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MATTI PUKKILA

Prognostic Factors in Pharyngeal Squamous Cell Carcinoma With Special Reference to Cell Adhesion, p53 Protein Expression and Nitric Oxide Synthase Expression

Doctoral dissertation

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ABSTRACT

Pharyngeal squamous cell carcinoma (PSCC) is a rare disease generally considered to be associated with smoking and alcohol overuse. Advancements in staging, histological diagnostics and treatment have not succeeded in elevating the low survival rates related to this disease. PSCCs are heterogenous tumors that can not be accurately classified by the present means and require new methods. Catenins are cytosolic adhesion proteins which also act as signal transducers. p53 is a nuclear phosphoprotein regulating gene transcription, DNA synthesis and repair, cell cycle coordination, apoptosis and angiogenesis. Inducible nitric oxide synthase (iNOS) is capable of generating substantial quantities of NO for prolonged time periods. It is expressed mainly in activated macrophages, but also in several other cell types as well as malignant tumors. Versican is a large extracellular matrix (ECM) proteoglycan associated in numerous biological processes. In the present work, the immunohistochemical expression of α-, β- and γ-catenins, p53 protein, inducible nitric oxide synthase (iNOS), and versican were studied in a PSCC cohort of 138 patients. The expression patterns were related to salient clinical and histological data, to each other, and patient survival. Of particular interest was their potential prognostic significance in PSCC.

At the time of diagnosis, the median age of the patients was 64 years, 105 (76%) were males and the vast majority were diagnosed with advanced disease: in 94 cases (68%), stage III or IV had been attained. In 88 patients (64%), PSCC originated in the oropharynx. The most prevailing treatment was radiotherapy alone (86 patients; 62%). The median disease-specific survival in the cohort was 22.3 (95% CI 15.4-29.2) months and the disease-specific survival rates for 3 and 5 years were 40% (95% CI 32-49) and 37% (95% CI 29-46), respectively.

Reduced membranous catenin expression was seen in 57 (49%), 32 (28%) and 30 (26%) tumors for α-, β- and γ-catenins, respectively. Reduced γ-catenin expression was significantly associated with poor histological tumor differentiation. Nuclear β-catenin was present in 27 (23%) tumors. The iNOS staining was mostly restricted to tumor cells. The obtained iNOS score was low in 57 (49%), but high in 61 (51%) tumors. iNOS scores were significantly lower in the largest (T4) tumors. A high iNOS score was significantly associated with a high nuclear p53 expression index and positive cytoplasmic p53 expression. Heterogeneous nuclear p53 expression was seen in all tumors and was accompanied by cytoplasmic tumor cell staining in 56 (46%) cases. Nuclear p53 overexpression was significantly more common in hypopharyngeal tumors. In carcinoma, strong stromal versican expression was graded high in 59 (50%), and low in 59 (50%) primary tumors. Cytoplasmic versican staining in carcinoma cells was present in 9 (8%) tumors. The strong stromal versican expression in the local metastases was statistically significantly more common than the stromal versican expression in primary tumors, and strong stromal versican staining was also more common in less advanced tumors. In the multivariate analysis of disease-specific survival, only the poor general condition of the patients, advanced stage of the disease, and nuclear β-catenin expression were independent predictors of unfavorable disease outcome in these patients.

In conclusion, nuclear β-catenin expression seems to be a potential new prognostic factor in PSCC.

National Library of Medicine Classification: QZ 365, WV 410
Medical Subject Headings: carcinoma, squamous cell; catenin; head and neck neoplasms; nitric-oxide synthase; pharyngeal neoplasms; prognosis; protein p53; proteoglycans; retrospective studies

To Satu and Maria

"Consummatum est"

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I wish to thank my mother Annikki Pukkila and my deceased father Tapani Pukkila for all the love and care over the years. Mother, it is a wonderful miracle and blessing that you are here sharing this day with me. I also want to express my deepest gratitude to my sister Leena and my brothers Jukka and Heikki for the shared days of happiness and sorrow.

I dedicate this book to the women of my life, Satu and Maria. Maria, daddy could have finished this work a bit earlier if you did not come and captivate us, but the light you have brought into our lives exceeds all other aspects! Beloved, Satu. My thesis would not have been finished without your love, patience and support during all these years. We made it. And now, let's get into something completely different!

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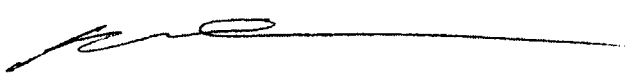

ABBREVIATIONS

ABC	avidin-biotin peroxidase complex
APC	adenomatous polyposis coli
ATM	p53 regulatory kinase
ATR	p53 regulatory kinase
Bax	oncogene, proapoptotic
Bcl-2	B-cell lymphoma-2 protein
BRCA1	tumor suppressor gene
CCND1	cyclin D1 gene
CD44	cell surface receptor glycoprotein
cdk	cyclin dependent kinase
cdki	cyclin dependent kinase inhibitor; cki
CI	confidence interval
CT	computed tomography
cyclin D1	cyclin regulatory protein D1
cyclin D1/PRAD-1	oncogene or proto-oncogene
DSS	disease specific survival
E2F	transcription factor
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase; NOS3
FGF	fibroblast growth factor
G1	globular end domain
G3	globular end domain
G1 phase	cell cycle phase G1
G1/S	cell cycle transition from G1 to S phase
G2 phase	cell cycle phase G2
GAG	glycosaminoglycan
Gr	grade (1-3), histopathological differentiation degree
GSK-3β	glycogen synthase kinase-3β
Gy	gray = joule/kg, a dose unit of absorbed ionizing radiation energy per mass of absorbing material
HA	hyaluronan; former hyaluronate or hyaluronic acid
HNC	head and neck cancer
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
hst-1	fibroblast growth factor 4 (FGF4)
IHC	immunohistochemistry
iNOS	inducible nitric oxide synthase, NOS2
int-2	fibroblast growth factor 3 (FGF3), KAI1
Karnofsky	Karnofsky performance status (0-100)
kD	kiloDalton
LEF/TCF	nuclear transcription factor
LSCC	laryngeal squamous cell carcinoma
M	M class; presence of distant metastases, M0-1
MDM2	negative p53 regulator
MMP	matrix metalloproteinase
MVD	microvascular density
N	N class; regional neck lymph node status, N0-3
N+	local lymph node metastasis present in the neck
NO	nitric oxide
NOS	nitric oxide synthase

nNOS	neural nitric oxide synthase, NOS1
OS	overall survival
p	p-value for statistical significance
$p14^{ARF}$	p53 regulatory kinase
$p15^{INK4b}$	cyclin-dependent kinase inhibitor
$p16^{INK4a}$	cyclin-dependent kinase inhibitor
$p21^{WAF1}$	cyclin-dependent kinase inhibitor, cited also as CIP1 or SDI1
$p27^{KIP1}$	cyclin-dependent kinase inhibitor
p53	p53 protein
PDGF	platelet derivative growth factors
pN	pathological N class
pRB	retinoblastoma protein
PRGF-1	receptor tyrosine kinase
PSCC	pharyngeal squamous cell carcinoma
pT	pathological T class
pTNM	pathological TNM classification
RB	retinoblastoma gene
RT	radiotherapy (external)
S	stage grouping based on TNM-classification; SI-IV
SCC	squamous cell carcinoma
SD	standard deviation
SND	selective neck dissection
T	T class, primary tumor size; T1-4
TGF-α	transforming growth factor receptor alfa
TGF-β	transforming growth factor receptor beta
TGF-α	transforming growth factor α
TNM	tumor-node-metastasis classification
TP53	p53 gene
TSG	tumor suppressor gene
v2	CD44 isoform
VEGF	vascular endothelial growth factor
wnt	regulatory glycoprotein family or signaling pathway
wt	wild-type, normal protein

LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I Pukkila MJ, Virtaniemi JA, Kumpulainen EJ, Pirinen RT, Johansson RT, Valtonen HJ, Juhola MT, Kosma V-M. Nuclear β catenin expression is related to unfavourable outcome in oropharyngeal and hypopharyngeal squamous cell carcinoma. J Clin Pathol 2001;54:42–7.

II Pukkila MJ, Kumpulainen EJ, Virtaniemi JA, Johansson RT, Halonen PM, Kellokoski JK, Kosunen AST, Nuutinen J, Kosma V-M. Nuclear and Cytoplasmic p53 Expression in Pharyngeal Squamous Cell Carcinoma: Prognostic Implications. Head Neck 2002;24:784-91.

III Pukkila MJ, Virtaniemi JA, Kumpulainen EJ, Johansson RT, Halonen PM, Kellokoski JK, Kosunen AST, Nuutinen J, Kosma V-M. Inducible Nitric Oxide Synthase Expression in Pharyngeal Squamous Cell Carcinoma; Relation to p53 Expression, Clinicopathological Data and Survival. Laryngoscope 2002;112:1084-8.

IV Pukkila MJ, Kosunen AST, Virtaniemi JA, Kumpulainen EJ, Johansson RT, Kellokoski JK, Nuutinen J, Kosma V-M. Versican Expression in Pharyngeal Squamous Cell Carcinoma; an Immunohistochemical Study. J Clin Pathol 2004;57:735–9.

This thesis also includes previously unpublished data.

CONTENTS

1. INTRODUCTION

Head and neck cancer (HNC) is the sixth most common malignancy world wide.[1] In the European Union, HNC covers ten percent of all malignant tumors in men and two percent in women.[2] One-fifth of these tumors originate in the oropharynx or hypopharynx. Histologically, most of them (over 90%) are squamous cell carcinomas (SCC), whereas mucoepidermoid carcinomas, lymphomas, and other tumor types are less frequently encountered.[1, 2] The annual incidence rate of pharyngeal cancer (lymphomas excluded) in Finland is about 1 per 100 000, which at present implies around 50 new cases every year in the whole country.[3] The main risk factors for pharyngeal squamous cell carcinoma (PSCC) are suggested to be tobacco and alcohol.[1, 4, 5] PSCC is typically diagnosed late with advanced disease.[6, 7] PSCC is treated with radiotherapy (RT), surgery and chemotherapy.[8] In the early stages, surgery and RT are equally effective, but in more advanced cases, the combination of surgery and RT has been common practice.[9-12] However, based on recent promising results, chemoradiotherapy may soon become the "golden standard" in treating PSCC.[13]

The prognosis for the head and neck squamous cell carcinoma (HNSCC) patients varies substantially, depending on the tumor site.[13] It is poorest in patients with hypopharyngeal, oral, and oropharyngeal tumors.[14, 15] The main clinical tool for assessing prognosis in PSCC is the TNM grading system. Even though histological diagnostics, staging and treatment of PSCC have progressed over the last decades, long-term survival rates have improved only slightly, and the survival rates in PSCC have remained low.[1] This is the result of both late diagnosis and characteristic heterogenic, unpredictable biological tumor behavior among these tumors.[16] To distinguish tumors more accurately, both for setting the prognosis and deciding upon appropriate treatment, new biological markers are needed in addition to the present clinical signs.[16]

In the present work, the immunohistochemical expression of α-, β- and γ-catenins, as well as inducible nitric oxide synthase (iNOS), p53 protein, and versican were studied in a PSCC cohort. The expression patterns were related to salient clinical and histological data, to each other, and patient survival. Of particular interest in this study was their potential prognostic value in PSCC. The nature of the neoplastic diseases of the nasopharynx differs considerably from that of the other pharyngeal regions.[17] Therefore, the nasopharynx is not discussed in this thesis.

2. REVIEW OF THE LITERATURE

2.1. Pharyngeal squamous cell carcinoma (PSCC)

2.1.1. General considerations

The anatomical region defined as the pharynx includes the nasopharynx, oropharynx and hypopharynx (Figure I). Pharynx is a part of the upper aerodigestive tract, which acts as a conduit for respiration, voice, fluids, and foods. Additionally, it contains immunologically important structures. The intricate anatomy, delicate and vulnerable functions, complex spatial relationships, as well as the nature and behavior of the squamous cell carcinoma form a challenging entity for all the professionals involved in the treatment of PSCC.[17]

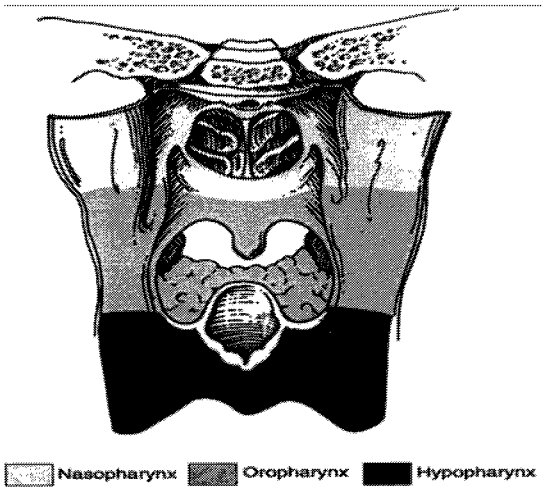

Figure I. Pharyngeal subsites, posterior view. Modified from Dicker, A. et al. Oropharyngeal cancer. In: Head and neck cancer; a multidisciplinary approach. Harrison, L.B., et al., editors. Lippincott-Raven publishers. Philadelphia 1999. p. 446.

2.1.2. Surgical anatomy of the pharynx

Oro- and hypopharyngeal subsites relevant to this thesis are presented in table 1 and in figures I and II.[17, 18] Of the several lymph node regions in the head and neck, five deep neck regions are strategic for PSCC (Figure III).[19] Clinically important are also the lateral and medial retropharyngeal, paratracheal, as well as paraesophageal and paratracheal lymph nodes.[20] In PSCC, the lymph nodes at levels II–IV are at the highest risk for metastasis.[21, 22]

Table 1. Oro- and hypopharyngeal subsites

Site
Oropharynx
1. Anterior wall (glosso-epiglottic area)
1.1. Base of the tongue (posterior to the vallate papillae or posterior third)
1.2. Vallecula
2. Lateral wall
2.1. Tonsil
2.2. Tonsillar fossa and tonsillar pillars
2.3. Glossotonsillar sulcus
3. Posterior wall
4. Superior wall
4.1. Inferior (anterior) surface of soft palate
4.2. Uvula
Hypopharynx
1. Pharyngo-oesophageal junction (postcricoid area, anterior wall): extends from the level of the arytenoid cartilages and connecting folds to the inferior border of the cricoid cartilage, thus forming the anterior wall of the hypopharynx
2. Pyriform sinus: extends from the pharyngo-epiglottic fold to the upper end of the esophagus. It is bounded laterally by the thyroid cartilage and medially by the hypopharyngeal surface of the aryepiglottic fold and the arytenoid and the cricoid cartilages.
3. Posterior pharyngeal wall: extends from the superior level of the hyoid bone (or floor of the vallecula) to the level of the inferior border of the cricoid cartilage and from the apex of one pyriform sinus to the other.

