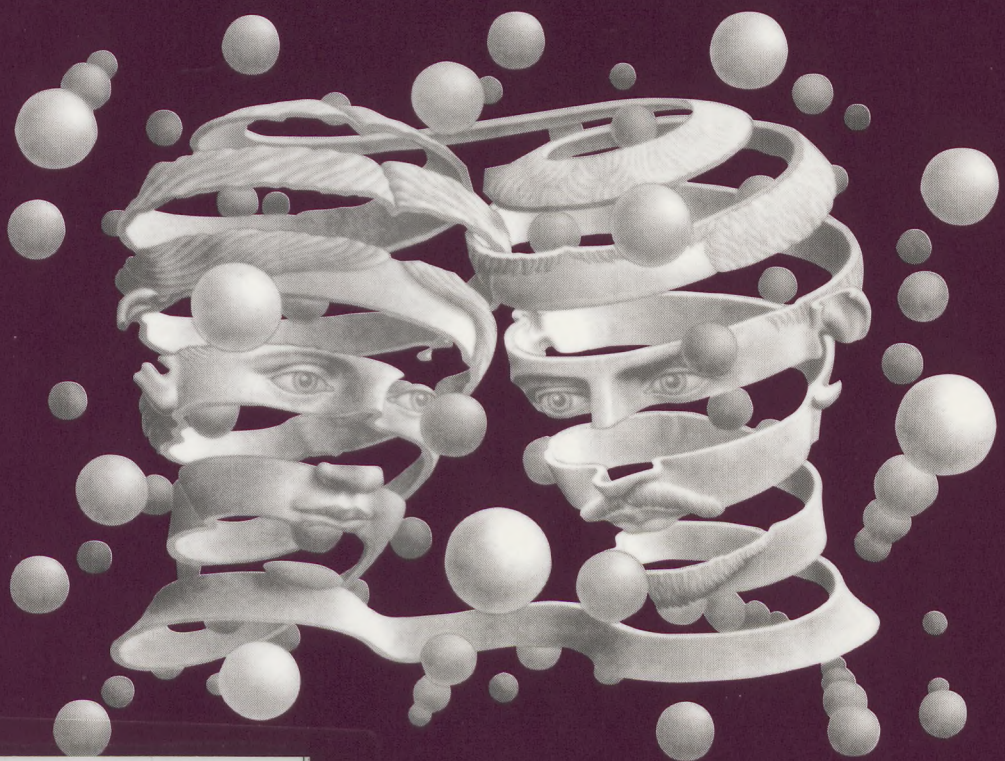
Oral Field Cancerization

Smoking induced mucosal alterations and their significance for the development of multiple head and neck tumors



Monique van Oijen

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Smoking induced mucosal alterations and their significance for the development of multiple head and neck tumors

Met roken samenhangende veranderingen in het mondslijmvlies
en hun belang voor de ontwikkeling van meerdere hoofd-halstumoren
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
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ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op dinsdag 26 oktober 1999, des namiddags te 16.15 uur

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Monique van Oijen

geboren op 8 april 1972 te Rossum

Promotor: Prof. Dr. P.J. Slootweg

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Dr. M.G.J. Tilanus

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"Te weten wat men weet en
te weten wat men niet weet,
dat is kennis"

Confucius

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Chapter 1

General introduction

Epidemiology

Next to cardiovascular diseases, cancer is the greatest cause of death in Western Europe and the U.S.A. in adults [1]. Cancer is defined as growth of tissue resulting from a continuous proliferation of cells with a selective growth advantage. This aberrant growth is supposed to occur after an accumulation of genetic alterations in oncogenes and tumor suppressor genes [2].

Head and neck cancer comprises cancers in the mouth (lip, gum, tongue, palate, floor of mouth and cheek), (para)nasal cavities, pharynx and larynx. It is worldwide the sixth most common malignancy in men and accounts for approximately 5% of malignant tumors in developed countries [1,3,4]. However, in parts of South East Asia, head and neck cancer is the most common malignancy accounting for up to 50% of malignant tumors in these regions [1,5]. These patterns reflect the prevalence of the specific risk factors in these geographic places; tobacco and alcohol in the developed countries and chewing of betel quid in South East Asia.

The average male to female ratio of the patients with head and neck cancer in developed countries is 3:1, but differs for the various anatomical locations [1]. In the last decades the incidence of head and neck cancer in females is rising, which seems to be related to an increase in smoking habits in women, whereas the incidence in males has stabilized [6]. In general these tumors develop from the fifth and sixth decade of life, with an increasing incidence at older ages [1].

Squamous cell carcinomas account for 90% of the head and neck cancers. According to the most recent data of the Dutch cancer registration, in 1995, 2029 new cases of head and neck squamous cell carcinomas (HNSCCs) occurred in the Netherlands with a population of 15.4 million inhabitants, whereas 619 patients died as a result of it in that same year [7]. This incidence is likely to rise in the future as a result of increasing mean age.

Prognosis and treatment of HNSCC

Survival of HNSCC patients depends on tumor extension, nodal stage and success of initial treatment. Survival rates of patients have improved remarkely since 1920, when less than 20% survived for more than 5 years. In general, nowadays a 5-year survival rate of 50-70% can be obtained, although some anatomic sites carry a less favorable prognosis than others. Unfortunately, overall survival rates have not improved very much in the last decades [8-11]. The incidence rate of second primary tumors is 10-

35%, depending on both the location of the first primary tumor and the age of the patient [12-15]. These second primary tumors adversely influence the prognosis of HNSCC patients [14].

Radiotherapy plays an important role in the treatment of HNSCC, sometimes in combination with surgery. Surgery, when indicated, can not only cause disfigurement but can also have a great impact on for example food intake and speech. Chemotherapy might be added as a treatment modality to radiotherapy in very advanced cases or as a sole palliative treatment to patients with locally recurrent disease or to patients with distant metastases [8,9,16,17].

Furthermore, the development of HNSCC could be prevented by detection and treatment of premalignant conditions. In theory, heavy burden of oral cancer, particularly in South-East Asia, could be reduced by programs of screening for pre-cancerous lesions [5,18-20]. The problem is that, to be effective, screening programs must be well organized and work in a defined, coordinated manner. These conditions usually are difficult to achieve in developing countries, where 78% of cases occur [1].

Aetiology

Tobacco and alcohol

Tobacco smoking and alcohol drinking are the two best recognized risk factors (Figure 1). Ninety percent of the HNSCC develop in smokers and drinkers. Studies of the epidemiology of HNSCC have identified tobacco and alcohol use as independent risk factors but when both factors are combined there is a synergistic effect [4,21-25]. Individuals that smoke more than 20 cigarettes a day and use more than 100 g of alcohol (equivalent of 1 liter wine) have a 200 times increased risk for head and neck cancer [21]. Among those tobacco products not only cigarettes and cigars are risk factors, but also snuffs and chews (with e.g. betel quid that consists of the leaf of the betal vine, areca nut, lime or tobacco) [4]. The more tobacco an individual uses per day and the longer this abuse persists, the more the risk for head and neck cancer will increase. Furthermore, the age when smoking began, also is a risk factor. People who started smoking before the age of 18 years had an increased risk of 1.5 [21]. Regarding alcohol abuse, it seems that the total amount of alcohol consumption is a more important risk factor than the type or constitution of alcohol beverage consumed [4,25].

Among the many components of cigarette smoke, polycyclic aromatic hydrocarbons are strongly implicated as carcinogens. Benzo[a]pyrene, which is present in amounts

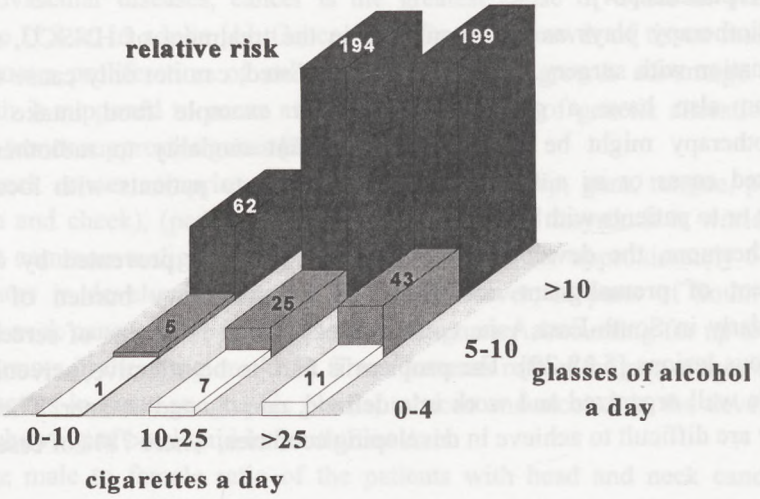

Figure 1. Risk factors of head and neck squamous cell carcinoma
Alcohol and tobacco are risk factors of HNSCC. The risk even increases more when both alcohol and tobacco are abused (adapted from Andre et al.[21]).

of 20-40 ng per cigarette is even one of the most potent mutagens known [26]. How elevated alcohol consumption results in increased risk is, however, still unclear, and several mechanisms have been suggested [4,25]. First, alcohol may simply act as a solvent, thus facilitating the passage of carcinogens through cellular membranes. Second, chronic alcohol consumption may affect the liver's ability to deal with toxic or potentially carcinogenic compounds. Furthermore, some metabolites of ethanol [27] or contaminants in beverages [28] can be carcinogenic. Part of the ethanol metabolism is even carried out in oral mucosa, which allows accumulation of the mutagen acetaldehyde [29]. Finally it has been suggested that alcohol may have an inhibiting effect on DNA repair mechanisms [30]. This suggests that individuals that abuse alcohol have an increased susceptibility to the mutagenic effects of environmental carcinogens.

Familial and genetic factors

Although the relationship between the abuse of tobacco and/or alcohol and the

development of HNSCCs seems clear, not all individuals with these habits develop cancer. Next to the chance factor, other factors also determine the individual risk for developing HNSCC. This risk might be affected by familial and genetic factors. Epidemiological studies have shown that HNSCCs have a tendency to occur more frequently in families of HNSCC patients [31,32].

Susceptibility to head and neck cancer might be associated with mutagen hypersensitivity [33]. The odd ratio to develop HNSCC was 11.5 in mutagen non-sensitive smokers and 44.6 in mutagen sensitive smokers [34]. This indicates that genetic factors can contribute to the mutagenic effects of certain carcinogens. For example, genetic polymorphisms in tobacco- and alcohol-metabolizing enzymes [35] or decreased expression of DNA mismatch-repair genes [36] can play a role in the effects of certain carcinogens in tobacco or alcohol. In families with syndromes like Ataxia-telangiectasia [37] and Xeroderma pigmentosum [38], that respectively are involved in DNA-damage recognition and DNA excision repair, an increased frequency of head and neck cancer was observed. Also Li-Fraumeni syndrome, with a high frequency of hereditary p53 mutations, can be accompanied with head and neck cancer [39].

Other factors

Viruses may play a role in the development of HNSCCs. The viruses that are considered to be of interest are human papilloma virus (HPV), herpes simplex virus (HSV), and Epstein-Barr virus (EBV) [40,41]. Factors, such as dietary deficiencies, particularly of pro-vitamin A, vitamins C and E, iron, and certain trace elements are thought to predispose to head and neck cancer [4,33]. Other risk factors include previous irradiation, asbestos and nickel industry, and poor hygiene [10].

Tumorigenesis

Premalignant lesions

HNSCC develops through a series of partly recognizable stages, reflecting the multistep process of tumorigenesis (Figure 2). This series may start with a precursor lesion in which the epithelial cells still function and look quite normal.This may be followed by precancerous lesions of the stratified squamous epithelium that are characterized by cellular atypia and loss of normal maturation, designated as dysplasia of which the severity -mild-moderate or severe- depends on the extent of cellular atypia and disordered epithelial maturation. When the lesion occupies the whole

thickness of the epithelium, but stromal invasion has not occurred, then the premalignancy is called a squamous cell carcinoma in situ. Finally, the term invasive carcinoma is used when carcinoma cells grow into the stroma. In this stage the blood and lymph system might be reached by the invasive cells which can result in local or distant metastases [42]. Multiple genetic alterations, which are described below, accumulate in the different stages (Figure 2) [43,44,45].

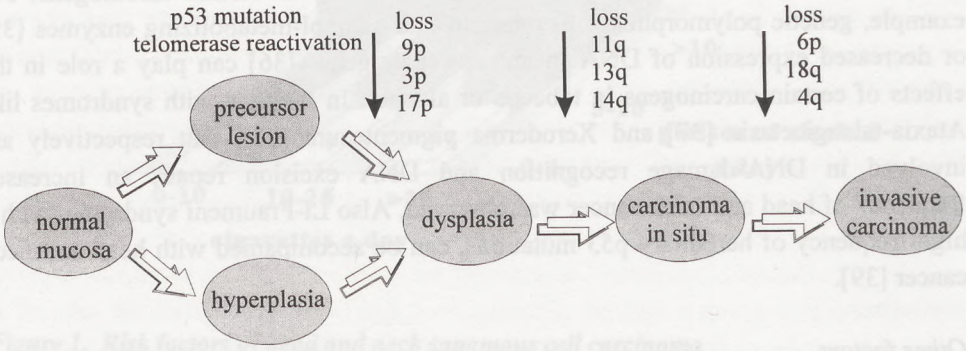

Figure 2. Multistep process of tumorigenesis

Recognizable stages in the multistep development of HNSCC. Important genetic changes that are associated with the histopathological progression of HNSCC are indicated [43,44,45].

Oncogenes and tumor suppressor genes

Cancer is a result from multiple genomic changes by carcinogens and hereditary alterations, that lead to the deregulation of the cell cycle machinery and to autonomous cell proliferation. Neoplastic transformation involves alterations in two types of genes: oncogenes and tumor suppressor genes. Oncogenes are activated and tumor suppressor genes are inactivated during tumorigenesis [46,47,48]. The frequency of alterations in these genes may differ between certain sites in the head and neck [49]. The most important genes involved in HNSCC will be discussed below.

Epidermal Growth Factor Receptor

One of the cellular oncogenes that play a role in HNSCC is the Epidermal Growth Factor Receptor (EGFR). This gene encodes for the receptor of both epidermal growth

factor and transforming growth factor alpha. Ligand binding to the extracellular domain of the EGFR causes receptor dimerization which activates tyrosine kinase function. This leads to autophosphorylation and subsequent phosphorylation of other intracellular target proteins which results in proliferation [50].

EGFR mRNA overexpression as well as protein overexpression has been demonstrated in nearly all HNSCCs [51-53]. EGFR expression increases during the development of normal epithelium to dysplastic epithelium and further increases when dysplasia becomes an invasive carcinoma [54]. This stepwise increase of EGFR coexists with an increased percentage of cells with polysomies of chromosome 7, where the EGFR is situated [55].

An increased number of EGFRs may interfere with the control of normal cell proliferation. Therefore an EGFR directed therapy could be useful for HNSCC patients. Trials have been conducted with anti-EGFR monoclonal antibodies [56] and retinoic acid, which downregulates the EGFR [57].

Cyclin D1

Cyclins are cell cycle regulators that are only functional when they are complexed with cyclin-dependent kinases (CDKs). Cyclin D1 regulates the G1/S transition in the cell cycle and is functional when it is complexed with either cdk4 or cdk6 [58]. Amplification of the 11q13 region which results in overexpression of the proto-oncogene cyclin D1 has been described in about half of the HNSCC [59-61]. Cyclin D1 amplification has been shown in premalignant lesions and the amplification frequency progresses from premalignant lesions to invasive carcinoma [60]. HNSCC patients with cyclin D1 amplification have a worse prognosis than those without amplification [61]. Antisense cyclin D1 inhibits proliferation of HNSCC cell lines [62]. Thus, cyclin D1 may play an important role in the growth and proliferation of HNSCC.

p16

p16 is an inhibitor of the D-type cyclin dependent kinases cdk-4 and cdk-6. By inhibition of the function of cyclin D-cdk complexes, p16 inhibits cell proliferation [63]. The tumor suppressor p16 can be inactivated by multiple genetic mechanisms: deletion, mutation or promoter methylation. HNSCCs often display an absence of p16 nuclear staining after immunohistochemistry [64]. Inactivation of p16 has also been shown in premalignant oral lesions [65,66]. Contradictary results were found regarding the mechanisms and frequency of absent p16 expression. One study revealed in 55% of the cases homozygous deletion of p16 [64]. Another study showed homozygous deletion of p16 in 20% of the HNSCCs [67]. The latter investigators also detected

methylation of the 5' CpG island at the p16 gene in 20% of the HNSCC and did not show any mutation [67], while the first study showed methylation in 17% of the HNSCC and mutations in 6% [64]. Despite these differences, both studies conclude that p16 is frequently inactivated in a significant proportion of the HNSCCs. Therefore p16 adenovirus-mediated gene therapy might be an interesting candidate for treatment of HNSCCs [68].

p53

The protein p53 controls cell cycle progression by acting as transcription factor for several genes that contain a p53 consensus sequence in their promoter region. After DNA damage, p53 becomes phosphorylated and upregulated. Subsequently p53 induces cell cycle arrest and/or apoptosis via the regulation of several proteins like the cdk-inhibitor p21(Waf1/Cip1), GADD45, and the apoptosis genes Bax and Bcl-2. p53 controls its own expression via MDM2 that allows targeting of p53 to the ubiquitin-mediated proteolytic machinery (Figure 3) [69].

Loss of function of p53 can result in uncontrolled cell division and progressive genomic instability [70]. Abnormalities of the p53 tumour suppressor gene are among the most frequent molecular events in cancer. More than 90% of the HNSCCs contain mutated p53 [71] and in 50% of the tumors loss of heterozygosity of p53 has been shown [72]. Furthermore, mutations in p53 are thought to be related to smoking [73,74].

Already more than thousand different p53 mutations have been described in human cancer, mainly in exons 4-9, which comprise the DNA binding core domain [75] However, some mutations occur outside this core domain [71]. Despite the fact that most mutations occur at different positions, a few hotspot areas are found in the most conserved areas of the gene [75,76].

Not every mutation in p53 has the same consequences for the function of the protein. Most p53 mutants can exert dominant negative effects on co-expressed wild-type p53, since p53 functions in a tetrameric complex [69,70,77]. However, besides losing the tumor suppressor function, some p53 mutants can also gain functions [69,78,79]. Two of these functions are increased expression of the multidrug resistance protein [78] and a disrupted spindle checkpoint [79]. The first may influence chemotherapy resistance and the latter may contribute to genomic instability.

Currently the role of mutant p53 in the prognosis of HNSCCs is unclear. It can be expected that this role becomes clearer when the notion that there are several types of p53 mutants that may exert different functions, is taken into account.

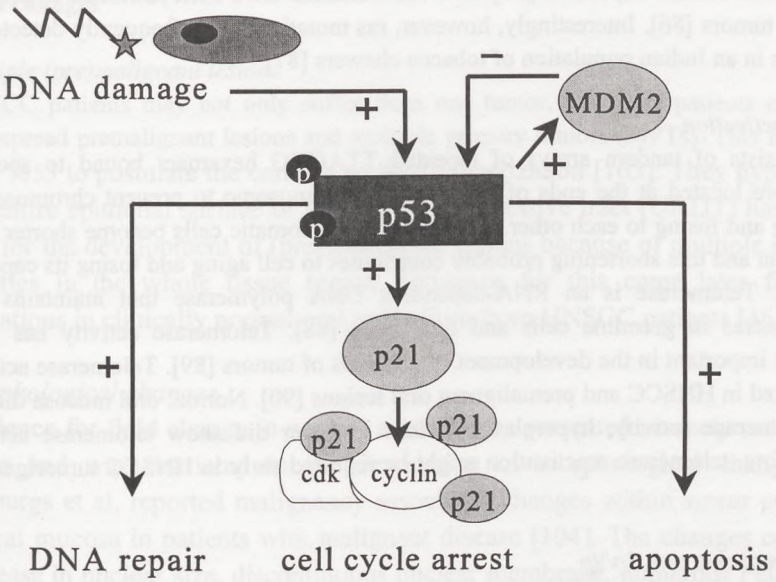

Figure 3. Functions of the tumor suppressor p53 after DNA damage
One of the functions of p53 after DNA damage is inducing cell cycle arrest. After up-regulation and phosphorylation of p53, the gene p21Waf1/Cip1 is induced. p21 inhibits several cyclin-cdk complexes which results in prevention of cell cycle progression. p53 also positively regulates MDM2 which targets the p53 protein for ubiquitination [69].

p53 is not only often found mutated in HNSCC but also in non-invasive lesions of the head and neck, which indicates that mutation of p53 is an early event in the development of HNSCC [45,80-82].

Since the presence of a mutant form of the tumor suppressor gene p53 has been correlated with disruption of programmed cell death (apoptosis) and reduced cell cycle arrest, it results in increased radiation resistance and cell survival. Wild-type p53 adenoviral gene transfer in HNSCC can both induce apoptosis and sensitize HNSCC to radiotherapy [83].

Ras

The ras genes (H-ras, K-ras, N-ras) encode guanosine-triphosphate (GTP)-binding proteins that attach to the inside of the cell membrane and transduce molecular signals to the nucleus. Certain mutations in ras result in constitutively active ras [84]. Nearly all relevant ras

mutations occur in codons 12, 13, 61 [85]. Few ras mutations have been identified in primary head and neck tumors [86]. Interestingly, however, ras mutations were frequently detected in oral carcinomas in an Indian population of tobacco chewers [87].

Telomerase reactivation

Telomeres consists of tandem arrays of repeated TTAGGG hexamers bound to specific proteins, and are located at the ends of each human chromosome to prevent chromosomes from degrading and fusing to each other. The telomeres in somatic cells become shorter with each cell dvision and this shortening probably contributes to cell aging and losing its capacity for replication. Telomerase is an RNA-dependent DNA polymerase that maintains and elongates telomeres in germline cells and fetal cells [88]. Telomerase activity has been suggested to be important in the development of all kinds of tumors [89]. Telomerase activity has been detected in HNSCC and premalignant oral lesions [90]. Normal oral mucosa did not show any telomerase activity, hyperplastic lesions however did show telomerase activity [90,91]. Therefore, telomerase reactivation might be required early in HNSCC tumorigenesis (Figure 2).

Losses on chromosome arm 3p/9p

Gains and losses of (parts of) chromosomes is a frequent event in tumors in general. In HNSCCs loss of heterozygosity is found frequently at loci from chromosomes 3p (3p14; 3p21; 3p25) and 9p (9p21-9p22) [92]. In addition, these losses are early events in HNSCC tumorigenesis since they are already frequently observed in premalignant lesions (Figure 2) [93,94]. Losses of loci at chromosomes 9p21 and 3p14 in oral premalignant lesions have value in cancer risk assessment [93]. 9p21 deletion correlates with recurrence of HNSCC [95].

It is interesting to know which genes are located at these loci. The tumor suppressor gene p16 is located at 9p21 [63]. However Waber *et al* found that genetic alterations of chromosome band 9p21-22 in HNSCC are not restricted to p16, since they showed frequent losses at three non-contiguous regions at 9p21-22 [96]. In addition Nakanishi *et al* also described other losses at this chromosome location [97]. Three distinct regions of deletions (3p14; 3p21; 3p25) on chromosome band 3p are supposed to be involved in the development of HNSCC [94,97]. The FHIT gene at 3p14.2 is considered as an involved candidate tumor suppressor [98-100]. Other candidate genes are currently under investigation [100,101]. Of course these losses on 3p and 9p are not the only genomic alterations in HNSCCs. Chromosome Y is lost in 53 % of the HNSCC of male patients [102]. A whole series of other, less frequent, gains and losses develops later during HNSCC tumorigenesis (Figure 2) [43,102].

Field changes

Multiple (pre)malignant lesions

HNSCC patients may not only suffer from one tumor, but these patients often also have widespread premalignant lesions and multiple primary tumors (MPTs). This led Slaughter *et al* in 1953 to postulate the concept of field cancerization [103]. They hypothesized that the entire epithelial surface of the upper aerodigestive tract (UADT) has an increased risk for the development of (pre-)malignant lesions because of multiple genetic abnormalities in the whole tissue region. Evidence for this came later from observed alterations in clinically normal oral epithelium from HNSCC patients [46].

Morphological changes

Evidence for field change in normal mucosa was presented scarcely until the last few years and was restricted to the description of morphological changes. In 1962, Nieburgs et al, reported malignancy-associated changes within smear cells of normal buccal mucosa in patients with malignant disease [104]. The changes consisted of an increase in nuclear size, discontinuous nuclear membrane, numerous Feulgen-negative areas, increased associated chromatin surrounding the clear areas, and absence of a single large nucleolus. The most consistent finding, nuclear enlargement could not be confirmed later by Ogden *et al* [105]. However, Incze *et al* confirmed an increase in nuclear area in normal oral mucosa remote from HNSCCs at an ultrastructural level in 1982 [106]. They also described an altered nuclear to cytoplasmic area ratio. A reduction in cytoplasmic area has been shown by Ogden *et al* [105]. Factors like chronic inflammation, irradiation and chemotherapy were all excluded.

Aneuploidy and chromosomal aberrations

In the last decade several publications have described other field changes. Despite the presence of nuclear enlargement, polyploid cells were not detected in normal tumor-distant mucosa [107]. Nevertheless in another study, aneuploidy was measured in hyperplastic/inflammatory mucosa that subsequently developed in an invasive carcinoma [108]. This aneuploidy was not detected in hyperplastic/inflammatory mucosa from healthy individuals. Hittelman *et al* reported that genomic instability somewhere in the upper aerodigestive epithelial field increased the risk to develop a HNSCC [109].

A whole series of chromosomal aneusomies was shown by fluorescence in situ hybridisation (FISH) in cells from brushes from macroscopically normal cheek opposite to the site of the HNSCC [110]. Chromosome aberrations were mainly seen in chromomomes 2, 3, 10, 12, 15, and X using centromeric and pericentromeric probes.

Despite other studies [43,93] which show early loss of chromosome 9 in HNSCC tumorigenesis, this loss was not observed here. Interestingly, the chromosome aneusomies found in the non-tumor specimens were also present in the corresponding tumor specimens. Nevertheless, the chromosomal imbalances appeared in lower frequencies of cells and in less complex combinations than in the tumors [110].

Polysomies of chromosome 7 and 17 were shown in tumor-adjacent mucosa (TAM) from HNSCC patients [55]. No polysomies were present in buccal epithelium from healthy individuals.

In another publication on chromosomal aberration the investigators used microsatellite analysis [111]. Allelic loss of chromosome 13 was detected in 10 of 16 informative tumor-adjacent mucosa samples when they were compared to blood samples.

Alterations in cytokeratin expression

Cytokeratins are the intermediate filament proteins found in the cytoplasm of all epithelial cells. There are at least 20 different keratin polypeptides that are expressed in different combinations depending on the type of epithelium and the degree of differentiation [112]. Aberrant expression of cytokeratins has been shown during the process of HNSCC carcinogenesis [113].

Cytokeratin 19 is normally present in minor amounts in the basal layer of noncornifying regions of oral epithelial [112]. Normal oral mucosa at the contralateral side of HNSCC had a more than three-fold increased expression of cytokeratin 19 as compared with healthy controls [114]. An increased expression of cytokeratin 19 was also observed in two other studies [115,116]. In the study of Bongers *et al*, this increase was shown in exfoliated cells [116]. It has been suggested that K19 expression, particularly in those oral sites where it is not usually seen, is related to inflammation [117]. However , Ogden *et al*, saw increased expression in cases without profound inflammatory change [115]. In another study it was noted that expression of cytokeratin 19 might be considered as a marker of premalignancy [118].

Cytokeratin 13 is usually present in moderate amounts in the suprabasal layer of non-cornifying, stratified squamous epithelia [112]. Copper *et al* found indications for an increase of this keratin in normal tumor-distant oral mucosa [114].

Cytokeratin 16 is expressed in keratinocyte pathologies associated with hyperproliferation and is therefore called a 'hyperproliferation' marker. This marker for fast cell turnover is predominantly expressed in the suprabasal cell layer of epithelial tissue [112,113]. A statistically significant increase in cytokeratin 16 expression was found in exfoliated cells from macroscopically normal mucosa from 6 different places

distant from the HNSCC [116].

Normally, cytokeratins 7 and 8 are not expressed by normal oral keratinocytes [112,115]. Nevertheless, these cytokeratins (identified by the antibody CAM 5.2) were expressed in the basal cells of approximately a third of the biopsies of normal mucosa from HNSCC patients [115].

Changes in histo-blood group antigens

Histo-blood group antigens are cell surface carbohydrates that show changes in expression related to tissue type, differentiation state and cell motility capacity. Type-2 chain ABH-carbohydrate structures are distributed broadly in epithelial and endothelial cells, independent of the patient's ABO-blood group. In normal oral and laryngeal epithelium, type-2 chain ABH-antigens are expressed on parabasal cells [119]. A 4-fold lower expression of type-2 chain ABH-antigen was shown in exfoliated cells from macroscopically normal mucosa from 6 different places distant from the HNSCC, compared with healthy individuals [116]. Since the ABH type-2 chain expression was always lower in the mucosa from the patients than in the mucosa from the healthy controls, this antigen may be promising as a negative marker for field change and risk indication.

Foci of cyclin D1 expression

Bartkova *et al* observed clearly defined foci of positivity of cyclin D1 protein in sections of normal mucosa adjacent to HNSCC that were not seen in sections of normal mucosa from healthy individuals [120].

Increased expression of the EGFR

Several studies have shown increased expression of the EGFR in tumor-adjacent mucosa [51,53,54,121-123]. In four of these studies an overexpression of the protein was observed [53,54,121,123] one study has described an elevated mRNA level [51], and one study has shown amplification of the EGFR gene [122]. Grandis *et al* showed that the EGFR protein expression was increased more if the investigated normal epithelium was located closer to the HNSCC [123].

Elevated TGF-α mRNA

Besides investigation of the EGFR also one of its ligands transforming growth factor alpha (TGF-α) was investigated. It was shown that the mRNA level of TGF-α was 5-fold increased in normal TAM compared with mRNA levels in control normal mucosa [51].

Increased proliferation

One of the characteristics of a tumor is an increased proliferation. Shin *et al* showed a sequential increase in proliferating cell nuclear antigen (PCNA) expression in head and neck tumorigenesis [124]. In addition, they showed an elevated number of PCNA positive cells in normal TAM [124], which was confirmed by Schwindt *et al* who used silver staining of nucleolar organizer regions as proliferation marker [125].

Elevated p53 expression and presence of p53 mutations

Mutant p53 has a higher stability than wild-type p53, which allows accumulation to levels detectable by immunohistochemistry [126,127]. The number of p53 positive cells gradually increases as oral epithelium progresses from normal to hyperplasia to dysplasia to carcinoma [128,129]. Focal p53 positivity was detected more often in normal TAM than in healthy control epithelium [45,128,130,131]. Polyclonal mutations in the p53 gene were identified in normal tumor-adjacent and tumor-distant mucosa [45,132].

Focal overexpression of p53 might reflect an increased risk of second primary tumors in these patients. Previously, it was shown that more p53 clusters were present in epithelium surrounding multicentric HNSCC than unicentric HNSCC [133]. Furthermore, p53 expression above the basal cell layer in oral mucosa has been found to be an early event of malignant transformation and has predictive value for the development of HNSCC [131]. This is in contrast with another study in which the investigators found that p53 overexpression in tumor-adjacent mucosa could not predict the likelihood of a second primary squamous cell carcinoma [134]. However, no distinction was made between basal and suprabasal p53 expression in this study.

Lack of bcl-2 expression

The homeostasis of normal tissue is a balance between cell proliferation and cell death. Alterations of both pathways contribute to a clonal expansion of cancer cells. Bcl-2 and its family play an important role in the regulation of the apoptotic pathway [135]. Apoptosis itself did not vary significantly in the different stages of HNSCC tumorigenesis, despite an increase in mitotic index [136]. However, in HNSCC and in normal tumor-adjacent mucosa there was lack of bcl-2 expression compared to control mucosa [136]. Bcl-2 is supposed to inhibit apoptosis [135], therefore one would expect an increase in bcl-2 expression during tumorigenesis and therefore the lack of bcl-2 expression is rather suprising. However, to estimate the bcl-2 activity, the expression of bcl-2 has to be interpreted in the context of levels of other bcl-2 family members.

Increased glutathione S-transferase

Glutathione S-transferases are detoxification enzymes with different isoclasses: α-, μ- and π-class [137]. They are multifunctional intracellular, soluble, or membrane-bound enzymes, which catalyze the conjugation of many electrophilic hydrophobic compounds with the tripeptide GSH. Glutathione S-transferase-μ is an isozyme with a marked specificity for catalyzing the conjugation of epoxides, such as benzo[a]-4,5-oxide and sterene-7-8-oxide, carcinogenic components in cigarette smoke [138]. Expression of glutathione S-transferase-μ is inherited and a two-fold higher risk of laryngeal cancer has been previously shown among smokers who lack the glutathione S-transferase-μ isoenzyme [139].

The expression of all glutathione S-transferase isoenzymes was significantly higher in the suprabasal and superficial layers of normal oral mucosa from HNSCC patients that subsequently developed a second primary tumor than in normal oral mucosa from HNSCC patients that who were minimally 7 years free of disease [139]. Also in cell scrapes of macroscopically normal tumor-adjacent mucosa elevated levels of glutathione S-transferase-μ and π-class were observed [140]. The reason for these increased levels is an intriguing fact, since elevated levels of detoxification enzymes would protect against carcinogenic attacks. It is possible that it reflects a futile response to carcinogenic metabolites. The reason for the high levels of glutathione S-transferase may not yet be clear, nevertheless it seems to have a predictive value for the development of a second primary tumor.

Presence of the proto-oncogene eIF4E

Control of gene expression at the translational level is important in cell growth and proliferation. A key participant in regulation of translation is the proto-oncogene eIF4E or mRNA 5' cap-binding protein [141]. This proto-oncogene has been found to be elevated in HNSCC [142]. Histologically normal surgical margins showed overexpression of eIF4E [142].

Protein tyrosine kinase and protein tyrosine phosphatase activity

Phosphorylation of proteins on tyrosyl residues is a key mechanism in signal transduction pathways that control growth, differentiation and cellular architecture of normal and malignant cells [143]. This phosphorylation is strictly regulated by protein tyrosine kinases and protein tyrosine phosphatases. Normal tumor-adjacent mucosa showed a 2.2-fold increase in protein tyrosine kinase activity compared to the control mucosa from healthy individuals. In addition, in the tumor-adjacent mucosa a 1.7-fold elevated ratio of protein tyrosine kinase activity over protein tyrosine phosphatase was

observed [144]. Preliminary results suggested that the proto-oncogene c-Src [145] could be responsible for the increased protein tyrosine kinase activity (personal communication; Dr. H.P. Verschuur *et al*).

Field cancerization

The conclusion can be drawn that many field changes have been observed in the TAM from HNSCC patients. These data support the field cancerization hypothesis which implicates that the whole UADT consists of multiple genetic abnormalities [103]. Nevertheless a lot of research is necessary to find out whether these changes have carcinogenetic significance or are merely epiphenomenal.

Field cancerization theories

The mucosal changes in the entire UADT were generally considered to be the result of exposure to carcinogens that caused multiple genetic abnormalities in the whole tissue region [103]. The multiple squamous cell lesions described in the oral field cancerization process were thought to have developed independently of each other.

An alternative theory for the occurence of multiple (pre)malignant lesions has been proposed in the last decade and is based on the premise that any transforming event is rare and that the multiple lesions arise due to widespread migration of transformed cells through the whole aerodigestive tract [43,146,147]. This last theory can be divided into two types of migration: a) migration of tumor cells for example by saliva (micrometastases), b) intraepithelial migration of the progeny of the initially transformed cells (Figure 4). If metastatic cells from a HNSCC move through the blood or lymph system they usually settle in the lung or in the first lymph node; that route does not lead to tumor deposits in the mucosal surface that lines the UADT.

Clinical relevance

The distinction between the migration theory and the independent transformation theory has clinical consequence. If the MPTs develop independently or after migration of progenitor cells then chemoprevention such as carcinogen blockers [148,149] should be an option for these patients. Furthermore, the patients should quit using tobacco and alcohol. If the MPTs develop after migration of tumor cells there would be no need for those patients to quit smoking and drinking, they would develop MPTs anyway and the chemoprevention [148] should be directed towards migration and proliferation. The different field cancerization theories also have impact on the

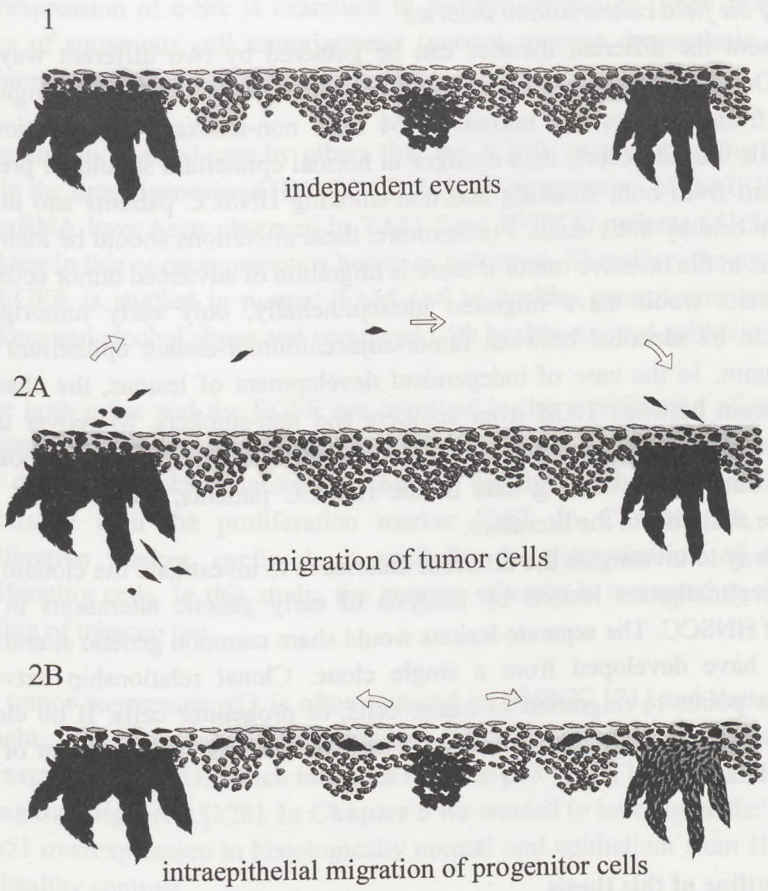

Figure 4. Different field cancerization theories
HNSCC patients frequently develop more than one primary tumor and often have widespread premalignant lesions. The different theories for the occurrence of these multiple lesions are depicted.

interpretation of positive tumor margins. If tumor margins are found positive for molecular changes [150] then one can either conclude that the tumor has not completely been resected or that independent events have caused molecular changes in the entire field. If the latter would be the case, it is not justified to conclude that the margins are tumor positive.

