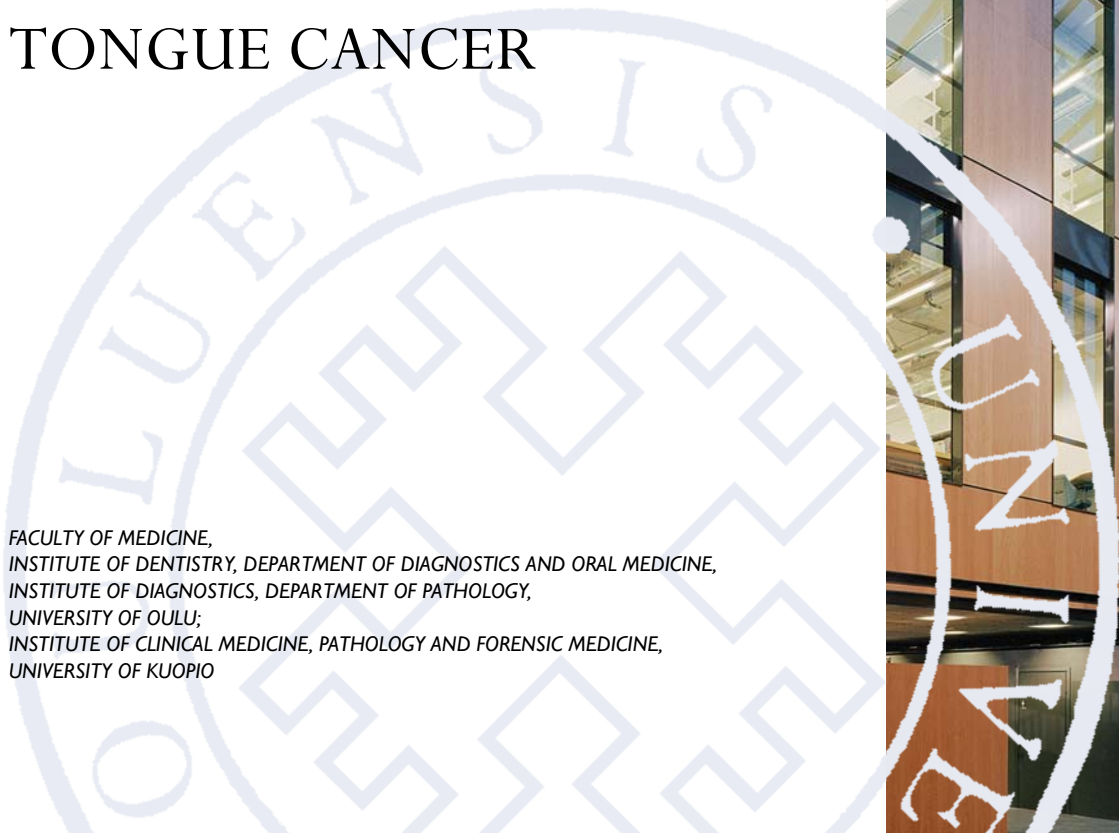


*Ibrahim O. Bello*

TIGHT JUNCTION PROTEINS  
AND CANCER-ASSOCIATED  
FIBROBLASTS IN AMELO-  
BLASTOMA, AMELOBLASTIC  
CARCINOMA AND MOBILE  
TONGUE CANCER

FACULTY OF MEDICINE,  
INSTITUTE OF DENTISTRY, DEPARTMENT OF DIAGNOSTICS AND ORAL MEDICINE,  
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UNIVERSITY OF KUOPIO





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*IBRAHIM O. BELLO*

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CANCER**

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**Bello, Ibrahim O., Tight junction proteins and cancer-associated fibroblasts in ameloblastoma, ameloblastic carcinoma and mobile tongue cancer.**

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***Abstract***

Squamous cell carcinoma (SCC) of the mobile tongue is the most common type of cancer of the oral cavity, accounting for 30-40% of oral cancers. It behaves aggressively and almost half of the affected patients still die of the disease despite great advances in its medical and surgical care. Ameloblastomas are the most common clinically significant type of odontogenic tumors, constituting approximately 1% of all cysts and tumors of the jaw. They are benign but locally invasive tumors with a strong tendency to recur after surgery. Ameloblastic carcinoma combines the histological features of ameloblastoma with cytologic atypia irrespective of the presence or absence of metastasis.

