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ANNA JOUPPILA-MÄTTÖ

*Epithelial-mesenchymal
Transition and Expression
of Its Transcription Factors
in Pharyngeal Squamous
Cell Carcinoma*

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ANNA JOUPPILA-MÄTTÖ

*Epithelial-mesenchyma transition and
expression of its transcription factors in
pharyngeal squamous cell carcinoma*

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Epithelial-mesenchymal transition and expression of its transcription factors in pharyngeal squamous cell carcinoma.

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ABSTRACT

Although pharyngeal squamous cell carcinoma (PSCC) is a rather rare disease its incidence has been increasing over past 3 decades now accounting for 130 000 new cases and 80 000 cancer deaths worldwide. The prognosis is one of the poorest of all the head and neck squamous cell carcinomas (HNSCC) and it has not improved to any significant extent despite the availability of multimodal therapies. The main risk factors for PSCC are the use of tobacco and alcohol. Recently the human papilloma virus (HPV) has been shown to be involved in evolution and prognosis of oropharyngeal squamous cell carcinoma (SCC). No other distinct biomarkers for PSCC patient survival have been established thus far.

Epithelial-mesenchymal transition (EMT) is a complex cellular process which is not only crucial for embryogenesis but it is also activated during tumor progression, enabling tumor cells to become invasive and to metastasize. Several transcription factors, like *snail*, *twist*, *sip1*, *slug* and *zeb1*, are fundamental in regulating EMT. The role of EMT-related transcription factors (EMT-RTFs) has not yet been established in PSCC.

In the present study, the immunohistochemical expressions of transcription factors *snail*, *twist*, *sip1*, *slug* and *zeb1* were analyzed in tumor cell, stromal and endothelial cell nuclei as well as in cytoplasm of PSCC samples in an effort to evaluate the association of their expressions to clinicopathological variables and patient prognosis. *Snail* expression in endothelial cell nuclei predicted a reduced disease specific survival (DSS). *Snail* expression in endothelial and stromal cell nuclei also associated with hypopharyngeal tumors, increased T-stage (T3-4) and poor general condition of the patient. Those tumors which displayed intense stromal staining of *twist* relapsed more frequently. In contrast, those tumors with negative immunostaining of *twist* and *snail* in stromal cell nuclei were smaller (T1-2), less advanced (Stage I-II) and located more often in the oropharynx. Those patients also had a better 5-year DSS and overall survival (OS). Tumors with positive epithelial nucleal *sip1* immunostaining were more advanced (Stage III-IV) and had more lymph node metastases. *Sip1* immunoreactivity significantly correlated with DSS and OS. Epithelial *sip1* was an independent prognostic factor in Cox proportional hazards model together with Karnofsky performance status score and T-stage. In the same multivariate analysis, co-expression of *snail*, *twist* and *sip1* in tumor epithelial cell nuclei predicted even poorer prognosis than *sip1* expression alone.

In conclusion, *sip1* seems to be a potential new prognostic factor in PSCC, and might be applicable for clinical use to indicate those patients with more ominous tumor behavior requiring more aggressive therapy and closer follow-up.

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TIIVISTELMÄ:

Nielun levyepiteelikarsinooma on varsin harvinainen sairaus, jonka esiintyvyys on kuitenkin noussut viimeisen 30 vuoden aikana. Uusia tapauksia ilmaantuu vuosittain 130 000 aiheuttaen 80 000 syöpäkuolemaa maailmanlaajuisesti. Taudin ennuste on huonoin kaikista pään ja kaulan alueen syövästä eikä ole parantunut viime aikoina huolimatta uusista hoitokeinoista. Tärkeimmät riskitekijät ovat tupakka ja alkoholi, joskin papilloomaviruksen on viime aikoina osoitettu vaikuttavan suunielun levyepiteelikarsinooman ilmiäsuun ja ennusteeseen. Yksiselitteisiä ennusteen arvioinnissa käytettäviä biomerkkiaineita ei toistaiseksi ole käytössä nielusyövässä.

Epitelialli-mesenkymaalinen transitio (EMT) on monimuotoinen prosessi, joka on välttämätön sikiökehitykselle mutta sen on todettu käynnistyvän myös syöpäsoluissa. Sen myötä kasvainsolut saavat kasvua ja metastasointia edistäviä ominaisuuksia. Snai1, twist, sip1, slug ja zeb1 ovat EMT:n säätelytekijöitä, joiden merkitystä nielusyövässä ei ole aiemmin selvitetty.

Tämän väitöskirjatyön tarkoitus oli selvittää yllä mainittujen säätelytekijöiden ilmentymistä kasvainsolujen, tukikudosten solujen sekä verisuonten endoteelisolujen tumissa sekä sytoplasmassa immunohistokemiallisin menetelmin ja arvioida ilmentymisen yhteyttä potilaan ennusteeseen ja kliinis-patologisiin muuttujiin. Snai1:n ilmentyminen kasvaimen endoteelisoluissa oli yhteydessä huonoon ennusteeseen, suureen kasvainkokoan (T3-4), kasvaimen sijaintiin alanielun alueella sekä potilaan alentuneeseen yleistilaan. Twist-positiivisuus kasvaimen tukikudoksessa liittyi suurempaan taudin uusimisriskiin. Kasvaimet, joiden tukikudossolut eivät ilmentäneet snai1:tä eivätkä twist:ä olivat pienempiä (T1-2) ja sijaitsivat useammin suunielussa. Näiden potilaiden ennuste oli merkitsevästi parempi. Pitkälle edenneiden (Stage III-IV), kaulalle etäpesäkkeitä jo tehneiden kasvainten tumissa todettiin usein sip1-proteiinia. Tämä ilmentyminen korreloi merkitsevästi potilaiden kuolleisuuteen ja oli myös itsenäinen ennustetekijä monimuuttuja-analyysissä yhdessä T-luokan ja potilaan yleistilan kanssa. Kun sekä snai1, twist että sip1 ilmentyivät yhtä aikaa kasvainsolujen tumissa, potilaan ennuste oli erittäin huono; tämä löydös oli niin ikään itsenäinen ennustetekijä monimuuttuja-analyysissä yhdessä T-luokan ja potilaan yleistilan kanssa.

Väitöskirjatyön perusteella sip1-proteiinia voidaan pitää mahdollisena nielusyövän ennustemerkkiaineena, joka voisi soveltua kliiniseenkin käyttöön osoittamaan aggressiivisesti käyttäytyvät kasvaimet.

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Ja Elsa: Nyt se on valmis!

Kuopio, October 2014

Anna Jouppila-Mättö

List of the original publications

This review is based on the following substudies. The Roman numerals below are used when reference is made to the original papers:

- I. Jouppila-Mättö A, Tuhkanen H, Soini Y, Pukkila M, Närkiö-Mäkelä M, Sironen R, Virtanen I, Mannermaa A, Kosma VM. Transcription factor snail expression and poor survival in pharyngeal squamous cell carcinoma. *Histol Histopathol* 26(4):443-9, 2011.
- II. Jouppila-Mättö A, Närkiö-Mäkelä M, Soini Y, Pukkila M, Sironen R, Tuhkanen H, Mannermaa A, Kosma VM. Twist and snail expression in pharyngeal squamous cell carcinoma stroma is related to cancer progression. *BMC Cancer* 11:350, 2011.
- III. Jouppila-Mättö A, Mannermaa A, Sironen R, Kosma V-M, Soini Y, Pukkila M. Sip1 predicts progression and poor prognosis in pharyngeal squamous cell carcinoma. *Histol Histopathol (accepted)*. In press.

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Abbreviations

CAF	Carcinoma-associated fibroblast	p16	Cyclin-dependent kinase inhibitor
CSC	Cancer stem cell	p53	Tumor suppressor gene, phosphoprotein p53
CT	Computer tomography	PAK1	p21-activated kinase-1
DSS	Disease specific survival	PBS	Phosphate buffered saline
ECM	Extracellular matrix	PDGF	Platelet derived growth factor
EGF	Epidermal growth factor	PSCC	Pharyngeal squamous cell carcinoma
EGFR	Epidermal growth factor receptor	RTK	Receptor tyrosine kinase
EMT	Epithelial-mesenchymal transition	SCC	Squamous cell carcinoma
EMT-RTF	EMT- related transcription factor	TGF- β	Transforming growth factor
EndMT	Endothelial-mesenchymal transition	TNM	Tumor-node-metastasis classification
FGF	Fibroblast growth factor	UICC	Union Internationale Contre le Cancer (International Union Against Cancer)
Gr	Grade (1-3), histopathological differentiation grade	WHO	World Health Organization
GSK3 β	Glycogen synthase kinase 3 β		
HIF1 α	Hypoxia-inducible factor-1 α		
HNC	Head and neck cancer		
HNSCC	Head and neck squamous cell carcinoma		
HPV	Human papilloma virus		
KPS	Karnofsky performance status		
MET	Mesenchymal-epithelial transition		
miRNA	Small, non-coding RNA		
MMP	Matrix metalloprotease		
MRI	Magnetic resonance imaging		
NF κ B	Nuclear factor κ B		
OS	Overall survival		

1 Introduction

Head and neck cancer (HNC) comprises a group of malignant tumors originating in several anatomical areas but over 90% of them are squamous cell carcinomas (SCC).(1) Pharyngeal squamous cell carcinoma (PSCC) includes nasopharyngeal, oropharyngeal and hypopharyngeal subsites. Nasopharyngeal carcinoma differs extensively both histologically and biologically from the other pharyngeal carcinomas and therefore it will not be discussed in this thesis. The incidence of PSCC has been increasing over the past three decades and now it accounts for 130 000 new cases and 80 000 cancer deaths worldwide.(2,3) The prognosis is one of the poorest among all head and neck squamous cell carcinomas (HNSCC) and has not improved despite the advances in multimodal therapies. PSCC is typically diagnosed at an advanced stage. The delay in diagnosis, appearance of second primaries and locoregional recurrences together with heterogenic, unpredictable tumor behavior all contribute to the poor prognosis.(4,5) The prognosis and recommended treatment modalities are determined mainly by the tumor-node-metastasis (TNM) status.(6) The main risk factors for PSCC are the use of tobacco and alcohol. Recently human papilloma virus (HPV) has been discovered to be involved in the appearance and prognosis of oropharyngeal SCC.(7-10)

Carcinogenesis is a multistep process characterized by the gradual accumulation of mutations in cancer cells. These transformed cells also modify their surrounding stromal tissue, a connective tissue framework with fibroblasts, inflammatory cells, fat cells and blood vessels creating a tumor growth supporting environment. Normal stromal fibroblasts are capable of inhibiting tumor growth but during tumor progression, the microenvironment shifts towards a growth-promoting nature and tumor associated fibroblasts become promoters of tumor progression. (11,12)

Epithelial-mesenchymal transition (EMT) is a complex cellular process crucial in embryogenesis but which is also activated during tumor progression and wound healing.(13,14) In the course of EMT, epithelial cells lose their organized arrangement and cohesion and acquire motile as well as invasive characteristics, enabling them to invade and metastasize. EMT is characterized by downregulation of certain epithelial adhesion molecules such as E-cadherin along with the upregulation of mesenchymal genes like N-cadherin, α -smooth muscle actin and vimentin.(15) EMT is driven by extracellular signalling such as that provided by soluble growth factors derived from surrounding tumor and stromal cells as well as environmental stimuli e.g. hypoxia.(16)

The EMT-process is regulated by several transcription factors including snail superfamily (snai1, snai2[l.slug] and snai3), twist with its family members as well as the zeb superfamily (zeb1 and zeb2 [l.sip1]). All of those factors induce EMT by provoking E-cadherin downregulation via binding to its promoter region.(17-20) These EMT-related transcription factors (EMT-RTFs) have also several other functions in tumorigenesis including blockade of the cell-cycle and preventing cells from undergoing apoptosis, as well as regulating cell polarity and angiogenesis. (21-27) The overexpression of EMT-RTFs has been associated with a poor outcome in many human cancers. Snail expression in laryngeal SCC associated with local recurrence and stromal immunoreactivity of snail correlated with curtailed disease specific survival (DSS) in patients with colon adenocarcinomas.(28,29) Similarly, slug overexpression has been related to aggressive tumor behavior and poor survival in patients with oesophageal SCC.(30) High twist expression associated with aggressive tumor properties and poor prognosis in oesophageal, breast and cervical SCC.(31-33) There is a report that sip1 was an independent prognostic factor in oral SCC.(34) There is also evidence of co-expression of EMT-RTFs resulting in a synergistic enhancement effect. The co-expression of twist and snail1 in hepatocellular

carcinoma and small-cell lung cancer predicted worse overall survival (OS) than either positive transcription factor alone.(35,36) However, according to other reports, the expression of EMT-RTFs does not seem to associate with tumor progression in several other carcinomas.(37-39) This highlights the versatile and complex roles of EMT-RTFs in distinct tumors. No studies addressing the role of EMT-RTFs in PSCC have been published previously.

In the present study, the immunohistochemical expressions of transcription factors *snai1*, *twist*, *sipl*, *slug* and *zeb1* were analyzed in tumor cells as well as stromal and endothelial cell nuclei and cytoplasm of PSCC samples in an attempt to evaluate the associations between their expressions, clinicopathological variables as well as with patient prognosis.

2 Review of the Literature

2.1 PHARYNGEAL SQUAMOUS CELL CARCINOMA

2.1.1 Anatomy of the pharynx

Pharynx is anatomically divided into three regions: nasopharynx, oropharynx and hypopharynx. Pharynx is a part of the upper aerodigestive track taking also part in voice formation and immunological defence. This thesis focuses on oro- and hypopharynx, which form a histopathologically and therapeutically distinct entity. Oropharynx is bounded proximally by the posterior edge of the hard palate and distally by the valleculae and hyoid bone. The posterior limit is defined by the muscular pharyngeal wall whereas the circumvallate papillae and palatoglossal muscle mark the anterior border. The lateral walls of the oropharynx are composed of the tonsillae and tonsillar fossae. (40) (Figure 1.) The hypopharynx lies posterior to the larynx extending from superior border of epiglottis to the inferior border of cricoid cartilage and further down to the superior oesophageal sphincter. The posterior border is formed by pharyngeal inferior constrictor muscles and laterally, forming lateral borders of pyriform sinuses are thyroid cartilage and thyrohyoid membranes. The subsites are presented in table 1 and Figure 2.

Table 1. Oro-and hypopharyngeal subsites. Modified from TNM classification of the malignant tumors. 7th edition. Sobin et al. (2010)

Site
OROPHARYNX
1. Anterior wall (glosso-epiglottic area)
1.1 Base of tongue (posterior to the vallate papillae or posterior third)
1.2 Vallecula
2. Lateral wall
2.1 Tonsil
2.2 Tonsillar fossa and tonsillar (faucial) pillars
2.3 Glossotonsillar sulci (tonsillar pillars)
3. Posterior wall
4. Superior wall
4.1 Inferior surface of soft palate
4.2 Uvula
HYPOPHARYNX
1. Pharyngo-oesophageal junction
2. Pyriform sinus
3. Posterior pharyngeal wall

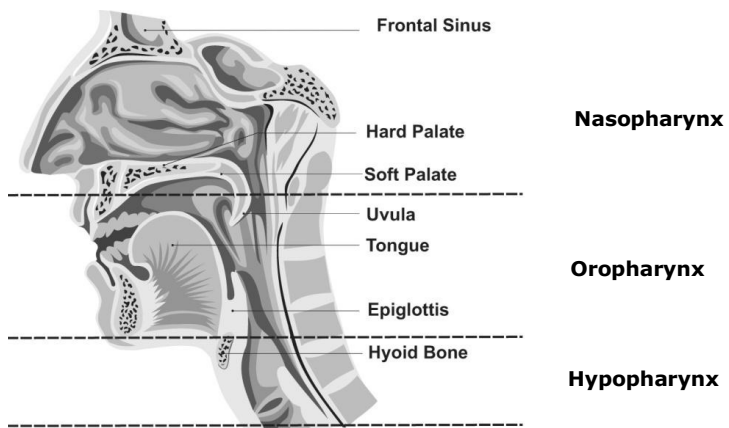


Figure 1. Anatomy of pharynx, lateral view. (Modified from www.greenfacts.org)

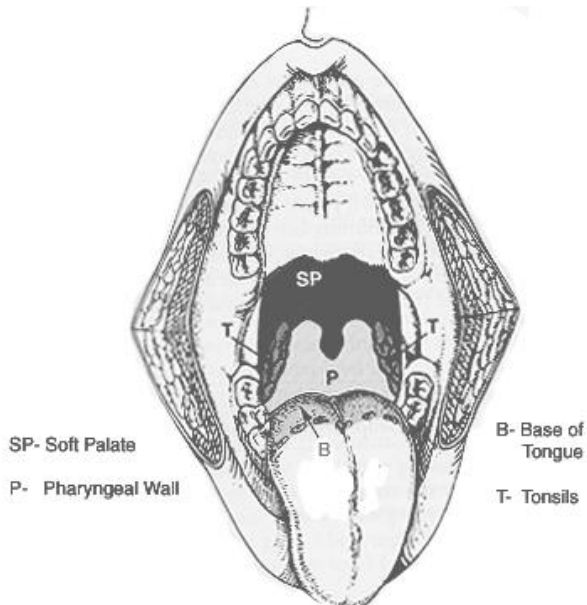


Figure 2. Oropharyngeal subsites, anterior view. (www.wehealny.org)

Cervical lymph nodes sites are divided into six regions. In PSCC, the lymph nodes in the regions II-IV have the highest probability for metastases.(41)

