Non-smoking and Non-drinking patients with Head and Neck Squamous Cell Carcinoma, A Distinct Population

ISBN: 978-90-39354971

Lay-out: Chris Bor, Medische fotografie en Illustratie, AMC, Amsterdam
Printed by: Buijten & Schipperheijn, Amsterdam

Non-smoking and Non-drinking patients with Head and Neck Squamous Cell Carcinoma, A Distinct Population

Niet rokende en Niet drinkende Patiënten met
Plaveiselcelcarcinomen van het Hoofdhalsgebied,
Een Specifieke Populatie
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge
het besluit van het college voor promoties in het openbaar te
verdedigen op vrijdag 4 februari 2011 des middags te 12.45 uur

door

Farzaneh Farshadpour
geboren op 11 januari 1976 te Teheran, Iran

Promotoren: Prof.dr. G.J. Hordijk
Prof.dr. R. Koole
Prof.dr. P.J. Slootweg

Dit proefschrift werd mede mogelijk gemaakt met financiële steun van:
Prof. dr. P. Egyedi Stichting ter bevordering van Mondziekten, Kaak-& Aangezichts-chirurgie te Utrecht, Stichting ORLU, ATOS Medical B.V., Olympus Nederland B.V., Specsavers, Stallergenes, Daleco Pharma B.V., Carl Zeiss B.V., ALK (Abelló)

Contents

Chapter 1

General introduction

General introduction

Head and neck squamous cell carcinomas (HNSCCs) comprise a heterogeneous group of malignancies of the epithelial lining of the upper aerodigestive tract. They are the world's 6th most common type of malignancies. The worldwide age standardized incidence rate (ASR) for tumors originating from the larynx, oral cavity and pharynx (excluding nasopharynx) were 33.3 per 100 000 male and 8.8 per 100 000 female individuals for the period 1998-2002[1].

There is a wide geographic variation in the incidence rates of these tumors. In Asian countries, such as Pakistan, India and Sri Lanka, oral cancer is the most common malignancy in men, and may contribute to 25% of all new cases of cancer[2]. Laryngeal tumors however, have the highest ASR in developed countries such as Spain, Italy and Brazil[1].

Trends in HNSCC incidence rates are diverse as well. An increase is seen for oral cavity cancer in Northern- and Eastern European countries and Japan. In Asian countries and in France these tumors are decreasing. Incidence of laryngeal tumors remain stable in France and Spain and Eastern Europe whereas an upward trend is reported in Denmark and Norway[1, 2].

In the Netherlands, the incidence of these malignancies in the year 2007 was 2090 (1409 men and 681 women). Furthermore, 702 deaths were reported due to these tumors. Between 1997 and 2007 the ASR of oral cavity tumors increased from 4.2 to 4.6 cases per 100 000 individuals and a decrease was reported for pharyngeal and laryngeal tumors, from 3.2 to 2.9 cases and from 4.7 to 3.5 cases per 100 000 individuals, respectively[3].

In general, HNSCC are treated with radiotherapy or surgery or a combination of both by means of a multidisciplinary approach. Chemotherapy is an additive treatment modality for advanced disease or as a palliative option. All treatment modalities have an enormous impact on patient's quality of life causing disfigurement and/or impairment of fundamental daily functioning such as speech, mastication and swallowing.

Second primary tumors (SPTs)

When compared to other malignancies, patients with HNSCC are at higher risk for development of SPTs with an estimated annual incidence of 3–4%[4-6]. In a recent Dutch study the 5-year and 10-year cumulative incidence of SPTs after treatment of a first primary oral or oropharyngeal squamous cell carcinoma was 13% and 21%, respectively[3, 7]. A multicenter study from 13 population-based cancer registries estimated a 20-year cumulative risk of 36% for all head and neck sites[8].

SPTs are mostly found in the head and neck region, lungs, and esophagus. Because of these localisations and the prior treatment received for the primary tumor, SPTs are difficult to manage and cause a decrease in survival[6].

The concept of field cancerization, first proposed by Slaughter et al[9] is subject of study to explain the pathogenesis of multiple primary tumor development. One theory based on this concept states that a large area of the aerodigestive tract mucosa is affected by long-term exposure to carcinogens such as tobacco and alcohol, leading to multiple, independently developing neoplastic lesions[10]. Other more recent studies suggest that at least a proportion of SPTs have a common clonal origin. Genetically altered (pre)malignant fields could migrate to a new site and transform to a new tumor. Additionally it is proposed that new primary tumors should be further analyzed using molecular classification aimed to differentiate between an independent or a common origin. Those which share a common origin with the primary tumor should be called second field tumors and those tumors which are independent should be described as SPTs[11-13].

Genetic alterations

The genetic alterations associated with HNSCC are abundant and involve a range of pathways. Aneuploidy (i.e. aberrant DNA content) and loss of heterozygosity (i.e. loss of genetic material, characterised by complete deletion, or loss of one allele) may result in inactivation of tumor suppressor genes, such as p53 and p16, and activation of oncogenes, such as epidermal growth factor receptor and subsequently lead to uncontrolled cell growth and metastasis[14-17].

Chromosomal aberrations which have often been related to HNSCC are chromosomal loss at 1p, 3p, 4p, 5q, 8p, 10p, 11q, 13q, 18q, and gains at 1q, 3q, 5p, 7q, 8q, 9q, 11q, 12p, 14q, and 15q[18, 19]. A tumor progression model was formulated which included genetic alterations leading to dysplasia (9p21, 3p21, 17p13), carcinoma in situ (11q13, 13q21, 14q31) and invasive tumors (4q26–28, 6p, 8p, 8q)[14, 16].

Microarray technology has become the method of choice to study gene expression patterns in HNSCC. Recently microarrays have been used to determine unique expression profiles associated to subgroups of patients with HNSCC who have different clinical behavior or characteristics. For instance, Roepman et al identified a 102-predictor gene set for lymph node metastases using microarray analysis[20].

It should be emphasized that in the majority of studies related to genetic alterations, no distinction is made between cases exposed to the mutagenic effects of the main risk factors for development of HNSCC, namely consumption of tobacco and alcohol and those who are not exposed to these risk factors.

Risk factors

More than 95% of patients with HNSCC smoke tobacco and consume alcohol. These substances are well established as independent and synergistic risk factors associated with HNSCC[21-27]. In a recent meta-analysis the highest relative risks (RRs) for tobacco smoking and the development of cancer were found for lung cancer (RR 8.96) and upper aerodigestive tract tumors; RR for laryngeal tumors was 6.98 (95% CI: 3.14-15.52; 10 studies),for pharyngeal tumors 6.76 (95% CI: 2.86-15.98; 8 studies) and for oral cavity tumors 3.43 (95% CI: 2.37-4.94; 12 studies)[23]. Many components of tobacco, for example nitrosamines have been proven to be carcinogenic[28]. Other products such as snuffs and chews (betel quid that consists of the leaf of the betel vine, areca nut, lime or tobacco), have also been identified as carcinogenic risk factors, especially in Asian countries such as India[29].

Consumption of three or more alcoholic beverages per day in non-smokers were shown to increase the risk for HNSCC to a RR of 2.04 (95% CI: 1.29-3.21) compared to never drinkers[24]. Amongst others, genetic polymorphisms in the alcohol-metabolizing enzyme aldehyde dehydrogenases have been associated with head and neck cancer[30, 31].

When combined the exposure of tobacco and alcohol leads to a multiplicative high risk for HNSCC. Individuals who smoke more than 20 cigarettes and consume more than 100 grams of alcohol a day were shown to have a 200 times increased risk[21].

Nonetheless, a small population of patients with HNSCC do not possess this traditional risk factor profile[32-35]. These non-smoking and non-drinking patients with HNSCC will be subject of study in this thesis.

Other factors have been identified to be involved in HNSCC. In the recent years Human papilloma virus (HPV) has been established as an etiological factor for development of squamous cell carcinoma especially originating from the oropharynx. These HPV-positive tumors seem to have a characteristic immunohistological profile, a better response to treatment and a favorable survival rate[36-38].

Poor oral hygiene, especially tooth loss indicating peridontal disease has been recognised as an etiological factor for head and neck cancer[39]. Dietary factors have been considered to be related to HNSCC; Western dietary patterns are reported to be strongly associated with the increased risk of laryngeal cancer, whereas a healthy dietary pattern was inversely associated[40]. Furthermore, a high fruit and vegetable intake is associated with reduced risk of head and neck cancer[41]. Other possible predisposing factors include gastrointestinal reflux[21, 42-44], low body mass index[45], low socioeconomic status[46], and presence of oral lichen planus or other mucosal lesions such as leukoplakia and erythroplakia which are considered as premalignant[47].

The aim and outline of this thesis

Non-smoking and non-drinking patients with HNSCC are uncommon and have not been studied often or in only small numbers. The pathogenesis for these patients without known risk factors is unclear. In this thesis a relatively large population of these non-smokers and non-drinkers with HNSCC will be described and analyzed with respect to different clinicopathological and etiological aspects. This knowledge may lead to better diagnostic and therapeutical strategies specifically focussed on these distinct patients so it can ultimately lead to an improvement of their prognosis.

In **chapter 2** the epidemiological aspects of 195 non-smoking and non-drinking patients in our center will be presented and compared to national data of all patients with HNSCC in The Netherlands.

In **chapter 3** univariate and multivariate analysis will be performed to assess overall and disease-specific survival data for non-smoking and non-drinking patients compared to smoking and drinking patients with HNSCC.

In **chapter 4** non-tumorous, tumor adjacent mucosa from 4 groups of patients will be immunohistochemically analyzed with regard to expression of dispersed single cells and clusters of p53 and suprabasal expression of Ki-67. We hereby aimed to compare mucosal field changes in the following groups of patients: non-smoking and non-drinking patients with multiple and single HNSCC and smoking and drinking patients with multiple and single HNSCC.

In **chapter 5** the presence and clinical implications of HPV in oropharyngeal tumors of non-smoking and non-drinking patients and matched smoking and drinking patients with HNSCC will be evaluated.

In **chapter 6** a distinct gene expression profile for non-smoking and non-drinking patients with HNSCC, found using microarray technique will be presented.

Reference List

(1) Curado MP, Hashibe M. Recent changes in the epidemiology of head and neck cancer. *Curr Opin Oncol.* 2009 May;21(3):194-200.

(2) Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol.* 2009 Apr;45(4-5):309-316.

(3) Netherlands Cancer Registry (2007 estimates) 2010Available from: URL: www.ikcnet.nl

(4) Jovanovic A, van der Tol I, Kostense PJ, et al. Second respiratory and upper digestive tract cancer following oral squamous cell carcinoma. *Eur J Cancer B Oral Oncol.* 1994 Jul;30B(4):225-229.

(5) Vaamonde P, Martin C, del Rio M., LaBella T. Second primary malignancies in patients with cancer of the head and neck. *Otolaryngol Head Neck Surg.* 2003 Jul;129(1):65-70.

(6) Leon X, Quer M, Diez S, Orus C, Lopez-Pousa A, Burgues J. Second neoplasm in patients with head and neck cancer. *Head Neck.* 1999 May;21(3):204-210.

(7) van der Haring I, Schaapveld MS, Roodenburg JL, de Bock GH. Second primary tumours after a squamous cell carcinoma of the oral cavity or oropharynx using the cumulative incidence method. *Int J Oral Maxillofac Surg.* 2009 Apr;38(4):332-338.

(8) Chuang SC, Hashibe M, Scelo G, et al. Risk of second primary cancer among esophageal cancer patients: a pooled analysis of 13 cancer registries. *Cancer Epidemiol Biomarkers Prev.* 2008 Jun;17(6):1543-1549.

(9) Slaughter D, Southwick H, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer.* 1953 Sep;6(5):963-968.

(10) van Oijen MG, Gilsing MM, Rijksen G, Hordijk GJ, Slootweg PJ. Increased number of proliferating cells in oral epithelium from smokers and ex-smokers. *Oral Oncol.* 1998 Jul;34(4):297-303.

(11) Braakhuis BJ, Tabor MP, Leemans CR, van der Waal I, Snow GB, Brakenhoff RH. Second primary tumors and field cancerization in oral and oropharyngeal cancer: molecular techniques provide new insights and definitions. *Head Neck.* 2002 Feb;24(2):198-206.

(12) Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res.* 2003 Apr 15;63(8):1727-1730.

(13) Tabor MP, Brakenhoff RH, van Houten V, et al. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. *Clin Cancer Res.* 2001 Jun;7(6):1523-1532.

(14) Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. *N Engl J Med.* 2001 Dec 27;345(26):1890-1900.

(15) Gold KA, Kim ES. Role of molecular markers and gene profiling in head and neck cancers. *Curr Opin Oncol.* 2009 May;21(3):206-211.

(16) Ha PK, Chang SS, Glazer CA, Califano JA, Sidransky D. Molecular techniques and genetic alterations in head and neck cancer. *Oral Oncol.* 2009 Apr;45(4-5):335-339.

(17) Molinolo AA, Amornphimoltham P, Squarize CH, Castilho RM, Patel V, Gutkind JS. Dysregulated molecular networks in head and neck carcinogenesis. *Oral Oncol.* 2009 Apr;45(4-5):324-334.

(18) Jin C, Jin Y, Wennerberg J, Annertz K, Enoksson J, Mertens F. Cytogenetic abnormalities in 106 oral squamous cell carcinomas. *Cancer Genet Cytogenet.* 2006 Jan 1;164(1):44-53.

(19) Singh B, Gogineni SK, Sacks PG, et al. Molecular cytogenetic characterization of head and neck squamous cell carcinoma and refinement of 3q amplification. *Cancer Res.* 2001 Jun 1;61(11):4506-4513.

(20) Roepman P, Wessels LF, Kettelarij N, et al. An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nat Genet.* 2005 Feb;37(2):182-186.

(21) Andre K, Schraub S, Mercier M, Bontemps P. Role of alcohol and tobacco in the aetiology of head and neck cancer: a case-control study in the Doubs region of France. *Eur J Cancer B Oral Oncol.* 1995 Sep;31B(5):301-309.

(22) Blot WJ, McLaughlin JK, Winn DM, et al. Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res.* 1988 Jun 1;48(11):3282-3287.

(23) Gandini S, Botteri E, Iodice S, et al. Tobacco smoking and cancer: a meta-analysis. *Int J Cancer.* 2008 Jan 1;122(1):155-164.

(24) Hashibe M, Brennan P, Benhamou S, et al. Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *J Natl Cancer Inst.* 2007 May 16;99(10):777-789.

(25) Jaber MA, Porter SR, Gilthorpe MS, Bedi R, Scully C. Risk factors for oral epithelial dysplasia--the role of smoking and alcohol. *Oral Oncol.* 1999 Mar;35(2):151-156.

(26) Lewin F, Norell SE, Johansson H, et al. Smoking tobacco, oral snuff, and alcohol in the etiology of squamous cell carcinoma of the head and neck: a population-based case-referent study in Sweden. *Cancer.* 1998 Apr 1;82(7):1367-1375.

(27) Polesel J, Talamini R, La Vecchia C., et al. Tobacco smoking and the risk of upper aerodigestive tract cancers: A reanalysis of case-control studies using spline models. *Int J Cancer.* 2008 May 15;122(10):2398-2402.

(28) Hecht SS. Progress and challenges in selected areas of tobacco carcinogenesis. *Chem Res Toxicol.* 2008 Jan;21(1):160-171.

(29) Sapkota A, Gajalakshmi V, Jetly DH, et al. Smokeless tobacco and increased risk of hypopharyngeal and laryngeal cancers: a multicentric case-control study from India. *Int J Cancer.* 2007 Oct 15;121(8):1793-1798.

(30) La Vecchia C., Zhang ZF, Altieri A. Alcohol and laryngeal cancer: an update. *Eur J Cancer Prev.* 2008 Apr;17(2):116-124.

(31) Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer.* 2007 Aug;7(8):599-612.

(32) Agudelo D, Quer M, Leon X, Diez S, Burgues J. Laryngeal carcinoma in patients without a history of tobacco and alcohol use. *Head Neck.* 1997 May;19(3):200-204.

(33) Andrews E, Seaman WT, Webster-Cyriaque J. Oropharyngeal carcinoma in non-smokers and non-drinkers: A role for HPV. *Oral Oncol.* 2009 Jun;45(6):486-491.

(34) Dahlstrom KR, Little JA, Zafereo ME, Lung M, Wei Q, Sturgis EM. Squamous cell carcinoma of the head and neck in never smoker-never drinkers: a descriptive epidemiologic study. *Head Neck.* 2008 Jan;30(1):75-84.

(35) Wiseman SM, Swede H, Stoler DL, et al. Squamous cell carcinoma of the head and neck in nonsmokers and nondrinkers: an analysis of clinicopathologic characteristics and treatment outcomes. *Ann Surg Oncol.* 2003 Jun;10(5):551-557.

(36) Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. *J Clin Oncol.* 2009 Apr 20;27(12):1992-1998.

(37) D'Souza G, Kreimer AR, Viscidi R, et al. Case-control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med.* 2007 May 10;356(19):1944-1956.

(38) Fakhry C, Gillison ML. Clinical implications of human papillomavirus in head and neck cancers. *J Clin Oncol.* 2006 Jun 10;24(17):2606-2611.

(39) Guha N, Boffetta P, Wunsch F, V, et al. Oral health and risk of squamous cell carcinoma of the head and neck and esophagus: results of two multicentric case-control studies. *Am J Epidemiol.* 2007 Nov 15;166(10):1159-1173.

(40) De Stefani E., Boffetta P, Ronco AL, eo-Pellegrini H, Acosta G, Mendilaharsu M. Dietary patterns and risk of laryngeal cancer: an exploratory factor analysis in Uruguayan men. *Int J Cancer.* 2007 Sep 1;121(5):1086-1091.

(41) Freedman ND, Park Y, Subar AF, et al. Fruit and vegetable intake and head and neck cancer risk in a large United States prospective cohort study. *Int J Cancer.* 2008 May 15;122(10):2330-2336.

(42) Freije JE, Beatty TW, Campbell BH, Woodson BT, Schultz CJ, Toohill RJ. Carcinoma of the larynx in patients with gastroesophageal reflux. *Am J Otolaryngol.* 1996 Nov;17(6):386-390.

(43) El-Serag HB, Hepworth EJ, Lee P, Sonnenberg A. Gastroesophageal reflux disease is a risk factor for laryngeal and pharyngeal cancer. *Am J Gastroenterol.* 2001 Jul;96(7):2013-2018.

(44) Bacciu A, Mercante G, Ingegnoli A, et al. Effects of gastroesophageal reflux disease in laryngeal carcinoma. *Clin Otolaryngol Allied Sci.* 2004 Oct;29(5):545-548.

(45) Peters ES, Luckett BG, Applebaum KM, Marsit CJ, McClean MD, Kelsey KT. Dairy products, leanness, and head and neck squamous cell carcinoma. *Head Neck.* 2008 Sep;30(9):1193-1205.

(46) Conway DI, Petticrew M, Marlborough H, Berthiller J, Hashibe M, Macpherson LM. Socio-economic inequalities and oral cancer risk: a systematic review and meta-analysis of case-control studies. *Int J Cancer.* 2008 Jun 15;122(12):2811-2819.

(47) van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. *Oral Oncol*. 2009 Apr;45(4-5):317-323.

Chapter 2

Non-smoking and non-drinking patients with head and neck squamous cell carcinoma: a distinct population

F. Farshadpour
G.J. Hordijk
R. Koole
P.J. Slootweg

Oral Diseases 2007 Mar;13:239-43

Abstract

Background. To recognise specific clinicopathological characteristics of non-smoking and non-drinking (NSND) head and neck squamous cell carcinoma (HNSCC) patients. This can increase our knowledge regarding a potentially different carcinogenesis in these patients.

Methods. Retrospective analysis of data for 195 NSND patients with HNSCC and comparison with data for patients with HNSCC obtained from the Netherlands Cancer Registry.

Results. Compared with all HNSCC patients in the Netherlands, our NSND patients with HNSCC were typically female (n=142; 73% vs 26%), old at disease presentation (mean 73 years vs 64 years), and had tumors mainly of the oral cavity (n=130; 67% vs 25%). Most tumors were stage I (n=67; 34%) and stage IVA (n=59; 30%). The incidence of second primary tumors (SPTs) was high (n= 32; 16%); mainly occurring in the oral cavity (n=26; 13%).

Conclusions. Our study confirms that NSND HNSCC patients have different clinicopathological characteristics from those of the overall HNSCC population; however, the frequency of SPTs is as high in NSND patients as in patients who smoke and drink alcohol. More research, and particularly molecular data is needed to obtain a better understanding of head and neck cancer in the NSND patients.

Introduction

Head and neck squamous cell carcinomas (HNSCCs) are malignant tumors arising from the mucosal membranes of the upper aerodigestive tract. HNSCC is a common malignancy, accounting for 5% of all newly diagnosed cancer cases world-wide. Despite significant improvements in treatment modalities, overall survival rates of HNSCC patients have only moderately improved[1]. This lack of progress in prognosis is mainly due to two factors. Firstly a high locoregional recurrence and distant metastases rate (10 to 30%) and second the high occurence of second primary tumors (SPTs) often located in the same or adjacent anatomical region. These SPTs occur at a constant rate of 2 to 3% per year for more than ten years[2].

The association between tobacco smoking and alcohol consumption and the development of cancer of the head and neck has been established in many studies. These substances are independent risk factors but exert a synergistic effect when combined[3, 4]. Individuals who smoke more than 20 cigarettes a day and use more than 100 grams of alcohol a day have a 200 times increased risk of developing head and neck cancer[3]. There is, however, a small population of HNSCC patients without these major risk factors. These non-smoking and non-drinking (NSND) patients are an interesting subgroup of HNSCC, but they have rarely been studied, or only in small numbers[5-7].

Analysis of this distinct group of HNSCC patients may shed light on several important issues: (A) specific clinicopathological features (i.e. gender, age, tumor localisation and tumor stage at disease presentation and the occurrence of second primary tumor), (B) additional risk factors and (C) possible changes in the non-neoplastic normal epithelium of these patients.

In the present study, we retrospectively described several clinicopathological characteristics of 195 non-smoking and non-drinking (NSND) patients with HNSCC at our centre. These data were compared with the same characteristics in all HNSCC patients in the Netherlands Cancer Registry.

Patients and methods

All newly diagnosed patients with head and neck cancer at the University Medical Center Utrecht (UMCU) have been prospectively registered in a database since 1980. This database contains information on patient characteristics, risk factors, and tumor classification, including development of recurrences and second primary

tumors. During the period 1980-2003, 4404 patients with squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and larynx were registered. Smoking and drinking habits were recorded. Smoking was categorised as no smoking (i.e. no history of smoking), quit smoking >2 years, quit smoking >1 year, quit smoking <1 year, and as 0-20 cigarettes/day or 20-40 cigarettes/day, and alcohol consumption was categorised as no alcohol consumption (i.e. no history of alcohol consumption), no daily alcohol consumption, 1 consumption/day, 2-4 consumptions/day, 5-9 consumptions/day, >9 consumptions/day.

Tumors were classified according to the International Classification of Diseases for Oncology (ICD-O) and the TNM classification (according to the criteria of the International Union Against Cancer). A second primary tumor was defined according to the criteria of Warren and Gates[8]: (1) each of the tumors must present a definite picture of malignancy, (2) each must be distinct, and (3) the probability of one being a metastasis of the other must be excluded. Local recurrence was defined as occurring less than 1.5 cm or 2 cm from the index tumor and within 5 years of detection of the index tumor.

Patient and tumor characteristics were compared with those of patients with HNSCC included in the Netherlands Cancer Registry (NCR). This population-based database, which has full coverage since 1989, contains information derived from the national computerized pathology databank and the hospital discharge databank, to which all Dutch hospitals annually provide information on the discharge diagnosis of admitted patients. Data on diagnosis, treatment, and follow-up for 6 months after cancer diagnosis are retrieved from patients' medical records.

Statistical analysis was performed using non-parametric χ^2 test for comparison of disease localisation in the two groups of patients (national database patients with HNSCC and the NSND patients with HNSCC). Student's *t* test was used for statistical analysis of age and gender.

Inclusion criteria

All HNSCC patients (oral cavity, oropharynx, hypopharynx, lip and larynx) who had no history of smoking or drinking were included in our study.

Results

Of the 4404 patients referred to the UMCU for cancer of the oral cavity, oropharynx, hypopharynx, or larynx in the period 1980 to 2003, 195 (4.4%) were eligible for inclusion in our study. Most patients were women (n=142; 73%). Mean age at initial diagnosis was 73 years (median 76, range 20-97). Sixteen patients (8%) had a history of another primary tumor other than HNSCC. These tumors were located in the breast (n= 7), uterus (n=3), skin (n=2), thyroid gland (n=1), abdomen (n=1), bladder (n=1), and pancreas (n=1). Sixty-two patients (32%) reported malignancy in first- or second-degree relatives, 10 of whom had head and neck malignancy (5%).

Table I shows tumor distribution according to pathological TNM classification and site. A substantial percentage of the tumors were located in the oral cavity (n=130; 67%). Most of the tumors were stage I (n=67; 34%) and stage IVA (n=59; 30%). Fifty patients (26%) had cervical lymph node metastases. Four patients (2%) had metastatic disease located in the lung and liver. Thirty-two patients (16%) developed second primary tumors (table II); 26 of these tumors were localised in the oral cavity. Three patients had a third primary tumor, one patient a fourth, one patient a sixth, and one patient a seventh. These tumors were all in the oral cavity.

