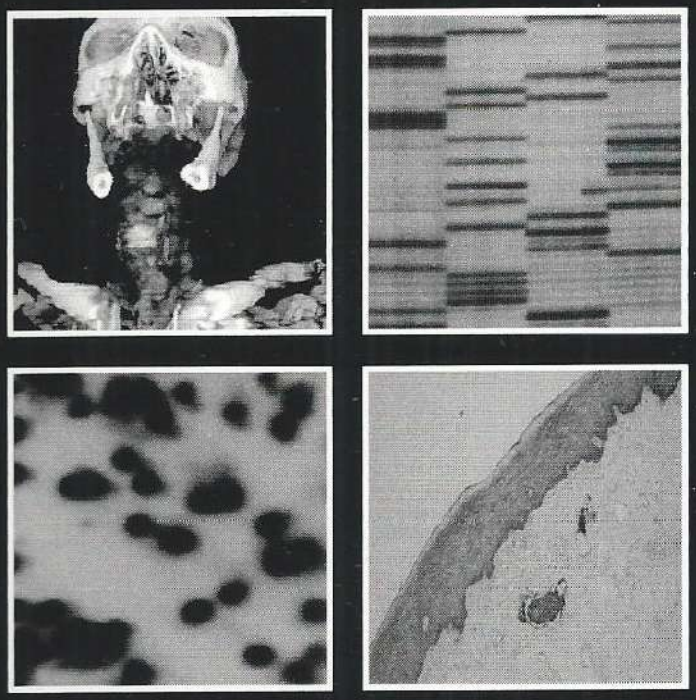
Molecular diagnosis and prognostic value of head and neck cancer in surgical margins



Viola van Houten

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Molecular diagnosis and prognostic value of head and neck cancer in surgical margins

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door

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geboren te 's-Hertogenbosch

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I am the singular
in free fall,
I and my doubles
carry it all:

Life's slim volume
spirally bound.
It's what I'm about
It's what I'm around

Contents

1

General introduction

Parts of this Chapter were published in:
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Recent Results in Cancer Research 157: 90-106 (2000)

Contents

1 Head and neck squamous cell carcinoma

1.1 Epidemiology

Head and neck squamous cell carcinoma (HNSCC) develops at different sites in the mucosal linings of the upper aerodigestive tract, including the oral cavity, oropharynx, nasopharynx, hypopharynx and larynx **[Fig 1]**. HNSCC accounts for approximately 5% of all newly diagnosed malignant tumors in Western Europe and the United States[1]. Annually there are approximately 500,000 new cases of HNSCC worldwide, and in 2000 approximately 2,500 cases in the Netherlands alone[2,3]. Geographically, there is a wide varation in the incidence of HNSCC, probably related to ethnic, cultural and socio-economical variables. In Southeast Asia, the incidence of nasopharyngeal tumors is highest, whereas in Western countries the preferential sites of HNSCC are the oral cavity, the pharynx and the larynx[2,4]. In general HNSCC develops in the middle-aged population, with an increasing incidence at rising ages. Male-female incidence ratios are relatively high, varying from about 2 for oral cancer, 3 for pharyngeal cancer and 12 for carcinoma of the glottis[5]. Over the last decade the incidence of HNSCC is increasing in females, presumably related to increased tobacco-smoking in females (see below).

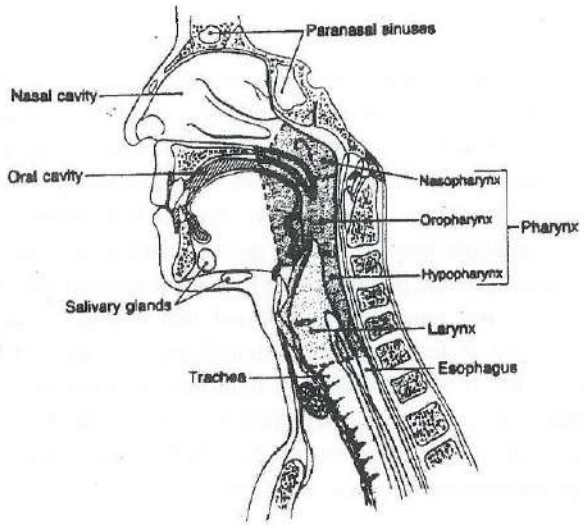

Figure 1. The upper aerodigestive tract (taken from Vokes et al. 1993)[2]

1.2 Etiology

Smoking and alcohol

Epidemiological data strongly indicated that the major risk factors for the development of HNSCC are the life-style factors smoking and alcohol consumption[6]. These factors have an independent carcinogenic potential but also have a synergistic effect (7-15x) when combined[2,7,8,9]. The relative risk also seems to be related to the time of exposure[10]. Over 4,000 chemicals have been identified in the smoke from cigarettes. The polycyclic aromatic hydrocarbons are strongly implicated as causative agents in the development of upper aerodigestive tract tumors. Benzo(a)pyrene and its reactive metabolites are by far the most carcinogenic[11]. Tobacco-related carcinogens play a crucial role in the induction of mutations in the DNA, characteristically found in the p53 gene of neoplastic cells in a relatively high percentage of (head and neck) cancers[12,13,14]. Another source of potent carcinogens originates from chewing tobaccos or betelnuts, but these habits are rare in Western countries[15].

Alcohol may damage the oral mucosa through a direct effect on cell membranes, removing lipids and increasing the permeability of the oral mucosa to noxious carcinogenic substances. More indirectly, alcohol seems to exert its carcinogenic function via metabolic processes, resulting in the activation of certain (pro)carcinogens[16]. The oral cavity (floor of mouth), the oropharynx, the supraglottic larynx and the hypopharynx are subsites with an increased risk associated with alcohol consumption[17].

Genetic susceptibility

Besides exposure to carcinogenic agents, also an intrinsic genetic susceptibility for head and neck cancer appears to play an important role as many heavy smokers and alcohol drinkers do not develop HNSCC. It is likely that patients who smoke and drink have an (inherited) predisposition that increases their risk for developing HNSCC. Using the clastogenic agent bleomycin Cloos et al. (1994) showed that HNSCC patients, and in particular patients with multiple primary tumors demonstrated a high mutagen sensitivity[18] and they suggested that this phenotype reflects the individual susceptibility for head and neck cancer. A later report of the same group indicated that a high mutagen sensitivity in combination with a history of heavy smoking significantly increases the risk to develop HNSCC with a relative risk of 60[19]. Besides this multifactorial inherited susceptibility there are some genetic defects associated with a high HNSCC cancer risk. Patients suffering from Fanconi anemia as a result of a defect in one of the FANC genes, have a very high risk to develop oral and oropharyngeal carcinoma at early age[20].

Viruses

Finally, also some viral infections have been found to be associated with the development of HNSCC. Human papillomaviruses (HPV) and Epstein-Barr virus (EBV) are viruses with well established oncogenic potential. HPV infection, particularly with the high-risk HPV subtype 16, has been associated with the development of a subgroup of HNSCC, especially in oropharyngeal (tonsillar) tumors[21,22,23]. However, the prevalence and etiological role of HPV in HNSCC is still not clear. HPV most likely exerts its carcinogenic effect by inactivation of cellular tumor suppressor gene products through interaction with the viral oncoproteins E6 and E7[24]. Besides HPV, EBV could play a role in oral carcinogenesis. EBV infections are common, with over 90% of adults being seropositive. EBV persistence seems to involve B-lymphocytes and it is found in a variety of malignant conditions, such as lymphomas, and a clear association was demonstrated with nasopharyngeal carcinomas[25].

1.3 Clinical and histopathological staging

Head and neck squamous cell carcinomas are classified according to the TNM system of the "International Union Against Cancer" (UICC)[26]. The TNM anatomical staging system is based on the assessment of three components: the extent of the primary tumor (T), the absence or presence and extent of regional lymph node metastases (N), and the absence or presence of distant metastases (M) **[Table 1A]**. Two classifications are used: (a) clinical classification (pre-treatment clinical classification, designated TNM or cTNM) and (b) pathological classification (post-surgical histopathological classification, designated pTN). The cTNM is based on evidence acquired before treatment, e.g. from physical examination, imaging, endoscopy, biopsy and ultrasound-guided fine needle aspiration cytology (USgFNAC). The pTN is based on histopathological examination of the resection specimen.

Based on the TNM classification, a stage grouping is developed to group tumors with comparable clinical prognosis. Patients are classified into four clinical stages I-IV **[Table 1B]**. Stage I and II represent early stages of the disease, with relatively small tumors and no lymph node metastases. Stages III and IV(A/B/C) represent the more advanced stages of HNSCC, with larger tumors that have spread to regional lymph nodes or secondary organs.

An integral part of the histopathological diagnosis is the examination of the surgical margins to screen for residual cancer as well as to assess the presence and grading of preneoplastic lesions (e.g. dysplasia). Assessment of the radicality of resection is an important parameter for clinical management and may indicate the need for more extended resection or post-operative radiotherapy. Also the assessment of the presence of epithelial dysplasia in the mucosal margins is usually used for clinical management. HNSCC originate from the mucosal epithelium and is sometimes preceded by precursor lesions that can be recognized by histology. The pathologist recognizes these precursor lesions as squamous epithelial dysplasia which is characterized by cellular atypia, and loss of normal differentiation[27]. The status of the epithelium is graded according to the standard criteria of the WHO international classification of tumors[28] as: (a) normal mucosa, (b) mild dysplasia, (c) moderate dysplasia and (d) severe

dysplasia or carcinoma *in situ* and (e) squamous cell carcinoma. It has been shown that patients with severe dysplasia and carcinoma *in situ* have a high risk for local recurrence and postoperative radiotherapy is often indicated for these patients. In contrast, the presence of mild and moderate dysplasia is not used for clinical management Histological grading, particularly grading of mild and moderate dysplasia is subjective and does not accurately predict the risk for cancer[29-31].

Table 1A. TNM-classification according to the UICC

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T1	Tumor ≤ 2 cm in greatest dimension
T2	Tumor > 2 cm, but ≤ 4 cm
T3	Tumor > 4 cm
T4	Tumor invades adjacent structures
NX	Regional lymph nodes can not be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node (≤ 3 cm)
N2a	Metastasis in a single ipsilateral lymph nodes (> 3 cm, but ≤ 6 cm)
N2b	Metastasis in multiple ipsilateral lymph nodes (none > 6 cm)
N2c	Metastasis in bilateral or contralateral lymph nodes (none > 6 cm)
N3	Metastasis in a lymph node (> 6 cm)
MX	Presence of distant metastasis can not be assessed
M0	No evidence of distant metastasis
M1	Distant metastasis

Table 1B. Stage grouping according to the UICC

Stage 0	Tis, N0, M0
Stage I	T1, N0, M0
Stage II	T2, N0, M0
Stage III	T3, N0, M0
	T1-3, N1, M0
Stage IV	T4, N0, M0
	Any T, N2-3, M0
	Any T, any N, M1

1.4 Treatment and prognosis

In general one third of the HNSCC patients present with small tumors that have not metastasized to the regional lymph nodes in the neck (TNM stages I or II). These tumors have a relatively good prognosis and the patients can often be cured with single modality treatment (surgery or radiotherapy), focused only at the tumor site. Whatever modality is chosen, cure will be achieved in >80% of these patients. Two thirds of the patients however, present with larger tumors or tumors that have already spread to the regional lymph nodes of the neck (TNM stages III and IV), and the prognosis of these tumors is worse. Patients with more advanced stages are treated with a combination of surgery and radiotherapy, focused both at the tumor site and the regional lymph nodes.

Despite significant advances in the treatment modalities over the last decades, the 5-year survival rates of patients with HNSCC have improved only moderately. A major reason for this discrepancy is the relatively high frequency of local recurrences: even when the surgical margins have been diagnosed as tumor-free by histopathology the local recurrence rate is still 10-30%[32]. Moreover, 10-20% of the patients develop regional recurrences and 15-25% distant metastases[33]. The moderate improvement in the 5-year survival rates can further be attributed to the changing pattern of failure as more patients now develop distant metastases.

1.5 Second primary tumors

Besides the clinical problems related to the index tumor HNSCC patients are at a high risk to develop second primary tumors, with an incidence of 2-3% new cases per year[34]. These second primary tumors (SPTs) can occur in the upper aerodigestive tract, the lungs and the

oesophagus. In 1953 Slaughter et al.[35] proposed the concept of field cancerization, implying that the mucosa of the upper aerodigestive tract is at risk for cancer development. This concept might explain a number of the clinical and histological observations in HNSCC patients including the frequently occuring second primary tumors. Obviously it is difficult to distinguish a SPT in the lung from a distant metastasis. Moreover, when a SPT arises in the same or adjacent anatomical area it is not easily distinguished from a local recurrence. In most studies the definition of a SPT is based on the criteria of Warren and Gates, published in 1932: (1) each of the tumors must present with 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[36]. Later these criteria were refined by Braakhuis et al. (2002) and references therein[37], stating that tumors in the same or adjacent anatomical site are considered SPTs when the distance to the index tumor is >2 cm, or the time of development > 3 years in between.

1.6 Locoregional recurrence

The relatively high incidence of locoregional recurrence in patients with histopathologically tumor-free resection margins strongly suggests that (pre)malignant cells have remained undetectable. As tumor cells spread locally by invasive growth, regionally to the lymph nodes via lymph vessels and to distant sites by hematogenic routes, it can be hypothesized that isolated tumor cells or micrometastases could be present locoregionally that are missed by routine histopathological examination. These tumor cells that are undetectable by routine diagnostic modalities such as histology and radiology have been defined as *minimal residual disease* (MRD)[38]. A second possibility is that preneoplastic mucosa, histopathologically graded as normal mucosa or mild and moderate dysplasia, remains unresected and undergoes subsequent malignant transformation to result in the development of a local recurrence. Further improvements in the cure rates of HNSCC patients can only be achieved if the factors responsible for local and distant treatment failure are critically examined. The limited sensitivity of the classical diagnostic methods in detecting small disseminated tumor deposits, triggered the development of novel sensitive molecular techniques for the detection of MRD, both systemically in blood and bone marrow, and locally, in surgical margins. This thesis will focus at the molecular analysis of surgical margins for the presence of cells clonally related to the primary tumor (minimal residual cancer or preneoplastic mucosal lesions), to assess the pathobiological and clinical relation with recurrence at the primary site.

2 Molecular diagnosis: selection of suitable markers

2.1 Tumor-specific markers

To date it is widely accepted that cancer arises as a result of the accumulation of (epi)genetic alterations in oncogenes and tumor suppressor genes. Some of these alterations occur specifically in genes that play a crucial role in the normal behavior of the cell, but often these changes appear in less important sequences and are therefore a mere reflection of the genetic instability of the tumors[39]. Hence, tumor cells harbor specific clonal genetic changes that can be used as molecular markers for the detection of cancer cells in clinical samples. There are a number of criteria determining the reliability of a molecular marker[40]. First, the molecular marker should be specific for tumor cells such that it correctly distinguishes between normal cells and tumor cells. Second, to qualify as a clonal marker, a genetic alteration should precede or occur at the stage of invasive cancer and be preserved during tumor progression and metastasis. And third, the marker has to be broadly applicable, i.e. the marker must be present in a large part of the study population.

Besides tumor-specific genetic alterations (DNA markers), also tissue-specific markers (RNA/protein markers) can be exploited, but with two important considerations: 1) the marker should still be expressed homogeneously in the tumors derived from the tissue, and 2) the marker should not be expressed in the clinical sample of interest. In this respect, detection of residual squamous cancer cells in the surgical margins of HNSCC patients is hampered by the presence of normal epithelial cells, which prohibits the use of squamous differentiation markers, and necessitates exploitation of tumor-specific DNA markers.

The first molecular progression model was described for colorectal cancer in which the accumulation of genetic alterations had been demonstrated[39]. The transitional stages of this model, ranging from normal epithelium, via adenoma to carcinoma *in situ* and metastases, are associated with mutations affecting oncogenes (e.g. K-ras) and tumor suppressor genes (e.g. p53). Moreover, it was shown that at these various stages cancer cells often display genetic instability which can be observed at the DNA level as amplifications, deletions or alterations of DNA repeat sequences, known as microsatellites[41,42]. A variant of this sequential DNA damage model was later described for squamous cell carcinoma of the head neck (HNSCC) carcinogenesis[43]. Since we aimed at exploring MRD in head and neck cancer, we currently focus on two established DNA markers: p53 mutations and HPV, which can both be exploited as tumor-specific markers in HNSCC, but also in other tumor types[44-50].

2.2 P53 mutations

Functional aspects of P53

The P53 tumor suppressor gene encodes a stress-induced transcription factor, a phosphoprotein steering cell cycle regulation and apoptosis. The p53 gene maps on chromosome 17p13.1 and contains 3 functional domains, a transcription activation domain, a mutations- and sequence-specific DNA-binding domain, and an oligomerization domain.

Within the amino acid sequence 5 evolutionary conserved regions are observed, termed I-V. The regions II-V (corresponding with exons 5-8) map to the sequence-specific DNA-binding domain in which most of the p53 mutations occur. The complete p53 gene consists of 11 exons, of which exon 1 is non-coding **[Fig 2]**. The resulting p53 protein product is a 53kD nuclear phosphoprotein with 393 amino acids[51,52]. The p53 protein functions as transcription factor, particularly influencing (activating/repressing) the transcription of genes that are involved in cell cycle control, cell growth and apoptosis. The p53 protein is upregulated after cellular exposure to genotoxic stress and acts as a "guardian of the genome": it safeguards the genetic integrity by controlling cell cycle progression at so called "checkpoints", the G1-S and G2-M transitions[53,54]. When the DNA of a cell is damaged, the p53 gene is activated. The increase in p53 switches on p21, a cyclin-dependent kinase inhibitor, resulting in underphosphorylation of pRB, the retinoblastoma gene product, causing cell cycle arrest in G1. In the subsequent time interval, damaged DNA can be repaired by cellular repair mechanisms before cell division, or, if the damage is too extended or irreversible, the cell enters the apoptotic pathway[55,56]. Clearly, these mechanisms of p53 control are crucial to prevent the propagation of genetic abnormalities to progeny cells. Loss of the physiological functions of p53 can result in uncontrolled cell division and progressive genomic instability. Loss of function of the p53 protein can be the result of either p53 gene mutations or posttranslational inactivation of the p53 protein, the latter being caused by binding to cellular proteins like mdm-2 or viral proteins[57], which target the protein for degradation. Many types of p53 mutations have been observed in a wide variety of cell lines and tumors, the most frequent being point mutations primarily in the central portion of the gene (exons 5-8), where the DNA-binding domains are located[13,14,58]. Mutations in a selective number of codons in this DNA-binding domain, the so called DNA contact mutations, act as dominant-negative mutations leading to direct functional inactivation of a remaining wild type allele[59-61]. In addition, the p53 mutation pattern shows specific changes associated with particular carcinogens, such as benzo[a]pyrene[62].

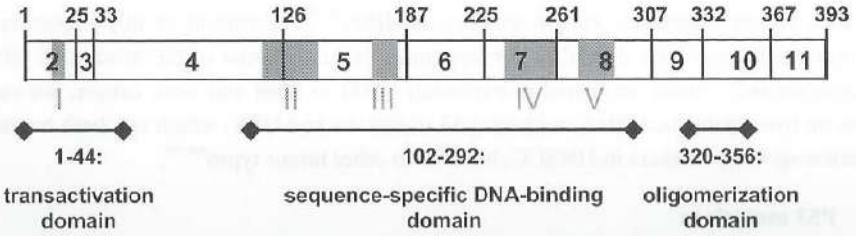

Figure 2. The structural organization of the p53 protein. *The numbered boxes between the vertical lines represent the 10 coding exons of the p53 gene with the amino acid numbers at the exon borders depicted at the top. The grey boxes (I-V) within the amino acid sequence represent the five evolutionary conserved regions. At the bottom, the three different functional domains are indicated.*

Mutated P53 as molecular marker for HNSCC

Alterations of the tumor suppressor gene p53 are the most common genetic events in human tumors, including head and neck cancer and they play an important role in the pathogenesis of HNSCC[12-14,63-66]. Based on a genetic analysis of mucosal lesions with apparent progressive histopathological appearance, Califano et al. (1996) presented a genetic progression model for HNSCC in which p53 plays an important role **[Fig 3]**[43]. According to this model, which is supported by other investigations, 17p LOH, which we consider to be concurrent with p53 inactivation or mutation, occurs in the transition from the early pre-invasive (hyperplastic) state to the invasive state[43,67,68]. Considering this model, p53 mutations appear to be an early event in HNSCC carcinogenesis, associated with the development of histologically recognizable dysplasia. The timing of p53 mutations in the genetic progression model of HNSCC therefore supports its suitability as molecular marker for (pre)cancer cells.

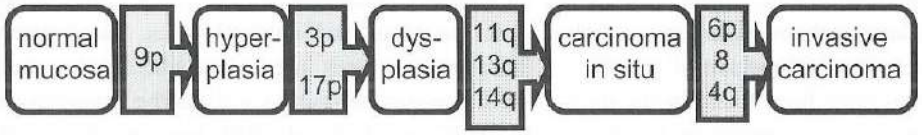

Figure 3. Genetic progression model for head and neck cancer according to Califano et al. (1996). *Genetic alterations involved in HNSCC carcinogenesis are shown within the arrows. The numbers indicate the chromosomal regions that show allelic losses, indicative for loss of tumor suppressor genes. P53 mutations (associated with 17p loss of heterozygosity) are considered to occur relatively early in progression.*

Notwithstanding, the suitability of p53 mutations as a clonal marker (usually determined by mutation screening of tumors and corresponding lymph node metastases, LNM) is being debated, and literature on this issue yields conflicting data. In a number of studies the clonal origin of p53 mutations in HNSCC tumor progression was confirmed[69,70], whereas in other studies it could not be demonstrated[71-73]. Reported figures range from 100% concordancy[69] to a mere 25% concordancy[73]. In this thesis, data are presented that support the effective use of p53 mutations as a clonal marker.

Exploitation of mutated p53 as a molecular marker for detection of (pre)cancer cells in HNSCC patients has also some drawbacks. First, the heterogeneity of p53 mutations necessitates sequencing of all individual tumors. Moreover, the different types of mutation makes it very difficult to find simple and sensitive assays for tumor cell detection (see paragraph 3). On the other hand, the heterogeneity reduces technical artefacts such as carry-over contamination. Finally, a limitation is that p53 mutations are present in only 50-60% of the head and neck cancers[14,66]. Its applicability seems therefore limited. It should be noted, however, that by screening more exons for mutations and using RNA as template the

frequency of mutations might reach up to 80%, whereas some reported even 100%[74]. To increase the frequency of mutations in our patient group, we analyzed the exons 2, 3, 4, 10 and 11 of the p53 gene of 21 HNSCC tumors that had no mutation in exon 5-9 by radioactive cycle sequencing and found two additional mutations in exon 4 and one in exon 3, leading to a an additional mutational frequency of 5% in the total study population (data not shown). By optimizing sequencing strategies p53 could serve as a broadly applicable clonal marker.

2.3 Human papillomavirus

HPV

Papillomaviruses are small double stranded DNA viruses and consist of a circular double stranded DNA molecule, approximately 7.9 kB in size, which is encapsidated in icosahedral protein coats[75]. More than 70 human papillovirus (HPV) types have been identified to date, distinguished based on sequence heterogeneity[76]. HPVs are epitheliotropic viruses and are commonly subdivided in cutaneous and mucosal types, based on their preference for the site of infection[77]. The cutaneous types infect skin, whereas the mucosal types have been found in the mucosa of the anogenital tract as well as the respiratory and upper aerodigestive tract. In addition, "low risk" and "high risk" HPV types are distinguished on the basis of their association with benign conditions or malignancies, respectively. Examples of benign lesions, associated with low risk HPV infection, are oral papillomas and genital condylomas, lesions that are often found in association with HPV 6 and HPV 11. In contrast, certain mucosotropic high-risk HPV types, like HPV 16, 18, 31 and 33, are associated with cervical cancer and cancer of the aerodigestive tract[21,78].

The HPV genome encodes 7 "early proteins" (E1-E7) that are supposed to be expressed before onset of viral DNA replication, and for 2 "late proteins" (L1,L2), that are the viral structural proteins of the capsid. The HPV E6 and E7 proteins of the high risk types function as oncoproteins and show transforming activity[79,80]. At the molecular level, HPV E6 is known to complex with the p53 tumor suppressor gene product and results in its degradation[81]. The E7 protein of several HPV subtypes is capable of binding and inhibiting the Rb gene product[82,83]. In this way, the high-risk HPV E6-mediated degradation of the p53 protein should be considered as an alternative pathway for "classical" mutation to knock-out the p53-regulated cell cycle control pathways. Hence, HPV infection may well disturb the normal well-regulated cell cycle control processes, causing genetic instability and predisposing patients to develop cancer. This also provides the biological basis to expect that tumors originating from HPV infection will show wild type p53, and this is supported by the observation that p53 mutations are absent in most cervical carcinomas[84,85]. However, the expression of E6/E7 genes seems not sufficient to induce and maintain a malignant phenotype. There is evidence that important additional events are involved in HPV-induced carcinogenesis. First, the virus needs to persist in the infected cell(s), as there is a long latency period between viral infection and appearance of cancer. Failure of the immune system to eradicate HPV infected cells therefore appears to play an important role. Second, malignant

transformation is accompanied by a shift of expression of the E6/E7 genes from the superficial, differentiating epithelial layers towards the more basal, proliferating, undifferentiated cell layers. The mechanisms underlying this shift are as yet unclear, but lead to an increased genetic instability in the proliferating (stem)cells. When the virus succeeds in overcoming the barriers, the E6/E7-induced genetic instability will cause accumulation of genetic alterations. The third requirement appears to be the occurrence of specific oncogenic mutations within the host cell genome, due to E6/E7 induced genetic instability.

HPV in HNSCC

Although HPV infection has mainly been implicated in the pathogenesis of cancer of the anogenital tract, in particular cervical cancer, there are strong indications for an etiological role of HPV in the development of HNSCC. HPV involvement in HNSCC has been suggested on the basis of histological and immunohistochemical studies, as well as using Southern blot and *in situ* hybridization techniques[86,87]. Initial studies showed that HPV DNA could be detected in subgroups of oral carcinomas[88]. The availability of PCR techniques led to a rapid increase in the number of studies but also caused a large variation in the prevalence rates. An important finding by Brandsma et al. (1989) was that different anatomic subsites within the upper aerodigestive tract have different susceptibilities to HPV infection or HPV-mediated carcinogenesis[89], and the highest occurrence rate of HPV was reported in tonsillar carcinomas. The data obtained so far suggest an association of high-risk HPV16 and a proportion of HNSCC[21,22,24,88]. However, clear relationships between HPV and HNSCC have not been established in great detail. If HPV infection indeed is an additional pathway for HNSCC carcinogenesis, it would be plausible to expect that in these patients "classical" p53 mutation is substituted by E6 mediated p53 degradation to knock out the p53 regulated pathways, and moreover that HPV is present in lymph node and distant metastases. However, in most studies on p53 mutations and HPV in head and neck cancer, HPV DNA presence and p53 mutations were overlapping[24,90,91]. More information on this subject is desirable to support the etiological role for HPV in HNSCC, and to enable its use as a molecular marker for minimal residual head and neck cancer.

HPV detection methods

HPV nucleic acids detection assays, which include hybridization techniques, like *in situ* hybridization, Southern blot hybridization and polymerase chain reaction (PCR), are currently being widely applied. Often, a combination of PCR followed by hybridization techniques is used as detection method, as PCR is superior in sensitivity (theoretically 1 HPV copy per sample), whereas for example the Southern blot method is still considered to be the most specific HPV DNA detection technique. The advantage of the PCR method is that it requires low amounts of input DNA. On the other hand, a major drawback is the possibility of laboratory contamination and unwanted positivity.

Two types of HPV DNA PCR assays are currently being used. General/consensus PCR, which allows the detection of a broad spectrum of HPV genotypes in a single reaction, or type-specific PCR, which employs specific primerpairs for each different HPV type, and

therefore generally used for the most common high-risk types. Detection and typing of the PCR products is usually performed by electrophoresis, followed by blotting and subsequent hybridisation of the products with consensus or type-specific probes[92,93]. Recently, a PCR-EIA (Enzyme immunoassay) has been developed for the general and type-specific detection[94]. Besides detection of HPV DNA, RT-PCR can be used for examining transcription of the major transforming viral genes E6 and E7[95].

3 Molecular diagnosis: methods of detection

3.1 P53 mutation identification

Mutation screening of the p53 gene should in theory be straightforward, but is in practice rather complex. This statement underlies the large differences reported in the mutational frequencies of p53: a considerable number of these differences can be explained by the method used for sequencing. Most researchers currently use direct DNA cycle sequencing, either with fluorescent chromophores or radioactive labels. In addition, also the p53 GeneChip assay (Affymetrix, Santa Clara, CA 95051, USA)[96] and MALDI-TOF mass spectrometry[97] are being used for (p53) sequencing. For allele discrimination in blood samples these methods seem equivalent, but for sequencing DNA derived from tumor tissue the method used becomes more critical. The amount of tumor cells is often low in tumor biopsies and the DNA of relatively poor quality, which might result in missing mutations or assigning sequencing artefacts as mutations. In general, both fluorescent and radioactive cycle sequencing are based on Sanger's dideoxynucleotide method[98]. As tumor samples are always contaminated by stroma, neoplastic areas in sections of the tumor need to be microdissected before DNA is isolated. Subsequently, the appropriate fragment of the p53 gene, either the domain encoded by exons 5-9 (1.8 kb) or separate exons are amplified by the polymerase chain reaction (PCR) using the tumor DNA as template. We and others have tested both solid phase fluorescent cycle sequencing and radioactive cycle sequencing for their effectiveness in detecting mutations in the p53 gene (ABI 373 sequencing technology; dye-labeled deoxynucleotides)[99,100], and we missed about half of the mutations detected by radioactive cycle sequencing (data not shown). A comparable observation (30% missed) was described by Ahrendt et al. (1999) in a very large study[96]. Notwithstanding, it should be noted that the recent introduction of capillary electrophoresis, and use of primers labeled with improved fluorescent dyes might have lead to an improvement of automated sequencing results, and that at present non-radioactive sequencing gives comparable results.

