Glutathione S-transferases and head and neck cancer

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Glutathione S-transferases and head and neck cancer

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Glutathione S-transferases and head and neck cancer

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Aan mijn ouders

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LIST OF ABBREVIATIONS

BHNL	benign head and neck lesions
°C	degrees Celcius
CI	Confidence Interval
CYP	cytochrome P 450
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked inmuno sorbent assay
EPHX	epoxide hydrolase
GSH	glutathion
GST	glutathione S-transferase (enzyme)
GST	<i>glutathione S-transferase (gene)</i>
H	hours
HN	all sites of head and neck malignancies
HNSCC	head and neck squamous cell carcinoma
Ile	isoleucine
LR	likelihood ratio
Min	minutes
N	number of patients or controls
n.s.	not specified
OR	Odds Ratio
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
SCC	squamous cell carcinoma
SSCP	single strand conformational polymorphism
V	volts
Val	valine
χ^2	chi square

INTRODUCTION TO THE CHAPTERS

Head and neck cancer is the sixth most common cancer in the world. The definition of head and neck cancer in this thesis includes squamous cell carcinomas of the oral cavity, pharynx, and larynx. Other head and neck tumor localisations and other histopathological types have been excluded from this study. The risk of head and neck squamous cell carcinoma (HNSCC) is strongly associated with smoking of tobacco and consumption of alcohol, both of which may be responsible for a considerable load of (pre)carcinogens. Metabolism and detoxification of such exogenous harmful compounds often occurs by phase I and phase II enzymes such as cytochrome P450 (CYP), microsomal epoxide hydrolase (EPHX) and glutathione S-transferase (GST).

Because genetic polymorphisms exist in the cytochrome *P4501A1*, glutathione S-transferase *M1*, *T1*, *P1* and *EPHX* genes, that may lead to complete absence (GSTM1 and GSTT1) or altered (CYP1A1, GSTP1 and EPHX) enzyme activity, individuals bearing such genetic polymorphisms and therefore lacking a (partially) normally functional enzyme, may be at higher risk of developing HNSCC. Therefore, next to smoking and drinking habits, it is believed that also genetic factors may contribute to the risk for HNSCC. The aim of this study is to determine whether genetic polymorphisms of the CYP, EPHX or GST detoxification enzyme systems influence the individual susceptibility for HNSCC.

Chapter 1 gives an overview of studies on genetic polymorphisms in biotransformation enzymes in relation to head and neck cancer. In chapter 2, genetic polymorphisms in *CYP1A1*, *GSTM1* and *GSTT1* are evaluated in relation to HNSCC susceptibility. Several years later, we discovered that demographic variation may influence this outcome. In chapter 3 we again evaluated the *GSTM1*, *GSTT1* and *GSTP1* genotypes, but now in control populations recruited from both the Nijmegen and Maastricht regions. Chapter 4 describes the quantification of blood plasma GSTP1-1 enzyme levels in patients with HNSCC to find out whether these values are suitable as tumor markers to monitor progression of HNSCC. Polymorphisms of the *GSTP1* gene in relation to HNSCC are investigated in chapter 5. A study on the association between plasma GSTP1-1 enzyme levels and the *GSTP1* genotype is presented in chapter 6. A study on the number of polymorphisms in the genes studied in this thesis in relation to HNSCC susceptibility, is presented in chapter 7. Chapter 8 is an international collaborative meta analysis on polymorphisms of the *GSTM1*, *GSTT1*, *GSTP1* and *CYP1A1* genes in relation to HNSCC susceptibility. The summary recapitulates the contents of the individual chapters.

In this thesis the genotypes and the enzymes of glutathione S-transferases are discussed. For a better distinction between these two, the genotypes or gene polymorphisms are printed in italic (e.g. *GSTP1*), whereas the enzymes are in normal print (e.g. GSTP1-1).

1

CHAPTER

GENERAL INTRODUCTION: GENETIC POLYMORPHISMS IN GLUTATHIONE S-TRANSFERASES AND HEAD AND NECK CANCER SUSCEPTIBILITY; A REVIEW

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Summary

Background The etiology of head and neck squamous cell carcinoma (HNSCC) may be closely related to toxification-detoxification processes of products that appear during smoking and/or alcohol consumption. The individual susceptibility to HNSCC may be determined by alterations of the detoxification process due to genetic polymorphisms of glutathione S-transferases (GSTs).

Methods Through an extensive search in PubMed and reference backtracking a selection was made of all English publications that evaluated one or more GST polymorphisms in relation to HNSCC.

Results A review is given on all literature to this date where one or more GST polymorphisms are evaluated in a HNSCC population. The site, size, race, and Odds Ratio are evaluated in the case and control populations.

Conclusions The studies on GST polymorphisms and HNSCC susceptibility published so far have reported contradicting results. Possible explanations in the variations of study results are given. Carefully designed, population-based studies will be necessary.

Introduction

The global incidence of head and neck squamous cell carcinoma (HNSCC) is approximately 5%. In several regions, the incidence of HNSCC is higher, which may be related to the consumption of specific substances, toxic or carcinogenic for head and neck tissues. Several individuals at risk for HNSCC can be readily identified by their excessive consumption of alcohol or tobacco, where it is also known that synchronous consumption of both substances increases the toxicity of the other (1). The incidence of HNSCC in the high-risk alcohol and tobacco consumer-group is significantly higher compared to non-consumers. Within this high-risk group however, the majority of the individuals ultimately do not develop HNSCC and therefore seem to be not susceptible to these hazardous substances. The question then arises: what is the explanation for this phenomenon; why does a person with a moderate or high intake of alcohol and tobacco contract HNSCC, whereas another person with comparable intake of both substances does not? It is hypothesized that this may be due to inter-individual differences in genetic susceptibility towards alcohol or tobacco.

One of the first steps in the initiation of head and neck cancer may be the binding of reactive metabolites of alcohol or tobacco smoke constituents to the DNA of mucosal cells. This process is strongly influenced by phase I and phase II enzymes which are involved in the toxification or detoxification of ingested compounds. For instance many polycyclic aromatic hydrocarbons (PAHs), chemicals that are present in large amounts in tobacco smoke (2), after ingestion may be first activated by phase I enzymes such as cytochromes P450 (CYP) or epoxide hydrolases (EPHX) to become potential carcinogens, which subsequently need to be detoxified by phase II enzymes such as the glutathione S-transferases (GSTs). An imbalance between the phase I and phase II drug metabolizing or detoxification enzymes may contribute to the individual susceptibility to HNSCC (see diagram 1).

Diagram 1 Schematic presentation of the detoxification of carcinogens by phase I and phase II enzymes (a. and b. respectively). (PAH: polycyclic aromatic hydrocarbons)

As a result of conjugation with glutathione, catalyzed by the glutathione S-transferases, the potential carcinogens are eliminated and DNA or other important biomolecules are protected against damage or adduct formation (3-6). In humans, the GST enzymes can be divided into four main classes: Alpha (GSTA), Mu (GSTM), Pi (GSTP) and Theta (GSTT). Each class consists of one or more isoenzymes with different but sometimes overlapping substrate specificities (5,6). At present genetic polymorphisms have been demonstrated in *GSTM1*, *GSTT1*, *GSTA1* and *GSTP1* (6-11).

Many polymorphisms in genes coding for risk modulating proteins or enzymes have been identified recently which appear to be related to the risk for a number of diseases. Therefore, it seems important to investigate whether the differences in susceptibility towards alcohol and tobacco as described above, can be attributed to genetic variations in systems that are involved in the detoxification of alcohol or tobacco-related carcinogens. Head and neck tissues, which are often exposed to alcohol or tobacco smoke, are protected by enzyme systems such as the glutathione S-transferases.

Glutathione and glutathione S-transferase system

Glutathione (GSH) is an intracellular thiol that neutralizes precarcinogenic and highly reactive electrophilic compounds, a process most commonly taking place by enzymatic conjugation catalyzed by glutathione S-transferases (GSTs). GSH is produced mainly in the liver (hepatocytes), and to a lesser extent in other cell types such as head and neck mucosal cells, by coupling of the amino acids glycine, cysteine and glutamic acid (12). When the GSH production is reduced or GSH is depleted, more electrophilic compounds may freely circulate and may cause damage to DNA or other important biomolecules. Since detoxification by GSH is strongly coupled to the GST enzymes, also a reduction or deficiency of GST isoforms in a similar way may result in higher levels of (DNA) damage.

Any factor that may disturb the metabolic balance of detoxification, can result in increased levels of carcinogenic compounds and in an increased risk for developing cancer. In this way GSH and GSTs may regulate the ability of each individual to metabolize environmental carcinogens.

Genetic polymorphisms, mostly resulting in significant reduction of corresponding enzyme activities, have been described in *GSTM1*, *GSTT1*, *GSTP1*, and *GSTA1* (8-11). For *GSTM1* and *GSTT1*, null polymorphisms may be present, resulting in complete absence of enzyme activity. The fact that *GSTM1* and *GSTT1* null genotypes in Caucasians are common (20-50%), implicates that their co-occurrence is also relatively common (approximately 10%), which suggests that their possible contribution to the susceptibility to HNSCC could be of considerable importance.

The polymorphism in *GSTP1* may result in considerable loss of the corresponding GSTP1-1 enzyme activity (13,14). The polymorphism in *GSTA1* probably is also widespread and may have significant consequences for the corresponding enzyme activities but it has hardly been studied yet since it was described only recently (11).

The studies performed so far have provided the following information: *GSTM1* and *GSTT1 null* genotype frequencies are highly variable in the HNSCC case populations which have been studied (range 43 - 78 % for *GSTM1 null*, and 9 - 59 % for *GSTT1 null*; see Table 1 and 2 respectively). However, similar variable frequencies are also seen in the control populations studied by the same researchers. When patients are selected according to HNSCC tumor site, also similar varying *GST null* frequencies are reported.

HNSCC susceptibility of individuals with the *GST null* genotype however, appears to be similar in most studies with non-*null* genotype individuals. However, it may be hypothesized that susceptibility is higher when interaction of GSTs with other factors such as tobacco smoking and/or alcohol consumption is considered. It appears however, that the contribution of the *GST null* genotypes in the susceptibility of HNSCC is small or even absent, especially when compared to the main risk factors described before (tobacco, alcohol).

A more detailed review on the literature with respect to studies on genetic polymorphisms in GSTs in relation to HNSCC is provided below.

GST genotypes and head and neck cancer

***GSTM1* genotype**

The *GSTM* enzyme family exists in different isoforms, where 5 have been isolated in humans. Specifically the *GSTM1* enzyme may be of interest for HNSCC, since it detoxifies metabolites of benzo [a] pyrene and other epoxides, which are tobacco related carcinogens (6). The corresponding gene *GSTM1* displays three different genotypes, two functional (*GSTM1 A* and *GSTM1 B*) and one nonfunctional genotype (*GSTM1 null*) have been identified. In Caucasian populations approximately 50% lacks the functional genotypes *GSTM1 A* or *GSTM1 B* (see Table 1). The *GSTM1 null* genotype frequencies however, do vary in and between countries and races, which makes it difficult for instance to compare the results of studies on Caucasians with those of other racial populations. Many studies reported a high frequency of *GSTM1 null* for other sites of cancer than head and neck. Considering the studies on HNSCC, as shown in Table 1, this seems not the case.

Kihara et al. (15) in Japanese smokers found that the *GSTM1 null* genotype was not associated with laryngeal cancer even though there was a link to the smoking history. In smokers with non-laryngeal HNSCC, however, *GSTM1 null* genotype was observed more frequently (62.7%) as compared to controls (48.7%) (OR 1.8; CI 1.0-3.0)(15). The same authors suggest that *GSTM1 null* genotype also may be a potential risk factor for HNSCC depending on smoking history.

Several research groups found no significant association between *GSTM1 null* genotype and any head and neck tumor localisation (16-22). Other researchers described an increased risk for HNSCC related to the *GSTM1 null* genotype (23-25). Nair et al. (26) in an Indian beetle quid or tobacco consuming population, found a significant higher frequency of *GSTM1 null* genotype in patients with oral leukoplakia, a possible precursor of oral squamous cell carcinoma. Cabelguenne et al. (27) reported no

Table 1. GSTM 1 null genotype frequencies in control and HNSCC patient populations

Author	Site	GSTM1 null Controls % of total (n)	GSTM1 null Cases % of total (n)	Population	Country	Odds Ratio (95% CI)
No association GSTM1 null genotype vs HNSCC						
Cabelguenne et al. (27)	HN	46.5 (264)	49.6 (162)	n.s.	France	1.0 (0.6-1.5)
Coutelle et al. (24)	Oropharynx	48.0 (37)	62.0 (21)	Caucasian	France	1.8 (0.5-6.2)
Gronau et al. (55)	HN	48.0 (60)	64.0 (83)	n.s.	Germany	n.s.
Deakin et al. (16)	Oral	54.7 (128)	55.0 (34)	Caucasian	UK	n.s.
Gonzalez et al. (51)	HN	54.0 (200)	58.0 (75)	Caucasian	Spain	1.0 (0.5-2.0)
Hung et al. (18)	Oral	57.7 (123)	58.5 (41)	Chinese mixed	Taiwan	n.s.
Jahnke et al. (30)	HN	52.0 (216)	56.2 (269)	Caucasian	Germany	n.s.
Jahnke et al. (29)	Larynx	57.7 (145)	50.8 (169)	Caucasian	France	0.9 (0.5-1.5)
Jourenkova et al. (36)	HN	52.3 (172)	47.9 (129)	Caucasian	France	0.8 (0.4-1.5)
	Oropharynx	52.3 (172)	44.8 (67)	Caucasian	France	1.3 (0.6-2.6)
	Oral	52.3 (172)	52.0 (50)	Caucasian	Japan	1.8 (1.0-3.3)
	Pharynx	46.3 (147)	58.7 (92)	Japanese	Japan	1.0 (0.5-1.8)
Katoh et al. (39)	Oral	48.7 (174)	48.1 (158)	Japanese	Japan	1.2 (0.8-2.0)
Kihara et al. (15)	Larynx	53.4 (219)	58.2 (126)	Caucasian	Germany	1.0 (0.7-1.5)
Matthias et al. (31)	Oral	53.4 (219)	57.0 (272)	Caucasian	Germany	0.2 (0.1-0.5)
	Larynx	53.4 (219)	57.4 (398)	Caucasian	Germany	1.0 (0.6-1.6)
	HN	46.5 (129)	46.2 (147)	Caucasian	USA	1.1 (0.6-2.0)
	HN	50.6 (164)	49.0 (145)	Japanese	Japan	1.2 (0.8-2.0)
	HN	45.0 (168)	50.0 (109)	Caucasian	USA	1.0 (0.7-1.5)
	HN	51.7 (207)	50.8 (185)	Caucasian	The Netherlands	1.1 (0.6-1.8)
	HN	51.7 (207)	50.0 (76)	Caucasian	The Netherlands	0.9 (0.5-1.6)
	HN	51.7 (207)	53.4 (73)	Caucasian	The Netherlands	1.0 (0.6-1.7)
	Oral / oropharynx	51.7 (207)	51.0 (135)	Caucasian	USA	1.3 (0.4-4.8)
	Larynx	51.0 (135)	49.0 (98)	Indian	India	0.4 (0.2-1.0)
	Oral	33.3 (60)	43.0 (100)	Japanese	Japan	n.s.
	Oral	42.0 (100)	n.s.	Caucasian	UK	n.s.
Park et al. (17)	Oral	n.s.	n.s.			
Sreelekha et al. (57)	Oral	n.s.	n.s.			
Tanimoto et al. (21)	Oral	n.s.	n.s.			
Worral et al. (19)	Oral	n.s.	n.s.			
Association GSTM1 null genotype vs HNSCC						
Buch et al. (53)	Oral	24.0 (450)	49.2 (297)	Indian	India	3.2 (2.4-4.3)
Cheng et al. (54)	HN	42.9 (315)	53.1 (162)	Mixed	USA	1.5 (1.0-2.2)
Coutelle et al. (24)	Larynx	48.0 (37)	78.0 (18)	Caucasian	France	4.7 (1.0-21.8)
Hanna et al. (50)	Larynx	50.0 (20)	80.0 (20)	n.s.	USA	n.s.
Hong et al. (49)	Larynx	52.4 (63)	68.3 (82)	Korean	Korea	3.5 (1.3-9.8)
Jourenkova et al. (23)	Larynx	52.3 (172)	60.5 (129)	Caucasian	France	1.6 (1.0-2.8)
Kihara et al. (15)	Larynx	48.7 (474)	62.7 (76)	Japanese	Japan	1.8 (1.0-3.0)
Sato et al. (56)	Non larynx	45.1 (142)	64.8 (100)	Japanese	Japan	2.2 (1.4-3.6)
Trizna et al. (25)	Oral	48.0 (42)	68.0 (186)	Mixed	USA	2.4 (1.2-4.7)

Patients or controls in group. HN = all sites of head and neck malignancies. CI = confidence interval

difference in frequency of *GSTM1* in a HNSCC total group or several subgroups, except for a subgroup of laryngeal cancer patients where an Odds Ratio of 2.6 (range 1.6-6.1) was found for patients with the *GSTM1 null* genotype.

***GSTT1* genotype**

Similar to research on the *GSTM1* genotype, the *GSTT1* gene is of interest because of the capacity of the corresponding *GSTT1-1* enzyme to detoxify alkyl halogenides, ethylene oxide, and other low molecular weight metabolic products. *GSTT1* also displays a genetic polymorphism resulting in a *GSTT1-1 null* phenotype being fully inactive. Since the detoxification of many toxic low molecular weight compounds may be deficient in individuals with the *GSTT1 null* genotype, such an individual may be potentially at risk. The *GSTT1 null* genotype occurs in approximately 20% of a normal Caucasian population. In other racial populations, this frequency may vary considerably as reported by Nelson et al. (28). For instance Hung et al. (18) in a Taiwanese study described *GSTT1 null* genotype frequencies of 52.9% and 58.5% in controls and cases respectively (see Table 2).

In contrast, there appears no racial difference in the data of Hung et al. (18) on *GSTM1 null* genotype rates compared to those of other investigators (see Table 1). However, in accordance with most results by other groups (see Table 2), Hung et al. did not find association of *GSTT1 null* genotype and HNSCC susceptibility. Furthermore, it is surprising that Jahnke et al. and Matthias et al. (29-31), who used overlapping patient and control samples, did reach different conclusions on the *GSTT1 null* genotype as a factor of HNSCC susceptibility.

There appears to be an interaction with the degree of tobacco smoke exposure in several studies. Olshan et al. (32) analysed tobacco users and non-users and revealed no indication of increased risk among moderate users with the *GSTT1 null* genotype. However *GSTT1 null* genotype individuals having smoked more than 40 pack years showed a higher OR compared to individuals with a functional gene. Also Hamel et al. (33) found a significant increase in OR for HNSCC in *GSTT1 null* light smoking individuals (OR 6.53; CI 2.25-19.0).

***GSTP1* genotype**

The enzyme *GSTP1-1*, encoded by the *GSTP1* gene, is also involved in the detoxification of tobacco related carcinogens (6). *GSTP1* displays two variant genotypes *GSTP1 AB* and *GSTP1 BB* next to the most common *GSTP1 AA* genotype. In a normal control population, the frequency of the *GSTP1 AA* genotype ranges from approximately 40-70%. The variant *GSTP1* genotypes show a decreased specific activity and affinity for substrates of the corresponding *GSTP1-1* enzyme, since the polymorphism results in an amino acid substitution near the substrate binding site area of the enzyme (8). Since the *GSTP1 BB* genotype occurs only in approximately 10% of the population, the two variant genotypes *GSTP1 AB* and *GSTP1 BB* have been combined in Table 3. As seen for *GSTT1*, for *GSTP1* also a strong correlation with race was found. Morita et al. (34) in a Japanese population found significant lower frequencies of the variant *GSTP1 AB* and *GSTP1 BB* genotypes for controls and cases as compared to Caucasian study groups. The genotype frequencies in another Japanese population with HNSCC described by Katoh et al. (35) are comparable to the frequencies found by other

Table 2. *GSTT1* null genotype frequencies in control and HNSCC patient populations

Author	Site	<i>GSTT1</i> null Controls	<i>GSTT1</i> null Cases	Race	Country	Odds Ratio (95% CI)
		% of total (n)	% of total (n)			
No association <i>GSTT1</i> null genotype vs HNSCC						
Buch et al. (53)	Oral	12.3 (450)	18.3 (297)	Indian	India	1.6 (1.0-2.6)
Cabelguenne et al. (27)	HN	19.4 (264)	18.1 (162)	n.s.	France	1.0 (0.6-1.7)
Deakin et al. (16)	Oral	18.5 (129)	11.8 (34)	Caucasian	UK	n.s.
Hanna et al. (50)	Larynx	20.0 (20)	15.0 (20)	n.s.	USA	n.s.
Hong et al. (49)	Larynx	36.5 (63)	57.3 (82)	Korean	Korea	1.8 (0.7-4.8)
Hung et al. (18)	Oral	52.9 (123)	58.5 (41)	Chinese mixed	Taiwan	1.2 (0.6-2.5)
Jourenkova et al. (23)	Larynx	15.7 (172)	19.4 (129)	Caucasian	France	1.4 (0.7-2.9)
Jourenkova et al. (36)	Pharynx	15.7 (172)	18.0 (50)	Caucasian	France	1.4 (0.6-3.7)
Kato et al. (39)	Oral	51.0 (147)	47.7 (92)	Japanese	Japan	0.7 (0.4-1.2)
Matthias et al. (31)	Larynx	22.2 (203)	19.4 (263)	Caucasian	German	0.9 (0.5-1.4)
	Oropharynx	22.2 (203)	27.7 (119)	Caucasian	German	1.5 (0.9-2.5)
	HN	22.2 (203)	22.0 (382)	Caucasian	German	n.s.
McWilliams et al. (52)	HN	18.3 (109)	16.9 (142)	Caucasian	USA	0.9 (0.5-1.5)
Olshan et al. (32)	HN	13.0 (168)	17.0 (109)	Caucasian	USA	1.4 (0.7-2.7)
Oude Ophuis et al. (20)	HN	20.3 (207)	19.5 (185)	Caucasian	The Netherlands	1.1 (0.6-1.7)
	Oral/oropharynx	20.3 (207)	19.7 (76)	Caucasian	The Netherlands	1.0 (0.5-2.0)
	Larynx	20.3 (207)	20.5 (73)	Caucasian	The Netherlands	1.0 (0.5-1.9)
Sreelekha et al. (57)	Oral	8.3 (60)	18.4 (98)	Indian	India	2.5 (0.3-21.7)
Trizna et al. (25)	HN	36.0 (42)	45.0 (186)	Mixed	USA	1.5 (0.7-3.0)
Worral et al. (19)	Oral	n.s.	n.s.	Caucasian	UK	n.s.
Association <i>GSTT1</i> null genotype vs HNSCC						
Cheng et al. (54)	HN	17.5 (315)	32.7 (162)	Mixed	USA	2.3 (1.4-3.6)
Hamel et al. (33)	HN	20.0 (101)	9.0 (70)	Mixed	Canada	2.5 (1.1-5.9)
Jahnke et al. (29,30)	Larynx	12.4 (145)	21.3 (169)	Caucasian	Germany	n.s.
Jourenkova et al. (36)	Oral	15.7 (172)	22.4 (67)	Caucasian	France	2.4 (1.0-5.5)
	HN	15.7 (172)	21.5 (121)	Caucasian	France	2.0 (1.0-4.0)

n.s. not specified, n = total number of patients or controls in group, HN = all sites of head and neck malignancies, CI = confidence interval

researchers in caucasian populations. This is however not so for their controls. All other studies were performed on Caucasian populations and show comparable frequency percentages.

Katoh et al. (35) observed the frequency of *GSTP1* AB/BB genotypes to be significantly higher in never smoking oral cancer patients than control individuals (OR 2.78; CI 1.1-7.5). This suggests that non-smoking individuals with *GSTP1* AB or BB genotype have a higher susceptibility for oral cancer.

Jourenkova et al. (36) reported no interaction of the *GSTP1* gene and oral or pharyngeal cancers separately, however, when these two patient groups were combined, only a borderline significant increase in risk of developing cancer of the oral cavity and pharynx was found (OR 1.6; CI 1.0-2.8). Matthias et al. (37) suggest that different sites of HNSCC may be associated with different GST polymorphisms, since the *GSTP1* AB/BB genotypes show lower frequencies in patients with laryngeal, but not with oropharyngeal malignancies.

Discussion

The reason for the variable results with respect to the genetic polymorphisms in GSTs and their association with HNSCC, as found in the many studies summarized in the Tables 1-3, is not clear. Possibly the effect of one variant genotype on HNSCC susceptibility is too weak. Perhaps the occurrence of more than one genetic polymorphism in phase I and/or phase II enzymes, acting as a cascade, will have more effect and may significantly influence individual susceptibility. Many conflicting reports on the cancer susceptibility of GST genotypes have been published in the past decades. Most of these studies have been conducted for other localisations than head and neck cancer. Possible explanations in such variations in study results will be given.

Several reports describe patients with *null* genotypes of *GSTM1* and/or *GSTT1* who had an increased oral cancer risk compared with individuals who had non-*null* genotypes of both *GSTM1* and *GSTT1*, suggesting that there are gene-gene and gene-environment interactions in the development of oral cancer (18). The combined *GSTM3* (AB or BB) and *GSTM1* *null* genotype conferred a 4-fold risk of laryngeal cancer as compared with the combined *GSTM3* AA and *GSTM1* positive genotypes (38). However, the effect of *GSTM3* (AB or BB) genotype was similar among individuals with *GSTM1* positive or *GSTM1* *null* genotypes (38).

Combined *null* genotypes of *GSTM1* and *GSTT1* prevailed in 60.2% of Indian cases with oral leukoplakia whereas no individuals with this combination were detected in a control group (26). This is a striking difference, which however may be due to racial differences or due to the use of different methods for the isolation of DNA, since control and patient material in this study was processed completely different (26).