Table I. pTNM pathological classification by tumor site

	Oral cavity	**Lip**	**Oropharynx**	**Hypopharynx**	**Larynx**	**Total**
	n=130	n=3	n=17	n=10	n=35	n=195
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
T1	45 (35)	1(33)	5 (28)	0 (0)	18 (51)	69 (35)
T2	43 (33)	0 (0)	4 (24)	1 (10)	8 (23)	56 (29)
T3	8 (6)	1(33)	4 (24)	2 (20)	4 (12)	19 (10)
T4	34(26)	1(33)	4(24)	7 (70)	5 (14)	51 (26)
N0	97 (75)	2 (67)	7 (41)	5 (50)	34 (97)	145 (74)
N1	18 (14)	0 (0)	2 (12)	2 (20)	0 (0)	22 (11)
N2	12 (9)	0 (0)	8 (47)	2 (20)	1 (3)	23 (12)
N3	3 (2)	1(33)	0 (0)	1 (10)	0 (0)	5 (3)
M0	127 (98)	3 (100)	16 (94)	10 (100)	35 (100)	191 (98)
M1	3 (2)	0 (0)	1(6)	0 (0)	0 (0)	4 (2)

Table II. Incidence of subsequent head and neck squamous cell carcinoma

Primary tumor	**Second primary tumor**	**Third primary tumor**	**Fourth primary tumor**	**Sixth primary tumor**	**Seventh primary tumor**
Oral cavity (n=130)	Oral cavity (n=23)	Oral cavity (n=3)	Oral cavity (n=1)	Oral cavity (n=1)	Oral cavity (n=1)
	Oropharynx (n=3)				
Lip (n=3)	0	0	0	0	0
Oropharynx (n=17)	Oral cavity (n=2)				
Hypopharynx (n=10)	0	0	0	0	0
Larynx (n=35)	Oral cavity (n=1)				
	Oropharynx (n=1)				
	Hypopharynx (n=2)				
Total (n=195)	32 (16%)	3 (2%)	1 (1%)	1 (1%)	1 (1%)

National data on HNSCC obtained from the NCR for the period 1989 to 2003, showed a male preponderance (74%) of the disease, with patients being on average 63.5 years old at initial diagnosis (table III). Most tumors were localised in the larynx (39%). The differences in gender, age and tumor localisation between the two groups were statistically significant.

Table III. National HNSCC* data (NCR†) versus NSND‡ HNSCC patients

	NSND		**NCR**		***P***
	N	%	N	%	
Gender					
♂	53	27	14128	74	<0.001
♀	142	73	4837	26	
Total	195	100	18965	100	
Age					
Mean	72,7		63,5		<0.001
Median	75,6		64		
Site					
oral cavity	130	66	4773	25	<0.001
Lip	3	2	2046	11	
oropharynx	17	9	3180	17	
hypopharynx	10	5	1498	8	
larynx	35	18	7468	39	
Total	195	100	18965	100	

* HNSCC Head and neck squamous cell carcinoma † NCR National cancer registry
‡ NSND Non-smoking and non-drinking

Discussion

The NSND HNSCC patients represented 4.4% of all patients with head and neck cancer treated at our centre. This proportion is consistent with a reported incidence of between 2.4% and 3.9%[5-7]. Compared with all HNSCC patients in the Netherlands, our NSND patients with HNSCC were mainly female (73% vs 26%), were older (73 vs 64 years), and had tumors mainly of the oral cavity (67% vs 25%).

Our study outcomes were consistent with those in the existing literature, although relatively few studies have evaluated NSND patients with HNSCC. Wiseman *et al.*[6] conducted a case series investigation of 40 NSND patients with HNSCC with a high percentage of older women (75%, mean age 60). Seventy-five percent of the tumors were in the oral cavity. Twenty-four percent of the patients developed a second primary tumor.

Agudelo *et al.*[5] retrospectively reviewed 933 patients with squamous cell carcinoma of the larynx, of whom 31 (3.3%) had no history of tobacco or alcohol use. Comparison of the NSND patients with their smoking and drinking counterparts showed that the NSND patients were on average 10 years older, that there was no male predominance, and that the lesions were mainly located in the glottis, which permitted early diagnosis and a better survival. None of the patients developed second primary tumors.

Constatinides *et al.*[7] described a cohort of 10 NSND elderly patients (inclusion criteria was age >59 years), 9 of whom were women. Their median age was 75 years (range 60-87). Lesions were confined to the oral cavity (n=6), oropharynx (n=3), and larynx (n=1). Three patients developed disease recurrence and two patients developed second primary tumors. De Boer *et al.*[9] examined 125 NSND women older than 40 years. These patients were 15 years older than their drinking and smoking counterparts, with most tumors localized in the oral cavity (73%).

Our findings and the current literature show that there are different populations of patients with HNSCC. NSND patients are mostly female, older, have more oral cavity tumors, and have a high incidence of second primary tumors. This suggests that carcinogenesis is possibly different in these two patient populations and that other tumorigenic factors may be relevant, such as Human Papilloma Virus (HPV), gastrointestinal reflux disease, oral lichen planus, poor diet, and familial predisposition.

HPV has been implicated in the development of oropharyngeal localised neoplastic lesions[10-12]. HPV infection rate in our NSND population, was not known. Gastrointestinal reflux disease and chronic voice abuse have been suggested as possible aetiological factors for laryngeal squamous cell carcinoma[13, 14], but not for oral cavity HNSCC as in the majority of our patients. A causal link between oral lichen planus and HNSCC has not been established, though some studies describe the transition of lichen planus to oral squamous cell carcinoma. The reported incidence of these tumors varies between 0.4% and 2.5%[15, 16]. We do not know whether patients in our study suffered from oral lichen planus. The possible association between nutritional deficiencies and HNSCC remains controversial, but a number of case-control studies have consistently shown patients with oral cancers to have a poor diet[17, 18]. Our patients' nutritional status was not available.

Only a few studies have evaluated the role of familial predisposition in HNSCC[19, 20]. One study showed the incidence of HNSCC in first-degree relatives of patients with new HNSCC to be significantly higher than in a control group[19]. In this study, most of the relatives of patients with oral and pharyngeal cancer had tumors of the upper digestive tract and most of the relatives of patients with laryngeal cancer had lung cancer. All patients and relatives had been exposed to the traditional HNSCC risk factors, namely tobacco and alcohol. To our knowledge, there are no studies of familial predisposition in NSND patients with HNSCC.

The absence of any known potential risk factor suggests that specific molecular and genetic mechanisms may be involved in the tumorigenesis of head and neck cancer in our population. Sorensen *et al*[21]. found no p53 mutations in 6 NSND patients younger than 40 years. Koch *et al.*[22] found a lower p53 mutation rate and a higher HPV infection rate in a non-smoking HNSCC population. They also found that nonsmokers were likely to have less loss of heterozygosity (LOH) at chromosomes 3p, 4q and 11q13 and a lower overall percentage of microsatellite alterations. Singh *et al*.[23] also reported that gains of 1p and amplification of 3q were significantly less common in NSND patients. These genetic differences in NSND patients suggest indeed a different pathogenesis. Further studies are needed to clarify this issue.

Strikingly, second primary tumors occurred in 16% of our NSND patients with HNSCC, which is approximately the same percentage as in HNSCC patients who smoke tobacco and drink alcohol[2, 24]. Two theories have been advanced to explain the pathogenesis of multiple HNSCC tumors. The first theory is based on the concept of field cancerisation, proposed by Slaughter *et al.*[25], which states that a large area of the aerodigestive tract mucosa is affected by long-term exposure to carcinogens, leading to multiple, independently developing lesions. Van Ooijen

et al.[26] studied this concept by investigating the proliferation index of epithelium from adjacent, histologically normal mucosa taken from smoking and non-smoking HNSCC patients. They observed a significantly increased proliferation index in epithelia from smoking patients, but not in epithelia from the non-smoking patients. The authors concluded that multiple tumors might be the result of continuous exposure to tobacco; however, this does not explain the high frequency of second primary tumors in NSND patients.

The second theory proposes that at least a proportion of multiple HNSCCs arise from one clonal cell population. Genetically altered fields containing the same p53 mutation as the index tumor have been detected in macroscopically normal mucosa surrounding the tumor and in some cases even extending beyond the surgical margins[27, 28]. Partridge *et al.*[29] examined a cohort of 11 patients with multiple HNSCC of which 9 NSND patients, and found some identical novel microsatellite alleles indicating early genetic aberrations and a common clonal origin. They suggested that multiple lesions in these patients arise due to lateral spread from a common precursor and thus are clonally related. Thus it appears that in NSND patients, HNSCC arise from clonal spread and not independently.

Despite the lack of exposure to tobacco and alcohol and for reasons not yet understood, the upper airway mucosa of NSND patients seems to have the same tendency to develop more than one tumor. To date, p53 mutations or an increased proliferation index, which are indicative of genetic alterations, have not been found, which suggests that other mechanisms must be responsible for this phenomenon. Future studies may show why epithelium not exposed to known carcinogens is still prone to develop multiple neoplastic lesions.

In conclusion, our study confirms the distinct clinicopathological characteristics of NSND patients with HNSCC. These patients are older, mainly female, and their tumors are predominantly localised in the oral cavity. Surprisingly, the rate of second primary tumor development is similar in patients with HNSCC who smoke and drink and in NSND patients with HNSCC. More knowledge of head and neck cancer in NSND patients is required to elucidate the mechanisms of their proneness to develop multiple tumors.

Reference List

(1) Vokes EE, Weichselbaum RR, Lippman SM, Hong WK. Head and neck cancer. *N Engl J Med.* 1993 Jan 21;328(3):184-194.

(2) Licciardello JT, Spitz MR, Hong WK. Multiple primary cancer in patients with cancer of the head and neck: second cancer of the head and neck, esophagus, and lung. *Int J Radiat Oncol Biol Phys.* 1989 Sep;17(3):467-476.

(3) Andre K, Schraub S, Mercier M, Bontemps P. Role of alcohol and tobacco in the aetiology of head and neck cancer: a case-control study in the Doubs region of France. *Eur J Cancer B Oral Oncol.* 1995 Sep;31B(5):301-309.

(4) Jaber MA, Porter SR, Gilthorpe MS, Bedi R, Scully C. Risk factors for oral epithelial dysplasia--the role of smoking and alcohol. *Oral Oncol.* 1999 Mar;35(2):151-156.

(5) Agudelo D, Quer M, Leon X, Diez S, Burgues J. Laryngeal carcinoma in patients without a history of tobacco and alcohol use. *Head Neck.* 1997 May;19(3):200-204.

(6) Wiseman SM, Swede H, Stoler DL, et al. Squamous Cell Carcinoma of the Head and Neck in Nonsmokers and Nondrinkers: An Analysis of Clinicopathologic Characteristics and Treatment Outcomes. *Ann Surg Oncol.* 2003 Jun 1;10(5):551-557.

(7) Constantinides MS, Rothstein SG, Persky MS. Squamous cell carcinoma in older patients without risk factors. *Otolaryngol Head Neck Surg.* 1992 Mar;106(3):275-277.

(8) Warren S, Gates O. Multiple primary malignant tumors. A survey of the literature and a statistical study. *Am J Cancer.* 1932;16:1358-1414.

(9) de Boer MF, Sanderson RJ, Damhuis RA, Meeuwis CA, Knegt PP. The effects of alcohol and smoking upon the age, anatomic sites and stage in the development of cancer of the oral cavity and oropharynx in females in the south west Netherlands. *Eur Arch Otorhinolaryngol.* 1997;254(4):177-179.

(10) Gillison ML, Koch WM, Shah KV. Human papillomavirus in head and neck squamous cell carcinoma: are some head and neck cancers a sexually transmitted disease? *Curr Opin Oncol.* 1999 May;11(3):191-199.

(11) Mineta H, Ogino T, Amano HM, et al. Human papilloma virus (HPV) type 16 and 18 detected in head and neck squamous cell carcinoma. *Anticancer Res.* 1998 Nov;18(6B):4765-4768.

(12) Wilczynski SP, Lin BT, Xie Y, Paz IB. Detection of human papillomavirus DNA and oncoprotein overexpression are associated with distinct morphological patterns of tonsillar squamous cell carcinoma. *Am J Pathol.* 1998 Jan;152(1):145-156.

(13) Freije JE, Beatty TW, Campbell BH, Woodson BT, Schultz CJ, Toohill RJ. Carcinoma of the larynx in patients with gastroesophageal reflux. *Am J Otolaryngol.* 1996 Nov;17(6):386-390.

(14) Morrison MD. Is chronic gastroesophageal reflux a causative factor in glottic carcinoma? *Otolaryngol Head Neck Surg.* 1988 Oct;99(4):370-373.

(15) Kaplan B, Barnes L. Oral lichen planus and squamous carcinoma. Case report and update of the literature. *Arch Otolaryngol.* 1985 Aug;111(8):543-547.

(16) Katz RW, Brahim JS, Travis WD. Oral squamous cell carcinoma arising in a patient with long-standing lichen planus. A case report. *Oral Surg Oral Med Oral Pathol.* 1990 Sep;70(3):282-285.

(17) Rossing MA, Vaughan TL, McKnight B. Diet and pharyngeal cancer. *Int J Cancer.* 1989 Oct 15;44(4):593-597.

(18) Winn DM, Ziegler RG, Pickle LW, Gridley G, Blot WJ, Hoover RN. Diet in the etiology of oral and pharyngeal cancer among women from the southern United States. *Cancer Res.* 1984 Mar;44(3):1216-1222.

(19) Copper MP, Jovanovic A, Nauta JJ, et al. Role of genetic factors in the etiology of squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg.* 1995 Feb;121(2):157-160.

(20) Goldstein AM, Blot WJ, Greenberg RS, et al. Familial risk in oral and pharyngeal cancer. *Eur J Cancer B Oral Oncol.* 1994 Sep;30B(5):319-322.

(21) Sorensen DM, Lewark TM, Haney JL, Meyers AD, Krause G, Franklin WA. Absence of p53 mutations in squamous carcinomas of the tongue in nonsmoking and nondrinking patients younger than 40 years. *Arch Otolaryngol Head Neck Surg.* 1997 May;123(5):503-506.

(22) Koch WM, Lango M, Sewell D, Zahurak M, Sidransky D. Head and neck cancer in nonsmokers: a distinct clinical and molecular entity. *Laryngoscope.* 1999 Oct;109(10):1544-1551.

(23) Singh B, Wreesmann VB, Pfister D, et al. Chromosomal aberrations in patients with head and neck squamous cell carcinoma do not vary based on severity of tobacco/alcohol exposure. *BMC Genet.* 2002 Nov 14;3(1):22.

(24) Panosetti E, Luboinski B, Mamelle G, Richard JM. Multiple synchronous and metachronous cancers of the upper aerodigestive tract: a nine-year study. *Laryngoscope.* 1989 Dec;99(12):1267-1273.

(25) Slaughter DP, Southwick H, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer.* 1953 Sep;6(5):963-968.

(26) van Oijen MG, Gilsing MM, Rijksen G, Hordijk GJ, Slootweg PJ. Increased number of proliferating cells in oral epithelium from smokers and ex-smokers. *Oral Oncol.* 1998 Jul;34(4):297-303.

(27) Bedi GC, Westra WH, Gabrielson E, Koch W, Sidransky D. Multiple head and neck tumors: evidence for a common clonal origin. *Cancer Res.* 1996 Jun 1;56(11):2484-2487.

(28) Tabor MP, Brakenhoff RH, van H, V, et al. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. *Clin Cancer Res.* 2001 Jun;7(6):1523-1532.

(29) Partridge M, Pateromichelakis S, Phillips E, Emilion G, Langdon J. Profiling clonality and progression in multiple premalignant and malignant oral lesions identifies a subgroup of cases with a distinct presentation of squamous cell carcinoma. *Clin Cancer Res.* 2001 Jul;7(7):1860-1866.

Chapter 3

Survival analysis of head and neck squamous cell carcinoma: influence of smoking and drinking

F. Farshadpour
H. Kranenborg
E. Van Beeck Calkoen
G.J. Hordijk
R. Koole
P.J. Slootweg
C.H. Terhaard

Head Neck. 2010 Aug 24. [Epub ahead of print]

Abstract

Background. Head and neck squamous cell carcinomas (HNSCCs) are associated with tobacco and alcohol, however the prognostic relevance of these substances is unclear.

Methods. Univariate and multivariate survival analysis were performed for patients with (n=1829) and without (n=183) substance use.

Results. HNSCC-specific survival (death due to primary- or recurrent HNSCC) and HNSCC/second primary tumor-specific survival (death due to primary- or recurrent HNSCC or second primary tumor) were not significantly different for patients who smoked and drank and those who did not (hazard ratio (HR) 1.26, 95%CI 0.86-1.85, HR 1.34, 95%CI 0.96-1.88, respectively). Overall survival was significantly affected; HR for smoking and drinking patients was 1.50 (95%CI 1.16-1.93).

Conclusions. Although tobacco and alcohol use are the main risk factors for development of HNSCC, disease outcome was comparable in patients who did, or did not use these substances. Tobacco and alcohol use affected overall survival, which emphasizes the importance of substance use cessation.

Introduction

The vast majority of head and neck squamous cell carcinomas (HNSCCs) are related to a history of smoking tobacco and drinking alcohol[1]; however, there is a small group of patients, approximately 3-4%, who have never smoked or drunk alcohol and who develop HNSCC[2-5]. In chapter two, we reported that the clinical characteristics of these non-smoking and non-drinking patients significantly differ from those of their smoking and drinking counterparts[3]. Non-smoking and non-drinking patients mainly have tumors of the oral cavity, tend to be older (mean age 72.7 yrs), and are more often female, (3:1 female to male ratio). HNSCC in the smoking and drinking population, in contrast, presents mostly in younger (mean age 63.5 yrs) male patients (female to male ratio 1:3) with tumors of the larynx. Because of their small numbers, there have been only a few studies of non-smoking and non-drinking patients with HNSCC[2-5]. Moreover, although clinical experience suggests that disease outcome and survival are better in non-smoking and non-drinking patients than in smoking and drinking patients, the literature is not consistent about this.

Several studies have addressed the importance of tobacco and alcohol as prognostic factors for survival; however, the results are contradictory and mostly concern a small group of non-smokers[6-12]. We chose to perform a survival analysis for HNSCC among patients who were lifelong non-users of both tobacco and alcohol or who had a history of smoking and drinking. We hypothesized that because of the synergistic effect of tobacco and alcohol on development of HNSCC when combined[1], a possible prognostic effect of these substances would be detected in these two 'extreme' groups. Disease outcome was calculated and compared after correction for other confounding factors, such as TN classification, site of origin, treatment, and age. To our knowledge, this is the first study to address HNSCC outcome in relation to tobacco and alcohol use in a large population of users and non-users.

Materials and methods

Patients

All patients with newly diagnosed HNSCC have been prospectively registered in a database of the Head and Neck Department at the University Medical Center Utrecht in the Netherlands since 1980. This database contains information on patient characteristics (e.g., gender and age), risk factors, tumor classification, and mode of treatment. Tumors were classified according to the International Classification of Diseases for Oncology (ICD-O) and the TNM classification (according to the criteria of the International Union Against Cancer, UICC). Between

1 January 1980 and 1 January 2004, 4607 patients were entered in our database. 2012 patients diagnosed with squamous cell carcinoma originating in the oral cavity, oropharynx, hypopharynx, and larynx were included in this study according to the following inclusion criteria: lifelong non-smoking and non-drinking patients (n=183) and patients who consumed both tobacco (quit smoking <1 year, and 1or more cigarettes/day) and alcohol (1 or more alcohol consumption/day), (n= 1829). Patients (n=2595) who were either smokers who did not drink or drinkers who did not smoke (n=2062) or former smokers and/or drinkers (n=533) were excluded for this analysis.

Follow-up

All patients were followed up in the University Medical Center Utrecht according to the following protocol. Patients were seen every 2 months in the first and second year after treatment, every 3 months in the third year, every 4 months in the fourth year and every 6 months in the fifth year. After 5 years of follow-up, patients without signs of recurrent disease were discharged from follow-up.

Second primary tumors in the lungs or esophagus were recorded, as were second primary tumors in the head and neck region, defined according to the criteria of Warren and Gates[13]: (1) each of the tumors must present a definite picture of malignancy, (2) each must be distinct, and (3) the probability of one being a metastasis of the other must be excluded. Recurrent HNSCC was defined as histologically confirmed tumor occurring less than 2 cm from the index tumor and within 5 years of detection of the index tumor. Disease recurrence and second primary tumor are presented actuarially (Kaplan-Meier) and in absolute figures.

Follow-up data were classified as follows: alive without HNSCC, death due to primary or recurrent HNSCC, death due to second primary tumor, death due to other (non-HNSCC related) causes, and follow-up status unknown. For this study, when follow-up status was unknown, a letter was sent to patients' general practitioner requesting the latest patient follow-up data according to the mentioned criteria above. HNSCC-specific survival time was defined as the time between the date of diagnosis of primary HNSCC and date of death due to primary HNSCC or recurrent HNSCC, date of last follow-up, or date of end of study (i.e. 1 June 2008). HNSCC/second primary tumor-specific survival time was defined as the time between diagnosis of primary HNSCC and time of death due to primary HNSCC or recurrent HNSCC, second primary tumor, date of last follow-up or date of end of study. Overall survival was defined as the time between the date of diagnosis of primary HNSCC and date of death due to any cause or date of the last follow-up or date of end of study.

Statistical analysis

Comparisons between baseline characteristics were performed using χ^2 tests for categorical data, and *t*-tests for continuous data. HNSCC-specific-, HNSCC/second primary tumor-specific, and overall survival probabilities were estimated for both the non-smoking and non-drinking and smoking and drinking groups and within strata of prognostic factors using the Kaplan-Meier approach and the actuarial method of Kaplan-Meier. Estimated survival curves were compared using the log-rank test. Multivariable analysis of HNSCC-specific survival, HNSCC/second primary tumor-specific survival and overall survival was performed using the Cox proportional hazards model. Because of the observational nature of this study, patients differ with respect to factors such as age, gender, tumor localization, (clinical) TN classification and treatment modality. These factors as well as tobacco and alcohol use were considered as potential confounding variables to be controlled in multivariable models. The results are reported in terms of hazard ratios (HRs) and their corresponding confidence intervals (CIs). All statistical analyses were performed using SPSS version 15.0. Values were considered statistically significant when p value was less than 0.05.

Results

Patient characteristics

The baseline characteristics of all patients are shown in Table 1. Non-smoking and non-drinking patients with HNSCC were significantly older, mostly female, and had mostly tumors of the oral cavity whereas smoking and drinking patients mainly had tumors of the larynx. The overall T- and N-classification distribution and treatment modalities were significantly different between the two groups. Distant metastasis was not significantly different. Non-smoking and non-drinking patients had mostly stage I tumors and smoking and drinking patients, stage II tumors ($p=0.003$).

Follow-up data

The mean follow-up was 4.6 years and the median 4.0 years (range, 1 month to 26.0 years). At the end of study 51.3% of the non-smoking and non-drinking and 44.3% of the smoking and drinking patients were alive without signs of disease and 21.9% and 27.9% had died of their disease (primary HNSCC or recurrence), respectively (Table 2). Four point nine percent of the non-smoking and non-drinking patients and 5.6% of the smoking and drinking patients had died of second primary tumor. Follow-up status was unknown for 8 patients (0.4%; address untraceable). No significant difference was found between both groups ($p=0.280$).

Table 1. Baseline characteristics of all patients (n=2012)

		NSND* (n=183)	(%)	SD† (n=1829)	(%)	p
Age (yrs)	mean	71.3		59.3		$p \leq 0.001$
	range	20-93		23-88		
Age (categories)	≤ 35	3	1.6	11	0.6	$p \leq 0.001$
	36-45	10	5.5	163	8.9	
	46-55	6	3.3	490	26.8	
	56-65	28	15.3	651	35.6	
	66-75	50	27.3	399	21.8	
	≥ 76	86	47.0	115	6.3	
Gender	Male	52	28.4	1452	79.4	$p \leq 0.001$
	Female	131	71.6	377	20.6	
Tumor localization	Larynx	35	19.1	833	45.5	$p \leq 0.001$
	Oral cavity	122	66.7	614	33.6	
	Oropharynx	16	8.7	231	12.6	
	Hypopharynx	10	5.5	151	8.3	
T-classification	1	74	40.4	480	26.3	$p \leq 0.001$
	2	57	31.1	626	34.2	
	3	18	9.8	267	14.6	
	4	34	18.7	456	24.9	
N-classification	0	144	78.8	1193	65.3	$p = 0.003$
	1	16	8.7	264	14.4	
	2	20	10.9	306	16.7	
	3	3	1.6	66	3.6	
M-classification	0	183	100	1814	99.2	NS‡
	1	0	0	15	0.8	
Stage	I	65	35.5	428	23.4	$p = 0.003$
	II	35	19.1	458	25.0	
	III	27	14.8	309	16.9	
	IV	56	30.6	634	34.7	
Treatment modality	RT§	53	29.0	856	46.8	$p \leq 0.001$
	Surgery	77	42.1	362	19.8	
	Surgery + RT	50	27.3	553	30.2	
	RT + CHT#	2	1.1	51	2.8	
	Surgery + RT + CHT	1	0.5	7	0.4	

*NSND = non-smoking and non-drinking, †SD = smoking and drinking, ‡NS = Non-Significant §RT = Radiation therapy, #CHT = Chemotherapy,

Disease recurrence occurred in 17.5% of the non-smoking and non-drinking patients and in 22.7% of the smoking and drinking patients; the localizations of these tumors were significantly different between groups (Table 3). Second primary tumors were found in 17.4% of the non-smoking and non-drinking patients, (3.3% of these second primary tumors were synchronous tumors) and in 18.4% of the smoking and drinking patients (3.5% of these second primary tumors were synchronous tumors).