Others have used transcript sequencing, which was claimed to detect p53 mutations in almost 100% of the HNSCC patients[74]. The possible advantage is that also mutations that are located outside the core domain are detected. A putative disadvantage is that more false-positive results might be obtained as a result of poor quality of the RNA.

Although radioactive cycle sequencing is not 100% reliable either, we have indications that the percentage of false-positive results is relatively low, approaching zero, whereas the percentage of false-negative results seems in the order of 10%. Based on own experience and

that of others, and taking into account the number of mutations outside the core domain and the indications of the false-negative rate, we estimate the mutation frequency in HNSCC at 70-80%.

3.2 Assays to detect DNA with point mutations

For p53 mutations as marker the mutational spectrum is very heterogeneous, which makes it difficult to find assays which are both sensitive, specific, robust and quantitative. For these reasons we have chosen the plaque hybridization assay as the gold standard. Our method has been slightly modified from the technique reported originally by Sidransky et al. (1991)[44]. The various steps are shown in [Fig 4].

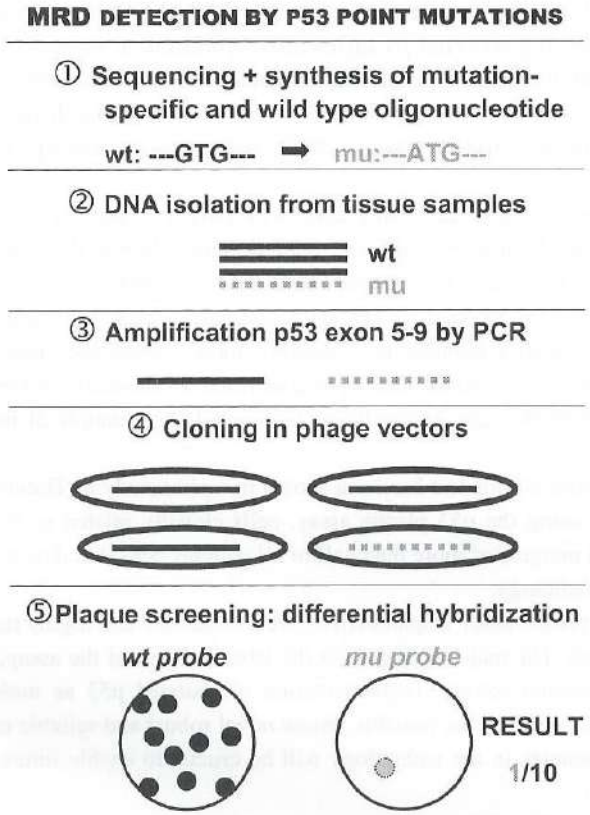

Figure 4. Schematic representation of the technical procedures of the plaque assay (for explanation see text)

In short, on the basis of the p53 gene sequence of the tumor DNA, a mutant-specific and corresponding wild type-specific oligonucleotide are selected. As a rule of thumb, 17-mer oligonucleotides are selected centrally across the mutation on the target strand, an important condition being that they do not contain a "dC" nucleotide at the 5'-end as these are very difficult to label by polynucleotide kinase (see Chapter 2 of this thesis)[101]. In theory, there might be thermodynamically more favorable locations for the mutated base resulting in a larger difference in melting temperature between the two oligonucleotides, but in practice the position around the center usually fulfills the requirements of discrimination. Using DNA from the specimen of interest as template, the p53 gene (exon 5-9 or separate exons) is amplified by PCR. Each PCR product is cloned into the arms of lambda phage DNA. After packaging *in vitro* to infectious phages, host bacteria (*E. coli* K12 LE392) are infected. Each phage has taken up a different PCR fragment, which will then be multiplied during infection. The lysed bacteria (plaques) contain large numbers of phages that are subsequently transferred to nylon membranes and analyzed by differential hybridization using the mutation-specific oligonucleotide and the wild type-specific oligonucleotide. Two dilutions of phages are hybridized usually: one with the tumor-specific probe and one with the wild type probe. Obviously, proper positive (primary tumor DNA) and negative (wild type DNA) controls are included.

As each plaque contains identical phages with only wild type or mutant DNA strands, the number of plaques hybridizing with the mutant oligonucleotide divided by the number of plaques hybridizing with the wild type oligonucleotide is a reliable measure of the tumor cell DNA load in the original sample. At this point we have added the confirmation of mutant positive plaques to identify putative false-positive signal. When the number of hybridizing plaques is low (between 1 to 5), the identity is confirmed by classical rescreening; the positive plaque is stabbed from the agar, replated and rescreened. An example of the plaque assay is shown in **[Fig 5]**.

The clinical value of this test has been shown in a pilot study by Brennan et al. (1995)[46]. They showed that, using the p53 plaque assay, cells clonally related to the tumor could be detected in surgical margins of more than half of all patients considered to be tumor-free using conventional histopathology.

Although the plaque assay is quantitative, well-controlled and highly reproducible, it has also some limitations. The major drawback is the laboriousness of the assay, and it is suitable only in the experimental setting. Implementation of mutated p53 as molecular marker in regular clinical care might not be possible unless novel robust and reliable methodologies are developed. Improvements in the technology will be crucial to enable future implementation.

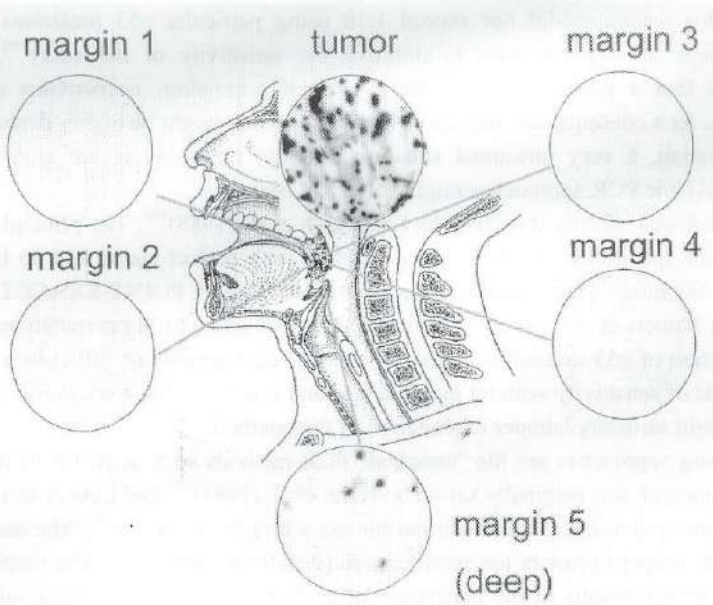

Figure 5. Typical example of MRD detection by the plaque assay using p53 as marker. *The mutation in the p53 gene was a nucleotide substitution located in exon 8, codon 272. DNA was isolated from resection margins, and exon 8 was amplified by PCR and cloned in lambda vectors. After host infection, the plaques were differentially hybridized with wild type and mutant oligonucleotides as probes. The positive plaques were stabbed, replated and rescreened (not shown). Tumor DNA was used as a positive control (tumor), and DNA from cell line UM-SCC-22A was used as a negative control (not shown).*

3.3 Alternative methods for tumor cell detection using point mutations as marker

The plaque assay is among numerous methods that are based on point-mutation detection in (clinical) samples, some of which have only very recently been developed and would be more rapid and less laborious alternatives including mutant-allele specific amplification (MASA)[102], oligonucleotide ligation assay (OLA)[103], POINT-EXACCT (a modified oligonucleotide ligation assay)[104], "enriched" restriction fragment length polymorphism PCR (RFLP-PCR)[105-106] and very recently also digital PCR[107] and PPEM (PNA-directed PCR, primer extension, MALDI-TOF)[108]. It is not possible to review all the various assays in detail and to indicate the pro's and con's extensively, but a few will be discussed below.

The MASA PCR-method was first outlined by Takeda et al. (1993) for the detection of (known) K-ras mutations in sputum of lung cancer patients[102]. The method is based on the efficient amplification using perfect match primers and inefficient amplification when using

mismatch primers. In various papers a sensitivity of at least 1:1,000 has been reported, but in our hands this technique did not exceed 1:10 using particular p53 mutations as marker. Although there are various ways to improve the sensitivity of the assay[109], the major drawback is that a particular assay for a particular mutation necessitates considerable optimization. As a consequence, the sensitivity of this assay might be highly dependent on the specific mutation, a very unwanted situation. Finally, the assay is not easy to quantify although RealTime PCR approaches might solve that issue.

The OLA was initially described by Landegren et al. (1988)[103]. The principle of this test is based on the specificity of DNA-ligases: ligation with perfect match and no ligation with mismatches. We tested a more extensive variant of this method, POINT-EXACCT, which was described by Somers et al. (1994)[104] for the detection of (known) K-ras mutations. However, for the detection of p53 mutations we have encountered a number of difficulties in reaching optimal levels of sensitivity without high background levels. Again, the heterogeneity of p53 mutations might seriously hamper exploitation of this method.

Promising approaches are the "enriched" PCR methods such as RFLP-PCR or REMS-PCR. This approach was originally set-up by Kahn et al. (1991)[105] and Levi et al. (1991)[106] for K-ras mutation detection and was modified for use with p53 mutations[107]. The method makes use of slightly adapted primers for amplification (usually in two steps). The result is that the wild type sequence results in the generation of a cleavage site for a specific endonuclease. Using alternate PCR steps and endonuclease digestion steps the mutant strands are enriched and can be visualized on an electrophoretic gel. Drawbacks of this technique are 1) the dependence of a mutation in a "near" restriction site limiting the use for heterogenous mutations such as in the p53 gene, 2) difficulties for quantitation, and 3) a very high risk for carry-over contamination (see also paragraph 3.4). The latter problem was in part solved by the use of thermostable endonucleases which are active during the PCR reaction[110,111]. Opening and closing of the vials can then be omitted. On the other hand these assays are theoretically sensitive and rapid, and might be suitable for implementation in the clinical setting.

Recently, a novel method, named digital PCR, has been introduced as an approach to identify predefined mutations in a minor fraction of a cell population[107]. The strategy involves the isolation of single molecules by limiting dilution and separate amplification of the individual template molecules so that the resultant PCR product is completely wild type or mutant. In fact, this strategy is comparable to the cloning of amplified fragments in phages used in the plaque assay, with the major difference that "cloning" is performed by limiting dilution before amplification. Although in theory a sensitivity of 1:1,000 could be reached, digital PCR seems mainly promising for a limited number of mutations (such as for K-ras), as for each mutation the molecular probes have to be optimized. What is more, for a required MRD detection level of 1:5,000, at least 10,000 separate PCR reactions have to be carried out, which requires extensive automatization, and which makes the assays expensive.

The most recent high-troughput method was described by Sun et al. (2002)[108]. The screening method was termed "PPEM", standing for PNA-directed, Primer Extension, MALDI-TOF. DNA samples are first amplified using peptide nucleic acid (PNA)-directed

PCR clamping reactions in which mutated DNA is preferentially enriched. The PCR-amplified DNA fragments are then sequenced through primer extension to generate diagnostic products. Finally, mutations are identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. This method has been claimed to detect as few as 3 copies of mutant alleles in the presence of a 10,000-fold excess of normal alleles in a robust and specific manner. Further improvement of this technique, including high-throughput automation and validation with large numbers of clinical samples, might support its implementation into clinical care.

3.4 Problems and pitfalls using point mutations as marker

Specificity (false-positivity)

Despite all the advantages of the described PCR based techniques for employement in MRD detection, these techniques are subject to certain (technical) problems, which could cause false-positive results. This false-positive signal arises in general from the sensitivity of the PCR reaction in amplifying not only target material, but also undesired contamination, even if the latter consists only of minute amounts of DNA or amplimers derived from prior PCR reactions. Although the positive signal detected in this way is often termed as "false-positivity", it is in fact "unwanted positivity". Contamination of patient material by tumor cells or tumor cells DNA can be introduced at different time points during sampling and technical processing, for example: 1) in the operating theatre, 2) during histopathological processing of the samples and 3) by DNA amplimer contamination in the PCR. Several precautions should be taken in every step to prevent contamination. For example, in our study, we tried to prevent tumor cell (DNA) contamination as follows. We decided to sample the margins in the operation theatre and not at the pathology department. Furthermore, we used one sample for histological control and one for DNA isolation. Moreover, before sampling the resection margins in the operation theatre, the operating field was extensively rinsed and the instruments changed. Particularly, tumor cell DNA contamination cannot be easily controlled for. DNA is a very stable molecular substrate and leakage from necrotic tumors and binding to the clinical sample of interest might give rise to false-positive results (see also Chapter 5 of this thesis). Finally, another source of contamination might be introduced during the process of PCR amplification. Contamination by DNA products from prior PCR reactions (amplimers), can be reduced and controlled by stringent measures, including performing PCR reactions in a separate laboratory room, maintaining clean reaction reagents and always checking for possible contamination with the use of proper negative control reactions.

Taq errors

A false-positive result in the plaque assay or any other assays using point mutations as marker could also be caused by the enzyme that is used to amplify the DNA: a thermostable DNA polymerase. It can be anticipated that the used enzyme (in our case *Taq* polymerase)

produces spontaneous single-base substitution errors at reported frequencies of 1/9,000 nucleotides polymerized[112,113]. This knowledge underlines the need to assess the degree to which random mutations introduced by the *Taq* polymerase during the amplification process, play a role in this type of analysis. In particular when archival, stained and fixed DNA is analyzed for point mutations as marker, the error frequencies considerably increase. Both *Taq* errors and DNA sequence damage are potential limitations of the use of point mutations as marker, but these are controllable by an adapted experimental set-up, additional measurements and statistical analysis (Nieuwenhuis et al., manuscript in preparation). In fact, from a number of experiments we have indications that Taq errors are a relatively small problem when the amount of input DNA in the PCR is sufficient (over 500 ng of DNA).

Sensitivity

The sensitivity of the p53 based plaque assay is determined by the amount of input DNA in the PCR amplification, the number of plaques screened and in some applications by the *Taq* error rate. The clinically relevant level of sensitivity is unknown. In this thesis approximately 5,000 plaques with p53 insert corresponding to 5,000 cells were screened. Still a single tumor cell in 5,000 normal cells is detectable. The level of detection might be a crucial issue in MRD detection, because it could determine the clinical significance of the molecular findings. The significant level of detection can only be determined if quantitative analyses in large studies have been completed and combined with (long-term) follow up data. Ideally, to gain insight in the clinically required sensitivity of MRD detection, margin assessment should be performed in patients who will not receive post-operative radiotherapy. The recurrence rate in these patients is then a proper reflection of the presence of MRD post-operatively assuming that MRD always develops into clinically manifest recurrence.

4 Scope of this thesis

In total 10-30% of HNSCC patients develop local recurrences after surgical treatment despite histological free surgical margins. This may in part result from minimal residual cancer or unresected preneoplastic mucosal lesions. It has become clear that sensitive molecular markers and methods are needed for an improved diagnosis of head and neck cancer and to investigate the pathobiology of recurrence at the primary site. Analysis of surgical margins for the presence of residual tumor cells or preneoplastic lesions using tumor-specific markers like p53 mutations and HPV16 E6 and statistical correlations with clinical outcome may lead to a better definition of patients at high risk for tumor recurrence. From the combination of pathobiological and clinical data it might eventually be possible to improve therapeutical approaches for head and neck cancer.

In Chapter 2, we describe a technical improvement of the plaque assay, and in Chapter 3 the value of p53 point mutations as clonal marker for the diagnosis of HNSCC. In Chapter 4,

we evaluated if HPV could serve as an alternative marker for the detection of cells clonally related to the tumor. In Chapter 5 the results of a pilot study (30 patients) are described in which we use p53 mutations as marker for MRD. The pathobiology of recurrence at the primary site was studied, using (immuno)histopathological methods. In Chapter 6, we present the results of the main prospective study of 128 HNSCC patients, in which we examined the prognostic value of molecular-positive surgical margins using p53 mutations and HPV16 E6 as molecular markers. The clinical course of disease parameters (local recurrence-free survival, disease-free survival and cause-specific survival) was evaluated and compared between patients with molecular-positive margins and molecular-negative margins. In Chapter 7, we discuss the prognostic value of the molecular margin status in relation to other clinicopathological and histopathological determinants of HNSCC, such as N-stage, number of tumor-infiltrated lymph nodes, dysplasia, perineural growth and others. In Chapter 8 a general discussion of the results presented in this thesis is provided.

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2

Labeling efficiency of oligonucleotides by T4 polynucleotide kinase depends on 5'-nucleotide

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Abstract

Phosphorus (5') end-labeling of oligonucleotides by T4 polynucleotide kinase is frequently applied when oligonucleotides are to be used as hybridization probes, primers for direct sequencing or primers for microsatellite analysis. For successful use of radioactively labeled oligonucleotides in differential hybridization experiments a labeling efficiency of more than 25% is needed. In this paper we show that a number of factors can influence the labeling efficiency: quality of the enzyme T4 PNK, the [γ-^{32}P]ATP used, purity of the oligonucleotide and the sequence of the oligonucleotide. The [γ-^{32}P]ATP used and the purity of the oligonucleotide seem to be the least important variables, the quality of the enzyme plays a more important role. However, our results clearly demonstrate that the sequence of the oligonucleotide largely determines the labeling efficiency. Oligonucleotides starting with a C at the 5'-end have labeling efficiencies which in general do not exceed 15%, whereas in contrast oligonucleotides starting with a G, T or A at the 5'-end reach labeling efficiencies of at least 40%.

Introduction

An important enzyme reaction in modern molecular biology is the transfer of phosphate groups to the 5'-end of DNA strands by T4 polynucleotide kinase (T4 PNK). The most frequent applications of this reaction are the generation of radioactively labeled oligonucleotides using [γ-^{32}P]ATP which can be applied as hybridization probes, primers for direct sequencing or primers for microsatellite analysis. Because synthetic oligonucleotides usually lack a 5' phosphate group they are easily labeled by transfer of the γ-phosphate of ATP from [γ-^{32}P]ATP to their 5'-end. When the reaction is carried out efficiently, the specific activity of these labeled oligonucleotides can reach at least 40%-50%[1,2]. Within our research programme "detection of minimal residual head and neck cancer by tumor-specific and tumor-associated molecular markers", we have adopted the method originally described by Sidransky et al. (1991)[3,4] for the detection of malignant squamous carcinoma cells in the resection margins of patients surgically treated for head and neck cancer. In short: tumor DNA is isolated and the p53 gene amplified by PCR and sequenced. Based on the mutation found, an oligonucleotide identical to the mutated sequence is synthesized. DNA from resection margins and lymph nodes is amplified, cloned in lambda phages, packaged, plated and differentially hybridized by the radioactively labeled oligonucleotide as a probe. Applying the labeling reactions on various oligonucleotides with T4 PNK we noticed considerable differences in labeling efficiency. Moreover we noticed that when the labeling efficiency drops below 25% (see Material & Methods) the probe is not suitable for differential hybridization. Theoretically four parameters could influence the labeling efficiency: 1) the quality of the enzyme T4 PNK, 2) the quality/purity of the oligonucleotide, 3) the sequence of the oligonucleotide and 4) the [γ-^{32}P]ATP used. In this paper we report the findings of an analysis of the influence of these parameters on the labeling efficiency of oligonucleotides.

Material and Methods

In each of the described labeling reactions (see Results, Tables 1-4) in total 10 pmoles of each oligonucleotide were labeled with 10 pmoles (3 µl) of [γ-^{32}P]ATP (Amersham, 's-Hertogenbosch, The Netherlands or NEN, Boston, USA; specific activity 3000 Ci/mmole; 10 mCi/ml in aqueous solution) using 9.5 U T4 polynucleotide kinase (Pharmacia, Roosendaal, The Netherlands or SpheroQ, Leiden, The Netherlands) in a final volume of 20 µl, according to the supplier of the enzyme. The oligonucleotide concentration was determined by OD 260 measurement (GeneQuant II RNA/DNA Calculator, Pharmacia Biotech) and calculated according to the following formula:

pmol/µl concentration = (OD 260 * dilution * 37 10E3)/MW

The molecular weight (MW) of the oligonucleotides was calculated based on the sequence by summarizing the molecular weights of the separate bases (313 for A, 289 for C, 329 for G and 304 for T).

The reaction mixtures were incubated at 37°C for 1 hour. Before column separation on Sephadex G50 columns (Pharmacia) 100 µl TE (10 mM TRIS-HCl, 0.2 mM EDTA pH 7.5) was added to the reaction mixtures and the total amount of radioactivity (total cpm) determined by liquid scintillation counting (WinSpectral™ 1414 LSC, Wallac) of a 2 µl aliquot. The mixtures were loaded on the columns and eluted with TE in fractions of 200 µl TE (approximately 9 or 10 fractions). The efficiency of [γ-^{32}P]ATP incorporation was measured by (radio)chromatography of the reaction mixtures through the columns. A typical example of the radioactivity in the various fractions is as depicted in Fig.1.

The appropriate fractions, containing radiolabeled oligonucleotides (fractions 5-8) were pooled and the total amount of radioactivity (incorporated cpm) measured as described. The efficiency of transfer of the radiolabel to the oligonucleotides was calculated by dividing the total amount of radioactivity in the pooled fractions after column separation (incorporated cpm) by the total amount of radioactivity in the reaction mixture before column separation (total cpm). All reactions were performed at least in duplicate.

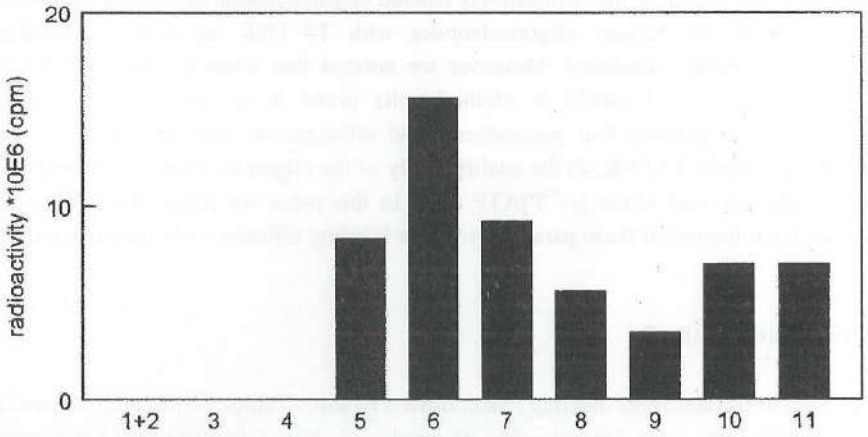

Figure 1. Chromatography pattern of radioactivity after labeling and column separation of oligonucleotide (5'-GTCACCACGAGCTGCCC-3'). *Fraction 1+2 were discarded. The labeled oligonucleotide is present in fraction 5-7, which were pooled for measurement of the total amount of radioactivity incorporated.*

Results

Initially, [γ-^{32}P]ATP of two manufacturers was compared but differences in labeling efficiency were not found (data not shown). Secondly the labeling efficiencies of T4 PNK enzymes obtained from different manufacturers (SpheroQ and Pharmacia) were compared using two oligonucleotides (Isogen, Maarssen, The Netherlands), purified by butanol extraction (standard purification method from the manufacturer): 5'-TGA GGA ATC AGA GGC CTG G-3' (int5A) and 5'-CCC TTC AGG TAC TAA GT-3' (22Awt). Labeling efficiencies are indicated in Table 1. The reaction was shown to be very inefficient when carried out with T4 PNK from SpheroQ.

Table 1. Labeling efficiencies of butanol purified oligonucleotides, using T4 PNK from either SpheroQ or Pharmacia

oligonucleotide sequence	code	labeling efficiency	
		T4 PNK SpheroQ	T4 PNK Pharmacia
5'-TGA GGA ATC AGA GGC CTG G-3'	int5A	12%	38%
5'-CCC TTC AGG TAC TAA GT-3'	22Awt	0.42%	7.0%

The results of this experiment showed a remarkable difference in enzymatic activity between T4 PNK from SpheroQ and Pharmacia using these oligonucleotides purified by butanol extraction as substrate. However, these figures could not explain the difference in labeling efficiency between the two oligonucleotides: 7% for 22Awt and 38% for int5A, respectively. We could not exclude minor differences in purity between these two oligonucleotides and therefore the 22Awt oligonucleotide was resynthesized and purified by either butanol extraction, anion exchange chromatography or reverse phase chromatography. Other conditions, such as the enzyme used (Pharmacia), were identical. Labeling efficiencies are as indicated in Table 2.

Table 2. Labeling efficiencies of oligonucleotide 22Awt purified by either butanol extraction, reverse phase chromatography or anion exchange chromatography.

oligonucleotide sequence	labeling efficiency		
	butanol	reverse phase	anion exchange
5'-CCC TTC AGG TAC TAA GT-3'	7%	11%	23%

Although the purity of the oligonucleotide indeed resulted into a markedly higher labeling efficiency (after anion exchange purification), the specific activity of this 22Awt oligonucleotide was still borderline (below 25%), and much less as compared to the butanol extracted oligonucleotide int5A (see Table 1; 38%). These experiments lead to the conclusion that some oligonucleotides were labeled efficiently, whereas in contrast others did not reach a high labeling efficiency, even not when the most active enzyme and superior oligonucleotide purification method were used. We therefore labeled a large set of randomly selected oligonucleotides, and the obtained labeling efficiencies are indicated in Table 3.

Table 3. Labeling efficiencies of randomly selected oligonucleotides

oligonucleotide sequence	code	labeling efficiency
5'-GTG GAG GAG ACC AAG GGT-3'	k9s	50%
5'-GGA GAG ACC GGC GCA CA-3'	9740pwt	51%
5'-GTA GGA ATT CAC TTG TGC CCT GAC TT-3'	p53 4s	43%
5'-ATT GCA CAG GTC CTC CTG GCA-3'	E48 258as	41%
5'-ATG GGA CAG GTA GGA CCT G-3'	p53 k8s	51%
5'-TGA GGA ATC AGA GGC CTG G-3'	p53 int5A	38%
5'-CTC ACC ACG AGC TGC CC-3'	9730pwt	17%
5'-CAT CGA ATT CTG GAA ACT TTC CAC TTG AT-3'	p53 9as	15%
5'-CCC TGC CCT CAA CAA GA-3'	96-63wt	8%
5'-CCC TTC AGG TAC TAA GT-3'	22Awt	7%

From the data in Table 3 we noted that oligonucleotides starting with a cytosine at the 5'-end were labeled much less efficiently than oligonucleotides starting with an adenine, guanine or thymine at the 5'-end (on average between 5-15% versus 40-65%). To confirm these findings and to exclude other differences in sequence or manufacturer we ordered four 17-mer oligonucleotides, differing only in the nucleotide at the 5'-end (N) being either a G, A, T or C (5'-NTCACCACGAGCTGCCC-3') and purified by routine butanol extraction (Isogen). These oligonucleotides were labeled according to the protocol described (Pharmacia enzyme) and the results are indicated in Table 4.

Table 4. Labeling efficiency of four oligonucleotides, differing only in the nucleotide at the 5'-end (N-oligonucleotide).

	N-oligonucleotide			
	G	A	T	C
labeling efficiency	60 ± 5%	45 ± 5%	45 ± 5%	10 ± 5%

The efficiency of transfer of [γ- ^{32}P]ATP to the oligonucleotide with a G at the 5'-end was 60% ± 5%, with an A and a T 45% ± 5%. In contrast the oligonucleotide with a C at the 5'-end only reached a labeling efficiency of 10% ± 5%.

Discussion

Our results clearly demonstrate that a number of factors influence the labeling efficiency of oligonucleotides with [γ-^{32}P] using the T4 PNK reaction. Two of these, the quality of the enzyme and the purity of the oligonucleotide were expected. The purity of the oligonucleotide seems to be the least important variable, the quality of the enzyme plays a more important role. However, the parameter largely determining the labeling efficiency is the nucleotide at the 5'-end of the oligonucleotide. Oligonucleotides with a C at the 5'-end have labeling efficiencies which in general do not exceed 15%, whereas in contrast oligonucleotides starting with a G, T or A at the 5'-end reach labeling efficiencies of at least 40%. This unexpected observation has a large technical impact. As described above, 5'-end labeled oligonucleotides are used for cycle sequencing, microsatellite analyses as well as differential hybridization. As we show here, a proper choice of the sequence of the oligonucleotide will enhance the labeling efficiency and thus increase the signal. What is more, it has been described by several authors that blunt-ended PCR products often cannot be cloned effectively into plasmid or phage vectors. Therefore other solutions have been introduced such as the addition of a restriction site or the construction of specially adapted vectors. It can be anticipated that a PCR product, amplified by two primers with a C at the 5'-end, cannot be cloned effectively when only 1% of the product would have gained a phosphate group at both ends. We therefore recommend for all applications to choose oligonucleotides starting with a G or alternatively a T or A, but never a C, for example by a shift in sequence.

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3

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 18/23 patients, a *TP53* mutation was detected in the primary tumor, and in all cases the same *TP53* mutation was present in the corresponding LNM and/or DM. In 9/20 patients with LNM and 3/7 patients with DM the LOH-pattern of the metastasis differed from that in the corresponding primary tumor at 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 significantly associated 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, 100% versus 27% (LNM) and 100% versus 0% (DM), respectively ($P = 0.008$ and $P = 0.029$; two-sided Fisher's 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) 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 not significantly improved during the last 20 years[2]. Important reasons for this lack of progress are the relatively high locoregional failure rate and the development of distant metastasis in patients with advanced stages[3].