Head and neck tumours. Modified from. TNM classification of the malignant tumours. 5th edition. Sobin, L.H. and Wittekind, Ch., editors. Wiley-Liss, Inc. New York 1997

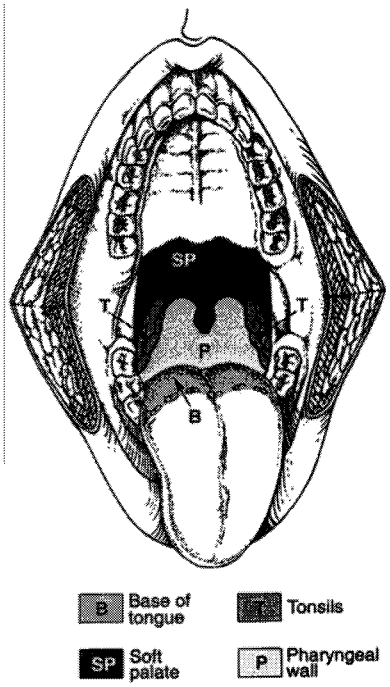

Figure II. Oropharyngeal subsites, anterior view. Modified from Dicker, A. et al. Oropharyngeal cancer. In: Head and neck cancer; a multidisciplinary approach. Harrison, L.B., et al., editors. Lippincott-Raven publishers. Philadelphia 1999. p. 446.

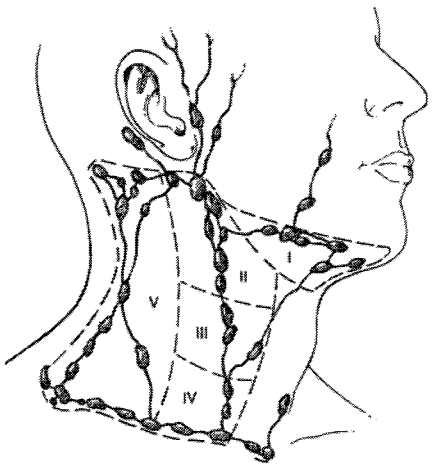

Figure III. Lymph node regions of the neck, lateral view. Modified from Dicker, A. et al. Oropharyngeal cancer. In: Head and neck cancer; a multidisciplinary approach. Harrison, L.B., et al., editors. Lippincott-Raven publishers. Philadelphia 1999. p. 447.

2.1.3. Epidemiology and risk factors

HNC is the sixth most common cancer worldwide.[1, 23] In the European Union, 10% of all malignant tumors in males and two per cent in females originate in the head and neck region.[2] The estimated age-standardized annual incidence of HNC in Europe is 35.4 per 100,000 for men and 4.5 per 100,000 for women, respectively.[2] One-fifth of these tumors originate in the oro- or hypopharynx. Oropharyngeal localization is twice as common as hypopharyngeal.[1] In Finland, the absolute annual number of new pharyngeal cancers seems to be slowly rising. The age-adjusted incidence rates of pharyngeal cancer, however, have remained fairly constant between years 1975 and 1999 (1.2-1.5 per 100,000 for males; 0.4-0.7 for females).[3] At the time of diagnosis, the vast majority of patients are older than 45 years.[3, 24]

The major risk factors of PSCC are tobacco use and alcohol drinking, which independently affect but together have a multiplicative effect.[1, 4, 5, 12] Moreover, human papillomaviruses (HPVs), especially types 16, 18 and 33, have been suggested to play a role in promoting PSCC.[25-28] The clinical picture of HPV associated HNSCC differs substantially from HPV-negative carcinomas as it is mostly seen in the tonsils and base of the tongue, and is associates with wild-type (wt) p53, p16 overexpression, increased proliferation (i.e., proliferation marker Ki-67 overexpression) and a better prognosis.[27, 29, 30]

2.1.4. Histopathology and grading

The pharynx is covered with a mucous membrane, with ciliated pseudostratified columnar or, as seen almost exclusively in the oro- and hypopharynx, with stratified squamous epithelium.[17] More than 90% of HNCs, the pharynx included, are SCCs originating from the epithelium of the mucosa.[1, 17]

The WHO histological grading of HNSCC has been presented by Shanmugaratnam.[31] Based on cellular structure, differentiation, nuclear polymorphism, and frequency of mitoses the assessable tumors are classified into four groups: well differentiated (Gr1), moderately differentiated (Gr2), poorly differentiated (Gr3), and undifferentiated (Gr4). Occasionally, the histological grade cannot be determined (GrX).

2.1.5. Presentation and diagnosis

PSCC often presents at an advanced stage.[6, 7] Frequent signs and symptoms of PSCC described in the literature include local mass (pharynx or neck), pain, numbness, dysphagia or odynophagia, aspiration, sudden change in denture, otalgia, bleeding, voice changes and hoarseness, speech difficulty (articulation or sound production), dyspnoea, airway symptoms, and weight loss.[12, 32, 33] Ulceration, bleeding, or pain often appear late and generally allude to advanced disease.[12, 32, 33] In the pharynx, pain results from glossopharyngeal or vagal irritation and may often refer to structures outside the pharynx, mostly the ear.[34] Shortening of the time interval between first perception of PSCC symptom and seeking medical consultation has been suggested as a means for improving the prognosis of PSCC.[35]

In most cases, complete clinical examination of the upper aerodigestive tract reveals the primary tumor and gives preliminary information on the tumor stage. Imaging with computed tomography (CT), magnetic resonance imaging (MRI), and ultrasonography (US) combined with fine needle aspiration, supplement clinical investigation.[12, 32, 33] In some patients careful panendoscopic examination under general anesthesia is recommended to assess the precise tumor status, and to exclude possible second primary tumors.[12, 33, 36]

In PSCC, distant metastases are diagnosed in 10% at presentation and appear later in about 5-20% of the patients.[37-39] Two-thirds of these are pulmonary, followed by bone (20%), liver (10%), skin, mediastinum and bone marrow.[40] Preoperative chest X-ray should be controlled, but in patients presenting with a large tumor with a high risk for pulmonary metastasis preoperative chest CT is warranted.[40] Routine screening of other possible metastatic sites is not indicated in PSCC.[40]

2.1.6. Staging

The TNM classification was originally developed by Pierre Denoix.[18] Since the 1940's it has been processed and improved to become an effective prognostic tool for assessing carcinomas originating in various locations. In this classification T characterizes the extent of the primary tumor, N describes the extent of the regional lymph node metastasis and M the presence of distant metastases. The extent of the disease is expressed as numbers after the three letters. In uncertain cases, a lower category must be selected. In all cases,

Table 2. TNM and stage; clinical classification for PSCC

TNM class and stage; clinical classification for PSCC				
T – Primary Tumor				
Oropharynx				
T1	≤2 cm			
T2	>2 to 4 cm			
T3	>4 cm			
T4	Invades adjacent structures			
Hypopharynx				
T1	≤2 cm and limited to one subsite			
T2	>2 to 4 cm or more than one subsite			
T3	>4 cm or with larynx fixation			
T4	Invades adjacent structures			
N – Regional Lymph Nodes (oropharynx and hypopharynx)				
N0	No regional lymph node metastasis			
N1	Ipsilateral single ≤3 cm			
N2	N2a	Ipsilateral single >3 to 6 cm		
	N2b	Ipsilateral multiple ≤6 cm		
	N2c	Bilateral or contralateral ≤6 cm		
N3	>6 cm			
Note:	Midline nodes are considered ipsilateral nodes			
M – Distant Metastasis				
MX	Distant metastasis can not be assessed			
M0	No distant metastasis			
M1	Distant metastasis exist			
Stage Grouping (oropharynx and hypopharynx)				
Stage 0	Tis	N0	M0	
Stage I	T1	N0	M0	
Stage II	T2	N0	M0	
Stage III	T1	N1	M0	
	T2	N1	M0	
	T3	N0 - N1	M0	
Stage IVA	T4	N0 - N1	M0	
	any T	N2	M0	
Stage IVB	any T	N3	M0	
Stage IVC	any T	any N	M1	

Head and neck tumours. Modified from. TNM classification of the malignant tumours. 5th edition. Sobin, L.H. and Wittekind, Ch., editors. Wiley-Liss, Inc. New York 1997

histological diagnosis must be confirmed before classification. The TNM and stage classification in PSCC are summed in table 2.

Clinical classification (TNM or cTNM) is based on data obtained prior to treatment by physical examination, imaging, endoscopy, biopsy, surgical exploration, and other necessary examinations. For pathological classification (pTNM), clinical data are complemented and adjusted according to knowledge obtained through surgery and with pathological investigation.[18] Various stages are based on T, N and M and/or pT, pN and pM categories.[18]

The weak point of TNM classification and staging lies in its poor prognostic validity in advanced-stage groups: while in stages I, II and III a reasonable number of classes are

defined, stage IV covers a wide range of categories. This has inspired development of supplementary classifications based on T and N data.[41, 42] This demonstrates the enduring need for reassessment and revision of TNM-based staging systems.[43] Despite its limitations, traditional TNM classification is a valid and widely used tool in clinical work. Molecular investigations of surgical resection margins and susceptible mucosal areas may give important information for assessing the risk of both local and distant recurrence, and for determining the cases which would benefit most from further surgery or adjuvant therapy.[44-47]

2.1.7. Treatment

There is no universally accepted treatment policy or convention for PSCC. Prevailing practice varies between different institutions, even in Finland. The main individual treatment modalities used are RT, surgery, and chemotherapy.[8] In early stages, surgery and RT are equally effective, but for treating more advanced tumors, the combination of surgery and RT is advisable.[9-12] Chemotherapy in combination with RT has been demonstrated to be effective at least for advanced SCC of the larynx, though accumulating evidence suggests good treatment results even with other HNSCCs, including those of the pharynx.[12, 48-55] According to recent reports, especially in advanced HNSCCs, concomitant chemoradiation seems to be a more effective treatment modality than RT alone.[56, 57]

In recent decades, surgical treatment of the neck in HNSCC has shifted towards less radical approaches.[58] The selective neck dissection (SND) has been shown to be suitable for both disease staging and treatment in N0 neck,[59, 60] as well as for treatment of N+ neck.[58] Moreover, the present data do not indicate that more radical surgery (radical or modified radical neck dissection) would yield any advantages compared to SND.[58] Optional SND guided by sentinel node biopsy might possibly represent the next step towards even more conservative surgical treatment.[61, 62] RT after neck dissection is recommendable for patients diagnosed with positive regional nodes, T3-4 primary tumor, macroscopically verified extracapsular growth in lymph node, and recurrent disease in previously unradiated patients.[63, 64]

Gene therapy techniques directed against the specific molecular causes of cancer are the therapeutic elements expected to brighten the so far murky prognosis of HNSCCs.[65-67] Different vectors (viral and nonviral) have been applied in HNSCC to transfer tumor suppressor genes, suicide genes, as well as immunologic and other types of genes into

cancer cells *in vitro* and *in vivo*.[68] In the most promising clinical trials, wt p53 gene introduction[69] or compounds blocking epidermal growth factor receptor (EGFR) overexpression have been studied.[70]

2.1.8. Follow-up and survival

The key purpose of follow-up in HNSCC is to provide early detection of loco-regional relapse and to reveal second primary tumors, even though the effect of this on long-term prognosis has been questioned.[71] Also evaluation of treatment results and sequelae as well as positive psychosocial effects are important.[72]

The prospects of HNSCC patients vary substantially, depending on the tumor site. It is poorest in patients with hypopharyngeal, oral, and oropharyngeal tumors.[14, 15] In Finland, the five-year relative survival rates of pharyngeal cancer, although slowly rising, have still remained around 50 percent.[73] Between 1975 and 1999 the mean age-adjusted mortality rates from pharyngeal cancer in Finland were 0.9 and 0.3 for males and females per 100,000, respectively.[3] In the study comprising all histological variants of pharyngeal malignancy, median disease-specific survival times of only 28 and 18 months were found for oro- and hypopharyngeal cancers, respectively.[74] Correspondingly, the five-year survival probabilities retrieved from a large cohort were 36% for the oropharynx and 31% for the hypopharynx.[15] Five-year overall survival rates from other large series also remain quite low: 33% for the hypopharynx and 48% for the oropharynx.[7, 10]

2.2. Clinical prognostic factors

2.2.1. Patient-related factors

Many distinct and disparate variables have been proposed as factors affecting prognosis and survival in HNSCC. These clinical prognostic factors can be categorized into patient-, tumor-, and treatment-related features. Established patient-derived prognostic factors in PSCC are age, sex, and general condition of the patient. Low pre-treatment hemoglobin concentration in association with RT is also a significant patient-related prognostic marker

Table 3. Karnofsky performance status

Definition	*%*	*Criteria*
Able to carry on normal activity and to work. No special care is needed.	100	Normal; no complaints; no evidence of disease.
	90	Able to carry on normal activity; minor signs of symptoms of disease.
	80	Normal activity with effort; some signs or symptoms of disease.
Able to work. Able to live at home, care for most personal needs. A varying amount of assistance is needed.	70	Cares for self. Unable to carry on normal activity or to do active work.
	60	Requires occasional assistance, but is able to care for most of his needs.
	50	Requires considerable assistance and frequent medical care.
Unable to care for self. Requires equivalent of institutional or hospital care. Disease may be progressing rapidly.	40	Disabled; requires special care and assistance.
	30	Severely disabled; hospitalization is indicated although death not imminent.
	20	Very sick; hospitalization necessary; active supportive treatment necessary.
	10	Moribund; fatal processes progressing rapidly
	0	Dead

in PSCC suggesting a dismal result.[6, 75] In general, the younger the patient is at presentation, the better the prognosis.[6, 15, 75-77] Only in solitary studies have the results has been the opposite,[78] or age has remained insignificant in prognostic validation.[79, 80] In most series, female gender is also favorable, even though quite often gender does not associate with prognosis in PSCC.[15, 79-81] Moreover, in a large HNSCC series treated with RT, including 174 PSCC patients, male gender favored a higher survival rate.[82]

The general condition of the patient at the time of diagnosis, as defined by Karnofsky performance status (Table 3),[83] is a common prognostic marker in PSCC that shows consistent results: a good general condition is invariably a good prognostic sign.[75, 79, 84-86] Nonetheless, when including only advanced cases, its predictive power may be lost.[87]

2.2.2. Tumor-related factors

Tumor-related clinical and pathological prognostic markers in PSCC include tumor site[15] and subsite,[6, 7, 88] as well as T,[6, 15, 75, 76, 84-86] N,[15, 75, 76, 80, 85, 89] and M status,[15] or stage.[7, 90] Categorically, higher TNM status or stage are signs of a less favorable outcome. In PSCC, a hypopharyngeal site or posterior oropharyngeal wall and pyriform sinus subsites are unpropitious tumor locations. Additional unfavorable prognostic variables reported in the

literature include large tumor size,[6, 78] positive pT and pN classes,[78, 80, 91] high pathological stage,[89] and positive pathological resection margins.[80, 91] Detected extracapsular tumor growth in regional lymph nodes may also be a devious sign,[91, 92] though the association with disease outcome remains somewhat questionable.[93] In a predictive sense, the degree of histological differentiation of the tumor is confusing in PSCC, as in most HNSCCs, possibly due to overruling by other more powerfull factors.[88] It may remain negligible,[78, 79, 81, 88] or better prognosis may associate with either better[82, 86] or poorer differentiation.[85] Finally, both lymphatic tumor invasion,[78] as well as, quite recently, high intratumoral lymph vessel density[94] have been described to allude towards reduced survival in PSCC.