Coutelle et al. (24) found that alcoholics (defined as individuals consuming more than 100g ethanol per day during at least 10 years) with the *GSTM1* *null* genotype who were also homozygous for a polymorphism in alcohol dehydrogenase (ADH), denoted as

Table 3. *GSTP1* genotype (*GSTP1* AB and *GSTP1* BB) frequencies in control and HNSCC patient populations

Author	Site	<i>GSTP1</i> AB/BB Controls	<i>GSTP1</i> AB/BB Cases Population		Country	Odds Ratio (95% CI)
		% of total (n)	% of total (n)			
No association of <i>GSTP1</i> AB/BB genotype and HNSCC						
Cabelguenne et al. (27)	HN	53.0 (264)	45.0 (162)	n.s.	France	2.8 (0.9-8.1)
Jahnke et al. (58)	Larynx	46.9 (216)	51.5 (n.s.)	Caucasian	Germany	n.s.
Jourenkova et al. (38)	Larynx	50.0 (172)	51.9 (129)	Caucasian	France	1.1 (0.7-2.0)
Jourenkova et al. (36)	Oral	50.0 (172)	58.2 (67)	Caucasian	France	1.5 (0.8-3.0)
	Pharynx	50.0 (172)	58.0 (50)	Caucasian	France	1.7 (0.8-3.4)
Kelders et al. (48)	HN	49.0 (25)	58.6 (51)	Caucasian	The Netherlands	
Matthias et al. (37)	Larynx	47.2 (180)	51.5 (260)	Caucasian	Germany	0.5 (0.2-1.0)
Matthias et al. (37)	Oropharynx	47.2 (180)	62.5 (120)	Caucasian	Germany	0.8 (0.4-1.4)
McWilliams et al. (52)	HN	53.2 (124)	58.9 (146)	Caucasian	USA	1.3 (0.8-2.0)
Morita et al. (34)	Larynx	31.1 (164)	18.9 (69)	Japanese	Japan	n.s.
	Pharynx	31.1 (164)	26.6 (45)	Japanese	Japan	n.s.
	HN	31.1 (164)	24.9 (145)	Japanese	Japan	n.s.
Olshan et al. (32)	HN	60.0 (168)	63.0 (109)	Caucasian	USA	1.2 (0.8-1.9)
Oude Ophuis et al. (47)	Oral/ oropharynx	56.1 (285)	53.5 (114)	Caucasian	The Netherlands	0.8 (0.4-1.6)
	Larynx	56.1 (285)	49.0 (100)	Caucasian	The Netherlands	0.9 (0.5-1.8)
	HN	56.1 (285)	50.6 (235)	Caucasian	The Netherlands	1.0 (0.6-1.7)
Association of <i>GSTP1</i> AB/BB genotype and HNSCC						
Jahnke et al. (58)	Oropharynx	46.9 (216)	62.5 (n.s.)	Caucasian	Germany	n.s.
Jourenkova et al. (36)	HN	50.0 (172)	58.7 (121)	Caucasian	France	1.6 (1.0-2.8)
Kato et al. (35)	Oral	23.8 (22)	37.3 (83)	Japanese	Japan	1.9 (1.0-3.6)

n = total number of patients or controls in group, n.s. = not specified, HN = all sites of head and neck malignancies, CI = confidence interval

ADH_3^1/ADH_3^1 which is associated with a rapid generation of acetaldehyde, had at least an 80% greater risk of developing laryngeal cancer than alcoholics who were *GSTM1* positive and who were not bearing the ADH_3^1/ADH_3^1 genotype.

Individuals with concurrent lack of both functional *GSTM1* and *GSTT1* genes had a tendency for a higher risk of laryngeal cancer as compared with those individuals having at least one of the above mentioned functional genes (OR 2.0; CI 0.8-5.2), and had an almost a 3-fold higher risk (OR 2.7; CI 1.0-7.4) when compared with individuals having both functional genes (23). These data suggest that a combination of genetic polymorphisms in detoxification enzymes, either phase I or phase II, could modulate the risk for malignant degeneration.

There are also studies that have shown an association between smoking and *null* genotype. Kihara et al. (15) found more individuals bearing the *GSTM1 null* genotype in the smoking patient group than in the healthy control group (56.7% vs 48.5%), whereas there was an equal distribution in gender. The same authors also described a smoking (n=128) and non-smoking (n=30) HNSCC group, and found a higher frequency of *GSTM1 null* genotype in the former group. HNSCC patients usually are smokers, and the higher frequency of *GSTM1 null* genotype in this group may suggest a role for *GSTM1* in the detoxification of tobacco smoke constituents.

However, associations found are not straightforward, since Park et al. (17) observed no association between the prevalence of *GSTM1 null* genotype and the levels of smoking or alcohol consumption in the HNSCC cases, whereas Jourenkova et al. (23) found that the *GSTM1 null* genotype was associated with an increased risk of larynx cancer among individuals who smoked 20 cigarettes/day or less but not among more heavier smokers. No interaction was found between *GSTM1 null* genotype and duration of tobacco consumption. For the *GSTT1 null* genotype, Jourenkova et al. (23) found the opposite result; no interaction between levels of tobacco consumption, but limited effect by long term smokers. Katoh et al. (39) found no effect of *GSTM1* and *GSTT1 null* genotypes on oral cancer in combination with smoking or drinking. The *GST* genotype of an individual may determine his smoking behavior in a similar way as the *ADH* genotype may influence alcohol consumption.

Differences in the way the *GST* system is assessed may explain part of the conflicting reports on the susceptibility of *GSTs*. There are studies that evaluated phenotypic expression of *GSTs* in relation to HNSCC (40-46), whereas others evaluated the genetic polymorphism (15,20,23,31,36-38,47) or a combination of both (48).

The collection of cell material for genotyping is different in the various studies. DNA isolation from peripheral lymphocytes/leukocytes (18,20,23,25,36,38,47-49) is the method of choice for genotyping. DNA isolation from tumor tissue, and especially paraffin embedded tissue (26), may not be as reliable due to possible mutations that may have occurred during carcinogenesis. Also, the use of DNA extracted from tissues processed in a different way, such as exfoliated (buccal mucosa) cells in the control group and paraffin embedded tissue in the patient group, as performed by Nair et al. (26) may introduce artefacts.

The moment of patient sampling (before, during, or after treatment) may influence the phenotypic, and to a much lesser extent the genotypic expression of the GST system, either locally or systemically, since it is well known that such therapies may generate new mutations. Results obtained from previously untreated patients therefore may be different compared to those having received earlier radio- or chemo-therapy. No mention of this has been made in any study published so far.

Note the extreme small groups of cases as well as controls by Hanna et al. (50), which makes it difficult to correctly interpret the results.

Combination studies with other than GST genes make interpretation of results more difficult and not transparent, for instance evaluation of GSTs with other genes such as p53 and p16 (22). However, it should be realized that GST enzymes most likely do not act as a stand alone system for carcinogenesis but probably act together with other enzymes or proteins.

In conclusion, we found that most studies conducted on the association between genetic polymorphisms in GSTs and the risk for HNSCC are difficult to interpret. Several factors, such as small sizes of control and patient populations studied, variations in the methods used, interracial-, demographic- or other differences in the patient and control populations studied may contribute to the variable results obtained so far. The finding that high consumption of alcohol and tobacco is a predominant factor for HNSCC has invariably been shown in most studies, whereas interaction with genotype could be demonstrated only now and then. New studies with much larger patient and control populations, or a meta analysis where all these contributing factors are critically evaluated and discussed would be highly desirable.

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2

CHAPTER

GLUTATHIONE S-TRANSFERASE M1 AND T1 AND CYTOCHROME P4501A1 POLYMORPHISMS IN RELATION TO THE RISK FOR BENIGN AND MALIGNANT HEAD AND NECK LESIONS

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Abstract

Background Susceptibility to head and neck cancer in a particular individual may depend in part on the metabolic balance between phase I enzymes such as cytochromes P450 (CYPs) and phase II enzymes such as glutathione S-transferases (GSTs). Genetic variability in CYP and GST isoenzymes may contribute to individual differences in susceptibility to chemical carcinogenesis. *GSTM1* and *GSTT1* null genotypes as well as polymorphic variants in the *CYP1A1* gene were described which may determine in part the risk for head and neck cancer.

Methods Polymorphisms of *GSTM1*, *GSTT1*, and *CYP1A1* in whole blood were detected by polymerase chain reaction method (PCR) in: 185 patients with head and neck squamous cell carcinoma (HNSCC), 78 patients with benign head and neck lesions (BHNL) and 207 blood donors.

Results *GSTM1* null genotype was demonstrated to be equally frequent in patients with HNSCC (50.8%), patients with BHNL (47.4%) and blood donors (51.7%). *GSTT1* null genotype occurred significantly more often in patients with BHNL (33.3%) as compared to blood donors (20.3%), demonstrating that lack of *GSTT1* may be a risk factor for BHNL. Presence of the rare valine in the *CYP1A1*/*NcoI* site was found in 33 (17.8%) patients with HNSCC, in 20 (25.6%) patients with BHNL and in 34 (16.4%) blood donors. For the presence of the rare cytosine nucleotide in the *CYP1A1*/*MspI* site these frequencies were 17.8, 15.4 and 15.9%, respectively.

Conclusions The occurrence of polymorphic variants in the *GSTM1*, *GSTT1*, and *CYP1A1* genes did not differ between the groups investigated, therefore indicating no significant contribution to the development of head and neck cancer.

Introduction

Head and neck squamous cell carcinoma (HNSCC) appears to be related to smoking and alcohol consumption (1-5). Polycyclic aromatic hydrocarbons (PAHs), present in tobacco smoke or metabolites of alcohol, are precursors of chemicals that increase the risk of cancer (6). One of the first steps in head and neck cancer may be the binding of exogenous electrophilic compounds to the DNA of mucosal cells (7-9). Most PAHs first require activation by phase I enzymes, such as cytochrome P4501A1 (CYP1A1), to become an ultimate carcinogen. These activated forms may be subjected to detoxification by phase II enzymes, especially glutathione S-transferases (GSTs). Thus, genetically determined susceptibility to head and neck cancer may depend on the metabolic balance between these enzymes.

Glutathione S-transferases are a family of dimeric enzymes, involved in the biotransformation of a large variety of compounds. GSTs catalyse the nucleophilic conjugation of glutathione to many different biologically active and potential carcinogenic compounds (10-13), which may be further metabolized to mercapturic acids. As a result the possible toxins or carcinogens are eliminated. In humans, the GST family can be divided into class Alpha (A), Mu (M), Pi (P) and Theta (T) enzymes, each class consisting of several isoenzymes with a (partly) overlapping substrate specificity (12,13). Genetic polymorphisms have been demonstrated for the genes coding for the *GSTM1* and *GSTT1* enzymes.

The *GSTM1* enzyme is involved in the detoxification of tobacco related carcinogens, like epoxides and hydroxylated metabolites of benzo[a]pyrene (13). Two functional alleles (*GSTM1**A and *GSTM1**B) and a non-functional *null*-allele (*GSTM1**O) have been identified in the *GSTM1* gene. Approximately 45% of the human Caucasian population lacks a functional allele (14-17). In other ethnic groups this proportion may be much higher (14). Carcinogenesis could be related to the absence of the functional *GSTM1* alleles resulting in an incapacity of detoxifying tobacco related carcinogens. Several studies have shown that the *GSTM1* polymorphism is associated with an increased risk of lung, bladder, gastric, colorectal, skin and larynx cancer (14,18-25). Other studies, however, revealed that the impact of the *GSTM1* trait may be low or even undetectable in different cancer populations (26-32).

The *GSTT1* gene also has a functional and a non-functional allele. Approximately 20% of the human Caucasian population lacks the functional allele (33), whereas in other ethnic groups this percentage may be different (23,34). Since the *GSTT1* enzyme is involved in the biotransformation of several low molecular weight toxins such as methyl-halogenids or ethylene oxide (13), part of the population lacking this enzyme might be more susceptible to smoking or alcohol related cancers. Lack of the *GSTT1* enzyme has been associated with bladder cancer (24), whereas other studies did not find an association with lung and skin cancer (32,35).

Many chemical compounds are metabolically activated to forms that have deleterious effects on organisms (36), and this metabolic activation is an obligatory initiation step in human carcinogenesis. Cytochrome P450 plays a major role in the formation of chemical carcinogens (37,38). Many isoenzymes of CYP have been found in humans.

Genetic variability in these isoenzymes may be responsible for the individual differences in susceptibility towards cancer (37,39). The CYP1A1 enzyme is involved in the activation of benzo[a]pyrene and other aromatic hydrocarbons in tobacco smoke (36-42). Two genetic polymorphisms have been demonstrated in the CYP1A1 gene, which both may result in an increased enzyme activity (43-45): (i) an isoleucine/valine substitution in exon 7, the heme binding region (CYP1A1/NcoI) (46,47), and (ii) a thymine/cytosine point mutation in the 3' flanking non-coding region (CYP1A1/MspI). Both genetic polymorphisms seem to be involved in an increased susceptibility to carcinogenesis of the lung (44,46-48). It has been suggested that homozygotes of the variant allele of each mutation are correlated with an enhanced susceptibility to smoking-related lung cancer in a Japanese population (31,44,46,47). No such relationship has been observed in black and non-black populations of Rio de Janeiro (49) or in Caucasian populations (50-52), where the occurrence of the susceptible alleles is very rare.

In the present study we investigated genetic polymorphisms in *GSTM1*, *GSTT1* and *CYP1A1* in patients with malignant and benign head and neck lesions (HNSCC and BHNLC respectively), in comparison with a group of healthy blood donors.

Materials and Methods

Patients and controls

A total of 185 patients with head and neck squamous cell carcinoma (HNSCC) were recruited preoperatively or postoperatively during their scheduled outpatient visit at the University Hospital Maastricht (The Netherlands) between 1994 and 1996 (mean age 59.6 ± 11.8 years; range 23 - 86). This group consists of 73 patients with larynx carcinoma (mean age 61.6 ± 10.6 years; range 36 - 86), 76 patients with oral and

Table 1. Classification of patients with HNSCC

Site - subsite of tumor		Number of patients
Oral cavity	floor of mouth	17
	tongue	11
	retromolar trigonum	7
	alveolar process	4
	mucosa cheek	2
Oropharynx	tonsil	12
	soft palate	6
	overlapping areas	5
	base of tongue	5
	lateral wall	3
	vallecular region	2
	posterior wall	2
Larynx	glottis	49
	supraglottis	24
Other	hypopharynx	16
	neck metastases of unknown primary	11
	nasopharynx	3
	sinus	2
	parotid gland	2
	skin	2
Total		185

oropharynx carcinoma (mean age 57.8 ± 12.0 years; range 23 - 82) and a remaining group of 36 patients (mean age 60.4 ± 12.6 years; range 28 - 86), consisting of carcinomas of the hypopharynx (n=16), nasopharynx (n=3), sinus (n=2), parotid gland (n=2), skin (n=2) and neck metastases of unknown primary origin (n=11). Table 1 shows the classification of the patients according to tumor risk. Table 3 shows a further specification.

Our second population consists of 78 patients with benign head and neck lesions (BHNL) such as polyps, cysts, and vocal cord nodules (mean age 49.2 ± 15.9 years; range 16 - 86). A further specification of these patients is presented in Table 2 and 3.

A group of 207 healthy blood donors (76 men; 131 women) recruited at the University Medical Center Nijmegen, (The Netherlands) served as a control group (mean age 34.3 ± 11.5 years; range 19 - 64).

At the time when informed consent was obtained, medical and other anamnestic data were obtained by interview. The investigations were approved by the Medical Ethical Review Committee.

Table 2. Classification of patients with BHNL

Site - type of lesion	Number of patients
Vocal cord - nodules / cysts	22
Parotid gland - pleiomorphic adenoma	16
Nose - polyps	13
Neck - cysts (medial / lateral)	9 (7/2)
Submandibular gland - sialolithiasis	8
Oral cavity - leucoplakia	4
Oral cavity - fibromas	3
Nose - cyst	2
Tonsil - retention cyst	1
Total	78

Table 3. Age and gender of the patients with HNSCC (total group as well as subclasses), BHNL, and controls

	HNSCC			BHNL	Controls
	Total group	Larynx carcinoma	Oropharynx carcinoma		
N	185	73	76	78	207
Age (yrs)					
Mean $\pm$ S.D.	59.6 ± 11.8	61.6 ± 10.6	57.8 ± 12.0	49.2 ± 15.9	34.3 ± 11.5
Range	23-86	36-86	23-82	16-86	19-64
Gender (%)					
Male	79.5	88.2	70.7	46.8	36.7
Female	20.5	11.8	29.3	53.2	63.3

Blood sampling and extraction of DNA

Blood was collected by venapuncture in sterile siliconized EDTA K3 (15%) 4 ml Vacutainer tubes (Becton Dickinson). Immediately after collection whole blood was stored at -20°C until use. Genomic DNA was isolated from whole blood using the Wizard™ genomic DNA purification kit, according to the instructions of the manufacturer (Promega, Madison Wisconsin, USA).

Genotyping

Genetic polymorphisms were detected by PCR and all primers were selected based on studies by others. The primers were synthesized by Pharmacia Biotech (Roosendaal, the Netherlands). All chemicals needed for PCR were purchased from Promega (USA). For *GSTM1* (primer set G2 and G3, ref. 27) and *GSTT1* (primer set T1 and T2, ref. 53) one primer was situated in the deleted area. Genetic polymorphism in exon 7 of the *CYP1A1* gene was determined by PCR (primer set *CYP1A1*/1 and *CYP1A1*/2, ref. 48) and restriction enzyme digestion according to Shields et al. (48). Here a primer was designed so that the presence of the rare G allele (valine) results in the loss of a *NcoI* restriction enzyme site. Genetic polymorphism in the 3' flanking region in the *CYP1A1* gene was detected by PCR and restriction enzyme digestion, using the primers C44 and C47 (44). Here, one of the primers was designed so that the presence of the rare C nucleotide results in the appearance of a *MspI* restriction enzyme site.

Fifty to hundred ng of DNA were amplified in a total volume of 25 μ l containing 10 mM Tris/HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$ (1.5 mM for *CYP1A1*/1 and *CYP1A1*/2), 200 μ M of deoxynucleotide triphosphate, 1 μ M of each primer, and 0.2 units of Taq polymerase, overlaid with one drop of mineral oil. PCR was carried out in a programmable Thermojet thermocycler (Eurogentec, Seraing, Belgium). After 5 min of pretreatment at 95°C, 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 59°C (*GSTM1* and *GSTT1*) or 55°C (*CYP1A1*) and 90 sec extension at 72°C.

For evaluating *GSTM1* and *GSTT1* polymorphisms, reaction mixture products were analysed by gel electrophoresis (2% agarose; 60 V; 1 h) for the presence of a 650-base pair product, which was indicative of the *GSTM1* gene or a 480-base pair product, indicative of the *GSTT1* gene. The lack of an amplification product is consistent with the null genotype. Detection of *CYP1A1* PCR products served as a positive control.

Amplification products of the *CYP1A1* gene were subjected to restriction fragment length digestion using *MspI* or *NcoI* (Promega; 10 units; 37°C, 1.5 h). Analysis of the *NcoI* site by gel electrophoresis (2% agarose; 60 V; 2.5 h) revealed a 163- and 32-base pair fragments for wild type alleles (isoleucine) or a single 195 base pair fragment when the mutation (valine) was present. Analysis of the *MspI* site by gel electrophoresis (2% agarose; 60 V; 1.5 h) revealed a 340-base pair fragment for mutant alleles (cytosine) or 200- and 140-base pair fragments for wild type alleles (thymine).

Statistical analyses

Statistical significance of differences for individual and combined polymorphisms between different groups was tested with a χ^2 analysis with Yates correction in 2x2 or 2x4 contingency tables, respectively.

Results

The distribution of the *GSTM1* and *GSTT1* genes in patients with HNSCC or BHLN and blood donors is summarized in Table 4. Presence of the *GSTM1* gene in patients with HNSCC (49.2%) did not differ significantly from the distribution in a population with

either benign head and neck lesions (52.6%, $p=0.71$) or healthy blood donors (48.3%, $p=0.95$). In subgroups based on tumor localisation (larynx and oral-oropharynx) these expression frequencies were maintained (46.6% and 50.0%, respectively) and no significant differences were obtained. The *GSTM1* polymorphism also did not show any significant difference between benign head and neck lesions and blood donors ($p=0.61$).

The BHL group showed a significantly lower expression of the *GSTT1* gene (66.7%) as compared to the blood donor group (79.7%; $\chi^2=4.6$, $p=0.03$) and the HNSCC group (80.5%; $\chi^2=5.1$, $p=0.02$). Compared to the blood donor population, the HNSCC group showed no significant difference in *GSTT1* expression (79.7% and 80.5%, respectively; $p=0.94$). In the HNSCC subgroups no differences in *GSTT1* gene distribution were noted.

Presence of both *GSTM1* and *GSTT1* genes occurred less frequently in patients with BHL as compared to patients with HNSCC ($\chi^2=10.2$, $p=0.01$), whereas no significant difference was found between healthy volunteers and patients with BHL or HNSCC.

Table 4. *GSTM1* and *GSTT1* expression in patients with HNSCC, BHL and in controls

	HNSCC (%)	BHL (%)	Controls (%)
<i>GSTM1</i> ⁺	91 (49.2)	41 (52.6)	100 (48.3)
<i>GSTM1</i> ⁻	94 (50.8)	37 (47.4)	107 (51.7)
<i>GSTT1</i> ⁺	149 (80.5)	52 (66.7)	165 (79.7)
<i>GSTT1</i> ⁻	36 (19.5)	26 (33.3)	42 (20.3)
<i>GSTM1</i> ⁺ <i>GSTT1</i> ⁺	79 (42.7)	26 (33.3)	81 (39.1)
<i>GSTM1</i> ⁺ <i>GSTT1</i> ⁻	12 (6.5)	15 (19.2)	19 (9.2)
<i>GSTM1</i> ⁻ <i>GSTT1</i> ⁺	70 (37.8)	26 (33.3)	84 (40.6)
<i>GSTM1</i> ⁻ <i>GSTT1</i> ⁻	24 (13.0)	11 (14.1)	23 (11.1)

⁺, gene present; ⁻, gene absent

Table 5 shows the expression rates of the polymorphic variants of the *CYP1A1/NcoI* and *CYP1A1/MspI* sites in patients with HNSCC, BHL patients and healthy blood donors. The expression rates of the different polymorphic variants were not statistically significant between the different groups. Presence of the rare G (valine) allele in the *NcoI* site ranges from 16.4% in the blood donor group to 25.6% in BHL patients. In the *MspI* site the rare C nucleotide occurred approximately equally frequent as the rare allele in the *NcoI* site and ranges from 15.4% in BHL patients to 17.8% in HNSCC patients. Chi square analyses gave p-values of 0.7114 and 0.9561 when comparing HNSCC or BHL with blood donors, and here again no statistically significant differences could be found.

In the BHL group, rare alleles in both the *CYP1A1/NcoI* and *CYP1A1/MspI* sites (Table 5) were found in 7 (9.0%) cases, whereas a rare allele in only one of the sites was found in 18 patients (23.1%) and no rare alleles in 53 patients (67.9%). In the HNSCC group these percentages were 8.1, 20.0 and 71.9%, respectively. Finally, in the blood donor group these rates were 7.2, 17.9 and 74.9%, respectively. The balance between the different polymorphic variants in the *CYP1A1/MspI* and *CYP1A1/NcoI* sites was significantly correlated in each population studied.

Table 5. Distribution of polymorphic variants in *CYP1A1/NcoI* and *CYP1A1/MspI* sites in patients with HNSCC, BHNL and in controls

	HNSCC (%)	BHNL (%)	Controls (%)
<i>NcoI</i> ^{wt}	151 (81.6)	58 (74.4)	173 (83.6)
<i>NcoI</i> ^{rare}	34 (18.4)	20 (25.6)	34 (16.4)
<i>MspI</i> ^{wt}	152 (82.2)	66 (84.6)	174 (84.1)
<i>MspI</i> ^{rare}	33 (17.8)	12 (15.4)	33 (15.9)
<i>NcoI</i> ^{wt} , <i>MspI</i> ^{wt}	133 (71.9)	53 (67.9)	155 (74.9)
<i>NcoI</i> ^{wt} , <i>MspI</i> ^{rare}	18 (9.7)	5 (6.4)	19 (9.2)
<i>NcoI</i> ^{rare} , <i>MspI</i> ^{wt}	19 (10.3)	13 (16.7)	18 (8.7)
<i>NcoI</i> ^{rare} , <i>MspI</i> ^{rare}	15 (8.1)	7 (9.0)	15 (7.2)
<i>GSTM1</i> ⁺ , <i>MspI</i> ^{wt}	78 (42.2)	40 (51.3)	84 (40.6)
<i>GSTM1</i> ⁺ , <i>MspI</i> ^{rare}	13 (7.0)	1 (1.3)	16 (7.7)
<i>GSTM1</i> ⁻ , <i>MspI</i> ^{wt}	74 (40.0)	26 (33.3)	90 (43.5)
<i>GSTM1</i> ⁻ , <i>MspI</i> ^{rare}	20 (10.8)	11 (14.1)	17 (8.2)

wt, wild type; rare, rare allele present

Combining polymorphic expressions of *GSTM1* and *CYP1A1/MspI*, *GSTT1* and *CYP1A1/MspI* or *GSTT1* and *CYP1A1/NcoI* only showed one statistically significant difference: The *GSTM1* null genotype and a rare allele in the *CYP1A1/MspI* site occurs more frequently in patients with BHNL than in blood donors ($\chi^2=8.8$, $p=0.03$).

Discussion

Extensive consumption of tobacco and/or alcohol are regarded as risk factors for head and neck cancer (1-5). Tobacco smoke contains several (pre)carcinogenic compounds. Enzymes such as *GSTM1*, *GSTT1* and *CYP1A1* are involved in the metabolism of several of these chemical (pre)carcinogens. In the assessment of individual cancer risk the identification of heritable variations in the expression of these enzymes, which could predispose subjects to such environmentally induced cancer, deserve considerable interest. *GSTM1* shows a polymorphic expression and about half the population in various racial groups is deficient of the *GSTM1* gene (14). Because the *GSTM1* enzyme can detoxify potential carcinogens like epoxides and aromatic hydrocarbons from tobacco smoke (13), smokers lacking *GSTM1* activity were suggested to be at high risk of developing lung cancer (17). The *GSTM1* null genotype was associated with high risk for lung, bladder, gastric, colorectal, skin, head and neck, and larynx cancer in several recent articles (18-26,54,55). However, other data were presented in which no contribution of the *GSTM1* null genotype for cancer risk was found (29,35,56-60).

Our data on the *GSTM1* null genotype in patients with HNSCC were in agreement with the latter reports. We found no difference in *GSTM1* gene expression between patients with HNSCC or BHNL and healthy blood donors. In the HNSCC subgroups of patients with larynx or oral-opharynx tumors, no differences could be observed either. Therefore, *GSTM1* polymorphism does not seem to influence the susceptibility for BHNL or HNSCC in the total group or in subgroups of larynx and oral-opharynx carcinoma. This suggests that presumed carcinogenic or toxic compounds causing either of these lesions are detoxified by enzymes other than *GSTM1*.

