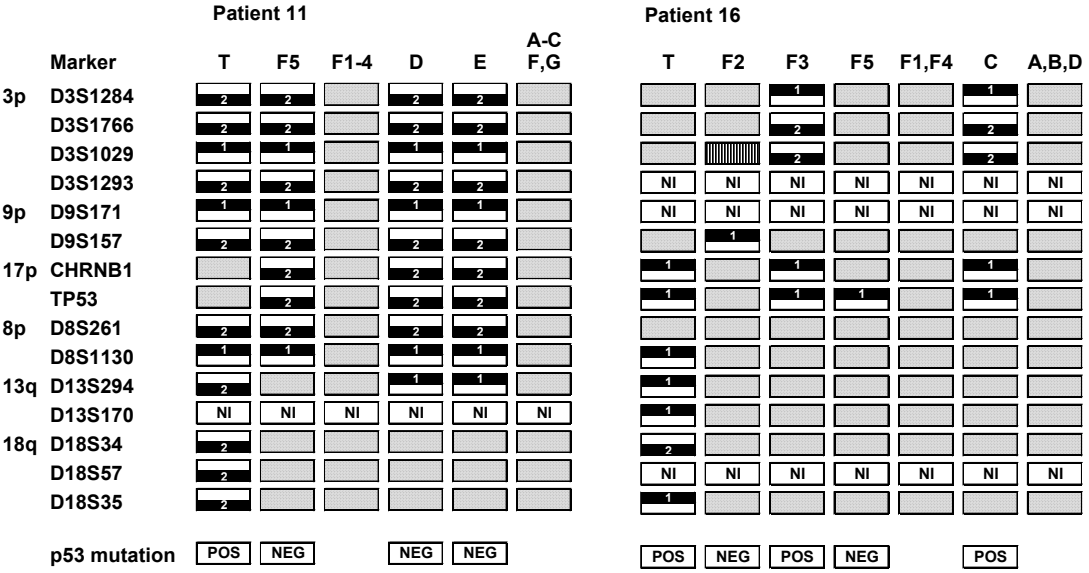
**Table 1.** *Frequency of LOH in HNSCC*[a]

| Locus | Map position[b] | LOH (%)[c] |
|---|---|---|
| D3S1284 | 3p12 | 17/21 (81) |
| D3S1766 | 3p14 | 18/23 (78) |
| D3S1029 | 3p21 | 13/22 (59) |
| D3S1293 | 3p24.3 | 16/22 (73) |
| D9S171 | 9p21 | 20/21 (95) |
| D9S157 | 9p22 | 20/25 (80) |
| CHRNB1 | 17p11-12 | 15/21 (71) |
| TP53 | 17p13.1 | 16/24 (67) |

[a]Results were generated from analysis of tumor DNA from 28 patients
[b]The map positions are based on the Genome Data Base (http://gdbwww.gdb.org/)
[c]Number of tumors with LOH/ total number of informative patients


Patient 1
Patient 2
Marker
T
F2
F1
F3, F4
A-D
F1
A
B
C-E
3p D3S1284
D3S1766
D3S1029
D3S1293
9p D9S171
D9S157
17p CHRNB1
TP53
8p D8S261
D8S1130
13q D13S294
D13S170
18q D18S34
D18S57
D18S35
NI
p53 mutation
POS


Patient 7
Patient 10
Marker
T
F1
F4
F2
F3, F5
A-D
F4
F1-3
B
A,C
D,E
3p D3S1284
D3S1766
D3S1029
D3S1293
9p D9S171
D9S157
17p CHRNB1
TP53
8p D8S261
D8S1130
13q D13S294
D13S170
18q D18S34
D18S57
D18S35
NI
p53 mutation
NEG
POS


Patient 11
Patient 16
Marker
T
F5
F1-4
D
E
A-C
F,G
F2
F3
F1,F4
C
A,B,D
3p D3S1284
D3S1766
D3S1029
D3S1293
9p D9S171
D9S157
17p CHRNB1
TP53
8p D8S261
D8S1130
13q D13S294
D13S170
18q D18S34
D18S57
D18S35
NI
p53 mutation
POS
NEG

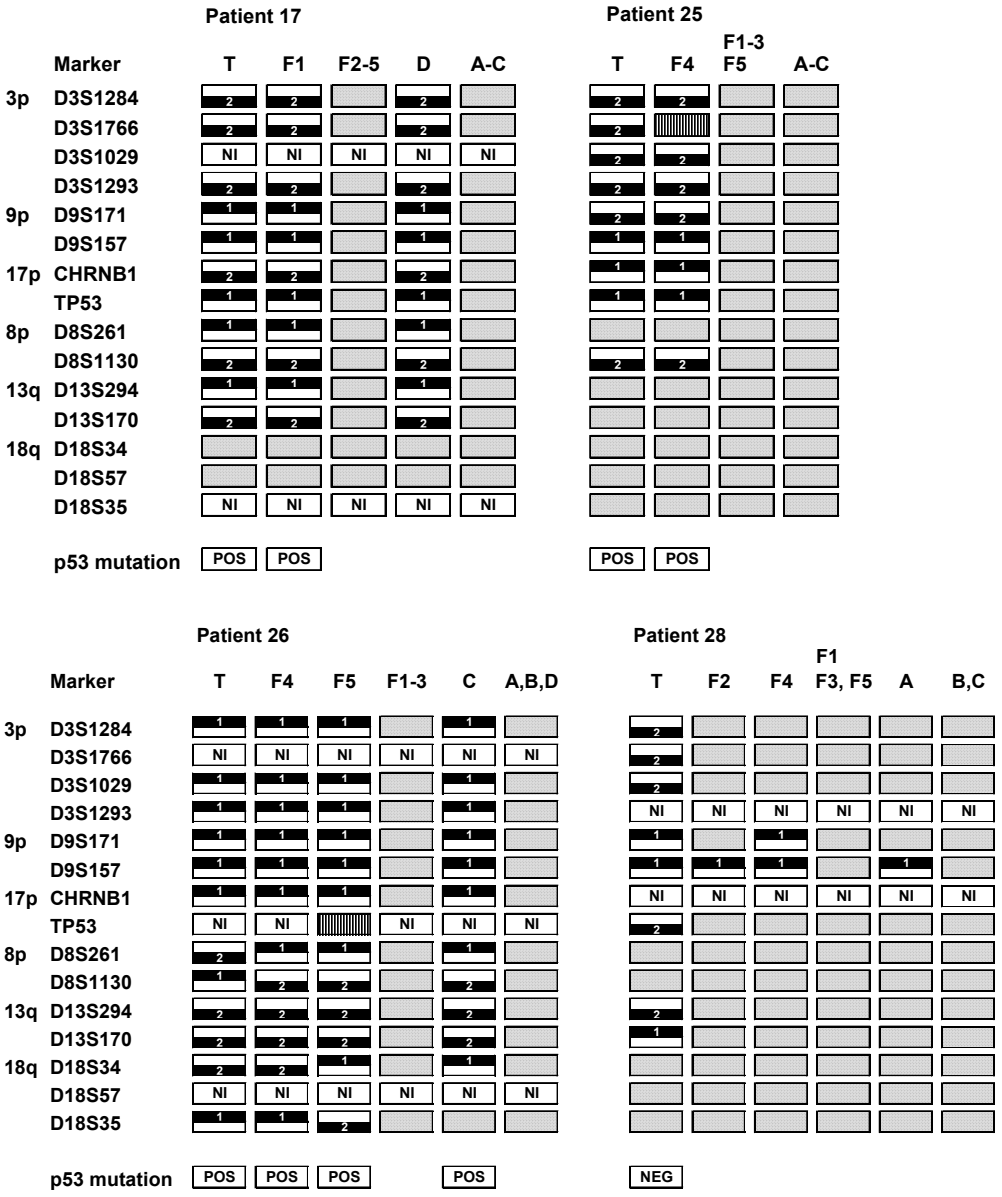

Patient 17
Marker
T
F1
F2-5
D
A-C
3p D3S1284
D3S1766
D3S1029
D3S1293
9p D9S171
D9S157
17p CHRNB1
TP53
8p D8S261
D8S1130
13q D13S294
D13S170
18q D18S34
D18S57
D18S35
p53 mutation
POS
Patient 25
T
F4
F1-3
F5
A-C
Patient 26
Marker
T
F4
F5
F1-3
C
A,B,D
Patient 28
T
F2
F4
F1
F3, F5
A
B,C
NEG
NI
Non Informative
NO LOH
Larger allele is lost
Smaller allele is lost
Microsatellite instability

**Figure 2.** *Results of the 10 patients who showed genetic evidence of field cancerization. F1-F5 represent the 5 mucosal biopsies taken of the surgical specimen (see Fig.1), and A-G represent the surgical margins (see Fig.1). The p53 mutations are indicated as NEG (negative) and POS (positive). The codon and type of mutation are listed in Table 2. In all cases, the surgical margins that showed genetic alterations corresponded to the localization of the mucosal biopsy (or biopsies) with LOH, making it likely that these genetically altered fields were connected. Please note that the margins were randomly coded by the pathologist, independent of the coding that was used for the mucosal biopsies. Therefore, each patient has a unique orientation of the mucosal biopsies with respect to the surgical margins (unlike the one that is presented in Figure 1). For example, in one case, F1 is located proximal to A, and in another case, F1 is located proximal to C, and so forth.*

**Table 2.** *Mutations of the p53 gene in patients with field cancerization*

| | **Tumor** | | | | | **Field** |
|---|---|---|---|---|---|---|
| Patient | Mutation | Exon | Change | Codon | Amino acid change | Mutation[a] |
| 1 | + | 6 | 1bp ins | 220-221 | Frameshift | Id. (F2) |
| 2 | + | 7 | G → A | 238 | Cys → Tyr | Id. (F1, F2, F4, A, B) |
| 7 | - | | | | | |
| 10 | + | 8 | G → T | 298 | Glu → Stop | Neg. (F4, B) |
| 11 | + | 5 | G → A | 175 | Arg → His | Neg. (F5, D, E) |
| 16 | + | 8 | C → G | 283 | Arg → Gly | Id. (F3, C) ; Neg. (F2, F5) |
| 17 | + | 5 | G → A | 141 | Cys → Tyr | Id. (F1) |
| 25 | + | 8 | 1bp del | 280 | Frameshift | Id. (F4) |
| 26 | + | 7 | C → T | 248 | Arg → Trp | Id. (F4, F5, C) |
| 28 | - | | | | | |

[a] *Id. means that the p53 mutation of the tumor was identical to the mutation of the field samples listed in parentheses. Neg. means that the mutation of the tumor was not confirmed in the field samples listed in parentheses. Confirmation was established by sequencing. Bp is basepair, ins means insertion and del, deletion.*

**Genetic Comparison between Field and Tumor.**

In 2 of 10 cases, the tumor and field showed similar genetic alterations (patients 7 and 17; Fig. 2). In 6 of 10 cases, the tumor showed additional losses, indicative of progression (patients 1, 2, 10, 11, 16, and 28; Fig. 2). In another 4 of 10 patients (patients 2, 11, 16, and 26), the field showed losses that were not present in the tumor, suggesting that these losses occurred after the divergence of two subclones, one of which progressed into invasive carcinoma. In some patients, the various mucosal biopsies showed the same genetic alterations, suggesting expansion of one clone. However, in 6 of 10 patients (patients 7, 10, 11, 16, 26, and 28), the various biopsies showed differences in the LOH pattern suggesting the expansion of different subclones within the field. Fig. 3 shows the genetic differences between the tumor and the various field subclones of patient 26. At chromosome 13q, the tumor and field samples showed the same losses. However, at chromosome 8p, the alternative allele is lost in the field samples as compared with the tumor. Moreover, at chromosome 18q, there are also genetic differences between the different field samples, clearly demonstrating the existence of various subclones.

**p53 Mutation Analysis of Field and Tumor.**

In all 10 patients with genetically altered fields, the tumors were sequenced for *p53* mutations in exons 5–9. In 8 of 10 patients, a *p53* mutation was found in the tumor (Table 2). To detect differences between the field and tumor, the DNA of the field was also sequenced to confirm the mutation of the tumor. In six of eight patients, the field had the same mutation as the tumor, whereas in two of eight patients, the field lacked the mutation found in the tumor.

**Persistence of Field Cancerization.**

In 7 of 10 patients, the field extended beyond the surgical margins (Fig. 2). In six of seven patients, a *p53* mutation was found in the tumor, and in three of six patients (patients 2, 16, and 26), the *p53* mutation could be confirmed in the resection margin (Table 2). Exfoliated cells taken from the scars of these three patients at 3 and 6 months after surgery were analyzed to follow the persistence of field cancerization. The exfoliated cells were analyzed for mutated DNA by the plaque assay using *p53* mutations as a marker. Patient 2 clearly showed *p53*-mutated cells at 3 and 6 months (0.17% and 0.56% of the screened plaques, respectively) after excision of the tumor (data not shown). However, patients 16 and 26 did not show *p53*-mutated cells in the exfoliated cell samples.

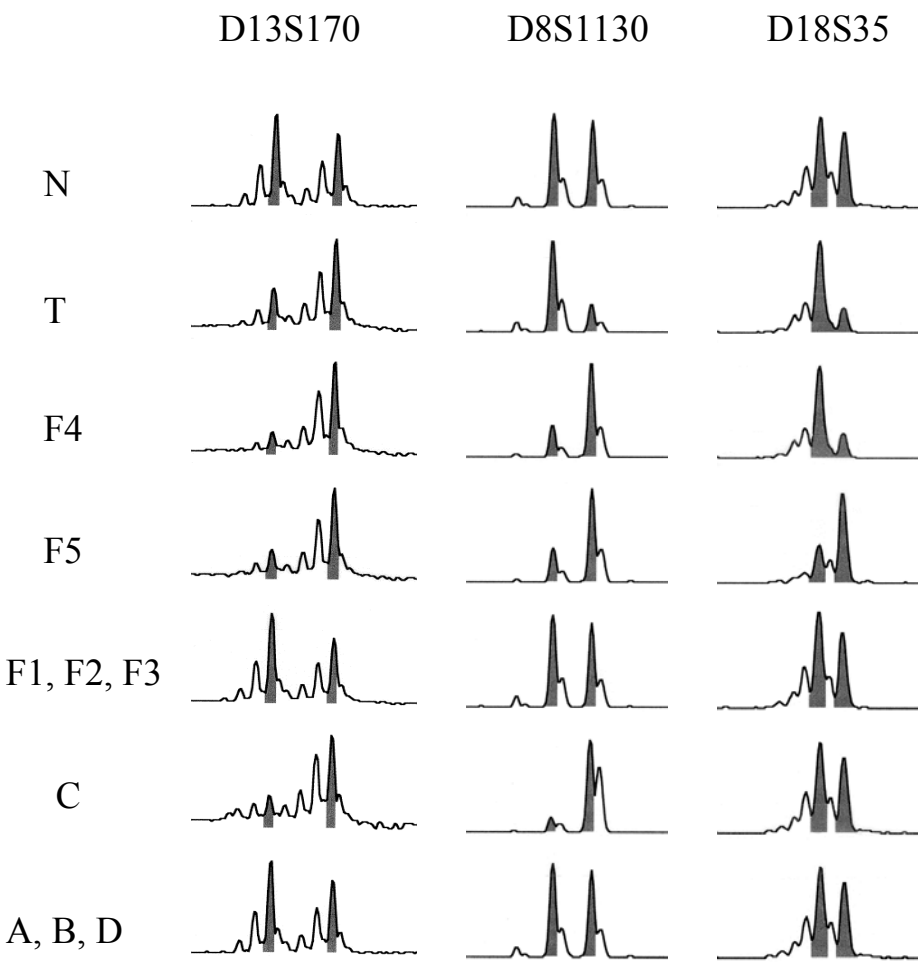



***Figure 3.*** *Results of microsatellite analysis of markers D13S170 (13q31), D8S1130 (8p23) and D18S35 (18q21) of patient 26 (see Fig. 2). Highlighted peaks show the two alleles that were used for calculation of LOH, as compared with normal DNA (N). Results of marker D13S170 showed that the tumor samples T, F4, F5 and C (see Fig. 1) were scored as LOH2 (loss of the smaller allele) and that F1-F3 and A, B, D were scored as having no loss. Results of marker D8S1130 showing genetic differences between tumor and field. Tumor showed loss of the larger allele (LOH1) whereas mucosal samples F4, F5 and margin C showed loss of the smaller allele (LOH2). Results of marker D18S35 show different subclones and genetic heterogeneity. Tumor and mucosal sample F4 showed LOH1; however, mucosal sample F5 showed loss of the other allele (LOH2), whereas margin C showed no loss at all.*

**Histology.**

All tumor and mucosa samples were reviewed and histopathologically classified and compared with the genetic analysis. In 12 patients, all mucosal biopsies were classified as normal mucosa, and in 16 patients, one or more mucosal biopsies were classified as mild, moderate, or severe dysplasia. The results of the genetic analysis and the histology of the 10 patients who showed genetic evidence of field cancerization are summarized in Table 3. The comparison between histological assessment and microsatellite analysis of all mucosal biopsies is depicted in Table 4A. In total, 132 mucosal biopsies were analyzed; 103 were classified as normal mucosa, 19 were classified as mild dysplasia, 8 were classified as moderate dysplasia, and 2 were classified as severe dysplasia. The presence of genetic alterations coincided in most cases with the results of the histopathological assessment; of the 17 mucosal biopsies that showed LOH, 14 were dysplastic, and 3 were normal. Conversely, the presence of dysplasia agreed less with the results of LOH analysis; 6 of the 19 mild dysplasias and 6 of the 8 moderate dysplasias showed LOH. For the 10 patients who showed genetic evidence of field cancerization, the paraffinembedded margins were also reviewed and classified. The results of histological assessment and microsatellite analysis are shown in Table 4B. In this material, the presence of genetic alterations was completely concordant with the histopathological assessment: all 9 margins that showed LOH were classified as dysplastic. The presence of dysplasia coincided well with LOH analysis in this selected group; four of the eight mildly dysplastic, all three moderately dysplastic, and both severely dysplastic margins showed LOH.

**Table 3** Comparison between histology and genetic alterations in the patients with field cancerization

| Patient no. | F1 | F2 | F3 | F4 | F5 |
|---|---|---|---|---|---|
| 1 | N / - [a] | Mild / + | N / - | N / - | |
| 2 | Mod / + | Mod / + | N / - | Mod / + | |
| 7 | Sev / + | N / - | N / - | Sev / + | N / - |
| 10 | N / - | N / - | N / - | Mod / + | |
| 11 | N / - | N / - | N / - | N / - | Mod / + |
| 16 | N / - | N / + [b] | Mild / + | N / - | N / + |
| 17 | Mod / + | N / - | N / - | N / - | N / - |
| 25 | N / - | N / - | N / - | Mild / + | N / - |
| 26 | N / - | Mild / - | N / - | Mild / + | Mild / + |
| 28 | N / - | N / + | Mild / - | Mild / + | Mod / - |

[a] *N, normal mucosa; Mild, mild dysplasia; Mod, moderate dysplasia; Sev, severe dysplasia. "–", no LOH; "+", LOH.*
[b] *Framed values represent an apparent lack of association between histology and genetic analysis*

**Table 4.** Comparison between histology and genetic alterations in 28 HNSCC patients
**A.** Comparison of 132 frozen mucosal biopises

| | Normal | Mild | Moderate | Severe | |
|---|---|---|---|---|---|
| + LOH | 3 | 6 | 6 | 2 | 17 |
| - LOH | 100 | 13 | 2 | 0 | 115 |
| | 103 | 19 | 8 | 2 | 132 |

**B**. Comparison of 43 paraffin-embedded surgical margins

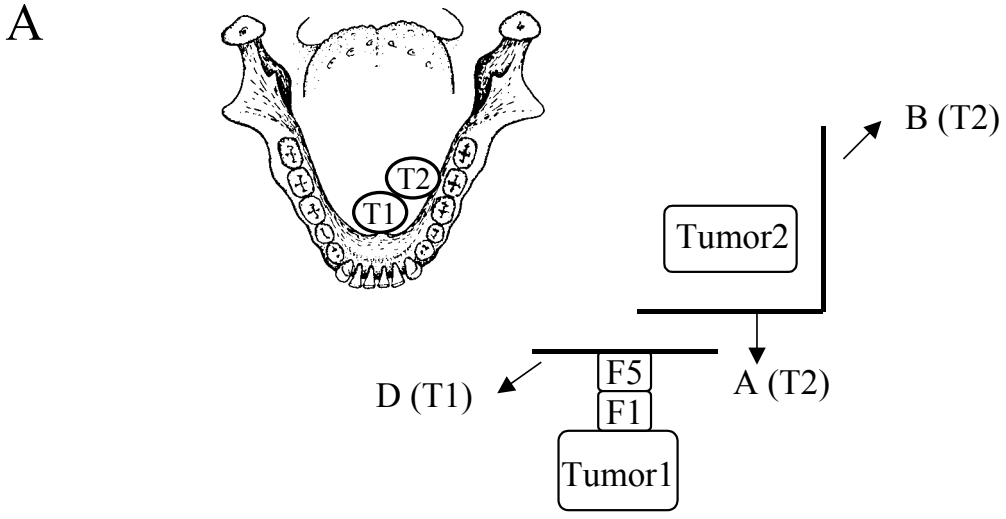
| | Normal | Mild | Moderate | Severe | |
|---|---|---|---|---|---|
| + LOH | 0 | 4 | 3 | 2 | 9 |
| - LOH | 30 | 4 | 0 | 0 | 34 |
| | 30 | 8 | 3 | 2 | 43 |

*In A, the comparison between histological assessment (degree of dysplasia) and LOH analysis of the 132 frozen mucosal biopsies obtained from all 28 patients is depicted. In B, the same comparison is shown of the 43 paraffin-embedded surgical margins obtained from the 10 field patients.*

**Clinical Implications.**

The clinical significance of the persistent fields could not be established in this study, mainly because the number of analyzed patients is rather small and, more importantly, because the follow-up period is rather short (range, 8–18 months; average follow-up, 12 months). To date, none of the seven patients whose surgical margins showed genetic alterations have developed a local recurrence or SPT. However, in the small pilot group in which only two biopsies adjacent to the tumor were analyzed, one patient did show a local recurrence in a field. This patient presented initially with a T1N0 floor of the mouth carcinoma and developed a clinically defined local recurrence at 1.5 cm from the scar after 28 months of follow-up. The results of the microsatellite analysis of the tumors as well as the intervening resection margins (additional markers at 9p and 13q were used) are shown in Fig. 4. The results strongly suggest that the local recurrence developed in a genetically altered field that had not been resected when the primary tumor was treated surgically. All biopsies shared the same genetic alterations at markers D9S1748 and D9S1751. Mucosal biopsy F5, surgical margin D of tumor 1, and local recurrence and surgical margin A as well as B (of tumor 2) showed the same additional hit at marker D13S158, which was not present in the first tumor. In fact, there were no differences in microsatellite alterations between the local recurrence (tumor 2) and surgical margin D of tumor 1. Mucosal biopsy F1 was classified as mild dysplasia, whereas mucosal biopsy F5 and all surgical margins [D (*T1*), A (*T2*), and B (*T2*)] were classified as moderate dysplasia. *p53* mutations were not detected in either of the tumors (*T1* and *T2,* Fig. 4).