The most important prognostic factor for HNSCC is the presence and number of lymph node metastases (LNM) in the neck. Patients without LNM have a risk of only 7% of developing distant metastasis (DM), whereas for patients with more than three positive lymph nodes this risk is almost 50%. Not only the presence and number of LNM, but also involved levels 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 a result of mutations and (epi)genetic 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 which are associated with invasive growth and metastatic behavior are less well-defined but may encompass in a number of tumors LOH at 18q and 8p[4,6,7,8]. Mutations in the *TP53* tumor suppressor gene are present in the majority of the head and neck cancers. It is well known that loss of *TP53* function is associated with increased genetic instability, as demonstrated in cell lines and *TP53*-deficient mice[9,10]. The *TP53* status might therefore play 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. The clinical experience indicates that lymphogenic metastases precede hematogenic metastases, suggesting that hematogenic metastases develop from lymphogenic metastases. However, there are some recent data 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 in the role of specific metastasis-suppressing or -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 the *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 have been 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 and/or DM from 23 HNSCC patients.

Materials and methods

Patients and tumor specimens

In total, 57 tissue samples of the primary tumor, LNM and/or DM were collected from 23 HNSCC patients. From 16 patients samples of the primary tumor and corresponding LNM were obtained, from 3 patients samples of the primary tumor and corresponding DM were collected and from 4 patients samples of the primary tumor and of both corresponding LNM and DM were obtained. Forty-two tissue samples were obtained as freshly frozen material and fifteen tissue samples as archival paraffin-embedded material, respectively. Patient characteristics are mentioned in Table 1. Tumor stages (pTN) were determined according to the International Union Against Cancer (UICC) criteria[19]. The DM were obtained from the skin (lower back; patient 19, and abdomen; patient 23), the lung (multiple lesions in both lungs; 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 mentioned in Table 1; all LNM were simultaneously resected with the primary tumor.

Microdissection and DNA extraction

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 de-paraffinized in xylene. In all cases, the first and last tissue sections were stained with hematoxylin-eosin (HE) 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. Dissected tissues were treated with 1 mg/ml proteinase K for 24 h at 52 °C 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 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, 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).

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

#	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*

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[4,20,21,22,23]. 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 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 °C for 1 minute, annealing at a temperature between 55 and 65 °C (depending on the primer set) for 1 minute, and extension at 72 °C for 2 minutes. 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 (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 greater than 50% in the tumor sample when compared with the same allele in normal control DNA, when necessary after stutter correction as described previously[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. (Sidransky et al., 1991). In short, an 1.8-kb fragment of the *TP53* gene, encompassing the exons 5 to 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 HE slides were examined by a pathologist (JAK) 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, 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 or MSI between primary tumors and corresponding metastases for the stratified groups were analyzed by the two-sided Fisher's exact test. Differences in the total number of discordant LOH or 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 to be significant.

Results

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

In 18/23 (78%) patients 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 and/or DM also lacked a *TP53* mutation (Figure 1).

Comparison of LOH/MSI patterns in primary tumors and metastases

In 9/20 patients differences in LOH patterns and microsatellite alterations were observed between primary tumors and LNM (Figure 1). In total, 24 discordant allelic losses and one discordant microsatellite alteration were observed among a total of 402 informative allelic comparisons. In the three cases (patients 1, 12, and 20) with multiple 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 observed 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 observed in the LNM but not in the primary tumor (patient 5).

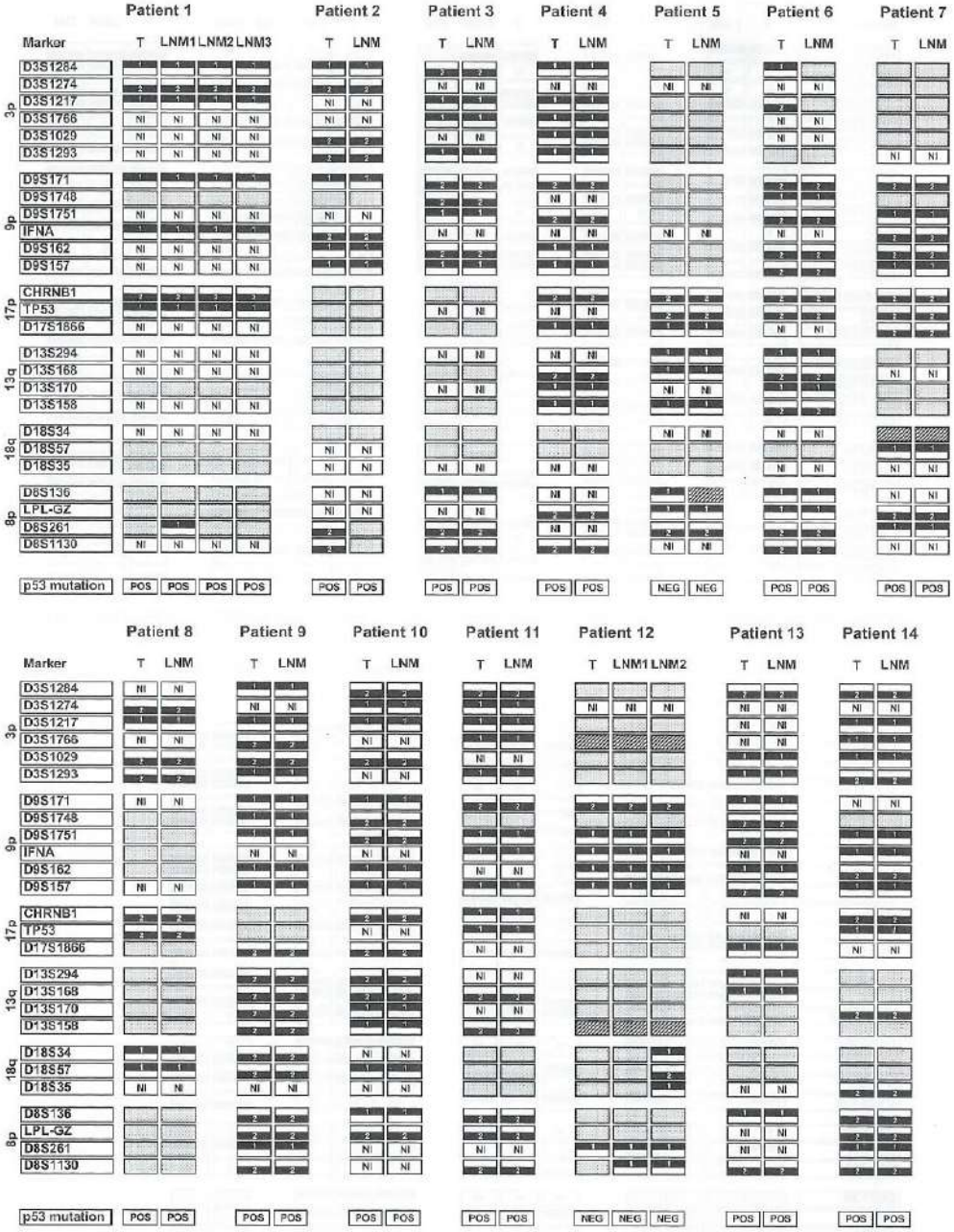
In 3/7 patients differences in LOH patterns and microsatellite alterations were observed between primary tumors and DM (Figure 1). In total, 18 discordant allelic losses and four discordant microsatellite alterations were observed among a total of 141 informative allelic comparisons (Figure 1). Patient 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 observed 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 observed 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 or 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 observed in the number of discordant allelic losses and microsatellite alterations between comparisons of primary tumor and corresponding LNM versus primary tumor and corresponding DM. From four patients (17, 19, 20, and 21), both LNM and DM could be obtained. Interestingly, in these four patients the genetic alterations of the LNM differed from those observed in the DM, suggesting that these two types of metastasis have arisen from different subclones or have acquired different alterations after separation from the primary tumor.



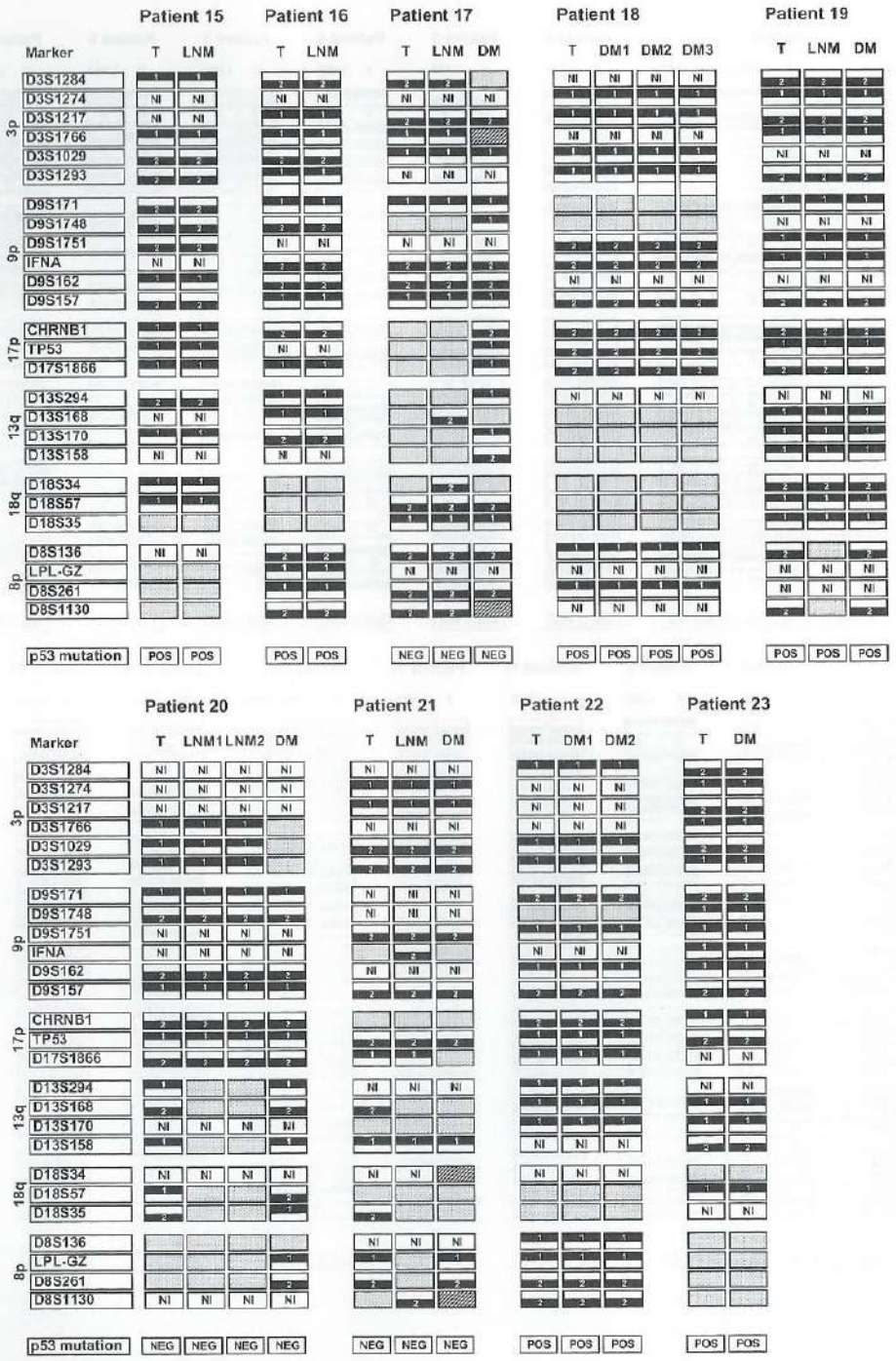


Figure 1. Results of the 23 primary HNSCC and corresponding LNM and/or DM. *The TP53 mutations are indicated as neg (negative) and pos (positive). The codon and type of mutation are listed in Table 1. T = tumor, LNM = lymph node metastasis and DM = distant metastasis.*

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

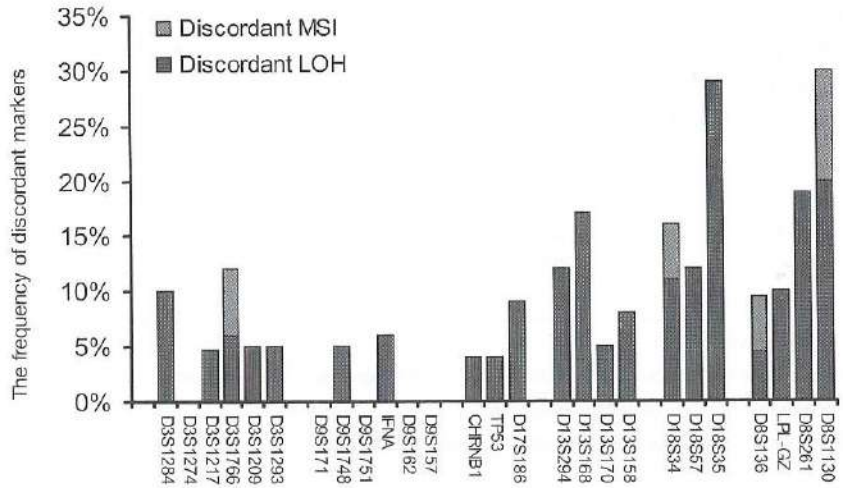


Figure 2. The frequency of discordant LOH or MSI between primary tumor and metastasis (LNM and DM) is shown per microsatellite marker. *Only informative markers were scored. When 2 or 3 LNM or DM were investigated, the LNM/DM that was the most discordant was scored. From pa tients with both LNM and DM, 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) as compared to micros ateltite markers located on chromosome arms 3p, 9p and 17p (proposed to be early markers in HNSCC carcinogenesis).*

Histological comparison between primary tumors and metastasis

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

Comparison between tumors with and without a *TP53* mutation

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

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

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

[a] *n, number of patients.*

[b] *Frequencies of patients with discordant LOH or MSI pattern when comparing primary tumor and corresponding metastasis.*

[c] *LNM = lymph node metastasis and DM = distant metastasis*

Differences in the LOH or MSI patterns of the LNM as compared to the primary tumor were significantly more common in *TP53* wild type than in *TP53* mutated tumors (100% versus 27%; P=0.008 with the two-sided Fisher's exact test). Differences in the LOH or MSI patterns of the DM as compared to the primary tumor were also significantly more common in *TP53* wild type than in *TP53* mutated tumors (100% versus 0%; P=0.029 with the two-sided Fisher's exact test).

Furthermore, the relative number of informative microsatellite markers that showed different results in primary tumor and 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 as compared to 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 observed in the metastasis but not in the primary tumor were only detected 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 frequency of discordant genetic alterations observed between comparisons of primary tumors and corresponding LNM and comparisons of 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 or, alternatively, points to further clonal evolution of metastatic cells after separation from the primary tumor. These findings further suggest that in a proportion of HNSCC patients hematogenic spread might occur already at an early stage, and that DM do not develop from LNM. These results confirm the data in recently published reports[11,12].

Microsatellite loci located at chromosome arms 13q, 8p and 18q scored the highest frequency 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[6,7,8,27]. Microsatellite markers located at chromosome arms 3p, 9p and 17p are suitable as clonal markers in HNSCC, as the LOH-pattern of these markers are relatively stable during tumor progression.

Discordant LOH patterns in metastases and primary tumors may be explained by low frequencies of the metastatic clones in the primary tumors. The existence of intratumoral heterogeneity has been demonstrated in many tumor types, *e.g.*, as melanomas[28] and ovarian tumors[29], supporting this theory. Another explanation for the observed genetic differences between the primary and the metastatic tumor is that additional genetic alterations accumulate in the metastatic cell clone after separation from the parental tumor. If this is the case, then time does not appear to be an important factor in this process since we did not observe an influence of the time period between the resection of the primary and metastatic tumor on the number of discordant alterations.

The most remarkable finding of this study was that the status of the *TP53* gene is associated with the frequency of discordant genetic alterations observed between primary

tumor and corresponding metastasis. The TP53 protein plays a central role in the cellular response to DNA damage. Increased levels and activity of TP53 result in the blocking of the replication of damaged DNA and in the elimination of cells that acquired DNA mutations[30]. In the majority of HNSCC, *TP53* is inactivated as a 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 is significantly associated with the degree of genetic differences between primary and corresponding metastatic tumor. A possible explanation is that tumor cell clones with *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 in order to metastasize. Following up on this, the implication of this explanation would be that head and neck tumors with a mutated *TP53* gene show a relatively poor prognosis. On this topic conflicting data have been reported. 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 significantly associated with the degree of genetic differences 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 is in these cases genetically identical to the clone with metastatic capacity. A comprehensive comparison of the genetic profiles of metastasizing and non-metastasizing *TP53* mutated tumors might then reveal specific genetic alterations involved in the metastatic process.

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4

Biological evidence that human papillomaviruses are etiologically involved in a subgroup of head and neck squamous cell carcinomas

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Abstract

High-risk human papillomaviruses (HPVs) have been proposed to be associated with a subset of head and neck cancers (HNSCC). However, clear biological evidence linking HPV-mediated oncogenesis to the development of HNSCC, is rare. An important biological mechanism underlying HPV-mediated carcinogenesis is the inactivation of p53 by the HPV E6 oncoprotein. In the present study we investigated this biological relationship between HPV and HNSCC. In total 84 HNSCC tumors were analysed for the presence of high-risk HPV nucleic acids by DNA PCR-EIA and E6 RT-PCR, respectively, and for the presence of mutations in the p53 gene. We found 20/84 cases HPV16 DNA-positive with DNA assays, 10 of which were consistently positive with all assays. Only 9/20 cases showed E6 mRNA expression, indicative for viral activity. Only these nine E6 mRNA-positive cases all lacked a p53 mutation, whereas the HPV DNA-positive and HPV-DNA negative tumors showed p53 mutations in 36% and 63% of the cases, respectively. Moreover, only in lymph node metastases of HPV E6 mRNA-positive tumors both viral DNA and E6 mRNA were present. Our study provides strong biological evidence for a plausible etiological role of high-risk HPV in a subgroup of HNSCC. Analysis of E6 mRNA expression by RT-PCR or alternatively, semiquantitative analyses of the viral load, seem more reliable assays to assess HPV involvement in HNSCC than the very sensitive DNA PCR analyses used routinely.

Introduction

Mucosotropic high-risk human papillomaviruses (HPVs), known to cause cervical and other anogenital cancers, have been proposed to play a role in the etiology of head and neck squamous cell carcinomas (HNSCC)[1]. The presence of high-risk HPV DNA in a subgroup of HNSCC has supported this hypothesis[2,3,4,5]. Molecular studies have provided important data on the role and oncogenic mechanism of high-risk HPV in carcinogenesis[6,7,8]. By expression of the viral oncoproteins E6 and E7 the virus dysregulates crucial cellular mechanisms such as the cell cycle and the apoptotic pathway. The E6 oncoprotein specifically inactivates wild type p53 and the E7 oncoprotein inactivates Rb, respectively. In this way the high-risk HPV E6-mediated degradation of the p53 protein should be considered as alternative pathway for "classical" mutation to knock-out the p53 regulated pathways, and provides the biological basis to expect that tumors which originate from HPV infection, will show wild type p53. Indeed, this general biological mechanism is supported by the finding that p53 mutations hardly occur in cervical carcinomas[9,10]. However, in most studies on head and neck cancer HPV DNA presence and p53 mutations were overlapping[2,11,12], an observation which gave rise to a long debate whether HPV is causally related to the development of a subset of these tumors.

On the other hand, the inconsistent observations might be explained by the methods and criteria that were used for HPV assessment and mutational analysis. HPV DNA detection by PCR is extremely sensitive, up to a level of a few DNA copies[13], and might lead to the detection of a few viral genomes that may not be clonally associated with the tumor. Moreover, the discrepancies in the data might be caused by the source of the tissue material, i.e. purified DNA or crude extracts of either cryosections or paraffin sections. Furthermore, p53 mutation frequencies were not determined in all cases by sequencing, but often based on immunohistochemical or single strand conformation polymorphism (SSCP) analysis. Finally, not all sequencing methods are equally reliable to detect mutations in tumor DNA[14].

In the present study, we investigated HPV involvement in HNSCC making use of the known biological properties of the virus in cervical carcinogenesis. We hypothesised that when the virus plays an important role in the genesis and progression of HNSCC: 1) HPV DNA should be present in the tumor, 2) the E6 viral oncogene should be expressed in the tumor, 3) the p53 gene should be wild type and 4) HPV DNA and E6 mRNA should be present in corresponding lymph node metastases.

Materials and Methods

Patients and tumor specimens

In total 84 patients who underwent surgical treatment for squamous cell carcinoma of the upper aerodigestive tract were included. The study was approved by the Institutional Review Board of the *Vrije Universiteit* Medical Center and informed consent was obtained from all patients. From each patient a fresh primary tumor sample was obtained, and, if present, a sample of each macroscopic lymph node metastasis. The tumor sample was directly snap-frozen into liquid nitrogen and stored at -80°C until further processing. Selection criteria used to include patients were tumor site (mainly oropharynx, oral cavity and hypopharynx) and size. The distribution of the tumors by anatomical site was as follows: 45 tumors were located in the oral cavity, 30 in the oropharynx, of which 18 were assigned as tonsil, 4 in the hypopharynx and 5 in the larynx. The age of the patients ranged from 40 to 77 years, with a mean of 58 years. In total 55 patients were male, 29 were female.

P53 sequencing

Routine haematoxylin-and-eosin staining was performed on 10 μm cryosections to confirm the presence of squamous cell carcinoma in the specimen sampled from the primary tumor and to guide microdissection. Neoplastic areas were microdissected and tumor DNA isolated. A 1.8-kb fragment of the p53 gene, encompassing the exons 5 to 9 was amplified by PCR[15]. Purified PCR products were sequenced directly by exon-specific primers using the radioactive dideoxynucleotide method (AmpliCycle Sequencing Kit, Perkin Elmer, Norwalk, CT, USA)[15], a very reliable sequencing method to detect p53 mutations[14]. For a subset of tumors (20) without a mutation in the exons 5 to 9, the remaining exons 2, 3, 4, 10 and 11 were sequenced in addition. Primer sequences and reaction conditions are available on request. To check the reliability of the sequencing method a plaque assay[15] was performed on 35/44 tumors. In all cases the sequenced mutation corresponded to the results with the plaque assay.

High-risk HPV DNA detection and typing

Detection of high-risk HPV DNA in HNSCC samples from all 84 patients was performed by general primer GP5+/GP6+-mediated PCR enzyme immunoassay (PCR-EIA) essentially as described previously[13]. This method allows the group-specific detection of 14 high-risk HPV genotypes by hybridization of HPV GP5+/6+ PCR products in an EIA format with an oligonucleotide-probe cocktail specific for HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. GP5+/6+ PCR products of positive cases (using a cut-off value of 3x background) were subsequently hybridized with the type-specific probes individually as described elsewhere[16]. The HPV test was only considered positive when both the EIA OD value reached at least 3x background and a fragment was visible after Southern blot hybridisation. All L1 negative tumor DNA's were subsequently tested for HPV16 E7 amplification using type-specific primers, again in an EIA format. High-quality purified DNA from microdissected tumor was used as starting material in all assays. All positive cases were confirmed on crude extracts of cryosections by the

same assays. As a control of the integrity of the target DNA all samples were subjected to a p53 or β-globin gene PCR. To prevent crossing-over contamination, all samples were handled carefully, cut on different blades, and DNA isolations as well as pre-PCR pipetting were performed in laboratories separate from post-PCR processing. In a number of cases negative tissues were cut between positive samples to check for crossing-over contamination in the cryotome. In all experiments a serial dilution of SiHa DNA was added as well as a number of non-template controls to adequately check both the sensitivity and specificity of the assays. In all experiments the controls showed the expected results.

HPV16 E6 RT-PCR

To assess the integrity of the RNA to be used for HPV16 transcript analysis, RNA samples were first pre-screened by RT-PCR specific for the U1 small nuclear ribonucleoprotein specific A protein (snRNP U1A)[17]. All samples showed the presence of snRNP U1A mRNA and were subsequently subjected to RT-PCR specific for the HPV16 E6 region. RT-PCR was performed as described previously[17], except that HPV16 E6-specific primers were used spanning nucleotides 204 to 525 of the HPV16 genome. These primers allow the detection of both full-length E6 transcripts and spliced E6*I mRNA, the latter being the major E7 encoding mRNA species. RNA was preincubated with 0,5 U RQ1 DNase (Promega, Leiden, The Netherlands) in RT reaction mix for 15 minutes at 37°C prior to reverse transcription to remove traces of genomic DNA that might be present. Reactions without RT were included as negative controls during cDNA synthesis. RT-PCR products were run on 1.5% agarose gels and blotted onto nylon membranes (GeneScreen Plus, NEN, Hoofddorp, The Netherlands). Hybridization was performed using an oligonucleotide-probe (nucleotide position 386 to 415) which specifically detects full-length E6 but not E6*I mRNA.

Results

In total 84 HNSCC cases were examined for the presence of HPV DNA by L1 GP5+/6+ general primer-mediated PCR-EIA[13] on purified DNA from microdissected tumor, the most appropriate template. In total 12/84 specimens (14%) were positive by this assay, all typed as HPV16. Subsequently, all positive cases were reanalysed on crude extracts of cryosections. Only 10/12 cases could be confirmed using these crude extracts as template. Because single integration events involving the L1 region might reveal false-negative results with this primer set, all negative cases were re-tested by PCR-EIA amplification of the HPV16 E7 region again using purified DNA as template. Eight additional tumors scored positive for HPV DNA, but none could be confirmed using crude extracts of cryosections as template. Revision of the L1 PCR-EIA data indicated that a number of the E7-positive cases also showed increased L1 values, but not on a level to exceed the threshold for a positive test (3x background[13]). In summary, in 20/84 cases one or more HPV DNA assays were positive, but only 10/20 showed consistent HPV DNA-positivity with all assays and templates used.

Subsequently, all carcinomas that were HPV DNA-positive (20/84) by either of these assays were examined for the presence of HPV16 E6 transcripts. In only 9/20 cases we could demonstrate expression of E6 mRNA (Table 1). These 9 cases were consistently positive with all the DNA PCR assays. All carcinomas showing HPV E6 mRNA expression were located either in the oropharynx (7/9, 4/7 being tonsillar carcinomas) or in the oral cavity (2/9) (Table 2). It has been suggested previously that HPV-positive HNSCC tumors have a basaloid morphology [18]. All 84 cases were revised. After histopathological review, indeed 6/9 HPV E6 positive cases showed a basaloid morphology. In contrast only 1/75 HPV E6 negative cases showed a basaloid morphology, a highly significant observation (P<0,0001, Fisher's exact test).

To further establish the role of the virus in tumor progression we analysed the DNA and RNA of 7 lymph node metastases (LNM) of 6 patients with HPV16 E6 RT-PCR positive carcinomas. In all 6 cases the LNM were shown to contain both HPV16 DNA as well as E6 transcripts. In Figure 1, representative results of HPV DNA and RNA assays are presented for two cases. Case 98-39 was unequivocally positive for HPV DNA by all assays but negative for E6 transcripts, and case 98-8 was unequivocally positive for HPV DNA and positive for E6 mRNA.

Table 1. Results of HPV DNA/RNA assays and p53 sequencing

Cases	HPV-DNA*	24 h L1 EIA value Mean (range)	E6 mRNA	P53 mutation	P-value#
9	+	2.53 (2.50-2.57)	+	0/9	0.0004
1	+	2.14	-	1/1	
10	+/-	0.18 (0.10-0.64)	-	3/10	0.183
64	-	0.11 (0.015-0.44)	-	40/64	

** Used DNA assays:*

1) GP5+/6+ PCR-EIA and subsequent Southern blot analysis of amplimers, both with purified DNA of microdissected tumor samples and crude extracts of cryosections as template

2) HPV16 E7 PCR-EIA and subsequent Southern blot analysis of amplimers both with purified DNA of microdissected tumor samples and crude extracts of cryosections as template

+ = consistently positive in all DNA assays

+/- = positive in one or more DNA assays, but not consistently in all DNA assays

- = negative in all assays with purified DNA as template

 # P-values were calculated by two-sided Fisher's exact test using the mutation frequency in the various groups against the frequency in the HPV DNA-negative group. A P-value < 0.05 was considered as significant.

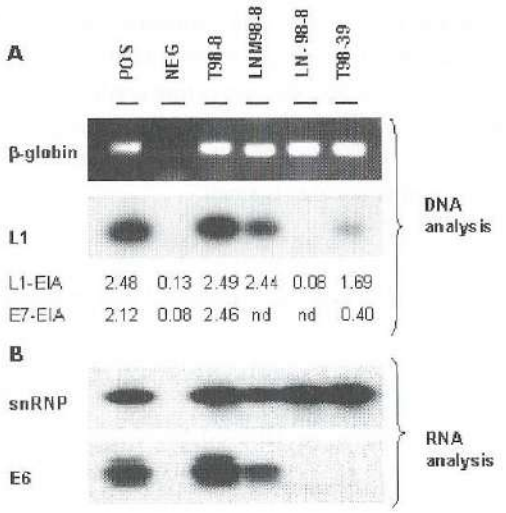

Figure 1. HPV DNA and RNA analysis

*In **panel A** a typical example of the used DNA-assays with crude extracts of cryosections as temp late is shown. In the upper part the gel image showing the beta-globin control of the DNA is depicted, and in the lower part the autoradiogram of the HPV L1 amplimers hybridized to the high-risk HPV probe. The figures below the autoradiogram indicate the E IA values with both the L1 PCR-EIA and the HPV16 E7 PCR-EIA after overnight incubation.*

*In **panel B** a typical example of the E6 RT-PCR data are shown. In the upper part the snRNP U1A amplimers used to check the integrity of the RNA are indicated. In the low er part the autoradiogram of the E6 amplimers hybridized with the HPV16 E6 probe are shown.*

POS = Positive control: nucleic acids isolated from the HPV16 positive SiHa cervical cancer cell line (10 ng DNA in A; 100 ng RNA in B). NEG means reactions without DNA or RNA template. LNM means a tumor-infiltrated and LN a tumor-free lymph node as determined by histopathological examination. T means tumor.