In addition, the recently discovered polymorphism of *GSTT1*, which is a result from a gene deletion (33) was investigated by PCR. The *GSTT1 null* genotype was found to be related to increased colorectal and larynx cancer susceptibility (56,57), whereas other reports found no relationship between *GSTT1 null* genotype and lung, oral and gastric cancer, and cutaneous basal cell carcinoma (35,56,58). However, no higher incidence of *GSTT1 null* genotype in benign lesions was reported before. We demonstrated an increase in *GSTT1 null* genotypes in patients with BHNL as compared to blood donors and patients with HNSCC. In the HNSCC subgroups as well as the total group of patients with HNSCC the *null* genotype frequency equalled that of the blood donor group. This suggests that *GSTT1* deficiency indicates an increased risk for BHNL, but not for HNSCC. This could implicate that BHNL and HNSCC may be caused by different mechanisms, and possibly one of the factors involved in BHNL may be a substrate for the *GSTT1* enzyme.

Because Chevenix-Trench et al. (54) recently suggested a trend of lower percentage of *GSTT1 null* genotypes in geriatric controls versus unselected (and most probably younger) controls, we looked for the same tendency in our control population. Thus, the control group was split up into two age groups (below 40 years and 40 years and above). We could not find a difference in the distribution of *GSTT1 null* genotypes between these two groups and a percentage of 24% *null* genotypes was found in the age group of 40 years and above. In addition, because in our study the number of *GSTT1 null* individuals in healthy volunteers matched very well with both data in the literature (12,54) and the frequencies found in patients with HNSCC, we believe the age difference between controls and patients may not be of any consequence for the genetic analyses as performed here.

Combined analysis of *GSTM1* and *GSTT1* polymorphisms demonstrated a statistically significant impact on head and neck carcinogenesis. It has previously been shown that combined *GSTM1* and *GSTT1 null* genotype was related to lung cancer risk (35). No relation, however, between *GSTM1* and *GSTT1 null* genotype and multiple cutaneous basal cell carcinoma (32) was found.

In addition to polymorphisms in phase II enzymes, genetic polymorphisms in the phase I enzyme *CYP1A1* gene were correlated to an increased susceptibility to chemical carcinogenesis (37,39,44,46-48). The *CYP1A1* enzyme is involved in the activation of benzo[a]pyrene and other aromatic hydrocarbons in tobacco smoke (36-42). Genetic variants in the *CYP1A1* gene investigated here can result in an enhancement of the *CYP1A1* enzyme activities (43,45). We demonstrated no difference in the distribution of genetic variants of the *CYP1A1/NcoI* nor of the *CYP1A1/MspI* site between patients with HNSCC, BHNL and blood donors, suggesting that these variations do not have a significant impact on the risk for these head and neck lesions and that compounds causing these lesions may not be activated by this enzyme. The *MspI* polymorphism of the *CYP1A1* gene was associated with a reduced survival in patients with non-small cell lung cancer (59). It was also an independent factor indicative for bad prognosis at the nonresectable advanced stage of this disease (59). In bladder cancer and squamous cell cancer of the lung, however, no impact of *CYP1A1/MspI* variants on cancer susceptibility was found. Genetic variation in the *CYP1A1/NcoI* site was weakly

but nonsignificantly associated with enhanced postmenopausal breast cancer risk (29), whereas another study reported a stronger association (30).

Individually, presence of the rare allele in the *CYP1A1/MspI* site or a *GSTM1 null* genotype had no effect on the development of squamous or small cell carcinoma of the lung (31). Combined, however, they were significant risk factors ($p < 0.01$) (31). We now found that having both *GSTM1 null* genotype and a rare allele in the *CYP1A1/MspI* site had a significant impact on the risk of BHL as compared to blood donors. This strongly suggests that generation of reactive metabolites by the *CYP1A1* enzyme and subsequent lack of detoxification by the *GSTM1* enzyme may be of importance in the etiology of BHL. Several other studies showed comparable results with respect to combined polymorphisms and increased risk for development of malignancies. Combination of *GSTM1 null* genotype and the rare *CYP1A1/MspI* allele showed an increased risk of squamous cell carcinoma of the lung when diagnosed before 66 years of age (61), and patients with non-small lung cell cancer carrying this combination of polymorphisms had remarkable shortened survival (59). The interactive effect of *GSTM1* and *CYP1A1* genotypes (45) suggests that the influence of detoxifying enzymes in mediating cancer risk may be modified by polymorphisms at other relevant loci as well. This is an important point which needs further attention in the future.

In summary, our study shows that (a) *GSTM1 null* genotype is a risk factor for BHL, but not for HNSCC, and (b) combined *GSTM1 null* genotype and a rare C nucleotide in the *CYP1A1/MspI* site was more often detected in patients with BHL as compared to blood donors, but not in patients with HNSCC.

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3

CHAPTER

DEMOGRAPHIC VARIATION IN THE DISTRIBUTION OF THE GLUTATHIONE S-TRANSFERASE T1 NULL POLYMORPHISM IN DUTCH HEALTHY CONTROLS; IMPLICATIONS FOR GENOTYPE RESEARCH IN HEAD AND NECK CANCER

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Abstract

Background We compared the *GSTM1*, *GSTT1* and *GSTP1* genotype frequencies in two groups of healthy blood donors, recruited from different, but neighboring regions in the Netherlands. The effect of cross linked comparisons for head and neck squamous cell carcinoma (HNSCC) on the outcome of a study previously published in Cancer is analysed.

Methods The *GSTM1*, *GSTT1* and *GSTP1* genotype frequencies in two Dutch Caucasian control populations ($n=207$ and $n=285$) from different but neighboring geographical regions (Maastricht/Nijmegen; distance, 125 km) were determined by polymerase chain reaction (PCR) related methods.

Results For the occurrence of the *GSTT1 null* genotype we found a significant difference ($p=0.003$) between the two control groups (20.3% vs. 33.0% *null* genotype in the Nijmegen and Maastricht control groups, respectively). Since the HNSCC patients are recruited from the Maastricht area, comparison with the Maastricht controls now reveals a significant difference for *GSTT1 null* rates, which are lower in patients vs. controls (OR=0.49, CI 0.32-0.76).

Conclusions Inter-regional differences exist, which may have significant effect in the distribution of GST polymorphism statuses. In addition to our earlier publications we now find that the *GSTT1 null* genotype appears to be involved in modulating the risk for HNSCC. The significance of the distribution patterns of GST polymorphisms in head and neck cancer patient populations can only be analyzed in comparison to a regional control group.

Introduction

In 1998 we investigated genetic polymorphisms in glutathione S-transferase M1 (*GSTM1*) and *GSTT1* in relation to head and neck squamous cell carcinoma (HNSCC) (1). In this study we reported that *GSTM1* and *GSTT1 null* genotypes were not related to HNSCC susceptibility, but that the *GSTT1 null* genotype occurred significantly more often in a patient population with benign head and neck lesions (BHNL). We therefore concluded that the *GSTT1 null* genotype was not a risk factor for HNSCC, but may be a risk factor for development of benign head and neck disease.

In this collaborative study between the University Hospitals of Nijmegen and Maastricht, the GST polymorphisms in healthy blood donors from the Nijmegen region were compared to patients with HNSCC from the Maastricht region. Since these two cities are separated geographically by only 125 km, we did not expect that significant differences in variant GST genotype rates could occur between two study groups originating from such close regions.

There have appeared many conflicting reports on the contribution of genetic polymorphisms in GSTs, in association with head and neck cancer or other malignancies, which may be partly due to mismatching of patient- and control populations. Therefore, we now compared the variant genotype frequencies of *GSTM1*, *GSTP1* and *GSTT1* in the control population of Nijmegen and a recently established control population of the Maastricht region. Also, we investigated whether these new data have any effect on our previously published results (1).

Materials and Methods

Populations studied

The first Dutch Caucasian control group (control group Nijmegen) consisted of 207 healthy blood donors (76 men; 131 women) and was recruited as described before (1) by advertising in a local paper at the University Medical Center Nijmegen (mean age 34 ± 12 years; range 19 - 64). A second Dutch Caucasian control group (control group Maastricht) consisted of 285 healthy blood donors (199 men, 86 women; mean age 50 ± 10 years; range 19 - 91) and was recruited from the same geographical area as the HNSCC and BHNL patients, at the Blood Bank of Maastricht. The geographical distance between the two cities Nijmegen and Maastricht is approximately 125 km. An extra control population (control group BHNL) consisted of 78 patients (mean age 49 ± 16 years; range 16 - 86) with benign head and neck lesions (BHNL) such as polyps, cysts, and vocal cord nodules, as described before (1).

The 185 Dutch Caucasian patients with HNSCC participating in the initial study (1) were recruited preoperatively or postoperatively as described previously during their scheduled outpatient visit at the University Hospital Maastricht between 1994 and 1996 (mean age 60 ± 12 years; range 23 - 86). This group consisted of 73 patients with laryngeal carcinoma (mean age 62 ± 11 years; range 36 - 86), 76 patients with

oral and oropharyngeal carcinoma (mean age 58 ± 12 years; range 23 - 82) and a remaining group of 36 patients (mean age 60 ± 13 years; range 28 - 86).

At the time when informed consent was obtained, medical and other anamnestic data were obtained by interview. The investigations were approved by both the Nijmegen and Maastricht local Medical Ethical Review Committees.

Genotyping by PCR Analyses

Blood was collected by venapuncture in EDTA vacutainer tubes, and blood was stored at -20°C until use. Genomic DNA was isolated from whole blood using the Wizzard genomic DNA isolation kit, according to the instructions of the manufacturer (Promega, Madison Wisconsin, USA). Genetic polymorphism rates were determined by initial PCR, followed by analysis on agarose gel.

Fifty to hundred ng of DNA were amplified by PCR in a total volume of 25 μl containing 10 mM Tris/HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM MgCl_2 , 200 μM of deoxynucleotide triphosphate, 1 μM of each primer, and 0.2 units of Taq polymerase, overlaid with one drop of mineral oil. PCR was carried out in a programmable Thermojet thermocycler (Eurogentec, Seraing, Belgium). After 5 min of pretreatment at 95°C , 35 cycles of 30 sec denaturation at 95°C and 30 sec annealing at 59°C were performed. All primers were selected based on studies by others (2-4). The primers were synthesized by Pharmacia Biotech (Roosendaal, The Netherlands). All chemicals needed for PCR were purchased from Promega (USA). For *GSTM1* (primer set G2 and G3) and *GSTT1* (primer set T1 and T2) one primer was situated in the deleted area. For evaluating *GSTM1* and *GSTT1* polymorphisms, reaction mixture products were analyzed by gel electrophoresis (2% agarose; 60 V; 1 h) for the presence of a 650-base pair product, which was indicative of the *GSTM1* gene or a 480-base pair product, indicative of the *GSTT1* gene. When at first analysis no product was found, always a second and eventually a third analysis was done, in parallel with a control PCR from the same DNA samples, so that a false negative result (apparent *null* genotype) was excluded. For studying the genetic polymorphism in *GSTP1* a primer set (105F and 105R) was designed so that the presence of the A to G substitution at codon 313 resulted in the appearance of an Alw261 restriction enzyme site (4).

Statistical analysis

Statistical analysis was performed by using the SPSS 8.0 statistical package. Odds Ratio analyses in 2x2 tables were used, as well as calculation of p-values.

Results

In Table 1 the frequencies of *GSTM1* and *GSTT1* genotypes of the two different normal control populations from Nijmegen and Maastricht are shown. The *GSTM1* and *GSTT1* genotypes of the group of patients with HNSCC and BHNH from the Maastricht region are included. For the *GSTM1* genotype, both control groups compared to each other, and the HNSCC patient group compared to the frequencies of the control groups from Nijmegen and Maastricht show no statistical difference (see Table 2).

The genotype frequencies for *GSTT1* however, show a significant difference between the two control groups from Nijmegen and Maastricht ($p=0.003$). The BHL group shows a frequency that matches the control group Maastricht.

For *GSTP1* there are no statistical differences between the two control groups from Nijmegen and Maastricht.

Table 2 shows risk estimates for the susceptibility of the various *GSTM1* and *GSTT1* genotypes on HNSCC when compared to different control groups. For the *GSTP1* genotype only a risk estimate is shown when compared to the two control groups. For *GSTM1* and *GSTP1* genotypes no significant differences were found. For the *GSTT1* genotype however, a significant difference was noted when the risk for HNSCC is estimated versus control group Maastricht, which matches with the patients with respect to recruiting area and race ($p=0.002$).

Table 1. Frequencies of *GSTM1* and *GSTT1* genotypes in the different control populations and in patients with BHL and HNSCC

	Controls Nijmegen (%)	Controls Maastricht (%)	BHL Maastricht (%)	HNSCC Maastricht (%)
<i>GSTM1</i> (null)	107 (51.7)	163 (57.2)	37 (47.4)	94 (50.8)
<i>GSTM1</i> +	100 (48.3)	122 (42.8)	41 (52.6)	91 (49.2)
<i>GSTT1</i> (null)	42 (20.3)	94 (33.0)	26 (33.3)	36 (19.5)
<i>GSTT1</i> +	165 (79.7)	191 (67.0)	52 (66.7)	149 (80.5)
<i>GSTP1</i> AB	88 (43.6)	122 (42.8)	#	#
<i>GSTP1</i> BB	25 (12.4)	37 (12.9)	#	#
<i>GSTP1</i> AA	89 (44.0)	126 (44.2)	#	#
Total	207 (202*)	285	78	185

* = controls for *GSTP1* (five DNA samples were not available any more); # = not evaluated

Table 2. Risk estimate of *GSTM1*, *GSTT1* and *GSTP1* genotype frequencies compared in two different control populations and HNSCC patients

	Odds Ratio (95% CI)	p
<i>GSTM1</i> null		
Control Maastricht vs. control Nijmegen	1.25 (0.86-1.78)	0.26
HNSCC vs. Nijmegen	0.96 (0.65-1.43)	0.94
HNSCC vs. Maastricht	0.78 (0.53-1.12)	0.21
<i>GSTT1</i> null		
Control Maastricht vs. control Nijmegen	1.92 (1.26-2.94)	0.003
HNSCC vs. Nijmegen	0.95 (0.57-1.56)	0.94
HNSCC vs. Maastricht	0.49 (0.32-0.76)	0.002
<i>GSTP1</i> AB/BB		
Control Maastricht vs. control Nijmegen	1.00 (0.69-1.43)	0.97

* = Odds Ratio was calculated by combining *GSTP1* AB and *GSTP1* BB genotypes

Discussion

In this study we found a highly significant difference in the rates of the *GSTT1 null* genotype between controls from the Nijmegen and Maastricht region. This has consequences for the interpretation of earlier results reported by us in 1998 (1), since now a significant lower risk for HNSCC of individuals with the *GSTT1 null* genotype is found. First of all, how can such inter-regional difference between controls be explained? Most probably the tendency to stay in the region of birth, may be a main reason, since it is well known that genetic inheritance contributes to the genetic profile of an individual.

Most studies that have been conducted on the role of polymorphisms in modulating the risk for HNSCC, do describe demographical data (see Table 3), with the exception of some papers (5-8) where no details on this are given. This does not mean however that demographic variation is present here. In several studies it is clearly mentioned that patients and controls were recruited from the same region, but not from the same hospital (9-12), thus similar to our earlier study (1). In other studies it was mentioned that patients and controls were recruited from different regions (1,13,14). Since there are many national and international collaborations between the different research groups with exchange of samples between different labs, it may often occur that controls and patients have been recruited from different (parts of) countries, which may have similar consequences as described in this paper.

Recently in a large international collaborative study Garte et al. (15) reported significant differences between different racial populations for *GSTM1* and *GSTT1* genotypes. For the *GSTM1 null* genotype, frequencies found were 53.1% for Caucasians, 52.9% for Asians and 26.7% for Africans. The total number of African patients included however, was only 4% (479 individuals) on a total number of 12,504 patients evaluated. For the *GSTT1 null* genotype, the frequencies for Caucasians were 19.7% compared to 47.0% for Asians (15).

Garte et al. (15) reported considerable differences in *GSTM1* and *GSTT1* genotype frequencies in Caucasian populations of several European countries, Canada or the USA (47-58% and 13-27% for *null* genotypes respectively). These findings suggest that these frequencies are rather variable in Caucasians all over the world. In addition, the data on *GSTT1 null* genotype frequency as found in our controls from the Maastricht region (33%) seem to be higher as compared to earlier reported data from European countries (13-26%) and are closer to those reported in the USA (27%) or Japan (35%) (15).

For the *GSTT1* genotype we found an unexpected significant difference ($p=0.003$) between the two Dutch control groups, collected at a distance of approximately 125 km. In our earlier report we even suggested a possible role for *GSTT1* genotype in the etiology of BBNL (1).

Table 3. Demographics of control and HNSCC patient populations and *GSTT1 null* genotype frequencies

Author	Site	<i>GSTT1 null</i> Controls % (n=total)	<i>GSTT1 null</i> Cases % (n=total)	Race	Country	Same demographics patients-controls	Odds Ratio (95% CI)
No association <i>GSTT1 null</i> genotype vs. HNSCC							
Buch et al. (17)	Oral	12.3 (450)	18.3 (297)	Indian	India	yes	1.6 (1.0-2.6)
Cabelguenne et al. (18)	HN	19.4 (264)	18.1 (162)	n.s.	France	yes	1.0 (0.6-1.7)
Deakin et al. (19)	Oral	18.5 (129)	11.8 (34)	Caucasian	UK	yes	n.s.
Hanna et al. (7)	Larynx	20.0 (20)	15.0 (20)	n.s.	USA	n.s.	n.s.
Hong et al. (20)	Larynx	36.5 (63)	57.3 (82)	Korean	Korea	yes	1.8 (0.7-4.8)
Hung et al. (21)	Oral	52.9 (123)	58.5 (41)	Chinese mixed	Taiwan	yes	1.2 (0.6-2.5)
Jourenkova et al. (22)	Larynx	15.7 (172)	19.4 (129)	Caucasian	France	yes	1.4 (0.7-2.9)
Jourenkova et al. (23)	Pharynx	15.7 (172)	18.0 (50)	Caucasian	France	yes	1.4 (0.6-3.7)
Kato et al. (24)	Oral	51.0 (147)	47.7 (92)	Japanese	Japan	yes	0.7 (0.4-1.2)
Matthias et al. (25)	Larynx	22.2 (203)	19.4 (263)	Caucasian	German	yes	0.9 (0.5-1.4)
	Oropharynx	22.2 (203)	27.7 (119)	Caucasian	German	yes	1.5 (0.9-2.5)
	HN	22.2 (203)	22.0 (382)	Caucasian	German	yes	n.s.
McWilliams et al. (26)	HN	18.3 (109)	16.9 (142)	Caucasian	USA	yes	0.9 (0.5-1.5)
Olshan et al. (27)	HN	13.0 (168)	17.0 (109)	Caucasian	USA	yes	1.4 (0.7-2.7)
Oude Ophuis et al. (1)	HN	20.3 (207)	19.5 (185)	Caucasian	The Netherlands	no	1.1 (0.6-1.7)
	Oral/oropharynx	20.3 (207)	19.7 (76)	Caucasian	The Netherlands	no	1.0 (0.5-2.0)
	Larynx	20.3 (207)	20.5 (73)	Caucasian	The Netherlands	no	1.0 (0.5-1.9)
Sreelekha et al. (8)	Oral	8.3 (60)	18.4 (98)	Indian	India	n.s.	2.5 (0.3-21.7)
Trizna et al. (30)	HN	36.0 (42)	45.0 (186)	Mixed	USA	yes	1.5 (0.7-3.0)
Worral et al. (28)	Oral	n.s.	n.s.	Caucasian	UK	yes	n.s.
Association <i>GSTT1 null</i> genotype vs HNSCC							
Cheng et al. (14)	HN	17.5 (315)	32.7 (162)	Mixed	USA	no	2.3 (1.4-3.6)
Hamel et al. (29)	HN	20.0 (101)	9.0 (70)	Mixed	Canada	yes	2.5 (1.1-5.9)
Jahnke et al. (5,6)	Larynx	12.4 (145)	21.3 (169)	Caucasian	Germany	n.s.	n.s.
Jourenkova et al. (23)	Oral	15.7 (172)	22.4 (67)	Caucasian	France	yes	2.4 (1.0-5.5)
	HN	15.7 (172)	21.5 (121)	Caucasian	France	yes	2.0 (1.0-4.0)
Oude Ophuis et al. (this article)	HN	33.0 (285)	19.5 (185)	Caucasian	The Netherlands	yes	2.0 (1.3-3.2)

n.s. not specified, n= total number of patients or controls in group, HN = all sites of head and neck malignancies. CI = confidence interval

However, when the risk for HNSCC is estimated versus the new control group, we found a significant reverse association between *GSTT1 null* genotype and HNSCC (OR=0.49, CI 0.32-0.76, p=0.002). We did not expect to find a difference in genotype frequency between such close regions, especially since the Netherlands is a very small country. Earlier, Roy et al. (16) found *GSTT1 null* genotype frequencies ranging from 3-39% in different Indian ethnic populations. They, however, did not mention distance, which may be much larger since India is a large country. In our study the different results can not be explained by ethnicity, since in The Netherlands geographically confined ethnic variations do not exist. We also can not explain this by a misinterpretation of PCR results, since the lack of an amplification product was always tested three times.

The association of genetic polymorphisms in toxification / detoxification enzymes and HNSCC is still a matter of controversy (see Table 3). We now have shown that significant demographic variations in genotype frequencies may exist, even in a small country. Demographic variations may be the key solution in explaining the differences in outcome in genetic polymorphism studies. In contrast to our earlier conclusion (1), we now must conclude that the *GSTT1 null* genotype appears to be less common in patients with HNSCC. A similar finding was published recently by London et al. (31) who found lower *GSTM1* and *GSTT1 null* genotype rates in patients with lung cancer, as compared to age and location matched controls. The authors could explain this finding as follows: consumption of cruciferous vegetables may protect against smoking induced cancers by their content of glucosinolates, precursors of isothiocyanates, the latter being potent inducers of glutathione S-transferases and other detoxification systems. However, since breakdown of isothiocyanates is also catalysed by GSTs, *GSTT1* and/or *GSTM1 null* individuals may have a worse clearance of isothiocyanates and may be better protected when consuming glucosinolate containing diets.

In conclusion: in contrast to the hypothesis that *GSTT1 null* genotype occurs more in patients with HNSCC we found the opposite; a less frequent occurrence of *GSTT1 null* genotype in HNSCC patients.

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4

CHAPTER

PLASMA GLUTATHIONE S-TRANSFERASE P1-1 LEVELS IN PATIENTS WITH HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Abstract

Background Many tumors contain high amounts of the detoxification enzyme glutathione S-transferase P1-1 (GSTP1-1). Elevated levels of GSTP1-1 have also been detected in serum and plasma from patients with gastrointestinal, lung or head and neck tumors. We evaluated the role of GSTP1-1 as a plasma tumor marker in patients with larynx-hypopharynx-oropharynx sites of head and neck squamous cell carcinoma (HNSCC), and benign head and neck lesions (BHNL).

Methods GSTP1-1 levels were measured in EDTA plasma using a recently developed sensitive and specific sandwich ELISA. An upper normal reference level of 21.8 μg GSTP1-1 per liter of plasma was calculated from results obtained from samples of 230 blood donors.

Results Median GSTP1-1 levels of 53 patients with oral/oropharyngeal SCC (10.6 $\mu\text{g}/\text{L}$; range 3.7-46.1 $\mu\text{g}/\text{L}$), 12 patients with hypopharyngeal SCC (11.9 $\mu\text{g}/\text{L}$; range 5.2-146.6 $\mu\text{g}/\text{L}$) and 28 patients with laryngeal SCC (14.4 $\mu\text{g}/\text{L}$; range 6.4-141.5 $\mu\text{g}/\text{L}$) were significantly elevated when compared to plasma GSTP1-1 levels from 45 patients with BHNL (8.1 $\mu\text{g}/\text{L}$; range 3.3-32.3 $\mu\text{g}/\text{L}$, $P < 0.0001$, $P < 0.01$ and $P < 0.0001$ respectively). However, only 6/53 patients (11%) with oral/oropharyngeal SCC, 1/12 patients (8%) with hypopharyngeal SCC, and 6/28 patients (21%) with laryngeal SCC had plasma GSTP1-1 levels above the upper normal reference level. Thus overall only 13/93 patients (14%) with HNSCC had elevated plasma GSTP1-1 levels. No significant relation between plasma GSTP1-1 levels and TNM classification of the tumors was detected.

Conclusions GSTP1-1 is not a suitable plasma tumor marker for HNSCC.

Introduction

Glutathione S-transferases (GSTs) are enzymes that catalyze the addition of glutathione to electrophilic centers of a wide variety of compounds. This reaction is the first step in the formation of mercapturic acids, a pathway mostly resulting in the elimination of potentially toxic compounds. GSTs also serve as transport proteins for a broad range of lipophilic compounds, such as bilirubin, bile acids, steroid hormones, and various xenobiotics (1,2). Based on structural, physicochemical, enzymatic, and immunological properties the family of enzymes is divided into four classes: Alpha, Mu, Pi and Theta (1-4).

Malignancies from colon, stomach, urinary bladder, uterine cervix, lung and larynx often contain increased amounts of GSTP1-1 when compared to the adjacent normal tissue (5,6). Three studies published in 1989 all indicated that a considerable number of patients with gastrointestinal malignancies had elevated serum GSTP1-1 levels (7-9).

Hirata et al. reported elevated plasma GSTP1-1 levels in 41/61 patients (76%) with primary oral cancer (10). These authors also reported an impressive decline in plasma GSTP1-1 levels after surgical resection of the tumor: 16 of 25 patients (64%) with good prognoses had elevated GSTP1-1 levels before surgery and all values normalized within eight weeks post-operatively. High GSTP1-1 levels were not only reported in plasma; immunohistochemical investigations also showed increased levels of GSTP1-1 in head and neck tumor tissue, which decreased after radiation therapy (11).

Because blood cells and platelets contain large amounts of GSTP1-1, the sampling procedures for serum or plasma are exceptionally critical (12-14). We recently reported that plasma samples had to be collected in special silicone coated ethylene diamine tetra-acetic acid (EDTA) blood collection tubes and should be processed under strict conditions to avoid spuriously high plasma GSTP1-1 levels (14).

In the current study, we investigated plasma GSTP1-1 levels in blood samples from patients with head and neck squamous cell carcinoma (HNSCC) and benign head and neck lesions (BHNL).