Figure 4



B

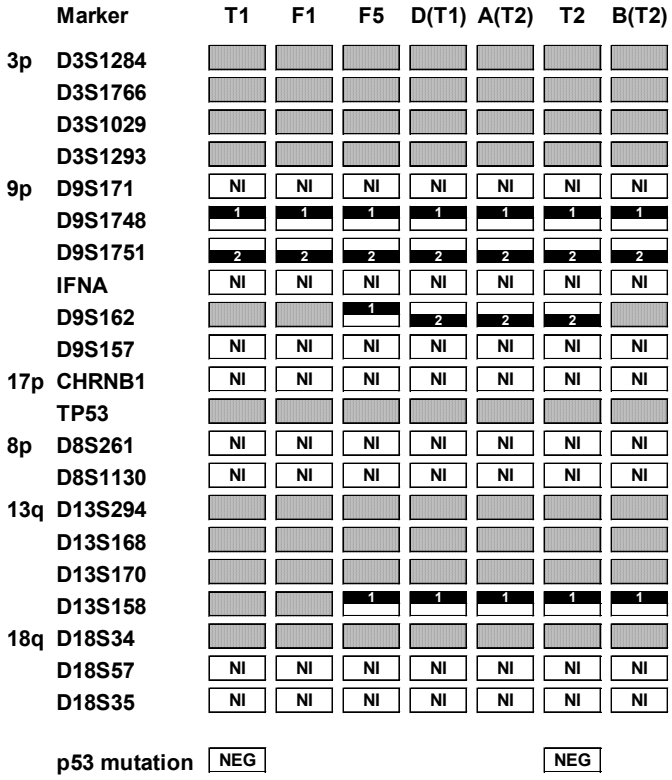



***Figure 4.*** *Field analysis of a patient with a T1N0 floor of mouth carcinoma (T1) who developed a (clinically) local recurrence (T2) 1.5 cm from the scar after 28 months follow-up. A, schematic drawing of the orientation of the surgical specimens. Biopsies of both tumors and intervening mucosa were taken from the surgical specimen [one (F1) <0.5 cm and one (F5)> 0.5 cm away from the edge of the tumor]. Also the paraffin-embedded resection margins of both surgical specimens [D (T1), A (T2) and B (T2)] were analyzed to study the extension of the field. B, the results of the microsatellite analysis suggest that the local recurrence has developed in the persistent field. All biopsies share the same genetic alterations at markers D9S1748 (9p21) and D9S1751 (9p21). Mucosal biopsy F5, surgical margin D of tumor 1, local recurrence (T2) and surgical margins A and B of tumor 2 show the same additional hit at marker D13S158 (13q32), which was not present in the index tumor (T1). For explanation of the other symbols, see Fig. 2.*

## Discussion

The present genetic analysis of macroscopically normal mucosa surrounding HNSCC has revealed a molecular basis for the process of field cancerization. There is molecular evidence for field cancerization in 36% (10 of 28) patients with HNSCC. The size of the field varies considerably between patients, and in 70% (7 of 10) of the patients, the field extended beyond the surgical margins.

The presence of genetic alterations in the mucosa samples and surgical margins was found to be associated with histopathological changes (Table 4). The majority of the genetically altered fields were classified as dysplastic, and in a few cases, genetic alterations were detected in histopathological normal mucosa, as also shown previously (22). The presence of dysplasia in the mucosal epithelium, however, was not always associated with genetic alterations because approximately half of the dysplastic lesions did not show LOH (Table 4). A possible explanation for this finding could be the difficulties of histopathological assessment of freshly frozen tissues because the histopathological classification of the paraffin-embedded samples was more concordant with genetic analysis. Moreover, interobserver variability in the scoring/grading of mild dysplasia could play a role (23) because the most cases without genetic alterations were observed in the mild dysplasia group. A third explanation is that mild dysplastic lesions harbor genetic alterations that have not been investigated. However, the markers used in this study are supposed to reflect the early transforming events in HNSCC (11).

Comparison of the spectrum of genetic alterations in premalignant field lesions provides insight into the carcinogenesis of HNSCC. In six of eight patients, the field had the same *p53* mutation as the tumor, strongly indicating a clonal relationship between the surrounding field(s) and tumor. Despite this clonal relationship, there were differences in most patients in the genetic alterations between field and tumor. In 6 of 10 patients, the tumor showed additional microsatellite alterations, and in 2 of 8 patients, *p53* mutations present in the tumor were not detected in the field. These additional genetic alterations are indicative of progression of the tumor according to the multistep model (11, 12). In contrast, in 4 of 10 patients, the field showed microsatellite alterations that were not present in the tumor. The picture emerges that all lesions share a common initiating event and that progression leads to the evolution of related subclones and genetic heterogeneity. One subclone eventually progresses into a tumor. The presence of different subclones throughout the field supports this hypothesis (Fig. 2). Other authors have described comparable findings (24, 25). The presented data support the following interpretation of the field cancerization concept: a single cell is genetically altered and gives rise to a large premalignant field that extends by clonal expansion and gradually replaces the normal mucosa. Subsequent progression of the different but related subclones in the field leads to

the development of (multiple) tumor(s).

In this study, not only was the presence of genetically altered fields established, but the persistence during clinical follow-up using the plaque assay. Microsatellite analysis is an insensitive molecular assay for tumor (or premalignant) cell detection, and a clinical sample should at least contain more than 50% aberrant cells to score LOH (see the formula in "Materials and Methods"). In all patients, the clinical aspect of the mucosa around the scar appeared normal after surgery. Therefore, it was not possible to select an area with a high percentage of genetically altered cells by visual inspection. The plaque assay is a highly specific and sensitive methodology for the identification of rare tumor (or premalignant) cells in clinical samples based on the detection of mutated *p53* (20). As an example, the exfoliated cells of patient 2 were analyzed for mutated DNA. This patient clearly showed *p53*-mutated cells at 3 and 6 months (0.17% and 0.56% of the screened plaques, respectively) after excision of the tumor (data not shown), indicating that these genetically altered fields can persist for at least half a year after removal of the tumor. In patients 16 and 26, however, we could not detect *p53*-mutated cells during clinical follow-up, although the mucosal biopsy as well as the resection margin showed the same *p53* mutation as seen in the tumor. An explanation could be that the fields remaining in these two patients were too small to be detected by the plaque assay. Only a small part of the single positive margins of patients 16 and 26 consisted of genetically altered cells, whereas the two resection margins of patient 2 consisted completely of genetically altered cells. Another even more interesting explanation could be that not all fields persist over time, but some might regress and disappear spontaneously [a phenomenon that is also observed in other premalignant lesions in the upper aerodigestive tract such as oral leukoplakia (26)].

A nonresected field could (in part) explain the high rate of local recurrence of HNSCC. A field that extends beyond surgical margins and thus has not been excised can progress after treatment into a new tumor at the same site. Such an example was seen in a patient of the pilot study (Fig. 4). This patient was surgically treated for a T1N0 tumor in the floor of the mouth and developed a local recurrence after 28 months. The results of the microsatellite analysis strongly suggest that the local recurrence developed in a genetically altered field that had not been resected when the primary tumor was treated surgically. According to the presently used clinical criteria, this new tumor in the field was classified as a local recurrence; however, the issue of whether it should be defined as a SPT can be discussed based on the molecular criteria.

Persistence of fields could also explain a subgroup of the clinically defined SPTs. Hypothetically, a persistent genetically altered field could extend into a large area of the mucosa. A new tumor developing in this field would then clinically be classified as a SPT, based on the distance (>2 cm) to the index tumor (27). Califano *et al.* (28) suggested that

two tumors (T1, hypopharynx; T2, lower esophagus) that were separated by 40 cm of normal-appearing mucosa but showed identical genetic alterations apparently originated by a process of clonal expansion of a single progenitor cell. The same notion was also reported by Worsham *et al.* (6) for two synchronous tumors of the floor of the mouth and piriform sinus that were separated by 4–6 cm. However, the presence of genetic alterations in the intervening mucosa was not confirmed in these studies.

A number of parameters may determine whether a field develops into a new tumor. A very important factor might be the follow-up period because a premalignant field may need a longer time to progress into a new tumor than a tumor that develops from remaining tumor cells. The follow-up time of the seven field patients varied from 8–18 months, with an average of 12 months, and to date, no new tumors have been observed. Mao *et al.* (29) and Rosin *et al.* (30) have shown that oral premalignant lesions might need up to 67 or 96 months, respectively, to progress to invasive cancer.

In addition, the pattern of LOH of the persistent fields might also play an important role in the progression to cancer (30). Rosin *et al.* (30) showed that patients with oral premalignant lesions with LOH at 3p and/or 9p had a 3.8-fold increased relative risk of developing cancer. In contrast, patients with additional losses of other chromosomes (4q, 8p, 11q, or 17p) showed up to a 33-fold increase in relative cancer risk. In our study, the surgical margins of some patients showed almost an identical LOH pattern as the tumor (Fig. 2).

In summary, our study provides the molecular basis for expanding fields and the presence of various genetically distinguishable subclones therein. Additional studies are needed to monitor the clinical implications of these persistent fields. Adequately identifying the extension of genetically altered fields and their risk for progression may have profound implications for future patient management.

## Acknowledgments

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# 4

# Comparative molecular and histological grading of epithelial dysplasia of the oral cavity and the oropharynx

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## Abstract

Histological grading of epithelial dysplasia in the oral cavity and oropharynx is used to predict the risk for cancer and to determine the treatment strategy. This grading, however, is subjective and not well reproducible. Recent publications have shown that molecular markers are promising in cancer risk assessment. The aim of the present study was to compare classical histological and molecular grading and to relate these to the proliferation rate by quantitative assessment of Ki-67 staining. Forty-three samples were analysed from the margins of patients who had undergone resection of their squamous cell carcinoma in the oral cavity/oropharynx. Three experienced pathologists performed the histological grading. With the consensus score, 12 samples were classified as normal and 31 as dysplastic (21 mild, six moderate, and four severe). Loss of heterozygosity (LOH) was assessed in the same samples with 15 microsatellite markers at chromosomes 3p, 9p, 17p, 8p, 13q, and 18q, and was present in 28 of the 43 samples. Twenty-four of the 28 cases (86%) with LOH were classified as dysplastic and four as normal. All ten samples with moderate and severe dysplasia and 14 of 21 samples with mild dysplasia contained LOH. In four of 12 biopsies classified as normal, LOH was found. A very striking and significant difference of the Ki-67 index was observed between LOH-positive and LOH-negative cases, 36.6 ± 11.1% versus 19.4 ± 2.8% positive cells, respectively. In mild dysplasia, 13 of 14 lesions containing LOH had a higher Ki-67 index than all seven lesions without LOH. Thus, in the oral cavity/oropharynx, LOH is more frequently found in the histologically higher-grade lesions (moderate dysplasia or worse) and in the lower grade lesions when a high proliferation rate is present. Assessment of proliferation with Ki-67 is a better surrogate for LOH than histological grading.

## Introduction

Despite advances in therapy leading to better local control, the long-term survival of patients with oral and oropharyngeal squamous cell cancer (OSCC) has only marginally improved in the last 20 years (1). An important approach to make headway with the management of OSCC is the early detection of lesions that have a high risk of developing into cancer. A major group of individuals at risk for cancer are those who have been treated for OSCC. After surgery alone or in combination with radiotherapy, a considerable local failure rate is found (2) that may be caused by the outgrowth of residual tumour cells that were not detected, despite histological examination of the resection margins. This phenomenon is referred to as 'minimal residual disease'. Another reason for tumour recurrence may be that a 'field' of premalignant cells has remained in the patient after resection of the primary tumour. A recent study has shown that in about one-quarter of OSCC patients with cancer-free surgical margins, premalignant lesions remain in the mucosa (3) and preliminary results have shown that these premalignant fields have the potential to develop into new tumours (3–5). In addition, individuals with primary dysplastic lesions in the oral cavity are at risk for OSCC. These lesions are sometimes visible as 'leukoplakia' (6).

For many years, histological grading of dysplasia has been the only method to determine the presence of a premalignant lesion and the risk of its progression into cancer. The WHO has defined histological changes that contribute to the diagnosis of dysplasia (7). In general, the severity of epithelial dysplasia is proportional to the risk of subsequent cancer development, but histological grading has limited value in the prediction of risk for the individual patient (8). There is a lack of uniformity among pathologists as to which of the morphological features might attribute most to the high-risk status of a lesion (9). Furthermore, morphological grading of dysplasia in general is hampered by subjectivity and low reproducibility (9–11) and is under considerable debate (12,13).

Molecular grading of premalignant lesions has been proposed to have additional value in predicting cancer. Considerable improvement in assessing cancer risk has been made. Mao *et al* (14) reported that loss of heterozygosity (LOH) at chromosome arms 3p and 9p is associated with an increased cancer risk. In a larger study, Rosin *et al* investigated the difference between cases (progressing leukoplakias) and controls (nonprogressing leukoplakias) (15). In this study, hyperplastic and low-grade dysplastic lesions from 116 patients were analysed for the presence of LOH at 19 microsatellite loci on seven chromosome arms. Loss at 3p or 9p was associated with a higher cancer risk, but in particular additional losses at 4q, 8p, 11q, and/or 17p appeared to indicate a 33-fold increase in cancer risk. Despite a difference in the cancer incidence between the groups

with dysplastic and hyperplastic lesions, the assessment of LOH greatly refined risk assessment in both groups. Partridge *et al* (16) have reported similar results.

To what extent molecular grading needs to be implemented, as it is labour-intensive and requires a well-equipped laboratory, is an important question. We therefore investigated correlations between molecular alterations, morphological grading, and the number of proliferating cells in lesions with a variable degree of dysplasia. The ultimate aim was to achieve an accurate and practical risk assessment of oral and oropharyngeal preneoplasia.

## Materials and methods

### Patients and tumour resection specimens

The material was derived from 34 tumour specimens from 25 patients who had undergone surgical treatment for a squamous cell carcinoma located in the oral cavity or in the oropharynx between January 1991 and January 2001 (nine patients developed two primary tumours in this time period). In total, 43 paraffin-embedded surgical margins (used for histological assessment of tumour margins) were selected: 28 samples with and 15 samples without genetic alterations. No more than two margins were taken from one tumour specimen and if one margin had genetic alterations, the other had not. Twenty of the 43 samples had been analysed as part of a previous study (3). During selection, we were not aware of the previous histological grade. Twenty-eight tumours were located in the oral cavity (12 in the tongue, six in the floor of mouth, six in the retromolar trigone, two in the lower alveolus, one in the upper alveolus, and one in the buccal mucosa) and six were located in the oropharynx (two in the anterior tonsillar pillar, two in the uvula, one in the soft palate, and one in the base of the tongue). Tumour stages (pTN) were determined according to the criteria of the International Union Against Cancer (UICC) (17): T1N0 (12 tumours), T2N0 (14 tumours), T2N1 (two tumours), T2N2b (three tumours), T3N2b (one tumour), T4N0 (one tumour), and T4N1 (one tumour). The age of the patients ranged from 38 to 82 years, with an average age of 61 years. In total, 14 patients were male and 11 were female.

### Microsatellite analysis

The presence of genetic alterations was assessed using 15 microsatellite markers located at chromosomes 3p, 9p, 17p, 8p, 13q, and 18q. These markers were selected because they frequently demonstrate LOH in head and neck squamous cell carcinoma (HNSCC) and precursor lesions (3,18). The following markers were used: D3S1284 (3p12), D3S1766 (3p14), D3S1029 (3p21), D3S1293 (3p24), D9S171 (9p21), D9S157

(9p22), CHRNB1 (17p11-12), TP53 (17p13.1), D13S294 (13q14.3), D13S170 (13q31), D18S34 (18q12), D18S57 (18q12), D18S35 (18q35), D8S261 (8p22), and D8S1130 (8p23). Primer sequences were obtained from the Genome Database for all of these markers (http://gdbwww.gdb.org/). The archival formalin-fixed and paraffin-embedded surgical margins were sectioned, placed on microscopic glass slides, and subsequently deparaffinized in xylene. For all cases, the first and last tissue sections were stained with haematoxylin and eosin (H&E) for histological grading of dysplasia and to guide microdissection. The other tissue sections were stained with 1% toluidine blue and 0.2% methylene blue, manually microdissected under a stereomicroscope, and used for genetic analysis. All microdissected mucosal areas were at least 4 mm in length. For normal DNA, connective tissue or muscle was microdissected from the same sections.

Microsatellite analysis was carried out on an automated ABI PRISM sequencer (310 Genetic Analyzer; Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) as described previously [3,19]. LOH was scored if one allele was decreased by more than 50% in the tumour sample when compared with the same allele in normal control DNA, when necessary after stutter correction as described previously (20).

**Grading of dysplasia**

All H&E-stained slides were examined by three experienced pathologists and scored according to the standard criteria of the World Health Organization international histological classification of tumours (7) without information on molecular data during histological classification. From each section, the same area was scored that had also been used for molecular assessment. For each case, each pathologist recorded the grade and details of the criteria on which the decision was based. Lesions were classified as (1) normal mucosa, (2) mild dysplasia, (3) moderate dysplasia, and (4) severe dysplasia or carcinoma *in situ*. The following additional criteria were scored: degree of maturation (normal, aberrant for lowest one-third, aberrant for two-thirds, aberrant for more than two-thirds), presence of mitotic figures (only in the (para)basal layer, up to one-third, up to two-third, in all layers), nuclear pleomorphism (normal, and moderately and severely aberrant), and nuclear-cytoplasmic ratio (normal and moderately and severely aberrant). In addition to the individual scores, a consensus score was also made. This was done in a single session and for each variable.

**Immunohistochemistry (IHC) and quantitative analysis**

Immunohistochemical staining of Ki-67 was performed on the 43 paraffin-embedded resection margins. The mouse monoclonal antibody MIB-1 (Immunotech, Marseille, France) was used. Five-micrometre sections were deparaffinized, placed in 0.3% methanolic peroxide (30 min) to block endogenous peroxidase activity, rinsed in

phosphate-buffered saline, subjected to antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0), and boiled for 25 min on a hot plate. After cooling overnight, the avidin–biotin complex methodology was used with diaminobenzidine (DAB) in H2O2 as chromogen (stained for 5 min). Sections were counterstained with haematoxylin, dehydrated, and mounted with xylene-substitute mountant.

The measurement of the number of Ki-67-positive cells was performed with the PROfessional Digitizing Interactive Tool, QPRODITR version 6.1 (Leica Imaging Systems Ltd, Cambridge, UK). As described by van Sandick *et al* (21), the automated scanning stage was used to enable electronic demarcation of the measurement area corresponding to the area of interest in the microscopic image, and to allow for systematic random sampling of fields of vision (22). Ki-67 immuno-quantitation was performed by means of the two-class immuno-scoring module of the QPRODIT system, with an electronic test grid and counters graphically overlaid on the microscopic image. The % positive nuclei were determined by scoring at least 100 nuclei in each measurement area. All features were scored in the full thickness of the mucosal epithelium. Intra- and inter-observer reproducibility of the quantitative analyses was assessed on the basis of the measurements performed on seven samples, selected at random. Reproducibility was high, as reflected by coefficients of variation (SD expressed as a percentage of the average) of 1.2% and 2.1% for the intra-observer and inter-observer comparison, respectively.

