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Risk factors and potential early markers

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RISK FACTORS AND POTENTIAL EARLY MARKERS OF CARCINOGENESIS FOR THE DEVELOPMENT OF SECOND PRIMARY TUMOURS IN HEAD AND NECK CANCER

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VRIJE UNIVERSITEIT

RISK FACTORS AND POTENTIAL EARLY MARKERS OF CARCINOGENESIS FOR THE DEVELOPMENT OF SECOND PRIMARY TUMOURS IN HEAD AND NECK CANCER

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Vivian Bongers

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Promotor: prof.dr G.B. Snow

Copromotor: dr B.J.M. Braakhuis

Referent: prof.dr C.J.L.M. Meyer

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CONTENTS

List of Abbreviations

BANA	N-benzoyl-DL-arginine-β-naphtylamine
BSA	Bovine serum albumin
CB	Cathepsin B
CK	Cytokeratin
CK-16	Cytokeratin 16
CK-19	Cytokeratin 19
CYFRA 21-1	Cytokeratin 19-fragments
GSH	Glutathione
GST	Glutathione S-transferase
HNSCC	Head and neck squamous cell carcinoma
hsp70	heat shock protein 70
NAC	N-acetylcysteine
PBS	phosphate buffered saline
SPT	Second primary tumour
TRAP	Total radical-trapping ability parameter
type 2-chain ABH	Histo-blood group H type 2-chain
Z-arg-arg-MEC	N-α-benzoyloxycarbonyl-L-arginine-7-amido-4-methylcoumarine

CHAPTER 1

General Introduction

1.1 The problem of second primary tumours in patients with head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma (HNSCC) originating from the mucous membranes of the upper aerodigestive tract accounts for approximately 5% of all malignant neoplasms in the Netherlands (Visser *et al.*, 1994). Worldwide more than 500,000 new cases are registrated annually and this incidence shows a tendency to increase (Blitzer *et al.*, 1988; Boring *et al.*, 1994). Moreover, pronounced functional deficits in e.g. speech and swallowing as well as cosmetic deformations associated with HNSCCs heighten their importance. In the last decades initial therapeutic and reconstructive modalities for HNSCC have markedly improved, but the overall survival rate (approximately 59%, Muir *et al.*, 1995) has only marginally increased. One of the chief causes of treatment failure in early stage HNSCC is the occurrence of second primary tumours (SPT). The great majority of these SPTs appear in the same organ or organ systems: the respiratory and upper digestive tract, including the oesophagus (De Vries *et al.*, 1986b). Slaughter *et al.* (1953) explain this phenomenon of multicentric tumour foci with their "field cancerization concept". This concept explains that the entire epithelial lining, covering the aero- and upper-digestive tract undergoes extensive cytologic changes as a result of exposition to repeated insults by the same carcinogens, like tobacco and alcohol metabolites, and as such is prone to multifocal cancers.

The original classification criteria for second primary tumours, as proposed by Warren *et al.* (1932), later modified by Hong *et al.* (1990), are at present still used. These are as follows: a second tumour has to be separate from the first by at least 2 cm of normal non-neoplastic mucosa. Besides this characteristic each tumour has to be malignant at histological examination. And in any case, the possibility that the second one is a metastasis of the first has to be eliminated. These cancers are also classified to their temporal sequence as synchronous if they develop simultaneously or within half a year of the first -so termed- index tumour and as metachronous if they become manifest more than half a year after the index tumour. The great majority (90%) of SPTs are metachronous tumours.

Epidemiological studies have established that SPTs appear with a continuing annual rate of 2,8%, depending on the site of the index tumour (Jovanovic *et al.*, 1994). The ultimate incidence of SPTs varies from 10 to as high as 40%, with subgroup incidences based on the site of the primary tumour, as shown by Schwartz *et al.* (1994) for the 5-year estimated cancer incidence (see table I).

Table I.: 5-year estimated incidence of second primary tumours, based on the site of the index tumour.

primary tumour	oral cavity	mobile tongue	tonsillae	base of tongue	pyriform sinus	larynx
SPT inc.	18%	10%	15%	46%	34%	23%

SPT inc. = incidence of second primary tumours. Ref.: Schwartz et al., 1994.

SPTs arise frequently at notoriously bad sites, such as the lungs and the oesophagus or in previously irradiated or operated areas and are therefore more difficult to treat with worser treatment outcomes than the corresponding index tumours. In this context early detection and chemoprevention strategies appear presently the best options to reduce morbidity and mortality related to multiple primary cancers.

Early detection of SPTs is until now limited to close clinical observation and radiographic investigations, like chest radiography. Ideally, panendoscopy is to be carried out with regularity, but clearly this is not feasible in all patients (Rachmat *et al.*, 1993). There is thus a great need for identification of high risk individuals.

Prevention, by means of quitting smoking and/ or alcohol consumption will contribute in only a minor way to reduce the risk on SPTs. Since HNSCC is in principal a disease of people in the sixth decade of their life, who have already been smoking and drinking for approximately thirty years, it will be clear that many injuries have already been caused before prevention will be started. However, avoidance of tobacco and alcohol is a desirable way to reduce the risk of second cancers of the aerodigestive system among long term survivors (Day *et al.*, 1994).

Chemoprevention, defined as the inhibition of cancer-development by administering a natural or synthetic compound, is an additional approach to improve the life expectation of HNSCC patients. The earliest randomized chemoprevention trial, concerning isotretinoin, as published by Hong *et al.* (1990) showed a decreased incidence of SPTs in the treated patients. In one of the current chemoprevention trials in Europe, the Euroscan trial (De Vries *et al.*, 1991), patients are treated with retinyl palmitate, N-acetylcysteine (NAC), both agents or no drugs. NAC is believed to act in the early stage of carcinogenesis, and is thought to protect against DNA damage, while vitamin A acts later in the promotion and progression stages. Theoretically, the combination covers nearly the entire carcinogenic process. A generally recognised problem with chemoprevention strategies is, that the assessment of the efficacy of a certain chemopreventive agent requires approximately ten years. However, biomarkers of carcinogenesis could be of value to predict the efficacy of a new chemopreventive agent. Established biomarkers are then to be used as surrogate

endpoints to shorten the follow-up period needed in chemoprevention trials. Moreover, such biomarkers could be used to identify patients at high risk for a second primary tumour, so that preventive measures can be applied in a more selected group of high risk cases making potential side-effects and toxicity of chemopreventive agents more acceptable.

In summary, the occurrence of SPTs, particularly during follow-up, is a major problem in HNSCC patients, especially those with early stage HNSCC. Early detection and chemoprevention are the best two options to reduce the morbidity and mortality. A prerequisite for both early detection and chemoprevention, however, is identification of high risk individuals. Although some insight has been gained in the kinds of patients who are most likely to be hit by a SPT, our knowledge of risk factors is still fragmentary. Therefore, this thesis addresses the identification of high risk factors for the development of SPTs at three levels: the constitutional level (*chapter 2*), the level of the mucosa at risk (*chapters 3, 4, 5 and 6*) and at the level of the serum (*chapters 7 and 8*). In addition, biomarkers of carcinogenesis could be applied to assess the effect of preventive drugs (*chapter 9*). The focus is on the mucosa at risk.

1.2 Constitutional risk factors

Recently Copper *et al.* (1994) showed in an epidemiological case-control study that, besides established risk factors like smoking and alcohol consumption, a genetic predisposition is an independent risk factor for head and neck carcinogenesis. They demonstrated that first degree relatives of HNSCC patients have a relative risk of 3.5 to develop a head and neck tumour. Cytogenetic studies, measuring the bleomycin-induced damage in cultured lymphocytes, support the existence of a constitutional risk factor for head and neck carcinogenesis (Schantz *et al.*, 1990; Cloos *et al.*, 1994). Moreover, in a retrospective study it was shown that bleomycin-induced chromosomal damage was significantly highest in HNSCC patients, who ultimately developed a SPT (Cloos *et al.*, 1994). With this background an epidemiological case-control study was performed within the framework of this thesis to demonstrate a genetic predisposition as a potential risk factor for the development of a SPT. This study is reported in *chapter 2*.

1.3 Selection of tissue (mucosa) markers to monitor head and neck squamous cell carcinoma evolution

Invasive squamous cell carcinoma is a result of a series of consecutive genetic and epigenetic events of which some do appear phenotypically as dysplasia. From morphological studies it has become clear that squamous cell carcinogenesis is a multistep process (Nees *et al*, 1993; Voravud

et al, 1993; Shin *et al*, 1994a). Tumour initiation starts with somatic mutations (Harris *et al*, 1991), causing an altered expression of specific genes involved in proliferation and differentiation of squamous epithelium. Assuming that head and neck tumorigenesis is a multistep process of transformation from normal tissue to malignant lesions, several groups (Nees *et al.*, 1993; Voravud *et al.*, 1993; Shin *et al.*, 1994a) are currently involved in the elucidation of these steps and the associated genetic and/ or phenotypic alterations. Comparing squamous cell carcinoma or premalignant tissues with normal squamous epithelium, identified alterations might be good candidates for biomarkers to disclose early carcinogenesis and to monitor chemoprevention trials. In general, genomic markers, proliferation and differentiation markers, and markers of detoxifying processes are potentially all good candidates for clinical markers. However, potential biomarkers have first to be validated in clinical trials, because a suitable biomarker has to meet several criteria. Such a marker should be detectable in tissue samples that can be easily and repeatedly obtained in a non-invasive way (see table II). For HNSCC patients it should be possible to obtain tissue samples from several sites of the aerodigestive tract to evaluate whether altered marker expression is a local or regional phenomenon. Repeated samples are needed when this screening method is used in prospective and follow-up studies. Biopsy samples are too harmful to fulfil these demands, but exfoliated cytology, using a non-invasive cytobrush, can provide repeated sampling in one individual from many sites of the aerodigestive tract without discomfort for the patient.

Potential markers, tested in the study under consideration, are selected on the basis of the forementioned multistage carcinogenesis concept. In epithelial lining tissues, like the mucosa of the head and neck, the first step in carcinogenesis might be generated by the covalent binding of exogenous electrophilic xenobiotics to the DNA. More and more evidence exists that glutathione S-transferases (GST) are important enzymes, concerned in detoxification processes of potential carcinogenic compounds, including electrophilic agents, which are potentially harmful to the DNA. To exert their ultimate carcinogenic effect many carcinogens require metabolic activation. Therefore, interindividual variation in carcinogen metabolism by activation and detoxification pathways are important determinants in cancer susceptibility (Harris, 1989). Absence of GSTM1 in 40-50% of the population, a GST-μ isoenzyme may be concerned in tobacco-smoke metabolism, has been thought to be such a risk factor in head and neck carcinogenesis (Seidegard *et al.*, 1988). Moreover, besides risk assessment by determining GSTM1 presence, up-regulated GST-subtypes, like GST-π and GST-α, could contribute to better risk assessment in HNSCC. Especially GST-π has already been identified as a tumour marker in many tumours (Tsuchida *et al.*, 1992). *Chapter 3* of this thesis outlines the current knowledge of glutathione S-transferases in HNSCC in a review. Moreover, in *chapter 4* the value of GST-μ, GST-π and GST-α as potential early markers of

Table II.: Detectability of markers by immunohistochemical studies in tissue blocks and exfoliated cytology, discussed in this thesis for monitoring cancer evolution.

Marker	Tissue blocks		Exfoliated cytology	Discussed in chapter of this thesis
	normal tissue	squamous cell carcinoma		
p53	-	+	-	*6*
Glutathione S-transferase π	+	+	+	*3, 4*
Glutathione S-transferase μ	+	+	+	*3, 4*
Glutathione S-transferase α	+	+	+	*3, 4*
Cytokeratin 16	+	+	+	*5*
Cytokeratin 19	+	+	+	*5*
Histo-blood group H type 2-chain antigen	+	+	+	*5*

carcinogenesis in the mucosa at risk of the head and neck, in both biopsy samples and exfoliated cells, is discussed.

During malignant transformation the expression of proteins concerning the cellular proliferation and differentiation processes can be modulated. Among the changes that occur in squamous epithelium as it progresses towards malignancy are changes in the expression of the various cytokeratins. These epithelium specific proteins are part of the cytoskeleton and belong to the class of intermediate filaments. The cytokeratin gene family consists of at least 20 different polypeptides, which can be divided into two subfamilies: the acidic or type 1 family and the neutral-basic or type 2 family. They are classified with a number according to their molecular weight and isoelectric point as described by Moll *et al.*, 1982. Epithelial cells express a set of keratins and the pattern is dependent on the site of the tissue and the presence of pathological conditions, such as inflammation and neoplastic transformation (Morgan *et al.*, 1987). Cytokeratin 16 is known to be associated with hyperproliferation in keratinocyte pathology (Van Der Velden *et al.*, 1993). This marker of fast cell turnover is predominantly expressed in the suprabasal layers of epithelial tissue. Cytokeratin 19 is expressed as a minor component in the basal layer of normal stratified epithelia. In dysplastic as well as in squamous cell carcinomas its expression is increased and is extended to the more superficial cell layers. Moreover, Copper *et al.* (1993) showed a more than three fold increased cytokeratin 19 expression in the healthy oral mucosa of HNSCC patients when compared to control individuals.

Tumorigenic transformation can be associated with incomplete synthesis of carbohydrates, deletion of complex structures and the accumulation of precursors (Hakomori, 1989). Carbohydrates of the epithelial cell membrane are of interest because of their functional role in cell-to-cell interactions and their role in regulating the cell cycle and replication. Among these oligosaccharides are blood group antigens, in particular these associated with the ABO blood group phenotypes. A core chain of four sugars attached to a lipid or a protein is the basis of this ABO system. Addition of a fucose residue to the terminal galactose results in the H antigen, which defines the group O phenotype. the antigens defining blood groups A and B are formed by the addition of terminal galactose residues to the sugar back bone. On epithelial and endothelial cells the H antigen is widely distributed as detected with an antibody directed against the H2 isomeric form of H (Bryne *et al.*, 1991). Detection of this H2 antigen is dependent of the blood group antigen, probably because the other antigens (A and/or B) are not formed in endothelial cells. Bryne *et al.* (1991) reported that the expression of this antigen, by monoclonal antibody research, in the most invasive parts of oral cavity tumours correlates with clinical behaviour. Interestingly, leukoplakias without histo-blood group type 2-chain ABH antigen expression, and showing no dysplasia according to conventional criteria, later developed into carcinoma (Auclair, 1984).

The value of cytokeratins and histo-blood group H type 2-chain antigen as potential early markers of carcinogenesis in exfoliated cells is discussed in *chapter 5*.

A limitation of exfoliative cytology is that the cell scrapes contain, in our hands, most times only living and differentiated non-living cells and no undifferentiated basal cells. Therefore, markers which may be potentially suitable in biopsies, like the p53 protein, may appear without value when analysing exfoliated cells. p53 is thought to be a valuable marker according to the hypothesis that carcinogen induced genetic alterations are the driving force in susceptible cells. When, eventually, cells have gained a selective growth advantage and have undergone clonal expansion, the life-threatening malignancy can be formed (Harris, 1991). Changes in mutations, like duplications and deletions of oncogenes and tumour suppressor genes are probably essential for this process, in which one of the most common changes at the gene level known so far is the p53 aberration (Hollstein *et al*, 1991). Mutations of the p53 tumour suppressor gene have been frequently reported in HNSCC, but the stage of carcinogenesis at which p53 mutation occurs is still contradictory, even though recent reports mention p53 mutations as an early event in carcinogenesis (Dolcetti *et al.*, 1992; Shin *et al.*, 1994b; Gallo *et al.*, 1995). The finding of Salam *et al* (1995) that p53 mutations were in the same range in patients with late TNM stages as in those with early TNM stages support this hypothesis. To evaluate the possible value of the expression of

p53 as a potential early marker for second primary head and neck tumours an antibody mediated study on mucosa biopsies has been carried out. This study is reported in *chapter 6*.

1.4 Circulating tumour markers in head and neck squamous cell carcinoma

In contrast to some other cancers for HNSCC, until now, no single useful circulating tumour marker is known. The development of a reliable circulating marker of neoplasia could assist in the evaluation of patients, by potentially expediating the detection of occult SPTs and could be used in monitoring response to therapy. The ideal tumour marker should be a sensitive indicator of disease such that a high percentage of patients would have serum levels above the established normal. It must also be specific in that normal controls will not have levels above the accepted standard. Serum concentrations should correlate with tumour burden and clinical outcome. They should return to normal with successful therapy and reappear prior to clinical recurrence. Unfortunately, such "ideal" tumour marker has not been established for HNSCC. However, there are several markers which have been found to be worthy of study in HNSCC, as recently reviewed by Rassekh *et al.* (1994).

Recently it has been suggested in laryngeal carcinoma patients, that serial analysis of serum cathepsin B-like activity may have clinical value in early warning of tumour recurrence and response to treatment (Krecicki *et al*, 1992). Numerous studies suggest that increased cysteine proteinase activities of the cathepsin B-like type are involved in the process of the invasion of a primary tumour in normal tissue. There are some papers dealing with this proteinase activity in serum as a useful tumour marker in breast and colorectal cancers (Recklies *et al*, 1980; Kolar *et al*, 1987; Hirano *et al*, 1993). However in HNSCC the preliminary speculative results have to be confirmed by more extensive studies. Such study is described in *chapter 7* of this thesis.

Another interesting serum tumour marker is called Cyfra 21-1, and measures a soluble fragment of cytokeratin 19. Since the development of monoclonal antibodies against cytokeratins, they are widely used for typing of malignant tissue. Until now little is known about the occurrence of soluble fragments of cytokeratins in serum. However, the new sandwich ELISA for the determination of soluble cytokeratin 19 fragments opens the way to measure fragments of cytokeratin 19 in the circulation. Referring to the recent results of Copper *et al.* (1993) about increased antibody dependent expression of cytokeratin 19 in the healthy mucosa (at risk) of HNSCC patients and to promising recent results with this serum assay in lung cancer patients (Pujol *et al.*, 1993; Stieber *et al.*, 1993), this assay looks of great interest for HNSCC patients. Therefore, we performed a limited study on the value of the expression of the soluble cytokeratin 19 fragment as a serum tumour marker in HNSCC patients, which is reported in *chapter 8*.

CHAPTER 2

The Relation between Cancer Incidence among Relatives and the Occurrence of Multiple Primary Carcinomas following Head and Neck Cancer

Vivian Bongers

Boudewijn J.M. Braakhuis

Hilde Tobi*

Herman Lubsen#

Gordon B. Snow

#*Dept. of Otolaryngology/ Head and Neck surgery, University Hospital Utrecht*

**Department of Epidemiology and Biostatistics, Free University, Amsterdam*

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ABSTRACT

Despite improvement in therapeutic modalities in head and neck squamous cell carcinoma (HNSCC) the overall survival rate has only marginally improved during the last decades. The occurrence of second primary tumours (SPT) in the respiratory and upper digestive tract is the main cause of treatment failure in early stage HNSCC. Identification of risk factors for the development of SPTs by epidemiological screening may lead to a better risk assessment in individual cases. Ninety-seven HNSCC patients, who ultimately developed a SPT (cases) and hundred HNSCC patients, who remained free of another carcinoma after treatment of the first for a period of minimally six years (controls) were interviewed about the incidence of respiratory and upper digestive tract carcinomas within parents and siblings (=brothers and sisters). All questioned patients were smokers.

Among the SPT positive patients 50 out of the 562 family members were reported to have suffered from cancer of the respiratory or upper digestive tract versus 16 out of the 629 family members of the SPT negative patients. All these 66 individuals were smokers. Statistical analysis (Stratified FisherÞs exact test) revealed a significant difference between cases and controls ($p=0.0001$). Neither age and sex of the patients, nor tumour stage influenced the occurrence of SPTs in this study. In conclusion, a positive family history of one or more relatives with respiratory and upper digestive tract cancer among patients with initial HNSCC was established as a risk factor (Odds Ratio 3.8) for the development of a SPT. These findings suggest that in addition to external carcinogens an intrinsic susceptibility determines the risk for the development of a SPT in HNSCC patients.

INTRODUCTION

Individual differences in susceptibility to chemically induced carcinomas are thought to be based on, at least in part, genetic variability in metabolic activation and detoxification of environmental procarcinogens (Harris, 1991; Nakachi *et al.*, 1993). For instance, tobacco and alcohol metabolites are identified as potential exogenous etiological factors in the induction and/ or progression of head and neck squamous cell carcinoma (HNSCC) (Talami *et al.*, 1990; Brugere *et al.*, 1986). A genetic predisposition could explain why only a few persons out of the many, who have been exposed to these external carcinogens develop HNSCC. In addition, the question arises why some HNSCC patients ultimately develop two or more primary malignant tumours in the respiratory and upper digestive tract, while others do not. This is an important question since second primary tumours (SPT) are known to be a major cause of treatment failure in patients cured for early stage HNSCC. The great majority of SPTs occur more than half a year after the first, so called index tumour and are designated metachronously. The constant annual rate is estimated to be nearly 3% (Jovanovic *et al.*, 1994). The ultimate incidence of second primary tumours varies from 10% to as high as 40%, depending on the site of the index tumour (Schwartz *et al.*, 1994). Second cancers in patients previously treated for HNSCC have enhanced morbidity and mortality, because of their appearance at notorious bad sites, like the lungs and the oesophagus, or at previously irradiated or operated areas. This clinical scenario has lead to the focus on early detection and (chemo)prevention strategies to reduce the morbidity and mortality rates. Taking into account, as has been said above, that SPTs occur with a constant rate over the years, intense follow-up should be continued for long periods, if not lifelong. One of the problems with such intense follow-up of cancer patients is the enormous work-load for a medical team. Passive prevention, leading to quit smoking and alcohol consumption has a doubtful contribution in the reduction of the risk on SPTs. Since HNSCC is in principal a disease of people in the sixth decade of their life, who have already been smoking and drinking for approximately thirty years, it will be clear that many injury has already been caused before prevention has been started (Day *et al.*, 1994). Chemoprevention by 13-cis retinoid acid has been demonstrated to be successful, but has a high amount of side-effects (Hong *et al.*, 1990). In this context it should be a logical approach to taylor the application of screening- and chemoprevention procedures to the estimated risk in each individual case. Until now, some insight has been gained which kinds of patients are at high risk to develop a SPT, but this knowledge is still fragmentary. A recent epidemiological case-control study supports that genetic predisposition is an important risk factor for head and neck carcinogenesis (Copper *et al.*, 1995). This study revealed that first degree relatives of a HNSCC patient have a relative risk of 3.5 to develop HNSCC (Copper *et al.*, 1995). Cytogenetic studies, measuring the bleomycin-induced damage in cultured lymphocytes, also support the existence of

constitutional risk factors for head and neck carcinogenesis (Schantz *et al.*, 1990; Cloos *et al.*, 1994). Moreover, sensitivity to bleomycin-induced chromosomal damage was significantly highest in HNSCC patients, who have ultimately developed a SPT (Cloos *et al.*, 1994). To optimize treatment strategies and reduce mortality rates in HNSCC patients, knowledge of events involved in cancer development are of great need to identify patients at high risk to develop a SPT. Therefore, the objective of the current study is to evaluate whether a positive family history for cancer of the respiratory and upper digestive tract is related to the development of a SPT.

PATIENTS AND METHODS

Study population: 97 HNSCC patients, who ultimately developed a SPT and 100 HNSCC patients, who remained free of another carcinoma after treatment for the first for a period of minimally six years were asked to collaborate in this study. All patients have had histopathologically proven squamous cell carcinomas. Patients from both study groups were interviewed during the follow-up period, when they consecutively visited our outpatient clinic. The median follow-up time was eight and six years from the diagnosis of the first carcinoma for the SPT negative and SPT positive group, respectively. The median time difference between the occurrence of the first and second primary tumor was two years. All patients were in good mental health , non-alcoholics and their families were intact. We classified SPTs according to the original criteria of Warren *et al.* (Warren *et al.*, 1932), as later modified by Hong *et al.* (Hong *et al.*, 1990). In short, a SPT has to be separate from the first by at least 2 cm of normal non-neoplastic mucosa. Each tumour has to be malignant at histologic examination. And, in any case, the possibility that the second one is a metastasis from the first has to be eliminated.

Data collection: A standard questionnaire was completed with the following data: year of birth, year of occurrence of the first tumour, smoking habits, and the incidence and site of cancer in parents and siblings (= brothers and sisters). Only parents and siblings were included in this study to reduce the possibility of recall-bias. For the relatives smoking was recorded as positive or negative, because of the unreliability of hetero-anamnestic counting of duration and intensity of smoking. Alcohol consumption was no part of the questionnaire, because of the unreliability of the reports by relatives.