Materials and methods

Patients

All blood samples from patients were collected at the Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Maastricht, the Netherlands. 53 patients with oral/oropharyngeal SCC, 12 patients with hypopharyngeal SCC and 28 patients with laryngeal SCC were studied. All diagnoses were confirmed by histology. Tumors were staged according to the TNM regulation of the International Union Against Cancer (15). The median age of the 93 HNSCC patients was 59 years (range 23-82 years) and 70 patients (75%) were males. Forty five patients with BHNL were studied (7 patients with parotid pleomorphic adenoma, 6 patients with submandibular disorders, 12 patients with rhinological disorders, 12 patients with vocal cord disorders, and 8 patients with neck disorders). The median age of this BHNL group was 47

years (range 16-86 years) and 26 patients (58%) were males. Patients with hepatic disorders were excluded from the study. As a control group served 230 blood donors, median age 35 years (range 18-69 years).

This study was approved by the local Medical Ethical Review Committee.

Plasma samples

Approximately 3 ml of blood was collected by venapuncture in vacuumed, silicone coated, K3 EDTA containing tubes (Vacutainer, order no. 606601 Becton Dickinson, Grenoble, France). Immediately after collection of the sample, blood was gently mixed with the EDTA solution. Tubes were centrifuged for 10 minutes at 3000 x g at room temperature within 2 hours. The upper two-third part of the plasma was collected and care was taken not to aspirate the platelets on top of the cell layer. Plasma samples were stored in portions at -20°C.

Assays

Plasma GSTP1-1 concentration was measured using a recently developed enzyme linked immuno assay (ELISA) (14). Briefly, microtiter plates were coated overnight with purified anti-GSTP1-1 monoclonal antibody. One hundred μ L of standard (0.4-100 μ g/L GSTP1-1), or 1:1 diluted plasma samples were added to the wells and incubated overnight. The next day the plates were washed and incubated with rabbit anti-GSTP1-1 serum, washed again, incubated with horseradish peroxidase labeled swine anti-rabbit and after a final wash, plates were stained with o-phenylenediamine- H_2O_2 . Standards and samples were measured in duplicate. A four parameter weighted logistic regression model was used to calculate standard curves and unknowns. The assay had no cross reactivity with GSTs of the Alpha or the Mu classes; the detection limit was 0.4 μ g/L GSTP1-1 and the intra- and inter-assay coefficients of variation were 5.8% and 10.9%, respectively.

Calculations and statistics

Plasma samples were categorized according to whether their GSTP1-1 content was below (normal) or above (elevated) the upper limit of the normal reference level. An upper normal reference limit of 21.8 μ g/L GSTP1-1 was calculated from plasma samples of the 230 healthy blood donors (14).

The statistical significance of differences between groups of patients and healthy controls was evaluated using the two-tailed Wilcoxon's rank-sum test. To avoid spurious differences, data were initially subjected to a Kruskal-Wallis analysis. All statistical analyses were performed using SPSS/PC+ statistical computer software version 5.0.

GSTP1-1 levels are expressed as medians with ranges, unless stated otherwise.

Results

The median GSTP1-1 levels for 53 patients with oral/oropharyngeal SCC (10.6 $\mu\text{g/L}$; 3.7-46.1 $\mu\text{g/L}$), 12 patients with hypopharyngeal SCC (11.9 $\mu\text{g/L}$; 5.2-146.6 $\mu\text{g/L}$) and 28 patients with laryngeal SCC (14.4 $\mu\text{g/L}$; 6.4-141.5 $\mu\text{g/L}$) were significantly elevated when compared to the median plasma GSTP1-1 levels for 45 BBNL patients (8.1 $\mu\text{g/L}$; 3.3-32.3 $\mu\text{g/L}$; $P<0.0001$, $P<0.01$ and $P<0.0001$, respectively). The median plasma GSTP1-1 levels for these three groups of patients with SCC were also significantly elevated when compared with the median plasma GSTP1-1 levels for 230 healthy controls (7.2 $\mu\text{g/L}$; 2.2-71.6 $\mu\text{g/L}$) ($P<0.0001$, $P<0.007$ and $P<0.001$, respectively).

However, plasma GSTP1-1 levels were above the upper limit of the normal reference level in only 6 of 53 patients (11%) with oral/oropharyngeal SCC, 1 of 12 patients (8%) with hypopharyngeal SCC and in 6 of 28 patients (21%) with laryngeal SCC (Figure 1). Overall 13 of 93 patients (14%) with HNSCC had elevated plasma GSTP1-1 levels, whereas only 1 of 45 patients (2.2%) with BBNL had an elevated plasma GSTP1-1 level (Figure 1). Plasma GSTP1-1 levels were not related to TNM stage grouping of the tumors (Table 1). Patients with HNSCC were significantly older than patients with BBNL (median 59 years, range 23-82 years and median 47 years, range 16-86 years, respectively; $P<0.002$).

Figure 1. Plasma glutathione S-transferase P1-1 (GSTP1-1) levels in patients with head and neck squamous cell carcinoma and patients with benign head and neck disorders. The dashed line denotes the upper normal reference limit.

Table 1. Plasma glutathione S-transferase P1-1 (GSTP1-1) levels in patients with head and neck squamous cell carcinoma according to stage of disease.

TNM Stage	Plasma GSTP1-1 ($\mu\text{g/L}$) median (range) (number of patients)			
	Oral/oropharyngeal SCC	Hypopharyngeal SCC	Laryngeal SCC	All sites HNSCC
I	9.3 (5.4-22.1) (9)	– (0)	14.6 (8.3-30.1) (6)	11.8 (5.4-30.1) (15)
II	9.4 (6.6-22.1) (12)	10.8 (9.5-12.1) (2)	17.6 (6.4-141.5) (6)	9.6 (6.4-141.5) (20)
III	10.8 (4.6-46.1) (11)	12.9 (12.0-13.8) (2)	14.0 (1)	11.2 (4.6-46.1) (14)
IV	10.9 (3.7-30.5) (20)	10.7 (5.2-24.2) (8)	11.7 (6.6-94.9) (13)	11.1 (3.7-94.9) (41)

Differences between TNM stage grouping were not significant. From one patient with an oral/oropharyngeal tumor and from two patients with laryngeal tumors the TNM classification and stage was not recorded.

Discussion

The ideal tumor marker is a tumor specific screening tool that assists in the workup and follow up of oncologic patients. It can also monitor the response to therapy, in which case it should be directly related to tumor bulk or tumor load. By these means, the marker should be able to detect tumor recurrence and/or regional or distant metastases before clinical presentation.

Hirata et al. reported elevated plasma GSTP1-1 levels in 41 of 61 patients (76%) with primary oral cancer and normal GSTP1-1 levels in patients with benign oral disease (10). These authors also reported an impressive decline in plasma GSTP1-1 levels after surgical resection of the tumor: 16 of 25 patients (64%) with good prognoses had elevated GSTP1-1 levels before surgery and all values normalized within eight weeks postoperatively. They also detected elevated plasma GSTP1-1 levels in most patients with tumor recurrence after surgery, even before this recurrence was detected clinically.

Because blood cells and platelets contain large amounts of GSTP1-1, the sampling procedures for serum or plasma are exceptionally critical (1,2,13,14,16). Recently we reported that even minor variations in the type of blood collection tubes or in the procedures used to handle and centrifuge the blood may substantially affect the resulting GSTP1-1 levels (14). This implies the need for identical sampling procedures for patients and controls. In our study the sampling, handling, storage, and assay procedures were identical for all patients and other donors.

Hirata et al. also reported that mean plasma GSTP1-1 levels were higher in patients with more advanced stages of SCC (10). More than 70% of patients with Stage III or IV oral cancer, in contrast to approximately 50% of those with Stage I and II disease, had elevated levels of GSTP1-1 in plasma. Niitsu et al. described elevated serum GSTP1-1 in more than 80% of patients with Stage III or IV gastric cancer, as opposed to only

about 50% of patients with Stage I and II (9). We could not confirm this relation in a larger group of patients (Hirata et al. (10) $n = 61$, Niitsu et al. (9) $n = 52$; this study $n = 93$). Median plasma GSTP1-1 levels were not significantly different among patients with Stage I, II, III or IV HNSCC. Furthermore, when median GSTP1-1 plasma levels were compared among tumor sites (oral-oropharynx, hypopharynx, larynx) and tumor stages, no significant differences were found (Table 1).

In most earlier studies, no differences in serum or plasma GSTP1-1 levels between males and females or among different age groups were observed (8-10,17,18). Various serum GSTP1-1 cutoff levels have been reported by different authors. Upper normal reference limits of 3.7 $\mu\text{g/L}$, 15.4 $\mu\text{g/L}$, 34.0 $\mu\text{g/L}$, 34.8 $\mu\text{g/L}$ GSTP1-1 in serum, 30.4 $\mu\text{g/L}$ GSTP1-1 in heparin plasma and 5.4 $\mu\text{g/L}$ and 13.99 $\mu\text{g/L}$ in EDTA plasma were reported in literature, based on samples from between 30 and 117 healthy controls (7-10,13,17,18). In agreement with the results reported by Takahashi et al., our control plasma GSTP1-1 levels also had to be log-transformed to obtain a nearly normal distribution (7). This transformation led to an upper normal reference level of 21.8 $\mu\text{g/L}$, as compared to a value of 21.9 $\mu\text{g/L}$, when calculated by taking mean value plus 2 times the standard deviation.

Our results also indicated that males had significantly higher GSTP1-1 levels than females. Among both males and females, a significant increase of GSTP1-1 levels was noted with increasing age (14). Males ages 20-40 years, 40-60 years or older than 60 years had median plasma GSTP1-1 levels of 8.2 $\mu\text{g/L}$, 9.1 $\mu\text{g/L}$ or 12.0 $\mu\text{g/L}$, respectively. The values for females in the same age groups were 5.5 $\mu\text{g/L}$, 6.6 $\mu\text{g/L}$ and 11.0 $\mu\text{g/L}$ respectively (14). The group of 93 HNSCC patients comprised 70 males and 23 females. The HNSCC group was also significantly older than the group of patients with benign head and neck disorders. These demographical variations may explain in part the difference in plasma GSTP1-1 levels between HNSCC and BHNH or control groups.

Hirata et al. also reported that the reappearance of elevated levels of GSTP1-1 in plasma occurred earlier than the clinical detection of recurrent disease in 10 of 13 patients with head and neck cancer (76.9%) (10). Several groups reported a decrease of GSTP1-1 to normal levels after radical surgical resection of the tumor (9,10).

Because we found that a small minority of patients (14%) had elevated GSTP1-1 levels in the diagnostic phase of our study, no collection of plasma for follow up studies was performed.

Earlier we studied 20 patients with gastrointestinal carcinomas before and after surgical resection of their tumors and found elevated plasma GSTP1-1 levels after resection of the tumor in some patients with normal preoperative levels (14). This indicates that, in patients with gastrointestinal tumors a postoperative GSTP1-1 elevation may well be caused by other mechanisms not related to tumorload. It is possible that this also occurs in patients with HNSCC; however, further studies are needed to ascertain this.

Perhaps manipulation of tumor and/or normal tissue may cause high intravascular GST levels. When tumor or adjacent normal tissue contains a very high level of

GSTP1-1, damage to these tissues during surgery could cause intravascular leakage resulting in increased plasma levels postoperatively.

Elevated GSTP1-1 levels were found in patients with non-malignant hepatic disorders (cirrhosis, hepatitis) suggesting that elevation of plasma or serum GSTP1-1 is not specific for malignant disease (8,9). In our study, patients were screened on hepatic disorders by liver enzyme counts. Patients with high entries have been excluded for this study. These criteria were not mentioned in most other reports.

In the current study, the clinical application of GSTP1-1 as a tumor marker for HNSCC was evaluated. The levels of GSTP1-1 in HNSCC were significantly higher than the levels in BHL and control groups. However, only 13 of 93 patients with HNSCC demonstrated elevated GSTP1-1 plasma levels. In contrast to earlier reports by others, we conclude that GSTP1-1 can not be used as a tool for the diagnostic screening of patients with HNSCC.

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5

CHAPTER

POLYMORPHISMS OF THE GLUTATHIONE S-TRANSFERASE P1 GENE AND HEAD AND NECK CANCER SUSCEPTIBILITY

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Abstract

Background Factors determining the individual susceptibility to head and neck squamous cell carcinoma (HNSCC) are still largely unknown. An imbalance between enzymes involved in the toxification and detoxification of (pre)-carcinogens closely related to HNSCC which may appear during smoking and alcohol consumption, may play a role. Genetic polymorphisms in glutathione S-transferases (GSTs) often result in altered detoxification, which may contribute to individual susceptibility to HNSCC.

Methods We studied the frequencies of polymorphic variants in the *GSTP1* gene in 235 patients with HNSCC and 285 healthy controls. In addition, data on exposure to alcohol and tobacco were recorded. DNA was extracted from whole blood and polymerase chain reaction (PCR) based methods were used to detect genetic polymorphisms.

Results In patients with HNSCC and control groups the homozygous *GSTP1 BB* genotype was observed in 12.3% and 13.6% respectively. No statistical differences were found for the *GSTP1 AA* and *GSTP1 AB* / *GSTP1 BB* genotypes.

Conclusions Our study showed that genetic polymorphisms of *GSTP1* are not associated with altered susceptibility to HNSCC.

Introduction

An imbalance in phase I drug metabolism enzymes such as cytochromes P450 (CYPs) or phase II detoxification enzymes such as glutathione S-transferases (GSTs) may contribute to the individual susceptibility to head and neck squamous cell carcinoma (HNSCC). These enzymes, for instance, are involved in the toxification and detoxification of metabolites of polycyclic aromatic hydrocarbons (PAHs), one of the primary carcinogens of tobacco smoke (1). Most PAHs first require activation by phase I enzymes to become potential carcinogens that are subsequently detoxified by phase II enzymes.

As a result of conjugation with glutathione, the potential carcinogens are eliminated and DNA or other important biomolecules are protected against damage or adduct formation (2-5). In humans, the GST enzymes can be divided into 5 main classes: Alpha (GSTA), Mu (GSTM), Pi (GSTP), Theta (GSTT), and Zeta (GSTZ). Each class consists of one or more iso-enzymes with different, but sometimes overlapping, substrate specificity (4,5). At present, genetic polymorphisms have been demonstrated for the *GSTM1*, *GSTT1*, and *GSTP1* genes (5-9).

Polymorphisms in the *GSTP1* gene consist of the variant genotypes *GSTP1 AB* and *GSTP1 BB* next to the wild type *GSTP1 AA*. The frequency of *GSTP1 AA* in healthy controls ranges from 42 – 69% (9-14). A transition of Adenine (A) to Guanine (G) at nucleotide 313 in exon 5 of the *GSTP1* gene results in substitution of isoleucine (Ile) to valine (Val) at position 104 in the amino acid sequence of the protein (7,15,16). The valine variants exhibit lower specific activity and affinity for the electrophilic substrates of the enzyme (7,13,15). The valine containing homozygous variant, *GSTP1 BB*, occurs in approximately 10 % of healthy controls, whereas the heterozygous Ile-Val variant, *GSTP1 AB*, occurs in approximately 35% of controls (9-14).

The *GSTP1* gene encodes for the iso-enzyme GSTP1-1. The GSTP1-1 enzyme level has been extensively studied in relation to tobacco associated malignancies. The GSTP1-1 enzyme is overexpressed in many pre-neoplastic and neoplastic lesions. Elevated tissue levels of GSTP1-1 enzyme have been found in stomach, colorectal, bladder, oral, pharynx, larynx, lung, skin and breast tumors compared with normal tissues of matched controls (5,17-22). The GSTP1-1 enzyme may be involved in the resistance to chemotherapeutic agents and radiotherapy (5,23-25). Since GSTP1-1 is also involved in the metabolism and subsequent removal of anti-cancer drugs, high levels of GSTP1-1 in tumors may contribute to drug resistance in several different cancers (26-28). However, there are also contradictory reports.

Because the amino acid change as result of the polymorphism close to the area of hydrophobic binding site for electrophiles, the homozygous *GSTP1 BB* and heterozygous *GSTP1 AB* gene products result in a decreased specific activity and affinity of the GSTP1-1 enzyme for electrophilic compounds (7). Thus, the altered metabolic activity of these enzymes could influence susceptibility to head and neck cancer. Earlier we investigated the possible involvement in HNSCC and in benign head and neck lesions (BHNLS) of genetic polymorphisms in *CYP1A1*, *GSTM1* and *GSTT1* but we found no enhanced rates of GST null genotypes in the cancer population (29). Earlier work from

our group showed that the GSTP1-1 enzyme, in a quantitative sense, is by far the most important detoxification enzyme in human head and neck tissues (30). Therefore we now investigated genetic polymorphisms in the GSTP1 gene in a large series of patients with HNSCC and in matched healthy controls. We also evaluated exposure to alcohol and tobacco of these subjects and evaluated the potential effect modification between GSTP1 polymorphisms and these environmental exposures.

Materials and Methods

Patients and controls

This case-control study was conducted in the patient referral region of Maastricht University Hospital. A total of 235 Caucasian patients (185 males, 50 females) with primary HNSCC were recruited during their scheduled admission at the Department of Otolaryngology, Head and Neck Surgery of the University Hospital Maastricht between 1994 and 1998 (mean age 59 years; range 23-86 years). This group consists of 100 patients with larynx carcinoma (mean age 60 years; range 35-85 years), 114 patients with oral-cavity/oropharynx carcinoma (mean age 58 years; range 23-86 years), and a group of 21 patients (mean age 61 years; range 41-82 years) with hypopharynx carcinoma.

From the same base population, a group of 285 healthy Caucasian blood donors (199 males, 86 females) were recruited at the Blood Transfusion Centre Limburg and served as a control group, frequency matched on age in 10 year age groups (mean age 50 years; range 19-91). The investigations were approved by the Medical Ethical Review Committee of the University Hospital of Maastricht and informed consent was obtained from all patients and controls according to the Helsinki Declaration II (1975, revised 1983).

Exposure assessment, blood sampling and genotyping

Information on tobacco use and alcohol consumption was collected for all patients and controls by interview. The amount of packyears (PY) was calculated as the number of years smoking x the number of packs a day (assuming 20 cigarettes = 1 pack). Individuals were defined as never smokers when they had smoked less than one pack year cumulatively.

Blood was collected by venapuncture in sterile siliconized EDTA K3 (15%) 4 ml vacutainer tubes (Becton Dickinson, San Jose, CA, USA). Immediately after collection whole blood was stored at -20 °C until use. Genomic DNA was isolated from whole blood using the Wizard™ genomic DNA purification kit, according to the instructions of the manufacturer (Promega, Madison Wisconsin, USA).

Genetic polymorphisms were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). For studying the genetic polymorphism in GSTP1 a primer set (105F and 105R) was designed so that the presence of the A to G substitution at codon 313 resulted in the appearance of an Alw261 restriction enzyme site (9). All primers were synthesized by Life Technologies (Breda, the Netherlands). All chemicals needed for PCR were purchased from Promega, Madison Wisconsin, USA.

Statistical analysis

The statistical program SPSS 8.0 for Windows was used. Information on *GSTP1* genotype, smoking or alcohol was missing in 0, 0, and 11 of the cases and 0, 3, and 1 of controls, respectively. For the purpose of the analysis, the PY of cigarette smoking were categorized as <1, 1-20, 21-40, 41-60, and 60+ PY or in increments of 10 PY. Alcohol consumption was categorized as 0-9, 10-50, 51+ gram/day. Because there were only 3 subjects who indicated never to have consumed alcohol, the first category consists of 0-9 grams per day. Analyses were conducted for the total group of patients with HNSCC, as well as for oral cavity/oropharyngeal and laryngeal carcinoma separately. Because of small numbers of hypopharyngeal carcinoma, this group was not evaluated separately.

The association between *GSTP1* polymorphisms, smoking, alcohol and HNSCC was modelled through multivariate logistic regression analysis, controlling for age and gender. First, analyses were conducted to examine the associations between *GSTP1* genotype and HNSCC, controlling for age, gender, smoking and alcohol. Specifically, we evaluated whether subjects with *GSTP1* AA are less susceptible to HNSCC than those with *GSTP1* AB/BB. Likewise associations between smoking and HNSCC or alcohol consumption and HNSCC were examined, controlling for age, gender and alcohol or smoking, respectively. The strength of the associations were estimated as Odds Ratios combined with 95% Confidence Intervals. Subsequently, logistic regression analyses were performed in which the association between smoking or alcohol and HNSCC (and its subgroups) was evaluated within subgroups of the *GSTP1* genotypes; the wildtype *GSTP1* AA group and the combined heterozygote/homozygote *GSTP1* AB/BB group. Likelihood ratio tests were conducted to test statistical significance of regression model terms, as well as for test for trends and interactions. Two-sided p values are reported throughout.

Finally, the interaction between smoking and alcohol was evaluated, specifically within subgroups of the *GSTP1* genotypes.

Results

Table 1 shows the frequency analyses for the A to G substitution at codon 313 in the *GSTP1* gene, which was examined in 235 HNSCC patients and 285 controls. The *GSTP1* BB frequency is low in the control group, as well as in the total HNSCC group or its subgroups. To maintain values for appropriate statistical analysis, we combined the frequencies for *GSTP1* AB and *GSTP1* BB (Tables 3-5).

Table 1. Frequency distribution of *GSTP1* genotypes in HNSCC cases and controls

<i>GSTP1</i> genotype	Controls N (%)	All HNSCC cases N (%)	Oral/oropharynx N (%)	Larynx N (%)
AA	125 (43.9)	116 (49.4)	53 (46.5)	51 (51.0)
AB	121 (42.5)	90 (38.3)	48 (42.1)	36 (36.0)
BB	39 (13.6)	29 (12.3)	13 (11.4)	13 (13.0)
Total	285	235	114	100

In Table 2, the frequency distribution is given on the smoking habits and the alcohol consumption in HNSCC cases, subgroups of HNSCC and the control group. The HNSCC group in total, as well as the two subgroups, show a significant higher tobacco exposure compared to the control group. The control group shows a significantly lower alcohol consumption than the HNSCC cases (total or subgroups). In the oral/oropharyngeal subgroup, the rate of daily alcohol consumption more than 51 gram/day is much higher compared to the laryngeal subgroup (54.1% vs 30.4% respectively). Alcohol consumption more than 51 gram/day hardly exists in the control group.

Table 2. Frequency distribution of smoking and alcohol consumption in HNSCC cases and controls

Covariable	Controls N (%)	All HNSCC cases N (%)	Oral/Oropharynx N (%)	Larynx N (%)
Smoking Packyears				
<1	86 (30.2)	13 (5.5)	7 (6.1)	3 (3.0)
1-20	130 (45.6)	36 (15.3)	20 (17.5)	13 (13.0)
21-40	60 (21.1)	113 (48.1)	52 (45.6)	53 (53.0)
41-60+	9 (3.2)	73 (31.1)	35 (30.7)	31 (31.0)
Total	285	235	114	100
Alcohol Gram/day				
0-9	139 (48.8)	18 (8.0)	7 (6.3)	10 (10.9)
10-50	142 (49.8)	106 (47.4)	44 (39.6)	54 (58.7)
51+	3 (1.1)	100 (44.6)	60 (54.1)	28 (30.4)
Total	284	224	111	92

Table 3 shows that the Odds Ratios (OR) for alcohol or smoking are similar in the HNSCC group as a total and the two subgroups, except for alcohol consumption more than 51 gram/day, where there is a higher OR in the oral/oropharyngeal group. To provide appropriate statistical values, smoking is displayed with an increment of 10 PY. When no adjustment is made for smoking and alcohol (but only for age and gender), the OR for *GSTP1* AA vs *GSTP1* AB/BB will be 1.31 in the total HNSCC group.

The association between alcohol and smoking and the *GSTP1* polymorphisms is shown in Table 4. For oral/oropharyngeal cases with the *GSTP1* AA genotype, alcohol consumption more than doubles the OR compared to the *GSTP1* AB/BB genotype. This is also shown for larynx carcinoma. In the oral/oropharyngeal cases, as well as the laryngeal cases with the *GSTP1* AB/BB genotype, Odds Ratios are increased by smoking compared with the *GSTP1* AA genotype. We tested for interaction of alcohol consumption between subgroups of *GSTP1* in the total HNSCC group; LR (Likelihood Ratio) $-\chi^2 = 0.96$, $df=2$, $p>0.05$. Also the interaction of smoking between subgroups of *GSTP1* in the total HNSCC group is calculated; LR $-\chi^2 = 6.04$, $df=1$, $p<0.025$.

Table 3. Association between alcohol, smoking and GSTP1 genotype and HNSCC (total group of cases and subgroups of oral/oropharyngeal and laryngeal cancer)

Risk factor	Total HNSCC N=235	Oral/oropharynx N=114	Larynx N=100
	OR (95% CI)	OR (95% CI)	OR (95% CI)
Alcohol Gram/day			
0-9	1.0 * (ref)	1.0 * (ref)	1.0 * (ref)
10-50	5.36 (2.68 - 10.72)	8.23 (3.05 - 22.20)	5.00 (1.97 - 12.68)
51+	194.16 (49.21 - 766.31)	420.03 (87.47 - 2016.92)	113.36 (22.91 - 560.84)
Test for trend, p-value	< 0.001	< 0.001	<0.001
Smoking Packyears			
Increment 10 PY	1.80 † (1.51 - 2.14)	1.63 † (1.34 - 1.97)	1.85 † (1.51 - 2.26)
GSTP1 AB/BB	1.0 § (ref)	1.0 § (ref)	1.0 § (ref)
GSTP1 AA	0.99 (0.58 - 1.71)	0.78 (0.38 - 1.60)	0.90 (0.46 - 1.79)

* Odds ratio, adjusted for age, gender, smoking, GSTP1 genotype

† Odds ratio, adjusted for age, gender, alcohol consumption, GSTP1 genotype

§ Odds ratio, adjusted for age, gender, alcohol consumption, smoking

ref = reference value

CI=confidence interval

Table 4. Association between alcohol consumption, smoking and HNSCC in subgroups of GSTP1 genotype

Risk factor	Total HNSCC OR (95% CI)	Oral/oropharynx OR (95% CI)	Larynx OR (95% CI)
Subgroup GSTP1 AA (108 cases, 122 controls)			
Alcohol Gram/day			
0-9	1.0 * (ref)	1.0 * (ref)	1.0 * (ref)
10-50	7.89 (2.75 - 22.68)	15.88 (2.66 - 94.66)	6.95 (1.76 - 27.50)
51+	232.24 (38.22 - 1407.08)	696.88 (70.08 - 6930.15)	140.16 (16.22 - 1211.12)
Test for trend, p-value	<0.001	<0.001	<0.001
Smoking Packyears			
Increment 10 PY	1.51† (1.21 - 1.80)	1.34 † (1.10 - 1.80)	1.63 † (1.21 - 1.97)
Subgroup GSTP1 AB/BB (116 cases, 159 controls)			
Alcohol Gram/day			
0-9	1.0 * (ref)	1.0 * (ref)	1.0 * (ref)
10-50	4.21 (1.65 - 10.70)	5.57 (1.64 - 18.89)	3.70 (1.07 - 12.84)
51+	191.67 (20.88 - 1759.33)	307.88 (26.42 - 3588.09)	74.82 (6.76 - 828.31)
Test for trend, p-value	<0.001	<0.001	<0.001
Smoking Packyears			
Increment 10 PY	2.28 † (1.73 - 3.01)	2.41 † (1.67 - 3.47)	2.46 † (1.74 - 3.49)

* adjusted for age, gender, smoking ref = reference value,

† adjusted for age, gender, alcohol CI = confidence interval

In Table 5 we show a significant interaction between smoking and alcohol consumption in HNSCC susceptibility when not adjusted for *GSTP1* genotype. The effect of smoking is stronger when more alcohol is consumed (OR increases from 1.27 to 3.53). However, because of small numbers (especially in high alcohol consumption category), interaction between alcohol and smoking and *GSTP1* genotype can not be fully calculated.