Subsequently all 84 tumor samples were analysed for the presence of p53 mutations. Sequencing of the exons 5 to 9 revealed a mutation in 41 samples (49%). Additional sequencing of the exons 2, 3, 4, 10 and 11 in the tumors without a mutation in exon 5-9 (20 cases) revealed only 3 mutations: one in exon 3 (oral cavity) and two in exon 4 (oropharynx and oral cavity), increasing the total mutation frequency to 52% (44/84) (Table 2). Interestingly, all HPV E6 mRNA-positive cases lacked a p53 mutation, a highly significant observation (two-sided Fisher's exact test P<0.001). Strikingly, 4/11 tumors that were positive for HPV DNA-PCR but negative

for E6 RT-PCR showed a p53 mutation (Table 1), a frequency not significantly different from the HPV DNA-PCR negative group.

Subsequently, tumor and patient data were compared between HPV-E6 positive tumors lacking a p53 mutation and HPV-E6 negative tumors displaying p53 mutations. Parameters analyzed were smoking history, sex, age, differentiation grade of the tumors and survival. We could not demonstrate any correlation between the HPV-status and these variables. Kaplan Meier analysis failed to show any difference in survival between the two groups (P=0.82). It should, however, be noted that the number of cases is small.

Table 2. Distribution of combined HPV status as assessed by E6 RT-PCR and p53 mutation by primary tumor site.

	P53 (+)		*P53 (-)*	
Site	HPV (+)	HPV (-)	HPV (+)	HPV (-)
Oropharynx-non-tonsil (n=12)	0	8	3	1
Oropharynx-tonsil (n=18)	0	7	4	7
Oral cavity (n=45)	0	22	2*	21
Hypopharynx (n=4)	0	4	0	0
Larynx (n=5)	0	3	0	2
Total (n=84)	0	44	9	31

* *The tumors of these two patients were located in the anterior floor of mouth and lateral floor of mouth/gingiva, respectively.*

Discussion

In this study we have clearly demonstrated an absolutely inverse correlation of HPV E6 mRNA expression and mutations in the p53 gene. The presence of p53 mutations in HPV DNA-positive HNSCC tumors has long overshadowed a clear etiological role of the virus in a subset of these tumors, although it cannot be exluded that the virus plays more often a role in the initial phase of carcinogenesis. The percentage of HPV positivity as assessed by E6 mRNA expression is, however, relatively low in comparison to many other studies[18,19]. This discrepancy might be explained by the fact that previous studies mainly considered the presence of HPV by DNA PCR only. The number of tumors containing solely HPV DNA as detected by PCR exceeds that of carcinomas which show also expression of HPV E6 mRNA and are likely to be clonally related to HPV[4]. Importantly, we have also shown that viral DNA and E6 mRNA is maintained in lymph node metastases of the HPV E6 RT-PCR positive cases, providing strong evidence for a key role of the virus in these particular tumors.

Our data suggest that the role of HPV in HNSCC can easily be overestimated when using

DNA assays only (20/84 DNA-positive versus 9/84 E6 mRNA-positive). The sensitivity of the HPV DNA-PCR is often so high that various types of misinterpretations might be the result due to technical artefacts (false-positive findings) or positive findings far below the minimal level of 1 HPV genome copy per tumor cell[20]. We thereby assume that when HPV indeed plays a role in tumor formation and maintenance the presence of virus should at least be 1 copy per tumor cell. This assumption is consistent with the findings of Snijders *et al.* (1997)[4] who showed that only carcinomas which contain relatively high copy numbers of viral DNA express the E6 mRNA. Possibly, quantitative or semi-quantitative PCR assays might already allow the discrimination of E6 mRNA-positive versus E6 mRNA-negative cases. This is supported by the observation that the HPV PCR-EIA OD values in this study read after overnight incubation were markedly higher in the HPV DNA-positive samples with E6 mRNA expression than in those samples without detectable E6 mRNA (Table 1).

In the recent study of Gillison *et al.* (2000)[18] evidence was presented for a causal association between HPV infection and HNSCC. Although the technical procedures appeared to be very consistent and solid, their data again did not establish a clear biological link between HPV and HNSCC. Various HPV-positive tumors appeared to have mutations in the p53 gene, and the overall mutation frequencies were not significantly different from the HPV-negative group. What is more, it was shown that in 43% of the cases the presence of HPV DNA as assessed by PCR could not be confirmed by Southern blotting, an alternative and less sensitive technique. Possibly, these authors also detected a number of putatively "biologically irrelevant" cases due the sensitivity of their DNA-PCR assays. The expression of the E6 oncogene, which confirms the etiological role of the virus in these tumors, might well be used to check for possibly confounding findings of HPV DNA-PCR assays. Still, we cannot exclude that in some E6 mRNA-negative cases the virus played a role in the initial phase of tumorigenesis according to a "hit and run" principle. This may imply that HPV-mediated degradation of p53 is substituted by mutational events during malignant progression. As an example, in one case we could confirm HPV DNA presence with all different assays on all material, but we failed to demonstrate E6 mRNA expression. This particular case also showed a p53 mutation. Since this patient did not show lymph node metastases we were not able to investigate the putative association in more detail. Besides this case there were a number of other E6 mRNA-negative cases which were HPV DNA-positive. However, in these cases the various DNA analyses were not unequivocally positive, which might have resulted from the sensitivity of particular primer sets and PCR strategies. Transient infection far below the level of one copy per cell might explain these observations.

The implications of our findings are that the large expectations with respect to vaccination programs for the treatment of HPV-induced head and neck cancers as suggested by Mc Neil *et al.* (2000)[21] should be interpreted with caution. Particularly in HPV DNA-positive cases where E6 expression is absent, the etiological role of the virus is questionable and the rationale for immunotherapy in our view is lacking, in these cases vaccination might still play a prophylactic role. For immunotherapy trials it seems a more appropriate approach to select patients by E6 RT-PCR or semiquantitative DNA PCR assays at a level of at least one copy per cell. In those cases

viral factors are apparently indispensable for the malignant state, and vaccination strategies might be successful.

Our study provides the "missing" biological link, which clearly demonstrates that high-risk HPVs are indeed responsible for a number of squamous carcinomas in the head and neck. There is some epidemiological evidence that this viral etiology could be related to sexual behavior[22,23]. Further research in these patient groups, combined with molecular data, should demonstrate whether specific sexual contacts with HPV-positive partners might cause the disease, possibly in concert with other risk factors such as smoking or drinking.

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5

Mutated p53 as molecular marker for the diagnosis of head and neck cancer

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Abstract

Background In total 10 to 30% of patients with head and neck squamous cell carcinoma (HNSCC) develop local recurrences despite seemingly adequate tumor resection. This may result from minimal residual cancer (MRC): small numbers of tumor cells left behind in the surgical margins, undetectable by routine histopathology. In recent studies p53 mutations have been considered as selective and sensitive DNA markers of cancer cells. There are two potential problems using mutated p53 DNA as marker: p53 mutations occur early in progression and might therefore detect unresected precursor lesions besides tumor cells. Moreover, DNA is a very stable biomolecule that might lead to false-positive results. These two potential problems have been evaluated in this study. **Methods** In total 50 patients with a radical tumor resection were included of whom 30 showed a p53 mutation in the primary tumor. Histopathologically tumor-free surgical margins were quantitatively analyzed for mutated p53 by molecular diagnosis (plaque assay) and subsequent (immuno)histopathology. **Results** P53 mutated DNA was detected in surgical margins of 19/30 patients. Immunohistochemistry confirmed the presence of small tumor foci in 2/19 mutated p53-positive cases. In 7/19 cases the tumor-specific p53 mutation was found in unresected dysplastic mucosal precursor lesions. Moreover, in a number of cases small p53-immunostained patches were detected, but the mutations found were never tumor-related. By screening contralateral exfoliated cells and plaque assays on RNA we could show that detection of mutated p53 DNA is prone to false-positive results. **Conclusion** Using p53 mutations as marker, both MRC and unresected mutated p53-positive mucosal precursor lesions are detected within surgical margins. Molecular assessment of surgical margins using p53 mutations enables selection of HNSCC patients at high risk for tumor recurrence, but tumor RNA seems at present a more specific biomolecule for analysis than tumor DNA.

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cancer in the world [1] and despite significant improvements in locoregional control by surgery and radiotherapy over the last decades, the 5-year survival rates of HNSCC patients only moderately improved. Even when the surgical margins have been diagnosed as tumor-free by histopathology, the local recurrence rate is still 10 to 30%.[2] This relatively high local recurrence rate can be explained in part by assuming that residual tumor cells in the surgical margins remain undetected by the current diagnostic methods (*minimal residual cancer*, MRC).[3] To improve the detection of residual tumor cells in HNSCC patients, molecular markers can be exploited that are sensitive enough to detect small numbers of tumor cells. Particularly detection of squamous cancer cells in the resection margins is hampered by the presence of normal epithelial cells, which prohibits the use of squamous differentiation markers, and necessitates exploitation of tumor-specific markers.

To date it is widely accepted that cancer arises as a result of the accumulation of (epi)genetic alterations in oncogenes and tumor suppressor genes.[4] The identification of a number of these genetic alterations, for example mutations in the p53 tumor suppressor gene, paved the way for their use as molecular markers. Mutations in the p53 gene frequently occur in many cancers and are present in 50-60% of head and neck cancers. It has been demonstrated that p53 mutations are reliable clonal markers as the mutations in lymph node metastases are always concordant with the mutations in the primary tumors.[5] Recently, the potential of molecular diagnosis using mutations in the p53 gene as tumor-specific marker has been demonstrated for the detection of occult cells clonally related to the tumor in surgical margins.[6,7] However, in these studies the source (cell type) of mutated p53 in the margins, which might be of clinical significance, was not further explored. It had been shown earlier that tumor-adjacent (hyperplastic or dysplastic) epithelium, sometimes demonstrates the same mutation as the primary tumor.[8] What is more, there are some indirect indications that mutated p53 when assayed on the DNA level might give rise to unwanted positive results. DNA is a very stable biomolecule and it has been shown that it leaks from tumors and can be used as a marker in serum.[9,10] The tumor DNA might therefore float in the saliva and lymph and cause unwanted positive results in margins.

In this study, we have evaluated the use of mutated p53 for the detection of minimal residual cancer in surgical margins of head and neck cancer patients. To determine the pathobiological source that gives rise to the mutated p53 signal in positive margins, we extensively screened the margins by histopathology and additional immunohistochemistry. We further showed by a number of different approaches that assays using tumor DNA as marker might reveal false-positive results. Our study clearly demonstrates the potential and limitations of mutated p53 as molecular marker for MRC detection.

Materials and Methods

Patients and tissue specimens

The study presented was approved by the Institutional Review Board of the *Vrije Universiteit* Medical Center and written informed consent was obtained from all patients. In total 50 HNSCC patients with a primary tumor in the oral cavity, oropharynx, hypopharynx or larynx who were scheduled for surgical treatment during August 1997 and October 1999 fulfilled all inclusion criteria including tumor-free resection margins as assessed by conventional histopathological examination. Patient and tumor characteristics are listed in Table 1A and 1B, whereas the study design is presented in Figure 1. At the time of surgery, 4-5 additional paired margin samples were taken from the edges of the surgical defect after excision of the tumor (3 or 4 superficial mucosal margins designated M1-M3/M4 and 1 deep connective/muscle tissue margin designated M5). One additional margin sample served as histopathological control and the paired sample was used for molecular analysis. In addition, exfoliated cells were taken from the contralateral cheek to check for p53-mutated DNA bound to the mucosa. To prevent tumor cell contamination of the samples during surgery, several precautions were taken. The operating field was rinsed extensively with Dakin's solution (0.5% sodiumhypochlorite pH 11.5 and 0.2% sodiumcarbonate) and all instruments and gloves were changed before sampling of the margins. From the resection specimen a fresh primary tumor sample was obtained immediately after surgery. The tumor sample and one of the paired additional margin samples (M1-M5) were directly snap-frozen into liquid nitrogen and stored at -80°C until further processing. The surgical specimen was formalin-fixed and routinely processed.

P53 sequencing

Routine haematoxylin-and-eosin staining was performed on 10 µm cryosections to confirm the presence of squamous cell carcinoma in the specimen sampled from the primary tumor and to guide microdissection. Neoplastic areas were microdissected and tumor RNA and DNA isolated. An 1.8-kb fragment of the p53 gene, encompassing the exons 5 to 9 was amplified from the DNA as described by Sidransky et al. (1991)[11] Purified PCR products were sequenced directly by exon-specific primers using the radioactive dideoxynucleotide method (AmpliCycle Sequencing Kit, Perkin Elmer, Norwalk, CT, USA).[11] The DNA of the 20 tumors that did not show a mutation within exons 5 to 9 were also sequenced for mutations in exons 2, 3, 4, 10 and 11. Primer sequences and reaction conditions are available on request.

P53 plaque hybridization assay on DNA from additional margin samples

Thirty patients with a p53 mutation in the DNA of the primary tumor were included in the subsequent (plaque assay) analyses. The technique used was essentially as described by Sidransky et al. (1991)[11] with a few minor modifications. In short, on the basis of the p53 gene sequence of the tumor DNA, a mutant-specific and corresponding wild type-specific

oligonucleotide were selected for each patient. DNA was isolated from the frozen part of the additional margin samples and the exon harboring the tumor-specific p53 mutation amplified by PCR, cloned into lambda GT11 vector arms and packaged *in vitro*. The packaged phages were infected onto host bacteria *Escherichia coli* K12 LE392 at different dilutions for wild type or mutant oligonucleotide hybridization. After overnight incubation at 37°C the plaques were transferred to nitrocellulose membranes. At least 1,000 plaques (containing insert) were analyzed for each margin by differential hybridization with tumor-specific and wild type-specific oligonucleotides as probes, end-labeled with [γ-^{32}P]ATP. Proper positive (primary tumor DNA) and negative (wild type DNA) controls were included. Finally, the number of plaques (containing insert) hybridizing with the mutant-specific oligonucleotide divided by the number of plaques hybridizing with the wild type-specific oligonucleotide was taken as a measure of the tumor cell DNA load in the original sample. When the number of hybridizing plaques was low (between 1 to 5), their identity was confirmed by classical rescreening: a positive plaque was stabbed from the agar, allowed to re-infect host bacteria, replated and rehybridized with the mutant-specific probe.[12]

Plaque assays on RNA were performed as described for DNA, except for the following steps. About 1 µg of RNA, isolated from surgical margins and tumor, was heated at 65°C for 10 min and cooled on ice. cDNA synthesis was performed with 25 pmol antisense primer, 2 U Superscript Reverse Transcriptase (Gibco Life Technologies, Breda, the Netherlands) and 2 U RNAsin (Promega, Leiden, The Netherlands) for 2 hours at 42°C in a buffer containing 60 mM KCl, 3 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.3). Then the cDNA fragment of interest (exons 5-6 or exons 7-9) was amplified as follows. 1 U Ampli Taq DNA polymerase (Perkin Elmer, Norwalk, CT), 25 pmol of sense and antisense primer and 5 µl of cDNA was added to a buffer containing 44 mM KCl, 1.2 mM $MgCl_2$ and 5 mM Tris-HCl (pH 8.3), in a total volume of 50 µl. The reaction mixture was subjected to a thermal cycling procedure of 5 minutes at 95°C, 40 amplification cycles (1 minute at 95°C, 1 minute at 60°C and 2 minutes at 72°C), and 5 minutes at 72°C. Primer sequences are available on request.

Histopathological review of surgical margins from the resection specimen

All paraffin embedded margins, taken from the resection specimen used for conventional histopathological examination as well as the paired additional margin samples were independently reviewed by two pathologists, as to whether residual tumor could be detected. There was no prior knowledge on the molecular data. Other histopathological features examined were adequacy of resection and presence of dysplasia as well as perineural and/or invasive growth. The adequacy of resection was determined by the closest distance of the tumor to the inked deep resection margins of the specimen. The cases were divided into 2 groups on the basis of this measurement: 1-5 mm (close margin) and ≥ 5 mm (adequate margin). Dysplasia was scored according to the standard criteria of the World Health Organization international classification of tumors.[13] 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. After independent classification of the margins by two pathologists (JAK, IvdW) the discordant cases were discussed to reach consensus. If no

consensus was reached, the highest grading of one of the pathologists was taken arbitrarily as the final grading.

Immunohistochemistry with anti-p53 and anti-cytokeratin antibodies

Immunohistochemical staining (IHC) was performed on the paraffin-embedded margins taken from the surgical specimen and used for the original histopathological evaluation to screen for residual cancer cells in the surgical margins. For patients with molecular-positive margins and p53-overexpressing tumors, sections were stained with monoclonal anti-p53 antibody DO7 (DAKO, Glostrup, Denmark). The method used for p53 staining was essentially as described by Cruz et al. (1998)[14] Briefly, 5 µm sections were deparaffinized, placed in 0.3% hydrogenperoxide in methanol (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) was followed by overnight incubation at 4°C with anti-p53 monoclonal antibody DO7 (1:500) (DAKO). Consecutive sections were incubated with irrelevant mouse myeloma IgG monoclonal antibody (1:500) (Zymed, San Fransisco, USA). Following incubation, slides were thoroughly washed and sections sequentially incubated with biotinylated rabbit anti-mouse antibody (1:500) (DAKO) for 30 min and streptavidin horseradish peroxidase immune complex (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. When the primary tumor did not show p53 overexpression, anti-CK staining was performed with rabbit anti-cytokeratin (1:3,000) (DAKO) to detect residual cancer cells in subepithelial tissues. The IHC method used in combination with this antibody is essentially as described by Pinkus et al. (1985)[15] using proteolytic digestion with trypsin reagent (Type II from porcine pancreas, 0.5 mg/ml); (Sigma, Zwijndrecht, The Netherlands) for 20 min at 37° C prior to staining to enhance signal in paraffin-embedded sections. A commercial preparation of anti-rabbit immunoperoxidase polymer (Histofine Simple Stain MAX PO, ITK, Uithoorn, The Netherlands) was used in combination with the rabbit anti-keratin antibody. Consecutive sections were incubated with normal rabbit serum (1:3,000) as a control (DAKO).

Immunohistological analysis and characterization of tumor-related precursor lesions in the resection margins

IHC-stained and parallel H&E margin sections were carefully reviewed by a pathologist and classified as minimal residual cancer (in subepithelial tissues) or p53-mutated precursor lesion (p53-immunostained epithelium: "field").

To confirm the tumor-related identity of the p53 mutation in the immunostained epithelial precursor lesions ("fields"), areas with p53 nuclear staining were microdissected from parallel sections and analyzed by plaque assay or sequencing. In all cases an (adjacent) non-stained area and the underlying submucosa were analyzed as negative control.

In four cases the primary tumor did not show overexpression of mutated p53 (nonsense mutations/frame shifts). To check whether in these four cases tumor-related p53-positive epithelial lesions were present, dysplastic areas were microdissected and analyzed for p53 mutations by sequencing or plaque assay. It had been noted that p53 immuno-positive mucosal lesions were always dysplastic, ranging from mild to severe, and therefore dysplasia was used as a surrogate indicator of a putative epithelial precursor lesion.

Based on the molecular and immunohistopathological results the cases with molecular-positive additional margin samples were classified as follows:

1) microscopically identified minimal residual cancer
2) proven tumor-related p53-mutated epithelial precursor lesion ("field")
3) unidentified minimal residual cancer (the presence of a tumor-related p53-mutated epithelial lesions in any of the resection margins was excluded)

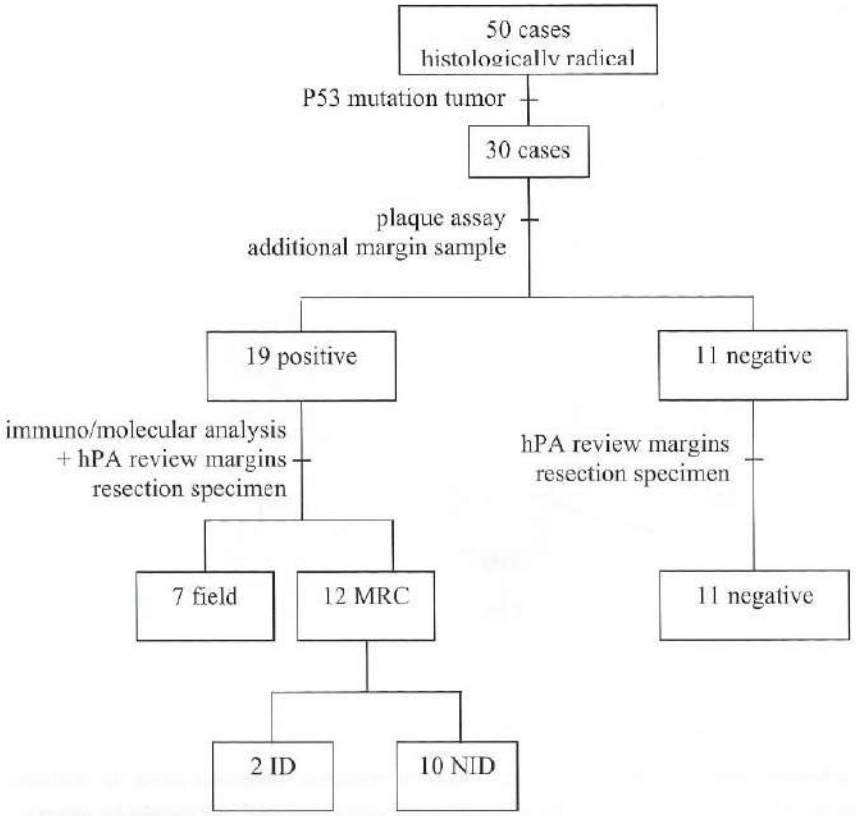

Figure 1. A schematic representation of the study design. *hPA review means histopathological review. ID means lesion microscopically id entified after immunostaining.*

Statistical analysis

Pearson Chi-square tests were performed on variables that could be correlated to the p53 status of the margins (adequacy of resection, dysplasia, perineural, and invasive growth). The influence of the degree of dysplasia was further tested with the linear-by-linear association test (StatXact). P-values below 0.05 were considered as significant.

Results

A. Molecular analysis of additional margin samples with DNA as template

The 50 tumor DNAs were analyzed for the presence of p53 mutations by direct sequencing. In total 30/50 patients (60%) showed a p53 mutation in the primary tumor (Table 2). The additional margin samples of these patients were analyzed using the p53 plaque hybridization assay. In total, 19/30 patients showed p53 mutated DNA in one or more additional margin samples. The percentage of mutated DNA ranged from 0.01 % to 15%. A representative patient example is shown in Figure 2. Characteristics of these 19 cases and details of the molecular analyses are provided in Table 1A, whereas the 11 patients with surgical margins negative for mutated p53 are described in Table 1B.

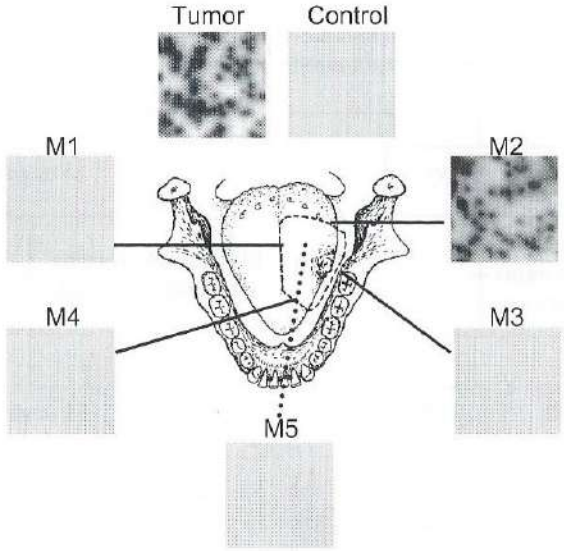

Figure 2. A schematic representation of case #25 staged by molecular diagnosis using the mutated p53 plaque assay. *This patient presented with a T1N0 tongue carcinoma and was treated by surgery. The patient had a C→G mutation in codon 267 of the p53 gene, leading to an arginine to glycine amino acid substitution. DNA from margins was analyzed as described. Note the hybridizin g plaques in the tumor as well as margin M2, indicative for DNA of cells clonally related to the tumor. After 11 months, this patient developed a local recurrence.*

Table 1A. Characteristics of the HNSCC patients with surgical margins positive for mutated p53

Case #	PT site[a]	TN	Postop RT	Pos surg margins muc/deep	% of mutant clones[b]	IHC[c]	Dysplasia[d]	Local relapse	F-up (m)
2	OP	T3N0	Y	2 m	0.03	MRC-NID♦	Mild	No	22
3	OP	T2N2b	Y	3 m	0.05	MRC-NID♦	Mild	No	35
4	OC	T2N0	N	1 m	0.01	*P53+ field*	Sev	No	17
5	OP	T3N1	Y	1 d	0.01	MRC-NID♦	Mild	**Yes**	21
7	OC	T1N0	N	1 m	4.4	*P53+ field*	Mod	No	12
10	OP	T3N2a	Y	3 m	0.05	MRC-NID♦	Mild	No	34
11	L	T3N2c	Y	1 m	0.03	**MRC-ID**	Mod	No	7
12	HP	T4N2b	Y	2 m	0.02	MRC-NID♦	Mod	No	14
17	OC	T4N2b	Y	5 m	0.08	MRC-NID	No	No	32
*19**	OP	T1N0	Y	5 m	3.6	*P53+ field*	Sev-CIS	No	32
*20**	OC	T1N0	N	5 m	0.08	*P53+ field*	Mod	No	24
21	OC	T3N2c	Y	5 m	0.28	MRC-NID	No	No	31
22	OC	T1N0	N	5 m	0.04	MRC-NID♦	Mod	No	30
24	OP	T2N0	N	1 d	2	**MRC-ID**	Mod	**Yes**	15
25	OC	T1N0	N	1 m	7	*P53+ field*	Mild	**Yes**	11
26	L	T4N2c	Y	3 m	15	*P53+ field*	Sev	No	17
27*	OC	T2N2b	Y	1 m	0.35	MRC-NID	No	**Yes**	6
28	OC	T2N0	N	2 m	0.02	*P53+ field*	Mod	No	20
29*	OP	T2N0	Y	1 m	0.5	MRC-NID	Mild	No	19

[a] *localization primary tumor: OP, oropharynx; OC, oral cavity; L, larynx, HP, hypopharynx*

[b] *if more than one margin was positive, the highest percentage measured for a margin is shown, m=mucosal margin, d=deep margin*

[c] *immunohistochemical analysis of mutated p53 -positive surgical margins. Cases indicated by * were immunostained by anti-cytokeratins. All other cases showed p53 overexpression.*

p53+field = mucosal field with tumor-specific p53 mutation; MRC-NID = microscopically unidentified minimal residual cancer. MRC-NID♦ =unidentified minimal residual cancer but presence of (multiple) patches without the tumor-specific p53 mutation. MRC-ID = microscopically identified minimal residual cancer

In cases #4, #7, #25, #26, and #28 fields were confirmed by IHC and subsequent molecular analysis of the microdissected epithelial layer. In cases #19 and #20 the field was confirmed by molecular analysis of the microdissected dysplastic epithelial layer only.

[d] *In cases with MRC or tumor, the highest histological grade in either of the margins is shown, whereas in cases with p53+ field the grade of dysplasia of the particular field margin(s) is shown, which was in all cases also the highest histological grade.*

Table 1B. Characteristics of the HNSCC patients with surgical margins negative for mutated p53

Case#	PT site[a]	TN	Dysplasia[b]	Local relapse	Follow-up (months)
1	HP	T2N1	Mild	No	38
6	OC	T3N2b	No	No	17
8	HP	T4N2b	Mod	No	9
9	OC	T2N0	No	No	35
13	L	T3N0	Mild	No	34
14	OC	T3N2b	Sev	No	24
15	OC	T3N2b	No	No	5
16	OC	T2N1	No	No	34
18	OC	T4N0	No	No	28
23	OC	T2N0	Mild	No	19
30	OC	T1N0	No	No	9

[a] *Localization primary tumor: OP, oropharynx; OC, oral cavity; L, larynx, HP, hypopharynx*
[b] *The highest histological grade in either of the margins is shown*

Table 2. Characteristics of p53 mutations

Case	Exon	Mutation	Codon	A-acid change	Immunohistochemistry Margin(s)	Local recurrence
1	6	1-bp ins	191	Frameshift		
2*	4	T→A	111	Leu→Gln	Patches	
3*	6	G→A	216	Val→Met	Patches	
4*	5	G→A	141	Ser→Stop	Field, mutation identical	+: ND
5*	8	G→T	272	Val→Leu	Patches	
6	5	T→A	163	Tyr→Asn		
7*	7	C→T	248	Arg→Trp	Field, mutation identical	
8	8	G→T	298	Glu→Stop		
9	6	A→G	205	Tyr→Cys		
10*	8	G→A	273	Arg→His	Patches	
11*	7	G→T	248	Arg→Leu	Tumor	
12*	7	6-bp ins	235	+AsnTyr	Patches, other mutations	
13	6	T→G	194	Leu→Arg		
14	6	C→G	193	His→Asp		
15	5	8-bp del	126	Frameshift		
16	9	94-bp del	307-321	Frameshift		
17*	5	T→C	127	Ser→Pro	NID	
18	3	2-bp del	29	Frameshift		
19*	9	C→T	317	Gln→Stop	Field, mutation identical	
20*	5	1-bp ins	151	Frameshift	Field, mutation identical	
21*	7	G→A	258	Glu→Lys	NID	
22*	8	G→A	273	Arg→His	Patches	
23	4	19-bp del + T→C	115-121	Frameshift		
24*	6	1-bp del	215	Frameshift	Tumor	+: ND
25*	8	C→G	267	Arg→Gly	Field, mutation identical	+: mutation identical
26*	7	G→A	244	Gly→Asp	Field, mutation identical	
27*	6	1-bp ins	220-221	Frameshift	NID	+: mutation identical
28*	7	G→A	238	Cys→Tyr	Field, mutation identical	
29*	8	G→T	298	Glu→Stop	NID	
30	8	C→G	283	Arg→Gly		

*Cases indicated with * had one or more p53 positive margin(s) in the plaque assay. NID means no field or tumor identified. ND means not determined. A-acid means amino acid. Mutation identical means p53 mutation in the lesion was identical to the mutation in the primary tumor.*

B. Morphological examination of surgical margins

Histopathological review

Histopathological review showed a small tumor clone in the deep additional margin sample (M5) of one of 19 molecular-positive cases (case #24, Table 1A). The margins of the resection specimens or the paired additional margin samples of the other cases remained tumor-free after review. Dysplasia was scored by two independent pathologists (JAK, IvdW). The inter-observer variability for the scoring of dysplasia was 30% before and 20% after consensus. Different gradings were mainly scored in the categories normal/mild dysplasia. Data on dysplasia versus the p53 status are shown in Table 1A and 1B, and summarized in Table 3A. In 16/19 (84%) patients with p53-positive margins at least one of the margins showed dysplasia whereas in only 5/11 (45%) patients with p53-negative margins dysplasia was seen in at least one margin. This difference was statistically significant (P=0.042). Furthermore, the relationship between the adequacy of tumor resection and the p53 status was examined. Among the p53-positive cases, 4/19 (21%) had close resection margins (1-5 mm), whereas among the p53-negative cases 3/11 (27%) had close margins. This difference was not statistically significant. Data are shown in Table 3B. Finally also the characteristics of perineural and invasive growth were compared to the p53 status. These tumor characteristics were equally distributed among both groups as shown in Table 3C.