Statistical analysis: Differences in occurrence of carcinomas in the respiratory and upper digestive tract in parents and siblings between cases and controls were tested for statistical significance using the Stratified version of the Fisherþs exact test. The Odds Ratio was used to measure the strength of association. For stratified data, the Mantel-Haenszel estimator was used. A 95% confidence interval was reported for the Odds Ratio.

RESULTS

The 197 patients, who were interviewed, reported 66 family members with cancer of the respiratory and upper digestive tract among parents and siblings. All these relatives with cancer of the respiratory and upper digestive tract were smokers. 50 (76%) of these patients were relatives of patients who developed a metachronous SPT, versus 16 (24%), who were relatives of patients, minimally six years free of another carcinoma after curative treatment for the first tumour. Stratification by family size was used to avoid bias by the number of family members. As shown in table 1, taking stratum into account a statistically highly significant difference in family related tumours between cases and controls existed (p=0.0001). Bias by smoking habits is minimal, since the percentages of smokers were nearly identical among the parents and siblings of cases and those of the controls. Bias by alcohol intake, the other established major risk factor for the development of HNSCC will also be minimal, since the majority of tumours in parents and siblings appeared in the lung (58%), and alcohol intake has not been established as a risk factor for lung cancer.

Table 1.: Frequency of cancer of the respiratory and upper digestive tract in parents and siblings, divided according to family size.

	2-3*		4		5		6-7		8-10		11 >	
	+ S	- S	+ S	- S	+ S	- S	+ S	- S	+ S	- S	+ S	- S
pos.	7	2	13	3	4	2	5	3	8	3	4	3
neg.	11	18	14	16	10	10	9	16	5	15	7	9

* *number of family members, + S= patients, who developed a second primary tumour. - S= patients, minimally six years free of carcinoma. pos.= number of families with one or more siblings affected by a respiratory or upper digestive tract carcinoma. neg.= number of families with none of the siblings affected by respiratory or upper digestive tract carcinoma.*

Taking stratum matching into account a highly significant difference in family related tumours between cases and controls existed (p= 0.0001, stratified Fisherþs exact test).

The Mantel-Haenszel estimate of the odds ratio for the development of a SPT in HNSCC patients with an affected parent or sibling was established at 3.8 with (95% confidence interval: 2.0-7.6). Among the 97 patients, who developed a SPT were 5 families with 2 affected relatives, whereas 1 family showed 5 lung or HNSCC cases out of the 7 siblings. Among the 100 patients, minimally six years free of another carcinoma after treatment for the first tumour only 1 family showed 2 affected relatives. However, the size of this study is too small to

calculate a cumulative risk factor for HNSCC when more than one family member is affected. The characteristics of the patients of both groups showed minor differences (table 2), but oral cavity carcinoma was more frequently represented among the SPT positive group, whereas laryngeal carcinoma was the preferential carcinoma in the other group.

Table 2.: Details of host and index tumour factors for patients with and without a second primary tumor.

Host factors primary		Patients with a SPT	Patients without a SPT
Total		97	100
Age at diagnosis of the index tumour	Mean	57	59
	< 60 years	60	53
	> 60 years	37	47
Sex	Male	72	82
	Female	25	18
% of smokers		100 %	100 %
Site of the index tumour	Larynx	39	71
	Oral cavity	58	29
T stage of the index tumour@	T 1	35	33
	T 2	31	28
	T 3	15	27
	T 4	16	12
N stage of the index tumour@	N 0	67	73
	N 1	20	22
	N 2	8	4
	N 3	2	1

SPT=second primary tumour; @=According to the T-N-M classification (Spiessl et al., 1992).

All 197 patients were smokers. The mean age of the patients, who developed a SPT was 57 ± 9 years, whereas the mean age of the patients, minimally six years free of disease was 59 ± 10 years. Therefore, these factors were not considered important confounders. The characteristics of the parents and siblings of the HNSCC patients with and without SPTs are reported in table 3. The increased frequency of squamous cell carcinoma among relatives of HNSCC patients with second primary tumours was observed both in parents and in siblings. A positive family history for respiratory or upper digestive tract carcinoma was less frequent in patients with laryngeal carcinoma than in patients with oral cavity carcinoma (26 versus 30%), but the differences are small. Relatives of this study population did not differ with respect to the other types of tumours. These were equally represented in both patient groups (table 3).

Table 3.: Characteristics of parents and siblings of head and neck squamous cell carcinoma patients with and without a second primary tumour.

	No.		% of ever smokers		No. of cancers of the RUDT		No. of cancers outside the RUDT	
	+ SPT	- SPT	+ SPT	- SPT	+ SPT	- SPT	+ SPT	- SPT
Total	562	629	71%	66%	50	16	45	44
Father	97	100	91%	89%	19	6	8	10
Mother	97	100	27%	31%	4	1	13	14
Brother	188	236	89%	82%	19	6	13	7
Sister	180	193	65%	51%	8	3	11	13

RUDT= respiratory and upper digestive tract. SPT= second primary tumour. + SPT: patients who ultimately developed a SPT (n=97). - SPT: patients, minimally six years free of carcinoma (n=100).

DISCUSSION

The development of SPTs is one of the major causes of treatment failure in patients suffering from HNSCC. Causative factors in HNSCC are undoubtedly multiple and complex. However, for strategies of early detection and chemoprevention to be successful, identification of high risk

individuals is essential. The work-load involved with the follow-up of patients treated for HNSCC, is to be accepted, as it has been clearly shown that further intensive long term follow-up may be of value for the detection and treatment of SPTs. Moreover, selecting patients who will benefit most from preventive agents justifies possible side-effects and prolonged administration of these agents.

Tobacco and alcohol consumption are known risk factors for the development of SPTs in the respiratory and upper digestive tract (Moore, 1971; Franco *et al.*, 1991, Day *et al.*, 1994). As for HNSCC, familial aggregation has been reported by Copper *et al.* (1995). The results of the present study, in addition, show that a positive family history is associated with the occurrence of a SPT. We estimated an Odds Ratio of 3.8 for the development of a SPT in patients with a parent or sibling, who suffered from carcinoma of the respiratory tract. Moreover, a strong family aggregation in the occurrence of multiple esophageal cancer has been found by a Japanese group (Morita *et al.*, 1994). To explain this increase in cancer risk two possibilities may be considered. First, a factor with a hereditary basis determines the cancer risk. The genetically controlled metabolism of tobacco (and perhaps alcohol) is a possible explanation why only a part of heavy smokers and drinkers develop HNSCC and especially only a part of the heavy smokers and drinkers develop a SPT. Variability in genetically determined detoxification pathways of procarcinogenic components of cigarette smoke by specific enzyme systems (eg. glutathione S-transferase μ and cytochrome p-450 enzymes) may be important in neoplastic transformation (Harris, 1991). Moreover, the results of an increased mutagen sensitivity, as recently shown for HNSCC and especially HNSCC patients who develop a SPT (Schantz *et al.*, 1990; Cloos *et al.*, 1994), could result from an inherited predisposition. A second explanation could be that the same risk habits such as smoking and drinking may be aggregated within the same family, leading to the observation of a close relationship between family history and the multiplicity of cancer. It was not possible to test this hypothesis in our study. The exact amount of tobacco and alcohol consumption was not recorded. An updated collection of these data had the risk to introduce recall bias. It must be emphasized that patients in both groups were all smokers (table 3) and the percentages of ever-smokers were nearly identical in the groups of relatives, with respect to parents and siblings. All individuals, that were interviewed were in good mental health and were non-alcoholics, so recall bias about family characteristics can be excluded.

In conclusion, our data indicate that the relative degree of risk in an individual to develop a SPT in the respiratory and upper digestive tract depends on an intrinsic factor. Combination of other identified risk factors like exposure to tobacco and alcohol, mutagen sensitivity and expression of specific enzyme systems in the mucosa of HNSCC patients (Bongers *et al.*, 1995c) could lead to further target opportunities for cancer prevention and control.

CHAPTER 3

The Role of Glutathione S-Transferases in Head and Neck Squamous Cell Carcinogenesis

A Review

Vivian Bongers

Gordon B. Snow

Boudewijn J.M. Braakhuis

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INTRODUCTION

Head and neck carcinogenesis is thought to be a multistep process (Voravud *et al.*, 1993; Shin *et al.*, 1994a). In first instance, carcinogen-induced genetic events are the driving force in susceptible cells, eventually, when cells have gained a selective growth advantage and have undergone clonal expansion, leading to the life-threatening malignancy (Harris, 1991). In epithelial lining tissues, like the mucosa of the head and neck, the first step in carcinogenesis is thought to be generated by the covalent binding of exogenous electrophilic xenobiotics to the DNA (Harris, 1991). More and more evidence exists that glutathione S-transferases (GST) are important enzymes, involved in detoxification processes of potential carcinogenic compounds, including electrophilic agents, which are potentially harmful to DNA.

Presently, the availability of antibodies directed against the many molecular forms of GSTs, molecular techniques, e.g. the polymerase chain reaction and specific enzyme activity measurements gives factual knowledge about this matter. In particular, antibody research is being investigated on their value in predicting cancer prognosis. In addition, serum GST levels are currently being tested as potential markers for cancer detection. With this background, we review recent progress in the knowledge of GSTs in relation to head and neck squamous cell carcinoma (HNSCC).

GLUTATHIONE

Glutathione (GSH) is the most ubiquitous and abundant intracellular thiol acting in concert with a wide spectrum of antioxidative agents, like vitamins C and E, and carotenoids as a universal protectant against electrophilic attacks. GSH plays a prominent part in antimutagenesis and anticarcinogenesis by donating a hydrogen atom to certain free radicals. Trapping of electrophiles are achieved by direct interaction with GSH or by enzymatic conjugation with GSTs (Mannervik *et al.*, 1988). GSH is synthesized within many cells from its constituent non-essential amino acids, glutamate, cysteine and glycine, which are synthesized by the body or obtained via the diet. Under conditions of GSH depletion the electrophilic load for DNA damage will increase. However, since GST isoenzymes catalyze GSH detoxification pathways, the geno- and cytotoxicity of electrophilic substrates is greatly reduced, even when GSH is scarcely available.

Tumour cells exhibit the (sometimes high) capacity to synthesize GSH, and exhibit elevated cellular GSH concentrations when compared to non-tumoral adjacent tissues (Meistner, 1994). The knowledge that notably drug- and radiation-resistant tumour cells exhibit high cellular GSH levels makes modulators of the GSH metabolism and GSH synthesis inhibitors of interest in increasing the efficacy of treatment resistant tumours. In summary, high cellular GSH levels could arrest effective cancer treatment by electrophilic drugs, but could, in a precancerous stage protect against tumour development.

Based on the knowledge that N-acetylcysteine has the ability to scavenge noxious free radicals by itself, and supports GSH biosynthesis, cancer chemoprevention research has directed its attention to the application of this antioxidant. At present, N-acetylcysteine is tested in the prospective Euroscan trial, a chemoprevention study in curatively treated patients with early stage oral, laryngeal and lung cancer. The aim of this trial is to prevent or delay the occurrence of second primary tumours (De Vries *et al.*, 1991). Recently, we showed in a relatively small group of patients, participating in the Euroscan trial, that treatment with N-acetylcysteine leads to increased thiol concentrations in blood plasma and erythrocytes (Bongers *et al.*, 1995b).

GLUTATHIONES-TRANSFERASES

GSTs are multifunctional intracellular, soluble or membrane-bound enzymes, which catalyze the conjugation of many electrophilic, hydrophobic toxins and carcinogens with the sulfur atom of the tripeptide GSH. The K_m values for most of the GST-catalyzed conjugations of alkylating agents with GSH are in the low micromolar range. Therefore these enzymes are still very good catalysts under conditions of considerable GSH depletion. Besides this function, they also act as binding proteins in various detoxification processes (Mannervik *et al.*, 1988). Removal of these xenobiotic GSH-conjugates from the interior of the cell into vesicles have recently been shown by studies concerning multiple drug resistance proteins to occur by energy dependent membrane transporters. The precise identity of these pumps is as yet unknown, but membrane proteins concerned with the efflux of GSH-conjugates are being identified (Awasthi *et al.*, 1994). Export from the cell of GSH-conjugates is sometimes, and only in some organs (e.g. the kidney), preceded by degradation along the mercapturic pathway, although this is not necessary for adequate excretion.

The cytosolic GSTs form a multigene family of dimeric proteins divided into four classes, on the basis of distinctive isoelectric focusing properties, amino acid sequence and immunoreactivity (Chasseaud, 1979; Meyer *et al.*, 1991). Although separate classes have been identified, they share homology and probably have evolved from a common ancestral gene. Separate classes exists of the multigene α, μ, π and θ classes. Class α are basic, class π acidic and class μ near neutral proteins (Mannervik *et al.*, 1992). Until now, most attention has been focused on the cytosolic GSTs, and less is known about the function of the membrane-bound GSTs. Although little structural homology between membrane-bound and cytosolic GSTs exists, any common functionality is not precluded (Chasseaud, 1979). A known function of the membrane-bound GSTs is the protection of membranes against lipid peroxidation by their glutathione peroxidase activity of hydroperoxides, thus protecting cells from oxidative stress (Mannervik, 1985). GSTs detoxifies not only exogenous metabolites, but also endogenous chemical compounds such as bilirubin, polycyclic aromatic hydrocarbons, and metabolites of arachidonic acid by direct binding (Chang *et al.*, 1987; Mosialou *et al.*, 1993).

Most of the exploratory work has been done in the rat, but as more has become known about human GST, it is apparent that much information obtained from the rat is relevant to man. The same multigene families are seen and there is considerable homology in primary structure across the two species (Ketterer, 1988). Whithin a species, homology between classes is 25-30%, and that between members of the same class is 75-95%. GSTs have catalytic activity either as homo- or as heteromers, which can be formed between the subunits belonging to the same subclass. Each subunit of a dimer exhibits catalytic activity independently of the other, possessing a hydrophobic binding site for electrophilic substrates (H-site) and a binding site for GSH (G-site). The H-site is presumed to be specific for each subunit, whereas the G-site may be similar for all GSTs (Adang *et al.*, 1990). Xenobiotics, including carcinogens and anticancer drugs can influence the transcriptional regulation of GST genes. Enhanced expression of GSTs has been found in many tumours. Discrepancy between GST-expression, as measured at the protein level and RNA level and enzyme activity studies has to our knowledge, until now not been described. These results suggest that increased enzyme expression correlates with increased detoxification potential. However, the anticipated role of GSTs in cancer prevention provides potential overlap with anticancer drug resistance, since toxic chemicals with electrophilic centers are used in cancer chemotherapy (Tew, 1994).

GLUTATHIONES-TRANSFERASEPI

GST-π appears to be the most ubiquitous of the human GST isoenzymes. The regulatory mechanism involved in the expression of GST-π, isolated from both rat (referred to as GST-P) and human placentas is of particular interest in carcinogenesis associated processes. It is present in many tissues with the highest concentrations in cells of the alimentary tract, lung, and erythrocytes (Tsuchida *et al.*, 1989; Moscow *et al.*, 1989). The expression of GST-π is not only tissue specific, but depends also on the stage of development. GST-π is inducible and frequently overexpressed in a wide variety of human (pre)neoplastic tissues when compared to their normal counterparts (Chasseaud, 1979; see for colon cancer ref. of Kodate *et al.*, 1986; for renal carcinoma ref. of Di Ilio *et al.*, 1987; and for lung cancer ref. of Di Ilio *et al.*, 1988). Developmental changes in enzyme expression, which may be re-activated by induction mechanisms, make altered GST-π expression in carcinogenesis an interesting regulatory mechanism and possible tumour marker. Early studies revealed that GST-P in the rat increases 30- to 50-fold during chemical hepatocarcinogenesis (Satoh *et al.*, 1985). Furthermore, GST-P has been found to be a good and early tumour marker in the hamster buccal pouch mucosa model, appearing in carcinogen-induced preneoplastic and neoplastic lesions as positive foci (Zhang *et al.*, 1992).

Since GST-π is supposed to have a protective role in carcinogenesis, more and more evidence exists that this same iso-enzyme is involved in multiple drug resistance. There is

presently little direct evidence that any anticancer drug is a direct substrate for GST-π. The concern of GST-π in multiple drug resistance is most likely a consequence of a pleiotropic stress response (Tew, 1994).

Immunohistochemical staining, using antibodies directed against GST-π can successfully be applied to detect early precancerous states, dysplasia, or differentiated carcinomas. Indeed GST-π is a tumour marker for many tumours (Tsuchida *et al.*, 1992). GST-π is usually the most common of the GSTs in tumour tissues and, therefore, the easiest to detect experimentally. The predominance of GST-π in the literature reflects this practical fact. However, this does not preclude a major role for other GSTs. Qualitative and quantitative increased immunohistochemical GST-π staining has already been reported in moderate and severe dysplasia and carcinoma in situ of the oral cavity and the oesophagus when compared to normal tissue (see table I) (Zhang *et al.*, 1994). Also in "normal" tissue surrounding an oesophageal carcinoma elevated GST-π levels have been detected, reflecting possibly a (pre)cancerous field effect (Sasano *et al.*, 1993). GST-π expression has been reported to be increased and to be the predominant isoenzyme among three classes of GSTs in non small cell lung cancer tumour tissue (Howie *et al.*, 1990a). In HNSCC the higher activity of GST-π might have a genetic basis, because the GST-π gene is located on band q13 of chromosome 11, which is often amplified in this tumour type (Lammie *et al.*, 1991).

GST-π levels in serum are considered to be a useful aid for early diagnosis, predicting tumour extent and as a follow-up marker. Investigations of GST-π content in sera of 61 patients

Table I: Quantitative tissue distribution of glutathione S-transferase subclasses in squamous cell carcinomas of the respiratory and upper digestive tract in comparison with the corresponding healthy mucosa based on cellular protein expression as visualized by immunohistochemical studies.

Tissue	GST-class			References
	α	μ	π	
Oral cavity	?	=/-@	++	*1*
Larynx	=	=/-@	+	*2*
Oesophagus	++	+/-@	=	*3*
Lungs	++	=/-@	++	*4,5,6*

? to our best knowledge, not reported in the literature, = comparable amounts in tumour and healthy tissue, + moderate higher levels in tumours, ++ strongly elevated levels in tumour, - absent, @ 40-50% of the individuals express the M0 allele.

1=Zhang et al., 1994; 2=Janot et al., 1993; 3=Peters et al., 1993; 4=Howie et al., 1990a; 5=Nazar-Stewart et al., 1993; 6=Di Ilio et al., 1988.

with oral cancer revealed significant elevated serum levels in 50% of cases with stage I and II disease and in 70% of cases with stage III and IV disease (Hirata *et al.*, 1992). Furthermore elevated levels were also discovered in most patients with tumour relapse before recurrence was detected clinically (Hirata *et al.*, 1992). However, more studies with more patients have to be carried out to prove the definitive value of this marker. Also in oesophageal carcinoma elevated levels of GST-π have been shown to be a tumour marker (Hirata *et al.*, 1992) and in non small cell lung cancer GST-π alone (sensitivity 41.3%) or in combination with CEA (sensitivity 64.5%) appear to be a superior tumour marker over other tested markers (Hida *et al.*, 1994).

GLUTATHIONES-TRANSFERASEMU

To date, five members of the μ class of GST have been identified in human tissues, designed as GSTM1-5 (Warholm *et al.*, 1981; Pearson *et al.*, 1993). The μ form shows higher efficiency for conjugating epoxides such as benzo[*a*]-4,5-oxide and styrene-7,8-oxide and other aromatic hydrocarbon epoxides and diolepoxides than either α or π forms (Chasseaud, 1979; Ross *et al.*, 1993; Warholm *et al.*, 1983). Specific GSTM1 activity can be detected by measurements with the substrate *trans*-stilbene oxide (Seidegard *et al.*, 1988). GSTM1 displays different functional alleles, GSTM1*a and GSTM1*b, and GSTM1*0, a null allelotype, expressed by a significant number of people, who are lacking a constitutive expression of the isoenzyme. The numbers vary from 40-50%, depending on the race and the methodology used, namely measurements with *trans*-stilbene oxide or genotyping by using PCR (Seidegard *et al.*, 1988; Seidegard *et al.*, 1985). Carcinogenesis could be related to the insufficient capacity to detoxify tobacco related carcinogens, like benzo[*a*]pyrene, by a GSTM1*0 carrier. Indeed, several studies have indicated that individuals homozygous for this null allele, have an increased risk of smoking-related cancers, such as laryngeal (Lafuente *et al.*, 1993), lung (Van Poppel *et al.*, 1993) and bladder cancer (Lafuente *et al.*, 1993). Odds Ratio analysis indicates that smokers with this polymorphic variant have an approximately 2-fold higher risk of developing these cancers. Lack of expression of GST-μ is related to increased DNA damage by mutagens in smoke (Van Poppel, *et al.*, 1993). It has even been shown that increasing GST-μ activity was associated with decreasing risk in a dose-response pattern (Nazar-Stewart *et al.*, 1993). The gene loci for GSTM1 and GSTM2 are closely linked on chromosome 1p. It has been suggested that the GST1*0 allele has resulted from an unequal cross-over between these two loci (Warholm *et al.*, 1981). Genetic polymorphism has not been described for GSTM2 or for any of the other μ class GST enzymes. Correlations between genetic characteristics and elevated susceptibility for environmental toxicities are a promising way of identifying patients at high risk of developing cancer in future. Another useful approach of markers like GSTs is the identification of high risk groups of treatment failure, as has been recently shown in a study of 71 children with acute lymphoblastic leukaemia. They showed

inferior relapse-free survival when lymphoblasts were positive for GST-μ (Hall *et al.*, 1994). In such high risk groups more aggressive therapy with more side effects is justified.

GLUTATHIONES-TRANSFERASEALPHA

Originally, GST-α was called ligandin, because its first known function is the transport of bilirubin within the hepatocyte (Smith *et al.*, 1977). Cytosolic class α isoenzymes generally exhibit high peroxidase activity. Characteristic properties of the class α GSTs include the catalysis of the isomerization of ketosteroids and the selenium-independent glutathione peroxidase activity towards C19 and C21 Δ5-3-ketosteroids (Benson *et al.*, 1976).

Class α consists of two different monomers, encoded by two different genes on chromosome 6, being identified as B1 and B2 (Hayes *et al.*, 1989). Developmental and age-dependent changes in the amounts of these isoenzymes have been observed (Monks *et al.*, 1990). In certain pathological conditions an increase of GST-α has been shown. The association of drug resistance between GST-α and nitrogen mustards has been described (Tew, 1994). In contrast, various tumour types exhibited a dramatic decrease in the level of GST-α enzymes (IARC, 1992).

GLUTATHIONES-TRANSFERASETHETA

GST-θ has been identified as a GST enzyme concerned in the detoxification of monohalomethanes, dichloromethane and ethylene oxide. Approximately 60-70% of the human population is able to carry out the conjugation reaction of monohalothanes with GSH. The others show a null phenotype, and are unable to execute this conjugation reaction of naturally occurring halothanes. The GSTM1 locus has attracted interest because of its absence in approximately 50% of the population and the observed elevated lung cancer risk. However, a positive GST-θ conjugator status is not necessarily beneficial because conjugation of monohalomethanes and ethylene oxide is detoxifying whereas conjugation of dichloromethane yields a mutagenic metabolite (Van Bladeren *et al.*, 1991). Dichloromethane metabolites can induce liver and lung tumours in mice and therefore GST-θ polymorphism is thought to be associated with a greater risk of cancer (IARC, 1992).

GLUTATHIONES-TRANSFERASESAND CARCINOGENESIS

It is conceivable that the increase of GST in (pre)neoplastic tissue of the head and neck is a response to ongoing carcinogenic damage during the multistep carcinogenic process. However, the crucial significance of elevated GST expression in preneoplastic tissues is not known until now. Increase in GST activity may be a response to exposure to noxious, potentially carcinogenic compounds. According to this hypothesis, the progression of preneoplastic alterations into more advanced neoplastic lesions could be prevented by elevated GST levels. However, the opposite

may also be true. It has been described that GSTs can convert substrates via conjugation with GSH, into either cytotoxic, genotoxic or mutagenic metabolites (Monks *et al.*, 1990). Another theory, also overshadowing the benefit of elevated GST tissue levels is the nonsubstrate ligand binding theory (Van Bladeren *et al.*, 1991). Usually the binding of substrates to GSTs ends in detoxification. However, a non-competitive inhibitor binds to a site distinct to the binding of GSH and the electrophilic second substrate and diminishes the rate of catalysis. At that time the turnover number of the enzyme decreases, while the proportion of enzyme molecules that have a bound substrate does not (Van Bladeren *et al.*, 1991). On the other hand, binding of substrates to a GST-site distinct of the GSH binding site can even enhance the GST-enzyme activity (Lippman *et al.*, 1989). Such findings need confirmation in additional experiments, comparing cellular GST expression patterns with its corresponding activity levels.