Table 2: Follow-up data

	NSND*		SD†		
	(n=183	(%)	(n=1829)	(%)	
Alive without HNSCC	94	51.3	810	44.3	
Death due to primay / recurrent HNSCC‡	40	21.9	510	27.9	
Death due to second primary tumor	9	4.9	103	5.6	NS§
Overall death (other causes)	40	21.9	398	21.8	
Follow-up status unknown	0	0	8	0.4	

*NSND = non-smoking and non-drinking, †SD = smoking and drinking, ‡HNSCC = head and neck squamous cell carcinoma, §NS = non-significant

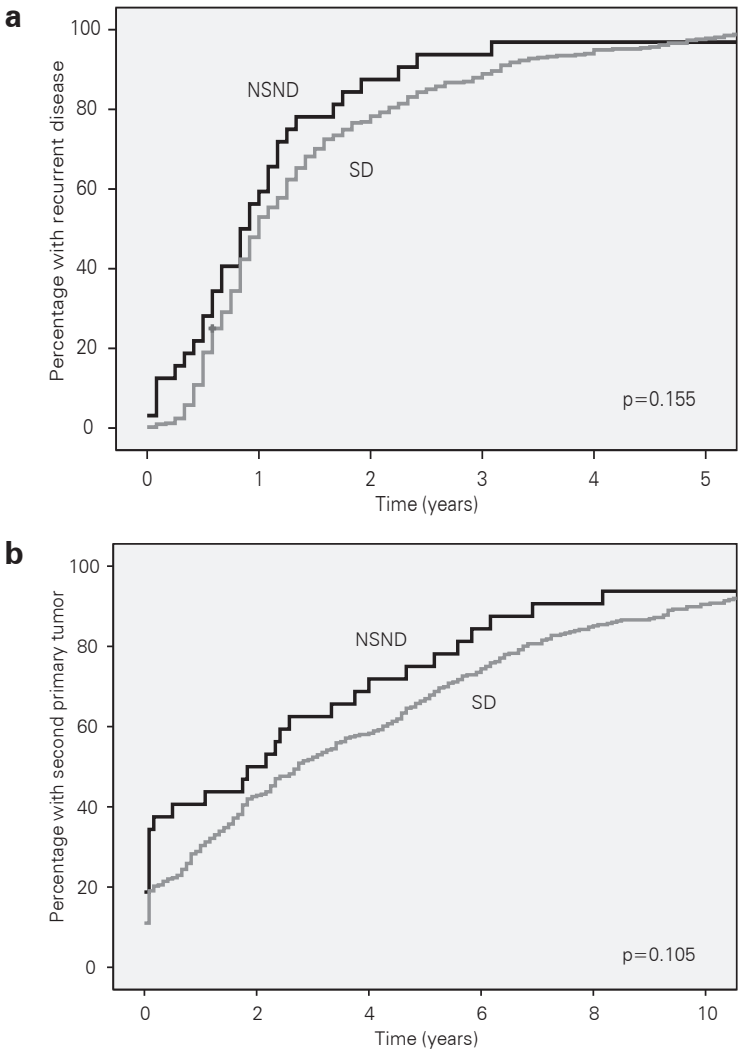

Figure 1. (a). Time to disease recurrence **(b).** Time to second primary tumor

Table 3: Recurrences and second primary tumors

		Overall				
		NSND*		SD†		
		(n=183)	(%)	(n=1829)	(%)	
Recurrence	Total	32	17.5	415	22.7	
	Tumor	22	12.0	310	16.9	p = 0.040
	Nodal	9	4.9	54	3.0	
	Distant	1	0.5	51	2.8	
Second primary tumor	Total	32	17.4	3336	18.4	
	Head & Neck	31	16.9	182	10	p ≤ 0.001
	Lung	1	0.5	137	7.5	
	Esophagus	0	0.0	17	0.9	

*NSND = non-smoking and non-drinking, †SD = smoking and drinking,

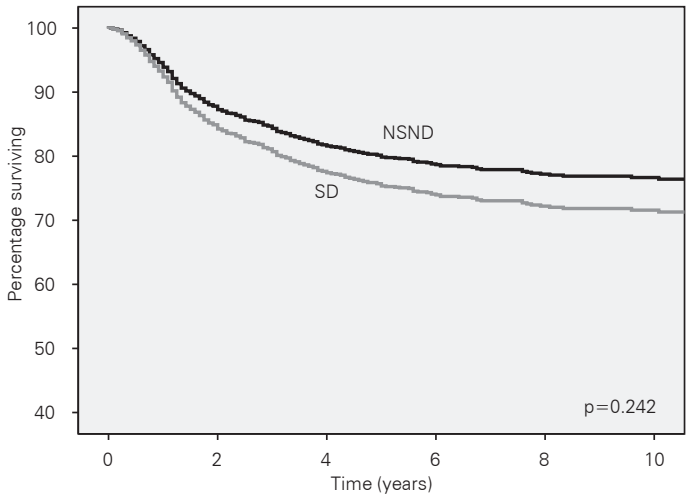

Figure 2. The adjusted impact of tobacco and alcohol on HNSCC-specific survival, i.e. death due to primary HNSCC or disease recurrence

The distribution of second primary tumors was different in the two groups. Nearly all second primary tumors of the non-smoking and non-drinking population were in the head and neck region whereas 8.4% of the smoking and drinking patients had second primary tumors in the lungs and esophagus (Table 3). Figure 1a and 1b show time to disease recurrence andsecond primary tumor, respectively, without a significant difference between the two groups.

Larynx		Oral cavity		Oropharynx		Hypopharynx	
NSND (%)	SD (%)	NSND (%)	SD (%)	NSND (%)	SD (%)	NSND (%)	SD (%)
20.0	26.3	17.2	16.6	18.8	20.8	10	30.5
20.0	21.0	9.8	11.2	12.5	15.6	10	19.9
0	2.8	6.6	3.6	6.3	1.7	0	3.3
0	2.5	0.8	1.8	0	3.5	0	7.3
14.3	14.9	20.5	21.7	12.5	22.5	0	17.9
11.4	5.9	20.5	14.0	12.5	14.7	0	8.6
2.9	8.8	0	6.4	0	6.5	0	6.6
0	0.2	0	1.3	0	1.3	0	2.6

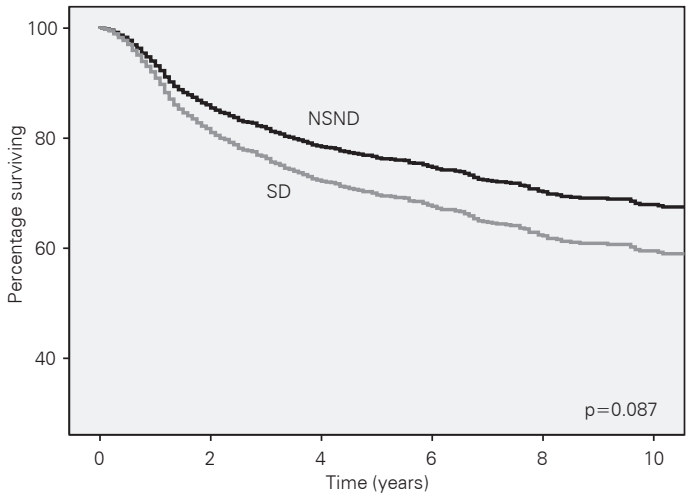

Figure 3. The adjusted impact of tobacco and alcohol on HNSCC/second primary tumor-specific survival, i.e. death due to primary HNSCC or disease recurrence or second primary tumor

HNSCC-specific survival

Univariate analysis showed that 91%, 81%, 72% and 69% of patients survived 1, 2, 5, and 10 years, respectively. There was no significant difference between the two groups ($p=0.112$). Most deaths due to primary HNSCC or recurrent disease occurred in the first year after tumor diagnosis (non-smoking and non-drinking 12.6% and smoking and drinking 11.1%). Multivariate analysis showed a slightly higher, but non-significant, -risk for primary HNSCC or recurrent disease among the smoking and drinking patients (HR 1.26, Table 4). Figure 2 shows the adjusted impact of tobacco and alcohol use on HNSCC-specific survival.

HNSCC/second primary tumor-specific survival

HNSCC/second primary tumor-specific survival for all patients was 90% after 1 year, 79% after 2 years, 68% after 5 years and 59% after 10 years. No significant difference was found between groups (univariate analysis, $p=0.072$). Multivariate analysis showed that the risk of death due to primary HNSCC, recurrent disease or second primary tumor was increased, but not significantly, by 34% in the smoking and drinking patients (HR=1.34, Table 4). Figure 3 shows the adjusted HNSCC/ second primary tumor- specific survival curve for both groups.

Overall survival

The median overall survival time was 5.9 years for all patients, it was 6.3 years for non-smoking and non-drinking patients and 5.9 years for smoking and drinking patients. The 1-, 2-, 5-, and 10-year cumulative survival rate was 85%, 75%, 59% and 44% for non-smoking and non-drinking patients, and 86%, 72%, 55% and 39% for smoking and drinking patients (univariate analysis, $p=0.284$). Most deaths occurred in the first year after tumor diagnosis (non-smoking and non-drinking 16.3% and smoking and drinking 13.6%). Multivariate analysis showed a significant difference in overall survival between smoking and drinking and non-smoking and non-drinking patients with HNSCC. Non-smoking and non-drinking patients had a 50% less risk of all cause death than smoking and drinking patients (Table 4; Figure 4).

Subgroup analysis

Because of the large number of oral cavity patients in the non-smoking and non-drinking population, we performed an additional subgroup analysis regarding this subsite only. Multivariate analysis showed no significant differences between both groups regarding all survival modalities. Smoking and drinking patients had a HR of 0.95 (95%CI 0.60-1.43) and 1.00 (95%CI 0.63-1.61) for HNSCC-specific- and HNSCC/second primary tumor-specific survival, respectively. Non-smoking and non-drinking patients had a 31% better overall survival (HR 1.31, 95%CI 0.92-1.86).

Table 4. The impact of tobacco and alcohol use based on multivariate analysis and adjusted for age, gender, c-TN-classification, tumor localization, and treatment modality HNSCC-specific survival

	HNSCC-specific survival			**HNSCC/second primary tumor-specific survival**			**Overall survival**		
	HR#	95% CI* for HR	p	HR	95% CI for HR	p	HR	95% CI for HR	p
NSND †	1.00			1.00			1.00		
SD‡	1.26	0.86-1.85	NS§	1.34	0.96-1.88	NS	1.50	1.16-1.93	0.002

*CI= Confidence interval, †NSND= non-smoking and non-drinking, ‡SD= smoking and drinking, §NS= Non-significant, #HR= Hazard ratio

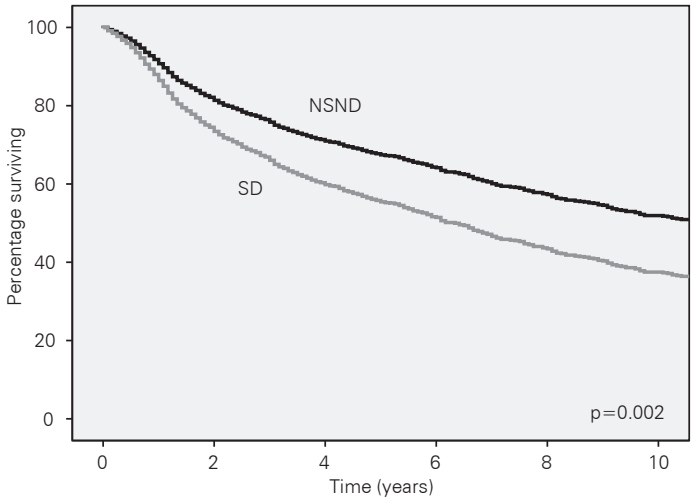

Figure 4. The adjusted impact of tobacco and alcohol on overall survival, i.e. death due to any cause

Discussion

In this study we performed a survival analysis on data for two large groups of patients registered with HNSCC in our center over a 24-year period. We intentionally chose the two most dissimilar groups with regard to tobacco and alcohol use, to increase the likelihood of detecting an impact of substance use on survival. To our knowledge, this is the first study addressing the survival outcome of non-smoking and non-drinking patients with HNSCC compared to smoking and drinking patients using uni- and multivariate analysis with a substantial population of non-smoking and non-drinking patients.

We earlier reported the unique characteristics of non-smoking and non-drinking patients with HNSCC. Remarkably, oral cavity is the most prevalent subsite in these ptients. Other predisposing factors than tobacco and alcohol for oral squamous cell carcinoma could be poor oral hygiene, dietary factors or premalignant oral lesions (such as lichen planus)[3].

Unexpectedly, we found that the incidence of subsequent primary tumors was similar in non-smoking and non-drinking patients and smoking and drinking patients (17.4% vs 18.4%, present study). Furthermore, the present study shows that these subsequent primary tumors were also mostly localized in the oral cavity, in the non-smoking and non-drinking patients and in the lung and esophagus in the smoking and drinking patients. Literature provides some evidence for clonal spread of genetic alterations in non-smoking and non-drinking patients with multiple

primary tumors[14] whereas oral field cancerization may explain the pathogenesis of multiple HNSCC in smoking and drinking patients[15].

We used multivariate analysis to assess HNSCC-specific and HNSCC/second primary tumor-specific survival time. After correction for known prognostic factors, such as TN classification, age, and treatment, we did not find tobacco and alcohol use to significantly affect either outcome. Literature on the prognostic effect of these substances is limited and usually concerns tobacco use only[4, 6, 8, 10, 12]. Pytynia et al. performed a matched-pair survival analysis for 50 never-smokers and ever-smokers and found a significant increase in risk of death due to disease and disease recurrence among smokers[11]. However, the 95% CIs were very wide. In addition, their survival outcomes were no longer significant after adjustment for alcohol consumption. They also excluded 33 non-smoking patients whom they were unable to match with smoking patients. These unmatched patients however had more advanced disease at the time of presentation and consequently worse survival outcome, which may have contributed to the impression that non-smokers have a better outcome. In a recent study by Harris et al., no difference in 10-year relapse-free survival was found for non-smoking and non-drinking patients (n=28) compared to smoking and drinking patients, but they did find a suggestion of improved 10-year overall survival for non-smoking and non-drinking patients (univariate analysis)[4]. In another study of the outcome of oral squamous cell carcinoma in Taiwan, the relative risks of patients with different oral habits were compared[9]. The combination of cigarette smoking, alcohol drinking, and betel quid chewing conveyed the highest relative risk of death (5.3), however the relative risk of death associated with tobacco and alcohol use was only 1.13. Several other studies were not able to find a significant effect of smoking on survival[7, 8, 12]. However these studies all had a small number of non-smokers, and failed to take alcohol use into consideration within a specific tumor subsite or special age category. In contrast, we included a large number of non-smokers and non-drinkers with tumors localized in all regions of the head and neck.

Overall survival was significantly affected by smoking and drinking, possibly because of the close relationship between these substances and other disorders, such as cardiovascular and chronic pulmonary disease, which we did not control for. Our results regarding overall survival are nonetheless similar to those of other studies. Agudelo et al., who assessed survival in patients with laryngeal squamous cell carcinoma using the Kaplan Meier method, found a better 5-year overall survival in non-smoking and non-drinking (n=33) than smoking and drinking patients[16]. Dikshit et al. performed a multicenter European study and reported an HR of 1.8 (overall survival) for smokers compared to non-smokers with laryngeal and hypopharyngeal cancer; this difference in survival was however not found for alcohol intake[6]. Beside gender, age, tumor localization, TN classification, and treatment modality,

other factors could have influenced survival, such as comorbidity and performance status[17]. Although we did not correct for these factors, we think they would be more important for overall survival than HNSCC-specific or HNSCC/second primary tumor-specific survival and probably would not influence our findings.

Human papilloma virus (HPV) has increasingly been associated with mainly oropharyngeal localized squamous cell carcinoma and possibly better prognosis[18, 19]. In a recent study, 78% of non-smoking and non-drinking patients (n=18) with oropharyngeal squamous cell carcinoma were reported to be infected with high-risk HPV[20]. Only 8.6% of our non-smoking and non-drinking population had oropharyngeal tumors, so a potential positive prognostic effect of HPV infection in our population would be small. It has been suggested that molecular differences in HNSCC, such as p53 mutations, are more common in patients who smoke than in patients who do not smoke and can lead to different clinical outcomes[8, 21]. In an earlier study, we did not find increased expression of p53 or Ki-67 in tumor adjacent mucosa, which would be indicative of pre-malignant mucosal alterations in the non-smoking and non-drinking patients, and therefore, the tendency of this group of patients to develop multiple tumors can not be attributed to mucosal genetic alterations related with overexpressions of these markers, as generally assumed[22]. Nutritional and socioeconomic status, hereditary factors (e.g., Plummer-Vinson Syndrome), occupational hazards and poor oral hygiene are other factors that could influence survival which we did not take into account[6, 23].

In conclusion, although tobacco and alcohol consumption are the most important risk factors for the development of primary HNSCC, the disease develops in a small population without these risk factors. However, after diagnosis these patients not only seem to have the same rate of disease recurrence and second primary tumor as smoking and drinking patients, but they also have a comparable disease outcome. More research is needed to understand the pathogenesis of HNSCC in this distinct population so that we can improve their survival. Furthermore, tobacco and alcohol influenced overall survival, which emphasizes the need to support patients in their efforts to stop using these substances.

References

1. Jaber MA, Porter SR, Gilthorpe MS, Bedi R, Scully C. Risk factors for oral epithelial dysplasia--the role of smoking and alcohol. Oral Oncol. 1999;35:151-156.
2. Dahlstrom KR, Little JA, Zafereo ME, Lung M, Wei Q, Sturgis EM. Squamous cell carcinoma of the head and neck in never smoker-never drinkers: a descriptive epidemiologic study. Head Neck 2008;30:75-84.
3. Farshadpour F, Hordijk GJ, Koole R, Slootweg PJ. Non-smoking and non-drinking patients with head and neck squamous cell carcinoma: a distinct population. Oral Dis. 2007;13:239-243.
4. Harris SL, Kimple RJ, Hayes DN, Couch ME, Rosenman JG. Never-smokers, never-drinkers: Unique clinical subgroup of young patients with head and neck squamous cell cancers. Head Neck 2009.
5. Wiseman SM, Swede H, Stoler DL, et al. Squamous cell carcinoma of the head and neck in nonsmokers and nondrinkers: an analysis of clinicopathologic characteristics and treatment outcomes. Ann.Surg.Oncol. 2003;10:551-557.
6. Dikshit RP, Boffetta P, Bouchardy C, et al. Lifestyle habits as prognostic factors in survival of laryngeal and hypopharyngeal cancer: a multicentric European study. Int.J.Cancer 2005;117:992-995.
7. Gorsky M, Epstein JB, Oakley C, Le ND, Hay J, Stevenson-Moore P. Carcinoma of the tongue: a case series analysis of clinical presentation, risk factors, staging, and outcome. Oral Surg.Oral Med.Oral Pathol.Oral Radiol.Endod. 2004;98:546-552.
8. Koch WM, Lango M, Sewell D, Zahurak M, Sidransky D. Head and neck cancer in nonsmokers: a distinct clinical and molecular entity. Laryngoscope 1999;109:1544-1551.
9. Lo WL, Kao SY, Chi LY, Wong YK, Chang RC. Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: factors affecting survival. J.Oral Maxillofac.Surg. 2003;61:751-758.
10. Pytynia KB, Grant JR, Etzel CJ, Roberts D, Wei Q, Sturgis EM. Matched analysis of survival in patients with squamous cell carcinoma of the head and neck diagnosed before and after 40 years of age. Arch.Otolaryngol.Head Neck Surg. 2004;130:869-873.
11. Pytynia KB, Grant JR, Etzel CJ, Roberts DB, Wei Q, Sturgis EM. Matched-pair analysis of survival of never smokers and ever smokers with squamous cell carcinoma of the head and neck. J.Clin.Oncol. 2004;22:3981-3988.
12. Verschuur HP, Irish JC, O'Sullivan B, Goh C, Gullane PJ, Pintilie M. A matched control study of treatment outcome in young patients with squamous cell carcinoma of the head and neck. Laryngoscope 1999;109:249-258.
13. Warren S, Gates O. Multiple primary malignant tumors. A survey of the litearture and a statistical study. Am.J.Cancer 1932;16:1358-1414.
14. Partridge M, Pateromichelakis S, Phillips E, Emilion G, Langdon J. Profiling clonality and progression in multiple premalignant and malignant oral lesions identifies a subgroup of cases with a distinct presentation of squamous cell carcinoma. Clin.Cancer Res. 2001;7:1860-1866.
15. van Oijen MG, Gilsing MM, Rijksen G, Hordijk GJ, Slootweg PJ. Increased number of proliferating cells in oral epithelium from smokers and ex-smokers. Oral Oncol. 1998;34:297-303.
16. Agudelo D, Quer M, Leon X, Diez S, Burgues J. Laryngeal carcinoma in patients without a history of tobacco and alcohol use. Head Neck 1997;19:200-204.

17. Hall SF, Groome PA, Rothwell D. The impact of comorbidity on the survival of patients with squamous cell carcinoma of the head and neck. Head Neck 2000;22:317-322.

18. Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. J Clin Oncol 2009;27:1992-8.

19. Ritchie JM, Smith EM, Summersgill KF, et al. Human papillomavirus infection as a prognostic factor in carcinomas of the oral cavity and oropharynx. Int J Cancer 2003;104:336-44.

20. Andrews E, Seaman WT, Webster-Cyriaque J. Oropharyngeal carcinoma in non-smokers and non-drinkers: A role for HPV. Oral Oncol. 2009;45:486-491.

21. Pukkila MJ, Kumpulainen EJ, Virtaniemi JA, et al. Nuclear and cytoplasmic p53 expression in pharyngeal squamous cell carcinoma: prognostic implications. Head Neck 2002;24:784-791.

22. Farshadpour F, Hordijk GJ, Koole R, Slootweg PJ. Head and neck squamous cell carcinoma in non-smoking and non-drinking patients with multiple tumors: etiologic significance of p53 and Ki-67 in non-tumorous epithelium. J.Oral Pathol.Med. 2008;37:549-554.

23. Boffetta P, Merletti F, Faggiano F, et al. Prognostic factors and survival of laryngeal cancer patients from Turin, Italy. A population-based study. Am.J.Epidemiol. 1997;145:1100-1105.

Chapter 4

Head and neck squamous cell carcinoma in non-smoking and non-drinking patients with multiple tumors: etiologic significance of p53 and ki-67 in non tumorous epithelium

F. Farshadpour
G.J. Hordijk
R. Koole
P.J. Slootweg

J Oral Pathol Med. 2008 Oct;37(9):549-54.

Abstract

Background. Non-smoking and non-drinking patients with head and neck squamous cell carcinoma have different clinical characteristics than their smoking and drinking counterparts. They are predominantly older female patients with oral cavity tumors, however both groups show the same percentage of second primary tumors. Expression of tumor suppressor gene p53 and proliferation marker Ki-67 in mucosal epithelial cells was analysed to study whether biomarker expression is associated with a history of smoking and drinking and with single and multiple tumors.

Methods. Non-smoking and non-drinking patients with multiple (n=18) and single tumors (n=15), smoking and drinking patients with multiple (n=15) and single tumors (n=14) were selected. For all groups, p53 and Ki-67 expression patterns in non-tumorous (tumor-adjacent) mucosa including positivity of dispersed single cells and clusters for p53 and for suprabasal expression of Ki-67 were immunohistochemically analysed and compared.

Results. p53 expression was significantly higher in users of tobacco and alcohol than in non-users. Ki-67 expression was not affected by tobacco and alcohol usage. Both Ki-67 and p53 were similarly expressed in the groups with single and multiple tumors and hence not significantly related to the number of tumors.

Conclusions. Non-smoking and non-drinking patients with squamous cell carcinoma have the same risk for developing multiple tumors as their smoking and drinking counterparts. As this occurs without an increased expression of p53 or Ki-67, the significance of these proteins as biomarkers indicating premalignant mucosal alterations is doubtful. Further research is needed to clarify this predisposition for developing multiple head and neck cancer.

Introduction

Head and neck squamous cell carcinomas (HNSCCs) are malignancies which are strongly associated with tobacco smoking and alcohol consumption [1-3]. There is, however, a distinct population of patients with HNSCC without these major risk factors. These non-smoking and non-drinking patients represent approximately 3% to 4% of all patients with head and neck cancer. Their clinical characteristics are different from those of their smoking and drinking counterparts; they are older, mainly female, and have tumors predominantly in the oral cavity. However, the rate of second primary tumor development is similar in the two groups of patients with HNSCC [4-7].

Alterations in non-malignant tumor-adjacent mucosa , such as expression of p53 and increased expression of proliferation markers have been studied in the past, because they were considered to shed light on the development of primary and subsequent HNSCC [8]. p53 is expressed in 50% to 96% of patients with HNSCC depending on the detection technique used [9-11]. The prevalence and spectrum of p53 mutations are significantly greater in smoking and drinking patients than in non-smoking and non-drinking patients [12]. Furthermore non-malignant mucosa adjacent to p53-positive carcinomas may show suprabasal p53 expression[13, 14]. Therefore suprabasal expression of p53 could be predictive for malignant transformation of potentially malignant oral lesions and may thus be useful as a risk marker for subsequent cancer development from oral leukoplakias [13]. Van Oijen et al. found more p53 expression in tumor-adjacent mucosa from smoking than in non-smoking patients with HNSCC [15].