### Statistics

The level of inter-observer agreement was determined by calculating Cohen's kappa values. Differences between groups were analysed with ANOVA. For both analyses, the SPSS program (version 7.5; SPSS Inc, Chicago, IL, USA) was used.

## Results

### LOH in lesions at the resection margins

Twenty-eight of the 43 samples had evidence of LOH. Details of the type of loss are shown in Table 1. In the case of loss, two or more microsatellite marker always appeared to be involved. 'No loss' indicates that none of the markers showed loss.

**Table 1**.

| Case no: | Patient[2] | Dysplasia score[1] | | | | LOH | | Ki-67 positive cells (%) |
|---|---|---|---|---|---|---|---|---|
| | | Obs I | Obs II | Obs III | Con-sensus | Presence | Location | |
| 1 | 1 (T1;M1) | 1 | 0 | 2 | 1 | Yes | 9p | 42.3 |
| 2 | 1 (T1;M2) | 1 | 0 | 0 | 1 | No | | 22.1 |
| 3 | 2 (T1;M1) | 2 | 0 | 1 | 1 | Yes | 3p, 9p, 17p, 13q | 28.5 |
| 4 | 2 (T2;M1) | 1 | 1 | 2 | 1 | Yes | 3p, 9p, 17p, 8p | 43.2 |
| 5 | 3 (T1;M1) | 2 | 1 | 2 | 2 | Yes | 3p, 9p, 13q, 18q | 32.4 |
| 6 | 3 (T1;M2) | 1 | 1 | 1 | 1 | No | | 22.6 |
| 7 | 4 (T1;M1) | 0 | 0 | 0 | 0 | Yes | 9p | 38.7 |
| 8 | 4 (T2;M1) | 0 | 0 | 0 | 0 | No | | 20.7 |
| 9 | 4 (T2;M2) | 2 | 1 | 2 | 2 | Yes | 9p, 8p | 42.4 |
| 10 | 5 (T1;M1) | 1 | 1 | 1 | 1 | Yes | 3p, 9p, 17p, 13q | 35.0 |
| 11 | 5 (T2;M1) | 1 | 1 | 0 | 1 | Yes | 3p, 9p, 17p, 13q | 39.4 |
| 12 | 6 (T1;M1) | 1 | 1 | 0 | 1 | No | | 17.8 |
| 13 | 6 (T1;M2) | 0 | 0 | 0 | 0 | No | | 17.4 |
| 14 | 7(T1;M1) | 3 | 3 | 3 | 3 | Yes | 3p, 9p, 17p, 13q, 8p | 68.3 |
| 15 | 7 (T1;M2) | 0 | 0 | 0 | 0 | No | | 16.1 |
| 16 | 8 (T1;M1) | 0 | 0 | 0 | 0 | No | | 20.3 |
| 17 | 8 (T1;M2) | 1 | 1 | 0 | 1 | Yes | 3p, 9p, 17p | 28.9 |
| 18 | 9 (T1;M1) | 1 | 1 | 1 | 1 | Yes | 9p, 17p, 8p | 33.2 |
| 19 | 10 (T1;M1) | 0 | 1 | 0 | 1 | No | | 21.3 |
| 20 | 10 (T1;M2) | 0 | 1 | 0 | 1 | No | | 19.9 |
| 21 | 10 (T2;M1) | 2 | 2 | 2 | 2 | Yes | 3p, 9p, 17p, 13q, 8p, 18q | 37.8 |
| 22 | 11 (T1;M1) | 0 | 0 | 0 | 0 | No | | 15.8 |
| 23 | 11 (T1;M2) | 1 | 1 | 1 | 1 | Yes | 3p, 9p, 17p, 13q, 18q, 8p | 31.1 |
| 24 | 12 (T1;M1) | 0 | 0 | 0 | 0 | Yes | 3p, 9p, 17p, 8p | 17.5 |
| 25 | 13 (T1;M1) | 0 | 0 | 0 | 0 | No | | 15.2 |
| 26 | 14 (T1;M1) | 2 | 1 | 1 | 1 | Yes | 3p, 9p, 8p | 37.6 |
| 27 | 14 (T2;M1) | 2 | 1 | 1 | 1 | Yes | 3p, 9p, 8p | 40.2 |
| 28 | 15 (T1;M1) | 1 | 0 | 0 | 0 | Yes | 3p, 9p, 13q | 21.5 |
| 29 | 15 (T2;M1) | 0 | 0 | 0 | 0 | Yes | 3p, 9p, 13q, 18q | 21.8 |
| 30 | 16 (T1;M1) | 2 | 1 | 2 | 2 | Yes | 3p, 9p, 17p, 13q, 8p | 41.7 |
| 31 | 16 (T2;M1) | 2 | 2 | 2 | 2 | Yes | 9p, 17p, 13q, 8p | 51.0 |
| 32 | 17 (T2;M1) | 3 | 3 | 3 | 3 | Yes | 3p,17p, 13q, 18q, 8p | 51.9 |
| 33 | 17 (T3;M1) | 3 | 3 | 3 | 3 | Yes | 3p, 17p, 8p | 50.9 |
| 34 | 18 (T1;M1) | 0 | 0 | 0 | 0 | No | | 17.1 |
| 35 | 18 (T3;M1) | 1 | 1 | 1 | 1 | Yes | 3p, 9p, 17p, 13q | 36.7 |
| 36 | 19 (T1;M1) | 1 | 0 | 1 | 1 | No | | 22.2 |
| 37 | 20 (T1;M1) | 3 | 3 | 2 | 3 | Yes | 3p, 9p, 17p, 13q, 8p | 41.8 |
| 38 | 21 (T1;M1) | 1 | 0 | 1 | 1 | Yes | 3p, 9p, 17p, 8p | 28.5 |
| 39 | 22 (T1;M1) | 1 | 1 | 1 | 1 | Yes | 3p, 17p | 32.6 |
| 40 | 22 (T1;M2) | 0 | 0 | 0 | 0 | No | | 18.9 |
| 41 | 23 (T1;M1) | 2 | 2 | 2 | 2 | Yes | 3p, 9p, 17p, 13q, 8p | 32.5 |
| 42 | 24 (T1;M1) | 1 | 1 | 1 | 1 | No | | 24.0 |
| 43 | 25 (T1;M1) | 1 | 1 | 1 | 1 | Yes | 17p, 13q | 16.4 |

***Table 1.*** *Degree of dysplasia: inter-observer agreement and correlation with molecular and immunohistochemical parameters.*

1) *Dysplasia score of the different observers (Obs): 0= no dysplasia; 1= mild; 2= moderate; and 3= severe dysplasia.*
2) *Patient characteristics: Shown is the patient number and in parentheses the primary tumor (T1) or second primary tumor (T2; T3) and the number of the margin analysed (M1 or M2).*

***Table 2.*** *Inter-observer agreement as reflected by kappa scores of the histopathological grading of lesions with a variable degree of dysplasia.*

| Observer comparison | Dysplasia | Differentiation | Mitoses | Nuclear pleomorphism | Nuclear-cytoplasmic ratio |
|---|---|---|---|---|---|
| I with II | 0.56 | 0.50 | 0.64 | 0.54 | 0.56 |
| I with III | 0.64 | 0.67 | 0.61 | 0.43 | 0.64 |
| II with III | 0.52 | 0.62 | 0.60 | 0.66 | 0.43 |
| Mean (SD) | 0.57 (0.06) | 0.58 (0.07) | 0.62 (0.02) | 0.54 (0.11) | 0.54 (0.11) |

*Details on the classification of the various parameters are shown in the Materials and methods section.*

***Table 3.*** *Sensitivity and specificity of various features for detecting presence of LOH*

| | Dysplasia* | Differentiation* | Mitoses* | Nuclear pleomorphism* | Nuclear-cytoplasmic ratio* | Ki-67 labelling index # |
|---|---|---|---|---|---|---|
| Sensitivity (%) | 86 | 82 | 75 | 79 | 86 | 86 |
| Specificity (%) | 53 | 73 | 47 | 87 | 87 | 100 |

** Consensus-score; normal vs. abnormal.*

*# 25% of Ki-67 positive cells were taken as the cut-off value (see the Results section).*

**Inter-observer agreement in the grading of dysplasia**

The results of histological grading by the three pathologists are also shown in Table 1. In 23 of the 43 cases (53%), there was total agreement on the grade of dysplasia. When the inter-observer agreement was analysed, an average kappa score of 0.58 was observed (Table 2). This falls into the category of a moderate strength of agreement (23). Table 1 also shows the consensus score. Twelve samples were classified as normal, and 21, six, and four as being mildly, moderately, and severely dysplastic, respectively. Between the three pathologists, there was the following agreement between the separate categories: 75% in severe dysplasia (3/4 cases), 50% in moderate dysplasia (3/6 cases), 38% in mild dysplasia (8/21 cases), and 92% (11/12) in cases without dysplasia. Taking the four separate grading criteria into account, there was a slight variation, with kappa values between 0.54 and 0.62 (Table 2). The assessment of the presence of mitotic activity showed the highest kappa value (0.62 ± 0.02) that is considered good agreement.

**Correlation between histological grading and LOH score**

Table 3 shows the correlation between the presence of LOH and the consensus score with respect to dysplasia and the various criteria used for dysplasia grading. The presence of LOH is used as the gold standard. Twenty-four of the 28 cases with LOH were classified as dysplastic, giving a sensitivity score (truly positive cases) of 86%. There were four cases with LOH that showed no signs of dysplasia (cases 7, 24, 28, and 29). Interestingly, three cases (Nos 24, 28, and 29) showed LOH at multiple arms and in only one case (No 28) was there disagreement among the pathologists. Eight of the 15 cases without LOH were classified as being non-dysplastic, yielding a specificity (truly negative cases) of 53%. Of the different morphological criteria, the nuclear-cytoplasmic ratio and the level of nuclear pleomorphism appeared to correlate best with LOH, as reflected by the highest sensitivity and specificity scores (Table 3). With respect to the different grades of dysplasia, all ten samples with moderate and severe dysplasia and 14 of 21 samples with mild dysplasia contained LOH. When moderate dysplasia was taken as the cut-off level, the sensitivity dropped to 36% and the specificity increased to 100%.

**Correlation between Ki-67 staining and LOH score**

There was a considerable variation in the percentage of Ki-67-positive cells between samples. The Ki-67 labelling index was related to the degree of dysplasia and differed significantly between the four grades of dysplasia ($p < 0.001$, ANOVA, with a significant linear trend, $p < 0.001$). The mean labelling indices (with SD) were as follows: no dysplasia, 20.1 ± 6.3; mild dysplasia, 29.7 ± 8.3; moderate dysplasia, 39.6 ± 7; and severe dysplasia, 53.2 ± 11 (Figure 1). The LOH samples had a significantly higher ($p < 0.01$) Ki-67 labelling index than the non-LOH group, with scores of 36.6 ± 11.1% and 19.4 ±

2.8% of Ki-67-positive cells, respectively. Interestingly, in the mild dysplasia group, two different categories could be distinguished on the basis of the Ki-67 index: a group of seven cases with a low Ki-67 index (21.4 ± 2) which did not exhibit LOH, and a group of 14 cases with a significantly higher Ki-67 index (33.8 ± 7), all of which showed LOH (Figure 1). In the latter group, only one case (No 43) had a low Ki-67 index of 16.4%. This case had a unique LOH pattern of 17p and 13q. Of the four cases without morphological signs of dysplasia but with LOH, only one case (No 7) had a high Ki-67 index (38.7%), while the other three cases (Nos 24, 28, and 29) displayed a low Ki-67 index. These three cases all displayed multiple losses. We have chosen 25% of Ki-67-positive cells as the upper cut-off level of normal proliferation based on the mean value in the non-LOH group (19.4) plus two times the SD (2.8). With this cutoff level, high proliferative activity correlated very well with the presence of LOH (sensitivity 86%, specificity 100%; Table 2) and appeared to be a much better surrogate for LOH than the histomorphological grading of dysplasia. No confounding influence was found between the presence of inflammation and the Ki-67 index (results not shown).

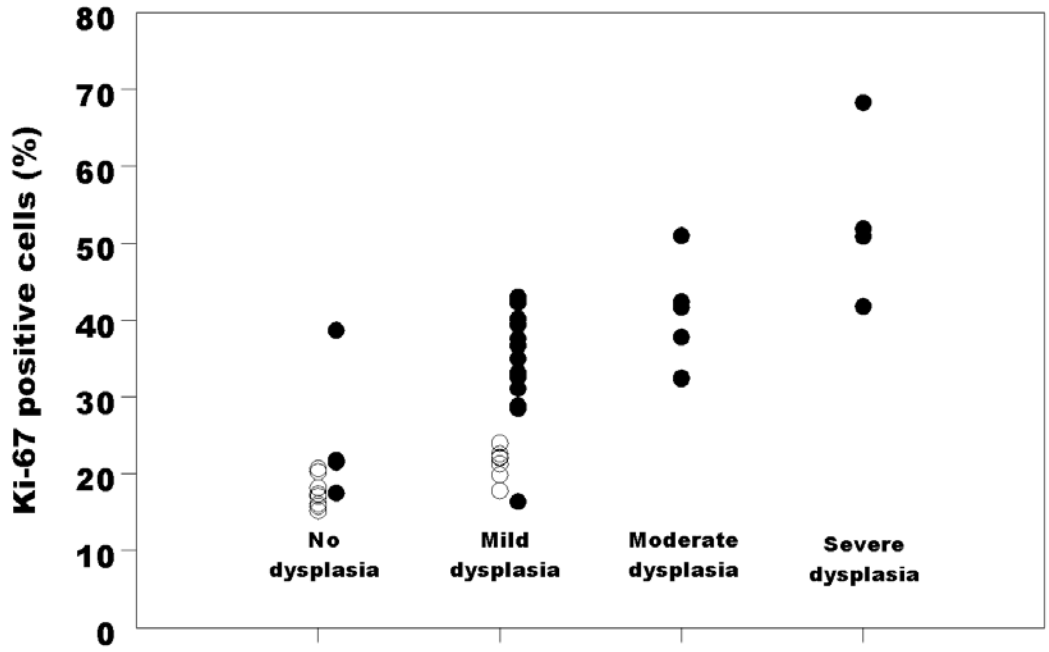


***Figure 1.*** *Ki-67 labelling index of resection margins of patients with oral/oropharyngeal squamous cell cancer in relation to the histological grade of dysplasia and the presence of LOH. The closed circles (●) are samples that contain LOH, while the open circles (○) do not contain LOH.*

## Discussion

This study was performed to investigate the correlation between histological and molecular characteristics of lesions showing various degrees of dysplasia, as it has been assumed that molecular markers will be valuable adjuncts to histological assessment in detecting cases that will progress to carcinoma. In fact, some studies have already shown that genetic alterations may improve risk assessment. Retrospective studies measuring LOH (15,16) and DNA ploidy (24) were used for this purpose. The present study tried to answer the question of whether and to what extent this molecular approach could replace routine histology. To ensure a valid comparison, we obtained an accurate histological diagnosis by gathering the judgement of three experienced pathologists. The inter-observer agreement of the dysplasia score (four groups: no, mild, moderate, and severe dysplasia) was substantial, with an unweighted kappa value of 0.57. This value is somewhat higher than values reported in other studies (10,11,25). None of the individual characteristics that were used by the pathologists to arrive at the diagnosis was superior with respect to interobserver agreement.

The molecular data were considered to be the 'gold standard' and for comparison, the consensus diagnosis was judged to be the most optimal score of dysplasia. When taking 'mild' as the lowest cut-off level for dysplasia, the sensitivity for the presence of LOH was high (86%). There were four lesions that had extensive losses, but no signs of dysplasia. Others have also reported this phenomenon [26] and to date, it is not known what the implications might be. With respect to the specificity, a significant number (47%) of lesions were classified as dysplastic, but no molecular alterations were identified. All of these cases were classified as mildly dysplastic.

The present study showed a good correlation between the Ki-67 labelling index and the presence of LOH. Ki-67 was chosen since a good correlation between the number of positive cells and the level of dysplasia has frequently been reported and is thought to improve reliable grading (27–32). Although the Ki-67 labelling index is a prognostic factor in many tumours (33), to our knowledge hardly any information is available that relates it to the presence of LOH or its value as a predictor of cancer risk in oral/oropharyngeal dysplasia. In the present study, the Ki-67 index proved to be a very good predictor for the presence of genetic alterations. Above the threshold of 25% positive cells, all lesions had genetic alterations. Within the group of mild dysplasia, the Ki-67 index identified all but one case that contained LOH. Somewhat in contrast with this finding was the fact that the specificity of nuclear pleomorphism and the nuclear-cytoplasmic area ratio for this detection of LOH seemed to be superior to that of the localization and presence of mitosis. This indicates that the detection of mitosis by the pathologist does not correlate well with the proliferation rate as measured by Ki-67 immuno-quantitation and that nuclear pleomorphism and the nuclear-cytoplasmic ratio

may be more important for reliable histological grading than the scoring of mitoses or maturation.

The relationship between a relatively high proportion of Ki-67-positive cells and the presence of genetically aberrant cells fits into the concept of 'the expanding preneoplastic field' (34). OSCCs often have an adjacent preneoplastic lesion that consists of cells that have tumour-related genetic alterations (3,16,35,36). This led to the proposition of the concept that a preneoplastic field precedes tumour formation. The eventual tumour develops within this field from a sub-clone that has emerged after additional genetic hits. These fields can be very large, over 6 cm in diameter, and may give rise to second primary tumours (37). One assumption in this model is that a field expands at the expense of the healthy surrounding mucosa. The present study provides evidence for this hypothesis. Although we cannot show that there is a process of active replacement, it is reasonable to assume that the higher proliferation rate of preneoplastic epithelium will gradually outweigh the normal healthy mucosa, given the time frame in which such a process gradually develops.

Molecular analysis will very likely improve the assessment of risk for lesions that will develop into OSCC (15), but future studies should demonstrate to what extent. This paper shows that Ki-67 staining is a good surrogate for LOH and can be used to preselect samples for further molecular studies that will lead to a reduction in materials and manpower.

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5

# Multiple Head and Neck Tumors Frequently Originate from a Single Preneoplastic Lesion

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## Abstract

The development of second primary tumors has a negative impact on the prognosis of head and neck squamous cell carcinoma. Previously, we detected genetically altered and tumor-related mucosal lesions in the resection margins in 25% of unselected head and neck squamous cell carcinoma patients [Tabor et al., Clin Cancer Res, 7: 1523–1532, 2001]. The aim of this study was to determine whether first and second primary tumors are clonally related and originate from a single genetically altered field. From 10 patients we analyzed the first tumor of the oral cavity or oropharynx, the >3-cm remote second primary tumor, and the mucosa from the tumor-free margins from both resection specimens. We compared *TP53* mutations and loss of heterozygosity profiles using 19 microsatellite markers at chromosomes 3p, 9p, 13q, and 17p. In all patients, genetically altered mucosal lesions were detected in at least one resection margin from both first and second primary tumor. Evidence for a common clonal origin of the first tumor, second primary tumor, and the intervening mucosa was found for at least 6 of 10 patients. Our results indicate that a proportion of multiple primary tumors have developed within a single preneoplastic field. Based on different etiology and clinical consequences, we propose that independent second primary tumors should be distinguished from second field tumors, that arise from the same genetically altered field the first tumor has developed from.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) comprises about 5% of all newly diagnosed cancer cases in the northern and western European countries and the United States (1). Despite advances in therapy, long-term survival of HNSCC patients has only moderately improved during the last 20 years (2). An important reason for this lack of progress is the development of second primary tumors in the upper aerodigestive tract (3). The reported incidence of second primary tumors varies, but on average they develop at a constant rate of 2 to 3% new cases per year (4). Patients at highest risk are those with early-stage disease, when control of the first (index) tumor, and therefore survival are greatest (3). It is thus critical to learn more about the molecular mechanisms of developing second primary tumors to establish new strategies to identify patients at risk and to prevent second primary tumor development.

The development of HNSCC is a multistep process involving the accumulation of genetic and epigenetic alterations in key regulatory genes (5). Deletion of somatic DNA at several tumor suppressor loci occurs early in HNSCC tumorigenesis and can be detected by microsatellite analysis (5, 6). Mutations in the *TP53* tumor suppressor gene are present in the majority of the head and neck cancers, and seem to occur early in HNSCC carcinogenesis (7-9).

Originally, it was thought that all second primary tumors develop independently after widespread epithelial exposure to carcinogens (10). By comparing *TP53* mutations and microsatellite alterations in primary tumors and corresponding second primary tumors, indications have been found that supported this theory of independent origin (11, 12). In contrast, several other reports suggested that at least a proportion of second primary tumors in HNSCC patients have arisen from one clonal cell population (13-17). Various mechanisms have been proposed to explain this proposed common clonal origin of second primary tumors such as shedding of (pre-)malignant cells into the saliva and implantation at other sites (14, 16) or lateral migration of isolated (pre-)malignant cells (13-17).