Investigation of the field cancerization theories

Information about the different theories can be gathered by two different ways of investigation. One way is to search for differences in alterations between histologically normal TAM from smokers and normal TAM from non-smokers. If migration of transformed cells indeed occurs, then changes in normal epithelium should be present in the epithelium from both smoking and non-smoking HNSCC patients and not in epithelium from healthy individuals. Furthermore, these alterations should be identical to the alterations in the invasive tumor if there is migration of advanced tumor cells but if progenitor cells would have migrated intraepithelially, only early tumorigenic alterations would be identical between tumor-adjacent/tumor-distant epithelium and the invasive tumor. In the case of independent development of lesions, the changes should be different between TAM from smokers and non-smokers. Moreover these differences should then also be present in normal mucosa from healthy smokers. Unfortunately, almost no smoking data of the HNSCC patients, whose TAM was investigated, are available in the literature.

The second way to investigate the different theories is to investigate the clonality of the multiple (pre)malignant lesions by analysis of early genetic alterations in the development of HNSCC. The separate lesions would share common genetic alterations if they would have developed from a single clone. Clonal relationship between multiple lesions points to migration of tumor cells, or progenitor cells. If no clonal relationship between multiple lesions can be found, independent development of the lesions is more likely.

The aim and outline of this thesis

In this thesis, alterations in histologically normal TAM from HNSCC patients are investigated in relation to smoking (and alcohol). Information about the role of smoking in relationship with field changes may provide evidence about the way of development of multiple (pre)malignant squamous cell lesions in the UADT. In addition, clonality of multiple squamous cell carcinomas in the UADT is investigated to make a choice between the different field cancerizations theories.

Preliminary results (personal communication; Dr. H.P. Verschuur *et al*) indicated that the proto-oncogene c-Src may be involved in the previously observed increased protein tyrosine activity in HNSCC and the normal tumor-adjacent mucosa. Therefore, we wanted to investigated if c-Src is indeed involved in the tumorigenesis of HNSCC.

Overexpression of c-Src is examined in paraffin-embedded tissue from the different stages of squamous cell tumorigenesis (normal mucosa, hyperplasia, dysplasia and carcinoma) by immunohistochemistry (**Chapter 2**).

It has already been shown by others that the EGFR, another tyrosine kinase, plays a role in the tumorigenesis of HNSCC [51,54]. Overexpression of the EGFR protein and the mRNA have been observed in TAM from HNSCC patients [51,54]. The role of smoking in this overexpression is however, unknown. Therefore, the overexpression of the EGFR is studied in normal TAM and in healthy control mucosa in relation to smoking and alcohol abuse and compared with healthy control epithelium **(Chapter 3)**.

Since both c-Src and the EGFR are involved in the proliferation of cells (Figure 5), proliferation is assessed in normal mucosa from HNSCC patients and healthy controls with different smoking habits. In **Chapter 4a**, the number of proliferating cells is established with the proliferation marker Ki67. In **Chapter 4b**, an alternative proliferation marker, cyclin A, is used for the determination of the number of proliferating cells. In this study, the number of cases is increased to further evaluate quitting of tobacco use.

The tumor suppressor p53 is often mutated in HNSCC [71] and these mutations are thought to be related to smoking [73]. Immunohistochemically detectable overexpression of p53, which indicates mutated p53 [127], has been shown to increase during tumorigenesis [128]. In **Chapter 5** we wanted to investigate the role of tobacco on p53 overexpression in histologically normal oral epithelium from HNSCC patients and healthy controls.

Immunohistochemically detectable overexpression of p53 in normal oral mucosa indicates the presence of mutant p53, however the possibility exists that it is wild-type p53 that is overexpressed [127]. Since sequencing of DNA from a low number of formalin-fixed and paraffin-embedded cells is very hard to perform, it was necessary to find another way to distinguish between overexpression of mutant p53 and wild-type p53. Therefore, we wanted to study the possibility of using the proliferation marker Ki-67. This marker is not present in quiescent cells in contrast to proliferating cells in which it is present during all cell cycle phases except for early G1 [157]. The hypothesis is that cells with overexpression of functional wild-type p53 are cell cycle arrested and that the marker Ki-67 will not be present in these cells. Cells with overexpression of mutant p53, however, should still proliferate and the marker Ki-67,

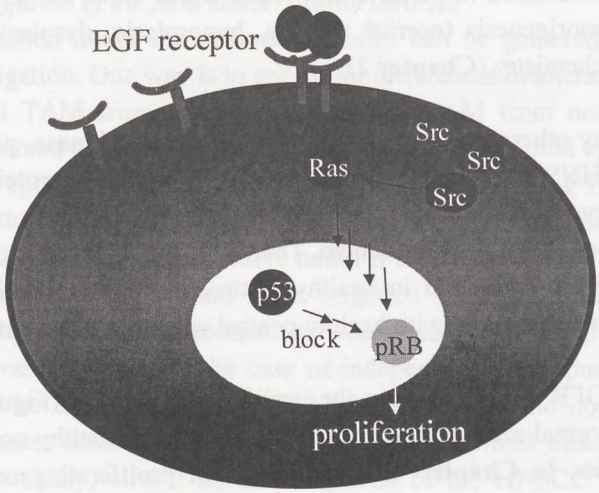

Figure 5. Regulators of proliferation
Proliferation is regulated by different proteins in different pathways [151-156]. Some of these proteins may be altered during tumorigenesis. One signal transduction pathway is depicted simplified.

will still be present in such cells. To test the hypothesis if cells with overexpression of wild-type p53 do not express Ki-67, the expression of Ki-67 is examined by immunohistochemistry when a cell line is in cell cycle arrest after induction of wild-type p53 (**Chapter 6**).

In addition, the p53-induced protein p21(Waf1/Cip1) is studied for application as a marker to discriminate between the presence of overexpressed mutant p53 and overexpressed wild-type p53 in normal oral mucosa (**Chapter 7**). It is investigated if immunohistochemical expression of p21 is indeed dependent on the presence of wild-type p53. Therefore, immunohistochemical expression of p21 is examined in HNSCC of which the p53 mutation state was assessed previously, by sequence analysis.

The clonality of multiple squamous cell carcinomas in the UADT is investigated in **Chapter 8** to gather evidence for the possible development of multiple HNSCC due to migrated transformed cells. This clonality is studied with mutations in the tumor suppressor p53 and microsatellite alterations on chromosome 3p, 9p and 17p as molecular markers. Beforehand, the applicability of these molecular markers is

evaluated.

Aneuploidy and numerical chromosome abnormalities have been shown frequently in HNSCC and their premalignant lesions [108,109]. Chromosome Y loss has been observed in half of the HNSCC from male patients [102, 158]. It is unknown whether a relation exists between this loss and the abuse of tobacco. As is mentioned before, information about the development of multiple (pre)malignant squamous cell lesions in the UADT may be provided by differences in alterations between histologically normal TAM from smokers and normal TAM from non-smokers. Therefore, numerical aberrations of chromosome Y are studied in TAM from HNSCC patients in relation to smoking by using fluorescence in situ hybridization (**Chapter 9**).

In **Chapter 10** all data are placed in their context. The data are discussed together with other published results within the scope of the different field cancerizations theories.

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Chapter 2

Overexpression of c-Src in areas of hyperproliferation in head and neck cancer, premalignant lesions and benign mucosal disorders

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Abstract

To examine which proteins are responsible for the elevated protein tyrosine kinase (PTK) activity in human head and neck squamous cell carcinoma (HNSCC) and adjacent histologically normal epithelium (Verschuur et al., Eur Arch Otorhinolaryngol 1993; 249: 466-469), *paraffin embedded sections of these tissues were stained for the PTK c-Src. We show with double labelling techniques, using antibodies again st both the proliferation marker Ki-67 and the PTK c-Src, that c-Src is overexpressed in areas of hyperproliferation in HNSCC, dysplastic epithelium, benign papillomas and inflamed normal tissue. Our data indicate that c-Src is (one of) the protein(s) responsible for the increased PTK activity in HNSCC. We could not demonstrate that c-Src expression is responsible for the increased PTK activity in tumor-adjacent normal epithelium. We assume that c-Src plays a role in the increased proliferation seen in (pre)malignant and benign epithelial lesions as well as in reactive inflammatory epithelial hyperplasia.*

Introduction

Head and neck squamous cell carcinomas (HNSCC) account for the sixth most common solid tumor worldwide and long-term survival rates are among the lowest of the major cancers. The key molecular changes in the multi-step progression of HNSCC are still unknown and a better understanding might lead to more rational therapy and improved survival of patients [1,2].

The concept of "field cancerisation" proposed by Slaughter *et al* in 1953 [3] introduced the hypothesis that the entire epithelial surface of the upper aerodigestive tract (UADT) is exposed to carcinogens that cause multiple genetic abnormalities and thereby an increased risk of cancer development. This theory is supported by clinical, histopathological, and recent molecular evidence [2]. Some of this evidence is gained by comparing normal control epithelium with tumor-adjacent dysplastic and histologically normal epithelium. Among others, differences have been found in proliferation [4,5], epidermal growth factor receptor (EGFR) expression [6,7], and p53 expression [8]. Furthermore, an increased protein tyrosine kinase (PTK) activity has been found in histologically normal mucosa from HNSCC patients as well as in HNSCC tumor tissues [9].

Phosphorylation of proteins on tyrosyl residues by protein tyrosine kinases is a key mechanism in signal transduction pathways that control growth, differentiation and cellular architecture of normal and malignant cells [10]. So to determine which protein causes the increased PTK activities in HNSCC and adjacent normal tissue is obviously an important task.

It has already been shown that one PTK, the EGF receptor, is elevated in HNSCC, in premalignant mucosa and in normal tumor-adjacent mucosa (TAM) [6,7]. However, other proteins with PTK activity may also play a role in the increased PTK activity in normal TAM, as in a previous study increased PTK activity could not be attributed to the EGFR [9]. In breast tumors the majority of PTK activity, measured with the same method, could be attributed to the presence of the proto-oncogene c-Src [11,12]. We therefore examined the presence of c-Src in HNSCC, adjacent dysplastic and histologically normal epithelium by immunohistochemistry and compared it with the expression found in normal control tissue from non-tumor patients. The expression of c-Src was analysed with respect to the proliferation state in these tissues, in benign papillomas and in inflamed tissues. Herefore, we applied double labelling of c-Src with the proliferation marker Ki-67, which is expressed in the nucleus during all stages of proliferation [13]. We show overexpression of c-Src in areas with hyperproliferation in HNSCC, adjacent dysplastic mucosa, benign

papillomas and epithelium of tissue with inflammatory alterations.

Materials and methods

Clinical material

The material for immunohistochemistry consisted of tissue specimens from patients who were treated surgically for HNSCC, benign papillomas, or mucosal inflammatory disease. Normal cheek mucosa was obtained from healthy individuals without tumors or mucosal inflammation. No patients had undergone any previous treatment other than biopsies as part of the diagnostic procedures. For immunohistochemical demonstration of c-Src protein and Ki-67, 4 µm sections were cut from selected paraffin blocks from 14 patients that contained invasive tumor, tumor-adjacent histologically normal mucosa and various degrees of dysplastic epithelium, 5 patients with a benign squamous papilloma, 5 patients with inflammatory alterations that resulted in reactive epithelial hyperplasia and 5 individuals with control normal mucosa. The clinical data of the patients is summarized in table 1.

Table 1. Clinical characteristics of the patients

tissue	**HNSCC with various degrees of dysplasias**	**benign papilloma**	**inflammatory mucosa**	**healthy mucosa**
n	14	5	5	5
mean age (range)	59 years (from 43 to 76)	48 years (from 26 to 67)	44 years (from 21 to 53)	43 years (from 24 to 62)
gender	11 ♂ 3 ♀	5 ♂ 0 ♀	4 ♂ 1 ♀	3 ♂ 2 ♀
location	12 oral cavity 2 laryngopharynx	3 oral cavity 2 laryngopharynx	5 oral cavity	5 oral cavity

Immunohistochemistry

The 4 µm sections were deparaffinised by xylene and dehydrated with 96% ethanol. Endogenous peroxidase activity was blocked by incubating the slides in 1,5% H_20_2 with methanol for 20 min. For antigen retrieval, the sections were boiled in citrate buffer (2.94 g/l sodium citrate, pH 6.0) for 15 min. and subsequently cooled down to 30 ^{0}C. After washing in phosphate buffered saline (PBS), the slides were preincubated with 10% non immune rabbit serum (NRS) for 15 min. Subsequently they were incubated for 60 min. with 1:800 diluted sheep-anti-Src polyclonal antibody (Affinity, Nottingham, UK; earlier used by Verbeek *et al* [11]), followed by washing in PBS and incubation with 1:100 diluted peroxidase labelled rabbit-anti-sheep antibody (DAKO a/s, Glostrup, Denmark) in 10 % AB^+ human serum for 30 min. After washing in phosphate-citrate buffer (17.5 g/l $Na_2HPO_4.2H_2O$ and 10.7 g/l citric acid, pH 5.8) the c-Src protein was visualized for light microscopy with DAB-reagent (0.06% 3,3-diaminobenzidine tetrahydrochloride and 0.03% H_20_2 in phosphate-citrate buffer). Sections were counterstained with haematoxylin for 3 min. In the negative control, the first antibody was omitted.

To study the expression of c-Src with respect to proliferation of the tissue the 4 µm sections were deparaffinised, dehydrated, boiled in citrate buffer and preincubated with NRS as described above. The sections were incubated for 60 min. with 1:100 diluted sheep anti-Src antibody together with a 1:25 diluted murine anti-Ki-67 monoclonal antibody (MIB-1, Immunotech, Marseille, France). After washing in PBS the sections were incubated for 30 min. with 1:100 diluted donkey-anti-sheep FITC conjugated antibody (ICN Biomedicals Inc, Ca, USA) and 1:50 diluted rabbit-anti-mouse TRITC conjugated antibody (DAKO) in 10% AB^+ serum. Sections were washed in PBS/Tween (0.05% Tween in PBS). The sections were covered with PBS/glycerol (9:1) and viewed with a confocal laser scanning microscope (CLSM MRC1000, Bio-Rad, Hercules, Ca, USA). By omitting respectively one of the two first antibodies in two controls, cross reaction by the antibodies was excluded.

Results

Overexpression of c-Src in HNSCC and adjacent premalignant epithelium

C-Src protein was detected in the cytoplasm of HNSCC and the adjacent dysplastic epithelium in sections of all 14 HNSCC patients by immunohistochemistry. Almost no c-Src could be detected in the histologically TAM and in control epithelium. In the adjacent premalignant dysplastic mucosa c-Src was expressed mainly in the atypical cells in the basal layers of the epithelium. In more severe dysplastic mucosa the expression was increased and was also found in the more superficial layers of the epithelium (Figure 1A). About the same expression of c-Src could be seen in the invasive carcinoma. C-Src was completely absent from the mature cells of the

squamous pearls in HNSCC and was exhibited only in the peripheral proliferative regions of these pearls (Figure 1B). Furthermore, the stromal cells were negative, in contrast to some lymphocytes that reacted positively for c-Src.

Expression of c-Src associated with proliferation

Previously mentioned sections were double labelled to show co-expression of the proliferation marker Ki-67 and c-Src through the confocal laser scanning microscope. In histologically normal TAM and control epithelium there was almost no c-Src expression, and only a few of the cells were positive for Ki-67 (Figure 2A). In dysplastic adjacent epithelium (Figure 2B) and invasive carcinoma (fig 2C) c-Src was highly expressed in the areas that contained many cells that were positive for Ki-67. Benign papillomas (Figure 2D) and inflammatory hyperplastic tissue (Figure 2E) both also showed an increased expression of c-Src in cells in the Ki-67-positive areas facing the stroma. However, not every cell showed co-expresssion of c-Src and Ki-67. Individual cells in the highly proliferating areas also expressed c-Src without Ki-67. The cells exhibiting c-Src positivity without Ki-67 positivity were especially numerous in two well-differentiated HNSCCs (Figure 2F). In almost all positive cells, c-Src was found in a granular pattern in the cytoplasm, mainly around the nucleus (Figure 2B).

Discussion

To determine which proteins are responsible for the increased PTK activity in HNSCC and TAM, we investigated the immunohistochemical expression of c-Src in several tissues. The data show that c-Src is overexpressed in areas of hyperproliferation in HNSCC and dysplastic mucosa, mucosa with inflammation, and benign papillomas. In normal mucosa c-Src was expressed in such a low amount that we could not detect any difference between normal epithelium adjacent to the HNSCC and the normal control epithelium. We conclude that c-Src is (one of) the PTK(s) that is overexpressed in HNSCC; however, this study cannot conclude that c-Src is involved in the increased PTK activity in histologically normal mucosal epithelium bordering HNSCC.

Increased c-Src expression and/or activity has previously been found in other cancers such as colon carcinomas [14], breast cancer [11], lung carcinomas [15], and bladder carcinomas [16]. Elevated c-Src expression or activity has also been found in premalignant epithelia of ulcerative colitis [17], premalignant epithelia of lung carcinomas [15] and in colonic benign polyps [14]. This is in accordance with our

results in HNSCC and premalignant epithelium.

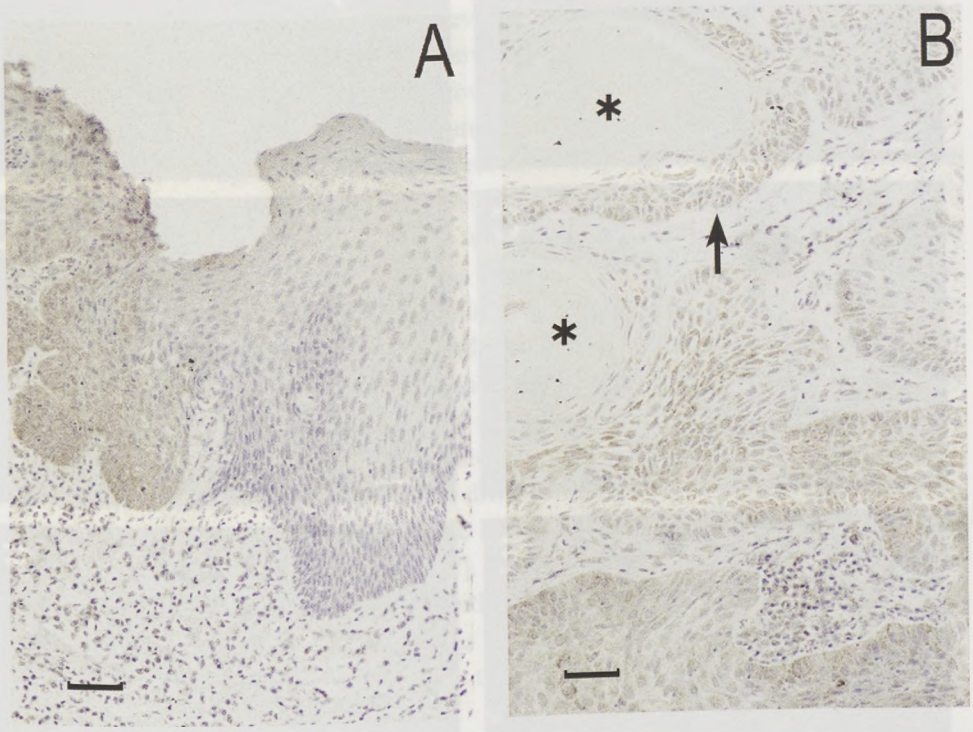
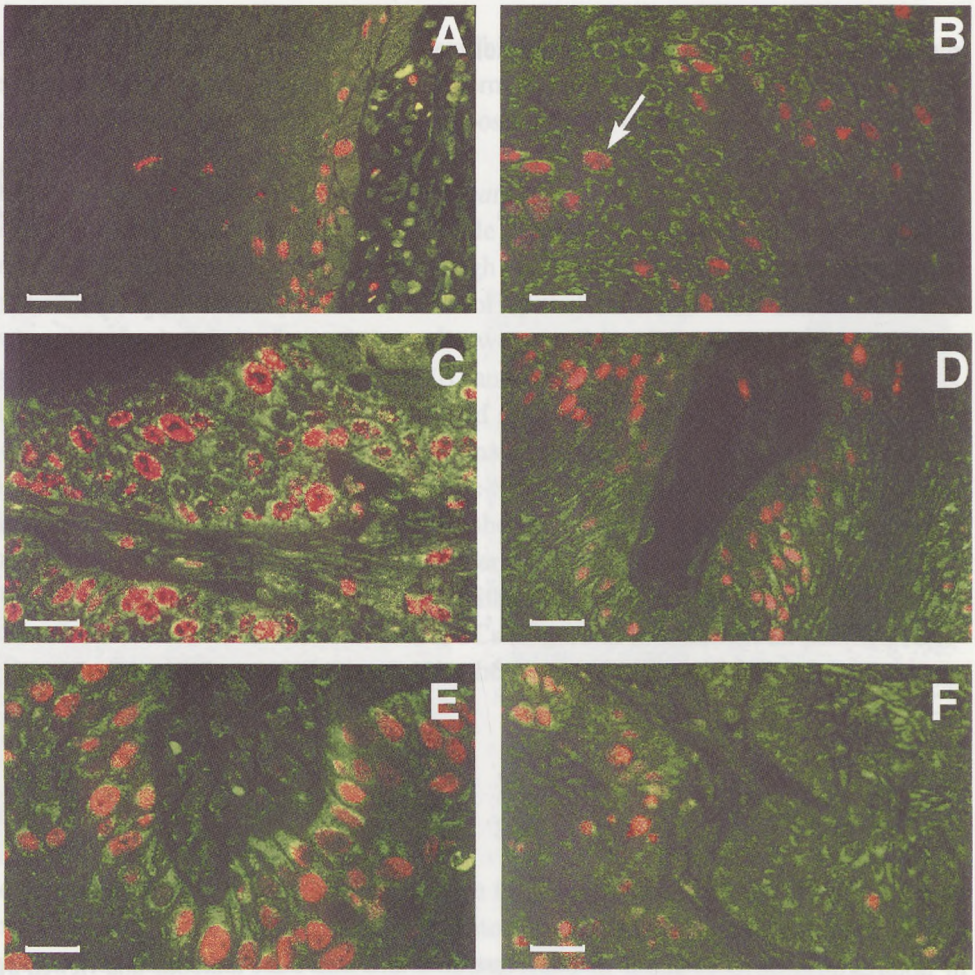


Figure 1. Oral tissues stained for c-Src
Sections of HNSCC and adjacent mucosa were incubated with c-Src antibody and viewed by light microscopy. A) Section showing the border between dysplastic (left) and normal mucosa (right). Expression of c-Src is present in the atypical cells of the dysplasia and in some lymphocytes of the underlying stroma. Bar is 39 μm. B) Invasive carcinoma with two squamous pearls (✱). Expression of c-Src is present in the tumor cells, however in the squamous pearls it is only present in the peripherally located layer with proliferating cells (➔). Bar is 29 μm.

Overexpressed c-Src in the tissues studied is not linked to normal proliferation but to hyperproliferation, as only overexpressed in highly Ki-67-positive areas and not in the proliferating basal cells in normal mucosa. We also found that the more severe the dysplasia, the more extensive the area of proliferating Ki-67-positive cells and the more cells were positive for c-Src. The level of c-Src activity in dysplastic colon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epithelia also correlated with the degree of dysplasia [17]. An increase in proliferation during the development of normal epithelium to severe dysplasia has been shown before by others [5]. Regarding the linkage of c-Src overexpression to hyperproliferation, it must be noted, however, that Ki-67-negative cells in these areas also showed c-Src overexpression in all investigated tissues; thus c-Src expression is not correlated with proliferation per se. However, this does not exclude the possibility that the presence of áctivated c-Src correlates with proliferation. The co-existence of c-Src and Ki-67 in a single cell could be the result of activated c-Src, while the presence of c-Src in a Ki-67 cell may indicate that c-Src is in an inactive state.

Two well-differentiated carcinomas with numerous Ki-67-negative cells also overexpressed c-Src in our study. This is not in accordance with our other results. However, it is known that c-Src can also regulate differentiation in addition to proliferation [18]. C-Src protein levels and activity are also increased in more highly differentiated human colon carcinomas and bladder carcinomas compared to poorly differentiated ones [18,16]. These data can possibly explain our finding that c-Src was also overexpressed in the well-differentiated tumors without high Ki-67 expression. However, we could not detect c-Src in the differentiating cells in normal epithelium.

The absence of c-Src overexpression in healthy TAM is surprising as we observed increased PTK activity in this mucosa [9]. We also found an increased proliferation, measured by Ki-67 staining, in normal epithelium adjacent to HNSCC compared with normal control epithelium [4] which has also been found by others using PCNA expression [5]. These findings suggest that the PTK c-Src, although undetectable by

Figure 2 (left page). Oral tissues stained for Ki-67 and c-Src
Sections of different tissues incubated with Ki-67 antibody (red nuclei) and c-Src antibody (green pattern) were viewed by confocal laser scanning microscopy. Some nuclei exhibit a yellow ring due to image distortion by the computer connected with the confocal laser scanning microscope. This must not be interpreted as co-localisation of c-Src and Ki-67. A) Normal mucosa of HNSCC patient; showing almost no c-Src expression. Some lymphocytes in the stroma show c-Src positivity. Bar is 21 μm. B) Dysplastic mucosa; showing granular c-Src expression predominantly perinuclear in the cytoplasm (➔) of atypical cells in the proliferating layers. Bar is 16 μm. C) Invasive carcinoma; showing c-Src expression in the Ki-67-positive as well in the Ki-67-negative tumor cells. Bar is 14 μm. D) Papilloma; showing c-Src expression in the basal proliferating area facing the stroma. Bar is 21 μm. E) Inflamed tissue; showing c-Src expression in the basal proliferating area facing the stroma. Bar is 12 μm. F) Highly differentiated invasive carcinoma; showing c-Src expression in the proliferating and differentiating tumor cells. Bar is 23 μm.

immunohistochemistry, may also be of importance in this TAM.

It is known that c-Src plays a role in the regulation of growth and malignant transformation of cells [12]. C-Src appears to be transiently activated not only at G0-G1 transition but also at the onset of mitosis [12], probably as an indirect consequence of serine and threonine phosphorylation by the Cdc2 kinase [19] that is also expressed in proliferative compartments of oral mucosa [20]. This is an indication for the presence of active c-Src in proliferative areas.

To constitute more evidence for a role for c-Src in the proliferation of HNSCC, we investigated the growth inhibiting response of the Src-like kinase inhibitor herbimycin A [21] on a HNSCC cell line. We found that herbimycin A dose-dependently decreased growth of the HNSCC cell line SSC-2, thereby reducing phosphotyrosine levels and without any direct toxic effects (results not shown). Treatment of these cells with the more general PTK inhibitor genistein [22] was found not to decrease the growth of the cells. These results support the involvement of tyrosine phosphorylation by Src-like PTKs in the proliferation of HNSCC, but are not conclusive, because of the debated specificity of the inhibitor herbimycin A.

The cause of overexpression of c-Src in all mentioned hyperproliferating tissues remains obscure. There is as yet no evidence for c-Src gene amplification in cancer. However, it has been reported that 5 out of 9 HNSCC cell lines exhibited gain of chromosome 20 [23] and that c-Src resides on the proximal long arm of chromosome 20 [24]. The gain of chromosome 20 could lead to an overexpression of c-Src.

The observation that c-Src is located in the cytoplasm and predominantly around the nucleus in all our c-Src-positive cells, might indicate a localization of c-Src at endosomal membranes or at cytoskeletal components [25]. Cytoskeletal localization might indicate an active form of c-Src. It has been reported that activated c-Src is translocated to the cytoskeleton upon activation by EGF or platelet-derived growth factor [26].

Our findings on c-Src localization coincide with observations in breast tumors [11]. However, we did not show a plasma membrane localization of c-Src. David-Pfeuty *et al* investigated the localization of c-Src in more detail in NIH-3T3 c-Src overexpressor cells [27]. They demonstrated by immunofluorescence microscopy that c-Src can be located at the plasma membrane, but during the G1 and S phases of the cell cycle it is mainly located perinuclearly in the cytoplasm around the centrosomes. During G2 phase and mitosis, however, they founda predominant cytosolic localization [27].

We [data not shown] and others have found that the EGFR, another PTK, is overexpressed in almost all HNSCC and is, like c-Src mainly expressed in the areas with proliferation [7]. This was determined without double-labelling technics.

Activation of c-Src is linked to EGFR overexpression or activation [28] and vice versa [29]. It has also been demonstrated that formation of a heterocomplex between c-Src and EGFR potentiates oncogenesis in a c-Src/EGFR double overexpressor cell line [30]. Probably, overexpression of EGFR and c-Src potentiate each other in the transformation of HNSCC cells.

Our results indicate a role for c-Src in the proliferation of HNSCC, premalignant epithelium and benign tumors but also in inflamed mucosa. A role for c-Src in the genesis of HNSCC is debatable, as it is unknown, whether overexpressed c-Src is a cause or a consequence of neoplastic transformation.

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Chapter 3

Increased expression of epidermal growth factor receptor in normal epithelium adjacent to head and neck carcinomas independent of tobacco and alcohol abuse

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Abstract

In this study we examined if expression of the epidermal growth factor receptor (EGFR) in normal epithelium adjacent to head and neck squamous cell carcinomas (HNSCC) is increased and if this increase is due to the use of tobacco and alcohol.Cut sections of formalin-fixed and paraffin-embedded material of histologically normal epithelium adjacent to HNSCC from 25 patients that smoke excessively and abuse alcohol, and 17 HNSCC patients that don't abuse tobacco and alcohol were compared with cut sections of normal epithelium from 27 control individuals. The sections were immunohistochemically stained for the EGFR. We show an elevation of the expression of the EGFR in patients who smoke and drink excessively, that could also be ascertained, to a lesser extent, in patients that do not have a history of smoking or drinking ($p=0.08$). We also find that the closer the epithelium lies to the HNSCC the higher the expression of the EGFR is. Expression of the EGFR is increased in tumor-adjacent mucosa (TAM) and this is not only due to the use of tobacco and/or alcohol. We suggest that paracrine effects of the HNSCC and migration of tumor cells may also play a role in this increased expression.

Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for the sixth most common solid tumor worldwide and long-term survival rates are among the lowest of the major cancers. The key molecular changes in the multi-step progression of HNSCC are still unknown and a better understanding might provide a rational therapy for a better survival of patients [1,2].

The development of HNSCC is related to the use of tobacco and alcohol [3]. Furthermore, it was noticed that multiple (pre)malignant lesions can arise in the same area. This led to the concept of "field cancerization", proposed by Slaughter *et al.* in 1953, who hypothesized that the entire epithelial surface of the upper aerodigestive tract is exposed to carcinogens which results in an increased risk of cancer development by causing multiple genetic abnormalities [4,5]. This theory is supported by clinical, histopathological, and recent molecular evidence [2]. Some of this evidence is gained by comparing parameters in normal control epithelium with those in histologically normal epithelium adjacent to the tumors. Among others, differences were found in proliferation [6], cytoplasmic areas [7], protein tyrosine kinase activity [8], p53 expression [9], and cytokeratin expression [10].

Several studies have shown increased epidermal growth factor receptor (EGFR) expression in histologically normal epithelium adjacent to HNSCC [11-16]. In addition an increased expression of EGFR was found in almost all HNSCC. The EGFR is a 170 kDa transmembrane glycoprotein with an intrinsic tyrosine-specific protein kinase activity that regulates growth in cells by transducing the mitogenic signal of its ligand across the cell membrane to the cytoplasm [17]. Four of previously mentioned studies have detemined elevated expression of the EGFR protein in the normal epithelium adjacent to HNSCC [11,12,14,16], one study [13] has described that the mRNA expression of the EGFR is increased, and a recent paper [15] has shown that this epithelium contains amplifications of the EGFR gene.

We immunohistochemically examined the expression of EGFR protein in morphologically normal epithelium adjacent to HNSCC compared with control epithelium in relation to tobacco and alcohol abuse. We found an increase of the EGFR expression in the epithelium adjacent to HNSCC in the group of patients that smoked and drank excessively as well as in the group that did not smoke or drink.

Materials and methods

Clinical material

The material for immunohistochemistry was taken from tissue specimens from three groups with 69 patients in total. Group 1 was composed of HNSCC patients that smoked excessively and most of them drank a lot of alcohol (Table 1). Group 2 consisted of HNSCC patients that had no smoking or regular drinking habits. We examined the histologically normal mucosa adjacent to the invasive tumors in these two groups, all selected by reviewing hematoxylin and eosin-stained histological slides with a pathologist. The selected sections contained both tumor and TAM. No patients had undergone any previous treatment other than biopsies as part of the diagnostic procedures. The third group was composed of patients that had no malignancy and that were surgically treated for other problems. The mucosa specimens of this latter group served as a control. About half of these control patients smoked and the other half did not smoke, none of them regularly used alcohol.

Immunohistochemistry

For immunohistochemical detection of the EGFR, 4 μm sections were cut from the formalin-fixed and paraffin-embedded mucosa samples. The sections were deparaffinised by xylene and incubated in 96% ethanol. Endogenous peroxidase activity was blocked by incubating the slides in 1,5% H_20_2 with methanol for 20 min. For antigen retrieval, the sections were incubated in a pepsin-glycine buffer ($2,5.10^6$ activity units pepsin/l and 7.5 g glycine/l, both Sigma, St Louis, USA) at 37 °C for 15 min. After washing with PBS, the sections were incubated with 10% non immune horse serum for 15 min. Subsequently they were incubated for 60 min. with 1:20 diluted and preheated (37°C) mouse-anti-EGFR monoclonal antibody (Clone E30, Biogenex, San Ramon, CA). This antibody is directed to the extracellular part of the EGFR and has been used before by others [16]. After washing in PBS, the sections were incubated with 1:500 diluted biotinylated horse anti-mouse antibody (Vector Laboratories Inc, Burlingham, CA) for 30 min. This was followed by washing in PBS again and the sections were incubated for 30 min. with a peroxidase-streptavidin conjugate (Immunotech. S.A., Marseille, France) in a 1:400 dilution The sections were washed in phosphate-citrate buffer (17.5 g/l $Na_2HPO_4.2H_2O$ and 10.7 g/l citric acid, pH 5.8) and the EGFR was visualized for light microscopy with DAB-reagent (0.06% 3,3-diaminobenzidine tetrahydrochloride and 0.03% H_20_2 in phosphate-citrate buffer). Sections were counter-stained with haematoxylin for 3 min.

As a negative control the first antibody was omitted. Some parts of the epithelium did not stain for the EGFR which could be used as a negative control as well.

Analysis of EGFR expression

The expression of the EGFR was classified into three categories depending on the extensiveness of EGFR staining in the epithelium breadthwise. Category A comprises

breadthwise staining of <25% of the epithelium from the basal layer. In category B 25-50% from the basal layer of the epithelium was EGFR positive, whereas >50% of the breadth was positive in category C comprises staining of >50% of the epithelium. That part of the epithelium, that was stained the most extensive, was noted. The distance between the tumor and different stained parts of the epithelium was measured in the section from that particular part of the epithelium to the nearest part of the tumor, by a ruler.

After measurement of the EGFR staining, the Chi-quadrate test was applicated to define the significance of the results with the P-value.

Table 1. Clinical characteristics of the patients

group	group 1: HNSCC patients smoking drinking	group 2: HNSCC patients non-smoking non-drinking	group 3: control patients
n	25	17	27
mean age	53 years	68 years	43 years
sex	16 men; 9 women	5 men; 12 women	13 men; 14 women
HNSCC	7 hypopharynx 2 oropharynx 2 tongue 8 floor of mouth 2 retromolar area 1 alveolar mucosa 2 larynx 1 palate	3 hypopharynx 1 oropharynx 7 tongue 2 floor of mouth 1 retromolar area 2 alveolar mucosa 1 cheek	no HNSCC
tobacco	20-100 cigarettes a day	no use of tobacco	-10 patients smoke 0-20 cigarettes a day -17 patients do not smoke
alcohol	-10 patients use 5-20 glasses of alcohol a day -10 patients use 2-4 glasses of alcohol - 5 patients do not use alcohol	no use of alcohol	no use of alcohol

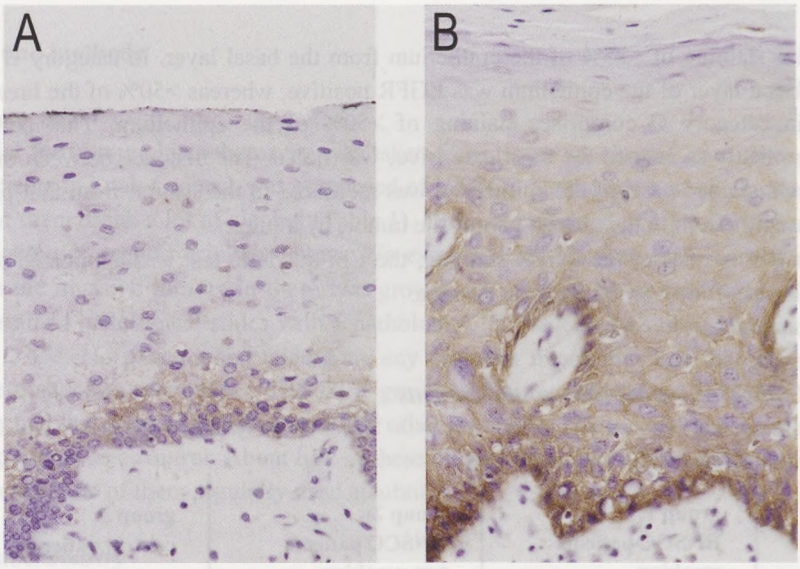

Figure 1. Immunohistochemical demonstration of EGFR expression in TAM of HNSC patients that do not smoke or drink

EGFR staining localized at the membrane and in the cytoplasm in the basal layers magnification 300 x. a) histologically normal epithelium 8 mm adjacent to HNSCC (<25% staining). b) histologically normal epithelium 1 mm adjacent to HNSCC (>50% staining).