Moreover, because an increase in epithelial proliferation has been detected in malignant and premalignant lesions from epithelia of the upper aerodigestive tract [16, 17], the expression of Ki-67, a nuclear antigen which is expressed during G1, S, G2 and the M phase of the cell cycle, has been assessed. Ki-67 expression has been found to be correlated with the degree of epithelial dysplasia and with genetic alterations in dysplastic epithelium [18-20]. However, Ki-67 may also be expressed in arrested cycling cells [21].

The absence of traditional risk factors associated with HNSCC in non-smoking and non-drinking patients provides a unique opportunity to study whether the pattern of expression of these biomarkers is indeed associated with a history of smoking and drinking or with single and multiple HNSCC. To this end, we analysed the expression of the biomarkers p53 and Ki-67 in tumor-adjacent mucosa of non-smoking and non-drinking patients with HNSCC and compared the results with biomarker expression in patients with HNSCC who did smoke cigarettes, and drink alcohol. We

hypothesized that the highest biomarker expression should be present in smoking and drinking patients with multiple HNSCC and the lowest in non-smoking and non-drinking patients with a single HNSCC.

Materials and methods

Clinical material

Patients were selected from the database of the Department of Head and Neck of the University Medical Center Utrecht. All patients with newly diagnosed HNSCC have been registered prospectively in this database since 1980. The data entered is taken from the medical records, at the first outpatient appointment and at admission. The database contains information on patient characteristics, risk factors, and tumor classification, including development of multiple tumors. When a second or subsequent primary tumor was diagnosed, all data for that patient was revised. During the period 1980-2003, 4404 patients with squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, larynx and lip were registered. Smoking and drinking habits were recorded. Smoking was categorized as no smoking (i.e. no history of smoking), quit smoking >2 years, quit smoking >1 year, quit smoking <1 year, and as 0–20 cigarettes/day or 20–40 cigarettes/day, and alcohol consumption was categorized as no alcohol consumption (i.e. no history of alcohol consumption), no daily alcohol consumption, 1 consumption/day, 2–4 consumptions/day, 5–9 consumptions/day, >9 consumptions/day. Tumors were classified according to the International Classification of Diseases for Oncology (ICD-O) and the TNM classification (according to the criteria of the International Union Against Cancer). A second primary tumor was defined according to the criteria of Warren and Gates [22]: (1) each of the tumors must present a definite picture of malignancy, (2) each must be distinct, and (3) the probability of one being a metastasis of the other must be excluded.

Of all 4404 patients referred to the UMCU, 195 (4.4%) met the criteria of no smoking and no alcohol consumption. Thirty-eight of these patients had multiple primary tumors. The clinicopathological aspects of these non-smoking and non-drinking patients have been described in a previous study from our center [6].
For the current study we selected four groups of patients with HNSCC who had only been treated surgically, and had not undergone previous radiotherapy or chemotherapy. Group A consisted of all eligible non-smoking and non-drinking patients with multiple HNSCCs (n=18). Of all 38 non-smoking and non-drinking patients with multiple HNSCC, 20 patients were excluded because of the following reasons: previous radiotherapy and/or chemotherapy (n=12), incomplete tissue

material and/or no tumor-adjacent mucosa available (n=8). Group B consisted of non-smoking and non-drinking patients with a single HNSCC, groups C consisted of patients with multiple HNSCCs who smoked (>20 cigarettes/day) and drank (>5 alcohol consumptions /day), and group D consisted of patients with a single HNSCC who smoked (>20 cigarettes/day) and drank (>5 alcohol consumptions /day). The groups B, C and D were chosen at random from the database after considering the criteria named above. Paraffin blocks of tumor tissue containing histologically normal margins, free from tumor, were selected.

Immunohistochemical analysis for detection of p53 and Ki-67 staining pattern

Immunohistochemical staining was performed using a two-step immuno-peroxidase technique on 4μm paraffin sections. Sections were mounted on silan coated glass-slides and stored overnight at 37°C. Prior immunohistochemical staining endogenous peroxidase blocking was performed. For antigen retrieval the sections were boiled in citrate buffer (2,94 g/l sodium citrate, pH 6.0) for 15 minutes and subsequently cooled down to room temperature. After being washed with PBS, the sections were incubated with mouse monoclonal antibody P53 (AM195-5M Biogenex, San Ramon, CA) or mouse monoclonal antibody Ki-67 Antigen (M 7240, Immunotech. S.A. Marseille, France) for 60 minutes. Then incubated with a horse anti mouse biotine -conjugated antibody (Vector laboratories, Burlingame CA) followed by a streptavidin-peroxidase conjugate (Immunotech, Margency France) both for 30 minutes. Visualisation of the peroxidase label was performed with DAB (3,3'-diaminobenzidine tetrahydrochloride) purchased from Sigma, St. Louis, MI. The sections were counterstained with Mayer's haematoxylin.

Analysis of p53 and Ki-67 staining patterns

All samples were carefully examined under low magnification by two researchers one of whom was an experienced pathologist (PS). Normal appearing tumor-adjacent mucosa from primary tumor mucosal samples without signs of dysplasia were chosen for analysis of p53 and Ki-67 staining pattern (figure 1).

Tumor-adjacent mucosa was analysed for the presence of dispersed single cell p53 expression (in an area of at least 100 nuclei) or focally overexpressed p53 in clusters of at least five p53 positive cells (in an area of at least 100 nuclei). When any of the two staining patterns were present tumor-adjacent mucosa was considered positive for that pattern, when none were present tumor-adjacent mucosa was considered negative for p53 expression.

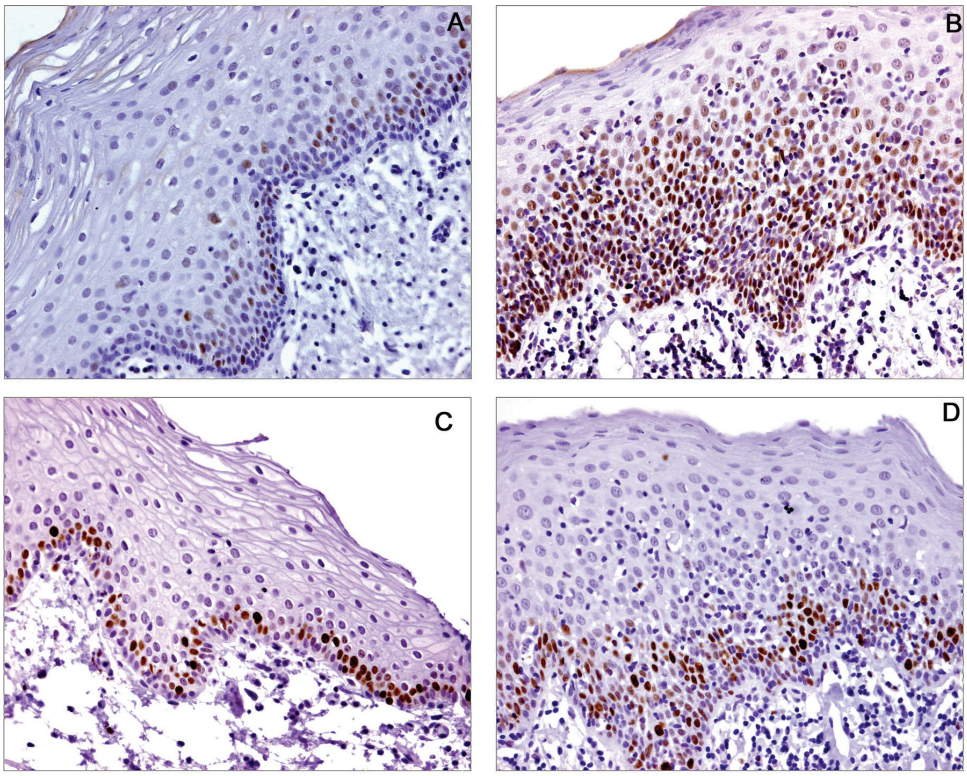

Figure 1. Immunohistochemical demonstration of p53 and Ki-67 in tumor-adjacent mucosa of HNSCC patients. Dispersed (single) p53 expression in the epithelium of tumor-adjacent mucosa from a patient with single HNSCC who does not smoke tobacco or use alcohol p53 (A), focal overexpression (clusters) in tumor-adjacent mucosa from a patient with single HNSCC who uses tobacco and alcohol (B), Ki-67 expression in the basal cell layer of tumor-adjacent mucosa from a patient with multiple HNSCC who does not smoke tobacco or use alcohol (C), Ki-67 suprabasal expression in tumor-adjacent mucosa from a patient with multiple HNSCC who uses tobacco and alcohol (D)

Ki-67 expression in the basal cell layers was ignored as this is universally seen. When Ki-67 was stained in nuclei above the two basal cell layers in at least three clusters composed of at least 5 cells, the sample was considered as positive suprabasal expression.

Statistical analysis

Staining patterns of different groups were compared using SPSS 12.0 software with Pearson's chi-square and Fisher's exact test. Outcome values were considered significantly different when p value was less than 0.05.

Results

the overall clinical characteristics of all groups (A-D) are summarized in Table 1. Non-smoking and non-drinking patients with HNSCC (groups A and B) were predominantly female and older, whereas smoking and drinking patients with HNSCC were predominantly male and younger. In both groups tumors mostly occurred in the oral cavity.

Table 1. Overall population description

Groups	**A**	**B**	**C**	**D**
Tobacco & alcohol consumption	NSND	NSND	SD	SD
No. of tumors	multiple	single	multiple	single
Total no.	18	15	15	14
Gender				
Male	2	1	11	9
Female	16	14	4	5
Age (yrs)				
Range	37-92	52-89	41-85	40-68
Median	76	80	58	55
Mean	72	75	60	55
Site primary tumor				
Oral Cavity	17	15	15	12
Oropharynx	0	0	0	2
Larynx	1	0	0	0
Distribution of multiple tumors				
SPT	18 oral cavity		8 oral cavity	
			2 oropharynx	
			5 hypopharynx	
3rd PT	5 oral cavity		3 oral cavity	
	1oropharynx		3 oropharynx	
			4 hypopharynx	
4th PT	2 oral cavity		2 oral cavity	
	1 oropharynx		3 oropharynx	
			3 hypopharynx	
5th PT	3 oral cavity		2 oral cavity	
6th PT	1 oral cavity		2 oral cavity	
7th PT	0		2 oral cavity	
8th PT	0		2 oral cavity	

NSND= Non-smoking and non-drinking, SD= smoking >20 cigarettes/day & drinking >5 alcohol consumptions /day, SPT= second primary tumor, PT= primary tumor

p53 staining pattern (figures 1A and 1B)

p53 staining pattern for all groups is summarized in Table 2. Tumor-adjacent mucosa from non-smoking and non-drinking patients with HNSCC showed dispersed single

Table 2. p53 and Ki-67 expression pattern for all groups

Groups	p53 expression pattern					
	Negative	(%)	Dispersed expression (single cell)	(%)	Focal overexpression (clusters)	(%)
A: TAM, NSND, multiple HNSCC	8	44%	1	6%	9	50%
B: TAM, NSND, single HNSCC	7	47%	3	20%	5	33%
C: TAM, SD, multiple HNSCC[a]	3	20%	6	40%	6	40%
D: TAM, SD, single HNSCC[a]	1	7%	2	14%	11	79%

cell p53 expression (single HNSCC 20% and multiple HNSCC 6%) and focally clusters overexpressing p53 (single HNSCC 33% and multiple HNSCC 50%). However tumor-adjacent mucosa from smoking and drinking patients with HNSCC had a significantly higher p53 staining pattern (A+B versus C+D, $p=0.02$, see footnote Table 2).

In tumor-adjacent mucosa from non-smoking and non-drinking patients, the expression of p53 was higher for patients with multiple tumors compared to patients with a single tumor; however this difference was not significant (A versus B). In smoking and drinking patients p53 expression was non-significantly higher in patients with a single tumor than in patients with multiple tumors (D versus C). p53 expression pattern was not significantly affected by the number of tumors present, regardless of the smoking and drinking habits of the patients (A+C versus B+D).

Ki-67 staining pattern (figures 1C and 1D)

Ki-67 staining pattern was not significantly different in tumor-adjacent mucosa from non-smoking and non-drinking patients with HNSCC compared to their smoking and drinking counterparts. This biomarker expression was also not significantly affected by the number of tumors present, regardless of the smoking and drinking habits of the patients (A+C versus B+D). Furthermore, we found no significant difference between groups with multiple and single tumor when considering the tobacco and alcohol usage; when comparing group A to B and group C to D.

Discussion

the development of multiple primary tumors adversely influences the prognosis of patients with HNSCC. It is one of the main reasons for lack of improvement in survival rates for these patients [23]. Different field cancerization theories have been postulated to explain the pathogenesis of multiple HNSCC. A continuous exposure to carcinogenic factors such as tobacco and alcohol may lead to premalignant lesions

Ki-67 expression pattern			
Negative	**(%)**	**Suprabasal expression**	**(%)**
14	88%	4	22%
13	87%	2	13%
11	73%	4	27%
11	79%	3	21%

TAM= Tumor-Adjacent Mucosa; NSND= Non-smoking and non-drinking, SD= smoking (>20 cigarettes/day) & drinking (>5 alcohol consumptions /day); HNSCC= Head and Neck Squamous Cell Carcinoma, [a]The SD groups have a significantly higher p53 expression pattern than the NSND groups: A+B (15 negative, 4 dispersed, 14 clusters) versus C+D (4 negative, 8 dispersed, 17 clusters) using Pearson's chi-square p =0.02

and ultimately to new primary tumors [8]. We remarkably found that patients with HNSCC without exposure to these substances have the same tendency to develop multiple tumors [6]. To gain insight in the pathogenesis of HNSCC in non-smoking and non-drinking patients, we compared the expression patterns of p53 and Ki-67 in tumor-adjacent mucosa in patients who did or did not smoke and drink and in patients with single or multiple tumors. In this way, we aimed to determine whether the expression of these biomarkers is correlated with a history of smoking and drinking and with the development of multiple tumors.

We found a significantly higher positivity for dispersed single cells and clusters of p53 in tumor-adjacent mucosa in smoking and drinking patients compared to non-smoking and non-drinking with HNSCC. This finding supports the relationship between tobacco and p53 overexpression, as reported in other studies [12,15,24]. However, we found no significant correlation between p53 expression and the number of tumors neither in smoking and drinking patients nor in non-smoking and non-drinking patients with HNSCC; in fact, we found that p53 expression was highest in tumor-adjacent mucosa from smoking and drinking patients with a single HNSCC. Hence, our hypothesis regarding an association between p53 expression and tobacco and alcohol usage was confirmed, however that this expression would be highest in smoking and drinking patients with multiple HNSCC was not confirmed.

The predictive value of p53 expression in tumor-adjacent mucosa for an increased risk of multiple HNSCCs is subject to debate. Ogden et al. found no predictive value of p53 overexpression in tumor-adjacent mucosa for second primary tumor after a 5-year follow-up [25], whereas Cruz et al. reported an association between suprabasal p53 expression and risk for progression and malignant transformation of premalignant oral lesions [13, 26]. This discrepancy in results may be due to differences in the examined mucosal samples, e.g. unlike Cruz et al. we did not take dysplastic lesions into account.

Whether the expression of p53 in tumor-adjacent mucosa from smoking and drinking patients with HNSCC represents overexpression of wild-type p53 due to genotoxic stress caused by chronic exposure to tobacco or the overexpression of mutated p53 can not be determined with certainty without sequence analysis of DNA or RNA isolated from the p53 positive cells.

Unpublished observations in our research lab by Tilanus et al. showed that p53 positive clusters may show mutations that are different from adjacent tumor which indicates the latter possibility, overexpression of mutated protein. However whether this applies in all clusters is debatable. In any event, p53 overexpression in smoking and drinking patients with HNSCC can be related either to the wild-type expression due to genotoxic stress or mutant due to genetic damage.

The reason for occasional p53 expression in tumor-adjacent mucosa in non-smoking and non-drinking patients with HNSCC is presently completely unclear. Unknown etiologic factors may either lead to wild-type expression or cause mutations resulting in expression of the mutant type protein.

We found no significant relation between Ki-67 expression and a history of smoking and drinking or single or multiple tumors. Tabor et al. found a correlation between the extent of Ki-67 positivity and the presence of loss of heterozygosity in dysplastic lesions of the oropharynx and concluded that Ki-67 may be a good predictor of cancer risk [20]. This disagreement may be because we investigated histologically normal tumor-adjacent mucosa, whereas Tabor et al. analyzed mucosa with different degrees of dysplasia. The predictive value of Ki-67 expression could be more pronounced in these dysplastic mucosal linings. Van Oijen et al. found a higher Ki-67 expression when investigating the number of proliferating cells in relation to tobacco consumption, however they did not take alcohol consumption into account [17].

In conclusion, our findings do not suggest an association between multiple HNSCCs and the level of expression of p53 and Ki-67 neither in smoking and drinking nor in non-smoking and non-drinking patients. This makes the predictive value of p53- and Ki-67 expression in tumor-adjacent mucosa for multiple HNSCC a subject to debate. Moreover, this lack of relationship between expression of p53 and Ki-67 in tumor-adjacent mucosa and subsequent tumor development in these tissues could indicate that these changes are not instrumental in causing recurrent tumors but just epiphenomenal. Additional research on other biomarkers or ploidy analysis should be performed to understand the carcinogenic pathway of HNSCC in non-smoking and non-drinking patients and especially why these patients have the same rate of second primary tumor disease as their smoking and drinking counterparts,

even without increased p53 or Ki-67 expression in the mucosa from which second primary tumors could originate.

Reference List

(1) Blot WJ, McLaughlin JK, Winn DM, et al. Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res.* 1988 Jun 1;48(11):3282-3287.

(2) La Vecchia C., Tavani A, Franceschi S, Levi F, Corrao G, Negri E. Epidemiology and prevention of oral cancer. *Oral Oncol.* 1997 Sep;33(5):302-312.

(3) Muir C, Weiland L. Upper aerodigestive tract cancers. *Cancer.* 1995 Jan 1;75(1 Suppl):147-153.

(4) Agudelo D, Quer M, Leon X, Diez S, Burgues J. Laryngeal carcinoma in patients without a history of tobacco and alcohol use. *Head Neck.* 1997 May;19(3):200-204.

(5) Constantinides MS, Rothstein SG, Persky MS. Squamous cell carcinoma in older patients without risk factors. *Otolaryngol Head Neck Surg.* 1992 Mar;106(3):275-277.

(6) Farshadpour F, Hordijk GJ, Koole R, Slootweg PJ. Non-smoking and non-drinking patients with head and neck squamous cell carcinoma: a distinct population. *Oral Dis.* 2007 Mar;13(2):239-243.

(7) Wiseman SM, Swede H, Stoler DL, et al. Squamous cell carcinoma of the head and neck in nonsmokers and nondrinkers: an analysis of clinicopathologic characteristics and treatment outcomes. *Ann Surg Oncol.* 2003 Jun;10(5):551-557.

(8) Tabor MP, Brakenhoff RH, van Houten VM, et al. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. *Clin Cancer Res.* 2001 Jun;7(6):1523-1532.

(9) Boyle JO, Hakim J, Koch W, et al. The incidence of p53 mutations increases with progression of head and neck cancer. *Cancer Res.* 1993 Oct 1;53(19):4477-4480.

(10) Kropveld A, Rozemuller EH, Leppers FG, et al. Sequencing analysis of RNA and DNA of exons 1 through 11 shows p53 gene alterations to be present in almost 100% of head and neck squamous cell cancers. *Lab Invest.* 1999 Mar;79(3):347-353.

(11) van Houten VM, Tabor MP, van den Brekel MW, et al. Mutated p53 as a molecular marker for the diagnosis of head and neck cancer. *J Pathol.* 2002 Dec;198(4):476-486.

(12) Brennan JA, Boyle JO, Koch WM, et al. Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. *N Engl J Med.* 1995 Mar 16;332(11):712-717.

(13) Cruz IB, Meijer CJ, Snijders PJ, Snow GB, Walboomers JM, van der Waal, I. p53 immunoexpression in non-malignant oral mucosa adjacent to oral squamous cell carcinoma: potential consequences for clinical management. *J Pathol.* 2000 Jun;191(2):132-137.

(14) Gonzalez-Moles MA, Galindo P, Gutierrez J, Rodriguez-Archilla A, Ruiz-Avila I, Sanchez-Fernandez E. Significance of p53 expression in non-tumoral epithelium adjacent to oral squamous cell carcinomas. *J Laryngol Otol.* 2002 May;116(5):355-358.

(15) van Oijen MG, van de Craats JG, Slootweg PJ. p53 overexpression in oral mucosa in relation to smoking. *J Pathol.* 1999 Mar;187(4):469-474.

(16) Roland NJ, Caslin AW, Bowie GL, Jones AS. Has the cellular proliferation marker Ki67 any clinical relevance in squamous cell carcinoma of the head and neck? *Clin Otolaryngol Allied Sci.* 1994 Feb;19(1):13-18.

(17) van Oijen MG, Gilsing MM, Rijksen G, Hordijk GJ, Slootweg PJ. Increased number of proliferating cells in oral epithelium from smokers and ex-smokers. *Oral Oncol.* 1998 Jul;34(4):297-303.

(18) Liu SC, Klein-Szanto AJ. Markers of proliferation in normal and leukoplakic oral epithelia. *Oral Oncol.* 2000 Mar;36(2):145-151.

(19) Macluskey M, Chandrachud LM, Pazouki S, et al. Apoptosis, proliferation, and angiogenesis in oral tissues. Possible relevance to tumour progression. *J Pathol.* 2000 Aug;191(4):368-375.

(20) Tabor MP, Braakhuis BJ, van der Wal JE, et al. Comparative molecular and histological grading of epithelial dysplasia of the oral cavity and the oropharynx. *J Pathol.* 2003 Mar;199(3):354-360.

(21) van Oijen MG, Medema RH, Slootweg PJ, Rijksen G. Positivity of the proliferation marker Ki-67 in noncycling cells. *Am J Clin Pathol.* 1998 Jul;110(1):24-31.

(22) Warren S, Gates O. Multiple primary malignant tumors. A survey of the literature and a statistical study. *Am J Cancer.* 1932;16:1358-1414.

(23) Vokes EE, Weichselbaum RR, Lippman SM, Hong WK. Head and neck cancer. *N Engl J Med.* 1993 Jan 21;328(3):184-194.

(24) Koch WM, Lango M, Sewell D, Zahurak M, Sidransky D. Head and neck cancer in nonsmokers: a distinct clinical and molecular entity. *Laryngoscope.* 1999 Oct;109(10):1544-1551.

(25) Ogden GR, Chisholm DM, Morris AM, Stevenson JH. Overexpression of p53 in normal oral mucosa of oral cancer patients does not necessarily predict further malignant disease. *J Pathol.* 1997 Jun;182(2):180-184.

(26) Cruz IB, Snijders PJ, Meijer CJ, et al. p53 expression above the basal cell layer in oral mucosa is an early event of malignant transformation and has predictive value for developing oral squamous cell carcinoma. *J Pathol.* 1998 Apr;184(4):360-368.

Chapter 5

Human papillomavirus and oropharyngeal squamous cell carcinoma: a case-case study regarding tobacco and alcohol consumption

F. Farshadpour
S. Konings
E.J.M. Speel
G.J. Hordijk
R. Koole
M. van Blokland
P.J. Slootweg
J.A. Kummer

Submitted

Abstract

Background. We aimed to determine the role of HPV in the pathogenesis and outcome of oropharyngeal squamous cell carcinoma (OSCC) in lifelong non-smoking and non-drinking patients with these tumors.

Methods. A case-case analysis was performed to compare presence of HPV-DNA in tumor cells of 16 non-smoking and non-drinking with 16 matched smoking and drinking patients (matching criteria: age at incidence, gender, tumor sublocation, tumor stage). HPV was detected using 2 PCR tests, FISH analysis and $p16^{INK4A}$ immunostaining.

Results. Non-smoking and non-drinking patients had more HPV-positive tumors than smoking and drinking patients (n=12; 75% versus n=2; 12.5%; $p<0.001$). All HPV-positive tumors showed $p16^{INK4A}$ overexpression, 1 HPV-negative tumor had $p16^{INK4A}$ overexpression, ($p<0.001$). Overall survival and disease-specific survival were higher for HPV-positive compared to HPV-negative cases ($p=0.027$, $p=0.039$, respectively).

Conclusions. HPV is strongly associated with OSCC of non-smoking and non-drinking patients. Specific diagnostic and therapeutic actions should be considered for these patients to achieve a better prognosis.

Introduction

The most important risk factors for developing head and neck squamous cell carcinoma in the Western countries are consumption of tobacco and alcohol[1]. However, there is a small population of non-smoking and non-drinking patients with head and neck squamous cell carcinoma, so other risk factors may be important[2]. Substantial evidence has shown that oncogenic human papillomavirus (HPV) which is the primary cause of uterine cervical cancer, is etiologically involved in the development of head and neck squamous cell carcinoma[3-10].It is estimated that up to 15-20% of all head and neck squamous cell carcinomas are associated with high risk HPV infection[3-10]. This prevalence varies broadly, depending on the sublocation of the tumor, the studied population, the detection method and the type of specimen used[4-10]. The highest rates of HPV-DNA (up to 70%) have been found in oropharynx squamous cell carcinomas (OSCCs), especially the tonsils. HPV type 16 has been detected in 90-95% of HPV-related OSCC, HPV-18 in some cases and HPV type 31, -33 and -35 in considerably less cases[7, 9-13].