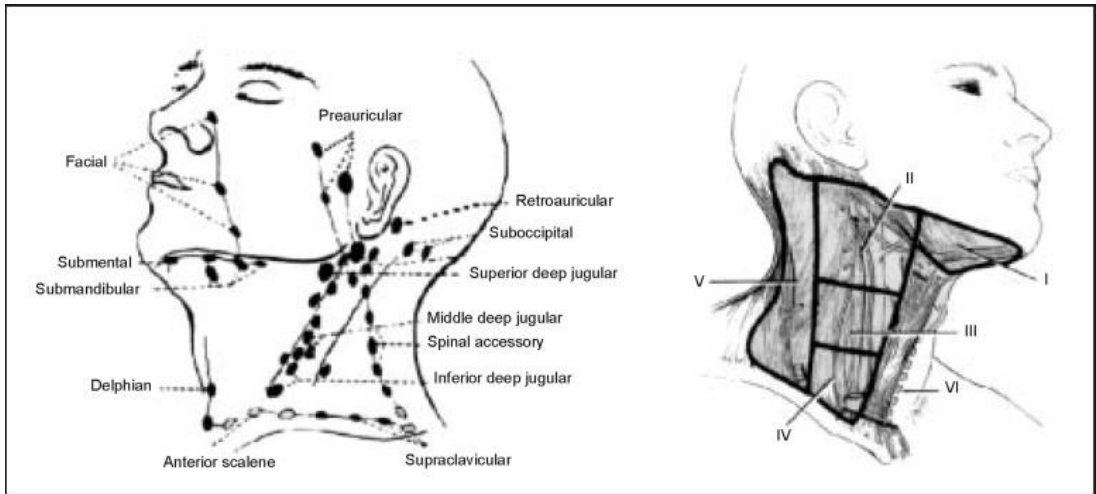


Figure 3. Cervical lymph node regions, lateral view. (www.sciencedirect.com)

2.1.2 Risk factors and epidemiology

The consumption of tobacco and alcohol are the best-known risk factors for pharyngeal carcinoma. Acetaldehyde, the major metabolite of ethanol, has been proved to be carcinogenic not only in animals but also in humans.(42) the levels of acetaldehyde in saliva are higher among smokers than non-smokers and smoking combined with alcohol consumption may increase the saliva acetaldehyde levels by as much as 7-fold.(43) Environmental tobacco smoke has also been reported to increase the risk for HNSCC.(44) Betel nut chewing is an important risk factor not only for oral but also pharyngeal SCC, especially in Asia.(45) Human papilloma virus (HPV), mainly type 16 and to a lesser extent 18, is a newly identified risk factor especially for carcinomas of the tonsils and the base of the tongue. According to the molecular analyses, 35-73% of oropharyngeal carcinomas are HPV positive and this percentage seems to be rising.(7-10) HPV16 genomic DNA was detected in up to 72% of the oropharyngeal cancers.(1,7) A diet poor in fruit and low in vitamins increases the risk for PSCC.(46) In contrast, recreational physical activity seems to protect from HNC, especially among smokers and drinkers.(47)

HNSCC, which can be located in several anatomic areas, is the sixth most prevalent malignancy in the world with approximately 900 000 cases diagnosed annually worldwide.(48) Of all HNSCCs, pharyngeal carcinoma constitutes less than one sixth accounting for 130 000 new cases and over 80 000 deaths yearly worldwide.(3,49,50) Incidence rates of oral and pharyngeal cancer among men are 2-4 higher than those among women and the vast majority of patients are over 45 years old at the time of diagnosis.(10)

However, there are increasing rates of cancer of the tongue being diagnosed in young men and women. The former disparity between sexes has decreased progressively during the recent decades in several HNCs.(4,10) The incidence of oropharyngeal carcinoma is increasing in spite of a decreasing prevalence of smoking.(2) In Finland the overall age-adjusted incidence of pharyngeal carcinoma was 0.89 per 100 000 inhabitants between years 1986 and 1996; 1.28 in men and 0.60 in women.(51) In the last 20 years, the incidence of oropharyngeal carcinoma increased from 0.66 to 1.36/100 000 person-years and the relative frequency of p16 positive PSCC has increased from 22% to 41% during the same time period.(2)

2.1.3 Histopathology and grading

Over 90% of the pharyngeal carcinomas are squamous cell carcinomas (SCC) originating from the mucosal epithelium. The World Health Organization (WHO) histological grading of SCC has been described by Shanmugaratnam.(52) Tumors are classified into three groups according to their cellular structure and differentiation, nuclear polymorphism and frequency of mitoses: well differentiated (Gr1), moderately differentiated (Gr2) and poorly differentiated (Gr3). The majority, 60% of oropharyngeal carcinomas are moderately differentiated, 20% are well differentiated and 20% are poorly differentiated.(53) Histological variants are rare and include verrucous, basaloid, spindle cell and adenosquamous carcinomas. (1) The other more rare pharyngeal malignancies include sarcomas, lymphomas, adenocarcinomas, mucoepidermoid carcinomas and melanomas. (54,55)

2.1.4 Presentation and diagnosis

The typical symptoms of PSCC are sore throat, tongue pain, poorly fitting dentures, otalgia, dysphagia, odynophagia, cough, mouth bleeding, voice changes, airway symptoms and weight loss. In the physical examination a pharyngeal mass or ulceration is often apparent. (1) Pain is usually a late symptom indicative of advanced disease and it is often transposed outside the pharyngeal area via vagal and glossopharyngeal nerve irritation.(1,56) The first manifestation of PSCC is unfortunately often a neck tumor as a sign of a lymph nodal metastasis. PSCC is often diagnosed at an advanced stage with the involved cervical lymph nodes.(57) The incidence of distant metastases at the time of diagnosis is approximately 10% the most common site being lung, followed by mediastinal lymph nodes, liver and bone.(1,58)

The diagnosis of pharyngeal carcinoma is based on thorough physical examination of the upper aerodigestive track and biopsy of the primary tumor. The role of computer tomography (CT) and magnetic resonance imaging (MRI) in evaluating the local extent, regional spread and distant metastases has been universally accepted. They both show similar sensitivities and specificities in detecting cervical lymph node metastases.(59) MRI is the most effective means for a local assessment of oropharyngeal cancer but CT is less sensitive to movement artifacts and is quicker to perform.(60) MRI is of better diagnostic value for mandibular invasion but both imaging techniques experience difficulties in precisely assessing cartilaginous extensions. (61),(62,63) Cervicothoracic CT is thought to be a part of the initial staging in oro-and hypopharyngeal cancer.(60) Routine screening of other possible metastatic sites is not warranted in PSCC.(64,65) Neck ultrasound with fine needle aspiration biopsy is an established method in confirming cervical lymph node metastases.(59) In some patients, panendoscopic assessment under general anesthesia including also the esophagus and tracheobronchial tree is recommended as a way to assess the precise tumor status. This technique is also valuable in cases of unknown primary tumor and also for excluding possible second primary tumors.(56) The incidence of second primaries has been around nine per cent in retrospective studies and in systematic panendoscopic explorations.(58,66,67) Positron emission tomography (PET) scanning

combined with CT is a method which is being increasingly used in PSCC diagnostics. It seems to be outstandingly effective in demonstrating primary tumors in cases of metastatic adenopathy and also in evaluating the response to chemoradiotherapy.(68,69) Narrowband imaging endoscopy uses a series of emission wavebands selected according to hemoglobin absorption spectra. This improves visualization of microvasculature and tissue micro-architecture giving assistance in the early diagnosis of cancerous and pre-cancerous mucosal lesions.(70)

2.1.5 Staging

TNM classification is a worldwide and universally accepted practise for reporting the extent of the malignant disease. The treatment modalities are determined mainly according to the classification and it is also a major tool in predicting the outcome for the cancer patient. TNM classification was defined by the International Union Against Cancer (UICC) over 50 years ago and is still widely applicable today as regularly updated and practical clinical tool.(6) In this classification, T characterizes the spread of the primary tumor, N describes the extent of regional lymph node metastases and M is the presence of distant metastases.(71) The clinical (cTNM) staging is based on clinical examination, biopsy and imaging prior to treatment. The pathological staging (pTNM) allows detecting occult metastases. Additionally extracapsular spreading of the primary tumor can be observed.(48) The numbering after the letters indicates the extent of the disease. In uncertain cases, lower class must be selected. The TNM classification and stage grouping in oro-and hypopharyngeal carcinomas are presented in table 2.

Table 2. TNM class and stage for PSCC, Modified from TNM classification of malignant tumors. 7th edition. Sobin et al. (2010)

T- Primary tumor	
OROPHARYNX	
T1	≤ 2 cm in the greatest dimension
T2	>2 cm to 4cm in the greatest dimension
T3	> 4 cm in the greatest dimension
T4a	Invades to larynx, deep/ extrinsic muscles of tongue, medial pterygoid, hard palate or mandible
T4b	Invades to lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base or encases the carotid artery
HYPOPHARYNX	
T1	Limited to one subsite and ≤ 2 cm or in the greatest dimension
T2	Invades more than one subsite or an adjacent site, or measures > 2 to 4cm
T3	> 4 cm in the greatest dimension, or with fixation of hemilarynx
T4a	Invades to thyroid/cricoid cartilage, hyoid bone, thyroid gland or oesophagus
T4b	Invades prevertebral fascia, encases carotid artery, or invades mediastinal structures

N – Regional lymph nodes (Oropharynx and Hypopharynx)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Single ipsilateral lymph node metastasis ≤ 3 cm
N2	N2a Single ipsilateral lymph node metastasis > 3 cm to 6 cm N2b Metastasis in multiple ipsilateral lymph nodes, ≤ 6 cm N2c Metastasis in bilateral or contralateral lymph nodes, ≤ 6 cm
N3	Metastasis in a lymph node > 6 cm

Note: Midline nodes are considered as ipsilateral nodes.

M- Distant metastasis

Mx	Cannot be assessed
M0	No distant metastasis
M1	Distant metastasis exists

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
	anyT	N2	M0
Stage IVB	anyT	N3	M0
Stage IVC	anyT	anyN	M1

2.1.6 Treatment

The main treatment modalities used in PSCC are surgery, radiotherapy and chemotherapy and their combinations. There are not globally, not even nationally standardized treatment protocols. Instead the therapy chosen depends upon each center's *modus operandi* and tradition as well as the patient's characteristics. Surgery is traditionally a standard treatment for HNSCC but is frequently limited by the anatomical extent of the tumor.(1) Surgical management provides a pathological staging of the primary tumor and regional lymph nodes for guiding adjuvant treatment decisions. The use of open surgery as the initial treatment of oro- and hypopharyngeal cancer has been declining over the past three decades.(72,73) Transoral robotic surgery (TORS) has been proposed to hold great potential for minimally invasive extirpation of oropharyngeal cancer. In selected cases it enables radical resection and reconstruction avoiding the side-effects of adjuvant therapy.(74-77) In addition, transoral laser microsurgery offers a minimally invasive endoscopic surgical approach for certain oropharyngeal tumors.(78) However, open surgery has an important

role as a salvage procedure after radiotherapy in cases where there are residual tumors or local or locoregional recurrence. In PSCC this often includes pharyngolaryngectomy. Free microsurgical flaps have considerably improved the functional and cosmetic results of surgery.(79) When surgery is a primary treatment, neck dissection is carried out in most cases as a part of surgical management.(40) The classic radical neck dissection has made way for the less morbidizing selective neck dissection, the operated nodal regions depending on the subsite and extent of the primary tumor.(48) In early N0 oropharyngeal SCCs, sentinel node biopsy seems to represent a promising alternative to elective neck dissection.(80) Postoperative radiotherapy is warranted in those cases with close or positive excision margins, nodal metastases, bone erosions as well as perivascular or perineural spread of the tumor are seen.(8,56)

In spite of advances in the surgical field, radiotherapy is still the treatment of choice in many oropharyngeal and nearly all hypopharyngeal carcinomas. Chemoradiotherapy with cisplatin, fluorouracil or the taxanes is the standard treatment for patients with advanced disease. Chemoradiotherapy, and surgery followed by radiotherapy has been reported to achieve equivalent results in locoregional control and survival in hypopharyngeal SCC.(81) The advantages of radiotherapy lie in organ preservation, as surgery in the pharyngeal area nearly always leads to major functional and cosmetic defects. Another advantage associated with radiotherapy is the simultaneous treatment of subclinical nodal spread.(57) Intensity modulated radiotherapy (IMRT) and altered fractionating schedules have diminished the side effects to salivary glands, pharyngeal muscles and bones and also improved the locoregional control.(1,56,72,82) HPV-virus positive oropharyngeal tumors have been shown to be highly radiation sensitive, for this reason radiation therapy could be the treatment of choice in those cases.(9)

The epidermal growth factor receptor (EGFR) has been identified as a therapeutic target for several malignomas, including HNSCC. EGFR can be targeted either by antibodies or by EGFR tyrosine kinase inhibitors.(8) Treatment with Cetuximab, a humanized mouse anti-EGFR monoclonal antibody has improved locoregional control and overall survival in combination with radiotherapy in locally advanced HNSCCs.(83,84)

2.1.7 Follow-up and survival

The aims of follow-up in PSCC are evaluation of therapeutic efficacy, management of impairments, detection of loco-regional relapses and second primary tumors as well as the provision of psychosocial support.(85) The early follow-up period starts one month after treatment and ends two years later. Morbidity and mortality during this period result either from locoregional recurrence or the complications of therapy.(58) The majority, 70% of new tumor manifestations have been reported to take place during that period.(85) The late follow-up period starts at the beginning of the third year and continues case-dependently, usually at least 5 years from treatment. Morbidity and mortality during that period are typically attributable to delayed regional or distant metastases, second primary tumors or other medical conditions.(58)

HNSCC is the sixth leading cause of cancer deaths worldwide and the 5-year survival is still as low as 40 to 50%.(8) In fact, of all HNSCCs, pharyngeal carcinoma has the poorest prognosis. The mean age-adjusted mortality rate of PSCC in Finland is 1.0 and the mortality rate has increased by more than 3 %-units during the last 10 years. The 5-year survival in Finland is 40% for men and 49% for women.(Finnish cancer registry, www.cancer.fi/syoparekisteri/) Survival depends on tumor stage and site. Hypopharyngeal carcinoma has the poorest prognosis of the pharyngeal subsites. In a Finnish survey, the median disease-free survival was 27.6 months for patients with oropharyngeal carcinoma and 17.7 months for those with hypopharyngeal carcinoma.(51) There are several reasons for PSCC treatment failures i.e. tumor recurrences at the primary site, delayed regional metastases or delayed distant metastases. According to a Danish study, a recurrent primary tumor is two times more common than a locoregional recurrence in oropharyngeal

carcinoma.(57) Hypopharyngeal tumors carry risks as high as 24% for delayed regional lymph nodal metastases and a 17% incidence of delayed distant metastases.(58,86) In a retrospective study conducted in 2550 patients, the incidence of second primaries in hypopharyngeal SCC was 8.9%.(58)

2.2 PROGNOSTIC FACTORS

2.2.1 Clinical prognostic factors

The general condition of the patient at the time of diagnosis, as defined by Karnofsky performance status (KPS) (table 3), is a common prognostic marker which provides consistent results in PSCC.(87,88) In a retrospective study of 3373 patients with HNSCC, the most decisive variable for prognosis was the T category.(89) According to the same study, other significant prognostic factors in multivariate analysis were alcohol consumption, KPS score, primary site and N class.(89) In a study of 289 patients with oropharyngeal SCC, T class also proved to be the most relevant prognostic factor together with the age of the patient as well as their gender and haemoglobin level. The stage did not exert such a major effect on prognosis, further highlighting the role of the primary tumor size over the lymphonodal status.(57) In neither of those studies did the histological grading play any significant role as a prognostic factor.(57,89) In oropharyngeal carcinoma, the subsite of the tumor also had an influence on prognosis, i.e. it is best in tumors in the posterior wall and worst when the tumor is in the base of tongue.(90) In a population-based study of pharyngeal cancer incidence and survival in northern Finland, the most important factor affecting poor prognosis was Stage IV tumor.(51) Furthermore, HPV-positivity and non-smoking status predicted a remarkably better prognosis in patients with oropharyngeal SCC.(7) Smoking seems to have even greater prognostic value; HPV positive non-smokers have significantly better DSS than their HPV positive smoking counterparts.(91)

Table 3. Karnofsky performance status. Modified from Karnofsky 1948.(87)

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

2.2.2 Molecular prognostic markers

There are several biological mediators and mechanisms that have been implicated in HNSCC progression. However, at present only a few of those have been shown to possess any clinical relevance. The most common genetic changes occurring in HNC progression are inactivation of tumor suppressor genes *p16* and *p53*. The *p16* gene inhibits cyclin-dependent kinase (CDK), thus its inactivation leads to uncontrolled cell-cycle progression as well as escape from senescence and apoptosis. In spite of its role as a tumor suppressor gene, *p16* overexpression also correlates highly with HPV positivity.(9,91) Clinically, the *p16* positive tumors display much of the same characteristics as the HPV positive tumors.(92) Thus, *p16* has continued to be investigated as an independent tumor marker in HNSCC. HPV and *p16* correlated with poorly differentiated histological grade as well as with nodal metastases in a study of HNSCC tumors.(92) Increased expression of EGFR has recently been associated with aggressive tumor behavior, resistance to chemotherapy and poor prognosis in oropharyngeal cancer.(93) However, in another oropharyngeal SCC material and in a HNSCC study it was not an independent prognostic factor.(94,95) The Transforming growth factor $\beta 1$ (*TGF β 1*) genotype has been associated with a favorable outcome in HNSCC patients, independently of *p16* expression.(95) Amplification of proto-oncogen cyclin D1 is seen in about one third of the HNC cases and this is usually associated with an invasive disease.(79)

2.3 EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

2.3.1 General considerations

Epithelial cells form layers of cells that are closely joined together by specialized membrane structures e.g. tight junctions, adherens junctions, desmosomes and gap junctions. In addition, epithelial cells display apical-basolateral polarization which is characterized by the polarized organization of the actin cytoskeleton and the presence of a basal lamina at the basal surface. Mesenchymal cells, on the contrary, do not form organized layers or have apico-basolateral polarization. Fibroblastic cells contact neighboring cells only focally and are not associated with the basal lamina. Unlike the cuboidal form of the epithelial cells, mesenchymal cells exhibit a spindle-shaped fibroblast-like morphology.(15) EMT is considered as a process during which epithelial cells lose their epithelial phenotype and acquire mesenchymal features.(13,96-98) These changes in cell connections and morphology lead to looser cell-cell -contacts and the acquisition of cellular motility, both of which enable invasion (fig. 4).