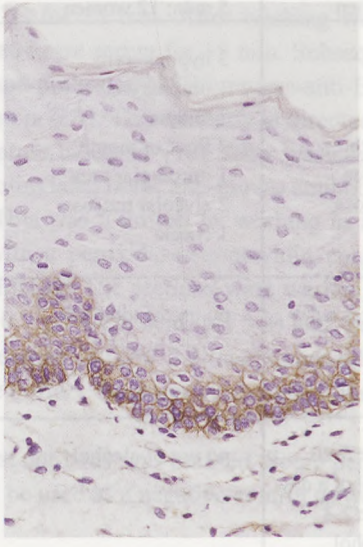

Figure 2. Immunohistochemical demonstration of EGFR expression in control epithelium

EGFR staining localized at the membrane (<25% staining), magnification 300x.

Results

All sections showed positive EGFR staining in the tumors. In the tumor-adjacent mucosa (TAM) and the control epithelium different extents of the epithelia were stained breadthwise. The staining of the EGFR was more extended in the normal epithelium adjacent to HNSCC of both the smoking/drinking and non-smoking/non-drinking group than in the epithelium of the control group (Figure 1 and 2; Table 2). Only 9 out of 27 cases from the control group showed more than 25% staining of the epithelium. However, 16 out of 25 cases from the smoking and drinking HNSCC patient group showed more than 25% staining and 9 out of 17 cases from the non-smoking and non-drinking HNSCC patient group showed more than 25% staining. So, the smoking and drinking HNSCC patient group contained more cases with increased EGFR expression than the non-smoking and non-drinking HNSCC patient group (p=0,08). No correlation could been found between the use of cigarettes and EGFR staining in the control group (results not shown).

Table 2. Extent of EGFR staining in histologically normal oral epithelium

normal epithelium	total	<25%	25-50%	>50%
group 1 adjacent to HNSCC$^{\Sigma}$ (smoking/ drinking)	n=25	9/25	13/25 *5/25	3/25 *1/25
group 2 adjacent to HNSCC$^{\Sigma}$ (non-smoking/ non-drinking)	n=17	8/17	5/17 *2/17	4/17 *3/17
group 3 control without tumor	n=27	18/27	7/27	2/27

The three groups were stained differently for the EGFR (p=0.08)

$^{\Sigma}$All tumors were positively stained for the EGFR

** Number of samples in which the expression of the EGFR increased more if the epithelium approached the tumor*

We observed in 11 cases of the HNSCC patients that the closer the normal epithelium was to the tumor the higher was the extent of staining (Figure 1). This was seen in both the HNSCC patient group that smoked and drank excessively and in the HNSCC patient group that did not abuse tobacco and alcohol (Table 2).

It was found that EGFR staining in the TAM was more localized in the cytoplasm than at the membrane, in contrast with the control epithelium (Figure 1 and 2). This could not be observed in all sections.

Discussion

The purpose of this study was to better understand why the EGFR expression is increased in histologically normal epithelium adjacent to HNSCC in relation to the concept of "field cancerization". Elevated expression of the EGFR protein in this epithelium has been found before [11,12,14,16]. However, in these studies the increased expression was found in patients that all smoked excessively and they did not look at a group of HNSCC patients without a smoking and drinking history. Neither did other studies in which amplification of the EGFR gene [15] or elevated levels of its mRNA [13] were studied in TAM. In this study, we confirmed an increased expression of the EGFR in epithelium of HNSCC patients that all smoked and almost all drank excessively compared with a control group. However, we also found an increased expression in the epithelium of non-smoking and non-drinking HNSCC patients, although in a lesser extent. In earlier studies, differences were observed in tumor-adjacent oral epithelium from HNSCC patients compared to healthy controls independent of smoking and drinking. In these studies a different expression of cytokeratins [10] and a reduction in cytoplasmic areas [7] were shown.

We could not determine any correlation between the overexpression of the EGFR and the use of tobacco in the control group. It must be noted however that individuals in the control group only smoked between 5-20 cigarettes a day and that the group did not contain more than 10 individuals that smoked. An earlier study has shown increased EGFR expression in normal control epithelium of individuals that had regular drinking and smoking habits [11]. However, the EGFR expression was determined in epithelium with chronic inflammation due to the abuse of alcohol and tobacco and it can not be excluded that the increased EGFR expression resulted from chronic inflammation instead of from tobacco and alcohol. We investigated histologically normal epithelium without inflammation. The observation that EGFR staining in the TAM was localized more in the cytoplasm than at the membrane in

contrast with the control epithelium, was consistent with findings in another study [14].

The increased expression of the EGFR in the TAM might be partially induced by tobacco and alcohol but also by paracrine effects of the tumor, as we also determined an increase in the HNSCC group that did not abuse tobacco and alcohol. Moreover in this study, the expression of the EGFR was elevated more if the epithelium was closer to the tumor, in concordance to the results of Grandis *et al* [14]. Furthermore, it was shown by others [18] that polysomies of chromosome 7, the chromosome that contains the gene for the EGFR [19], occur more frequently in tumor-adjacent epithelium than in tumor-distant epithelium. The elevated EGFR protein expression [11,12,14,16] and the polysomies of chromosome 7 [18] are, next to the adjacent-epithelium, very obviously present in the tumors itself. In all our sections, tumors were stained EGFR-positive. Based on these findings, our results do not exclude the idea that the local phenomenon of "field cancerization" could also involve the intra-epithelial expansion and lateral migration of cells from the tumor itself [5].

Cells with an increased EGFR expression take a growth advantage compared with other cells. Especially because it was shown by others that the expression of transforming growth factor alpha (TGFα), a ligand for the EGFR, is also increased at the mRNA and protein level in TAM from HNSCC patients [14,20]. The observation of elevated EGFR expression in TAM may provide a basis for the development of an early recurrence or second primary tumor after surgery. Therefore an EGFR directed therapy could be suggested for these patients. Trials have been taken place with anti-EGFR monoclonal antibodies [21] and retinoic acid [22], that down-regulates EGFR mRNA levels [20].

Thus, it is shown that changes, in TAM, generally attributed to smoking and drinking habits, also occur in patients that do not smoke or drink. So, we suggest that paracrine effects of the tumor or migration of tumor cells might also play a role in "field cancerization".

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Chapter 4a

Increased number of proliferating cells in oral epithelium from smokers and ex-smokers

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Abstract

To analyse initial tobacco-related cellular alterations in the upper aerodigestive tract, we investigated the proliferation state in paraffin embedded samples of tumor-adjacent histologically normal mucosa from head and neck squamous cell carcinoma (HNSCC) patients and normal buccal mucosa from healthy individuals. The proliferation index (PI) was assessed by indirect immunohistochemical staining for the proliferation marker Ki-67.Ki-67. Only a slight rise in PI was seen in the normal epithelium from non-smoking HNSCC patients in comparison with the epithelium from non-smoking healthy individuals. The epithelia from the smoking HNSCC patients and from the healthy smoking individuals both showed an increased PI compared with epithelia from the non-smoking HNSCC patients and healthy individuals (P=0.001). In addition, the ex-smokers in both groups still showed a trend towards increased PI. Increased PI after cessation of smoking could indicate permanent epithelial alterations. Our findings provide new evidence for the concept of field cancerization.

Introduction

Head and neck squamous cell carcinoma accounts for the sixth most common solid tumor worldwide and long-term survival rates are among the lowest of the major cancers. The key molecular changes in the multi-step progression of HNSCC are still unknown and a better understanding might provide a rational therapy for a better survival of patients [1,2].

The development of HNSCC is related to the use of tobacco and alcohol [3,4,5,6,7]. The finding of multiple (pre)malignant lesions in the upper aerodigestive tract (UADT) has led to the concept of field cancerization, as proposed by Slaughter *et al* in 1953. They hypothesized that the entire epithelial surface of the UADT is exposed to carcinogens which results in an increased risk of cancer development by causing multiple genetic abnormalities in the whole tissue region [8]. An alternative theory for the occurence of multiple (pre)malignant lesions is based on the premise that any transforming event is rare and that the multiple lesions arise due to widespread migration of the progeny of the initially transformed cells through the whole UADT [9,10].

Field cancerization is supported by clinical, histopathological, and recent molecular evidence [2]. Evidence was among others gained by comparing parameters in normal control epithelium with those in histologically normal epithelium adjacent to the tumors. Differences were found in epidermal growth factor receptor expression [11,12], p53 mutations [13], copy numbers of chromosome 7 and 17 [14], ultrastructural morphology [15], cytoplasmic areas [16] and protein tyrosine kinase activity [17].

Increased proliferation in the whole UADT epithelium could represent an early stage in the multistep process of the development of HNSCC. To test this hypothesis, the proliferation index (PI) in apparently healthy oral and laryngopharyngeal epithelium from HNSCC patients as well as in normal oral epithelium from healthy individuals was determined by staining for the proliferation marker Ki-67. This nuclear antigen is expressed during G1, S, G2 and the M phase of the cell cycle and has been extensively used as an indicator of cell proliferation [18,19]. We show that an increasing number of proliferating cells in the tumor-adjacent mucosa (TAM) and in normal epithelium is correlated with the use of tobacco. Even individuals who cessated smoking showed a trend towards an increased number of proliferating cells in their epithelium.

Materials and methods

Clinical material

The material to assess proliferation in relation to tobacco use was taken from tissue specimens from six selected groups with 153 patients in total after reviewing hematoxylin and eosin-stained histological slides. In the first three groups, we examined the histologically normal mucosa adjacent to HNSCC which was obtained from HNSCC resection specimens with or without adjacent dysplasias. The patients had not undergone any previous treatment other than biopsies as part of the preoperative diagnostic procedures. Group 1 consisted of HNSCC patients that had no smoking habits. Group 2 was composed of HNSCC patients that smoked excessively. Group 3 was composed of HNSCC patients who had stopped smoking longer than 1 year before they were operated for their invasive cancer (Table 1).

The next three groups consisted of patients that had no malignancy. Buccal mucosa was removed during surgical treatment for non-tumor related intra-oral conditions. Patients from group 4 did not have a history of smoking, patients from group 5 smoked and patients from group 6 had stopped smoking longer than 3 months before surgery. It must be remarked that the HNSCC patients in group 2 besides excessive smoking, almost all also used extremely large amounts of alcohol. This was inevitable, because of the highly related occurence of both smoking and alcohol abuse in these patients. In the other groups we could choose non-alcohol users.

The use of mentioned tissues for this research was approved by the ethical comittee for scientific research on human beings of the Utrecht University Hospital. HNSCC patients were operated at the Department of Otorhinolaryngology or at the Department of Oral and Maxillofacial Surgery of the Utrecht University Hospital. Data of smoking and alcohol usage were obtained from the clinical records of the patients. The mucosal samples from the healthy individuals were obtained from oral surgeons working in private practice. These patients were interviewed by use of a standardized questionnaire for the data concerning their smoking and drinking habits.

The material for assessment of proliferation between different sites in oral mucosa consisted of buccal mucosa samples from 37 healthy non-smoking and non-drinking individuals (mean age 27 years) and tonsil mucosa samples from 26 healthy non-smoking and non-drinking individuals (mean age 25 years).

Immunohistochemistry

For immunohistochemical detection of the proliferation marker Ki-67, 4 μm sections were cut from the formalin-fixed and paraffin-embedded mucosa samples. The sections were deparaffinised by xylene and incubated in 96% ethanol. Endogenous peroxidase activity was blocked by incubating the slides in methanol with 1,5% H_2O_2 for 20 min. For antigen retrieval, the sections were boiled in citrate buffer (2.94 g/l sodium citrate, pH 6.0) for 15 min. and subsequently cooled down to 30 ^{0}C. After washing with PBS, the sections were incubated

Table 1. Clinical characteristics of the patients

	HNSCC patients			healthy individuals		
	group 1 non-smoking	**group 2 smoking**	**group 3 quit-smoking**	**group 4 non-smoking**	**group 5 smoking**	**group 6 quit-smoking**
number	20	22	29	29	34	19
mean age	57 years	43 years	72 years	29 years	33 years	42 years
sex	5 ♂ 15♀	13 ♂ 9♀	19 ♂ 10 ♀	19 ♂ 9 ♀	28 ♂ 8 ♀	14 ♂ 5 ♀
tumor presence and location	18 oral 2 larynx	14 oral 5 larynx 2 hypophx 1 orophx	15 oral 5 larynx 6 hypophx 3 orophx	no tumor, buccal mucosa	no tumor, buccal mucosa	no tumor, buccal mucosa
tobacco use a day	no tobacco use in past or at present	20-100 cigarettes	quit smoking longer than 1 year ago	no tobacco use in past or at present	15-30 cigarettes	quit smoking longer than 3 months ago
mean pack-years	0	unknown	unknown	0	16	18
alcohol use a day	no or only social alcohol use	-10 patients use 5-20 units -10 patients use 2-4 units -2 patients do not use	no or only social alcohol use	no or only social alcohol use	no or only social alcohol use	no or only social alcohol use

hypophx=hypopharynx, orophx=oropharynx

The number of packyears, a measure of cumulative smoking, was calculated as the number of years smoked multiplied by the number of packs of cigarettes (20 cigarettes a pack) smoked daily. A unit of alcohol is defined as one alcoholic beverage. Social use means no more than two alcoholic consumptions per day.

with 10% non immune horse serum for 15 min. Subsequently they were incubated for 60 min. with 1:200 diluted mouse-anti-Ki-67 (MIB-1, Immunotech. S.A., Marseille, France) followed by washing in PBS and incubation with 1:500 diluted biotinylated horse anti-mouse antibody (Vector Laboratories Inc, Burlingham, CA) for 30 min. After washing in PBS the sections were incubated for 30 min. with peroxidase-streptavidin conjugate (Immunotech.) in a 1:400 dilution. The sections were washed in phosphate-citrate buffer (17.5 g/l $Na_2HPO_4.2H_2O$ and 10.7 g/l citric acid, pH 5.8) and Ki-67 was visualized for light microscopy with DAB-reagent (0.06% 3,3-diaminobenzidine tetrahydrochloride and 0.03% H_20_2 in phosphate-citrate buffer). Sections were counterstained with haematoxylin for 3 min.

Analysis of Ki-67 positivity

The proliferation index (PI) was determined by counting the number of Ki-67 positive cells in a epithelial area defined by a length of 100 basal cells. The chosen histologically normal epithelial areas were free of atypia, hyperplasia and inflammation. Furthermore, they were all lightly keratinized. After counting the number of Ki-67 positive nuclei, both analysis of variance and the two-sided Student's t-test were performed to define the significance of the results.

Results

To assess the number of proliferating cells in normal epithelium, paraffin sections were stained for Ki-67 with the antibody MIB-1. All tissue samples showed Ki-67 positivity mainly in the nuclei of the parabasal and basal cells of the oral and laryngopharyngeal epithelium (Figure 1). The various mean proliferation indices (PI) were determined in normal epithelium adjacent to HNSCC and in normal buccal epithelium of healthy individuals in relation to the usage of tobacco (Figure 2; Table 2). The PI of the epithelium from non-smoking HNSCC patients (mean PI= 59.3) was slightly higher than the mean PI of the healthy non-smoking individuals (mean PI= 52.4). The epithelium from the smoking HNSCC patients (mean PI= 84.2) contained a 1.6 times higher PI compared with the epithelium from the healthy non-smoking individuals and a 1.4 times higher PI compared with the epithelium from the non-smoking HNSCC patients. Furthermore, a 1.3 times higher PI was found in the epithelium from smoking individuals of the healthy group (mean PI=68.4) compared with the non-smoking individuals in this same group. However, the PI in the smoking healthy individuals seemed to be less increased than the PI of the epithelium from smoking HNSCC patients.

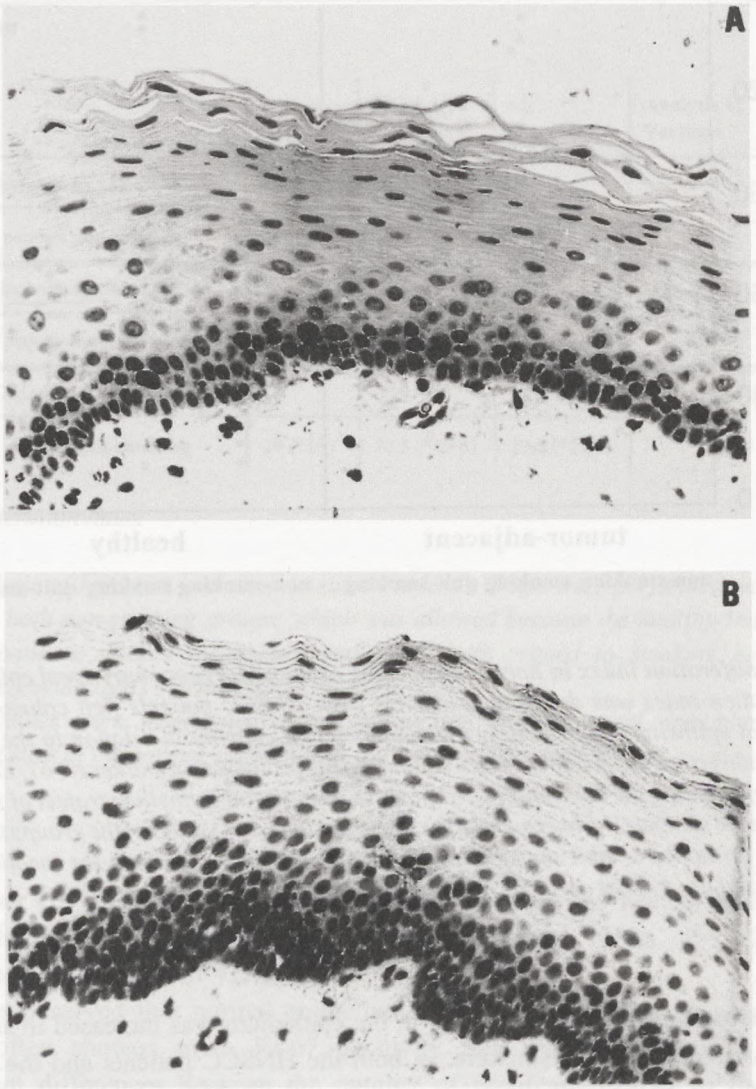

Figure 1. Immunohistochemical demonstration of Ki-67 in TAM of HNSCC patients
Ki-67 positivity is seen in the nuclei of basal and parabasal cells of the histologically normal epithelium after indirect immunohistochemical staining and a light counterstaining with haematoxylin (original amplification 200x). A) TAM from a patient who does not use tobacco. B) TAM from a patient who excessively abuses tobacco.

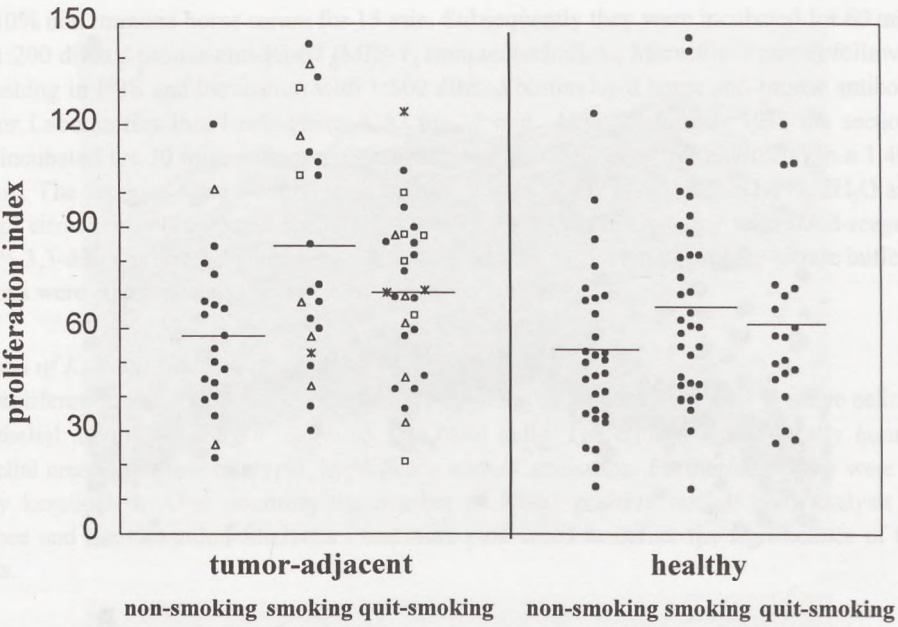

Figure 2. Proliferation index in normal appearing oral and laryngopharyngeal epithelium
The proliferation index was determined in TAM from HNSCC patients (left column) and in normal buccal epithelium from healthy individuals (right column) in relation to the usage of tobacco, by indirect immunohistochemical staining of the proliferation marker Ki-67. Each point is the result of counting the number of Ki-67 positive nuclei in an epithelium traject of 100 basal cells in one tissue sample. The horizontal lines represent the mean values of the groups that never smoked, still do smoke or quit smoking cigarettes. The symbols represent the location of the normal epithelium: (●) oral cavity, (Δ) larynx, (□) hypopharynx, (✳) oropharynx.

After ascertaining the fact that the PI in the epithelium was increased in smokers, the PI was determined in ex-smokers. In both the HNSCC patients and the healthy individuals the PI of the epithelium in ex-smokers was still increased, although to a lesser extent than in the epithelium from smokers. It must be remarked, however that this increase in the ex-smokers was not completely significant (Table 2). The epithelium from the ex-smoking HNSCC patients (mean PI= 70.5) appeared to contain a higher PI in comparison with the epithelium from the healthy ex-smoking individuals (mean PI= 60.9). The mean PIs in non-smoking, smoking and quit-smoking healthy individuals and HNSCC patients were significantly different from

Table 2. Statistics of the proliferation indices in normal oral and laryngopharyngeal epithelium

	n	mean PI	sd	Analysis of Variance	Student's T-test
Healthy individuals **non-smoking**	29	52.4	24.3		
HNSCC patients **non-smoking**	20 (*18)	59.3 (*58.7)	24.6 (*22.6)		
Healthy individuals **smoking**	34	68.4	31.2	P< 0.001 (*P=0.002)	P=0.001 (*P=0.004)
HNSCC patients **smoking**	22 (*14)	84.2 (*79.9)	35.2 (*34.1)		
Healthy individuals **quit-smoking**	19	60.9	26.9	P=0.09 (*P=0.13)	P=0.024 (*P=0.071)
HNSCC patients **quit-smoking**	29 (*15)	70.5 (*69.6)	23.8 (*21.7)		

PI=proliferation index

The Analysis of Variance and the two-sided Student's T test were performed with respect to the PI in both non-smoking groups, which was allowed because the healthy individuals and tumour patients showed the same trend in PI with regard to smoking habits as was determined by Analysis of Variance (P=0.72).

**These values were determined after omitting the oropharyngeal, hypopharyngeal and laryngeal sites of the normal epithelium in the tumor patients group.*

each other (P=0.021; data not shown), but within both the healthy and the tumor group the same trend was shown regarding smoking habits (P=0.72), as was determined by Analysis of Variance.

It was observed that normal epithelium from the oropharynx, hypopharynx and larynx often showed more Ki-67 positivity than buccal epithelium. To assess significant differences between the number of proliferating cells in these tissues, paraffin sections from 37 buccal mucosas and 26 tonsil mucosas from healthy non-smoking and non-drinking individuals were stained for Ki-67. The mean PI (115.2) for tonsil mucosa was almost twice as high (P<0.001; data not shown) as the mean PI (58.8) for buccal mucosa (Figure 3).

Taking into consideration that the location of the epithelium might be of importance for the PI, our previous results were re-evaluted (Table 2). However, the

main findings did not differ after this re-evalution.

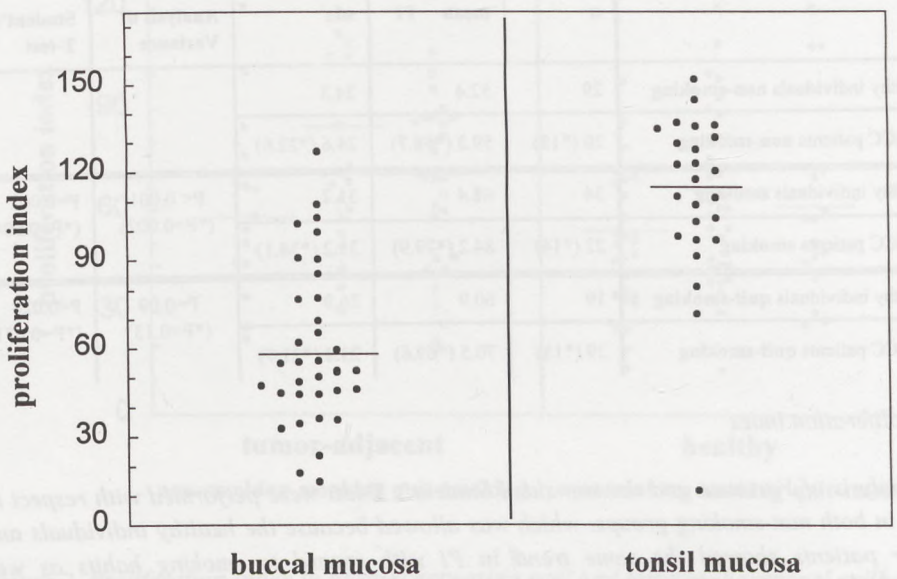

Figure 3. Proliferation index in normal buccal and tonsil epithelium
The proliferation index was determined in normal buccal and tonsil epithelium by indirect immunohistochemical staining of the proliferation marker Ki-67. Each point is the result of counting the number of Ki-67 positive nuclei in an epithelium traject of 100 basal cells in one tissue sample. The horizontal lines represent the mean values of the groups.

Discussion

In this study the number of proliferating cells in epithelium adjacent to HNSCC and oral epithelium of healthy individuals is determined in relation to smoking habits to obtain evidence for tobacco related mucosal alterations preceding cancer development. Indirect immunohistochemical staining of the proliferation marker Ki-67 was used to determine the PI.

Two previous studies have found an increased proliferation in tumor-adjacent normal epithelium by staining of two other proliferation markers (a) proliferating cell nuclear antigen [20] and (b) nucleolar organizer regions [21]. We also found a slightly

increased proliferation in TAM compared to control epithelium. However, both previous studies did not consider the relation with tobacco in the increase. They only mentioned an increased proliferation in TAM compared with normal epithelium from healthy control individuals.

We could prove the significance of tobacco use for the main increase in number of proliferating cells in TAM as this increment is not seen in the same amount in the TAM of non-smokers. Therefore, this increment of PI can not only be explained by paracrine effects of the tumor or by lateral intraepithelial migration of the progeny of the initially transformed tumor cells. The deleterious role of tobacco is further substantiated by an increase in number of proliferating cells in normal oral epithelium from healthy smokers. Even HNSCC patients and healthy individuals who cessated smoking cigarettes showed a trend towards an increased PI in their epithelium. Thus, exposure to tobacco during a long time can alter cell cycle progression. If these smoking-induced alterations are considered as (one of) the first steps to premalignany, our results add evidence for the conservative theory of field cancerization [8,9,10] that says that the multiple (pre)malignant lesions often found in UADT epithelium are caused by the prolonged exposure to carcinogens.

The increased PI that was found in smokers and ex-smokers in both tumor-adjacent and control mucosa is not due to differences in age neither by gender differences as differences in age and gender do not correlate with the differences in PI. This does not mean that differences in age and gender can not play a role at all in proliferation states in epithelia. However regional differences in UADT epithelium could play a greater role as we observed that the number of proliferating cells in tonsil mucosa was almost twice as high as the number in buccal mucosa. Alterations in proliferation between different sites in the UADT were also found by Shin *et al* in TAM [20]. Though, differences in location did not create a bias in our results with respect to smoking habits. Furthermore, the increased PI in relation to smoking habits is also demonstrated in the group of healthy individuals in which all tissue samples were obtained from the same site. Thus, smoking does increase the proliferation in UADT epithelium.

The PI in TAM from smokers appears to be higher than the PI in epithelium from healthy smokers. This is probably due to the higher daily use of cigarettes by the HNSCC patient group than by the patients in the healthy group but can also be explained by the fact that HNSCC patients almost all drank extremely large amounts of alcohol next to their smoking habits (Table 1). It was shown before that chronic ethanol consumption by rats caused oral mucosal athrophy associated with hyper-regeneration [22]. Furthermore, it is known that alcohol intake increases the risk for

HNSCC and potentiates the noxious effects of tobacco [3,5]. It seems to be that the PI of TAM in ex-smokers is also increased in comparison with the epithelium from healthy ex-smokers. This could also be explained by the probably higher use of cigarettes by the HNSCC patients before cessation this habit or by a possible abuse of alcohol in earlier years.

The relevance of an increasing number of proliferating cells for carcinogenesis is substantiated by the observations of Shin *et al* [20]. They found that the various steps presumed to play a role in HNSCC tumorigenesis: epithelial hyperplasia, epithelial dysplasia, and HNSCC, were associated with a sequentially increasing PI. The significance of tobacco in inducing epithelial hyperplasia and dysplasia has been shown by others [23]. Therefore, the role of tobacco in HNSCC carcinogenesis may well manifest itself in two ways: by causing histomorphologically visible alterations and by an increased number of proliferating cells that is present in not yet morphologically altered epithelium.

One should rule out the possibility that the increased PI in morphologically normal epithelium from smokers is only an adaptive tissue response without having any significance in terms of carcinogenesis. Indeed, a concentration-dependent wave of proliferation was shown in rat tracheal epithelium after acute exposure to tobacco smoke [24] which could be explained by a local regenerative response to compensate for increased cell loss or damage by the tobacco. However, we think that the increased PI in smokers is not only due to this regenerative effect, but also due to protracted other effects of smoking, like mutated cell growth controlling genes [2]. It is known that tobacco contains many carcinogens [25]. An indication for presence of protracted effects is the higher PI in ex-smokers in both the TAM and normal epithelium from healthy individuals. Additional evidence that tobacco-induced cellular alterations persist after abandoning smoking comes from clinical studies which showed that cessation of smoking only partly reduces the risk for developing second primary tumors in the UADT. There is only a 50% reduction of the oral cancer risk when individuals cessated smoking longer than 9 years [6]. No reduction of risk for second primary tumors was seen after two years of cessation [7]. In addition, approximately the same number of precancerous lesions was present in ex-smokers as in light smokers [23].

Cells of heavy smokers have been found to be mutagen-hypersensitive by determination of the number of chromatid breaks per cell of cultured lymphocytes treated with bleomycin [4]. Furthermore, an increased proliferation rate in cells raises the susceptibility for the development of cancer, because dividing cells become more sensitive for mutagenesis by carcinogens [26]. This means that the epithelium from

smokers and ex-smokers is more susceptible to mutagenic effects than the epithelium from non-smokers and mutations in important genes controling cell growth could start the multi-step progression of HNSCC [2]. In addition, the higher incidence of oropharynx tumors than buccal tumors in HNSCC patients [27] may partially be explained by the higher number of proliferating cells in tonsil mucosa than in buccal mucosa.

We demonstrated that smoking leads to an increased number of proliferating cells in UADT-epithelium, which adds new evidence to the concept of field cancerization. The possible persistence of the increased PI after cessation of smoking, could indicate permanent epithelial alterations and may refute the idea that this increased PI in smokers merely represents a reversible epithelial response to a noxious agent. As this increase in proliferating cells occurs without the development of histologically visible epithelial alterations, we consider this feature as the first step on the way to HNSCC. The higher proliferative activity in smokers and ex-smokers may well constitute a fertile soil for genetic events culminating in the development of HNSCC. Further studies will hopefully reveal which cellular mechanisms regulate the disturbance of proliferation by the influence of tobacco.

Acknowledgements

We would like to thank the oral surgeons and otorhinolaryngologists who provided the tissue samples and patient data for the HNSCC patient group. We also express our gratitude to the oral surgeons who interviewed and biopsied the healthy individuals. Hanneke van de Craats is also thanked for her assistance. Furthermore, we are indebted for the statistical help from the University Centre for Biostatistics.

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Chapter 4b

Increased number of cyclin A positive cells in oral epithelium from smokers

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Abstract

Previously, we showed an increase in the number of Ki-67 positive cells in normal oral epithelium from smoking healthy individuals and smoking head and neck squamous cell carcinoma patients (Oral Oncology 1998; 34: 297-303). *Ex-smokers showed a trend towards an increased proliferation index, which could indicate permanent epithelial alterations. In this report the proliferation index was assessed with cyclin A staining. The same results were obtained. This supports the idea that the increased proliferation index persists for a time after abandoning this habit.*

Introduction

The development of head and neck squamous cell carcinoma (HNSCC) is related to the use of tobacco and alcohol [1,2,3,4]. The finding of multiple (pre)malignant lesions in the upper aerodigestive tract (UADT) has led to the concept of field cancerization, as proposed by Slaughter *et al.* in 1953. They hypothesized that the entire epithelial surface of the UADT is exposed to carcinogens which results in an increased risk of cancer development by causing multiple genetic abnormalities in the whole tissue region [5].

Previously, we showed that the number of Ki-67 positive cells in oral epithelium from both smoking healthy individuals and smoking HNSCC patients was increased [6]. Ex-smokers showed a trend towards an increased proliferation index which indicated permanent epithelial alterations [6]. Since the incidence of p53 mutations increased with the progression of HNSCC [7] and p53 mutations are related to smoking [8], we investigated if p53 overexpression was associated with the increased proliferation in oral mucosa from smokers [9]. However, no relationship could be found [9]. Thus probably other alterations than p53 mutations cause the increased proliferation in oral mucosa from smokers.

In this report, the trend towards increased proliferation in normal oral mucosa from ex-smokers is further investigated in HNSCC patients. This time we used another proliferation marker, named cyclin A. This cyclin is expressed during S-phase and G2-phase [10]. Furthermore, we selected HNSCC patients who did not abuse alcohol to prevent a bias from this [11]. In addition, we increased the number of patients for this investigation.

Materials and Methods

Clinical material

The material to assess proliferation in relation to tobacco use was taken from tissue specimens from three selected groups with 94 patients in total after reviewing hematoxylin and eosin-stained histological slides (Table 1). We examined histologically normal mucosa adjacent to HNSCC which was obtained from HNSCC resection specimens with or without adjacent dysplasias. The chosen histologically normal epithelial areas were free of atypia, hyperplasia and inflammation. Furthermore, they were all lightly keratinized. The patients had not undergone any previous treatment other than biopsies as part of the preoperative diagnostic procedures.

The use of mentioned tissues for this research was approved by the ethical comittee for

scientific research on human beings of the Utrecht University Hospital. HNSCC patients were operated at the Department of Otorhinolaryngology or at the Department of Oral and Maxillofacial Surgery of the Utrecht University Hospital. Data of smoking and alcohol usage were obtained from the clinical records of the patients.

Immunohistochemistry

For immunohistochemical detection of the proliferation marker cyclin A in the sections from the formalin-fixed and paraffin-embedded mucosa samples, we followed the same procedure as before [6] with the following changes. For antigen retrieval, the sections were boiled in Tris/EDTA buffer (4.84 g/l Tris, 0.37 g/l EDTA; pH 8.0) for 15 min. and cooled down to 30 ^{0}C. They were incubated for 60 min. with 1:50 diluted mouse-anti-cyclin A (cyclin A, Novocastra, New Castle, UK), followed by a biotine-streptavidin dectection method [6].

Table 1. Clinical characteristics of the HNSCC patients

	group 1 non-smoking	**group 2 smoking**	**group 3 quit-smoking**
number	26	28	40
mean age	68 years	58 years	66 years
sex	7 ♂ 19 ♀	19 ♂ 9♀	28 ♂ 12 ♀
tumor site	23 oral 0 larynx 2 hypophx 1 orophx	20 oral 5 larynx 2 hypophx 1 orophx	22 oral 8 larynx 6 hypophx 4 orophx
tobacco use a day	no tobacco use in past or at present	20-100 cigarettes	10-100 cigarettes, quit smoking longer than 1 year ago

hypophx=hypopharynx, orophx=oropharynx

patients do not drink more than 2 alcoholic consumptions a day

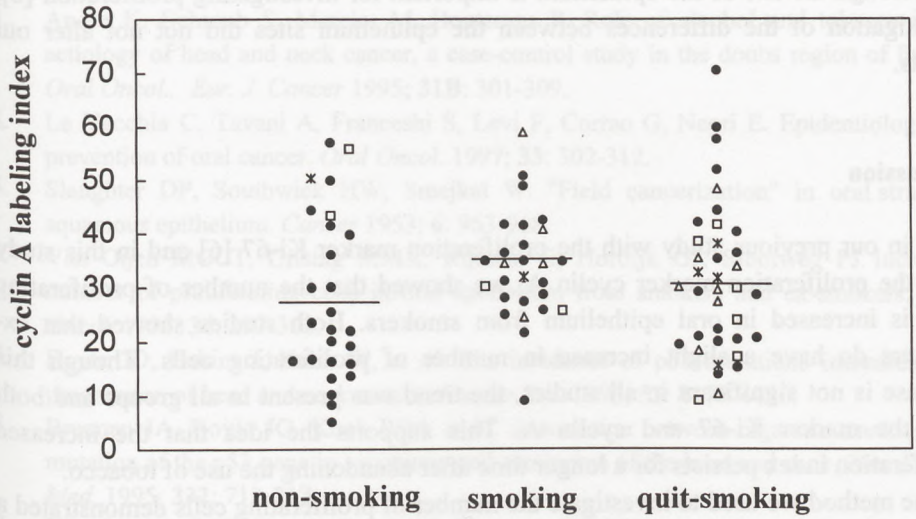

Figure 1. Proliferation index in normal appearing oral and laryngopharyngeal epithelium
The proliferation index was determined in normal epithelium adjacent to HNSCC in relation to the usage of tobacco, by indirect immunohistochemical staining of the proliferation marker cyclin A. The chosen histologically normal epithelial areas were free of atypia, hyperplasia and inflammation. Each point is the result of counting the number of cyclin A positive nuclei in an epithelium traject of 100 basal cells in one tissue sample. The horizontal lines represent the mean values of the groups that never smoked, still do smoke or quit smoking cigarettes. The symbols represent the location of the normal epithelium: (●) oral cavity, (Δ) larynx, (□) hypopharynx, (✳) oropharynx.