Table 5. Interaction between alcohol and smoking regarding risk of HNSCC, in total group and in subgroups defined by *GSTP1* genotype

Alcohol category	Smoking OR (95% CI) per 10 Packyears
Total group <i>GSTP1</i> AA/AB/BB (224 cases, 281 controls)	
Alcohol Gram/day	
0-9	1.27 (1.03 - 1.57)
10-50	2.46 (1.90 - 3.20)
51+	3.53 (1.27 - 9.81)
Test for interaction smoking/alcohol, χ^2 (p-value)	18.2 (p<0.01)
Subgroup <i>GSTP1</i> AA (108 cases, 122 controls)	
Alcohol Gram/day	
0-9	1.24 (0.96 - 1.60)
10-50	2.08 (1.44 - 3.01)
51+	n.p.
Test for interaction smoking/alcohol, χ^2 (p-value)	-
Subgroup <i>GSTP1</i> AB/BB (116 cases, 159 controls)	
Alcohol Gram/day	
0-9	1.43 (0.91 - 2.28)
10-50	2.85 (1.96 - 4.16)
51+	0.57 (0.06 - 5.48)
Test for interaction smoking/alcohol, χ^2 (p-value)	3.5 (p>0.05)

n.p. = no estimation possible

CI = confidence interval

Discussion

Glutathione S-transferases are involved in the detoxification of a wide variety of chemical carcinogens, including those derived from tobacco smoke (5).

The enzyme *GSTP1-1* is known to catalyze the detoxification of PAH in vitro, and may be a major enzyme involved in the detoxification of tobacco related metabolic products in vivo (5). The expression in tissues may vary depending on site or localisation, and immunohistochemical studies have shown a constant and widespread *GSTP1-1* expression throughout pharynx and larynx squamous cell epithelium in both normal and tumor tissue (10). Earlier we showed that *GSTP1-1* is the most important GST enzyme in human head and neck tissues (30). Therefore, we studied the occurrence of polymorphisms of this gene (*GSTP1*) in patients with different head and neck tumors and controls.

We previously reported that the *GSTM1* and *GSTT1 null* genotypes are not associated with an increased susceptibility for HNSCC (29). In one of the largest studies performed so far, we found no significant association between risk of HNSCC and occurrence of genetic polymorphisms in the *GSTP1* gene. This was also found for oral/oropharyngeal cancers and laryngeal cancers separately.

The *GSTP1-1* enzyme is overexpressed in many neoplastic lesions, such as malignancies of stomach, colorectum, bladder, oral tissue, pharynx, larynx, lung, skin and breast, compared with corresponding normal tissues (5,17-22,30). However, when estimating *GSTP1-1* serum levels in patients with head and neck cancer, we found significantly higher levels compared to controls, but no association with *GSTP1* genotype was observed (31). This may indicate that the overexpression of *GSTP1-1* in head and neck cancer is not dependent on genotype but most probably transcriptionally regulated.

We found a strong association between smoking and alcohol consumption and HNSCC susceptibility. Assessment of smoking or alcohol consumption is difficult, especially with respect to patient history for the total accumulative exposure. Exposure usually takes place over many years and is not always consistent. In addition, different cigarette brands may yield completely different nicotine and tar exposures. High consumption of alcohol often is not revealed by patients because of its social inacceptance. It therefore would help much to determine additional biomarkers such as serum gamma glutamyltransferase, which could be indicative of recent consumption of alcohol. Development of biomarkers which could indicate the exposure and the period of consumption would be more helpful.

In our control population, *GSTP1 BB* occurs in 13.6%, and *GSTP1 AB* in 42.5%. Our data for the healthy controls are in agreement with the genotype frequencies in the control populations reported by other researchers (9-14). Similar observations were found for the HNSCC group (12.3% and 38.3% respectively).

Our data for HNSCC patients are also in agreement with several other studies performed. Lin et al. (14) found that polymorphisms in *GSTP1* were not associated with increased risk for esophageal cancer. They even found a slightly lower occurrence of *GSTP1 AB* or *BB* genotypes in cancer and hyperplasia cases than in controls (14). Jourenkova et al. (32) and Matthias et al. (10) found no association between the *GSTP1 AB* or *BB* genotypes and risk of larynx cancer. Harries et al. (9) reported an association between occurrence of the *GSTP1* polymorphism and susceptibility to bladder or testicular cancer, but found no association with breast or colon cancer. In other studies no association was found between the *GSTP1* polymorphism and colorectal, lung or breast cancer (33,34).

Several other studies, however, revealed a significant correlation between occurrence of *GSTP1* polymorphisms and pre-neoplastic and neoplastic lesions. Lung cancer patients had a significantly higher frequency of *GSTP1 BB* genotype (15.9%) and a lower frequency of *GSTP1 AA* genotype (38.4%) than controls (9.1% and 51.5% respectively) (12). Homozygous *GSTP1 BB* was found significantly more often in patients with Barrett's esophagus and esophageal adenocarcinoma (35). Matthias et al. (10) have

reported that the *GSTP1* polymorphism influences susceptibility to pharyngeal but not to laryngeal squamous cell carcinoma. However, when comparing our patient groups in a similar way as Matthias et al. (10) (oral cavity/oropharyngeal SCC and hypopharyngeal SCC versus laryngeal SCC), we did not find a significant difference in the genotype distributions.

Ryberg et al. (12) reported that patients with the *GSTP1* *BB* genotype had higher DNA adduct levels in lung tissue than the *GSTP1* *AA* genotypes. They also found a linear trend between DNA adduct levels and occurrence rates of *GSTP1* *B* genotypes.

The variant genotypes *GSTP1* *AB* and *GSTP1* *BB* showed a different specific activity and affinity for several electrophilic substrates but were identical in their affinity for glutathione (7,15). The properties of the variant genotypes were conducted in "in vitro experiments" with recombinant proteins by Ali-Osman et al. (7), which however, does not necessarily provide adequate information on the enzymes and their reactions "in vivo." In agreement with the in vitro results, Watson et al. (13) and van Lieshout et al. (35) reported a significantly lower conjugating activity with 1-chloro-2,4-dinitrobenzene (CDNB) in lung and esophageal tissue samples, respectively, in patients with *GSTP1* *AB* and *GSTP1* *BB* compared to *GSTP1* *AA* genotypes.

In summary, we found no evidence for a possible genetic predisposition to HNSCC due to a genetic polymorphism in the *GSTP1* gene. This suggests that the carcinogens involved in the etiology of HNSCC may not be critically dependent on detoxification by the *GSTP1*-1 enzyme. Further studies on other combinations of polymorphic genotypes in detoxification enzymes and their relation to local and systemic enzyme concentration seem to be justified.

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CHAPTER

THE ASSOCIATION BETWEEN GLUTATHIONE S-TRANSFERASE P1 GENOTYPE AND PLASMA LEVEL IN PATIENTS WITH HEAD AND NECK CANCER

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Abstract

Background Glutathione S-transferase P1 (GSTP1-1) is an important enzyme as it plays a major role in many detoxification reactions, including tobacco related metabolic products. GSTP1-1 is the most abundant of all GST enzymes in normal human head and neck epithelium, whereas it is overexpressed in head and neck malignancies. At least three different GSTP1 genotypes exist, AA, AB and BB, the latter two which have been correlated with reduced enzyme activity. Many authors have studied the GSTP1 genotypes in relation to the risk for human head and neck squamous cell carcinoma (HNSCC). A correlation between GSTP1 genotype and GSTP1-1 enzyme plasma levels has not been made before.

Materials and Methods GSTP1 genotype and GSTP1-1 plasma levels were established in 87 HNSCC patients and 51 patients with benign head and neck lesions (BHNL) who served as controls.

Results For all GSTP1 genotypes (AA, AB and BB) in HNSCC patients the mean corresponding plasma GSTP1-1 values were significantly higher compared to the controls. There was no significant difference in the plasma GSTP1-1 levels between the different genotypes in HNSCC patients.

Conclusion There is no association between GSTP1 genotype and GSTP1-1 plasma levels in patients with head and neck cancer.

Introduction

Squamous cell carcinoma of the head and neck region (HNSCC) is the 5th most common malignancy in the world. Most cases of HNSCC are associated with alcohol abuse or smoking of tobacco but only a small number of individuals do develop HNSCC. This suggests the influence of genetic components in the susceptibility to HNSCC. Some of the factors that could explain this difference in susceptibility may be the presence of polymorphisms in genes coding for enzymes involved in carcinogen metabolism, such as the glutathione S-transferase (GST) enzymes. GSTs remain a focal point for research because of their role as defense mechanism against xenobiotics such as polycyclic aromatic hydrocarbons (PAHs), which are an important class of carcinogens present in tobacco smoke (1). Most precarcinogens have to be activated by phase I enzymes such as cytochrome P450 1A1 (CYP1A1) to become a carcinogen in the form of reactive epoxide intermediates such as benzo[a]pyrene diol epoxide (BPDE) (1). These carcinogens subsequently may be detoxified by various phase II enzymes such as GST enzymes.

Human GSTs can be subdivided into five classes, Alpha (A), Mu (M), Pi (P), Theta (T) and Zeta (Z). Each class includes one or more isoenzymes. Glutathione S-transferase P1-1 (GSTP1-1) is a major enzyme in the detoxification of tobacco related metabolic products (2) and because it is quantitatively the most abundant GST enzyme in the human head and neck area (3), many researchers have tried to link its genotype to the risk for HNSCC (4-7).

Several genotypes of the *GSTP1* gene have been described, one resulting in isoleucine versus valine amino acid substitution at position 104 in the corresponding enzyme (8). This 104 valine or *GSTP1 B* variant is homozygously present in 10% of the Caucasian population. Approximately 55% of the Caucasian population is homozygous for the 104 isoleucine or *GSTP1 A* allele, whereas approximately 35% is heterozygous, containing both alleles (Ile and Val).

Homozygosity for the wildtype *GSTP1 A* allele has been found in a lower incidence in patients with oral and pharyngeal carcinoma in a Caucasian population (4,5), whereas a higher incidence of the *GSTP1 A* allele in pharyngeal and laryngeal cancer patients in a Japanese study has been described (6). Another study revealed no significant differences in *GSTP1* genotypes in laryngeal cancer patients as compared to healthy control subjects (7). Our group found that genetic polymorphisms of *GSTP1* are not associated with altered susceptibility to HNSCC (9).

GSTP1 genotypes and their possible influence on the risk of other forms of malignant and benign tumors have been evaluated in patients with lung cancer, where one study showed a significantly higher incidence of the *GSTP1 BB* genotype in the cancer group (1) whereas another study revealed no difference (10). In urogenital malignancies significantly higher incidence of the *GSTP1 B* genotype was found (11) whereas in gastrointestinal malignancies no higher incidence was found (11,12). In the premalignant Barrett's esophagus, however, a higher incidence of the *GSTP1 B* genotype was demonstrated (14). In breast carcinoma a higher incidence of the *GSTP1 B* genotype was found as well (15).

Since the GSTP1-1 enzyme was shown to be overexpressed in a wide variety of tumors (16), another field of interest is the relevance of plasma GSTP1-1 levels as tumor-marker in several categories of cancer patients (17-21). However, some arguments pleading against a role of GSTP1-1 as a plasma tumor marker in patients with HNSCC and gastrointestinal cancer were recently given by us (22,23). We found significantly higher GSTP1-1 plasma levels in HNSCC patients as compared to a group of benign head and neck lesions (BHNL) as controls. However, only 14 % of patients with HNSCC had plasma levels above the upper normal reference level of 21.8 µg/L, which was estimated after analyzing samples of 230 healthy blood donors. In the past, several investigators have correlated GSTP1 genotype and GSTP1-1 tissue levels, e.g. in lung malignancies (24), in human placenta (25) and in Barrett's esophagus (14). They all found significant differences between the individual genotypes GSTP1 AA, AB and BB. An association between the GSTP1 genotype and GSTP1-1 plasma levels in patients with head and neck malignancies has however never been studied.

Since GSTP1-1 enzyme activity may be associated with GSTP1 genotype (14,24,25), we now want to investigate whether patients with elevated GSTP1-1 plasma levels all belong to a certain GSTP1 genotype. Therefore we investigated whether one of the genotype subcategories differed significantly in mean GSTP1-1 plasma level. We also compared the mean plasma levels in 51 controls and 87 HNSCC patients in each of the three genotype subcategories (GSTP1 AA, AB or BB).

Materials and Methods

A total of 138 patients were included preoperatively during their scheduled admission at the department of Otorhinolaryngology, Head and Neck Surgery of the University Hospital of Maastricht between 1995 and 1998. The group consisted of 51 patients with BHNL who served as controls [23 females (45 %) and 28 males (55 %)] and 87 patients with HNSCC [21 females (24 %) and 66 males (76 %)], all with no previous history of cancer.

In the BHNL group the mean age was 49 ± 18 years, range 16-86 years. In the HNSCC group the mean age was 58 ± 12 years, range 23-82 years. All sites of tumor expression are given in Table 1.

Table 1. Site of tumor origin in HNSCC.

Site of tumor origin	Number of cases
Oral cavity	25
Oropharynx	23
Hypopharynx	12
Larynx	27

Informed consent was obtained and medical and other anamnestic data were obtained by interview. The Medical Ethical Review Committee approved the investigations.

Blood sampling

Approximately 3 ml of blood was collected by venapuncture in vacuumed, silicone-coated, K3 EDTA-containing tubes (Vacutainer, Order No. 606601, Becton Dickinson, Grenoble, France). Immediately after collection, blood was gently mixed with the EDTA solution. Approximately 0.5 ml of whole blood was stored at -20°C for genotyping. The remaining was centrifuged for 10 minutes at 3000 X g at room temperature within 2 hours. The upper two-third of the plasma was collected, and care was taken not to aspirate the platelets on top of the cell layer. Plasma samples were stored at -20°C until use.

Assays

GSTP1 genotyping

Genomic DNA was isolated from whole blood using the Wizard™ genomic DNA purification kit, according to the instructions of the manufacturer (Promega, Madison Wisconsin, USA). Genetic polymorphisms in *GSTP1* were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, using a primer set (105F and 105R), designed so that the presence of the rare B allele (valine) resulted in the appearance of an *A/w261* restriction enzyme site according to Harries et al. (11). All primers were synthesized by Pharmacia Biotech (Roosendaal, the Netherlands). All chemicals needed for PCR were purchased from Promega.

Plasma GSTP1-1 measurement

Plasma GSTP1-1 levels were measured using a recently developed enzyme linked immunosorbent assay (ELISA) (23). Briefly, microtiter plates were coated overnight with purified anti-GSTP1-1 monoclonal antibody. One hundred µL of standard (0.4-100 µg/L GSTP1-1), or 1:1 diluted, plasma samples were added to the wells and incubated overnight. The next day the plates were washed and incubated with rabbit anti-GSTP1-1 serum, washed again, and incubated with horseradish peroxidase-labeled swine anti-rabbit antibody; and after a final wash, plates were stained with O-phenylenediamine- H₂O₂. Standards and samples were measured in duplicate. A four-parameter weighted logistic regression model was used to calculate standard curves and unknowns. The assay had no cross-reactivity with GSTs of the Alpha or Mu classes; the detection limit was 0.4 µg GSTP1-1 /L, and the intra- and inter-assay coefficients of variation were 5.8% and 10.9%, respectively.

Statistical analysis

For statistical analysis we used the statistical package SPSS for Windows 8.0. The statistical significance of differences between the individual means for the control and HNSCC groups for each genotype was tested with an independent t-test for equality of means. Possible unreliable variables were detected by the Cooks test.

The significance of the difference of means for the different genotypes in each category of disease (benign, malignant or total) was tested with a One-way Analysis of Variances, combined with a Levene's test for equality of variances and completed with Bonferroni and Tamhane post-hoc tests.

Results

The results of the assays and different comparative tests are shown in Tables 2-4.

The distribution of genotypes in the whole study group (HNSCC and BHNH controls) was 62 individuals (44.9%) with the *GSTP1* AA genotype, 56 (40.6%) with the AB genotype and 20 (14.5%) with the BB genotype. For the control group this was 26 individuals (51.0%) with *GSTP1* AA, 18 (35.3%) with AB and 7 (13.7%) with the BB genotype. For the HNSCC group the distribution was 36 individuals (41.4%) with *GSTP1* AA, 38 (43.7%) with AB and 13 (14.9%) with the BB genotype.

Table 2 shows the mean *GSTP1*-1 plasma levels according to *GSTP1* genotype in the two study groups (HNSCC and controls). For all *GSTP1* genotypes (AA, AB and BB) in HNSCC the mean and median values were higher as compared to corresponding controls. The highest mean values were observed in the heterozygotic *GSTP1* AB groups in both study groups.

Table 2. Plasma *GSTP1*-1 levels.

Group	<i>GSTP1</i> Genotype	Mean*	N	Sd*	Minimum*	Maximum*	Median*
Control	AA	9.1	26	3.2	5.1	17.9	8.3
	AB	9.9	18	6.1	4.2	32.3	8.2
	BB	9.3	7	4.5	3.3	15.9	7.4
	Total	9.4	51	4.5	3.3	32.3	8.2
HNSCC	AA	12.0	36	5.1	4.6	26.8	10.7
	AB	19.8	38	26.1	3.7	141.5	11.8
	BB	15.0	13	5.3	9.3	26.8	14.5
	Total	15.9	87	17.9	3.7	141.5	11.2
Total	AA	10.8	62	4.6	4.6	26.8	9.5
	AB	16.6	56	22.1	3.7	141.5	10.0
	BB	13.0	20	5.6	3.3	26.8	12.8
	Total	13.5	138	14.8	3.3	141.5	10.0

* Values are given as µg/L.

N: number of subjects in each category.

Sd: standard deviation.

Table 3 shows the results of the independent samples t-tests in order to compare the mean *GSTP1*-1 plasma values in each genotype subgroup. When comparing the HNSCC to the control group for the *GSTP1*AA genotype, plasma *GSTP1*-1 values were significantly higher in the HNSCC-group (12.0 and 9.1 µg/L respectively, $p = 0.008$). A similar result was obtained for the AB genotype, even when corrected for one abnormal high value (141.5 µg/L) which appeared unreliable according to Cook's test (19.8 vs. 9.9 µg/L, $p = 0.031$). The *GSTP1* BB genotype also showed a difference that was significant (15.0 vs. 9.3 µg/L, $p = 0.028$). The difference in *GSTP1*-1 plasma values for the combined control and HNSCC groups was significant as well (9.4 vs. 15.9 µg/L, $p=0.002$).

Table 3. Comparison of mean GSTP1-1 enzyme plasma levels in the GSTP1 genotype subgroups (T-tests.)

GSTP1 genotype	t-test Control vs. HNSCC (p-values)
AA	0.008
AB	0.031
AB, exclusion of highest value	0.035
BB	0.028
Total	0.002

(Difference in GSTP 1-1 plasma level between control and HNSCC for the different genotypes)

Table 4 shows the results of one-way analyses of variance to compare the mean GSTP1-1 plasma concentrations for the different genotypes in each study group. The difference between the mean plasma GSTP1-1 levels for each GSTP1 genotype in the control group was not significant (9.1 (AA); 9.9 (AB) and 9.3 µg/L (BB) respectively, $p=0.862$). Neither was it for the HNSCC group (12.0; 19.9 and 15.0 µg/L, $p=0.170$) and the total of both groups (both controls and HNSCC) (10.8; 16.6 and 13.0 µg/L, $p=0.100$).

Table 4. Comparison of mean GSTP1-1 enzyme plasma levels for the different GSTP1 genotypes in the control and HNSCC population (One-way analysis of variances.)

Group	ANOVA significance (p-values)
Control	0.862
HNSCC	0.170
Total	0.100

(Differences in mean GSTP1-1 plasma level between the genotypes in control, HNSCC and both)

Discussion

The purpose of this study was to evaluate the possible association between the genetic polymorphisms in the GSTP1 gene and the phenotypic expression (GSTP1-1 plasma levels) in patients with malignant head and neck tumors.

No previous studies have been performed on the correlation between GSTP1 genotype and GSTP1-1 plasma level. However, a correlation between enzyme function and GSTP1 genotype has been made before (8,14,24,25). Ali-Osman et al. and Zimniak et al. have provided evidence of polymorphisms in the human GSTP1 gene locus, resulting in functionally different GSTP1-1 proteins (8,25). Watson et al. and van Lieshout et al. found significant differences in GST enzyme conjugating activity between the GSTP1 AA wildtype and the AB and BB genotypes in lung and esophageal tissues, respectively (14,24).

We found no significant correlation between the different GSTP1 genotypes and the corresponding GSTP1-1 plasma levels. This was the case when the control- and HNSCC-groups were investigated separately and also when they were combined. This means that both in patients with HNSCC and controls, no direct relation was present

between *GSTP1* genotype and *GSTP1-1* phenotype, as was found earlier in lung or esophageal tissue of patients with corresponding lung or esophageal lesions (14,24).

In a previous study on HNSCC we found that *GSTP1-1* is not a plasma tumor marker for individual patients, despite the fact that median plasma levels in HNSCC were significantly higher as compared to controls, but only a small part of HNSCC patients had plasma levels elevated above the normal reference level of 21.8 µg/L (22).

When comparing HNSCC to controls, significantly different *GSTP1-1* plasma levels were found for all *GSTP1* genotype categories. These findings show that there is no difference in the influence of the genotypes on *GSTP1-1* plasma levels between HNSCC and controls. The control group had a slightly higher percentage of *GSTP1* AA genotypes than the HNSCC group (51.0% vs. 41.4%) and a lower percentage of *GSTP1* AB genotypes (35.3% vs. 43.7%). The clinical significance of this difference has to be investigated in a larger patient group.

Hirata et al. considered estimation of *GSTP1-1* plasma levels a useful aid for early diagnosis, prediction of tumor extent and determining treatment efficacy and prognosis for oral cancer (17). We did not find arguments for such a role of *GSTP1-1* for our HNSCC patients (22). The question is whether this difference can be explained by either methodological, racial or demographic factors or by the fact that Hirata et al. limited their investigation to oral cancers (17). Another explanation could be that the Japanese and Dutch patient groups investigated have a different distribution of *GSTP1* genotypes. The *GSTP1* frequency distribution in the Dutch population is 55% for *GSTP1* AA, 35% for *GSTP1* AB and 10% for the *GSTP1* BB genotype. Hirata et al. (17) give no data on the genotype distribution, but another Japanese study by Morita et al. (6) indicates a frequency distribution of 69% for *GSTP1* AA, 29% for the AB and 2% for the BB genotype, which indeed differs considerably as compared to corresponding data in our Dutch population.

We conclude that there is no clear association between *GSTP1* genotype and *GSTP1-1* phenotypic expression in the form of plasma levels in patients with head and neck lesions as was found previously for GST enzyme activity in tissues of patients with lung cancer (24) or Barrett's esophagus (14). Perhaps further investigation of the corresponding *GSTP1-1* tissue enzyme activity in head and neck patients will reveal such a correlation.

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7

CHAPTER

MULTIPLE GENETIC POLYMORPHISMS IN DETOXIFICATION ENZYMES AND HEAD AND NECK CANCER SUSCEPTIBILITY

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Abstract

Background Genetic polymorphisms in single biotransformation enzymes may modulate the risk for head and neck squamous cell carcinoma (HNSCC). A pilot study evaluating the effect of combined polymorphisms in the glutathione S-transferase (GST) or the epoxide hydrolase (EPHX) genes was performed in order to determine whether such a combination of polymorphisms influences the risk for HNSCC.

Materials and Methods Genotyping by PCR or PCR-RFLP of the *GSTM1* and *GSTT1* null polymorphisms, the *GSTP1 B* variant and the Tyr 113 His variant of exon-3 of *EPHX* was successfully performed from 225 patients with HNSCC and 266 healthy controls, recruited at the University Hospital Maastricht.

Results An analysis on the simultaneous occurrence of multiple polymorphisms in any of the genes described above, without adjusting for age, gender, smoking or alcohol consumption, revealed no statistical differences between patients with HNSCC and healthy controls.

Conclusion A combination of multiple polymorphisms in the *GST* and *EPHX* genes is not associated with a higher susceptibility for HNSCC.

Introduction

The risk of head and neck squamous cell carcinoma (HNSCC) is strongly associated with smoking of tobacco and consumption of alcohol (1), which may be responsible for a considerable load of (pre)carcinogens. Metabolism and detoxification of such exogenous harmful compounds often occurs by phase I and phase II enzymes such as epoxide hydrolase (EPHX) and glutathione S-transferase (GST).

Because genetic polymorphisms exist in glutathione S-transferase *GSTM1*, *GSTT1*, *GSTP1* and *EPHX*, which may lead to complete absence of (*GSTM1* and *GSTT1*) or partially deficient (*GSTP1* and *EPHX*) corresponding enzyme activity, individuals bearing such genetic polymorphisms and therefore lack of a (partially) functional enzyme, may be at a higher risk of developing tobacco and alcohol related cancers (2-4). We have earlier investigated involvement of single polymorphisms in biotransformation enzymes in modulation of HNSCC risk (2-4) and found that the *GSTT1* null genotype may modulate the risk of head and neck cancer (HNSCC) (Chapter 3). Reports in the literature are very conflicting on the significance of GST polymorphisms in HNSCC susceptibility (5-12). Possibly, this is due to: 1. the relatively small patient and control study groups in most papers, 2. insufficient matching of patients and controls, and 3. the use of different methods for genotyping between the various research groups and sometimes even within the same study.

Since chemical modification of compounds to be detoxified is very complex and often requires several subsequent chemical reactions catalyzed by several different enzymes, we evaluated whether the simultaneous lack or dysfunction of one or more GST and EPHX enzymes as a result of a polymorphism in the corresponding gene, may influence the susceptibility for HNSCC.