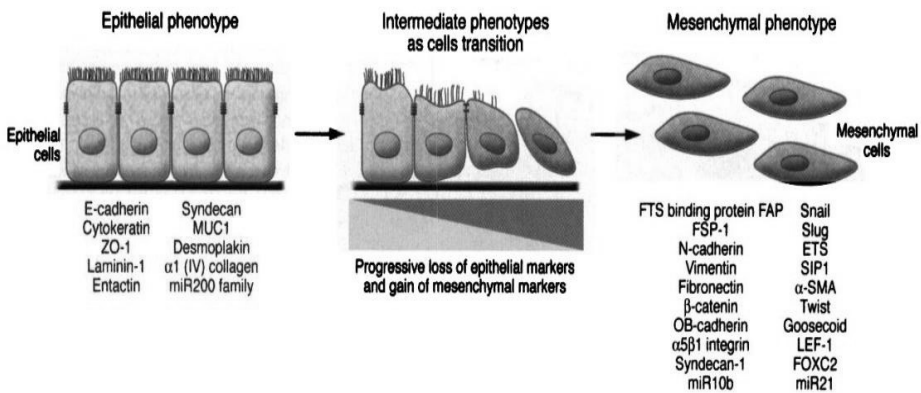


Figure 4. Epithelial and mesenchymal phenotypes and commonly used cell markers characterized to each type. (Kalluri et al. 2009, reprinted by permission of publisher)

There are three types of EMT which become manifest under different circumstances. Type 1 occurs during implantation, embryo formation and organ development and it is a self-contained process. Primary EMT events take place during the implantation of embryo into the uterus, during gastrulation and during neural crest formation.(99,100) This primary mesenchyme can be re-induced to form secondary epithelia by a mesenchymal-epithelial transition (MET) which is a reverse process and crucial in forming distinct cell types as a part of organogenesis during secondary and tertiary EMT processes.(96,100) Type 2 is associated with wound healing and tissue regeneration and it ceases once repair has been achieved and inflammation is attenuated. In the setting of organ fibrosis, EMT can continue to respond to ongoing inflammation and lead to organ destruction.(99,101) Type 3 occurs in epithelial cancer cells due to genetic and epigenetic alterations affecting oncogenes and

tumor suppressor genes which make the cells especially responsive to heterotypic signals originating from tumor microenvironment. Cells generated by type 3 EMT are typically seen at the invasive front of the primary tumor and considered to be cells that may invade and metastasize leading to systemic manifestations of cancer progression.(13,15,99,101)

2.3.2 Biomarkers for EMT

Presence of E-cadherin is one of the main features of the epithelial phenotype. E-cadherin is the main constituent of adherens junctions but it promotes also the formation of desmosomes.(13) Its expression is decreased during EMT. Moreover, the loss of E-cadherin promotes EMT by inducing the expression of twist and zeb1.(102) There are other markers for epithelial phenotype for example cytokeratin, mucin-1, desmoplakin, occludins, claudin-1 and claudin-7.(15) In addition to the loss of epithelial markers, an increase in the expression of mesenchymal markers such as N-cadherin, vimentin, fibronectin and smooth-muscle actin is also a characteristic of EMT (fig. 4).(99,103) The cadherin switch from E-cadherin to N-cadherin has often been used to monitor the progress of EMT.(99,103) β -catenin is localized in the cell membranes in normal epithelial cells linking cadherins to the intracellular cytoskeleton. During EMT, as a result of E-cadherin loss, β -catenin relocates to either cytoplasm or nucleus.(15) Matrix metalloproteases (MMP) are secreted proteases that can digest the components of the extracellular matrix (ECM) and other junctional proteins involved in cell-cell as well as in cell-ECM adhesion.(104) They have been designated also as mesenchymal markers which are frequently overexpressed in tumor cells.(105)

2.3.3 EMT signaling

EMT is triggered by the interplay of extracellular signals, including soluble growth factors, components of the extracellular matrix as well as by intracellular cues (Fig. 5).(97) During development, EMT is induced by receptor tyrosine kinases (RTKs), which are activated by several soluble growth factor signals, such as TGF- β , fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet derived growth factor (PDGF) and epidermal growth factor (EGF).(106,107) In most cellular models, EMT is induced by the interaction between overexpressed RTKs and TGF- β signaling.(108) Growth factors are produced either by tumor cells or by the surrounding stromal cells and their production can be influenced by many environmental factors.(16,109)

TGF- β is one of the most prominent EMT inducing cytokines that activates a wide array of transcription factors.(97) TGF- β signaling activates ras-dependent and Smad-dependent mechanisms and these promote the translocation of the Smad complex into the nucleus where it then activates EMT-RTFs.(16,110) TGF- β binding initiates Par6 phosphorylation and the activation of the E3-ubiquitin ligase, Smurf-1. The activated Smurf-1 promotes the degradation of RhoA, resulting in tight junction dissociation and the blocking of cell adhesion.(16) Par6 phosphorylation also causes the loss of apical-basal polarity.(96)

FGF receptor activation switches on two different pathways, Src and Ras. Src activation phosphorylates cytoskeleton-associated proteins, adherens junction proteins and focal adhesion components. The Ras pathway activates mitogen-activated protein kinase (MAPK) which is translocated to nucleus and thus can activate snail and slug. (13) Three signaling pathways which are essential for stem cell function and early embryogenesis, namely Wnt/ β -catenin, Notch and Hedgehog signaling pathways, have also a major impact on EMT.(108) MMPs are induced by TGF- β and are capable of cleaving E-cadherin and disrupting the cadherin-mediated cell-cell contacts thus promoting EMT.(16) In addition, EMT processes often require the co-expression of integrin receptors.(15) Integrins are cell-surface receptors which link ECM to the intracellular actin cytoskeleton.(16) Integrins have a dual influence on EMT process: they can induce E-cadherin downregulation and inhibit E-cadherin mediated signals to integrins by activation of the small GTPase Rap1.(15)

EMT is also regulated by multiple environmental factors.(98,109) The influence of inflammatory components on the regulation of EMT has been highlighted recently. For instance the induction of *snai1* through the cyclo-oxygenase2 (COX2) pathway as well as the induction of *snai1* and *zeb1* through nuclear factor κ B (NF- κ B) and by some interleukins has been reported.(111,112) By secreting TGF- β , tumor associated macrophages provoke *snai1* overexpression via NF- κ B activation.(109) Furthermore it is known that hypoxia promotes EMT by direct stimuli of transcription factors via hypoxia-inducible factor-1 α (HIF 1 α). (16,97)

There is a significant cross-talk between the EMT-inducing signals and transcription factors that can lead to stable reprogramming of epithelial cells to mesenchymal states.(96,113). There is also a growing understanding of the epigenetic regulation of EMT involving three types of changes: DNA methylation, histone modifications and micro-RNAs (miRNA).(114) Each of these mechanisms has been shown to play a major role in controlling EMT. MiRNAs are short non-coding RNAs that repress gene-expression at the post-transcriptional level. Some miRNAs are reduced in tumors as compared to normal tissue and they have been shown to restrain the EMT process. The miR-200 family inhibits the repressors of E-cadherin and thus helps in maintaining the epithelial phenotype.(105,113,115) In contrast, some miRNAs like miR21, miR25 and miR31 facilitate EMT by downregulating tumor suppressor genes .(101,105,113)

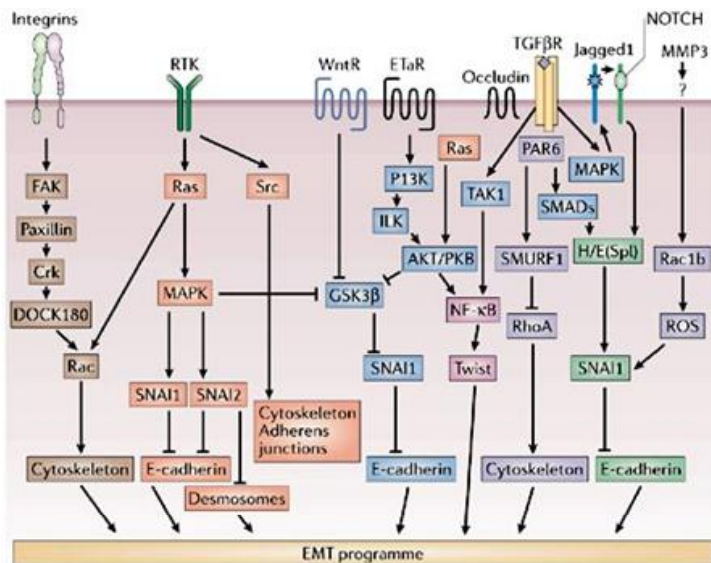
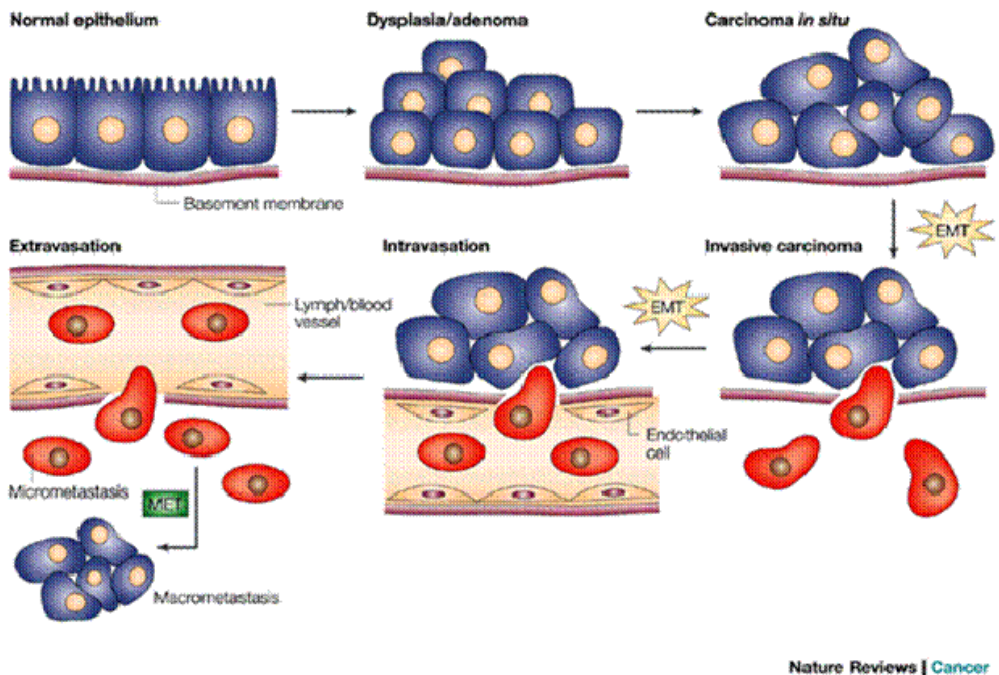


Figure 5. Overview of the signaling pathways that regulate EMT. (Thiery and Sleeman 2006, reprinted by permission of publisher)

2.3.4 EMT and epithelial-stromal interaction in cancer progression

Tumor progression and the formation of metastases involve several distinct steps: emigration of tumor cells from the primary tumor, penetration through basement membrane, invasion into neighboring tissues and into blood or lymphatic vessels, cell survival detached from the tumor mass, extravasation, formation of the micrometastatic nodule as well as adaptation and reprogramming of the surrounding stroma to form macrometastases (Fig. 6).(116) Tumor cells are capable of going through those steps by adopting the mesenchymal phenotype. The manipulation of E-cadherin has a dual effect on tumor progression. Firstly, the loss of E-cadherin disrupts adhesion junctions which allow

the detachment of malignant cells from the epithelial-cell layer. Secondly, the loss of E-cadherin has direct effects on signaling pathways, including the Wnt signaling pathway and Rho-family GTPase mediated modulations, involved in tumor cell migration and tumor growth.(16) Tumorigenic processes involve genetically abnormal cells that progressively lose their responsiveness to normal growth-regulatory signals.(96) Tumor tissues typically lack the coordinated and orderly induction of a complete EMT and the EMT markers are only present in a certain part of tumor tissue and cells. This might be due to the presence of genetic heterogeneity within the tumor and highly variable environmental signals.(117) In the absence of growth factors that actively promote the induction and continued expression of EMT derived by activated stroma, cells will revert back to the epithelial state. MET is believed to be involved in the formation of distant metastases by this mechanism.(96,109,113)



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Figure 6. EMT and MET in the emergence and progression of carcinoma. During tumor progression, epithelial cells gain mesenchymal features by EMT which enables them to emigrate from the primary tissue, penetrate through the basement membrane and enter into the blood or lymphatic vessels. At the metastatic site, they extravasate and form metastases by MET.(Modified from Thiery 2002)

There is solid evidence to suggest that EMT plays a significant role during tumor progression. The expression of mesenchymal markers as well as the loss of epithelial markers correlate with tumor progression and a poor prognosis in multiple carcinomas.(113,118-120) Genetic analyses have revealed that carcinoma cells contribute to the stromal-fibroblast population in tumors indicating that tumor cells have gone through EMT.(121-123) However, the *in vivo* demonstration of EMT has turned out to be challenging due to heterogeneity of the tumors, the phenotypic complexity within tumor cells and the reversible nature of EMT.(98)

The cross-talk between the microenvironment and the tumor cells has been emphasized in recent studies.(98,109,124) The behavior of carcinoma is influenced by the tumor microenvironment consisting of ECM, diffusible growth factors and cytokines, blood vasculature, inflammatory cells and fibroblasts. Normal stroma contains a low number of fibroblasts in association with a physiological ECM. Instead, activated stroma has an increased number of fibroblasts, enhanced capillary density and type-1-collagen and fibrin deposition.(125) This reactive stroma provides oncogenic signals to facilitate tumorigenesis. In the absence of aberrant microenvironmental stimuli, the genetic and epigenetic alterations in tumor cells are believed to be insufficient to induce tumor progression.(109) An invasive tumor is often associated with the expansion of tumor stroma and increased deposition of ECM and in many carcinomas the stroma comprises most of the tumor mass.(12)

Cancer cells can modulate their own microenvironment by producing stroma-modulating growth-factors, which include basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), PDGF, EGF, interleukins and TGF β . These factors act in a paracrine manner to induce angiogenesis and to evoke inflammatory responses. The factors also activate surrounding stromal cells to secrete additional growth factors and proteases.(12,124,126) Activated fibroblasts are an important sources of ECM-degrading proteases such as MMPs.(125) The cancer cells also start to produce proteolytic enzymes, which remodel ECM and the basement membrane provoking pro-invasive and pro-migratory environment.(12) Carcinoma associated fibroblasts (CAF) are fibroblasts that have been modulated by the influence of neighboring tumor cells.(127) They are mesenchymal cells that share the characteristics of fibroblasts and smooth-muscle cells and are often identified by the expression of α -smooth muscle actin.(128) The presence of CAFs in activated stroma has been observed in many cancer types.(128,129) It is believed that the oncogenic signals from the CAFs can stimulate the progression of epithelial cells into their tumorigenic counterparts by expressing a range of cytokines and growth factors, promoting tumor-cell survival and migration.(12,98) CAFs also further activate tumor stroma by stimulating angiogenesis and recruiting inflammatory cells (Fig. 7).(11)

EMT may also lead to the formation of stromal cells in addition to solitary invasive carcinoma cells.(13) These non-tumorigenic carcinoma-derived fibroblasts have displayed a remarkable property of enhancing tumorigenity in mice since they possess both tumor growth and EMT promoting properties.(16,98,122) Furthermore, tumor associated macrophages, representing a major component of the tumor microenvironment, can promote EMT by producing TGF- β .(98) Many factors favoring EMT in carcinomas are components of heterotypical signaling pathways which originate in the tumor-associated stroma.(96,130) Although there are several of these microenvironment derived signals, Wnt and TGF β have been the most extensively studied. EMT and endothelial-mesenchymal transition (EndMT) are ways for converting epithelial and endothelial cells into fibroblast-like cells with an altered genome.(125) However, according to the genetic studies, only a fraction of the CAFs are derived from EMT.(122)

Hypoxia, often present in large tumor masses, can induce EMT in tumors through multiple distinct mechanisms including upregulation of HIF1 α , activation of the Notch or NF- κ B pathways or direct activation of *snai1* and *twist*.(96,109)