2.2.3. Treatment-related factors

The different curative treatment modalities, including RT, surgery, and adjuvant chemotherapy, also have their own prognostic references. In RT, treatment time,[78, 80] duration of the RT breaks,[80] total radiation dose,[75, 78, 87] average weekly radiation dose,[80] and fractioning protocol,[95, 96] whereas in surgery pT,[91] pN,[78, 80] and incomplete surgical resection with macroscopic recidual tumor have been implicated.[80, 97] Combination of concomitant chemotherapy with RT has also been shown to improve prognosis in HNSCC.[56, 57]

2.3. Molecular prognostic markers

2.3.1. General considerations

Over the last decades, histological diagnostics, staging and treatment of HNSCC have improved. Long-term survival rates, however, have shown only exiguous improvement, leaving HNSCC still among the cancers with dubious and often dismal prognosis.[1] The unpredictable nature of HNSCC is, at least to some extent, due to the characteristic heterogenic biological tumor behavior among these tumors.[16]

To be able to discriminate tumors more accurately for setting prognosis and deciding upon treatment, independently of the present clinical signs used (stage, site, N and M status), a search for new biological markers is crucial.[16] A solid theoretical basis for this lies in the HNSCC molecular development model, referred to by the apropos term "field

cancerization": a progressive multistep model starting from epithelial hyperplasia and dysplasia and leading eventually to invasive carcinoma with special genetic and molecular events occurring at each phase.[29, 98, 99] This has been recently complemented by the transcriptional HNSCC progression model of Ha et al.[100] Genetic alterations are followed by malignant phenotype characteristics including unresponsiveness to proliferation and differentiation arrest signals, incessant proliferation, escape from apoptosis, invasion and angiogenesis.[101, 102] These carcinogenesis and tumor progression associated incidents have been disclosed by molecular cancer biology and can be roughly separated into oncogene activation, tumor suppressor gene (TSG) inactivation, immortalization, invasion, and metastasis development.[103] This categorization is somewhat arbitrary and complex interactions between various routes are common.

Potential HNSCC related oncogenes include *ras*, *myc*, *int-2*, *hst-1*, *cyclin D1/PRAD-1*, *cyclin E*, *Bcl-2* and *Bax*, and *EGFR*.[16, 66, 101, 103, 104] Implicated HNSCC associated tumor suppressor genes are retinoblastoma (*RB*), *TP53*, $p21^{WAF1}$, $p16^{INK4a}$, and transforming growth factor receptors (*TGF-α* and *-β*).[16, 66, 101, 103, 104] Moreover, suggested participatory cell-cell and cell-matrix interaction mediators include members of the immunoglobulin-like super family, cadherins and catenins, integrins, receptor tyrosine phosphatases, selectins, hyaluronan (HA), HA receptors (e.g., CD44) and some matrix metalloproteinases (MMPs).[16, 103, 105] Further molecular markers implicated in HNSCC are angiogenesis-related factors[104] and nitric oxide synthases (NOSs).[106, 107]

2.3.2. Oncogenes

Proto-oncogene are normal cellular genes that govern cellular growth and proliferation through intracellular signaling pathways. Upon mutation or activation by another mechanism, the proto-oncogene is transformed into an oncogene. In carcinogenesis, oncogenes may function as growth factors, growth factor receptors, signal transducers, cell cycle regulators, or as apoptosis inhibitors.[108]

The cell cycle consists of four consecutive phases: the preparation phase G1, DNA replication phase S, preparation phase G2, and the mitosis phase M, which is again followed by the G1 phase. This cycle is controlled at various points by both external (growth factors, cellular adhesion, and stress) and internal signals (DNA damage, mitotic spindle emergence, and cell cycle synchronization). At various points, cell cycle arrest, protein

degradation, or apoptosis may be induced. This cell cycle control is one of the key points affected in malignant cells, involving proliferation, differentiation, and apoptosis.[109]

2.3.2.1. Cyclin D1/PRAD-1

The cyclin D1 proto-oncogene belongs to the cyclin regulatory protein family. Cyclin-dependent functions are mediated through interactions with cyclin-dependent kinases (cdks) that control cell cycle transitions.[66] Various growth factors and attachment of cells onto the extracellular matrix induce cyclin D1 expression.[109] It associates with cdk4 and cdk6, but in cell cycle phase G1 the main partner of cyclin D1 is cdk4.[103] During the G1 phase, the retinoblastoma protein (pRB) is phosphorylated by cyclin D1-cdk4 complex resulting pRB dissociation and release of transcription factor E2F, which eventually allows the cell cycle to shift through the G1/S checkpoint.[66, 109-111] E2F is also involved in DNA synthesis during the S phase, where it is capable of inducing cdk inhibitors (cdkis) $p16^{INK4a}$, $p21^{WAF1}$, $p27^{KIP1}$ and p53, which then inhibit cell cycle progression.[109]

Cyclin D1 gene, *CCND1*, has been located in the 11q13 chromosomal region (chromosome 11, long [q] arm, region 1, band 3), together with other presumed proto-oncogenes. These include, in addition to *CCND1*, *int-2* (fibroblast growth factor 3, FGF3), *hst-1* (FGF4), and *EMS1*.[16, 112] The proto-oncogenes *int-2* and *hst-1* belong to the FGF family and upon activation induce cell proliferation and angiogenesis.[112] The 11q13 chromosome region has been shown to be frequently amplified in head and neck carcinomas, but only cyclin D1 has shown consistent overexpression.[103] Both gene amplification and induced protein expression have been demonstrated in HNSCC,[66] and both have also been suggested to be unfavorable prognostic signs in carcinomas of this region.[66, 113, 114] The *cdk4* gene has also been reported to be overexpressed in laryngeal SCC (LSCC), where simultaneous overexpression of both proteins resulted in poorest survival.[103]In the larynx, cyclin D1 overexpression correlated strongly with cyclin gene amplification, thus suggesting this as the main mechanism of its overexpression, at least in LSCC.[103] *CCDN1* amplification in the oro- and hypopharynx, as well as in the larynx seems to be more common than in other head and neck sites.[112]

Differences in molecular expression at different sites (e.g., cyclin D1) might be one factor explaining differences in clinical behavior between various sites in HNSCC.[112]

2.3.2.2. Bcl-2 and Bax

Programmed cell apoptosis is essential for normal development, tissue homeostasis, and defense against pathogens. Its important role in autoimmune and degenerative diseases, as well as in many neoplasms has also been suggested.[115-117]

Human follicular lymphoma associated 14;18 translocation revealed the first human Bcl-family member Bcl-2 (B-cell lymphoma-2), which eventually led to recognition of a new oncogenic Bcl-2 family, all important regulators of apoptosis.[118, 119] Through complex mechanisms, members of this family register information on intracellular disturbances (for example, DNA damage), integrate and interpret the data, and judge between life or death; incorporated subfamilies either oppose (Bcl-2) or favor apoptosis (Bax, BH3).[115] The anti-apoptotic Bcl-2 protein forms heterodimers with pro-apoptotic Bax, and the proportional concentration of the counterparts in this reaction series determines whether a cell survives or is degraded by caspase enzyme.[66, 115, 120]

Among the various sites in HNSCC, Bcl-2 expression has been reported to be strongest in nasal, paranasal, and nasopharyngeal sites, followed by the oropharynx, hypopharynx and larynx, while oral lesions seem to have weakest expression.[116, 121] In HNSCC, Bcl-2 overexpression has been shown to associate with poorer differentiation[116, 121, 122] and advanced local lymph node status.[116] Several authors have reported ambiguous results concerning the prognostic significance of Bcl-2 in HNSCC. In various studies covering all head and neck sites, high Bcl-2 expression was associated with either favorable[116, 123, 124] or unfavorable[125-128] prognosis. Finally, in most series, Bcl-2 expression did not relate to disease outcome.[121, 129-136] Furthermore, based on the results showing that Bcl-2 overexpression together with simultaneous p53 mutation cumulatively reduces the OS, Gallo *et al.* proposed cross-talk between p53 and Bcl-2 in HNSCC.[137] In line with this, the series of 88 LSCCs by Jackel et al.[127] found that survival was especially ominous when Bcl-2 overexpression occurred in combination with p53 overexpression. However, this interrelation was not corroborated by a third series of HNSCC.[133]

The association between Bax expression and prognosis has been studied even less in HNSCC. In four studies, Bax did not interrelate with survival.[121, 131, 132, 135] In a series reporting Bax expression in LSCC, a combination of high Bax and low Bcl-2 expression in the tumor was a significant predictor of bad OS.[138] Finally, in oral and oropharyngeal SCC, poor OS associated with low Bax expression, which in this series interlinked with p53 overexpression.[139] In conclusion, the value of both Bcl-2 and Bax remains contradictory, once more demonstrating the complex biology of HNSCC.

2.3.3. Tumor suppressor genes (TSGs)

The purpose of the TSG system is to protect cells from unregulated growth, cell proliferation and division. TSGs can be separated into two categories: "gatekeepers" and "caretakers". The first group directly regulates cell proliferation, whereas the second group does not directly control cell cycle but coordinates cellular responses to DNA damage, and when mutated, accelerates neoplastic transformation of the cell.[140] Observable phenotypic changes in somatic cells require inactivation of both TSG alleles.[101, 140]

Well-characterized human TSGs include adenomatous polyposis coli (APC), *BRCA1*, *p73*, *p53*, $p21^{WAF1}$, $p16^{INK4a}$, $p15^{INK4b}$, $p14^{ARF}$, *RB*, *TGF-α* and *TGF-β*.[16, 66, 101, 103, 104, 140] Implicated HNSCC associated tumor suppressor genes are *RB*, *TP53*, $p21^{WAF1}$, $p16^{INK4a}$, *TGF-α* and *TGF-β*.[16, 66, 101, 103, 104]

2.3.3.1. p53

The loss of normal p53 tumor suppressor gene function due to genetic alterations is a major factor behind human cancers, in general. The *TP53* gene on chromosome 17p13 (chromosome 17, short [p] arm, region 1, band 3), codes for a nuclear phosphoprotein p53 which regulates gene transcription, DNA synthesis and repair, cell cycle coordination, apoptosis, and angiogenesis.[141, 142]

Normally, the p53 system is dormant but is readily activated upon cellular stress, like DNA damage, hypoxia, oncogene activation, viral replication and shortage of ribonucleotides. The complex cellular machinery connected with p53 metabolism and function has lead to the concept of a p53 network. Different mechanisms activate p53 through different routes, including specific upstream p53 regulatory kinases: ATM and DNA-dependent kinase in one, ATR and caseine kinase II in another, and $p14^{ARF}$ in the third oncogenic route. They all modify p53 and its negative regulator MDM2, resulting in reduced p53 degradation, p53 stabilization, increased nuclear concentration, binding to DNA, and finally targeted gene activation. Downstream targets for activated p53 include numerous genes involved in growth arrest (e.g., $p21^{WAF1}$), apoptosis (e.g., Fas and Bax), senescence ($p21^{WAF1}$), and in angiogenesis (e.g., maspin and KAI1). Additionally, p53 induces MDM2 synthesis by creating a negative feed-back loop between them.[142, 143] Furthermore, recent data suggest that cytoplasmic p53 might be involved in yet another Bax-dependent apoptosis pathway.[144]

TP53 is the gene most often mutated in human cancer, occurring in over 50% of the malignant tumors. Mutated p53 is unable to bind DNA and thus lacks the aforementioned activator capacity. Other proposed ways for p53 inactivation and possibly overexpression—also in HNSCC—are alterations in its downstream target genes, complexing with other proteins, or abnormal degradation.[142, 143] The half-life of wt p53 is short, with the protein degrading in 20-30 minutes.[145] Genetic or other structural changes transform the wt p53 conformation, resulting in protein stabilization and accumulation in cells, and allowing its immunohistochemical detection.[16, 142] Both *TP53* mutation and/or p53 overexpression are common findings in HNSCC.[143, 146] Interestingly, in HPV associated PSCC, *TP53* mutations are less frequent, but wt p53 is readily degradated by the viral E6 oncoprotein.[29, 30]

The literature concerning p53 in HNSCC is evolving fast. Moreover, the potential role of p53 expression as an independent prognostic marker in HNSCC has been studied by many groups, but with conflicting conclusions. This literature has recently been reviewed by several authors.[16, 66, 104, 147] To summarize these data, abnormal or overexpressed p53 may be an adverse prognostic sign in HNSCC.

To further clarify the role of p53 in HNSCC, more well prepared studies with large patient materialss are needed. It has been suggested that complete p53 status evaluation should include both *TP53* genotyping for the hole coded gene sequence (exons 2-11). Furthermore, p53 immnunohistochemistry (IHC), analyses of $p21^{WAF1}$ as well as MDM2 expression should be studied simultaneously.[143, 146, 148]

2.3.3.2. $p21^{WAF1}$

$p21^{WAF1}$, cited also as CIP1 or SDI1,[149] was identified as a downstream target of p53 in the p53-mediated tumor suppressor cascade.[150] It is a protein with cdki-activity,[151] whitch is activated by wt p53, but not by the mutant type.[150] DNA damage results in a gain in wt p53 expression, wt p53 protein accumulation in the nucleus, and subsequently induced $p21^{WAF1}$ expression. The ensuing cell cycle arrest in the G1/S transition results from the inhibitory effect of $p21^{WAF1}$ on cdks as well as on cyclin D1 activation. The physiological meaning of this is to allow nuclear mechanisms to repair DNA damage as well as to enhance genomic stability and integrity. It has, however, been implicated in cell cycle control even later in the G2 phase.[152] As p53 directly controls $p21^{WAF1}$ transcription, it has been proposed to be an indirect indicator of p53 activity.[153] However, several other factors, e.g., epidermal growth factor (EGF), FGF, platelet-derivative growth factor (PDGF), BRCA1, $p16^{INK4a}$, and pRB

have also been shown to induce $p21^{WAF1}$ expression, regardless of p53.[154-157] This alludes to more than just one $p21^{WAF1}$-related apoptotic pathway.[146, 157] In all, the role of $p21^{WAF1}$ in cell cycle control is apparently complicated and still partly unclear. Exposed $p21^{WAF1}$ functions in cell senescence, apoptosis inhibition, and in inducing the production of several extracellular matrix components further obscures the picture.[149]

While in some series, including colorectal, cervical and lung cancers, the lack of $p21^{WAF1}$ expression correlated with unfavorable prognosis, similar results for cancers of the prostate, ovary, cervix, breast and esophagus related to induced $p21^{WAF1}$ expression. In many series, $p21^{WAF1}$ expression did not have prognostic value at all.[149] Accordingly, in HNSCCs $p21^{WAF1}$ overexpression has been reported to be either a good,[158, 159] bad,[160] or insignificant[146, 161] prognostic sign. Thus, the role of $p21^{WAF1}$ in cancer is anything but clear.