The effectiveness of tight junction proteins (claudins 1, 4, 5, 7 and occludin) and cancer-associated fibroblasts (CAFs) as prognostic markers in OTSCC and as markers of malignancy in ameloblastomas was studied. Abundance of CAFs and Claudin 7 derangement was found to be associated with poor disease-specific survival in oral (mobile) tongue cancer. Appearance of CAFs within the epithelial islands of ameloblastoma was found to be a marker of malignancy in the tumor. The prognostic predictability of CAF density, Ki-67 (cell proliferation marker), maspin (tumor suppressor marker) and tumor DNA content (tumor ploidy using image cytometry) in tongue cancers was also tested. CAF density was the only marker strongly predictive of prognosis. In ameloblastomas,  $\alpha$ -SMA (for CAFs), Ki-67, epithelial membrane antigen (EMA) and DNA content (using image and flow cytometry) were assessed as markers of ameloblastic carcinoma. Only  $\alpha$ -SMA was able to predict ameloblastic carcinoma when found in the epithelial islands. In conclusion, staining for  $\alpha$ -SMA and claudin 7 seems to be beneficial for prognostication in tongue cancer, while  $\alpha$ -SMA staining may be beneficial in differentiating ameloblastoma from ameloblastic carcinoma.

***Keywords:*** ameloblastic carcinoma, ameloblastoma, cancer-associated fibroblasts, prognosis, tight junction proteins, tongue cancer



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Oulu, November 2009

Ibrahim Olajide Bello

## Abbreviations

AEC	3-amino-9-ethylcarbazol
AI	apoptotic index
AJ	adherens junction
BCL2	B-cell lymphoma/leukemia 2
(b)FGF	(basic) fibroblast growth factor
CAF	carcinoma-associated fibroblast
CA-9	carbonic anhydrase-9
CAR	coxscakie virus and adenovirus receptor
CI	confidence interval
CT	computed tomography
CTGF	connective tissue growth factor
CXCL	chemokine, CXC motif, ligand
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EGF(R)	epidermal growth factor (receptor)
EMA	epithelial membrane antigen
EMT	epithelial-mesenchymal transition
endMT	endothelial-messenchymal transition
FA	focal adhesion
FAP	fibroblast-activated protein
FCM	flow cytometry
FNAC	fine needle aspiration cytology
<i>FOS</i>	FBJ murine osteosarcoma viral oncogene homolog
FSP-1	fibroblast specific protein-1
GJ	gap junction
GLUT	glucose transporter
HA	hyaluronan
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
HR	hazard ratio
HSC-3	human tongue squamous cell carcinoma cell line
IARC	International Agency for Research on Cancer
ICM	image cytometry

IGF	insulin-like growth factor
JAM	junctional adhesion molecule
Ki-67	antigen identified by monoclonal antibody Ki-67
KOT	keratocystic odontogenic tumor
LI	labeling index
LML	log minus log
Maspin	<i>mammary serine protease inhibitor</i>
MET	mesenchymal-epithelial transition
MF	myofibroblast
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MVD	microvascular density
NG2	neuron-glia antigen-2
OSCC	oral squamous cell carcinoma
OTSCC	oral (mobile or anterior) tongue squamous cell carcinoma
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PET	positron emission tomography
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFRP1	secreted frizzled-related protein 1
<i>Snail</i>	zinc finger phosphoprotein
SPARC	secreted protein, acidic, rich in cysteine (osteonectin)
SPSS	Statistical Package for the Social Sciences
TGF- $\beta$	transforming growth factor-beta
TJ	tight junction
TNF (R)	tissue necrosis factor (receptor)
TNFR1A	tissue necrosis factor receptor superfamily, member 1A
TNM	tumor, node, metastasis
TSN	tobacco-specific nitrosamine
WHO	World Health Organization
VEGF	vascular endothelial growth factor
ZO	zonula occludens
$\alpha$ -SMA	alpha-smooth muscle actin