Table 3. Results of the histological grading of surgical margins compared to p53 status.

3A. Dysplasia.

	Histological classification of dysplasia				
P53 status	no	Mild	Moderate	severe/CIS	total
Positive	3	8	5	3	19
Negative	6	3	1	1	11
Total	9	11	6	4	30

CIS=Carcinoma in Situ. For histological classification see Material & Methods. For each patient the highest grade of dysplasia in the mucosal epithelium of one or more margins is taken. When mild and moderate dysplasia are combined, then P=0.081 (linear-by-linear association). When dysplasia (in total) is compared to no dysplasia, P=0.042.

3B. Adequacy of tumor resection

	Tumor-free margin		
P53 status	close (<5 mm)	adequate (≥5 mm)	Total
Positive	4	15	19
Negative	3	8	11
Total	7	23	30

The adequacy of tumor resection is measured by the closest distance of tumor to the i nked deep resection margin. Pearson Chi-square test, two-sided P=1.000 (exact)

3C. Tumor characteristics

	Perineural growth		
P53 status	Yes	No	Total
Positive	6	13	19
Negative	3	8	11
Total	9	21	30

Pearson Chi-square test, two-sided P=1.000 (exact)

	Invasive growth		
P53 status	Yes	no	Total
Positive	6	13	19
Negative	5	6	11
Total	11	19	30

Pearson Chi-square test, two-sided P=0.696 (exact)

Immunohistochemistry for residual subepithelial cancer

All margins of the resection specimens obtained from the 18 molecular-positive cases that were tumor-free after review were subsequently analyzed by additional IHC. Staining with anti-p53 monoclonal antibody DO7 on paraffin sections of the matched primary tumors showed p53 overexpression in 14/18 cases. This was in concordance with the type of p53 mutation: the tumors with missense mutations gave rise to p53 overexpression whereas the 4 tumors with nonsense/frame-shift mutations did not. The surgical margins of the resection specimen of these 14 cases with p53-overexpression were analyzed with anti-p53 DO7. In one patient (case #11, Table 1A) minimal residual tumor was identified with anti-p53 IHC that could be confirmed by anti-cytokeratin IHC (Figure 3). This tumor cell cluster was not reported in both the first histopathological assessment as well as in the histological review of both pathologists. Subsequent staining with antibody CD31 showed that the tumor cell cluster was located in a vessel. In none of the other patients residual tumor was observed in the surgical margins.

The surgical margins of the resection specimen of the 4 cases that did not show p53 overexpression in the primary tumor (#19, 20, 27 and 29, indicated with an asterisk in Table 1A) were analyzed with anti-cytokeratin (anti-CK) antibodies. None of these cases showed subepithelial residual tumor.

Characterization of p53 stained epithelial lesions in the margins of the resection specimen

In 5 patients with p53-overexpressing tumors one or more of the margins of the resection specimen showed large contiguous p53-positive mucosal precursor lesions (5-20 mm) ("fields"). All these large "fields" were graded as dysplastic ranging from mild to severe. A representative example of such a p53-positive mucosal field is shown in Figure 4A. In all 5 cases the microdissected p53-positive lesion showed the same mutation as the tumor. The plaque assay analysis of case #25 is shown in Figure 4A as a representative example.

In the four cases that lacked p53 overexpression in the tumor (cases #19, 20, 27, and 28, indicated with an asterisk in Table 1A) the presence or absence of a p53-positive mucosal field could not be established by p53 immunostaining. As we had noticed that all tumor-related p53-immunostained epithelial lesions were graded as dysplastic, we decided to use dysplasia as a surrogate indicator for field in cases that the tumor did not show p53 overexpression. In cases #19, 20 and 29 dysplasia was present in one or more of the resection margins, and in those cases the dysplastic areas were microdissected and screened for p53 mutations by plaque assay or sequencing. In cases #19 and #20 a tumor-related p53 mutation-positive mucosal field could be demonstrated. Case #29 appeared to be p53 wild type.

Remarkably, in 6 cases no large contiguous positive p53 fields were seen in the margins of the resection specimen but small and dispersed p53 IHC positive mucosal "patches". These were never larger than 200 cells in diameter. The brown nuclear staining of p53-positive cells appeared quite specific in all cases. An example of a p53-positive patch is shown in Figure 4B. Analysis of microdissected small p53-positive patches never showed the presence of the tumor-specific mutation, but other p53 mutations were seen instead. Consequently, the p53 signal obtained in the initial plaque assay can not be derived from these p53-positive patches

and their source remains inconclusive. Sequencing analysis of a microdissected patch of case #12 is shown in Figure 4B. All findings are summarized in Figure 1.

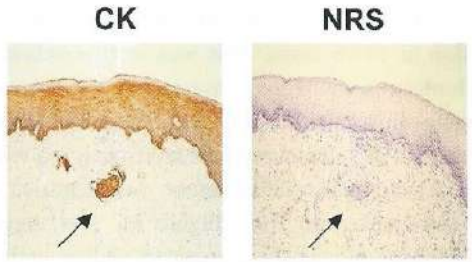

Figure 3. Immunohistochemical staining of p53 in primary tumor and surgical margin of case#11 using anti-cytokeratins. *In histopathologically "tumor-free" surgical margin H, immunostained with anti-cytokeratins, a small tumor embolus is seen. The tumor embolus was m issed by routine histopathological examination and review. Only after immunoguidance the embolus was recognized and classified as carcinoma. Right: same margin stained with normal rabbit serum .*

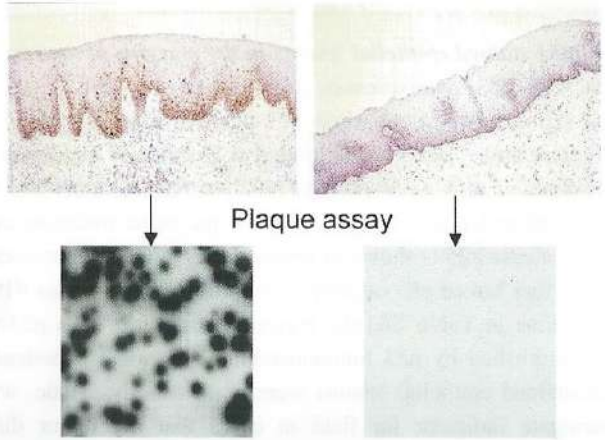

Figure 4A. Representative example of p53 immunohistochemical staining, microdissection and plaque assay of p53-positive fields in surgical margins of case #25.
Upper left panel: histopathologically tumor-free surgical margin A, with moderate dysplasia, anti-p53 DO7 staining. Note the extensive suprabasal staining. Lower left panel: p53 plaque hybridization of the microdissected positive mucosa of the same margin. The number of mutant clones in the p53 IHC-positive area was 80%. Upper right panel: histopathologically tumor-free surgical margin D of the same patient, with mild dysplasia, anti-p53 DO7 staining. Lower right panel: p53 plaque hybridization of the mucosa of the same margin. No mutant clones were observed.

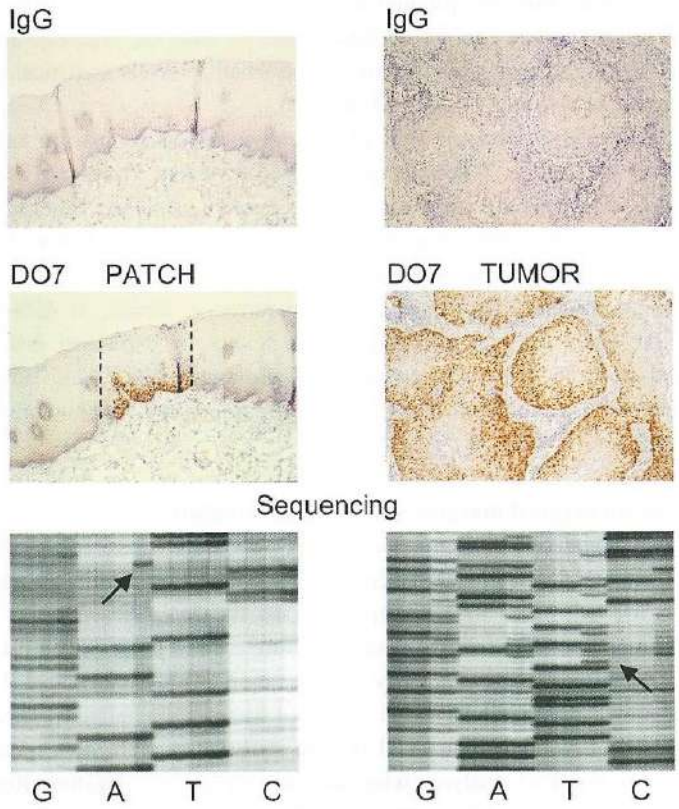

Figure 4B. Representative example of p53 immunohistochemical staining, microdissection and sequencing of p53-positive patches in surgical margins of case #12
Upper left panel: histopathologically tumor-free surgical margin with mild dysplasia, irrelevant IgG antibody. Middle left panel: same margin, anti-p53 DO7 staining. Note the patch with suprabasal staining. Lower left panel: autoradiograph of p53 sequencing of the microdissected positive patch of the same margin. Sequencing was performed as described in Material and Methods. The lanes represent G, A, T and C of exon 7. Four samples are run in parallel, where each fourth lane corresponds to the DNA of the patch. A point mutation consisting of a G to A base substitution at codon 237, leading to a amino acid change of methionine to isoleucine, is evident. On the right panels the staining and sequence analysis of the corresponding tumor is shown. Upper right panel: SCC of patient #12, irrelevant IgG staining. Middle right panel: SCC of patient #12, anti-p53 DO7 staining. Lower right panel: p53 sequencing of exon 7 of the microdissected tumor. Three samples are run in parallel, each third lane corresponds to the DNA of the tumor. The other two samples were wild type. The tumor showed a 6-nucleotide insertion in codon 235. Note that the mutation in the DNA of the patch is not similar to the mutation in the DNA of the primary tumor.

C. Correlation of molecular/morphological analyses and recurrence at the primary site

The risk for local relapse might be determined by both the presence and the type of residual lesion. Although the number of cases is too small to detect statistical significance, a few observations are noteworthy (Tables 1A and 1B).

First, none of the patients with mutated p53-negative additional margin samples (11/30) developed a local recurrence (follow-up period 5-38 months). From the 19 patients with mutated p53-positive additional margin samples 11 could be explained either histologically or clinically: 7 cases showed a mutated p53-positive mucosal precursor lesion, 2 cases showed microscopically identified minimal residual cancer, and two cases of the group that could not be identified microscopically developed a local recurrence. Hence, in 42% (8/19) of the molecular-positive cases the margin status could neither be explained histologically nor clinically. Based on these data it was considered that plaque assays using (amplified) tumor DNA might give rise to false-positive results either by tumor cell contamination or by tumor DNA contamination

D. Plaque assay on surgical margins with RNA as template

To exclude tumor cell contamination RNA was used as template for the plaque hybridization assay. RNA of surgical margins of four patients was available and analysed by plaque assay. Case #26 was molecular-positive in the DNA-based assay and showed morphologically a p53-positive field, and was therefore used as positive control. Case #6 was molecular-negative in the DNA-based assay, and was used as negative control. Two cases that were molecular-positive in the DNA-based assay but were immunohistochemically classified as unidentified MRC (case #3 and case #10) were analyzed as well. As expected, case #26 remained positive in the RNA analysis, whereas case #6 remained negative. Remarkably, the cases #3 and #10 that were positive in the tumor DNA-based plaque assay were negative in the RNA analysis (data not shown).

E. Analysis of exfoliated cells from the contralateral site

False-positive results might also occur when DNA leaks from (necrotic) tumors via the saliva and contaminates the mucosa. To examine this hypothesis contralateral exfoliated cells of 14/19 margin molecular-positive patients were tested by a DNA plaque assay. Indeed, 8/14 patients showed p53-mutated DNA in the contralateral exfoliated cells (data not shown).

Discussion

In this study we evaluated mutated p53 as molecular marker by a systematic molecular and morphological analysis of the surgical margins of head and neck cancer patients. Patients with cells clonally related to the tumor in the surgical margins could be classified in two different groups: 7 cases with mutated p53-positive mucosal fields and 12 cases with putative minimal residual cancer, 2 of which could be identified microscopically.

In 7/19 cases a positive plaque assay could be explained by the presence of a tumor-related p53-mutated epithelial field in a margin. A high percentage of mutant clones (>3%) in the plaque assay seems to be an accurate indicator for the presence of a p53-mutated field, although the percentage of epithelium in the mucosa sample is obviously of influence. These mutated p53-positive mucosal fields are most likely contiguous with the tumor and usually extend over thousands of cells in the basal- and suprabasal layers. In fact these might be the precursor lesion from which the tumor arose. Recently, Tabor et al. (2001)[16] performed a comprehensive analysis to determine the presence, persistence and extension of these fields, using 3p, 9p and 17p LOH as marker. In total 25% of the HNSCC patients appear to have a tumor-related genetically altered field surrounding the tumor that extends into the surgical margins, as determined by LOH analysis. The authors provided evidence that in these fields new cancers develop that are clinically assigned either as local recurrences or as second primary tumors. We detected similar fields using mutated p53 as molecular marker (in approximately 40% of the molecular-positive patients). This observation clearly shows that mutated p53 DNA in surgical margins is not derived from residual tumor cells only but can also be explained from non-invasive epithelial precursor lesions. In one case of seven a tumor arose in the field that was clinically assigned as "local recurrence". This patient showed mild dysplasia in the mutated p53-positive margin. One explanation is that a second tumor developed in the unresected epithelial precursor lesion. The relative low incidence of new tumors in these fields might be due to the limited follow-up period. It was indicated earlier by Mao et al. (1996)[17], Rosin et al. (2000)[18] and Partridge et al. (2000)[19] that the progression of precursor lesions to invasive cancer may take 5 to 10 years. Obviously, it needs to be considered that besides the field, this patient also might have had residual tumor in the margin that could explain the local recurrence.

In some patients, we observed small and dispersed p53-positive mucosal patches (<200 cells) instead of large contiguous fields. Also in patients with wild type p53 tumors, these patches were sometimes observed in the margins. It was already shown by Waridel et al. (1997)[20] that p53 mutations are present in mucosal biopsies of HNSCC and particularly frequent in patients with multiple primary head and neck tumors. Also Park et al. (1999)[21] showed by LOH analysis in the normal bronchial epithelium of lung cancer patients small patches with clonal alterations different from the primary tumor. We explained the patches that we observed as the units of stem cells, amplifying cells and differentiated cells that make up the normal mucosa and skin[22]. When the stem cell acquires a genetic alteration, its derived clonal "patch" will contain the same change, explaining the p53-immunostained patch. These changes most likely frequently occur in the mucosa of heavy smokers. Particularly when additional genetic hits take place, the clone might gain growth advantage, laterally displacing the normal mucosa, and causing a "field" to develop, thereby enormously increasing the cancer risk. These observations have been summarized in a biological progression model depicted in Figure 5.

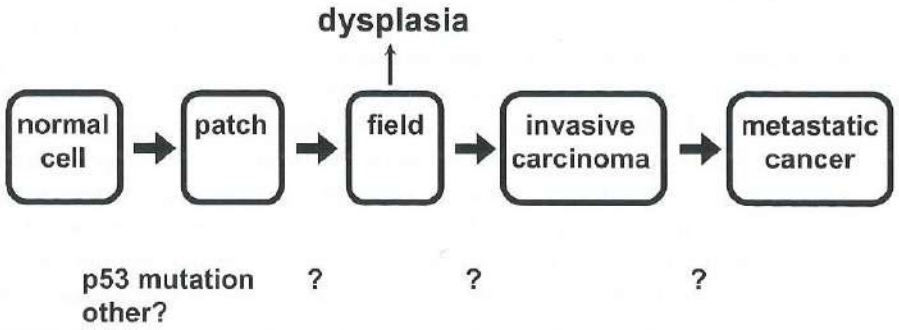

Figure 5. Schematic drawing of a biological progression model for head and neck cancer based on the observations presented in this article. *The various stages have been indicated. Fields can be recognized in some cases histologically as dysplastic lesions. Our model starts with a mutation in the p53 gene as this was the marker used to visualize the various stages. It should be noted that also "patches" might be present that are characterized only by p16 or other tumor suppressor gene inactivation. The genetic alterations that characterize t he different steps from patch to field to invasive carcinoma to metastatic cancer are currently not exactly known.*

The 12 patients who did not show field in the surgical margins were classified as MRC cases. Residual tumor could be identified microscopically in 2/12 MRC cases (MRC-ID cases in Table 1A). In these 2 MRC cases the plaque assay results could be explained by the morphological analyses. However, in a substantial number of MRC patients residual tumor at the primary site was not identified by morphological analysis (10 MRC-NID cases, Table 1A) whereas the percentage of local recurrence in this group is considerable (2/10). This suggests that morphological analysis is not always sensitive enough to detect all dispersed tumor cells. In case #11 we detected a small tumor "embolus" that after a few additional sections disappeared. Sensitive assays like the plaque assay in which larger volumes of tissue are analyzed allow detection of these tumor emboli. Serial or stepwise sectioning combined with IHC might allow identification of more cases with tumor emboli in (lymphatic) vessels, and we therefore analyzed the resection margins of two molecular-positive MRC-NID cases who developed a local recurrence by additional stepwise histological evaluation. Despite immunostaining of five sections serially cut every 50 micrometer, no additional tumor could be identified.

The number of patients with mutated p53 positive margins that could neither be explained morphologically nor clinically (8 MRC-NID cases without a local recurrence, 42%) was relatively high. It could be argued that in all these cases MRC was present at molecular diagnosis, but that postoperative radiotherapy successfully eradicated the residual tumor cells. However, one patient was treated with surgery only (case #22, Table 1A) and did not develop a local relapse so far (follow-up period 30 months). We therefore considered that false-

positive results were obtained. One of the causes of false-positive results might be contamination of tumor (derived) material in the additional margin samples despite the stringent control measures taken to minimize the risk for false-positive results both at the level of sampling and pre/post PCR processing. To formally exclude tumor cell contamination we screened the margins of two "MRC-NID" cases by RNA plaque assays using appropriate positive and negative controls. These two cases were negative in the RNA analysis suggesting that not tumor cells were detected in these two cases by the DNA-based assay, but naked tumor DNA, leaking from the tumors via the saliva and lymphatics[10]. This hypothesis is supported by the observation that tumor DNA in serum can be determined in head and neck cancer patients and used as marker[9]. Indeed, analysis of contralateral exfoliated cells indicated that tumor DNA floating in the saliva might have contaminated the margins. These findings indicate that tumor DNA is a biomolecule that might give rise to false-positive results and hamper exploitation of DNA markers for margin analyses. Based on our data it could be solved by using RNA as biomarker.

In summary, in this study we have extensively investigated the use of p53 as molecular marker to detect residual head and neck cancer and its relation to morphological parameters. Molecular analysis was shown to be more sensitive than morphological techniques for the detection of residual tumor cells, but molecular analysis alone must be treated with care as DNA markers might give rise to false-positive results. At present we do not know whether our findings will influence prognostic studies.

Acknowledgements

We are greatly indebted to Dr. I. Cruz and Ing. T. Tadema (Dept. of Pathology, Vrije Universiteit Medical Center, Amsterdam) for technical assistance and to Dr. S. Scholnick (Dept. Of Otolaryngology-Head and Neck Surgery, Washington University School of Medicine, St Louis) and Dr. B.J.M. Braakhuis (Dept. of Otolaryngology/Head and Neck Surgery, Vrije Universiteit Medical Center, Amsterdam) for critically reading the manuscript.

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6

Molecular diagnosis of surgical margins and survival of patients with head and neck cancer

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(Submitted)

Abstract

Background. Despite histologically radical tumor resection ten to thirty percent of patients with head and neck squamous cell carcinoma (HNSCC) develop local recurrences. This may result from either minimal residual cancer (MRC) or unresected tumor-related mucosal precursor lesions in the surgical margins. In this prospective study, mutated p53 and human papillomavirus (HPV) were used as molecular markers to determine the presence and the clinical significance of MRC or precursor lesions in the resection margins.

Methods. Seventy-nine of 128 eligible patients fulfilled the criteria of histopathologically radical tumor resection and presence of mutated p53 (69 cases) or HPV (10 cases) in the primary tumor. Surgical margins of these 79 patients were analyzed by molecular techniques to determine the presence of cells clonally related to the tumor. Immunostaining revealed whether these cells were present as precursor lesion. The molecular margin status was correlated to clinical outcome.

Results. At least one surgical margin of 47/79 (60%) patients was molecular-positive, of which 10 as result of an unresected precursor lesion. Statistical analysis showed that molecular-positive surgical margins were significantly associated with decreased local recurrence-free survival (P=0.017), disease-free survival (P=0.010) and cause-specific survival (P=0.015). Whether the cells were present as MRC or precursor lesion seemed not relevant. Tumor status (mutated p53, HPV or none) was not related to outcome. Presence of a molecular-positive margin was an independent marker for prognosis.

Conclusions. The molecular margin status determines not only local recurrence-free survival but also disease-free and cause-specific survival in HNSCC enabling selection of patients at high risk for tumor recurrence, and to guide clinical management.

Introduction

Head and neck squamous cell carcinoma (HNSCC) comprises approximately 5% 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 in part due to the relatively high local recurrence rate observed in HNSCC patients. Even when the surgical margins are diagnosed as tumor-free by histopathology, the local recurrence rate is still 10 to 30%.[2] There are two explanations for this clinical observation. Minimal residual cancer (MRC) that is not detectable by routine histopathological examination is left behind in these patients and might cause local recurrences. Alternatively, (tumor-related) mucosal precursor lesions that have not been surgically resected might give rise to new invasive carcinomas. It is generally accepted that HNSCC develops through a number of precursor stages, histopathologically often recognized as mild, moderate and severe dysplasia. These lesions can have large dimensions and are left behind after tumor resection in 25% of the cases.[3] Detection of cells clonally related to the tumor but present either as an unresected mucosal precursor lesion ("field") or MRC may improve the clinical management of HNSCC patients. Moreover, it might be of clinical significance to make a distinction between the two types of lesions.[4]

To allow detection and distinction of MRC and "field" we used a two-step approach. Molecular markers were selected that 1) are present at the earlier stages of cancer progression, 2) allow sensitive detection at the level of a single (tumor) cell in 10^3-10^4 normal cells, and 3) are present in separate tumors to increase the number of evaluable cases. Based on these criteria a promising molecular marker is mutated p53. Mutations in the p53 tumor suppressor gene are present in 50-60% of head and neck cancers[5], and we have demonstrated that these are reliable clonal markers for HNSCC.[6] It is known from other studies that p53 mutations are early genetic alterations in HNSCC that are often present in (dysplastic) mucosal precursor lesions.[3,7] Therefore, mutated p53 in the margins may reflect both (dysplastic) precursor lesions and residual cancer cells. The potential value of mutated p53 as marker for assessment of histopathologically staged surgical margins has been demonstrated in previous studies[8,9], but the number of patients included in these studies did not allow to demonstrate a prognostic value in relation to cause-specific survival and disease-free survival. Moreover, the distinction between MRC and "field" was not investigated.

A second promising molecular marker seems human papillomavirus (HPV) nucleic acids. High-risk HPV types, in particular HPV16 and -18, can induce immortalization of human epithelial cells, an initial step in cancer progression. The viral E6 oncoprotein inactivates the p53 protein, thereby causing an increased risk for cancer development. Originally HPV infection was related to cervical carcinogenesis, but recent data showed involvement of HPV in 10 to 20% of HNSCC.[10,11] Previously we demonstrated that particularly HPV16-positive tumors that show expression of the viral E6 oncogene do not have mutations in the p53 gene. Moreover, lymph node metastases corresponding to these tumors also showed HPV16-E6 expression, indicating a consistent viral involvement in these

tumors. HPV-E6 transcripts therefore seem to have similar potential as mutated p53 to serve as molecular marker for specific and sensitive detection of cells clonally related to the tumor.

Here, we present a prospective study with long-term follow-up involving 79 HNSCC patients with a histopathologically radical tumor resection to assess the clinical significance of cells clonally related to the tumor in surgical margins using mutated p53 and HPV-E6 as molecular markers. Using immunostaining and subsequent molecular analyses a distinction was made between minimal residual cancer (MRC) and unresected tumor-related mucosal precursor lesions ("fields").

Materials and Methods

Patients and tissue specimens

The study protocol was approved by the Institutional Review Board of the *Vrije Universiteit* Medical Center, and written informed consent was obtained from all patients. Clinicopathological parameters were obtained from patient records and pathology reports. The enrolment started in September 1997 and ended in September 2000. In total 179 HNSCC patients who were scheduled for surgical treatment consented to enrolment in the study. The criterion for analysis was tumor-free surgical margins as assessed by routine histopathological examination. In total 143 of 179 patients met the inclusion criterion, and from 128 of 143 cases sufficient material was available for molecular analysis (Figure 1).

At the time of surgery, 4 to 5 paired additional margin samples were taken from the edges of the surgical defect after tumor excision (3 or 4 superficial mucosal margin samples designated M1 to M3/M4 and 1 deep connective/muscle tissue margin sample designated M5). One additional margin sample served as histopathological control and the paired margin sample was used for molecular analysis. To prevent tumor cell contamination, the operating field was rinsed extensively with Dakin's solution (0.5% sodiumhypochlorite pH 11.5 and 0.2% sodiumcarbonate), and the instruments and gloves were changed before sampling. Immediately after surgery and before routine (histological) processing of the resection specimen, a sample of the primary tumor and connective tissue was obtained. These samples and one of the paired additional margin samples (M1 to M5) were directly snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Nucleic acids of the 128 tumors were analyzed for p53 mutations and HPV-E6 expression as described earlier.[11,12] In total 69 of 128 tumors (54%) showed a p53 mutation in the DNA and 10 demonstrated HPV DNA, all typed as HPV16, and viral E6 transcripts. Patient characteristics of these 79 cases are summarized in Table 1.