2.3.3.3. $p16^{INK4a}$

$p16^{INK4a}$, a 16-kDa protein designated also as p16 TSG, CDKN2A or MTS1, locates on chromosome 9p21 and has been found to be the commonest site of genetic abnormality in HNSCC. It is a member of an important group of cdkis, and together with $p15^{INK4b}$, $p21^{WAF1}$ and $p27^{KIP1}$. $p16^{INK4a}$ binds to cdk4 and cdk6 to restrain their association with cyclin D1, thus inhibiting the catalytic activity of the cyclin D1-cdk complex, preventing pRB phosphorylation and the release of E2F. Eventually, cellular $p16^{INK4a}$ expression leads to a stalled cell cycle in the G1/S phase transition. Inactivation of $p16^{INK4a}$ can take place by several mechanisms, including mutation, homozygous deletion, and promoter hypermethylation leading to gene silencing. As a result of genetic alterations in up to 50-70% of the HNSCCs, $p16^{INK4a}$ is inactivated. Frequent deletions resulting in the loss of heterozygosity (LOH) on the short arm of chromosome 9 (9p21–22) have been reported, not only in head and neck carcinomas but also in dysplasia and carcinoma in situ, suggesting the involvement of this region in the early stages of HNSCC. The high incidence of $p16^{INK4a}$ inactivation in HNSCC indicates that this gene plays an important role in the development of the disease. At present, however, the diagnostic or prognostic value of $p16^{INK4a}$ remains to be determined, and further studies are necessary to clarify the role of $p16^{INK4a}$ in HNSCC.[16, 103, 162-166] Furthermore, in HPV-related PSCCs, $p16^{INK4a}$ is often upregulated as a result of viral E7 oncoprotein-induced pRB inactivation.[30]

2.3.4. Adhesion-related factors

The extracellular matrix (ECM) is a functional complex of macromolecules that lies beneath epithelial cells and encircles connective tissue cells. The metabolism and tasks of ECM involve active molecular mechanisms and interactions that direct cellular migration, attachment, differentiation, and organization. ECM not only provides a passive structural frame for the cellular tissue component, but also plays a highly interactive role in several ECM-related processes. The major constituents of ECM include collagens, structural glycoproteins, and proteoclycans which form structural entities, such as basement membranes and elastic fibers.[167]

Cell adhesion molecules act as connectors between the cells interior and exterior. They are transmembrane glycoproteins with extracellular, intramembranous and cytoplasmic domains. These adhesion molecules work through their specific external partners, including other cells or ECM components which in binding alter the structure of the molecule and modulate its actions. All adhesion molecules link with other molecules inside the cell and are capable of regulating cell functions. The six transmembrane cell adhesion molecule families are: an immunoglobulin-like super family, cadherins, integrins, receptor tyrosine phosphatases, selectins and hyaluronan receptors.[105]

2.3.4.1. Cadherins and catenins

Cadherins are integral transmembrane glycoproteins with sites for both binding Ca^{2+} and adhesion. They control cell motility, migration, sorting and differentiation. Cadherins play critical roles in embryogenesis and myogenesis, as well as in muscular and neural functions.[105] Cadherins were first recognized as transmembrane glycoproteins involved in Ca^{2+}-dependent intercellular adhesion. Their central roles in development, cell polarity, and tissue morphology have been revealed later. The best characterized member of this family is epithelial E-cadherin responsible for homotypic zipper-like binding with E-cadherin molecules of the adjacent epithelial cells in adherens junctions. E-cadherin is coded by gene *CDH1* located on chromosome 16q22.[16, 168, 169]

Catenins, namely α-, β-, γ- (also known as plakoglobin), and δ-catenin (also known as $p120^{CTN}$ or p120-catenin), are essential for normal E-cadherin function. The genes for α-, β-, and δ-catenin are located on genes 5q31 (*CTNNA1*), 3p21 (*CTNNB1*), and 11q11

(*CTNND1*), respectively.[169] The gene for γ-catenin has not yet been located.[169] The extracellular E-cadherin domain links with the neighboring cadherin. The cytoplasmic domain of E-cadherin associates with either β- or γ-catenins, whereas α-catenin connects the cadherin-bound β- or γ-catenins to the actin of the cellular cytoskeleton. α-catenin binds actin directly, or indirectly through actin associated proteins (i.e., α-actinin or vinculin). δ-catenin binds to intracellular E-cadherin closer to the intramembranous domain and promotes cadherin clustering. The intracellular part of the E-cadherin-catenin complex may be conjoined with receptor tyrosine kinases (e.g., c-erbB2, c-met, PRGF-1, EGFR), non-receptor tyrosine kinases (e.g., src), receptor protein tyrosine phosphatases (RPTP), or episialin (MUC-1).[168, 170] Free cytosolic β-catenin is bound by the cytoplasmic macromolecule complex enclosing the APC protein, adaptor protein axin or its homologue conductin, GSK-3β (glycogen synthase kinase-3β), and EB1 (microtubule end binding protein).[168, 170] Catenins can also act as signal transducers. The most investigated route associated with catenins is the wnt signaling pathway[171] which is another route for nuclear β-catenin translocation and target gene activation.

Based on *in vitro* models and experiments, the E-cadherin gene might act as either an invasion-suppressor or tumor-suppressor gene. E-cadherin may be downregulated at gene, transcription or protein level, resulting in a dysfuctional E-cadherin-catenin complex.[168] In HNSCC, reduced E-cadherin expression using IHC has been reported with histological dedifferentiation and metastasis.[172-175] In more recent reports, reduced E-cadherin has also been found to be a significant prognostic factor for poorer OS.[176, 177]

In cancer, pathological stabilization of β-catenin, nuclear translocation, LEF/TCF induction, and oncogenic activation may result from wnt overexpression or from mutations of various components in different steps of the wnt signaling pathway. Even though wnt overexpression seems to be uncommon in human cancers,[170] components of the wnt signaling pathway have been implicated in several tumors,[178-181] as well as in experimental cancer models.[182, 183] Recently, mutations of β-catenin and/or APC have been reported, for example, in colon,[178] anaplastic thyroid,[179] and hepatocellular cancer,[180, 184] as well as in malignant melanoma.[181] In all these reports, mutations accompanied nuclear localization of β-catenin.[179, 180, 181, 184] In clinical HNSCC materials, reduced catenin expression, shown by IHC, has related to increased probability for local lymph node metastasis[176, 177, 185] and poorer histological differentiation.[186-189] Furthermore, the loss of β-catenin expression has been reported to be more profound in the area of active invasion.[190] In one paper cytoplasmic localization of the α-catenin tended to indicate poorer survival.[191] This is,

however, the only direct implication of an association between aberrant catenin expression and prognosis in HNSCC, excluding the nasopharyngeal primary site.

2.3.4.2. Versican

Versican is a large chondroitine sulphate (CS) proteoglycan carrying several active domains which enable versatile interactions in a great variety of biological processes.[192-195] It is a member of the aggrecan gene family.[194] The fundamental parts of versican include globular end domains G1 and G3, and a central domain situated between them.[192] In either G1 or G3 end, the molecule holds specific binding domains for polysaccharide HA and for EGF, as well as a complement regulatory domain.[193, 195] Versican exists as four different isoforms generated through alternative splicing of versican mRNA, which results in four differently constructed central domains of versican (V0: αGAG + βGAG; V1: βGAG; V2: αGAG; V3: none).[192, 194]

Versican has been shown to be an important factor in controlling embryogenesis.[196] It is, however, expressed also in a wide variety of human adult tissues.[197-199] It influences several biological and pathological processes and plays a role in anti-adhesion, proliferation, migration, and extracellular matrix fabrication.[192]

Versican is expressed in several malignant tumors, suggesting versican involvement in the development and progression of cancer. Elevated versican levels have been reported in melanoma,[200] mesothelioma,[201] sarcoma,[202] brain tumours,[203] and in carcinomas of the gastrointestinal tract,[204] breast,[205, 206] ovary,[207] and prostate.[208] Apparently, versican promotes tumor growth by destabilizing focal cell contacts, which will repress cell adhesion and stimulate cell proliferation.[201, 209-211] Versican has also been implicated in vascular pathology and angiogenesis.[199] These findings encouraged investigators to even propose possibilities for new anti-angiogenic cancer therapies based on versican tumor biology.[212]

In HNSCC, the roles of versican remain unrevealed and further investigations are warranted.

2.3.4.3. Hyaluronan (HA)

HA (previously known as hyaluronate or hyaluronic acid), an unbranched polysaccharide constructed from repeating N-acetyl-glucosamine and glucuronic acid disaccharide units, is

a macromolecule with multifaceted functions in cell migration, embryogenic proliferation and differentiation, wound healing and inflammation.[213] The membrane-bound hyaluronan synthase (HAS) produces HA on the inner cell surface.[214] HA can be found in almost all human tissues.[215] The ECM compounds binding HA include proteoglycans, glial binding protein, hyaluronectin, collagen VI, and fibronectin. Elevated HA expression has been reported in gastric,[216] breast,[217] lung,[218] colorectal,[219] and prostatic[220] cancers. In all these adenocarcinomas, elevated HA expression has been associated with poor survival. In HNSCC, however, reduced or irregular HA expression was found to be related to poorer survival.[221, 222]

2.3.4.4. CD44

CD44 is a cell surface glycoprotein participating in many cellular processes. It is coded by a single gene on 11p13 and exists as several isoforms generated by alternative exon splicing and posttranslational glycosylation. CD44 has been shown to act as a receptor for cell-cell and cell-matrix adhesion, as a signal transmitter, and as a growth factor-presenting molecule; the first revealed CD44 function was lymphocyte homing, but several other physiological and pathological affiliations, including metastasis, have since been revealed. It is widely distributed in various human cells and tissues, also in squamocellular ones. CD44 binds several ligands involved in cell adhesion, including collagen, fibronectin, laminin and HA. CD44 is also involved in HA internalization and intracellular HA degradation.[223-226]

Numerous IHC-based analyses using different monoclonal antibodies against different CD44 isoforms and molecular biological techniques have shown that CD44 is overexpressed in many tumor types, suggesting a role for CD44 in tumor development and progression. In contrast to several other malignancies, CD44 splice-variant isoforms are often down-regulated in squamous cell carcinomas of the head and neck.[226] In various HNSCC sites, reduced CD44 expression (total, or isoforms v2, v6 or v9) has been associated with shorter survival rates.[221, 227-230] The findings from other series showing no association between unaltered or reduced CD44 expression (total, or isoforms s or v2) in HNSCC, nevertheless, complicates the picture.[231-233] The clinical significance of CD44 in squamous cell carcinomas of the head and neck as a tumor marker for cancer diagnosis and prognosis remains to be resolved.

2.3.4.5. Matrix metalloproteinases (MMPs).

The most important degradatory proteolytic enzymes are the matrix metalloproteinases (MMPs). To date, over 20 different members of the MMP family have been characterized. They are fundamental for several physiological processes, such as embryogenesis, female cyclus, development of placenta, differentiation, tissue remodeling, and angiogenesis, but are also activated in pathological conditions including cancer.[234] MMPs are zinc metalloenzymes which are divided by their structure and substrates into secreted collagenases (MMP-1, MMP-8, MMP-13, MMP-18), gelatinases (MMP-2, MMP-9), and stromelysins (MMP-3, MMP-10, MMP-11). The fourth transmembrane group is called membrane-type MMPs (MT-MMP).[235] MMPs digest collagens, gelatin, elastin, fibronectin, proteoglycans, and several other ECM components. MMP function is normally tightly controlled at several levels through regulation of their transcription, translation and proenzyme activation, as well as through specific inhibition by tissue inhibitors of metalloproteinases (TIMPs).[234]

MMPs have been shown to be important participators in promoting invasion and metastasis in several cancers.[234-236] In HNSCC, the exact roles of MMPs are only now beginning to emerge. At least MMP-2, -9,-13 and to some extent MMP-7, as well as MMP-12, have been related with HNSCC progression. Potential members of this family alluding to less favorable prognosis in association with their overexpression are MMPs 2, 7, 9, and 12.[234, 237-241] In PSCC, poorer disease outcome has been shown with the overexpression of MMP-2.[237] Poorer disease outcome has also been implicated with overexpression of MMP-2 in hypopharyngeal SCC.[237]

To sum up, the extracellular activities of MMPs and their tissue inhibitors linked to pathological tissue destruction are complex. To elucidate the role of MMPs, as well as their prognostic power in HNSCC, will require additional data.

2.3.5. Angiogenesis-related factors

Angiogenesis, the multistep process starting from local endothelial activation, sprouting of neovasculature from existing endothelium and eventually formation of new confluent capillary network, is fundamental to tumor growth.[242, 243] The angiogenic activity of a tumor can be assessed either by IHC using antibodies against endothelial antigens (e.g., factor VIII, CD-31, CD-34) and assessing the microvascular density (MVD), or with antibodies

against factors promoting angiogenesis.[104] Even though MVD has been shown to be an independent prognostic factor in several malignant tumors, in HNSCC the significance of angiogenesis-related data obtained by MVD evaluation is inconsistent.[104, 243-246] Different tumors also express several angiogenic cytokines.[247] In HNSCC, the prognostic ability of at least vascular endothelial growth factor (VEGF), basic FGF (bFGF), PDGF-AB and -BB, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), MMP-2, MMP-9, and tenascin-C (TnC) have been investigated.[248-253] Only VEGF has been linked with poor prognosis in HNSCC,[250] though contradictory results have also been reported.[248] Additionally, small HA-derived fragments, as well as inducible nitric oxide synthase (iNOS) expression have been related to induced angiogenesis.[254, 255] Furthermore, a non-angiogenesis-dependent pathway for tumor vascularization and growth has been recently described.[256]

The acquired answers concerning cancer vascularization seem to evoke new questions, leaving the picture still far from complete and clearly warranting additional studies.

2.3.6. Nitric oxide synthase

Nitric oxide synthases (NOS) are enzymes synthesizing nitric oxide (NO) from the amino acid L-arginine.[257] Three NOS isoenzymes have been revealed: endothelial (eNOS, NOS3) and neuronal (nNOS, NOS1), as well as iNOS (NOS2).[257] eNOS and nNOS mainly mediate physiological processes (vascular tone regulation and synaptic events), whereas iNOS is capable of generating substantial quantities of NO for prolonged time periods.[257, 258] Physiologically, iNOS is mainly expressed in activated macrophages, though also in several other cell types.[259] The 37-kb gene coding iNOS has been located in human chromosome 17q12.[260]

A high NO concentration has cytotoxic effects and is involved in nonspecific immunity.[257] NO has been shown to regulate both growth of primary tumors and metastases in human cancer.[258, 261] Neoplastic cells exposed to high NO concentrations produced by activated macrophages (iNOS) in the tumor site show cytotoxic damage and are driven towards apoptosis,[107, 262] whereas a lower concentration originating from other stromal cells or tumor cells associates with tumor promotion and metastasis.[106, 107]

Several human cancers have been reported to express iNOS.[255, 263-270] Tumoral iNOS expression has been implicated with stage,[266, 270] histological differentiation,[265-267] cell

proliferation,[266] induced angiogenesis,[255] p53 expression or mutation,[263, 269] and survival.[266, 267, 270]

In line with other tumor locations, iNOS expression has also been described in HNSCC.[271-278] The elevated metastatic potential in association with iNOS expression in HNSCC was suggested by Gallo et al.,[271] and was later confirmed in another HNSCC material.[274] A transition towards iNOS as the principal NO source in HNSCC has also been suggested.[272] Brennan et al.[273] demonstrated an interrelation between p53 expression and iNOS. In the larynx, iNOS induction has been associated with induced angiogenesis in the progression from dysplasia into invasive SCC.[275] An interesting connection between higher iNOS activity and MMP-9 overexpression, as well as the presence of mutated p53 in tumor samples, was stated by Franchi et al.[276] Furthermore, induced iNOS activity and protein expression have been associated with concurrent induction of cyclo-oxygenase-2 pathway (COX-2) in HNSCCs.[278] Both induced iNOS and COX-2 expression were also significantly more common in association with mutated p53 in the samples, and were inhibited by wt p53 *in vitro*.[278]

In both physiology and pathology, the role of iNOS seems to be complex and requires further effort to gain more understanding of this interesting and, apparently, powerful synthase, also in relation to HNSCC.