Results

To assess the number of proliferating cells in TAM, paraffin sections were stained for cyclin A. All tissue samples showed cyclin A positivity mainly in the nuclei of the parabasal and basal cells of the oral and laryngopharyngeal epithelium. The various mean proliferation indices (PI) were determined in relation to the usage of tobacco (Figure 1). The PI of the normal epithelium from smoking patients (mean PI=35.2) was significantly increased (P=0.04; Welch's test) compared with non-smoking patients (mean PI=28.0). The PI in the epithelium from the quit-smoking patients

(mean PI=31.3) was not significantly different from either the non-smoking or the smoking patients.

Although the site of the epithelium is important for investigating proliferation [6], investigation of the differences between the epithelium sites did not not alter our results.

Discussion

Both in our previous study with the proliferation marker Ki-67 [6] and in this study with the proliferation marker cyclin A, we showed that the number of proliferating cells is increased in oral epithelium from smokers. Both studies showed that ex-smokers do have a slight increase in number of proliferating cells. Though this increase is not significant in all studies, the trend was present in all groups and both with the marker Ki-67 and cyclin A. This supports the idea that the increased proliferation index persists for a longer time after abandoning the use of tobacco.

The method we used to investigate the number of proliferating cells demonstrated a uniformly increased number of cyclin A positive cells in the (supra)basal layers. This indicates that the increase in proliferation is probably not due to genetic events, since genetic events most likely would have created a focal increase in cyclin A positive cells. In addition, the best candidate for such an early genetic alteration, p53, was not involved in the observed increase in number of proliferating cells, as the number of p53 overexpressing cells in the epithelium from smokers was not increased similar to the uniform pattern of the cyclin A positive cells [9].

The increase in proliferation in ex-smokers is not as high as in smokers, in all our investigated groups from this study and the previous study [6], which indicates that at least part of the increase in proliferation is due to a local regenerative response to compensate for increased cell loss or damage by the tobacco. However, this can also be explained by a possible lower number of tobacco packyears.

Cessation of smoking only partly reduces the risk for developing second primary tumors in the UADT [2]. The higher proliferative activity in (ex-)smokers may constitute a fertile soil for genetic events accumulating in the development of HNSCC.

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Chapter 5

p53 overexpression in oral mucosa in relation to smoking

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Abstract

The tumor-suppressor p53 is mutated in many head and neck squamous cell carcinomas (HNSCC). In this study it was immunohistochemically assessed that similar numbers of p53 overexpressing cells were uniformly spread over normal oral epithelium despite different smoking habits or the presence of an adjacent HNSCC. Previously, we have observed an increased number of proliferating cells in normal oral mucosa from (ex-)smoking individuals (Oral Oncology 1998; 34: 297-303). *We can conclude that overexpression of p53 does not play a role in this increase. In contrast to the uniformly spread p53 overexpression, focally overexpressed p53 occurred more frequently ($p<0.05$) in the tumor-adjacent normal mucosa (TAM) from smoking HNSCC patients (50%) than from non-smoking HNSCC patients (20%). This increase in focal p53 overexpression might represent an early alteration in the development of HNSCC. However, it could not be detected in mucosa from healthy smokers. This indicates that besides the abuse of tobacco, other environmental factors and/or genetic factors must contribute to the presence of p53 positive clusters in TAM. Abuse of alcohol is an additional factor in our HNSCC patients and might play a role in the development of the p53 positive clusters together with the abuse of tobacco.*

Introduction

The development of head and neck squamous cell carcinoma (HNSCC) is generally accepted to be a multistep process in which alterations in oncogenes and tumor-suppressor genes play an important role [1,2]. Epidemiologic data have strongly linked tobacco and alcohol consumption to the development of HNSCC [3,4].

The finding of multiple (pre)malignant lesions in the upper aerodigestive tract (UADT) has led to the concept of field cancerization, as proposed by Slaughter *et al.* in 1953 [5]. They hypothesized that the entire epithelial surface of the UADT is exposed to carcinogens which results in an increased risk of cancer development by causing multiple genetic abnormalities in the whole tissue region. An alternative theory for the occurrence of multiple (pre)malignant lesions is based on the premise that any transforming event is rare and that the multiple lesions arise due to widespread migration of the progeny of the initially transformed cells through the whole UADT [6,7].

Field cancerization is supported by clinical, histopathological, and recent molecular evidence [1]. In histologically normal tumor-adjacent mucosa (TAM), alterations were found among others in proliferation [8,9], extent of cytoplasmic areas [10], genetic state and expression of the tumor suppressor p53 [11,12], epidermal growth factor receptor expression [13], protein tyrosine kinase activity [14], and cytokeratin expression [15]. Some of these changes appeared to relate to the use of tobacco, such as increased proliferation [9] and expression of cytokeratin 8/cytokeratin 7 [15].

Previously we have observed an increased number of proliferating cells in normal oral mucosa in relation to smoking [9]. In this study we wanted to focus on the relation between p53 alterations and the use of tobacco. The tumor suppressor gene p53 is thought to play a role in the carcinogenesis of a lot of malignancies [16]. The protein p53 is believed to control cell cycle progression via the regulation of several other proteins like the cyclin-dependent kinase inhibitor p21(Waf1/Cip1), MDM2, Bax, Bcl-2 and GADD45 [16]. p53 is often found mutated in HNSCC, non-invasive lesions of the head and neck and TAM [11,12,17,18]. Moreover, it is known that the occurrence of p53 mutations is often associated with smoking [17,19].

Mutant p53 has a higher stability than wild-type p53, which allows accumulation to levels detectable by immunohistochemistry [20,21,22]. We used immunohistochemical techniques to investigate p53 overexpression in normal oral mucosa from HNSCC patients and healthy individuals in relation to the use of tobacco.

Materials and methods

Clinical material

The material for detection of p53 in relation to smoking habits was taken from tissue specimens from six groups with 153 patients in total, all selected by reviewing hematoxylin and eosin-stained histological slides (Table 1). The material is the same as used in our previous study in which we assessed the increase in number of positive Ki-67 cells in oral mucosa from smokers and ex-smokers [9]. In the first three groups, we examined the histologically normal mucosa adjacent to HNSCC which was obtained from HNSCC resection specimens. Group 1 consisted of HNSCC patients that had no smoking habits. Group 2 was composed of HNSCC patients that smoked excessively. Group 3 was composed of HNSCC patients who had stopped smoking longer than 1 year before they were operated for their invasive cancer. The patients had not undergone any previous treatment other than biopsies as part of the preoperative diagnostic procedures.

The next three groups consisted of patients that had no malignancy. Buccal mucosa was removed during surgical treatment for non-tumor related intra-oral conditions. Patients from group 4 did not have a history of smoking, patients from group 5 smoked and patients from group 6 had stopped smoking longer than 3 months before surgery. It must be remarked that the HNSCC patients in group 2 besides excessive smoking, almost all also used extremely large amounts of alcohol. This was inevitable, because of the highly related occurrence of both smoking and alcohol abuse in these patients. In the other groups we could choose non-alcohol users.

The use of mentioned tissues for this research was approved by the ethical comittee for scientific research on human beings of the Utrecht University Hospital. HNSCC patients were operated at the Department of Otorhinolaryngology and the Department of Oral and Maxillofacial Surgery of the Utrecht University Hospital. Data of smoking and alcohol usage were obtained from the clinical records of the patients. The mucosal samples from the healthy individuals came from oral surgeons working in private practice. These patients were interviewed by use of a questionnaire for the data concerning their smoking and drinking habits.

As the initial number of the investigated mucosas from healthy individuals was rather small to detect possible differences in focal p53 expression, additional mucosa samples from 52 non-smokers, 43 smokers (18.9 mean pack-years) and 49 quitters (18.3 mean pack-years) were collected for this particular part of the research.

Immunohistochemistry

For immunohistochemical detection of the tumor suppressor p53, 4 μm sections were cut from the formalin-fixed and paraffin-embedded mucosa samples. The sections were deparaffinised by xylene and incubated in 96% ethanol. Endogenous peroxidase activity was

Table 1. Clinical characteristics of the patients

	HNSCC patients			healthy individuals		
	group 1 non-smoking	**group 2 smoking**	**group 3 quit-smoking**	**group 4 non-smoking**	**group 5 smoking**	**group 6 quit-smoking**
number	20	22	29	29	34	19
mean age	57 years	43 years	72 years	29 years	33 years	42 years
sex	5 ♂ 15♀	13 ♂ 9♀	19 ♂ 10 ♀	19 ♂ 9 ♀	28 ♂ 8 ♀	14 ♂ 5 ♀
tumor presence and location	18 oral 2 larynx	14 oral 5 larynx 2 hypophx 1 orophx	15 oral 5 larynx 6 hypophx 3 orophx	no tumor, buccal mucosa	no tumor, buccal mucosa	no tumor, buccal mucosa
tobacco use a day	no tobacco use in past or at present	20-100 cigarettes	quit smoking longer than 1 year ago	no tobacco use in past or at present	15-30 cigarettes	quit smoking longer than 3 months ago
mean pack-years	0	unknown	unknown	0	16	18
alcohol use a day	no or only social alcohol use	-10 patients use 5-20 units -10 patients use 2-4 units -2 patients do not use	no or only social alcohol use	no or only social alcohol use	no or only social alcohol use	no or only social alcohol use

hypophx=hypopharynx, orophx=oropharynx

The number of packyears, a measure of cumulative smoking, was calculated as the number of years smoked multiplied by the number of packs of cigarettes (20 cigarettes a pack) smoked daily. A unit of alcohol is defined as one alcoholic beverage. Social use means no more than two alcoholic consumptions per day.

blocked by incubating the slides in 1,5% H_20_2 with methanol for 20 min. For antigen retrieval, the sections were boiled in citrate buffer (2.94 g/l sodium citrate, pH 6.0) for 15 min. and subsequently cooled down to 30 ^{0}C. After washing with PBS, the sections were incubated with 10% non immune goat serum for 15 min. Subsequently they were incubated for 60 min. with 1:300 diluted rabbit-anti-p53 (CM1, Biogenex, San Ramon, CA) followed by washing in PBS and incubation with 1:1000 diluted biotinylated goat anti-rabbit antibody (Vector Laboratories Inc, Burlingham, CA) for 30 min. After washing in PBS the sections were incubated for 30 min. with peroxidase-streptavidin conjugate (Immunotech. S.A., Marseille, France) in a 1:400 dilution. The sections were washed in phosphate-citrate buffer (17.5 g/l $Na_2HPO_4.2H_2O$ and 10.7 g/l citric acid, pH 5.8) and p53 was visualized for light microscopy with DAB-reagent (0.06% 3,3-diaminobenzidine tetrahydrochloride and 0.03% H_20_2 in phosphate-citrate buffer). Sections were counterstained with haematoxylin for 1 min.

The polyclonal antibody CM1 was chosen, because it recognizes epitopes at both the N and the C terminus of the p53 protein. As positive controls, two HNSCC with known p53 point mutations were used (1: position 839 in exon 8, arg to lys, 2: position 583 in exon 6, ile to phe). In our negative controls the first antibody was omitted.

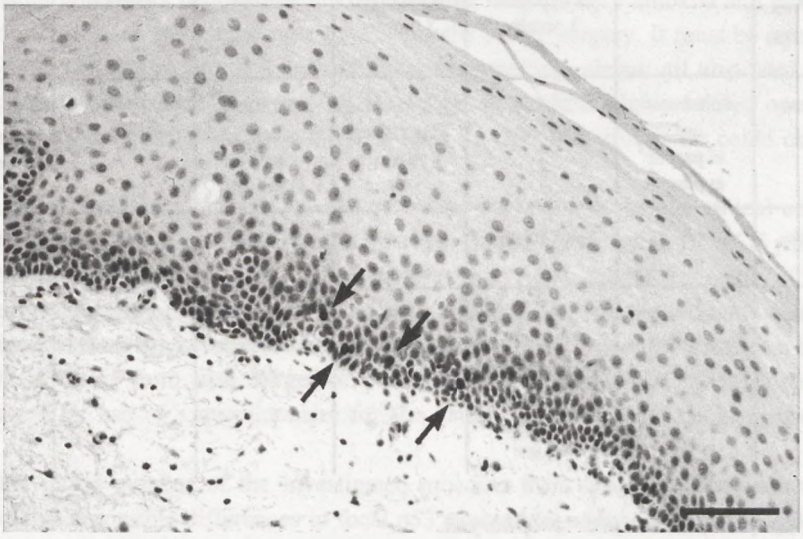

Figure 1. p53 overexpressing cells uniformly spread in normal oral mucosa
A paraffin section from normal mucosa from a non-smoking healthy individual was stained for p53 with CM-1 antibody by using indirect immunohistochemistry. The section was counterstained with haematoxylin. The figure shows some p53 positive cells (↗) in the basal layer of the epithelium. Bar represent 60 μm.

Analysis of p53 positivity

The p53 labelling index (LI) was determined by counting the number of p53 positive nuclei in an epithelial area defined by a length of minimal 100 basal cells. The chosen histologically normal epithelial areas were free of atypia, hyperplasia and inflammation. Inflammation was excluded as we have detected an increased p53 overexpression in mucosa with inflammation compared with mucosa without mucosa [results unpublished]. After counting the number of p53 positive cells, the two-sided Student's *t* test was applied to define the significance of the results.

For detection of focal p53 overexpression, the whole present normal epithelium without inflammation, was viewed by two independent persons. The epithelium was called focally positive if minimal one cluster of at least ten p53 positive cells next to each other or on top of each other was present and the surrounding normal epithelium was less p53 positive. The Chi-quadrate test was applied to assess the significance of the results.

Results

p53 overexpression in normal oral epithelium

p53 protein expression was immunohistochemically investigated in paraffin-embedded histologically normal oral epithelium from HNSCC patients and healthy individuals with different smoking habits. Earlier we have shown that the normal epithelium from (ex-)mokers has an increased number of uniformly spread Ki-67 positive cells [9]. Almost all normal tissue samples showed some p53 positivity in the nuclei of the basal and parabasal cells after antigen retrieval (Figure 1). The p53 LI (labelling index) in the epithelium and the means of the different groups were assessed by counting the p53 positive cells that were uniformly spread in the epithelium (Figure 2). The mean p53 LI was about 13 and comparable in all groups. The use of tobacco does not increase the number of p53 positive cells that are uniformly spread over the epithelium. Furthermore, differences in location of the UADT did not alter the p53 LI.

Focal p53 overexpression in normal tumor-adjacent epithelium from smokers

Instead of counting the overexpression of p53 in an arbitrary chosen small epithelium traject, we also looked for focal overexpression of p53 in longer epithelium trajects, in relation to smoking and presence of tumor.

Focal p53 overexpression was detected in the TAM group (Figure 3A). The focal p53 expression was obvious and comprised also expression above the basal layers of the epithelium. The TAM from the smokers and non-smokers contained one or more

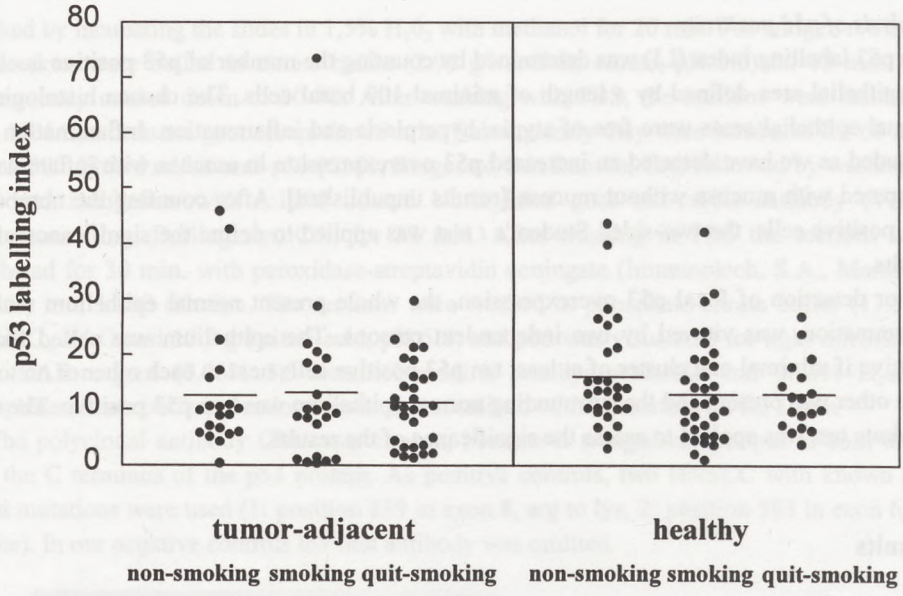

Figure 2. p53 labelling-index in normal oral epithelium
The p53 LI was determined in normal epithelium adjacent to head and neck squamous cell carcinomas (left column) and in normal oral epithelium of healthy individuals (right column) in relation to the usage of tobacco, by indirect immunohistochemical staining of the tumor suppressor p53 with CM-1 antibody. Each point is the result of counting the number of p53 positive nuclei in an epithelium traject of 100 basal cells in one tissue sample. The horizontal lines represent the mean values of the groups that never smoked, still do smoke or quit smoking cigarettes.

p53 clusters in 50% and 20% of the cases respectively (Table 2). The number of cases with focal p53 expression was significantly higher ($p<0.05$) in the smoking HNSCC patients compared to the non-smoking patients. In 32% of the HNSSC patients that cessated smoking one or more p53 clusters were observed. This was not significantly different from both the smoking and the non-smoking group of HNSCC patients. Clusters of p53 overexpression in TAM were present independently of p53 overexpression in the tumor (Table 2).

In the group of healthy individuals mostly less extensive p53 positive clusters were seen (Figure 3B). Clusters of p53 expression in the healthy group were found in 13% of the non-smoking and smoking group and in 12% of the epithelia from quit-smoking

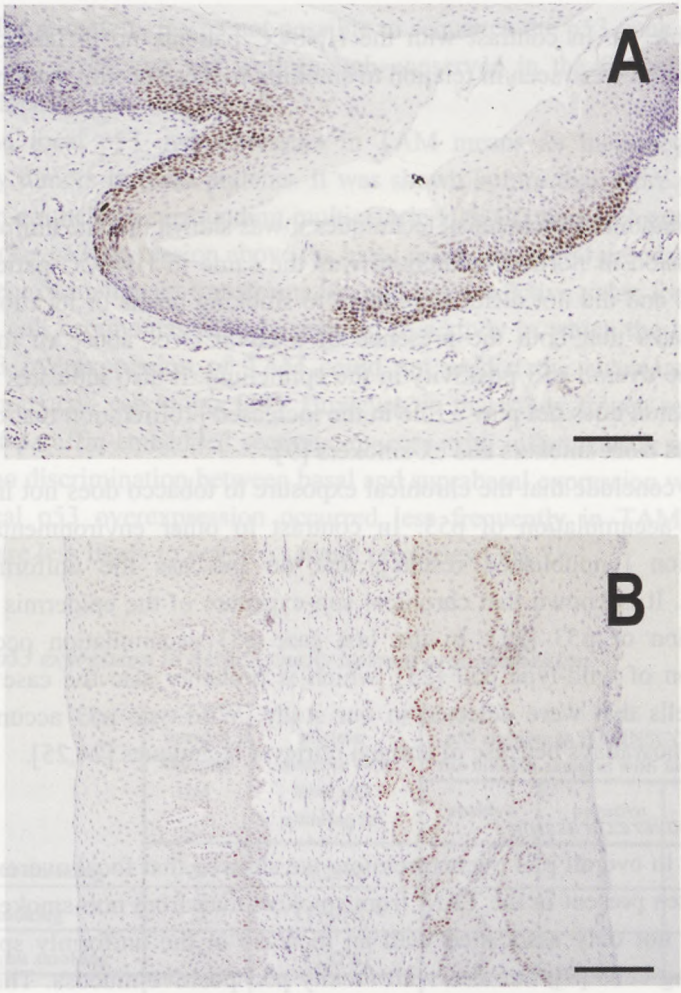

Figure 3. Focal p53 overexpression in histologically normal oral epithelium
Paraffin sections from normal oral mucosa were stained for p53 with CM-1 antibody by using indirect immunohistochemistry. The sections were counterstained with haematoxylin. A) Extensive focal p53 overexpression in both the basal and suprabasal layers of TAM from a smoking patient. B) Normal buccal mucosa from a healthy non-smoker with less extensive focal p53 overexpression only in cells of the basal layer of the right epithelium and not of the left epithelium. The figure shows two epithelial surfaces as a consequence of tissue folding. Bars represent 120 μm.

group (Table 2). In contrast with the HNSCC patients no differences in focal p53 overexpression were seen in relation to smoking in these healthy patients.

Discussion

By using immunohistochemical techniques it was shown that the uniformly spread p53 overexpression in normal epithelium was the same in HNSCC patients and healthy individuals and did not differ in relation to smoking habits or location in the UADT. This indicates that both the presence of a HNSCC or abuse of tobacco does not increase the overall p53 positivity in the epithelium. It also indicates that p53 protein overexpression does not play a role in the increased proliferation that occurs overall in oral mucosa from smokers and ex-smokers [9].

We can conclude that the chronical exposure to tobacco does not induce an overall detectable accumulation of p53. In contrast to other environmental stresses, like inflammation [unpublished results], that do increase the uniformly spread p53 expression. It is known that chronical sun-exposure of the epidermis does also cause accumulation of p53 [23]. In the last case p53 accumulation occurs because of stabilization of wild-type p53 [23], which is probably also the case in the few p53 positive cells that were detected in our study. Wild-type p53 accumulation is easy detectable nowadays because of antigen retrieval techniques [24,25].

Focal p53 overexpression

In contrast to overall p53 overexpression, we showed that focal overexpression of p53 is more often present in the TAM from smokers than from non-smokers. The abuse of tobacco is not only associated with an increase in the uniformly spread number of proliferating cells [9], but also with focally p53 positive mucosa. These p53 positive clusters might be an indication that smoking induces early mucosal alterations in the development of HNSCC.

The number of p53 positive clusters in normal epithelium from HNSCC patients that cessated smoking was in between the non-smoking and smoking group, though not significantly different, which may indicate that part of the p53 clusters has disappeared after quitting the use of tobacco.

It has been shown before that focal p53 overexpression in TAM is often due to p53 mutations [12]. Furthermore, smoking is associated with p53 mutations in HNSCC and premalignant lesions [17,19,26]. The increase in focal p53 expression in TAM from smoking patients as found in our study may well represent the presence of mutated p53

cell clusters. Unfortunately, it was not possible to sequence the p53 gene in these cell clusters, since the DNA was not well enough conserved in the formalin-fixed and paraffin-embedded tissues.

Possibly, the focal p53 overexpression in TAM means an increased chance of second primary tumors in these patients. It was shown before that more p53 clusters were present in epithelium surrounding multicentric HNSCC than unicentric HNSCC [27]. Furthermore, p53 expression above the basal cell layer in oral mucosa is found to be an early event of malignant transformation and has predictive value for developing oral squamous cell carcinomas [28]. In contrast to a study in which the investigators found that p53 overexpression of TAM could not predict the chance of a second primary squamous cell carcinoma [29]. However, in that study frozen sections were used instead of paraffin-embedded sections, mucosa with inflammation might not be excluded and no discrimination between basal and suprabasal expression was made. In our study, focal p53 overexpression occurred less frequently in TAM from non-smokers, who are less likely to develop second primaries [30].

Table 2. Focal p53 expression in histologically normal oral epithelium

	mucosa samples (n)	mucosa samples with focal p53 staining (n)	p53 staining in the HNSCCs belonging to the mucosa samples with focal p53 staining		
			positive	negative	not done
HNSCC patients **non-smoking**	20	4 (20%)	3	1	0
HNSCC patients **smoking**	22	11 (50%) *	4	6	1
HNSCC patients **quit smoking**	29	7 (32%)	3	1	3
healthy individuals **non-smoking**	80	10 (13%)			
healthy individuals **smoking**	77	10 (13%)			
healthy individuals **quit smoking**	68	8 (12%)			

Focal expression of p53 was observed in histologically normal epithelium without inflammation by indirect immunohistochemistry. In addition p53 expression was assessed in the HNSCC

n=number

**=significantly different from the non-smoking HNSCC patients ($p<0.05$: Chi-quadrate test).*

Focal p53 overexpression has been shown before in TAM [12,28,31,32]. Unfortunately, in these studies the difference between smoking patients and non-smoking patients was not investigated. Nakanishi *et al* [27], found focal p53 overexpression in TAM from smokers and not in epithelium from non-smokers, in accordance with our results. However, inflamed mucosa which could have contributed to this focal p53 overexpression [unpublished results] was not excluded in this study. We proved an increase in focal p53 overexpression in relation to smoking while mucosa with inflammation was excluded.

Regarding the field cancerization process, there are two theories explaining the presence of focal p53 overexpression in TAM: A) migration of progeny cells from the tumor [6,7], B) independent occurrence of a genetic lesion [5]. Our results are in favour of the latter one for three reasons 1) smoking is associated with more focal p53 overexpression in the TAM, 2) p53 negative tumors also express p53 positive clusters in their TAM, 3) the p53 positive clusters are often present at large distance from the tumor.

Other environmental and genetic factors that play a role in p53 overexpression

Abuse of tobacco alone is probably not enough to cause focal p53 overexpression because the increased focal expression could not be detected in the healthy smoking individuals. Probably, other environmental factors or genetic factors also play a role in the development of these clusters. Susceptibility to head and neck cancer is known to be associated with mutagen hypersensitivity [33]. The crude odd ratio to develop HNSCC was 11.5 in mutagen non-sensitive smokers and 44.6 in mutagen sensitive smokers [34]. This indicates that genetic factors can contribute to the mutagenic effects of certain carcinogens. For example, genetic polymorphisms in tobacco- and alcohol-metabolizing enzymes [35] or decreased expression of DNA mismatch-repair genes [36] can play a role in the effects of certain carcinogens in tobacco or alcohol. This probably explains why not every heavy smoker develops HNSCC and in our case p53 positive clusters.

The consumption of alcohol potentiated the crude odd ratio for HNSCC in hypersensitive smokers [33], probably because alcohol inhibits the DNA repair [37]. Most of our smoking HNSCC patients also abused alcohol, which means that p53 mutations created by carcinogens from tobacco cannot be repaired anymore in the HNSCC patients. The healthy smokers did not abuse alcohol. A possible involvement of alcohol in p53 overexpression was already suggested in the study of Colcucci *et al* [38]. They investigated primary cultures of normal oral mucosa from healthy

individuals. In contrast to our results they did find p53 mutations and protein overexpression more often in mucosa from healthy smokers than from healthy non-smokers. It must be noted however that investigating cell cultures is different from investigating fixed tissues.

From this study can be concluded that chronic exposure of mucosa from the UADT to tobacco does not increase the uniformly spread number of p53 positive cells. However, the abuse of tobacco is linked to an increase in focal overexpression of p53 in HNSCC patients. Since this increase is not observed in healthy smokers, other factors like the abuse of alcohol or genetic mutagenic sensitivity probably also play a role in the development of these p53 positive clusters.

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Chapter 6

Positivity of the proliferation marker Ki-67 in non-cycling cells

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Abstract

Ki-67 is a proliferation marker that is often used to estimate the growth fraction of tumors and other tissues. This antigen is expressed during all phases of the cell cycle but not in quiescent G_0 cells. Many studies fail to indicate that the Ki-67 antigen can be expressed, even when DNA synthesis is blocked. In this study the expression of the antigen Ki-67 was investigated in cycle-arrested osteosarcoma cells. It is shown that these cells are positive for Ki-67 even when they are arrested in G_1/S or G_2/M by using synchronizing inhibitors, by inducing p21(Waf1/Cip1) in a tetracycline-regulated expression system or by inducing wild type p53 and p21 after inflicting DNA-damage. From our results it can be concluded that not all cells containing the Ki-67 antigen are actively proliferating cells and we advice against the use of Ki-67 in studies on cells that overexpress p53 or p21.

Introduction

In tumor cells and premalignant lesions multiple genetic changes have lead to the deregulation of cell proliferation. Cell proliferation can among others be measured by determination of the expression of the cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67 as first was described by Gerdes et al. in 1983 [1[. Despite extensive studies and wide use of the Ki-67 antibody, little is known about the function of the Ki-67 antigen. It is known that it is a non-histone protein assembled by two polypeptide chains with an apparent molecular weight of 345 and 395 kDa. Furthermore, it constitutes part of the nuclear matrix during interphase and is associated with the chromosomes during mitosis [reviewed in 2,3]. It was also shown that the Ki-67 antigen is required for DNA synthesis in vitro [4].

The Ki-67 antibody (or MIB-1, its equivalent for use in paraffin-embedded tissues) has been repeatedly used to assess cell proliferation in different kind of tumors or other lesions in order to correlate its expression to prognosis [5,6,7,8]. It was assumed in these studies that the growth rate of the tumors can be estimated by calculating the percentages of Ki-67 positive cells and indeed a linear relationship was observed between labeling indices of Ki-67 and bromodeoxyuridine (BrdU) in human malignant tumors [9]. However, Ki-67 labeling indices often do not correlate with prognosis and sometimes they are not similar to other proliferation markers [10,11,12]. The use of Ki-67 in growth rate estimation of tumors has been discouraged several times [12,13]. One study revealed that the Ki-67 fraction can be invariably higher than the BrdU positive fraction, measured in cell lines after prolonged BrdU labeling [12]. The Ki-67 antigen is expressed during all phases (G_1-S-G_2-M) of the cell cycle but not in quiescent G_0 cells or cells stimulated to enter G_1 phase [14,2]. The higher Ki-67 positive fraction than BrdU positive fraction in cell lines might be due to retainment of the antigen in non-proliferating cells [12].

It was also demonstrated that the Ki-67 antigen can be expressed when DNA synthesis is blocked [14]. To analyze if this last phenomenon may increase numbers of Ki-67 positive cells without a real increase in proliferation we investigated if Ki-67 indeed stains positive in cells that are blocked either in G_1/S or G_2/M phase of the cell cycle. Therefore, we arrested cells by using synchronizing inhibitors and by inducing p21(Waf1/Cip1) in a tetracycline-regulated expression system. We also mimicked physiological cell cycle arrest by inducing wild type p53 and p21 after inflicting DNA-damage.

In response to DNA-damaging agents wild type p53 induces a G_1/S cell cycle arrest via the cyclin-dependent kinase inhibitor p21. This enables the cell to repair the DNA

damage or to induce apoptosis. In addition p53 can also induce a G_2/M cell cycle arrest independently of p21 to prevent aneuploidy [15,16]. Recently, it was found that p21 can also induce an arrest in G_2/M phase [17]. As mentioned above, the Ki-67 antigen is expressed in G_1, S, G_2 and M. Since cells arrested in response to p53 are blocked either at the G_1/S transition or somewhere in G_2/M, one could expect these arrested cells to stain positive for Ki-67. Therefore, this sheds a different light on studies in which authors tried to establish a correlation between the expression of p53 and Ki-67 or between p21 and Ki-67. In previous studies these correlations were based on the assumption that expression of wild type p53 or p21 gives rise to Ki-67 negative cells [18,19]. The fact that Ki-67 can be positive in those phases of the cell cycle where a p53-mediated arrest occurs means that discriminations between wild type p53 and mutant p53 in premalignant lesions [20,21] can not be made. Namely, both mutated p53 and wild type p53 could occur together with Ki-67 positivity.

We show that Ki-67 is still expressed in U2OS-derived cells that are arrested in G_1/S by hydroxyurea, in G_2/M by nocodazole, in G_1/S-G_2/M after induction of p21 via stable transfection, or in G_1/S/G_2 by induction of wild type p53 and p21 after DNA damage. Moreover, we demonstrate co-expression of p53 protein and Ki-67 not only in dysplastic but also in normal oral mucosa with inflammation from healthy non-smokers.

Materials and methods

Inducible p21 expression in U2OS cells

To obtain cells in which the expression of p21 could be regulated independently of p53 we cloned a 526 bp fragment of p21 human cDNA into a tetracyline-repressible expression vector as is described before [17]. UTA6 cells, derived from the human osteosarcoma cell line U2OS and expressing the tetracycline controlled transactivator (tTA) hybrid protein, were stably transfected with the p21 expression vector [17]. These cells were called UTA21.15 cells and express p21 in the absence of tetracyline and do not express p21 in the presence of tetracycline. Furthermore, these cells express functional retinoblastoma protein and p53 [22].

Cell culture

UTA21.15 cells were routinely cultured in Dulbecco's Modified Eagle Medium (Gibco, Middlesex, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM L-glutamine (Gibco), 100 µg/ml pencillin/streptomycin (Gibco) and 1 µg/ml tetracyline (Boehringer Mannheim, Mannheim, Germany) in a 5% humidified CO_2 incubator

at 37°C. Almost confluent dishes with asynchronous UTA21.15 cells were washed twice with phosphate buffered saline (PBS) before trypsinization. Cells were diluted five times, replated in 10 cm^2 dishes with round cover slips on the bottom and cultured in the presence or absence of 1 μg/ml tetracycline. After 24 hours the medium of the tetracycline free dishes was replaced by fresh medium and the medium of the other dishes was replaced for fresh medium with 1 μg/ml tetracycline alone or with both tetracycline and with 0.01 M hydroxyurea (ICN Biomedicals Inc, Costa Mesa, California), 0.25 μg/ml nocodazole (ICN Biomedicals Inc), 3 mM camptothecin (Sigma, St. Louis, Maryland), or 10 μg/ml cycloheximide (Sigma).

Analysis by flow cytometry

After a total of 48 hours of cell growth, the coverslips with UTA21.15 cells were removed for immunocytochemistry and the DNA content and DNA replication were analyzed of the remaining cells in the dishes by flow cytometry. To this end, cells were pulsed with 1 μM BrdU (Sigma) for 10 min and washed twice with PBS before trypsinization. After washing, the cells were fixed for minimal 2 hours in 70% ethanol at 4^0C. The ethanol was washed away with PBS and the cells were treated with 0.1 N HCl containing 0.5 mg/ml pepsin (Merck, Darmstadt, Germany) for 20 min at room temperature. Then the cells were washed again and treated with 2N HCl for 12 min at 37^0C, followed by addition of borate buffer (10 g/l boric acid adjusted to pH 8.5 with 0.1 M borax=disodiumtetraborate). After washing with PBS/ Tween-20/BSA (0.5% Tween and 0.1% bovine serum albumine in PBS), the samples were incubated with 1:20 diluted fluorescein isothiocyanate (FITC)-conjugated anti-BrdU (Becton-Dickinson, San Jose, California) for 60 min at 4^0C. All samples were washed and counterstained with PBS containing 10 μg/ml propidium iodide (Sigma) and 10 μg/ml DNase-free RNaseA at 37^0C for 15 min The stained cells were analyzed on a fluoresence-activated cell sorter using Lysis II software flow cytometry analysis (Becton-Dickinson).

Immunocytochemistry

After a total of 48 hours of cell growth, the coverslips with UTA21.15 cells were transfered to a 24 well plate and washed twice with cold PBS. Cells were fixed for 10 min in 3.7% formaldehyde and washed four times in PBS. The cells were incubated for 60 min with 1:50 diluted murine anti-Ki-67 monoclonal antibody (MIB-1, Immunotech S.A., Marseille, France). After washing three times in PBS/Tween/BSA the cells were incubated for 30 min with 1:20 diluted goat-anti-mouse FITC-conjugated antibody (Becton-Dickinson) and counterstained for 5 min with propidium iodide (5 μg/ml). The cells were washed in PBS/ Tween, covered with PBS/glycerol (9:1) and analyzed using a confocal laser scanning microscope (CLSM MRC-1000, Bio-Rad, Hercules, California). As a negative control the first antibody was omitted.

For double labeling Ki-67 with p21 or p53, the cells were fixed for 5 min in methanol. The cells were incubated for 60 min with 1:200 diluted murine MIB-1 antibody and either 1:250 diluted rabbit anti-p21 polyclonal antibody (C19, Santa Cruz Biotechnology Inc, Santa Cruz,

California) or 1:250 diluted rabbit anti-p53 polyclonal antibody (CM1, Biogenex, San Ramon, California). After washing, the cells were incubated for 30 min with 1:250 diluted goat-anti-rabbit biotine-conjugated antibody (Vector Laboratories Inc, Burlingham, California). The cells were washed again and finally incubated with 1:100 diluted tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin (Zymed, San Francisco, California) and 1:20 diluted goat-anti-mouse FITC-conjugated antibody. By omitting respectively one of the two first antibodies in two controls, cross reaction of the antibodies was excluded.