An increase of GST enzymes in serum of cancer patients, as has also been described for HNSCC patients, can either be a result of the release by cancer cells or an acute-phase response of the host against cancer cell proliferation. An observation that elevated serum GST-π levels revert to the normal range after surgical removal of the tumour, is suggestive that the enzymes were produced by the cancer cells (Hirata *et al.*, 1992). In general, whatever the mechanism underlying elevated serum GST levels might be, when GST serum levels follow the clinical outcome, return to normal with successful therapy and re-appear prior to clinical recurrence, they might be of value in clinical cancer care. The lack of tumour and organ specificity makes the determination of serum GSTs unsuitable as a screening tool for primary respiratory and upper digestive tract cancer detection, but could be of great value in monitoring therapy efficiency and the discovery of recurrent disease in a very early stage. Also its application in the prediction of the development of second primary tumours in the respiratory and upper digestive tract, another major cause of treatment failure in early stage HNSCC (Lippman *et al.*, 1989), could be of great value. However, to establish the applicability of GSTs as tumour markers a large prospective trial has to be conducted.

Other non-invasive screening methods for early cancer detection consist of the analysis of broncho-alveolar lavage fluid and cytology specimens obtained by brushing the mucosa. Both screening methods might be of value for especially early detection of second primary tumours. Broncho-alveolar lavage fluids are easy obtainable during bronchoscopy, but bronchoscopies are quite incriminating. Radio-immuno assays for four subfractions of GST (π, α (B1 and B2) and μ) on broncho-alveolar lavage fluid showed statistically significant elevated monomer fractions of the α class GST (GST-B1 and B2) in the lavage fluid. Elevation of GST-B1 and GST-B2 levels in the lavage fluid were found before a tumour was visualized or detected by cytology (Howie *et al.*, 1990). Therefore, these parameters could be of value in the early diagnosis of lung cancer. In

summary, extensive validation of this technique might lead to a new modality to detect and monitor cancer.

A non-invasive screening procedure executed in our own laboratory concerns the application of GST antibodies on exfoliated cytology (Copper *et al.*, 1993; Bongers *et al.*, 1995a). This non-invasive technique can easily be repeated during follow-up. The great need for such screening methods in long term cancer follow-up, screening for second primary tumours at an early stage and monitoring of chemoprevention drugs makes exfoliated cytology an attractive approach. Therefore, we tested GST antibodies on cell scrapes of six different sites of the upper aerodigestive tract from HNSCC patients and comparable non-cancer individuals. We observed a significantly higher expression of GST-π and GST-μ in cell scrapes of the healthy mucosa of HNSCC patients at high risk to develop a second primary tumour (Bongers *et al.*, 1995c). For GST-α we found a more heterogenous expression pattern in exfoliated cells and a not significant higher expression in patients (Bongers *et al.*, 1995c). Moreover, these findings support the field cancerization theory, like the findings of Janot *et al.*, who found that tumours and adjacent mucosa of HNSCC patients had similar enhanced GST-levels and activity (Janot *et al.*, 1993). However, further research must be carried out before the value of GST expression in exfoliated cells as a predictive biomarker for early second primary tumour detection in clinical follow-up studies and during chemoprevention trials can be assessed.

CONCLUSIONS

Smoking and alcohol consumption are well known risk factors for the development of HNSCC (Brugere *et al.*, 1986). However, the fact that only a proportion of smokers develop cancer suggests that interindividual differences exist in dealing with carcinogens. The ultimate amount of carcinogen-induced damage results from activation, detoxification and DNA repair pathways. These intrinsic factors are now thought to have a genetic basis, that may underlie differences in cancer predisposition. Recently, epidemiologic data have provided that a genetic predisposition to HNSCC exists (Copper *et al.*, 1995). Also the fact that HNSCC patients have an intrinsic relatively low ability to deal with bleomycin-induced damage supports the concept of cancer predisposition (Cloos *et al.*, 1994). Most evidence for genetic predisposition for cancer is found at the level of xenobiotic metabolism since genetic polymorphism has not only been described for GSTs, but also for certain P-450 subtypes, e.g. CYP1A1. The combination of a certain cytochrome P-450 enzyme genotype, which activates environmental carcinogens to their electrophilic intermediates, and GSTM1 deficiency, which detoxifies reactive metabolites, has been associated with a synergistically increased risk for squamous cell lung carcinoma (Nakachi *et al.*, 1993). This correlation has been demonstrated for lung carcinoma (Nakachi *et al.*, 1993) and looks

also to be of value in HNSCC with respect to the corresponding etiological carcinogenic processes for lung and HNSCC. The need for complementary research in such interacting metabolising pathways still exists. A better understanding of the interaction of known single risk factors could contribute to better individual risk assessment. Besides risk assessment by determining GSTM1 presence, up-regulated GST-subtypes in HNSCC could be of additional help in early detection. Especially GST-π looks to be of value as a tumour serum marker or as a tumour marker in exfoliated cells.

In conclusion the findings of GST research have led to a better understanding of the many events involved in head and neck carcinogenesis. On the basis of the present results further studies to assess the practical value of GSTs as markers for the early detection and/ or efficacy of chemopreventative agents of second primary tumours in HNSCC patients are warranted.

CHAPTER 4

Second Primary Head and Neck Squamous Cell Carcinoma Predicted by the Glutathione S-transferase Expression in Healthy Tissue in the Direct Vicinity of the First Tumour

Vivian Bongers

Gordon B. Snow

Nico de Vries

Alex R. Cattan*

Andy G. Hall*

Isaac van der Waal#

Boudewijn J.M Braakhuis

*Department of Hematology, University of Newcastle upon Tyne, The Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne NE1 4LP, United Kingdom

#Department of Maxillofacial Surgery and Oral Pathology, Free University Hospital

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ABSTRACT

BACKGROUND: Glutathione S-transferases (GSTs) are known to play a role in the detoxification of carcinogens. Individual isoenzymes of the α-, μ- and π-class vary in substrate specificities, tissue distribution, and activities among individuals. GST-π expression has been shown to be increased in preneoplastic and neoplastic lesions. GST-μ is known to play a role in detoxification of epoxides released from cigarette smoke, and individuals with low GST-μ activity have a relatively high risk to develop smoking-related lung and laryngeal cancer. The occurrence of a second primary tumour (SPT) in the whole respiratory and upper aerodigestive tract is an important factor for mortality in head and neck squamous cell carcinoma (HNSCC), and, at present, there are no markers that are available to predict which patient has increased chances in developing an SPT. Risk-assessment by use of biomarkers, particularly the ones that can be obtained with non-invasive techniques, are of great value in predicting prognosis and hence possible more aggressive treatment and follow-up in selected patient groups.

EXPERIMENTAL DESIGN: In a nested case control study, 20 patients who had previous history of oral cancer were used; 10 of the 20 had developed an SPT while the other 10 patients were minimally 7 years free of disease. The expression of GST-π, GST-μ and GST-α was immunohistochemically analyzed using apparently normal oral mucosa, free of tumour or dysplasia, obtained from the resection edges around the primary tumour. In another experiment, the three GST isoenzymes were immunohistochemically analyzed using exfoliated cells, obtained non-invasively from several sites of the upper aerodigestive tract of the apparently normal-looking mucosa of HNSCC patients ($n=25$) and of matched control individuals ($n=10$).

RESULTS: The expression of all GST was significantly higher ($p<0.001$) in the suprabasal and superficial layers of the mucosa at risk. Also in cell scrapes of clinically healthy mucosa of HNSCC patients, we observed a significantly higher expression ($p<0.001$) of GST-π and GST-μ compared with their controls. For GST-α, we observed a more heterogenous expression pattern in these exfoliated cells.

CONCLUSIONS: Expression of GST-π, -μ and -α in normal tissue in the direct vicinity of the first tumour seems to have predictive value for the development of an SPT.

INTRODUCTION

Head and neck squamous cell carcinogenesis is generally accepted to be a multi-step process (Shin *et al.*, 1994; Voravud *et al.*, 1993), analogous to that shown for other tumours (Vogelstein *et al.*, 1988; Minna *et al.*, 1986). At the morphological level, several steps are described, i.e., hyperplasia, metaplasia, dysplasia, carcinoma *in situ*, and invasive carcinoma. Despite conventional screening studies for early detection, the overall survival of patients with head and neck squamous cell carcinoma (HNSCC) has not significantly improved during the last decade. The development of second primary tumours (SPT) in the upper aerodigestive tract and the lungs is the main cause of mortality for patients with early stage HNSCC (Lippman *et al.*, 1989). The general accepted explanation for this phenomenon is the field cancerization theory, first proposed by Slaughter *et al.* in 1953 (Slaughter *et al.*, 1953). This theory proposes that there is a predisposition of an entire field of tissue to the development of SPTs through repeated carcinogenic insults. The development of the cancer is a multi-step process (Voravud *et al.*, 1993) starting with a somatic mutation in the initiation stage. It is conceivable that detoxification enzymes, like glutathione S-transferases (GST), are of crucial importance in the early stages of carcinogenesis.

GSTs are multifunctional intracellular, soluble, or membrane-bound enzymes, which catalyse the conjugation of many electrophilic hydrophobic compounds with the tripeptide glutathione (GSH) and may also act as binding proteins in various detoxification processes (Mannervik *et al.*, 1988). Individual isoenzymes of the α-, μ- and π-class vary in substrate specificities, tissue distribution, and activities among individuals (Mannervik, 1985). A particular interest in the π class enzymes originated from studies demonstrating increased immunohistochemical expression of GST-π in preneoplastic and neoplastic lesions in various human tissues, including the oral cavity, the oesophagus, and the lungs (Zhang *et al.*, 1994; Tsuchida *et al.*, 1989; Di Ilio *et al.*, 1988). In patients with oral cancer, GST-π has been reported to be a useful serological marker for early diagnosis of the primary tumour and recurrent disease (Hirata *et al.*, 1992). GST-μ is an isoenzyme with a marked specificity for catalysing the conjugation of epoxides, such as benzo[*a*]-4,5-oxide and sterene-7,8-oxide, carcinogenic components in cigarette smoke (Warholm *et al.*, 1983). Expression of GST-μ is inherited as an autosomal dominant trait. An increased risk of lung cancer (Seidegard *et al.*, 1886) and about a 2-fold higher risk of larynx cancer (Lafuente *et al.*, 1993) has been previously shown among smokers who phenotypically lack (<10 ng/ml blood) the GST-μ isoenzyme.

The first objective of this study was to determine the value of GST-enzymes as markers for the prediction of SPTs using archival tissue specimens. The second aim of our study was to investigate if these enzymes can be used as markers in exfoliated cell samples. The non-invasiveness of this method makes repeated long term follow-up sampling possible, which is an important issue in cancer screening and prevention.

EXPERIMENTAL DESIGN

Comparison of GST enzymes in HNSCC patients with and without SPTs was performed in a nested case control study on normal tissue, surrounding the index tumour. The expression of GST-π, GST-μ and GST-α was analyzed immunohistochemically and compared between HNSCC patients who subsequently developed a SPT (n=10) and HNSCC patients who were minimally 7 years free of disease (n=10). The 10 patients, who developed a SPT were selected from our file of all carcinoma patients, who were admitted to our hospital since 1987. Next, 10 suitable controls, who were minimally seven years free of disease after eradication of the first tumour, were also selected. Cases and controls were matched for age, gender, alcohol, and tobacco habits (table I). The expression of GST-π, GST-μ and GST-α was analyzed in exfoliated cell samples obtained from the mucosa of 25 other HNSCC patients and 10 controls. In addition, biopsies from tumours of a subgroup of HNSCC patients and normal mucosa of healthy patients were analyzed as a reference. In a subgroup of patients the GST expression in tumours was compared with that found in exfoliated cells.

METHODS

Patients

Normal tissue, surrounding the index tumour, free of carcinoma and dysplasia from patients with an oral cavity tumour, who were minimally 7 years free of tumour were compared to the same tissue from people who developed a SPT during follow-up. Patient and tumour characteristics are shown in table I. The definition of SPTs is according to the international criteria, as defined by Hong *et al.*, 1990. The slides were assessed for dysplasia by one of us (I van der Waal, oral pathologist). Tongue tissue of healthy controls was obtained during post-mortem examination within 6 hours after death. Biopsies of the lateral border of the tongue were taken. The tissue blocks were formalin fixed and paraffin embedded. Paraffin sections (4 μM) were deparaffinized and rehydrated before immunohistochemistry was performed.

Cytological preparations were obtained from 25 patients with HNSCC, who underwent a diagnostic panendoscopy. Patients and tumour characteristics are shown in table IV. Exfoliated cells were obtained by scraping with a Cytobrush^R, (Medscand AB, Malmö, Sweden) from healthy appearing mucosa of the oesophagus (20 cm from the upper alveolar ridge), the vocal fold, the ventricular fold, the whole floor of mouth, the middle part of the buccal mucosa and the dorsal tongue. In tumour patients the scrapes were obtained from the contralateral site of the carcinoma.

Table I: Characteristics of patients and primary tumours from whom apperently normal tissue was studied for the expression of GSTs.

No.	Age	Gender	Characteristics of the primary tumour			SPT loc.[4]	Smoking[5]	Alcohol[6]
			Loc.[1]	pTNM[2]	Diff.[3]			
1	51	M	fom.	pT2N0M0	p/m	-	+	+
2	61	M	fom.	pT3N1M0	m/w	-	+	+
3	64	F	fom.	pT1N0M0	w	-	+	+
4	45	F	tongue	pT1N0M0	m	-	-	+
5	65	M	tongue	pT2N0M0	w	-	+	+
6	55	M	fom.	pT3N1M0	m	-	+	+
7	60	M	fom.	pT2N2M0	w	-	+	+
8	55	M	fom.	pT2N2M0	m	-	+	+
9	71	F	fom.	pT1N0M0	m	-	+	+
10	59	M	fom.	pT3N1M0	m	-	+	+
11	71	M	fom.	pT1N0M0	w	lung	+	+
12	62	M	fom.	pT2N0M0	m/w	lung	+	+
13	55	F	tongue	pT3N0M0	m	pharynx	+	+
14	57	F	fom.	pT2N0M0	w	lung	+	+
15	38	M	tongue	pT3N2M0	w	lung	+	+
16	48	M	tongue	pT2N0M0	m/w	lung	+	-
17	42	M	fom.	pT1N0M0	p/m	tongue	+	-
18	59	M	fom.	pT3N2M0	p/m	lung	+	+
19	54	M	tongue	pT1N0M0	m	larynx	+	+
20	65	F	fom.	pT2N0M0	m	oesophagus	+	+

[1] *loc.=localisation, fom= floor of mouth*

[2] *pTNM=tumour classification, according to the new T-N-M classification (Spiessl et al., 1992)*

[3] *diff.=degree of differentiation: p=poorly, m=moderate, w=well*

[4] *SPT loc.=localisation of the second primary tumour (-=no SPT)*

[5] *Smok.=smoking habits, -= never smoked, += more than 10 cigarettes/day, for at least 20 years*

[5] *Alc.=alcohol habits, - no, + yes (more than five alcoholic beverages per day)*

The brushes were stirred in 3 ml phosphate buffered saline (PBS), allowing the cells to be released from the brush, washed with PBS, and cytological preparations were made on poly-L-lysine (Sigma, St.Louis, USA) coated slides by the cytocentrifuge (Shandon, Cheshire, England). The slides were air dried for 60 min, fixed with 100% methanol for 10 min, and stored at -70°C until use.

Tumour samples taken from a subgroup of 10 patients, from whom we sampled exfoliated cells were also immunohistochemically processed.

Immunohistochemistry

Expression of π, α, and μ class GSTs was assessed using the immunoperoxidase technique with an avidin biotin complex (Vectastain ABC-kit, Vector laboratories, Burlingame, CA). Polyclonal GST antisera (Novocastra laboratories, Newcastle upon Tyne, U.K.) were raised in rabbits (Cairns *et al.*, 1992; Hall *et al.*, 1990). Antisera specificity has been checked earlier, as reported by Cairns *et al.* (1992). The μ antibody was found to recognize both M1 and M2 and possibly M3 and M4. The antibodies were diluted in PBS with 1% bovine serum albumin (BSA, Sigma). Cytocentrifuge preparations were incubated with the primary antibodies for 60 min at room temperature. Non-specific staining was blocked with normal swine serum (DAKO, Glostrup, Denmark) prior to the first incubation with antibody and with endogenous peroxidase activity by 0.06% H_2O_2 in methanol, before the second antibody. After incubation with the first antibody, biotinylated affinity isolated swine immunoglobulins to rabbit immunoglobulins (DAKO) were added and the peroxidase label was developed with diaminobenzidine tetrahydrochloride (DAB, Sigma). Counterstaining was performed with haematoxylin (Merck, Darmstadt, Germany). The stained slides were finally mounted with "Kaiser's glycerine gelatine" (Merck). A negative control was made for each slide of each person, using PBS/ BSA 1% instead of the first antibody. Cytocentrifuge preparations from patients and controls were stained simultaneously. All samples were stained with the same batch of antibody. In each staining session a positive control was included.

Evaluation was performed by light microscopy on coded slides by two independent observers. For each coded tissue slide 100 cells per cell layer (basal, suprabasal and superficial cell layer) were counted in 2 separate fields. A three-point scale was used: ++, + and -. For statistical analysis we added all the scores for 200 cells, giving a value of 2 to a ++-cell and 1 for a +-cell. For exfoliated cells slides at least 200 cells were determined. There was a very clear-cut difference between positive and negative cells. The inter- and intra-observer variations were less than 10%. The data from patients and controls were compared, using Student's t test. A p value of less than 0.05 was considered to be significant. Possible correlations between age, gender, current smoking, and alcohol consumption with the immunohistochemical test results and in addition of the test results between different sites were determined by the Spearman's rank correlation test. To test the possible bias of smoking in the exfoliated cells experiments multiple linear regression was used.

RESULTS

GST EXPRESSION IN NORMAL MUCOSA IN ORAL CAVITY CARCINOMA PATIENTS WITH OR WITHOUT SPTS:

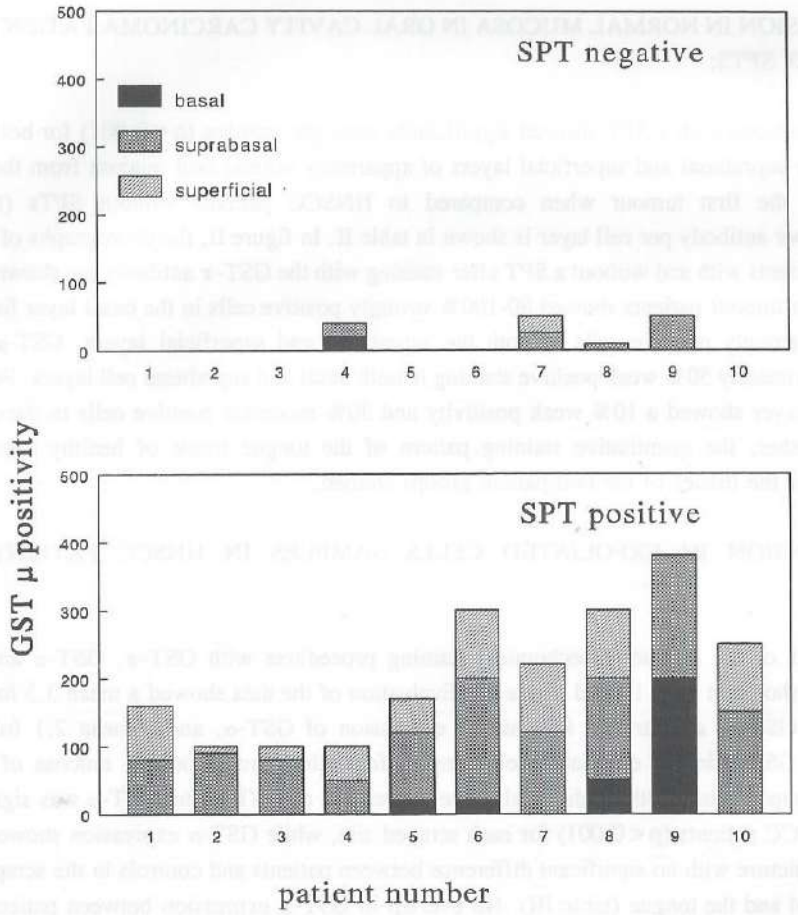
HNSCC patients with a SPT showed significantly stronger staining ($p<0.001$) for both GST-μ, π and α in the suprabasal and superficial layers of apparently normal oral mucosa from the surgical specimens of the first tumour when compared to HNSCC patients without SPTs (figure I). Specification per antibody per cell layer is shown in table II. In figure II, the photographs of resection edges from patients with and without a SPT after staining with the GST-π antibody are shown. Tongue biopsies of non-tumour patients showed 90-100% strongly positive cells in the basal layer for GST-π, and 50-90% strongly positive cells in both the suprabasal and superficial layers. GST-μ staining showed approximately 50% weak positive staining in both basal and suprabasal cell layers. For GST-α the basal cell layer showed a 10% weak positivity and 30% moderate positive cells in the other two layers. Altogether, the quantitative staining pattern of the tongue tissue of healthy controls was between that of the tissues of the two patient groups studied.

GST EXPRESSION IN EXFOLIATED CELLS SAMPLES IN HNSCC PATIENTS AND CONTROLS:

The results of the immunohistochemical staining procedures with GST-π, GST-α and GST-μ antibodies are shown in table III and figure III. Evaluation of the data showed a mean 3.3 fold higher expression of GST-π, a mean 1.5 fold higher expression of GST-α, and a mean 2.1 fold higher expression of GST-μ in the exfoliated cell samples from the normal-looking mucosa of HNSCC patients, as compared to healthy individuals. The expression of GST-π and GST-μ was significantly higher in HNSCC patients ($p<0.001$) for each scraped site, while GST-α expression showed a more heterogenous picture with no significant difference between patients and controls in the scrapes of the ventricular fold and the tongue (table III). No overlap in GST-π expression between patient scrapes and that of the control individuals exists. For all tested markers there was a considerable variation in expression between sites of a given patient (the standard deviation varied from 1.0 to 13.2 for GST-μ, from 2.7 to 12.1 for GST-π, and from 2.53 to 11.6 for GST-α). No statistical significant correlation between the level of expression of all GST-markers at all sites and age, gender, current and cumulative smoking, alcohol consumption, localization and stage of the index tumour was found ($p>0.05$, table IV). When corrected for smoking with multiple regression analysis, the significance level of the differences did not change.

To answer the question whether staining results of exfoliated cells correlate with that of the

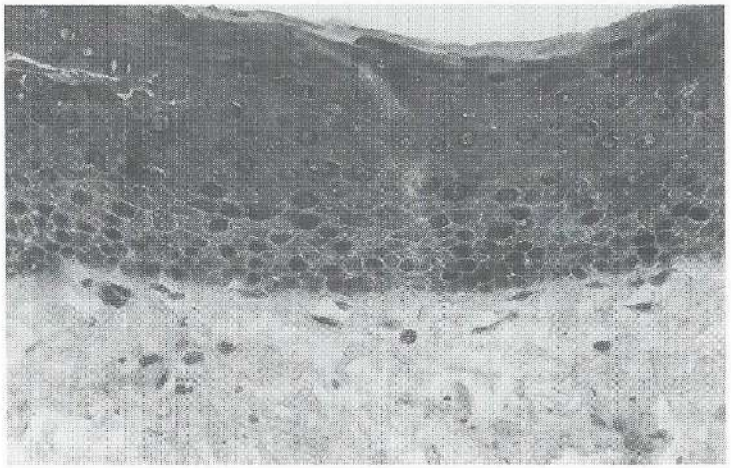
Figure I.:



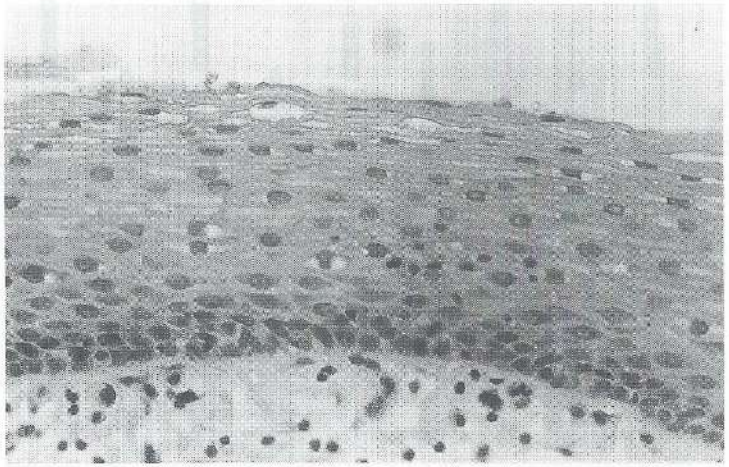
Immunohistochemical glutathione S-transferase-μ expression in normal mucosa, surrounding the index tumour, free of tumour or dysplasia. 10 patients minimally seven years free of tumour (considered as second primary tumour (SPT) negative) were compared to 10 patients who developed a SPT (considered as SPT positive). 100 cells were counted per layer. Positivity of a cell was scored semiquantitatively on a three-point scale: ++ positive cells got a factor 2, + positive cells got a factor 1 and negative cells were scored as 0.