3. AIMS OF THE STUDY

Among all the head and neck carcinomas, cases originating in the oro- or hypopharynx have been reported to have the worst prognosis. Predicting patient survival and selecting treatment is at present solely based on clinical characteristics, i.e., stage, histological differentiation of the tumor and general condition of the patient. The purpose of the present study was to identify new potential prognostic tools to find the patient groups which might benefit from new, more aggressive treatment modalities or combined therapies, thus helping to brighten the dark reputation of PSCC. The specific aims of the present study were:

1. To assess the prognostic power of traditional clinical and histopathological variables in PSCC

2. To examine the expression and prognostic value of α-, β- and γ-catenins in PSCC

3. To explore the expression of p53 protein and its association with survival in PSCC

4. To investigate iNOS expression, as well as the relationship between it and prognosis in PSCC

5. To evaluate the expression and the prognostic role of versican in PSCC

4. PATIENTS, MATERIALS AND METHODS

4.1. Study design

In the present work, a longitudinal, retrospective, population-based cohort design was used. All patients diagnosed with primary PSCC in Eastern Finland between the years 1975 and 1998 were identified from the records of all hospitals in the Area of Regional Responsibility for the Kuopio University Hospital, including the Kuopio University Hospital, as well as the Central Hospitals of Central Finland (Jyväskylä), Northern Karelia (Joensuu), Southern Savo (Mikkeli) and Eastern Savo (Savonlinna). Simultaneously, the same population was searched for PSCC through the population-based Finnish Cancer Registry. Primary investigations revealed 161 cases fulfilling the search criteria. The mean population in the district during the study period (1975-1998) was 870,000.

4.2. Clinical data and follow-up

The clinical data from all 161 cases were reviewed by one oncologist and two otolaryngologists. Among these patients, the disease originated from the oral cavity in three cases and in the other three cases from the larynx. A further four cases had to be excluded due to insufficient clinical data. The remaining 151 tumors were staged according to the Union Internationale Contre le Cancer (UICC) classification (1997), based on written hospital records of clinical otolaryngological status, endoscopy, and chest X-ray.[18] Karnofsky performance status at the time of diagnosis was coded according to hospital charts. All patients were regularly followed up by an otolaryngologist and/or oncologist until death or May 1999. The cause of death was defined from the hospital records, or from death certificates. None of the patients was lost from the follow-up.

4.3. Tumor samples and histology

All tissue samples available, both original hematoxylin and eosin (H&E) stained slides, as well as all available archival paraffin embedded blocks from the primary tumors and from all local metastases, were retrieved from the archives of each hospital. All tissue samples used in these studies were originally fixed in 10% formalin (buffered, pH 7.0) and embedded in

paraffin. Of the 151 cases 11 were excluded from the study due to non-squamous histology, and two due to carcinoma in situ histology. Finally, 138 cases with histologically verified invasive squamous cell carcinoma of oro- or hypopharynx with sufficient clinical data were studied. All the sections were evaluated, and histological differentiation of the primary tumor was determined according to the World Health Organization (WHO) criteria by one experienced pathologist.[31] The most representative tumor block was chosen and cut into 5-µm thick sections for immunohistochemical stainings (Table 4). In 14 cases, additional tissue samples from regional lymph node metastases were available for immunohistochemical stainings.

4.4. Immunohistochemistry

The immunoperoxidase method used for immunohistochemical stainings was practically identical in all studies I-IV, with necessary modifications in antigen retrieval and primary antibodies. Firstly, the sections cut from paraffin embedded samples were rehydrated. To optimize the accessibility of the antigen, the epitopes were unmasked by microwave boiling in buffer, as indicated in table 4 (antigen retrieval). Potential endogenous peroxidase activity present in samples was blocked with 5% H_2O_2. To block any unspecific binding of the primary antibody, the samples were treated with 1.5% normal horse serum (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, U.S.A.) in PBS at 20 °C for 25 to 35 minutes. Protein specific monoclonal mouse IgG primary antibodies were used as indicated in table 4. The samples were then incubated with biotinylated secondary antibody against mouse IgG and were bathed in avidin-biotin peroxidase reagent. Finally, to demonstrate the bound secondary antibody, diaminobenzidine tetrahydrochloride solution was used as a cromogen. The samples were counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted with DePex. Each staining batch included both positive and negative controls. For catenins, normal epithelium and glandular tissue served as internal controls. Other tissues used for both positive and negative controls are presented in table 4. For negative controls, the primary antibody was omitted.

Table 4. Specific antibodies used for immunohistochemical stainings

Marker (study number)	*Number of cases*	*Antigen retrieval**	*Monoclonal primary antibody*	*Manufacturer*	*Dilution*	*Control tissues*
α-catenin (I, II, IV)	116	-600 W 3 x 5 min -0.05 mol/L Tris/HCl buffer (pH 9.7)	Mouse anti-α-catenin, clone 5	Transduction Laboratories, Lexington, KY, U.S.A.	1:200	-Lung -Intestine
β-catenin (I, II, IV)	116	-600 W 3 x 5 min -0.05 mol/L citrate buffet (pH 6.0)	Mouse anti-β-catenin, clone 14	Transduction Laboratories, Lexington, KY, U.S.A.	1:1000	-Lung -Intestine
γ-catenin (I, II, IV)	116	-600 W 3 x 5 min -0.05 mol/L citrate buffet (pH 6.0)	Mouse anti-γ-catenin, clone 15	Transduction Laboratories, Lexington, KY, U.S.A.	1:200	-Lung -Intestine
p53 (II, III, IV)	123	-600 W 3 x 5 min -0.01 mol/L citrate buffet (pH 6.0)	Mouse anti-human-p53, clone DO7	Dako A/S, Glostrup, Denmark	1:1000	-Intestine
iNOS (III)	118	-600 W 6 x 5 min -0.01 mol/L citrate buffet (pH 6.0)	Mouse anti-human-iNOS, clone 6	Transduction Laboratories, Lexington, KY, U.S.A.	1:200	-Intestine
Versican (IV)	118	-800 W 3 x 5 min -0.01 mol/L citrate buffet (pH 6.0)	Mouse anti-human-versican clone 2B1	Seikagaku Corporation, Tokio, Japan	1:500	-Skin

*Microwave boiling and buffer

4.5. Immunoreactivity grading

Evaluation of IHC was always done unaware of the clinical and histopathological data. The entire assessable tumor area, excluding necrotic parts, was analyzed in each study (I-IV). Additionally, also peritumoral stroma was included in the analysis when appropriate. For catenins, the primary evaluation was performed by three investigators, and for p53, iNOS, as well as versican by two investigators. The results were compared, and samples not agreed upon (less than 10% of the tumors in each series) were re-evaluated with a dual-head microscope, and the final scores were settled.

For α-, β- and γ-catenins, the percentage of tumor cells showing membranous staining was first evaluated on a continuous scale. For statistical analysis, median percentage was used to separate normal (≥90%) and reduced (<90%) membranous catenin staining. The

reduced membranous catenin expression was described as discontinuous or absent, with or without cytoplasmic staining. Nuclear β-catenin was also detected in some tumors, and when nuclear staining for β-catenin was seen in more than 10% of the nuclei of the neoplastic cells, the tumor was graded positive for nuclear β-catenin.

The fraction of tumor cells expressing nuclear p53 was assessed on a continuous percentage (0%-100%) scale and was graded as weak (1), moderate (2) or strong (3). Simultaneously, the cytoplasmic p53 staining was graded as negative or positive. Finally, a semi-quantitative nuclear p53 expression index was calculated by multiplying the stained nuclei percentage (%) by the intensity. This index (range 0-300) was divided into tertiles: in the statistical analyses low and intermediate tertiles were combined and studied against the highest tertile.

In accordance with p53 evaluations, the proportion of tumor cells expressing iNOS were also evaluated on a continuous percentage (0%-100%) scale. At the same time, the intensity of iNOS expression was graded as negative (0), weak (1), moderate (2), or strong (3). The percentage of stained cells was categorized into four classes (0-25% = 1, 26-50% = 2, 51-75% = 3 and 76-100% = 4). A semi-quantitative iNOS expression score, taking into account both staining intensity and the proportion of stained cells, was then calculated by adding together percentage category (1-4) and staining intensity (0-3). For statistical analyses, the iNOS expression score was considered low (1-3) or high (4-7).

For the stromal versican expression, the percentage proportion of strong versican staining intensity (comparable with the strong expression seen in positive dermal control tissues) from the total intra- and peritumoral stromal area was again assessed on a continuous percentage (0%-100%) scale. The median percentage (10%) was used to divide the strong stromal versican expression into two categories for statistics: in the low fraction, the percentage was <10%, and in the high fraction ≥ 10%. At the same time, the intracellular versican staining evident in some tumors was graded as either negative or positive.

4.6. Statistical methods

As descriptive statistics for continuous variables, means with standard deviations (SD) or medians with ranges were used for normal distribution or non-normal (skewed) distribution, respectively. Statistical associations between classified variables were tested with the chi-square (χ^2) test for independence. In cases not fulfilling presumption criteria for χ^2 test, the

Fisher's exact test for independence was used instead. The distributions of the variables were tested with the nonparametric Mann-Whitney U test (two samples) or with nonparametric Kruskal-Wallis H test (several samples) in cases of non-normal interval or ratio scale variables or ordinal variables. Wilcoxon's nonparametric test for two related samples was used to compare the groups. Spearman's nonparametric rank order correlation coefficient (r_s) was used to test the linear association between non-normal variables.

Disease-specific survival (DSS) was calculated from the date of the primary diagnostic biopsy to the end of follow-up or death. The univariate analyses of DSS were based on the Kaplan-Meier estimation (log rank test).[279] Multivariate DSS analyses (Cox proportional hazards model)[280] were performed in a stepwise manner. The enter limit was $p<0.05$ and the removal limit $p\geq0.1$. The baseline covariates used in the model were age, sex, site of the primary tumor, histological differentiation, T class, N class, M class or stage, as well as Karnofsky performance status. Other immunohistochemical variables used in the survival analyses were membranous catenin expression (normal vs. reduced), nuclear β-catenin expression (positive vs. negative), nuclear p53 expression index (low/intermediate vs. high), cytoplasmic p53 expression (positive vs. negative), iNOS expression score (high vs. low), strong stromal versican expression (high vs. low) and intracellular versican expression (positive vs. negative).

Of the original cohort of 138 patients, a varying number of cases had to be excluded from studies I-IV because tumor material was not available for the IHC analyses (Table 4). The potential distortion of patient material in studies I-IV was excluded by testing the representativeness of the included subgroups of the original cohort. The categorical variables (sex, tumor site, histological grade, stage, Karnofsky performance status) were tested with the nonparametric qhi-square (χ^2) goodness-of-fit test, and the continuous variables (age, length of symptomatic period prior to diagnosis) with the parametric one-sample t test. Due to the long study period (1975-1998), the possibility that the disparity in the storage time of the tumor samples might affect the studied histological variables had to be excluded. For this reason, the study cohort was chronologically divided into ten subgroups. The independence between the histological variables and storage time was tested with Fisher's exact test for the categorical histological variables (histological differentiation and all immunohistochemical variables). All statistical calculations were performed with SPSS for Windows (Release 8.0 - 10.0) (SPSS Inc., Chicago, IL, U.S.A.) software in an IBM compatible computer. Results with $p<0.05$ were regarded as statistically significant.

4.7. Ethics

The research plan was approved by The Research Ethical Committee of the University of Kuopio and the Kuopio University Hospital (decision No 102/97), and permission for obtaining data from the Finnish Cancer Registry and from hospital records was conferred by the Finnish Ministry of Social Affairs and Health (permission No 88/08/97). Due to the retrospective nature of the present study the patients or their families have not been contacted. The data obtained during the study have not been included in hospital records.

4.8. Conflict of interest and financial support

This study was financially supported by the Kuopio University Hospital EVO Funds, The Savo Cancer Fund, The Finnish Foundation for Cancer Research, The Finnish Cultural Foundation of Northern Savo and The Ear Research Foundation of Finland. The author of this thesis or any of the co-authors in each original publication had no conflicts of interests in relation to the methodology, results or any other sector of studies I-IV.

5. RESULTS

5.1. Patient characteristics, follow-up and treatment

5.1.1. General remarks

The summary of the clinical and histological data for the whole patient group are presented in table 5. A subpopulation of 116-123 cases with sufficient histological material available were included in studies I-IV. There was no statistically significant difference in the distributions of the baseline characteristics (sex, age, Karnofsky performance status, tumor differentiation, stage) between the original patient group (n=138) or any of the subgroups in the studies I-IV (χ^2 test for goodness-of-fit and one-sample t test; data not shown). The long study period had no significant effect on the immunohistological or histological variables studied (membranous catenins, nuclear β-catenin, nuclear p53 expression index, cytoplasmic p53 expression, iNOS expression index, strong stromal versican expression, cytoplasmic versican expression, strong versican expression in metastases, or histological differentiation; Fisher's exact test for independence; data not shown).

5.1.2. Age, sex and symptoms

Data on age, sex and symptoms are presented in table 5. The male-female ratio in the cohort was 3:1, and the median age at the time of diagnosis was 63 years. One of the three most prevailing symptoms, i.e., local pain in pharynx or neck, palpable neck tumor or dysphagia, was reported by over 80% of the patients (Table 6). Forty-eight (35%) patients mentioned more than one prediagnostic symptom. The duration of the symptomatic period preceding diagnosis, i.e., delay from the first recognition of a symptom until histological diagnosis, was significantly shorter in patients diagnosed with stage I disease, as compared with patients diagnosed with higher stages (II-IV) (Mann-Whitney U test, p=0.010). Moreover, the length of the prediagnostic symptomatic period tended to be longer in females (Mann-Whitney U test, p=0.083). The duration of symptoms did not relate to age, site, T, N or M class.

Table 5. Clinical and histopathological patient data (n=138).