Materials and Methods

Data and results of GST and EPHX genotyping obtained by polymerase chain reaction method (PCR) in material collected from HNSCC patients and healthy blood donors from earlier studies was used (2-4,13). The PCR methods for genotyping of *GSTM1*, *GSTT1*, *GSTP1* are described in detail in our earlier studies (Chapters 2, 3 and 5) (2-4,13). The PCR method for EPHX is described in detail by Zusterzeel et al. (14) and will be briefly described below. The polymorphism Tyr 113 His in exon 3 was examined. DNA samples were amplified by PCR. The PCR mixture contained approximately 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris HCl pH 8.0, 2 mM MgCl₂, 1.5 u Taq polymerase, and 1 µM of amplification primers in a total volume of 50 µL. Denaturing of DNA during 5 min at 94°C. Thirty amplification cycles. PCR product digestion with Tht111 and run on 3% agarose gel. Identification of 113 His by 2 DNA bands (209 and 22 base pairs), and detection of 113 Tyr by an undigested DNA band of 231 base pairs.

Chi square and Odds Ratio (OR) analysis was made to compare polymorphisms of one or more of these genes in 225 patients with head and neck squamous cell carcinoma (HNSCC) and 266 healthy controls. Statistical analysis was performed by using SPSS 8.0 software.

Results

In patients as well as in controls, an analysis on the number of genetic polymorphisms (0-4) in *GST* or *EPHX*, co-occurring in the same individual was performed. The results are shown in Table 1. As the Odds Ratios clearly show, no significant difference is found between the group of HNSCC patients and the healthy controls with respect to the co-occurrence of 0-4 different genetic polymorphisms in the detoxification enzymes studied here.

Table 1. Number of genetic polymorphisms in *GSTM1*, *GSTT1*, *GSTP1* or *EPHX* in patients with HNSCC and healthy controls

Polymorphisms	HNSCC N=225 (%)	Controls N=266 (%)	Odds Ratio OR (CI 95%)
0	11 (5)	15 (6)	
1	52 (23)	64 (24)	1.1 (0.5-2.6)
2	71 (32)	90 (34)	1.1 (0.5-2.5)
3	70 (31)	77 (29)	1.2 (0.5-2.9)
4	21 (9)	20 (7)	1.4 (0.5-3.9)

Discussion

Tobacco smoke may contain a considerable number of carcinogens, and the metabolism of each carcinogen may be very complex, involving the interaction of several biotransformation enzymes, such as cytochrome P450, epoxide hydrolase, glutathione S-transferases and many others. In the studies we have performed so far, we have not found evidence that single genetic polymorphisms in *CYP* or *GSTs* are major modulators for the risk for head and neck cancer. Individually however, these metabolic polymorphisms may be very weak risk factors, explaining also the variable result in the literature. But in combination with other polymorphisms, which each confer a very small risk, the sum up of those small risk factors may become large enough to be visible. However, we found no evidence in our study for such a sum up effect of the four polymorphisms investigated here.

We conclude that co-occurrence of up to four different polymorphisms in the *GST* and *EPHX* genes are not associated with a higher susceptibility for HNSCC. The results of this pilot study remain in agreement with the results presented in the other chapters, since no effect at all or only a slightly protective effect of the *GSTT1* null genotype was found. DNA mapping and evolving techniques may create new possibilities for the simultaneous investigation of many polymorphisms in biotransformation enzymes in the near future.

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8

CHAPTER

META- AND POOLED ANALYSES OF GSTM1, GSTT1, GSTP1, CYP1A1 POLYMORPHISMS AND RISK OF HEAD AND NECK CANCERS

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Abstract

Background Variants in genes coding for phase I and II enzymes such as the cytochrome P450 (CYP) family and glutathione S-transferases (GST) may alter individual susceptibility to cancer. *GSTM1*, *GSTT1*, *GSTP1* and *CYP1A1* polymorphisms may be associated with head and neck cancers though evidence from previous studies has not been consistent.

Materials and Methods We conducted a meta-analysis of 30 published case-control studies on these polymorphisms including 4,286 head and neck cancer cases and 5,440 controls. Subsequently, in a pooled analysis of original data from 9 published and 2 unpublished case control studies (2,324 cases and 2,766 controls), we explored the same associations as well as potential gene-gene and gene-environment interactions.

Results The summary Odds Ratios from the meta-analysis were 1.24 (95%CI=1.11, 1.38) for the *GSTM1 null* genotype, 1.20 (95%CI=1.00, 1.44) for the *GSTT1 null* genotype, 1.10 (95%CI=0.92, 1.31) for the *valine* genotype of the *GSTP1* polymorphism at codon 104 and 1.27 (95%CI=0.99, 1.63) for the *valine* genotype of the *CYP1A1* polymorphism at exon 7. Sources of heterogeneity included sample size, geographic region, year of publication and matching status. The results of the pooled analysis also supported an association of the *GSTM1 null* genotype (OR=1.32, 95%CI=1.07, 1.62) and *GSTT1 null* genotype (OR=1.35, 95%CI=1.13, 1.61) with head and neck cancers. An association was suggested for multiple GST genotypes and head and neck cancer risk.

Conclusion Our meta analysis results support the concept of a moderate association of *GSTM1*, *GSTT1*, *CYP1A1* polymorphisms and the risk of head and neck cancers.

Introduction

The study of low penetrance genes in head and neck cancers is of importance since even low risk genes can be a substantial public health burden when the polymorphism is common (1). Variants in genes coding for phase I and phase II enzymes such as the cytochrome P450 (CYP) and glutathione S-transferases (GST) families may potentially alter individual susceptibility to cancer. Several studies have been conducted on potential genetic risk factors for head and neck cancer and a recent review on *GSTM1* and *GSTT1* polymorphisms summarized the results of case-control studies as inconclusive (2). Of the 21 studies reviewed for the *GSTM1* null genotype, 13 studies reported ORs between 0.9 and 1.3, while 8 studies reported ORs between 1.4 and 3.9 (2). For the *GSTT1* null genotype, 8 studies reported ORs from 0.5 to 1.2, while 6 reported ORs from 1.4 to 2.6 (2). Since these metabolic enzymes may be among numerous genes involved in the multistage pathway of cancer, they are expected to be low to moderate risk factors that may be difficult to detect. However, even a moderate effect on cancer risk would be of biological and medical importance because of the possibility of identifying high risk individuals for target prevention activities.

Tobacco smoke contains a range of different carcinogens including polycyclic aromatic hydrocarbons (PAH), aromatic amines and nitrosamines (3). The amount of carcinogenic exposure to the upper aerodigestive tract will depend on whether the carcinogen is activated by phase I enzymes and whether it is detoxified by phase II enzymes. Variants in genes coding for these enzymes may therefore alter the individual's exposure to tobacco carcinogens. The *CYP1A1* gene codes for a phase I enzyme that activates tobacco procarcinogens such as benzo[a]pyrene and aromatic amines, into their carcinogenic forms (4). A point mutation in *CYP1A1* exon 7, resulting in an amino acid replacement of isoleucine by valine in codon 462, which is strongly linked to 3801T>C in the 3'-flanking region, was reported to be associated with increased enzyme activity (5,6). The variant genotype is suggested to be harmful possibly by increasing carcinogen activation and generating reactive oxygen species (7). Moreover, smokers with the *CYP1A1* variant polymorphism were shown to have elevated DNA adduct levels (8).

The GST family includes phase II enzymes that detoxify carcinogens and reactive oxygen species (9). Individuals who have homozygous deletions for the *GSTM1* and *T1* gene have no *GSTM1*-1 and *T1*-1 enzyme activity. Lack of these enzymes may potentially increase cancer susceptibility because of a decreased ability to detoxify carcinogens such as benzo[a]pyrene-7,8-diol epoxide, the activated form of benzo[a]pyrene. The *GSTP1* polymorphism at codon 104 results in the replacement of isoleucine by valine due to an A → G transition. The valine variant of the *GSTP1* polymorphism may be 2-3 times less stable (10) and may be associated with a higher level of DNA adducts (11).

The previous review on *GSTM1*, *GSTT1* and head and neck cancer included papers written in English and published between 1993 - 2000 (2). Since papers on 12 additional study populations not included in the review are available (12-23), we conducted an updated meta-analysis of head and neck cancer case-control studies on *GSTM1*, *GSTT1*, and additionally on *GSTP1*, *CYP1A1* to assess whether the available evidence supports an association and to determine the sources of heterogeneity

among the study results. In addition, we pooled the raw dataset from 11 case-control studies on these metabolic genes and head and neck cancer, to explore the main effect of the genes, as well as gene-gene and gene-environment interactions.

Materials and Methods

Meta-Analysis

A MedLine search was conducted for case-control studies reported up to March 2003 of *GSTM1*, *GSTT1*, *GSTP1* and *CYP1A1* on the risk of head and neck cancer, including oral, pharyngeal and laryngeal cancers. We focused on the *null* polymorphism of the *GSTM1* and *GSTT1* genes, the *Ile104Val* polymorphism of the *GSTP1* gene, and the *Ile462Val* polymorphism of the *CYP1A1* gene. The following keywords for genotypes were used in the MedLine search: "Glutathione," "GSTM1," "GSTT1," "GSTP1" and "CYP1A1." In addition, we reviewed the literature cited by each of the papers that we identified. A total of 36 publications were identified including 30 different populations (12-48).

We focused on studies that genotyped individuals for the polymorphisms by PCR (polymerase chain reaction) and excluded 23 studies that assessed gene expression via measurement of protein levels. Positive controls for the *GSTM1* and *GSTT1* genotyping were mentioned for the majority of studies. *GSTP1* was genotyped in all studies by RFLP (Restriction Fragment Length Polymorphism)-PCR. For *CYP1A1* genotyping, 3 studies used allele specific PCR (14,33,46), 1 study used PCR-SSCP (single-strand conformational polymorphism)(43) and the other studies used RFLP-PCR. When several papers were identified for the same population, we referred to the most updated information source. Studies that did not report Odds Ratios (ORs) and 95% confidence intervals (CIs) and did not provide enough data to estimate crude ORs were excluded (49-51).

For each study we abstracted the date of publication, the country where the study was conducted, the organs within the head and neck cancer studied, the control source (hospital, hospital-healthy, population-based or mixed), the number of cases and controls, and whether controls were matched to cases (matching, frequency matching, no matching). Healthy subjects recruited from hospitals as controls were categorized as 'hospital-healthy.'

Statistical Analysis

We abstracted crude Odds Ratios (ORs) from the studies when available. We calculated for each study, crude ORs and 95% CIs when the study: 1) did not estimate the OR but presented enough data for estimation, 2) reported an OR adjusted for various factors, 3) reported only ORs stratified by factors such as race or site. For a study from France, we combined the data for cancers of the oral cavity, pharynx and larynx that had been presented in separate publications (30-32).

When both the data and crude ORs were presented, we checked the calculation for the crude ORs and 95% CIs. In the case of discrepancy (22), we retained the crude OR that we calculated, since the OR presented in the original publication may have been ad-

justed for some factors. We did not perform a meta-analysis of the adjusted ORs because adjustment was not comparable among the studies. For the *GSTP1* and the *CYP1A1* polymorphisms, we combined the heterozygous and homozygous polymorphisms due to the limited number of subjects who were homozygous mutant.

Whenever possible we estimated or extracted study-specific ORs separately by organ within the head and neck (oral cavity, pharynx, larynx), and by smoking status (never, ever smokers). For 4 studies, we were unable to separate cancer cases of the nasopharynx, nasal cavity, sinus or salivary gland from other head and neck cancers (28,36,39,42). The histology of head and neck cancer cases was squamous cell carcinoma (SCC) in most studies, but 2 studies (22,29) did not specify the histology and one study (36) included 20 out of 156 cases with "other miscellaneous histologies."

Summary ORs were estimated with the statistical program STATA by inverse-variance weighting, using a random effects model that includes a term for heterogeneity between studies (52). Tests for heterogeneity between the studies were conducted for each analysis. Publication bias was assessed with Begg's funnel plots (53) and Egger's regression asymmetry test (54).

We conducted an influence analysis, where each study was excluded one at a time to determine the magnitude of influence on the summary estimate. We excluded several studies that appeared to be less consistent with the other studies in order to ensure that a few studies were not responsible for excessively influencing the summary estimate. We also excluded some studies that resulted in asymmetry in Begg's funnel plot, in an attempt to minimize publication bias. Both summary estimates including all studies identified and excluding several studies were presented.

Summary ORs were presented by specific organs within the head and neck and by smoking status. The meta-analysis was also stratified by geographic region (Asia, North America, Europe), period of publication (1995-1999, 2000-2002), number of subjects (<100 cases or <100 controls, ≥ 100 cases & ≥ 100 controls), matching of cases and controls (matched, frequency matched, not matched), and control source (hospital, hospital-healthy or population based). Summary ORs were estimated when there were at least 3 risk estimates available.

Pooled Analysis

The data on *GSTM1*, *GSTT1*, *GSTP1*, *CYP1A1* and head and neck cancers for the pooled analysis was extracted from the International Collaborative Study on Genetic Susceptibility to Environmental Carcinogens (GSEC) database (55,56), which contains individual level data from case-control studies on genes that metabolize environmental carcinogens. Investigators who had published their results from case-control studies on genetic polymorphisms and cancers were identified through a MedLine search and requested to provide published and unpublished original data from their studies. Our data included 11 case-control studies, of which 9 had been published and included in the meta-analysis (12,25,26,28,30-32,34,35,37,38,42-45). Cases of cancer of the nasopharynx, maxillary sinus and salivary glands were excluded from the analysis. Of the 2,324 head and neck cancer cases included in the analysis, there were 840 oral cavity cancers, 501 pharyngeal cancers, 904 laryngeal cancers and 79

cases with cancer at an unspecified organ within the head and neck. The control group included 2,766 subjects.

Statistical Analysis

In order to assess the association of the polymorphisms with head and neck cancer, the logistic regression model was used to estimate study-specific ORs and their 95% CIs. We estimated a crude OR and an OR adjusted for age, sex and race for each study. In the case of *GSTM1* and *CYP1A1*, we combined heterozygous and homozygous mutant genotypes for consistency with the meta-analysis approach. ORs estimated for individual studies and numbers of cases and controls may not precisely match those reported in the publications. Heterogeneity between studies was assessed with the test for heterogeneity while publication bias was assessed with the Begg's funnel plots (53) and Egger's regression asymmetry test (54). A summary OR was estimated by inverse-variance weighting, with the random effects model because of the heterogeneity detected among studies (52). Studies identified from the influence analysis and Begg's funnel plots were excluded in an attempt to minimize bias. Stratified analysis was conducted by cancer site and smoking status, as well as for Caucasians and cases with squamous cell carcinoma only. In the pooled analysis, we did not assess the effect of study characteristics due to the small number of studies available.

Results

Study-specific crude ORs and overall summary ORs from the meta-analysis of *GSTM1*, *GSTT1*, *GSTP1*, *CYP1A1* polymorphisms are shown in table 1. The distribution of the 4 polymorphisms among the head and neck cancer cases and controls from the 30 populations are presented in table 2. The frequency of the genotypes varied between geographic regions among controls: 24.7 - 57.8% for the *GSTM1 null*, 8.3 - 52.8% for *GSTT1 null*, 23.8 - 64.6% for the *GSTP1 valine* genotype and 5.8 - 39.5% for the *CYP1A1 valine* genotype. The percentage of Caucasians in the U.S. studies were: 88.9% in cases and 87.9% in controls (24), 95.6% in cases and 93.3% in controls (39), 62% in cases and 86% in controls (41), 100% in cases and controls (43), 66.2% in cases and 67.3% in controls (44), 61.5% in cases and controls (45), or not specified (15,48).

In the meta-analysis of the *GSTM1* polymorphism, 29 studies were identified with a total of 4,096 cases and 5,146 controls (Table 1, Figure 1). The overall summary OR was 1.36 (95%CI=1.17, 1.58) when adjusted for study center (Table 3). Two studies were excluded as they were less consistent with the other studies (12,17) and 4 studies were excluded to minimize the publication bias suggested by the funnel plot shown in Figure 2 (15,18,20,25). These 4 studies were of a small sample size and had all identified a strong positive association. In the final model including 23 studies (3,407 cases & 4,373 controls), the OR for the *GSTM1 null* genotype was 1.24 (95%CI=1.11, 1.38) and it was particularly high for oral cavity cancer and among ever smokers. The ORs for the *GSTM1 null* genotype was higher for studies with smaller sample sizes than for larger studies ($p=0.072$). Differences between ORs in other strata were not identified.

Figure 1. Study specific odds ratios and 95% confidence intervals from 29 case-control studies in the meta-analysis of head and neck cancers and *GSTM1 null* genotype, by year of publication

Figure 2. Begg's funnel plot of 29 studies in the meta-analysis of the *GSTM1 null* genotype and head and neck cancer (darkened circle = study excluded from final model)

Table 1. Summary of case-control studies included in meta-analysis of GSTM1, GSTT1, GSTP1 (ILE104VAL), CYP1A1 (ILE462VAL) polymorphisms and head and neck cancers

Ref no	Authors	Year	Country	Control Source	Site			Cases	Controls	GSTM1 (null) Crude OR (95%CI)	GSTT1 (null) Crude OR (95%CI)	GSTP1 (any val) Crude OR (95%CI)	CYP1A1 (any val) Crude OR (95%CI)
					Oral	Pharynx	Larynx						
(12)	Buch	2002	India	Hospital, Healthy	X			297 ^{1,2}	450 ²	2.95 (2.16, 4.04)* ⁶	1.60 (1.06, 2.41)*		
(24)	Cheng	1999	USA	Hospital	Head & Neck, Site NS			162	315	1.51 (1.03, 2.21)	2.30 (1.48, 3.56)*		
(25)	Coutelle	1997	France	Alcoholic clinic		X	X	39 ^{2,3,4}	37 ^{2,3,4}	2.38 (0.93, 6.06)* ⁶			
(26)	Deakin	1996	UK	Hospital	X			40	577	1.01 (0.53, 1.92)*	0.59 (0.20, 1.71)*		
(27)	Gonzalez	1998	Spain	Hospital, healthy	X	X	X	75 ⁴	200 ⁴	1.34 (0.78, 2.29)*			
(13)	Gronau	2000	Germany	Hospital, healthy		X	X	83	60	1.89 (0.96, 3.71)*			
(14)	Hahn	2002	Germany	Hospital, healthy	X			94	92	1.29 (0.72, 2.31)*			0.64 (0.17, 2.34)*
(28)	Hamel	2000	Canada	mixed	X	X	X	90 ^{1,5}	90	0.96 (0.53, 1.73)	2.57 (1.12, 5.90)		
(15)	Hanna	2001	USA	Hospital			X	20	20	4.00 (0.98, 16.27)* ⁶	0.71 (0.14, 3.66)* ⁶		
(16)	Hong	2000	Korea	Hospital			X	82 ^{2,4}	63 ^{2,4}	1.96 (0.99, 3.86)*	2.34 (1.19, 4.58)*		
(29)	Hung	1997	Taiwan	Population based	X			41 ⁴	123 ⁴	1.03 (0.50, 2.12)*	1.26 (0.61, 2.58)*		
(17)	Jaskula-Sztul	1998	Poland	NS, healthy			X	171	180	0.71 (0.46, 1.08)* ⁶	0.77 (0.45, 1.31)		
(31,32)	Jourenkova1	1999	France	Hospital	X	X	X	250 ²	172 ²	1.09 (0.74, 1.60)*	1.38 (0.82, 2.30)*		
(30,31)	Jourenkova2	1999	France	Hospital	X	X	X	250 ²	172 ²			1.23 (0.86, 1.82)*	
(33)	Kao	2002	Taiwan	Hospital	X			106	146				5.42 (2.83, 10.38)* ⁶
(35)	Kato1	1999	Japan	Hospital, healthy	X			92	147	1.65 (0.98, 2.80)	0.88 (0.52, 1.48)		1.29 (0.76, 2.18)
(34)	Kato2	1999	Japan	Hospital, healthy	X			83	122			1.91 (1.04, 3.52)*	
(18)	Kietthubthaw	2001	Thailand	Population based	X			53	53	3.02 (1.36, 6.71)* ⁶	0.58 (0.26, 1.26)* ⁶		
(36)	Kihara	1997	Japan	Hospital, healthy	X	X	X	156 ⁵	472	1.29 (0.90, 1.86)*			
(19)	Ko	2001	Germany	Hospital, healthy	Head & Neck, Site NS			312	300	1.22 (0.88, 1.67)*	1.01 (0.68, 1.50)*		
(37)	Matthias1	1998	Germany	Hospital	X	X	X	380	193				1.04 (0.64, 1.70)*
(38)	Matthias2	1999	Germany	Hospital	X	X	X	398 ⁷	219 ⁷	1.18 (0.82, 1.68)*	0.99 (0.66, 1.49)*	1.39 (0.98, 1.96)*	
(39)	McWilliams	2000	USA	Hospital	X	X	X	160 ^{5,7}	149 ⁷	0.99 (0.62, 1.59)	0.91 (0.47, 1.74)	1.26 (0.78, 2.04)*	0.42 (0.18, 0.99)* ⁶
(40)	Morita	1999	Japan	Hospital, healthy	X	X	X	145	164	0.94 (0.60, 1.46)*		0.73 (0.44, 1.21)*	0.88 (0.55, 1.41)*

(20)	Nomura	2000	Japan	Hospital	X	X		109 ⁵	33	2.43 (1.10, 5.38)* ⁶			
(41)	Olshan	2000	USA	Hospital	X	X	X	182 ⁷	202 ⁷	0.96 (0.64, 1.46)*	1.47 (0.84, 2.58)*	1.25 (0.81, 1.92)*	1.33 (0.58, 3.06)*
(42)	Oude Ophuis1	1998	Nether-lands	Hospital, healthy	X	X	X	185 ^{1.5}	207	0.97 (0.65, 1.44)*	0.95 (0.58, 1.56)*		1.15 (0.68, 1.93)*
(21)	Oude Ophuis2	2003	Nether-lands	Hospital, healthy	X	X	X	235	285			0.80 (0.57, 1.13)*	
(43)	Park1	1997	USA	Hospital	X	X	X	131	131				2.58 (1.17, 5.66)*
(44)	Park2	1999	USA	Hospital	X	X	X	154	246			0.91 (0.60, 1.38)*	
(45)	Park3	2000	USA	Hospital	X	X	X	164	344	1.34 (0.92, 1.95)*			
(46)	Sato	2000	Japan	Hospital, healthy	X			142	142	2.24 (1.40, 3.61)			1.88 (1.17, 3.03)*
(22)	Sreelekha	2001	India	Hospital, healthy	X			98	60	1.92 (0.99, 3.74)*	2.48 (0.87, 7.06)*		5.21 (2.37, 11.43)* ⁶
(47)	Tanimoto	1999	Japan	Hospital	X			100	100	1.04 (0.59, 1.83)*			
(23)	To-Figueras	2002	Spain	Hospital, healthy			X	204 ⁷	203 ^{2,7}	0.92 (0.62, 1.35)*	0.67 (0.41, 1.09)* ⁶	1.01 (0.68, 1.49)*	
(48)	Trizna	1995	USA	Hospital, healthy	X	X	X	186 ⁷	42 ⁷	2.37 (1.20, 4.67)	1.47 (0.71, 3.02)		
Number of studies (cases/controls) identified										29 (4,096/5,146)	19 (2,914/3,439)	9 (1,768/1,699)	11 (1,683/1,592)
Number of studies (cases/controls) included in meta-analysis										23 (3,407/4,373)	15 (2,679/3,104)	9 (1,768/1,699)	8 (1,340/1,265)
Summary OR (95%CI)										1.24 (1.11, 1.38)	1.20 (1.00, 1.44)	1.10 (0.92, 1.31)	1.27 (0.99, 1.63)
Test for heterogeneity										0.22	0.11	0.13	0.19
Publication bias (Egger's test)										0.11	0.31	0.52	0.93

1. prevalent & incident cases, 2. all ever tobacco smokers and/or chewers, 3. all drinkers, 4. all males, 5. includes nasopharyngeal, maxillary sinus, and/or salivary gland cancers, 6. Studies excluded from final model, 7. Number of cases and controls genotyped varied for each polymorphism, thus overall numbers are presented, *Odds Ratio calculated, NS = not specified

The summary OR for the 19 studies identified for the meta-analysis of *GSTT1* and head and neck cancers was 1.18(95%CI=0.97, 1.44) (Table 3). Three studies (15,18,24) were excluded since they appeared to be divergent from the other studies and another (18) was excluded to minimize the potential publication bias that was identified in Begg's funnel plot (Figure 3). Including 2,679 cases and 3,104 controls from 15 studies, the summary OR for the *GSTT1* null genotype on the risk of head and neck cancers was 1.20 (95%CI=1.00, 1.44). The small number of available studies limited the analysis stratified by cancer site. The summary ORs were higher for studies from Asia ($p=0.061$), studies published from 2000 to 2002 ($p=0.031$) and matched studies ($p=0.011$).

A small number of publications was identified on *GSTP1* and *CYP1A1* polymorphisms and the risk of head and neck cancers. For *GSTP1*, we identified 9 case-control studies with 1,768 head and neck cancer cases and 1,699 controls (Table 4). None of the studies was excluded since there were no strong indications of excessive influence or publication bias. There was some suggestion that the valine genotype of *GSTP1* may elevate the risk of head and neck cancer (summary OR=1.10, 95%CI=0.92, 1.31), and specifically the risk of oral cancer. Summary ORs for some strata could not be estimated due to the small number of studies.

Eleven case-control studies were identified for the association between the *CYP1A1* polymorphism and head and neck cancers, with a summary OR of 1.50 (95%CI=1.00, 2.26). Three studies (22,33,39) were excluded as they appeared to be less consistent with the other studies. According to the meta-analysis of the remaining 8 studies with 1,340 cases and 1,265 controls, the risk of head and neck cancers may be increased moderately by the *CYP1A1* valine polymorphism (OR=1.27, 95%CI=0.99, 1.63).