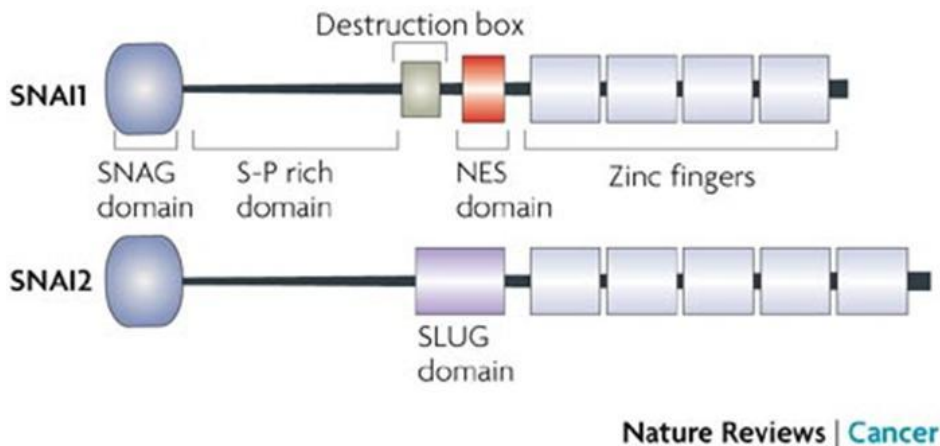
In addition to promoting invasion and metastatic dissemination, EMT contributes to the acquisition of therapeutic resistance of cancer cells by protecting cells from drug- and radiation -induced apoptosis and cell death.(131,132) In a breast cancer cell line, EMT was found to be involved in mediating multidrug resistance (MDR).(133)

2.3.5 EMT related transcription factors (EMT-RTFs)

2.3.5.1 Snai1 and Slug

Snail was first identified in *Drosophila* where it downregulates E-cadherin to control gastrulation.(137) To date, three members of snail superfamily have been described; snail (originally identified as snail), snai2 (also known as slug) and snai3 (smuc). These all are zinc-finger transcription factors sharing a common structure, i.e. a highly conserved C-

terminal region, four to six zinc fingers and a divergent amino-terminal region (Fig. 8).(112,138) Snail-deficient mouse embryos fail to gastrulate which eventually leads to death of the embryos.(139) Snail genes are also involved in mouse and chick palatal fusion.(140) During tumorigenesis, *snai1* has several cellular functions in addition to the direct repression of E-cadherin. It down-regulates other epithelial markers but, on the other hand activates the expression of some mesenchymal-like and pro-invasive genes.(141,142) Snail expressed cells are resistant to direct apoptotic stimuli and to DNA damage and they also promote angiogenesis.(21,22) Snail may also accelerate metastasis through immunosuppression.(143)



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Figure 8. Main structural domains of *Snai1* and *Snai2* (Peinado 2007, reprinted by the permission of publisher)

The transcriptional activation of *snai1* is mediated by several pathways including TGF- β , Notch and WNT-pathways as well as hypoxia. It is known that TGF- β can induce the activity of *snai1* promoter and triggers EMT by a mechanism dependent on mitogen-activated protein kinase (MAPK) signaling pathway.(144) Notch signaling induces *snai1* directly by activating its promoter and indirectly through HIF-1 α mediated activation. WNT signaling can increase *snai1* function by preventing its nuclear export and degradation. On the other hand, *snai1* can promote WNT signaling by ensuring that E-cadherin remains downregulated thus making β -catenin available to promote WNT signaling.(119)

At the posttranscriptional level, the activity of the *snai1* protein is regulated by phosphorylation, which in turn influences its subcellular localization. Snail is a very unstable protein and it is considered to be active only when it is localized in the nucleus.(145) There are at least five kinases that can phosphorylate *snai1* protein in distinct regions.(97) Glycogen-synthase kinase-3 β (GSK3 β) phosphorylates two Ser residues on *snai1*, one of which targets *snai1* for ubiquitination and degradation, whereas the other promotes its nuclear export.(145) Mutations in GSK3 β -mediated phosphorylation produce a stabilized form of *snai1* that becomes localized in nucleus and promotes EMT.(15) Activation of EGF receptor signals to *snai1* through p21-activated kinase-1 (PAK1). PAK1 phosphorylates *snai1* on a different Ser residue than GSK3 β which also results in *snai1*'s accumulation within the nucleus. Additional protein modifications by lysyl oxidases 2 and

3 further stabilize snail protein and promote EMT as well as tumor invasion.(146) Proinflammatory cytokine tumor necrosis factor α (TNF α) stabilizes snail through the NF- κ B pathway thus promoting inflammation-mediated metastases in breast cancer.(147) There is a report that Snail mediates MMP-induced EMT(104), and it also indirectly upregulates MMP2.(142) Snail can bind to and repress its own promoter, evidence for the presence of an autoregulatory loop.(112) Snail is repressed by MiR-29b and let-7d, MiRNAs that block EMT and invasiveness in HNSCC. Members of the miR-30 family also target snail.(134)

Snail overexpression correlates with tumor progression in breast, endometrial and ovarian carcinomas.(148-150) Its expression in tumor stroma but not in epithelium correlates with tumor progression, metastasis and patient survival in colorectal tumors.(29) There are only a few studies examining snail expression in HNC. Snail expression in HNSCC predicted metastases but did not correlate with the levels of p16 or EGFR expression.(151) In oral SCC, snail was mainly detected in tumor stroma and in some endothelial cells but displayed no correlation with the histological grade or nodal metastasis.(37) In laryngeal carcinoma, the nuclear or cytoplasmic expression of snail was associated with local recurrence.(28) Snail overexpression in HNSCC correlated with metastases and worse prognosis.(152) In a recent study of nasopharyngeal carcinoma, nuclear expression of snail predicted poor survival while cytoplasmic expression had no correlation with any of the studied clinicopathological variables.(153)

Slug is involved in neural crest development of chicken and *Xenopus* embryos.(138) Slug deficient mice are viable but slow-growing and display craniofacial malformations as well as coloring defects.(154) In humans, slug-mutations are connected to the Waardenburg syndrome with deafness and impaired melanocyte function.(155) Slug expression promotes E-cadherin downregulation, although slug is a much weaker repressor of E-cadherin than snail.(156) Slug expression is not always associated with E-cadherin downregulation but it may act synergistically with other E-cadherin repressors.(157) Slug expression also promotes programmed cell death.(23) TGF β 1 increases slug levels in malignant oral SCC.(158) In that study induction of slug did not repress E-cadherin levels but instead increased MMP-9 expression. Slug is a molecular target of transmembrane tyrosine kinase receptor c-kit signaling pathway.(159) In addition, Wnt signaling upregulates slug and promotes E-cadherin repression.(160) Exogenous slug expression in HNSCC cells causes changes in the assembly of the adherens junction and the desmosome characterized by a classical cadherin switch.(161) Slug downregulation promoted apoptosis and decreased invasion capability in esophageal SCC.(162) Slug antagonizes p53 mediated apoptosis in haematological precursors.(163) There is little knowledge about post-transcriptional regulation of slug. However, the partner of paired (Ppa) protein modulates its stability and degradation.(164) MiR-1 and MiR-200 inhibit slug expression and are repressed by binding of slug to their promoters. Slug is also inhibited by MiR-203.(134)

In esophageal cancer, slug expression is related to lymph node metastasis, invasion and poor prognosis.(30) In addition, in colorectal carcinoma, slug expression has been reported to be an independent prognostic factor for poor survival.(165) However, in oral SCC slug expression did not correlate with any investigated clinicopathological variable or survival.(38)

2.3.5.2 Twist

Twist is a basic helix-loop-helix (bHLH) transcription factor consisting of two parallel α -helices joined by a loop which is required for dimerization. It was first reported to play a key role in mesoderm formation and proper gastrulation in *Drosophila*.(166) Twist is also an important factor in mammalian embryogenesis. Mutational inactivation of the *twist* gene is postulated to be responsible for the Saethre-Chotzen syndrome which is an autosomal dominant disorder characterized by the premature fusion of cranial sutures, skull deformation, limb abnormalities and facial dysmorphisms.(167) Twist expression is

upregulated by EGF and transcriptionally regulated by an oncoprotein STAT3.(168) Hypoxia and HIF-1 α also induce twist upregulation and at the same time twist is critical in hypoxia-induced EMT.(169) NF κ B has been implicated in EMT since it can promote expression of twist.(110) At the post-transcriptional level, p38 kinase mediated phosphorylation regulates the homodimerization and DNA binding capacity of twist while p42 and p44 kinases are involved in twist degradation in response to Notch signaling.(112) Several miRNAs have been reported to target twist, including miR-214, miR-580 and let-7d.(97)

Twist induces EMT by promoting the downregulation of E-cadherin and several catenins but also by upregulating mesenchymal markers.(20). Yang et al linked twist to the early phases of metastasis formation, namely tumor cell intravasation. Although twist was highly expressed in metastatic cell line its suppression did not affect cell proliferation and tumor formation but it did reduce significantly lung metastases from a mouse mammary gland tumor model.(20,170) A recent study reported that activation of twist was sufficient to promote carcinoma cells to undergo EMT and disseminate into blood circulation.(171) Twist has also been shown to possess antiapoptotic properties. It inhibits oncogene-dependent cell death and it bypasses p53-induced growth arrest thus facilitating tumor progression.(24) Twist also allows cancer cells to override premature senescence and it cooperates with oncoproteins such as Ras and ErbB2 to promote complete EMT.(172) Bmi1, a polycomb-group repressor complex protein, which is often upregulated in stem cells and cancer seems to be a direct downstream target gene of twist. (97,173) Upregulation of twist has been also associated with cellular resistance to the taxol and vincristine, two important anticancer drugs.(174) The resistance is mediated through twist-induced protection against apoptosis being regulated through Akt phosphorylation.(175) Akt, a known proto-oncogen, is a downstream target of twist, which binds to the E-boxes on its promoter and transactivates its expression.(176) Twist can also exert its downstream effects in cancer cells through the modulation of several MiRNAs. (177)

Twist is absent in normal epithelium but is known to be induced in several human solid tumors. Twist expression significantly shortened the metastasis-free period in one HNSCC series(169) and in another HNSCC trial, the extent of twist expression correlated with a higher frequency of lymph node metastases as well as tumor progression.(178) Twist overexpression has been associated with aggressive tumor properties and poor survival also in oesophageal and cervical SCC.(31,33) However, in a recent study of oral SCC, the extent of twist expression did not correlate with any clinicopathological variables or survival.(179) Twist was postulated to mediate taxol resistance through protection against apoptosis in nasopharyngeal carcinoma.(174,175)

2.3.5.3. Zeb1 and Sip1

The human zeb family of transcription factors consists of two members, zeb1 (Zinc-finger E-box binding homeobox 1, also known as TCF8 and δ EF1) and zeb2 (Smad interacting protein 1, sip1, also known as ZFXH1B). These proteins are highly homologous and are characterized by two clusters of zinc fingers separated by a homeodomain.(18) During embryogenesis, zeb1 influences the development of neural, chondroid and haematolymphoid tissues.(180) Sip1 is expressed in embryonic neural crest cells and the neural tube and is responsible for the fate of neuronal progenitor cells as well as the myelination of the central nervous system. (18) (181) Sip1 is also critical for neural crest epithelial sheet formation.(182) Zeb family members were initially found to be mutated in developmental diseases including Hirschsprung's disease and posterior polymorphous corneal dystrophy.(183,184)

While zeb1 and sip1 have many overlapping characteristics, they still display distinct expression patterns. TGF β factors induce both zeb molecules which bind to Smad proteins and antagonistically regulate their transcriptional properties; zeb1 activates smad-mediated transcription whereas sip1 represses it.(185) Zeb1 is induced by TGF β 1, NF- κ B and the

notch signaling pathways.(115,186) Zeb molecules are believed to act as either transcriptional repressors or activators depending on the target gene and tissue as well as on posttranscriptional modifications.(187) Zeb1 and sip1 are repressed by miR-200 family and miR-200 family members, in turn, can be repressed by zeb-proteins, thus forming regulatory loops maintaining cells in either the epithelial or mesenchymal states.(105,115). Overexpression of miR-200 ensures the maintenance of epithelial traits, inhibits EMT and reduces tumor invasion. Surprisingly, however, elevated miR-200 can also promote metastatic colonization and be present in malignant cancer stem cells.(97,113)

In addition to downregulating E-cadherin, zeb1 also affects epithelial cell polarity by downregulating several polarity markers and by inducing the selective loss of basement membrane components.(25,26) Since it is an inhibitor of the epithelial phenotype, zeb1 is not expressed in normal epithelium, although it is found in isolated fibroblasts and immune cells in the interstitial stroma.(134) However, zeb1 is highly expressed in the invading cancer cells of many tumors. Spoelstra et al discovered, that zeb1 was not expressed in normal endometrial epithelium but overexpressed in malignant epithelium and also in tumor-associated stroma.(188) In gastric carcinoma, zeb1 overexpression correlated with higher TNM class, deeper invasion and lymph node metastases.(189) Zeb1 overexpression predicted also larger tumor size, metastases and poorer survival in hepatocellular carcinoma patients.(190) However zeb1 expression did not correlate with survival in either patients with lung tumors(191) or bladder carcinoma.(39)

Sip1 downregulates E-cadherin transcription by binding to the e-boxes of its promoter. The level of sip1 expression is elevated in fibroblasts and invasive carcinoma cell-lines and the extent of the expression strongly and inversely correlates with E-cadherin expression.(19) Sip1 expression also downregulates the major constituents of different cell junctional complexes such as tight junctions, adherens junctions, desmosomes and gap junctions.(180) Furthermore, sip1 exerts an anti-apoptotic effect on the response to combat DNA damage.(27) Sip1 expression has also been shown to induce several MMPs, especially MMP2.(192) Its post-transcriptional regulation is organized by proprotein convertase 2 - mediated sumoylation of sip1 which prevents its interaction with c-terminal binding protein by promoting its cytoplasmic localization. The functional consequences of this process for protein stability remain to be clarified.(112) The amount of expression and subcellular localization of sip1 varies considerably between different tissues and tumor types.(193) In a study of 47 OSCC samples, the expression of sip1 was detected in tumor epithelium and stromal cells in 28% of the samples and it exhibited an inverse correlation with E-cadherin expression. In that study, sip1 expression proved to be an independent prognostic factor for poor DSS.(34) Furthermore, in tongue SCC, sip1 expression correlated with E-cadherin repression and predicted vascular invasion as well as delayed lymphonodal metastases.(194) In bladder cancer, the sip1 expression was found to represent an independent predictor of poor DSS and preserved cells from DNA-damage-induced apoptosis thus protecting cancer cells from radiation.(27) In addition, sip1 expression predicted lower OS in lung cancer.(195)

2.3.6 Cooperation between EMT-RTFs

All the above-described transcription factors induce EMT by repressing E-cadherin and other epithelial markers and by enhancing the expression of mesenchymal proteins.(113) Why are there so many distinct transcription factors involved? One reason may be that they are activated in many different steps of embryogenesis and their expression depends on a diverse array of contextual signals which are present in different tissues and act in various stages of development. Another explanation is that none of these transcription factors can regulate the EMT on their own but they are expressed in various combinations in different cell and cancer types.(196) Snai1 could be considered as an early marker of EMT and it sometimes contributes to the induction of the other factors. In contrast, slug, zeb1, sip1 and twist may be responsible for the maintenance of the migratory cell behavior and other

tumorigenic properties.(112) Knockdown experiments in several cancer lines with the co-expression of EMT-RTFs have shown that ablation of only one transcription factor may be sufficient to block EMT, either partially or even totally.(113)

TGF β has been considered as a key molecule in orchestrating the interplay of EMT-RTFs in tumor progression.(107) Sip1 mediated E-cadherin repression does not seem to be dependent on snail expression but instead snail could be an upstream mediator of TGF β -induced zeb1 expression.(18,19) Snail increases zeb1 protein levels through both transcriptional and post-transcriptional mechanisms and the protein can exist in its induced level for several weeks after snail has been eliminated.(134,197) In addition, slug activates zeb1 by direct binding to its promoter. Twist needs a direct transcriptional induction of slug to promote EMT. Slug knockdown completely blocks the ability of twist to suppress E-cadherin transcription and the entire EMT process.(198)

As a sign of the interaction between EMT-RTFs in different human cancers, there is often enhanced tumor progression and poorer prognosis, when several EMT-RTFs are expressed simultaneously. The co-expression of snail, twist and HIF-1 α in HNSCC correlated with metastases and poorer prognosis as compared with expression of only one or two markers.(169) Furthermore, in hepatocellular carcinoma and in non-small cell lung cancer, snail and twist co-expression predicted lower survival.(35,36) Co-expression of twist2 and sip1 together with HIF-2 α correlated with a higher risk for distant metastases and a lower survival rate in patients with adenoid cystic carcinoma of the salivary gland.(199)

2.3.7 Endothelial-mesenchymal transition (EndMT)

EndMT, analogous to EMT, is characterized by delamination of endothelial cells from the organized cell layer and the acquisition of mesenchymal markers as well as invasive and migratory properties. During embryogenesis EndMT is important in the organization of the endocardial cushion and heart valves.(101) The endothelial cells associated with tumor microvasculature can also contribute to the formation of mesenchymal cells via EndMT. Mouse endothelial cells can acquire a fibroblast-like phenotype when exposed to TGF β .(200) EndMT can also be modulated by Notch pathway.(201) Angiogenic vessels can undergo EndMT and thus it may play an important role in stabilizing neovasculature during angiogenesis.(201) It has been postulated that EndMT is an important source of carcinoma associated fibroblasts. Indeed, endothelial cells are believed to contribute to the accumulation of fibroblasts within the microenvironment of angiogenic tumors.(200)

2.3.8 Cancer stem cells (CSC)

EMT can be manifested in only a subset of tumor cells, often located at the invasive front of the tumor, and the adjacent stromal cells.(202) These cells may be referred to as CSC with a particular propensity for invasion and metastases.(11,203) CSCs are considered to be a subpopulation of tumor cells capable of regenerating the entire tumor with metastatic properties.(204) They remain in a quiescent state thus becoming resistant to chemo- and radiation therapies and therefore they play an important role in cancer relapses and metastases.(204,205) However, the existence and significance of CSCs is still rather controversial.