Variable	*All*	*Range*	*95% CI**
Number of patients	138		
Mean age at the time of diagnosis, years (SD)	64 (10.9)	36-89	
Median duration of the symptoms, months	3	0-76	2-4
Sex, (female-male ratio)	1 : 3.2		
Male (%)	105 (76)/		
Female (%)	33 (24)		
Site of the primary tumor			
Oropharynx (%)	88 (64)		
Hypopharynx (%)	50 (36)		
T class (%)			
T1	23 (17)		
T2	46 (33)		
T3	25 (18)		
T4	44 (32)		
N class (%)			
N0	81 (59)		
N1	21 (15)		
N2	32 (23)		
N3	4 (3)		
M class (%)			
M0	132 (96)		
M1	6 (4)		
Stage (%)			
S I	16 (12)		
S II	28 (20)		
S III	26 (19)		
S IV	68 (49)		
Differentiation, grade (%)			
1 Good	34 (25)		
2 Moderate	64 (46)		
3 Poor	40 (29)		
Karnofsky performance status (%)			
≥ 70	91 (66)		
< 70	47 (34)		
Primary treatment (%)			
Radiotherapy only	86 (62)		
Radiotherapy and surgery	38 (28)		
Surgery only	8 (6)		
No cancer specific treatment	6 (4)		
Relapse (%)			
No	45 (33)		
Yes	51 (37)		
No remission	42 (30)		
Second primary (%)			
No	121 (88)		
Yes	17 (12)		
Median OS, months	20.2	1-332	15.7-24.7
Median DSS, months	22.3	1-125	15.4-29.2

* CI, confidence interval

Table 6. The most prevalent prediagnostic symptoms

Symptom	*All (n=138) (%)*	*OP (n=88) (%)*	*HP (n=50) (%)*
Pain in the throat or neck	50 (36)	38 (43)	12 (24)
Neck mass	33 (24)	22 (25)	11 (22)
Dysphagia	32 (23)	10 (11)	22 (44)
Globus sensation	14 (10)	7 (8)	7 (14)
Odynophagia	10 (7)	3 (3)	7 (14)
Tumor found incidentally	8 (6)	7 (8)	1 (2)
Ear ache	6 (5)	6 (7)	0 (0)
Visible tumor or ulcer	5 (4)	5 (6)	0 (0)
Blood in sputum or saliva	5 (4)	3 (3)	2 (4)
Hoarseness	5 (4)	2 (2)	3 (6)

OP, oropharynx; HP, hypopharynx

5.1.3. General condition and other diseases

The Karnofsky performance status coded at the time of diagnosis ranged between 90 and 40, and the median value was 70.The distribution of Karnofsky performance status was, as follows: 90 in eight (6%), 80 in 21 (15%), 70 in 62 (45%), 60 in 29 (21%), 50 in 15 (11%), and 40 in three (2%) cases. Karnofsky performance status decreased significantly with increasing age of the patient (Kruskal-Wallis H test, $p=0.008$; Spearman's rank order correlation coefficient $r_s= -0.30$, $p<0.001$). The general condition of the patient related significantly to T class: Karnofsky performance status decreased as the T class rose (Fisher's exact test for independence, $p=0.015$). Karnofsky performance status did not associate with primary site, duration of the symptoms, sex, alcohol consumption, smoking, N or M class, or stage.

In the study cohort, there were altogether nine second-primary HNSCCs; two oral cavity, two supraglottic and four glottic carcinomas were diagnosed before or during the study period. Thirty-four (25%) patients were otherwise healthy, and in one (1%) case data were not available. The remaining 101 (74%) suffered from chronic diseases: in 42 (30%) one disease, 30 (22%) two, 20 (14%) three and in 11 (8%) four or more chronic disorders had been diagnosed. The most prevalent chronic conditions were as follows: coronary heart disease (n=43, 31%), other atherosclerosis (n=28, 20%), hypertension (n=20, 14%), other heart problem (n=17, 12%), chronic bronchitis (n=12, 9%), neurological disease (n=11, 8%), diabetes (n=8, 6%), psychiatric disorder (n=7, 5%), asthma (n=5, 4%), alcoholism (n=5, 4%), and chronic anemia (n=5, 4%).

5.1.4. Smoking and alcohol drinking

Hospital records did include data concerning patient's smoking habits and alcohol consumption in 77% and 59% of the cases, respectively (Table 7). Regular smoking associated significantly with heavier drinking (Fisher's exact test for independence, $p<0.001$).

Table 7. Smoking and alcohol drinking in the study cohort (n=138)

Variable	Number (%)
Smoking	
No	21 (15)
Occasionally	7 (5)
Regularly	79 (57)
No data available	31 (23)
Alcohol consumption	
None	18 (13)
Reasonable	35 (25)
Profuse	79 (21)
No data available	29 (41)

5.1.5. Tumor location, TNM status, stage and histological grade

The tumor site data are presented in table 8. In general, tumors were large at the time of diagnosis. (Table 5). T class did not associate with sex, age, smoking, alcohol consumption or duration of the symptoms. N class was positive in 57 (41%) cases (Table 5) but it did not associate with T class, sex, age, smoking, alcohol drinking, or location of the primary tumor.

At the time of the diagnosis, almost half of the cases had already advanced into stage IV (Table 5). Age, sex or primary site did not associate with stage. The histological differentiation of the primary tumor was good in 34 (25%), moderate in 63 (46%), and poor in 41 (29%) cases. The histological grade did not associate with patient's age, sex, smoking habits or with the duration of the symptoms.

Table 8. Tumor primary site and side (n=138)

Primary sites and sides	*n (%)*
Site	
Oropharynx	88 (64)
Tonsil	37 (26)
Tonsillar fossa and palatoglossal arches	2 (2)
Glossotonsillar sulcus	1 (1)
Base of the tongue	32 (23)
Posterior wall of the oropharynx	7 (5)
Soft palate	3 (3)
Uvula	6 (4)
Hypopharynx	50 (36)
Posterior wall of the hypopharynx	11 (8)
Fossa pyriformis	36 (25)
Pharyngo-esophageal junction	3 (3)
Side	
Right	64 (47)
Left	57 (41)
Middle	17 (12)

Table 9. Treatment in the whole patient group (n=138)

Treatment	*n (%)*
Modality	
Radiotherapy (RT)	86 (62)
RT alone	62 (45)
RT and combined chemotherapy	24 (17)
RT and surgery	38 (28)
Preoperative RT	3 (2)
Sandwich (pre- and postoperative) RT	3 (2)
Postoperative RT	32 (24)
Surgery alone	8 (6)
Surgery	7 (5)
Surgery and combined chemotherapy	1 (1)
No cancer specific treatment	6 (4)
Basic care only	4 (3)
Palliative chemotherapy	2 (2)
Surgery of the primary tumor	**46** (33)
Local excision	32 (23)
combined with pharyngolaryngectomy	4 (4)
combined with laryngectomy	3 (3)
combined with resection of mandible	2 (2)
combined with hemiglossectomy	1 (1)
Reconstruction at the primary location	**46** (33)
None, direct closure	39 (27)
Free skin graft	1 (1)
Local muscle flap	4 (3)
Microvascular free tissue craft	2 (2)
Surgery of the ipsilateral neck	**46** (33)
None	23 (16)
Radical neck dissection	19 (14)
Supraomohyoidal neck dissection	4 (3)
Surgery of the contralateral neck	**0 (0)**

5.1.6. Treatment

In this cohort, 134 (97%) patients received cancer-specific active treatment, while four (3%) patients received basic care only due to their poor general condition. The treatment was intended to be curative in 120 (87%) and palliative in 14 (10%) cases. The treatment data are presented in table 9. Surgery included excision of the primary tumor in all 46 (33%) operated cases and was extended to also include the larynx, pharynx, mandible or oral tongue, when appropriate. Reconstruction after the tumor resection was required in only 7 cases. Dissection of the ipsilateral neck was performed in 46 (33%) patients. In all irradiated cases, the primary location was treated. The ipsilateral neck was radiated in 102 (74%) patients and contralateral neck in 98 (71%) patients. The median tumor dose was 66 Gy (range 16-80), median ipsilateral neck dose 56 Gy (range 0-72), and median contralateral neck dose 52 Gy (range 0-66).

5.2. Immunohistochemistry (IHC)

5.2.1. Catenins (I)

The median percentage of tumor cells with positively stained plasma membranes was 90 (range 5-100) for all three catenins. The expression was graded as reduced in 57 (49%) tumors for α-catenin, in 32 (28%) for β-, and in 30 (26%) tumors for γ-catenin. β-catenin was expressed in the tumor cell nuclei in 27 (23%) tumors (Figure IVA). The membranous expression patterns (normal or reduced) of various catenins were significantly interrelated with similar trends (χ^2 test for independence; α vs. β, $p<0.001$; α vs. γ, $p<0.001$; β vs. γ, $p=0.025$). Reduced γ-catenin expression was associated significantly with poor tumor differentiation (χ^2 test for independence, $p=0.028$). Membranous and nuclear β-catenin expression did not associate with each other nor was any association detected between other catenin expression patterns and recorded clinicopathological variables.

5.2.2. p53 protein (II)

Heterogeneous nuclear p53 expression was seen in all tumors and was accompanied by cytoplasmic tumor cell staining in 56 (46%) cases. Nuclear p53 overexpression (index > 240) was significantly more common in hypopharyngeal than in the oropharyngeal primary site (χ^2 test for independence, p=0.009), but was not associated with any other clinicopathological patient characteristics (age, sex, histological differentiation, Karnofsky performance status or stage). Cytoplasmic p53 expression was associated with nuclear p53 overexpression (χ^2 test for independence, p<0.001). It was also more common in males (χ^2 test for independence, p=0.017), though no association was detected with other clinicopathological variables (age, primary site, histological differentiation, Karnofsky performance status, stage; data not shown). The tumor stroma remained negative for p53 in all cases. In the normal tissues adjacent to the carcinoma, faint nuclear p53 staining was occasionally visible in the dysplastic epithelium, as well as in the basal and parabasal cells of the normal epithelium. Nuclear p53 overexpression is shown in figure IVB.

5.2.3. Inducible nitric oxide synthase (iNOS; III)

The iNOS staining was mostly restricted to tumor cells (Figure IVC). Occasional faint granular coloring was seen in mononuclear inflammatory cells present in the samples. Positive staining was also present in parts of the glandular ductal epithelium in some samples. The median iNOS score was 4 (range 1-7). The lower values (1-3) were considered to represent weak iNOS expression, and higher values (4-7) strong iNOS expression in the tumor. The obtained iNOS score was low in 57 (41%), high in 61 (45%) and not available in 20 (14%) tumors. iNOS scores were significantly lower in the largest (T4) tumors than in the smaller ones (T1-3) (χ^2 test for independence, p=0.043). No association was seen between iNOS score and N or M class, tumor stage or histological differentiation (data not shown). However, in all four cases presenting with distant metastasis (M1), the iNOS score in the primary tumor was low (Fisher's exact test for independence, p=0.051).

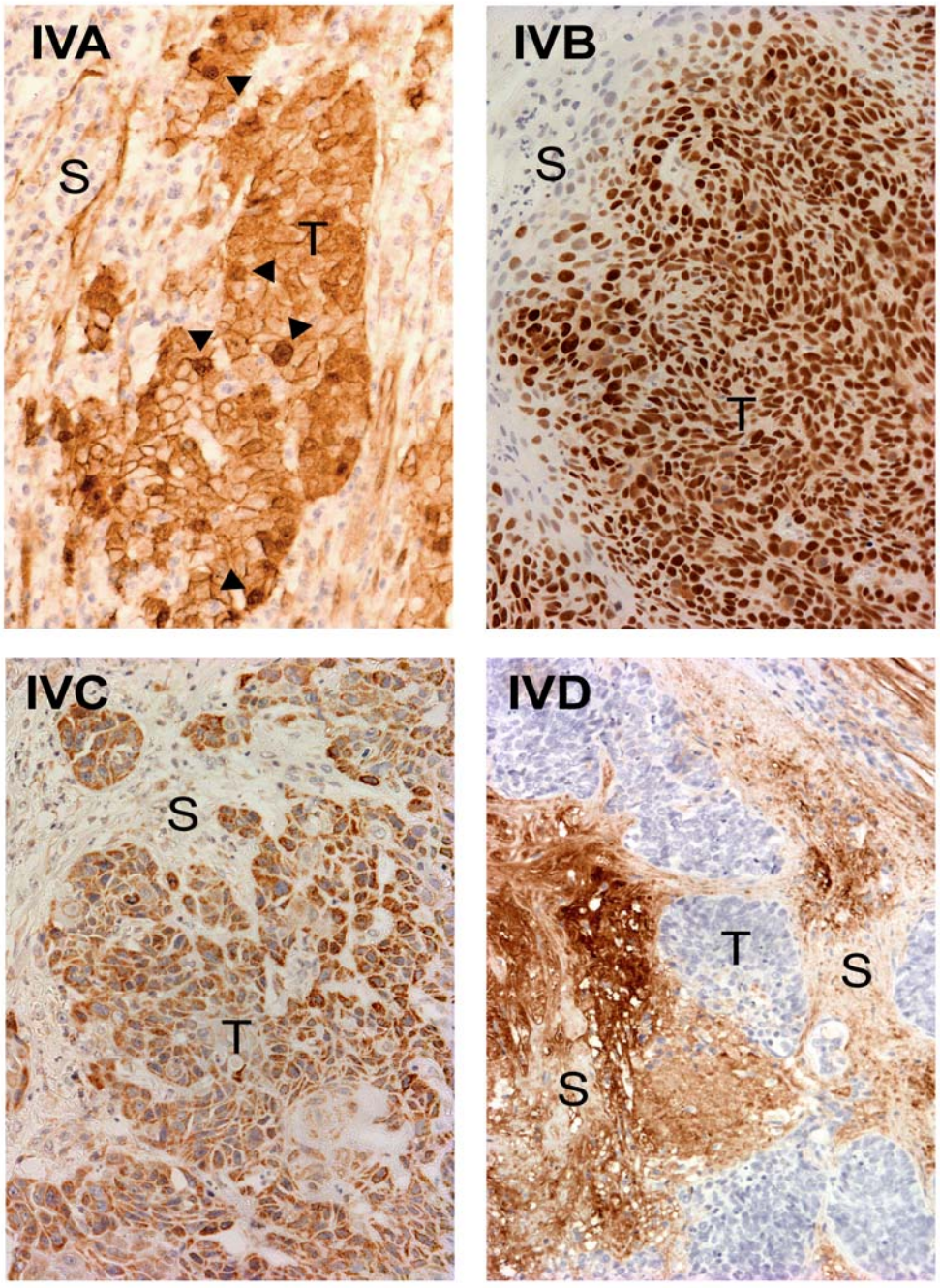

Figure IVA-D. IHC stainings in PSCC; S, stroma; T, tumor. (A) Nuclear β-catenin expression (*arrowheads*). (B) Nuclear p53 expression. (C) Cytoplasmic iNOS expression. (D) Stromal versican expression.

5.2.4. Versican (IV)

In the tumor surroundings, there was immunoreactivity for versican in the blood vessel walls and in the peritumoral connective tissue. In carcinoma, strong stromal versican expression was graded according to median percentage (10%). It was high in 59 (50%) (Figure IVD), but low in 59 (50%) primary tumors. Cytoplasmic versican staining was present in the carcinoma cells in 9 (8%) tumors. Intracellular versican accumulation was more common in association with high strong stromal versican expression, though this association was statistically insignificantly. Of the 14 local lymph node metastases, in only one case was strong stromal versican expression graded low (<10%), while in the remaining 13 cases, the grade was high (≥10%). Strong stromal versican expression in the local metastases was statistically significantly more common than that in the primary tumors (Wilcoxon's nonparametric test for two related samples, p=0.018). A high percentage of strong versican staining was also more common in less advanced tumors (SI-II vs. SIII-IV; χ^2 test for independence, p<0.001) as well as in oropharyngeal tumors (χ^2 test for independence, p=0.013).