Figure II.:

(a):

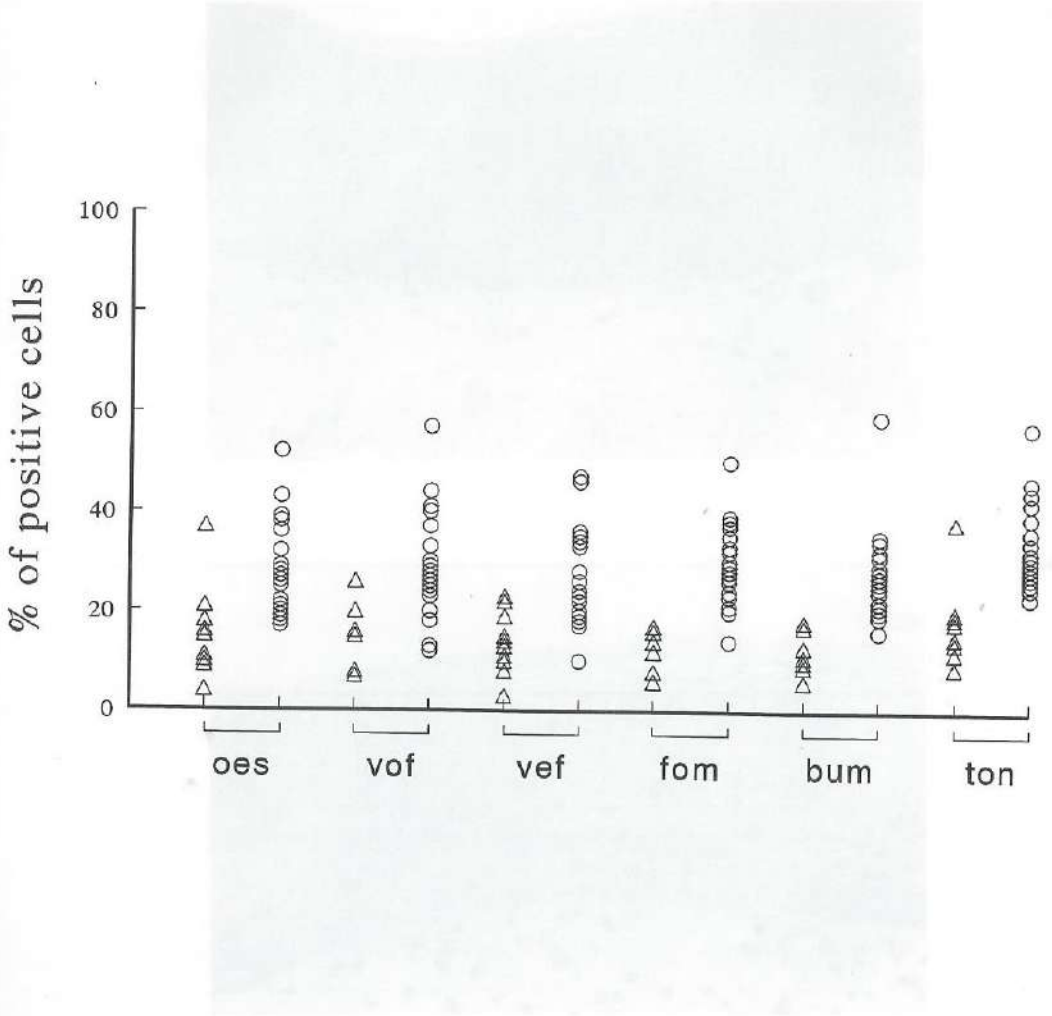


(b):



Glutathione S-transferase-π expression in a resection edge free from tumour or dysplasia from a patient who developed a SPT (a), and in a resection edge of a patient free of disease (b). There is strong positive staining in suprabasal and superficial cell layers from the patient who developed a SPT and lack of staining in the patient free of disease. Original magnification 400x.

Figure III.:



Glutathione S-transferase-μ expression in exfoliated cells of the mucosa of non-cancer patients (△) (n=10) and of the "normal" mucosa of head and neck squamous cell carcinoma patients (○) (n=25).
Abbreviations: oes: the oesophagus; vof: the vocal fold; vef: the ventricular fold; fom: the floor of mouth; bum: the buccal mucosa; ton: the tongue.

Table II: GST expression in apperently normal tissue, free of tumour or dysplasia, obtained during surgical resection of the index tumour.

GST	SPT-/+[1]	% positive cells[2]								
		Basal layer			Suprabasal layer			Superficial layer		
		++	+	-	++	+	-	++	+	-
π	- SPT	16 (± 28)	12 (± 21)	72 (± 33)	17 (± 21)	36 (± 32)	47 (± 31)	9 (± 17)	25 (± 38)	60 (± 35)
	+ SPT	37 (± 38)	5 (± 7)	58 (± 36)	75 (± 28)	24 (± 28)	1 (± 3)	55 (± 35)	12 (± 24)	33 (± 30)
μ	- SPT	0 (± 0)	2 (± 6)	98 (± 6)	0 (± 0)	10 (± 17)	90 (± 17)	0 (± 0)	2 (± 8)	98 (± 8)
	+ SPT	11 (± 31)	7 (± 16)	82 (± 31)	28 (± 34)	62 (± 28)	12 (± 16)	10 (± 21)	36 (± 36)	54 (± 30)
α	- SPT	0 (± 0)	0 (± 0)	100 (± 0)	1 (± 3)	4 (± 7)	95 (± 10)	0 (± 0)	3 (± 7)	96 (± 10)
	+ SPT	0 (± 0)	5 (± 16)	95 (± 16)	10 (± 9)	58 (± 19)	32 (± 22)	0 (± 0)	43 (± 39)	57 (± 39)

1 *SPT-/+: -: patients, who are minimally seven years free of disease (n=10), +: patients, who developed a second primary tumour (SPT) during follow-up (n=10).*

2 *++/+/- scores were performed semiquantitavely on a three point scale, means (± SD).*

Table III: GST expression in exfoliated cells

	GST-π		GST-α		GST-μ	
	Controls	Patients	Controls	Patients	Controls	Patients
Oesophagus	10 ± 2	32 ± 8	15 ± 11	30 ± 11	15 ± 9	29 ± 9
Vocal fold	8 ± 3	31 ± 8	18 ± 9	28 ± 9	13 ± 7	28 ± 10
Ventricular fold	9 ± 3	28 ± 7	21 ± 11	26 ± 9	14 ± 6	27 ± 10
Floor of mouth	9 ± 3	28 ± 6	18 ± 10	28 ± 9	12 ± 4	29 ± 8
Buccal mucosa	9 ± 3	28 ± 5	18 ± 10	29 ± 5	11 ± 4	27 ± 8
Tongue	10 ± 4	33 ± 7	19 ± 14	26 ± 5	17 ± 8	33 ± 8

The percentage of positive cells are given (means ± the standard deviation, Controls, n=10; Patients, n=25)

tumour, we extended our study as follows. 10 tumour biopsies, derived from the patients from whom we obtained exfoliated cells of healthy mucosa. All tumours showed positivity, in changing amounts, for the tested GST antibodies. Comparison of GST-expression in exfoliated cells of condemned mucosa and tumours (performed by the Spearman correlation test), belonging to the same patients showed only a significant correlation for GST-μ ($p<0.03$).

DISCUSSION

The most important question in HNSCC biomarker research projects is whether these markers are of value in predicting the development of a SPT during follow-up. Such high risk patients could then have more intense follow-up and possibly can receive adjuvant chemopreventive drugs. The present study showed that GST-isoenzyme expression in normal tissue of head and neck squamous cell carcinoma patients may be usable to predict the development of a SPT during follow-up. This study showed a significantly higher expression of all GST-enzymes in suprabasal and superficial cell layers of patients who developed a SPT. GST-μ was the most promising marker in this panel, followed by GST-π and, to a lesser extent, by GST-α. It is worth mentioning that in this study, archival tissue specimens of tumours with adjacent normal tissue were used. These specimens are available from every radical surgical resection and can be obtained without extra burden for the involved patients.

Since these antibodies provided positive results in superficial cell layers of tissue blocks, we investigated GST expression in exfoliated cells of healthy mucosa of head and neck squamous cell carcinoma patients. This non-invasive method has advantages over biopsies, especially in follow-up studies. The study results, comparing GST expression between patients and controls, show increased GST expression of the μ and π class in cancer patients. These results confirm Slaughter's field cancerization theory and are in agreement with earlier results, reporting increasing amounts of GST-π in successive precarcinogenic stages of oral squamous cell carcinoma in comparison to control specimens (Zhang *et al.*, 1994). The high local levels of GST-μ could be of crucial importance in protecting the epithelium against cytogenetic damage, especially those caused by smoke metabolic intermediates. However, the reason for these high GST levels in precancerous tissue is not yet clear. It has been often suggested that elevated levels of detoxification enzymes, like GSTs, would protect against carcinogenic attacks. The high GST levels in tissue of patients who develop a SPT challenge this hypothesis. It is possible that increased GST expression may reflect a futile response to carcinogenic metabolites or even that they may be involved in the formation of carcinogens, as described earlier by Monks *et al.*, 1990. Another proposed hypothesis, possibly explaining the ongoing carcinogenic process despite high GST levels, is the nonsubstrate ligand binding theory (Van Bladeren *et al.*, 1991). A non-competitive inhibitor binds simultaneously with GSH and the electrophilic

substrate to distinct binding sites of the GST molecule. Such as yet unidentified non-competitive inhibitor diminishes the rate of catalysis by decreasing the turnover rate of the enzyme, rather than by diminishing the proportion of enzyme molecules that have a bound substrate. Our results of a moderate expression in healthy tissue and high expression in healthy tissue of patients who eventually did develop an SPT is in agreement with this non-substrate binding theory. The relatively low GST expression in patients who did not develop an SPT remains an intriguing fact. For these patients the GST levels appeared to be too low to prevent the first tumour, but apparently high enough to avert a second one.

A direct comparison between histological and cytological findings obtained from tissues of healthy controls could not be made. First, the experiments were done in two patient sets and second, the biopsies originated from the lateral border, whereas the scrapes were obtained from the dorsum of the tongue.

The negative GST-μ phenotype has been reported to be a risk factor for laryngeal cancer (Hussey *et al.*, 1987). This can not be confirmed by our results in exfoliated cells with these polyclonal antibodies. Partial sequence identity between the different isoenzymes of the μ-class might explain this discrepancy. Amino acid sequences are > 60% identical within a class and about 30% identical among classes (Pickett *et al.*, 1989). Cross reaction of the antibody used here with other μ class isoforms is therefore possible. The real predictive value of GST expression in exfoliated cells can only be assessed in a prospective study, as we have already started in our laboratory.

In chemoprevention there is a great need for predictive biomarkers, especially in tissues obtained by a non-invasive way (as earlier reported by our research group, see for ref. Copper *et al.*, 1993). Most evidence for the potential of chemopreventive agents comes from studies in which retinoids are used (Sporn *et al.*, 1976). Recently, growing interest has originated in chemopreventive agents with the potential to scavenge free oxygen radicals, like thiols. Presently, N-acetylcysteine is being tested in the Euroscan chemoprevention trial (De Vries *et al.*, 1991). GSTs may be of value in monitoring the efficacy of free oxygen scavenging agents, concerning glutathione metabolism. However, further research must be executed before the value of GST expression in exfoliated cells as a predictive biomarker during follow-up studies in chemoprevention trials can be assessed.

Table IV: Patient characteristics and individual staining results of buccal mucosa exfoliated cells.

No.	Age (years)	Gender	Tumour loc.[1]	TNM clas.[2]	Diff.[3]	Cum. smoking (py)[4]	Current smoking (no./day)[5]	Alcohol (U/day)[6]	Positive cells in buccal mucosa scrapes (%)		
									GST-π	GST-μ	GST-α
Patients											
1	62	M.	fom.	pT1N0M0	m.	60	50	4	34	32	25
2	77	M.	glot.	pT4N0M0	m.	30	6	6	26	26	31
3	66	M.	fom.	pT2N0M0	w.	25	12	4	26	32	30
4	56	M.	fom.	pT1N0M0	m.	15	10	5	38	26	28
5	68	M.	fom.	pT4N1M0	w.	14	10	1	36	28	23
6	20	F.	tongue	pT3N2cM0	m.	2	10	0	29	28	22
7	56	F.	tongue	pT3N2bM0	w.	34	20	5	22	29	23
8	58	M.	s. glot.	T4N1M0	m.	80	10	6	31	31	25
9	66	M.	tongue	pT1N0M0	w./m.	60	45	2	35	26	26
10	64	M.	tongue	pT3N0M0	w./m.	10	5	3	30	25	31
11	70	M.	pharynx	pT3N3M0	m./p.	2	1	2	32	30	31
12	68	M.	fom.	pT1N0M0	w./m.	40	20	5	33	42	39
13	69	F.	s. glot.	pT2N1M0	p.	50	30	3	28	36	34
14	57	M.	fom.	pT2N0M0	p.	68	10	2	31	39	32
15	67	M.	tongue	pT4N2cM0	m.	28	20	8	46	36	25
16	56	F.	tongue	pT2N0M0	m.	44	30	3	33	23	27
17	44	M.	tongue	pT2N1M0	m./p.	10	10	2	28	44	28
18	62	F.	tongue	pT3N2bM0	w./m.	0	0	0	35	46	31
19	68	M.	s. glot.	pT2N0M0	p.	45	20	0	29	25	22
20	75	M.	tonsil	pT3N0M0	m.	2	1	1	30	27	29
21	47	F.	tonsil	pT2N0M0	w.	20	25	1	35	34	33
22	39	M.	s. glot.	pT3N2bM0	p.	13	8	3	36	34	35
23	39	M.	tongue	pT1N0M0	w.	12	15	1	22	28	24
24	64	M.	tongue	pT3N2bM0	m./p.	22	0	3	57	57	39
25	70	M.	glot.	pT4N0M0	m.	10	5	3	41	42	37
MEAN	60	19M/6F	-------	------	--	28	15	3	33	33	29

Tabel IV continued:

No.	Age (years)	Gender	Motive for panendoscopy	Cum. smoking (py)	Current smoking (py)	Alcohol (u/day)	Positive cells in buccal mucosa scrapes (%)		
							GST-π	GST-μ	GST-α
Controls									
1	79	M.	ZenkerPs diverticula	6	4	2	8	9	17
2	80	F.	Oesophagusperforation	12	5	1	11	15	9
3	40	M.	Carcinofobia, no abnormalities	20	25	4	10	12	6
4	71	F.	Unknown pain, no abnormalities	25	13	1	9	19	33
5	69	F.	Fish bone removal	7	3	1	2	20	10
6	36	M.	Persistent hoarseness after trauma	7	10	2	13	38	11
7	63	F.	Scar tissue after intubation	16	10	1	13	18	31
8	34	M.	Floor of mouth fistula	6	4	0	8	14	12
9	40	F.	Stab-wound in the neck	20	22	0	10	14	26
10	47	F.	Chicken-bone swallowed	6	5	1	14	15	26
MEAN	56	4M/6F	----------	13	10	1	10	17	18

[1] *Tumour loc. = tumour localization: fom. =floor of mouth, glot. =glottic larynx, s. glot. =supraglottic larynx.*

[2] *Tumour classification, according to the new T-N-M classification (Spiessl et al., 1992).*

[3] *diff. =degree of differentiation: p. =poorly, m. =moderately, w. =well differentiated.*

[4] *Cum. smoking= cumulative smoking in py= pack years: number of years, multiplied by the number of cigarette-packs daily smoked (assuming that one pack contains 25 cigarettes).*

[5] *Current smoking: actual smoking status in number of cigarettes per day.*

[6] *Alcohol habits in U/day= number of alcoholic beverages per day.*

CHAPTER 5

Potential Early Markers of Carcinogenesis in the Mucosa of the Head and Neck using Exfoliative Cytology

Vivian Bongers

Gordon B. Snow

Nico de Vries

Boudewijn J.M. Braakhuis

J. Pathol., 1995

ABSTRACT

Patients with head and neck squamous cell carcinoma (HNSCC), who are thought to be cured, are at high risk to develop a second primary tumour in the mucosa of the upper aerodigestive tract and the lungs. This phenomenon is in agreement with the concept of "field cancerization", that implies that the whole mucosa is condemned to develop into neoplasia. We hypothesized that early markers of carcinogenesis are present in all cells of the mucosa of a patient with a HNSCC. Therefore, we analyzed the expression of cytokeratin 16, cytokeratin 19 and histo-blood group antigen H (ABH), type 2-chain by means of immunocytochemistry on exfoliated cells taken from six sites of the upper aerodigestive tract of the "healthy" mucosa of previously untreated HNSCC patients (n=25) and of control individuals (n=10). The mucosal expression of these markers was highly different between patients and controls. Since no overlap in ABH-type 2-chain expression existed between patients and controls and the expression between sites in a given individual highly correlated, this marker was the most promising of the tested markers. These data suggest that cytokeratin 16, cytokeratin 19 and ABH-type 2-chain are markers of field cancerization in easily available exfoliated cells, which may be applied to monitor and/ or predict the occurrence of second primary tumours.

INTRODUCTION

The development of head and neck squamous cell carcinoma (HNSCC) is generally accepted to be a multistep process (Voravud *et al.*, 1993). Accordingly, carcinogen exposure is followed by multiple genetic alterations that initiate phenotypic changes. At the morphological level the multistep carcinogenic process is phrased in the terms hyperplasia, metaplasia, dysplasia, carcinoma in situ and invasive carcinoma. The occurrence of multiple, independent foci of tumor in one individual can be explained by assuming that the whole mucosa of the upper aerodigestive tract and the lungs has been exposed to the same carcinogens. This phenomenon has been already described by Slaughter *et al.* in 1953 as the "field cancerization" process. This concept explains the development of second primary tumours (SPTs), one of the most important factors negatively influencing the survival rate of HNSCC patients (Lippman *et al.*, 1989). Monitoring of the carcinogenic process and chemoprevention are among the most promising modalities to increase the survival rate of HNSCC patients in the near future. Therefore the intention of this study was to find markers for early detection of HNSCC in exfoliated cells of the upper aerodigestive tract. We used exfoliated cells for this study, because they are easy available, the technique is non-invasive and can be employed in follow-up studies.

On the basis of the reported aberrant expression of cytokeratin 19 (CK-19) (Copper *et al.* 1993), cytokeratin 16 (CK-16) (Morgan *et al.*, 1987) and histo-blood group H (ABH) type 2-chain (Stenersen *et al.*, 1992) in (pre)malignant tissue, these markers were selected for the present study. Because of the high degree of tissue specific expression of many markers, we compared the marker expression in the mucosa at various sites in HNSSC patients to those in healthy individuals.

Cytokeratins (CKs) are intermediate cell filament proteins, expressed during normal epithelial differentiation as part of the cytoskeleton of the cell. Twenty different CKs can be distinguished according to their molecular weight and isoelectric point in two-dimensional electrophoresis (Debus *et al.*, 1984). Aberrant expression of subtypes has been observed during the process of carcinogenesis (Morgan *et al.*, 1987; Moll *et al.*, 1982; Van Der Velden *et al.*, 1993).

CK-16 is expressed in keratinocyte pathologies associated with hyperproliferation and is therefore called a "hyperproliferation" marker. This marker of fast cell turnover is predominantly expressed in the suprabasal cell layer of epithelial tissue (Van Der Velden *et al.*, 1993; Wetzels *et al.*, 1992).

CK-19 is found in a broad range of epithelial tissues. It is expressed as a major component in cells of many simple epithelia and usually as a minor component in the basal layer of stratified epithelia (Van Der Velden *et al.*, 1993). In dysplastic stratified epithelia as well as in squamous cell carcinomas its expression increases and is extended to the suprabasal layers

(Cintorino *et al.*, 1990). Moreover, Copper *et al.* (1993) showed that CK-19 expression is increased in the normal mucosa of HNSCC patients.

Blood group antigens of the ABH system are cell surface carbohydrates. Type 2 chain ABH-antigens are distributed broadly in human epithelial and endothelial cells, independent of the patients blood group (Dabelsteen *et al.*, 1988). In normal oral and laryngeal epithelium type 2 chain ABH-antigens are expressed on parabasal cells (Stenersen *et al.*, 1992; Dabelsteen *et al.*, 1988). Tumorigenic transformation can be associated with synthesis of new carbohydrates, deletion of complex structures, and the accumulation of precursors (Hakomori, 1989). There is evidence that the expression of the type 2-chain ABH antigen in the deepest parts of oral cavity tumours correlates with clinical behaviour (Bryne *et al.*, 1991). Interestingly, leukoplakias without the ABH type 2 antigen expression, and showing no dysplasia according to conventional criteria, later developed into carcinoma (Auclair, 1984).

PATIENTS AND METHODS

Between October 1992 and April 1993, 25 previously untreated patients with histologically proven HNSCC, stage T1-T4 (Spiessl *et al.*, 1992) underwent a diagnostic panendoscopy. Also 10 patients with benign disease underwent a panendoscopy. Detailed characteristics of patients and control individuals are listed in Table I.

The exfoliated cells were obtained by scraping with a Cytobrush plus^{R} (Medscand AB, Malmö, Sweden) from normal, clinically healthy mucosa of the oesophagus (20 cm from the upper alveolar process), vocal fold, ventricular fold, (whole) floor of mouth, (middle part of the) buccal mucosa and dorsal tongue (at 1/3 of the lateral border). In tumour patients the preparations were obtained from the contralateral site of the carcinoma. The brushes were stirred in 3 ml phosphate buffered saline (PBS), allowing the cells to be released from the brush, washed with PBS, and cytological preparations were made on poly-L-lysine coated glass slides using a cytocentrifuge (Shandon, Cheshire, England). The slides were air dried for 60 min, fixed with 100% methanol for 10 min, and stored at -70° until use.

The immunocytochemical staining was performed by means of the immunoperoxidase technique with an avidin-biotin complex (Vectastain ABC-kit, Vector laboratories, Burlingame, CA) using the mouse monoclonal antibodies: LL025 (Wetzels *et al.*, 1992) (kindly provided by Dr. E.B. Lane, Dundee, UK), recognizing CK-16, an antibody directed against CK-19, (Boehringer Mannheim, Germany), and an antibody directed against blood group type 2 chain ABH-antigen, (DAKO, Copenhagen, Denmark). Cytocentrifuge preparations were incubated with these monoclonal antibodies for 60 min at room temperature. Non-specific staining was blocked with

Table I: Patient characteristics and individual staining results of buccal mucosa exfoliated cells.