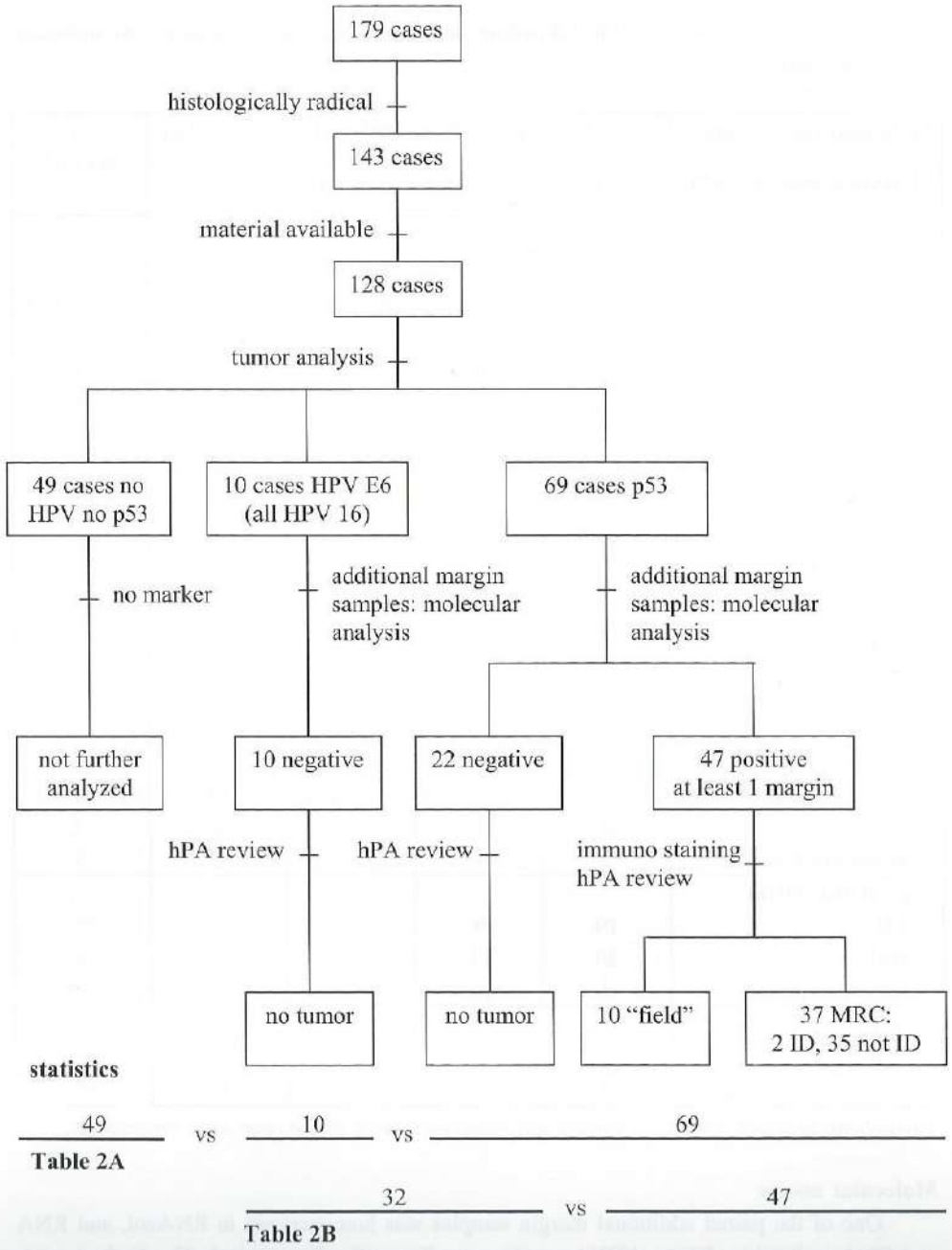

Figure 1. A schematic representation of the study design. *hPA review means histopathological review. ID means tumor identified by IHC in margins from the resection specimen*

Table 1. Patient characteristics of the 128 patients included in the study, in relation to the molecular tumor- and margin status

Molecular *tumor* status	Mutated p53		HPV16-E6	No marker	TOTAL
Molecular *margin* status	Positive	Negative	Negative	-	
Overall	47	22	10	49	128
Median age [range]	57.2 [45.4-77.8]	56.9 [19.9-78.5]	51.9 [40.7-72.1]	58.6 [38.4-81.3]	57.2 [19.9-81.3]
Gender					
Male	32	11	8	27	78
Female	15	11	2	22	50
Tumor site					
Oral Cavity	29	17	5	41	92
Oropharynx	14	2	5	7	28
Larynx	3	1	-	1	5
Hypopharynx	1	2	-	-	3
Tumor stage					
T_1/T_2	26	11	10	32	79
T_3/T_4	19	11	-	14	44
Recurrent tumor*	2	-	-	3	5
Lymph node metastases					
N_0	19	12	2	27	60
N+	21	9	7	14	51
Delayed N+	5	1	1	5	12
Recurrent tumor*	2	-	-	3	5
Stage (UICC 1998)					
I/II	19	9	3	24	55
III/IV	26	13	7	22	68
Recurrent tumor*	2	-	-	3	5
Therapy					
Surgery	17	10	3	25	55
Surgery+Radiotherapy	30	12	7	24	73

*Two patients indicated with an * presented with recurrent tumors. Th ese cases were not restaged.*

Molecular assays

One of the paired additional margin samples was homogenized in RNAzol, and RNA and DNA isolated by RNAzol/DNAstat (Campro Scientific, Veenendaal, The Netherlands). Plaque assays for mutated p53 were performed according to Sidransky et al. (1991)[13] with a few minor modifications as described previously.[12] Proper positive (primary tumor DNA) and negative (wild type DNA) controls were included. On average 1,000 to 10,000 plaques for each margin were screened and the ratio of mutant/wild type plaques calculated. A representative example of the assay is shown in Figure 2.

Detection of HPV16-E6 transcripts was performed using 400 to 500 nanograms total RNA from additional margin samples as described previously.[11] To assess the sensitivity of the HPV16-E6 RT-PCR assay, RNA isolated from the HPV16-containing cervical cancer cell line SiHa was serially diluted in a background of 500 ng RNA of human primary keratinocytes (Figure 3).

A case was designated "molecular-positive" when p53-mutated DNA or HPV16-E6 transcripts were detected in one or more surgical margins.

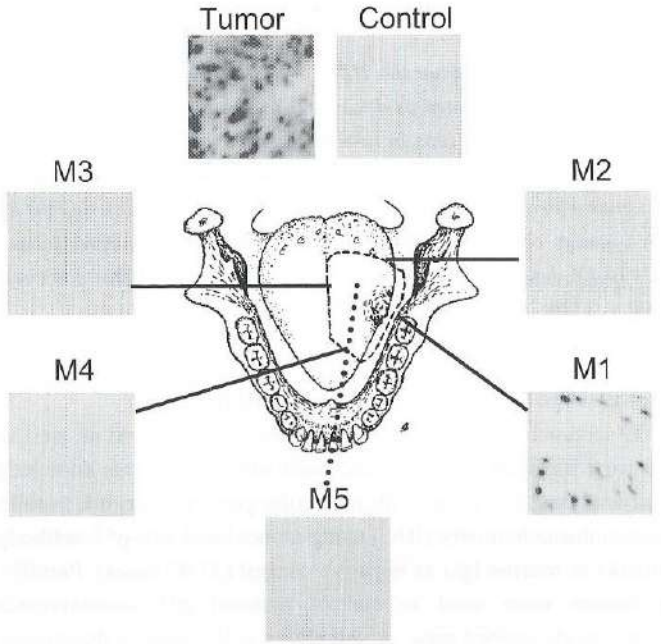

Figure 2. A schematic representation of one of the patients staged by molecular diagnosis using the mutated p53 plaque assay. *This patient (99-7) presented with a T2N2B tongue tumor and was treated by surgery and postoperative radiotherapy. The patient had a frame -shift mutation (insertion of a T) in codon 220 of the p53 gene. DNA from the margins was analyzed as described. Note the hybridizing plaques in the tumor as well as in margin M1, indicative for tumor DNA. This patient developed a local recurrence after 6 months.*

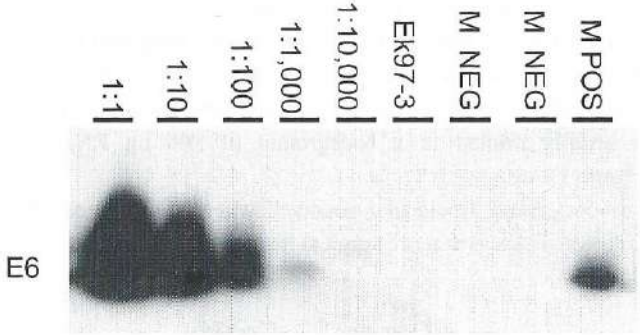

Figure 3. Serial dilution of RNA from the HPV16-positive cell line SiHa in normal keratinocyte RNA. *HPV-E6 RT-PCR was performed as described. To demonstrate the suitability of the assay for the detection of HPV16-E6-positive cells in resection margins we included one histological irradical margin of a patient with HPV16-E6 in the tumor (positive control) and two histological irradical margins from a patient with a p53 mutation in the tumor (negative control). SnRNP RNA amplification was used for RNA quality control and showed strong signal for all samples (data not shown). The sensitivity of HPV16-E6 detection is between 1: 10^3 to 10^4 dilution. This was confirmed in a serial dilution of 1:1,000, 1:3,000, 1:6,000 and 1:10,000.*

Identification of cases with "field" by (immuno)histopathology

All paraffin sections of margins from the surgical specimens as well as the additional margin samples were histopathologically reviewed without previous knowledge of the results of the molecular analyses. For cases with molecular-positive margins, parallel sections were analyzed by immunohistochemistry (IHC) using monoclonal anti-p53 antibody DO7 (DAKO, Glostrup, Denmark) or murine IgG as negative control (37/47 cases). Paraffin sections of the corresponding tumors were used to control mutated p53 overexpression. Cases that demonstrated a positively stained para- or suprabasal cell cluster in the mucosal epithelium of the margin(s) were further investigated. The stained areas (always >20% of a margin) were microdissected from parallel sections and analyzed by plaque assay or sequencing to confirm that the mutation was identical to that in the corresponding tumor. These cases were assigned as "field". It was further noted that mutated p53 immuno-positive "fields" were always classified histologically as dysplastic. In cases when the tumors showed no mutated p53 overexpression (10/47 cases, mostly frame-shift mutations), dysplastic regions in the surgical margins were microdissected and the DNA sequenced to confirm the mutation identified in the corresponding tumor. Based on these results margins were either classified as "field" or MRC. The latter is in fact equivalent to "not-field".

Statistical analysis

The major statistical endpoints of this study were local recurrence-free survival (*LRfs*), disease-free survival (*Dfs*) and cause-specific survival (*Css*) in relation to the molecular status of the tumor and surgical margins. Time to recurrence or death was measured from the date of panendoscopy and histological confirmation of malignant tumor, usually 2-4 weeks prior to surgery. Patients who developed a second primary tumor were censored for all outcomes at the incidence date of the second tumor (3 of 128 cases). Delayed lymph node metastases that developed in an untreated neck during follow-up were not regarded as a clinical relapse and were therefore not considered as statistical endpoint. These cases are indicated by "delayed N+" in Table 1.

Kaplan-Meier estimates and log-rank tests were computed with BMDP software[14]. To investigate the influence of other parameters on the association between p53 margin status and disease, the Cox Proportional Hazards model for covariates was used. Patients with delayed lymph node metastases were staged as N+ in the multivariate analysis. P values below 0.05 were considered significant.

Results

Molecular analysis of surgical margins

The histopathologically tumor-free additional margin samples (M1 to M5) of 79 patients with a p53 mutation (69 cases) or HPV16-E6 transcripts (10 cases) in the primary tumor were analyzed using the p53 plaque hybridization assay (Figure 2) or HPV16-E6 RT-PCR, respectively (Figure 3). The molecular data and the main clinical characteristics of the patients are summarized in Table 1. In total 47 of 79 (60%) patients showed one or more molecular-positive additional margin samples. All additional margin samples analyzed by HPV16-E6 RT-PCR were molecular-negative whilst 47 of 69 analyzed for mutated p53 were molecular-positive. This difference was not statistically significant (Fisher's exact test). The percentage of p53-mutated DNA in the molecular-positive margins ranged from 0.01 % to 15%.

Identification of cases with "fields" by (immuno)histopathology

All margins of the resection specimens that had been used for routine histological examination obtained from the 47 molecular-positive cases, were re-analyzed by immunohistochemistry and histopathological review. In total 37 of 47 cases showed p53 overexpression in the corresponding primary tumors that allowed margin screening with anti-p53 DO7 to detect "field". In 10 of 47 cases a (tumor-related) "field" was identified in one or more margins from the resection specimen. In 8 cases it was visualized by P53 IHC (Figure 4A) and in 2 cases it was identified by DNA sequencing of dysplastic mucosa. The fields were always >20% of a margin, usually >5 mm.

Remarkably, in 2 cases, both not identified as "field", a small clump of tumor cells was observed that could be confirmed histologically as invasive carcinoma, in one case only after immunoguidance (Figure 4B). In the remaining cases no pathobiological substrate could be

detected that explained the positive plaque assay. Together, all cases (37) that could not be explained by the presence of "field" (10) were classified as MRC. In 2 of 37 MRC cases tumor could be confirmed. All findings are summarized in Figure 1.

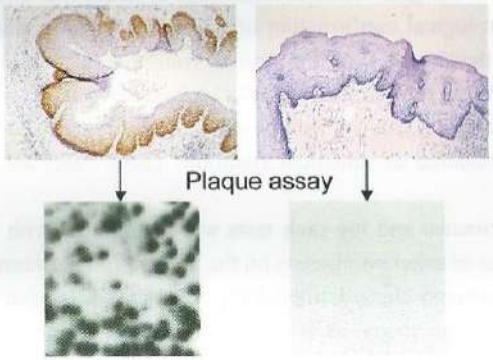

Figure 4A. An example of a mutated p53 "field" (case 99-9). *The epithelial layer was microdissected and analyzed by plaque assay to confirm the tumor-related identity of the precursor lesion. As control non-stained mildly dysplastic mucosa of another margin of the same patient was a nalyzed in parallel.*

Figure 4B. A small tumor embolus immunostained with anti-p53 DO7. *It was observed during the screening for "field" cases (case 97-68). A parallel section was stained with anti-CD31 to prove that the embolus was located in a vessel (data not shown). The tumor embolus was not diagnosed by routine histopathological examination as well as review. Only after immunoguidance the embolus was recognized and classified as invasive carcinoma.*

Molecular analyses and clinical outcome

Presence of a p53 mutation in the *tumor*, presence of HPV16-E6 transcripts in the *tumor*, or absence of both did not influence outcome as determined by Kaplan-Meier analysis for both local recurrence-free survival (*LRfs*: P=0.21), disease-free survival (*Dfs*: P=0.52) and cause-specific survival (*Css*: P=0.51). Clinicopathological features in relation to the tumor status are summarized in Tables 1 and 2A.

Table 2A. Clinical outcome in 128 HNSCC patients in relation to tumor status.

	Mutated TP53 in tumor (69 cases)	HPV in tumor (10 cases)	No marker in tumor (49 cases)	P-value Log rank test
Local recurrence	8	0	2	0.21
Recurrent disease	16	1	11	0.53
Dead of disease	16	1	9	0.51

Subsequently the association of the molecular *margin* status (margin molecular-positive [MRC and "field"] versus margin molecular-negative) with outcome was determined. The median follow-up was 21,5 months (range 3,7 to 46 months). Kaplan-Meier estimates and the associated log-rank tests showed that the *LRfs* was significantly worse in the group with molecular-positive margins (P=0.017: Figure 5A). The median time to local recurrence was 10 months. Intriguingly, also the *Dfs* and *Css* were significantly worse in the group with molecular-positive margins (P=0.010 and 0.015 respectively: Figure 5B/C). Patient, tumor and margin characteristics were comparable in the margin molecular-positive and -negative group (Table 2B).

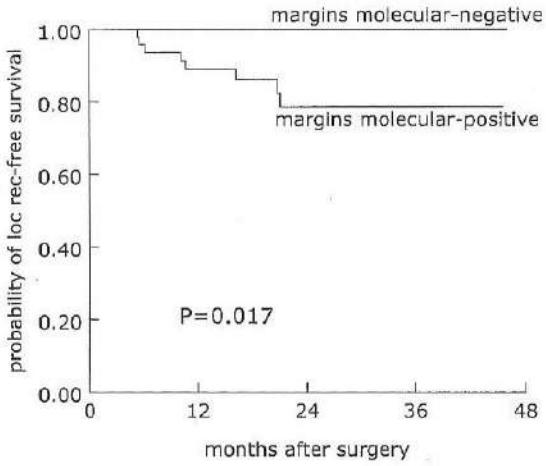

Figure 5A. Kaplan-Meier curve showing local recurrence-free survival in relation to the molecular margin status assessed by a plaque assay for mutated p53 or HPV-E6 RT-PCR. *Patients who remained disease-free were censored in the analysis at the date of last clinical follow-up examination or death. Patients who developed other recurrences were not censored at the date of r ecurrence as other relapses were not considered to have influence on local recurrence development. LRfs of patients with molecular-positive margins was significantly worse than that of patients with molecular-negative margins.*

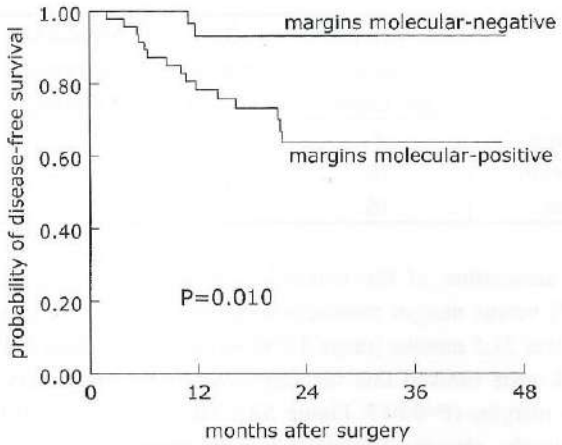

Figure 5B. Kaplan-Meier curve showing disease-free survival in relation to the molecular margin status assessed by a plaque assay for mutated p53 or HPV-E6 RT-PCR. *Patients who did not develop a relapse (local recurrence, regional recurrence, distant metastases) were censored in th e analysis at the date of their last clinical follow-up examination or death. Dfs of patients with molecular-positive margins was significantly worse than that of patients with molecular-negative margins.*

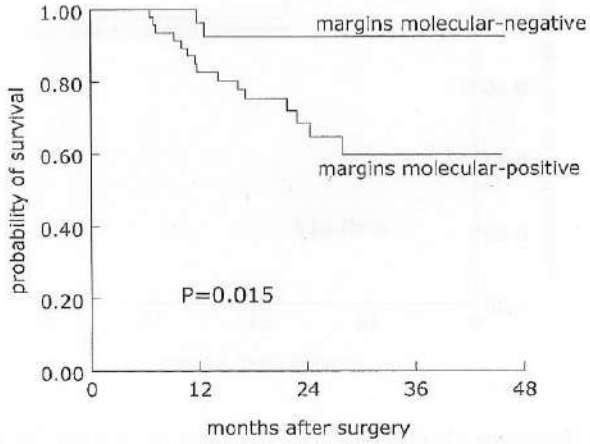

Figure 5C. Kaplan-Meier curve showing cause-specific survival in relation to the molecular margin status assessed by a plaque assay for mutated p53 or HPV-E6 RT-PCR. *Patients who died of other causes or remained alive were censored in the analysis until their date of death or last clinical follow - up examination, respectively. The cause-specific survival of patients with molecular-positive margins was significantly worse than that of patients with molecular-negative margins.*

Table 2B. Clinical outcome in 79 HNSCC patients with mutated p53 or HPV-E6 in primary tumor in relation to molecular margin status.

	Margin molecular-positive (47 cases)	Margin molecular-negative (32 cases)	P-value Log rank test
Local recurrence	8	0	0.017
Recurrent disease	15	2	0.010
Dead of disease	15	2	0.015

The influence of other parameters on the association between molecular margin status and disease was investigated with the Cox Proportional Hazards model. Clinical covariates that were associated with *LRfs*, *Dfs* and *Css* comprised T-stage, N-stage, stage, number of lymph node metastases, and extranodal spread. The multivariate relative risk of the presence of a molecular-positive margin is 5.2 [95% CI=1.18-22.66] for *Css* and 5.5 [95% CI= 1.27-24.26] for *Dfs*. It could not be determined for *LRfs* as local recurrences did not occur in the group with molecular-negative margins. Neither T-stage, N-stage nor stage significantly influenced the impact of molecular margins status on both *Css* and *Dfs*.

Moreover, Kaplan-Meier analysis of the three separate groups (MRC-positive, "field"-positive, margin molecular-negative) and other comparisons showed that the MRC-positive and "field"-positive groups had similar poor outcome. These observations indicated that the distinction between "field" and MRC was clinically not significant.

Discussion

In the present study, we have assessed the clinical significance of the presence of cells clonally related to the tumor in the surgical margins of HNSCC patients, using both mutated p53 and HPV16-E6 transcripts as molecular markers.

The clinical outcome of patients with or without p53 mutation or HPV16-E6 transcripts in the primary tumor was not significantly different for *LRfs*, *Dfs* or *Css*, allowing statistical analysis for other prognosticators including molecular margin status. Intriguingly, a molecular-positive margin status was not only associated with a high rate of local recurrences, but also with a high rate of regional recurrence and distant metastases. This association suggests that residual tumor cells are detected in surgical margins that travel through vessels, particularly lymphatic vessels, which we indeed found in one case. We performed additional stepwise histological analysis in other cases (molecular-positive margin + local recurrence) but did not find tumor (Van Houten et al., in press). Molecular analysis seems therefore at present superior over (immuno)histochemical analysis for the detection of MRC.

In 10 of 47 cases a molecular-positive margin could be explained by the presence of a unresected tumor-related mucosal precursor lesion ("field"). The presence of these tumor-related "fields" surrounding HNSCC has already been reported earlier[3,15], supporting the

hypothesis that patients with these "fields" are at risk for developing local recurrence and second primary tumors. In our study the distinction between "field" (a precursor lesion) or MRC (invasive carcinoma) appeared not to be of clinical significance. This could be related to the relatively small "field" group. A second explanation for this observation might be that besides the identified "field", these patients might have had also residual cancer cells in the margin that could not be detected separately.

Our data indicate that molecular analysis of resection margins is of large importance for the staging of HNSCC patients but there are some limitations at present that hamper implementation. Particularly the laboriousness of the plaque assay to assess mutated p53 hampers clinical use. Moreover, the number of MRC-positive cases was relatively high: 37 of 79 (47%). It could be argued that in all these MRC-positive cases residual cancer cells were present and that these were successfully eradicated by postoperative radiotherapy. However, 9 of 11 patients with MRC-positive margins who were treated with surgery only did not develop a relapse so far (follow-up period 10.6 to 43.0 months, median = 23.6 months). Due to stringent control measures during sampling and processing of the margins, technical problems are unlikely. An explanation might be that these small numbers of cells do not always give rise to local tumor regrowth. Finally, it needs to be considered that in a few cases DNA leakage from necrotic tumors via saliva and/or lymph might have caused false-positive results. Tumor DNA can also be detected in serum in some cases[16,17].

Despite a few limitations to the presented molecular approaches, our data strongly indicate that the molecular status of the margins provides important information for clinical management. For breast cancer it has been shown that disseminated tumor cell detection in bone marrow aspirates seems most promising for improved staging[18], whereas for head and neck cancer (pre)cancer cell detection in the resection margins seems most important, not only in relation to risk for local relapse, but also in relation to total relapse and even disease-related death. Particularly when the molecular margin status is combined with clinical parameters high risk groups can be identified (molecular-positive margin/N+ group: 12/21 [57%] relapses and tumor-related deaths). These patients are therefore eligible for adjuvant therapy protocols. Secondly, as no local recurrences were observed in the patient group with negative margins, a group of patients can be identified that might be spared postoperative radiotherapy and thus significant morbidity despite their advanced T-stage. In this study 50% of the molecular margin-negative patients were treated with postoperative radiotherapy.

Summarized, our data show that molecular analysis of surgical margins is superior over (immuno)histopathology to identify (subgroups) of patients who are at high risk to develop (local) relapse and tumor-related death. Furthermore, patients with molecular-free margins might be spared postoperative radiotherapy. Future effort should be focused on the identification of "field"-specific and tumor-specific molecular markers that can be measured by more cost-effective methods.

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We are greatly indebted to Ing. F. Denkers, J. Dijkstra, D. Kramer and W. de Jong, (*Vrije Universiteit* Medical Center, Amsterdam) for technical assistance. Dr. M. van den Brekel (Dept. Otolaryngology/Head and Neck Surgery, Netherlands Cancer Institute) for clinical assistance, and prof. Dr. D. Sidransky (Head and Neck Cancer Research Division, The Johns Hopkins University School of Medicine, Baltimore USA) for help with sequencing and plaque assay.

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7

Prognostic significance of molecular and clinicopathological markers in head and neck cancer

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Abstract

Background In a recent study it was shown that molecular diagnosis of cells clonally related to the tumor (minimal residual cancer (MRC) or preneoplastic lesions) in surgical margins by HPV or mutated p53 as markers has independent prognostic value to identify patients with decreased local recurrence-free survival (*LRfs*). The molecular margin status (MMS) also revealed prognostic information for disease-free survival (*Dfs*) and cause-specific survival (*Css*). In the current study it was investigated which clinico-pathological parameters determine clinical outcome and interact with the MMS. **Methods** Seventy-nine of 128 eligible patients who fulfilled the criterion of histopathologically radical tumor resection showed the presence of mutated p53 (69 cases) or HPV (10 cases) in the primary tumor. Surgical margins of these 79 cases were diagnosed by molecular assays and the clinical significance of the MMS compared to the impact of other clinicopathological and histological parameters as examined by a multivariate Cox Proportional Hazards model. **Results** Statistical analysis showed that a positive MMS (or MRC status for *LRfs*) has an independent multivariate relative risk of 8.1 for *LRfs* (P=0.014), 5.2 for *Css* (P=0.0081) and 5.5 for *Dfs* (P=0.0055). The MMS was not related to *DMfs*. For all outcomes, except for *LRfs*, the presence of three or more tumor-infiltrated lymph nodes was the strongest prognosticator, causing a RR of 13.8 (P<0.0001) for *Dfs* and 14.0 (P<0.0001) for *Css*. **Conclusion** The MMS is more important than any clinicopathological parameter for predicting *LRfs*, whereas for all other outcomes, particularly those related to metastases, the presence of three or more tumor-infiltrated lymph nodes seems to be the most important prognosticator. The risk for LR did not relate to histopathological parameters, such as perineural growth, invasive growth, grade of dysplasia or histological differentiation.

Introduction

Despite significant improvements in surgery and radiotherapy, prognosis of patients with squamous cell carcinomas of the head and neck (HNSCC) has only moderately increased. Locoregional recurrences occur in about 10-30% of the HNSCC patients with histopathologically tumor-free surgical margins, whereas 15-25% develop distant metastases.[1,2] Residual tumor cells, not detectable by routine histopathology and often referred to as *minimal residual disease* (MRD)[3], are assumed to play a crucial role in the relatively high number of recurrences observed in these patients. Recent studies have shown that molecular diagnosis allows improved staging of head and neck cancer.[4,5] In a prognostic study using p53 mutations and HPV as tumor-specific markers we have shown that both residual tumor cells (minimal residual cancer, MRC) and unresected tumor-related mucosal precursor lesions ("fields"), are present in the surgical margins of HNSCC patients (Van Houten et al. in press[6]). Moreover, we showed that a positive molecular margin status is an independent prognosticator for local recurrence-free survival, disease-free survival and cause-specific survival, independent from N-stage or T-stage (Van Houten et al. submitted[7]). In the current study we used a multivariate model to evaluate the clinical significance of the molecular margin status (MMS) as compared to other clinicopathological and histological parameters, with regard to local recurrence-free survival (*LRfs*), locoregional recurrence-free survival (*LRRfs*), distant metastases-free survival (*DMfs*), disease-free survival (*Dfs*) and cause-specific survival (*Css*).

Several prognostic factors have been described, of which the extent of the primary tumor (T-stage) and the histopathological status of the neck nodes (N-stage) are among the most significant. Leemans et al. (1994)[1] reported that patients with T3 and T4 disease have a three fold increased risk for local recurrence as compared to patient with stages T1 and T2 disease. In addition, the presence of more than three positive nodes on histopathological examination was a strong independent predictor of recurrence at the primary site. In another report Leemans et al. (1993)[2] showed that patients with more than three positive lymph nodes have a five-fold increased risk for developing distant metastases (stratified for T-stage). Moreover, in the group of patients with nodal involvement, cases with extranodal spread showed a threefold increase in the incidence of distant metastases as compared to patients without this feature (P=0.017).

In addition to these conventional clinical parameters, some histopathological parameters have often been proposed to have predictive value in head and neck cancer, although not always concordant results have been obtained. The grade of epithelial dysplasia (reviewed by Warnakulasuriya et al., 2001[8]) was in some cases shown to be related to the risk of malignant progression and/or recurrence,[9-12] whereas others reported a poor or no correlation.[13,14] Other histopathological parameters that have been implicated to be associated with a poor prognosis in HNSCC are perineural invasion and invasive growth pattern.[15-20] Most studies show an association between perineural growth and/or invasive growth and increased locoregional recurrence and/or decreased survival.

Genotypic changes in the primary tumor, particularly mutations in the p53 gene, which are very common in HNSCC, have also been suggested to have additional prognostic value for HNSCC.[21-24] Moreover, their localization in the DNA contact region was shown to provide prognostic information.[25-27] Recently, also the presence of high-risk human papillomavirus types (e.g. HPV16) as detected by PCR techniques has been proposed to play a role in the development and prognosis of HNSCC.[23, 29-34]

We present a prospective follow-up study involving 128 HNSCC patients with a histopathologically radical tumor resection, to evaluate the relative importance of the molecular margin status for the prediction of recurrence and survival as compared to several clinicopathological and histopathological parameters. The independent prognostic value of these parameters was determined in a multivariate Cox Proportional Hazards model for a number of clinical outcomes.

Material and Methods

Patients and tissue specimens

The study presented was approved by the Institutional Review Board of the *Vrije Universiteit* Medical Center, and written informed consent was obtained from all patients. Clinicopathological parameters from these patients were obtained from patient records and pathology reports. The enrolment period started in September 1997 and ended in September 2000. During this period 128 HNSCC patients who underwent histopathological radical surgical removal of the primary tumor met the inclusion criteria. Criteria for postoperative radiotherapy were based on standard clinicopathological parameters; i.e. multiple nodes or extranodal spread.

At the time of surgery 4 or 5 paired additional margin samples were taken from the edges of the surgical defect after tumor excision (3 or 4 superficial mucosal margin samples and 1 deep connective tissue margin sample). One additional margin sample served as histopathological control and the paired margin sample was used for molecular analysis. Immediately after surgery and before routine histological processing of the resection specimen, a fresh sample of the primary tumor was obtained. These samples and one of the paired additional margin samples were directly snap-frozen into liquid nitrogen and stored at -80°C until further processing. The remaining resection specimen was formalin-fixed and embedded in paraffin for histopathological assessment.

Molecular parameters: tumor- and margin status

Nucleic acids of the 128 tumors were analyzed for p53 mutations and HPV-E6 expression as described earlier.[4,30,35,36] In total 69 of 128 tumors (54%) showed a p53 mutation in the DNA, and 10 demonstrated HPV DNA, all typed as HPV16, as well as viral E6 transcripts. P53 mutations in codons 120, 241, 248, 273, 276, 277, 280, 281 and 283 were classified as contact mutations according to Powell et al. (2000).[26]

The 79 patients showing either a p53 mutation or HPV in the primary tumor were eligible for subsequent molecular analysis of surgical margins as described previously.[30,35,36] Molecular margin status (MMS) was defined on the basis of the presence or absence of cells clonally related to the primary tumor. Using immunohistochemistry and (subsequent) molecular analyses a distinction was made between molecular-positive **field** and molecular-positive **MRC** (minimal residual cancer). A molecular-positive field was defined as the presence of an unresected tumor-related precursor lesion in one or more surgical margins. MRC was defined as a molecular-positive margin while a tumor-related field was absent. Molecular characteristics of the 128 eligible cases are shown in Table 1.