In the pathogenesis of HPV-related cancer, integration of the viral genome into the cellular DNA and, as a result, upregulation of the viral oncoproteins E6 and E7 seem to be crucial events. These oncoproteins subsequently cause dysfunction of amongst others tumor suppressor proteins, p53 and pRb, respectively, leading to cell proliferation, impaired apoptosis and ultimately chromosome instability[14].

Immunohistochemical detection of $p16^{INK4A}$ overexpression, a product of tumor suppressor gene CDKN2A, has been associated with HPV-related head and neck squamous cell carcinoma and in some studies used as a surrogate biomarker for HPV detection[6, 15, 16]. Recent studies have characterized a subset of HPV-related OSCC in which $p16^{INK4A}$ overexpression predicts the presence of oncogenic HPV infection and identifies those with a better prognosis[17, 18]. Moreover, deletion of the CDKN2A locus together with functional inactivation of the tumor suppressor protein $p16^{INK4A}$ have been detected in head and neck squamous cell carcinoma without a relationship with HPV infection[19, 20].

HPV-positive head and neck squamous cell carcinomas are predominantly poorly differentiated and show a characteristic basaloid morphology in comparison with HPV-negative tumors[4, 5]. Furthermore, patients with HPV-positive tumors are less likely to consume large amounts of tobacco and alcohol[9, 15, 21, 22] and seem to have a better response to radiotherapy and a favorable survival rate[4, 11, 18, 23, 24]. So there are signs that these tumors form a separate entity within the heterogeneous group of head and neck squamous cell carcinomas.

The correct determination of HPV's involvement in the pathogenesis and prognosis of OSCC is dependent on several patient- and tumor-related co-factors, such as tobacco- and alcohol use, TNM-stage and treatment modality. Although most

investigators have found a trend between HPV and lesser amount of tobacco and alcohol use, the definitions of the used amounts are not always clear. Furthermore to date no matched analysis with smoking and drinking patients has been performed. In addition, previous studies have often used only one assay to determine the biological association of HPV infection with tumorigenesis.
In this study we aimed to determine the role of HPV in carcinogenesis and disease outcome for non-smoking and non-drinking patients with OSCC. Therefore, we performed a case-case study of a well defined population of 16 non-smoking and non-drinking and 16 matched, smoking and drinking patients with OSCC for the presence of HPV DNA and overexpression of biomarker $p16^{INK4A}$. The presence of HPV DNA was analysed using three different methods, i.e. fluorescence in situ hybridization (FISH) and two polymerase chain reaction (PCR)-based assays (Amplicor® and Linear Array® HPV detection kits).

Material and methods

Patients were selected from a database at the University Medical Center Utrecht, in which all patients with newly diagnosed head and neck squamous cell carcinoma are prospectively registered since 1980. This database contains information on patient characteristics, risk factors, tumor classification, treatment modalities and follow-up data including number of recurrences and subsequent primary tumors. Patients were classified as non-smoking and non-drinking, when they had no history of smoking tobacco and alcohol consumption. Patients were classified as smoking and drinking, when they actively smoked tobacco and consumed alcohol. Former smokers or drinkers were not included. All patients were treated according to institutional protocols, final decision was made in consultation with the patient. Follow-up time (in months) was considered from date of diagnosis (i.e. first proven biopsy) to date of death or date of last follow-up (January 1, 2009). Seventeen non-smoking and non-drinking patients with a primary head and neck squamous cell carcionma located in the oropharynx (ICD-codes 141.0, 145.3, 145.4, 146.0, 146.1, 146.2, 146.3, 146.6) were found in the database of which 16 were selected because of absence of tumor tissue in 1 case. These patients were matched with smoking and drinking patients on gender, age (+/- 5 years), sublocation of tumor and tumor stage. A case-case analysis was performed to compare the prevalence of HPV DNA and overexpression of $p16^{INK4A}$ in both groups.

Tissue specimens

32 formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks from either biopsy or surgical resection specimens were obtained. Two experienced head and neck pathologists (JAK, PS) examined H&E-stained slides to select the areas in which tumor cells were present and evaluated the morphological appearances. Both pathologists were blinded to the smoking and drinking status. Tumor grade was recorded as well, moderate, or poor according to the criteria of the World Health Organization[25]. In addition, tumors were assessed for the absence or presence of hyperkeratosis, vasoinvasive- and perineural growth and typical basaloid features, i.e. small, dark cells with scant cytoplasm, hyperchromatic nuclei, marked mitotic activity, a predominant lobular pattern of growth, and the absence of prominent keratinisation[26].

HPV analysis

DNA isolation and PCR analysis

For DNA extraction tumor areas from FFPE slides were isolated by microdissection. After deparaffinization, the tissue fragments were digested in 150 μl 50mM Tris/HCL (pH 8.0) 0,5% (v/v) Tween-20 with proteinase K (final concentration 2 mg/ml). After 1 hour incubation at 56°C the lysates were boiled to inactivate the proteinase K, and subsequently centrifuged. Supernatants were transferred into clean eppendorf tubes, and directly used for PCR. PCR was performed using the Amplicor® HPV Test kit (Amplicor HPV Amplification kit: 03610799 190, Amplicor HPV Detection kit: 03610799 190, Amplicor HPV Controls Kit: 03610756 190; Roche, Basel, Sz) as well as the Linear Array® HPV Genotyping Test (Linear Array HPV Genotyping Kit: 03378179 190, Linear Array HPV Detection Kit: 208693; Roche). Both tests were carried out according to the manufacturer's recommended protocol including positive and negative controls. The Amplicor® test is a qualitative in vitro test which uses amplification of target DNA by PCR and nucleic acid hybridization for the detection of high-risk HPV DNA genotypes (i.e., HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68). It uses primers to define a sequence of nucleotides within the L1 region of the HPV genome that is 150 base-pair (bp) long. This test also features a concurrent isolation and amplification of the human β-globin gene to assess DNA integrity for each tested specimen. The Linear Array® test uses the same detection technique, however it targets a HPV genome sequence of 450 bp and is able to detect high-risk (same types as mentioned above) as well as low-risk HPV-DNA (i.e., HPV types 6, 11, 40, 42, 43 and 44).

FISH

FISH was performed on 4μm-thick tissue sections as described previously[6, 15]. Briefly, sections were deparaffinized, pretreated with 85% formic acid/0.3% H_2O_2, 1 M NaSCN and 4 mg/ml pepsin in 0.02 M Hcl, post-fixed in 1% formaldehyde in PBS, dehydrated in an ethanol series and hybridized with a digoxigenin-labeled HPV 16-specific probe (PanPath, Amsterdam, The Netherlands) according to the manufacturer's instructions. After hybridization the preparations were washed stringently in 50% formamide, 2×SSC, pH 7.0 at 42°C (2 times 5 min). The probes were detected by application of mouse anti-digoxin (Sigma, St. Louis, MO), peroxidase-conjugated rabbit anti-mouse IgG and peroxidase-conjugated swine anti-rabbit IgG (both Dako; Glostrup, Dk), and visualized by a peroxidase reaction using rhodamin-labeled tyramide. Preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 4,6-diamidino-2-phenyl indole (DAPI; Sigma: 0.2 ug/ml). Microscope images were recorded with the Metasystems Image Pro System (black and white CCD camera; Sandhausen, Germany) mounted on top of a Leica DM-RE fluorescence microscope equipped with DAPI and rhodamin filters. Evaluation of nuclear hybridization signals was performed by two investigators (FF and EJMS) according to previously described criteria[15]: punctate and/or diffuse signals throughout the nucleus indicating integrated and episomal HPV DNA, respectively, and granular FISH pattern if >1 nuclear signals, varying significantly in size and intensity, were observed. Control hybridizations were performed as described previously[15].

Immunohistochemical detection of p16^{INK4A}

4μm-thick tissue sections were deparaffinized with xylene and rehydrated by serial ethanol dilutions. Endogenous peroxidase activity was blocked by incubation for 30 minutes with 0.3% (v/v) H_2O_2 in methanol followed by antigen retrieval by boiling in 0.01 M sodium citrate buffer pH 6 for 15 minutes in a microwave oven. Slides were then incubated with a p16^{INK4A}-specific primary mouse monoclonal antibody (Neomarkers, Fremont, USA), diluted 1:160 for one hour at room temperature followed by a secondary visualisation reagent for 45 minutes (Powervision Goat-anti-Mouse/Rabbit/Rat labelled with horseradish peroxidase, ImmunoLogic, ImmunoVision Technologies, Brisbane, USA). After each incubation step, slides were washed in phosphate-buffered saline containing 3% (w/v) BSA. Peroxidase activity was visualized by incubation with diaminobenzidine/ H_2O_2 and cell nuclei were counterstained with hematoxylin. All p16^{INK4A}-positive cases were assessed for nuclear and/or cytoplasmic staining pattern. The staining patterns were scored semi-quantitatively for the percentage of p16^{INK4A}-positive tumor cells. The sections were graded as positive (+) when at least 75% of the tumor cells showed p16^{INK4A}-

positivity and as negative (-) when no staining was visible. Only one case (Table 2) showed 25% $p16^{INK4A}$-positive tumor cells and was considered as ±.

Statistics

The association between HPV status and other variables was tested using Chi-square and Fisher's exact test. Disease-specific survival (i.e. death due to primary tumor, tumor recurrence or subsequent primary tumor) and overall survival (i.e., mortality due to all causes) were determined for HPV-positive and HPV-negative cases, non-smoking and non-drinking and smoking and drinking groups and for cases with and without $p16^{INK4A}$ overexpression using an univariate approach (i.e. Kaplan-Meier) method as patients were matched on possible confounding factors. Estimated survival curves were compared using log-rank test. A p value ≤ 0.05 was considered statistically significant.

Table 1. Basic characteristics of all cases

	Non-smoking and non-drinking	**Smoking and drinking**
	n	**n**
Gender		
Male	3	3
Female	13	13
Age at tumor incidence (years)		
Mean	64.8	63.0
Range	45-83	50-78
Tumor stage		
II	3	3
III	6	4*
IVA	7	9*
Year of initial diagnosis		
1982-1986	2	1
1987-1991	2	3
1992-1996	6	5
1997-2001	2	5
2002-2006	4	2
Tumor location (ICD-code)		
Base of tongue (141.0)	6	6
Tonsil (146.0)	5	5
Tonsillar fossa (146.1)	3	3
Vallecula (146.3)	2	2

*Best possible match for 2 cases was stage IVA instead of III

Results

Sixteen non-smoking and non-drinking patients with OSCC were matched with 16 smoking and drinking patients according to the above mentioned criteria. The smoking and drinking patients used the following amounts of tobacco and alcohol at the time of diagnosis: 2-4 units of alcohol/day (n=10), 5-9 units of alcohol/day (n=4) and >9 units of alcohol/day (n=2); ≤20 cigarettes/day (n=5) and >20 cigarettes/day (n=11). For 2 non-smoking and non-drinking patients the best possible match was disease stage IVA instead of III. The incidence dates ranged from 1980 to 2005. Table 1 summarizes the basic clinical characteristics of all cases.

HPV status for all cases was determined using two PCR-based test kits and FISH analysis (Table 2). The Amplicor® PCR test showed 12 HPV-positive and 15 HPV-negative cases and was in 5 cases inconclusive due to negative β-globin gene results. The Linear Array® PCR test showed 7 HPV-positive and 19 HPV-negative

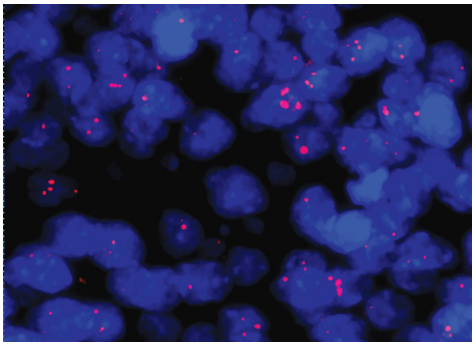

Figure 1a. HPV16-specific FISH analysis showing viral integration

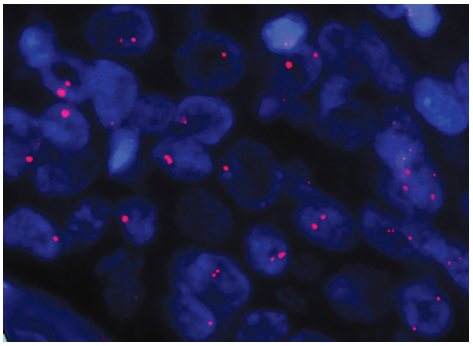

Figure 1b. HPV16-specific FISH analysis showing granular staining pattern

cases and 6 cases that were inconclusive due to negative β-globin gene results. The FISH analysis revealed 12 HPV 16-positive cases of which 1 with a very low signal intensity (4B), and 19 cases without a detectable signal and 1 case which was inconclusive due to insufficient tissue material. Eight of the FISH-positive cases showed punctate signals in the tumor cell nuclei indicating integrated HPV DNA and 4 showed granular nuclear staining (Figure 1). Based on these outcomes (see also discussion) we determined the HPV-status as follows: 12 of 16 non-smoking and non-drinking cases (75%) had a positive HPV status versus 2 of 16 smoking and drinking controls (12.5%, $p<0.001$, Tables 2, 3).

Immunohistochemical analysis for biomarker $p16^{INK4A}$ was detected as shown in Tables 2 and 3. $p16^{INK4A}$ overexpression (at least 75% of cells with positive staining) was found in 14 cases (44%), in 1 case (1B) 25% of cells stained positive (3%) and 17 cases (53%) were negative. All positive cases had strong nuclear as well as

Table 2. HPV and p16INK4A results of all cases

Case-case	p16INK4A overexpression	HPV			Final HPV-outcome
		PCR (Amplicor®)	PCR (Linear Array®)	FISH	
1A*	+	Present	HPV-33/52,33,35,58	Absent§	positive
1B†	±	Present	Absent	Absent	positive
2A	+	Present	Absent	Present	positive
2B	+	Present	HPV-16	Present	positive
3A	+	Present	HPV-16	Present	positive
3B	-	Absent	Absent	Absent	negative
4A	+	Present	HPV-16	Present	positive
4B	-	Absent	Absent	Present¥	negative
5A	+	Present	Absent	Present	positive
5B	-	Absent	Absent	Absent	negative
6A	+	Present	HPV-16	Present	positive
6B	-	Absent	Absent	Absent	negative
7A	+	Present	HPV-16	Present	positive
7B	-	Absent	Absent	Absent	negative
8A	+	Present	HPV-16	Present	positive
8B	-	Absent	Absent	Absent	negative
9A	+	NO‡	NO‡	Present	positive
9B	-	Absent	Absent	Absent	negative
10A	+	Present	NO‡	NO‡	positive
10B	-	Absent	Absent	Absent	negative
11A	+	Present	NO‡	Present	positive
11B	-	Absent	Absent	Absent	negative
12A	+	NO‡	NO‡	Present	positive
12B	-	NO‡	NO‡	Absent	negative
13A	-	Absent	Absent	Absent	negative
13B	-	Absent	Absent	Absent	negative
14A	-	Absent	Absent	Absent	negative
14B	-	Absent	Absent	Absent	negative
15A	-	Absent	Absent	Absent	negative
15B	-	Absent	Absent	Absent	negative
16A	-	NO‡	NO‡	Absent	negative
16B	+	NO‡	Absent	Absent	negative

*A: Non-smoking and non-drinking
†B: Smoking and drinking
‡Not Obtained (for PCR tests for example due to a negative β-globin PCR)
§HPV-16 specific FISH-probe
¥poor signal

cytoplasmic staining except case 16B which showed predominantly cytoplasmatic staining. All HPV-positive cases had $p16^{INK4A}$ overexpression whereas 17 of 18 HPV-negative cases had no detectable $p16^{INK4A}$, ($p<0.001$, Table 3).

Table 3. Characteristics of all cases according to HPV-status

Variable	HPV n (%)		p-value
	Positive (n=14)	Negative (n=18)	
Tobacco and Alcohol			<0.001
Non-smoking and non-drinking	12 (86)	4 (22)	
Smoking and drinking	2 (14)	14 (78)	
$p16^{INK4A}$ overexpression			<0.001
+	13 (93)	1 (6)	
-	0	17 (94)	
±	1 (7)	0	
Tumor location (ICD-code)			NS*
Base of tongue (141.0)	4 (29)	8 (45)	
Tonsil (146.0)	4 (29)	6 (33)	
Tonsillar fossa (146.1)	4 (29)	2 (11)	
Vallecula (146.3)	2 (15)	2 (11)	
Tumor			NS
T1	3 (21)	1 (6)	
T2	6 (43)	7 (39)	
T3	3 (21)	6 (33)	
T4	2 (15)	4 (22)	
			NS
T1-T2	9 (65)	8 (45)	
T3-T4	5 (35)	10 (55)	
Nodal involvement			NS
N0	4 (29)	7 (39)	
N1	3 (21)	4 (22)	
N2	7 (50)	7 (39)	
Stage			NS
II	2 (15)	4 (22)	
III	5 (35)	5 (28)	
IVA	7 (50)	9 (50)	
Year of initial diagnosis			NS
1982-1986	1 (7)	2 (11)	
1987-1991	2 (15)	3 (17)	
1992-1996	6 (43)	5 (28)	
1997-2001	2 (15)	5 (28)	
2002-2006	3 (21)	3 (17)	
Treatment modality			NS
Radiotherapy	6 (43)	6 (33)	
Chemotherapy + Radiotherapy	2 (15)	0	
Surgery + Radiotherapy	5 (35)	9 (50)	
Surgery	0	2 (11)	
Chemotherapy	0	1 (6)	
Supportive	1 (7)	0	

Table 3. *Continued*

Variable	HPV n (%) Positive (n=14)	Negative (n=18)	p-value
Tumor grade			NS
Moderate	7 (50)	4 (22)	
Poor	7 (50)	14 (78)	
Perineural growth			NS
Yes	4 (29)	2 (11)	
No	10 (71)	16 (89)	
Vasoinvasive growth			NS
Yes	3 (21)	1 (6)	
No	11 (79)	17 (94)	
Keratinization			0.025
Yes	3 (21)	11 (61)	
No	11 (79)	7 (39)	
Basaloid features			0.039
Yes	9 (65)	5 (28)	
No	5 (35)	13 (78)	
Tumor recurrence			NS
Yes	3 (21)	3 (17)	
No	11 (79)	15 (83)	
Second primary tumor			NS
Yes	1 (7)	4 (22)	
No	13 (93)	14 (78)	

*Non-significant

The associations between HPV status and tumor subsite, T- or N-classification, tumor stage, year of initial diagnosis, treatment and vasoinvasive- and perineural growth were not significant (Table 3). In contrast HPV-positive tumors showed significantly less often keratinisation (p=0.025) and more often basaloid features (p=0.039, Table 3). Tumor recurrence was found in 3 HPV-positive (2 locoregional and 1 distant) and 3 HPV-negative cases (all locoregional) and in 2 non-smoking and non-drinking and 4 smoking and drinking patients. Second primary tumor was found in 1 HPV-positive (in the oral cavity) and 4 HPV-negative cases (1 oral cavity, 3 oropharynx and 1 lung) and in1 non-smoking and non-drinking patient and 5 smoking and drinking patients (Table 3, no significant correlations).

Survival data

Follow-up time ranged from 5.9 to 182.1 months. Median follow-up time was 61.1 months. The 5-year overall and disease-specific survival for all cases was 53% and 64%, respectively. Cause of death in 20 deceased patients was as follows: due to

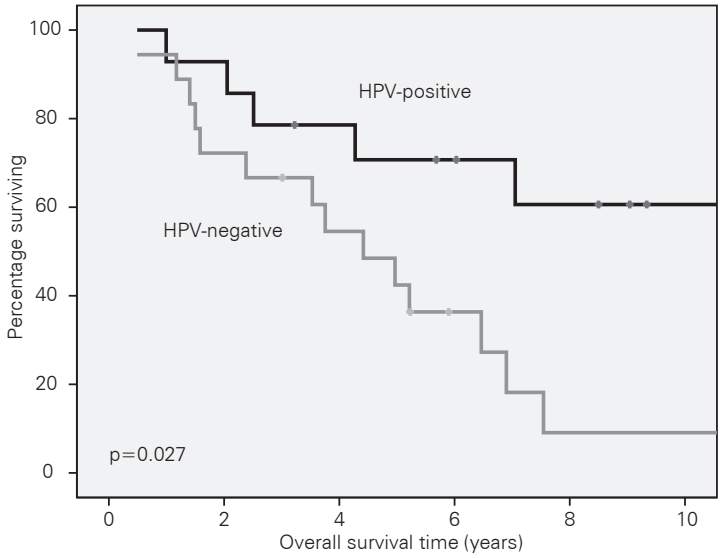

Figure 2a. Overall survival for HPV-positive compared to HPV-negative cases

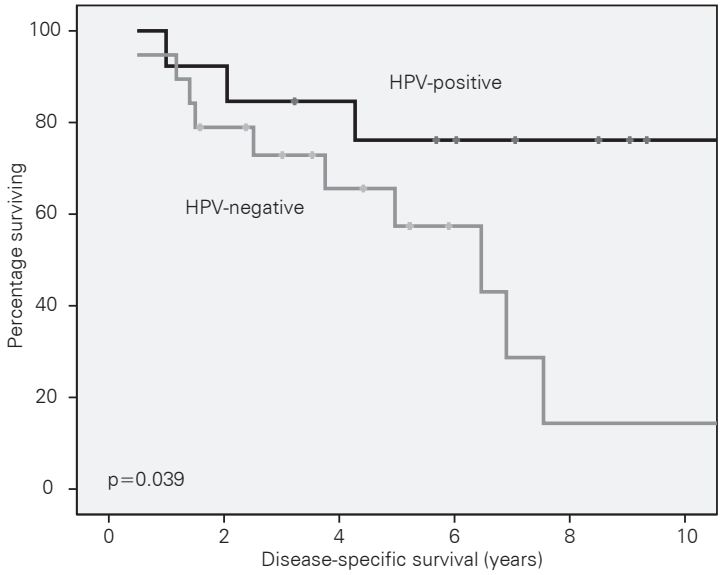

Figure 2b. Disease-specific survival for HPV-positive compared to HPV-negative cases

primary tumor (n = 4; 1 non-smoking and non-drinking, 3 smoking and drinking), other causes (n = 7; 4 non-smoking and non-drinking, 3 smoking and drinking) of which 5 cardial and 2 pulmonary disease, recurrent disease (n = 6; 2 non-smoking

and non-drinking, 4 smoking and drinking) and second primary tumor (n = 3 smoking and drinking). For HPV-positive and HPV-negative cases the 5-year overall survival was 71% and 42% and 5-year disease-specific survival was 76% and 57%, respectively. Overall and disease-specific survival were both significantly higher for HPV-positive compared to HPV-negative cases (p=0.027, p=0.039, respectively, Figure 2), for non-smoking and non-drinking patients compared to the smoking and drinking counterparts (p=0.037, p=0.013, respectively) and for cases with $p16^{INK4A}$ overexpression compared to those without detectable $p16^{INK4A}$ overexpression (p=0.028, p=0.030, respectively).

Discussion

To date this study is the first that analyses the role of HPV in the pathogenesis and clinical behavior of OSCC in non-smoking and non-drinking patients in comparison with matched smoking and drinking patients. HPV was strongly associated with OSCC in the absence of tobacco and alcohol use. HPV was found in 75% of the non-smoking and non-drinking patients compared to 12.5% of the smoking and drinking patients. Our results are consistent with other studies, although they mostly have shown this association separately in a group of nonsmokers or in a group of nondrinkers. Lindel et al. found HPV in 62 percent of nonsmokers and 38 percent of nondrinkers with oropharyngeal tumors[9]. Tachezy et al. demonstrated HPV-positive oropharynx and oral cavity tumors in all nonsmokers and 69% of nondrinkers, and in a recent study non-smoking and non-drinking patients with OSCC were reported to be 6.1 times more likely to be infected with high risk HPV[22, 27]. Increasing evidence shows a particular risk factor profile for HPV-related head and neck squamous cell carcinoma with not only less consumption of tobacco and alcohol but also a different sexual behavior and higher use of marijuana in mostly younger patients (<55 years[28]) compared to non HPV-associated head and neck squamous cell carcinoma[21, 28, 29]. We do not have patient data regarding sexual behavior and use of drugs in our studied population.

Additional characteristics of our studied HPV-positive tumors included the presence of basaloid features and lack of keratinisation which has been reported by previous studies[4, 5]. Likewise in this study as well as numerous other studies HPV-related tumors proved to be associated with not only a better overall survival but also a better disease-specific survival[4, 9, 16, 23, 24]. The underlying mechanism for this prognostic effect of HPV is unclear. Although only one HPV-positive case had a second primary tumor compared to three HPV-negative cases, this difference was not significant. Also no correlation was found between recurrent disease or different treatment modalities and HPV-positivity. Nevertheless a better response on treatment like

an increased sensitivity for radiotherapy possibly due to remaining amounts of p53 function in HPV-associated tumors might also explain the favorable prognosis. So it seems important to recognize patients with HPV-related head and neck squamous cell carcinoma to customize therapeutic decisions. Moreover, combination of HPV with recently identified prognostic indicators such as loss of chromosome 16q and the presence of $p21^{CIP1/WAF1}$ or nuclear survivin expression, holds further promise to select patients for this purpose[19].