We have recently found evidence for the existence of large genetically altered mucosal lesions (referred to as “fields”) in a significant proportion of patients with an oral or oropharyngeal tumor (7). In 10 of 28 (36%) patients genetic alterations were detected in macroscopically normal mucosa surrounding the tumor, and in 7 of these 10 patients (25% of the total number) these mucosal lesions extended beyond the surgical resection margins. Genetic analysis strongly indicated a clonal relationship between tumor and accompanying genetically altered field. The picture emerges that these tumors have developed within a pre-existing preneoplastic field. In this model, a single genetically altered cell gives rise to a proliferating clone that develops into an expanding

preneoplastic field and gradually replaces the normal mucosa. During progression within this genetically altered field various clones develop with additional genetic alterations. This is a continuous process of evolution and eventually multiple clones exist that are genetically different but share a common origin. One clone ultimately develops into a carcinoma. From the clinical point of view it is important to realize that after surgery of the first primary tumor, the nonresected field may progress further into a second primary tumor in adjacent areas. If this hypothesis is valid, the first and second primary tumors should have a common clonal origin and should share specific genetic alterations that are also present in the intervening genetically altered mucosa.

To test our hypothesis, we analyzed multiple primary oral and oropharyngeal tumors and their corresponding surgical resection margins from 10 patients. The clonal origin of the tumors was examined by studying patterns of allelic loss and *TP53* mutations in the tumor samples and the intervening mucosa.

## Materials and Methods

### Patients and Tumor Specimens

Ten HNSCC patients who developed a second primary tumor in the period of 1995 to 2000 formed the basis of the present study. All patients fulfilled the following criteria: 1) all tumors must have been located in the oral cavity and/or in the oropharynx; 2) all tumors must have been of the squamous cell type; 3) all tumors must have been surgically resected to obtain mucosa of the routinely paraffin-embedded resection margins (used for histopathological assessment of complete tumor excision); 4) the first primary tumor must have been completely resected as assessed by conventional histopathological examination of the resection margins; 5) the second primary tumor and the first tumor must have been separated by at least 3 cm of nonneoplastic epithelium based on clinical and surgical findings [the distance between the tumors is an additional criterion to the ones proposed by Warren and Gates (18) and was larger than the 2 cm used by Hong and colleagues (19)], and for the present study, it appeared that the first and second primary tumors were separated by a distance that varied between 3 and 6 cm; and 6) sufficient material should be available in the samples for DNA extraction and subsequent analysis. A total of 22 tumor samples were collected from these 10 patients. Seventeen tumor samples were obtained as archival paraffin-embedded tissue and five tumor samples as frozen material. The resection margins were obtained as archival paraffin-embedded tissue. From two patients (patients 4 and 7) with two synchronous HNSCCs an additional mucosal biopsy located between the two tumors was obtained during surgery. Patients' characteristics are summarized in Table 1. Tumor stages (pTN) were determined according to the International Union Against Cancer criteria (20).

***Table 1.*** *Patient characteristics and clonal relationship of primary tumor and second primary tumor*

| Patient | Sex | Lesion | Date | Site[a] | pTN | Clonality[b] |
|---|---|---|---|---|---|---|
| 1 | F | t1 | 7/97 | R retromolar trigone | T2N2b | Clonal |
| | | t2 | 12/97 | Anterior floor of mouth | T1N0 | |
| 2 | M | t1 | 2/97 | L retromolar trigone | T2N0 | Not clonal |
| | | t2 | 2/97 | R mobile tongue | T2N0 | |
| | | t3 | 4/98 | Uvula | T1N0 | |
| 3 | M | t1 | 7/94 | L-lateral border of tongue | T2N0 | Clonal |
| | | t2 | 3/99 | L retromolar trigone | T1N0 | |
| 4 | F | t1 | 5/99 | L base of tongue | T2N1 | Not clonal |
| | | t2 | 5/99 | R anterior tonsillar pillar | T1N0 | |
| 5 | M | t1 | 11/92 | R lower alveolus | T4N1 | Clonal |
| | | t2 | 12/98 | R upper alveolus | T2N0 | |
| 6 | M | t1 | 3/93 | R lower alveolus | T3N2b | Not clonal |
| | | t2 | 5/98 | R retromolar trigone | T2N0 | |
| 7 | M | t1 | 11/00 | L retromolar trigone | T2N0 | Clonal |
| | | t2 | 11/00 | Anterior floor of mouth | T2N1 | |
| 8 | M | t1 | 10/91 | L-lateral border of tongue | T1N0 | Clonal |
| | | t2 | 9/95 | Anterior floor of mouth | T1N0 | |
| 9 | F | t1 | 3/95 | R buccal mucosa | T2N0 | Clonal |
| | | t2 | 4/96 | Soft palate | T2N0 | |
| 10 | M | t1 | 2/88 | Anterior floor of mouth | T3N1 | Not clonal |
| | | t2 | 8/95 | L retromolar trigone | T4N0 | |
| | | t3 | 8/95 | Uvula | T1N0 | |

*a L, left; R, right.*

*b Clonal relationship of the primary tumor and second primary tumor revealed by molecular analysis. All tumors that were clonally related originated from a single precursor lesion.*

**Microdissection of tumor and mucosa samples**.

Freshly frozen tumor samples were cut on a cryo-microtome, and tissue sections (10 μm) were mounted on microscopic glass slides. From the paraffin-embedded tumor samples 10-μm sections were obtained, placed on microscopic glass slides, and subsequently deparaffinized in xylene. For all cases the first and last tissue sections were stained with hematoxylin-eosin (H&E) for histological analysis and to guide microdissection. The other tissue sections were stained with 1% toluidine blue and 0.2% methylene blue and manually microdissected under a stereomicroscope. All microdissected samples contained > 80 % of tumor cells.

From every tumor at least two paraffin-embedded margins were microdissected. We selected margins that were localized opposite to the other tumor and were nearest to the virtual line that connects the tumors. When one of these two margins showed genetic alterations, we did not study any other margin. When both margins did not show genetic alterations, all other margins were studied as well. Histopathology and TP53 immununostaining were used to guide microdissection (see below). First, all H&E slides of the resection margins were examined and histopathologically abnormal mucosa was microdissected. Second, mucosa that showed clear suprabasal staining for TP53 was microdissected separately. When no histopathologically abnormal or TP53-positive stained mucosa was present, a part of the normal mucosa was selected at random and microdissected. All mucosal areas selected for microdissection were continuous in a given sample and encompassed at least 25% of the total mucosa of the resection margin.

**Selection of chromosomal loci for microsatellite analysis**

To assess genetic relationships between first tumor, second primary tumor, and both accompanying fields, we investigated loss of heterozygosity (LOH) by using 19 microsatellite markers located at chromosomes 3p, 9p, 17p, and 13q. These markers were selected because they frequently demonstrate LOH in HNSCC and precursor lesions (5, 6, 21). The following markers were used: D3S1284 (3p12), D3S1274 (3p12), D3S1217 (3p13), D3S1766 (3p14), D3S1029 (3p21), D3S1293 (3p24), D9S171 (9p21), D9S1748 (9p21), D9S1751 (9p21), IFNA (9p21), D9S162 (9p22), D9S157 (9p22), CHRNB1 (17p11-12), TP53 (17p13.1), D17S1866 (17p13.3), D13S294 (13q14.3), D13S168 (13q14.3), D13S170 (13q31), and D13S158 (13q32). Primer sequences were obtained from the Genome Database for all of these markers (http://gdbwww.gdb.org/).

**DNA extraction and microsatellite analysis**

Dissected tissues were treated with 1 mg/ml of proteinase K for 24 h at 52 ºC in a 100-μl buffer containing 100 mM TRIS-HCL (pH 9.0), 10 mM NaCl, 1% SDS, and 5 mM EDTA. The DNA was purified by phenol-chloroform extraction and collected by ethanol

precipitation using 2 µg of glycogen as carrier. The DNA was redissolved in LoTE-buffer (3 mM TRIS; 0.2 mM EDTA, pH 7.5). Normal DNA was isolated from blood samples obtained at the time of surgery, from connective tissue or from muscle microdissected from the sections. The DNA concentration was measured by microfluorometry with the Hoefer Dynaquant (Amersham/Pharmacia Benelux NV, Roosendaal, the Netherlands).

Microsatellite analysis was performed on an automated ABI PRISM sequencer (310 Genetic Analyzer; Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). One primer (Isogen Bioscience, Maarssen, the Netherlands) of each marker was end-labeled with one of the fluorescent dyes FAM, HEX, or NED (Applied Biosystems). DNA (10 ng) was amplified by multiplex polymerase chain reaction (PCR) (involving two or three markers) in a total volume of 10 µl containing 2 pmol of each labeled and unlabeled primer. Details of the multiplex PCR are available on request. The PCR buffer included 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ , 0.2 mM deoxynucleotide triphosphate and 0.5 U of *Taq* DNA polymerase (AmpliTaq, Perkin Elmer, Gouda, the Netherlands). PCR amplifications for each primer set were performed for 35 cycles consisting of denaturation at 94º C for 1 minute, annealing at a temperature between 55 and 65 º C (depending on the primer set) for 1 minute, and extension at 72 º C for 2 minutes. The amplified product was diluted in sterilized water, usually fivefold. For analysis, 12 µl of deionized formamide were combined with 0.5 µl of Genescan-350 [ROX] size standard (Applied Biosystems) and 1 µl of diluted PCR product in a tube. The samples were loaded on the automated sequencer and run following the supplier's protocol. The data were analyzed with GeneScan Analysis software (version 1.2; Applied Biosystems). LOH was scored if one allele was decreased by more than 50% in the tumor sample when compared with the same allele in normal control DNA, when necessary after stutter correction as described previously (22).

**Immunohistochemistry with anti-TP53 antibodies**

Immunohistochemical staining was performed on the primary tumor samples and on the accompanying paraffin-embedded resection margins. The method used for TP53 staining was essentially as described by Cruz *et al.* (23). Briefly, 5-µm sections were deparaffinized, placed in 0.3% methanolic peroxide (30 min) to block endogenous peroxidase activity, rinsed in PBS (phosphate-buffered saline) and subsequently subjected to antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven (600 W at 100°C for 10 minutes). Preincubation with normal rabbit serum (NRS 1:50; DAKO, Copenhagen, Denmark) was followed by overnight incubation at 4°C with anti-TP53 monoclonal antibody DO7 (1:500; DAKO). Consecutive sections were incubated with mouse myeloma IgG monoclonal antibody (1:500; Zymed, San Francisco, USA) as a negative control. After incubation, slides were thoroughly washed and sections

sequentially incubated with biotinylated rabbit anti-mouse antibody (1:500; DAKO) for 1 hour. Diaminobenzidine in $H_2O_2$ was used as chromogen (stained for 5 min). Sections were counterstained with haematoxylin, dehydrated and mounted with xylene-substitute mountant. The staining pattern of the tumor samples was assessed and was classified positive if more than 50% of the tumor cell nuclei were stained. The mucosa of the resection margins was scored positively when there was a clear suprabasal staining.

***TP53* sequencing**

All tumors of the 10 HNSCC patients were sequenced for exons 5-9 of the *TP53* gene. Sequencing was performed as described by Sidransky *et al*. (24). In short, an 1.8-kb fragment of the *TP53* gene, encompassing the exons 5-9, was amplified from DNA of microdissected frozen tumor specimens. For paraffin-embedded material the exons were amplified separately. Purified PCR products were directly sequenced by exon-specific primers using the radioactive dideoxynucleotide method (Applied Biosystems) (24). Primer sequences and reaction conditions are available on request. When no mutation was found in exon 5-9 of the *TP53*-gene, exon 4 was sequenced in addition. When a *TP53* mutation was detected in DNA of the primary tumors, DNA isolated from the mucosa of the accompanying resection margins was subsequently sequenced for mutations in that particular exon.

**Histopathological classification**

All H&E stained slides were examined by an experienced pathologist (JEvdW) and scored according to the standard criteria of the World Health Organization international histological classification of tumors (25). Lesions were classified as: 1) normal mucosa, 2) mild dysplasia, 3) moderate dysplasia, 4) severe dysplasia or carcinoma in situ, and 5) squamous cell carcinoma. The pathologist had no information on molecular data during histopathological classification.

**Methodological Analysis**

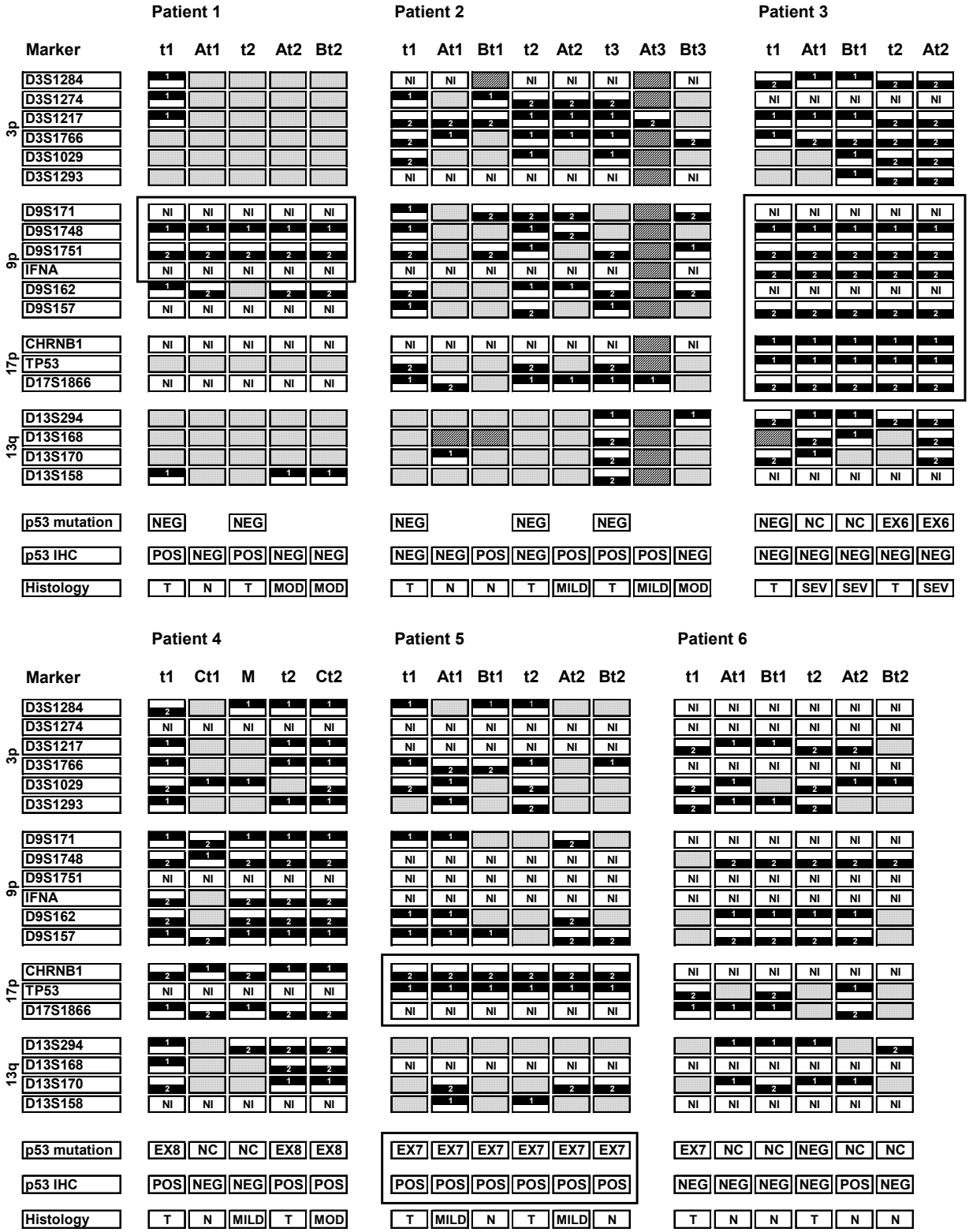
We tested the hypothesis that one genetically aberrant cell has evolved into a large field consisting of various subclones with related genetic patterns. Within this field two subclones developed into two carcinomas. As a consequence, all cells in the field and the tumors share one or more genetic aberration(s) characteristic for the primordial clone. To be considered “field” two criteria were applied: the dissected mucosal lesion should not be malignant according to routine histopathological criteria and LOH should be present in at least one marker. The likelihood that the various lesions (first tumor, field of first tumor, field of second primary tumor, and second primary tumor) are genetically related, and thus have a common clonal origin, was based on the comparison of TP53 mutations and

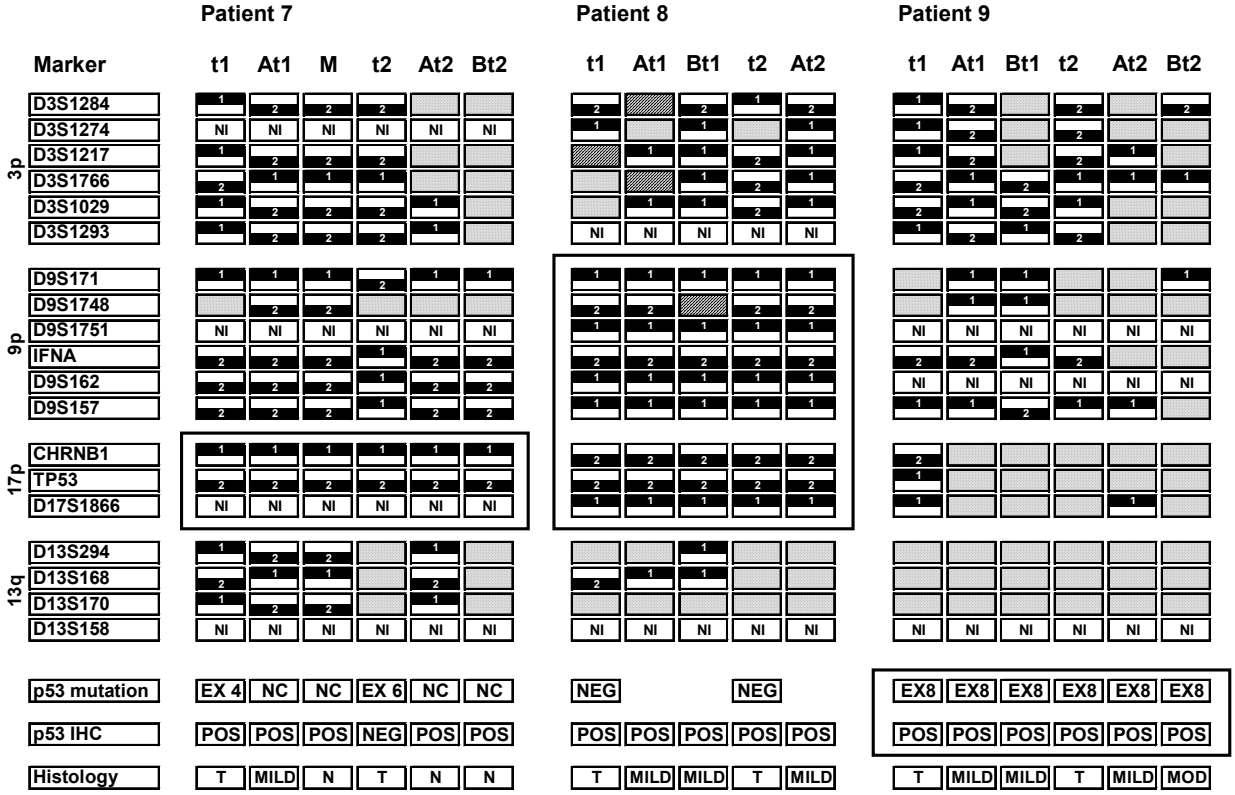
LOH patterns. When the TP53 mutation was identical in primary tumor, field of primary tumor, field of second primary tumor, and second primary tumor, the clonal relationship was considered proven. As for the LOH pattern, the probability that this similarity arose by chance was calculated. The chromosomal locus 9p21 (markers D9S171, D9S1748, D9S1751, and IFNA) and/or the chromosomal arm of 17p (markers CHRNB1, TP53, and D17S1866) were selected because these loci particularly demonstrated LOH in genetically altered and tumor-related fields as has been shown previously (5, 7, 26, 27). When LOH patterns at 9p21 and/or 17p appeared to be similar then the most telomeric informative marker of either of these chromosomal loci was selected for probability calculation based on the LOH frequencies reported for these loci. Based on previous work, frequencies of LOH in HNSCCs at 9p21 and 17p were estimated as 0.93 and 0.70, respectively (7, 9). The probability that the same (paternal or maternal) allele was lost is therefore 0.5 x 0.93 = 0.465 and 0.5 x 0.70 = 0.35 for both markers. Genetic alterations at either chromosomal locus 9p21 or 17p were considered to be independent, allowing the multiplication of the probabilities when both loci were involved. The probabilities that a similar LOH pattern arose by chance in the four lesions investigated are calculated will be either $(0.465)^3$ = 0.10 (only 9p21 involved), $(0.35)^3$ = 0.04 (only 17p involved), or $(0.465)^3$ x $(0.35)^3$ = 0.004 (both 9p21 and 17p involved).