Clinicopathological parameters

The following clinicopathological parameters as summarized in Table 1 were analysed for their relative prognostic importance as compared to the MMS in 79 patients: gender, T-stage, N-stage, Stage, number of tumor-positive lymph nodes (nN), extranodal spread (R), histological differentiation, grade of dysplasia, invasive growth pattern, perineural growth and tumor distance to the deep margin.

To investigate dysplasia, all paraffin-embedded surgical margins of the resection specimen of the 79 cases were re-examined closely by two independent pathologists who had no prior knowledge on clinical data before screening. The grade of dysplasia was scored according to the standard criteria of the World Health Organization international classification of tumors.[37] Lesions were graded as: (0) normal mucosa, (1) mild dysplasia, (2) moderate dysplasia, (3) severe dysplasia or (4) carcinoma in situ. In addition, a dysplasia "score" was calculated for each patient, as we hypothesized that the cancer risk would depend on both the grade of dysplasia and the extension (% per margin) of the dysplastic lesions. The dysplasia score was calculated as follows. Per margin, the grade of dysplasia observed (ranging from 0 to 4) was multiplied by the percentage, rendering a figure between 0 and 400. If two grades were observed in a margin, only the highest grade was taken into account. This figure was calculated for all margins per patient, summarized and the mean score/number of margins was taken.

The tumor distance to the deep margin could be determined for 54 cases by measuring the closest distance of the tumor to the inked deep resection margin in one or more sections taken through the central part of the tumor. The cases were divided into 2 groups on the basis of this measurement: 1-5 mm (close margin) and ≥ 5 mm (adequate margin).

Table 1. Patient characteristics of the 128 patients included in the study, in relation to the molecular tumor and margin status

Molecular *tumor* status		Mutated p53 69		HPV16-E6 10	No marker 49	
Molecular *margin* status		Positive	Negative	Negative	-	TOTAL
Overall		47	22	10	49	128
Gender	Male	32	11	8	27	78
	Female	15	11	2	22	50
T-stage (T)	T_1	10	4	2	14	30
	T_2	16	7	8	18	49
	T_3	11	8	-	9	28
	T_4	8	3	-	5	16
	Recurrent tumor*	2	-	-	3	5
N-stage (N)	N0	11	5	-	10	26
	N1	3	2	1	5	11
	N2A	1	0	3	0	4
	N2B	10	7	3	9	29
	N2C	6	0	0	0	6
	N3	1	0	0	0	1
	Delayed N+	5	1	1	5	12
	No ND (= cN0)	8	7	2	17	34
	Recurrent tumor*	2	-	-	3	5
Extranodal spread (R)						
Yes		17	5	4	12	38
No		4	3	3	2	12
Delayed N+		5	1	1	5	12
no ND/N0/NK		19	13	2	27	60
Recurrent tumor*		2	-	-	3	5
No of pos. lymph nodes (nN)						
0		11	5	-	10	26
1		4	2	4	5	15
2		3	3	2	3	11
3		3	3	0	1	7
4		3	0	1	2	6
>4		8	1	0	3	12
Delayed N+		5	1	1	5	12
no ND		8	7	2	17	34
Recurrent tumor*		2	-	-	3	5

Molecular *tumor* status		Mutated p53 69		HPV16-E6 10	No marker 49	
Molecular *margin* status		Positive	Negative	Negative	-	TOTAL
Overall		47	22	10	49	128
Stage (UICC 1998) (S)						
I		10	4	2	13	29
II		9	5	1	11	26
III		6	3	1	8	18
IVA/B/C		20	10	6	14	50
Recurrent tumor*		2	-	-	3	5
Differentiation (DF)						
Poor		5	3	1	-	9
Moderately		31	15	7	39	92
Well		8	3	1	6	18
NK		1	1	1	1	4
Recurrent tumor*		2	-	-	3	5
Grade of dysplasia (DS)						
No		9	8	4		
Mild		20	7	5	NA	NA
Moderate		11	1	1		
Severe-CIS		7	6	0		
Invasive growth (IG)	Yes	14	8	2	13	37
	No	33	14	8	36	91
Perineural growth (PG)	Yes	14	4	0	8	26
	No	33	18	10	41	102
Tumor distance deep margin						
TDDM < 5mm (close)		13	7	6	NA	NA
TDDM ≥ 5 mm (adequate)		13	12	3		
NK		21	3	1		
Postop. radiotherapy	Yes	30	12	7	24	73
	No	17	10	3	25	55
DNA contact mutation	Yes	9	4	NA	NA	NA
	No	38	18			

*No ND = no neck dissection. NK=not known. Patients indicated with an * presented with recurrent tumors. These cases were not restaged*

Statistical analysis

The major statistical endpoints of this study were *LRfs*, *LRRfs*, *DMfs*, *Dfs* (defined as all recurrent disease) and *Css*. Kaplan-Meier analyses and log-rank tests were used to estimate the (univariate) clinical significance of a positive molecular tumor status (MTS), a positive MMS and covariates (n=79 cases). The multivariate prognostic value of the MMS and/or covariates was evaluated by Cox Proportional Hazards analysis using BMDP software (n=79 cases). P-values below 0.05 were considered significant. Time to recurrence was measured from the date of surgery. Patients who developed a second primary tumor were censored at the incidence date of the second tumor. In all cases only the first recurrence in time was taken into account.

Bivariate models with MMS and each covariate were used to evaluate the dynamic interplay between both parameters. A change of the P-value relative to the threshold (P=0.05) was considered an "interaction". Possible "interactions" were defined as: mutual influence (MI) if both P-values change, dominancy (D) if a significant parameter causes a change of the P-value of the other parameter, or masking (M) if a non-significant parameter changes the P-value of the other parameter.

In the Cox Proportional Hazards analysis for *LRfs* the MMS could not be determined as no LR occurred in the MMS-group resulting in degenerated models. In this analysis MMS was represented by the MRC status only (thus adding the fields to the molecular-negative group).

For the parameters T-stage and N-stage, in addition to the separate clinical classes (T-score and N-score) caesurae were analysed to determine the threshold for an increased relative risk. For T-stage, the caesurae were defined as: >T1, >T2 (=T1+2 versus T3+4) and >T3. For N-stage, the caesurae were defined as: >N0 (=N0 versus N+), >N1, >N2A, >N2B and >N2C. In addition the number of involved lymph nodes (nN) was analysed, with the following caesurae: nN>0, nN>1, nN>2, nN>3, and nN>4.

In particular for the parameters related to lymph node staging, missing values had to be taken into account. Lymph node metastases that developed in an untreated neck during follow-up (n=12 in 128 cases, n=7 in 79 cases) and were treated by delayed neck dissection were not regarded as a clinical relapse and indicated by delayed N+ in Table 1. For the analysis "N0 versus N+" these patients were categorized as (clinically) N+, but as "missing values" for the other N-stage caesurae, nN caesurae and extranodal spread. The histopathological N-stage of patients who did not initially have their neck dissected, remained free of delayed lymph node metastases and did not receive postoperative radiotherapy (n=16, median follow-up = 21 months, range 5-32 months) was substituted by the clinical N0-stage (in all N-stage caesura), to prevent selection bias of a relatively favourable group. They remained "missing" for the parameters number of positive lymph nodes and extranodal spread as no information (neither clinically, nor histopathologically) was available. Two patients with recurrent tumors have been excluded for all T-stage and lymph node parameters.

Results

Univariate (log-rank) analysis of molecular tumor status and outcome

First, the clinical significance of a p53 mutation or HPV in the primary tumor was analysed. In total, 69/128 eligible tumors analysed contained a mutation in the p53 gene, whereas 10 other tumors showed the presence of HPV16-E6 transcripts. However, neither the presence of a p53 mutation in the tumor, nor the presence of HPV16-E6 transcripts in the tumor, or absence of both did influence the outcome as determined by Kaplan-Meier analysis for *LRfs* (P=0.21), *LRRfs* (P=0.29), *DMfs* (P=0.73), *Dfs* (P=0.53) and *Css* (P=0.51). A summary of the molecular tumor parameters and log-rank tests is shown in Table 2A, whereas the Kaplan-Meier curves of *Dfs* and *Css* are shown in Figure 1A and 1B.

In addition, we analysed the type of p53 mutation in relation to prognostic significance. Kaplan-Meier analysis and log-rank tests for 69 p53-tumor-positive cases showed that clinical outcome of patients with P53 contact mutations was not significantly different from patients with non-contact mutations (Table 2B).

Table 2A. Clinical outcome in 128 HNSCC patients in relation to molecular tumor status

	Mutated TP53 in tumor (69 cases)		HPV in tumor (10 cases)	No marker in tumor (49 cases)	P-value Log rank test
	MMS+	MMS-			
Local recurrence	8	0	0	2	0.21
Loco-regional recurrence	11	1	0	6	0.29
Distant metastases	4	0	1	5	0.73
Recurrent disease	15	1	1	11	0.53
Dead of disease	15	1	1	9	0.51

Table 2B. Clinical outcome in 69 HNSCC patients in relation to DNA contact mutations

	Mutated TP53 Contact (13 cases)	Mutated TP53 NON-Contact (56 cases)	P-value Log rank test
Local recurrence	1	7	0.66
Loco-regional recurrence	1	11	0.33
Distant metastases	1	3	0.75
Recurrent disease	2	14	0.49
Dead of disease	2	14	0.56

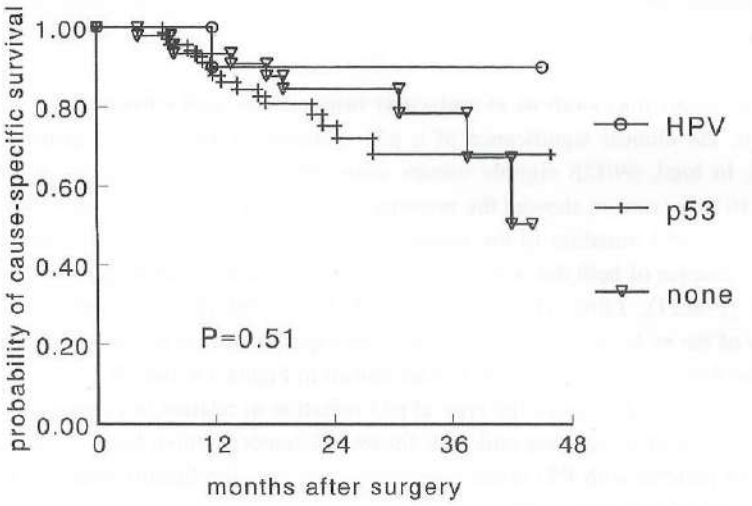

Figure 1A. Kaplan-Meier curve showing disease-free survival in relation to the molecular tumor status assessed by sequencing of P53 or HPV-E6 RT-PCR for 128 cases. Patients who did not develop a relapse (local recurrence, regional recurrence, distant metastases) were censored in the analysis at the date of their last clinical follow-up examination or death. The disease-free survival of patients with a molecular-positive tumor status was not significantly different than that of patients with molecular-negative tumors.

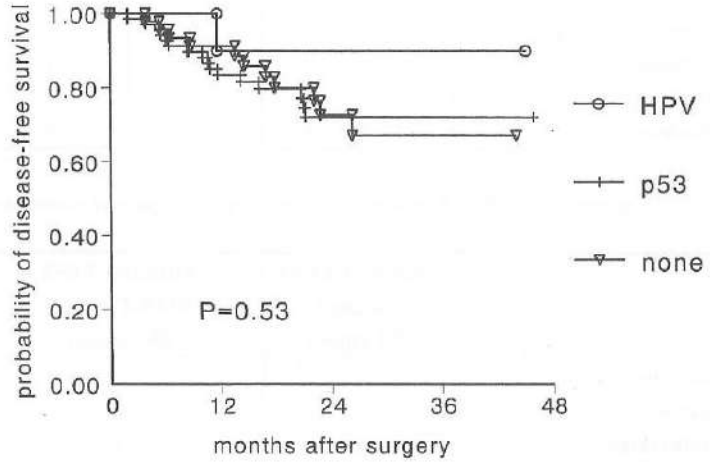

Figure 1B. Kaplan-Meier curve showing cause-specific survival in relation to the molecular tumor status assessed by sequencing of P53 or HPV-E6 RT-PCR for 128 cases. Patients who died of other causes or remained alive were censored in the analysis at the date of their last clinical follow-up examination or death, respectively. The cause-specific survival of patients with a molecular-positive tumor status was not significantly different than that of patients with molecular -negative tumors.

Univariate (log-rank) analysis of MMS and outcome

As outcome was independent of the marker status of the primary tumor, molecular analyses of surgical margins was not confounded by the type of marker used (HPV or p53). Of the 79 cases with a molecular-positive tumor status (P53+ or HPV+), 47 patients showed the presence of marker-positive cells clonally related to the primary tumor in the surgical margins (47 P53+, 0 HPV+). Ten of these 47 cases with molecular-positive margins were shown to represent mucosal precursor lesions (fields) whereas the remaining 37 cases were classified as MRC cases. Kaplan-Meier estimates and log-rank tests showed that the *LRfs* (P=0.017), *LRRfs* (P=0.017), *Dfs* (P=0.010) and *Css* (P=0.015) were significantly worse in the group with molecular-positive margins (submitted). For *DMfs* (P=0.31) no significant difference was observed.

Multivariate analysis of molecular margin status and covariates

The relative risk of a positive molecular margin status as compared to clinico-pathological parameters and possible interactions were analysed for 79 patients showing either mutated p53 or HPV in the primary tumor, using Cox Proportional Hazards models for all clinical outcomes.

A. Local recurrence-free survival

The relative risk (RR) of the molecular margin status could not be determined for *LRfs* as no local recurrences occurred in the group with molecular-negative margins. It could, however, be determined for MRC status when the molecular-positive field cases (n=10) were included in the molecular-negative group. Using this strategy, an RR of 8.1 (P=0.014) could be determined, as one local recurrence developed from a tumor-related mucosal precursor lesion (Table 3). In addition to the MRC status, the presence of more than two tumor-positive lymph nodes (nN>2) as a single parameter contributed significantly to *LRfs* (RR=5.22, P=0.033). However, in bivariate analysis, the MRC status (RR=6.10, P=0.047) was dominant over nN>2 (RR=3.39, P=0.11). This means that the MRC status already explains the outcome by itself, whereas nN>2 does not add significant prognostic value. Grade of dysplasia or tumor distance to the deep margin were not related to *LRfs*. Table 4A summarizes the findings with regard to *LRfs*.

Table 3. Relative risks and 95% CI for a positive molecular margin status as independent parameter in Cox Proportional Hazards analysis

Outcome	Relative Risk	95% CI lower	95% CI upper	P-value
LRfs [a]	8.1	0.96	68.9	0.014
LRRfs	8.1	1.00	65.4	0.0083
DMfs	3.0	0.32	27.8	0.2840
Dfs	5.6	1.23	25.0	0.0055
Css	5.2	1.15	23.4	0.0081

[a] *For LRfs, MRC status was analysed instead of MMS to avoid a degenerated model*

Table 4. Cox Proportional Hazards analyses for molecular margin status and clinicopathological covariates

A. Local recurrence-free survival

	Single			Bivariate				
	RR	95% CI	P	RR covar	P covar	RR MRC	P MRC	Interplay
MRC[a]	8.10	0.96-68.9	0.014	-	-	-	-	-
N0/N+	2.49	0.49-12.8	0.23	2.33	0.28	7.92	0.016	No
T1+2/T3+4	1.91	0.46-7.93	0.37	1.37	0.66	7.68	0.020	No
nN>3	4.67	1.05-20.8	0.060	2.77	0.20	6.35	0.045	No
nN>2	5.22	1.17-23.3	0.033	3.39	0.11	6.10	0.047	D_{mrc}[c]
R+/R-[b]	1.52	0.25-9.16	0.64	NA	NA	NA	NA	-

[a] MRC status was analysed instead of molecular margin status as no events were observed in the molecular margin negative group [b] R=extranodal spread. No events were observed in the MRC-/R- group, interaction could not be analysed (NA). [c] D_{mrc} = dominancy of MRC status

B. Locoregional recurrence-free survival

A positive MMS significantly influenced *LRRfs*, with an RR of 8.1 (P=0.0083) as single parameter (Table 3). Other parameters that were significantly related to the *LRRfs* were an advanced T-stage (T3+T4), a high N-stage (N+) and the presence of more than two nodal metastases (nN>2). nN>2 was the strongest prognosticator for *LRRfs* (RR=10.7, P=0.0001). In a bivariate model, this parameter was independent from the MMS. None of the histopathological parameters significantly contributed to *LRRfs*. A summary of the parameters contributing to *LRRfs* is shown in Table 4B.

B. Loco-regional recurrence-free survival

	Single			Bivariate				
	RR	95% CI	P	RR covar	P covar	RR MMS	P MMS	Interplay
MMS[a]	8.10	1.00-65.4	0.0083	-	-	-	-	-
N0/N+	4.22	0.90-19.9	0.033	4.43	0.028	8.93	0.0053	No
T1+2/T3+4	3.89	1.14-13.3	0.021	3.33	0.042	7.52	0.012	No
nN>3	8.78	2.70-28.6	0.0006	5.87	0.0049	5.52	0.052	$D_{nN>3}$[b]
nN>2	10.7	2.99-38.5	0.0001	8.15	0.0006	5.91	0.035	No
R+/R-	2.87	0.57-14.4	0.15	2.32	0.26	7.61	0.015	No

Other parameters that showed significant P-values were T>3, all N-stage caesurae, all nN caesurae. [a] MMS = molecular margin status [b] $D_{nN>3}$=dominancy of nN>3

C. Distant metastases-free survival

The MMS was not a significant prognosticator for *DMfs* in multivariate analysis (P=0.28) (Table 3). The parameter most significantly determining the risk for distant metastases was the presence of more than two nodal metastases (nN>2) (RR=25.7, P=0.0011). In bivariate analysis, this parameter did not show interaction with the MMS. Neither of the histopathological covariates significantly contributed to *DMfs*. Table 4C gives a summary of the findings with regard to *DMfs*.

C. Distant metastases-free survival

	Single			Bivariate				
	RR	95% CI	P	RR covar	P covar	RR MMS	P MMS	Interplay
MMS[a]	2.97	0.32-27.8	0.28	-	-	-	-	-
N0/N+	3.49	0.37-32.7	0.21	3.67	0.20	3.34	0.23	No
T1+2/T3+4	3.14	0.50-19.7	0.21	2.83	0.25	2.81	0.31	No
nN>3	15.1	2.34-97.4	0.0051	13.3	0.010	1.40	0.78	No
nN>2	25.7	2.56-256	0.0011	22.7	0.0021	1.54	0.70	No
R+/R-[b]	NA	NA	NA	NA	NA	NA	NA	-

Other parameters that showed significant P-values were caesurae N>N1, N>N2A, nN>1
[a] MMS = molecular margin status [b] R=extranodal spread. No events were observed in the R- group, multivariate RR or interaction could not be analysed (NA).

D. Disease-free survival

Patients with a positive MMS showed a significantly decreased *Dfs* (RR=5.6; P=0.0055) (Table 3). But also an advanced T-stage (T3+T4), a high N-stage (N+), a high Stage (III+IV), extranodal spread (R+) and the presence of more than two nodal metastases (nN>2) were important prognosticators for *Dfs*. No interaction was observed between the MMS and nN>2 in bivariate analysis. Although in this analysis the relative risk of the MMS (RR=3.7, P=0.048) was three times lower as the RR of nN>2 (RR=10.7, P<0.0001) it was still a significant and independent prognostic factor for *Dfs*. Histopathological covariates did not play a significant role in the models. A summary of the model parameters is shown in Table 4D. A Kaplan-Meier curve and log-rank test is shown for nN≤ 2 vs nN>2 (n=79 cases) (Figure 2A).

D. Disease-free survival

	Single			Bivariate				
	RR	95% CI	P	RR covar	P covar	RR MMS	P MMS	Interplay
MMS[a]	5.55	1.23-25.0	0.0055	-	-	-	-	-
N0/N+	3.99	1.12-14.3	0.013	4.27	0.0103	6.16	0.0030	No
T1+2/T3+4	3.63	1.31-10.1	0.0092	3.15	0.0207	5.18	0.0085	No
nN>3	10.6	3.93-28.6	0.0000	7.50	0.0002	3.41	0.080	$D_{nN>3}$[c]
nN>2	13.8	4.57-41.8	0.0000	10.7	0.0000	3.70	0.048	No
R+/R-[b]	4.23	0.90-19.9	0.031	3.52	0.0656	4.66	0.019	D_{MMS}[d]

Other parameters that showed significant p-values were T>T3, all other N-stage caesurae, Stage (I+II/III+IV), all other nN-caesurae

[a] MMS = molecular margin status [b] R= extranodal spread [c] $D_{nN>3}$= dominancy of nN>3

[d] D_{MMS}=dominancy of molecular margin status

E. Cause-specific survival

The MMS was significantly related to a decreased *Css* (RR=5.2, P=0.0081) (Table 3). However, the presence of more than two nodal metastases (nN>2) involved the highest relative risk for *Css* (RR=14.0, P<0.0001). Other covariates, which significantly contributed to *Css*, were an advanced T-stage (T3+T4), a high N-stage (N+), a high Stage (III+IV) and extranodal spread (R+). When analysed in a bivariate model nN>2 (RR=10.9, P<0.0001) was dominant over the MMS (RR=3.1, P=0.097). Histopathological covariates did not show significant influence on *Css*. Relative risks of all parameters related to *Css* are shown in Table 4E. A Kaplan-Meier curve and log-rank test is shown for nN>2 (n=79 cases) (Figure 2B).

E. Cause-specific survival

	Single			Bivariate				
	RR	95% CI	P	RR covar	P covar	RR MMS	P MMS	Interplay
MMS[a]	5.18	1.15-23.4	0.0081	-	-	-	-	-
N0/N+	4.18	1.17-15.0	0.011	4.75	0.0057	6.22	0.030	No
T1+2/T3+4	3.49	1.26-9.68	0.011	2.97	0.028	4.76	0.014	No
nN>3	9.65	3.60-25.9	0.0000	6.95	0.0003	2.94	0.14	$D_{nN>3}$[c]
nN>2	14.0	4.64-42.3	0.0000	10.9	0.0000	3.10	0.097	$D_{nN>2}$[d]
R+/R-[b]	4.30	0.91-20.2	0.029	3.51	0.067	4.31	0.028	D_{MMS}[e]

Other parameters that showed significant p-values were T>T3, all other N-stage caesurae except N>2C, Stage, clinical nN, nN-score, all nN-caesurae

[a] MMS = molecular margin status [b] R = extranodal spread [c] $D_{nN>3}$= dominancy of nN>3

[d] $D_{nN>2}$= dominancy of nN>2 [e] D_{MMS}= dominancy of molecular margin status

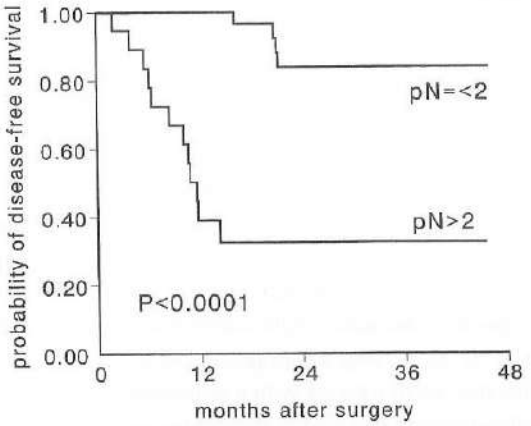

Figure 2A. Kaplan-Meier curve showing disease-free survival in relation to the number of positive lymph nodes (nN > 2 versus nN ≤ 2) for 79 cases. *Patients who died of other causes or remained alive were censored in the analysis at the date of their last clinical follow -up examination or death, respectively. Patients who were not treated by neck dissection, who developed a delayed lymph node metastasis or who were included with a recurrent tumor had missing values for this parameter. The disease-free survival of patients with more than two positive lymph nodes was significantly lower compared to patients with two or less positive nodes.*

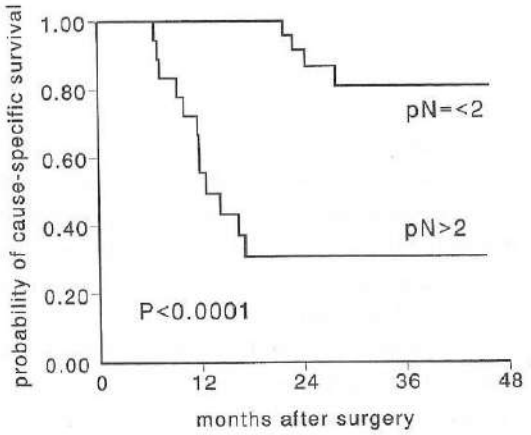

Figure 2B. Kaplan-Meier curve showing cause-specific survival in relation to the number of positive lymph nodes (nN > 2 versus nN ≤ 2) for 79 cases. *Patients who died of other causes or remained alive were censored in the analysis at the date of their last clinical follow -up examination or death, respectively. Patients who were not treated by neck dissection, who developed a delayed lymph node metastasis or who were included with a recurrent tumor had missing values for this parameter. The cause-specific survival of patients with more than two positive lymph nodes was significantly lower compared to patients with two or less positive nodes .*

Conclusions

The prognosis of HNSCC patients has improved only moderately during the recent decades. The locoregional recurrence rates of histopathologically radically treated patients varied between 10-30%[1] and in our series of 79 (128) patients it is 15% (14%). Some progress in patient management has been made after the identification of more sensitive diagnostic tools for the early detection of (minimal) residual head and neck cancer in surgical margins. We recently showed that molecular diagnosis of surgical margins using p53 mutations and HPV E6 as tumor-specific marker has prognostic value (submitted), and we suggested that this new molecular-diagnostic parameter might contribute to a more detailed risk assessment of head and neck cancer by identifying subgroups of patients who require specific treatment strategies. More specifically, as all patients with a molecular-negative margin status remained disease-free (13/32 without having received postoperative radiotherapy), it seems warranted to initiate trials to withhold post-operative radiotherapy from these patients.

So far, the prognosis of patients is still largely based on the conventional T and N staging parameters and particularly the presence and extent of cervical lymph node metastases[38]. In earlier studies these parameters were shown to be associated with a poor prognosis, not only in terms of locoregional recurrence, but also in terms of distant metastases and survival.[1,2]

We showed that for *LRfs* a positive MRC status was the strongest prognosticator, independent from nN>2 and other clinicopathological covariates in multivariate analysis. For all other outcomes, in particular *DMfs*, nN>2 was the most important prognostic parameter. In addition to nN>2, MMS also independently contributed to *LRRfs* and *Dfs*. The prognostic value of the MMS for *LRRfs* and *Dfs* could be explained by its profound effect on predicting local recurrence (for *LRRfs* 8/12 relapses were LR and for *Dfs* 8/17 relapses were LR). In addition, we have strong indications that the MMS partly reflects the status of the regional lymph nodes in the neck. In a previous study (submitted)[7] tumor emboli were detected in (lymphatic) vessels of MRC-positive surgical margins by immunohistochemistry. Obviously, the presence of these lymphatic emboli could reflect the lymph node status, but this was not supported by the proportional hazards analysis of regional recurrence solely, which showed no independent prognostic value for MMS (data not shown).

We have shown that for *DMfs* not the MMS but nN>2 is an important prognostic factor (RR=25.7, P=0.0011). Strikingly, in the present study *DMfs* highly decreased when more than two lymph nodes were present, in contrast to the studies of Leemans et al. (1994,1993)[1,2] who reported increased risks for both local recurrence and distant metastases if more than three nodal metastases were present. There are a number of explanations for this difference. First, the number of patients in our study is limited, and the percentage of distant metastases in our study was rather low (5/79 = 6%). Second, the follow-up time is still limited (DM are likely to occur up to three years after primary treatment). Third, for local recurrence the difference might be explained by postoperative radiotherapy. During the time interval from the previous study (1973-1986) and the current study (1997-2000) the indication for postoperative

radiotherapy has changed. Previously, postoperative radiotherapy was applied if three or more tumor-positive nodes were found in the specimen, wheras today postoperative radiotherapy is indicated also for patients with two histologically positive nodes (with or without extranodal spread).

In a number of studies the significance of extranodal spread as an independent prognostic factor for tumor recurrence has been confirmed.[39-47] Postoperative radiotherapy is likely to be effective whenever the tumor has grown through the capsule of the lymph node. Extranodal spread might have confounded the relation between the presence of nodal metastases and outcome in our study. It might be argued that extranodal spread is more frequently observed with higher nodal stages. A test for linear trend confirmed this finding (P=0.0004). In fact, all cases with more than two positive lymph nodes showed extranodal spread. However, as the relative risks of extranodal spread were generally much lower or not significant as compared to the number of nodes, it did not seem to have a strong influence on clinical outcome. For *DMfs*, an increased proclivity of the tumor cells to spread to more nodes and the blood stream seems more important than passing the capsule. The number of involved lymph nodes is a stronger predictor of clinical outcome than the standard N-staging parameters (N0-N3).

In addition to the clinicopathological parameters a high number of histopathological parameters have been analysed in this study, but none of these appeared to have significant prognostic value in this patient group. Our findings for the grade of dysplasia are in contrast with the recent retrospective study of Weijers et al. (2002).[12] In a small and carefully selected patient population (37 cases with tumor-free margins, no severe dysplasia, no postoperative radiotherapy and maximal follow-up of 5 years) they showed that the presence of mild or moderate epithelial dysplasia in the margins of surgically removed oral SCC carries a significant risk for the development of local recurrence (P<0.01). Possible explanations why we did not find a significant relation between the grade of dysplasia and local recurrence might be: 1) the heterogeneity of the study population (e.g. with regard to tumor locations and postoperative radiotherapy, although these factors did not statistically differ between the MMS+ and MMS- groups) and 2) the relatively short follow-up time, as field recurrences might still occur after 10 years.[48] Obviously, another problem that might have occurred is the subjectivity in evaluating oral dysplasia, limiting the achievement of a clinically reliable diagnosis on the basis of epithelial dysplasia.[49-51] Therefore in recent years the more objective grading using genetic alterations or DNA ploidy is under investigation.[14,48,52,53]

In addition to the histopathological features, the molecular tumor status did also not provide prognostic information in our study. The relationship between p53 mutation and HNSCC prognosis is controversial, since conflicting results have been published. Some studies reported that p53 mutations/p53 overexpression were not associated with survival and/or recurrence rates.[54-56] whereas others demonstrated prognostic significance and/or an association with patterns of treatment failure.[22-24] However, the majority of the studies on this subject did not investigate p53 mutations by sequencing but only examined p53 protein expression in relation to disease parameters. It is known that immunohistochemical detection

of p53 expression is not always correlated to its mutation status.[55] Moreover, some studies have shown that DNA contact mutations were associated with higher tumor stages, a higher incidence of lymph node metastasis, a shortened recurrence-free survival and cause-specific survival in head and neck cancer patients.[25-27] In our study, neither the presence nor the type of p53 mutations (DNA contact mutations) seemed to influence clinical outcome.