5.2.5. Interrelations between variables

A high iNOS score was significantly associated with a high nuclear p53 expression index (χ^2 test for independence, p=0.006) and positive cytoplasmic p53 expression (χ^2 test for independence, p=0.025). Nuclear p53 overexpression (index>240) or cytoplasmic p53 expression was not related to either membranous catenin expression (α-, β-, or γ-catenin) or nuclear β-catenin expression. No association was seen between versican expression and p53, catenins, or any other tested variable.

5.3. Disease-specific survival (DSS)

Complete follow-up data were available for the whole cohort (n=138). The median follow-up time was 20 months (range 1-332; 95% CI 11-29). Of the 138 patients, 45 (33%) remained free of the disease during the follow-up. Forty-two (30%) patients did not achieve complete

Table 10. The location of the relapses in the whole cohort (n=138)

Relapse location	*All (n=51)*	*OP (n=30) (%)*	*HP (n=21) (%)*
Local	29 (57)	15 (50)	14 (70)
Neck	13 (25)	10 (31)	3 (15)
Distant	9 (18)	6 (19)	3 (15)

OP, oropharynx; HP, hypopharynx

response, and in 51 (37%) patients, the cancer relapsed after a disease-free period. In most cases, the carcinoma recurred in the site of the primary tumor (Table 10). The primary site (oropharynx vs. hypopharynx) (χ^2 test for independence, p=0.28) or primary treatment (Fisher's exact test for independence, p=0.27) did not predict the location of the relapse (primary site vs. local lymph node).

At the end of follow-up, only 21 (15%) of the original 138 patients were alive, and 117 (85%) had deceased. In 87 (63%) cases, the cause of death was PSCC, while the remaining 30 (22%) patients had died due to a condition unrelated to PSCC. Ten patients in the last group had died of a secondary malignant tumor. These included four pulmonary carcinomas as well as carcinomas of the oral cavity, esophagus, stomach, rectum,

Table 11. Associations between various prognostic factors and low DSS (n=138; Log rank test)

Variable (cut-off)	*Significance, p*	*Log rank*
Higher age (median)	0.30	1.09
Female sex	0.32	1.0
Hypopharyngeal site (OP-HP)[a]	0.081	3.04
Longer symptomatic period, months (median)	0.75	0.10
Heavier smoker	0.71	1.38
Higher alcohol consumption	0.83	0.87
Low Karnofsky performance status (median)	**<0.001**	**27.74**
Presence of other diseases (no-yes)	0.34	0.90
Higher T class (T1-T2-T3-T4)	**<0.001**	**38.37**
Higher N class (N0-N+)	**0.030**	**4.74**
M class (M0-M1)	**0.0019**	**9.62**
Higher stage (SI-SII-SIII-SIV)	**<0.001**	**39.21**
Lower grade (low-moderate-high)	0.71	0.67
Lower grade (low or moderate vs. high)	0.54	0.38
Palliative goal of the treatment	**<0.001**	**76.10**
Reduced membranous α-catenin	0.48	0.50
Reduced membranous β-catenin	0.87	0.03
Reduced membranous γ-catenin	0.66	0.20
Positive nuclear β-catenin	**0.0013**	**10.35**
Higher nuclear p53 expression index	**0.014**	**6.06**
Positive cytoplasmic p53 expression	**0.040**	**4.23**
Low iNOS expression index	0.78	0.07
Low strong stromal versican expression	0.17	1.84
Negative intracellular versican expression	0.18	1.81

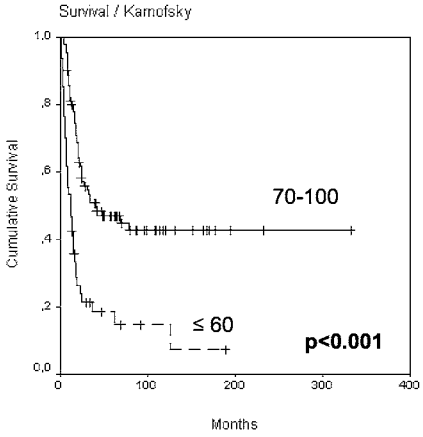

Figure V. DSS, Karnofsky performance status high or low; +, censored case.

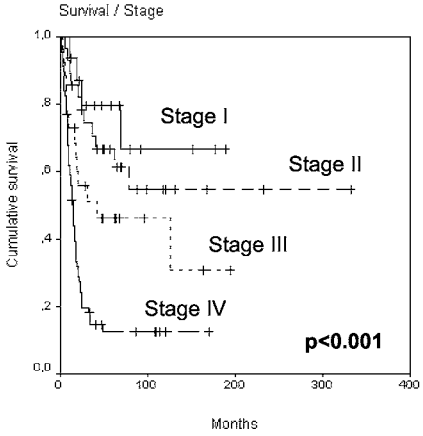

Figure VI. DSS, stage I-IV; +, censored case.

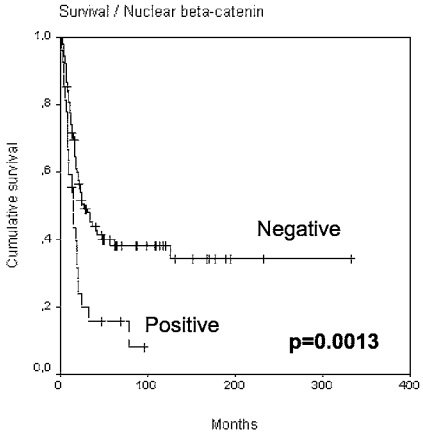

Figure VII. DSS, nuclear β-catenin positive or negative; +, censored case.

pancreas and prostate. In general, the prognosis of the PSCC in this material was inauspicious. The median survival in the whole cohort was 20 months (range 1-332, 95% CI 16-25). The median disease-specific survival (DSS) for the whole cohort was 22.3 (range 1-126; 95% CI 15.4-29.2) months. The DSS rates for 3 and 5 years were 40 (95% CI 32-49) and 37 (95% CI 29-46) percent, respectively. The median DSS was 24.8 months (range 1-126; 95% CI 4.7-44.9) for the oropharynx and 20.9 (range 1-64; 95% CI 14.3-27.5) months for the hypopharynx. The results of univariate survival analyses in the original cohort (n=138) are presented in table 11. In univariate analysis, better DSS was predicted by the post cricoid and oropharyngeal subsites, high Karnofsky performance status (Figure V), low T, N and M classes, low stage (Figure VI), surgical treatment alone or combination of surgery and RT, curative treatment intention, absent nuclear β-catenin (Figure VII), low nuclear p53 expression index, and absence of cytoplasmic p53 expression (Original Publication II, Figures 1 and 2).

In the multivariate analysis (Table 12), only the general condition of the patient (Karnofsky performance status), stage of the disease, and nuclear β-catenin expression predicted DSS and were independent predictors of disease outcome in this cohort. When T class was used in the model as a categorical variable instead of stage, lower T class statistically significantly predicted better prognosis without changing the power of other significant markers (data not shown).

Table 12. Predictors of DSS in Cox proportional hazards model.

Variable (cut-off)	*Hazard ratio*	*95% CI*	*Significance, p*
Age (median)			
Median age or younger*	1.0		
Older	1.10	0.62-1.95	0.75
Sex			
Male*	1.0		
Female	1.23	0.68-2.21	0.50
Site			
Oropharynx*	1.0		
Hypopharynx	1.12	0.66-1.92	0.67
Karnofsky (median)			
High (70-100)*	**1.0**		
Low (40-60)	**0.36**	**0.21-0.63**	**<0.001**
Stage (SI-SII-SIII-SIV)†			**0.001**
S I*	**1.0**		
S II	**1.86**	**0.39-9.0**	**0.44**
S III	**4.35**	**0.89-21.3**	**0.070**
S IV	**7.55**	**1.75-32.6**	**0.007**
Grade (low or moderate vs. high)			
Low or moderate*	1.0		
High	0.97	0.54-1.76	0.92
Membranous α-catenin			
Normal*	1.0		
Reduced	1.17	0.66-2.06	0.59
Membranous β-catenin			
Normal*	1.0		
Reduced	1.04	0.56-1.94	0.89
Membranous γ-catenin			
Normal*	1.0		
Reduced	0.79	0.42-1.52	0.48
Nuclear β-catenin			
Negative*	**1.0**		
Positive	**1.86**	**1.01-3.43**	**0.047**
Nuclear p53 expression index			
Low*	1.0		
High	1.12	0.55-2.29	0.75
Cytoplasmic p53 expression			
Negative*	1.0		
Positive	1.09	0.56-2.14	0.80
iNOS expression index			
High*	1.0		
Low	1.18	0.68-2.03	0.56
Strong stromal versican expression			
High*	1.0		
Low	1.03	0.61-1.75	0.92
Intracellular versican expression			
Negative*	1.0		
Positive	0.62	0.20-1.92	0.41

* Reference

†Categorical variable, reference class stage I

6. DISCUSSION

6.1. The study cohort and clinical data

The present population of 138 PSCC patients includes all the cases from a single geographical area diagnosed with this disease during the study period of 1975–1998, and there was no patient selection at the starting point. Both clinical and histological materials were carefully reviewed. Moreover, the nasopharyngeal primary site was excluded in an attempt to homogenize the original material. Only patients with definitive carcinoma and with accurate as well as sufficient clinical records were included. Knowing the variable nature of HNSCCs in the various sites, incorporation of both oro- and hypopharyngeal tumors into the study material might be somewhat ambiguous. However, similar site selection has been previously done in prognostic studies,[87] while in several other studies even a greater number of sites have been included.[60, 92, 94, 281-283] Additionally, in a large material of 1396 HNSCC cases, the 5-year survival probability for both these sites was fairly similar.[15] Accordingly, pooling together both oro- and hypopharyngeal SCCs into the same material should not have affected or distorted the obtained survival data. This is further supported by the fact that, even though oropharyngeal tumors tended to have better prognosis, the site was not an independent prognostic factor in this cohort.

In the present material, a typical patient diagnosed with PSCC was an older man with a history of tobacco and alcohol abuse. The disease stage at the time of diagnosis was commonly high (SIII-IV 68%), which is fully in line with earlier data for PSCC.[6, 7] Moreover, the symptom spectrum was quite similar to that reported in the literature.[12, 32, 33] Furthermore, the mean age of 64 years (SD 10.9) in the cohort is also similar to that reported in other comparable material.[74] Of the potential clinical prognostic markers, the general condition of the patient,[75, 79, 84-86] T class,[6, 15, 75, 76, 84-86] and stage[7, 90] remained independent prognostic factors in the multivariate analysis. Furthermore, the prevailing treatment strategies and modalities remained fairly constant throughout the whole investigated time period. However, the long study period of 23 years and the resulting wide variation in time of preserving archival tissue samples might be a potential source of error for the studied histological and immunochemical variables. Statistical testing, nevertheless, excluded this possibility because the stainings did not vary according to length of the filing period. Moreover, variations in the fixation processes of the histological samples may have led to differences in the immunogenity of the studied immunohistochemical variables. This

possibility is difficult to exclude and interpret in retrospective studies such as the present work. However, an attempt was made to minimize the effect of this potentially confounding factor, as only well preserved areas in the samples were studied and all necrotic areas were omitted. Finally, the 5-year disease-specific survival of 37% is in accordance with previous data.[7, 10, 15]

The presented data confirm that the current cohort is representative and is qualified for the type of prognostic investigations performed in this series.

6.2. Catenins

Both qualitative and quantitative changes in catenins have been reported to associate with dedifferentiation, dissemination of tumor cells from primary location, and prognosis in various malignant tumors,[179, 284-291] including HNSCC.[187, 191, 292-294] However, the exact mechanisms behind changes in catenin expression in cancer are still unclear. Some reports have demonstrated the important role of catenins in the normal function of cadherin-mediated adhesion and tumor behavior,[292] while in other series, catenin loss has been considered to be less important.[293] The combination of α-catenin with β- or γ-catenin is required for normal E-cadherin mediated adhesion. The reduced expression of a single catenin can thus affect the function of the whole cadherin-catenin complex. For this, α-catenin is crucial, but only β- and γ-catenins can ensure normal and fully functional E-cadherin-mediated adhesion.[295, 296] Similar irregular catenin expression as has been reported earlier in other malignant tumors, such as esophageal,[285] colorectal[286] and nasopharyngeal[294] carcinomas, was evident in the tumors of this series. In the present material, the expression of at least one catenin was reduced in 60 percent (n=69) of the tumors, and the catenin expression patterns were significantly associated with each other. In other head and neck cancer materials coordinated expression of catenins has not been investigated,[185-187, 297] or discovered.[176, 292, 293] However, a significant association was detected between membranous expression patterns of β- and γ-catenins in breast carcinoma, and between α- and γ-catenins in gastric carcinoma,[284, 290] which parallel the results of the present study. In this series, reduced γ-catenin expression was significantly related to poorer histological differentiation. Similarly, in other HNSCC materials, reduced membranous γ-catenin expression has also been related to tumor dedifferentiation.[186, 187] In the present cohort, however, no association was found between membranous α- or β-

catenin expression patterns and tumor grade. Such associations have been reported in HNSCC materials by other investigators.[186-188, 293] This finding is, however, inconsistent, as suggested by the results of several other studies in which differentiation did not associate with catenin expression.[176, 185, 292, 297] In oral SCC, γ-catenin expression has also been associated with invasion, suggesting altered catenin expression to be more profound in the invading tumor areas.[186] This was, however, not clearly evident in the present PSCC cohort. In oral HNSCCs, reduced catenin expression was associated significantly with N+ local lymph node status,[176, 185, 190] but no other pattern, specific for oral, pharyngeal or laryngeal tumor sites, has been reported in the literature.