Immunohistochemistry

Tissue specimens were obtained from 5 patients that were surgically treated for HNSCC and contained dysplasias adjacent to the tumor. These patients had not undergone any previous treatment other than biopsies as part of the diagnostic procedures. Five buccal mucosal specimens with high p53 expression, apparently related with inflammation in the underlying stromal tissue, were obtained from healthy non-smoking/non-drinking individuals who were treated for dental problems. Use of these tissues was approved by the ethical comittee for scientific research on human beings of the Utrecht University Hospital.

Sections (4 μm) of the tissues were deparaffinized by xylene and dehydrated with 96% ethanol. For antigen retrieval, the sections were boiled in citrate buffer (2.94 g/l sodium citrate, pH 6.0) for 15 min and subsequently cooled down to 30 ^{0}C. After washing in PBS, the slides were incubated for 60 min with both 1:50 diluted rabbit anti-p53 polyclonal antibody (CM1) and 1:25 diluted murine MIB-1 antibody, followed by washing in PBS and incubation for 30 min with 1:250 diluted goat-anti-rabbit biotine-conjugated antibody. The sections were washed again, blocked with 10% rabbit serum (DAKO, Glostrup, Denmark) for 15 min and incubated with 1:50 diluted FITC-conjugated streptavidin (Zymed) and 1:50 diluted rabbit-anti-mouse TRITC-conjugated antibody (DAKO) for 30 min The sections were covered with PBS/glycerol (9:1) and analyzed using a confocal laser scanning microscope. By omitting respectively one of the two first antibodies in two controls, cross reaction of the antibodies was excluded.

Results

Ki-67 positivity in G_1/S and G_2/M arrested cells by hydroxyurea and nocodazole

To determine if Ki-67 antigen is still present in cells in G_1/S cell cycle arrest or in G_2/M arrest, osteosarcoma-derived UTA21.15 cells were incubated respectively with hydroxyurea (a DNA synthesis inhibitor) and nocodazole (a microtubule inhibitor) for 24 hours. The cell cycle profiles were analysed by incubation of the cells with propidium iodide and using flow cytometry. As expected, treatment with hydroxyurea

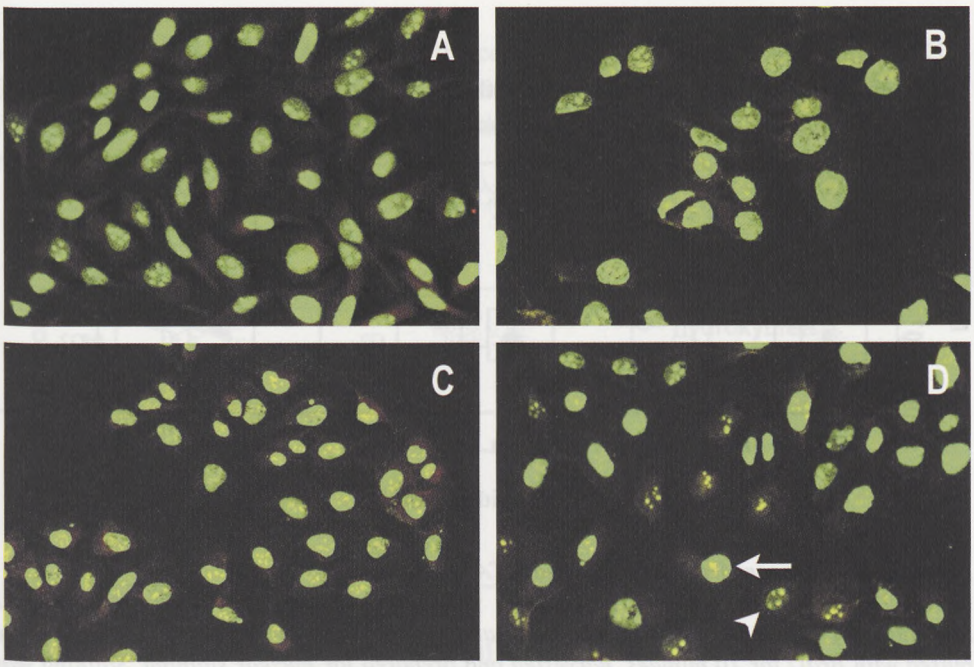


Figure 1. Ki-67 positivity in arrested UTA21.15 cells

Coverslips with UTA21.15 cells were fixed in formaldehyde. The cells were stained for Ki-67, counterstained with propidium iodide and analyzed using a confocal laser scanning microscope. ***A)*** *Strong Ki-67 expression in cells incubated for 24 hours with hydroxyurea (G_1/S arrest).* ***B)*** *Strong Ki-67 expression in cells incubated for 24 hours with nocodazole (G_2/M arrest).* ***C)*** *Strong Ki-67 expression in control cells incubated for 48 hours with tetracyline.* ***D)*** *Variable Ki-67 expression in cells incubated for 48 hours without tetracycline, which resulted in p21 expression. Sixty five percent of the cells expressed strong Ki-67 (arrow) and 35% showed a low expression level of Ki-67 (arrow head). Amplifications are 400x.*

caused the cells to accumulate for 83% in G_1/S phase (data not shown). However, the cells still showed intense staining of the Ki-67 antigen (Figure 1A) as was shown by immunocytochemistry. In addition, treatment with nocodazole resulted in an accumulation (87%) of cells in G_2/M phase (data not shown). The G_1/S and G_2/M arrested cells appeared equally positive for Ki-67 (Figure 1A, 1B). As can be expected the nuclei of the cells treated with nocodazole were almost all twice as big as the nuclei of the cells treated with hydroxyurea.

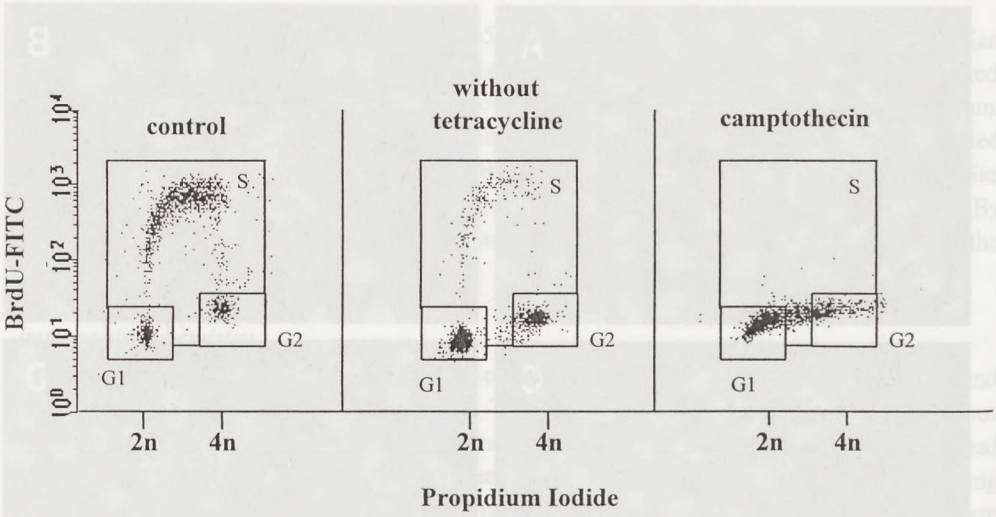


Figure 2. BrdU incorporation in arrested UTA21.15 cells
UTA21.15 cells were cultured in medium with (control) or without tetracycline (p21 induction) for 48 hours or with tetracycline and camptothecin for 24 hours. The cells were pulsed with BrdU for 10 minutes, harvested and prepared for nuclear staining. Staining was performed by incubation with propidium iodide and BrdU-FITC antibody. Cell cycle profiles were then determined using bivariate flow cytometry. 2n and 4n indicate the number of chromosomes in one nucleus.

Ki-67 positivity in p21-expressing cells

To mimick normal p21 induction we created stably transfected UTA21.15 osteosarcoma cells with inducible p21 expression. These cells express the p21 upon rtemoval of tetracycline from the culture medium. After 4 hours in tetracycline-free culture p21 can be first detected and p21 continues to rise up to 72 hours (data not shown). These levels of p21 caused an almost complete inhibition of cell proliferation (data not shown). Only 10% of the cells, cultured for 48 hours in tetracycline-free medium and thus with p21 expression, incorporated BrdU as is shown by flowcytometry, the rest of the cells accumulated in G_1/S and G_2/M phase (Figure 2). In contrast with the control cells cultured in medium with tetracycline, of which 60% incorporated BrdU. The Ki-67 antigen expression in the UTA21.15 cells was determined by using immunocytochemistry. Almost all cells stained positive for Ki-67 in the control (Figure 1C) and after analyzing 300 cells, it appeared that 65% of the cells still stained strongly positive after p21 induction (Figure 1D). To investigate whether the Ki-67 staining correlated with the expression of p21, we double stained

the cells for Ki-67 and p21. And indeed the cells with p21 expression also showed Ki-67 expression, although 44% of the p21 positive cells stained for Ki-67 at a very low level (Figure 3A, page 119). This indicates that cells stain positive for Ki-67 in a cell proliferation block.

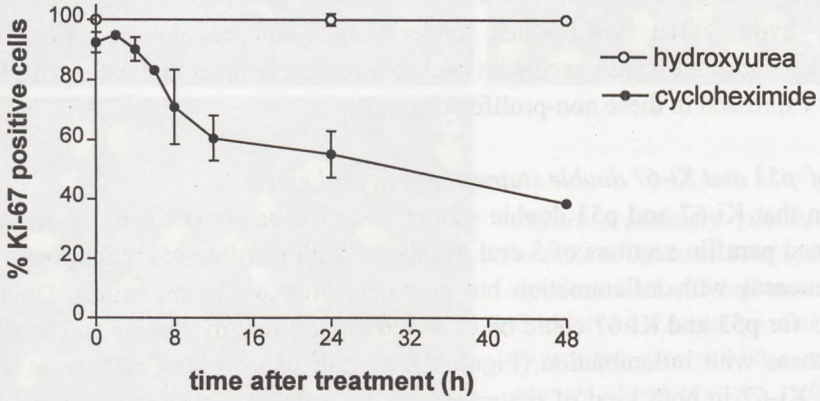


Figure 4. Immunocytochemical half-life of Ki-67
UTA21.15 cells were cultured in medium with tetracyline and cycloheximide or with tetracycline and hydroxyurea. After 0, 2, 4, 6, 8, 12, 24, 48 hours the cells were fixed in methanol and stained for Ki-67. The percentage positively stained cells was determined of 300 cells. Weakly stained cells were not counted as Ki-67 positive cells. SEMs were determined after three experiments.

Ki-67 positivity in cells expressing wild type p53 and p21 induced by DNA damage
Now that we had shown Ki-67 positivity in p21-arrested cells in a tetracycline-regulated expression system, we wanted to know whether these cells still stained positive for Ki-67 by inducing wild type p53 and p21 by inflicting DNA damage. Therefore these cells were treated with camptothecin which causes DNA breaks by inhibition of topoisomerase I. It is known that these cells contain wild type p53 [22] so that DNA damage should result in expression of both p53 and p21. Cells treated with camptothecin for 24 hours arrested in their proliferation as is shown by flow cytometric determination of BrdU incorporation (Figure 2). Double labeling of these cells with p53 and Ki-67 or p21 and Ki-67 showed that these cells can express p53 and p21 simultaneously with the Ki-67 antigen (Figure 3B en Figure 3C, page 119).

Immunohistochemical half-life of Ki-67

After treatment of UTA21.15 cells with cycloheximide, which inhibits protein synthesis, staining for the Ki-67 antigen gradually decreased. After 24 hours only about half of the cells still stained strongly positive for Ki-67 (Figure 4). In contrast 24 hours after treatment with hydroxyurea all the cells still stained strongly positive (Figure 1A; Figure 4). Even after 60 hours all these cells still stained positive for Ki-67, while there was no BrdU incorporation. Therefore, positivity of Ki-67 in cells arrested by hydroxyurea lasted much longer than could be expected from the estimated half-life of Ki-67 assessed with cycloheximide treatment, indicating that Ki-67 is newly expressed in these non-proliferating cells.

Detection of p53 and Ki-67 double stained cells in oral epithelium

To ascertain that Ki-67 and p53 double stained cells frequently occur in tissues, we double stained paraffin sections of 5 oral dysplasias with possible p53 mutations and 5 buccal mucosas with inflammation but probably without p53 mutations. Double stained cells for p53 and Ki-67 could be observed in all 5 oral dysplasias and in all 5 buccal mucosas with inflammation (Figure 5). Not all p53 positive cells were also positive for Ki-67 in both kind of tissues. It can be concluded that cells expressing p53 can simultaneously express the antigen Ki-67 in these tissues.

Figure 3 (upper right page). Double labeling of p21/ Ki-67 and p53/ Ki-67 in UTA21.15 cells
Coverslips with UTA21.15 cells were fixed in methanol and incubated with murine Ki-67 antibody (MIB-1) and rabbit-p21 antibody or rabbit-p53 antibody. The stained cells were analyzed using a confocal laser scanning microscope. ***A)*** *p21 expression (red) and Ki-67 expression (green) in cells incubated for 48 hours in tetracycline-free medium. About 56% of the cells expressing p21 showed a strong expression level of Ki-67 (yellow cells, closed arrow) and about 44% had a low expression level of Ki-67 (closed arrow head). There were also cells that did not express p21 (open arrow).* ***B)*** *p53 expression (red) and Ki-67 expression (green) in cells incubated for 24 hours with tetracycline and camptothecin. Most cells expressing p53 showed a strong Ki-67 expression level (yellow cells, closed arrow) but some cells had a low or no Ki-67 expression level (closed arrow head). There were also cells that did not express p53 (open arrow).* ***C)*** *p21 expression (red) and Ki-67 expression (green) in cells incubated for 24 hours with tetracycline and camptothecin. Most cells expressing p21 showed a strong expression level of Ki-67 (yellow cells, closed arrow) but some cells had a low expression level (closed arrow head). There were also cells that did not express p21 (open arrow). Amplifications are 400x.*

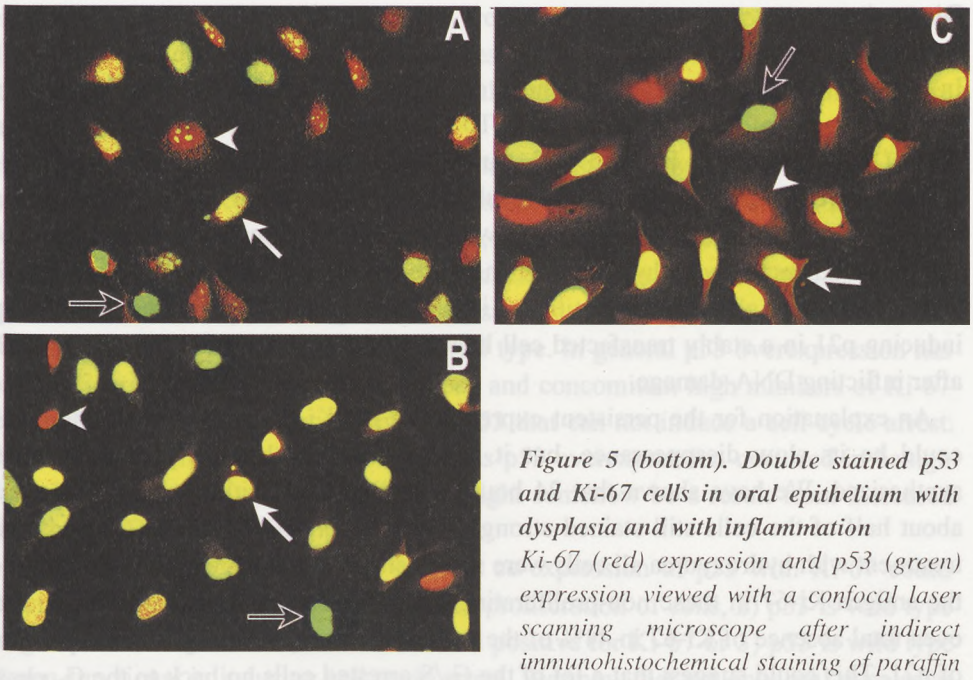
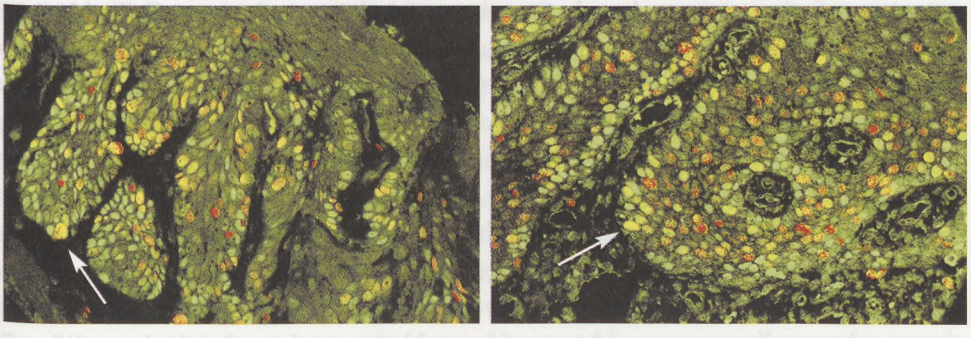


Figure 5 (bottom). Double stained p53 and Ki-67 cells in oral epithelium with dysplasia and with inflammation

Ki-67 (red) expression and p53 (green) expression viewed with a confocal laser scanning microscope after indirect immunohistochemical staining of paraffin sections. The arrows point to double stained (yellow) cells. ***A)*** *oral dysplasia with possible p53 mutations and* ***B)*** *buccal epithelium with inflammation and probably without p53 mutations. Amplifications 400x.*



Discussion

In a lot of studies the proliferation index of tumors has been estimated by calculating the percentages of Ki-67 positive cells. The fact that Ki-67 can be expressed, even when DNA synthesis is blocked [14], can cause problems. Arrested cells could also stain positive for Ki-67, which would result in an overestimation of the real number of actively proliferating cells. We have shown that U2OS-derived cells arrested in G_1/S and G_2/M, respectively by hydroxyurea and nocodazole, still stain strongly positive for Ki-67. We have also shown positive Ki-67 staining in non-proliferating cells, by inducing p21 in a stably transfected cell line and by inducing wild type p53 and p21 after inflicting DNA-damage.

An explanation for the persistent expression of the Ki-67 antigen in arrested cells could be its slow disappearance, but it is also possible that the protein is newly synthesized. We have shown that 24 hours after treatment with cycloheximide only about half of the cells still stained strongly positive for Ki-67, while 60 hours after treatment with hydroxyurea all cells were still positive. This suggests new synthesis of the antigen Ki-67 in these non-proliferating cells. We observed a weaker staining or even total absence of Ki-67 in 44% of the cells with tetracycline-regulated expression of p21. This could suggest that a lot of the G_1/S arrested cells go back to the G_0 phase after being arrested for a longer time. However, this effect was not seen in the G_1/S arrested cells treated with hydroxyurea probably because they arrest later in G_1.

Other studies have shown contradicting results about the half-life of Ki-67 positivity in arrested cells. Van Dierendonck et al. have found that S/G_2 arrested MCF-7 breast cancer cells, induced by methotrexate also retained the antigen for at least 60 hours [12]. However, only half of the cells treated with tamoxifen, which caused G_1 accumulation, retained the antigen for 60 hours. Littleton et al. have shown that presence of the antigen lasted for about 24 hours after an G_1/S block by desferrioxamine [13]. They showed, like us, that only 50% of the cells stained positive for Ki-67, 24 hours after cycloheximide treatment [13]. Using flow cytometry it was shown in another study, that the half-life of the Ki-67 antigen estimated to be less than one hour [23]. However, this difference in Ki-67 half-life might very well be due to the technique used in this study.

From our results it can be concluded that not all cells containing the Ki-67 antigen are actively proliferating cells, which means that in certain tissues a high number of Ki-67 positive cells does not always represent a large growth fraction. Therefore, in tissues where cells express wild type p53 or p21 the number of actively proliferating cells can be overestimated. However, this can also occur in other situations. In

fibrosarcoma tumors a lot of hypoxic cells are non-cycling but are in all phases of the cell cycle [24] and colon carcinoma cell lines can be arrested in all stages of the cell cycle during quiescence [25]. These cells could stain positive for Ki-67 while they are not proliferating.

Even more profound mistakes can be made in studies when one tries to draw conclusions from correlations between expression of wild type p53 or p21 and Ki-67. In a number of studies, e.g. in oral dysplasias, snuff-induced lesions and papillomas an attempt was made to correlate immunohistochemical overexpression of p53 with the expression of Ki-67 [20,21,26]. In these studies it is important to know if the overexpressed p53 protein is mutated or wild type. In general p53 overexpression has been considered indicative of p53 mutations and concomitant high numbers of Ki-67 were explained by the presence of mutant p53 that can not induce a cell cycle arrest. On the contrary, Ingle *et al* suggested that as p53 overexpression occurred in benign lesions in the absence of mutated p53, it might somehow be a result from increased epithelial proliferation activity [27].

The consequences of our results are that co-expression of p53 with Ki-67 could mean 1) p53 is mutated and does not inhibit proliferation of cells, 2) p53 is wild type and gives rise to arrested cells which are still positive for Ki-67 or 3) p53 is wild type but non-functional in inducing cell cycle arrest. We show that not only oral dysplasias but also oral mucosa with inflammation, contains double stained p53 and Ki-67 cells. The mucosa with inflammation was obtained from healthy individuals who did not use tobacco or alcohol, which implicates that the overexpressed p53 is not mutated. Therefore it must be concluded that immunohistochemical correlations between p53 and Ki-67 do not allow a discrimination between overexpression of wild type p53 and mutant p53. One can neither conclude that a simultaneous increase in p53 positive and Ki-67 positive cells indicates a relation between p53 and active proliferation.

Moreover, immunohistochemical studies also do not allow conclusions to be drawn on the role of p21 overexpression in tumors as shown by Maestro *et al.* They claimed that non-Hodgkin lymphomas overexpressing wild type p53 and p21, somehow escape a growth arrest because the cells are Ki-67 positive [19]. This is not necessarily true, because the cells can be temporarily arrested and remain Ki-67 positive. Furthermore, others tried to establish a correlation between p21 and Ki-67 expression in tumors to investigate the role of p21 in proliferation and differentiation [18,28,29,30]. In these studies the correlations between p21 and Ki-67 were discussed, as if p21 would always cause a negative expression of Ki-67, which is not the case.

From our results it can be concluded that not all cells containing the Ki-67 antigen are actively proliferating cells, since we show that a lot of arrested cells can be

positive for Ki-67. Therefore, we advice against the use of Ki-67 to assess proliferative activity in tissues overexpressing p53 or p21.

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Chapter 7

Expression of p21(Waf1/Cip1) in head and neck cancer in relation to proliferation, differentiation, p53 status and cyclin D1 expression

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Abstract

p21(Waf1/Cip1) is a critical downstream effector in the p53-dependent pathway of growth control and causes growth arrest through inhibition of cyclin-dependent kinases. In this study 67% of 43 head and neck squamous cell carcinomas (HNSCCs) and 60% of 15 tumor-adjacent oral dysplasias overexpressed p21 by immunohistochemical staining. Overexpression of p21 in HNSCCs was independent of functional p53, as assessed by p53 mutation analysis, loss of heterozygosity of the p53 gene and p53 immunohistochemistry. Rather, the expression pattern of p21 was associated with differentiation. Furthermore, in most tumors, the p21 positive cells did not incorporate bromodeoxyuridine (BrdU), which indicates inhibition of proliferation by p21 in these cells. Nevertheless,in some tumors, p21 was also expressed in proliferating cells. In these latter tumor cells, cyclin D1 was frequently expressed as well. Therefore, we suggest that expression of cyclin D1 might overcome the inhibitory effect of p21 these cells.

Introduction

The development of head and neck squamous cell carcinoma (HNSCC) is generally accepted to be a multistep process in which alterations in oncogenes and tumor suppressor genes play an important role [1]. Genetic alterations in the tumor suppressor gene p53 [2] occur in up to 85% of HNSCC [3,4]. Furthermore, amplification and/or overexpression of the proto-oncogene cyclin D1 [5] have been described in about half of the HNSCC [6,7]. Cyclins are only functional when they are complexed with cyclin-dependent kinases (CDKs); cyclin D1 is functional when it is complexed with either cdk4 or cdk6 [5].

p21(Waf1/Cip1) is induced by p53 and causes growth arrest through inhibition of the function of cyclin-dependent kinases (cdk) by integration in the cyclin-cdk complexes [8,9]. p21 performs its inhibitory functions by blocking the interaction between cdk-substrates and the cyclin-cdk complexes. p21 binds to the cyclin-cdk complexes with two conserved cyclin-recognition motifs of which one is located in the N-terminus of p21 and one in the C-terminus [9]. The inhibitory function of p21 depends on the number of p21 molecules in the cyclin-cdk complexes [10]. It is thought that a single p21 molecule in the complex does not overcome the activities of the cyclin-cdk complexes, in contrast to multiple p21 molecules that do overcome the activities of the cyclin-cdk complexes. Overexpression of p21 can suppress tumor growth [reviewed in 8] and therefore, p21 seems to act as a tumor suppressor. However, mutations and deletions in the p21 gene have been very rarely seen in HNSCC or in any of the at least 14 other kinds of tumor types that have been investigated [11,12]. On the contrary, polymorphisms could be detected in these tumors, including HNSCC [11,12,13]. These p21 variants occurred more frequently in tumor patients (20.4%) than in healthy individuals (10.7%) [11,12,13]. Furthermore, the polymorphisms were present more often in cancers without p53 mutations [11,13]. Another role of p21 is induction of differentiation, which is independent of p53 [14,15].

In this study, we investigated expression of p21 in HNSCC in relation to cell differentiation, p53 status, expression of cyclin D1 and proliferation. Our results indicate that p21 expression in HNSCC is independent of p53 and is often associated with cell differentiation. We suggest that expression of cyclin D1 overcomes the inhibitory effect of p21 in proliferating cells that express p21.

Materials and methods

Clinical material

Two patient groups formed the basis for this study. The first group consisted of 28 HNSCC patients that had been used to investigate the presence of p53 mutations in the entire p53 gene [16]. HNSCC tissue specimens and venous blood were collected from these patients. The HNSCC came from various sites (Table 1). They had not undergone any previous treatment other than biopsies as part of the preoperative diagnostic procedure. Part of each tumor was frozen immediately after surgery and the other parts including normal epithelium and dysplasias were fixed in formalin and embedded in paraffin. The mean age of the patients was 56 years (range 28-90 years); 6 patients were women. Most tumors were moderately differentiated. No undifferentiated tumors were present (Table 1). Peripheral blood lymphocytes were used for DNA extraction as control for the detection of loss of heterozygosity (LOH) and mutations of the p53 gene. The frozen material was used for assessment of p53 mutations and loss of heterozygosity (LOH) of p53 and the paraffin-embedded material was used for immunohistochemical staining for p21, p53, cyclin D1 and Ki-67. Paraffin-embedded tumor-adjacent dysplastic lesions from 15 of mentioned HNSCC patients were selected by reviewing hematoxylin and eosin-stained histological slides and were also used for immunohistochemical staining for p21, p53, cyclin D1 and Ki-67.

The second group consisted of 15 other patients with all laryngeal squamous cell carcinomas. They had not undergone any other previous treatment than an intravenous injection with sterilised bromodeoxyuridine (BrdU, 100 mg in 10 ml of 0.9% saline) in the Department of Radiotherapy (Dr. H. Struikmans) two hours before surgical removal of the HNSCC. This injection was with the patients' informed consent and with approval of the ethical comittee for scientific research on human beings of the Utrecht University Hospital. The mean age of these patients was 61 years (range 49-76 years); 4 patients were women. After surgery, the tumors and adjacent normal epithelium were formalin-fixed and paraffin-embedded and used for immunohistochemical staining for p21, cyclin D1 and BrdU. The p53 status was not determined in these tumors.

RNA/DNA extraction and amplification

RNA was extracted from frozen tumor samples by using Trizol LS reagent (Life Technologies, Paisley, UK). cDNA was synthesized from 3 μg RNA with 1 μg Oligo-dT_{15} primer (Promega, Madison,Wi, USA) and 200 units Superscript™ RNAse H-reverse transcriptase (Life Technologies). Full length p53 cDNA was amplified in 3 overlapping PCRs, basically according to the method of Sjörgen *et al* [17] with minor modifications [16].

DNA was extracted from frozen tumor samples by using the QiaAmp Tissue Kit (Qiagen GmbH, Hilden, Germany). Furthermore, DNA was extracted from peripheral blood lymphocytes from the corresponding blood samples by the conventional salting-out method. All 11 p53 exons including 2 introns were amplified in 9 PCRs, basically according to the

Table 1. p21 expression in HNSCC in relation to differentiation, p53 status, and cyclin D1 expression

		p53 mutation			immunohistochemistry			
patient	location	exon;position	result	LOH p53	p53	p21	cyclin D1	differen-tiation
1	larynx		wild-type p53	no	+/-	+++	-	m
2	oropharynx		wild-type p53	no	+	++	-	p
3	oral cavity		wild-type p53 #	yes	+/-	++	+	m
4	oral cavity	exon 1	"wild-type p53"	no	+/-	+++	+++	m
5	oral cavity	intron 2	"wild-type p53"	ni	-	-	-	m
6	larynx	exon 6; ss	G →A; unknown	yes	-	-	+++	m
7	oral cavity	exon 7; AA 255	T → G; Ile → Ser	yes	++	+++	+	m+w
8	oral cavity	exon 8; AA 280	G → A; Arg → Lys	yes	+++	+/-	-	m
9	larynx	exon 5; AA 164	A → T; Lys → stop	yes	+/-	++	+++	m
10	oropharynx	exon 5; alt. splic.	del. exon 5	no	+	+/-	nd	p
11	oral cavity	exon 6; AA 195	A → T; Ile → Phe	no	+++	++	+++	m+w
12	oral cavity	exon 7	del. 6 bp; Arg/Pro/Ile → Asp	yes	+++	+++	+++	m
13	oral cavity	exon 7; AA 237	G → C; Met → Ile	yes	++	+++	+	p
14	oral cavity	exon 6; ss	G → C; downstr. stop	yes	-	+++	nd	m
15	oral cavity	exon 4; AA 148	ins. C; downstr. stop	nd	-	+++	+/-	m
16	hypopharynx	exon 4; AA 113	T → C; Phe → Ser	yes	++	++	-	m
17	oropharynx	exon 5; AA 179	C → G; His → Asp	no	++	+	+++	m
18	oropharynx	exon 10	ins. 6 bp; Leu → Stop	no	++	+++	+	m
19	larynx*	exon 5; alt. splic.	del. exon 5	yes	+	+/-	+/-	m
20	oral cavity	exon 10; ss	G → A; downstr. stop	yes	+++	+	+++	m
21	larynx	exon 5	del. exon 5	yes	++	+	nd	w
		exon 7; AA 249	G → T; Arg → Ser					
22	oral cavity	exon 5	del. 6 bp; Lys/Thr/Cys → Asn	no	+++	++	-	m
23	oral cavity	exon 7; AA 237	G → T; Met → Ile	ni	+++	++	-	m
24	oropharynx	exon 4; AA 100	C → T; Gln → stop	yes	+/-	+/-	+++	p
25	larynx		nd#	no	+++	+++	+++	m
26	oral cavity		nd#	no	+/-	+	nd	m
27	metastasis*		nd#	no	+++	+++	+	m+w
28	oral cavity		nd#	ni	++	++	++	m

*p=poorly-differentiated, m=moderately-differentiated, w=well-differentiated, nd=not determined, ni=non-informative, #=incomplete DNA or mRNA analysis, *=patient is irradiated before surgery, AA=amino acid, bp=base pairs, del.=deletion, ins.=insertion, ss=splice site, alt. splic.=alternative splicing, downstr.=downstream, (-) 0-1%, (+/-) 2-5%, (+) 6-20%, (++) 21-50%, (+++) >50% positively stained cells.*

method of Lehman *et al* [18] with minor modifications [16].

All primers were elongated with templates for universal sequencing primers. The PCRs were performed in 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ (Perkin Elmer, Norwalk, CT, USA), 1 U Ampli Taq DNA Polymerase (Perkin Elmer), 10 pmol of tailed primers, 0.2 mM each dNTP (Promega) and 500 ng DNA (or 3 µl cDNA). PCR conditions for the amplifications were identical for DNA and cDNA, except for the PCR of exon 6 DNA that was performed with a concentration of 1.0 mM $MgCl_2$. The annealing temperature was 60 ^{0}C for all PCRs except for exon 10 DNA which was performed at 56 ^{0}C.

Sequencing and analysis of loss of heterozygosity

The PCR-products were sequenced using the Dye Primer Cycle Sequencing Ready Reaction Kit with Taq FS (ABI, Perkin Elmer), separated on a 6% polyacrylamide gel using an automated DNA sequencer (373A, ABI) and compared by using multi locus sequence analysis software.

p53 allelic deletions were reflected by loss of a polymorphic 2 basepair repeat at the human p53 locus, TP53CA, as desribed by Jones and Nakamura [19]. The DNA from tumor tissues and peripheral blood lymphocytes was amplified with a $^{5'}$6-carboxy-fluorescein (FAM) labelled 5' primer $^{5'}$AGGGATACTATTCAGCCCGAGGTG$^{3'}$ and a 3' primer $^{5'}$ACTGCCACTCCTTGCCCCATTC$^{3'}$. The reaction mix contained 1x PCR buffer (Perkin Elmer), 1.5 mM $MgCl_2$ (Perkin Elmer), 0.2 mM each dNTP (Promega), 2 pM of both primers, 0.5 U Ampli Taq Polymerase (Perkin Elmer) and 50 ng DNA. The PCR was performed at an annealing temperature of 58 ^{0}C. After separating the amplified products on a 6% polyacrylamide gel on an automated DNA sequencer, possible loss of one p53 allele was determined by using GeneScan Analysis 1.0.0 and Genotyper 1.0 software (ABI).

Antibodies

The following antibodies were used for immunohistochemistry: anti-p53 (1:300 dilution, polyclonal CM1; biogenex, San Ramon, CA), anti-p21 (1:50 dilution, monoclonal Ab3; Neomarkers, Fremont, CA), anti-Ki-67 (1:500 dilution, monoclonal MIB-1; Immunotech, Marseille, France), anti-BrdU (1:20 dilution, monoclonal BrdU; Roche Diagnostics; Basil, Switzerland), anti-cyclin D1 (1:10 dilution, monoclonal NCL-cyclin D1-GM; Novocastra, New Castle, UK).

Immunohistochemistry

For immunohistochemical detection, 4 µm parallel sections from the paraffin-embedded samples were used immediately after cutting. The detection of the antigens was applied as described before for p53 [20]. After deparaffinisation and blocking endogenous peroxidase activity, antigen retrieval was performed. For detection of p53, p21, Ki-67 and cyclin D1 the sections were preheated in citrate buffer. For detection of BrdU, the sections were incubated in a pepsin-glycine buffer ($2{,}5.10^6$ activity units pepsin/l and 7.5 g glycine/l, both Sigma, St

Louis, USA) at 37 °C for 20 min. After washing with H_2O, the sections were incubated for 30 min in H_2O with 3.3% HCl. After washing with H_2O, the sections were preincubated for 15 min. with either 10% horse serum (for p21, BrdU, Ki-67 and cyclin D1) or 10% goat serum (for p53). After an one hour incubation with the diluted primary antibodies, the sections were incubated with 1:500 diluted biotinylated horse-anti-mouse antibody (Vector Laboratories Inc, Burlingham, CA) for 30 min. For detection of p53, the sections were incubated with 1:1000 diluted biotinylated goat-anti-rabbit antibody (Vector Laboratories). Finally, the sections were incubated for 30 min. with peroxidase-streptavidin conjugate (Immunotech. S.A., Marseille, France) in a 1:400 dilution and developed in DAB-reagent (0.06% 3,3-diaminobenzidine tetrahydrochloride and 0.03% H_20_2 in phosphate-citrate buffer). Sections were counterstained with haematoxylin for 1 min.

As a general positive control tumor-adjacent normal oral mucosa was stained in the same sections as the tumor tissues. The negative controls consisted of replacing the first antibody with another primary antibody of the same IgG subclass. Furthermore, as an additional positive control, the antibody against p21 was tested in a U2OS-derived cell line in which p21 was stably transfected and the antibody against p53 was tested in U2OS cells in which p53 was induced by DNA-damage [20]. A positive control for cyclin D1 was a tumor with known cyclin D1 overexpression.

Evalution of immunostaining involved screening of the entire section. The staining of p53, p21, BrdU and cyclin D1 was classified in five categories of percentages of positively stained nuclei: (-) 0-1%, (+/-) 2-5%, (+) 6-20%, (++) 21-50%, (+++) >50%. Overexpression of p53, p21 or cyclin D1 was concluded when more than 20% of the cells stained positive.