No.	Age	Gender	Tumor loc.	TNM clas.	Diff.	Cum. smoking	Current smoking	Alcohol	Positive cells in buccal mucosa scrapes (%)		
	(years)					(py)	(no/day)	(U/day)	CK-19	CK-16	ABH2*
PATIENTS											
1	62	M.	fom.	pT1N0M0	m.	60	50	4	40	33	0
2	77	M.	glot.	pT4N0M0	m.	30	6	6	16	37	7
3	66	M.	fom.	pT2N0M0	w.	25	12	4	17	30	34
4	56	M.	fom.	pT1N0M0	m.	15	10	5	25	45	24
5	68	M.	fom.	pT4N1M0	w.	14	10	1	16	45	29
6	20	F.	tongue	pT3N2cM0	m.	2	10	0	54	41	20
7	56	F.	tongue	pT3N2bM0	w.	34	20	5	17	34	10
8	58	M.	s. glot.	T4N1M0	m.	80	10	6	29	27	17
9	66	M.	tongue	pT1N0M0	w./m.	60	45	2	33	24	33
10	64	M.	tongue	pT3N0M0	w./m.	10	5	3	24	22	25
11	70	M.	pharynx	pT3N3M0	m./p.	2	1	2	19	36	22
12	68	M.	fom.	pT1N0M0	w./m.	40	20	5	16	32	1
13	69	F.	s. glot.	pT2N1M0	p.	50	30	3	24	25	29
14	57	M.	fom.	pT2N0M0	p.	68	10	2	18	27	23
15	67	M.	tongue	pT4N2cM0	m.	28	20	8	20	37	12
16	56	F.	tongue	pT2N0M0	m.	44	30	3	27	27	20
17	44	M.	tongue	pT2N1M0	m./p.	10	10	2	21	28	2
18	62	F.	tongue	pT3N2bM0	w./m.	0	0	0	23	47	18
19	68	M.	s. glot.	pT2N0M0	p.	45	20	0	22	24	7
20	75	M.	tonsil	pT3N0M0	m.	2	1	1	35	41	24
21	47	F.	tonsil	pT2N0M0	w.	20	25	1	38	23	23
22	39	M.	s. glot.	pT3N2bM0	p.	13	8	3	24	24	10
23	39	M.	tongue	pT1N0M0	w.	12	15	1	34	29	14
24	64	M.	tongue	pT3N2bM0	m./p.	22	0	3	36	39	7
25	70	M.	glot.	pT4N0M0	m.	10	5	3	59	43	17
MEAN	60	19M/6F	------	------	–	28	15	3	27	33	17

Table 1 continued:

No.	Age (years)	Gender	Motive for panendoscopy	Cum. smoking (py)	Current smoking (py)	Alcohol (U/day)	Positive cells in buccal mucosa scrapes (%)		
							CK-19	CK-16	ABH2
CONTROLS									
1	79	M.	ZenkerPs diverticula	6	4	2	8	19	72
2	80	F.	Oesophagusperforation	12	5	1	8	12	80
3	40	M.	Carcinofobia, no abnormalities	20	25	4	8	15	78
4	71	F.	Unknown pain, no abnormalities	25	13	1	8	11	80
5	69	F.	Fish bone removal	7	3	1	2	14	71
6	36	M.	Persistent hoarseness after trauma	7	10	2	9	24	86
7	63	F.	Scar tissue after intubation	16	10	1	16	16	72
8	34	M.	Floor of mouth fistula	6	4	0	13	12	73
9	40	F.	Stab-wound in the neck	20	22	0	14	10	95
10	47	F.	Chicken-bone swallowed	6	5	1	11	12	78
MEAN	56	4M/6F	----------	13	10	1	10	15	79

*Gender: M.=male, F.=female. Tumor classification, according to the new T-N-M classification (Spiessl et al., 1992). Tumor loc.= tumor localization. fom.=floor of mouth, glot.=glottic larynx, s. glot.=supraglottic larynx. diff.=differentiation grade. p.=poorly, m.=moderately, w.=well differentiated. * ABH2= type 2 chain ABH-antigen. Smoking habits in py= pack years; number of years, multiplied by the number of cigarette-packs daily smoked (assuming that one pack contains 25 cigarettes). Cum. smoking= cumulative smoking, total number of cigarette packs smoked until tumor development. Current smoking: actual smoking status in number of cigarettes per day. Alcohol habits in U/day= number of alcohol units used daily.*

normal horse serum (Vector Laboratories) before the first antibody was attached and endogenous peroxidase activity by 0.06% H_2O_2 in methanol, before the second. After incubation with the second antibody, biotinylated horse-anti-mouse immunoglobulin (Vector Laboratories) was used and the peroxidase label was developed with diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO). Counterstaining was performed with haematoxylin. The stained slides were finally mounted with "Kaiser's glycerine gelatine" (Merck, Darmstadt, Germany). A negative control was made for each site of each person, using PBS instead of the first antibody. Cytocentrifuge preparations from patients and controls were stained simultaneously. A first antibody to an irrelevant antigen was not included, because a previous study of our laboratory has shown complete lack of staining using several mouse monoclonal antibodies (Copper *et al.*, 1993).

Evaluation was performed by light microscopy on coded slides by two independent observers (V.B. & B.J.M.B.). There was a very clear-cut difference between positive and negative cells (figure I). The percentage of positive cells was determined on at least 200 cells per coded slide.

Counting was performed in well preserved areas, where individual cells could be analyzed. Evaluation in a subset of specimens by two independent observers showed a coefficient of variation of less than 10%, similar to that after repeated counting by a single individual.

The data from the patients and the controls were compared using the StudentPs t-test. A p value of less than 0.05 was considered significant. Possible correlations between age, gender, pack years, current smoking and alcohol consumption with the test results and in addition between test results of different sites were determined with the Spearman Rank correlation test.

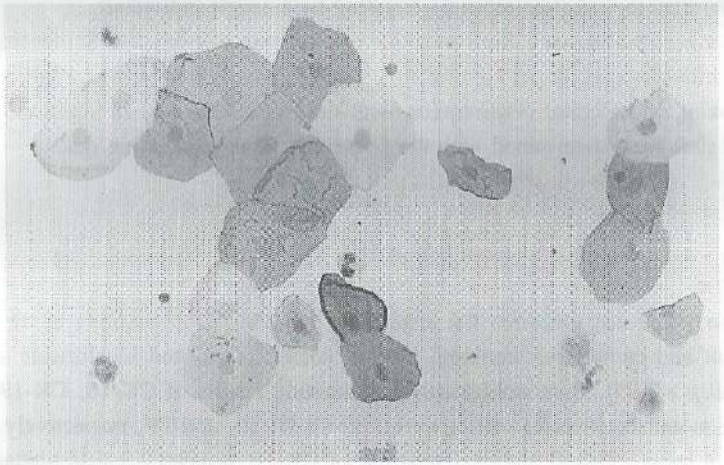
RESULTS

No correlation was found between the level of expression of all markers at all sites versus age, gender, current and cumulative smoking, alcohol consumption, and localization and stage of the index tumor (all $p>0.05$). Immunohistochemical staining results of CK-16, CK-19 and type 2 chain ABH-antigen in exfoliated cells are shown in figures II, III, and IV, respectively. Evaluation of the data showed a mean 2.5 fold higher expression of CK-16, a mean 2.3 fold higher expression of CK-19 and a mean 4.1 fold lower expression of type 2 chain ABH antigen in the "normal" mucosa of patients with HNSSC, as compared to the ones from healthy individuals ($p<0.001$ for each site and for each marker). Individual data of the buccal mucosa samples in relation to patient characteristics are shown in table I. A summary of the results of all sites is shown in table II. Type 2 chain ABH-antigen expression pattern shows no overlap between patients and control individuals. In contrast to controls, exfoliated cells obtained from patients show less or no positive ABH type 2 chain staining (figure I).

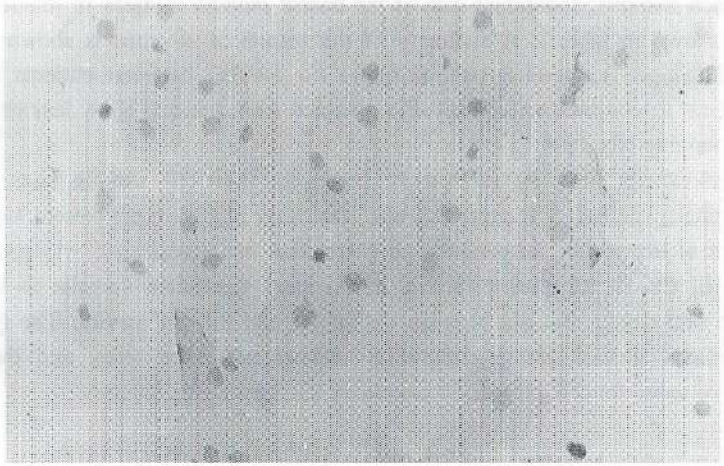
In healthy individuals variation of marker expression between sample sites within one individual was minimal, whereas in patients this was somewhat higher. Each patient had his or her own specific pattern of expression. In patients, no correlation of expression of various sites could be found for CK-16. CK-19 expression showed a statistical significant correlation between the oesophagus and the oral cavity, but not between the larynx and the oral cavity. The type 2 chain ABH-antigen expression showed strong correlation between all tested sites, and therefore one sample has predictive value for the expression pattern of the whole oral mucosa.

Figure 1.:

(a):



(b):



Type 2 chain ABH-antigen expression in exfoliated cells of the ventricular fold of a control individual (a) and of the same sample site of a head and neck squamous cell carcinoma patient (b). There is strong positive staining in most cells of the control individual and lack of staining in most of the exfoliated cells of a head and neck squamous cell carcinoma patient. Original magnification was 400x.

Table II: Expression of histo-blood group antigen H type 2-chain, CK-16 and CK-19 in exfoliated cells.

	ABH2*		CK-16		CK-19	
	Controls	Patients	Controls	Patients	Controls	Patients
Oes.	83 ± 9	19 ± 15	15 ± 7	27 ± 8	11 ± 4	32 ± 13
Vocal fold	86 ± 8	21 ± 14	8 ± 4	28 ± 8	8 ± 4	28 ± 8
Vent. fold	83 ± 10	20 ± 15	7 ± 5	25 ± 9	7 ± 2	30 ± 10
Fl. mth.	83 ± 8	19 ± 12	10 ± 4	27 ± 8	12 ± 4	33 ± 10
Buc. muc.	79 ± 7	17 ± 10	10 ± 5	27 ± 10	13 ± 6	30 ± 10
Tongue	80 ± 8	23 ± 15	15 ± 4	33 ± 8	10 ± 4	27 ± 11

* *ABH2=type 2 chain ABH-antigen; CK=cytokeratin; Oes.=oesophagus; Vent.=ventricular;Fl. mth.=floor of mouth; buc. muc.=buccal mucosa.*

All values (% of positive cells) are given as the means ± the standard deviation. (Controls, n=10; Patients, n=25.)

DISCUSSION

More and more evidence support the field cancerization theory of Slaughter (1953). Recently Ogden *et al.* visualized field changes in the oral cavity by means of immunohistochemical keratin expression (Ogden *et al.*, 1990). Furthermore, Copper *et al.* (1993), reported about elevated CK-19 values in exfoliative cells of the healthy tongue mucosa of HNSCC patients. Voravud *et al.* (1993), found also evidence for field cancerization at the chromosomal level and placed this in perspective of the concept that head and neck tumorigenesis is a multistep process. Comparable results were found by Shin *et al.*(1994), who investigated the expression of epidermal growth factor. Our finding of a highly significant increase in expression of both CK-16 and CK-19 and a highly significant decrease in type 2 chain ABH-antigen expression ($p<0.001$) in the mucosa of HNSCC patients, as compared to controls is also in line with the field cancerization theory. The question arises whether cellular expression of ABH type 2 chain is a potential biological marker that can be used to predict SPTs. The incidence of the development of a SPT, recently established for the Dutch situation is 2.8 % per year (depending on the site of the primary tumor) (Jovanovic *et al.*, 1994). Eventually 20-40% of the treated patients will develop a SPT during follow-up. ABH type 2 chain expression is a potential marker, that can be used to identify individual patients at risk to develop a SPT. A negative correlation between expression and the

development of the SPT may be anticipated. Hypothetically, the mean value minus the standard deviation of the number of positive cells in one or more scrapes can be taken a cut-off level to estimate risk. It appeared that 36% of the patients had values beneath this cut-off level, making them, hypothetically, most at risk. Identification of the high risk patients is important, because they can be targeted for intensive intervention strategies, like chemoprevention and more intense follow-up. CK-16 and CK-19, are thought not to have a predictive value as a single marker, because more than half of the patients have one or more scrapes with extreme high values.

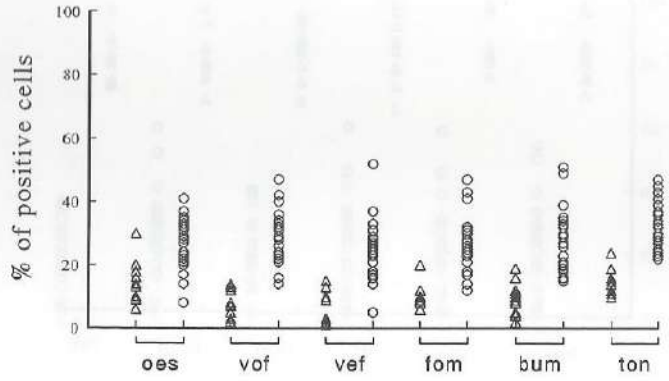
Exfoliated cells, as used in our study, are easy accessible in a non-invasive way and are therefore of benefit in clinical follow-up studies. However, a disadvantage on this approach may be that it is until now unknown whether these superficial samples (down to the suprabasal cell layer) are predictive for what is going on in deeper (basal) cell layers.

Using this approach in follow-up studies it is also important that repeated cell scrapes can be taken in time from easy accessible sample locations, like the oral cavity and that these samples have predictive value for what is going on in the rest (oro- and hypopharynx, larynx, lungs, oesophagus) of the upper aerodigestive tract. Statistical analysis of our results showed in this perspective a significant correlation of ABH-type 2-chain expression between all tested different sites and the buccal mucosa ($p<0.001$). For CK-16 and CK-19 this correlation between sites within one individual could not be found in our study. On the other hand, this could imply that the tumor could develop at the site with the most aberrant expression. It is in this respect important to note that there was a large inter-site variation within one individual.

A promising approach to prevent the development of SPTs is chemoprevention. However, an important limiting factor in chemoprevention research is the long follow-up time needed before a conclusion can be drawn about the effectivity of an agent. Therefore the research on putative biomarkers as an intermediate endpoint has gained in popularity since 1988. Because of different expression of CK-16, CK-19 and ABH-type 2-chain in exfoliated cells of healthy tissue and tissue at risk, they may serve as markers for identifying people for whom chemoprevention is recommended and for monitoring the efficacy of chemopreventive intervention. As in every follow-up situation serial laryngo-broncho-oesophagoscopies are too incriminating for treated patients and only material from the oral mucosa will be obtained during chemoprevention studies. In this way ABH-type 2-chain looks to be the most useful marker of the three tested markers in this study for monitoring chemoprevention by scraping the buccal mucosa during follow-up as a reflection of the status of the mucosa of the upper aerodigestive tract.

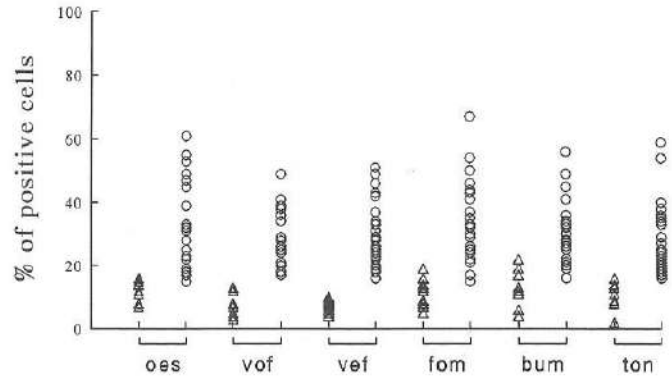
In conclusion ABH-type 2-chain and in a lesser extend CK-16 and CK-19 are possibly useful markers in monitoring the carcinogenic process of patients who are at risk to develop a SPT in the near future.

Figure II.:



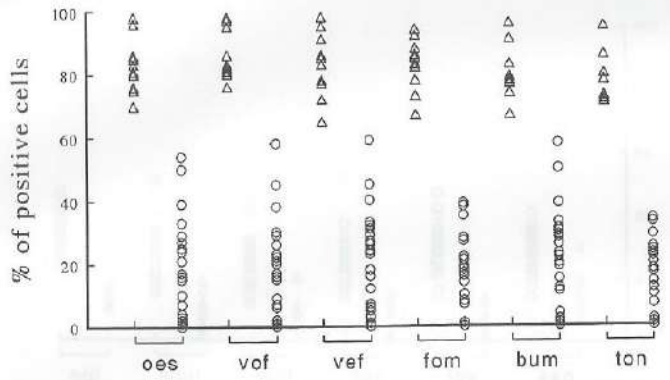
Cytokeratin 16 expression in exfoliated cells of the mucosa of non-cancer patients (△) (n=10) and of the "normal" mucosa of head and neck squamous cell carcinoma patients (ñ) (n=25).
Oes represents the oesophagus; vof, the vocal fold; vef, the ventricular fold; fom, the floor of mouth; bum, the buccal mucosa and ton, the tongue, respectively.

Figure III.:



Cytokeratin 19 expression in exfoliated cells of the mucosa of non-cancer patients (△) (n=10) and of the "normal" mucosa of head and neck squamous cell carcinoma patients (ñ) (n=25).
Oes represents the oesophagus; vof, the vocal fold; vef, the ventricular fold; fom, the floor of mouth; bum, the buccal mucosa and ton, the tongue, respectively.

Figure IV.:



Type 2 chain ABH-antigen expression in exfoliated cells of the mucosa of non-cancer patients (△) (n=10) and of the "normal" mucosa of head and neck squamous cell carcinoma patients (ñ) (n=25).
Oes represents the oesophagus; vof, the vocal fold; vef, the ventricular fold; fom, the floor of mouth; bum, the buccal mucosa and ton, the tongue, respectively.

CHAPTER 6

Value of p53 Expression in Oral Cancer and Adjacent Normal Mucosa in Relation to the Occurrence of Multiple Primary Carcinomas

Vivian Bongers

Gordon B Snow

Isaac van der Waal[#]

Boudewijn JM Braakhuis

[#] *Department of Maxillofacial Surgery and Oral Pathology, Free University Hospital.*

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ABSTRACT

Paraffin embedded, formalin fixed tissue sections from patients suffering from a primary oral squamous cell carcinoma were immunohistochemically investigated for the presence of p53 expression using the Bp53-11 antibody. The aim of this study was to determine the predictive value of p53 expression as a biomarker for the development of a second primary tumour (SPT) in the respiratory and upper digestive tract. In a nested case control study neoplastic and normal tissue sections of 44 patients who had a previous history of cancer were used. 15 of the 44 had developed a SPT, while the other 29 were minimally 7 years free of disease. Additionally, 9 SPTs were included in this study to establish whether concordance exists in tumours that develop in the same field. Ten of the 29 patients (34%), free of tumour during follow-up had p53 positive tumours. Eight of 15 patients (53%), who developed an SPT had a p53 positive primary tumour. This difference is not statistically different (χ_2-test). 40% of the total group of primary oral cavity tumours showed p53 positivity. When comparing the first and the second tumour, discordance in p53 expression between the first and second tumour was seen in 4 out of 9 cases. None of the cases showed p53 positivity in adjacent normal mucosa. In conclusion, p53 immunoreactivity in neoplasia, dysplasia and normal tissue does not predict the development of an SPT. In addition, multiple primary tumours do not have identical p53 expression.

INTRODUCTION

Review of treatment outcomes over the last 30 years in head and neck squamous cell carcinoma (HNSCC) reveals progress in initial treatment, but only marginal increase of long term survival. The development of a second primary tumour (SPT) in the respiratory and upper digestive tract is the chief cause of treatment failure and death in patients who present with early stage disease (Lippman *et al.*, 1989). Head and neck carcinogenesis has been proposed to be a multistep process in an anatomic field repeatedly exposed to carcinogens. Recent findings of geno- and phenotypic alterations in histologically normal epithelium of cancer patients support the "field cancerization" theory (Slaughter *et al.*, 1953; Voravud *et al.*, 1993; Shin *et al.*, 1994a).

Activation of cellular protooncogenes or inactivation of tumour suppressor genes are most probably genetic alterations, involved in this multistep process of carcinogenesis. The transcription factor p53 is the product of a tumour suppressor gene. The wild type form has a half-life time of 6 to 30 minutes. In contrast, proteins from mutated genes form complexes with a heat shock protein (hsp70) that result in a metabolically stable protein, that has a half life time of many hours (Finlay *et al.*, 1988). This forms the basis for the detection of mutated p53 by immunohistochemistry, because wild type p53 does not accumulate in high enough levels for detection by immunohistochemistry (Langdon *et al.*, 1992). Recently, the group of Bartek described Bp53-11 as an useful antibody for immunohistochemistry on paraffin sections (Bartek *et al.*, 1993; Bartkova *et al.*, 1991). In HNSCC, like in a number of other human solid tumours (Nigro *et al.*, 1989), p53 gene mutations have been reported to be a common implicated genetic event. By immunohistochemical detection techniques increased p53 levels have been found in 35-90% of HNSCCs (Bartek *et al.*, 1993; Warnakulasuriya *et al.*, 1992; Slootweg *et al.*, 1994; Maestro *et al.*, 1992; Ogden *et al.*, 1992; Field *et al.*, 1991; Somers *et al.*, 1992; Dolcetti *et al.*, 1992). In studies concerning oral cavity carcinomas percentages of 35 (Warnakulasuriya *et al.*, 1992) and 54 (Ogden *et al.*, 1992) p53 positivity have been reported. Moreover, p53 has been recently proposed to be an early event in head and neck carcinogenesis since immunohistochemical studies showed p53 positivity in histologically inconspicuous epithelia at significant distance from the primary tumour and premalignant lesions (Nees *et al.*, 1993; Shin *et al.*, 1994b). The forementioned studies did not report about the prognostic value of early p53 expression in the clinical development of SPTs. Recently, it has been shown that p53 positivity in surgical margins, histopathologically free of tumour, was correlated with local tumour recurrence (Brennan *et al.*, 1995). Because there is a great need for early markers to identify patients who are at high risk to develop an SPT, we

applied the immunohistochemical staining procedure against p53 on both tumours and the normal mucosa of patients who were surgically radical treated for their primary oral cavity tumour. From these patients the smoking and alcohol history was known and they had not undergone any previous cancer therapy. We performed this study as a nested case control study to establish the possible predictive value of p53 expression for the development of an SPT in both primary tumours and clinico-pathologically normal tissue, obtained during surgery for the index tumour.

PATIENTS AND METHODS

From our archives we selected 15 formalin-fixed paraffin embedded tissue blocks of primary oral carcinomas of patients that were initially treated between 1978-1988. These patients eventually developed an SPT. The control group consisted of 29 patients from the same period that had not developed an SPT during the follow-up time of minimally seven years. Patients with a local recurrence were excluded from the study. None of the patients had been exposed to previous radio- or chemotherapy. Individual patient characteristics are reported in table I. From 9 patients, who had developed a second primary tumour during follow-up also the second tumour was investigated. All tumour specimens and normal tissues were reviewed on haematoxylin and eosin stained histological sections by one of the authors (IvdW). Dysplasia was identified by criteria described earlier (Pindborg, 1980). Paraffin sections of 4 μM on poly-L lysine (Sigma, St. Louis, USA) coated slides were deparaffinized and rehydrated before immunohistochemistry was performed. p53 staining was performed using the immunoperoxidase technique with an avidin biotin complex (Vectastain ABC-kit, Vector laboratories, Burlingame, USA).The monoclonal mouse Bp53-11 antibody is a highly sensitive antibody recognizing an antigenic determinant in the aminoterminal region of the p53 protein and reacting with both wild-type and mutant forms of p53 (Nees *et al.*, 1993), and was kindly provided by Dr. Bartek (Bartek *et al.*, 1993). The antibody was diluted 1:10 in PBS to which 1% bovine serum albumin (BSA, Sigma) was added. The slides were incubated with the Bp53-11 antibody for 60 min at room temperature. Non-specific staining was blocked with normal horse serum (Vector Laboratories) before incubation with the primary antibody and endogenous peroxidase activity by 0.006% H_2O_2 in methanol, before incubation with the secondary. After incubation with the primary antibody, biotinylated horse-anti-mouse immunoglobulin (Vector Laboratories) was used and the peroxidase label was developed with diaminobenzidin tetrachloride (DAB, Sigma). Counterstaining was performed with haematoxylin (Merck, Darmstadt, Germany). The stained slides were finally mounted with "Kaiser's glycerine gelatine" (Merck). A negative control was made using PBS/ BSA (1%) instead of the primary antibody.

Evaluation of p53 positivity was performed by light microscope on coded slides by two independent observers. Nuclei with a clear brown color were regarded as positive for p53.

Statistical analysis was performed by the chi square test and by the Spearman rank correlation test.