Figure 3. Begg's funnel plot of 19 studies in the meta-analysis of the *GSTT1* null genotype and head and neck cancer (darkened circle = study excluded from final model)

Results of the pooled analysis of *GSTM1*, *GSTT1*, *GSTP1*, *CYP1A1* and head and neck cancers are shown in Table 5. The race/ethnicity distribution for the Lazarus study was 64.3% Caucasian and 35.7% African-American among cases, and 61.8% Caucasian and 38.2% African-American among controls. For the Romkes study, the cases were 97.4% Caucasian and 2.6% African-American, while the controls were 78.9% Caucasian, 15.5% African-American, 4.2% Latino and 1.4% Asian. All 11 studies in the pooled analysis had data on *GSTM1*, but two studies were excluded since their study estimates were less consistent with respect to the other studies (12,26). When including all studies, the OR was 1.32 (95%CI=1.07, 1.62) adjusting for study center. After excluding the 2 studies, the OR for the *GSTM1 null* genotype, adjusted for study center was 1.35 (95%CI=1.13, 1.61) and the OR adjusted for study center, age, sex and race was 1.32 (95%CI=1.04, 1.69). The *GSTM1 null* genotype was associated with an increased risk of head and neck cancers among smokers and Caucasians. The risk of head and neck cancer was also elevated by the *GSTT1 null* genotype (OR adjusted for study center=1.35, 95%CI=1.13, 1.61; OR adjusted for study center, age, sex and race=1.41, 95%CI=1.00, 1.57), but not by the *GSTP1* and *CYP1A1* polymorphisms, according to the pooled analysis. There appeared to be heterogeneity among the studies, especially in the case of the *GSTP1* polymorphism.

Gene-gene interactions were assessed for all combinations of *GSTM1*, *GSTT1*, *GSTP1* and *CYP1A1* on the risk of head and neck cancers in the pooled analysis, but strong interactions were not identified. We also analyzed the data for possible gene-environment interactions between each polymorphism and smoking, but again interactions were not obvious. However, we did see an increasing risk of head and neck cancers with multiple GST polymorphisms in an analysis of a subset of studies that had data on all 3 polymorphisms (906 head and neck cancer cases and 543 controls) (26,30-32,37,38,42). The *null* genotypes *GSTM1* and *GSTT1*, and the *valine* polymorphism of *GSTP1* were considered high risk relative to subjects who had low risk genotypes for all 3 GSTs. Taking subjects with all low risk GST polymorphisms as the reference, the OR was 1.13 (95%CI=0.83, 1.53) for subjects who had 1 high risk GST polymorphism, 1.19 (95%CI=0.87, 1.63) for subjects who had 2 high risk GST polymorphisms and 1.69 (95%CI=0.99, 2.88) for subjects with all 3 high risk GST polymorphisms when adjusted for study center (test for trend, p-value=0.08). When further adjusted for age, sex, and race, the OR was 1.16 (95%CI=0.83, 1.63) for 1 high risk GST polymorphism, 1.23 (95%CI=0.86, 1.75) for 2 high risk GST polymorphisms, and 2.06 (95%CI=1.11, 3.81) for having 3 high risk GST polymorphisms (test for trend, p-value=0.04). When stratified by smoking, the results were not statistically significant.

Table 2. Distribution of GSTM1, GSTT1, GSTP1 (ILEVAL), CYP1A1 (ILEVAL) genotypes among head and neck cancer cases and controls

Reference	Country	% GSTM1 Null		% GSTT1 Null		% GSTP1 any val		% CYP1A1 any val	
		Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Asia									
(12)	India	49.2	24.7	18.2	12.2				
(22)	India	49.0	33.3	18.4	8.3			51.0	16.7
(35)	Japan	58.7	46.3	47.8	51.0			45.7	39.5
(34)	Japan					37.3	23.8		
(36)	Japan	55.1	48.7						
(40)	Japan	49.0	50.6			24.8	31.1	33.8	36.6
(20)	Japan	67.0	45.5						
(46)	Japan	64.8	45.1					52.1	36.6
(47)	Japan	43.0	42.0						
(16)	Korea	68.3	52.4	57.3	36.5				
(29)	Taiwan	58.5	57.7	58.5	52.8				
(33)	Taiwan							86.8	54.8
(18)	Thailand	56.6	30.2	34.0	47.2				
	Overall in Asia	55.0	41.7	30.9	27.7	29.3	28.0	52.7	39.5
Europe									
(25)	France	69.2	48.6						
(31,32)	France	54.4	52.3	20.4	15.7				
(30,31)	France					55.2	50.0		
(13)	Germany	63.9	48.3						
(14)	Germany	59.6	53.3					4.3	6.7
(19)	Germany	53.2	48.3	20.5	20.3			15.0	14.5
(37)	Germany								
(38)	Germany	57.4	53.4	22.0	22.2	55.0	46.9		
(42)	Netherlands	50.8	51.7	19.5	20.3			18.4	16.4
(21)	Netherlands					50.6	56.1		
(17)	Poland	49.1	57.8	17.5	21.7				
(27)	Spain	58.7	51.5						
(23)	Spain	47.1	49.3	17.2	23.6	50.7	50.2		
(26)	UK	55.0	54.8	11.8	18.5				
	Overall in Europe	54.3	52.4	19.8	20.1	53.3	51.4	14.0	13.2
North America									
(28)	Canada	56.7	57.8	10.0	22.2				
(24)	USA	53.1	42.9	32.7	17.5				
(15)	USA	80.0	50.0	15.0	20.0				
(39)	USA	46.3	46.5	16.9	18.3	58.9	53.2	6.5	14.0
(41)	USA	43.6	44.6	18.6	13.5	66.3	61.1	7.6	5.8
(43)	USA							17.6	7.6
(44)	USA					62.3	64.6		
(45)	USA	43.3	36.3						
(48)	USA	68.3	47.6	44.9	35.7				
	Overall in North America	52.5	43.1	25.0	18.2	62.7	60.9	9.4	7.8
	OVERALL	54.1	46.6	23.6	21.6	52.7	50.6	26.6	23.0

Table 3. Meta-Analysis of case-control studies for *GSTM1*, *GSTT1* and head and neck cancer by cancer site, smoking status and study characteristics

	GSTM1				GSTT1			
	No. of studies	OR ^a for null genotype (95%CI)	Test for Heterogeneity	Egger's Test	No. of studies	OR ^a for null genotype (95%CI)	Test for Heterogeneity	Egger's Test
Overall	29	1.36 (1.17, 1.58)	0.00	0.20	19	1.18 (0.97, 1.44)	0.00	0.98
Excluding several studies	23 ¹	1.24 (1.11, 1.38)	0.22	0.11	15 ²	1.20 (1.00, 1.44)	0.11	0.31
Cancer site								
Oral	9 ³	1.31 (1.02, 2.69)	0.13	0.79	6 ⁶	1.28 (0.93, 1.76)	0.23	0.73
Pharynx	3 ⁴	1.14 (0.77, 1.67)	0.58	0.17	—	—	—	—
Larynx	7 ⁵	1.17 (0.90, 1.54)	0.10	0.10	3 ⁷	1.13 (0.70, 1.81)	0.09	0.81
Smoking status								
Never smokers	3 ⁸	1.00 (0.57, 1.75)	0.69	0.66	—	—	—	—
Ever smokers	6 ⁹	1.27 (0.98, 1.64)	0.07	0.49	4 ¹⁰	1.31 (0.90, 1.89)	0.17	0.86
Region								
Asia	8	1.42 (1.19, 1.79)	0.14	0.62	5	1.48 (1.03, 2.13)	0.15	0.58
North America	6	1.24 (0.98, 1.57)	0.17	0.76	4	1.43 (0.97, 2.09)	0.28	0.47
Europe	9	1.13 (0.99, 1.30)	0.76	0.27	6	0.98 (0.81, 1.20)	0.64	0.33
Year of Publication								
1995 - 1999	13	1.27 (1.09, 1.47)	0.17	0.44	8	1.01 (0.83, 1.23)	0.65	0.93
2000 - 2002	10	1.20 (1.02, 1.40)	0.34	0.12	7	1.49 (1.11, 2.00)	0.12	0.18
Number of cases & controls								
<100 cases or <100 controls	10	1.45 (1.19, 1.77)	0.49	0.54	7	1.45 (0.98, 2.14)	0.09	0.54
≥100 cases & ≥controls	13	1.17 (1.03, 1.32)	0.26	0.99	8	1.11 (0.93, 1.33)	0.35	0.68
Control Source								
Hospital Healthy or Population	13	1.32 (1.11, 1.57)	0.08	0.08	8	1.13 (0.90, 1.40)	0.24	0.44
Hospital	10	1.17 (1.02, 1.34)	0.66	0.96	7	1.32 (0.96, 1.81)	0.10	0.70
Matching								
Matched	5	1.32 (1.04, 1.67)	0.47	0.70	3	1.82 (1.29, 2.57)	0.50	0.18
Frequency matched	4	1.12 (0.91, 1.37)	0.70	0.78	3	1.38 (0.99, 1.93)	0.95	0.52
Not matched	14	1.28 (1.09, 1.49)	0.08	0.07	9	1.01 (0.83, 1.24)	0.31	0.77

1. excluded Buch, Coutelle, Hanna, Jaskula-Sztul, Kietthubthew, Nomura, 2. excluded Cheng, Hanna, Morita, Nomura, Sreelekha, Tanimoto 4. included Gronau, Jourenkova, Morita, 5. included Gronau, Hong, Jaskula-Sztul, Jourenkova, Kihara, Matthias, Morita, To-Figueras, 6. included Buch, Deakin, Hung, Jourenkova, Katoh, Sreelekha 7. included Hong, Jourenkova, Matthias, 8. included Kihara, Olshan, Park, 9. included Hong, Jourenkova, Kihara, Ko, Olshan, Park, 10. included Hong, Jourenkova, Ko, Olshan

a. OR adjusted for study center, b. ever smoking was categorized by different criteria in the studies: smoked at least 5 years (29), smoked at least 5 cigarettes/day for 4 years (19), smoked at least 5 cigarettes/day for 5 years (30-32), smoked at least 100 cigarettes in a lifetime (41, 45) and not specifically defined (34,36).

Table 4. Meta-analysis of case-control studies for GSTP1 (ILEVAL), CYP1A1 (ILEVAL) and head and neck cancer by cancer site, smoking status and study characteristics

	GSTP1				CYP1A1			
	No. of studies	OR ^a for any val (95%CI)	Test for Heterogeneity	Egger's Test	No. of studies	OR ^a for any val (95%CI)	Test for Heterogeneity	Egger's Test
Overall	9	1.10 (0.92, 1.31)	0.13	0.52	11	1.50 (1.00, 2.26)	0.00	0.76
Excluding several studies	—	—	—	—	8 ¹	1.27 (0.99, 1.63)	0.19	0.93
Cancer site								
Oral	3 ²	1.52 (1.05, 2.20)	0.63	0.22	4 ⁴	1.16 (0.69, 1.94)	0.09	0.60
Pharynx	—	—	—	—	—	—	—	—
Larynx	5 ³	0.94 (0.73, 1.20)	0.20	0.42	3 ⁵	1.03 (0.65, 1.62)	0.29	0.07
Smoking status								
Never smokers	—	—	—	—	—	—	—	—
Ever smokers ^b	3 ⁶	1.15 (0.87, 1.51)	0.49	0.86	—	—	—	—
Region								
Asia	—	—	—	—	3	1.29 (0.83, 2.00)	0.09	0.93
North America	3	1.11 (0.86, 1.43)	0.48	0.56	—	—	—	—
Europe	4	1.08 (0.85, 1.38)	0.14	0.94	3	1.05 (0.74, 1.48)	0.71	0.26
Year of Publication								
1995 - 1999	5	1.15 (0.87, 1.53)	0.08	0.97	5	1.18 (0.89, 1.57)	0.23	0.01
2000 - 2002	4	1.02 (0.82, 1.27)	0.33	0.07	3	1.49 (0.91, 2.43)	0.28	0.09
Number of cases & controls								
<100 cases or <100 controls	—	—	—	—	—	—	—	—
≥100 cases & ≥controls	8	1.06 (0.90, 1.24)	0.24	0.68	6	1.31 (0.96, 1.78)	0.12	0.38
Control Source								
Hospital Healthy or Population	4	0.98 (0.70, 1.17)	0.07	0.36	5	1.21 (0.89, 1.65)	0.19	0.50
Hospital	5	1.21 (1.01, 1.45)	0.65	0.56	3	1.44 (0.84, 2.46)	0.16	0.46
Matching								
Matched / Frequency matched	3	1.12 (0.89, 1.42)	0.47	0.60	3	1.48 (0.72, 3.03)	0.17	0.34
Not matched	4	1.18 (0.90, 1.55)	0.12	0.90	5	1.21 (0.93, 1.57)	0.24	0.98

1. excluded Kao, McWilliams, Sreelekha, 2. included Jourenkova, Katoh, Morita, 3. included Jourenkova, Matthias, Morita, Oude Ophuis, To-Figueras, 4. included Hahn, Katoh, Morita, Sato, 5. included Matthias, Morita, Park, 6. included Jourenkova, Katoh, Olshan

a. OR adjusted for study center b. ever smoking was categorized by different criteria in the studies: (30, 31), smoked at least 100 cigarettes in a lifetime (41) and not specifically defined (34).

Table 5. Pooled analysis of case-control studies on the *GSTM1*, *GSTT1*, *GSTP1*, *CYP1A1* polymorphisms and head and neck cancer

PI	Reference	Country	Control Source	Site			Cases	Controls	<i>GSTM1</i> OR (95%CI)	<i>GSTT1</i> OR (95%CI)	<i>GSTP1</i> Any Val OR (95%CI)	<i>CYP1A1</i> Any Val OR (95%CI)
				Oral	Pharynx	Larynx						
Benhamou	Jourenkova (30-32)	France	Hospital	X	X	X	250	172	1.09 (0.74, 1.60)	1.38 (0.82, 2.30)	1.23 (0.83, 1.82)	—
Bhisey	Buch (12)	India	Hospital, Healthy	X			300 ^{1,2,4}	678 ^{2,4}	1.73 (1.49, 1.99) ⁵	1.61 (1.11, 2.33)	—	1.06 (0.76, 1.48)
Cascorbi	unpublished	Germany	Hospital, Healthy		X	X	505 ⁶	223 ⁶	2.01 (1.34, 3.01)	1.26 (0.65, 2.43)	—	1.02 (0.51, 2.04)
Coutelle	Coutelle (25)	France	Alcoholic Clinic		X	X	39 ^{2,3,4}	76 ^{2,3,4}	2.37 (1.05, 5.36)	—	—	—
Hamel	Hamel (28)	Canada	Mixed	X	X	X	196 ^{1,6}	199 ⁶	1.09 (0.74, 1.62)	1.15 (0.67, 1.98)	—	—
Kato	Kato (34,35)	Japan	Healthy	X			45	91	1.59 (0.78, 3.27)	—	—	—
Lazarus	Park (43-45)	USA	Hospital	X		X	185 ⁶	367 ⁶	1.40 (0.96, 2.03)	—	0.87 (0.57, 1.33)	—
Manni	Oude Ophuis (21,42)	Netherlands	Hospital, Healthy	X	X	X	245 ^{1,6}	159 ⁶	1.33 (0.77, 2.29)	0.56 (0.30, 1.05)	0.77 (0.52, 1.14)	0.63 (0.33, 1.19)
Romkes	unpublished	USA	Healthy		Head & Neck		40 ⁶	70 ⁶	0.79 (0.36, 1.73)	0.84 (0.25, 2.84)	—	—
Strange	Deakin (26)	USA	Hospital	X			107 ⁶	493 ⁶	0.69 (0.45, 1.05) ⁵	1.38 (0.84, 2.27)	1.53 (0.94, 2.48)	2.11 (0.85, 5.24)
Strange	Matthias (37,38)	Germany	Hospital	X	X	X	422 ⁶	238 ⁶	1.31 (0.94, 1.83)	1.54 (0.91, 2.63)	1.56 (1.11, 2.19)	0.87 (0.55, 1.38)
No of studies (cases/controls) in data									9 (1,820/1,352) ⁷	8 (1,929/1,830)	5 (1,164/982)	5 (1,558/1,467)
Summary OR ^a									1.35 (1.13, 1.61)	1.25 (1.00, 1.57)	1.15 (0.86, 1.53)	0.98 (0.75, 1.29)
Test for heterogeneity									0.27	0.22	0.04	0.28
Publication bias (Egger's test)									0.78	0.14	0.75	0.79
Oral									1.24 (0.97, 1.57)	1.34 (0.99, 1.82)	1.37 (0.88, 2.14)	0.97 (0.56, 1.69)
Pharynx									1.25 (0.98, 1.61)	1.11 (0.66, 1.87)	1.10 (0.58, 2.05)	0.77 (0.47, 1.25)
Larynx									1.53 (1.17, 2.00)	1.10 (0.81, 1.49)	1.08 (0.81, 1.44)	0.93 (0.64, 1.34)
Never smokers									1.37 (0.84, 2.23)	1.29 (0.83, 1.99)	1.38 (0.46, 4.12)	0.95 (0.62, 1.45)
Ever smokers									1.30 (1.07, 1.59)	1.23 (0.77, 1.94)	1.01 (0.76, 1.33)	0.87 (0.50, 1.51)
Caucasians									1.29 (1.04, 1.59)	1.17 (0.91, 1.50)	1.15 (0.86, 1.54)	0.95 (0.64, 1.43)
SCC									1.25 (1.05, 1.47)	1.17 (0.88, 1.55)	1.13 (0.83, 1.54)	—

1. prevalent & incident cases, 2. all ever tobacco smokers and/or chewers, 3. all drinkers, 4. all males, 5. studies excluded from final model, 6. number of cases and controls genotyped varied for each polymorphism, thus overall numbers are presented, 7. excluded Buch, Deakin

a. OR adjusted for study center

Discussion

The results from the meta-analysis supported the hypothesis that *GSTM1*, *GSTT1* and *CYP1A1* polymorphisms increase the risk of head and neck cancers. Sources of heterogeneity included sample size among the *GSTM1* studies, and geographic region, period of publication, and matching among the *GSTT1* studies. The pooled analysis confirmed the association of head and neck cancers with *GSTM1* and *GSTT1* but the associations with *GSTP1* or *CYP1A1* polymorphisms were not clear. The pooled analysis was based on a subset of published studies from the meta-analysis that tended to report no associations or weak associations. Gene-gene and gene-environment interactions were not identified but a relationship was suggested between an increasing number of high risk GST genotypes and the risk of head and neck cancer.

Case-control studies with small sample size (less than 100 cases or 100 controls) may be reporting inflated ORs for the *GSTM1 null* genotype. These results may suggest caution in the interpretation of small case-control studies. Our summary estimates on the effect of the *GSTT1* polymorphism may differ for the 3 general regions where the studies were conducted. A higher summary estimate was seen from studies in Asia for the *GSTT1* genotype. The distribution of *GSTT1 null* in controls was low in the studies from India (12,22) and when we excluded the 2 Indian studies, the summary estimate for Asia was no longer elevated (OR=1.34, 95%CI=0.75, 2.39). A detailed assessment of the role of this polymorphism in India goes beyond the scope of this study.

Studies on *GSTT1* and head and neck cancers that were published since the year 2000 report an elevated summary risk, whereas studies published before 2000 did not. Though publication bias may be suspected for the more recent studies, it was not suggested by Egger's test ($p=0.18$). A change in genotyping methods could be a possible explanation, but all studies had used PCR to amplify the *GSTT1* gene and most studies mentioned using positive controls. Furthermore, since we were unable to control for matching factors in the meta-analysis, we may have bias in our study-specific effect estimates. However, matched studies did not seem to have ORs that were biased toward *GSTT1 null* as might be expected, when compared to unmatched studies. The heterogeneity observed between studies due to year of publication and matching status may reflect other study characteristics.

Carcinogen metabolism is complex, involving the interaction of numerous carcinogens and enzymes. The GSTs have a variety of substrates including environmental carcinogens, pesticides, drugs and endogenous molecules of lipid peroxidation as well as inducing agents, some of which double as substrates including polycyclic aromatic hydrocarbons, phenolic antioxidants, isothiocyanates, and reactive oxygen species (57). The metabolic action of GST enzymes may differ by tissue type, as the *GSTP1* enzyme has been observed to be at the highest concentration in oral and pharyngeal tissues and the *GSTM1* enzyme at the highest concentration in laryngeal tissue, relative to the other GSTs (2). GST enzyme expression levels may also differ by general controls of gene expression, such as the rates of transcription, translation and degradation as well as possible posttranslational modifications.

Individually these metabolic polymorphisms may be weak to moderate risk factors, explaining the inconsistent results seen in epidemiologic studies. The meta-analysis supported the hypothesis that *GSTM1*, *GSTT1*, and *CYP1A1* polymorphisms are moderate risk factors for head and neck cancer. On the other hand, combinations of genotypes that each confers a small relative risk may sum to a large enough relative risk that can be observed in epidemiologic studies. Our pooled analysis supported the idea that multiple GST polymorphisms combined may confer a greater risk of head and neck cancers. Future epidemiologic studies focusing on combinations of polymorphisms within the same gene family or other related gene families may be helpful in identifying individuals at high risk for head and neck cancers and in elucidating gene-gene interactions.

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SUMMARY, CONCLUSIONS, DISCUSSION AND FUTURE PERSPECTIVES

The incidence of head and neck squamous cell carcinoma (HNSCC) is strongly related to the consumption of alcohol and tobacco. However, the majority of the individuals consuming alcohol and tobacco ultimately do not develop HNSCC and therefore seem to be not susceptible to these hazardous substances. The explanation may be that considerable inter-individual differences exist in the detoxification capacity for alcohol and carcinogens of tobacco smoke. Several complex families of phase I and phase II detoxification enzymes exist, the levels of which may vary from one individual to another, as a result of induction or inhibition by dietary or lifestyle factors. In addition, genetic differences between individuals may also contribute to the actual differences in detoxification enzyme levels. The latter differences may be largely caused by genetic polymorphisms in detoxification enzymes, the knowledge on which is rapidly evolving during the last decade.

The overall aim of this thesis was to provide more information on the occurrence of genetic polymorphisms in cytochrome P4501A1 (CYP1A1) and glutathione S-transferase (GST) detoxification enzymes in both patients with HNSCC as well as healthy controls, in order to be able to get insight in the eventual modulating effect of such polymorphisms on the susceptibility for HNSCC. The summary recapitulates the contents of the individual chapters.

In chapter 1 the mechanism of glutathione conjugation to a potential carcinogen compound is briefly described. Subsequently, an overview is presented on the literature published to this date concerning the GST polymorphisms in *GSTM1*, *GSTT1* and *GSTP1* and HNSCC susceptibility. The studies so far published have reported contradicting results, and possible explanations for these varying results are given.

In chapter 2 the genetic variability in the corresponding cytochrome P4501A1 (CYP1A1), *GSTT1* and *GSTM1* isoenzymes, which may contribute to individual differences in HNSCC susceptibility, is discussed. By Polymerase Chain Reaction / Restricted Fragment Length Polymorphism (PCR/RFLP) techniques, the different *CYP* and *GST* genotypes could be detected in DNA isolated from whole blood, in 185 patients with HNSCC, 207 healthy blood donors and an additional control group of 78 patients with benign head and neck lesions (BHNL). The *GSTM1 null* genotype was demonstrated equally frequent in patients with HNSCC, BHNL and blood donors,

whereas the *GSTT1 null* genotype occurred significantly more often in patients with BHNL as compared to blood donors. This could mean that the *GSTT1 null* genotype may be a risk factor for BHNL.

In chapter 3 we had to revise our findings on the *GSTT1 null* genotype, as published before in chapter 2, due to demographic variations in the controls. By comparing the *GSTM1*, *T1* and *P1* genotype frequencies of two healthy blood donor groups from the Nijmegen and Maastricht regions, we found inter-regional differences. The *GSTM1*, *GSTT1* and *GSTP1* genotype frequencies in two control populations ($n=285$ and $n=207$) from different but neighboring (Maastricht/Nijmegen) geographical regions, were determined by PCR/RFLP methods. For the *GSTT1 null* polymorphism we found a significant difference ($p=0.003$) between the two control groups. When the risk for HNSCC (all patients are from the Maastricht area) is estimated versus the Maastricht control group, we now found significantly less HNSCC patients with the *GSTT1 null* genotype as compared to the controls. In contrast to our earlier conclusion, we now have to conclude that *GSTT1 null* genotype appears to be involved in the modulation of the risk for HNSCC and not for BHNL.

In chapter 4 we evaluated the role of the *GSTP1-1* enzyme plasma levels as a tumor marker in patients with different sites of HNSCC. *GSTP1-1* enzyme levels were measured in plasma of patients and controls, using a sensitive and specific sandwich Enzyme Linked Immuno Sorbent Assay (ELISA). Median *GSTP1-1* plasma levels of patients with oral/oropharyngeal, hypopharyngeal and laryngeal sites of HNSCC were significantly elevated when compared to *GSTP1-1* levels from patients with BHNL, who served as controls. Overall however, only 13 out of 93 patients with HNSCC had elevated plasma *GSTP1-1* levels, which makes *GSTP1-1* not suitable as plasma tumor marker for HNSCC.

In chapter 5 we studied the frequencies of polymorphic variants in the *GSTP1* gene, in patients with HNSCC and healthy controls. In patients with HNSCC and controls, the homozygous *GSTP1 BB* genotype was observed in 12.3% and 13.6% respectively. Also no statistical differences were found for the distribution of the *GSTP1 AA* and *GSTP1 AB* genotypes between patients and controls. This study showed that genetic polymorphisms in *GSTP1* are not associated with an altered susceptibility to HNSCC.

Many authors have studied the *GSTP1* genotypes in relation to the risk for HNSCC. In chapter 6, the correlation between *GSTP1* genotype and *GSTP1-1* enzyme plasma levels was evaluated. *GSTP1* genotype and *GSTP1-1* plasma levels were measured in HNSCC patients and patients with BHNL who served as controls. For all *GSTP1* genotypes (*AA*, *AB* and *BB*) in HNSCC patients, the mean plasma *GSTP1-1* values were significantly higher compared to the corresponding control groups. However, there was no significant difference in the plasma *GSTP1-1* levels between the different groups of *GSTP1* genotypes in HNSCC patients.

Since chemical modification of compounds to be detoxified is very complex and often requires several subsequent chemical reactions, with corresponding enzymes, we evaluated in chapter 7, whether the concurrent lack or dysfunction of one or more *GST* and Epoxide Hydrolase (*EPHX*) genes, influences the susceptibility for HNSCC. No sig-

nificant difference was found between the group of HNSCC patients and the healthy controls with respect to the co-occurrence of zero to four (0 – 4) different genetic polymorphisms. We conclude that multiple polymorphisms in the *GST* and *EPHX* genes are not associated with a higher susceptibility for HNSCC.

In chapter 8 a meta-analysis on the association between HNSCC and genetic polymorphisms in *CYP1A1*, *GSTM1*, *GSTT1* and *GSTP1* is presented in the form of an international collaborative study, including the results of 30 published case-control studies, including 4,286 head and neck cancer cases and 5,440 controls. Subsequently, in a pooled analysis of original data from 9 published and 2 unpublished case control studies (2,324 cases and 2,766 controls), the same associations as well as potential gene-gene and gene-environment interactions were explored. The summary Odds Ratios (OR) from the meta-analysis and the pooled analysis suggest a weak association for the *GSTM1*, *GSTT1* and *CYP1A1* genotypes and head and neck cancer risk.