3 Aims of the Study

Despite the recent advances in cancer diagnostics and treatment, PSCC still has a poor prognosis. Concurrently, the incidence of PSCC is increasing. Until now, the prognosis of the patient and selection of the treatment modality has been determined mainly by clinical factors. To improve the diagnostics and thus also the treatment of the patients, more specific prognostic markers will be needed. EMT is a well characterized phenomenon providing potential predictors for tumor behaviour but it has not been studied in PSCC. The specific aims of this study were:

1. To analyse the prognostic value of clinical variables in the current PSCC material (I-III)
2. To examine the expression of transcription factor *snai1* and its effect on the prognosis in PSCC (I)
3. To evaluate the expression and prognostic role of *twist* in PSCC (II)
4. To investigate *sip1*, *zeb1* and *slug* expression and their influence on survival in PSCC (III)
5. To assess the co-expression of EMT-RTFs and its prognostic value in PSCC (II-III)

4 Patients, materials and methods

4.1 Study design

This study was a retrospective, longitudinal research with population-based cohort design. The primary cohort incorporated all patients diagnosed with PSCC between the years 1975 and 1998 in the Area of Regional Responsibility of the Kuopio University Hospital including Central Hospitals of Central Finland (Jyväskylä), Northern Karelia (Joensuu), Southern Savo (Mikkeli) and Eastern Savo (Savonlinna) together with Kuopio University Hospital. The patients were identified from the hospital records and the Finnish Cancer Registry (www.cancerregistry.fi). Initially there were a total of 161 patients fulfilling the criteria. The mean population in the district during the study-period was 870 000.

4.2 Clinical data and Follow-up

The clinical data from all 161 patients were reviewed by one oncologist and two otolaryngologists. Ten cases had to be excluded because the cancer had originated outside the pharyngeal area or because of insufficient clinical data. The remaining 151 tumors were staged according to the UICC classification (1997), based on written hospital records of clinical otolaryngological status, endoscopy and chest x-ray. (206) Karnofsky performance status (KPS) at the time of diagnosis was coded according to the case history. All the patients had been regularly followed up by an otolaryngologist and/or oncologist until death or April 2009. The cause of death was obtained from hospital records or from death certificates. None of the patients was lost from the follow-up.

4.3 Tumor samples and histopathology

All tissue samples available were retrieved from the archives of each hospital. They had been all originally fixed in 10% formalin (buffered, pH 7.0) and embedded in paraffin. Of the 151 cases 138 were histologically verified invasive squamous cell carcinomas of oro- or hypopharyngeal origin for which sufficient clinical data was available. All the sections were evaluated and histological differentiation of the primary tumor was determined according to the WHO criteria by one experienced pathologist. (52) Sufficient material for tissue microarrays and immunohistochemical analyses were available from 110 original tumor samples for *snai1* staining, 109 for *twist* staining and 108 for *sip1*, *zeb1* and *slug* stainings.

4.4 Tissue microarray and immunohistochemistry

Two most representative areas of the paraffin-embedded tissue blocks were chosen by an experienced pathologist (V-M Kosma or Y Soini) and marked for microarrays which were constructed using 1.0 mm core Manual tissue arrayer I (Beecher Instruments, Silver Spring, MD, USA). Four- μ m-thick sections were first deparaffinized and rehydrated in a routine manner. Then the sections for *snai1* analysis were heated in a microwave oven (800W) for 2 x 5 minutes in Tris EDTA buffer (pH 9.0) and the other sections in 0.01 molar citrate buffer (pH 6.0) for 2 or 3 x 5 minutes (3 for *twist*, two for *sip1*, *zeb1* and *slug*), incubated in the last buffer for 18 minutes and washed twice for 5 minutes in phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked with hydrogen peroxide (5%, 5 minutes) followed by washing with water 2 x 5 minutes and with PBS for 2 x 5 minutes. Non-specific binding was blocked with 1.5% normal serum in PBS for 25-35 minutes at room temperature. All the sections were incubated overnight at 4°C with specific antibodies as indicated in table 4. In the negative controls, the primary antibody was omitted. The slides were then washed with PBS for 2 x 5 minutes. *Snai1*, *twist*, *sip1* and *zeb1* stained slides

were incubated with the biotinylated secondary antibody (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) for 35 minutes at room temperature. Thereafter, the slides were washed twice in PBS for 5 minutes, incubated for 45 minutes in preformed avidin-biotinylated peroxidase complex solution (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) and washed with PBS for 2 x 5 minutes. In slug sections, Dako REAL™ EnVision™ secondary antibody (K5007) was used and incubated slides for 30 minutes. The color was developed by diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO, USA). The samples were counterstained with Mayer's haematoxylin, washed, dehydrated, cleared and mounted with Depex (BDH, Poole, UK). Ovarian tumor tissue with known positive expression was used as a positive control for *snai1*. Strongly positive pharyngeal tumor tissue samples for *twist*, *slug*, *sip1* and *zeb1* antibodies were identified in preliminary test stainings and were then used as positive controls in each definitive staining series.

Table 4. Specific antibodies used for immunohistochemical stainings

Marker	Number of cases	Primary antibody	Manufacturer	Dilution
<i>snai1</i>	110	mouse monoclonal anti <i>snai1</i>	non-commercial, (207,208)	1:750
<i>twist</i>	109	mouse monoclonal anti <i>twist</i>	Abcam, Cambridge, UK	1:100
<i>sip1</i>	108	rabbit polyclonal anti <i>sip1</i>	Santa Cruz Biotech. Inc, Dallas, USA	1:200
<i>slug</i>	108	rabbit polyclonal anti <i>slug</i>	Abnova, Taipei City, Taiwan	1:100
<i>zeb1</i>	108	mouse monoclonal anti <i>zeb1</i>	GenWay Biotech. Inc, San Diego, USA	1:500

4.5 Evaluation of the expression

All the array spots were separately evaluated by two or three observers: A Jouppila-Mättö, H Tuhkanen and Y Soini for *snai1*, A Jouppila-Mättö, R Sironen and Y Soini for *twist*, A Jouppila-Mättö and Y Soini for *sip1* and *slug* and A Jouppila-Mättö and R Soini for *zeb1*. The observers were unaware of the clinical and histopathological data. The number of *snai1* immunostained tumor epithelial, stromal and endothelial cell nuclei was counted on a continuous scale, tumor epithelial cell nuclei from two spots and stromal and endothelial cell nuclei from one array spot. In *twist*, *sip1*, *slug* and *zeb1* array spots, the percentage of stained tumor epithelial and stromal cell nuclei was counted and classified into 5 categories: 0-5%=1, 6-25%=2, 26-50%=3, 51-75%=4, 76-100%=5. The number of array spots with detectable endothelial cell nuclear positivity was counted in *sip1*, *slug* and *zeb1* stainings and marked as 2 if both spots were positive, 1 if one spot was positive and 0 if there were no positive endothelial cell nuclei present. In *sip1* and *slug* samples, also cytoplasmic staining in tumor epithelia and stromal tissue was observed. It was classified into 4 groups: no staining=0, weak staining=1, moderate staining=2, intense staining=3. In the classified variables, the observers evaluated together all of these spots where the scores were diverging by more than one class in order to reach a consensus. The mean value between the two array spots was then calculated. For continuous variables, the interobserver agreement was evaluated by the Spearman test. The median value of every variable was counted and the samples were divided accordingly into positive and negative subgroups. All the median values are represented in table 5.

Table 5. Median values of the positive imunohistochemical stainings

	snai1(continuous scale)	twist (classified)	sip1(classified)	slug(classified)	zeb1(classified)
Tumor epithelial cell nuclei	2	1.0	1.0	2.25	0.5
Tumor stromal cell nuclei	3	2.0	2.0	1.75	3.0
Endothelial cell nuclei	7	-	1.0	1.0	1.0
Tumor epithelial or stromal cytoplasm	-	-	1.25	2.1	0

4.6 Statistical analyses

Descriptive statistics were used for continuous variables, i.e. means with standard deviations were used for normal distribution whereas medians with ranges were used for values with non-normal (skewed) distribution, respectively. Mann-Whitney test was used to examine the associations between continuous variables. Statistical associations between classified variables were tested with the chi-square (χ^2) test for independence. Frequency tables with three variables were calculated with one-way ANOVA –test. Spearman's nonparametric rank order correlation coefficient was used to test the linear association between non-normal variables. The disease specific survival was defined as the time period between the date of primary diagnostic biopsy and the date of death due to pharyngeal cancer or to the end of follow-up; overall survival due to death of any cause. Univariate survival analyses were evaluated using the Kaplan-Meier method. The statistical differences between the curves were analyzed using the log-rank test. Multivariate survival analysis was performed using Cox's proportional hazards model in a stepwise manner. Values of $p < 0.05$ were considered as significant. The statistical analyses were performed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA).

4.7 Ethics

The research plan of the original research material had been approved by the ethical committee of Kuopio University and Kuopio University Hospital (decision No 102/97), and permission for accessing data from the Finnish Cancer Registry and from the hospital records was obtained from the Finnish Ministry of Social Affairs and Health (permission No 88/08/97). Due to the retrospective nature of the study, the patients or their families did not need to be contacted. The data obtained have not been incorporated into hospital records.

5 Results

5.1 REMARKS ON COHORT AND TREATMENT

5.1.1 Patient and tumor characteristics

The summary of the clinical and histopathological data as well as treatment characteristics are presented in table 6. The mean age of the patients at the time of diagnosis was 65 years and three out of four patients were male. The median duration of the symptoms before diagnosis was three months with the most prevalent prediagnostic symptoms being throat pain, neck mass and dysphagia. According to the retrospective hospital records, 67 (62%) of the patients were smokers and 53 (49%) had abundant alcohol consumption. The KPS score coded at the time of diagnosis ranged between 40 and 90 and in 66% of the patients, the score was 70% or better. At the time of diagnosis most of the tumors (88%) were classified as at least T2 and 43% of the patients presented with locoregional lymph node metastases. Almost 70% of the tumors were diagnosed at an advanced stage (SIII-IV) and 76% displayed moderate or a poor degree of differentiation. Sixty three percent of the tumors were of oropharyngeal origin.

5.1.2 Treatment and follow-up

In this cohort 99 patients received radiotherapy either as a primary treatment (63%) or postoperatively as adjuvant therapy (28%). The median tumor dose of irradiation was 61 Grays (range 16-80). The primary tumor was operated in 36 cases (33%) and ipsilateral neck dissection was performed in 18 patients (17%). The treatment was intended to be curative in 95 (87%) and palliative in 11 (10%) cases. Four patients received basic care only due to their poor general condition. In 78 cases (72%), the treatment was successful while 31 (28%) tumors did not respond.

Complete follow-up data were available for the whole cohort. Median OS was 20.9 months, 24.8 for oropharyngeal and 17.9 for hypopharyngeal carcinomas. Median DSS was 22.4 months (CI 12.7-32.2). The 5-years OS was 31.2% and DSS 41.3%. During five year follow-up, there were 41 recurrences (38%) and 10 (9%) second primaries. In most cases, tumors relapsed at the site of the primary tumor (n=22, 54%). There were 10 neck lymph nodal (24%) and 9 distant relapses (22%). The median recurrence free period was 9.6 months (range 1.8 to 54.8 months). At the end of follow-up period, 67 patients (62%) had died from pharyngeal carcinoma while only 10 (9%) were alive and diseasefree.

Table 6 . Clinicopathological features of the patients with pharyngeal squamous cell carcinoma (n = 108)

Variable	n (%)
Mean age at the time of presentation, years	65 (std.dev. 10,5)
Median duration of the symptoms, months	3 [0-76]*
Sex	
Male	81 (75)
Female	27 (25)
Site of primary tumor	
Oropharynx	68 (63)
Hypopharynx	40 (37)
T category	
T1	13 (12)
T2	39 (37)
T3	21 (19)
T4	35 (32)
N category	
N0	62 (57)
N1	16 (15)
N2	27 (25)
N3	3 (3)
M category	
M0	104 (96)
M1	4 (4)
Stage	
S I	9 (8)
S II	24 (22)
S III	21 (19)
S IV	54 (50)
Histologic differentiation	
Gr 1	25 (24)
Gr 2	48 (44)
Gr 3	35 (32)
Karnofsky performance status score	
≥ 70 %	71 (66)
< 70 %	37 (34)
Primary treatment	
Radiotherapy	68 (63)
Surgery and radiotherapy	31 (28)
Surgery	5 (5)
No cancer specific treatment	4 (4)
Relapse	
No	36 (33)
Yes	41 (38)
No response	31 (29)
Second primary tumor	
No	98 (91)
Yes	10 (9)
Median OS, months	20.9 [1.1-401.3]*

*Values in square brackets indicate range.

5.2 IMMUNOHISTOCHEMISTRY

5.2.1 Snai1

Snai1 expression was detected in epithelial, tumor stromal fibroblast and endothelial cell nuclei of PSCC samples. Epithelial positivity was detected in 75 (68%) of the tumors. In all, 49 (46%) of tumor stromal cells and 51 (48%) of endothelial cells showed positive immunostaining (Fig. 9). Stromal expression significantly associated with expression in endothelium and epithelium (p -values <0.001) while expression in endothelium was related to immunoreactivity in epithelium ($p = 0.002$). In less than ten samples, snai1 expression was detected adjacent to necrotic and inflammatory areas. Cytoplasmic snai1 expression in epithelium was present in only 4 (4%) of 110 samples. Snai1 protein expression in epithelial cells was more abundant in tumors with hypopharyngeal origin ($n=32$, $p = 0.044$) and patients with low KPS score ($n=30$, $p = 0.039$). The positive snai1 immunoreactivity in epithelial cells predicted an earlier local relapse ($p = 0.042$). Stromal snai1 expression was more common in hypopharyngeal tumors ($n=23$, $p = 0.038$), with increasing T category (T3-4, $n=31$, $p = 0.037$), and in samples of patients with poorer general condition (KPS score < 70 ; $n=22$, $p = 0.039$). In endothelial cells, positive snai1 immunoreactivity was related to hypopharyngeal primary site ($n=26$, $p = 0.01$), increasing T category (T3-4, $n=35$, $p = 0.005$), advanced stage (III-IV, $n=44$, $p = 0.005$) and poor histopathological differentiation (gr.2-3, $n=46$, $p = 0.012$). No association was observed between snai1 immunoreactivity and age, gender or neck lymph node metastases.

5.2.2 Twist

Twist expression was detected in tumor epithelial and stromal fibroblast cell nuclei. However, the expression in stromal cell nuclei was more common than epithelial expression (40% and 35%, respectively) (Fig. 10). Tumors with abundant stromal expression relapsed more frequently ($p=0.04$). Stromal expression was more common in hypopharyngeal than in oropharyngeal tumors ($p=0.015$). Stromal expression of twist did not correlate with patients' age or gender, tumor size, stage, histological grade or smoking. However, there was a tendency between stromal twist immunoreactivity and larger tumor size (T3-4) and advanced stage (SIII-IV) ($p=0.064$ and $p=0.086$ respectively). In the current cohort, epithelial expression of twist did not correlate statistically significantly with any of the clinicopathological variables. However, patients with twist epithelial immunoreactivity tended to have a worse general condition (KPS score < 70 ; $p=0.077$) and they also had more neck lymph node metastases ($p=0.099$).