We also found better overall and disease-specific survival for non-smoking and non-drinking cases and those with $p16^{INK4A}$ overexpression compared to their counterparts. We consider these results to be related to HPV-positivity. In another recent study by our studygroup regarding disease outcome for all head and neck squamous cell carcinoma in our center, we found no difference in survival between those who smoke and drank and those who did not[30].

Some controversy exists concerning the most reliable way to determine biologically relevant HPV infection in FFPE tissue. Therefore, it has been proposed to use at least more than one method to identify a firm association of the virus with the tumor cells. Most studies agree upon the use of the surrogate marker $p16^{INK4A}$ followed by a HPV-specific test, such as HPV DNA PCR[16, 18], HPV E6 RT-PCR[17] or HPV FISH[15, 31]. We used four methods to detect the HPV-status, i.e. $p16^{INK4A}$ -immunostaining, PCR using two different test kits, and FISH analysis, which strongly correlated with each other. In 4 cases (9A, 12A, 12B, 16A) the β-globin gene could not be amplified by both PCR tests, hence the FISH data were used to determine the HPV-status, which corresponded with the presence of $p16^{INK4A}$ overexpression in case of HPV-positivity. Nevertheless, also some discrepancies were found between the different tests used. In cases 5A and 2A the Amplicor® test was positive for HPV, whereas the Linear Array® test was negative, probably due to the large fragments that need to be amplified in the latter assay. As a consequence, the Amplicor® and the FISH results were used to proof HPV-positivity for these cases. In case 1A PCR revealed the presence of HPV DNA of types 33/52, 33, 35, 58 with corresponding $p16^{INK4A}$ overexpression, which explains the negative outcome of the HPV type 16-specific FISH analysis. Only in cases 1B and 4B FISH analysis did not correlate with PCR and $p16^{INK4A}$ immunostaining, and in these cases we decided to consider a positive $p16^{INK4A}$ and PCR status as signs for HPV-positivity. However, the opposite may also be true as one considers the very high sensitivity of HPV DNA PCR, which may lead to false-positive results[32], as well as the fact that $p16^{INK4A}$ can be overexpressed without the presence of HPV e.g., case 16B and a study by Hafkamp et al[15]. On the other hand the $p16^{INK4A}$ staining pattern in case 16B was purely cytoplasmic in contrast to the other $p16^{INK4A}$ -positive cases in which cytoplasmic and nuclear pattern was seen. This may point to other reasons than HPV for upregulation of this

biomarker. Furthermore, the results as mentioned in Table 3 and survival curves would not be affected by opposite results of cases 1B and 4B.

We conclude that HPV is strongly associated with oropharyngeal tumors, especially in lifelong nonsmokers and nondrinkers. With better and more valid detection techniques it is likely that these patients will be recognized as a specific entity within the heterogeneous group of head and neck cancer. Diagnostic and therapeutic actions will then be more focussed on this distinct group and may lead to better prognosis.

Acknowledgments

The authors thank A. Haesevoets (Department of Molecular Cell Biology, Maastricht University Medical Center, The Netherlands) and J.M.S.A.A. Straetmans (Department of Otorhinolaryngology, Maastricht University Medical Center, The Netherlands) for outstanding technical support of the FISH experiments.

References

1. Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. N.Engl.J.Med. 2001;345:1890-1900.
2. Farshadpour F, Hordijk GJ, Koole R, Slootweg PJ. Non-smoking and non-drinking patients with head and neck squamous cell carcinoma: a distinct population. Oral Dis. 2007;13:239-243.
3. D'Souza G, Kreimer AR, Viscidi R, et al. Case-control study of human papillomavirus and oropharyngeal cancer. N.Engl.J.Med. 2007;356:1944-1956.
4. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J.Natl.Cancer Inst. 2000;92:709-720.
5. Hafkamp HC, Manni JJ, Speel EJ. Role of human papillomavirus in the development of head and neck squamous cell carcinomas. Acta Otolaryngol. 2004;124:520-526.
6. Hafkamp HC, Speel EJ, Haesevoets A, et al. A subset of head and neck squamous cell carcinomas exhibits integration of HPV 16/18 DNA and overexpression of p16INK4A and p53 in the absence of mutations in p53 exons 5-8. Int.J.Cancer 2003;107:394-400.
7. Herrero R, Castellsague X, Pawlita M, et al. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. J.Natl.Cancer Inst. 2003;95:1772-1783.
8. Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol. Biomarkers Prev. 2005;14:467-475.
9. Lindel K, Beer KT, Laissue J, Greiner RH, Aebersold DM. Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. Cancer 2001;92:805-813.
10. Strome SE, Savva A, Brissett AE, et al. Squamous cell carcinoma of the tonsils: a molecular analysis of HPV associations. Clin.Cancer Res. 2002;8:1093-1100.
11. Fakhry C, Gillison ML. Clinical implications of human papillomavirus in head and neck cancers. J.Clin.Oncol. 2006;24:2606-2611.
12. Klussmann JP, Weissenborn SJ, Wieland U, et al. Prevalence, distribution, and viral load of human papillomavirus 16 DNA in tonsillar carcinomas. Cancer 2001;92:2875-2884.
13. Mork J, Lie AK, Glattre E, et al. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. N.Engl.J.Med. 2001;344:1125-1131.
14. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nat. Rev.Cancer 2002;2:342-350.
15. Hafkamp HC, Manni JJ, Haesevoets A, et al. Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas. Int.J.Cancer 2008;122:2656-2664.
16. Klussmann JP, Gultekin E, Weissenborn SJ, et al. Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. Am.J.Pathol. 2003;162:747-753.
17. Smeets SJ, Hesselink AT, Speel EJ, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int.J.Cancer 2007;121:2465-2472.

18. Weinberger PM, Yu Z, Haffty BG, et al. Prognostic significance of p16 protein levels in oropharyngeal squamous cell cancer. Clin.Cancer Res. 2004;10:5684-5691.

19. Klussmann JP, Mooren JJ, Lehnen M, et al. Genetic Signatures of HPV-related and Unrelated Oropharyngeal Carcinoma and Their Prognostic Implications. Clin.Cancer Res. 2009;15:1779-1786.

20. O'Regan EM, Toner ME, Smyth PC, et al. Distinct array comparative genomic hybridization profiles in oral squamous cell carcinoma occurring in young patients. Head Neck 2006;28:330-338.

21. Gillison ML, D'Souza G, Westra W, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J.Natl.Cancer Inst. 2008;100:407-420.

22. Tachezy R, Klozar J, Salakova M, et al. HPV and other risk factors of oral cavity/oropharyngeal cancer in the Czech Republic. Oral Dis. 2005;11:181-185.

23. Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. J.Clin.Oncol. 2009;27:1992-1998.

24. Ritchie JM, Smith EM, Summersgill KF, et al. Human papillomavirus infection as a prognostic factor in carcinomas of the oral cavity and oropharynx. Int.J.Cancer 2003;104:336-344.

25. Barnes L, Everson JW, Reichart P, Sidransky D. Pathology and genetics of head and neck tumours (WHO classification of tumours): IARC press Lyon; 2005.

26. Wain SL, Kier R, Vollmer RT, Bossen EH. Basaloid-squamous carcinoma of the tongue, hypopharynx, and larynx: report of 10 cases. Hum.Pathol. 1986;17:1158-1166.

27. Andrews E, Seaman WT, Webster-Cyriaque J. Oropharyngeal carcinoma in non-smokers and non-drinkers: A role for HPV. Oral Oncol. 2009;45:486-491.

28. Smith EM, Ritchie JM, Summersgill KF, et al. Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. Int.J.Cancer 2004;108:766-772.

29. Chaturvedi AK, Engels EA, Anderson WF, Gillison ML. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. J.Clin.Oncol. 2008;26:612-619.

30. Farshadpour F, Kranenborg H, Calkoen EV, et al. Survival analysis of head and neck squamous cell carcinoma: Influence of smoking and drinking. Head Neck 2010.

31. Begum S, Cao D, Gillison M, Zahurak M, Westra WH. Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma. Clin.Cancer Res. 2005;11:5694-5699.

32. van Houten V, Snijders PJ, van den Brekel MW, et al. Biological evidence that human papillomaviruses are etiologically involved in a subgroup of head and neck squamous cell carcinomas. Int.J.Cancer 2001;93:232-235.

Chapter 6

Microarray analysis identifies a different gene expression profile for non-smoking and non-drinking patients with head and neck squamous cell carcinoma, a preliminary report

F. Farshadpour
P. Roepman
G.J. Hordijk
R. Koole
P.J. Slootweg

Submitted

Abstract

Background. A small subset of patients with head and neck squamous cell carcinoma (HNSCC) are non-smoking and non-drinking and have distinct clinical chracteristics. We aimed to identify a possible different genetic profile for these patients when compared to their smoking and drinking counterparts.

Methods. The gene expression data previously detected from primary tumors located in the oral cavity and oropharynx, using DNA microarray were analyzed for their differential expression between non-smoking and non-drinking patients (n=15) and smoking and drinking patients (n=89). Student's T-test ($p<0.05$) and 10-fold cross-validation procedure (100 times repeated) were performed to determine differentially expressed genes.

Results. Non-smoking and non-drinking patients were older, mostly female and had oral cavity localised tumors whereas smoking and drinking patients were younger male patients with 81% oral cavity and 19% oropharynx tumors. A set of 49 differentially expressed genes were detected. Amongst others, 7 genes related to Interferon-γ were downregulated and 2 genes linked to NFKB pathway were upregulated.

Conclusions. Differentially expressed genes in non-smoking and non-drinking patients with HNSCC possibly indicate the presence of a different cellular response to carcinogenic events in these patients. Further studies are warranted to validate this gene set and explore possible therapeutic implications to improve prognosis for these patients.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is highly associated to tobacco and alcohol use[1]; however, it is also diagnosed in a small group of non-smoking and non-drinking patients[2,3]. In previous studies we described the clinicopathological characteristics and disease outcome for these patients[3,4]. When compared to the smoking and drinking population with HNSCC, the non-smoking and non-drinking patients are more frequently female (3:1 female to male ratio), older at disease presentation (mean age 72.7 yrs), and have mainly oral cavity tumors. Whereas those with tobacco and alcohol use are mostly younger (mean age 63.5 yrs) male patients (female to male ratio 1:3) with laryngeal tumors. Furthermore, we found that the number of disease recurrence and second primary tumor (SPT) in both groups was similar; namely, 17.2% versus 21.7% disease recurrence, and 16.7% versus 18.4% SPT in the non-smoking and non-drinking and smoking and drinking patients, respectively. However, nearly all recurrences and SPTs in the non-smoking and non-drinking patients are located in the head and neck region; in contrast the smoking and drinking patients also develop distant recurrences and SPTs in the lung and esophagus. Moreover, we analyzed survival for both groups and found no significant difference in HNSCC-specific outcome. Hence, it seems that unknown mechanisms lead to development of HNSCC in a subgroup of patients without the most common risk factors. These tumors present differently compared to those from patients who use both tobacco and alcohol, which may be attributed to an underlying molecular and/or genetic profile. Research regarding this issue is scarce, but necessary to enable us to improve disease outcome for these patients.

In a previous study from our center a predictive gene expression signature for lymph node metastases was detected from primary tumors located in the oral cavity and oropharynx, using DNA microarray profiling[5]. The generated full-genome gene expression data was used in the current study to analyse whether non-smoking and non-drinking patients show unique gene expression pattens compared to the smoking and drinking population to confirm our hypothesis that this population's distinct clinical characteristics are based on different genetic profile compared to the smoking and drinking patients with HNSCC.

Material and methods

Tumor tissue samples

All patients with newly diagnosed HNSCC have been prospectively registered in a database of the Head and Neck Department at the University Medical Center

Utrecht in the Netherlands since 1980. This database contains information on patient characteristics (e.g., gender and age), risk factors, tumor classification, and mode of treatment. Smoking and drinking habits were recorded. Smoking was categorized as no smoking (i.e. no history of smoking), quit smoking >2 years, quit smoking >1 year, quit smoking <1 year, and as 0–20 cigarettes/day or 20–40 cigarettes/day, and alcohol consumption was categorized as no alcohol consumption (i.e. no history of alcohol consumption), no daily alcohol consumption, 1 consumption/day, 2–4 consumptions/day, 5–9 consumptions/day, >9 consumptions/day. 119 patients were selected randomly from this database, according to the inclusion criteria: biopsy- proven primary HNSCC located in the oropharynx and oral cavity with tumor samples with at least 50% tumor cells. Of these 119 tumors, 104 samples could be successfully analyzed on DNA microarrays and were included in the study. Risk factors of these 104 patients were reviewed from the database, 15 patients had no history of smoking of which 8 patients had no alcohol consumption and 7 no daily alcohol consumption. These patients were considered non smoking non drinking in the current study.

Microarray gene expression

Full-genome gene expression data was previsouly generated[5]. In brief, total RNA was isolated from 2-3 tumor sections (20 μm), DNase treated and amplified by in vitro transcription using T7 RNA polymerase and including 5-(3-aminoallyl)-UTP. Next, Cy3 or cy5 fluorophores were coupled to the generated cRNA and hybridized on custom-made oligonucleotide (70-mer) arrays against a common reference sample that consisted of a pool of HNSCC tumors RNAs. Microarrays were scanned and cy5 signals were quantified and normalized (lowess, VSN).

Analysis for determination of differential genes

Of the 21,329 genes on the microarray, 6221 were excluded based on aberrant signal and spot morphology. All remaining gene probes were analyzed for their differential expression between non-smoking and non-drinking patients (n=15) and smoking and drinking patients (n=89). Initally a Student's T- test was performed for each across both patient groups. This analysis resulted in a set of 116 genes that were found to be significantly different ($p < 0.05$). However due to the relative small and unbalanced group sizes, we can expect that a part of the identified differentially expressed genes are caused by a high false-discovery rate (FDR). For a more robust differential analysis that is likely to have a lower FDR we applied a 10-fold cross validation procedure in which both patient groups were more balanced in size. Within each cross-validation (CV) loop, thirteen randomly selected samples of the 15 non-smoking and non-drinking patients were compared to 26 randomly selected

samples of the 89 smoking and drinking patients and analyzed for the differentially expressed genes (Student's T-test $p<0.05$). This CV-procedure was repeated 100 times and resulted in a final set of 49 differentially expressed genes. These 49 genes were all part of the set of 116 initially found genes.

Results

Table 1 shows basic characteristics of all patients in both groups. Non-smoking and non-drinking patients were older, mostly female and had only oral cavity localised tumors whereas smoking and drinking patients were younger male patients with 81% oral cavity and 19% oropharynx tumors.

Using a 10-fold cross validation procedure (see methods for details) we have identified a set of 49 genes that were differentially expressed between the non-smoking and non-drinking patients (n=15) and smoking and drinking patients (n=89). Figure 1 and Table 2 show the 49 differentially expressed genes. Of the 49

Table 1. Baseline characteristics of all patients (n=104)

		NSND[1]		SD[2]	
		(n=15)	(%)	(n=89)	(%)
Age (yrs)	mean	69		60	
	range	40-87		37-83	
Age (categories)	36-45	1	6	6	7
	46-55	2	13	23	26
	56-65	2	13	41	46
	66-75	5	34	15	17
	≥ 76	5	34	4	4
Gender	Male	2	13	60	67
	Female	13	87	29	33
Tumor localisation	Oral cavity	15	100	72	81
	Oropharynx	0	0	17	19
T-classification	1	5	34	12	14
	2	6	40	34	38
	3	2	13	10	11
	4	2	13	33	37
N-classification	0	10	67	46	52
	1	5	33	28	31
	2	0	0	15	17
M-classification	0	15	100	89	100
	1	0	0	0	0

[1]Non-smoking and non-drinking, [2]Smoking and drinking

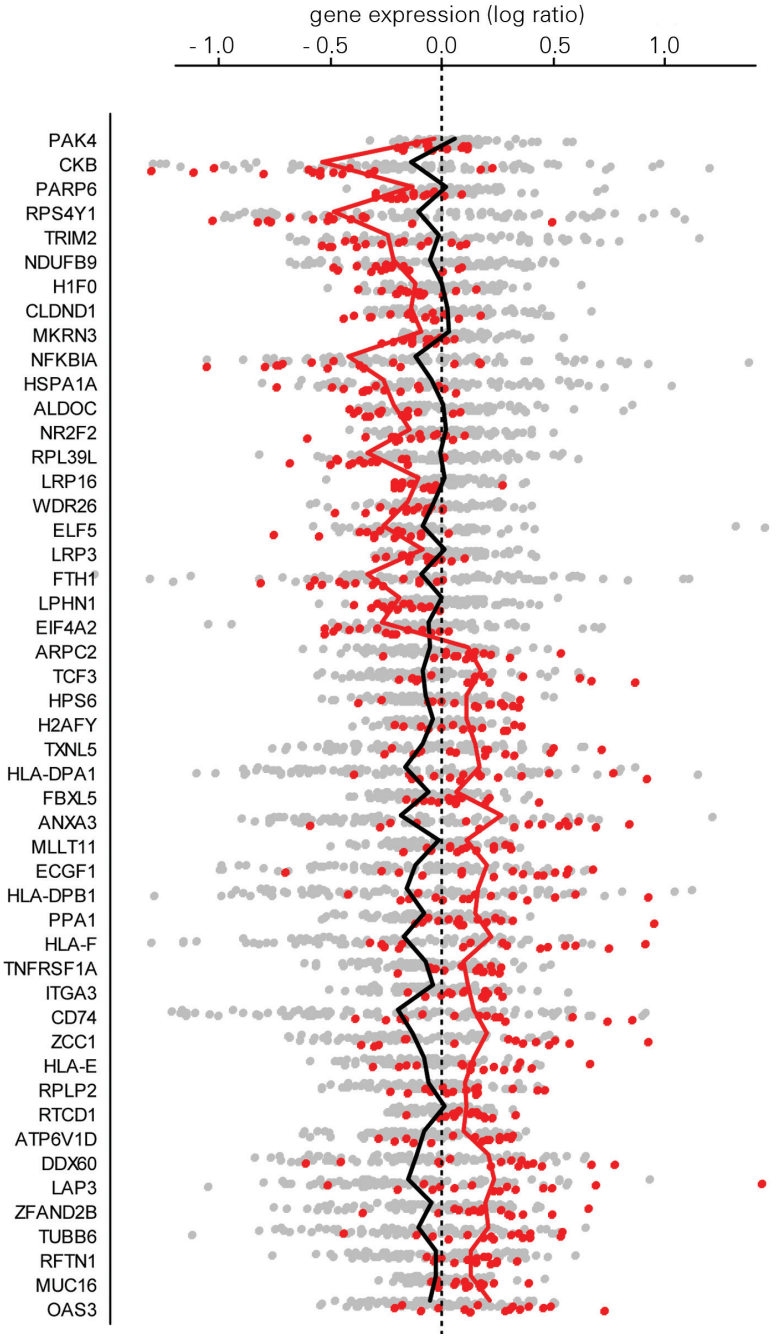

Figure 1. Differential gene expression (logratio) of the 49 genes for 15 non-smoking and non-drinking patients (red) and 89 smoking and drinking patients (gray). Genes are ordered according to table 2. Mean expression of each group is indicated by the solid red and black lines.

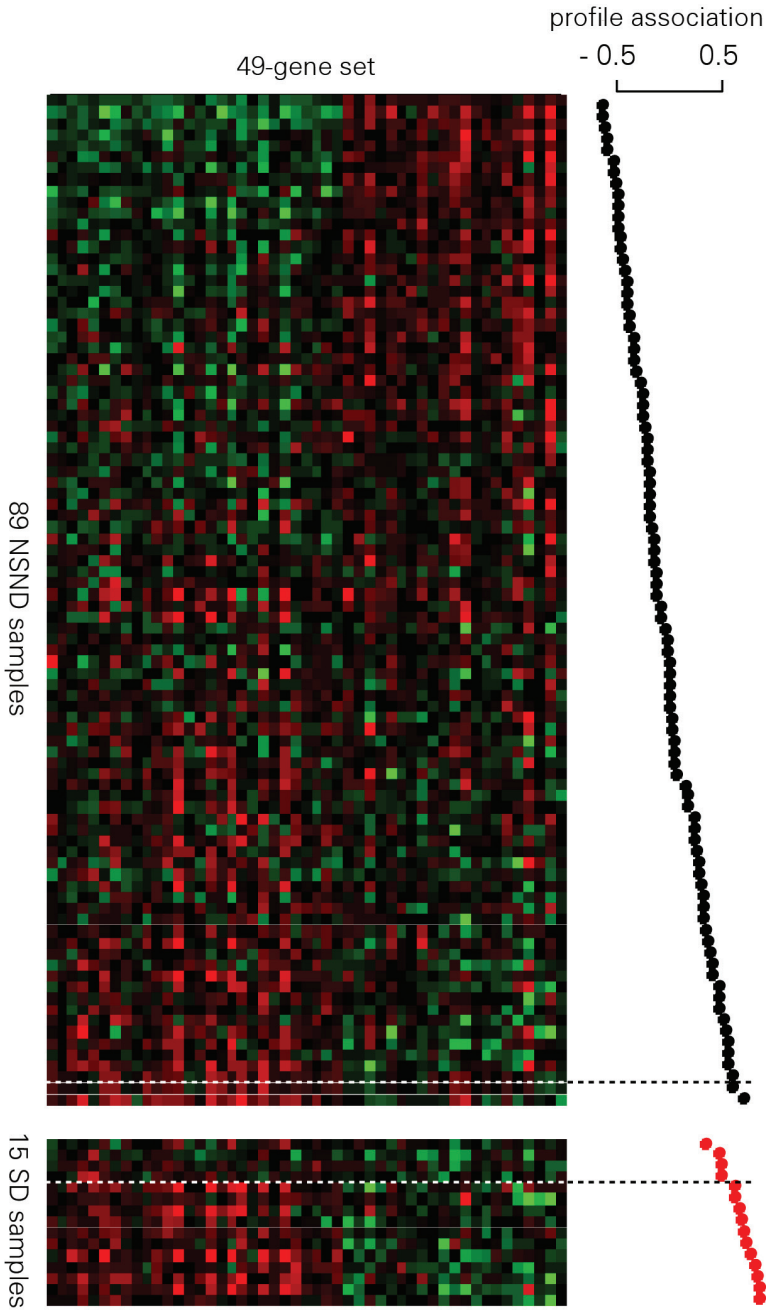

Figure 2. Classification of samples by the 49 non-smoking and non-drinking gene profile. Samples are ordered according to their 49-gene profile and grouped and coloured according to the patients smoking and drinking status with red indicating non-smoking and non-drinking (NSND) and black smoking and drinking (SD) patients. The 49 genes are shown in the same order as in table 2 and figure 1. Red indicated relative high expression, green indicated relative low expression.