Another tumor marker that has been investigated in this study is the HPV status of the primary tumor. Some studies indicated that a positive HPV status of the primary tumor is a significantly favorable prognostic factor in HNSCC (mainly tonsillar tumors).[23,28,32,33] Recently, it was reported that HNSCC patients with HPV16 in the primary tumor showed a better local control and survival which was attributed to an increased radiosensitivity.[34] However, HPV was also not a significant prognostic factor in the current study.

In summary, our findings of different prognostic values in HNSCC may supplement clinical assessment. The presence of more than two nodal metastases is the most important prognosticator for *LRRfs*, *DMfs*, *Css*, and *Dfs*. A molecular-positive margin status is the strongest and only prognosticator for *LRfs*, and thereby has predictive value for *LRRfs*, *Css* and *Dfs*. These findings might be clinically used for a more individualized risk assessment and patient management.

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8

General Discussion

General discussion

Despite the improvements in surgery and radiotherapy over the last decades, the 5-year survival rates of patients with head and neck squamous cell carcinoma only moderately increased. A major reason for this discrepancy is the relatively high frequency of local recurrences: even when the surgical margins have been diagnosed as tumor-free by histopathology the local recurrence rate is still 10 to 30%.[1] These data suggest that either residual tumor cells (minimal residual cancer) remain undetected in the surgical margins by the current diagnostic methods, or the risk for progression of preneoplastic lesions in the surgical margins is underestimated. The routine histopathological examination of surgical margins serves the two-fold aim of screening for residual tumor as well as to assess the presence and grading of preneoplastic lesions. If residual tumor is detected, re-resection and/or post-operative radiotherapy are considered. Besides the screening for tumor, the pathologist inspects the epithelium for dysplasia. Epithelial dysplasia is a premalignant condition morphologically characterized by aberrant differentiation, atypia, and mitotic activity in more superficial layers. According to the WHO classification dysplasia is graded as mild, moderate and severe[2]. This grading is, however, highly subjective and associated with a large intra- and inter-individual variation[3-5]. Contradictory results have therefore been obtained with regard to the prognostic significance of dysplasia in resection margins[6-12], and only the presence of severe dysplasia in resection margins is currently considered for treatment planning. The presence of mild or moderate dysplasia in resection margins is not used for clinical management of HNSCC patients.

The research presented in this thesis focuses on the prognostic value of the molecular detection of cells clonally related to the tumor in surgical margins of HNSCC patients, and elucidation of the underlying pathobiology of recurrence at the primary site. Two molecular markers were exploited in these studies: p53 mutations and HPV16 E6 transcripts. Our investigations support their suitability as tumor-specific clonal markers (Chapters 3 and 4). Furthermore, in a pilot study of 30 patients it was shown that using p53 mutations as marker minimal residual cancer and preneoplastic lesions are detected. Moreover, it was shown that DNA-based plaque assays might lead to false-positive observations (Chapter 5). Nevertheless, in a subsequent large prospective clinical study of 128 patients with histologically tumor-free resection margins it was demonstrated that the molecular margin status significantly predicted local recurrence-free survival, disease-free survival and cause-specific survival (Chapter 6). Multivariate analysis indicated that the molecular margin status was the single most important prognosticator for local recurrence-free survival, whereas for other outcomes the presence of more than two lymph nodes was the most important parameter (Chapter 7). In general, our results strongly support the exploitation of molecular diagnosis for a more accurate assessment of surgical margins.

Molecular diagnosis of minimal residual disease

Circulating tumor cells have been noticed by clinicians and investigators since 1869[13]. However, the number of circulating tumor cells is generally low, and only since the development of techniques with a high sensitivity and specificity this field has increasingly obtained attention. A major breakthrough was the implementation of anti-cytokeratin immunocytochemistry (ICC) of blood and bone marrow aspirates which was exploited in a large number of studies dealing on a variety of solid tumors[14-19]. Particularly the bone marrow has been identified as an important site for detection of hematogeneously disseminated cancer cells. Some studies showed a large clinical impact for the assessment of disseminated tumor cells in bone marrow aspirates[20-22]. Recently, Braun et al. (2000)[22] demonstrated the clinical significance of detection of disseminated tumor cells (DTC) in bone marrow of patients with breast cancer. The presence of DTC in the bone marrow was significantly correlated to a poor survival and disease-free survival, and provided prognostic information comparable to the lymph node status. However, the techniques for rare cell detection are difficult and small methodological details such as the antibody, the staining substrate etc. determine the sensitivity, specificity and robustness of the assay. Variations in methodology can often explain discordant results between studies[23].

In recent years, molecular diagnosis has not only gained attention for assessment of hematogeneous spread of cancer cells to secondary organs such as the bone marrow, but also for analysis of tumor cells in other tissues or body fluids such as resection margins, lymph nodes, urine and saliva[24-26]. For these purposes, attention has shifted towards the use of tumor-specific markers, usually DNA markers, in combination with DNA amplification methods based on the polymerase chain reaction. However, these promising new molecular assays and the exploited markers still need to be validated in large clinical trials. The final aim of this research field is to exploit molecular techniques for tailoring adjuvant therapy, and to increase the knowledge on the pathobiology of recurrence and metastasis. In this thesis research was focused particularly on the molecular diagnosis and prognostic value of minimal residual head and neck cancer in surgical margins. Moreover, our data gave also interesting clues on HNSCC etiology, carcinogenesis and the pathobiology of recurrence at the primary site.

HNSCC carcinogenesis

Although most patients present with invasive carcinomas, several lines of evidence suggest that head and neck cancer develops through a number of histologically well-defined precursor lesions. Califano et al. (1996)[27] demonstrated that the progressing histopathological changes run in parallel with an increase in the number of genetic changes. This elegant genetic progression model described the early and late genetic events in the progression to invasive carcinomas, facilitating the selection of genetic markers for molecular diagnosis and carcinogenesis research. The drawback of this model is that it uses histological grading as "gold standard", and that it does not distinguish between the different biological stages that are of clinical relevance. Particularly the development of large preneoplastic fields is a distinct biological stage in HNSCC carcinogenesis with important clinical consequences. On the basis

of the data presented in Chapter 5 we deduced a more clinically and biologically oriented model for HNSCC carcinogenesis in which four biological stages are distinguished: 1) the switch from a normal epithelial stem cell to a genetically altered stem cell, 2) the switch from a single genetically altered stem cell into a large preneoplastic field of genetically altered cells, 3) the progression of a clone in the field into invasive carcinoma, and 4) the development of metastatic carcinoma. In addition to the p53 mutated "fields", we demonstrated with p53-immunostaining the presence of small p53 mutated "patches". These patches most likely correlate with the units of stem cells, amplifying cells and differentiated cells that make up the normal mucosa and skin. When a stem cell acquires a genetic alteration, its derived daughter cells will contain the same change, explaining the observed p53-immunostained patch. The mutations found in these patches were never identical to the mutations determined in the corresponding tumor. The development of these patches might frequently occur in the mucosal epithelium of heavy smokers or other risk groups[28]. When additional genetic hits take place in the genetically altered stem cell, it might gain growth advantage, laterally displacing the normal mucosa and causing a field to develop that can extend as large as 10 cm in diameter. Individuals with genetically altered patches and fields in the mucosa will have an enormously increased risk for development of invasive carcinomas. In this respect it is important to consider that normal mutation rates are extremely low (approximately 10^{-5} per generation)[29] and that at least 5-10 genetic events are required for progression into cancer[27,30]. This would suggest that cancer development is an extremely rare event, hardly occurring in the world population. Two important factors cause an enormous increase in cancer risk despite the low normal mutation rates. First, genome stability is decreased by the initial genetic hits, accelerating the mutation rate. Second, the development of large preneoplastic lesions such as the fields in HNSCC oncogenesis, increases the number of genetically altered cells that might undergo subsequent transformation[31,32]. Much more cells are therefore present with an increased risk for progression while their genomic stability has decreased. When an invasive carcinoma develops in a field that is diagnosed and treated, then part of the field might remain unresected causing a high risk for new tumors to develop. The p53-immunostained fields that were observed in our studies in the resection margins all showed the same mutation as the corresponding tumor, indicating a monoclonal origin. The new carcinomas that might develop in these unresected fields can be diagnosed as local recurrences or second primary tumors depending on the distance from the index tumor (>2 cm) and the interval (>3 years). The challenge of the coming years will be to determine the critical molecular steps in this model.

Molecular diagnosis of HNSCC in surgical margins

In Chapters 5-7 the prognostic impact of molecular assessment of surgical margins in patients with head and neck squamous cell carcinoma was presented. The most important clinical finding was that local recurrence-free, disease-free and cause-specific survival were all significantly worse in patients with molecular-positive margins when compared to patients with molecular-negative margins. For local recurrence-free survival, the molecular margin

status was the most important independent prognostic factor (RR=8.1, P=0.014), whereas for other outcomes, particularly distant metastases-free survival, the presence of more than two lymph node metastases was the most important risk factor (distant metastases-free survival: RR=25.7, P=0.0011). Particularly when the molecular margin status is combined with the N-stage, risk groups can be identified that are eligible for different (experimental) adjuvant therapy protocols.

A special risk group encompasses the patients presenting with tumor-related mucosal precursor lesions (fields) that show a clonal relationship with the primary tumor. These mucosal precursor lesions are often not completely resected and might give rise to new invasive carcinomas. In one patient this seemed to be the case. Theoretically, it might be of clinical significance to make a distinction between the two types of residual lesions, fields or cancer cells (MRC), as the presence of remaining cancer cells in the resection margins would logically lead to a higher local recurrence rate in a limited time frame. In our study the distinction between field or MRC appeared not to be of clinical significance. This could be related both to the relatively small field group, differences in treatment between the groups, or that besides the identified field, these field patients might have had also residual cancer cells in the margin that could not be detected separately. Notwithstanding, a positive molecular margin was the only strong prognostic factor for local recurrence-free survival.

Perspectives

The follow-up period of our study is still relatively short, particularly as patients remain at risk for local failure for at least two years following initial diagnosis and therapy. Therefore a longer follow-up interval might reveal more patients who develop a local recurrence or other failures. Moreover, patients with fields might develop new invasive carcinomas after prolonged time intervals (>3 years) only. It might also be worthwhile to increase the sample size and consider the p53 group as a separate entity, excluding the HPV cases. Several studies have indicated that tumors caused by HPV infection have a better prognosis. Although this observation could not be confirmed in this study, it was remarkable that we did not observe local recurrences in any of the HPV patients.

There are a few limitations to the approaches used in this thesis. P53 mutations are present in 50-80% of the head and neck cancers and therefore not all patients are eligible for study of the margins. This observation triggered the search for additional markers, and in Chapter 4 it was shown that HPV16 DNA and RNA are present in an additional 10-20% of the head and neck cancers, cases all negative for p53 mutations, which solves part of this problem.

The second limitation is that we need to consider that DNA-based assays cause false-positive results. There are three observations that indicated this problem. First, the high frequency of positive molecular margins using mutated p53 as marker that could neither be explained histologically nor on the basis of clinical outcome (29/47: 62%). Also when using HPV DNA as marker 5 out of 8 (63%) patients analyzed were positive in one or more resection margins while none developed a local recurrence. Second, nine patients who were

molecular-positive (mutated p53) and negative for fields, and who did not receive post-operative radiotherapy, still remain disease-free (follow-up 10-39 months, mean 22 months). Third, samples of exfoliated cells taken contralaterally were positive for tumor DNA in most of the analyzed patients, suggesting contamination via the saliva. Surgical margins might also be contaminated by DNA via the lymph. A solution might be to use RNA instead of DNA for analysis. Using RNA-based assays, we could demonstrate that the frequencies of positive results are much lower. For example 0/10 cases analyzed with HPV E6 RT-PCR showed a positive molecular margin-status, which was in agreement with the clinical outcome, whereas 5/8 cases were positive using DNA-based assays, a significant difference (P=0.007, Fisher's exact test). Also margins of patients with p53-mutated tumors that were shown to be positive in DNA-based assays became negative when RNA-based assays were exploited, again in agreement with the clinical outcome. These observations support our hypothesis that DNA-based analysis might give rise to false-positive results, which might be overcome by RNA-based analysis.

A third drawback of the use of p53 mutations as clonal marker is that the current detection techniques are too laborious and complex for routine implementation. Only HPV-E6 transcript analysis seems a reliable and simple technique that might deserve implementation for MRD detection. The development of novel robust and reliable methodologies to detect mutated DNA in a background of wild type DNA is necessary to ensure acceptance of molecular diagnosis of surgical margins in the clinical management of head and neck cancer patients. The most recent development, PPEM (PNA directed, Primer Extension, MALDI-TOF) which is a variant of a mass spectrometry detection method[33] seems a promising technology which might pave the way for large and clinically applicable MRD detection programs in upcoming trials.

Future implications for patient management

The clinical data presented in Chapters 6 and 7 might be of value for different groups of patients. First, it is noteworthy that none of the patients with a molecular-negative margin status developed a local recurrence (follow-up 4-46 months, mean 23 months). Particularly important in this respect is that 13 of 32 molecular-negative patients who did not receive postoperative radiotherapy all remained disease-free (follow-up 11-42 months, mean 23 months). These encouraging data might allow setting up a subsequent clinical trial to tailor adjuvant radiotherapy on the basis of molecular margin analysis. The group of patients that might benefit from this tailored approach are those with a T3-T4 carcinoma in oral cavity and oropharynx with a pN0 or pN1 neck (without extranodal spread). At our hospital, like in many centers around the world, these patients currently receive postoperative radiotherapy with its associated morbidity based on T-stage. A subgroup of these patients might be spared post-operative radiotherapy on the basis of molecular margin analysis. Moreover, as HNSCC patients are at risk for second primary tumors, the treatment option of radiotherapy is retained.

A second group of patients that might benefit from molecular margin analysis are those who show a positive MRC status (molecular-positive margins but no p53-mutated field). Our

data are in support of adjuvant treatment (surgery + post-operative radiotherapy) for these patients. However, the rate of false-positive findings when using DNA-based plaque assays causes a problem. From the 47 patients (of in total 79 patients analyzed) with a positive molecular margin status 17 were not treated by post-operative radiotherapy. In total 8 of these 17 cases were correctly assigned as molecular-positive as they developed a local recurrence (3) and/or showed a field (6). Nine molecular-positive cases who were not treated by post-operative radiotherapy could neither be explained histologically nor clinically and might be considered as false-positive (53%) (follow-up 11-39 months, mean 22 months). It is difficult to decide whether the percentage of overtreatment that would result from this false-positive rate is acceptable against the percentage of patients who might have had benefit from adjuvant post-operative radiotherapy (3/17: 18%) based on molecular analysis.

A third group of patients that might benefit from molecular assessment of the surgical margins are the patients who show a mutated p53 field. These patients are at increased risk to develop local recurrences and second primary tumors and should be followed at increased frequencies for prolonged time intervals also after 5 years of follow-up[34]. In the Netherlands control visits usually decrease in frequency after five years of follow-up, while in many foreign countries follow-up visits are always relatively infrequent. For HNSCC patients with mutated p53 fields, lifelong follow-up visits at regular intervals (e.g. 3 times/year) seem indicated. Although development of new carcinomas cannot be prevented currently, early diagnosis allows curative treatment with less invasive procedures. To identify a subgroup of these patients with p53-mutated fields a p53 immunostaining could be included in the histopathological analysis of the resection margins besides the routine haematoxylin-eosin staining.

From the patients who show genetically altered fields in the resection margins only a subgroup will demonstrate fields that can be detected by mutated p53 overexpression. Using LOH analysis Tabor et al. (2001)[32] showed in 25% of the HNSCC patients tumor-related genetically altered fields in the resection margins, and only in 4/7 these fields also showed tumor-related p53 mutations. It will be a challenge for the future to develop innovative methods for identifying individuals with genetically altered fields and to allow accurate grading of these fields. Detailed profiling using genetic markers[32] or DNA aneuploidy[12] measurement shows promise in this respect. Therapeutic treatment of these fields might be reached by the application of oncolytic viruses such as ONYX-015[35-37], viruses that specifically eradicate cells with inactivated damage response pathways.

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Summary

Summary

Chapter 1 comprises a general introduction to the field of molecular diagnosis in head and neck cancer research and the problems currently encountered in the diagnosis of HNSCC. Highlighted are the properties of molecular markers, particularly focused on mutations in the p53 gene and HPV.

In **Chapter 2** a technical improvement of the plaque assay, the molecular method used in this thesis, is described. The labeling of various oligonucleotides by polynucleotide kinase was analysed, thereby varying the source of kinase, purity of the oligonucleotide and the 5' terminal nucleotide. It was shown that the labeling efficiency of oligonucleotides strongly depends on the 5' nucleotide, a dC at the 5' end rendered very low efficiencies (10 ± 5%) while a dG at the 5' end rendered the highest efficiencies (60 ± 5%).

In **Chapter 3** the suitability of p53 mutations and other genetic alterations as clonal markers is described. A number of discordant reports were described in the literature. By sequencing the p53 DNA of both primary HNSCC (n=23) and the corresponding lymph node metastases (n=25) and/or distant metastases (n=10), we could show that in all cases the same p53 mutation was detected, supporting its use as a clonal marker.

In **Chapter 4** the suitability of HPV as clonal marker for HNSCC was investigated in cases where the p53 gene is not mutated. We demonstrated that HPV16 appears to play an etiological role in 9/84 (10%) of head and neck tumors, and is usually overestimated by the very sensitive HPV-DNA assays. Moreover, we demonstrated that the virus remains actively present in lymph node metastases of these tumors, supporting its etiological role in these tumors and its use as tumor-specific clonal marker for molecular detection.

In **Chapter 5** the use of mutated p53 as molecular marker using genomic DNA as template for the detection of cells clonally related to the tumor in surgical margins of 30 head and neck cancer patients is evaluated. Using molecular and immunohistochemical analyses both residual tumor cells and unresected tumor-related mucosal precursor lesions ("field") were detected within molecular-positive surgical margins. Further drawbacks of using genomic DNA as template were investigated. Moreover, p53 mutated "patches" were detected in the mucosal margins which might be the precursor stage of field lesions. On the basis of these data we proposed an adapted HNSCC progression model.

In **Chapter 6** a prospective study of 128 radically treated head and neck cancer patients was reported. Using p53-mutations and HPV16-E6 transcripts as molecular markers, cells clonally related to the tumor were detected in histopathologically tumor-free surgical margins. This study demonstrated that a molecular-positive margin status is significantly associated with a decreased local recurrence-free survival, disease-free survival and cause-specific survival as determined by Kaplan-Meier analysis and log-rank tests.

In **Chapter 7** the prognostic value of a positive molecular margin status was determined in relation to other clinico- and histopathological prognosticators for HNSCC, such as lymph node status. Cox Proportional Hazards models showed that for local recurrence-free survival the molecular margin status is the strongest independent prognosticator, whereas for other outcomes, in particular distant metastases-free survival, the presence of more than two lymph node metastases is the most important prognosticator of survival and disease-free survival. These data support our previous findings that the molecular margin status is an important tool to select patients at high risk for local tumor recurrence, which supports its future use for a more individualized treatment planning.

Nederlandse samenvatting

Samenvatting

In **Hoofdstuk 1** wordt de hedendaagse problematiek rond de diagnose en behandeling van het hoofd-halsplaveiselcelcarcinoom (HHPCC) geschetst, alsmede een algemene inleiding in de moleculaire diagnostiek gegeven. Belangrijke aandachtspunten zijn de eigenschappen van moleculaire markers, in het bijzonder mutaties in het p53 gen en humaan papillomavirus (HPV).

In **Hoofdstuk 2** wordt een technische verbetering van de plaque assay beschreven, de moleculaire methode die gebruikt wordt in dit proefschrift. De radioactieve labeling van verschillende oligonucleotides met behulp van het enzym polynucleotide kinase werd geanalyseerd, waarbij afwisselend het enzym, de zuiverheid van het oligonucleotide en het 5' nucleotide werden gevarieerd. Aangetoond werd dat de labelingsefficiëntie van oligonucleotiden sterk afhangt van het 5' nucleotide, waarbij een dC aan het 5' uiteinde hele lage efficiënties ($10 \pm 5\%$) oplevert, terwijl een dG aan het 5' uiteinde de hoogste efficiënties ($60 \pm 5\%$) laat zien.

In **Hoofdstuk 3** wordt de bruikbaarheid van p53 mutaties en andere genetische veranderingen als klonale markers aan de orde gesteld. In de literatuur zijn hierover tegenstijdige studies gepubliceerd. Door basevolgorde bepaling van het p53 gen van zowel primaire HHPCC (n=23) als bijbehorende halskliermetastasen (n=25) en/of afstandsmetastasen (n=10), konden wij aantonen dat in alle gevallen dezelfde p53 mutatie voorkomt, hetgeen een ondersteuning vormt voor het gebruik van p53 mutaties als klonale marker.

In **Hoofdstuk 4** is de bruikbaarheid van HPV als klonale marker voor HHPCC onderzocht in die gevallen waarin het p53 gen niet gemuteerd was. Wij toonden aan dat HPV16 een etiologische rol blijkt te spelen in 9/84 (11%) van de hoofd-halstumoren, en dat de rol van het virus gewoonlijk wordt overschat in het onstaan van deze tumoren door de zeer gevoelige HPV-DNA tests. Bovendien blijkt dat het virus aktief aanwezig blijft in halskliermetastasen van deze tumoren. Dit ondersteunt de etiologische rol van het virus in deze tumoren en ook het gebruik van HPV als tumor-specifieke klonale marker voor moleculaire detektie.

In **Hoofdstuk 5** wordt ingegaan op het gebruik van gemuteerd p53 als moleculaire marker. Hierbij werd gebruik gemaakt van genomisch DNA als template voor de detektie van cellen, klonaal gerelateerd aan de tumor in de chirurgische snijvlakken van 30 HHPCC patienten. Gebruikmakend van moleculaire en immuunhistochemische analyses werden zowel residuele tumorcellen als achtergebleven tumor-gerelateerde mucosale precursor lesies ("veld") waargenomen in moleculair-positieve chirurgische snijvlakken. Voorts werden enige nadelen van het gebruik van genomisch DNA als template geëvalueeerd. Bovendien werden "patches" van cellen met p53 mutatie(s) waargenomen, die de voorloper stadia zouden kunnen zijn van de veldlesies zoals gezien in de chirurgische snijvlakken. Deze data vormden de basis voor een aanpassing van het progressie model voor HHPCC.

In **Hoofdstuk 6** wordt een prospectieve studie beschreven van 128 hoofd-halskankerpatiënten die histologisch radicaal werden behandeld. Gebruikmakend van p53 mutaties en HPV16-E6 transcripten als moleculaire markers werden cellen, klonaal gerelateerd aan de tumor, gedetekteerd in histopathologisch tumor-vrije chirurgische snijvlakken, en deze bevinding werd gekorreleerd aan het klinisch beloop. Deze studie toonde aan dat een positieve moleculaire margin significant geassocieerd is met een verlaagde locaal recidief-vrije overleving, ziekte-vrije overleving en oorzaak-specieke overleving na Kaplan-Meier analyse en log-rank tests.

In **Hoofdstuk 7** wordt de prognostische waarde van een moleculair-positieve status van de snijvlakken in vergelijking met andere klinische- en histopathologische voorspellende factoren- voor het beloop van HHPCC zoals de halsklierstatus, bepaald. Cox Proportional Hazards modellen toonden aan dat de moleculaire status van de snijvlakken de sterkste voorspellende factor is voor een verlaagde locaal recidief-vrije overleving, terwijl voor andere parameters van het klinisch beloop, in het bijzonder afstandsmetastasen-vrije overleving, de aanwezigheid van twee of meer halskliermetastasen de belangrijkste voorspellende factor is. Deze data onderbouwen onze eerdere bevinding dat de moleculaire status van de snijvlakken het belangrijkste instrument is om patiënten te selekteren die een hoge kans hebben op het ontwikkelen van een lokaal recidief. We hopen op basis hiervan in de toekomst een meer individueel behandelingsplan te kunnen ontwikkelen.

Dankwoord

Nu u de moed hebt gehad om dit proefschrift te lezen of althans door te bladeren tot deze pagina, is de tijd rijp om ***iedereen*** te bedanken die een bijdrage heeft geleverd aan de totstandkoming van dit levenswerk. Het beschreven onderzoek was slechts mogelijk door de inzet van velen, waarvan ik er toch een aantal apart wil noemen ondanks het risico mensen te vergeten.

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Op een gedeelde eerste plaats wil ik graag Ruud Brakenhoff, mijn copromotor, bedanken. Beste Ruud, jouw niet te stuiten drang om het maximale uit dit "p53 onderzoek" te halen heeft mijn enthousiasme voor de wereld van de moleculaire biologie en het kankeronderzoek vanaf het begin aangewakkerd en gestimuleerd. Jouw niet geringe wetenschappelijke inbreng heeft ertoe bijgedragen dat ik nu met trots dit boek kan presenteren, iets waarvoor ik je zeer erkentelijk ben. Jouw onderzoekersspirit en nieuwe ideeën hebben op mij altijd zeer motiverend gewerkt. Ik deel inmiddels je grenzeloze enthousiasme om ooit de New England te behalen! Ruud, bedankt voor alle ondersteuning bij het afronden van dit proefschrift, ook (en meestal) op onmogelijke tijdstippen.

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Maar naast Fedor verdient ook Janny Dijkstra hier een speciaal plekje. Janny, jij bent nu heel druk met de "laatste restjes". We gaan het redden!

Na deze "specials" natuurlijk dank aan alle (ex)collega's van de afdeling Tumorbiologie. Bedankt voor de fijne samenwerking en jullie hulp, maar zeker zo belangrijk, voor de gezellige onderlinge sfeer, op het lab (wie heeft dit nu weer opgemaakt!), op de AIO kamer (mag ik nu even achter de PC?), tijdens de lab-uitjes (nat pak) en de

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Ook de pathologen, Paul van Diest, Johan Westerga, Jacqueline van der Wal en met name Alain Kummer, hebben een prominente rol gespeeld in dit onderzoek. Jullie hebben de niet altijd even dankbare taak op jullie genomen na OK tijd een "p53 defect" uit te snijden. Ik kon jullie op de meest exotische tijdstippen komen lastigvallen met een preparaat en jullie hebben deze taak altijd met veel inzet uitgevoerd. Alain en Jacqueline, ook enorm veel dank voor de (eindeloze) revisies. Ik heb door dit onderzoek veel respect gekregen voor jullie werk als patholoog! In dit kader wil ik ook Wim de Jong niet vergeten. Beste Wim, dankzij jouw hulp bij het uitsnijden hoefden we niet altijd tot in de avonduurtjes door te werken!

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Bij de Universiteit van Maastricht ben ik door Erik Thunnissen en Veerle Somers gastvrij ontvangen om experimenten te kunnen uitvoeren aan P53 point exacct. Dit "uitstapje" naar het zuiden was een welkome afwisseling in de dagelijkse plaque assay beslommeringen.

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Binnen de VU wil ik verder met name de gewaarde hulp van de fotografen Hans en Jaap (PA) en René den Engelsman (AVC) niet onvermeld laten. Met jullie hulp waren mijn presentaties toch altijd weer net op tijd klaar! Op deze plaats wil ik ook Mieke en de andere medewerk(st)ers van de KNO poli enorm bedanken voor hun hulp bij het verzamelen van alle patiëntenstatussen. En niet te vergeten Renée van Wegen van het secretariaat KNO, bedankt voor je logistieke ondersteuning en het up-to-date houden van mijn "dossier".

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Viola

Curriculum Vitae

Viola van Houten werd op 1 februari 1975 geboren te 's-Hertogenbosch. In 1993 behaalde zij haar gymnasium diploma aan het St. Jans Lyceum te 's-Hertogenbosch. In datzelfde jaar begon zij met de studie Biomedische Gezondheidswetenschappen aan de Universiteit Nijmegen. Zij behaalde haar Propaedeuse cum laude en koos vervolgens voor de specialisatierichting Toxicologie. Tijdens de doctoraalfase deed zij twee stages. De eerste stage liep zij op het Provinciaal Bureau Medische Milieukunde (Drs. H. Jans) te Breda alwaar zij een onderzoek uitvoerde naar verbetering van de registratie van milieugezondheids-klachten. De hoofdstage deed zij op Laboratorium voor Effectenonderzoek (Dr. H. J. van Kranen) van het Rijks Instituut voor Volksgezondheid en Milieuhygiëne (RIVM) te Bilthoven. Hier heeft zij met behulp van sequentie analyse van het p53 gen onderzoek gedaan naar de invloed van UV straling op het ontstaan van huidkanker bij muizen. In Augustus 1997 werd het doctoraal diploma Gezondheidswetenschappen behaald. In september van datzelfde jaar begon zij aan het promotieonderzoek, beschreven in dit proefschrift, bij de afdeling KNO-Tumorbiologie van het VUMC te Amsterdam, onder begeleiding van Prof. Dr. G.B. Snow en Dr. R.H. Brakenhoff. Sinds november 2001 is zij werkzaam als Clinical Research Associate bij NDDO Oncology B.V.

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