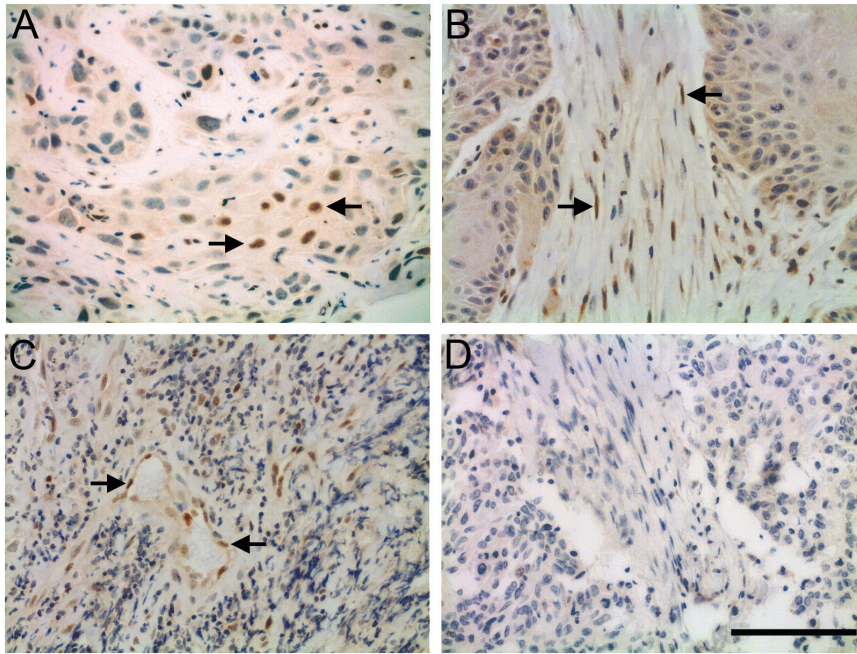


Figure 9. Expression of transcription factor snai1 in pharyngeal squamous cell carcinoma. Positive immunostaining in the nuclei of malignant epithelium (A), spindled stromal cells (B) and the vascular endothelium (C). Lack of expression is also illustrated (D). (Original magnification of x 400, scale bar 100µm)

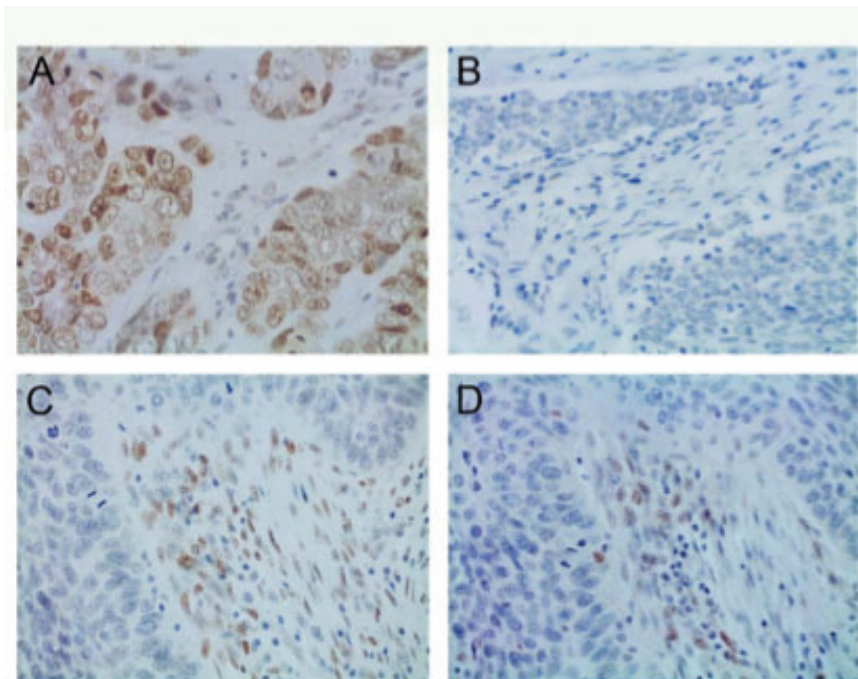


Figure 10. Expression of twist and snai1 nuclear transcription factors in pharyngeal squamous cell carcinoma. Positive (A) and negative (B) twist immunostaining and positive snai1 staining (C) in the spindled stromal cells (arrows). (Original magnification of x400)

5.2.3 Sip1

Sip1 expression was abundant in tumor epithelial nuclei especially at the invasive front and it was also frequently seen in tumor stromal fibroblasts and endothelial cell nuclei as well as in the cytoplasm of all the cell types. Forty four of 108 (41%) samples showed epithelial cell nuclear sip1 positivity and cytoplasmic positivity (41%). The stromal cells were sip1 positive in 38 of 103 cases (37%) and 63 samples (58%) displayed positive endothelial cell nuclei (Fig 11). There was also a correlation between sip1 cytoplasmic and nuclear immunostaining ($p<0.001$ for each cellular compartment). Furthermore, endothelial and stromal nuclear stainings correlated statistically significantly ($p<0.001$). Tumors with positive sip1 immunostaining in epithelial cell nuclei were more advanced (SIII-IV $n=75$, $p=0.02$) and had more often lymph node metastases (N1-3 $n=46$, $p=0.04$) as compared to sip1 negative tumors. There were also more second primaries diagnosed in this group ($n=10$, $p=0.048$). Hypopharyngeal tumors were more often sip1 positive as compared with oropharyngeal tumors (epithelial nuclei $p=0.002$, stromal nuclei $p=0.03$, cytoplasm $p<0.001$). Better differentiated tumors (grade 1-2 vs. 3) had often positive sip1 immunostaining in epithelial ($n=36$, $p=0.006$), stromal ($n=30$, $p=0.048$), endothelial ($n=51$, $p<0.001$) cell nuclei or cytoplasm ($n=35$, $p=0.02$). Tumors with positive stromal staining of sip1 relapsed significantly more often than sip1 negative tumors ($n=32$, $p=0.007$). (Table 7)

Table 7. Clinicopathological variables and sip1 expression (Significant values marked bold)

	positive epithelial cell nuclei, cases	negative epithelial cell nuclei, cases	p-value	positive stromal cell nuclei, cases	negative stromal cell nuclei, cases	p-value
age <65	26	33		16	41	
age >65	18	31	0.440	22	24	0.039
KPS score<70	16	21		17	19	
KPS score≥70	28	43	0.702	21	46	0.111
gr. 1-2	36	36		30	39	
gr. 3	8	28	0.006	8	26	0.048
T1-2	18	34		15	34	
T 3-4	26	30	0.212	23	31	0.208
S I-II	8	25		9	22	
S III-IV	36	39	0.021	29	43	0.278
N0	20	42		20	39	
N1-3	24	22	0.037	18	26	0.466
M0	41	63		37	63	
M1	3	1	0.155	1	2	0.897
oropharyngeal origin	20	48		18	45	
hypopharyngeal origin	24	16	0.002	20	20	0.028
remission	11	25		6	27	
no remission or relapse	33	39	0.128	32	38	0.007
no second primary	37	61		33	60	
second primary tumor	7	3	0.048	5	5	0.366

5.2.4 Slug

Tumor epithelial cell nuclear immunostaining of slug was apparent in 57 of 108 (53%) PSCC tissue samples. Stromal fibroblasts nuclear staining was detected in 60 of 106 samples (57%), tumor epithelial and stromal cytoplasmic in 55 of 108 array spots (51%) and 55% of the array spots showed endothelial nuclear cell positivity for slug (54 of 98 samples)(Fig. 11). Tumors with positive slug expression in tumor epithelial nuclei located more often in hypopharynx than in oropharynx ($n=27$, $p=0.02$). In addition, slug positive tumors were more often well or moderately differentiated (epithelial nuclei $n=45$, $p=0.004$; endothelial nuclei $n=41$, $p=0.049$; cytoplasm $n=43$, $p=0.01$). Younger patients did not usually have slug immunostaining in the stromal cell nuclei of the tumor samples ($n=32$, $p=0.007$).

5.2.5 Zeb1

Epithelial zeb1 immunopositivity was scarce. Thus, only single positive cell nuclei were detected in 34 of 108 tumor samples (31%). Stromal positivity was detected in 38 (35%) and positive endothelial cells in 61 spots (56%). There was no cytoplasmic staining of zeb1 in any of the samples (Fig 11). Zeb1 immunoreactivity did not correlate with any of the clinicopathological variables.

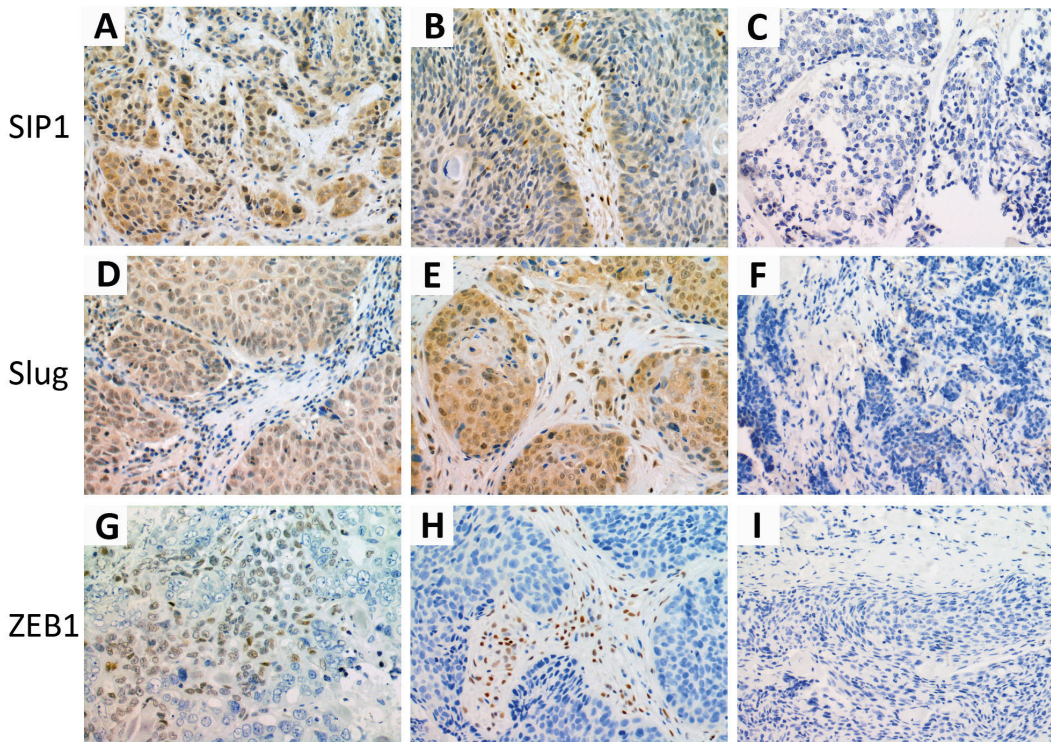


Figure 11. Immunohistochemical detection of sip1, slug and zeb1 in pharyngeal squamous cell carcinoma. Epithelial (A ,D,G), stromal (B,E,H) and negative immunostainings (C ,F,I) of sip1, slug and zeb1 respectively. The stromal component includes endothelial and fibroblastic cells (Original magnification of x200).

5.2.6 Co-expression of *snai1* and *twist*

A strong association between *twist* and *snai1* expression in stromal cell nuclei was detected ($p=0.001$, Spearman's correlation coefficient 0.33) while in the epithelial cancer cell nuclei, a similar association was absent ($p=0.206$, Spearman's correlation coefficient 0.10). In experiments evaluating tumors undergoing *twist* and *snai1* related EMT a group was selected where both *twist* and *snai1* were positive and renamed the subgroup as EMT+ ($n=29$, 27%). All of these EMT+ tumors were at least Stage II ($p=0.05$) and were located more often in hypopharynx ($p=0.035$). *Twist* and *snai1* co-expression did not correlate with histological grade, metastases or smoking habits. Respectively, tumors lacking both *twist* and *snai1* stromal immunoreactivity ($n=42$, 38.5%) were renamed EMT-. These tumors were significantly smaller (T1-2, $n=27$, 64%, $p=0.008$), less advanced (SI-II, $n=18$, 43%, $p=0.031$) and located more often in oropharynx ($n=33$, 79%, $p=0.007$). Furthermore, non-smokers had more EMT- tumors ($n=11$, 26%, $p=0.034$). It was possible to detect either *snai1* or *twist* stromal immunoreactivity in thirty eight (35%) patients. The tumors were larger according to the number of positive transcription factors ($p=0.034$) (Table 8).

Table 8. Expression of *twist* and/or *snai1* in tumor stroma in different T categories

T classification	Stromal expression of <i>twist</i> and/or <i>snai1</i>			<i>p</i>
	EMT- n (%)	<i>twist</i> or <i>snai1</i> + n (%)	EMT+ n (%)	
T1 and T2	27 (25)	15 (14)	11 (10)	0.034
T3 and T4	15 (14)	23 (21)	18 (16)	

5.2.7 Co-expression of other transcription factors

There was a distinct association between tumor epithelial and especially stromal staining of *sip1*, *slug* and *zeb1*. The co-expression of all five EMT-RTFs is detailed in Table 9. We stratified a subgroup of tumors ($n=17$, 16%) where *snai1*, *twist* and *sip1* all co-expressed in tumor epithelial cell nuclei. In this subgroup, all tumors were at least stage III ($p=0.003$). The tumors located more commonly in hypopharynx ($n=11$, 65%, $p=0.009$), were larger (T3-4, $n=13$, 76%, $p=0.02$), and had more lymph node metastases (N1-3 $n=11$, 65%, $p=0.04$). Almost all of these tumors were well or moderately differentiated ($n=15$, 88%, $p=0.04$). There were 19 samples where none of the three transcription factors were expressed in the tumor epithelium. However no significant correlation with any of the clinicopathological variables was seen in this subgroup. In 17 samples (15.6%) *snai1*, *twist* and *sip1* were all expressed in tumor stroma. In this subgroup, only two patients recovered whereas the other 15 either relapsed or did not attain remission ($p=0.05$). In 31 samples, none of those transcription factors were expressed in tumor stroma (28.4%). In that latter group, most of the tumors were small (T1-2) at presentation ($n=21$, 68%, $p=0.007$).

Table 9a. Correlations of positive tumor epithelial cell nuclear stainings

		sip1 n=44	slug n=57	zeb1 n=34	twist n=38
sip1 n=44					
slug n=57	SpCC	0.22 *			
	p	0.02			
zeb1 n=34	SpCC	-0.10	-0.04		
	p	0.30	0.71		
twist n=38	SpCC	0.17	0.00	0.25 *	
	p	0.08	0.99	0.01	
snai1 n=75	SpCC	0.21 *	0.36 **	0.22 *	0.08
	p	0.03	0.00	0.03	0.42

Sp.CC Spearman's correlation coefficient

*. Correlation is significant at the 0.05 level.

**. Correlation is significant at the 0.01 level.

Table 9b. Correlations of positive tumor stromal cell nuclear stainings

		sip1 n=38	slug n=60	zeb1 n=38	twist n=44
sip1 n=38					
slug n=60	SpCC	0.58 **			
	p	0.00			
zeb1 n=38	SpCC	0.37 **	0.50 **		
	p	0.00	0.00		
twist n=44	SpCC	0.50 **	0.40 **	0.64 **	
	p	0.00	0.00	0.00	
snai1 n=49	SpCC	0.34 **	0.07	0.28 **	0.40 **
	p	0.00	0.49	0.01	0.00

Sp.CC= Spearman's correlation coefficient

**. Correlation is significant at the 0.01 level.

5.3 SURVIVAL ANALYSES

5.3.1 Clinicopathological factors

A summary of univariate analysis of clinicopathological factors and DSS is presented in table 10. The most important prognostic factors in this analysis were tumor size (T class) and stage together with the general condition of the patient. Hypopharyngeal carcinomas had a significantly poorer prognosis than oropharyngeal carcinomas and distant metastases naturally deteriorated the prognosis. Age, gender, consumption of tobacco or alcohol, or length of time period between first symptoms and diagnosis or histological grading did not have any effect on the prognosis.

Table 10. Kaplan-Meier univariate analysis of associations between various clinicopathological factors and DSS (Significant values marked bold)

variable (n)		number of deaths in PSCC, 5-years follow-up	p-value
gender	male (82)	46 (56%)	0.515
	female (27)	18 (67%)	
age	<64,6 (60)	34 (57%)	0,423
	≥64,6 (49)	30 (61%)	
no tobacco smoking (19)		9 (47%)	0.226
tobacco smoking (67)		40 (60%)	
no alcohol consumption (14)		8 (57%)	0.703
alcohol consumption (53)		28 (53%)	
KPS score	< 70 (37)	29 (78%)	0.000
	≥ 70 (72)	35 (49%)	
Duration of symptoms before dg. <5.3 months (78)		46 (59%)	0.605
≥5.3 months (31)		18 (58%)	
BMI	<20 (13)	9 (69%)	0.054
	20-25 (50)	32 (64%)	
	>25 (41)	21 (51%)	
T class	1 (13)	4 (31%)	0.000
	2 (40)	15 (38%)	
	3 (21)	13 (62%)	
	4 (35)	32 (91%)	
N class	0 (63)	32 (51%)	0.059
	1-3 (46)	32 (70%)	
M class	0 (105)	60 (57%)	0.030
	1 (4)	4 (100%)	
Stage	I (9)	2 (22%)	0.000
	II (25)	6 (24%)	
	III (21)	11 (52%)	
	IV (54)	45 (83%)	
Histological grade	1 (26)	15 (58%)	0.954
	2 (48)	28 (58%)	
	3 (35)	21 (60%)	
Primary site	oropharynx (69)	35 (51%)	0.035
	hypopharynx (40)	29 (73%)	

5.3.2 EMT-related transcription factors

In Kaplan-Meier univariate analysis, positive *snail* immunoreactivity in endothelial cell nuclei strongly predicted poor five-year DSS ($p = 0.009$). Additionally, there was a trend noted between stromal nuclear *snail* expression and poor DSS ($p = 0.067$). In contrast, epithelial nuclear expression of *snail* was not related to prognosis. Neither epithelial nor stromal cell nuclear expression of *twist* alone associated with DSS or OS in the univariate analysis. Instead, the EMT- group experienced a statistically significantly better 5-year survival as compared to patients who had positive *twist* or *snail* stromal expression (DSS $p=0.037$; OS $p=0.014$). The 5-year prognosis tended to worsen as the number of positive transcription factors detected increased (DSS $p=0.113$; OS $p=0.043$).

Tumor epithelial cell nuclear *sip1* immunoreactivity correlated significantly with five-year DSS and OS, the estimated disease specific survival time being 37 months for patients with *sip1* negative and 26 months for *sip1* positive tumors (DSS $p=0.012$, OS $p=0.003$). Similarly, stromal cell nuclear *sip1* positivity also correlated statistically significantly with DSS and OS ($p=0.018$ and $p=0.003$, respectively). No such correlation with survival was found for *slug* or *zeb1* immunostainings (DSS, $p=0.16$ and $p=0.44$, respectively).

Cox proportional hazards model included age, KPS score, T-class, N-class, histopathological grade and the EMT-RTFs as variables. Positive epithelial cell nuclear staining of *sip1* was an independent prognostic factor for DSS and OS together with KPS score and T-class (DSS $p=0.046$, $p=0.001$, $p<0.001$ and OS $p=0.023$, $p<0.001$, $p<0.001$, respectively)(Fig.12),(Table12).