Results

p21(Waf1/Cip1) expression HNSCC, in dysplastic and normal oral epithelium

Expression of p21 was detected in the suprabasal (Figure 1A) and sometimes also in the superficial layers of normal oral epithelia. No expression of p21 was observed in the fully differentiated cells of the normal epithelium. More than 20% of the cells stained p21-positive in 20/28 tumors of the first group (Table 1) and in 9/15 tumors of the second group with 15 BrdU-labelled tumors (Table 2). Therefore, in 67% of the HNSSC p21 overexpression could be concluded. Three of these positively stained tumors are shown (Figure 1B, 1C, 1D). In dysplasias, overexpression of p21 was present in 60% of the 15 samples (Table 3). In some tumors and dysplasias the p21 staining in the nuclei was also more intense than in the normal adjacent tissue (Figure 4B).

p21(Waf1/Cip1) overexpression in HNSCC is independent of p53

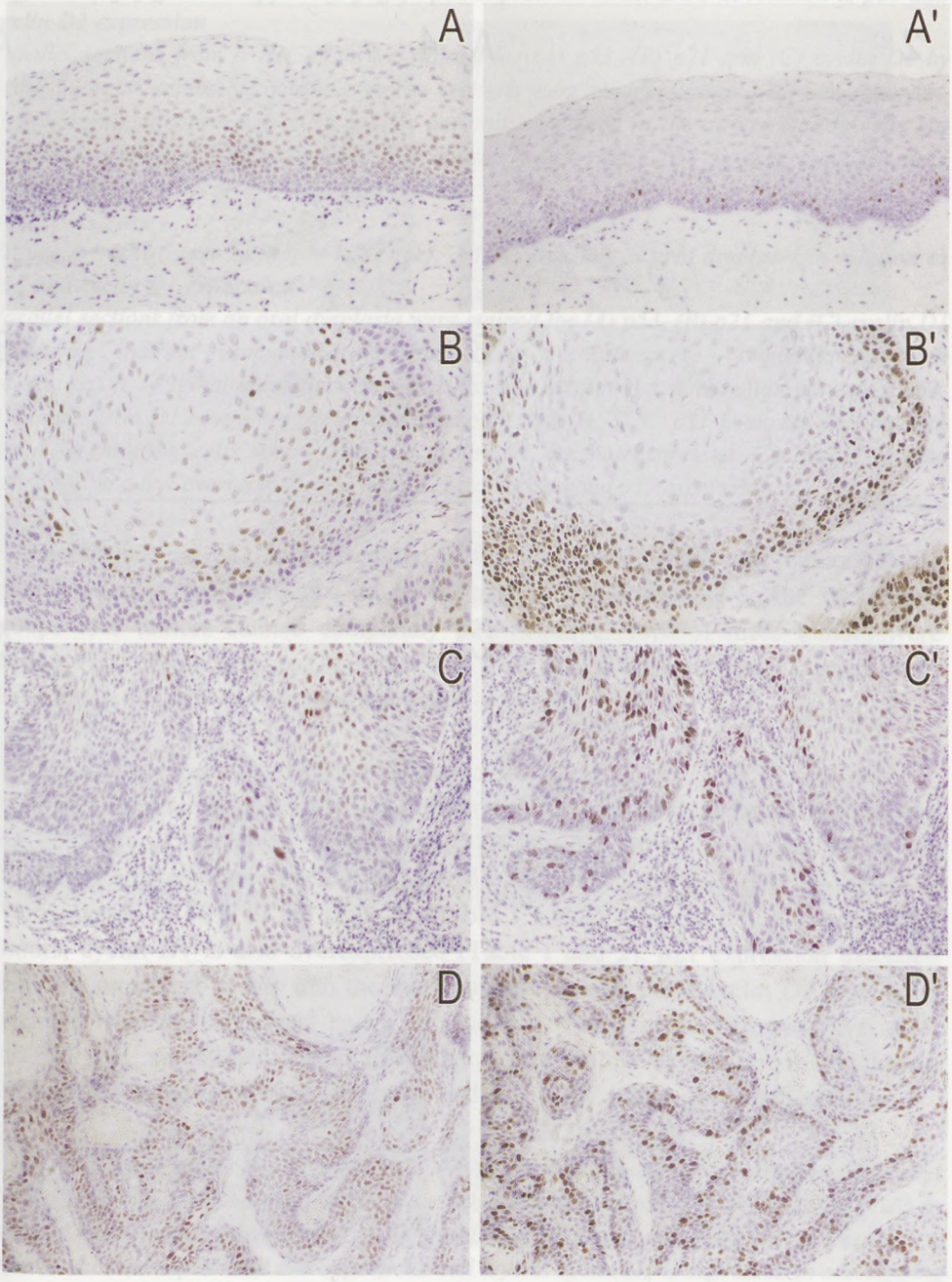
The p53 status of the first group of 28 tumors was assessed by sequencing of both the entire p53 gene, with all exons and exon-adjacent intron areas, and the p53 mRNA as well as determination of p53 allelic loss. It appeared that almost all tumors (83%) contained p53 mutations and 50% had also lost one p53 allele (Table 1). For example, one patient (nr 14) had lost one allele and contained a nonsense mutation in exon 6 which assumes lack of a functional p53 protein in this tumor (Table 1). Despite this, almost all tumor cells expressed p21. Another tumor (nr 12) with a point mutation in exon 7 of one p53 gene (amino acid 237: Met-Ile), p53 allelic loss and immunohistochemical overexpression of the mutated gene, also still stained positive for p21 (Table 1). Thus, p21 overexpression occurs in tumors without functional p53. Other evidence for p53-independent expression of p21 came from the topographical distribution of p53 and p21. In most tumors expression of p53 was observed in the proliferating cells, as was assessed by BrdU incorporation, while p21 was expressed in the non-proliferating cells (Figure 1B, 1B' and 1C, 1C'). Thus, in most cells with p53 overexpression no p21 expression could be detected. However, not all tumors with mutated p53 stained positive for p53 (Table 1).

p21(Waf1/Cip1) overexpression in HNSCC is associated with differentiation

In normal oral squamous epithelium p21 expression is associated with the initiation of differentiation and is not expressed in the proliferating BrdU labelled cells (Figure 1A and 1A'). p21 expression in well-differentiated parts of the carcinomas showed a topographic distribution similar of that seen in normal squamous epithelium, which indicates that p21 is associated with differentiation in these tumors (Figure 1B). In the moderately and poorly differentiated tumors, a link between p21 and differentiation could not be assessed because of lack of a differentiation pattern. However, it could

Figure 1 (right page). Immunohistochemical expression of p21 in normal oral mucosa and HNSCC in relation to BrdU, p53, and Ki-67 expression

Paraffin sections from normal oral mucosa and HNSCC were stained for p21, BrdU, p53 and Ki-67 by using indirect immunohistochemistry. The sections were counterstained with haematoxylin. Original magnification was 200x. A) p21 expression in the differentiating layers of normal oral mucosa, A') BrdU expression in the basal layers of a parallel section. B) p21 expression in a well-differentiated HNSCC in the differentiating layers, B') p53 expression in the basal layers of a parallel section. C) p21 expression in some parts of HNSCC, C') BrdU expression in other parts of a parallel section. D) p21 expression in HNSCC, D') Ki-67 expression in a parallel section.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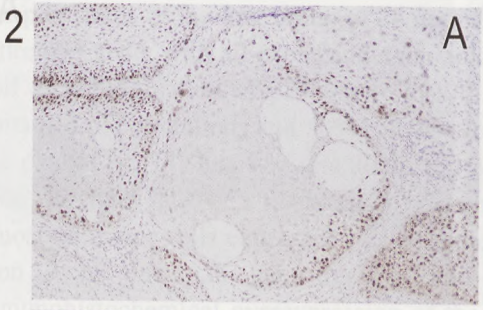
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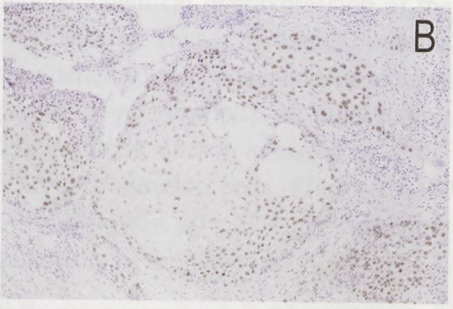
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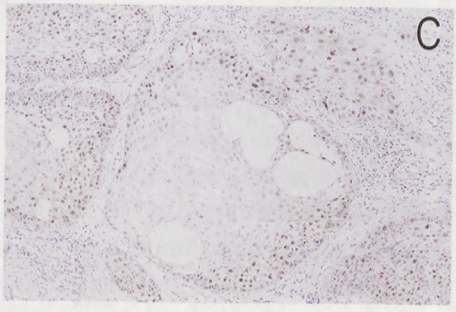
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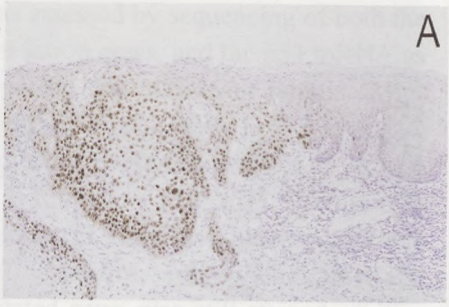
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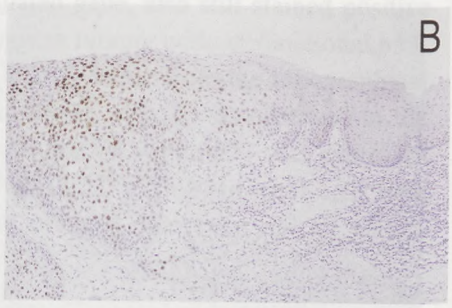
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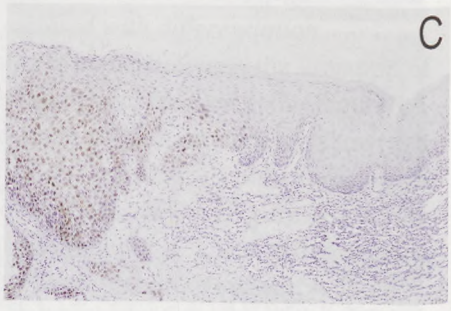
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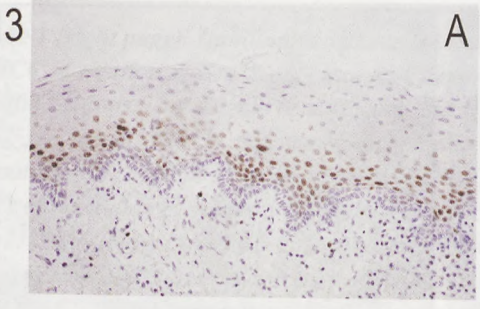
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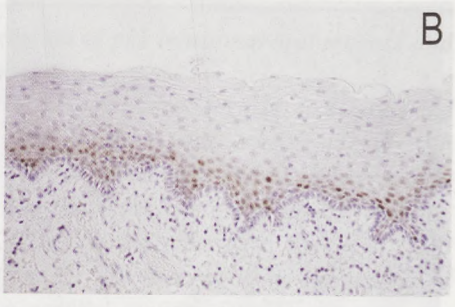
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Figure 2 (left figure on upper left page). p21 expression in HNSCC in relation to p53 and cyclin D1 expression
Parallel sections from a HNSCC were stained for (A) p53, (B) p21 and (C) cyclin D1 by using indirect immunohistochemistry. The sections were counterstained with haematoxylin. Original magnification was 100x. p21 is not only expressed in the differentiating cells but also in the proliferating peripheral suprabasal layers where p53 is expressed. In these proliferating cells cyclin D1 is also expressed.

Figure 4 (right figure on upper left page). p21 expression in oral dysplasia in relation to p53 and cyclin D1 expression
Parallel sections from an oral dysplasia were stained for (A) p53, (B) p21 and (C) cyclin D1 by using indirect immunohistochemistry. The sections were counterstained with haematoxylin. Original magnification was 100x. The topological distribution pattern of p53, p21 and cyclin D1 resembles the distribution pattern in HNSCC. p21 is mostly expressed in the differentiating cells where p53 and cyclin D1 are not expressed. Cyclin D1 is also expressed in a few overlapping cells with p21 expression and p53 expression.

be established that p21 was expressed in the non-proliferating cells without BrdU incorporation in such tumors (Figure 1C and 1C'). Furthermore in the two tumor groups four tumors stained completely p21 negative. Two of these tumors were moderately differentiated and the other two were poorly differentiated. Thus no well-differentiated HNSCC were completely p21-negative.

p21(Waf1/Cip1) expression in proliferating cells in HNSCC
Most p21 expressing cells were not proliferating as was assessed by BrdU labelling and detection of the proliferation marker [21] Ki-67. However, some cells in the proliferating layers expressed p21 (Figure 1D,1D' and Figure 2A, 2B). Cyclin D1 is overexpressed in 60% of the HNSCC (Table 1 and 2). In most p21 overexpressing tumors, cyclin D1 is also overexpressed (Table 1 and 2). p21 expression extended from the suprabasal layers into the more superficial layers and cyclin D1 expression extended from the suprabasal layers into the more basal layers. However, cyclin D1 and p21 were sometimes co-expressed suprabasally in the well-differentiated tumors (2B and 2C) in the same layers as where BrdU or Ki-67 staining was present. This

Figure 3 (bottom figure on left page). p21 and cyclin D1 expression in normal oral mucosa
Parallel sections from normal oral mucosa were stained for p21 (A) and cyclin D1 (B) by using indirect immunohistochemistry. Original magnification was 200x. p21 expression is expressed in the suprabasal and superficial cell layers, cyclin D1 is expressed in overlapping layers with p21 expression and in suprabasal layers without p21 expression.

Table 2. p21 expression in BRDU-labelled HNSCC in relation to cyclin D1 expression

	immunohistochemistry			
patient	p21	cyclin D1	BrdU	differentiation
1	-	+/-	+	p
2	+++	++	++	m
3	+	+	-	m
4	+	nd	+	m+w
5	++	++	-	m+w
6	++	++	+	w
7	+++	+	++	m
8	+	+/-	++	m
9	-	-	+	p
10	++	++	-	p
11	++	++	+	m+w
12	++	++	+	m+w
13	+	++	-	p
14	++	++	+/-	m
15	++	+	-	m+w

p=poorly-differentiated, m=moderately-differentiated, w=well-differentiated, nd=not determined

was also seen in normal oral epithelium (Figure 3). In a few HNSCC, cells with p21 expression had also incorporated BrdU. However, in the same areas of these tumors, most cells expressed cyclin D1. Thus, p21 is mainly expressed in non-proliferating cells and when it is expressed in proliferating cells, cyclin D1 is often expressed as well.

p21(Waf1/Cip1) expression in oral dysplasia

p21 is overexpressed in 60% (9 out of 15) of the dysplasias (Table 3). This p21 overexpression is probably also independent of p53 as was determined by using immunohistochemistry: p21 was often expressed in the upper more differentiating cells in mild dysplasias and in most dysplasias there was a mutually exclusive expression pattern with p53 (Figure 4A and 4B). In the more severe dysplasias p21 was expressed throughout the whole thickness of the epithelium. Cyclin D1 was also overexpressed in 57% (4 out of 7) of the stained dysplasias (Table 3). Unfortunately, there was not enough material to stain the other 8 dysplasias for cyclin D1. Overexpression of cyclin D1 occurred in the same dysplasias as overexpression of p21. In some dysplasias the expression of p21 was located in the upper layers and expression of cyclin D1 in the basal and suprabasal layers of the epithelium. In other

dysplasias the expression pattern of p21 and cyclin D1 overlapped. Overexpression of p21 in dysplasia seems to have the same features as in HNSCC, regarding differentiation, expression of p53 and expression of cyclin D1.

Table 3. p21 expression in oral dysplasia in relation to p53 and cyclin D1 expression

	immunohistochemistry		
patient	**p53**	**p21**	**cycline D1**
1	nd	-	nd
2 dysplasia a	-	+	nd
dysplasia b	-	-	nd
3	-	-	nd
4	-	+	+
5	+ down	+ up	+ down
6	+	+	nd
7	-	+/- up	+/- down
8	-	+	-
9	+	+	+ down
10	nd	-	nd
11	-	+	nd
12	+ down	-	-
13	-	+	nd
14	+	-	nd
15	+ down	+ up	+

These dysplasias were obtained from 15 patients from Table 1. Patient 2 had two independent dysplasias. nd=not determined, up=expression only in the upper differentiating cells in the dysplasia, down=expression only in the more basal cells in the dysplasia

Discussion

In this study we have shown that p21 protein overexpression is very common in HNSCC and oral dysplasia. Overexpression of p21 has been observed in HNSCC before, as was published in two articles that appeared during preparation of this manuscript [22,23]. Nadal *et al* [22] detected in HNSCC, besides an increase in p21 protein, an increase in p21 mRNA, in contrast to ERBER et al who did not detect an increase in p21 mRNA [23]. It was suggested in both studies that the increased expression of p21 was regulated post-transcriptionally, which has been investigated in

more detail before in leukemic cells [24].

In the recent years p21 expression has been investigated in different tumor types [reviewed in 25,26]. In a lot of tumors, like non-small cell lung carcinomas, breast carcinomas and carcinomas of the oesophagus, p21 was expressed independent of p53 and was associated with differentiation [15,27]. In colorectal carcinomas p21 mRNA was decreased instead of increased [28]. In other tumors, like in anaplastic astrocytomas [29] and in adenomas/adenocarcinomas of the colon [30], p21 overexpression seemed dependent on functional p53. Thus, p21 overexpression might be regulated differently in various tumors [reviewed in 24]. p21 overexpression was also variable in our HNSCC. At present we cannot explain the mechanisms behind this.

In HNSCC, p21 is expressed independent of functional p53 since almost all our HNSCC contained mutated p53. The remaining p53 allele could have induced enough wild-type p53 protein to induce p21 [31], however p21 was also overexpressed in HNSCC with both mutated p53 and allelic loss of p53. Another reason for p53-independent expression of p21 is that p21 was mostly expressed in other cells than p53. At first sight the immunohistochemical results seemed not to be in concordance with the p53 mutation status as in some tumors with mutated p53 no overexpression of p53 was present. However, this can be explained by presence of nonsense mutations that cause undetectable truncated p53 [32] and presence of mutations that do not increase the stabilization of p53 [33].

In the two other studies on p21 expression in HNSCC [22,23], it was also suggested that p21 expression in HNSCC was independent of p53 gene status. However, in contrast to these other studies, we sequenced full length p53 mRNA and as a control all 11 exons at the genomic level and found 30% of the p53 mutations outside the core domain from exon 5 to exon 8 [further described in 16]. Together with our p53 LOH analysis this gives a more complete view on the p53-independent expression of p21. Furthermore, p21 also seems to be expressed independent of p53 in dysplasias as was shown by the mutually exclusive immunohistochemical expression pattern with p53.

Overexpression of p21 in HNSCC and dysplasia is probably associated with differentiation as was shown by the topological distribution of p21 in well-differentiated HNSCC and mild dysplasias. Since, our clinical material did not contain undifferentiated carcinomas, a good correlation between p21 expression in HNSCC and differentiation grade could not be determined in contrast to the study of Nadal *et al* [22]. In the poorly and moderately differentiated HNSCC, the association of p21 with differentiation was not as obvious as in the well-differentiated HNSCC, however

p21 was expressed in the suprabasal layer that did not proliferate anymore and these cells might have started the initial step of differentiation. Unfortunately, cytokeratin expression patterns could not prove this as the differentiation-associated cytokeratins, like cytokeratin 13, disappeared in HNSCC and dysplasias, even in the well-differentiated tumors [unpublished results, 34].

Other mechanisms than p53 induction of p21 must be active in HSNCC as p53 is often non-functional while p21 is still induced. p21 can indeed be induced independent of p53 by serum or a variety of purified growth factors including transforming growth factor β, platelet-derived growth factor, fibroblastic growth factor and epidermal growth factor [26,35,36]. Also nerve growth factor and MyoD can induce p21 and differentiation, independent of p53 [37,38]. Recently a p53-related protein named p73 was found to replace p53 to induce p21 [39].

In most normal, dysplastic and tumor cells, p21 seemed functional in inducing cell cycle arrest and initiating differentiation as these cells did not incorporate BrdU. However in some cells, p21 was also expressed in proliferating cells, which means that p21 is not functional in arresting all cells. In the study of Erber *et al* the same phenomenon was observed in the HNSCC [23]. Though, they only analysed proliferation by using the proliferation markers Ki-67 and PCNA, which can give false results in p21 expressing tissues. The antigen Ki-67 can still stain positive in G1/S arrested cells by p21 [20] which also applies to PCNA [unpublished results]. In our study the tissues were labelled with BrdU, which avoids false positivity of proliferating cells. Nevertheless, also when BrdU was used as proliferation marker some proliferating cells still expressed p21.

The precise mechanism which allows proliferation in the presence of accumulated p21 is unclear. Low amounts of p21 have been detected in various proliferating normal and transformed cells in culture [15,40]. This suggests a treshhold model of growth-inhibitory function of p21. The number of p21 molecules in a cyclin-cdk complex determines the inhibitory effect of p21 [10]. In many p21 overexpressing HNSCCs, we found that cyclin D1 was also overexpressed. Co-expression of p21 and cyclin D1 was restricted to the proliferating suprabasal cells. The same phenomenon was observed in some normal oral tissues and oral dysplasias. This suggests that if cyclin D1 is high enough it can overcome the inhibitory effect of p21 and it can induce proliferation. It has been shown by others that cyclin D1 can even induce p21, which occurs without altering cell cycle progression [40].

Besides in proliferating cells, cyclin D1 was also expressed in the more differentiating cells in some normal oral mucosas and HNSCC. Probably, cyclin D1 does not overcome the effect of p21 here and these cells do not proliferate anymore

but start to differentiate. Possibly, other factors play a role in the fact that cyclin D1 does not cause proliferation in these cells. It has been shown before that cyclin D1 is increased together with p21 in differentiating PC12 cells [37]. Specific roles for cyclin D1 in differentiation have also been proposed, like complex formation with unfunctional cdks [41].

In conclusion: p21 overexpression occurs frequently in HNSCC and oral dysplasia. This expression is independent of functional p53 and is often associated with differentiation. In the cases where p21 is also expressed in proliferating cells, we suggest that expression of cyclin D1 might overcome the inhibitory effect of p21.

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Chapter 8

The origin of multiple squamous cell carcinomas in the aerodigestive tract

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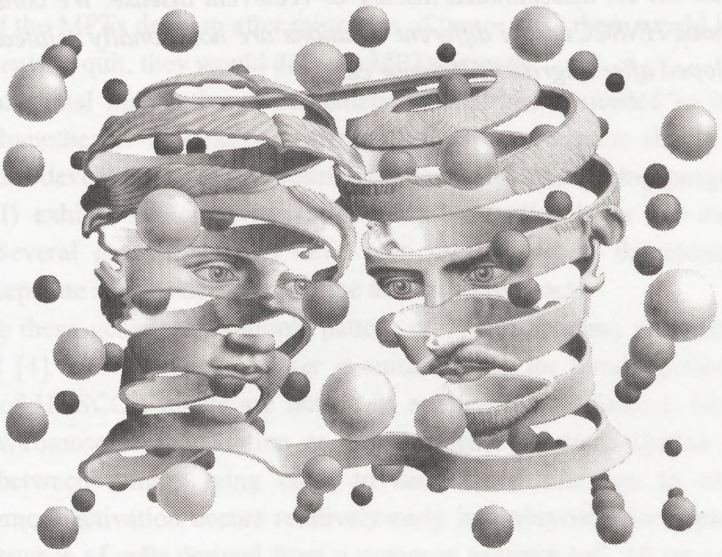

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Abstract

Chemoprevention, quitting smoking and quitting abusing alcohol may prevent the development of multiple primary tumors (MPTs) in the aerodigestive tract, if new MPTs arise independently but are of no benefit if MPTs are due to migration of tumor cells. We investigated 9 patients with MPTs in the head and neck for clonality, by employing mutation analysis of the entire coding region of p53 and loss of heterozygosity (LOH) pattern analysis of microsatellite markers on chromosome arms 3p, 9p, and 17p. Beforehand, we established the reliability of these clonal markers in 16 cases of primary head and neck squamous cell carcinomas (HNSCCs) with matched metastases. Thereafter, 11 patients with diagnostic uncertainties about their multiple tumors were analyzed in a similar way. Both p53 gene mutations and LOH patterns were stable during tumor progression. Furthermore, the variability of p53 gene mutations was high. More than 90% of the tumors contained a p53 mutation. A particular mutation never occurred more than twice in a total of 69 primary HNSCCs. Five cases had a common mutation. In contrast, LOH patterns showed less variability, these being identical in 5 out of 16 cases. The metachronous HNSCCs from 9 patients showed all different p53 mutations and in the three cases that were subjected to LOH analysis different patterns were observed. All 11 diagnostic cases could be categorized as having either MPTs, disseminated disease or recurrent disease. We conclude that the metachronous HNSCCs from different locations are not clonally related and thus have not developed after migration of tumor cells.

Introduction

In up to 20% of the patients affected by HNSCC, the mucosa of the aerodigestive tract shows widespread precancerous lesions and additional cancers within 5 years [1,2]. This phenomenon has led Slaughter *et al* to propose the field cancerization theory, which claims that after repeated carcinogenic exposures, the mucosa accumulates genetic alterations resulting in the induction of multiple, independent malignant lesions [3].

An alternative theory for the occurrence of multiple (pre)malignant lesions is based on the premise that any transforming event is rare and that the multiple lesions arise due to widespread migration of transformed cells through the whole aerodigestive tract [4,5]. This second theory can be divided into two types of migration: a) migration of tumor cells for example by saliva (micrometastases), b) intraepithelial migration of the progeny of the initially transformed cells.

Recently several molecular studies were aimed to investigate these theories [4-13]. However, distinction between the two alternative migration theories was never made. For the classification and subsequent treatment of risk patients it seems to be important to know how multiple primary tumors (MPTs) develop. If the MPTs develop independently or after migration of the progenitor cells then chemoprevention should be an option for these patients. Furthermore, the patients should quit using tobacco and alcohol. If the MPTs develop after migration of tumor cells there would be no need for those patients to quit, they would develop MPTs anyway.

A good clonal marker based on genetic alterations, is needed to investigate the different hypotheses. To qualify as a marker, such an alteration should I) occur very early in the development of the lesion, II) be maintained during progression of the lesion, III) exhibit sufficient variability, IV) be applicable in the majority of the lesions. Several genetic markers have been used to assess the clonal relationship between separate squamous lesions in the aerodigestive tract.

Among these genetic alterations, pattern of X-chromosome inactivation has been employed [4]. However, this marker is suitable only for female patients and as the majority of HNSCC patients are males, its applicability is limited. Moreover, when using X-chromosome inactivation as clonal marker for investigation of the clonal relation between tumors lying close to each other, one has to realize that X-chromosome inactivation occurs relatively early in embryonic development, resulting in large patches of cells derived from a common ancestor cell, all having the same X chromosome inactivated [14]. In addition, X-chromosome inactivation might be non-

random or cell-type specific [15].

Also, a combination of fluorescence in situ hybridization and karyotyping of tumors with chromosome rearrangements has been used as a clonal marker [8]. However, not every tumor has such specific chromosome rearrangements that they can be used as clonal marker. Moreover, karyotyping depends on the succes of short-term culture.

Thirdly, LOH patterns at different loci have been used as clonal markers [5,6,7]. LOH at loci on chromosome 3p and 9p have been shown to occur early in carcinogenesis [13,16,17].

Finally, p53 mutations have been employed as a clonal marker [11,12,13,18]. p53 mutations seem to be an early event in the development of HNSCC since they are already present in normal tissue distant from tumors [10,19,20], in normal tissue from healthy smokers [21] and in premalignant lesions [20].

Since both p53 mutations and LOH in chromosome 3p/9p are early events during HNSCC, these seem both to be promising for the investigation of the field cancerization theory. In the first part of this study, we assess the stability, variability and applicability of these clonal markers. In the second part of the study we investigate the different field cancerization hypotheses using mutations in the p53 gene and LOH patterns of chromosome arms 3p, 9p and 17p as clonal markers. In the final part of this study, we illustrate the use of these clonal markers for the diagnoses of patients with uncertainties about their second squamous cell carcinoma in the aerodigestive tract being either a second primary tumor, a recurrence, or a metastasis. Clonal markers had to be used since the relationships between these tumors could not be determined on clinical, radiographic or even histopathologic grounds.

Materials and methods

Clinical material

Three different patient groups were selected. The first group consisted of 15 HNSCC patients (patient 1-15) with matched lymph node metastases (Figure 1) [22]. and one autopsied HNSCC patient (patient 16) with widespread metastases (liver, kidney, adrenal gland, pleura and lymph node). This group served to establish whether the selected clonal markers were stable during tumor progression and metastasizing. The second group consisted of 9 patients (patients 17-25) with metachronous HNSCCs at different sites (Table 1). Together with this group, the first group of 16 cases and a group of 24 other HNSCC patients [18] the variability of the clonal markers was established. The different field cancerization hypotheses were also tested with these 9 patients with metachronous HNSCCs. The third group consisted of 13

patients: 2 patients (patient 26 and 27) with 2 synchronous HNSCCs from the same location and only separated by dysplastic mucosa, and 11 patients (patients 28-37 and patient 16) with uncertainties about their second squamous cell carcinoma in the aerodigestive tract being a second primary tumor, a recurrence, or a metastasis (Table 2). Since patient 16 (the autopsied case with widespread metastases) also had lung lesions, this patient was also added in this series for investigation of the origin of the lung lesions. This group served to assess the applicability of the clonal markers in clinical practice. In this group, p53 gene mutations were analysed in all tumors. LOH patterns were only investigated in inconclusive cases.

Squamous cell carcinoma tissue specimens and venous blood were collected from all patients, with approval of the ethical comittee for scientific research on human beings of the University Medical Center, Utrecht. Furthermore, informed consent was obtained from all patients. From almost all cases, the lesions were frozen immediately after surgery. From the other cases formalin-fixed and paraffin-embedded tissue was available.

RNA and DNA extraction

Before RNA or DNA isolation, hematoxylin and eosin-stained histological sections were evaluated for the presence of at least 40% tumor cells. RNA was extracted from 20 μm frozen tumor sections by using Trizol LS reagent (Life Technologies, Paisley, UK). cDNA was synthesized from 3 μg RNA with 1 μg Oligo-dT_{15} primer (Promega, Madison,Wi, USA) and 200 units Superscript™ RNAse H-reverse transcriptase (Life Technologies).

DNA from frozen tumor sections was extracted by using the QiaAmp Tissue Kit (Qiagen GmbH, Hilden, Germany). DNA from paraffin embedded sections was extracted as follows. The sections were deparaffinised twice by xylene (each 30 minutes) and rehydrated in four different percentages of ethanol (100%-96%-70%-0%).They were incubated twice for 30 minutes in each percentage of alcohol. The sections were incubated overnight at 55 ^{0}C in an 1 ml solution (100 mM NaCl, 10 mM Tris-HCl (pH 8), 0.25 mM EDTA (pH 8), 0.5% SDS) to which 300 μg proteinase K (Roche Diagnostics, Basel, Switzerland) was added. Subsequently, 300 μg proteinase K was added again, followed by a second overnight incubation at 55 ^{0}C. A phenol-chloroform purification was performed followed by ethanol precipitation of the DNA with help of the carrier glycogen (Roche Diagnostics).

DNA from peripheral blood lymphocytes was extracted from the corresponding blood samples by the conventional salting-out method [23]. This DNA was used as a control for polymorphisms and hereditary mutations in the p53 gene. It was also used as a control for microsatellite alterations in tumors.

p53 mutation analysis

Full length p53 cDNA was amplified in 3 overlapping PCRs with 5' and 3' primers basically according to the method of Sjörgen *et* al [24] with minor modifications [18]. All 11 p53 exons were amplified from DNA templates in 9 PCRs if no mutations were detected on RNA level or when no RNA was available [18]. All 5' and 3' primers were elongated with respectively -

21M13 and M13 sequencing templates.

All amplifications were performed as described before [18]. Except for the amplification of DNA from paraffin sections, where 1 U Ampli Taq Gold DNA polymerase (Perkin Elmer, Norwalk, Conneticut) was used with 2 mM $MgCl_2$. In some cases re-amplification was necessary with the DNA from paraffin sections. The first PCR was performed with primers without the sequencing templates. In the re-PCR with 1 µl of the first amplified product, primers were used with sequencing templates.

The PCR-products were sequenced and subsequently analysed as described before [18]. The mutated codons were numbered from the first coding nucleotides.

LOH analysis

The following 10 microsatellite markers were used: D3S1304, D3S1263, D3S1277, D3S1289, D3S1300, D3S1285, D9S171, D9S161, D17S831 and TP53. The fluorescence-labelled primers for the first 9 microsatellite markers were commercialy available from ABI PRISM, Linkage mapping set 2 (Perkin Elmer). The DNA from tumor tissues and peripheral blood

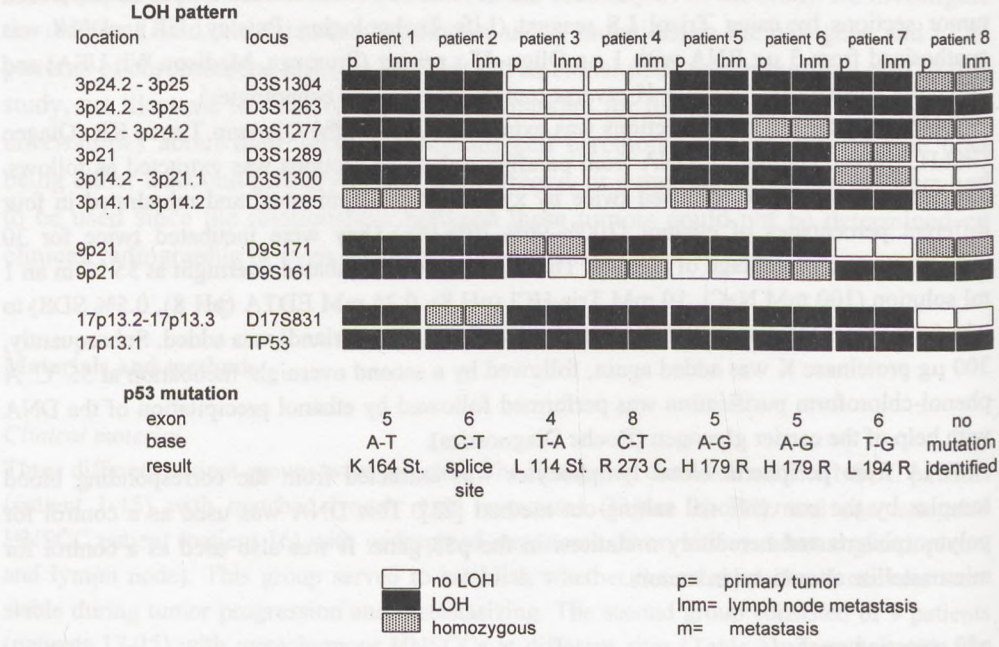
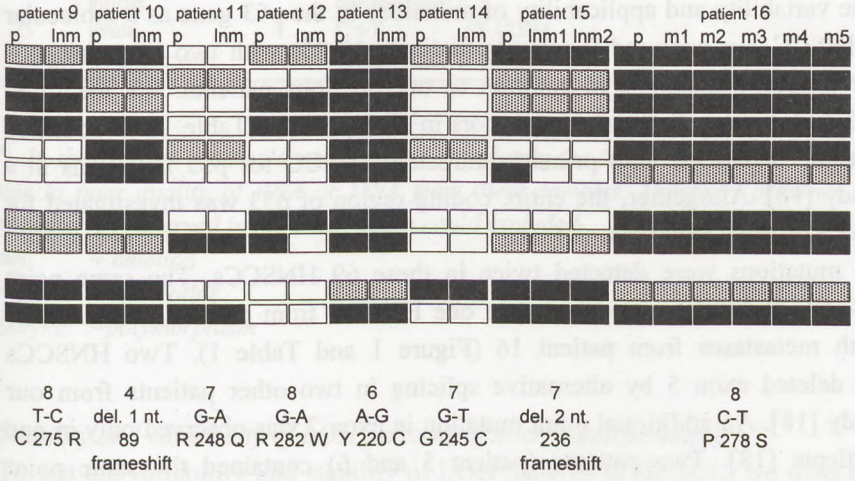


***Figure 1 (continues on the right page). LOH patterns and p53 mutations in patients with** Patient 1 was also used for mutation anlysis in the study from Kropveld et al., 1999 [18]. [22]. St.=stop codon, del.=deletion, nt.=nucleotide*

lymphocytes was amplified according to the instructions of the manufacturer (Perkin Elmer).

The microsatellite marker TP53 is first described by Jones and Nakamura [25]. Amplification of TP53 was performed with the $^{5'}$6-carboxy-fluorescein (FAM) labelled upstream primer $^{5'}$AGGGATACTATTCAGCCCGAGGTG$^{3'}$ and the downstream primer $^{5'}$ACTGCCACTCCTTGCCCCATTC$^{3'}$. The reaction mixture contained 1x PCR buffer (Perkin Elmer), 1.5 mM $MgCl_2$ (Perkin Elmer), 0.2 mM each dNTP (Promega), 2 pM of both primers, 0.5 U Ampli Taq Polymerase (Perkin Elmer) and 50 ng DNA. The PCR was performed at an annealing temperature of 58 ^{0}C.

The amplified products of all microsatellite markers were separated on a 6% polyacrylamide gel on an automated DNA sequencer (373A, Perkin Elmer). LOH was determined by using GeneScan Analysis 2.0.0 and Genotyper 1.1 software (Perkin Elmer) by comparing the allele ratio in the control DNA with the allele ratio in the tumor DNA. Since in some of our sections only 40% of tumor cells were present and because of the heterogeneity in some tumors, LOH was conferred if the ratio of one allele was reduced >25% to prevent false negatives. The 25% boundary did not cause false positives, since the allele ratios from DNA



primary tumors and matched metastasis

P53 mutations of patients 1 - 15 were published before in the study from Tjebbes et al., 1999

from frozen normal mucosa samples were never reduced more than 10% [unpublished data].

Results

Stability and variability of p53 gene mutations

The stability of p53 gene mutations was examined in the autopsied HNSCC patient with widespread metastases (Figure 1). In this patient (patient 16), the mutation (exon 8 C⇨T : P 278 S) was detected in the primary HNSCC and was maintained during the metastatic process in all 5 metastases (liver, kidney, adrenal gland, pleura, and lymph node).

We investigated p53 mutations in primary HNSCC with matched lymph node metastasis in 15 other patients (patients 1-15: Figure 1) [22]. We detected p53 mutations in 14 patients. In all cases of primary HNSCC and matched lymph node metastasis the mutations proved to be identical. Thus, p53 mutation pattern is very stable and is maintained during metastasizing.

To test the variability and applicability of mutations in the p53 gene as a molecular marker in HNSCC, we analysed 9 patients (patients 17-25) with two metachronous HNSCCs at different locations, in addition to our previous patients. We identified different p53 mutations in both primary tumors in all 9 patients (Table 1). In addition, we investigated 25 patients with primary untreated HNSCC for p53 mutations in a previous study [18]. Altogether, the entire coding region of p53 was investigated for mutations in 69 primary HNSCCs.