Table I: Patient characteristics and individual p53 data.

no.	gender	age	primary tumour		SPT[3]	p53 positivity (+/-)				psm[6]	csm[7]	alc[8]
			loc.[1]	TNM[2]		normal	dyspl.[4]	primT[5]	SPT.[3]			
1	F	79	Rtongue	pT1N0	Ltongue	-	n.a.	+	+	-	-	+
2	F	57	Ltongue	pT2N0	Rtongue	-	n.a.	-	-	++	+	++
3	M	45	Ltongue	pT2N0	Rtongue	-	±	+	+	++	+	++
4	M	38	Rtongue	pT3N1	Sinus pf	-	n.a.	-	-	-	-	-
5	M	67	Ltongue	pT1N1	lung	-	n.a.	-	n.a.	+	?	+
6	F	49	Rtongue	pT2N0	pharynx	-	±	+	-	+	+	+
7	M	38	Rtongue	pT3N2	lung	-	n.a.	+	n.a.	++	?	+
8	M	50	Rtongue	pT1N0	glottis	-	n.a.	-	-	+	+	+
9	M	59	Fom	pT3N2c	lung	-	n.a.	-	n.a.	++	?	++
10	M	42	Palatum	pT1N0	tongue	-	+	+	-	++	-	-
11	M	48	Ltongue	pT2N0	lung	-	n.a.	+	n.a.	+	?	-
12	F	57	Fom	pT2N1	Rtongue	-	n.a.	+	-	+	+	-
13	M	56	Trig.	pT2N0	bronch.	-	-	-	n.a.	++	?	++
14	M	55	Rtongue	pT3N0	Btongue	-	n.a.	-	+	++	?	++
15	M	71	Fom	pT1N0	lung	-	n.a.	+	n.a.	++	?	++
16	F	43	tongue	pT1N0	-	-	n.a.	+	n.ap.	-	?	-
17	M	56	tongue	pT3N0	-	-	n.a.	-	n.ap.	++	?	++
18	F	59	tongue	pT1N0	-	-	n.a.	-	n.ap.	+	?	+
19	M	58	tongue	pT3N0	-	-	n.a.	+	n.ap.	+	?	+
20	F	77	tongue	pT2N0	-	-	n.a.	-	n.ap.	-	?	+
21	M	60	tongue	pT1N0	-	-	n.a.	-	n.ap.	+	?	-
22	F	71	tongue	pT2N0	-	-	n.a.	+	n.ap.	-	?	-
23	M	74	tongue	pT2N1	-	-	-	-	n.ap.	+	?	+
24	M	79	tongue	pT1N0	-	-	n.a.	+	n.ap.	-	?	-
25	M	75	tongue	pT2N1	-	-	-	+	n.ap.	+	?	+
26	M	66	tongue	pT1N0	-	-	-	-	n.ap.	-	?	+
27	M	50	tongue	pT3N1	-	-	n.a.	+	n.ap.	++	?	++

28	M	40	tongue	pT3N0	-	-	-	-	n.ap.	++	?	++
29	F	65	tongue	pT1N0	-	-	-	-	n.ap.	+	?	+
30	M	52	tongue	pT3N1	-	-	±	+	n.ap.	++	?	++
31	M	46	tongue	pT3N0	-	-	-	-	n.ap.	++	?	++
32	M	71	tongue	pT2N0	-	-	-	-	n.ap.	+	?	+
33	M	54	tongue	pT3N0	-	-	n.a.	-	n.ap.	++	?	++
34	M	50	tongue	pT3N1	-	-	n.a.	-	n.ap.	++	?	++
35	F	79	tongue	pT1N0	-	-	n.a.	+	n.ap.	-	?	-
36	F	45	Tongue	pT1N0	-	-	n.a.	-	n.ap.	-	?	+
37	M	69	fom	pT3N1	-	-	n.a.	-	n.ap.	++	?	+
38	M	51	fom	pT2N0	-	-	n.a.	-	n.ap.	++	?	++
39	F	64	fom	pT1N0	-	-	-	-	n.ap.	-	?	-
40	F	82	fom	pT2N1	-	-	n.a.	+	n.ap.	-	?	-
41	M	60	tongue	pT2N0	-	-	n.a.	-	n.ap.	+	?	+
42	M	59	fom	pT1N0	-	-	n.a.	-	n.ap.	++	?	+
43	F	58	fom	pT2N0	-	-	n.a.	+	n.ap.	+	?	+
44	M	62	tongue	pT1N0	-	-	n.a.	-	n.ap.	+	?	+

[1]Loc. = localisation, [2]TNM: pTNM stage of the primary tumour, according to the new TNM classification (Spiessl et al., 1992), [3]SPT=second primary tumour, [4]dyspl. =dysplasia, by earlier described criteria (Pindborg, 1980); p53 positivity: - negative, ± scarce positive cells, + fields of positive cells. [5]primT= primary tumour, [6]psm=previous smoking habits, [7]csm=current smoking habits, [8]alc. =alcohol habits.

Rtongue= right side of the tongue, Ltongue= left side of the tongue, Btongue= base of tongue. Trig.= retromolar trigonum, fom= floor of mouth. Bronch. = bronchus.

n.a. = not available

n.ap. not applicable

Smoking habits: ++ > 20 cig/day; + < 20 sig/day; - never smoker.

Alcohol habits: ++ > 4 units/day; + 1-4 units/day; - no alcohol usage.

RESULTS

In a nested case control study p53 expression has been determined in tumour and normal mucosa of 44 patients with an oral cavity tumour. No statistical difference in frequency of p53 positivity between patients who eventually developed an SPT and patients who did not, could be observed, as shown in table II. We also found discrepancy between p53 expression in the index

tumour and the corresponding SPT in 4 out of 9 patients (table I).

In all, but one, cases (n=13), where the invasive tumour exhibited p53 protein expression, the adjacent dysplastic areas also showed p53 positivity in the basal cell layer. In 3 of the 12 cases the dysplastic area showed only sparse p53 positive cells. The others showed an overall p53 positive basal cell layer. When the tumours were p53 negative, the corresponding dysplastic areas were negative too. No expression of p53 protein was found in adjacent, clinico-pathological normal-looking mucosa of all cases.

Earlier, a significant correlation between smoking habits and p53 expression has been reported in HNSCC (Field *et al.*, 1991). The most common mutation in p53, a G to T transversion has been linked to tobacco usage, which implicates that specific carcinogens may induce mutations in the p53 gene (Somers *et al.*, 1992). However, a significant correlation between p53 expression and smoking habits (p=0.28), alcohol consumption (p=0.32), tumour T and N stage (p=0.8 and p=0.46, respectively) and age (p=0.91) could not be found in our study.

Table II: p53 staining of the primary oral cavity tumour of patients who developed a second primary tumour and patients who did not develop another carcinoma.

	p53 positive	p53 negative
SPT* + (n=15)	8 (53%)	7 (47%)
SPT* - (n=29)	10 (34%)	19 (66%)

* *SPT= Second primary tumour.*

DISCUSSION

The purpose of this study was to establish whether immunohistochemical expression of mutated p53 in the tumour or peritumorous normal mucosa of HNSCC patients could be correlated with the eventual development of an SPT. In 1993 and 1994 two reports about p53 expression in histologically normal epithelium adjacent to the tumour of HNSCC patients appeared and it was proposed that mutations in the p53 gene are an early event in head and neck carcinogenesis (Nees *et al.*, 1993; Shin *et al.*, 1994b). More recently, Brennan *et al.* showed that detection of p53 positivity by molecular analysis in histopathological tumour free surgical margins and lymph nodes was correlated with the development of local tumour recurrence (Brennan *et al.*, 1995).

In general, we also found p53 positivity in the basal cell layers of dysplastic tissue adjacent to positive tumours. Thus, our study supports the concept that p53 mutation may occur in an early phase of carcinogenesis. However, it must be added that in a significant proportion of tumours (60%) p53 does not occur at all. This percentage of lack of p53 immunohistochemical staining is in agreement with reports of other authors (Slootweg *et al.*, 1994; Chen *et al.*, 1994).

In our study, in histopathologically normal mucosa of 44 HNSCC patients, obtained during surgical removal of the index tumour p53 protein could not be detected. Nees *et al.* (1993). showed p53 positivity in several tumour distant biopsies of normal mucosa of HNSCC patients, using the same antibody as we did. Also, Shin *et al.* (1994b) found p53 expression in histologically normal epithelium adjacent to the tumour in 21% of the specimens. In contrast to these forementioned two studies in our study patients with local recurrences were excluded.

The key finding of our study is that the immunohistochemical expression of p53 by Bp53-11 in neoplastic, dysplastic and normal peritumorous mucosa has no value in predicting the development of an SPT. However, the absence of antibody reactivity does not rule out genetic alterations of p53. Frameshift mutations as well as nonsense mutations, that may result in the production of truncated forms of p53, can lead to altered p53 undetectable by available monoclonal antibodies (Chen *et al.*, 1994). Recently, 40% discrepancy between single strand conformational polymorphism analysis for the detection of p53 mutations in exons 5-9, the most common sides for p53 abnormalities, and immunohistochemistry has been shown (Xu *et al.*, 1994). Both overexpression without apparent mutations and mutations without detectable p53 immunostaining were observed. Recognizing mutations in tumour surrounding biopsies, always obtained during radical surgical resections, could otherwise be of great value in identifying patients at high risk to develop an SPT and could eventually improve their prognosis. However, the now known discrepancy between molecular genetic and antibody based studies caution against drawing a definitive conclusion about the value of p53 overexpression by using a single detection modality. The approximately positive predictive value of p53 detection in tumours by immunohistochemistry is 63% (Greenblatt *et al.*, 1994). It is still an option that detecting p53 mutation at the genomic level has some value in predicting the development of an SPT.

We also observed that p53 mutations, detected by the Bp53-11 antibody, in multiple primary tumours within one individual, are not always similar. This shows a biologically diversity in tumour suppressor genes, that are probably involved in the development of an SPT in HNSCC. This finding of difference in mutation pattern of multiple primary tumours in HNSCC supports the "field cancerization" theory, that states that dietary or environmental agents cause wide-spread genetic damage leading to multifocal neoplasia (Slaughter *et al.*, 1953). The discordance of p53 mutations in multiple primary tumours strongly favour a multifocal, polyclonal process, as earlier reported by Chung *et al.* (1993).

CHAPTER 7

Serum Proteinase Activities in Head and Neck Squamous Cell Carcinoma Patients

Vivian Bongers

C. Henk Konings*

Anne-Marie Grijpma*

Ivar Steen

Boudewijn J.M. Braakhuis

Gordon B. Snow

Dept. of Clinical Chemistry, Free university hospital, Amsterdam

Submitted to Anticancer Res.

ABSTRACT

Proteinases are known to be involved in carcinogenesis and various substrates are available now to measure the activity of these enzymes. For head and neck squamous cell carcinoma (HNSCC) at this moment no suitable tumour marker exists. Therefore, we compared proteinase activity in serum of 20 untreated HNSCC patients with that of 20 non-cancer individuals. When N-benzoyl-DL-arginine-ß-naphtylamine (BANA) as the substrate, proteinase activity seemed higher among patients, but this difference disappeared after adjustment for alcohol and tobacco consumption. Applying N-α-benzoyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methylcoumarine (ZAAM) as the substrate no difference was found. Additon of E-64, an inhibitor of cysteine proteinase showed that cathepsin B contributed minimally to the ZAAM-specific activity.

INTRODUCTION

Despite improvements in therapeutic and reconstructive modalities in head and neck squamous cell carcinoma (HNSCC), the 5-year survival rate has only marginally improved during the last decades. Local and regional recurrence remains a problem in patients with HNSCC, particularly in those with advanced stage disease. In addition, the occurrence of multiple primary tumours in the respiratory and upper digestive tract is an important limitation for an improved survival rate in radically treated HNSCC patients with early stage disease. Moreover, early detection is often difficult in previously operated or irradiated areas. The development of a reliable tumour marker could assist in monitoring response to therapy and in early detection of disease, whereas the follow-up of HNSCC patients is until now limited to close clinical observation and radiographic investigations. Unfortunately, in contrast with some other cancer types no single, useful circulating tumour marker is known for HNSCC (Rassekh *et al.*, 1994). However, there are several new tumour markers have recently been discovered, which may be of value for HNSCC. Tumour invasion is associated with release of lysosomal proteinases, including collagenases, plasminogens and the cysteine proteinases, in the extracelluar environment (Recklies *et al.*, 1980). The increase in extracelluar enzyme activity appeared to correlate with the ability of a tumour to invade normal tissue (Sloane *et al.*, 1990). Moin *et al.* (1992) have shown that the cysteine proteinase cathepsin B (CB) is capable of actively degrading extracelluar proteins like laminin and fibronectin. Recently, it has been reported that N-benzoyl-DL-arginine-ß-naphtylamine (BANA) sensitive proteinase-activity can be employed as an useful serum tumour markers in breast cancer (Recklies *et al.*, 1980). When N-α-benzoyloxycarbonyl-L-arginyl-L-arginine-7 amido-4-methylcoumarine (ZAAM) is used as the substrate, it has been shown that the proteinase-activity was elevated in serum of patients with gastrointestinal malignancies (Kolar *et al.*, 1987). Elevated serum proteinase-activities have been measured in primary lung tumours when compared to the adjacent normal lung parenchyma (Hirano *et al.*, 1993; Krepela *et al.*, 1990; Ebert *et al.*, 1994). With respect to HNSCC, proteinase activity was measured with BANA as the substrate, according to the protocol of Barrett (1976). Enzyme levels, as measured by this assay, were proposed by (Krecicki *et al.*, 1992) to be of value for monitoring the individual response to treatment of laryngeal carcinoma. These authors claimed this BANA sensitive proteinase-activity as "cathepsin B (CB)-like" activity. However, other investigators suggest that BANA has a low specificity for CB-activity (Recklies *et al.*, 1980; Sloane *et al.*, 1990). Therefore, we feel that, when BANA is used as the substrate, it is preferable to refer to "serum-proteinase-activity".

The aim of this study was to affirm increased serum BANA-sensitive proteinase-activity in HNSCC patients in comparison to non-cancer controls. In addition, we measured in the same

samples, the value of cysteine proteinase activity by using ZAAM as the substrate, referred to as "CB-like" activity (Sloane *et al.*, 1990). We also performed an inhibitory study by applying the inhibitor trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), an irreversible inhibitor and active site titrant of CB that is ineffective against other proteinases (Barrett *et al.*, 1982).

MATERIAL AND METHODS

The study included 20 previous untreated HNSCC patients, referred to our hospital for treatment of their primary tumour. All tumours were histopathologically proven squamous cell carcinomas. None of the patients were earlier exposed to radiation- or chemotherapy. The clinicopathological features of the patients are reported in Table I. A non-cancer control group existed of 20 volunteers, admitted for non-inflammatory, benign disease.

Venous blood (10 ml) was drawn, allowed to clot and centrifuged at room temperature. Serum samples were stored at -70 °C until analysis.

BANA-sensitive proteinase activity was measured in duplicate by Barretts photometric method (Ebert *et al.*, 1994). The BANA (purchased from Bachem, Liestal, Switzerland) concentration in the assay was 30 mM. The release of 2-naphtylamine by the hydrolysis of BANA was measured at 520 nm a Pharmacia 4060 spectrophotometer (Cambridge, United Kingdom). A calibration line was made by analysing serial dilutions of the naphtylamine (Sigma, St Louis, MI, USA) solution. Enzyme activities were expressed in U/l (1 U of activity corresponds with the release of 1 μmol of naphtylamine/min. at 37° C). ZAAM-sensitive proteinase activity was determined in duplicate according to the spectrofluorometric method of Kolar *et al.* (1987), in the absence and presence of 10 μM E-64 (Sigma). Minor modifications of this method were the higher concentration (300 μM) of the ZAAM (Bachem, Budendorf, Switzerland) in the assay and a lower pH (5.5) of the reaction solution. The fluorescence of the reaction product was measured in a spectrofluorometer (Kontron, Milan, Italy). We used 7-amino-4-methylcoumarin (Bachem) for calibration. Enzyme activity was expressed in U/l (1 U of activity corresponds with the release of 1 μmol of 7-amino-4-methylcoumarin per min at 37°C). The assays were performed in duplicate.

The mean $\pm$ the standard deviation (SD) of BANA-sensitive and ZAAM-sensitive proteinase activities in patients and control individuals were calculated and compared, using the Student's *t*-test. A p value less than 0.05 was considered to be significant. Proteinase activities, were correlated with age, gender, smoking- and alcohol consumption, and TNM tumour stage using the Spearman's correlation test. To test the possible bias of age, smoking- and alcohol habits in the serum proteinase activities multiple linear regression was used.

Table I: Patient characteristics, and serum proteinase activities as measured with two different substrates.

	Gender	Age (years)	tumour characteristics		Smoking* py	Alc.** U/day	Proteinase activity	
			pTNM#	location			BANA (U/l)[1]	ZAAM (U/l)[2]
Patients								
1	M	67	T1bN0	larynx	30	2	53	0.9
2	M	57	pT3N2	o.c.	32	4	73.7	1.1
3	M	48	pT2N0	o.c.	15	3	27.6	0.9
4	M	61	pT3N2	o.c.	40	6	46.5	1.1
5	M	56	pT3N1	o.c.	35	2	23.9	1.7
6	F	43	pT4N0	o.c.	10	1	6.4	1.0
7	M	72	T1bN0	larynx	22	2	19.8	1.2
8	M	60	TxN2	unknown	45	3	14	1.4
9	F	66	T2N0	pharynx	15	0	17.6	1.0
10	F	70	pT3N2b	o.c.	20	2	9.8	1.3
11	M	74	T1aN0	o.c.	25	4	15.1	1.1
12	M	71	pT3N1	o.c.	23	6	22.4	1.2
13	F	52	pT3N1	o.c.	30	3	31	1.7
14	M	62	pT3N3	pharynx	31	5	23.5	1.1
15	M	77	pT1N0	o.c.	39	3	22.9	0.8
16	M	69	pT3N2b	o.c.	20	0	16.2	1.1
17	M	54	pT3N2b	o.c.	25	3	22.9	1.6
18	M	68	pT3N0	larynx	35	3	26.5	1.4
19	M	70	pT2N0	o.c.	20	10	12.3	1.2
20	M	60	pT3N2b	larynx	0	0	27.3	0.8
MEAN	16 M/4 F	63	------	-----	26	3	25.6	1.2
SD	-----	9	-----	-----	11	2	15.9	0.3
Controls (n=20)								
MEAN	14 M/6 F	53	-----	-----	16	2	15.1	1.2
SD	-----	9	-----	-----	19	2	11.1	0.3

* smoking habits in py=pack years. Pack year is defined as the number of years multiplied by the number of cigarette packs smoked per day, assuming that one pack contains 25 cigarettes. ** drinking habits in U alcohol used per day.
[1]BANA= serum proteinase activity, measured with N-benzoyl-DL-arginine-β-naphtylamine as the substrate. [2]ZAAM= serum proteinase activity, measured with N-α-benzoyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methylcoumarine as the substrate, without E-64.
# pTNM stage of the primary tumour, according to the new T-N-M classification (Spiessl et al., 1992). o.c.= oral cavity.

RESULTS

BANA-sensitive proteinase activity showed significantly ($p<0.05$) higher values in serum of previously untreated HNSCC patients when compared to non-cancer control individuals (Table I). However, multiple linear regression analysis proved that tobacco and alcohol consumption influenced the results. After correction for these factors, the significant difference did not hold any longer. In addition, no significant difference was found between serum proteinase activity of HNSCC patients and controls as measured by the ZAAM assay, without the use of E-64. Age, gender and TNM-stage did neither correlate with proteinase activity by the BANA assay nor by the ZAAM assay. Individual proteinase activities in the serum samples of patients and controls, as measured with the two substrates did not correlate. The enzyme activity obtained when E-64 was present in the ZAAM assay was subtracted from the enzyme activity when E-64 was absent, giving a much better approach for measuring CB-activity (Barett *et al.*, 1982). We observed a very low (< 5%) inhibition of the cleavage of ZAAM by E-64. This showes that CB-activities are near or just above the detection limit both in patients and in control serum samples.

DISCUSSION

The main finding of our study is that the serum BANA-sensitive proteinase activity in HNSCC patients and controls did not differ significantly, when the results are corrected for smoking and alcohol consumption. Our results are in contrast with a Polish group in which BANA was also used as the substrate (Krecicki *et al.*, 1992). These authors (Krecicki *et al.*, 1992) observed very high levels of the so called CB-like activity in patients with laryngeal carcinoma and concluded that the BANA-assay is of predictive value in their study population. In their study the correlation between smoking and drinking and the BANA-sensitive enzyme activity has not been analysed (Krecicki *et al.*, 1992). The discrepancy in data between the Polish study and ours derives possibly from the use of different analytical procedures. Krecicki *et al.* (1992) modified the original assay (Barrett, 1976) by extending the reaction time of 10 min (40 °C) to 12 hours (37 °C) and used a 0.6 times lower substrate concentration in the test. In our study the BANA assay was unmodified. In addition, differences in characteristics of the study population of Krecicki *et al.* (1992) and ours may be another explanation for the discrepancy in results of the two studies.

In addition, it has to be concluded from our study results that proteinase activity in serum of HNSCC patients does not differ from non-cancer individuals when the ZAAM assay is used. It is well known that ZAAM (a synthetic low molecular weight peptide) belongs to the most specific substrates for measuring the so-called CB-like activity (Barrett *et al.*, 1981), because cathepsin H has essentially no activity against this substrate (Barrett *et al.*, 1981), whereas cathepsin L and S

have activity more than two orders of magnitude less activity CB with ZAAM as the substrate (Kirschke *et al.*, 1984). However, proteinases other than cysteine proteinases may also cleave the ZAAM substrate and therefore we measured CB activity in the presence and absence of E-64. The enzyme activity obtained when E-64 was present in the ZAAM assay was subtracted from the enzyme activity when E-64 was absent, giving a method for specifically measuring CB-activity (Kolar *et al.*, 1987). Regrettably, this approach resulted in a CB activity that was near the detection limit and this was found for controls as well as for patients. This finding is in agreement with the data of Kolar *et al.*, (1987) who used a similar method and also reported low CB activities in control subjects. Apparently, CB is only a minor proportion of the proteinase activity in the blood serum that is measured when ZAAM is used as the substrate.

CHAPTER 8

Circulating Fragments of Cytokeratin 19

In patients with Head and Neck Squamous Cell Carcinoma

Vivian Bongers

Boudewijn J.M. Braakhuis

Gordon B. Snow

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ABSTRACT

In head and neck squamous cell carcinoma (HNSCC) a reliable serum marker of carcinogenesis should be of predictive value for the development of recurrent disease or a second primary tumour. At the moment, for HNSCC such suitable tumour marker is not available. Recently, elevated levels of cytokeratin 19-fragments (Cyfra 21-1) have been detected in serum of patients with lung cancer, in particular with squamous cell carcinoma. Cytokeratin 19 is an intermediate cell filament protein expressed in simple epithelia and their malignant counterparts. Squamous cell carcinomas of the upper aerodigestive tract and the lungs are carcinomas, that reveal an increased expression of cytokeratin 19 with increasing grade of malignancy. Therefore, in this prospective study, a standardized sandwich enzyme-linked immunosorbent assay against soluble cytokeratin 19 fragments was tested in serum of 20 patients with previously untreated HNSCC. The results were compared to that of 20 non-cancer control individuals. Patients and controls did not differ significantly with respect to age, gender, alcohol and drinking habits. From 12 patients who were minimally three months in remission of HNSCC we measured Cyfra 21-1 levels during follow-up.

Our results showed significantly higher Cyfra 21-1 concentrations in serum of cancer patients (10.21 ± 3.03 ng/ml) when compared to non-cancer controls (7.2 ± 2.63 ng/ml). After radical treatment the marker levels significantly dropped to 1.65 ± 1.07 ng/ml. In conclusion, cyfra 21-1 appears to be of value as a circulating tumour marker for HNSCC, especially in monitoring disease control.

INTRODUCTION

Despite improvements in therapeutic and reconstructive modalities in head and neck squamous cell carcinoma (HNSCC), the 5-year survival rate has only marginally improved during the last decennia. Local and regional recurrence of disease remains a problem in patients with HNSCC, particularly in those with advanced stage disease. Moreover, early detection is often difficult in previously operated-on or irradiated areas. The development of a reliable circulating tumour marker could assist in monitoring response to therapy and in early detection of recurrent disease. In contrast with some other cancers no useful tumour marker exists for HNSCC until now (Rassekh *et al.*, 1994).