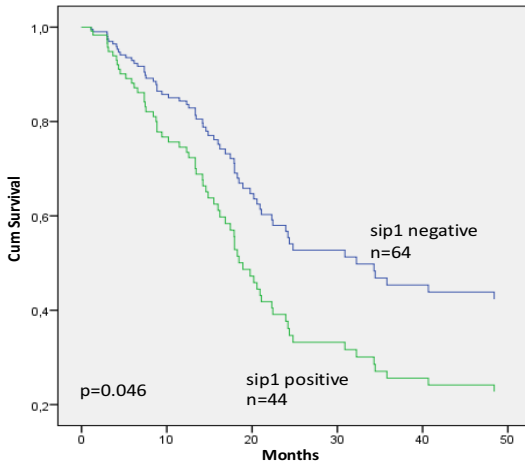


Figure 12. Cox proportional hazards model of 5-year survival analysis of pharyngeal squamous cell carcinoma. *Sip1* expression in tumor epithelial cell nuclei predicts poorer disease specific survival ($p=0.046$).

In the group where *snai1*, *twist* and *sip1* were all co-expressed in the epithelial cell nuclei, both DSS and OS were very poor ($p<0.001$) (Fig. 13). None of these patients was alive after 5 years and the mean survival time was a mere 12 months. The result remained similar also in Cox proportional hazards model i.e. the epithelial co-expression of these three transcription factors was an independent prognostic factor for poorer DSS and OS ($p=0.002$ and $p<0.000$) in PSCC, together with KPS score and T-class ($p=0.002$ and $p<0.001$, respectively). The DSS and OS were significantly better if no expressions of *snai1*, *twist* or *sip1* were detected in tumor stroma ($p=0.05$, $p=0.02$ respectively) (Fig. 14). However, this was not an independent prognostic factor in the Cox proportional hazards model. A summary of the univariate analysis of all EMT-RTF expressions and co-expression in association with DSS is presented in table 11. All statistically significant predictors of DSS in Cox proportional hazards model are shown in table 12.

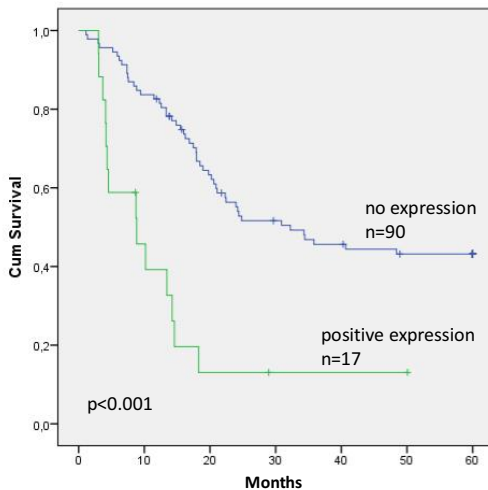


Figure 13. Kaplan-Meier univariate 5-year survival analysis of pharyngeal squamous cell carcinoma. In the group where *snai1*, *twist* and *sip1* are all expressed in tumor epithelial nuclei DSS is significantly poorer ($p<0.001$).

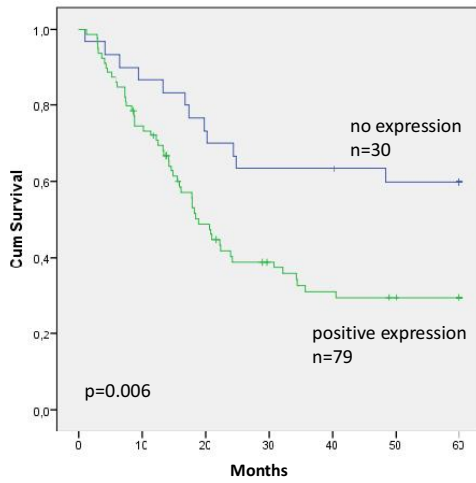


Figure 14. Kaplan-Meier univariate 5-year survival analysis of pharyngeal squamous cell carcinoma. When there is no expression of *snai1*, *twist* or *sip1* in tumor stroma, the DSS is significantly better ($p=0.006$).

Table 11. Kaplan-Meier univariate analysis of association of EMT-marker nuclear expressions and DSS (Significant values marked bold)

EMT-marker/group of markers	Number of deaths in PSCC, 5-years follow-up	p-value
snai1 tumor epithelium pos. (75)	44 (59%)	0.387
neg. (34)	20 (59%)	
snai1 tumor stroma pos. (49)	32 (65%)	0.067
neg. (57)	30 (53%)	
snai1 endothelium pos. (54)	38 (70%)	0.009
neg. (55)	26 (47%)	
twist tumor endothelium pos. (38)	24 (63%)	0.265
neg. (69)	40 (58%)	
twist tumor stroma pos. (44)	28 (63%)	0.253
neg. (65)	36 (55%)	
sip1 tumor epithelium pos. (44)	31 (70%)	0.012
neg. (64)	33 (52%)	
sip1 tumor stroma pos. (38)	27 (71%)	0.018
neg. (65)	35 (54%)	
sip1 endothelium pos. (63)	37 (59%)	0.833
neg. (46)	27 (59%)	
sip1 cytoplasm pos. (44)	30 (68%)	0.181
neg. (64)	34 (53%)	
slug tumor epithelium pos. (57)	36 (63%)	0.158
neg. (51)	28 (55%)	
slug tumor stroma pos. (60)	35 (58%)	0.618
neg. (46)	27 (59%)	
slug endothelium pos. (54)	35 (65%)	0.240
neg. (55)	29 (53%)	
slug cytoplasm pos. (55)	35 (63%)	0.291
neg. (54)	29 (54%)	
zeb1 tumor epithelium pos. (34)	22 (65%)	0.338
neg. (75)	42 (56%)	
zeb1 tumor stroma pos. (38)	23 (61%)	0.496
neg. (71)	41 (58%)	
zeb1 endothelium pos. (61)	37 (61%)	0.942
neg. (48)	27 (56%)	
snai1+twist stroma pos. (EMT+) (29)	18 (62%)	0.314
snai1+twist stroma neg. (EMT-) (42)	20 (48%)	0.037
snai1+twist+sip1 epit.pos. (17)	14 (82%)	0.000
snai1+twist+sip1 epit. neg.(19)	10 (52%)	0.368
snai1+twist+sip1 str. pos. (17)	12 (71%)	0.141
snai1+twist+sip1 str neg. (31)	15 (48%)	0.049

Table 12. Statistically significant predictors of DSS in Cox proportional hazards model

variable	p-value
T-class (T1-2/T3-4)	<0.001
KPS score (≥ 70 / < 70)	0.001
sip1 epithelial nuclear expression (neg./pos.)	0.045
snai1, twist and sip1 epithelial nuclear co-expression (neg./pos.)	0.002

6 Discussion

6.1 THE STUDY DESIGN, COHORT AND CLINICAL DATA

6.1.1 Cohort and methods

The original population of 138 cases consisted of all the oro- and hypopharyngeal SCC patients diagnosed in the Eastern Finland area during the study period. Sufficient tissue material for microarrays and immunohistochemical stainings was available from 110 to 108 samples. Two array spots had diminished to too small an area to allow a reliable interpretation of the stainings. The utilization of array spots instead of the whole tumor prepartate diminishes the investigated area and carries a possible risk for mis-interpretation. The spots were, however, meticulously selected by an experienced pathologist so that they would contain a representative area of the tumor invasive front and stromal tissue. Array spots have been widely used in several previous immunohistochemical studies.(39,193,209,210) In a study of thyroid carcinomas, the immunohistochemical stainings were conducted for both whole sections and array spots and the results were reported to be closely matched.(211) The use of immunohistochemistry as the only study method has to be considered as a deficiency of this thesis. However, immunohistochemistry is a widely used and well documented method exploited in many clinical trials with survival analyses.(27,30,34-36,38)

HNC is a very diverse set of malignomas originating from various anatomical sites. The classification of the sites between studies has varied extensively complicating the comparison of the obtained results. The decision to exclude nasopharyngeal carcinoma from this study was made in an attempt to homogenize the material since nasopharyngeal carcinoma differs from oro- and hypopharyngeal carcinomas remarkably in its etiology, histopathology, clinical features and prognosis.(55) However, oro- and hypopharyngeal SCCs share the same risk factors, histopathology and to some extent also clinical features and treatment protocols. Although hypopharyngeal tumors had a worse prognosis as compared to their oropharyngeal counterparts, the site was not an independent prognostic factor suggesting that combination of these subgroups does not distort the survival data. In several earlier studies, all the HNCs or HNSCCs originating from various sites have been combined into the same material.(151,170,178,212-214)

The study period is very long with the earliest samples being over 30 years old. PSCC is however a rather rare disease and the study period has to be prolonged to gather a material large enough into permit reliable statistical analyses. In order to avoid the effect on different storage times on histological stainings, the material was previously chronologically divided to ten subgroups and the independence of histological variables was compared with Fisher's exact test.(215) There was no statistical difference between those subgroups.(215) Moreover, all the immunohistochemical stainings succeeded technically well with consistent staining results and they were interpreted without any time delay. The diagnostic criteria, classification and treatment protocols have remained very stable throughout the study period.

6.1.2 Clinical variables and prognostic factors

In the present material, a typical patient at the time of PSCC diagnosis was an elderly man with advanced disease. That description is in line with previous studies about pharyngeal carcinoma.(51,55,90,216) Moreover, the symptom spectrum in this study was rather similar

in comparison with the literature.(55,216,217) The delay from symptoms to diagnosis was three months, slightly shorter than that described in a Spanish material from the same decades (4,5 months).(55) This might suggest that our health care system is rather comprehensive. Nonetheless, the delay from primary symptoms to diagnosis is still longer in PSCC than in many other cancers due to its nonspecific symptoms and also due to lifestyle of the typical patient. In the present cohort, 62% of the patients reported that they were current or previous smokers and 49% declared modest to profound alcohol consumption. Neither of these parameters correlated with survival or other clinicopathological variables, although benzo[a]pyrene in cigarettes has shown to induce EMT and promote disease progression in lung cancer patients.(218) However, information of smoking and alcohol consumption in the present study was gathered from the medical records without any specific confirmation. Moreover, the information of smoking was missing in 23 cases and that on alcohol consumption was not known in 42 cases. Therefore, that information should be viewed with caution. The number of nodal metastases was lower in the present material (43%) than in previous studies on oropharyngeal and pharyngeal SSCs (70% and 60%, respectively).(55,90)

In the current material, 100 (91%) of the patients received radiotherapy either as a primary treatment (63%) or as an adjuvant therapy (28%). Primary surgery had been performed in 36 cases (33%) and neck dissection in 17% of the cases. In a Finnish nationwide study of oropharyngeal carcinoma conducted during 1995-1999, primary radiotherapy was given to 14% and combined radiotherapy with surgery to 76% of the patients. In that study, neck dissection was performed in 70% cases.(90) In hypopharyngeal carcinomas, surgery is rarely the primary treatment, which explains the differences between the results in comparison to the present study. DSS survival was 22.4 months in the current material, 40.7 for oropharyngeal and 17.9 for hypopharyngeal carcinoma, with the median OS being 20.9 months. These data are very well in line with previous studies with Finnish population.(51,90) With respect to the clinical factors studied, T-class or stage as well as KPS score remained independent prognostic factors in multivariate analysis which is fully in agreement with previous studies.(7,57,89-91) KPS score which reflects the patient's general condition was rather low in the present material since in 34% of the patients the score was less than 70. Pharyngeal carcinoma causes pharyngeal pain and dysphagia affecting nutritional intake and thus commonly resulting in deterioration of a general condition of the patient. The low BMI values, less than 20, revealed a poorer DSS also in the present material. In addition, many patients are alcoholics with a poor life style and possibly with other untreated medical conditions.

Interestingly histological grade had no association with survival in the current material. That observation has been made also in several other HNC materials.(34,38,57,89,179,219) Traditionally tumor differentiation is often believed to describe disease aggressiveness and prognosis, as also in PSCC. However, according to the present results, it is not very suitable for that purpose. Tumor size has a much stronger effect on the patient's survival and it is not dependent on cell differentiation. Furthermore, sip1, found to be an independent prognostic factor in the present study, is usually expressed in well differentiated tumors. This proposal is still not definitive but certainly needs and deserves to be further investigated.

6.1.3 HPV

In the 1980's a Finnish research team noted that 40% of oral SCCs in their study contained similarities with HPV-associated lesions.(220) This discovery initiated a new way of thinking: there are two different types of HNCs and especially oropharyngeal carcinomas taking notice of risk factors and clinical features. Patients with HPV-positive tumors are often non-smokers and non-drinkers with a smaller tumor size and better survival. HPV-positive tumors are also more sensitive to radiation and chemotherapy than HPV negative tumors.(7,8,91) P16 is an established biomarker for the function of the HPV E7 oncoprotein

and it is widely used for detection of HPV positive tumors.(9,91) The p16 status was also evaluated in the present oro- and hypopharyngeal SCC material. In the whole material, p16 positivity was detected in only 19 (18%) of the whole study group (n=107) and in ten (25%) samples of oropharyngeal origin. This incidence is slightly less than that reported in earlier publications.(7,91) The current material is rather old and the prevalence of HPV infection may have risen during the recent years which, at least partly, explains the difference.(8) The p16 positivity correlated with small tumor size (T1-2), poor differentiation grade (Gr 3) and lymph nodal metastases such as in previous studies.(91,92) There was, however, no correlation with expression of EMT-RTFs and p16. In Kaplan-Meier univariate analysis, p16 negative patients had lower DSS and OS ($p=0.012$ and $p=0.002$, respectively) but it was not an independent prognostic factor in multivariate analysis unlike sip1 epithelial expression, T-class and KPS score (data not shown). Thus, according to the current study, sip1 seems to be a more important prognostic marker for survival than p16 in PSCC.

6.2 EMT AND TRANSCRIPTION FACTORS

6.2.1 EMT and tumor-stroma interference

In addition to normal embryogenesis, EMT occurs in many pathological situations such as wound healing, fibrosis and acquisition of the invasive phenotype in epithelial tumors.(13) Tumor cells undergoing EMT lose their epithelial features and gain motile and invasive characteristics. Successful induction of EMT in tumor progression seems to be dictated by three factors: the differentiation program inherited from the normal cell origin, genetic and epigenetic changes accumulated during tumor progression and the signals that tumor cell is receiving from the nearby activated stroma.(221) Initiation of signal transduction cascades can be manifest in disparate outcomes in different cell and tumor types.

The EMT process has assumed to be fundamental in tumor progression and metastasizing. There has been, however, skepticism regarding the pathological relevance of EMT on grounds of its infrequent occurrence in several tumors and also the lack of mesenchymal markers in metastases.(110,222) It has also been claimed that the complete transition to mesenchymal phenotype is not required for invasion and metastases but tumor cells can invade by collective migration even while still displaying an epithelial phenotype.(110) It is difficult to obtain in vivo proof of existence and importance of EMT in cancer progression but, nevertheless, there is accumulating evidence to support its significant role in the immensely complex context of cancer development and progression.(13,15,96-98,101,113)

Equally controversial is also the existence and significance of CSC in tumorigenesis. It has been postulated that there is a subgroup of cancer cells which possesses the ability to self-renew and to differentiate into all cell types. (131,223) They remain in a quiescent state and are endowed with enhanced DNA repair mechanisms thus becoming resistant to chemo- and radiation therapies. Therefore they play an important role in cancer relapses and metastases.(204,205) EMT can trigger reversion to a CSC-like phenotype through TGF- β and WNT pathways, thus contributing tumor progression.(131)

EMT is a reversible process which is balanced between complicated regulatory networks and microenvironmental signals.(113) MET is prerequisite for disseminated tumor cells to proliferate and form distant metastases. When the disseminated cells no longer receive signals from the primary tumor surroundings, the cellular phenotype reverses back to its epithelial state, thus resulting the epithelial phenotype of metastases.(96,97,101,117,171) Due to the reversible nature of EMT process, it is unlikely that EMT-inducing transcription factors are permanently altered at the genomic level but, instead, are variably expressed while being controlled at both the transcriptional and translational levels.(171) Epigenetic

regulation encompasses three types of changes, DNA methylation, histone modifications and miRNAs, each of which has been shown to play an important role in controlling EMT.(114) These epigenetic modifications are reversible, contributing to plasticity in EMT-MET –processes.(113) However, there are also somatic, irreversible mutations in tumor growth regulating genes which can act in conjunction with epigenetic regulation.

The EMT markers are often present in tumor stroma close to the invasive front, as was also seen in the present study. A proportion of stromal cells might represent transformed tumor cells which had gone through EMT. Furthermore, in a response to growth factors, transcription factors might stimulate the conversion of stromal fibroblasts into more motile myofibroblasts.(125) There are also non-neoplastic activated fibroblastic cells in tumor stroma which may express transcription factors due to their interactions with epithelial cancer cells. These stromal cells might also express transcription factors and cannot be distinguished by immunohistochemistry alone from EMT transformed stromal cells..