Five p53 mutations were detected twice in these 69 HNSCCs. The same point mutation (C-->T: P 278 S) was detected in one HNSCC from patient 18 and in the HNSCC with metastases from patient 16 (Figure 1 and Table 1). Two HNSCCs contained a deleted exon 5 by alternative splicing in two other patients from our previous study [18]. An additional point mutation in exon 7 was observed only in one of those patients [18]. Two patients (patient 5 and 6) contained the same point mutation (H 179 R) in their primary HNSCC (Figure 1). In the HNSCC from patient 11 and the second HNSCC from patient 17 also an identical point mutation (R248Q) was identified (Figure 1 and Table 1). Finally, the first HNSCC from patient 17 contained the same point mutation (Y220C) as the HNSCC from patient 13 (Figure 1 and Table 1). Thus p53 is mutated in more than 90% of the HNSCCs and shows variability in its mutations.

Table 1. p53 mutations in multiple primary tumors

patient	location	p53 mutation			second p53 mutation		
		exon	base	result	exon	base	result
17	hypopharynx	6	A⇨G	Y 220 C			
	oropharyhnx	7	G⇨A	R 248 Q			
18	**mandible**	**8**	**C⇨T**	**P 278 S**			
	hypopharynx	**8**	**C⇨G**	**P 278 R**			
19	floor of mouth left	6	A⇨G	N 200 S			
	tongue right		not identical *				
20	**tongue**	**4**	**del. 1 nt**	**frameshift**			
	pharynx	**5**	**C⇨T**	**H 179 Y**			
21	tongue	4	G⇨A	V 54 I	4	splice site	del. of 200 nt
	palate		not identical *				
22	**hypopharynx**	**7**	**ins. 1 nt**	**frameshift**	**4**	**C⇨A**	**P 60 Q, polym.**
	floor of mouth		**no mutation identified**		**4**	**C⇨A**	**P 60 Q, polym.**
23	tongue right	5	C⇨G	P 128 A	5	G⇨T	V 157 F
	tongue left	6	T⇨G	V 216 G			
24	**palate**	**5**	**C⇨A**	**P 151 H**			
	glottis		**not identical ***				
25	uvula	4	C⇨T	T 125 M			
	floor of mouth		not identical *				

* *In these cases, we could not examine completely the full length p53 cDNA and all exons due to poor quality of RNA or DNA from these samples. However, presence of the same mutation as observed in the other tumor could excluded.*

del. = *deletion*
nt. = *nucleotide*
polym. =*polymorphism*

Stability and variability of LOH patterns of microsatellite markers

To test the variability and stability of LOH patterns in HNSCC, we used the same 15 HNSCC with matched lymph node metastasis and the HNSCC patient with widespread metastases as were used for the p53 mutation analysis (patient 1-16). Only in three patients, the lymph node metastasis showed one discordant locus compared with the primary HNSCC (Figure 1): the differences were detected in locus D9S161 from patient 3, locus D9S161 from patient 12 and locus D3S1285 from patient 15). All other loci showed complete concordance. Thus LOH of these microsatellite markers is

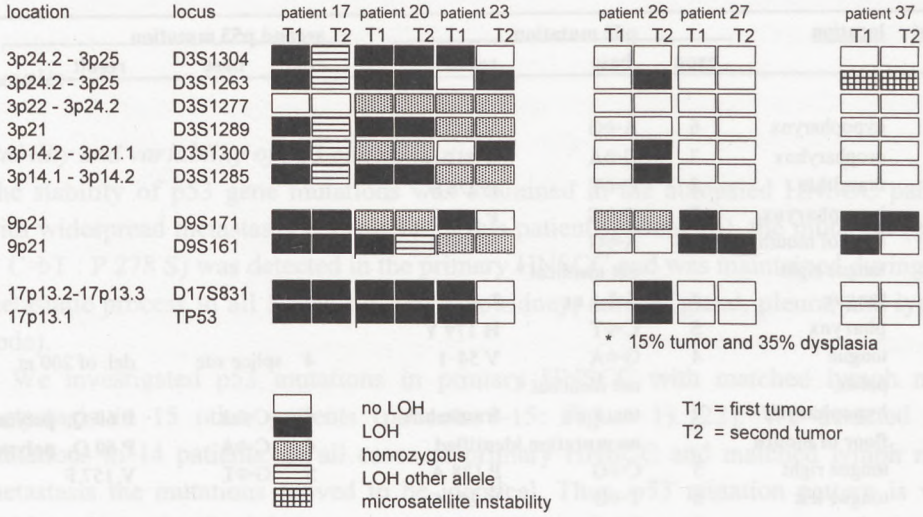


Figure 2. LOH patterns in patients with multiple tumors

almost completely maintained during the progression of the tumor and during metastasizing. However, the LOH patterns were exactly the same in 5 of the 16 primary HNSCC (patient 1, 6, 10, 13 and 16; Figure 1). All 10 markers showed LOH in these HNSCCs. Thus, the variability of LOH patterns is not as high as the mutations in p53. Furthermore, 30 alleles in the 10 loci studied of 16 patients were not informative, because of homozygosity.

Investigation of the field cancerization theory

Nine patients (patient 17-25) with metachronous HNSCCs from different locations were investigated for p53 mutations in their HNSCCs (Table 1). All 9 patients contained different p53 mutations in their two HNSCCs. In three of these patients (patient 17, 20 and 23), we were also able to perform LOH analysis. From the other patients either one of the HNSCC was formalin-fixed or one of the HNSCC samples did not contain an sufficient amount of tumor. All three analysed patients contained different LOH patterns in their two HNSCCs (Figure 2). Patient 17 showed loss of the short allele in one tumor and loss of the long allele in the other tumor (Figure 3). In patient 20 the patterns of chromosome arm 3p and 17p were exactly the same in both

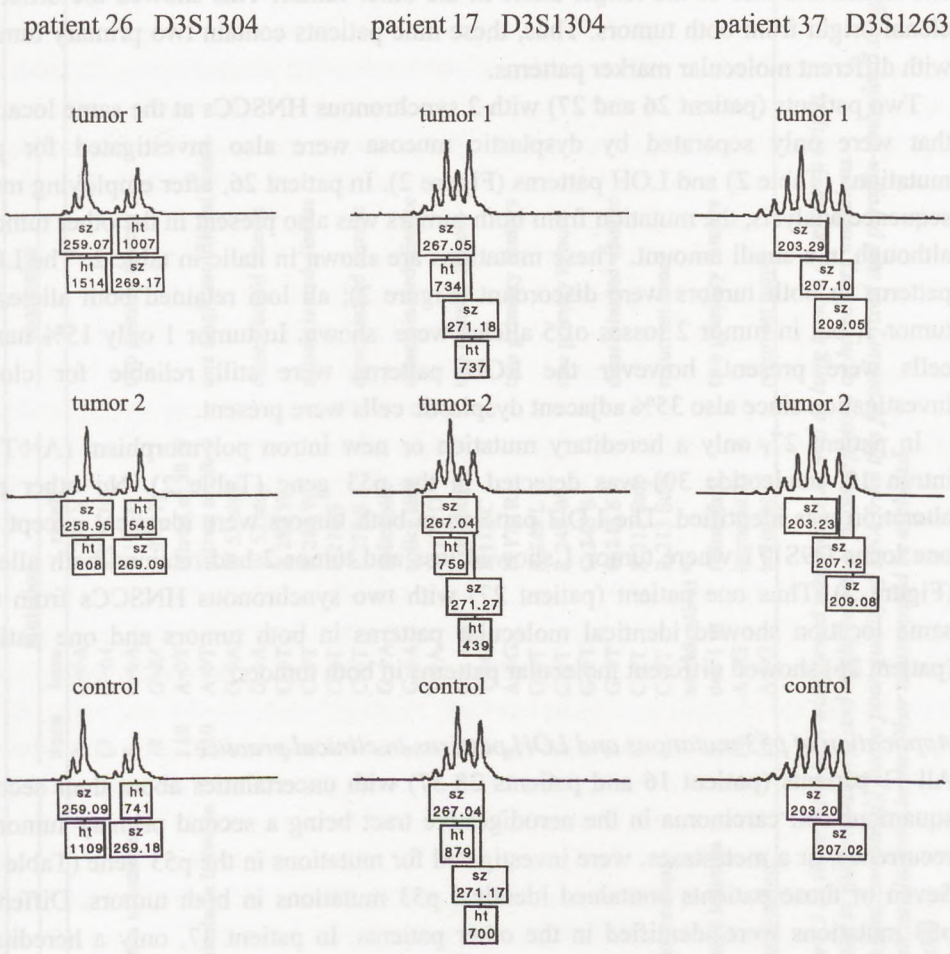


Figure 3. Microsatellite analysis

The alleles of short tandem repeats from 3 patients are shown. The two allele peaks are preceded by smaller stutter peaks. Patient 26 showed no LOH at locus D3S1304. Patient 17 showed LOH of the short allele in tumor 1 and LOH of the long allele in tumor 2. Patient 37 showed microsatellite instability of locus D3S1263. ht=height of peak, sz=size of short tandem repeat.

tumors, however D9S161 on chromosome arm 9p showed LOH of the short allele in one tumor and loss of the longer allele in the other tumor. This showed the different clonal origin from both tumors. Thus, these nine patients contain two primary tumors with different molecular marker patterns.

Two patients (patient 26 and 27) with 2 synchronous HNSCCs at the same location that were only separated by dysplastic mucosa were also investigated for p53 mutations (Table 2) and LOH patterns (Figure 2). In patient 26, after employing multi sequence analysis, the mutation from both tumors was also present in the other tumors, although in a small amount. These mutations are shown in italic in table 2. The LOH patterns in both tumors were discordant (Figure 2); all loci retained both alleles in tumor 1, but in tumor 2 losses of 5 alleles were shown. In tumor 1 only 15% tumor cells were present, however the LOH patterns were still reliable for clonal investigation since also 35% adjacent dysplastic cells were present.

In patient 27, only a hereditary mutation or new intron polymorphism (A⇨T in intron 10, nucleotide 30) was detected in the p53 gene (Table 2). No other p53 alteration was identified. The LOH patterns in both tumors were identical except for one locus D9S171 where tumor 1 showed loss and tumor 2 had retained both alleles (Figure 2). Thus one patient (patient 27) with two synchronous HNSCCs from the same location showed identical molecular patterns in both tumors and one patient (patient 26) showed different molecular patterns in both tumors.

Application of p53 mutations and LOH patterns in clinical practice

All 11 patients (patient 16 and patients 28-37) with uncertainties about their second squamous cell carcinoma in the aerodigestive tract being a second primary tumor, a recurrence, or a metastases, were investigated for mutations in the p53 gene (Table 2). Seven of those patients contained identical p53 mutations in both tumors. Different p53 mutations were identified in the other patients. In patient 37, only a hereditary mutation or new intron polymorphism (A⇨G intron 6, nucleotide 31) was found in the p53 gene. No other mutation was identified. In this case the LOH pattern was also established (Figure 2). Both tumors showed the same microsatellite alteration at locus D3S1263 and were only discordant at one locus (D9S161) in their LOH pattern (Figure 3).

Table 2. Application of p53 mutations in the diagnosis of multiple squamous carcinomas

patient	location	mutation				conclusion
		exon	base	result		
26	HNSCC	8	G⇨A	C 275 Y		
		(5	*G⇨A*	*R 175 H)*		
	synchronous HNSCC, with dysplasia in between	5	G⇨A	R 175 H		two primary tumors
		(8	*G⇨A*	*C 275 Y)*		
27	**HNSCC**	**i 10**	**A⇨T**	**intron nt 30**	**#**	
	synchronous HNSCC, with dysplasia in between	**i 10**	**A⇨T**	**intron nt 30**	**#**	**one single multifocal lesion**
28	HNSCC	4	G⇨A	W 53 stop		
	lung	4	G⇨A	W 53 stop		disseminated disease
29	**HNSCC**	**8**	**C⇨T**	**R 282 W**		
	lung	**8**	**C⇨T**	**R 282 W**		**disseminated disease**
16^	HNSCC	8	C⇨T	P 278 S		
	lung	8	C⇨T	P 278 S		disseminated disease
30	**HNSCC**	**9**	**G⇨A**	**splice site**		
	lung	**9**	**G⇨A**	**splice site**		**disseminated disease**
31	lung left	5	T⇨A	V 172 D		
	synchronous lung carcinoma right	8	del. 1 nt	frameshift		two primary tumors
32	**HNSCC**	**4**	**T⇨G**	**F 113 C**		
	lung	**5**	**A⇨G**	**H 179 R**		**two primary tumors**
33	HNSCC	7	G⇨T	R 248 L		
	lung	7	C⇨T	R 248 W		two primary tumors
34	**lung**	**7**	**G⇨T**	**G 245 S**		
	synchronous lung carcinoma	**7**	**G⇨T**	**G 245 S**		**disseminated disease**
35	HNSCC	6	C⇨T	R 213 Stop		
	metachronous HNSCC, about same location	6	C⇨T	R 213 Stop		recurrent tumor
36	**HNSCC**	**not identical**		*		
	metachronous HNSCC, about same location	**5**	**del. 1 nt**	**frameshift**		**two primary tumors**
37	HNSCC with margin dysplasia	i 6	A⇨G	intron nt 31	#	
	metachronous HNSCC, about same location	i 6	A⇨G	intron nt 31	#	recurrent tumor

^ *This patient was also included in Figure 1 as there were both widespread metastases as well as lung tumors of unknown origin.*

\# *These p53 alterations were also detected in the lymphocytes and might be either polymorphisms or hereditary mutations.*

* *In this case we could not examine all exons completely due to poor quality of DNA from this formalin-fixed and paraffin-embedded sample. However, presence of the same mutation as observed in the other tumor could be excluded.*

del. = deletion, i = intron, nt = nucleotide

Discussion

Stability and variability of p53 mutations and LOH patterns

To investigate if MPTs in the aerodigestive tract develop independently or after cell migration, a good clonal marker is required. In this study we showed that mutations in the p53 gene are maintained during the metastasizing process. The 69 completely investigated primary HNSCCs showed p53 mutations in more than 90%. The chance that two lesions are not clonally related and both do not show a p53 mutation is less than 1%. In addition, the identical p53 mutations were in all cases only detected twice, which means that only in 2 out of 69 investigations (3%) a false clonal relation may be found. Thus p53 mutations are very useful as clonal marker since they are 1) stable during progression, 2) variable and 3) applicable in nearly all cases.

LOH patterns of 10 microsatellite markers on chromosome arms 3p, 9p and 17p were stable during progression of the tumor. In all 16 investigated cases, the lymph node metastasis showed almost the same LOH pattern as the primary HNSCC, which is in concordance with the results of Sun *et al* [26]. In all 3 of our discordant cases, LOH was detected in the primary tumor and not in the metastasis. This indicates selective growth advantage for a clonal population without this LOH.

The variability of the LOH patterns was not as high as of the p53 mutations, since in 5 out of 16 cases the LOH pattern was the same. The identical patterns probably occur because of complete loss of chromosome arms. If one would use only the 6 microsatellite markers on chromosome arm 3p, even more cases (11 out of 16) would show identical patterns of complete loss. In other studies, loss of chromosome 3p in HNSCC was also frequently detected [27]. Additional microsatellite markers on other chromosome arms than 3p, 9p, 17p might have to be used to get a more discriminative potention for clinical applications. However, their patterns may be less stable since these loci might get lost later during tumor progression. At least from the markers on 3p, 9p and 17p it is known that they are early genetic events [5,13,16,17].

Employment of LOH patterns as clonal marker is only possible in the cases without complete loss of the investigated chromosome arms. Cases in which loss of the short allele is observed in one lesion and loss of the long allele in the other tumor are informative. The microsatellite markers also provide additional information if microsatellite instability is present (as is shown in patient 37).

From our results we conclude that the mutation pattern in the p53 gene is the best clonal marker for investigation of the origin of multiple tumors in the aerodigestive tract. In those cases in which p53 mutations are not conclusive, analysis of

microsatellite markers could be informative.

Field cancerization theories

In the 9 patients (patient 17-25) with metachronous HNSCCs from different locations, the p53 mutations were different in both tumors from all patients (Table 1). Furthermore, both tumors contained different LOH patterns in the three patients that were investigated (Figure 2). Thus, the second tumors in these HNSCC patients have not developed from seeding cells from the first tumor. Likely these tumors were developed independently from each other.

Nevertheless, the possibility that MPTs have arosen from separate outgrowth of migrated progenitor cells bearing not yet identified genetic events occurring still earlier than the until now known early changes: LOH at 3p/9p/17p and p53 mutations, can not be excluded. However, it is difficult to envisage how progenitor cells, with only a minimal genetic alteration, have the capacity to migrate intraepithelially over such a large distance. Nor, it is very plausible that these progenitor cells are displaced by saliva whereafter they settle elsewhere for outgrowth, since these cells can not have any invasive or metastatic capabilities.

Alternatively, progenitor cell spread could have taken place during embryonic development. A somatic mutation could have occurred at that time and a widespread field of progenitor cells can then be present. If tumors would develop from those cells than the further development of these tumors would be independent and abusing tobacco and alcohol would cause an additional risk. Therefore, it is important for HNSCC patients to quit abusing tobacco and alcohol after the diagnosis of their first primary tumor. Epidemiological studies support this finding; quitting smoking and abusing alcohol decreases the risk of a second primary tumor [2,28].

In several other studies, MPTs or dysplastic lesions, that were remote from each other, were investigated for clonal relationships. In these studies a few patients contained the same genetic alterations in their multiple lesions. Partridge *et al* detected 1 patient (out of 15) with a dysplasia and a tumor located remote from each other with the same microsatellite alteration at locus D3S192 [7]. Furthermore, Scholes *et al* observed 1 patient with two HNSCCs that were from opposite sites [6]. This patient was investigated with 10 informative microsatellite markers at chromosome arm 3p, 9p and 17p and showed concordant LOH patterns in these two different tumors. After analysing tumor karyotypes and fluorescence in situ hybridisation, Worsham *et al* identified a patient with two synchronous HNSCCs that had a common clonal origin [8]. Franklin *et al* investigated 1 individual with widespread dysplastic changes at

different locations in the respiratory epithelium and detected the same p53 mutation in most of the dysplastic mucosas [29]. These studies illustrate that in a few cases separated MPTs can be clonally related. Nevertheless, in most cases separated tumors seem to have developed independently [7,9,11,12,30], although the possibility remains that they are derived from one single clone defined by not yet known common genetic changes. For the time being, the analyzed clonal markers that occur early in a pre-invasive state indicate independency.

Multicentric tumors however, or tumors that are separated only by a small area of normal mucosa or surrounded with dysplasia more often have the same clonal origin. This was the case in 1 of the 2 patients (patient 26 and 27) that we investigated and in several cases in other studies [5,6,7,9]. Patient 27 only showed a hereditary mutation or a new intron polymorphism in the p53 gene and no other mutation (Table 2). Regarding LOH data, both lesions had identical patterns, except for one locus (Figure 2). As this sometimes also was the case in our control HNSCC with the matched lymph node metastasis the conclusion can be drawn that both tumors were developed from one lesion.

In patient 26 both synchronous tumors contained different p53 mutations with the mutation from the other tumor being present at a small level (Table 2). The LOH patterns showed the different origin of the two HNSCC (Figure 2). The dysplasia probably belongs to tumor 1 and tumor 2 has developed separately from the other tumor.

Our results imply that local migration of tumor cells can cause the development of a second tumor, but this does not necessarily have to occur.

Clinical applicability

Separating primary lung carcinomas from lung metastases is of clinical importance and the treatment depends on this distinction. Using only the mutation pattern in the p53 gene, we conclude from our 8 investigated patients (patients 28-34 and patient 16; Table 2), that 5 patients had disseminated disease and the other 3 patients had two primary tumors. p53 mutations apparently are very useful in the discrimination between a second lung carcinoma and a lung metastasis.

Three patients with metachronous HNSCCs at about the same location (patient 35-38; Table 2) were also investigated with mutations in the p53 gene to make the distinction if the second tumors were recurrences or not. The mutation pattern in the p53 gene was conclusive in 2 patients. The second tumor was a recurrence in patient 35 and a second primary tumor in the patient 36. In the tumors from patient 37 a

hereditary mutation or a new intron polymorphism was detected. Since, no other mutations in p53 were identified, conclusions about the second tumor could not be drawn only with the p53 state. After microsatellite analysis it became clear that both tumors contained the same microsatellite instability of the microsatellite marker D3S1263. Based on these results it was concluded that the second tumor was a recurrence from the first tumor. Therefore, we conclude that employment of p53 mutation analysis and in some cases additional microsatellite analysis is very useful in clarifying the origin of second tumors.

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Chapter 9

Smoking related loss of chromosome Y in normal epithelium adjacent to head and neck squamous cell carcinomas

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A preliminary report

Abstract

In patients with a head and neck squamous cell carcinoma (HNSCC), the entire epithelial surface of the upper aerodigestive tract is supposed to have an increased risk for the development of (pre-)malignant lesions, due to multiple genetic abnormalities in the whole tissue region. In this study, numerical chromosome 1 and Y aberrations were investigated in histologically normal tumor-adjacent mucosa (TAM) from 10 smoking and 9 non-smoking HNSCC patients. The number of chromosomes were compared with those in epithelia of 10 healthy individuals. No differences were observed in the mean observed numbers of chromosome 1 between the three groups. Nevertheless, loss of chromosome Y was shown in the TAM from smoking patients. The non-smoking patients did not show chromosome Y loss. Thus, a smoking related numerical chromosome abnormality was present in TAM which supports the field cancerization theory.

Introduction

The incidence rate of second primary tumors in head and neck squamous cell carcinoma (HNSCC) patients is 10-35%, depending on both the location of the primary tumor and the age of the patient [1,2,3]. These second primary tumors adversely influence the prognosis of HNSCC patients [2]. HNSCC patients often have also widespread premalignant lesions. This led Slaughter *et al* to postulate the concept of field cancerization [4]. They hypothesized that the entire epithelial surface of the upper aerodigestive tract (UADT) has an increased risk for the development of (pre-) malignant lesions caused by multiple genetic abnormalities in the whole tissue region. Evidence for this hypothesis was based on observed alterations in histologically normal oral epithelium from HNSCC patients [5]. Changes were found among others in p53 expression [6], epidermal growth factor receptor expression [7], expression of cytokeratins [8], expression of proliferation markers [9], size of cytoplasmic area [10], and cyclin D1 expression [11].

Originally, the mucosal changes in the entire UADT were widely accepted to be the result of a multistep process, driven by acquired mutations due to exposure to carcinogens of the whole tissue region [4]. The multiple squamous lesions described in the oral field cancerization process were thought to have developed independently of each other.

An alternative theory for the occurrence of multiple (pre)malignant lesions has been proposed in the last decade and is based on the premise that any transforming event is rare and that the multiple lesions arise due to widespread migration of transformed cells through the aerodigestive tract [12,13,14]. To be able to find methods to prevent [15] second primary tumors in future, it is important to know how they develop.

One way is to search for differences between histologically normal tumor-adjacent mucosa (TAM) from smokers and normal TAM from non-smokers. Smoking is the main risk factor in the development of HNSCC [1]. In the case of independent development of lesions, changes present in mucosa from smoking HNSCC patients should differ from changes in mucosa from non-smoking HNSCC patients. If migration of transformed cells indeed occurs, then changes in normal TAM should be present in both smoking and non-smoking HNSCC patients and not in epithelium from healthy individuals.

Aneuploidy and a whole series of gains and losses of chromosomes are frequently present in cells from HNSCC and from its preneoplastic lesions [16-21]. In a high percentage of male HNSCC patients, the tumor has lost chromosome Y [17,21]. The

aim of this study was to obtain support for either the time-honoured theory of oral field cancerization or the recently proposed migration theory, therefore we studied the prevalence of numerical aberrations of chromosome Y in histologically normal TAM from smoking and non-smoking HNSCC patients. Chromosome 1 was used as a control for more general chromosomal alteration.

Materials and methods

Clinical material

Formalin-fixed and paraffin-embedded histologically normal oral and laryngeal mucosa was selected from three groups of patients after reviewing hematoxylin and eosin-stained histological slides.

The first group comprised 9 cancer-free individuals (mean age 26 years; 8 male, 1 female) that underwent tonsillectomy in private practice. Tonsil mucosa was sampled from the obtained tonsils. The cancer-free individuals were interviewed by use of a questionnaire for the data concerning their smoking and drinking habits. All individuals that ever smoked or abused alcohol were excluded from this control group. Consumption of more than 3 glasses of alcohol was considered as alcohol abuse.

The second group consisted of 10 HNSCC patients (mean age: 67 years; all male) that smoked more than 20 cigarettes each day but only socially consumed alcohol. The third group consisted of 10 HNSCC patients (mean age: 66 years; 5 male, 5 female) that had never smoked or abused alcohol. No patient had undergone any previous treatment other than biopsies as part of the preoperative diagnostic procedures. The normal mucosa was sampled from peripheral parts of the tumor resection specimen. The patients were operated at the Department of Otorhinolaryngology or at the Department of Oral and Maxillofacial Surgery of the University Hospital Utrecht. Data of smoking and alcohol usage were obtained from the clinical records of the patients.

The use of mentioned tissues for this research was approved by the ethical comittee for scientific research on human beings of the Utrecht University Hospital and informed consent was obtained from the involved patients.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed according to Kaa et al. with the modifications as are mentioned below [22].

Slide preparation

For detection of chromosomal abnormalities, 5 μm sections were cut from the formalin-fixed and paraffin-embedded mucosa samples. The sections were fixed overnight at 58 ^{0}C on silan-

coated slides. The sections were deparaffinised by xylene and rehydrated in a series of decreasing percentages of ethanol. Subsequently, they were digested in pepsin-glycine buffer ($5x10^6$ activity units pepsin/l and 7.5 g glycine/l; both Sigma, St Louis, USA) at 37 °C for 10-20 min, washed in aqua dest, dehydrated in increasing of ethanol, air-dried and heated at 80 ^{0}C for 30 min.

Probe labeling and hybridization

The following pericentromeric probes were used: 1) chromosome Y, pHU14/SRY [23], obtained from Amersham Pharmacia Biotech (Uppsala, Sweden 2) chromosome 1, PUC1.77 [24], generously provided by the Department of Medical Genetics, University Medical Center Utrecht. The probes were labeled with digoxygenin using a nick translation reaction (Roche Diagnostics, Basel, Switzerland). The hybridization mixture consisted of 50% deionized formamide, 2 X Saline Sodium Citrate (2 x SSC:0.3M NaCl, 30mM NaCitrate, pH 7.0), 10% dextran sulphate, 100 ng/µl herring sperm DNA and 1 µg/µl tRNA, at probe concentrations of 3 ng/µl mixture. The hybridization mixture was denatured at 100 ^{0}C for 5 min. After adding the mixture on the section, a coverslip was placed. The slides were incubated at 96 ^{0}C for 10 min. to denature the target DNA. This was followed by overnight incubation of the slides at 37 ^{0}C in a humidified chamber.

Detection

The coverslip was removed by rinsing the slides in 50% formamide, 2 x SSC containing 0.05% Tween-20 at room temperature, which was repeated 3 times for 5 min. Subsequently, the slides were washed 3 times 5 min. in 2 x SSC containing 0.05% Tween-20 at room temperature and once in PBS with 0.05% Tween-20.

The sections were incubated at room temperature with 1:500 diluted mouse-anti-digoxygenin antibody (Roche Diagnostics) for 60 min., followed by 2 times 10 min. washing in PBS with 0.05% Tween-20. A biotinylated horse-anti-mouse antibody (Vector Laboratories Inc, Burlingham, CA) was added for 30 min. in a 1:500 dilution. Subsequently, the sections were rinsed 2 times 10 min. in PBS with 0.05% Tween-20 and incubated for 30 min. with 1:100 diluted fluorescein-labeled streptavidin (Roche Diagnostics). The nuclei were counterstained for 45 sec. with 20 ng/ml propidium iodide (Sigma) and washed for 5 min. in PBS with 0.05% Tween-20.

Evaluation of the FISH signals

The stained sections were evaluated using a fluorescence microscope (Leica DMR, Leica, Wetzlar, Germany) equipped with single- (Leica I3 and Leica N2.1) and double-band pass (Leica G/R) filters to detect fluorescein and propidium iodide. The number of signals was counted in at least 200 nuclei per section with a 10x ocular and a 63x 0.75 objective. The following criteria were used: a) overlapping nuclei were not counted, b) signals should have the same size and intensity which excludes aspecific signals, c) minor binding sites were not

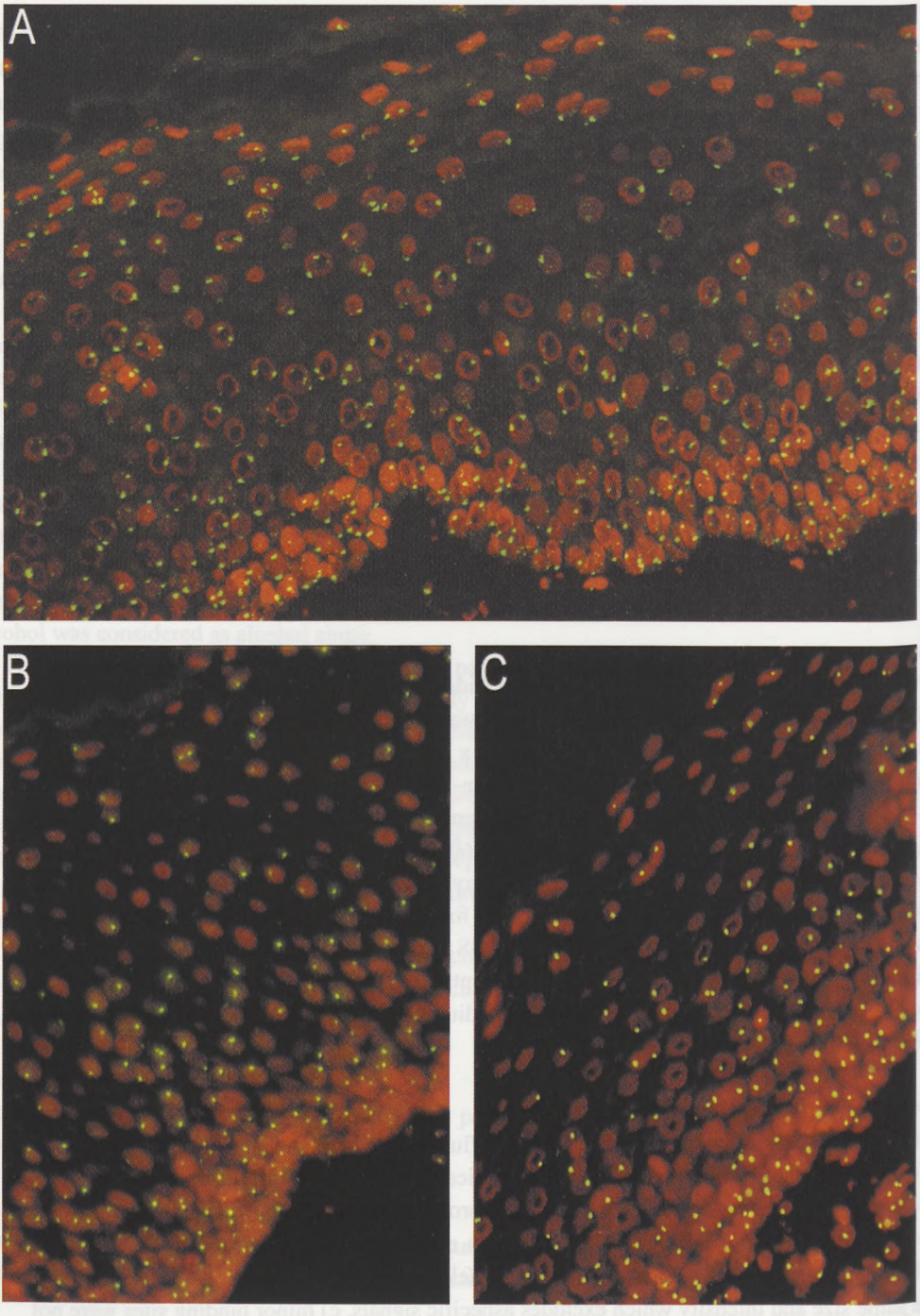
A
B
C

Figure 1 (left page). Fluorescence in situ hybridization of normal tumor-adjacent oropharyngeal epithelium.
Pericentromeric staining of chromosome 1 (green spots) in paraffin-embedded normal mucosa A) from a healthy control individual. The red fluoresence ring-like structures in some nuclei are fixation artefacts and not damaged nuclei.
Pericentromeric staining of chromosome Y (green spots) in paraffin-embedded histologically normal mucosa adjacent to a HNSCC B) from a male smoker C) from a male non-smoker.

counted and d) damaged nuclei were excluded. The mean proportion of signals per cell for each probe was calculated. The chromosome index of diploid cells measured in truncated nuclei in tissue sections is always smaller than it should be, therefore statistics were necessary. Differences between mean chromosome indexes of the groups were assessed with one-way analysis of variances ANOVA, according to Bonferroni with a significance level of 0.05.

Results

The sections from normal oropharyngeal epithelium were stained with pericentromeric probes for respectively chromosome 1 and Y (Figure 1). Not all nuclei contained the normal number of spots since not the entire nuclei are present in the 5 µm thin sections, which causes an underrepresentation of the normal number of chromosomes. After counting the percentage of cells with expected number of chromosomes per section (1 spot for chromosome Y in males and 2 spots for chromosome 1) , the mean percentages per group were assessed (Figure 2).

The mean percentage of the cells with diploid chromosome 1 were about the same in all investigated groups: the control group had a mean of 84.0% (with sd=4.2%), the smoking HNSCC patient group had a mean of 82.7% (with sd=4.0%), and the non-smoking HNSCC patient group had a mean of 83.6% (with sd=5.6%). One non-smoking HNSCC patient showed loss of chromosome 1. The percentage of epithelial cells with diploid chromosome 1 was only 73% in this patient.

A significant loss of chromosome Y could be shown in the normal TAM from the male smoking HNSCC patients (p=0.03). The mean percentage of cells with one chromosome Y was no more than 73.3% (with sd=8.0%). Most other cells did not show staining for chromosome Y while only a few cells showed two chromosome Y signals. This is in contrast to the mean percentage of 81.5% (with sd=1.8%) of cells

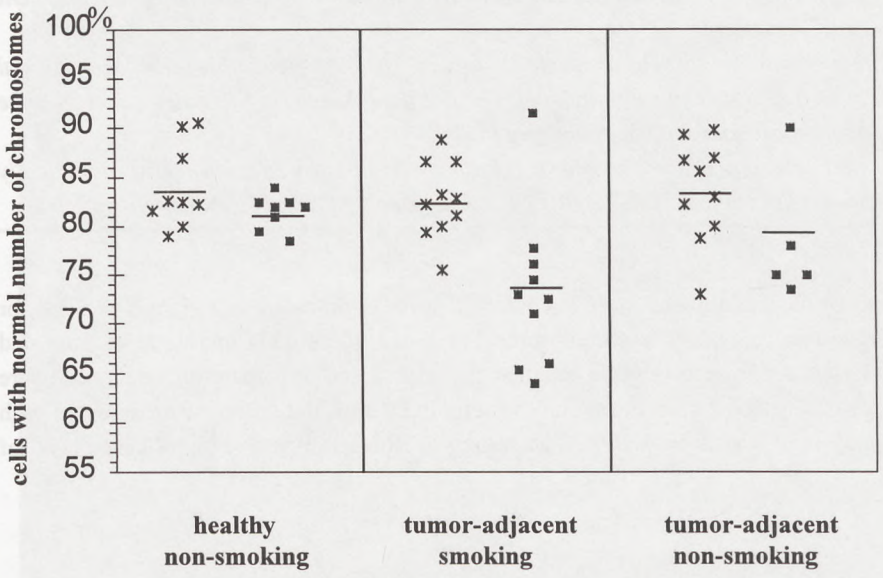

Figure 2. Fluorescence in situ hybridization of normal oropharyngeal epithelium.
Each point is the result of the percentage of cells with the expected number of chromosomes from one section (two chromosomes 1 and one chromosome Y). The number of pericentromeric spots from chromosome 1 (✳) and chromosome Y (■) were counted in at least 200 nuclei per section. The horizontal lines represent the mean values of the groups.

with the normal number of chromosome Y in the control group.

Unfortunately, only five patients in non-smoking HNSCC group were male, which gave us a low number of samples for chromosome Y staining. The mean percentage of cells with one chromosome Y in the epithelium from the non-smoking patients group was 79.5% (with sd=7.1%) which was not different (p=1.0) from the control group. However, one HNSCC patient might have gain of chromosome Y in his tumor-adjacent epithelium. The percentage of epithelial cells with one chromosome Y was 90% in this patient. This was also the case in one HNSCC patient from the smoking group (Figure 2).

Discussion

Numerical chromosome 1 and Y aberrations were investigated in histologically normal TAM from smoking and non-smoking HNSCC patients. We have shown that a normal diploid number of chromosome 1 is present in epithelial cells from these HNSCC patients. Only one non-smoking HNSCC patient showed loss of chromosome 1. This is in contrast to the chromosome 1 gain that has been shown in some invasive HNSCC [20].

Since no increase in the number of chromosome 1 was observed in the epithelium from most HNSCC patients, complete polyploidy seems not to occur in histologically normal TAM. Aneuploidy has been shown in some mucosa samples with inflammatory changes from HNSCC patients and also in preneoplastic lesions by others [18,19].

Gain of chromosome Y was observed in one smoking HNSCC patient and in one non-smoking patient. However, we have shown loss of chromosome Y in the epithelium from the smoking HNSCC patients and not in the non-smoking patients, which indicates a smoking induced field change. Chromosome Y loss has previously been documented in HNSCC from male patients [17,21] and also in other tumors [25]. The implication of loss of chromosome Y in the development of HNSCC however is unclear, since no important tumor suppressor gene has been identified on chromosome Y yet.