Cyfra 21-1 is a new immunochemical assay, that measures a fragment of cytokeratin 19 in serum. In this assay cytokeratin 19 fragments are recognized by the monoclonal antibodies, KS 19-1 and BM 19-21. The serum level of the cytokeratin subunit 19 fragment, Cyfra 21-1, is an independent prognostic factor for squamous cell lung carcinoma (Pujol *et al.*, 1993). It has been shown that the sensitivity of Cyfra 21-1 correlates clearly with tumour size and stage (Stieber *et al.*, 1993). Moreover, Van Der Gaast *et al.* established the value of Cyfra 21-1 in disease monitoring of squamous cell lung cancer (Van Der Gaast *et al.*, 1994).

In head and neck epithelia the number of cytokeratin 19 positive cells increases with growing malignancy grade (Van Der Velden *et al.*, 1993; Moll *et al.*, 1982). Moreover, slight increase of intracellular cytokeratin 19 expression has also been demonstrated in the healthy mucosa of HNSCC patients (Copper *et al.*, 1993, Bongers *et al.*, 1995a) This finding is in agreement with the field cancerization concept (Slaughter *et al.*, 1953), a generally accepted theory for the occurrence of second primary tumours in the respiratory and upper digestive tract, a major cause of treatment failure in early stage HNSCC.

The aim of the present study is to evaluate the clinical usefulness of Cyfra 21-1 in serum of HNSCC patients. A valuable tumour marker for monitoring recurrent disease would first of all return to normal after successful therapy. For that purpose we repeated the measurement of serum Cyfra 21-1 levels in patients, minimally three months in remission of their index HNSCC.

PATIENTS AND METHODS

Patients: The study included 20 previously untreated HNSCC patients, referred to our hospital for evaluation and treatment of their primary tumour. All tumours were histopathologically proven squamous cell carcinomas. None of the patients have been earlier exposed to radiation- or chemotherapy. The clinicopathological features of the patients and the tumours are reported in

table I. From 12 of these patients serum samples were collected, when they were clinically minimal three months in remission of their disease. A non-cancer control group existed of 20 healthy volunteers, admitted for non-inflammatory, benign disease.

Measurement of the cytokeratin 19 fragment by the Cyfra 21-1 assay: Venous blood (10 ml) was drawn, allowed to clot and centrifuged at room temperature. Serum samples were stored at -70°C until analysis. The serum cytokeratin 19 fragment was determined by using the Cyfra 21-1 EIA Enzymun test (kindly provided by Boehringer Mannheim, Mannheim, Germany). The test principle is based on a two-step sandwich assay using the streptavidin technology and performed at room temperature. Solutions of 0.00, 2.90, 7.80, 18.90 and 53.60 ng/ml were used to prepare a standard curve and two control samples, as enclosed by the manufacturer were included in each measurement series. 35 μl standard or sample plus 700 μl of incubation solution together with biotinylated antibody (monoclonal antibody 19.1) were incubated in streptavidin coated polystyrene tubes for 30 minutes, followed by aspiration and washing. Incubation solution, 700 μl, together with antibody-POD conjugate (monoclonal 19-21 antibody) was added again for 30 minutes. The unbound conjugate was removed by another aspiration and washing procedure. After addition of 700 μl hydrogen peroxide and chromogen ABTS (Boehringer Mannheim) and incubation for 60 minutes the absorbence was measured photometrically (Labsystems Multiscan biochromatic, type 348, Pharmacia LKB, Woerden, The Netherlands) at 414 nm and the Cyfra 21-1 concentrations were calculated from the standard curve. The calibration curve showed a linear relationship between titrations of Cyfra 21-1 and absorbance at 414 nm, (R=0.998).

Statistics: The mean Cyfra 21-1 level of the patients and control group were calculated and compared, using Student's *t*-test. The data before and after therapy were compared by using the paired *t* test. A p value < 0.05 was considered to be significant. The Cyfra 21-1 levels were correlated with age, gender, smoking-, alcohol habits, and TNM-stage using the Spearman's correlation test. To test the possible bias of age, smoking and alcohol habits in the Cyfra 21-1 serum values, multiple linear regression was used.

Table I. Patient characteristics and results of pre- and posttreatment serum Cyfra 21-1 levels.

	Gender	Age (Years)	Tumour characteristics			Smo-k.* py	Alcohol** U/day	CYFRA[1] ng/ml	CYFRA[2] ng/ml
			pTNM#	Loc.	Diff.				
Patients									
1	M	67	T1bN0	larynx	w.	30	2	5.7	n.d.
2	M	57	pT3N2	o.c.	m.	32	4	12.3	4.38
3	M	48	pT2N0	o.c.	w.	15	3	10	0.53
4	M	61	pT3N2	o.c.	p.	40	6	16.9	1.09
5	M	56	pT3N1	o.c.	m.	35	2	10.2	0.56
6	F	43	pT4N0	o.c.	w.	10	1	6.3	2.86
7	M	72	T1bN0	larynx	m.	22	2	7.1	2.08
8	M	60	TxN2	-	p.	45	3	15.7	n.d.
9	F	66	T2N0	pharynx	p.	15	0	8.7	0.99
10	F	70	pT3N2b	o.c.	m.	20	2	8.2	1.28
11	M	74	pT1aN0	o.c.	w.	25	4	10.9	1.31
12	M	71	pT3N1	o.c.	m.	23	6	6.6	n.d.
13	F	52	pT3N1	o.c.	m.	30	3	9.7	1.43
14	M	62	pT3N3	pharynx	w.	31	5	11.4	1.65
15	M	77	pT1N0	o.c.	m.	39	3	9.9	n.d.
16	M	69	pT3N2b	o.c.	m.	20	0	12.9	n.d.
17	M	54	pT3N2b	o.c.	p.	25	3	8.2	n.d.
18	M	68	pT3N0	larynx	m.	35	3	9.6	n.d.
19	M	70	pT2N0	o.c.	w.	20	10	9.6	n.d.
20	M	60	pT3N2b	larynx	m.	0	0	14.2	1.65
MEAN	16M/4F	63	----	----	---	26	3	10.21	1.65
SD	----	9	----	----	---	11	2	3.03	1.07

(Table I. continued)
Controls (n=20)

MEAN	14M/6F	53	----	----	16	2	7.2	n.d.
SD	----	9	----	----	19	1	2.63	n.d.

* smok.=smoking habits in py=pack years. Pack years is a unit of cumulative smoking and is defined as the number of years, multiplied by the number of cigarette-packs smoked daily, (assuming that one pack contains 25 cigarettes). ** Alc.=drinking habits in U alcohol used daily. CYFRA[1]: serum CYFRA 21-1 level in previously untreated patients. CYFRA[2]: serum CYFRA 21-1 level at least 3 months after radical treatment for primary carcinoma. # pTNM stage of the primary tumour, according to the new T-N-M classification (Spiessl et al., 1992). Loc.=Localisation of the index tumour. Diff.=degree of differentiation, p.=poor, m.=moderate, w.=well differentiated. o.c.=oral cavity carcinoma. n.d.=not determined.

RESULTS

Cyfra 21-1 serum levels in previously untreated HNSCC patients were significantly ($p<0.001$) higher when compared to non-cancer individuals (table I). Patients who were minimally 3 months in remission of their HNSCC had a significant decrease of serum Cyfra 21-1 values ($p<0.001$, figure I). Age, gender, site, TNM stage (Spiessl *et al.*, 1992) and degree of differentiation of the index tumour did not correlate with serum Cyfra 21-1 values. Since the odds ratios did minimally change when corrected for possible bias by age and smoking, correction was not necessary.

DISCUSSION

At the cellular level cytokeratin 19 is particularly abundant in most HNSCC (Van Der Velden *et al.*, 1993). In contrast to cytokeratins themselves, fragments of intermediate filaments are soluble in serum. These fragments are detectable by a sandwich enzyme-linked immunosorbent assay in serum, the Cyfra 21-1 test (Bodenmüller *et al.*, 1992).

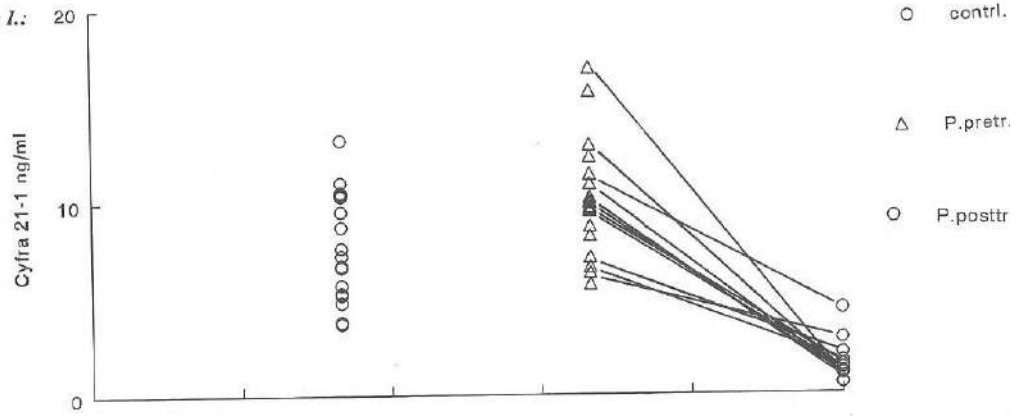
Recently, in HNSCC a significant difference in serum levels of Cyfra 21-1 between HNSCC patients and non-cancer individuals has been detected (Doweck *et al.*, 1995) as was earlier established for lung cancer of different histologic origin and in a lesser extent for colon carcinoma and other solid tumours (Stieber *et al.*, 1993). Moreover, it has been shown in HNSCC patients that serum Cyfra 21-1 levels show a tendency to follow the clinical course of the disease, making it a possible useful serum tumour marker. However, cytokeratins are not organ specific and they can be derived from all sorts of epithelial derived tumours. This lack of tumour specificity makes the determination of the cytokeratin 19 fragment in serum a limited suitable marker for primary HNSCC tracing. However, the Cyfra 21-1 test could be of value to the monitoring of therapy

efficiency and to the detection of recurrent squamous cell carcinoma in an early stage. Detection of recurrent disease is often difficult in previously operated or irradiated areas. In our study, patients in remission of primary HNSCC showed reduced serum Cyfra 21-1 levels. Serum values of patients in remission of HNSCC were also far lower than those of the non-cancer individuals. Scar tissue formation and poor lymphatic drainage after surgery or irradiation can be the cause of this phenomenon.

Doweck *et al.* (1995) showed in their pilot study a significant relation between TNM stage and serum Cyfra 21-1 values. In our study the lack of correlation between tumour stage and Cyfra 21-1 levels is possibly due to the relatively small patient population with a great variation in tumour stages and sites within the head and neck region. However, patients with stage IV disease and lymphatic spread showed a tendency to higher serum values than patients with early stage disease.

In conclusion, we could establish that the Cyfra 21-1 test shows elevated levels in serum of a number of HNSCC patients and has the potential to be a valuable serum marker in the follow-up of HNSCC patients. The data of this study and of Doweck *et al.* (1995) justify further investigation. However, to establish the value of Cyfra 21-1 for the prediction of recurrent disease a large prospective study has to be conducted. During such prospective study for the establishment of the usefulness of Cyfra 21-1 in long-term monitoring, also its predictive value for the development of second primary tumours, another major cause of treatment failure in HNSCC, can be investigated.

Figure I.:



Serum Cyfra 21-1 levels in non-cancer individuals, patients with HNSCC and patients in remission from HNSCC. Contrl.=Control individuals (n=20), P.pretr.=Previously untreated HNSCC patients with a histopathologically proven squamous cell carcinoma (n=20). P.posttr.=HNSCC patients clinically minimal three months in remission from their primary tumour.

CHAPTER 9

Antioxidant Related Parameters in Patients Treated for Cancer Chemoprevention with N-Acetylcysteine

Vivian Bongers

Jan de Jong*

Ivar Steen

Nico de Vries

Aalt Bast*

Gordon B. Snow

Boudewijn J.M. Braakhuis

** Department of Pharmacochemistry, Free University hospital, Amsterdam.*

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ABSTRACT

N-acetylcysteine (NAC) is an antioxidant, possibly effective in the early steps of carcinogenesis, and is applied to prevent second primary tumours in the upper aerodigestive tract and the lungs. In this study, we evaluated the pharmacodynamic profile of 600 mg NAC treatment, given daily for 3 months. Treatment caused a significant increase of the non-protein-SH concentration in blood plasma (38%) and erythrocytes (31%). Glutathione levels in exfoliated buccal mucosa cells appeared not to be influenced by treatment. The total radical-trapping ability parameter (TRAP) of blood plasma showed no change. *In vitro*, the addition of glutathione, but not of NAC did increase the TRAP-value. In addition, when peroxyl radicals were generated *in vitro* NAC was shown to be consumed more rapidly than glutathione. This suggests that NAC prevents early damage, while glutathione functions over a longer time period.

INTRODUCTION

The occurrence of second primary tumours (SPT) is the major cause of treatment failure in early stage, radically treated, head and neck squamous cell carcinoma (HNSCC) patients (Vikram, 1984). Therefore, the interruption of the carcinogenic process by chemopreventve drugs seems an attractive strategy to prevent the occurrence of SPTs.

N-acetylcysteine (NAC) is a thiol that is intracellularly deacetylated to cysteine that efficiently supports glutathione (GSH) biosynthesis (Holdiness, 1991; Meistner *et al.*, 1983), and is currently being clinically tested as a chemopreventive agent. NAC and GSH function as anti-mutagenic and anti-carcinogenic agents. Properties of these compounds can be ascribed to multiple protective mechanisms, such as the scavenging of reactive oxygen species, and the modulation of detoxification and DNA repair processes (De Flora *et al.*, 1991a; De Flora *et al.*, 1991b; De Caro *et al.*, 1989). Prevention of carcinogenesis by NAC has been shown *in vitro* (De Flora *et al.*, 1991a) and *in vivo* (De Flora *et al.*, 1991b; De Caro *et al.*, 1989). Based on these preclinical data NAC was chosen for the EUROSCAN trial, a prospective chemoprevention study in curatively treated patients with early stage oral, laryngeal and lung cancer (De Vries *et al.*, 1991).

A serious limitation of chemoprevention studies is the long follow-up time needed for the assessment of the efficacy of chemopreventive agents. If good biomarkers for the prediction and monitoring of therapy response were available, we could test many more chemopreventive agents, in less time, with fewer patients in future. For NAC both systemic and site (the tissue where tumours may develop) specific markers, related to antioxidant activity would be relevant. Moreover, solid data on the pharmacodynamic profile of NAC in humans are scarce.

PATIENTS AND METHODS

From ten patients blood plasma and exfoliated cells were taken before and after three months of 600 mg NAC therapy daily on AN empty stomach. NAC (Fluimucil^R), was kindly donated by Zambon (Milan, Italy). Blood plasma was obtained not more than 2 hours after NAC intake using a heparinised tube. Exfoliated cells were collected by scraping the buccal mucosa with a cytobrush^R (Medscand AB, Malmö, Sweden) and washed with phosphate buffered saline. Both samples were stored in nitrogen at -70°C until use. The patients included had been cured for early stage oral cavity (n=2) and laryngeal (n=8) cancer, classified as T_1-T_2, N_0 and M_0 by the TNM classification from 1987 (Spiessl *et al.*, 1992). There were seven males and three females, with a median age of 66 years (range 52-81). Three patients had never smoked, and the others had smoked for between 11 and 90 pack-years. Pack years are defined as the number of years

multiplied by the number of cigarette-packs smoked daily, assuming that one pack contains 25 cigarettes.

Collected plasma samples were analyzed with the total radical-trapping ability parameter (TRAP) assay (Wayner *et al.*, 1987). This assay reflects the synergistic protection of various antioxidants against the biological damage caused by peroxyl radicals in blood plasma. Ascorbate was measured by HPLC (Speek *et al.*, 1984) and uric acid was assayed enzymatically (Town *et al.*, 1985). GSH in plasma was measured by HPLC using precolumn derivatisation with ortho-phtalaldehyde and fluorometric detection (Neuschwonder-Tetri *et al.*, 1989). Non-protein thiol in erythrocytes was determined according to Beutler *et al.* (Beutler *et al.*, 1963). The glutathione (reduced and oxidized GSH) determinations on exfoliated cells of the buccal mucosa were performed enzymatically using a sensitive flow injection analysing system (Redegeld *et al.*, 1988).

Statistical analysis was performed by the paired StudentPs t-test.

RESULTS

The GSH concentration in plasma after a 3 month treatment period of 600 mg NAC increased significantly ($p<0.001$) by 38%. The non-protein SH levels in erythrocytes also increased significantly ($p<0.001$) by 31% (table I). In erythrocytes an upper limit of approximately 2 mM seemed to be attained. The TRAP value of plasma, uric acid levels and ascorbate levels showed no significant change after this treatment period (table I).

Table I. Important anti-oxidant related parameters before and after 3 months treatment with NAC.

	Erythrocytes		Blood plasma							
	[NP-SH][a]* (mM)		TRAP* (μM)		[GSH]* (μM)		[Ascorbic acid] (μM)		[Urate] (μ	
	t=0	t=3[b]	t=0	t=3[b]	t=0	t=3[b]	t=0	t=3[b]	t=0	t
mean[1]	1.40	1.83	1068	1032	3.2	4.4	13	16	410	4
sd[1]	0.33	0.19	436	400	0.9	1.6	7	10	56	7

[a] *NP-SH: non protein bound SH groups.* [b] *t=3: samples after 3 months NAC treatment.*

* *samples determined in duplicate.*

[1] *The present results are based on measurements in ten patients.*

The GSH concentrations in exfoliated cells of the buccal mucosa were around the detection limit. In six of 20 samples the levels were below the detection limit (50 pmol/ cell pellet). The available data did not indicate an effect of NAC treatment. In most cases a change in GSH levels was observed. The day-to-day variation, measured in a series of control persons, showed a similar variability (table II). Oxidised GSH could not be detected in any of the samples.

Table II. GSH values during follow-up in exfoliated buccal mucosa cells of healthy individuals.

Number	[GSH] in exfoliated cells (pmol/mg protein).	
	day 1	day 10
Control 1	457 ± 157*	208 ± 11*
Control 2	187 ± 68	208 ± 78
Control 3	47 ± 12	80 ± 38
Control 4	134 ± 43	153 ± 27
Control 5	151 ± 28	103 ± 9
Patient 1	113 ± 16	48 ± 15
Patient 2	u.d.l.	u.d.l.
Patient 3	53 ± 0	u.d.l.
Patient 4	93 ± 6	147 ± 31
Patient 5	u.d.l.	u.d.l.
Patient 6	154 ± 29	412 ± 35
Patient 7	52 ± 6	69 ± 3
Patient 8	u.d.l.	112 ± 20
Patient 9	69 ± 18	51 ± 26
Patient 10	62 ± 67	150 ± 14

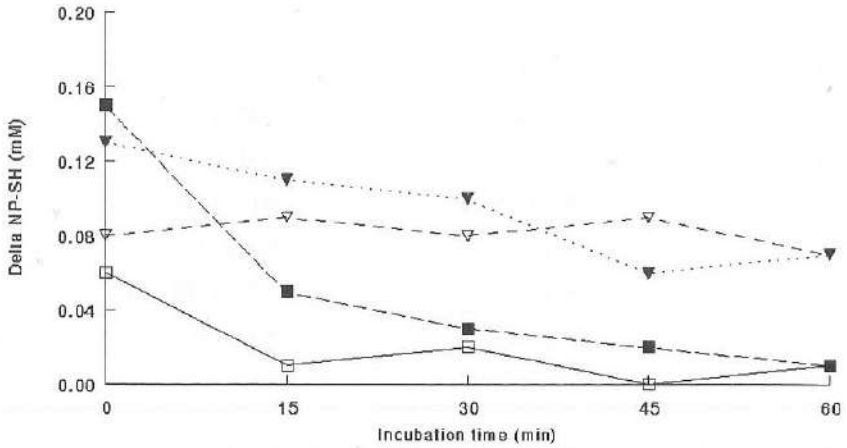
* *Mean value of two samples with standard deviation. u.d.l.: under detection limit.*

To explain the lack of effect of NAC treatment on TRAP values, we extended our experiments by adding relatively high concentrations of NAC and GSH *in vitro* to plasma of untreated patients. The mean TRAP value of untreated plasma was 786 μM (n=2), 795 μM and 794 μM after addition of 100 and 200 μM NAC, respectively (n=5). Despite repeated measurements, no marked

alterations were observed after NAC addition, but the TRAP increased to 825 μM and 886 μM after adding 100 and 200 μM GSH, respectively (n=2), as was shown in earlier studies (Spiessl *et al.*, 1992). *In vitro* incubations of the dithiols, dihydrolipoic acid [TRAP value with 100 μM: 798 μM (n=2), with 200 μM: 782 μM (n=2)] and dithiotreitol [TRAP value with 100 μM: 779 μM (n=2), with 200 μM: 788 μM (n=2)] to blood plasma did not increase the TRAP value. The assays were performed in the same individuals. However, an explanation for this phenomenon was found after addition of GSH to heated plasma. Plasma heated for 2 min at 90°C prevented increase of the TRAP value after GSH addition. The TRAP value of untreated plasma was 546 μM (n=2) and 531 and 548 μM when 100 and 200 μM GSH were added, respectively (n=2). To determine which enzymatic pathway was activated by GSH, we subsequently added catalase to heated plasma. Interestingly, catalase did not increase the TRAP value (546 versus 533 μM).

Further studies on thiol concentrations during *in vitro* incubation of plasma with 2,2'-azobis (2-amidinopropane).HCl (ABAP), to generate free radicals and NAC or GSH showed that NAC was undetectable within 15-20 min, whereas GSH could be measured over more than 1 h, covering the whole period of the TRAP assay (figure 1). Spontaneous oxidation of non-protein-SH groups in the plasma samples with buffer, NAC or GSH, in non-ABAP exposed control samples was nihil.

Figure 1.:



In vitro exposure of a blood plasma sample to ABAP with addition of 100 μM NAC (□), 100 μM GSH (▽), 200 μM NAC (■), or 200 μM GSH (▼). NP-SH was measured each 15 min during 1 hour. Δ NP-SH reflects the difference between the samples with NAC or GSH addition and the control sample, without drug. The coefficient of variation was less than 6%; determinations were performed in duplicate.

DISCUSSION

One of the mechanisms that supports the choice of NAC as a cancer preventing agent is its ability to scavenge free oxygen radicals (De Caro *et al.*, 1989). The TRAP assay has yielded useful information on the protective role of the synergistic action of various endogenous antioxidants particularly against peroxyl radicals. The TRAP values of this patient population after radical treatment for their HNSCC showed a broad range (1068 $\pm$ 436 μM), but did not differ significantly from a population of healthy volunteers described previously (Wayner *et al.*, 1987). In the present study, NAC treatment did not lead to significant changes in the TRAP values. Because of the large contribution of uric acid and ascorbate to the TRAP (Town *et al.*, 1985), we could exclude possible interference of changes of these metabolites by measuring their concentrations in the plasma samples (table I). A possible explanation for the lack of effect of NAC on the TRAP is that the relative contribution of GSH and NAC to the TRAP is too small to lead to a change (1 mole GSH can trap 0.3 mole peroxyl radicals) (Wayner *et al.*, 1987). Apparently, NAC traps peroxyl radicals with an efficiency like GSH and this system does not allow the detection of the relatively small increase in antioxidants in human blood plasma, as observed in this study. However the GSH reservoir in erythrocytes increased in most cases (Table I), which may also physiologically contribute to antioxidant delivery into blood plasma.

To test the ability of NAC to act as a precursor of GSH, GSH levels before and after NAC treatment were determined. GSH is an important intracellular defence system against reactive oxygen species. The GSH concentration in plasma after NAC therapy increased significantly. Our measurements of non-protein-bound thiols in erythrocytes, which gives a reflection of the intracellular whole body situation, also showed a significant increase, and supports the theory that most of the nonprotein bound thiols are storaged in the erythrocytes. In exfoliated cells, changes by NAC treatment were seen, but these were insignificant because in controls there was wide intra-individual variation and therefore no definitive conclusion could be drawn (table II). However, in any case it can be concluded that the available data did not show a consistent effect of NAC treatment.

To explain the lack of effect of NAC treatment on TRAP values, we extended our TRAP experiments by adding relatively high concentrations of NAC and GSH to plasma of untreated patients in the test tube. Despite repeated measurements, no marked alterations were observed after NAC addition, but the TRAP increased after GSH addition. *In vitro* addition of the dithiols dihydrolipoic acid and dithiothreitol to blood plasma did not increase the TRAP value. An explanation for this phenomenon was found after addition of GSH to heated plasma. Heating of the plasma prevented the increase of the TRAP value following GSH addition, suggesting that the GSH effects on TRAP may be enzyme mediated. Two glutathione dependent peroxidases could be

involved, i.e., the Se-dependent and Se-independent forms. Both organic and inorganic hydroperoxides can be reduced in this way. Interestingly, catalase did not increase the TRAP value. This suggests that the GSH dependent increase of the TRAP value occurs via enzymatic reduction of organic hydroperoxides.