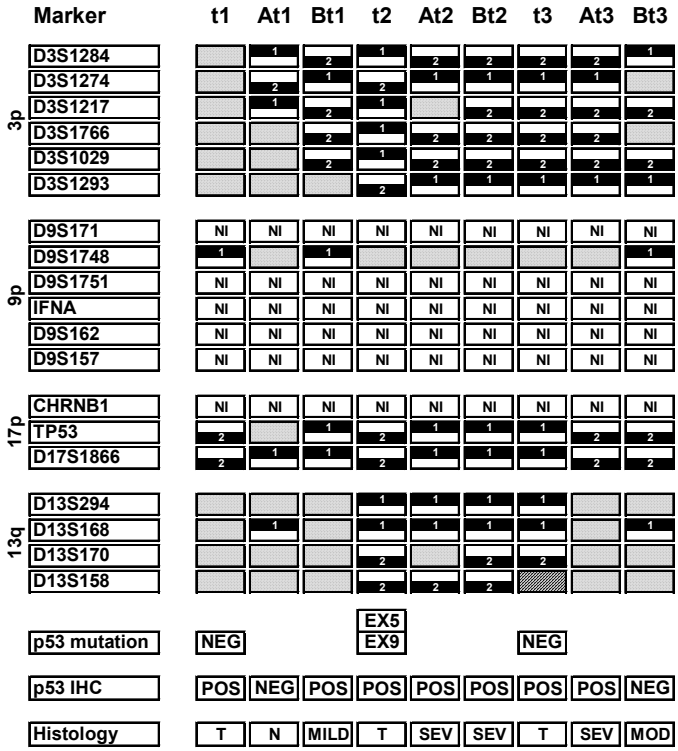
## Results

### Detection of Genetically Altered Fields in Resection Margins

In all 10 patients, genetically altered fields were detected in at least one resection margin from both first tumor and second primary tumor (Figure 1). In nine patients, genetic alterations were detected in the one or two margins facing the other tumor. Only in patient 4, the two margins facing the other tumor did not show genetic alterations. However, in other margins of both tumors of this patient genetic alterations were detected. The genetic alterations were detected in mucosal lesions that encompassed at least 25% of the total epithelial layer of a surgical margin and were at least 4 mm in length. In most cases, the genetically altered mucosa showed similar genetic alterations when compared to the corresponding tumor, suggesting a clonal relationship. In a few cases the tumor and the corresponding resection margins, had no genetic alterations in common (eg, first tumor of patient 4 and corresponding margin Ct1 and patient 10 and margin At1).


Patient 1
Patient 2
Patient 3
Marker
t1 At1 t2 At2 Bt2
t1 At1 Bt1 t2 At2 t3 At3 Bt3
t1 At1 Bt1 t2 At2
3p
D3S1284
D3S1274
D3S1217
D3S1766
D3S1029
D3S1293
9p
D9S171
D9S1748
D9S1751
IFNA
D9S162
D9S157
17p
CHRNB1
TP53
D17S1866
13q
D13S294
D13S168
D13S170
D13S158
p53 mutation
p53 IHC
Histology
Patient 4
Patient 5
Patient 6
Marker
t1 Ct1 M t2 Ct2
t1 At1 Bt1 t2 At2 Bt2
t1 At1 Bt1 t2 At2 Bt2
3p
D3S1284
D3S1274
D3S1217
D3S1766
D3S1029
D3S1293
9p
D9S171
D9S1748
D9S1751
IFNA
D9S162
D9S157
17p
CHRNB1
TP53
D17S1866
13q
D13S294
D13S168
D13S170
D13S158
p53 mutation
p53 IHC
Histology


Patient 7
Patient 8
Patient 9
Marker
t1 At1 M t2 At2 Bt2
t1 At1 Bt1 t2 At2
t1 At1 Bt1 t2 At2 Bt2
3p
D3S1284
D3S1274
D3S1217
D3S1766
D3S1029
D3S1293
9p
D9S171
D9S1748
D9S1751
IFNA
D9S162
D9S157
17p
CHRNB1
TP53
D17S1866
13q
D13S294
D13S168
D13S170
D13S158
p53 mutation
EX 4 NC NC EX 6 NC NC
NEG NEG
EX8 EX8 EX8 EX8 EX8 EX8
p53 IHC
POS POS POS NEG POS POS
POS POS POS POS POS
POS POS POS POS POS POS
Histology
T MILD N T N N
T MILD MILD T MILD
T MILD MILD T MILD MOD


Patient 10
Marker
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3p
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D3S1274
D3S1217
D3S1766
D3S1029
D3S1293
9p
D9S171
D9S1748
D9S1751
IFNA
D9S162
D9S157
17p
CHRNB1
TP53
D17S1866
13q
D13S294
D13S168
D13S170
D13S158
p53 mutation
NEG
EX5
EX9
NEG
p53 IHC
POS NEG POS POS POS POS POS POS NEG
Histology
T N MILD T SEV SEV T SEV MOD

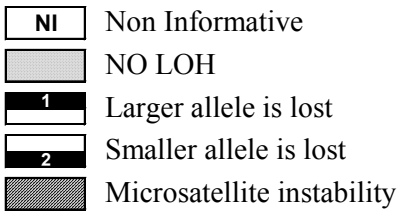
NI
Non Informative
NO LOH
1
Larger allele is lost
2
Smaller allele is lost
Microsatellite instability

***Figure 1.*** *Results of the genetic analysis of the multiple primary tumors from 10 patients. t1, t2, and t3, represent the first tumor, second primary tumor, and third tumor, respectively. At1 and Bt1 represent the resection margins of the first primary tumor that were localized in the opposite direction to the second primary tumor and were nearest to the virtual line that connects both tumors. At2 and Bt2 represent the resection margins of the second primary tumor that were localized in the opposite direction to the first primary tumor. Only the margins that showed genetic alterations are presented. When no genetic alterations were detected in the two margins facing the other tumor, all other margins (with prefix Ct) were studied as well. Only in patient 4 were genetic alterations detected in other margins than the two margins facing the other tumor. M represents a mucosal biopsy that was located between the two synchronous tumors of patients 4 and 7. The TP53 mutations are indicated as NEG (negative) and EX4-9 when a mutation was detected in that particular exon. The codon and type of mutation are listed in Table 2. NC indicates that TP53 mutation of the tumor could not be confirmed in the corresponding resection margins. The immunohistochemical staining for the TP53 protein (p53 IHC) was indicated as NEG (negative) and POS (positive). The samples were histopathologically classified as normal mucosa (N), mild dysplasia (MILD), moderate dysplasia (MOD), severe dysplasia (SEV), and squamous cell carcinoma (T). Genetic alterations that were present in the first and second primary tumor and both resection margins are placed in a* ***framed box****. In all these cases, the margins with genetic alterations were facing the other tumor, suggesting that these margins are part of a single preneoplastic field.*

***Table 2.*** *Results of TP53-immunostaining (TP53-IHC) and TP53 mutation analysis in primary tumor and second primary tumor*

| Patient | Lesion[a] | TP53-IHC | Change[b] | Exon | Codon | Aa-change[c] | Resection margins[d] |
|---|---|---|---|---|---|---|---|
| 1 | t1 | + | Wt | | | | |
| | t2 | + | Wt | | | | |
| 2 | t1 | – | Wt | | | | |
| | t2 | – | Wt | | | | |
| | t3 | + | Wt | | | | |
| 3 | t1 | – | Wt | | | | |
| | t2 | – | G → A | 6 | Intron | Splice site | Id. (At2) |
| 4 | t1 | + | G → A | 8 | 273 | Arg → His | Neg. (Ct1, M) |
| | t2 | + | G → A | 8 | 273 | Arg → His | Id. (Ct2) |
| 5 | t1 | + | G → A | 7 | 244 | Gly → Asp | Id. (At1, Bt1) |
| | t2 | + | G → A | 7 | 244 | Gly → Asp | Id. (At2, Bt2) |
| 6 | t1 | – | G → A | 7 | Intron | Splice site | Neg. (At1, Bt1) |
| | t2 | – | Wt | | | | |
| 7 | t1 | + | 1 bp ins | 4 | 109 | Frameshift | Neg. (At1, M) |
| | t2 | – | C → T | 6 | 196 | Arg → Stop | Neg. (At2, Bt2) |
| 8 | t1 | + | Wt | | | | |
| | t2 | + | Wt | | | | |
| 9 | t1 | + | A → T | 8 | 280 | Arg → Stop | Id. (At1, Bt1) |
| | t2 | + | A → T | 8 | 280 | Arg → Stop | Id. (At2, Bt2) |
| 10 | t1 | + | Wt | | | | |
| | t2 | + | G → A | 5 | 175 | Arg → His | ND |
| | | | A → T | 9 | Intron | Splice site | ND |
| | t3 | + | Wt | | | | |

*a t1 and t2 represent the first tumor and second primary tumor (SPT)*
*b wt, wild type; ins, insertion.*
*c Aa-change = amino-acid change*
*d Id. means that the TP53 mutation of the tumor was identical to the mutation of the corresponding resection margins listed in parentheses. Neg. means that the mutation of the tumor was not confirmed in the samples of the corresponding margins listed in parentheses. ND means not determined, since the corresponding resection margins showed loss of the opposite allele at the TP53 locus (Figure 1) when compared to the primary tumor. For the codes of the samples listed in parentheses see Figure 1.*

**TP53 Mutation Analysis of the First Tumor, Second Primary Tumor, and Corresponding Resection Margins**

Ten primary tumors and 12 second primary tumors from 10 patients were sequenced for TP53 mutations in exons 5 to 9. In nine tumors a single TP53 mutation was detected in exons 5 to 9, and in one tumor (patient 10, t2) two mutations were detected (Table 2). In 12 tumors no mutation was found in exons 5 to 9 of the TP53 gene, and by additional sequencing of exon 4 an extra mutation could be identified in 1 of these 12 tumors (patient 7, Table 2).

From the 11 tumors in which a *TP53* mutation was detected, we also tried to confirm the presence of the mutation in the mucosa of the corresponding resection margins (Table 2). In six comparisons, the *TP53* mutation of the tumor could also be detected in one or more resection margin(s). Three patients showed the same *TP53* mutations in both first primary tumor and second primary tumor. In two of these three patients (patients 5 and 9), the same *TP53* mutation could be detected in the mucosa of the resection margins of both the first and second primary tumor (Table 2, Figure 1). To exclude the possibility that these concordant *TP53* mutations were germ-line mutations, DNA of connective tissue and muscle from both patients was sequenced in addition. In both cases, the DNA of connective tissue and muscle showed wild-type *TP53*. In the third patient (patient 4), the *TP53* mutation could only be detected in the mucosa of the resection margin of the second primary tumor and not in the resection margins of the first tumor. Furthermore, a mucosal biopsy of this patient (located between the primary tumor and second primary tumor) also lacked the *TP53* mutation.

**LOH Analysis of First Tumor, Second Primary Tumor, and Corresponding Resection Margins**

From all 10 patients the first tumor, the second primary tumor, and mucosa of the corresponding resection margins of both tumors were analyzed for LOH. The results of the LOH analysis are summarized in Figure 1. All tumors analyzed showed LOH at two or more chromosomal loci. For none of the 10 patients was the LOH pattern of the first tumor completely identical to the LOH pattern of the corresponding second primary tumor, and it differed at least at five microsatellite markers. Five of 10 patients showed concordant LOH at one or more chromosomal loci in the first tumor, second primary tumor, and the corresponding margins of both tumors (Figure 1). Patient 1 showed loss of the same alleles at 9p21 in the first tumor, its resection margin A, the second primary tumor, and its resection margins A and B (Figure 1). Patients 3 and 8 showed loss of the same allele at 9p21, 9p22, and 17p in the first tumor, second primary tumor, and the resection margins of both tumors. Patient 7 showed the same LOH pattern at chromosome 17p in the first tumor, resection margin A of the primary tumor, mucosal biopsy M

(located between the primary tumor and second primary tumor), resection margins A and B of the second primary tumor, and the second primary tumor. Finally, patient 5 showed the same LOH pattern at chromosomal loci 17p in the first tumor, the resection margins A and B of the first tumor, resection margin A and B of second primary tumor, and the second primary tumor (also the same TP53 mutation was detected in all samples of this patient, see above).

**Interpretation of the Results and Molecular Assessment of Clonality**

The hypothesis that the first tumor and second primary tumor have a common clonal origin and thus have developed from a large common premalignant field was considered to be confirmed in patients 5 and 9. Both tumors and accompanying resection margins of these patients showed the same TP53 mutation.

In contrast to *TP53* mutations, LOH patterns are less specific and are much more likely to be identical by chance. For four cases (patients 1, 3, 7, and 8) concordant LOH at 9p21 and/or 17p13 was observed between the first tumor, the second primary tumor, and the intervening mucosa. The probability that this concordant LOH pattern arose by chance was calculated. As an example in patient 7 the resection margins closest to the virtual line between the tumors were At1 and At2. The mucosa of these margins was assigned as field-based on the presence of LOH. The first tumor, margin At1, margin At2, and second primary tumor showed loss of the same alleles at 17p. Microsatellite marker TP53 was used for calculation using LOH in the primary tumor as proband, and the probability that the pattern arose by chance was calculated as 1 x 0.35 x 0.35 x 0.35 = 0.043 or 4.3% (see Materials and Methods). The probabilities that the LOH patterns arose by chance were 0.43% for patient 3, 10% for patient 1, and 0.43% for patient 8. In this study we performed calculations based on the presence of a common LOH. The absence of LOH, however, was found at the chromosomal loci 3p14-3p24, 17p, and 13q14 for patient 1 and at chromosomal loci 13q14-13q32 for patient 9. If we had used this information in our calculations as well, then the probabilities that the similarity of LOH patterns arose by chance would have been lower for these two patients. We judged that sufficient evidence is provided that for 6 of 10 patients the first tumor and second primary tumor are clonally related and arose from a single primordial field. The molecular diagnosis of clonality is summarized in Table 1.

**Histology and Immunohistochemistry**

All tumor samples and corresponding resection margins were reviewed and histopathologically classified and compared with the genetic analyses. A total of 37 paraffin-embedded surgical margins showing genetic alterations were histopathologically classified as: 13 as normal mucosa, 12 as mild dysplasia, 6 as moderate dysplasia, and 6

as severe dysplasia (see Figure 1). The two freshly frozen mucosal biopsies (located between the primary tumor and second primary tumor) that both showed LOH were classified as normal mucosa (patient 7) and mild dysplasia (patient 4). All tumor samples and corresponding resection margins were analyzed by immunohistochemistry for overexpression of the TP53 protein. Fifteen of the 22 analyzed tumors showed intense TP53 staining of most tumor nuclei (Figure 1, Table 2). In 8 of the 15 positively stained tumors, a TP53 mutation was identified (Table 2). Twenty-three of the 37 surgical margins that showed genetic alterations demonstrated a clear suprabasal staining for the TP53 protein (Figure 1).

## Discussion

Second primary tumors are a major problem in head and neck oncology. Their development has a profound impact on long-term survival, particularly for patients with early-stage disease. Current understanding of the origin of these second tumors is limited. In the present study molecular techniques have been used to better understand the pathobiology of these lesions. An important finding of this study was that the frequency of genetically altered field is very high in the total patient group. In all ten cases proof for field was detected, surrounding both first tumor and SPT. This prevalence of a genetically altered field is much higher than the 25% we have observed for a group of 28 unselected patients with a single oral or oropharyngeal tumor (7). It should be noted, that although we have evaluated the same chromosomal loci, we used more markers in the current study; when the analysis is limited to the markers of the previous study one of the patients (number 1) would have been judged to be without a field. The overall results of the present study suggest that the presence of large genetically altered fields in one or more resection margin(s) is a risk factor for the development of SPT. Therefore, analyzing margins for the presence of genetically altered mucosa might identify the subgroup of patients at high risk for developing SPT. Whether (and which type of) an unresected genetically altered field eventually will develop into SPT, is not clear at this time and should be determined in a carefully designed study with larger patient groups. Other parameters should be taken into account as well, such as follow-up time (26, 28), smoking behavior (29, 30) and specific genetic alterations (28). If such a high-risk patient group can be identified chemoprevention or gene therapy are potential approaches to prevent the development of SPT.

Evidence was provided that genetically altered fields could be large. For six of the ten patients two primary tumors have developed in a contiguous genetiallly altered field. In all these six patients the first and second primary tumor were separated by 3 to 6 cm,

indicating the large extension of these fields. Taking also the surface of the tumors into account, fields can have a diameter of over 7 cm.

On the basis of the results of the present and the previous study (7), we propose a revised model for head and neck carcinogenesis. Large areas of normal mucosa are replaced by a population of genetically altered cells of monoclonal origin (referred to as an expanding field). Within such a field additional genetic hits may lead to the emergence of multiple genetically related subclones, and one eventually develops into cancer. In case of a very large field (> 3 cm diameter) SPT can develop. The mode by which these genetically altered cells replace the normal mucosa is unclear, but this expanding population of cells probably has a growth advantage as compared with the normal epithelial stem cells, and therefore overgrow the normal stem cells by a higher proliferation rate. Indeed, we have recently obtained proof that lesions with genetically altered cells have a higher proliferation rate than normal epithelial cells (31). The presence of a large preneoplastic field to explain the mechanism of a common origin of first and second primary tumor make the other proposed mechanisms unlikely (13–17).

For four of the ten patients, no or in our opinion too little evidence of a single precursor lesion was found suggesting that these SPT were of independent origin. However, it should be emphasized that this may be an over-estimation and the possibility of a common initiating genetic alteration can not be completely excluded, as only a limited number of genetic markers were analyzed. To stress this point, patient 9 showed only an identical *TP53* mutation in both tumors and their corresponding margins, whereas the LOH-patterns would never have been considered convincing evidence for a common clonal origin. As discussed above, all the four patients with seemingly unrelated tumors had genetically altered fields in margins of the first and second tumor. Some of these fields did not have a single genetic alteration similar to the tumor, suggesting an independent origin of tumor and field. Such independent fields have been reported before (7).

We have detected concordant *TP53* mutations in the first tumor and second primary tumor in three cases. This is in contrast to other authors who failed to show similar *TP53* mutations in the first tumor and second primary tumor (11, 12). In two cases (patients 5 and 9) the *TP53* mutation was also present in the intervening mucosa of both tumors. Thus, for two cases convincing evidence was found that the first tumor and second primary tumor had the same clonal origin and that they had developed within a single preneoplastic lesion. In the third case (patient 4) an identical *TP53* mutation was detected in both primary tumors, but this mutation could not be detected in any of the resection margins of the first tumor. Thus, the criterion for establishing the common origin of both tumors and the field in between was not fulfilled. Of notice, this *TP53* mutation resulted in strong positive immunohistochemical staining of the tumors. The mucosa of the

margins from the first tumor, however, did not show TP53 immunopositivity, which in our view is indicative for the absence of the common genetically altered field. It should be realized that this *TP53* mutation was located at a hot spot (codon 273) in one of the most conserved areas of the *TP53* gene. Mutations at this hot spot are detected at a percentage of 6 to 9% in primary tumors [our own unpublished data and Walker and colleagues (32)]. We therefore decided that these tumors do not have a common clonal origin, and do not share a common field. In support of this statement is that the LOH analysis revealed that the tumors had lost the opposite allele at the *TP53* locus. There are, however, mechanisms, such as gene conversion and crossing over, which might explain the LOH of the opposite allele at the TP53 locus. So, it cannot be excluded completely that the tumors are genetically related, apparently without a common field.

The results of the present study suggest that inactivation of the tumor suppressor genes *p14ARF*, *p16* (both at 9p21), and *TP53* (located at 17p13) play a key role in the early malignant progression of a cell and are the initiating events in the mechanism of clonal expansion of a field. All three of these tumor suppressor genes play a crucial role in cell cycle regulation and/or apoptosis and are interacting at various levels. These tumor suppressor genes seem therefore to be the most promising molecular targets for prevention strategies.

The present results give further insight into the molecular mechanisms of second primary tumor development. Thus far, and also in the present study, the definition of second primary tumor is based on clinical parameters. Intriguingly, it is at this moment possible to discriminate between different ways of development of second primary tumor and the question arises whether every second tumor should be called a second primary tumor. In a recent article (33) we proposed a new classification of second tumors in adjacent sites for HNSCC, based on the genetic profile of the tumors and the intervening mucosal field. We propose to allocate the term “second primary tumor” for the second tumor that has developed independently from the first tumor. This type of cancer comes closest to the original clinical concept of second primary cancer. When a second tumor arises from the same field as the first tumor has developed, we propose to designate it as a “second field tumor.” It is more than a question of semantics; based on the different etiology, we believe it is important to make this discrimination. Moreover, there may be clinical consequences, eg, additional field tumors may require other screening and therapeutic approaches than real second primary tumors.

In conclusion, our study provides evidence that in a proportion of patients the primary tumor and second primary tumor develop from a single contiguous genetically altered field and thus have a common clonal origin. In other patients the first and second primary tumors develop independently from genetically unrelated fields. The picture emerges that in the second primary tumor patients large areas of the normal mucosa have

been replaced by one or more monoclonal cell populations. Adequately identifying the presence of genetically altered fields and their risk for progression may have profound implications for the effective prevention of second primary tumors.

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# 6

# Genetically Altered Fields as Origin of Locally Recurrent Head and Neck Cancer: a Retrospective Study

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Clinical Cancer Research, in press

## Abstract

*Purpose*: Surgeons treating patients with head and neck squamous cell carcinoma (HNSCC) heavily rely on histology to decide whether the resection margins were tumor-free and subsequent adjuvant treatments can be omitted. However, despite the presence of tumor-free margins ten to thirty percent of HNSCC patients still develop a locally recurrent tumor. Evidence is available that recurrent cancer develops from either 1) outgrowth of a relatively small number of tumor cells that have not been detected by the pathologist or 2) a precursor lesion in which additional genetic alterations have led again to invasive cancer.