Table 2. Differentially regulated genes for non-smoking and non-drinking patients with HNSCC

Gene	Refseq ID	Biological function and pathways	Chromosome	Up/down regulated
PAK4	NM _ 005884	Cellular component movement, signal transduction, protein amino acid phosphorylation	19q13	down
CKB	NM _ 001823	Brain development, cellular chloride ion homeostasis, creatine metabolic process	14q32	down
PARP6	NM _ 020213	DNA repair, transcriptional regulation, apoptosis	15q23	down
RPS4Y1	NM _ 001008	Encodes small ribosomal subunit	Yp11	down
TRIM2	NM _ 015271	Metal ion-, zinc ion- and protein binding	4q31	down
NDUFB9	NM _ 005005	Transprt of electrons	8q13	down
H1F0	NM _ 005318	Nucleosome assembly	22q13	down
CLDND1	NM _ 019895	Regulating membrane permeability	3q12	down
MKRN3	NM _ 005664	Metal ion-, zinc ion- and protein binding	15q11	down
NFKBIA	NM _ 020529	Cell adhesion, immune and proinflammatory responses, apoptosis, cell differentiation and growth	14q13	down
HSPA1A	NM _ 005345	stabilized proteins, involved in the ubiquitin-proteasome pathway	6p21	down
ALDOC	NM _ 005165	Brain cell apoptosis and metabolism	17q11	down
NR2F2	NM _ 021005	Regulation of apolipoprotein A-I gene transcription	15q36	down
RPL39L	NM _ 052969	Encodes ribosomal protein L39	3q27	down
LRP16	NM _ 014067	Member of the macro domain superfamily	11q11	down
WDR26	NM _ 025160	Apoptosis, cell transduction	1q42	down
ELF5	NM _ 001422	Signal transduction	11p13	down
LRP3	NM _ 002333	Possibly signal transduction	19q13	down
FTH1	NM _ 00203	Iron homeostasis	11	down
LPHN1	NM _ 014921	Signal transduction, neronal activities	19q13	down
EIF4A2	NM _ 001967	Cell growth, profiferation, differentiation, and oncogenic transformation	3q28	down
ARPC2	NM _ 005731	Cellular component movement, positive regulation of actin filament polymerization	2q36	up
TCF3	NM _ 003200	Cell differentiation	19p13	up
HPS6	NM _ 024747	Organelle organization, melanocyt differentiation	10q24	up
H2AFY	NM _ 004893	Transcription regulation, DNA repair, RNA replication and chromosomal stability	5q31	up
TXNL5	NM _ 032731	TNF mediated signalling pathway	17p13	up
HLA-DPA1	NM _ 033554	Assoiciated with MHC class II	6p21	up
FBXL5	NM _ 012161	Iron homeostasis, modification-dependent protein catabolic process, protein ubiquitination	4p15	up
ANXA3	NM _ 005139	Regulation of cellular growth, signal transduction	4q13	up
MLLT11	NM _ 006818	Fused with a number of translocation partners in cases of leukemia	1q21	up
ECGF1	NM _ 001953	Diverse physiological effect on most types of cells and tissues, differentiation of endothelial cells	1p21	up
HLA-DPB1	NM _ 002121	Assoiciated with MHC class II	6p21	up
PPA1	NM _ 021129	Phosphate metabolism of cells	10q11	up
HLA-F	NM _ 018950	Assoiciated with MHC class I	6p21	up
TNFRSF1A	NM _ 001065	TNF mediated pathways	12	up

Table 2. *Continued*

Gene	Refseq ID	Biological function and pathways	Chromosome	Up/down regulated
ITGA3	NM _ 005501	Integrin-mediated signalling pathway, cell adhesion	17q21	up
CD74	NM _ 004355	Associated with MHC class II	5q32	up
ZCC1	NM _ 022750	Metal ion binding, nucleic acid binding, transferase activity	7q34	up
HLA-E	NM _ 005516	Associated with MHC class I receptor activity	6p21	up
RPLP2	NM _ 001004	Protein synthesis	11p15	up
RTCD1	NM _ 003729	RNA processing	1p21	up
ATP6V1D	NM _ 015994	ATP synthesis, ion and protein transport	14q23	up
DDX60	NM _ 017631	Nucleic acid binding	4	up
LAP3	NM _ 015907	Turnover of intracellular proteins	4p15	up
ZFAND2B	NM _ 138802	Nucleic acid binding	2q35	up
TUBB6	NM _ 032525	Intracellular protein traffic, chromosome segregation, cell structure and motility	18p11	up
RFTN1	NM _ 015150	Cell migration and proliferation	3p24	up
MUC16	NM _ 024690	Cell surface associated	19p13	up
OAS3	NM _ 006187	Nucleoside, nucleotide and nucleic acid metabolism, interferon-mediated immunity	12q24	up

genes, 21 genes showed lower expression levels in non-smoking and non-drinking patients and 28 genes showed increased expression in these patients. Genes with descreased expression included those associated with signal transduction (ie *PAK4*, *ELF5*, *LPHN1*) and apoptosis (ie *ALDOC*, *WDR26*). Furthermore, there seems to be a downregulation of genes related to the NFKB pathway (*NFKBIA*, *HSPA1A*). Increased expression levels were observed in genes that are involved in major histocompatibility complex molecules (ie *HLA-DPB1, HLA-DPA1, HLA-F, CD74, HLA-E*), protein synthesis (ie *RPLP2, ATP6V1D, LAP3, TUBB6*) and nucleic acid binding (*ZCC1, DDX60, ZFAND2B, OAS3*). In addition, non-smoking and non-drinking patients seem to have an increased Interferon-γ (IFN-γ) activation. As many as 7 genes that could be linked to IFN-γ were upregulated (ie *HPS6, ECFG1, CD74, HLA-E, LAP3, MUC16, OAS3*).

Next, we have developed a non-smoking and non-drinking specific gene profile to classify all samples with non-smoking non-drinking related gene expression patterns (nearest-mean classifier, similary as described by Roepman et al.[5]). Classification by this 49-gene profile resulted in an accuracy of 94% with a sensitivity of 73% and a specificity of 98% (Figure 2). Interestingly, four of the non-smoking and non-drinking patients showed a gene profile that was more representative of a smoking and drinking phenotype, while on the other hand, two tumors from smoking and drinking patients showed a non-smoking and non-drinking profile.

Discussion

In this study we found 49 diffferentially expressed genes in a group of patients with HNSCC without the most commonly associated lifestyle habits, namely tobacco smoking and regular alcohol drinking. We earlier found HNSCC in non-smokers and non-drinkers to be clinically distinct[3,4] and hypothesized a possible different genetic expression profile from their smoking and drinking counterparts.

Seven upregulated diffferentially expressed genes were associated with IFN-γ. IFNs are powerful regulators of antiviral activities and cell proliferation and differentiation[6]. The associations between these genes and IFN-γ have been studied as follows. *LAP3* has been reported to be part of a apoptosis-related gene family. IFN-γ was shown to modulate p53-independent apoptotic pathways by directly and indirectly inducing select apoptosis-related genes such as *LAP3*[7]. *MUC 16*, a cell membrane associated mucin is thought to provide a protective, lubricating barrier against particles and infectious agents at mucosal surfaces[8]. In a study by Albertsmeyer et al. IFN-γ was shown to modulate the expression of *MUC16* at transcriptional level[8]. Nguyen et al. showed that IFN-γ produced by natural killer cells regulates the level of expression of *HLA-E*[9]. Overexpression of *HLA-E* prevented the destruction of healthy cells by immature natural killer cells[9]. In addition, another study showed that IFN-γ is able to regulate other HLA genes such as *HLA-F*[10]. Finally, *HPS6* was related to the IFN-γ pathway through another (WNT) signaling pathway in bone[11] and *ECHF1*, also known as Thymidine phosphorylase, was related to IFN-γ and correlated with the amount of different types of infiltrating immune cells in breast cancer[12]. So, IFN-γ plays an important role in controlling the immune response upon pathogenic challenge. Our findings suggest that the non-smoking and non-drinking patients with HNSCC have a different immune response to their tumor when compared to their smoking and drinking counterparts which may reflect a different underlying carcinogenetic mechanism.

Two downregulated genes were *NFKBIA* and *HSPA1A,* both connected to the NFKB pathway. This pathway is a key regulator in TRD/TRAIL-mediated apoptosis[13] and has been implicated in the development of HNSCC from premalignancy and progression to invasion and metastasis[14]. It seems to have direct carcinogenic effects in HNSCC cells and may enable these cells to disable or evade immune control[15], but to date no specific relation to tobacco or alcohol use was investigated.

Other possible factors involved in pathogenesis of HNSCC in non-smoking and non-drinking patients include prevalence of Human Papilloma Virus (HPV). HPV seems to play an important etiological role in mostly oropharyngeal localised

tumors[16]. In the present study all non-smoking and non-drinking patients had oral cavity tumors, but a role for HPV in this group can not be ruled out. Schlecht *et al*. compared gene expression patterns between HPV-positive and -negative tumors using cDNA microarrays and found a multiple-gene signature which was able to predict HPV16 prevalence (n=11) in primary HNSCC[17]. They also performed a subgroup analysis for the non-smoking patients (n=7 of which 3 were HPV16 positive) and identified a subset of 123 genes predictive of HPV16 infection. Remarkably, they detected a down regulation of IFN related proteins versus an up regulation in our data set, though these were not the same genes (i.e. IFIT1, IFITM1-3, IFI6-16, IFI44L, and OAS2).

Although we were able to identify specific up- or downregulated genes for a particular group of patients with HNSCC, we cannot rule out the possibility of confounding because of an unequal distribution of patients and only a small sample size of non-smoking and non-drinking patients. The patterns identified here should be evaluated on new, independent and larger cohorts to further determine whether they have true functional roles in the development of HNSCC in these patients. To this end a multicentric approach is required because of the small incidence of these cases. After finding a validated gene cluster translational implications for therapeutic interventions should be addressed to achieve specific and selectively targeted approaches. For instance, cis-platinum, a frequently used cytotoxic chemotherapeutic agent in HNSCC has also been shown to inhibit NFKB activation and expression of antiapoptotic genes.

In conclusion, in this preliminary report we identified 49 genes related to HNSCC patients without tobacco and regular alcohol use. Further validation of these genes could lead to new insights in tumorigenesis, selected therapeutic options and possibly a better prognosis for these patients.

References

1. Jaber MA, Porter SR, Gilthorpe MS, Bedi R, Scully C. Risk factors for oral epithelial dysplasia--the role of smoking and alcohol. *Oral Oncol* 1999;35(2):151-6.
2. Dahlstrom KR, Little JA, Zafereo ME, Lung M, Wei Q, Sturgis EM. Squamous cell carcinoma of the head and neck in never smoker-never drinkers: a descriptive epidemiologic study. *Head Neck* 2008;30(1):75-84.
3. Farshadpour F, Hordijk GJ, Koole R, Slootweg PJ. Non-smoking and non-drinking patients with head and neck squamous cell carcinoma: a distinct population. *Oral Dis.* 2007;13(2):239-243.
4. Farshadpour F, Kranenborg H, Calkoen EV, Hordijk GJ, Koole R, Slootweg PJ, et al. Survival analysis of head and neck squamous cell carcinoma: Influence of smoking and drinking. *Head Neck* 2010.
5. Roepman P, Wessels LF, Kettelarij N, Kemmeren P, Miles AJ, Lijnzaad P, et al. An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nat.Genet.* 2005;37(2):182-186.
6. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annu. Rev.Immunol.* 1997;15:749-795.
7. Ossina NK, Cannas A, Powers VC, Fitzpatrick PA, Knight JD, Gilbert JR, et al. Interferon-gamma modulates a p53-independent apoptotic pathway and apoptosis-related gene expression. *J.Biol.Chem.* 1997;272(26):16351-16357.
8. Albertsmeyer AC, Kakkassery V, Spurr-Michaud S, Beeks O, Gipson IK. Effect of pro-inflammatory mediators on membrane-associated mucins expressed by human ocular surface epithelial cells. *Exp.Eye Res.* 2010;90(3):444-451.
9. Nguyen S, Beziat V, Dhedin N, Kuentz M, Vernant JP, Debre P, et al. HLA-E upregulation on IFN-gamma-activated AML blasts impairs CD94/NKG2A-dependent NK cytolysis after haplo-mismatched hematopoietic SCT. *Bone Marrow Transplant.* 2009;43(9):693-699.
10. Gobin SJ, van ZM, Woltman AM, van den Elsen PJ. Transactivation of classical and nonclassical HLA class I genes through the IFN-stimulated response element. *J.Immunol.* 1999;163(3):1428-1434.
11. Alam I, Sun Q, Koller DL, Liu L, Liu Y, Edenberg HJ, et al. Differentially expressed genes strongly correlated with femur strength in rats. *Genomics* 2009;94(4):257-262.
12. Ruckhaberle E, Karn T, Engels K, Turley H, Hanker L, Muller V, et al. Prognostic impact of thymidine phosphorylase expression in breast cancer--comparison of microarray and immunohistochemical data. *Eur.J.Cancer* 2010;46(3):549-557.
13. Daigeler A, Brenzel C, Bulut D, Geisler A, Hilgert C, Lehnhardt M, et al. TRAIL and Taurolidine induce apoptosis and decrease proliferation in human fibrosarcoma. *J.Exp.Clin. Cancer Res. 2008;27:82.*
14. Lee TL, Yang XP, Yan B, Friedman J, Duggal P, Bagain L, et al. A novel nuclear factor-kappaB gene signature is differentially expressed in head and neck squamous cell carcinomas in association with TP53 status. *Clin.Cancer Res.* 2007;13(19):5680-5691.
15. Loercher A, Lee TL, Ricker JL, Howard A, Geoghegen J, Chen Z, et al. Nuclear factor-kappaB is an important modulator of the altered gene expression profile and malignant phenotype in squamous cell carcinoma. *Cancer Res.* 2004;64(18):6511-6523.
16. Andrews E, Seaman WT, Webster-Cyriaque J. Oropharyngeal carcinoma in non-smokers and non-drinkers: a role for HPV. *Oral Oncol* 2009;45(6):486-91.

17. Schlecht NF, Burk RD, Adrien L, Dunne A, Kawachi N, Sarta C, et al. Gene expression profiles in HPV-infected head and neck cancer. *J.Pathol.* 2007;213(3):283-293.

Chapter 7

General Discussion

General Discussion

A head and neck surgeon will occasionally encounter patients with head and neck squamous cell carcinoma (HNSCC) who do not match the most common risk profile, namely that of a patient with high use of tobacco and alcohol. These non-smoking and non-drinking patients will raise questions in relation to epidemiological, aetiological, therapeutic and prognostic aspects of their disease. These questions are difficult to answer due to the rarity and as a consequence the small number of studies concerning this distinct population. In this thesis we addressed some of these questions by means of analyzing the non-smoking and non-drinking patients with HNSCC in our center during the last 24 years and reviewing the literature regarding this issue. In this section the following questions will be discussed:

1. *What are the epidemiological features of a non-smoking and non-drinking patient with HNSCC? Are these features any different from the smoking and drinking patient?*
2. *What other etiological factors could have caused HNSCC?*
3. *Does a non-smoking and non-drinking patient have a better prognosis than a patient who uses both substances?*
4. *Are there any immunohistological characteristics in tumor adjacent mucosa from a non-smoking and non-drinking patient when compared to the smoking and drinking patient?*

1. What are the epidemiological features of a non-smoking and non-drinking patient with HNSCC? Are these features any different from the smoking and drinking patient?
In a 24-year period we found 198 non-smoking and non-drinking patients with HNSCC which comprise 4.3% of the total population of HNSCC in our center (this thesis). Incidence rates of this population in the current literature are scarce but comparable (between 2.9 and 3.9%) with our findings [1, 2]. Seven out of 10 non-smoking and non-drinking patients in our population were female with a high mean age of 72 years (this thesis). When compared to Dutch national data from the National Cancer Registry the gender distribution was reversed with 7 out of 10 patients being male. Furthermore the mean age for all patients with HNSCC was 10 years younger than all Dutch patients with HNSCC. Similar opposing data was found when comparing the non-smoking and non-drinking patients to those with smoking and drinking habits in our center (this thesis). Another striking difference between both groups of patients was the localisation of the tumor; while the larynx was the most common site in our center's smoking and drinking group and in the total Dutch HNSCC population (44% and 39%, respectively), oral cavity tumors had the highest frequency (66%) in the non-smoking and non-drinking population (this thesis). The TN-classification and disease stage were also differently distributed between patients with and

without substance use with non-users having mostly early stage cancer. The reason for this difference is unclear, perhaps patient delay is a determining factor in this issue. Non-users could be more aware of their complaints and seek medical consultation sooner than smokers and drinkers. In conclusion, we found that the typical non-smoking and non-drinking patient with HNSCC in our center presents as an older female patient with stage I oral cavity tumor. Other studies regarding these epidemiological data all found the female preponderance[1-5], a high mean age[1, 2, 5, 6] and mostly early stage oral cavity localised tumors[1-6].

However, a recent study by Dahlstrom et al. in which 172 non-smoking and non-drinking patients in the US were analyzed, showed not only very old patients (18% 70 years or older), but also a high percentage of young patients (13% younger than 40 years) with a mean age of 55 years[4]. Furthermore most of the tumors were localised in the oropharynx (48%) followed by the oral cavity (42%). Their typical non-smoking and non-drinking patients were either women under 50 years of age with oral tongue cancer, men under 60 with oropharyngeal cancer or women 70 years or older with gingivobuccal cancer. These differences with our population of non-smoking and non-drinking patients could partially be caused by prevalence of other ethnic backgrounds of the patients in the US (including Asian-, Hispanic- and African Americans) and possibly a high percentage of HPV related (oropharyngeal) tumors. Our population had very little other ethnic background than Dutch and HPV seemed to play a minor role as further discussed below.

One other remarkable aspect of our center's abstainers was the incidence of second primary tumors (SPTs) which was similar to patients with substance use (this thesis). SPTs were found in 16.7% of the non-smoking and non-drinking patients, (4,5% of these SPTs were synchronous tumors) and in 18.4% of the smoking and drinking patients (5% of these SPTs were synchronous tumors). However, the distribution of SPTs was different in the two groups. Nearly all SPTs of the non-smoking and non-drinking population were in the head and neck region, especially the oral cavity whereas almost half of SPTs of the smoking and drinking patients were localized in the lungs and esophagus. The literature provides some clues that multiple HNSCCs are at least partly clonally related although reliable and precise methods to determine the origin of these tumors are yet to be found[7]. It is furthermore conceivable that the thus far unknown carcinogenic factors in the non-users are specially present and active in the oral cavity, though literature regarding the incidence and pathophysiology of SPTs among non-smokers and non-drinkers is scarce[8].

2. What other etiological factors could have caused HNSCC?

Substantial evidence has shown that oncogenic human papillomavirus (HPV) which is the primary cause of uterine cervical cancer, is etiologically involved in the development of HNSCC, especially in the oropharynx. So we analyzed the role of

HPV in the pathogenesis (and outcome) of non-smoking and non-drinking patients with oropharyngeal tumors.

We performed the first matched pair analysis in which presence of HPV-DNA in tumor cells of 16 non-smoking and non-drinking patients with oropharyngeal squamous cell carcinoma (OSCC) was compared with 16 patients with a history of smoking and drinking. There is some discussion concerning the best way to detect high risk HPV in formaline fixed paraffin embedded tumor tissue. To achieve the most reliable outcome, we used four different methods, i.e. fluorescence in situ hybridization (FISH), two polymerase chain reaction (PCR)-based assays (Amplicor® and Linear Array® HPV detection kits), and $p16^{INK4A}$-immunostaining. HPV was found in 75% of the non-smoking and non-drinking patients compared to 12.5% of the smoking and drinking patients. The literature also shows an association between patients with HPV-positive tumors and consumption of smaller amounts of tobacco and alcohol[9-12]. Additionally a recent study showed that non-smoking and non-drinking patients with oral and oropharyngeal tumors were 6.1 times more likely to have HPV DNA in their tumors compared to benign biopsy controls[13]. One explanation for this tendency for HPV associated tumors in non-smokers and non-drinkers could be that smoking may have a protective effect on HPV infection due to increased keratinization[14]. We could confirm this hypothesis, our HPV-positive cases had significantly less keratinisation than the HPV-negative cases (21% vs 61%). Moreover, we found patients with HPV-positive tumors have their own specific immunohistological characteristics (including the presence of basaloid features and lack of keratinisation) and more importantly a better overall and disease-specific survival compared to their smoking and drinking counterparts. These findings have also been reported by previous studies and underscore the significance of recognition of perhaps a different entity within the heterogeneous group of HNSCC[11, 15-19]. Furthermore, it is also important to state that not all oropharyngeal tumors are related to HPV. In our population, 25% of the non-smoking and non-drinking patients and 87.5% of the smoking and drinking patients with oropharynx cancer were not HPV-positive. So other aetiological factors should also be considered.

We furthermore identified a different genetic profile for non-smoking and non-drinking patients (n=15) compared to smoking and drinking patients (n=89). A set of 49 differentially expressed genes were detected. Some genes found were related to controlling and regulating the immune response upon pathogenic challenges. These genes could (partially) explain the underlying mechanisms leading to development of HNSCC in this specific group of patients. When validated on larger cohorts, they could also initiate specifically selected therapeutic agents.

As mentioned in the general introduction other factors have been identified in the pathogenesis of HNSCC, such as poor oral hygiene, dietary factors, gastrointestinal reflux and the presence of premalignant mucosal lesions. Also the role of

environmental exposure to tobacco smoke for especially female non-smoking and non-drinking patients with HNSCC should be considered[20]. Unfortunately data concerning these factors were not available for our population.

3. Does a non-smoking and non-drinking patient have a better prognosis than a patient who uses both substances?

To answer this question we performed multivariate and univariate survival analysis for all non-smokers and non-drinkers and all patients who had a history of smoking and drinking (n=2181) in our center during a 24-year period of follow-up. Tobacco and alcohol use did not significantly affect disease specific survival (i.e. death due to primary HNSCC or recurrence or SPT) after correction for known prognostic factors, such as TNM classification, age, and treatment. Literature regarding a possible negative prognostic effect of tobacco and alcohol is limited and usually merely involves nonsmokers without taking alcohol consumption into account or concerns a specific tumor localization or age category[3, 21-26]. A matched-pair survival analysis for 50 never-smokers and ever-smokers found a significant increase in risk of death due to disease and disease recurrence among smokers, but survival outcomes were no longer significant after adjustment for alcohol consumption and cancer associated symptom index[27]. In addition no difference in 10-year relapse-free survival was found for non-users compared to smoking and drinking patients in another study[3]. So it seems that although tobacco and alcohol are major risk factors for development of HNSCC, a small group of non-users may also suffer from these tumors and these patients seem to have a similar disease specific outcome compared to their smoking and drinking counterparts.

We did however find that overall survival was significantly better for patients without substance use as was reported in other literature[2, 6, 21]. This is most likely due to presence of tobacco- and alcohol related comorbidities such as cardiovascular and pulmonary disease. Therefore for daily clinical practice it remains crucial to help patients in their efforts to quit tobacco and (excessive) alcohol use to optimize their quality of life and survival.

4. Are there any immunohistological characteristics in tumor adjacent mucosa from a non-smoking and non-drinking patient when compared to the smoking and drinking patient?

Expression patterns of biomarkers p53 and Ki67 were immunohistochemically analyzed in non-malignant tumor adjacent mucosa from the non-smoking and non-drinking patients with single and multiple tumors. The same analysis was done for a group of smokers and drinkers with single and multiple HNSCC and all data were compared. We found a significant association between substance use and a higher positivity for dispersed single cells and clusters of p53 which has been shown

in other studies as well[23, 28, 29]. This association was not found for Ki67 in contrast to other reports where alcohol was not taken into account or different degrees of mucosal dysplasia was investigated[30, 31]. This difference may be because we investigated healthy mucosa and the predictive value of Ki-67 expression may be more pronounced in later stages of mucosal alteration in which dysplastic features are visible. Surprisingly, no relationship was found between expression of both biomarkers and multiple HNSCCs for both users and non-users. Hence a predictive value of p53 and Ki67 for multiple HNSCC could not be confirmed by our study. Literature regarding this subject is contradictory[32-34]. In our opinion it seems that expression of these markers are not crucial for recurrent HNSCC, but merely epiphenomenal.

Future perspectives

For a better understanding of the pathogenesis and a prognostic improvement for non-smoking and non-drinking patients with HNSCC, a prospective multicentric study should be considered, mainly because of the small incidence rates of these specific cases. A database consisting of information regarding other possible etiological factors including oral hygiene and presence of premalignant lesions is important in further studying this distinct group of patients within the heterogeneous group of patients with HNSCC. To be able to determine whether subsequent tumors in these patients are true second primary tumors, recurrences or second field tumors as described in other studies[35, 36], the exact locations of new tumors and assessment of clonality markers such as allelic imbalance and p53 mutations should be analyzed. It is also recommendable to perform an HPV analysis for all tumor subsites in these patients, starting with a $p16^{INK4A}$-immunostaining and in presence of overexpression, a subsequent determination of HPV-status using (fresh frozen material) PCR or FISH analysis. In addition microarray analysis in a larger cohort of non-users with HNSCC could validate our already found specific gene set and possibly lead to specific therapeutic strategies.

Reference List

(1) Constantinides MS, Rothstein SG, Persky MS. Squamous cell carcinoma in older patients without risk factors. *Otolaryngol Head Neck Surg.* 1992 Mar;106(3):275-277.

(2) Agudelo D, Quer M, Leon X, Diez S, Burgues J. Laryngeal carcinoma in patients without a history of tobacco and alcohol use. *Head Neck.* 1997 May;19(3):200-204.

(3) Harris SL, Kimple RJ, Hayes DN, Couch ME, Rosenman JG. Never-smokers, never-drinkers: unique clinical subgroup of young patients with head and neck squamous cell cancers. *Head Neck.* 2010 Apr;32(4):499-503.

(4) Dahlstrom KR, Little JA, Zafereo ME, Lung M, Wei Q, Sturgis EM. Squamous cell carcinoma of the head and neck in never smoker-never drinkers: a descriptive epidemiologic study. *Head Neck.* 2008 Jan;30(1):75-84.

(5) Wey PD, Lotz MJ, Triedman LJ. Oral cancer in women nonusers of tobacco and alcohol. *Cancer.* 1987 Oct 1;60(7):1644-1650.

(6) Wiseman SM, Swede H, Stoler DL, et al. Squamous cell carcinoma of the head and neck in nonsmokers and nondrinkers: an analysis of clinicopathologic characteristics and treatment outcomes. *Ann Surg Oncol.* 2003 Jun;10(5):551-557.

(7) Pateromichelakis S, Farahani M, Phillips E, Partridge M. Molecular analysis of paired tumours: time to start treating the field. *Oral Oncol.* 2005 Oct;41(9):916-926.

(8) Partridge M, Pateromichelakis S, Phillips E, Emilion G, Langdon J. Profiling clonality and progression in multiple premalignant and malignant oral lesions identifies a subgroup of cases with a distinct presentation of squamous cell carcinoma. *Clin Cancer Res.* 2001 Jul;7(7):1860-1866.

(9) Gillison ML, D'Souza G, Westra W, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. *J Natl Cancer Inst.* 2008 Mar 19;100(6):407-420.

(10) Hafkamp HC, Manni JJ, Haesevoets A, et al. Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas. *Int J Cancer.* 2008 Jun 15;122(12):2656-2664.

(11) Lindel K, Beer KT, Laissue J, Greiner RH, Aebersold DM. Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. *Cancer.* 2001 Aug 15;92(4):805-813.

(12) Tachezy R, Klozar J, Salakova M, et al. HPV and other risk factors of oral cavity/oropharyngeal cancer in the Czech Republic. *Oral Dis.* 2005 May;11(3):181-185.