Interestingly, β-catenin was expressed in the tumor cell nuclei of 27 (23%) tumors. It was also an independent predictor of poor survival in this material. Such a connection has not been previously reported for HNSCC. The aberrant localization of β-catenin in the cell nucleus has been found in many tumors, such as thyroid,[179] hepatocellular,[180, 184] breast,[183] colorectal,[298, 299] endometrial,[300] and esophageal[301] carcinomas, melanomas,[181] and colorectal adenomas,[299] as well as in benign intestinal polyps.[302] In line with the results obtained in the present study, an association between nuclear β-catenin expression and shorter survival has also been reported in colorectal and hepatocellular carcinoma.[184, 298] Previously, nuclear β-catenin expression has been detected in the head and neck region in only nasopharyngeal carcinoma, but not in other regions and it was lacking prognostic significance.[294] Cytoplasmic β-catenin has an important role in both E-cadherin-mediated cell-cell adhesion and in the wnt pathway as a downstream signaling molecule.[168, 170] Normally, intracellular β-catenin is bound by the cytosolic complex, phosphorylated by GSK-3β, and then introduced to the E3/SCF-ubiquitin ligase complex for ubiquination. The ubiquinated β-catenin is thereafter readily degraded in proteosomes. If either the β-catenin phosphorylation site or the APC-β-catenin binding site become mutated, β-catenin is not dismantled and the resulting excess β-catenin is transported into the nucleus where it may activate transcription in association with pontin52, TBT (TATA box binding protein) and LEF/TCF transcription factors. (lymphoid enhancer binding factor / T cell transcription factor).[168, 170] Both β-catenin and APC mutations resulting in nuclear localization of β-catenin have been reported in several cancers.[179-181, 184] Similar alterations could also be speculated to be responsible for the nuclear β-catenin expression and unfavorable prognosis in this material. Another possible route might be induced wnt signaling. Wnts are secreted embryogenetic cell growth, motility and differentiation regulating glycoproteins. They function in a paracrine way and activate numerous intracellular signaling pathways. The

significant role of wnt pathway related factors in cancer is only now begining to be revealed. One of the three main wnt routes, the classical or canonical wnt pathway involves β-catenin. As extracellular wnts bind to the transmembrane receptors, called frizzleds, in the presence of LRP co-receptors, an intracellular mediator called dishevelled is induced. Dishevelled blocks β-catenin phosphorylation, β-catenin is stabilized, the cytoplasmic β-catenin concentration raises and, finally, β-catenin translocates into the nucleus.[170] Interestingly, cyclin D1 has been shown to be a target gene of the wnt/APC/β-catenin pathway, and cyclin transactivation, secondary to APC or β-catenin mutations, might participate in colon cancer initiation.[110] Several other investigators have also reported catenin-cyclin interactions in cancer.[303, 304] Overexpression of the integrin-linked kinase induces β-catenin stabilization, which may also indicate an association between wnt and integrin signaling pathways as well as oncogenic transformation.[171]

As stated above, catenins may entail a malignant phenotype through several intricate mechanisms, thus promoting numerous cellular features typical of malignant cells. The exact mechanisms between nuclear β-catenin expression and dismal prognosis detected in the PSCC at hand remain to be hypothesized.

6.3. p53

The role of p53 in HNSCC is contradictory. In the present material, both nuclear p53 overexpression and cytoplasmic p53 expression suggested a more aggressive disease course and outcome, though not independently. The monoclonal p53 antibody used in the present study was D07, which reacts with both wt and mutated p53 proteins.[121, 227, 305-312] In HNSCC, the expression of nuclear p53 demonstrated by IHC is a common, though rather confusing, finding as it has alluded towards both favorable[305, 307, 312] and unfavorable[227, 308, 310] disease outcome. In our material, in line with earlier reports,[227, 313] a high nuclear p53 expression index was a sinister prognostic marker. Cytoplasmic accumulation of p53 has previously been described in HNSCC[305, 306, 313] along with other malignant tumors.[314-320] Hirvikoski *et al.*[305] reported cytoplasmic accumulation of p53 in 20% of the laryngeal carcinomas using the same monoclonal antibody as in the present study. In our PSCC material, nuclear p53 expression was accompanied by supplementary cytoplasmic p53 staining in as many as 45% of the tumors. Our results suggested that in PSCC, cytoplasmic accumulation of p53 protein also points towards more aggressive tumor behavior. The

prognostic impact of cytoplasmic p53 is also controversial, and has been shown to associate with both favorable[320] and unfavorable[316, 317] prognosis. Association between cytoplasmic p53 expression and prognosis has not been previously reported in HNSCC.

Active carrier-mediated transport of p53 protein between the cytoplasm and nucleus has been shown to exist in both directions.[321] Moving p53 in and out of the nucleus has been suggested to be a fast and sensitive way of regulating its activity.[322] However, cytoplasmic p53 expression does not seem to be an unequivocal sign of p53 inactivation in tumors, as in neuroblastoma cells, p53-mediated functions are not affected in spite of wt p53 sequestration in the cytoplasm.[323] Additionally, the mutated p53 protein has also been demonstrated in tumor cell cytoplasm. In colorectal cancer, Jansson *et al.*[315] have shown cytoplasmic p53 accumulation to associate with mutations similar to those seen with nuclear p53 overexpression. The immediate p53 function, namely transcriptional activation of target genes, takes place in the nucleus. In the absence of p53 induction by cellular stress, p53 activity may be suppressed by hampered transportation into the nucleus or accelerated moving outwards from there. In tumors lacking a p53 mutation, disturbances in these mechanisms may explain nonfunctional p53 status.[322] Alterations in p53 degradative systems is another mechanism implicated in inhibition of appropriate p53 activation.[322]

In bladder carcinoma, reduced membranous β-catenin expression was associated with nuclear p53 overexpression.[324] Moreover, β-catenin overexpression has been demonstrated to induce p53 accumulation in cell culture, apparently by blocking its proteolysis.[325] Miyagishi *et al.*[326] have shown in their recent *in vitro* study that a stable β-catenin mutant can competitively suppresses the p53-dependent pathway. Furthermore, Wang *et al.*[327] have reported downregulation of an intracellular regulatory cascade involving β-catenin (wnt signaling pathway) by p53. These results suggest an association between β-catenin and p53. However, in this clinical material, we found no sign of such an interrelationship on a protein level. Finally, p53 has also been shown to associate with the cyclin pathway.[328, 329]

The exact means resulting in cytoplasmic or nuclear p53 expression, as detected by DO7 monoclonal antibody, in this PSCC material remains speculative. Apparently, both reflect a summation of several mechanisms involving mutations, as well as disordered synthesis, cytoplasmic and nuclear transportation, and/or degradation. It can be concluded that in the present large PSCC material, nuclear p53 overexpression was associated with unfavorable prognosis. Interestingly, positive cytoplasmic p53 expression was also clearly associated with a dismal prognosis. However, additional studies applying new techniques will be necessary in order to reveal the exact role of p53 in both HNSCC and PSCC.

6.4. Inducible nitric oxide synthase (iNOS)

The source of iNOS expression in cancer is controversial. In the current PSCC material, iNOS staining was mostly seen in the tumor cells, but sparse granular coloring was also infrequently visible in mononuclear inflammatory cells infiltrating tumors. These observations support earlier results from HNSCC,[271, 273, 330] and suggest that in these tumors most of the NO is produced by iNOS situating in tumor cells. In colon carcinoma, iNOS activity has been localized mostly in tumor-infiltrating inflammatory cells.[263] The reasons for this difference remain to be elucidated.

In some cancers, iNOS expression has been reported to decrease along with tumor dedifferentiation.[265, 267] In this PSCC cohort, however, iNOS expression did not associate with histological differentiation, as has also been reported before in both HNSCC[271, 273, 330] and other cancers.[264, 266, 331] In the current PSCCs, identical to the results in oral SCC,[273] changes in iNOS expression were not associated with the primary tumor site.

The relation between tumor size and iNOS expression has not yet been resolved. In the present cohort, iNOS expression was weakest in the largest (T4) tumors. The association is quite the opposite to that found in local prostate cancer where strong iNOS expression was associated with higher pT class.[266] In oral squamous cell carcinoma, changes in iNOS expression did not relate to T class.[273, 330] In addition, our results suggest that iNOS expression does not depend on N class in PSCC. Analogous results have been reported for malignant tumors originating in the esophagus,[264] prostate,[266] and in melanoma.[332] In some HNSCC materials,[271, 330] as well as in breast cancer,[331] stronger iNOS expression has been reported to favor local metastasis. Moreover, in HNSCC, higher N class has been associated with lower iNOS expression.[272, 274]

The inhibition of iNOS expression at an early stage of tumor progression has been proposed as a means for delaying tumor growth in head and neck carcinomas.[272] In both HNSCC[272] and colorectal carcinoma,[263, 267] iNOS activity has been reported to decline with advancing tumor stage. However, higher iNOS activity in more advanced HNSCCs has also been reported by Gallo *et al.*[271] In this material, iNOS expression was not associated with tumor stage. iNOS expression was not either linked with prognosis in the present study, whereas in colorectal carcinoma reduced iNOS expression has been reported to imply a dismal prognosis.[267]

In the present PSCC cohort, a high iNOS score was statistically significantly associated with both high nuclear p53 expression and cytoplasmic p53 expression. Similarly, an association between iNOS and p53 expression has been reported for oral

SCC.[273] The apparent association between iNOS and p53 has been indicated in both carcinogenesis and tumor progression.[333] It has been suggested that stimulated iNOS expression and subsequent NO production might prejudice DNA, thus inducing p53 mutations in human colon tumors.[263] Accordingly, NO seems to be capable of causing DNA damage both directly and indirectly by inhibiting DNA repair mechanisms.[258] In addition, NO has been shown to induce cellular p53 accumulation in human cells *in vitro*.[334, 335] Moreover, in the work of Forrester *et al.*,[334] the cellular accumulation of wt p53 was found to repress iNOS gene promoter activity, resulting in down-regulation of iNOS expression in those cells and thus suggesting a negative feedback loop between iNOS overexpression and p53. When studying the influence of iNOS expression in head and neck tumors, Gallo *et al.*[271] found that elevated tumor NOS activity prompted angiogenesis and metastatic phenotype in HNSCC. These findings, along with knowledge of the high p53 mutation frequency in HNSCC and the aforementioned results, drove Gallo and colleagues[271] to suggest that in HNSCC, elevated NOS activity and consequently induced tumor angiogenesis were due to p53 mutation. However, Ambs *et al.*[263] have proposed induced iNOS expression to be the preliminary step leading to secondary DNA damage and eventually mutated, non-functional p53 tumor suppressor protein. More recently, they have, nevertheless, presented results supporting the possibility that p53 mutation might be the cause for induced iNOS expression in HNSCC.[278] Taken together, the causal relationship between induced expression of both iNOS and p53 in cancer still remains to be elucidated.

6.5. Versican

In malignant tumors, versican constrains cell adhesion, and promotes as well proliferation, invasion, and migration.[201, 211] In some tumors, intense versican expression is associated with more aggressive tumor behavior.[203, 205, 207, 208] In malignant breast,[205] prostate,[208] and ovarian tumors,[207] and possibly also in gliomas,[203] a gain in stromal versican expression has been associated with a more aggressive clinical course of the disease. However, in the present study, versican expression patterns were not prognostic features.

In breast carcinoma[206] and melanoma,[200] the expression of versican has been reported both in primary tumors and metastases. Interestingly, a significantly higher percentage of strong stromal versican expression was found in local metastases than in the primary tumors in the present PSCC cohort. Similar differences in the intensity of versican expression between primary tumors and metastases have not been previously described.

This finding, nevertheless, is still solitary and requires confirmation from other tumor cohorts. However, the detected high versican expression in the metastases of this material presumably reflects the importance of versican-related mechanisms—inhibited adhesion,[210] precipitated proliferation,[209, 211] and induced migration[210]—in metastasis formation and growth.

In this PSCC material, versican was expressed in tumor stroma inside and around the tumor. This finding is supported by several previous reports.[200-208] Intermediators secreted by tumor cells have been suggested as a means for inducing versican production in tumor stroma.[205, 336, 337] In cancers, versican deposits have been demonstrated both in tumor stroma[206] and tumor cells.[200, 201, 338] mRNA analyses have also shown that tumor-associated versican is synthesized both in the peritumoral stroma and tumor cells.[201, 206] In addition to stromal versican staining, some tumors in the current series showed supplementary clear intracellular versican accumulation. Intracellular versican accumulation may result from aberrations in versican production, storage, degradation, or cellular uptake. The causes and mechanisms promoting intracellular versican expression, however, need further investigations.

In vitro studies have shown that versican binds to CD44.[339] and that the amino-terminal domain (G1) of versican binds hyaluronan with high affinity.[195] Versican and HA expression have also been linked in cancer, as stromal versican has been shown to co-occur with HA in ovarian carcinoma.[207] The increased amounts of versican together with ECM macromolecules, such as polysaccharide HA, increase pericellular matrix volume and distend the ECM, thereby permitting tumor invasion.[192] Versican may also stimulate stroma synthesis in tumors through induced fibroblast proliferation, which has been illustrated in experimental cell models *in vitro*.[209] Apart from a tumor promoting effect, Cattaruzza *et al*.[340] have recently demonstrated that HA may inhibit versican-induced cell proliferation in sarcoma cells. In addition to HA, both versican and membranous catenins participate in cellular adhesion. It has been suggested that the extracellular matrix (including, e.g., versican) may play an important role in modulating β-catenin stability.[341] Moreover, the results in studies by Xu and Yu interestingly show that E-cadherin, a key binding partner of catenins, negatively regulates CD44-hyaluronan interactions and CD44 functions.[342] They demonstrated that induced E-cadherin expression may impede CD44-mediated tumor cell invasion into HA-rich ECM.[342] Even though the aforementioned implications bind E-cadherin and β-catenin to versican, these implications could not be validated in the present clinical PSCC material.

The versican gene is regulated by the tumor suppressor p53 *in vitro*, as demonstrated

by Yoon *et al.*[343] Their results show that versican gene expression is directly induced by p53 at least in a controlled exprerimental laboratory environment. Inspired by this finding, immunohistological data of versican and p53 expression were combined in the present study. Traces of an interface were, however, not revealed. The results indicate that no significant associations (inductive or suppressive) exist between versican and p53 at the protein expression level in PSCC. This may be because of the absence of a true functional interrelationship. It is also possible that p53 is mutated or otherwise nonfunctional in carcinoma cells. Other possibilities include otherwise inhibited or altered versican transcription and protein expression or function.

The cumulative data suggest that one fundamental mechanism, through which versican may advance cancer progression is angiogenesis promotion and fabrication of intensely vascular stroma in the tumor surroundings.[206, 212, 344] Proangiogenic tumor growth factor β1 (TGF-β1) derived from prostate cancer cells has been demonstrated to increase versican synthesis in cultured fibroblasts.[337] Moreover, in prostate cancer, versican has been suggested as a factor promoting angiogenesis in cooperation with other factors.[344] Zheng et al. (2004) have recently shown, how the G3 domain of versican can excite fibronectin and VEGF synthesis in astrocytoma cells and form active complexes with them.[212] Interactions incorporating these three fragments generated changes promoting angiogenesis, including enhanced endothelial cell adhesion, proliferation, and migration.

Versican, as indicated by its name, seems to have numerous efficient ways to work in cancer. However, further studies will be required to clarify both the general roles and exact mechanisms of action of versican in human neoplasia, and particularly in HNSCC.

7. SUMMARY AND CONCLUSIONS

PSCC is a rare disease strongly associated with tobacco and alcohol use. Advances in histological diagnostics, staging and treatment have not succeeded in elevating the low survival rates characterizing this disease. PSCCs are heterogeneous tumors that can not be accurately classified by the present means and thus requires new methods. In the present work, the immunohistochemical expression of α-, β- and γ-catenins, p53 protein, inducible nitric oxide synthase (iNOS), and versican were studied in a PSCC cohort consisting of 138 patients. The expression patterns were related to salient clinical and histological data, to each other, and to patient survival. Of particular interest was their potential prognostic significance for PSCC.

The main findings in the present study with 138 patients are:

1. clinical TNM class, stage and the general condition of the patient were the most important prognostic factors

2. nuclear β-catenin was a significant prognostic factor for poor overall survival

3. both nuclear and cytoplasmic p53 expressions were associated with unfavorable disease outcome

4. iNOS expression was significantly weaker in large tumors.

5. versican was expressed more actively in the earlier stages of PSCC, and in local neck lymph node metastases

In conclusion, along with more traditional prognostic factors, nuclear β-catenin expression can also be used as a prognostic marker in PSCC.

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