*Experimental design*: In a retrospective study on 13 HNSCC cases we analyzed the primary tumor, its surrounding histologically tumor-free resection margins and the local recurrences for loss of heterozygosity (22 microsatellite markers on 6 chromosomes) and *TP53* mutations to determine the origin of the recurrent cancer.

*Results*: A precursor lesion was absent in five of 13 (39%) cases and the genetic similarity of the primary and recurrent cancer was high, providing evidence that residual cancer cells were the origin of recurrence. For the remaining eight cases (61%) a genetically related precursor lesion (field) was detected and for five of these cases evidence was found that both the primary and recurrent carcinoma originated from this field. The remaining three cases were less conclusive.

*Conclusions:* This study explains the pathobiology of locally recurrent HNSCC in patients with histologically tumor-free resection margins, and indicates that the development of novel therapies to decrease the local recurrence rates in HNSCC should not only be focused on eradicating residual cancer cells but also on the precursor lesions that are left behind.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) comprises approximately 5-10% of all newly diagnosed cancer cases in Europe and the United States (1). Despite significant advances in surgery and radiotherapy over the last decades, the 5-year survival rates of HNSCC patients only moderately improved partly due to the relatively high local recurrence rate. Even when the surgical margins are diagnosed as tumor-free by histopathology the local recurrence rate is still 10 to 30% (2-4). A local recurrence is defined according to clinical criteria as the occurrence of another carcinoma within three years after and less than two cm away from the primary carcinoma (5). There are two theoretical explanations for these unexpected local recurrences. First, cancer cells have remained in the patient, which were not detected in the resection margins by the pathologist. This is likely to concern a relatively small number of cancer cells and this source of local recurrence can therefore be designated local minimal residual cancer (MRC) (6). Second, it has recently been suggested that tumor-related mucosal precursor lesions, 'fields' of genetically altered cells surrounding the tumor, may be left behind, and these might give rise to new invasive carcinomas (7-9). These new carcinomas are clonally related to the primary tumor because they develop from a common genetically altered field and therefore have been designated 'second field tumors' (SFT) (5).

These fields can be large, a diameter of over seven cm has been reported for the oral cavity and oropharynx (10). These dimensions indicated that likewise second primary tumors, defined according to the clinical criteria that they develop more than two cm away from the primary tumor or minimally three years after occurrence of the primary tumor (5), might develop in these fields left behind. Indeed is was shown that the majority of second primary tumors in the same or adjacent anatomical region develop from such a precursor field left behind (10).

Most patients present with invasive carcinomas, but there is ample evidence to suggest that HNSCC develops through a number of histologically defined precursor lesions designated as mild, moderate and severe dysplasia. Califano *et al.* demonstrated that the progressing stages characterized by these histological changes run more or less parallel with an increase in the number of genetic alterations (11). Within this progression model loss of heterozygosity (LOH) of chromosomes 3p, 9p and 17p could be defined as early, and LOH of 8p, 18q and 13q as late alterations (7, 11-13). Responsible tumor suppressor genes or oncogenes have been identified on some of these chromosomal regions, such as the *TP53* gene on 17p and the *CDKN2A* locus on 9p21. Mutations in the *TP53* tumor suppressor gene are present in the majority of HNSCC (14) and evidence has been provided that *TP53* mutations are early genetic alterations in HNSCC (9, 13, 15). In a recent publication the fields of genetically altered cancer cells were discussed in the

perspective of multi-step HNSCC genesis (13). The process of clonal selection is continuing in a field and eventually a subclone develops into carcinoma, characterized by invasive growth and metastatic potential. Hence, a clonal relationship exists between a field and the carcinomas that develop within the field.

Knowledge of the type and sequence of genetic alterations involved in HNSCC genesis and the involvement of field as a precursor lesion allows us to reconstruct the origin of recurrent cancer after resection of HNSCC with curative intent. Aim of the present study was to reveal the origin of locally recurrent cancer. With the help of genetic markers we set out to determine whether local MRC or a precursor field was the cause of recurrent cancer.

## Materials and methods

**Patients and tissue samples**.

Clinicopathological information was obtained from patient records and pathology reports. Patient records were screened between 1993-2000. Inclusion criteria were: i) the primary tumor was located in the oral cavity or oropharynx, treated by surgery (with or without post-operative radiotherapy) and resection margins were histologically tumor-free, ii) the local recurrence developed within two cm from the treated area and within three years, according to published criteria (5, 16), iii) the local recurrence was biopsied or treated by surgery, and iv) sufficient paraffin-embedded material was available to allow molecular studies. In total 13 cases fulfilling all criteria were collected (Table 1).

**Genetic analyses.**

Microsatellite analysis at 3p, 9p, 17p, 8p, 13q and 18q and mutations in the *TP53* gene were used as genetic markers of clonality. DNA was sequenced for *TP53* mutations in exons 4 to 10 as described previously (17). LOH analysis was performed as described previously (10, 18). In short, 22 microsatellite markers on chromosomes 3p, 9p, 17p, 8p, 13q and 18q were selected for LOH analysis. Paraffin embedded tissue blocks were used that have been prepared for routine histopathology. Neoplastic areas were manually microdissected from both primary tumors and local recurrences. Moreover, all resection margins, minimally four and maximally seven, surrounding the primary tumor were screened for dysplasia by an experienced pathologist (JAK). All areas with signs of dysplasia were manually micro-dissected. When no morphological changes were found for a particular patient, the epithelium of each margin was divided randomly and dissected. DNA of the stroma was used as control to assess LOH and was taken from the same paraffin block the tumor or margins was dissected from.

**Establishment of common genetically altered field**.

We used genetic markers to discriminate SFT from the MRC-derived local recurrence. We started the analysis by defining whether the primary tumor was surrounded by a field and whether this had a clonal origin common with both the primary tumor and the local recurrence. To establish a clonal relationship published criteria were used (19): the primary tumor, local recurrence and the epithelial layer of one of the margins of the primary tumor should contain either i) a similar TP53 mutation and/or ii) a similar microsatellite shift, an alteration in the length of a microsatellite also referred to as MSI and/or ii) a similar pattern of LOH (based on all markers and at least two informative markers per arm) in a single chromosomal arm. The probability that any of these alterations occur by chance in all these three lesions can considered to be low. With respect to the analysis of the margins: when more than one margin showed concordant losses, the one with the highest level of concordance was selected for further comparison and calculation.

**Assessment of clonal relationship in the absence of field.**

The absence of a genetically field was considered an indication that the recurrence developed from MRC. It could, however, not be excluded that the field remained unnoticed and therefore additional confirmation based on the comparison of the genetic profiles of primary tumor and recurrence was performed. An identical TP53 mutation or an identical microsatellite shift in the primary and recurrent tumor was considered proof that MRC was the origin of the recurrence (18, 19). In addition, LOH profiles of the primary tumor and local recurrence were compared for this purpose and we calculated the chance that the genetic pattern of the local recurrence was identical to that of the tumor by coincidence. Starting point for the calculation was the reported LOH frequency for the different chromosome arms (3p: 76%, 9p: 82%, 17p: 74%, 13q: 46%, 18q: 35%, 8p: 70%). These frequencies have been determined in primary HNSCCs (median number of carcinomas of 50, range 40-81) from previous studies (9, 10, 18). Microsatellites on a given chromosome arm do not detect LOH in an independent manner and therefore not the individual microsatellite markers, but all markers on a chromosome arm were considered to represent one locus. Information was also obtained from the arms that showed no LOH at all. The probability (in percentage) was calculated that a similar pattern (yes or no LOH in multiple arms) occurred in both lesions by coincidence. If this was below 5%, the genetic concordance between primary tumor and local recurrences was considered high enough to support the option that MRC was the origin of the recurrence.

**Assessment of clonal relationship in the presence of field.**

In the cases that a precursor lesion, a genetically altered field surrounding the tumor was observed, the genetic patterns of the primary tumor and the local recurrence were compared. Even when such a field is present, an MRC origin of the recurrent cancer is an option. A genetic similarity between a primary and recurrent tumor can be considered evidence for a MRC origin of the recurrent cancer, while a genetic dissimilarity indicates that the recurrent cancer has progressed independently in the preneoplastic field. *TP53* mutations, microsatellite shifts and LOH profiles of the primary tumor and local recurrence were compared for this purpose. To allow for this comparison the genetic markers that defined the presence of the common precursor lesion had to be excluded, since these lack discriminative power on theoretical grounds. A concordant MSI or *TP53* mutation (not shared with the field), was judged to be in favor of the vision that the local recurrence originated from local MRC. Without concordant *TP53* mutations or microsatellite instability the probability was calculated that the LOH pattern of the local recurrence was identical to that of the tumor by coincidence. We used for this calculation the reported LOH frequency for the different chromosome arms, as discussed above. All microsatellites at one arm were considered to represent one locus and information was also obtained from the arms that showed no LOH at all. A value below 5% was considered statistical evidence to assume that the LOH pattern of the recurrence was concordant with that of the primary tumor, and that MRC was the source of the local recurrence.

***Table 1.*** *Patient characteristics and origin of local recurrence*

| Patient number | Gender | Age | Site | TNM | RT[a] | Dysplasia[b] | Interval[c] (months) |
|---|---|---|---|---|---|---|---|
| 1 | F | 50 | Mobile tongue, L [d] | T1N0 | N | Mild | 10 |
| 2 | M | 75 | Mobile tongue, R | T1N0 | N | Mild | 9 |
| 3 | M | 46 | Mobile tongue, L | T2N2B | Y | No | 6 |
| 4 | F | 52 | Floor of mouth, Ant | T2N0 | N | No | 6 |
| 5 | M | 65 | Floor of mouth, L | T1N0 | N | Mild[e] | 25 |
| 6 | F | 67 | Base of tongue, L | T2N1 | Y | No | 9 |
| 7 | M | 74 | Proc alv sup, R | T2N0 | N | Mild | 10 |
| 8 | M | 81 | Mobile tongue, L | T2N0 | N | Severe | 30 |
| 9 | M | 72 | Base of tongue, R | T3N2B | N[f] | No | 5 |
| 10 | F | 83 | Mobile tongue, L | T1N0 | N | No | 7 |
| 11 | F | 86 | Mobile tongue, R | T1N0 | N | Mild | 29 |
| 12 | F | 64 | Floor of mouth, Ant | T4N2C | Y | No | 11 |
| 13 | M | 44 | Floor of mouth, R | T3N1 | N | No | 15 |

*a) RT means treated by postoperative radiotherapy.*

*b) The grade of dysplasia indicated corresponds to the margin that was considered to contain the tumor-related 'field'. When other margins showed a higher grade, it was indicated separately.*

*c) Interval means time to local recurrence.*

*d) L means left, R means right, Ant means anterior. Proc alv sup indicates processus alveolaris superior.*

*e) Margin A (see Fig. 1) was graded as moderate dysplasia. However, the genetic profile of the mild dysplastic lesion of margin E showing more concordance is shown.*

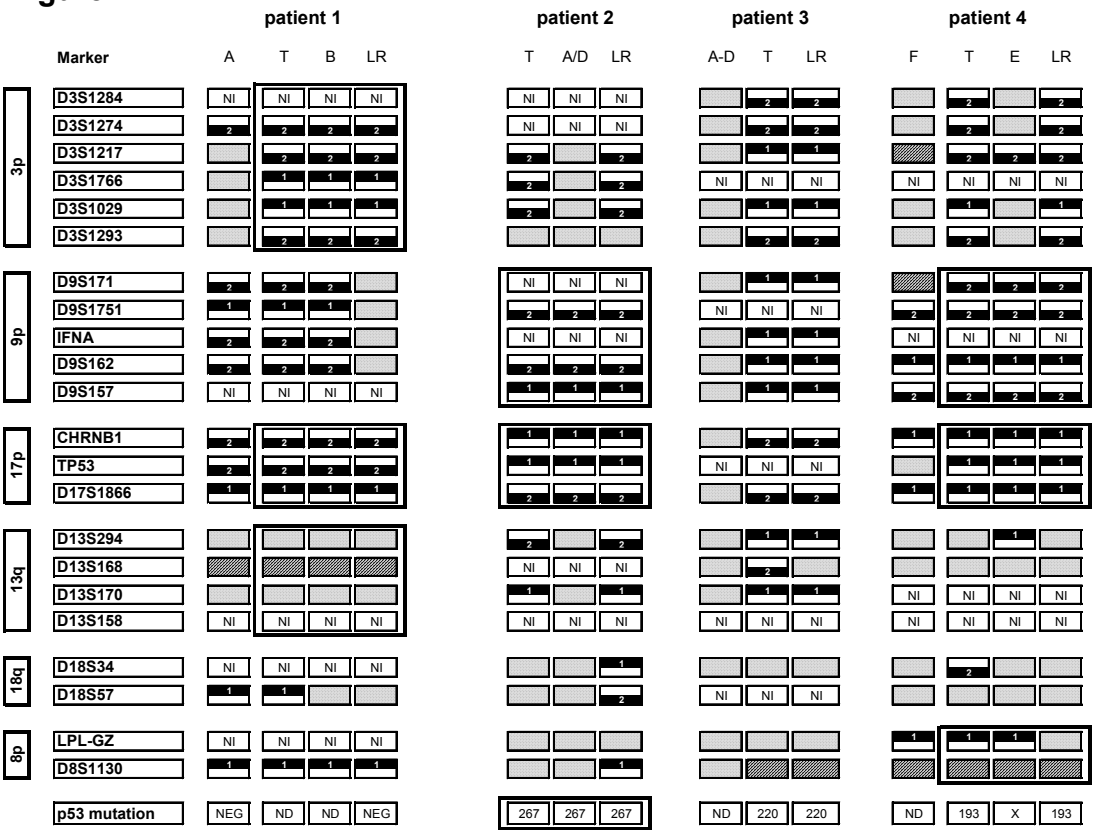
*f) Patient refused postoperative radiotherapy*

***Table 2.*** *Genetic patterns to decide whether a local recurrence has developed from MRC or precursor lesions (based on data from fig. 1)*

| | | | Comparison genetic patterns primary tumor and local recurrence | | | |
|---|---|---|---|---|---|---|
| | Presence tumor related field | | Other than LOH; similar markers | LOH | | |
| Patient number | Yes/no | If yes, based on | | Probability of similarity by coincidence (%) | If similar, based on | Decision origin of local recurrence |
| 1 | Yes | LOH at 3p, 17p, 13q, 8p | N.A. | No similarity | N.A. | Precursor lesion |
| 2 | Yes | *TP53* mutation, LOH at 9p, 17p | N.A. | 9 | LOH at 3p, 13q | Precursor lesion |
| 3 | No | | MSI at 8p, *TP53* mutation | <1 | LOH at 3p, 9p, 17p | MRC |
| 4 | Yes | MSI at 8p, 9p, 17p | *TP53* mutation | | | ? |
| 5 | Yes | MSI at 3p, LOH at 9p | N.A. | 52 | No LOH at 13q | Precursor lesion |
| 6 | Yes | *TP53* mutation, LOH at 3p and 9p | N.A. | 1 | LOH at 8p, no LOH at 17p, 18q, 8p | ? |
| 7 | Yes | TP53 mutation, LOH at 17p | MSI at 8p | 8 | LOH at 3p, 18q | ? |
| 8 | Yes | LOH at 9p, 17p, 8p | N.A. | 18 | LOH at 18q | Precursor lesion |
| 9 | No | | *TP53* mutation | <1 | LOH at 3p, 9p, 17p, 8p | MRC |
| 10 | No | | *TP53* mutation, MSI at 3p and 18q | 1 | LOH at 17p, 8p, 18q and no LOH at 13q | MRC |
| 11 | Yes | LOH at 9p | | 14 | LOH at 3p, 17p | Precursor lesion |
| 12 | No | | *Tp53* mutation | <1 | LOH at 3p, 9p, no LOH at 17p, 13q, 18q, 8p | MRC |
| 13 | No | | *Tp53* mutation | <1 | LOH at 3p, 9p, 17p, 13q, 8p, no LOH at 18q | MRC |

*N.A.; not applicable*

## Figure 1A


patient 1
patient 2
patient 3
patient 4
Marker
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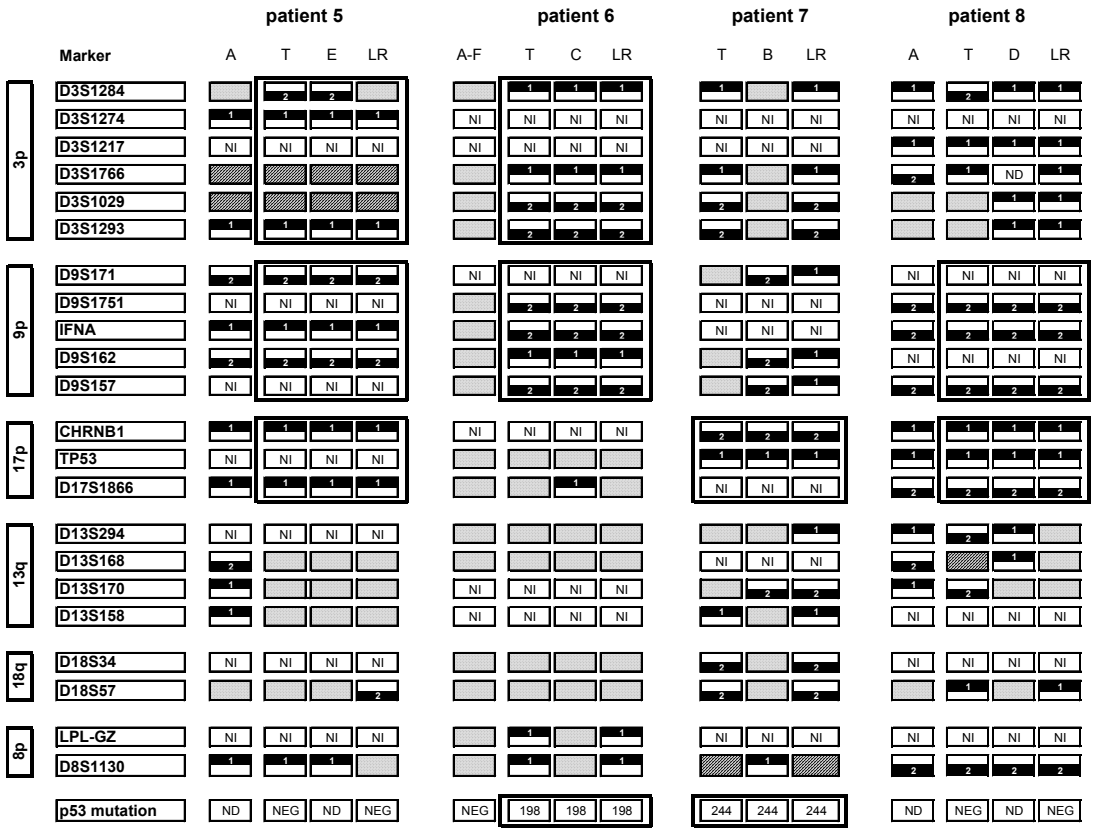
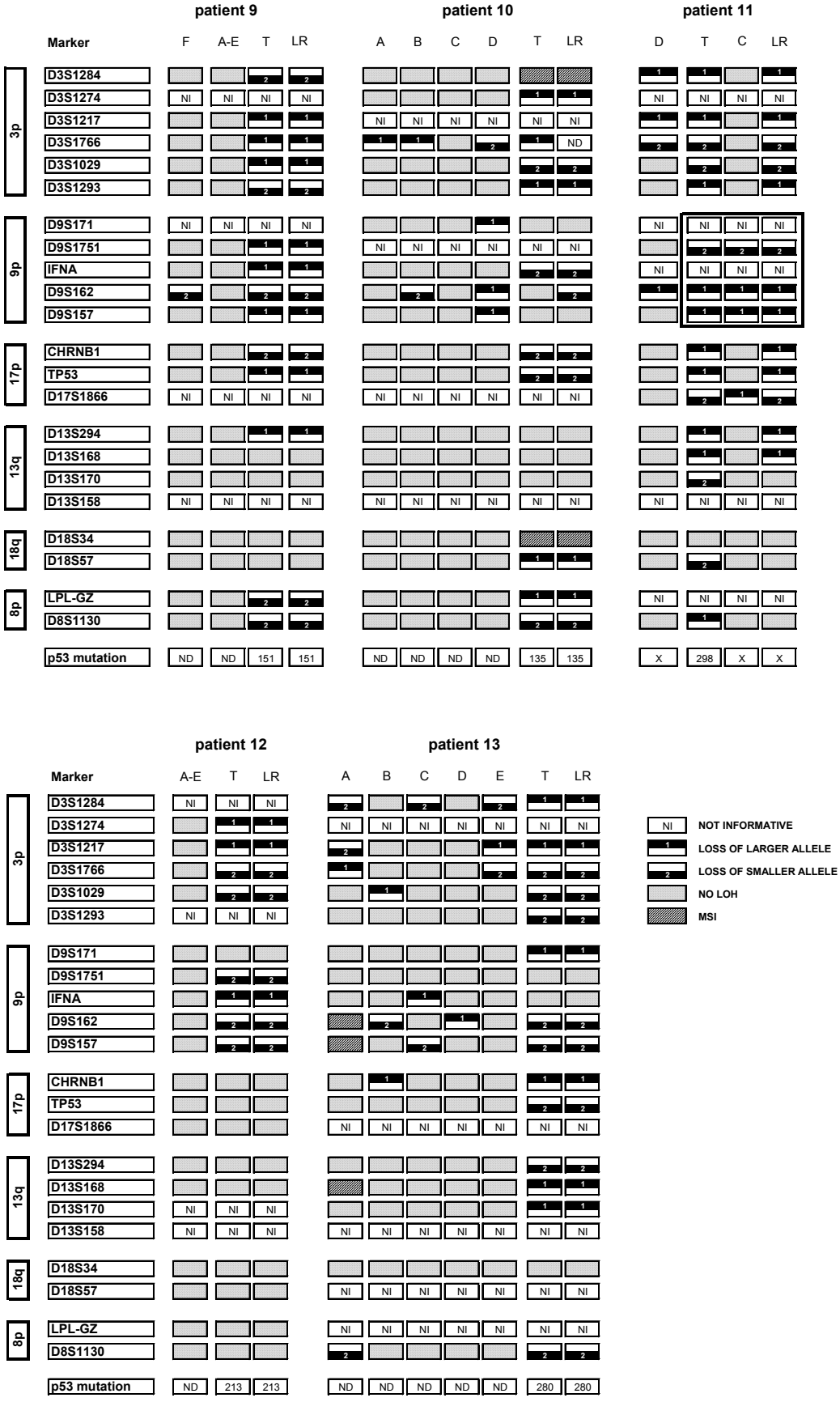
T A/D LR
A-D T LR
F T E LR
D3S1284
D3S1274
D3S1217
D3S1766
D3S1029
D3S1293
3p
D9S171
D9S1751
IFNA
D9S162
D9S157
9p
CHRNB1
TP53
D17S1866
17p
D13S294
D13S168
D13S170
D13S158
13q
D18S34
D18S57
18q
LPL-GZ
D8S1130
8p
p53 mutation
NEG ND ND NEG
267 267 267
ND 220 220
ND 193 X 193


patient 5
patient 6
patient 7
patient 8
Marker
A T E LR
A-F T C LR
T B LR
A T D LR
D3S1284
D3S1274
D3S1217
D3S1766
D3S1029
D3S1293
3p
D9S171
D9S1751
IFNA
D9S162
D9S157
9p
CHRNB1
TP53
D17S1866
17p
D13S294
D13S168
D13S170
D13S158
13q
D18S34
D18S57
18q
LPL-GZ
D8S1130
8p
p53 mutation
ND NEG ND NEG
NEG 198 198 198
244 244 244
ND NEG ND NEG