Conclusions

1. Distribution of *GSTT1* genotypes in The Netherlands is variable.
2. Research on the associations between genetic polymorphisms and the modulation of risk for HNSCC therefore should be conducted under strict conditions with respect to including both cases and controls.
3. Plasma *GSTP1*-1 can not be used as a tumor marker for the detection or follow-up of HNSCC patients.
4. The *GSTP1* genotypes and the levels of plasma *GSTP1*-1 enzyme are not associated in patients with HNSCC.
5. Meta-analysis and pooled analysis in an international collaborative study show a weak association of *GSTM1*, *GSTT1* and *CYP1A1* genotypes and head and neck cancer, which becomes weaker when adjusted for study center, age, gender and race. For polymorphisms in *GSTP1*, when adjusted for study center, age, gender and race no such correlation is seen.

Discussion

Our hypothesis that polymorphisms in biotransformation enzymes could increase the risk of HNSCC is not supported by our case-control studies. In one of the first studies (chapter 2), we found no association between polymorphisms of the *GSTM1*, *GSTT1* and *CYP1A1* genes and a higher risk for head and neck cancer. In the same study, contradictory to the hypothesis, we initially found a significantly higher number of *GSTT1* null genotypes in patients with BHNH, which suggested that the *GSTT1* null genotype may be a risk factor for BHNH. This conclusion, however, was based on a case population recruited from a different geographical area (Maastricht) than the control population (Nijmegen). The BHNH group, also patients recruited from the Maastricht area, served as an additional control group. Later on (chapter 3) we recruited a second control group, this time from the same region (Maastricht) as our HNSCC and BHNH patients, since we missed data on alcohol consumption and smoking in the Nijmegen controls, and we did not expect to find significant differences in the *GSTT1* genotype

distribution between controls collected approximately 125 kilometers apart. The frequencies for the *GSTT1 null* genotype in this Maastricht control group, were comparable to the BHNH patient group, but not to the HNSCC patient group which meant that the BHNH group in fact served as a representative control group. More important however, was that these data were in contradiction with already published results (chapter 2). The *GSTT1 null* genotype frequencies were now significantly lower in HNSCC patients as compared to controls, suggesting involvement of *GSTT1 null* in the modulation of risk for HNSCC.

Chapter 4 evaluates the use of GSTP1-1 enzyme levels in the diagnostic stage of head and neck disease. Several studies have shown elevated GSTP1-1 enzyme levels in plasma of head and neck cancer patients in the first stage of disease, which declined after surgical resection of the tumor. In our patient-control groups we found no relation between HNSCC in any stage and GSTP1-1 plasma levels. We however found, that the methods for treatment of blood and preparing the plasma are very critical, since GSTP1-1 is present in large amounts in many blood cells, which may be released into the plasma when not properly processed. Thus, the procedures carried out in this study are very critical and the question remains, even if there would have been a positive correlation, whether such a critical procedure would be of clinical value.

Even though we found that measurement of GSTP1-1 enzyme plasma levels has no value as a tumor marker, there have been several reports on the GSTP1-1 enzyme to be present in high concentrations in several types of tumor tissue (head and neck tumors included).

We also evaluated whether a polymorphism of the *GSTP1* gene may be involved in head and neck cancer (chapter 5), but found no correlation. The strong associations between smoking and laryngeal cancer, and between alcohol intake and oral/oropharyngeal cancer, was expected because of the different ways carcinogen exposure may take place. Tobacco smoke is passing the larynx as most narrow site of the airway during in- and exhalation, whereas alcohol containing liquids remain temporarily in the oral cavity even after swallowing.

Having determined plasma GSTP1-1 enzyme levels and *GSTP1* genotype in HNSCC patients and controls, in chapter 6 a combination of the results of the previous two chapters was made. We hypothesized that the elevated GSTP1-1 plasma levels in HNSCC patients may be caused by one of the *GSTP1* genotypes. The fact that no association between *GSTP1* genotype and phenotype was found, supports our view that the *GSTP1* polymorphism alone appears to have minimal effect on GSTP1-1 enzyme activity in head and neck tissues and HNSCC susceptibility.

A study combining several polymorphisms of the *GST* and *EPHX* genes, also shows no evidence for an effect of these polymorphisms on the risk for HNSCC (chapter 7). In this study, we only studied the number of polymorphisms of any of these genes in patients and controls, without adjusting for age, gender, smoking or alcohol consumption.

In chapter 8, an international multicenter meta- and pooled analysis was conducted. Only weak associations with HNSCC and *GSTM1*, *GSTT1* and *CYP1A1* genotypes were

found. The conclusions of this meta analysis support the view of this thesis, that genetic polymorphisms in carcinogen metabolizing enzyme systems like glutathione S-transferases appear to be of minor importance in the development of HNSCC.

Future perspectives

The process of carcinogenesis is very complex. Even when main sources of the carcinogens are known, which are alcohol and tobacco smoke in many of the HNSCC cases, then the exact ways of the "route of carcinogenesis" are difficult to reconstruct. The metabolism of alcohol is relatively straightforward, but tobacco smoke contains many toxic and potentially carcinogenic compounds, the metabolism of each of those may involve many different enzymes, and a lot of research remains to be done before eventually important modulating enzymes may be characterized.

Alcohol dehydrogenase, acetaldehyde dehydrogenase, several other members of the cytochrome P450 and glutathione S-transferase families, UDP-glucuronosyltransferases which have recently been associated with HNSCC, N-acetyltransferases and many other enzymes or polymorphisms in the corresponding genes, may be investigated on their involvement in the modulation of the HNSCC risk.

To be able to perform this research properly, it is very important to have a large blood, tissue and databank from patients with HNSCC and the proper controls, which has already been set up. Tissue research may also provide valuable information on local enzyme activities.

In all our studies the statistical analyses were limited due to the large contrast in smoking and alcohol consumption behavior between patient and control groups. The number of non-smoking HNSCC patients is scarce, whereas in the healthy control groups there are almost no heavy smokers and/or alcoholics. Especially these extreme groups (heavy smoking and heavy drinking healthy elderly controls, and non-smoking, non-drinking elderly HNSCC patients) may be of particular interest for head and neck cancer research, since these individuals may have an extreme good, or an extreme bad functioning detoxification system.

In this thesis we have shown that head and neck cancer, which is strongly related to high tobacco and alcohol consumption, does not appear to be modulated by polymorphisms in some enzymes that detoxify tobacco carcinogens or alcohol, but many enzymes need to be investigated before definitive conclusions can be drawn.

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SAMENVATTING, CONCLUSIE, DISCUSSIE EN TOEKOMSTIGE MOGELIJKHEDEN

De mate van voorkomen van plaveisel cel carcinoom van het hoofd hals gebied (head and neck squamous cell carcinoma, HNSCC) is sterk gerelateerd aan de consumptie van alcohol en tabak. Echter, bij het merendeel van de personen die alcohol gebruiken of roken ontstaat uiteindelijk geen hoofd hals tumor. Deze personen lijken niet vatbaar voor de kwalijke gevolgen van deze stoffen. Het lijkt erop dat er behoorlijke verschillen bestaan tussen personen onderling in de ontgiftings-capaciteit van alcohol en de kankerverwekkende (carcinogene) stoffen uit tabaksproducten. Er bestaan enkele complexe families binnen de fase I en fase II detoxificatie enzymen. Bij deze enzymen kan de functie tussen personen onderling variëren door verschil in diët of levensgewoonten. Ook genetische verschillen tussen personen kunnen bijdragen aan verschillen in functie van detoxificatie enzymen. Deze laatste verschillen kunnen vooral worden veroorzaakt door genetische polymorphismen in detoxificatie enzymen. De afgelopen tien jaar is hier veel over bekend geworden.

Dit proefschrift heeft tot doel meer informatie te verschaffen over de verschillende genetische polymorphismen van cytochroom P450 1A1 (CYP1A1) en glutathion S-transferase (GST) detoxificatie enzymen in relatie tot de vatbaarheid voor HNSCC. Deze samenvatting beschrijft de inhoud van de individuele hoofdstukken.

In hoofdstuk 1 wordt het mechanisme van koppeling (conjugatie) van glutathion aan een potentiële kankerverwekkende stof kort beschreven. Tevens wordt een overzicht gegeven van de literatuur die tot nu toe is gepubliceerd betreffende de GST polymorphismen in *GSTM1*, *GSTT1* en *GSTP1* en de vatbaarheid voor HNSCC. Tot op dit moment zijn veel tegenstrijdige resultaten gerapporteerd en hiervoor wordt een aantal mogelijke verklaringen gegeven.

In hoofdstuk 2 wordt een studie beschreven waarin gekeken is naar de genetische variatie van de corresponderende cytochroom P450 1A1 (CYP1A1), *GSTT1* en *GSTM1* isoenzymen die een mogelijke bijdrage leveren aan de individuele verschillen in vatbaarheid voor HNSCC. Via polymerase ketting reactie methoden (polymerase chain reaction, PCR) werden de verschillende CYP en GST genotypen aangetoond in DNA, verkregen uit bloed monsters van 185 patiënten met HNSCC, 207 gezonde bloed-donoren en een extra controlegroep van 78 patiënten met goedaardige hoofd hals aandoeningen (benign head and neck lesions, BHNL). Het *GSTM1 null* genotype kwam even vaak voor bij patiënten met HNSCC, BHNL en de bloed-donoren. Het

GSTT1 null genotype kwam significant vaker voor bij patiënten met BHNL dan bij bloed-donoren. Als gevolg hiervan zou het *GSTT1 null* genotype een risico-factor kunnen zijn voor het ontstaan van BHNL.

In hoofdstuk 3 komen wij terug op onze bevindingen ten aanzien van het *GSTT1 null* genotype, zoals reeds gepubliceerd in hoofdstuk 2, vanwege demografische verschillen in controlegroepen. Bij vergelijking van *GST M1*, *T1* en *P1* genotype frequenties van twee groepen gezonde bloed-donoren uit de regio's Nijmegen en Maastricht, bestonden aanwijzingen voor mogelijke regionale verschillen. De frequenties van de *GSTM1*, *GSTT1* en *GSTP1* genotypen in twee controle populaties ($n=285$ en $n=207$) uit verschillende, maar aangrenzende geografische regio's (Maastricht/Nijmegen) werden bepaald door middel van de PCR methode. Bij vergelijking van deze controlegroepen werd een significant verschil ($p=0,003$) gevonden voor het *GSTT1 null* genotype. Als de groep patiënten met HNSCC uit de regio Maastricht wordt vergeleken met de nieuwe controlegroep, die eveneens uit Maastricht komt, worden nu significant minder HNSCC patiënten met het *GSTT1 null* genotype gevonden. In tegenstelling tot onze eerdere opvatting, moeten wij nu concluderen dat het *GSTT1 null* genotype betrokken lijkt bij de beïnvloeding van het risico op HNSCC en niet op BHNL.

In hoofdstuk 4 werd gekeken naar de bruikbaarheid van *GSTP1-1* enzym plasma waarden als tumor marker in patiënten met verschillende typen hoofd hals tumoren. *GSTP1-1* plasma spiegels werden bepaald in plasma van patiënten en een controlegroep door middel van een gevoelige en specifieke sandwich Enzyme Linked Immuno Sorbent Assay (ELISA). De mediane *GSTP1-1* plasma spiegels bij patiënten met HNSCC van de mond/mondbodem/oropharynx, hypopharynx en larynx waren significant verhoogd ten opzichte van een controlegroep patiënten met BHNL. Slechts 13 van 93 patiënten met HNSCC had een verhoogde plasma spiegel van *GSTP1-1*. *GSTP1-1* is derhalve niet geschikt als plasma tumor marker voor hoofd hals tumoren.

In hoofdstuk 5 werden de polymorphismen van het *GSTP1* gen bij patiënten met HNSCC vergeleken met een gezonde controlegroep. Het homozygote *GSTP1 BB* genotype kwam in deze groepen respectievelijk 12,3% en 13,6% voor. Ook voor *GSTP1 AA* en *GSTP1 AB* werden geen significante verschillen gevonden tussen patiënten en de controlegroep. Dit onderzoek heeft aangetoond dat genetische polymorphismen in het *GSTP1* gen niet lijken bij te dragen aan veranderde vatbaarheid voor het ontstaan van HNSCC.

Er is door veel onderzoeksgroepen onderzoek gedaan naar een relatie tussen het *GSTP1* genotype en het risico voor HNSCC. In hoofdstuk 6 is gekeken naar een relatie tussen het *GSTP1* genotype en de *GSTP1-1* enzym plasma spiegels bij patiënten met HNSCC en patiënten met BHNL. Voor alle *GSTP1* genotypen (*AA*, *AB* en *BB*) in patiënten met HNSCC waren de gemiddelde *GSTP1-1* plasma spiegels significant hoger ten opzichte van de corresponderende BHNL groepen. Er werd echter geen significant verschil gezien van de *GSTP1-1* plasma spiegels tussen de verschillende *GSTP1* genotypen in patiënten met HNSCC.

Chemische verandering van stoffen die ontgift moeten worden is erg complex. Het vereist dikwijls een aantal opeenvolgende chemische reacties die vaak onder invloed van

enzymen plaatsvinden. In hoofdstuk 7 hebben wij gekeken naar de relatie tussen gelijktijdige afwezigheid danwel functieverlies van één of meer GST en Epoxide Hydro-lase (*EPHX*) genen en de vatbaarheid voor HNSCC. Ten aanzien van het voorkomen van nul tot vier verschillende genetische polymorphismen werd geen significant verschil gevonden tussen de groep patiënten met HNSCC en de gezonde controlegroep. Wij concluderen dat meervoudige polymorphismen van de GST en *EPHX* genen de vatbaarheid voor HNSCC niet lijkt te beïnvloeden.

In hoofdstuk 8 wordt een meta-analyse gepresenteerd tussen HNSCC en genetische polymorphismen van *CYP1A1*, *GSTM1*, *GSTT1* en *GSTP1*. Dit is een internationaal samenwerkingsverband, waarin de resultaten van 30 gepubliceerde studies zijn opgenomen (4286 patiënten met HNSCC, 5440 controlepersonen). Hierna zijn de resultaten van 9 gepubliceerde en 2 ongepubliceerde studies (2324 HNSCC patiënten, 2766 controles) onderworpen aan een pooled analyse. Hierbij zijn ook mogelijke gen-gen en gen-omgevings interacties bekeken. De samenvattende Odds Ratios (OR) van meta-analyse en pooled analyse suggereren een zwakke relatie tussen *GSTM1*, *GSTT1* en *CYP1A1* genotypen en risico voor hoofd hals kanker.

Conclusies

1. De spreiding van *GSTT1* genotypen is in Nederland variabel.
2. Onderzoek naar het verband tussen genetisch polymorphismen en het risico voor HNSCC dient derhalve onder gestandaardiseerde condities plaats te vinden voor zowel patiënten als controlegroepen.
3. Plasma *GSTP1*-1 kan niet als tumor marker worden gebruikt voor diagnostiek of follow-up van patiënten met HNSCC.
4. De genotypen van *GSTP1* en de *GSTP1*-1 enzym plasma spiegels staan niet in relatie tot elkaar bij patiënten met HNSCC.
5. Meta-analyse en pooled-analyse in een internationaal samenwerkingsverband laten een zwakke relatie zien tussen *GSTM1*, *GSTT1* en *CYP1A1* genotypen en hoofd hals kanker. Deze relatie wordt nog zwakker na correctie voor studie centrum, leeftijd, geslacht en ras. Voor de polymorphismen van het *GSTP1* gen werd een dergelijke relatie niet gezien.

Discussie

Onze hypothese dat polymorphismen in biotransformatie enzymen het risico van HNSCC verhogen wordt niet ondersteund door onze patiënt-controle studies. In één van onze eerste onderzoeken (hoofdstuk 2), kon geen verband worden aangetoond tussen polymorphismen van de *GSTM1*, *GSTT1* en *CYP1A1* genen en een verhoogd risico op hoofd hals kanker. In dezelfde studie werd, in tegenstelling tot onze hypothese, aanvankelijk een significant verhoogd *GSTT1 null* genotype in patiënten met BHNL gevonden, hetgeen suggereerde dat het *GSTT1 null* genotype wellicht een risico factor kon zijn voor BHNL. Echter, deze bevinding was gebaseerd op een patiënten populatie uit Maastricht en een controle populatie uit Nijmegen. De BHNL groep bestond eveneens uit Maastrichtse patiënten en diende als een extra controle groep.

Op een later moment (hoofdstuk 3) werd een tweede controle groep uit Maastricht samengesteld vanwege het feit dat data betreffende alcohol consumptie en rookgedrag ontbraken bij de Nijmeegse controle groep. Wij verwachtten geen significante verschillen in *GSTT1* genotype aan te treffen tussen twee controle groepen uit regio's die geografisch circa 125 kilometer van elkaar zijn gescheiden. De frequenties voor het *GSTT1 null* genotype in de Maastrichtse controle groep waren vergelijkbaar met die van de BHNL groep, echter niet met die van de HNSCC patiënten groep. Dit betekende dat de BHNL groep in feite een representatieve controle groep was. Belangrijker echter, was dat deze resultaten tegenstrijdig waren met reeds gepubliceerde resultaten (hoofdstuk 2). Nu waren de *GSTT1 null* frequenties significant lager in patiënten met HNSCC ten opzichte van de controle groep, waaruit blijkt dat *GSTT1 null* genotype wel betrokken zou kunnen zijn bij het risico op HNSCC.

Hoofdstuk 4 beoordeelt de bruikbaarheid van GSTP1-1 enzym plasma spiegels in de diagnostische fase van hoofd hals kanker. Enkele onderzoeken hebben aangetoond dat bij patiënten waarbij zojuist HNSCC was aangetoond, een aanvankelijk verhoogde GSTP1-1 spiegel in het plasma zou dalen na chirurgische verwijdering van de tumor. In onze patiënt-controle groepen kon geen verband tussen HNSCC (in welk stadium dan ook) en GSTP1-1 plasma spiegels worden aangetoond. Wel vonden wij de methoden voor de behandeling van het bloed en de bereiding van het plasma kritiek omdat GSTP1-1 in hoge mate aanwezig is in veel bloedcellen en in het plasma wordt uitgescheiden indien de bereiding niet correct plaatsvindt. Deze procedures zijn dus erg complex waarbij de vraag blijft bestaan of, zelfs bij een duidelijke correlatie, een dergelijke procedure van klinische betekenis zou kunnen zijn.

Ook al hebben wij aangetoond dat GSTP1-1 enzym plasma spiegels geen nut hebben als tumor marker, zijn er toch studies die hoge concentraties GSTP1-1 enzym beschrijven in verschillende typen tumor weefsel (inclusief hoofd hals tumoren).

Wij hebben geen verband aangetroffen tussen het polymorfisme van het *GSTP1* gen en hoofd hals kanker (hoofdstuk 5). Wel verwachtten wij conform de resultaten een duidelijke relatie tussen roken en larynx kanker en tussen alcoholgebruik en mond/mondbodemkanker vanwege de verschillende manier waarop blootstelling van de schadelijke stoffen plaatsvindt. Tabaksrook passeert tijdens in- en uitademing de larynx, zijnde het meest nauwe deel van de bovenste luchtwegen; alcoholhoudende dranken verblijven tijdelijk in de mondholte, zelfs na het doorslikken hiervan.

Nadat de GSTP1-1 enzym plasma spiegels en het *GSTP1* genotype waren bepaald in dezelfde patiënt- en controlegroepen, werden de resultaten in hoofdstuk 6 met elkaar in verband gebracht. De werkhypothese was dat de verhoogde GSTP1-1 plasma spiegels bij patiënten met HNSCC wellicht werden veroorzaakt door één van de *GSTP1* genotypen. Een verband tussen *GSTP1* genotype en fenotype kon niet worden vastgesteld, hetgeen past bij onze visie dat alleen het *GSTP1* polymorfisme een slechts minimaal effect lijken te hebben op de GSTP1-1 enzym activiteit in hoofd hals weefsel en op de vatbaarheid voor HNSCC.

Een onderzoek waarbij verschillende polymorphismen van de *GST* en *EPHX* genen worden gecombineerd levert ook geen bewijs op dat deze polymorphismen invloed uitoe-

fenen op het risico op HNSCC (hoofdstuk 7). In dit onderzoek werd het aantal polymorfismen van elk van deze genen bepaald bij zowel patiënten met HNSCC als in de controle groep. Hierbij werd niet gecorrigeerd voor leeftijd, geslacht, rookgedrag of alcohol consumptie.

In hoofdstuk 8 werd door middel van een internationaal multicenter meta- en pooled analyse een zwak verband gevonden tussen HNSCC en *GSTM1*, *GSTT1* en *CYP1A1*. De bevindingen van deze meta analyse ondersteunen de visie van dit proefschrift; genetische polymorfismen in carcinogeen metaboliserende enzym systemen zoals glutathion S-transferasen lijken van ondergeschikt belang in het ontstaan van hoofd hals kanker.

Toekomstige mogelijkheden

Het proces van carcinogenese is ingewikkeld. Zelfs al zijn de belangrijke bronnen van carcinogenen bekend, zoals alcohol en tabaksrook in veel gevallen van HNSCC, dan nog is reconstructie van wat zich exact afspeelt tijdens carcinogenese moeilijk. De metabole omzetting van alcohol is relatief eenvoudig, maar tabaksrook bevat veel toxische en mogelijk carcinogene stoffen, waarbij het metabolisme van elk van deze stoffen door veel verschillende enzymen beïnvloed wordt. Voordat mogelijk belangrijke enzymen volledig in kaart zijn gebracht is meer onderzoek noodzakelijk.

Alcohol dehydrogenase, acetaldehyde dehydrogenase, enkele anderen van de cytochrome P450 en glutathion S-transferase families, UDP-glucuronosyltransferasen welke recentelijk in relatie zijn gebracht met HNSCC, N-acetyltransferasen en veel andere enzymen of polymorfismen in de corresponderende genen, kunnen nog worden onderzocht op hun invloed op het ontstaan van hoofd hals kanker.

Om in staat te zijn een gedegen onderzoek te verrichten is het heel belangrijk om een grote bloed-, weefsel- en databank te hebben van patiënten met HNSCC en van de juiste controles. Dit project is reeds gestart. Weefsel onderzoek kan nog belangrijke informatie verschaffen over lokale enzym activiteiten.

De statistische analyse in alle onderzoeken van dit proefschrift was beperkt vanwege het grote verschil in rook- en alcoholconsumptie gedrag tussen patiënt- en controle-groepen. Het aantal niet-rokende patiënten met HNSCC was klein, terwijl in de gezonde controle groepen vrijwel geen zware rokers en/of drinkers waren. Met name deze extreme groepen (zwaar rokende en zwaar drinkende gezonde oudere controle personen en niet rokende, niet drinkende oudere patiënten met HNSCC) zijn van bijzonder belang voor hoofd hals kanker onderzoek, omdat deze personen wellicht een extreem goed of extreem slecht functionerend detoxificatie systeem hebben.

In dit proefschrift hebben wij laten zien dat hoofd hals kanker, dat duidelijk een relatie heeft met overmatig roken en alcoholgebruik, niet beïnvloed lijkt te worden door polymorfismen in een aantal enzymen die tabak en alcohol detoxificeren. Nog veel meer enzymen zullen moeten worden onderzocht voordat hierover een definitieve uitspraak gedaan kan worden.

DANKWOORD

De laatste loodjes wegen het zwaarst. Dit zullen velen hebben ervaren rondom de afronding van een proefschrift. Dan eindelijk is het moment aangebroken om na te denken over het dankwoord. Dit blijkt dan ook nog eens één van de drukste momenten te zijn, waarbij het gevaar bestaat dat bepaalde mensen worden vergeten. Daarom wil ik iedereen bedanken die bij heeft gedragen op welke manier dan ook aan de totstandkoming van dit proefschrift. Een aantal mensen wil ik in het bijzonder bedanken voor hun belangrijke bijdrage.

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Lieve Marianne (Beest), alleen jij weet echt hoeveel tijd dit onderzoek ons heeft gekost. De afgelopen 3 jaar hebben wij in sneltreinvaart een serie "life-events" meegeemaakt, waarbij deze één van de hekkensluiters zal zijn. *Behind every able man is an able woman*. Dit adagium is door jou ontstaan! Ik heb veel respect voor de manier waarop jij de zaken in ons uitbreidende gezin steeds weet te regelen, temeer daar ik nogal eens " bezig was " voor het onderzoek. Jouw trouwe steun, geduld en liefde zullen nu ook eindelijk worden beloond. Beest bedankt!

CURRICULUM VITAE

Michèl Oude Ophuis werd op 4 mei 1967 geboren te Hillegersberg in Rotterdam. Van 1979 tot 1985 was hij woonachtig in McLean, Virginia, USA. Aldaar heeft hij op Langley High School gezeten tot 1983. Met het oog op een studie in Nederland werd vanaf 1983 de opleiding voortgezet aan de Washington International School te Washington DC, waar hij met succes het International Baccalaureate Diploma heeft behaald in 1985. Vervolgens werd gestart met de studie Geneeskunde aan de Katholieke Universiteit Nijmegen. Het propaedeutisch examen werd in 1986 en het doctoraal examen in 1989 behaald. Tot aan zijn co-assistentenschappen in 1990 werden extra-curriculaire stages Heelkunde / Plastische Chirurgie gevolgd aan het Mubarak al Kabeer Hospital in Kuwait, alsmede een onderzoekstage binnen de afdeling Plastic and Reconstructive Surgery (Prof. dr. D.A. McGrouther) van University College London. In 1993 werd het artsexamen behaald. Na werkzaam te zijn geweest als arts-assistent cardiochirurgie niet in opleiding in het Universitair Medisch Centrum Nijmegen (Prof. dr. L.K. Lacquet), startte hij op 1 januari 1995 met de opleiding tot Keel-, Neus- en Oorarts in het Academisch Ziekenhuis Maastricht onder leiding van Prof. dr. J.J. Manni. De B-opleiding vond plaats in het Atrium Medisch Centrum te Heerlen bij Dr. T.D. Zijlker. Tijdens zijn opleiding werd onder leiding van Prof. dr. J.J. Manni en Dr. W.H.M. Peters gestart met het wetenschappelijk onderzoek dat uiteindelijk heeft geleid tot dit proefschrift. Sinds 1 januari 2000 is hij werkzaam als Keel-, Neus- en Oorarts in VieCuri Medisch Centrum voor Noord-Limburg. Hij is getrouwd met Marianne Steenmetz. Zij hebben twee kinderen, Martijn en Janneke.