It is known that clonal chromosome abnormalities may accumulate with age in UADT mucosa [26]. In addition, chromosome Y loss also occurs in normal bone marrow from elderly men [27]. The mean age (26 years) of our control group was lower than of our two HNSCC patient groups. However, our non-smoking HNSCC patient group with a mean age of 66 years (ranged from 32 to 89 years) did not show a significant chromosome Y loss in contrast to our smoking HNSCC patient group with about the same mean age of 67 years (ranged from 48 to 74 years). Furthermore, the elder HNSCC patients did not show lower percentages of chromosome Y [data not shown].

In contrast with loss of chromosome Y, polyploidy was not observed. Numerical chromosomal aberrations occur probably earlier in the tumorigenesis than total polyploidy, which was also suggested by Carey *et al.* [28].

In another study, a whole series of other chromosomal abnormalities was shown by FISH in cells from brushes from macroscopically normal cheek mucosa opposite to the site of the HNSCC [29]. Chromosome aberrations were mainly seen in chromomomes

2, 3, 10, 12, 15, and X using centromeric and pericentromeric probes. Interestingly, the chromosome aneusomies found in the non-tumor specimens were also present in the corresponding tumor specimens. Nevertheless, the chromosomal imbalances appeared in lower frequencies of cells and in less complex combinations than in the tumors [29]. It must be noted that cell brushes from normal mucosa may be contaminated by wandering tumor cells. Any relationship with smoking was not investigated in this study.

Polysomies of chromosome 7 and 17 were shown in TAM from HNSCC patients by others, while no polysomies were present in buccal epithelium from healthy individuals [30]. The polysomies were absent in tumor-distant epithelium [31]. Almost all HNSCC patients smoked which suggests smoking-induced polysomies. The absence of polysomies in tumor-distant epithelium might be explained by a more intense exposure to tobacco close to the tumor than at more remote sites.

Numerical chromosome abnormalities were present in TAM which supports the field cancerization theory. Both Hittelman *et al* and Högmo *et al* showed a relationship between genomic instability in premalignant lesions and the risk to develop a HNSCC [18,32]. We found a relationship between chromosome Y loss and smoking which illustrates that a field change present in smoking HNSCC patients is absent in non-smoking HNSCC patients. This is in agreement with two of our previous studies in which we respectively showed an increased proliferation [9] and focal overexpression of the tumor suppressor p53 in the epithelium from smoking HNSCC patients [6]. Thus, it appears that TAM from smoking HNSCC patients shows alterations not found in non-smoking patients. We can not exclude that TAM from non-smoking HNSCC patients contains migrated cells that not betray themselves by showing any of the above mentioned features due to differences in genetic alterations between HNSCC from smoking and non-smoking patients. Nevertheless, our results rather support the idea that tumor-adjacent mucosal changes are carcinogenic induced independent events than the result of migrated transformed cells.

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Chapter 10

General discussion and summary

Field changes

In this thesis we investigated whether multiple (pre)malignant squamous cell lesions in the upper aerodigestive tract (UADT) develop independently from each other (time-honoured hypothesis) or that they arise from migrating transformed cells and thus share a common source (migration theory). To address this issue, alterations in histologically normal tumor-adjacent mucosa (TAM) from head and neck squamous cell carcinoma (HNSCC) patients were studied (Table 1).

Table 1. Field changes

marker	chapter	method	field changes
c-Src	2	IHC	c-Src not detectable in TAM
EGFR	3	IHC	EGFR overexpressed in TAM
Ki-67/cyclin A	4	IHC	Number of proliferating cells increased in TAM
p53	5	IHC	Clusters of p53 positive cells in TAM
chromosome Y	9	FISH	Loss of chromosome Y in TAM

Potential field changes were investigated in tumor-adjacent mucosa (TAM) by immunohistochemistry (IHC) or fluorescence in situ hybridisation (FISH)

An increase in protein tyrosine kinase (PTK) activity in TAM was shown in a previous study [1]. We investigated by immunohistochemistry whether the proto-oncogene c-Src was the PTK responsible for this increased activity. c-Src could be demonstrated in hyperproliferating mucosal lesions, in which it showed co-expression with the proliferation marker Ki-67. However, c-Src protein expression levels were too low to detect in TAM and normal control epithelium. Therefore, we could not draw any conclusions about the background of the increase in PTK activity in TAM.

We did show an immunohistochemically detectable overexpression of the epidermal growth factor receptor (EGFR) in TAM, which was in agreement with results from other studies [2,3]. An increased number of proliferating cells in TAM by using PCNA [4] and silver staining of nucleolar organizer regions has been shown previously [5]. We confirmed this finding both with the proliferation marker Ki-67 and with the proliferation regulator cyclin A. Loss of chromosome Y often occurs in HNSCC [6,7].

We observed loss of chromosome Y as a field change in TAM. Another field change was found by studying p53 immunohistochemically. We showed focally distributed overexpression of p53 in TAM.

Distinction between mutant p53 and wild-type p53

Clusters with overexpression of p53 in TAM could represent mutant p53 as well as wild-type p53, since occasionally p53 cells overexpress wild-type p53 [8]. As sequencing of the p53 gene is often very hard to perform on small clusters of cells in formalin-fixed and paraffin-embedded sections, another method was required to investigate the p53 gene status in these clusters.

In **Chapter 6**, the possibility to use the proliferation marker Ki-67 [9] for discrimination between overexpression of mutant p53 and wild-type p53 was investigated. It was hypothesized that normally functioning wild-type p53 in contrast to mutant p53 would cause cell cycle arrest with loss of immunohistochemically dectable expression of the proliferation marker Ki-67. However, the results showed that the Ki-67 is also positive in arrested cells, which means that Ki-67 can not distinguish between wild-type p53 and mutant p53. Therefore, in **Chapter 7** the p53-induced protein p21(Waf1/Cip1) [10] was investigated for the applicability as marker to discriminate between the presence of mutant or wild-type p53 in the cells with p53 overexpression. Normal functioning wild-type p53 should induce p21 while mutant p53 should not. This study showed that p21 can be expressed independent of p53, which means that neither p21 is a good marker to distinguish between wild-type p53 and mutant p53. Thus, no alternative method for sequencing the p53 gene has been found to ascertain the p53 state in p53 overexpressing cell clusters. Unfortunately, we did not succeed in sequencing enough of these interesting clusters to draw any conclusions about their p53 status [data not shown]. Employing more suitable material, others showed that focal p53 overexpression in TAM is often due to p53 mutations [11].

Evidence for smoking-induced field cancerization

The role of smoking (and alcohol) was examined in the earlier described observed field changes in TAM. If these field changes are related with abusing tobacco and/or alcohol and are not detected in non-smoking patients this would support the time-honoured theory of carcinogen-induced field cancerization.

Overexpression of the EGFR was almost as high in TAM from non-smoking/non-drinking HNSCC patients as in TAM from smoking/drinking HNSCC patients (**Chapter 3**). Furthermore, no increase in EGFR expression was observed in mucosa from cancer-free smokers compared to cancer-free non-smokers. The EGFR expression in the mucosa from the HNSCC patients was less elevated when the epithelium was located more distant to the tumor. These results suggest a paracrine effect on the EGFR expression due to factors released by the tumor.

In **Chapter 4**, we showed an increased number of proliferating epithelial cells in the TAM from smoking HNSCC patients and to a lesser extent in TAM from ex-smoking HNSCC patients. No increase was observed in TAM from non-smoking HNSCC patients. This increase in proliferating cells was not only observed in TAM but also in mucosa of the UADT from healthy smokers. Also a trend towards an increased proliferation was shown in the mucosa from healthy ex-smokers. Thus, the elevated proliferation persists for some time after quitting smoking and might represent early genetic alterations. However, if it are genetic alterations that play a role in this increased proliferation, one expects to detect only clusters of proliferating cells whereas we detected a more uniform distribution of proliferating cells. Moreover, the higher proliferative activity in smokers and ex-smokers may well constitute a fertile soil for genetic events culminating in the development of HNSCC. Either one or the other, both support the field cancerization hypothesis of lesions developing independently of each other by carcinogen-induced alterations.

The possible role of altered p53 in this overall increased epithelial proliferation in smokers was examined by immunohistochemistry (**Chapter 5**). No differences in uniformly spread overexpression of p53 were observed between mucosa from smokers / non-smokers and ex-smokers; neither in mucosa from the HNSCC patients nor in the mucosa from healthy control individuals. Therefore, altered p53 does not seem to play a role in the increased epithelial proliferation in smokers.

In contrast to the uniformly spread p53 overexpression, focally overexpressed p53 occurred more frequently in normal epithelium from smoking HNSCC patients (50%) than from non-smoking HNSCC patients (20%) and from healthy control individuals (12 %) (**Chapter 5**). Since the presence of p53 overexpressing cell clusters is not elevated in epithelium from non-smoking patients, this field change is probably not due to migrating transformed cells. This finding again implies smoking-induced field cancerization. However, the elevated number of p53 positive clusters could not be detected in mucosa from healthy smokers (13 %). This indicates that besides the abuse of tobacco, other environmental factors and/or genetic factors must contribute to the

presence of p53 positive clusters in TAM. Abuse of alcohol was an additional factor in these HNSCC patients and might have played a role in the development of the p53 positive clusters together with the abuse of tobacco.

Possibly, this carcinogen-induced focal p53 overexpression represents an early alteration in the development of HNSCC. One study showed that p53 expression above the basal cell layer in oral mucosa is an early event of malignant transformation and has predictive value for developing oral squamous cell carcinomas [12]. Another study showed that more p53 clusters were present in epithelium surrounding multicentric HNSCC than unicentric HNSCC[13].

A significant loss of chromosome Y was only detected in TAM from smoking HNSCC patients and appeared not to be present in the non-smoking patients **(Chapter 9)**. A whole series of chromosomal aneusomies was shown by fluorescence in situ hybridisation (FISH) in cells from brushes from macroscopically normal cheek opposite to the site of the HNSCC by others [14]. Unfortunately, only one patient from the 10 investigated patients did not smoke. This patient did not show chromosomal changes in the normal cells, however one patient is obviously not enough to draw any conclusion about the role of tobacco in these chromosomal aberrations. The mucosa from cancer-free control smokers was compared with mucosa from cancer-free non-smokers. No significant chromosome aneusomies were detected, although a trend in aneusomies of chromosome 2, 6, and Y was observed in the mucosa from smokers [14].

In another study, polysomies of chromosome 7 and 17 were observed in TAM from HNSCC patients [15]. Almost all patients smoked, which might suggest that the polysomies were smoking-induced. However, the polysomies were significantly higher in tumor-adjacent mucosa than in tumor-distant mucosa which refutes this idea.

Many other field changes, as mentioned in the introduction, have been observed in TAM from HNSCC patients. However, the relationship between these changes and smoking or alcohol abuse was often not investigated. The role of tobacco was only shown in a few studies. Ogden *et al* described an altered nuclear to cytoplasmic area ratio in TAM and suggested that tobacco might play a role in this alteration since healthy smokers also showed a slight increase in nuclear area compared to healthy non-smokers [16]. Furthermore, expression of cytokeratins 7 and 8 in TAM occurred more frequently in the smoking group of patients than in the non-smoking group [17].

Most of the studies performed by others are in agreement with our own results **(Chapters 4, 5 and 9)** which showed that increased proliferation, focal overexpression of the tumor suppressor p53 and loss of chromosome Y were only present in the TAM

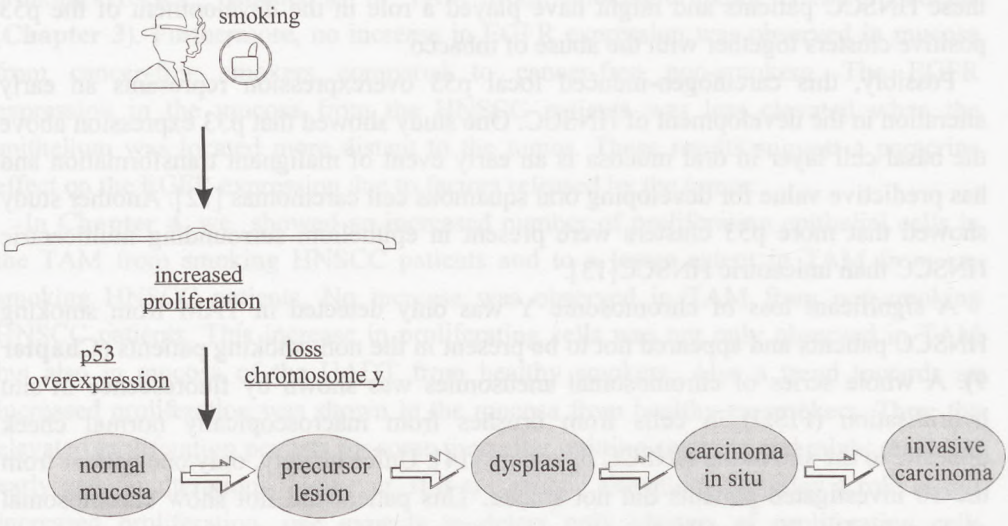

Figure 1. Smoking-induced field changes

The abuse of tobacco is related to three field changes in TAM from HNSCC patients: 1) focal p53 overexpression, 2) increased proliferation, 3) loss of chromosome Y. These smoking-induced field changes might be the beginnings of new independently developing lesions

from smoking HNSCC patients. Thus, it appears that TAM from smoking HNSCC patients shows alterations not found in non-smoking patients (Figure 1), which implies that TAM in smoking patients harbours altered cells being absent in non-smoking patients. If these cells have migrated from adjacent tumors, one would expect them to be present also in TAM from non-smoking patients which is not supported by our findings. Moreover, one observed field change, the increased proliferation, was already present in healthy individuals without a tumor being present as source for migrating cells (**Chapter 4a**). Therefore, unless we assume that TAM in both smoking and non-smoking patients contains migrating cells that do not betray themselves by showing yet unknown alterations, our results support the idea that tumor-adjacent mucosal changes are carcinogen-induced independent events rather than the result of migrated transformed cells.

It has been shown that the risk of developing second primary HNSCCs is higher in

smokers/drinkers than in non-smokers/non-drinkers [18,19]. In addition, this risk decreases when the patient quits smoking and abusing alcohol [19]. This observation also perfectly fits into the time-honoured field cancerization theory in which the mucosal changes in the entire UADT were considered to be the result of exposure to carcinogens that caused multiple genetic abnormalities in the whole tissue region.

Polyclonality of multiple primary tumors in the head and neck

The different field cancerization theories were also investigated in patients with multiple primary tumors (MPTs) in the head and neck. If multiple tumors would have developed due to migration of tumor cells then these tumors have to show identical genetic alterations. Clonality data of these tumors were obtained with two clonal markers (**Chapter 8**). The p53 mutation in the first primary tumor was used as a clonal marker and loss of heterozygosity (LOH) pattern of 10 microsatellite markers on chromosome arms 3p, 9p and 17p was used as the other clonal marker.

The reliability of these markers was assessed beforehand (**Chapter 8**). From the literature it was known that alterations in these markers occur very early during HNSCC development. p53 mutations and LOH pattern were both stable during tumor progression and metastasizing. P53 mutations were present in more than 90% of the HNSCCs and a particular mutation never occurred more than twice in a total of 69 primary HNSCCs. In contrast, LOH patterns showed less variability, being identical in 5 out of 16 cases. Therefore, we concluded that a p53 mutation was the best molecular clonal marker. The LOH pattern was only useful when complete loss of the same chromosome arm had not occurred in both cases. Therefore LOH pattern analysis was only applied in cases in which p53 analysis was inconclusive.

The p53 mutations and LOH patterns were different in each analyzed couple of two remote primary tumors from 9 investigated HNSCC patients (**Chapter 8**). This excludes the possibility that the remote second primary tumors had developed from migrated tumor cells, although they could have developed by migration of progenitor cells with a yet unknown genetic alteration that occurs even earlier than LOH at 3p/9p/17p or a p53 mutation. However, it is difficult to envisage how progenitor cells, with only a minimal genetic alteration, have the capacity to migrate intraepithelially over such a large distance. Neither is it very plausible that these progenitor cells are displaced by salivary flow whereafter they settle elsewhere for outgrowth, since progenitor cells do not have any invasive or metastatic capabilities. In addition it has been shown that a few transformed cells that are surrounded by normal cells do not

succeed in the development of a lesion [20].

Two patients with synchronous HNSCC tumors lying close to each other were also investigated for clonality (**Chapter 8**). One patient showed an identical p53 alteration and LOH pattern in both tumors and the other patient showed differences. This indicates that in the first patient intraepithelial migration of tumor cells might have occurred.

Several other studies showed polyclonality between MPTs or dysplastic lesions that were remote from each other [21-25]. Nevertheless, in some studies a sole patient contained the same genetic alterations in his multiple lesions [21,26]. However, tumors that were located very close to each other, more often showed identical changes [21,22,26,27) and might have developed from the same clone. It is unclear whether the few multiple tumors that have a clonal relationship have developed after tumor cell migration or after progenitor cell migration. As is mentioned before, the latter seems not very plausible.

Presence of identical or polyclonal p53 mutations in TAM also provides evidence for one or the other field cancerization theory. In two previous studies, others have sequenced the DNA of biopsies from frozen sections with histologically normal TAM [11,28]. In both studies, the various p53 mutations identified in TAM differed from the p53 mutation in the matched HNSCC. This excludes that early lesions in TAM occur due to migration of progenitor cells with a p53 mutation.

One exception to the observed polyclonal p53 mutations was described by Franklin *et al*, who showed one individual with widespread dysplastic changes at different locations in the respiratory epithelium with the same p53 mutation in most of the dysplastic mucosal samples [29]. Migration of p53 mutated progenitor cells could have occurred in this patient.

Clinical relevance and conclusions

The finding that field changes frequently occur in TAM creates a different view on tumor excision margins that contain molecularly altered cells [30,31]. The conclusion that the margin is tumor positive if it shows presence of genetically altered cells, can not be drawn with all molecular markers. The molecular marker has to be specific for that particular tumor. For example, identical p53 mutations in the margins as in the tumor might be useful [32]; however overexpression of eIF4E in the margins is not specific for that tumor and may as well be a field change caused by smoking [33].

Most field changes appear to be smoking-induced which implies carcinogen-

induced field cancerization instead of field cancerization due to migrated transformed cells. Therefore, patients with a HNSCC better quit smoking to reduce the risk of MPTs, especially since HNSCC patients seem to be more susceptible [18,34] to tumor development than cancer-free smokers.

From the clonality studies in this thesis and results by others we conclude that almost no remote multiple tumors develop due to migration of tumor cells. They seem to develop independently as a result of the continuous carcinogenetic influence of alcohol and/or tobacco. Although migration of progenitor cells with not yet identified early alterations can neither be refuted nor confirmed, clinically this is not important since further development of these imaginary altered cells to tumors would be independent and due to continuation of the abuse of tobacco and alcohol. That these imaginary altered cells can develop into cancer independently from ongoing carcinogenetic stimulation is refuted by epidemiologic data [18,19] that shows a strong correlation between occurrence of MPTs and continuation of abusing alcohol and tobacco. The conclusion that multiple (pre)malignant squamous cell lesions mostly develop independently of each other and appear to be carcinogen-induced (Figure 2), supports the ongoing of chemoprevention trials with carcinogen blockers [35,36].

Physicians sometimes have difficulties to categorize a second squamous cell carcinoma in the aerodigestive tract from a HNSCC patient as a new primary tumor,

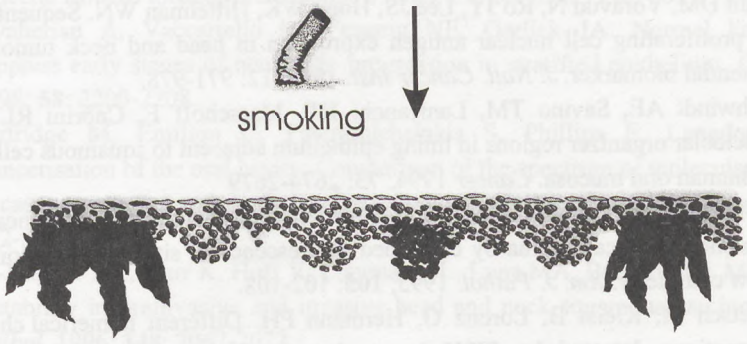

Figure 2. Multiple (pre)malignant lesions

Multiple remote (pre)malignant lesions are carcinogen-induced and develop independently. Therefore, HNSCC patients better quit smoking and abusing alcohol even after development of a HNSCC. Independent development of lesions also supports the continuation of chemoprevention trials with carcinogen-blockers.

disseminated disease or recurrent disease. The relationships between these tumors can not be determined on clinical, radiographic or even histopathologic grounds while the treatment of the patient depends on proper classification. Since, the use of a p53 mutation as clonal marker was reliable and LOH pattern analysis of 10 microsatellite markers on chromosome arms 3p/9p/17p was applicable as clonal marker under restricted conditions in the cases in which p53 analysis was inconclusive, these clonal markers were applied in 11 diagnostic uncertainties **(chapter 8)**. With the help of these clonal markers we could successfully distinguish a new primary tumor from disseminated disease or recurrent disease.

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Nederlandse samenvatting

voor niet-ingewijden

Inleiding

Na hart- en vaatziekten is kanker de meest voorkomende doodsoorzaak in Nederland. Een kankergezwel ontstaat door abnormale groei van cellen. Deze abnormale groei komt tot stand door veranderingen in stukjes erfelijk materiaal (genen) die de vermenigvuldiging van cellen regelen. De genetische veranderingen betreffen toegenomen functionele activiteit van groeistimulerende genen, ofwel oncogenen, en verlies van functie van groeiremmende genen, ofwel tumor-suppressorgenen.

Ook kanker van de slijmvliezen in het hoofd-halsgebied, zoals keelkanker en mondkanker (bv tongkanker en wangkanker), ontstaat op deze manier. Bij mannen staat deze vorm van kanker op de zesde plaats in de rij van de meest frequent vóórkomende maligniteiten.

De belangrijkste twee risicofactoren voor de ontwikkeling van hoofd-halskanker zijn roken en alcohol drinken. Hoogstwaarschijnlijk zorgen de stoffen in tabaksrook en alcohol voor de eerdergenoemde genetische veranderingen in het erfelijk materiaal. Vervolgens kan een reeks van veranderingen plaatsvinden in de cellen, waardoor het slijmvlies via verschillende voorstadia kan veranderen in een kwaadaardig gezwel.

Vraagstelling

Het is bekend dat zo'n 20% van de mensen met hoofd-halskanker meer dan één gezwel in de slijmvliezen van het hoofd-halsgebied ontwikkelt. Bij deze patiënten zijn meestal ook voorstadia van hoofd-halskanker te vinden. Dit verschijnsel is 'Field Cancerization' genoemd. Er zijn twee verschillende theorieën die 'Field Cancerization' verklaren.

☞De eerste theorie dateert reeds uit 1953. Deze theorie houdt in dat de verschillende gezwellen en voorstadia onafhankelijk van elkaar ontstaan door de voortdurende invloed van roken en/of alcohol drinken op het gehele slijmvlies in het hoofd-halsgebied.

☞De tweede theorie is pas 10 jaar geleden ter sprake gekomen. In deze theorie wordt het ontstaan van de diverse gezwellen en voorstadia geweten aan rondzwervende kwaadaardige cellen van het eerste gezwel.

In dit proefschrift is onderzocht welke van de twee theorieën de juiste is. Hiertoe is onder andere gekeken of er al veranderingen waarneembaar zijn in het gezond-ogende slijmvlies van patiënten met hoofd-halskanker, welke veranderingen dat zijn, en of deze veranderingen gerelateerd zijn aan het rookgedrag van de patiënten.

Met roken samenhangende veranderingen in hoofd-halsslijmvlies

Deze veranderingen zijn op verschillende manieren te detecteren. Voor ons onderzoek is gebruik gemaakt van twee technieken waardoor met behulp van de microscoop de afwijkingen zichtbaar gemaakt kunnen worden. De eerste techniek heet immunohistochemie en daarmee kunnen allerlei regulerende stoffen (eiwitten) in het weefsel aangetoond worden. De tweede techniek heet fluorescente in situ hybridisatie en met deze techniek kunnen veranderingen in het erfelijk materiaal, in dit geval de chromosomen, aangetoond worden. Met behulp van een merkstof voor delende cellen, Ki-67 genaamd, en de techniek immunohistochemie, is gevonden dat celvermenigvuldiging vaker vóórkomt in normale slijmvliezen van patiënten met een hoofd-halstumor dan in slijmvliezen van gezonde controle mensen. Na verder onderzoek bleek dat deze toegenomen celvermenigvuldiging gerelateerd was aan roken. Zelfs patiënten met een hoofd-halstumor die gestopt waren met roken hadden een licht toegenomen celvermenigvuldiging. Ook bij gezonde mensen die veel roken, kon deze toename waargenomen worden.

Verder is met behulp van de immunohistochemie aangetoond dat er vaker clusters cellen met veranderingen in het tumor-suppressoreiwit p53 worden gevonden in de normale slijmvliezen van de patiënten met hoofd-halskanker dan in die van gezonde controle mensen. Ook deze verandering bleek gerelateerd aan het rookgedrag van de patiënten.

Met de andere techniek, de fluorescente in situ hybridisatie, is gevonden dat het mannelijke geslachtschromosoom Y vaak verloren is gegaan in de normale slijmvliezen van mannen met hoofd-halskanker. Dit verlies kwam alleen voor bij de rokende patiënten.

Er kan dus geconcludeerd worden dat er veranderingen in het gehele gezond-ogende slijmvlies van patiënten met hoofd-halskanker te detecteren zijn en dat deze veranderingen door roken geïnduceerd worden. Daardoor is de oudste theorie van 'Field Cancerization', het meest aannemelijk.

Verschillende genetische veranderingen in tumorenparen

De vraagstelling over de twee 'Field Cancerization' theorieën is ook nog op een andere manier onderzocht. Er is gekeken of de genetische afwijkingen in verschillende kankergezwellen van één patiënt hetzelfde zijn. Mochten deze afwijkingen namelijk steeds in beide kankergezwellen hetzelfde zijn, dan zou de meest recente theorie van 'Field Cancerization' over de rondzwervende tumorcellen, aannemelijk worden. De

cellen van het eerste gezwel hebben dan namelijk bepaalde afwijkingen, die behouden zouden blijven, nadat de cellen van plaats veranderd zijn en ergens anders weer een nieuw gezwel gevormd hebben.

Enkele afwijkingen die al vroeg in de ontwikkeling van hoofd-halskanker voorkomen en daarna niet meer veranderen, zijn verlies van delen van chromosoomgebieden 3p, 9p en 17p. Ook genetische veranderingen in het tumor-suppressorgen p53 horen daarbij. Met behulp van moleculair-biologische technieken zijn verschillen in deze afwijkingen aan te tonen tussen gezwellen van verschillende patiënten. In dit proefschrift zijn deze verschillen bekeken tussen twee kankergezwellen van steeds één patiënt. Er zijn 9 patiënten onderzocht en tussen de tumorenparen werden geen enkele keer dezelfde afwijkingen gevonden. Uit dit onderzoek blijkt dat rondzwervende kwaadaardige cellen waarschijnlijk niet de oorzaak zijn van het ontstaan van meerdere hoofd-halskankergezwellen en de voorstadia ervan.

Conclusie

Uit alle resultaten tezamen wordt geconcludeerd dat de verschillende gezwellen en voorstadia in patiënten met hoofd-halskanker onafhankelijk van elkaar ontstaan, door de voortdurende invloed van roken (en/of alcohol drinken) op het gehele slijmvlies in het hoofd-halsgebied. Dit houdt in dat het nog steeds belangrijk is voor deze patiënten om na de diagnose en behandeling van het eerste gezwel te stoppen met roken (en het drinken van veel alcohol). Ook kan het nut van medicijnen die de kwaardaardige invloeden van roken en alcohol tegengaan verder onderzocht worden om het uitgroeien van een tweede kankergezwel te voorkomen.

"Zelfs een weg van duizend mijl begint met één enkele stap"

Oosterse wijsheid

"Niemand is zo blind,
als hij die niet wil zien"

Oosterse wijsheid

Aangepast van de Stivoro-website

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"Je hebt zoveel, je bent zo rijk, vaak zonder 't je te realiseren
Geniet ervan zolang het kan, 't geluk kan altijd keren"

Onbekend

Curriculum vitae

Monique van Oijen werd geboren op 8 april 1972 te Rossum. In 1990 behaalde zij het VWO-diploma aan de middelbare school "Buys Ballot" te Zaltbommel, waarna zij van 1990 tot 1995 Biologie (medische richting) studeerde aan de Katholieke Universiteit Nijmegen. Ze studeerde af met het judicium 'Met Genoegen' en tijdens deze studie zijn twee hoofdvakstages gevolgd. Gedurende de eerste stage bij de afdeling Celbiologie van de Katholieke Universiteit Nijmegen (Prof. Dr. E.J.J. van Zoelen) lag de nadruk op de rol van groeifactoren bij celproliferatie. De tweede stage werd gevolgd bij de afdeling Haematologie, Academisch Ziekenhuis Nijmegen St. Radboud (Dr. F.W.M.B. Preijers). Gedurende deze stage werden versterkende stoffen gezocht voor een immunotoxine tegen B-cel tumoren. Na deze stages werkte zij een periode in Londen, bij het National Institute for Medical Research (Dr. E. Dzierzak), om mutanten van het TAT-gen in HIV te maken met als doel om het gemuteerde virus in de toekomst als decoy-therapie in HIV-geïnfecteerde patiënten toe te passen. In augustus 1995 werd zij aangesteld als AIO bij de faculteit Geneeskunde van het Academisch Ziekenhuis Utrecht op een gezamenlijke onderzoeksproject van de afdelingen Pathologie (Prof. Dr. P.J. Slootweg) en Haematologie (Dr. G. Rijksen). Het onderzoek had als doel om te onderzoeken hoe meerdere hoofd-hals plaveiselceltumoren en premaligne afwijkingen kunnen ontstaan in patiënten met hoofd-hals kanker en wat voor rol het rookgedrag hierbij speelt. In oktober 1999 werd dit onderzoeksproject met een promotie afgerond. Tijdens haar aanstelling als AIO behartigde zij de belangen van de AIO's en OIO's betrokken bij de onderzoeksschool Ontwikkelingbiologie. Sinds september 1999 is zij werkzaam als post-doc bij de afdeling Immunologie van het Nederlands Kanker Instituut in Amsterdam (Prof. Dr. G.C. de Gast, Dr. J.B. Haanen). In dit project wordt onderzocht hoe bij patiënten met kanker (niercelcarcinomen, melanomen of B-cel tumoren) de immuunrespons tegen de tumorcellen versterkt kan worden. Hiervoor zullen antigen-specifieke T-cellen geselecteerd worden uit de patiënt of uit een donor met behulp van MHC-tetrameren.

List of publicaties

Articles

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3. Frasier C, Abraham DA, van Oijen M, Cunliffe V, Irvine A, Craig R, Dzierzak EA. Inhibition of Tat-mediated transactivation and HIV replication with Tat mutant and repressor domain fusion proteins. *Gene Therapy* 1998; **5**: 946-954.
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5. van Oijen MGCT, Rijksen G, ten Broek FW, Slootweg PJ. Increased expression of epidermal growth factor receptor in normal epithelium adjacent to head and neck carcinomas independent of tobacco and alcohol abuse. *Oral Dis.* 1998; **4**: 4-8 .
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7. van Oijen MGCT, Medema RH, Slootweg PJ, Rijksen G. Positivity of the proliferation marker Ki-67 in non-cycling cells. *Am. J. Clin. Pathol.* 1998; **110**: 24-31 .
8. van Oijen MGCT, Tilanus MGJ, Medema RH, Slootweg PJ. Expression of p21(Waf1/Cip1) in head and neck cancer in relation to proliferation, differentiation, p53 status and cyclin D1 expression. *J. Oral Pathol. Med.* 1998; **27**: 367-375.
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10. van Oijen MGCT, Leppers vd Straat FGJ, Tilanus MGJ, Slootweg PJ. The origin of multiple squamous cell carcinomas in the aerodigestive tract. *Submitted to Cancer.*
11. van Oijen MGCT, Slootweg PJ. Oral field cancerization. Carcinogen-induced

independent events or micrometastatic deposits? A review. *Submitted to Cancer Epidem. Biom. Prev.*

12. van Oijen MGCT, Slootweg PJ. Gain-of-function mutations in the tumor suppressor gene p53. A minireview. *Submitted to Clin. Cancer Res.*
13 van Oijen MGCT, Poorts LK, van Mansum M, de Weger RA, Slootweg PJ. Smoking related loss of chromosome Y in normal epithelium adjacent to head and neck squamous cell carcinomas. *Submitted to Oral Oncol.*
14. Dullens HFJ, van Oijen MGCT, Schroten N, Strik A, Slootweg PJ. DNA measuring by confocal laser scan microscopy to detect aneuploidy. *Submitted to J. Clin. Pathol.*

Abstracts

1) van Oijen MGCT, Medema RH, Rijksen G, Slootweg PJ. Positiviteit van de proliferatiemarker Ki-67 in niet-delende cellen. *Ned. Tijdschr. Geneeskd.* 1998; **142**: 2278.
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3) Van Oijen MGCT, Poorts LK, Slootweg PJ. Mucosal changes in head and neck cancer. Incipient tumors or micrometastases? *J. Cancer Res. Clin. Oncol.* 1999: **125**, suppl 2: S105.
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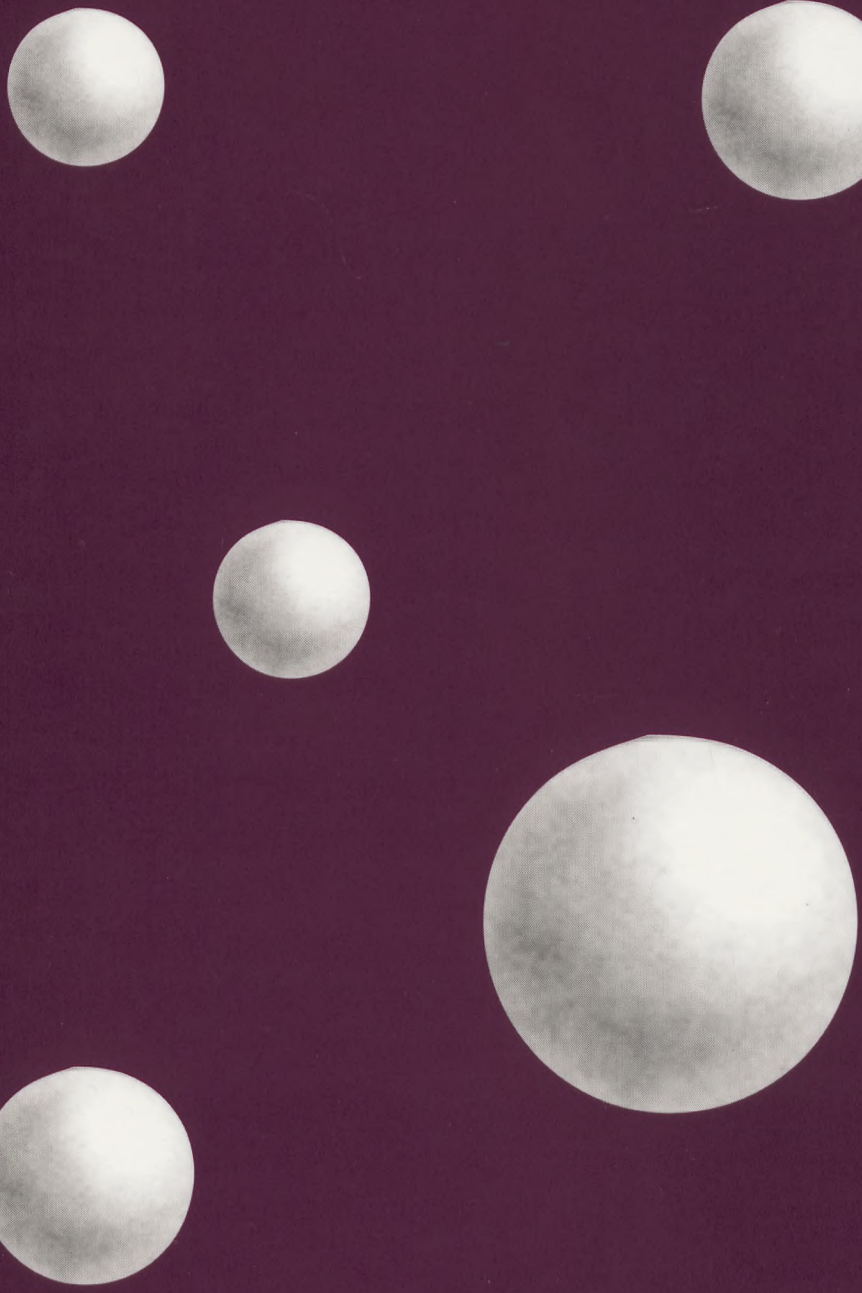
Abbreviations

BrdU	bromodeoxyuridine
BSA	bovine serum albumine
CDK	cyclin-dependent kinase
CLSM	confocal laser scanning microscope
DAB	3,3-diaminobenzidine tetrahydrochloride
EDTA	ethylene diamine tetra-acetic acid
EGFR	epidermal growth factor receptor
FAM	carboxy-fluorescein
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
HNSCC	head and neck squamous cell carcinoma
LOH	loss of heterozygosity
MPTs	multiple primary tumors
NRS	non-immune rabbit serum
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PI	proliferation index
PTK	protein tyrosine kinase
SD	standard deviation
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TGF	transforming growth factor
TAM	tumor-adjacent mucosa
TRITC	tetra-ethylrhodamine thiocyanate
UADT	upper aerodigestive tract



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