## Figure 1B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patient 10
patient 11
Marker
F A-E T LR
A B C D T LR
D T C LR
3p
D3S1284
D3S1274
D3S1217
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D3S1029
D3S1293
9p
D9S171
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IFNA
D9S162
D9S157
17p
CHRNB1
TP53
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13q
D13S294
D13S168
D13S170
D13S158
18q
D18S34
D18S57
8p
LPL-GZ
D8S1130
p53 mutation
ND ND 151 151
ND ND ND ND 135 135
X 298 X X
patient 12
patient 13
Marker
A-E T LR
A B C D E T LR
p53 mutation
ND 213 213
ND ND ND ND ND 280 280
NI NOT INFORMATIVE
LOSS OF LARGER ALLELE
LOSS OF SMALLER ALLELE
NO LOH
MSI

***Figure 1 (A and B).***
*Genetic profiles of the primary tumors, corresponding resection margins and local recurrences. In the first column the chromosome arms are shown and in the second the microsatellites located at those arms. For each patient the pattern of the primary tumor (T) and of the local recurrence (LR) is shown. The characters A through F indicate the margins of the primary tumor (A-E stands for A through E, meaning that 5 margins have been analysed and A/D means that margin A and D are shown). When a tumor-related field is found (according to the criteria in the Materials and Methods) the genetic profile of the margin corresponding to that field was depicted between those of the primary tumor and the local recurrence. A box emphasizes the genetic alterations that have led to decide in favour of a tumor-related field.*
*NI=not informative*
*ND=not determined*
*NEG : no mutation in the TP53 gene*
*X: no confirmation of the TP53 mutation as found in the corresponding tumor*

## Results

**Local recurrence in the absence of tumor related field.**

The different profiles are indicated in Fig. 1 and further details on the motivation for decisions can be found in Table 2. In 5 of the 13 cases (38%: patients no. 3, 9, 10, 12 and 13) we could not detect a genetically altered field that was clonally related to the primary tumor. No concordant genetic aberrations encompassing a whole chromosome arm could be found in the margins. The absence of field was strongly indicative but not conclusive to decide that these recurrences were derived from local MRC. When comparing the genetic profiles of the recurrent and the primary tumors, however, no or only minor differences were observed. All five pairs share an identical *TP53* mutation, two a common MSI pattern and in all combinations the probability that the LOH pattern was identical by coincidence was 1% or less. These results were considered to be evidence that in all 5 cases the local recurrences originated from MRC.

**Local recurrence in the presence of a tumor related field**

In 8/13 cases (62%) a tumor-related genetically altered field could be detected. In 3 of these cases (patients 2, 6 and 7) the decision was based on the common *TP53* mutation in primary tumor, one of its margins and the local recurrence. In 2 cases (patients 1 and 4) a common MSI was observed and in the remaining 3 patients (5, 8 and 11) a common LOH pattern of at least one chromosomal arm indicated the presence of a field clonally

related to the tumor.

**Precursor field as origin of local recurrence**.

It should be noted that in the presence of a tumor-related field the local recurrence could still could have developed from MRC. To distinguish local recurrences originating as result of histologically undetected MRC cells from new tumors arising in a tumor-related genetically altered field, we used the concordance of genetic markers between tumor and recurrence that were not shared with the field. In 5 cases we could not find evidence for a concordant pattern of the remaining set of genetic markers (patients 1, 2, 5, 8 and 11). There were neither common *TP53* mutations nor microsatellite shifts. In addition, we could not exclude that a common LOH pattern in a given chromosomal arm had originated by coincidence. Based on the observed differences we therefore concluded that these five cases are likely to be local recurrences that have developed from a precursor field. As an example, we discuss the characteristics of patient 8. Margin D is similar to the primary tumor and the local recurrence based on the pattern of LOH at 9p, 17p and 8p confirming the presence of a clonally related field. When comparing primary tumor and local recurrence a similar LOH pattern was only present at 18q. The probability that this has occurred by chance is 0.5 (LOH may occur at one of the two alleles) multiplied with 35% (chance to observe LOH in this arm) is 18%. The primary and recurrent tumor might therefore have this similar pattern by chance. The local recurrent tumor was therefore not considered to originate from MRC and consequently most likely arose as new tumor from the unresected genetically altered field.

For 3 additional patients (patients 4, 6 and 7) it was difficult to decide about the origin of the recurrence. The recurrent tumor was genetically very similar to the original tumor: a common *TP53* mutation (patient 4), a common MSI (patient 7) and a convincingly similar LOH pattern (patient 6). These alterations were not shared with the detected field. Despite the presence of a precursor lesion surrounding the primary tumor, the genetic profiles indicate that MRC is the likely origin of recurrent cancer. The possibility that the recurrences of these three cases were derived from a precursor lesion, however, could not be excluded conclusively.

## Discussion

The aim of this study was to reveal the pathobiology of locally recurrent HNSCC. Patients were selected who had locally recurrent cancers defined by present clinical criteria: the development of another cancer within a period of three years after the first tumor and at a distance less than two cm separated from the first tumor. Only patients who

had tumor-free margins when the primary tumor was resected were included.

The present study suggests that in approximately 40% of patients a limited number of cancer cells (MRC) is left behind after surgery and this leads to the outgrowth of a new genetically identical carcinoma at the same site. An important finding is that in the other 60% of cases fields of genetically altered cells were detected, that had the same clonal origin as the primary and the recurrent tumor. It is known that new carcinomas develop in such a field (7, 9, 13). Indeed, for the majority of cases with a tumor-related field surrounding the primary tumor, the genetic dissimilarity between the primary and recurrent tumor provided evidence that the local recurrence has originated from the field with genetically altered cells. Thus, approximately half of the local recurrences (as defined according to clinical criteria) originate from genetically altered fields and may be considered SFT (5). In a previous study we could show that a similar proportion (60%) of second primary tumors had developed from a precursor field (10). In that study the clinical criteria for a second primary tumor were used: it should develop more than two cm away from the primary tumor or after more than three years after the primary tumor. The other 40% of second primary tumors in that study was genetically completely unrelated to the primary tumor.

The data of the present study suggested a relation between the origin of locally recurrent tumor and the time interval between the development of the primary and recurrent cancer. The five cases with a likely MRC origin showed an average interval of 8.8 months, whereas this was 20.6 months for the five cases for which a precursor lesion was the most likely cause. Apparently, a few cancer cells take less time to grow into a clinically detectable cancer than a field of preneoplastic cells needs for further progression to invasive cancer.

For some cases we have presently studied, too limited data was available for reliable classification and misclassification may have occurred. Despite the presence of an unresected tumor-related precursor lesion, for three cases we could not find definitive evidence that the preneoplastic cells were indeed the source of recurrent cancer. The primary and recurrent tumor were genetically very similar, suggesting that the locally recurrent tumor might well have developed from a few cancer cells left behind after resection that were not detected by the pathologist. Nonetheless, for these three cases it also remains possible that the tumor-related precursor lesion was the origin for the recurrent tumor, since the presently used technique may not be sensitive enough to detect differences between preneoplastic and neoplastic cells. In addition, when chromosomal breakpoints would have been taken into account, the local recurrence of patient 2 could also be classified to originate from local MRC. The details of the 3p alterations have not been included in our evaluation, but the absence of LOH at D3S1293 suggests a breakpoint in the 3p arm that is present in the primary tumor and the local recurrence but

not in the field. Since we do not know the likelihood of a break between that particular microsatellite and D3S1029, we have not been able to use this observation in the evaluation.

The studied precursor lesions, the fields with genetically altered cells, are macroscopically not visible for the surgeon, which in part explains that they are often left behind. The pathologist, however, can recognize most of these lesions as dysplasia. It is important to note that in most cases (4 out of 5: 80%) the SFT arose in tumor-related precursor lesions that were graded as mild dysplasia, confirming previous reports that histology seems suitable for identification of genetically aberrant mucosal epithelium, but of very limited use for risk assessment (20, 21). Since it is known that fields can be large (10), also part of the second primary tumors defined according to the clinical criteria should be classified as SFT. These molecular studies have led to new insights and a new classification of local recurrences, SFT and second primary tumors on basis of genetic criteria has been proposed (5).

Our observations indicate that reliable methods should be developed for molecular grading of the margins of surgically treated HNSCC patients. In previous studies addressing the risk for progression of leukoplakia lesions in the oral cavity, it was shown that a specific pattern of allelic imbalance and ploidy are important markers to predict progression (21-23). These approaches may be very well suited also for accurate risk assessment of the clinically not visible fields that surround the tumors. Once a more reliable risk assessment has been developed, it can be exploited to identify the high risk fields. Presence of such a high risk field in a surgical margin should be followed by a lifelong surveillance at regular intervals. Moreover, it could indicate a more conservative approach as to adjuvant radiotherapy as far as the primary site indications are concerned. Patients with a preneoplastic field are at risk for SFT and this therapeutic modality should thus be kept in reserve if at all possible. In addition, current treatment modalities (surgery and/or (chemo-)radiotherapy) are very effective to eradicate tumor cells, but these may not be indicated for the treatment of relatively large fields of preneoplastic cells. There is a need for the development of targeted treatment strategies for patients at risk for SFT (24).

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# 7

# Summary, General discussion and Future perspectives

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## Summary

In the introduction (**Chapter 1**) it is outlined that despite significant advances in HNSCC treatment modalities, long-term survival of HNSCC patients has only moderately improved during the last 20 years (1). Important reasons for this lack of progress are the relatively high recurrence rates observed in these patients and the development of second primary tumors (SPT) in the upper aerodigestive tract (2, 3). Local recurrences are thought to result from minimal residual cancer (4). As for SPT, Slaughter et al. proposed the concept of field cancerization, and discussed its clinical significance for the development of new tumors (5). According to this concept, a large area of the aerodigestive tissue has been affected by long-term exposure to carcinogens. In this preconditioned epithelium multiple carcinomas can develop and these would thus be genetically unrelated (6, 7). Recently, evidence has been found for a common clonal origin of multiple primary tumors (8-12).

The aim of this thesis was to investigate the field cancerization concept and its relation to the development of local recurrences and SPTs after therapy with curative intent of the primary index tumor.

In order to learn more of the pathobiology of recurrent cancer, genetic alterations can be used to study field cancerization and to assess a relationship between the first primary tumor, a local recurrence and/or a corresponding SPT. In **Chapter 2** the reliability of TP53 mutations and patterns of "loss of heterozygosity" (LOH) to assess clonal relationships for HNSCC are described. The selection of these genetic alterations was based on the publication describing a multistep carcinogenesis model for HNSCC (13). A number of discordant reports on the reliability of these genetic alterations was found in the literature, but these could have been attributed to differences of used techniques. We compared 23 primary HNSCC with 25 corresponding lymph node metastases (LNM) and 10 corresponding distant metastases (DM) with respect to TP53 mutations and patterns of loss of heterozygosity (LOH) based on 26 microsatellite markers on six chromosome arms (3p, 9p, 17p, 13q, 8p and 18q). In all cases the same TP53 mutation was detected in the primary tumor as in the corresponding LNM and/or DM. These findings make TP53 mutation a very suitable clonal marker for HNSCC. The LNM and DM from HNSCC shared most of the allelic losses of the corresponding primary tumors but differed from them at a limited number of loci. Microsatellite loci located at chromosome arms 13q, 8p and 18q scored the highest rate of discordance. Microsatellite markers located at chromosome arms 3p, 9p and 17p showed almost no discordance, supporting the notion that alterations at these loci occur early in HNSCC

carcinogenesis. Therefore, microsatellite markers located at chromosome arms 3p, 9p and 17p are most suitable to assess clonal relationships in HNSCC.

In **Chapter 3** we analyzed genetic alterations in HNSCC and adjacent macroscopically normal mucosa in order to study the process of field cancerization. In 28 HNSCC patients loss of heterozygosity (LOH) was determined in tumor and five noncontiguous mucosal biopsies using eight microsatellite markers at 3p, 9p and 17p. From patients who showed genetic alterations in their mucosal biopsies all margins of the surgical specimen were subsequently analyzed to determine the extension of the field. Genetically altered fields were detected in 10 of the 28 (36%) HNSCC patients. The field varied in size between patients, and consisted of genetically different, but related subclones. In 7 of 10 cases, the field extended into the surgical margins. Genetic analysis strongly indicated a clonal relationship between tumor and accompanying field. The picture emerged that these tumors develop within a pre-existing preneoplastic field.

Thus far, clinical management of preneoplastic lesions is based on histological grading, but its limitations are well known. Molecular grading of premalignant lesions has been proposed to have additional value in predicting cancer. In **Chapter 4** the relations between molecular alterations, morphological grading and the number of proliferating cells in preneoplastic proliferating fields were described. Forty-three samples were analysed from the margins of patients who had undergone resection of their squamous cell carcinoma in the oral cavity/oropharynx. Three experienced pathologists performed histological grading and established a consensus score. Loss of heterozygosity (LOH) was assessed in the same samples with 15 microsatellite markers at chromosomes 3p, 9p, 17p, 8p, 13q and 18q. The proliferation rate was assessed by quantitative assessment of Ki-67 staining. This study showed: (a) a relatively large inter-observer variability of histopathological grading; (b) a genetically altered field can occur with normal histology; (c) all moderately and severely dysplastic lesions, and about two-third of the mildly dysplastic lesions show genetic alterations; and (d) genetically altered cells in a field show a high proliferative capacity, as determined with Ki-67 staining.

In order to test our hypothetical model as described in Chapter 3, we studied whether first and second primary tumors are clonally related and originate from a single genetically altered field. In **Chapter 5** we analyzed from ten patients the first tumor of the oral cavity or oropharynx, the > 3 cm remote second primary tumor, and the mucosa from the tumor-free margins from both resection specimens. We compared *TP53* mutations and loss of heterozygosity (LOH) profiles using 19 microsatellite markers at 3p, 9p, 17p, and 13q. In all patients, genetically altered mucosal lesions were detected in at least one

resection margin from both first and second primary tumor. Evidence for a common clonal origin of the first tumor, SPT and the intervening mucosa was found for at least six of ten patients. This study indicated that a proportion of multiple primary tumors develop within a single preneoplastic field.

In **Chapter 6** the pathobiology of locally recurrent HNSCC in patients with histologically tumor-free resection margins was described. In a retrospective study on 13 HNSCC cases we used loss of heterozygosity (22 microsatellite markers on 6 chromosomes) and *TP53* mutations to determine the origin of recurrent cancer. A precursor lesion was absent in five of 13 (39%) cases and the genetic similarity of the primary and recurrent cancer was high, providing evidence that residual cancer cells were the origin of recurrence. For the remaining eight cases (61%) a genetically related precursor lesion (field) was detected and for five of these cases evidence was found that both the primary and recurrent carcinoma originated from this field. The remaining three cases were less conclusive.

## General Discussion

### Genetically Altered Fields

In this thesis evidence is provided for the existence of genetically altered fields in HNSCC patients. Detailed analysis of the alterations between these fields and the corresponding tumors revealed a genetic relationship for almost all cases. We therefore propose the following model of HNSCC carcinogenesis: a single cell is transformed and gives rise to one large extended premalignant field by clonal expansion and gradual replacement of normal mucosa. During progression within this genetically altered field various clones develop with additional genetic alterations. This is a continuous process of evolution and these clones are genetically different but share a common origin. One clone ultimately develops into a carcinoma. From the clinical point of view it is important to realize that after surgery of the first primary tumor, part of the field may remain in the patient.

On the basis of the new insights described in this thesis, we propose the following definition of field cancerization: “the presence of one or more areas consisting of epithelial cells that have genetic alterations”. A field lesion (or shortly “field”) has a monoclonal origin and does not show invasive growth and metastatic behavior, two hallmarks of cancer. A field lesion is preneoplastic by our definition; it may have histological aberrations characteristic for dysplasia and high proliferative capacity (Chapter 4).

**New paradigm: second field tumors**

The concept of the expanding field in carcinogenesis has important clinical consequences. The presence of a field with genetically altered cells appears to be a continuous risk factor for cancer. The results provided in this thesis indicated that a proportion of multiple primary tumors in the same or adjacent anatomical area have developed within a single pre-neoplastic field. It should be noted that the decision whether the tumors and the intervening mucosa of the field were of common clonal origin was based on strict criteria. There is thus a possibility that in reality the percentage of clonally related tumors is even higher. Additionally, it was shown that a part of the local recurrences originate from unresected genetically altered fields and a part from residual cancer cells.

The realization of a genetically altered field as a cancer risk factor provide a new paradigm, the definitions of "local recurrence" and SPT need a molecular addendum. The term "SPT" was proposed in this thesis to be allocated for the second tumor that has developed independently from the first tumor (Chapter 5). When a second tumor arises from the same field in which a first tumor has developed, it was proposed to designate it as a "second field tumor"(SFT). It is important to make this discrimination, because a different etiology may have clinical consequences. SFTs will relatively easily be followed by third and fourth field tumors. In addition, patients at risk for SFT may need an adjusted follow-up, characterized by more frequent and more focused screening. Likewise, the clinical definition of "local recurrence" needs to be reconsidered in molecular terms. This type of lesion can also be the result of local remnants of a field that may develop into cancer (Chapter 6). Therefore, a local recurrence can be a SFT as well. In this case, the knowledge whether there is a field at risk may have the same consequences. The proposed molecular classification is summarized in Figure 1.

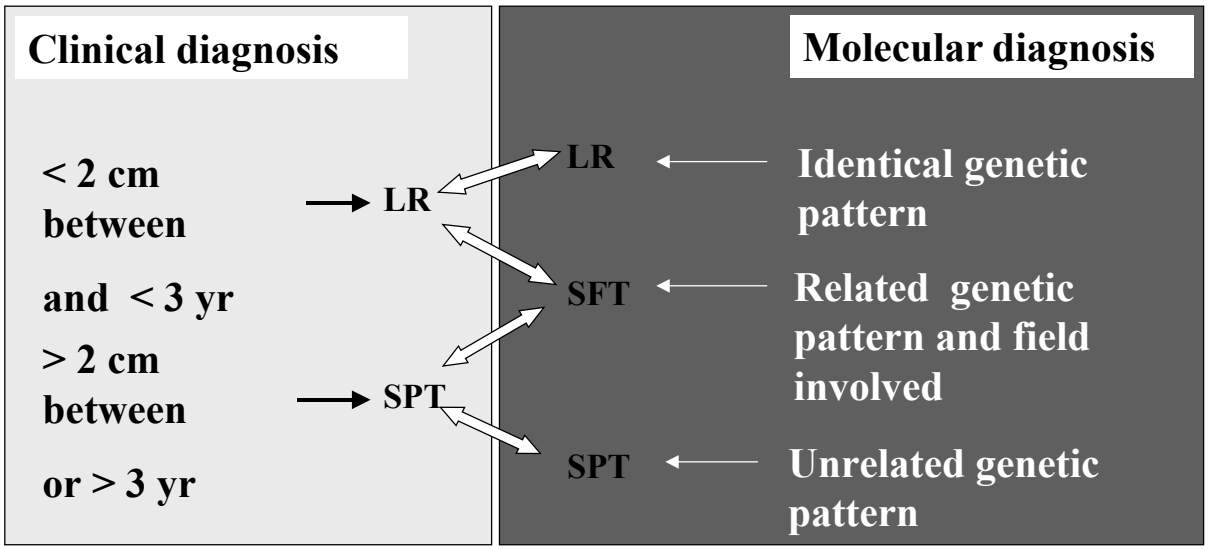


***Figure 1**. Second field tumors: a new paradigm?*

## Future Perspectives

The realization that many, if not all, HNSCC are preceded by precursosr lesions opens new possibilities for early diagnosis and intervention. Clinical investigations are hampered by the fact that the majority of genetically altered fields are clinically invisible and need to be detected with molecular biological techniques (Chapter 3). As these fields are clinically not recognizable, there is an urgent need to improve the clinical detection of field. Diagnostic procedures, such as the staining with toluidine blue and fluorescence imaging might provide improved detection possibilities (14, 15). A promising option may be screening for precancerous lesions by brushing cells of the oral mucosa at different sites and subsequent genetic analyzing the DNA of these cells (16).