Our further *in vitro* experiments showed that thiols added to a blood plasma sample are rapidly consumed after peroxidation exposure (fig. 1). In these *in vitro* studies NAC is more rapidly consumed than GSH, which suggests that NAC prevents early damage while GSH functions over a longer period.

The present study in a relatively small group of patients shows a possible beneficial effect of NAC. No relevant changes in GSH levels could be detected in the target tissue, the exfoliated cells of the buccal mucosa. Therefore the GSH levels in exfoliated cells are not a useful parameter for monitoring chemoprevention efficacy. However, the thiol status in blood plasma and erythrocytes for the efficacy of thiol containing chemopreventive agents increased and may have a beneficial effect on the oxidative stress status. The next step will be to use these parameters as biomarkers to predict the occurrence of SPTs. But this can only be assessed after evaluation of the ongoing clinical trial.

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CHAPTER 10

SUMMARY AND CONCLUSIONS

Summary

Chapter 1.

Patients suffering from a head and neck squamous cell carcinoma (HNSCC) frequently develop a second primary tumour (SPT) in the mucosa of the respiratory and upper digestive tract inclusive of the oesophagus. For this phenomenon of multicentric tumour foci the term "field cancerization" has been introduced. This concept reflects that the entire mucosal lining covering the aero- and upper digestive tract is on the way to develop extensive cytologic changes as a result of exposition to repeated insults of the same carcinogens, like tobacco and alcohol metabolites. The majority of SPTs arise over more than half a year after the first, so called, index tumour with a constant rate of 2.8% per year. SPTs have a bad prognosis, because of their occurrence at notoriously bad sites - the lung and the oesophagus - or in earlier treated areas with the consequence that the development of SPTs has a very serious impact on the survival of patients, initially curatively treated for an early stage HNSCC. To reduce the morbidity and mortality due to the development of SPTs early detection and (chemo)prevention are the most attractive options. Both strategies are, however, only feasible in high risk individuals. Knowledge about risk factors for the development of SPTs is until now scarce. This thesis addresses the identification of high risk factors for the development of SPTs at three levels: the constitutional level, the level of the mucosa at risk and the level of the serum.

Chapter 2.

An epidemiologic analysis to elucidate whether a genetic predisposition is a risk factor for the development of a SPT showed interesting results. Among HNSCC patients who ultimately developed a SPT significantly more first degree relatives reported to have had cancer of the respiratory and upper digestive tract than first degree relatives of HNSCC patients who did not develop another carcinoma. A relative risk of 3.8 was calculated in this study. This study supports the hypothesis that apart from exogenous carcinogenic factors, endogenous constitutional factors play a role in the etiology of HNSCC.

Chapter 3.

Based on the "field cancerization" concept early markers of carcinogenesis should be demonstrable in a majority of the mucosal cells of the airways and the upper digestive tract, if a SPT will develop in that mucosa in the near future. Head and neck squamous cell carcinogenesis is considered to be a multistep process: repeated carcinogenic insults will ultimately lead to genetic alterations, resulting in selectively expanding malignant cells. In this process a crucial role can be ascribed to endogenous metabolic activation and detoxification processes of potential carcinogenic agents. Glutathione S-trasferases (GST) are important detoxifying enzymes. Utilizing antibodies

directed against different molecular subgroups of the GSTs insight can be obtained in quantitative local enzym concentrations at the mucosal level, and also at level of the serum. In chapter 3 of this thesis a review is presented about the current knowledge of the role of GSTs in head and neck carcinogenesis.

Chapter 4.

Comparison of archival tissue blocks of histopathological normal mucosa surrounding a head and neck tumour both of patients who ultimately developed a SPT and of patients who remained free of another carcinoma may give an indication whether expression of GSTs (π, μ and α) is of predictive value for the development of a SPT in the head and neck area. In such a study, it could be shown that patients who ultimately developed a SPT showed a significantly higher expression of GST π, μ and α in their non-cancer tissue blocks.

Clearly, it is desirable to assess mucosal alterations during the course of time. Unfortunately, it is not feasible to take biopsies at frequent intervals, as these are too harmful for patients. However, the use of cell scrapes of the mucosal lining offers an opportunity to carry out follow-up studies without discomfort for the patient. The expression of GST-π, μ and α was determined in exfoliated cells of HNSCC patients and comparable control individuals. Hopeful results were obtained. A significantly higher expression of GST-π and μ in the mucosa of HNSCC patients were seen. GST-α shows a more heterogenous expression pattern both among patients and among control individuals and therefore appears to be of less value as a single follow-up marker.

Chapter 5.

In another study the expression of cytokeratin 16, cytokeratin 19 and histo-blood group antigen H type 2-chain in exfoliated cells has been investigated by means of immunohistochemical analysis. For each of these antigens a significant difference in expression was found in the "healthy" mucosa of HNSCC patients (n=25) when compared to exfoliated cells obtained from the same mucosal locations in non-cancer individuals (n=10). Histo-blood group antigen H type 2-chain looks the most promising marker of this panel, as no overlap in expression level between patients and controls was seen. Moreover, a significant correlation between expression of this marker among cell scrapes of different locations of the head and neck area in a given person was found. This is in accordance with the "field cancerization" concept and makes this marker attractive, as its expression in scrapes from the mucosa of the easily accessable oral cavity can be considered to reflect alterations in the mucosa of the head and neck in general. The definitive predictive value of these markers, just as that of GSTs, has to be established in a large prospective study extending over many years.

Chapter 6.

Until now, no consensus exists whether p53 can be considered as an early marker of carcinogenesis. To evaluate whether the expression of p53 is of possible predictive value for the development of SPTs, p53 expression in tissue biopsies of histopathological normal mucosa, dysplastic mucosa and tumour biopsies from HNSCC patients, who ultimately developed a SPT and who remained free of carcinoma, was determined by means of immunohistochemical analysis. In addition, in part of the group of the patients, who developed a SPT, p53 expression was assessed in both the index tumour and the SPT. From this study it can be concluded that the expression of p53 does not appear to be of any value for the prediction of the development of a SPT in HNSCC patients.

Chapter 7.

Until now, no useful serum tumour marker for HNSCC is available. Serum proteinase level has been found to be a useful marker in various other malignant tumours. Moreover, recently hopeful results with this marker have been obtained in laryngeal squamous cell carcinoma patients. In this thesis the possible value of serum proteinase level, measured by different substrates has been studied. No significant difference in serum proteinase level between HNSCC patients and controls was found after correction for smoking and alcohol habits.

Chapter 8.

Another potential serum tumour marker which has recently been found to be of value in lung cancer patients, is Cyfra 21-1. Cyfra 21-1 is directed against a soluble fragment of cytokeratin 19. In contrast to the entire cytokeratin 19 molecule, this fragment is present in the serum. Although a substantial overlap in the serum levels of this marker between patients and control individuals was found, Cyfra 21-1 serum levels were significantly higher in HNSCC patients. In addition, curatively treated HNSCC patients showed a remarkable decrease of this parameter in their serum. This marker, therefore holds promise for screening for residual disease or a SPT.

Chapter 9.

In general, cancer incidence can be reduced by minimalization of the exposition to carcinogens. However, to what extent the incidence of SPTs in HNSCC patients can be reduced by stopping smoking and drinking alcohol, is debatable, because these people have been exposed to these metabolites for decades before their HNSCC appeared. Chemoprevention, defined as inhibition of the carcinogenic process before or during the preneoplastic period by farmacological intervention is an attractive supplemental approach to prevent the development of SPTs. N-acetylcysteine (NAC) is one of the currently tested chemopreventive agents in a large clinical

chemoprevention trial (the Euroscan study). NAC is applied as a chemopreventive agent, because of its anti-oxidative properties. One of the major limitations in chemoprevention research is the long follow-up time needed before definitive conclusions about the actual activity of an agent can be drawn. If good biomarkers for the prediction and monitoring of therapy response were available, many more chemopreventative agents could be tested in less time, with fewer patients. In chapter 9 of this thesis the possible value of glutathione levels in blood and exfoliated buccal mucosa cells, as biomarkers for the activity of NAC, are described. The thiol status in blood plasma and erythrocytes increased significantly during NAC therapy. Glutathione levels in exfoliated cells of the buccal mucosa, one of the sites at which SPTs may develop, yielded no useful parameter for therapy control. The utility of serum and erythrocyte glutathione levels as a biomarker for early detection of SPTs can be established only after definitive evaluation of this chemoprevention trial at the end of the nineties.

Conclusions

The prevalence of a positive family history for head and neck and/ or lung cancer is an independent risk factor for the occurrence of a SPT in the mucosa of the respiratory and upper digestive tract, including the oesophagus.

The studies which have been carried out to identify potential early markers for the development of a SPT at the the level of the mucosa at risk have resulted in several interesting markers. The best potential marker in exfoliated cells of the mucosal lining, at this moment, appears to be the histo-blood group antigen H type 2-chain. Lack in overlap of expression of this marker between patients and controls, as well as significant correlation in expression among cell scrapes of different locations of the head and neck area in a given person, make this marker of potential value for early detection of a SPT. Moreover, in about 20% of the tested patients this marker showed extremely low expression values. This percentage is in agreement with the percentage of patients, in which a SPT can be expected. However, the ultimate predictive value of potential early markers of carcinogenesis, like histo blood group H type 2-chain, has to be established in large prospective follow-up studies extending over many years.

At the serum level Cyfra 21-1 looks to be of potential value for monitoring radically treated HNSCC patients. However, it is essential for a predictive serum marker, that in case of recurrence or a SPT elevated serum levels appear before tumour can be detected by standard clinical examination and investigations. Again, large long term follow-up studies are needed to allow for the definitive assessment of the predictive value of serum tumour markers, like Cyfra 21-1, for the development of a SPT.

CHAPTER 11

SAMENVATTING

EN

CONCLUSIES

Samenvatting

Hoofdstuk 1.

Bij patiënten met een plaveiselcelcarcinoom uitgaande van het slijmvlies in het hoofd-hals gebied (hoofd-hals plaveiselcel carcinoom - HHPCC) komen frequent tweede primaire tumoren (TPT) voor in het slijmvlies van de luchtwegen en dat van de bovenste voedselweg, inclusief dat van de slokdarm. Men spreekt wel van het "field cancerization" concept, welke wordt toegeschreven aan een min of meer gelijke blootstelling van de betreffende slijmvliezen aan carcinogenen zoals tabak en alcohol metabolieten. De grote meerderheid van de TPT doet zich langer dan een half jaar na de behandeling van de eerste (index) tumor voor, met een constante frequentie van 2,8% per jaar. TPT hebben over het algemeen een slechte prognose, omdat zij of op ongunstige plaatsen - long of slokdarm - of in eerder behandeld gebied voorkomen. Vroege detectie en chemopreventie lijken op dit moment de beste opties ter vermindering van de morbiditeit en de mortaliteit ten gevolge van TPT. Beide strategieën zijn echter alleen uitvoerbaar bij die patiënten, die een hoog risico lopen om TPT te ontwikkelen. Over risicofactoren voor het ontwikkelen van TPT is echter weinig bekend. In dit proefschrift worden, met het uiteindelijke doel om patiënten met een hoog risico op een TPT in een vroeg stadium te kunnen identificeren, risicofactoren op drie niveaus beschreven, het constitutionele niveau, het niveau van het slijmvlies "at risk" en dat van het serum.

Hoofdstuk 2.

Epidemiologisch onderzoek werd verricht om inzicht te verkrijgen in hoeverre een belaste familie anamnese een risicofactor vormt voor het optreden van een TPT. Op basis van anamnestisch onderzoek werd vastgesteld, dat onder patiënten, bij wie zich een TPT ontwikkelde significant vaker een belaste familieanamnese voor hoofd-hals en/ of longkanker bestond in vergelijking tot patiënten, die minimaal 7 jaren na curatieve behandeling van hun HHPCC geen TPT hadden ontwikkeld. Uit deze resultaten kon berekend worden, dat een belaste familieanamnese als een onafhankelijke risico factor voor het ontwikkelen van een TPT beschouwd kan worden (Odds ratio: 3,8). Dit resultaat ondersteunt de hypothese dat bij het ontstaan van HHPCC exogene carcinogenen een rol spelen, maar dat tevens een genetisch bepaalde verhoogde gevoeligheid voor die carcinogenen van belang is.

Hoofdstuk 3.

Uitgaand van het "field cancerization" concept, zouden vroege markers van carcinogenese in een meerderheid van de cellen van het slijmvlies van de luchtwegen en de bovenste voedselweg aantoonbaar moeten zijn, wanneer zich in de toekomst een TPT in die mucosa gaat ontwikkelen. Omdat hoofd-hals carcinogenese als een meerstaps proces beschouwd kan worden, waarin door

carcinogenen geïnduceerde genetische veranderingen uiteindelijk leiden tot selectief groeiende maligne cellen, kan aan endogene metabole activatie en detoxificatie processen van exogene carcinogenen een belangrijke rol in dit meerstaps proces worden toegekend. Glutathione S-transferases (GST) zijn belangrijke enzymen in detoxificatie processen van potentiële carcinogene agentia. Gebruik van momenteel beschikbare antilichamen, gericht tegen verschillende moleculaire vormen van GSTs, kunnen inzicht verschaffen in kwantitatieve enzym concentraties in de lokale slijmvliezen en ook in serumspiegels. In hoofdstuk 3 van dit proefschrift wordt een overzicht gegeven van de meest recente inzichten in deze materie met betrekking tot hoofd-hals kanker.

Hoofdstuk 4.

Vergelijking van archief materiaal van histopathologisch niet afwijkende mucosa rondom een primaire hoofd-hals tumor van zowel patiënten bij wie zich een TPT ontwikkelde als van patiënten die tumor vrij bleven kan een initiële aanwijzing geven of antilichaam gemedieerde expressie van GSTs (π, μ en α) potentiële waarde heeft voor de voorspelling van het ontstaan van een TPT in het hoofd-hals gebied. In een op deze wijze uitgevoerd onderzoek werd in de mucosa van patiënten, die uiteindelijk een TPT ontwikkelden, een significant hogere GST π, μ en α expressie gezien.

Het herhaald nemen van biopten is echter belastend voor patiënten. Vervolgen van slijmvlies veranderingen in de tijd daarentegen is gewenst gedurende de follow-up. Het gebruik van celuitstrijkjes van het slijmvlies biedt wel de mogelijkheid op weinig belastende wijze vervolg-onderzoek uit te voeren. Teneinde meer inzicht te krijgen in de potentiële bruikbaarheid van antilichaam gemedieerde expressie van markers in uitgestreken cellen, werd de expressie van GST-π, μ en α vastgesteld in uitgestreken cellen van zowel hoofd-hals kanker patiënten als vergelijkbare controle individuen. Hoopvolle resultaten werden verkregen. Er werd een significant hogere expressie van GST-π en μ in de mucosa van kanker patiënten zien ten opzichte van gezonde controle individuen. GST-α laat een sterk heterogeen patroon binnen zowel patiënten als controles zien en lijkt in elk geval als singuliere marker van weinig waarde.

Hoofdstuk 5.

Een tweede studie ter validering van potentiële markers in celuitstrijkjes werd verricht gebruik makend van immunohistochemische analyse met behulp van monoclonale antilichamen gericht tegen de celskeletelementen cytokeratine 16 en cytokeratine 19, en tegen bloed groep antigeen H-type 2, een element van de celmembraan. De expressie van elk van deze antilichamen, op zich, liet een significant verschillende expressie zien tussen de "gezonde" mucosa van de oncologie patiënten (n=25) en vergelijkbare controlepersonen (n=10). Het bloed groep antigeen H-type 2 lijkt uit dit geteste panel antilichamen de marker met de meeste potentie voor vroege

detectie. Er bleek namelijk geen overlap in mate van expressie tussen patiënten en controle personen te bestaan. Bovendien correleerde de expressie van dit antilichaam wanneer celuitstrijkjes, verkregen van verschillende plaatsen binnen het hoofd-hals gebied werden vergeleken. Dit komt overeen met het veronderstelde "field-cancerization" concept voor het ontwikkelen van TPT in het hoofd-hals gebied en maakt deze marker attractief, omdat haar expressie in celuitstrijkjes uit bijvoorbeeld de goed toegankelijke mondholte als afspiegeling beschouwd kan worden van wat gaande is in de mucosa van het hoofd-hals gebied in het algemeen. Evenals voor de antilichamen gericht tegen GSTs, zal de uiteindelijke predictieve waarde van de expressie van deze differentiatie karakteristieken voor het ontstaan van TPT moeten worden vastgesteld in een grootschalige prospectieve studie.

Hoofdstuk 6.

Tot op heden bestaat geen consensus of p53 al dan niet als vroege marker van carcinogenese te beschouwen is. Met antilichaam onderzoek wordt hoofdzakelijk gemuteerd p53 gedetecteerd, daar het genproduct normalerwijze een halfwaarde tijd van 6 tot 30 minuten heeft, terwijl het eiwit afkomstig van het gemuteerde p53 gen enkele uren aantoonbaar aanwezig blijft. Om uit te maken of p53 expressie in biopsie materiaal eventuele voorspellende waarde heeft voor het ontstaan van een TPT werd aan de hand van archief materiaal de expressie van p53 in niet-afwijkende mucosa van patiënten met een HHPCC, in dysplatisch epitheel en in tumor biopten bekeken. In deze studie werden zowel patiënten met een primaire tong tumor, die uiteindelijk een TPT ontwikkelden, als patiënten, die gedurende minimaal 7 jaren na genezing van de eerste tumor vrij bleven van een carcinoom op de expressie van het p53 antigen beoordeeld. Ook werd bij een beperkt aantal patiënten naast de index tumor ook de tweede primaire tumor op p53 expressie onderzocht. Op grond van de resultaten van deze studie kan p53 niet als marker voor het optreden van TPT worden aangemerkt.

Hoofdstuk 7.

Tot op heden is geen serummarker voor de detectie van HHPCC beschikbaar. Verhoogde serum proteinase spiegels zijn beschreven voor verschillende maligne tumoren en een recent onderzoek bij larynx carcinoom patiënten liet hoopvolle resultaten zien. In aansluiting op die resultaten, wordt in dit proefschrift de mogelijke waarde van serum proteinase activiteit, gemeten met verschillende assays beschreven. Bij vergelijking van HHPCC patiënten met overeenkomstige controle individuen werd geen verschil in serum proteinase activiteit gevonden nadat correctie voor het gebruik van tabak- en alcoholproducten had plaatsgevonden.

Hoofdstuk 8.

Een andere recent met succes, bij longkanker patiënten, geteste serumtumormarker betreft de Cyfra 21-1 test. Cyfra 21-1 is gericht tegen een cytokeratine 19 fragment, dat, in tegenstelling tot het volledige cytokeratine 19, in serum wordt uitgescheiden. In long en hoofd-hals epitheel blijkt het aantal cytokeratine 19 positieve cellen toe te nemen bij oplopende maligniteits-graad. Hoewel een duidelijke overlap bestond tussen de Cyfra 21-1 expressie in serum van patiënten en vergelijkbare controle individuen, kwam Cyfra 21-1 in significant hogere concentraties voor in het serum van de HHPCC patiënten. Met name de waarneming dat Cyfra 21-1 spiegels significant daalden, nadat radicale tumorbehandeling had plaatsgevonden, maakt de Cyfra 21-1 test een hoopvolle additionele marker om HHPCC patiënten gedurende klinische follow-up op een recidief dan wel een TPT te screenen.

Hoofdstuk 9.

In het algemeen zou met het minimaliseren van de blootstelling aan carcinogenen kankerpreventie deels gerealiseerd kunnen worden. In hoeverre echter met het stoppen van roken en van het gebruik van alcohol het optreden van TPT kan worden teruggedrongen is discutabel, omdat deze mensen veelal decennia lang in hoge mate hebben blootgestaan aan deze metabolieten alvorens HHPCC optreedt. Inhibitie van het carcinogene proces vóór of tijdens de preneoplastische periode door middel van farmacologische interventie is daarom een aantrekkelijke aanvullende modaliteit voor de preventie van TPT. N-acetylcysteine is één van de momenteel in een klinische trial (Euroscan chemopreventie trial) geteste farmaca. N-acetylcysteine wordt toegepast als chemopreventicum vanwege de aan dit middel toegeschreven anti-oxidatieve eigenschappen. De vrije SH-groep van N-acetylcysteine zelf speelt daarin een cruciale rol, maar zeker zo belangrijk is de omzetting ervan in intracellulair gereduceerd glutathion, een belangrijke lichaamseigen stof met anti-oxidatieve eigenschappen. Omdat één van de grootste beperkingen van klinische chemopreventie studies de lange follow-up tijd is, alvorens de daadwerkelijke werkzaamheid van een chemopreventicum bekend wordt en de beschikbaarheid over biomarkers deze tijdsduur aanzienlijk zou kunnen bekorten, wordt in hoofdstuk 9 de mogelijke waarde van het meten van glutathion spiegels in bloed en uitgestreken wangslijmvlies cellen beschreven. De thiol status in bloedplasma en erythrocyten van de onderzochte patiënten nam significant toe onder N-acetylcysteine therapie. Glutathion bepalingen in uitgestreken cellen uit de mondholte, één van de doelorganen van dit chemopreventicum, leverden geen bruikbare parameters op voor betrouwbare therapie controle. De bruikbaarheid van de glutathion spiegels als biomarker voor het vroegtijdig voorspellen van het optreden van TPT kan echter pas worden vastgesteld na de definitieve evaluatie van deze chemopreventie studie eind jaren negentig.

Conclusies

Het voorkomen van een positieve familie anamnese voor hoofd-hals of longkanker is een onafhankelijke risicofactor voor het optreden van een TPT in het slijmvlies van de luchtwegen en de mondholte en de slokdarm.

Het verrichtte onderzoek ter identificatie van potentiële markers voor het ontwikkelen van een TPT op het niveau van het slijmvlies "at risk" heeft verscheidene interessante markers opgeleverd. De meest hoopvolle marker in uitgestreken cellen van het slijmvlies lijkt op dit moment het bloed groep antigen H-type 2. Het ontbreken van overlap in expressie van deze marker tussen geteste patiënten en controles en het bestaan van correlatie in expressie van deze celkarakteristiek in celuitstrijkjes van verschillende slijmvlies locaties in de luchtwegen en de bovenste voedselweg binnen één individu, geven deze marker potentie voor vroege detectie in de toekomst. Patiënten tonen een significant lagere expressie van dit antigen dan gezonde personen. Bovendien kwam het antigen bij circa 20% van de geteste patiënten in extreem lage waarden voor. Dit percentage komt overeen met het percentage patiënten, van wie op grond van eerder verricht epidemiologisch onderzoek verwacht mag worden, dat zij uiteindelijk een TPT zullen ontwikkelen. De werkelijke predictieve waarde van deze potentiële vroege markers zal echter uit grootschalige studies met lange follow-up duur moeten blijken. Eenmaal gevalideerde biomarkers kunnen vervolgens ook worden toegepast om de effectiviteit van chemopreventie op carcinogenese te vervolgen.

Op het niveau van het serum lijkt de Cyfra 21-1 test van potentiële waarde voor monitoring van patiënten met een curatief behandeld HHPCC. Essentieel daarvoor is echter dat deze marker in het serum in verhoogde concentratie verschijnt alvorens klinische detectie van een recidief danwel TPT mogelijk is. Ook hier is voor definitieve evaluatie een grootschalige prospectieve studie met lange follow-up duur noodzakelijk.

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Curriculum vitae

De auteur van dit proefschrift werd op 14 april 1966 geboren te Bussum.
In 1984 behaalde zij het eindexamen gymnasium aan het gemeentelijk gymnasium te Hilversum. In datzelfde jaar werd begonnen met de studie geneeskunde aan de Rijks Universiteit Utrecht. Het artsexamen werd in 1992 behaald, waarna gestart werd met het onderhavige promotieonderzoek op de afdeling Keel-, neus- en oorheelkunde, Vrije Universiteit te Amsterdam (hoofd: Prof. Dr. G.B. Snow).
Sinds 1 augustus 1995 is zij werkzaam als arts-assistente op de afdeling nucleaire geneeskunde van het Academisch Ziekenhuis te Utrecht (opleider: Dr. P.P. van Rijk).