6.2.2 Snai1

There are a large number of studies with snai1 in diverse set of carcinomas presenting occasionally contradictory expression data.(112,224) That could be partly due to the undefined specificity of commercial anti-snai1 antibodies. The anti-snai1 antibody used in the current study is one of the recently developed non-commercial monoclonal anti-snai1 antibodies that have been well-characterised and show evident nuclear staining.(207,208,225) There is also a considerable variation between methods used in each study and also in classifying of positive staining results. All that in addition to sometimes inaccurate discrimination between nuclear and cytoplasmic staining makes the comparison of the results between published studies very challenging. In the present study, cytoplasmic staining was seen in only 4 samples. The significance of cytoplasmic reactivity is, however, unclear and because snai1 activity is controlled by its subcellular location, only nuclear snai1 is believed to be active and stable.(145)

Immunoreactivity of snai1 has been detected previously in epithelial cancer cells in HNSCC (28,37,226) as it has also in several other cancer types. (22,29,136,148,149,207,225) As shown here, snai1 expression is more abundant in stromal cells, in agreement with reports from various other malignant tumors.(29,37,136,207,226,227) The expression of snai1 in epithelial cells is thought to take place already in the early phases of tumor development. In a previous study by our group, the snai1 protein expression was examined during ovarian tumor development in the transition from precursor lesions into carcinomas.(136) In benign tumors, no epithelial or stromal staining was observed in contrast to the situation in borderline tumors and especially in carcinomas, supporting a role for snai1 in early tumorigenesis. There is increasing evidence revealing that early and late state EMT may have different molecular profiles. Subcellular localization may be an indicator for early EMT whereas elevated total levels of markers could be evidence of late EMT.(228)

According to many reports investigating several human carcinomas, epithelial nuclear expression of snai1 protein is not associated with survival, (29,136,149,225) except in a recent study, where epithelial snai1 overexpression was claimed to significantly decrease the survival of patients with lung adenocarcinoma but not in lung SCC.(22) However, in colorectal carcinomas, snai1 expression in tumor stroma associated with lower DSS and the presence of distant metastases.(29) In the present study, those patients with stromal snai1 expression in their tumors displayed a trend towards poorer survival. These findings indicate that snai1 is expressed in the stroma of locally advanced PSCC tumors where it may modify the tumor microenvironment facilitating further cancer progression. Snai1 is present in activated mesenchymal cells pointing to its relevance in the communication between the tumor and the stroma.(207) Mesenchymal expression of snai1 has been suggested to stimulate invasion and angiogenesis in tumor.(229) Snai1 expression in stromal cells in the vicinity of tumor might also partly represent epithelial tumor cells that

have undergone EMT.(207) Furthermore, the interaction between epithelial and stromal cells during tumorigenesis may stimulate stromal cells to express *snail*.(37)

In the present study, *snail* expression was detected in a subset of endothelial cells and it associated statistically significantly with poor DSS in these PSCC patients. This is the first study to indicate that endothelial cell staining is related to poor survival in PSCC, although it was not an independent prognostic factor in the multivariate analysis. *Snail* expression in endothelial cells may be evidence that the protein takes part in the endothelial-mesenchymal transition (EndMT) which has been shown to be an important source of cancer-associated fibroblasts.(200) As reported by Kokudo et al.,*snail* mediates the actions of endogenous TGF β signals and these have been shown to induce EndMT.(230) EndMT has been suggested to play a role in activation of angiogenesis thus promoting tumor growth.(201)

In these samples, *snail* expression was occasionally detected adjacent to necrotic and inflammatory areas. *Snail* expression and function may be triggered by hypoxia.(112,231) This kind of activation of *snail* may take place also in large, poorly vascularised PSCC tumors and it may possibly explain the more intense *snail* expression in hypopharyngeal tumors, which are usually larger at the time of diagnosis. Staining in or close to necroinflammatory areas could reflect the anti-apoptotic functions of *snail*.(119) Proinflammatory mediators like interleukin 1 β are shown to upregulate *snail* and this way inflammation is supposed to promote EMT and tumor progression.(232) Inflammation induced factors also possibly stabilizes the *snail* protein.(147)

6.2.3 Twist

Twist has several properties that facilitate tumor progression including the triggering of EMT, inhibition of apoptosis and the enhancement of angiogenesis.(172,233) Twist has also been shown to induce EMT through chromatin remodeling.(173) Twist overexpression correlates with E-cadherin downregulation in HNSCC cell line.(173) However, in a study of nasopharyngeal carcinoma, *snail* expression was associated with repressed E-cadherin expression while twist expression had no influence on the expression of E-cadherin.(234) Thus, EMT regulators show varied expression profiles and distinct roles in different kinds of carcinomas. In the present study, the nuclear staining intensity of twist was detected in the stromal component more often than in epithelial cancer cells. The same phenomenon has been observed with *snail* in several carcinomas.(29,37,136,207,226,227) There was a trend towards a more pronounced twist staining intensity in larger and more advanced stage tumors. In a recent study in patients with oral SCC, twist was expressed in carcinomas but not in benign tissue samples.(179) Furthermore in ovarian tumors twist expression increased stepwise in normal, borderline and malignant tumors.(135) Consequently, the expression of twist seems to intensify with tumor progression.

Stromal expression of twist was more frequent in hypopharyngeal than in oropharyngeal tumors. It remains unclear whether this is due to the more advanced disease stage at the time of diagnosis with its possibly more hypoxic conditions, or if it represents a true feature of the hypopharyngeal tumors with a very poor overall prognosis. Interestingly, the patients with stromal twist and *snail* negative tumors were more often non-smokers. In bladder cancer, most of the patients with positive twist expression were current smokers.(235) This agrees with the previous hypothesis that smoking could modulate the expression of EMT markers. The aromatic hydrocarbon B[a]P which is present in tobacco smoke has been shown to cause EMT-like, partly irreversible, dynamic changes in gene expression including twist up-regulation. (218)

To date, there are only a few other studies investigating the association between twist expression and clinicopathological features in HNSCC. In the studies of Fan and Gasparotto, twist expression showed no association with either prognosis or

clinicopathological variables.(179,214) In a rather small cohort of various head and neck squamous cell carcinomas (n=50), nuclear and cytoplasmic staining of twist correlated with histologic differentiation grade, advanced stage and large neck lymph node metastases.(178) The comparison of these data with the present study has to be conducted with care. Since transcription factors are considered to be active only when located in the cell nuclei (145), cytoplasmic staining was not included into the present evaluation. In this study, twist expression alone did not associate with survival. There is some evidence of positive staining of twist and poor survival in HNSCC and similarly in oesophageal SCC.(31,170) In addition, twist expression in the epithelial compartment of breast carcinoma was associated with poor survival.(236) Evidently, tumors from different sites and with different histology vary in their twist expression profile. There is also variations in the classification strategies used in different publications. The cut-off points between positive and negative values diverge and in some studies also staining intensity has been included in the evaluation. This makes the direct comparison of the results rather challenging.

According to previous studies, twist has been postulated to be responsible for regulation of metastasis development. In breast tumor cell lines, twist has been shown to promote the early steps of tumor metastasis, where tumor cells gain access to the circulation with no apparent benefit to the growth of the primary tumor.(20) Twist expression also correlates with distant metastases, for example in esophageal SCC.(237) In the present study, patients with epithelial cell nuclear expression of twist tended to have more neck lymph node metastases although the difference was not statistically significant. The metastatic event is a complex process with many diverse phases and there are certainly also several other factors regulating it.

There are no published data about the half-life of twist or on its stability within the cell nucleus but *snai1* is known to be highly unstable.(145) As is typical for transcription factors it is probable that twist is expressed in tumor cells for only a very short time period which might explain why only one third of the epithelial cancer cells expressed twist in their cell nucleus. Some of the stromal cells may represent transformed epithelial cancer cells undergoing EMT.(96) That could be one explanation for the stromal staining of twist observed also in the present material.

6.2.4 Sip1, slug and zeb1

E-cadherin downregulation has been considered as a principal landmark of EMT. Sip1 binds to the promoter area of E-cadherin, inducing its downregulation.(19) However, the association between E-cadherin and sip1 is somewhat ambiguous. In OSCC, there is no significant inverse correlation between these factors.(194) This raises the possibility that sip1 has functions other than downregulation of E-cadherin. Sip1 expression causes also downregulation of other major constituents of tight junctions, adherens junctions, desmosomes and gap junctions at the transcriptional level.(180) In squamous cell carcinoma, the presence of sip1 also protects the cells from DNA-damage-induced apoptosis.(27) Different levels of regulation influence the spatio-temporal expression of sip1 protein which may point to an ability for this protein to play diverse roles in different contexts.(18) There are several studies investigating sip1's role in the development of multiple cancers.(27,195,238) However, as far as is known no previous studies have examined the role of sip1 in PSCC.

Sip1 expression in human tissues and tumors varies considerably and both nuclear and cytoplasmic expressions have been described in various sites.(27) In the present study, sip1 was expressed abundantly in both tumor epithelial cell nuclei and cytoplasm. There was also profuse stromal and endothelial staining in these tissue samples. Sip1 has been previously detected in tumor cell nuclei, stromal fibroblasts or cytoplasm in 28% of oral squamous cell carcinoma samples (34) and in cell nuclei of 40% of head and neck spindle

cell carcinoma samples.(239) Thus, the present results are in line with these previous findings. Snail has shown to be very unstable and thus only nuclear protein is again considered to be active.(145) The half life of sip1 has not been reported. Cytoplasmic expression of sip1 was as abundant as nuclear staining but the expression did not correlate with tumor progression or survival. Therefore, these results support the view that only nuclear expression of sip1 is significant.

This is the first study to demonstrate, that sip1 alone and together with other transcription factors can enhance tumor progression in PSCC. Epithelial expression of sip1 in the nuclei of carcinoma cells was associated with advanced stage and higher lymph node status. Sip1 expression also had a major impact on the patient's DSS. This may be partly caused by advanced stage and lymph node metastases but epithelial expression of sip1 remained an independent prognostic factor also in the Cox proportional hazards model, together with KPS and T-class. A similar association between increased sip1 expression and decreased survival has been earlier shown in oral SCC.(34) Furthermore, sip1 has a negative effect on survival in urothelial and non-small cell lung cancer.(27,195) This implicates sip1 as a potential marker of aggressiveness of such carcinomas. In the present material, sip1 expression was increased in less-differentiated carcinomas, a phenomenon also found in oral SCC by Maeda et al.(34) Histological grading alone had no effect on survival in neither of the analyses. Thus, it is possible that sip1 directly enhances tumor progression and metastases independently of cellular differentiation.

In the present study, the stromal nuclear expression of sip1 correlated with elevated risk of tumor relapse. In oral SCC, sip1 overexpression predicted a delayed appearance of neck metastases. (194) It has been proposed that snail reflects early EMT alterations and other transcription factors, like sip1, could be responsible for maintenance of the migratory cell behavior.(112) Accordingly, the immunostained stromal cells might, at least partially, represent the transformed tumor cells that have undergone EMT. These motile cells would thus be capable of invading adjacent tissues and vessels to promote metastases and recurrences. On the other hand, non-neoplastic stromal fibroblasts may express transcription factors by interacting with adjacent epithelial cancer cells. The significance of stromal tissue in tumor progression has been studied and emphasized in recent years.(109,124,127,130,240) In this way, these modified stromal fibroblasts may be not only enablers, but even potential inducers of malignancies.

Sip1 and slug expression was more frequent in hypopharyngeal than in oropharyngeal tumors as also established with twist. It still remains unclear whether this is due to the more advanced disease stage at the time of diagnosis or whether it represents a true feature of the hypopharyngeal tumors. There are different expression patterns of EMT related transcription factors in carcinomas of various origins.(157,193) On the other hand, transcription factors facilitate tumor growth by triggering EMT, inhibiting apoptosis and enhancing angiogenesis and thus their expression often reflects advanced tumors.(19,27,233)

Even though slug has been associated with cancer progression in colorectal carcinoma and esophageal SCC,(30,165) in the present study, the expression of slug did not correlate with prognosis. Furthermore in another study investigating oral SCC, slug expression failed to display any association with clinicopathological variables and survival.(38) This again implies that there are different roles of EMT related transcription factors in distinct tumors. In a very recent study with HNC cell lines, there were poor survival rates when both zeb1 and sip1 were highly expressed.(213) As far as is known there are no other previous studies about zeb1 in head and neck carcinoma. However, in the current study, there was no correlation between zeb1 expression and clinicopathological variables or survival.

6.2.5 Co-expression of EMT-RTFs

The present study revealed a moderate co-expression of all the transcription factors in tumor epithelium and the expression of *snai1* and *slug* correlated clearly, possibly due to their common origins and structure. The stromal expressions of all five transcription factors were strongly associated to each other. A strong correlation between the EMT-RTFs has been shown also in ovarian carcinoma.(135) In order to analyze previously published data about the correlations of gene expressions of the EMT related transcription factors the GeneSapiens database was utilized (<http://www.gene.sapiens.org>). It was possible to detect 34 analyses in head and neck carcinoma revealing a significant positive correlation between the expression levels of *sip1* and *slug* ($p=0.047$), *zeb1* and *twist* ($p<0.001$) and a clear trend suggestive of a correlation between *snai1* and *sip1* ($p=0.056$). No correlation was seen between the transcription factors in normal oral or pharyngeal tissue samples implying that the cooperation between the transcription factors takes place particularly in malignant tissue, as also reported by Kilpinen et al.(241) During embryogenesis, many EMT related transcription factors are often activated simultaneously.(198) However, in distinct tumors these transcription factors also seem to work separately. There is also a certain hierarchy between the factors, for example, *twist* needs direct induction from *slug* to induce EMT.(198) In the diffuse type of gastric carcinomas, *slug*, *snai1* and *sip1* seem to complement each another.(242)

In the present study, the tumors that did not express either *twist* or *snai1* in stroma were smaller, of lower stage and were more often situated in the oropharynx. Those tumors also had a significantly better 5-year DSS. On the contrary, positive *snai1* and *twist* stromal expression associated with larger tumor size and more advanced stage. Other previous studies with *twist* and *snai1* also support the hypothesis that these two transcription factors act in collaboration to promote EMT and tumor progression, even though they are regulated independently.(35,36,169) In the present material, tumors which expressed simultaneously three transcription factors, *snai1*, *twist* and *sip1*, in the epithelial nuclei were larger and more advanced. The prognosis of the patients found with these tumors was dismal the mean DSS being only 12 months. The result was significant also in the multivariate analysis together with T-class and KPS. These results reveal a remarkable propensity of the transcription factors to co-operate and intensify one another's function in the present PSCC cohort. It is evident that elevated nuclear expression of transcription factors is indicative of ongoing EMT. There is growing evidence that *snai1* could be an early marker of EMT while other EMT-RTFs may be responsible for the maintenance of tumorigenic properties.(112) TGF β is a key molecule that might promote the interplay between the transcription factors during EMT.(112) However, hypoxia is also thought to be a significant factor co-ordinating the interplay which possibly relates to the observation of accumulation of EMT-RTFs in association with tumor growth.(112,169) Nevertheless, the inductive cooperative potential of the transcription factors in cancer development and progression seems to be even greater than simply the sum of their individual effects.

In the current study a subgroup was selected where *snai1*, *twist*, *sip1* and *slug* were all co-expressed in tumor epithelium. However, the number of positive cases remained too small to permit any reliable statistical analyses.

6.3 Clinical implications

The expressions of EMT-RTFs, especially *sip1*, seem to have significant prognostic value in PSCC. Immunohistochemical staining is a relatively fast, cheap and simple process highlighting the possibility that it could be potential prognostic marker also for clinical use. Detecting *sip1* expression in PSCC could help us to select those patients who would benefit from more aggressive treatment modalities and to follow-up them more carefully for recurrences and second primaries.

Furthermore, activation of EGF-receptors has been shown to induce EMT.(243,244) At present, EGFR inhibitors are the only targetted therapy approved for the treatment of HNSCC; this drug selectively acts against epithelial cells and thus tumor cells which have gone through EMT are protected from this chemotherapeutic agent.(132,244) EMT also promotes DNA repair and suppresses radiation-induced apoptosis. Thus, the inhibition of the EMT process could make tumors more sensitive to both radiation and chemotherapy. Moreover, the inhibition of EMT process might also restrict tumor invasion and metastasizing. EMT and its transcription factors in addition to its epigenetic regulation as well as the role of numerous miRs which can influence EMT may create considerable number of potential targets for new cancer therapeutics in the future by for example silencing oncogenes or forced-expression of tumor suppressor genes. One major challenge will be to identify those agents that selectively target mesenchymal cells and/or impact the EMT process reversing the cellular alteration back towards the epithelial phenotype.

7 Summary and conclusions

The prognosis of PSCC has remained poor, poorest of all HNCs, in spite of advances in diagnostics and treatment. Prognosis is still based almost solely by TNM-classification and histological grading. New biomarkers are needed to select the patients with aggressive tumors and poor survival prospects in order to target the treatment and follow-up resources more effectively. The EMT process and its transcription factors provide several possibilities for assessing tumor behavior.

Based on the results the following conclusions could be drawn:

1. T-class and general condition of the patient were the most important prognostic factors. Histological grade offered no prognostic value.
2. Endothelial nuclear expression of *snai1* predicted lower DSS. *Snai1* expression in other cell nuclei or cytoplasm had no statistically significant effect on survival.
3. Twist expression alone displayed no correlation with survival. Simultaneously, lack of both *snai1* and twist expression in tumor stromal cell nuclei predicted better 5-years DSS.
4. Sip1 expression in tumor epithelial cell nuclei was an independent prognostic factor in PSCC. Furthermore, stromal nuclear expression of sip1 predicted poorer DSS. The expression of slug or zeb1 did not associate with survival.
5. Co-expression of *snai1*, twist and sip1 in tumor epithelial nuclei is an independent prognostic factor for poor survival in PSCC. The absence of stromal nuclear co-expression of the three transcription factors was also significantly associated with better survival.

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ANNA JOUPPILA -MÄTTÖ
*Epithelial-mesenchymal
Transition and Expression of
Its Transcription Factors in
Pharyngeal Squamous Cell
Carcinoma*



Epithelial-mesenchymal transition (EMT) is a complex cellular process which is activated in tumorigenesis and enables tumor cells to invade and metastasize. It is regulated by several transcription factors i.e. *snai1*, *twist*, *sip1*, *slug* and *zeb1*. In the present retrospective study we evaluated the expression of these transcription factors in pharyngeal squamous cell carcinoma. The expression of *sip1* in carcinoma cells and tumor stroma associated with advanced tumor stage and predicted poorer DSS.



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