(13) Andrews E, Seaman WT, Webster-Cyriaque J. Oropharyngeal carcinoma in non-smokers and non-drinkers: A role for HPV. *Oral Oncol.* 2009 Jun;45(6):486-491.

(14) Klussmann JP, Weissenborn SJ, Wieland U, et al. Prevalence, distribution, and viral load of human papillomavirus 16 DNA in tonsillar carcinomas. *Cancer.* 2001 Dec 1;92(11):2875-2884.

(15) Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst.* 2000 May 3;92(9):709-720.

(16) Hafkamp HC, Manni JJ, Speel EJ. Role of human papillomavirus in the development of head and neck squamous cell carcinomas. *Acta Otolaryngol.* 2004 May;124(4):520-526.

(17) Klussmann JP, Gultekin E, Weissenborn SJ, et al. Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. *Am J Pathol.* 2003 Mar;162(3):747-753.

(18) Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. *J Clin Oncol.* 2009 Apr 20;27(12):1992-1998.

(19) Ritchie JM, Smith EM, Summersgill KF, et al. Human papillomavirus infection as a prognostic factor in carcinomas of the oral cavity and oropharynx. *Int J Cancer.* 2003 Apr 10;104(3):336-344.

(20) Dahlstrom KR, dler-Storthz K, Etzel CJ, et al. Human papillomavirus type 16 infection and squamous cell carcinoma of the head and neck in never-smokers: a matched pair analysis. *Clin Cancer Res.* 2003 Jul;9(7):2620-2626.

(21) Dikshit RP, Boffetta P, Bouchardy C, et al. Lifestyle habits as prognostic factors in survival of laryngeal and hypopharyngeal cancer: a multicentric European study. *Int J Cancer.* 2005 Dec 20;117(6):992-995.

(22) Gorsky M, Epstein JB, Oakley C, Le ND, Hay J, Stevenson-Moore P. Carcinoma of the tongue: a case series analysis of clinical presentation, risk factors, staging, and outcome. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2004 Nov;98(5):546-552.

(23) Koch WM, Lango M, Sewell D, Zahurak M, Sidransky D. Head and neck cancer in nonsmokers: a distinct clinical and molecular entity. *Laryngoscope.* 1999 Oct;109(10):1544-1551.

(24) Lo WL, Kao SY, Chi LY, Wong YK, Chang RC. Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: factors affecting survival. *J Oral Maxillofac Surg.* 2003 Jul;61(7):751-758.

(25) Pytynia KB, Grant JR, Etzel CJ, Roberts D, Wei Q, Sturgis EM. Matched analysis of survival in patients with squamous cell carcinoma of the head and neck diagnosed before and after 40 years of age. *Arch Otolaryngol Head Neck Surg.* 2004 Jul;130(7):869-873.

(26) Verschuur HP, Irish JC, O'Sullivan B, Goh C, Gullane PJ, Pintilie M. A matched control study of treatment outcome in young patients with squamous cell carcinoma of the head and neck. *Laryngoscope.* 1999 Feb;109(2 Pt 1):249-258.

(27) Pytynia KB, Grant JR, Etzel CJ, Roberts DB, Wei Q, Sturgis EM. Matched-pair analysis of survival of never smokers and ever smokers with squamous cell carcinoma of the head and neck. *J Clin Oncol.* 2004 Oct 1;22(19):3981-3988.

(28) Brennan JA, Boyle JO, Koch WM, et al. Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. *N Engl J Med.* 1995 Mar 16;332(11):712-717.

(29) van Oijen MG, van de Craats JG, Slootweg PJ. p53 overexpression in oral mucosa in relation to smoking. *J Pathol.* 1999 Mar;187(4):469-474.

(30) Tabor MP, Braakhuis BJ, van der Wal JE, et al. Comparative molecular and histological grading of epithelial dysplasia of the oral cavity and the oropharynx. *J Pathol.* 2003 Mar;199(3):354-360.

(31) van Oijen MG, Gilsing MM, Rijksen G, Hordijk GJ, Slootweg PJ. Increased number of proliferating cells in oral epithelium from smokers and ex-smokers. *Oral Oncol.* 1998 Jul;34(4):297-303.

(32) Ogden GR, Chisholm DM, Morris AM, Stevenson JH. Overexpression of p53 in normal oral mucosa of oral cancer patients does not necessarily predict further malignant disease. *J Pathol.* 1997 Jun;182(2):180-184.

(33) Cruz IB, Snijders PJ, Meijer CJ, et al. p53 expression above the basal cell layer in oral mucosa is an early event of malignant transformation and has predictive value for developing oral squamous cell carcinoma. *J Pathol.* 1998 Apr;184(4):360-368.

(34) Cruz IB, Meijer CJ, Snijders PJ, Snow GB, Walboomers JM, van der Waal, I. p53 immunoexpression in non-malignant oral mucosa adjacent to oral squamous cell carcinoma: potential consequences for clinical management. *J Pathol.* 2000 Jun;191(2):132-137.

(35) Braakhuis BJ, Tabor MP, Leemans CR, van der Waal I, Snow GB, Brakenhoff RH. Second primary tumors and field cancerization in oral and oropharyngeal cancer: molecular techniques provide new insights and definitions. *Head Neck.* 2002 Feb;24(2):198-206.

(36) Califano J, van der RP, Westra W, et al. Genetic progression model for head and neck cancer: implications for field cancerization. *Cancer Res.* 1996 Jun 1;56(11):2488-2492.

Summary

Summary

In *chapter one* a short general introduction and the aim and outline of this thesis is given.

In *chapter two* 195 patients with head and neck squamous cell carcinoma (HNSCC) who had no history of smoking tobacco or drinking alcohol are described. The data is retrieved from the database at the University Medical Center Utrecht (UMCU) where all newly diagnosed patients with head and neck cancer have been prospectively registered since 1980. This database contains information on patient characteristics, risk factors, and tumor classification, including data on development of recurrences, second primary tumors and follow-up. During the period 1980-2003, 4404 patients with squamous cell carcinoma of the lip, oral cavity, oropharynx, hypopharynx and larynx were registered. The clinicopathological characteristics of 195 (4.4%) non-smoking and non-drinking patients were analyzed and compared with data for all Dutch patients with HNSCC obtained from the Netherlands Cancer Registry. Non-smoking and non-drinking patients with HNSCC had distinct characteristics. They were typically female (n=142; 73% versus (vs) 26%), had advanced age at disease presentation (mean 73 years vs 64 years) and had tumors mainly of the oral cavity (n=130; 66% vs 25%). Most tumors were stage I (n=67; 34%) and stage IVA (n=59; 30%). The incidence of second primary tumors (SPTs) was high (n= 32; 16%), mainly occurring in the oral cavity (n=26; 13%).

In *chapter three* the prognostic relevance of tobacco and alcohol for patients with HNSCC was addressed. To this end, univariate and multivariate survival analysis were performed for 2012 patients with HNSCC from our center (UMCU), including 183 non-smoking and non-drinking patients and 1829 patients who consumed both tobacco (quit smoking <1 year, and 1 or more cigarettes/day) and alcohol (1 or more alcohol consumption/day). HNSCC-specific survival (death due to primary- or recurrent HNSCC) was not significantly different for patients who smoked and drank and those who did not (hazard ratio (HR) 1.26, 95% confidence interval (CI) 0.86-1.85). This was also the case for HNSCC/second primary tumor-specific survival (death due to primary- or recurrent HNSCC or second primary tumor) (HR 1.34, 95%CI 0.96-1.88). Overall survival was significantly affected by tobacco and alcohol; HR for smoking and drinking patients was 1.50 (95%CI 1.16-1.93).

In *chapter four* expression of tumor suppressor gene p53 and proliferation marker Ki-67 in non-tumorous (tumor-adjacent) mucosal epithelial cells were analyzed to study whether biomarker expression is associated with a history of smoking and drinking and with single and multiple HNSCC. Non-smoking and non-drinking

patients with multiple (n=18) and single tumors (n=15), smoking and drinking patients with multiple (n=15) and single tumors (n=14) were selected. For all groups positivity of dispersed single cells and clusters for p53 and for suprabasal expression of Ki-67 were immunohistochemically analyzed and compared. p53 expression was significantly higher in users of tobacco and alcohol than in non-users. Ki-67 expression was not affected by tobacco and alcohol use. Both Ki-67 and p53 were similarly expressed in the groups with single and multiple tumors and thus not significantly related to the number of tumors. Hence, the significance of these proteins as biomarkers indicating premalignant mucosal alterations in HNSCC was considered as doubtful.

In *chapter five* a case-case analysis was performed to compare presence of HPV-DNA in tumor cells of 16 non-smoking and non-drinking with 16 matched smoking and drinking patients with oropharyngeal localized tumors (matching criteria: age at incidence, gender, tumor sublocation, tumor stage). HPV was detected using 2 PCR tests, FISH analysis and $p16^{INK4A}$ immunostaining. Non-smoking and non-drinking patients had more HPV-positive tumors than smoking and drinking patients (n=12; 75% versus n=2; 13%; $p<0.001$). All HPV-positive tumors showed $p16^{INK4A}$ overexpression, 1 HPV-negative tumor had $p16^{INK4A}$ overexpression, ($p<0.001$). Overall survival and disease-specific survival were better for HPV-positive compared to HPV-negative cases ($p=0.027$, $p=0.039$, respectively). So, HPV was strongly associated with oropharyngeal squamous cell carcinoma of non-smoking and non-drinking patients. Specific diagnostic and therapeutic actions should be considered for these patients to achieve a better prognosis.

In *chapter six* the gene expression data previously detected from primary tumors located in the oral cavity and oropharynx, using DNA microarray was analyzed for their differential expression between non-smoking and non-drinking patients (n=15) and smoking and drinking patients (n=89). Student's T-test ($p<0.05$) and 10-fold cross-validation procedure (100 times repeated) were performed to determine differentially expressed genes. A set of 49 differentially expressed genes were detected. Amongst others, 7 genes related to Interferon-γ (IFN-γ) were downregulated. These genes play an important role in controlling the immune response upon pathogenic challenge. Moreover, 2 genes linked to NFKB pathway, which is a key regulator in TRD/TRAIL-mediated apoptosis were upregulated. Our findings suggest that the non-smoking and non-drinking patients with HNSCC have a different immune response to their tumor when compared to their smoking and drinking counterparts which may reflect a different underlying carcinogenetic mechanism.

In *chapter seven* a general discussion has been written regarding reported findings in this thesis. Furthermore suggestions for additional research have been proposed in future perspectives.

Nederlandse samenvatting

Nederlandse samenvatting

In *hoofdstuk een* wordt een algemene introductie gegeven aangaande dit proefschrift.

In *hoofdstuk twee* worden 195 patiënten met plaveiselcelcarcinomen uitgaande van het hoofdhalsgebied beschreven die nooit tabak gerookt hebben of alcohol hebben gedronken. De hiervoor benodigde data is verzameld uit een database van het Universitair Medisch Centrum Utrecht waarin alle patiënten met hoofdhalstumoren sinds 1980 prospectief zijn geregistreerd. Deze database bevat onder andere informatie over klinische parameters zoals risico factoren, tumor stadium, ontwikkeling van recidieven of nieuwe primaire tumoren en follow-up gegevens. Gedurende de periode 1980-2003, werden 4404 patiënten met plaveiselcelcarcinomen uitgaande van de lip, mondholte, oropharynx, hypopharynx en larynx geregistreerd. Hiervan werden 195 patiënten zonder tabak- of alcoholgebruik (4.4% van het totaal) geselecteerd. De klinische en pathologische parameters van deze groep werd vergeleken met die van alle patiënten met plaveiselcelcarcinomen van het hoofdhalsgebied verkregen via de Nederlandse Kanker Registratie. Niet rokende en niet drinkende patiënten waren vooral vrouwen (n=142; 73% versus (vs) 26%), hadden een hogere leeftijd ten tijde van incidentie (gemiddeld 73 jaar vs 64 jaar) en hadden veel tumoren uitgaande van de mondholte (n=130; 66% vs 25%). Het betrof vooral stadium I (n=67; 34%) en stadium IVA (n=59; 30%) tumoren. Tevens was er sprake van een hoge incidentie van tweede primaire tumoren (n= 32; 16%) met name uitgaande van de mondholte (n=26; 13%). Concluderend, niet rokende en niet drinkende patiënten met plaveiselcelcarcinomen hebben typische klinische eigenschappen, maar ze hebben wel evenveel tweede primaire tumoren als de rokende en drinkende patiënten.

In *hoofdstuk drie* wordt de prognostische waarde van tabak- en alcoholgebruik voor patiënten met plaveiselcelcarcinomen uitgaande van het hoofdhalsgebied geanalyseerd. Hiertoe werden multivariate en univariate survival analysen voor 2012 patiënten uit onze database verricht waarvan het 183 niet rokers en niet drinkers en 1829 rokers en drinkers betrof. Ziekte specifieke survival, dat wil zeggen sterfte door primaire tumor of recidief en sterfte door primaire tumor of recidief of tweede primaire tumor waren niet significant beïnvloed door tabak- en alcoholgebruik (hazard ratio (HR) 1.26, 95% betrouwbaarheids interval (BI) 0.86-1.85, HR 1.34, 95%BI 0.96-1.88, respectievelijk). De overall survival (sterfte ongeacht de oorzaak) daarentegen was wel significant beter voor de niet rokers en niet drinkers vergeleken met de rokers en drinkers (HR 1.5, 95%BI 1.16-1.93). Dus ondanks het feit dat tabak- en alcoholgebruik de belangrijkste risico factoren zijn voor het ontwikkelen van

hoofdhalstumoren, is de ziekte specifieke overleving vergelijkbaar voor patiënten met en zonder inname van deze stoffen. Desalniettemin is het adviseren van stoppen van roken en drinken belangrijk daar de algemene overleving wel beïnvloed wordt door tabak- en alcoholgebruik.

In *hoofdstuk vier* wordt een analyse verricht naar de immunohistochemische expressie van biomarkers p53 en Ki67 in normale mucosa naast tumoreus veranderd mucosa. Dit wordt voor 4 verschillende groepen patiënten met plaveiselcelcarcinomen van het hoofdhalsgebied gedaan, namelijk: patiënten zonder tabak- en alcoholgebruik met enkele (n=18) en multipele tumoren (n=15) en patiënten met tabak- en alcoholgebruik met enkele (n=14) en multipele tumoren (n=15). Het doel van dit onderzoek is om te beoordelen of er een relatie bestaat tussen deze biomarkers en tabak- en alcoholgebruik en tussen deze biomarkers en ontwikkeling van multipele tumoren. Er wordt voor alle groepen gekeken naar immunohistochemisch verspreid voorkomen dan wel clusters van p53 en naar het voorkomen van suprabasale expressie van Ki67. p53 toont een hogere expressie bij rokers en drinkers vergeleken met niet rokers en niet drinkers. Ki-67 expressie wordt niet beïnvloed door tabak- en alcoholgebruik. Voorts tonen beide biomarkers een vergelijkbare expressie in patiënten met een enkele tumor en met multipele tumoren. Derhalve wordt de significantie van deze biomarkers voor het aanduiden van premaligne mucosale veranderingen in plaveiselcelcarcinomen van het hoofdhalsgebied betwijfeld.

In *hoofdstuk vijf* wordt een 'case-case'analyse verricht waarbij de aanwezigheid van Humaan pailloma virus (HPV) in tumorcellen uitgaande van de oropharynx van 16 patiënten zonder en 16 patiënten met tabak- en alcoholgebruik wordt onderzocht. De groepen werden gematcht op geslacht, leeftijd ten tijde van incidentie, sublocatie en stadium van de tumor. HPV detectie werd middels vier verschillende technieken onderzocht, namelijk: 2 verschillende PCR tests, FISH en immunohistochemische bepaling van biomarker $p16^{INK4A}$. Patiënten zonder tabak- en alcoholgebruik hadden meer HPV-positieve tumoren (n=12; 75% versus n=2; 13%; $p<0.001$). Alle HPV-positieve tumoren en één HPV-negatieve tumor toonden overexpressie van $p16^{INK4A}$. Overall- en ziekte-specifieke survival waren hoger voor patiënten met HPV-positieve vergeleken met patiënten met HPV-negatieve tumoren ($p=0.027$, $p=0.039$, respectievelijk).

In *hoofdstuk zes* wordt de gen expressie data, eerder gevonden bij primaire plaveiselcelcarcinomen uitgaande van de mondholte en oropharynx middels DNA microarray techniek gebruikt, teneinde een differentiële expressie in groepen met (n=89) en zonder tabak- en alcoholgebruik (n=15) te analyseren. Een student's T-test en een 10-fold cross-validation procedure (100 keer herhaald) werden

uitgevoerd om differentiële genen te identificeren. Een set van 49 genen met een verschillende expressie in beide groepen werden gevonden. Onder andere 7 genen gerelateerd aan Interferon-γ (IFN-γ) waren 'downregulated'. Deze genen spelen een belangrijke rol in het controleren van het respons van het immuunsysteem op pathologische processen. Tevens werden 2 genen verbonden aan de NFKB pathway gedetecteerd met een 'upregulation'. NFKB beheerst in belangrijke mate de TRD/TRAIL-gemedieerde apoptosis. Op basis van de gevonden genen lijken de niet rokende en niet drinkende patiënten met plaveiselcelcarcinomen een andere immuun respons te hebben op het ontwikkelen van een maligniteit dan patiënten met tabak- en alcoholgebruik. Dit wijst mogelijk op een andere onderliggende carcinogenese bij deze groep patiënten.

In *hoofdstuk zeven* wordt een algemene discussie aangaande de onderwerpen van dit proefschrift weergegeven. Tevens worden er suggesties gedaan voor het verrichten van nader onderzoek voor deze specifieke groep van patiënten met plaveiselcelcarcinomen.

Dankwoord

Dankwoord

Dit proefschrift is tot stand gekomen door ondersteuning, vertrouwen, en inspiratie van vele mensen om mij heen.
In de eerste plaats ben ik bijzonder dank verschuldigd aan mijn promotoren. Ik heb het voorrecht gehad om begeleid te worden door drie deskundigen vanuit drie verschillende disciplines. Kenmerkend waren onze regelmatige bijeenkomsten waarin ieder vanuit zijn eigen vakgebied de zaak bekeek en becommentarieerde. Het was voor mij zaak om goed te luisteren en snel aantekeningen te maken. Op cruciale momenten gaven deze bijeenkomsten mij het vertrouwen en enthousiasme om verder te gaan. Ook heb ik het erg gewaardeerd dat ik in het algemeen de vrijheid en vertrouwen kreeg om een eigen planning van de voortgang van het onderzoek te maken.
Professor Hordijk, u nam mij aan voor de opleiding KNO en voor het verrichten van dit onderzoek. Daarmee heeft u aan het begin gestaan van mijn carrière in alle opzichten. Ik ben u zeer dankbaar voor uw vertrouwen en voor uw altijd geduldige en zachtaardige begeleiding, soms vanuit verre oorden, van mijn opleiding en onderzoek.
Professor Slootweg, in de afgelopen jaren zijn uw deskundige en efficiënte opmerkingen over de verschillende projecten bepalend geweest. Zonder uitzondering was ieder manuscript binnen 48 uur gelezen en geretourneerd met belangrijke en leerzame verbeteringen.
Professor Koole, ook al was ik niet van de 'kaak', heb ik me door u op vele wijzen altijd zeer gesteund gevoeld. Ik denk aan het tot stand komen van vele (deel) projecten door financiële ondersteuning vanuit de kaakchirurgie. Maar ook aan uw aanwezigheid en steun bij presentaties en uw interesse en begrip voor persoonlijke omstandigheden.
Dr J.A. Kummer, beste Alain, samenwerken met jou betekende een hoge dosis aan enthousiasme voor wetenschap en streven naar perfectie tot in details; vooral zal ik de gevleugelde woorden 'nog één test..' niet snel vergeten. Het HPV project heeft daardoor lang geduurd; des te meer gelegenheid voor leuke gesprekken en gezelligheid die ongetwijfeld nog vaak zullen voorkomen.
Mijn mede auteurs, Saskia Konings, Hanneke Kranenborg en Eveline van Beeck Calkoen, toen nog coassistenten, nu artsen waaronder twee KNO-artsen in opleiding, dank voor jullie belangrijke en gedegen bijdrage aan dit proefschrift. Daarnaast was het voor mij een leerzame ervaring om jullie te mogen begeleiden. Dr. C.H. Terhaard, beste Chris, het was iedere keer even zoeken voordat ik je kamer vond, maar dat werd beloond met constructieve opmerkingen waardoor het 'survival' stuk vele malen overzichtelijker en leesbaarder werd. Veel dank! Dr. E.J.M. Speel, beste Ernst-Jan, dank voor jouw specifieke expertise en bijdrage. Ik heb goede hoop dat

we ons 'HPV' project in de toekomst kunnen voortzetten. Dr. P. Roepman, beste Paul, ondanks je zeer drukke baan, vond je tijd om een en ander voor mij uit te zoeken (met name op het laatste moment..) waarvoor mijn hartelijke dank.
Op de afdeling pathologie hebben veel mensen mij op verschillende manieren geholpen. Marina Vervaasdonck, Sabrina Elshof, Gladys Mancipe, Domenico Castigliego, Dick van Wichen, en andere medewerkers; jullie lab heb ik enige tijd onveilig gemaakt door gebrek aan specifieke vaardigheden en te vaak afgaande pieper. Desondanks heb ik door jullie gastvrijheid en hulp vele coupes gesneden, gewassen, gekleurd, bekeken, gefotografeerd, wat uiteindelijk heeft geleid tot een deel van dit proefschrift. Marja van Blokland, veel dank voor je grote inzet voor de HPV studie. Jurryt van der Gugten, André Huisman en professor van Diest, hartelijk dank voor jullie bijdrage aan het 'CLSM' project. Het is misschien geen 'hoofdstuk' geworden, maar jullie inzet en precisie waren leerzaam en inspirerend.
Verder zou ik graag alle (oud)arts-assistenten, stafleden en medewerkers van de afdeling KNO-heelkunde van het UMCU, Gelre ziekenhuizen Apeldoorn en Kennemer Gasthuis willen danken voor hun interesse en collegialiteit in de afgelopen jaren.
Mijn paranimf Karin Oomen, als 'laatsten van Hordijk' mochten we door van de 'wetenschapscommissie'; dat schiep een band. Maar vooral je bescheidenheid, groot gevoel voor collegialiteit en gezelligheid waren een grote steun in de afgelopen jaren en hebben geleid tot onze vriendschap. Ik wens je veel succes met jouw aankomende promotie en andere avontuurlijke plannen. Ik kijk uit naar jouw boekje!
Mijn paranimf, Marloes Bongers en ik kan in dit kader niet anders dan onze gezamelijke vriendin Anne Brecht Francken ook te noemen. Jullie vriendschap is van bijzonder grote waarde; ook voor dit onderzoek. Inspirerend waren jullie vele pagina's tellende en kwalitatief zeer goede proefschriften (waaronder één cum laude) en trots heb ik naast jullie gestaan op die bewuste dagen. Dat waren beslissende momenten, ook voor mijn onderzoek. Jullie streven naar het beste in al jullie bezigheden is altijd stimulerend. Ik zou willen zeggen lang leve (Haaf) weekenden met de kinderen en de mannen!
Natuurlijk was ook dit project niet mogelijk geweest zonder de steun, interesse en liefde van alle vrienden en vriendinnen en familie. Ik beschouw mezelf als een zeer bevoorrecht mens met zo'n sterke achterban! Lieve familie VVVB, lieve Hanneke en Paul, Susanne, Anton en Lex, Alexandra en Wytse, wat geniet ik altijd van jullie humor, creativiteit, verstandigheid en gezelligheid; jullie zijn mijn Nederlandse wortels. Lieve familie F, lieve Pary en Hasan, Farshad, Carolijn en Victor, Farnaz en Jelle, we hebben altijd alles voor elkaar over en dat is een grote rijkdom; eigenlijk kan geen een project ooit echt mislukken met jullie aan mijn zijde. Liefste Piet, "dit is jouw werk en je moet er van houden" om maar één verstandige opmerking van jou in dit verband te noemen. Leven met jou was al een feest, en inmiddels onwaarschijnlijk meer door de komst van onze dochter, Sophia.

Curriculum Vitae

Curriculum Vitae

Farzaneh van Voorst van Beest-Farshadpour werd op 11 januari 1976 geboren te Teheran, Iran. Op haar elfde verhuisde ze met haar familie naar Nederland, waar ze in 1994 haar VWO diploma aan 't Hooghe Landt college te Amersfoort behaalde. Ze werd nadien 2 keer uitgeloot voor de studie geneeskunde; het eerste jaar maakte ze een wereldreis, het tweede jaar begon ze met de studie geneeskunde in Diepenbeek, België. In 1996 kon ze aan de Rijks Universiteit Groningen met geneeskunde starten waar ze onder andere een wetenschappelijke stage begeleid door professor H.J. Hoekstra afrondde en op 30 januari 2003 haar artsexamen behaalde. In hetzelfde jaar begon zij met de opleiding KNO-heelkunde in het Universitair Medisch Centrum Utrecht onder professor G.J. Hordijk. Tevens werd gestart met het traject van het onderhavige proefschrift. Een deel van de opleiding werd gevolgd in Gelre Ziekenhuizen lokatie Apeldoorn onder dr. P.P.G. van Benthem en dr. Tj.D. Bruintjes. Momenteel is ze als KNO-arts werkzaam in het Kennemer Gasthuis te Haarlem. Zij is getrouwd met Pieter Paul van Voorst van Beest; zij hebben samen een dochter, Sophia.