A number of parameters may determine whether a field develops into a new tumor. Besides host factors, such as smoking, biological characteristics of the field may be of importance for HNSCC development (17). With respect to molecular markers, preliminary investigations with oral leukoplakia show interesting results. It appeared that the presence of allelic loss at 3p and 9p is associated with an increased cancer risk (18, 19). Additional losses of other chromosomes (4q, 8p, 11q, or 17p) dramatically increased cancer risk (18). Another study by Sudbo *et al*. showed that DNA contents in cells of oral leukoplakia can be used to predict the risk of oral carcinoma (20). Patients who have been surgically treated for HNSCC and are at risk for SFT can be enrolled to study the risk profile of a genetically altered field. Such a study has an obvious advantage: it is known approximately where the lesion will develop (where the initial tumor has been) and it is possible to monitor the disease process (for instance by brushing cells). Furthermore, knowledge of the genetic alterations that precede the development to cancer will provide a basis for a rational therapy (e.g., a gene-therapy based approach) of these preneoplastic lesions (21).

**In conclusion:** the presence of a field with genetically altered cells is a risk factor for cancer. These persisting fields also explain the high incidence of development of another tumor after surgery of the initial carcinoma. Detection and monitoring of genetically altered fields may have profound implications for future cancer prevention.

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# Samenvatting, discussie en perspectieven

## Samenvatting

Vijf procent van alle kwaadaardige tumoren die jaarlijks worden gediagnosticeerd in West Europa en de Verenigde staten, betreft een plaveiselcelcarcinoom uitgaande van het slijmvlies in het hoofd-halsgebied (HHPCC) **(Hoofdstuk 1).** Ondanks belangrijke vooruitgang in de behandelingsmogelijkheden van HHPCC is de lange termijn overleving van deze patiënten groep de afgelopen 20 jaar nauwelijks gestegen (1). Belangrijke redenen voor hiervoor zijn onder andere het optreden van lokaal recidiverende tumoren en het ontstaan van tweede primaire tumoren (2, 3). Over het algemeen wordt aangenomen dat een lokaal recidief ontstaat uit achtergebleven tumorcellen (4). Als verklaring voor het onstaan van tweede primaire tumoren stelde Slaughter en coauteurs het concept van "field cancerization" op (5). Volgens dit concept heeft het gehele slijmvlies van de bovenste lucht- en voedselwegen een verhoogd risico op het ontwikkelen van kanker als gevolg van langdurige blootstelling aan carcinogenen (o.a. roken). In dit beschadigde epitheel kunnen multipele tumoren ontstaan, waarvan lange tijd gedacht werd dat ze onafhankelijk van elkaar ontstonden (6, 7). Met de nu ter beschikking staande technieken zijn er recent aanwijzingen gevonden voor een gemeenschappelijke oorsprong van multipele primaire tumoren (8-12).

Het doel van het onderzoek beschreven in dit proefschrift was om de genetische basis van het concept van "field cancerization" te onderzoeken en de relatie met het ontstaan van een lokaal recidief en tweede primaire tumor vast te stellen.

Kanker ontstaat door een accumulatie van genetische veranderingen in tumorsuppressorgenen en oncogenen (13). Deze veranderingen in het DNA kunnen worden gebruikt om de eerste primaire tumor genetisch te vergelijken met een lokaal recidief of een tweede primaire tumor. In **hoofdstuk 2** werd de bruikbaarheid van mutaties in het p53 tumorsuppressor-gen en "loss of heterozygosity" (LOH) als klonale markers voor HHPCC onderzocht. We vergeleken hierbij de p53 mutaties en LOH-patronen in zowel primaire HHPCC (n=23) als bijbehorende halskliermetastasen (n=25) en/of afstandsmetastasen (n=10). In alle gevallen werd dezelfde p53 mutatie gevonden in de primaire tumor als in de bijbehorende halskliermetastasen en/of afstandsmetastasen. Deze bevindingen maken p53 mutaties erg geschikt voor gebruik als klonale marker. De LOH-patronen van de primaire HHPCC kwamen eveneens voor het grootste deel overeen met de LOH-patronen van de bijbehorende halskliermetastasen en/of afstandsmetastasen. De LOH-patronen van chromosoom armen 3p, 9p en 17p kwamen bijna altijd geheel overeen. Onze bevindingen ondersteunen het gebruik van p53 mutaties en LOH-patronen van chromosoom armen 3p, 9p en 17p als markers om "field cancerization" te onderzoeken, en het klonale verband tussen multipele primaire tumoren vast te stellen.

In **hoofdstuk 3** werd het concept van "field cancerization" bestudeerd door genetische veranderingen in HHPCC en nabijgelegen macroscopisch normaal slijmvlies te onderzoeken. Van 28 HHPCC patiënten werden de genetische veranderingen (LOH-patronen) bepaald in de primaire tumor en 5 nabijgelegen slijmvlies-biopten. In 10 van de 28 HHPCC patiënten (36%) werden genetische veranderingen gedetecteerd in het omringende slijmvlies. De dimensies van het genetisch veranderde slijmvlies (aangeduid met "veld") leken per patiënt te verschillen. In 7 van de 10 patiënten, strekte het genetische veranderde slijmvlies zich uit tot in de chirurgische snijvlakken. De genetische veranderingen toonde een duidelijk klonaal verband aan tussen de primaire tumor en het omringende genetisch veranderde slijmvlies. Dit waren aanwijzingen dat de primaire tumor ontstaan was vanuit één preneoplastisch veld.

In **Hoofdstuk 4** wordt de relatie tussen moleculaire veranderingen, histologische veranderingen en het aantal delende cellen in preneoplastische velden beschreven. Tot nu toe is de behandelingsstrategie van preneoplastische laesies gebaseerd op histologische gradering, ondanks de beperkingen hiervan. Drieënveertig snijvlakken van patiënten met een HHPCC werden bekeken door drie ervaren pathologen. Dezelfde samples werden onderzocht op genetische veranderingen (LOH-patronen). De delende celfractie werd bepaald met behulp van kwantitatieve analyse van een immuunhistochemische Ki-67 kleuring. Deze studie toonde aan: (a) dat er een relatief hoge variabiliteit tussen verschillende waarnemers ten aanzien van histologische gradering bestaat, (b) dat er genetische veranderingen in het slijmvlies kunnen voorkomen zonder histologisch waarneembare veranderingen, (c) dat alle matige en sterke dysplastische laesies en 2/3 van de mild dysplastische laesies genetische veranderingen bevatten, en (d) dat de genetisch veranderde cellen van een veld gekenmerkt worden door een relatief hoge delingsactiviteit.

In **Hoofdstuk 5** werd onderzocht of eerste en tweede primaire tumoren in hetzelfde of aanliggende anatomische gebied klonaal aan elkaar gerelateerd zijn en of ze waren ontstaan uit een groot gemeenschappelijk preneoplastisch veld. We analyseerden van 10 HHPCC patiënten de eerste primaire tumor, de op een afstand van meer dan 3 cm verder gelegen tweede primaire tumor en het daartussen gelegen slijmvlies. Alle samples werden onderzocht op genetische veranderingen (p53 mutaties en LOH-patronen). In alle patiënten werden genetische veranderingen gedetecteerd in het tussen beide tumoren in liggende slijmvlies. Door analyse van de patronen van genetische veranderingen kon in 6 van de 10 gevallen worden aangetoond dat beide tumoren uit één relatief groot gemeenschappelijk preneoplastisch veld waren ontstaan en dus klonaal aan elkaar zijn gerelateerd.

In **Hoofdstuk 6** wordt de relatie tussen genetisch veranderde velden en het ontstaan van lokaal recidieven beschreven. Deze lokaal recidieven ontstonden binnen twee centimeter van de verwijderde primaire tumor en binnen drie jaar. In een retrospectieve studie werden 13 HHPCC patiënten onderzocht die een lokaal recidief hadden ontwikkeld, terwijl de snijvlakken histologisch tumor-vrij waren. De primaire tumor, het lokaal recidief en de snijvlakken werden onderzocht op genetische veranderingen (p53 mutaties en LOH-patronen). Er werd aangetoond dat 5 van de 13 lokaal recidieven waren ontstaan uit achtergebleven tumorcellen. In 5 van de 13 gevallen waren de lokaal recidieven ontstaan uit een achtergebleven preneoplastisch veld. De overige drie gevallen waren minder duidelijk. Kortom: een aanzienlijk deel van de lokaal recidieven ontstaan uit genetisch veranderde velden die onvermoed achter worden gelaten in de patiënt na chirurgische behandeling van de primaire tumor.

## Discussie

### Genetisch veranderde velden

In dit proefschrift is bewijs geleverd voor het bestaan van genetisch veranderde velden in HHPCC patiënten die macroscopisch niet zichtbaar zijn. Door genetische analyse werd een duidelijk klonaal verband aangetoond tussen de primaire tumor en het omringende genetisch veranderde slijmvlies. We introduceren daarom een nieuw carcinogenese model om het ontstaan van HHPCC te verklaren. In dit model ondergaat een enkele slijmvliescel genetische veranderingen en geeft door klonale expansie en verdringing van normaal slijmvlies, aanleiding tot het ontstaan van één groot genetisch veranderd veld. Tijdens progressie van dit veld ontstaan verschillende klonen met additionele genetische veranderingen. Dit is een continu proces van evolutie en ofschoon deze klonen gedeeltelijk genetisch divergeren, hebben ze één gemeenschappelijke klonale oorsprong. Eén kloon ontwikkelt zich uiteindelijk tot een tumor. Vanuit klinisch oogpunt is het belangrijk te realiseren dat na chirurgische verwijdering van de eerste primaire tumor, het achtergelaten genetisch veranderde veld aanleiding kan geven tot nog een tumor. Afhankelijk van de afstand tot de verwijderde index tumor en het moment waarop deze nieuwe tumor ontstaat, wordt deze volgens de huidige klinische criteria aangeduid als lokaal recidief of tweede primaire tumor.

Op grond van de nieuwe inzichten beschreven in dit proefschrift stellen we de volgende definitie van "field cancerization" voor: "de aanwezigheid van één of meer gebied(en) bestaande uit genetisch veranderde slijmvliescellen". Een genetisch veranderd veld heeft een monoklonale oorsprong en vertoont geen tekenen van invasieve groei of metastasen, twee kenmerken van kanker. Een genetisch veranderd veld is preneoplastisch

en kan histologische veranderingen tonen passend bij dysplasie. Het genetisch veranderd veld heeft een verhoogde prolifererende capaciteit (Hoofdstuk 4).

**Nieuw paradigma: tweede veld tumoren**

Het concept van genetische veranderde velden in HHPCC patiënten heeft belangrijke klinische consequenties. Uit onze retrospectieve studies blijkt dat de aanwezigheid van zo'n veld een continue risicofactor is voor het ontwikkelen van kanker. Een gedeelte van de multipele primaire tumoren in hetzelfde of aanliggende anatomische gebied ontstaan uit een groot preneoplastisch veld dat achterblijft wanneer de index tumor wordt verwijderd. Additioneel bleek dat ook een aanzienlijk deel van de lokaal recidieven ontstaan uit onvermoed achtergebleven genetisch veranderde velden.

De ontdekking van genetische veranderde velden als risicofactor geeft aanleiding tot het formuleren van een nieuw paradigma: de klinische definitie van "lokaal recidief" en "tweede primaire tumor" moeten van een moleculair addendum worden voorzien. De term "tweede primaire tumor" moet worden gereserveerd voor de groep tweede tumoren die zich geheel onafhankelijk hebben ontwikkeld van de eerste primaire tumor (Hoofdstuk 5). Wanneer een tweede tumor ontstaat uit hetzelfde genetisch veranderde veld waaruit de eerste primaire tumor zich heeft ontwikkeld, is de term "tweede veld tumor" beter op zijn plaats. Het is belangrijk om dit onderscheid te maken, daar in het geval van een "tweede veld tumor" er een grote kans is op het ontstaan van een derde tumor. Het achtergebleven genetisch veranderde veld is immers nog steeds aanwezig in de patiënt en kan op ieder moment weer aanleiding zijn voor het ontstaan van een nieuwe tumor! Deze groep patiënten zou wellicht gebaat zijn bij een meer frequente en intensievere controle. Een vroege diagnose van de nieuwe tumor zou de behandeling vergemakkelijken en de prognose verbeteren. Ook de klinische definitie van "lokaal recidief" moet herzien worden vanuit moleculair oogpunt. Een deel van deze tumoren ontstaat ook uit achtergebleven genetisch veranderde velden en zouden dus ook als "tweede veld tumor" kunnen worden geclassificeerd. Ook hier geldt dat deze patiënten gebaat zouden kunnen zijn bij een meer frequente en intensievere controle. De voorgestelde moleculaire classificatie is samengevat in Figuur 1.

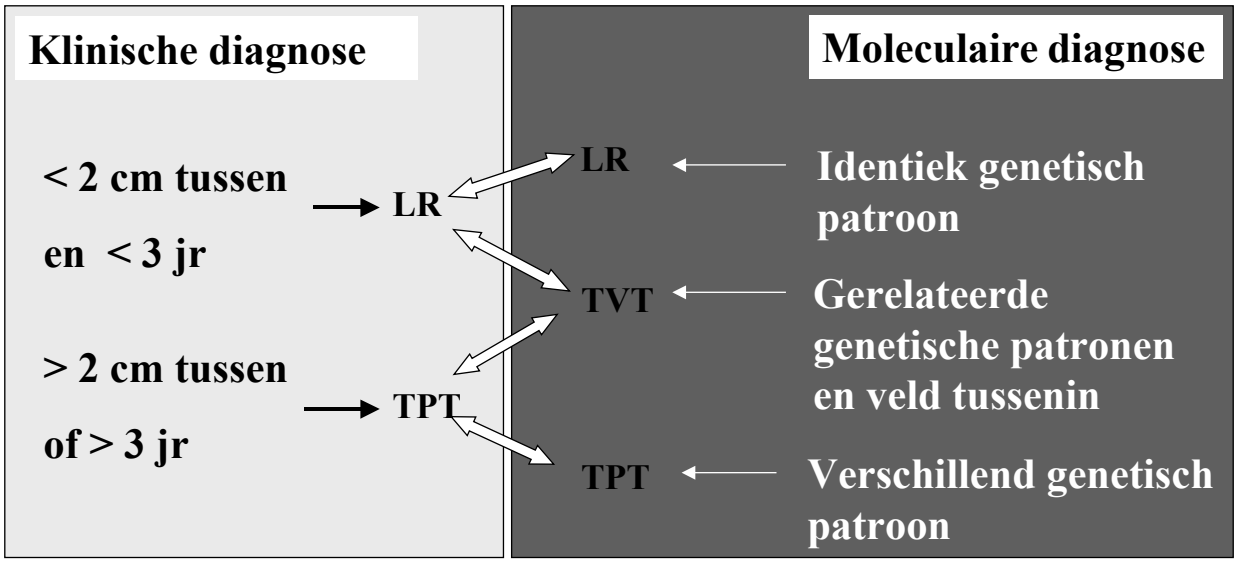


***Figure 1*** *Tweede veld tumor: een nieuw paradigma? LR = lokaal recidief, TPT = tweede primaire tumor, TVT = tweede veld tumor.*

## Hoe nu verder?

Het gegeven dat bij veel, zo niet alle patiënten met hoofd-hals kanker de tumor wordt voorafgegaan door een genetisch veranderd veld, opent perspectief voor vroegdiagnose, en misschien zelfs screening. Klinisch onderzoek wordt helaas bemoeilijkt door het feit dat deze velden klinisch niet waarneembaar zijn in de patiënt. Slechts een subgroep van deze velden is waarneembaar als een witte (leukoplakie) of rode (erythroplakie) plek in de mond- en keelholte. Een bijkomend probleem is dat de histologische gradering een niet erg goede inschatting geeft van de kans op kanker. Er is dus een dringende behoefte aan eenvoudige technieken die zowel de detectie van het veld verbeteren als de gradering. Diagnostische procedures, zoals een toluidine blauw kleuring of autofluorescentie technieken, kunnen hopelijk hierbij een rol spelen (14, 15). Een andere mogelijkheid is het uitstrijken van cellen van het mondslijmvlies op plaatsen waar een grote kans bestaat dat er een tumor ontstaat (16). Van deze cellen kan het DNA worden geanalyseerd op genetische veranderingen.

Een aantal variabelen kan invloed hebben op het ontstaan van een tumor in een genetisch veranderd veld. Naast gewoontes van de patiënt zoals (doorgaan met) roken, kunnen ook de biologische eigenschappen van het veld van belang zijn voor het ontwikkelen van een nieuw carcinoom (17). Met betrekking tot deze biologische eigenschappen, zijn er enkele interessante bevindingen gedaan in relatie tot de progressie van orale leukoplakie. Het bleek dat LOH op chromosoomarmen 3p en 9p geassocieerd was met een verhoogd risico op het ontwikkelen van kanker (18, 19). Additionele LOH op andere chromosoomarmen (4q, 8p, 11q, of 17p) verhoogde deze kans op kwaadaardige

ontaarding nog verder (18). Een studie van Sudbo en coauteurs liet zien dat de DNA hoeveelheid in de cellen van een orale leukoplakie goed kan worden gebruikt om de kans op het ontwikkelen van een tumor te voorspellen (20). Vergelijkbare studies zullen moeten worden uitgevoerd om het risico profiel van achtergebleven velden vast te stellen in patiënten die behandeld zijn voor een eerste tumor. Zo'n studie heeft duidelijke voordelen: het is bekend waar de nieuwe tumor zich ongeveer zal ontwikkelen (in de buurt van de eerste tumor) en het is mogelijk om de progressie te volgen (bijvoorbeeld door het uitstrijken van cellen op een steeds dezelfde potentieel verdachte plek). De kennis, welke genetische veranderingen aan kwaadaardige ontaarding voorafgaan, zal helpen om een rationele therapie te ontwikkelen (bijvoorbeeld gentherapie) om deze preneoplastische laesies te bestrijden (21).

**Samenvattend:** de aanwezigheid van genetische veranderde velden is een risicofactor en misschien zelfs een *conditio-sine-qua-non* voor het ontwikkelen van HHPCC. Deze velden die macroscopisch meestal niet zichtbaar zijn verklaren de hoge incidentie van meerdere tumoren die klinisch worden aangeduid als lokaal recidief en tweede primaire tumor. Het detecteren en vervolgen van deze velden kan grote implicaties hebben voor toekomstige kankerpreventie. De kennis van de precieze genetische veranderingen die het veld en later de tumor veroorzaken zal van grote waarde blijken om zowel de diagnostiek als de behandeling van deze preneoplastische laesies te verbeteren, en hopelijk leiden tot een verlaagde sterfte aan HHPCC.

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## Curriculum Vitae

De auteur van dit proefschrift werd geboren op 3 juni 1971 te Angerlo. In 1990 behaalde hij zijn diploma V.W.O. aan het Liemers College te Zevenaar. In datzelfde jaar begon hij aan de studie Medische Biologie aan de Universiteit Utrecht en behaalde de propedeuse. In 1991 begon hij aan de studie Geneeskunde aan de Universiteit Utrecht. Tijdens de studie deed hij zijn wetenschappelijke stage gedurende 8 maanden bij het Research Laboratorium Neurologie (UMC) onder supervisie van prof.dr. P.R. Bär. Een deel van deze stage werd uitgevoerd in Cincinnati (Verenigde Staten) onder supervisie van dr. C.E. Thomas. Hij haalde zijn artsexamen in januari 1998. In april 1998 startte hij vervolgens zijn promotieonderzoek op de afdeling Keel-, Neus- en Oorheelkunde van de Vrije Universiteit in Amsterdam. Dit werd uitgevoerd onder begeleiding van prof.dr. C.R. Leemans, dr. B.J.M. Braakhuis en dr. R.H. Brakenhoff. Voor dit onderzoek werd hem in 2003 de Chris Meijer prijs uitgereikt. In november 2001 startte hij met zijn opleiding tot Keel-, Neus- en Oorarts in het Vrije Universiteit Medisch Centrum te Amsterdam (opleider prof.dr. C.R. Leemans).