## List of original publications

This thesis is based on the following articles which are referred to in the text by their roman numerals

- I Bello IO, Soini Y, Slootweg PJ & Salo T (2007) Claudins 1, 4, 5, 7 and occludin in ameloblastomas and developing human teeth. *J Oral Pathol Med* 36: 48–54.
- II Bello IO, Vilen S-T, Niinimaa A, Kantola S, Soini Y & Salo T (2008) Expression of claudins 1, 4, 5, 7 and occludin and relationship with prognosis in squamous cell carcinoma of the tongue. *Hum Pathol* 39: 1212–1220.
- III Bello IO, Alanen K, Slootweg PJ & Salo T (2009) Alpha-smooth muscle actin within epithelial islands is predictive of ameloblastic carcinoma. *Oral Oncol* 45: 760–765
- IV Bello IO, Vered M, Dobriyan A, Yahalom R, Alanen K, Nieminen P, Kantola S, Läärä E, Dayan D & Salo T (2009) Increased density of carcinoma-associated fibroblasts strongly predicts poor prognosis in mobile tongue cancer. Manuscript.



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# 1 Introduction

Squamous cell carcinoma of the oral (mobile) tongue (OTSCC) is associated with a fairly unpredictable clinical course. It accounts for the largest share of all oral cancers and is particularly aggressive mainly because of its high propensity for metastasizing to the regional lymph nodes (Silver & Moisa 1991; Yasumatsu *et al.* 2001). A relatively high proportion of such metastases is undetectable at the time of presentation (van den Brekel *et al.* 1998). Clinical staging using TNM classification has long been used as the standard tool for treatment planning and predicting the prognosis of the disease. However, this staging method does not give sufficient predictive information for optimal treatment that will be beneficial for the individual patient (Högmo *et al.* 1999). There has therefore been a continuous search over the years for other prognostic markers that may have more reliable predictive potential.

Ameloblastoma is regarded as the most clinically significant tumor of odontogenic origin since it is locally aggressive and has a very high recurrence rate after inadequate or conservative treatment (Ghandhi *et al.* 2006). The direct malignant counterpart is ameloblastic carcinoma, which is associated with a poor prognosis (Dhir *et al.* 2003).

In this study, the prognostic predictability potential of tight junction proteins (claudins 1, 4, 5, 7 and occludin) and cancer-associated fibroblasts (CAFs) in OTSCC and as markers of malignancy in ameloblastomas was examined. The effectiveness of CAF density, Ki-67 (cell proliferation marker), DNA content and maspin (serine protease inhibitor and tumor repressor marker) as prognostic markers in OTSCC was compared. The effectiveness of CAF density, Ki-67, DNA content and epithelial membrane (EMA) antigen in differentiating between benign ameloblastoma and ameloblastic carcinoma was also evaluated.



## **2 Review of the literature**

### **2.1 Oral squamous cell carcinoma**

Oral cancer ranks as the eighth most common cancer worldwide, although it shows epidemiologic variations between geographic regions (Petersen 2003). Apart from the perennially high incidence rates in south-central Asia, where it ranks among the three most common cancers, sharp increases have also been reported in many countries in Europe, Australia and the USA (Steward & Kleihues 2003). At least 90% of all malignant neoplasms in the oral cavity are squamous cell carcinoma (SCC), representing about 5% of all cancers in men and 2% in women worldwide (Parkin *et al.* 2003). In the USA, as many as 43% of patients have regionally spread disease at the time of diagnosis, in addition to a further 9% presenting with distant metastasis, thereby resulting in overall poor prognosis (CDC 1998). Overall mortality still remains as high as 50% despite great advances in management (Walker *et al.* 2003).

The highest age-standardized incidence rates are found in India and Thailand (Petersen 2003). France, the French-speaking part of Switzerland, Northern and Central Europe and some parts of Latin America have overly high rates amongst men (Barnes *et al.* 2005). Males are affected more often than females although there has been a gradual increase in women affected over the long term due to increased smoking. In the USA, the male to female ratio decreased from 6:1 to 2:1 in less than 50 years (Silverman 1998). Women in India have had a higher incidence over time because of heavy chewing of tobacco (Barnes *et al.* 2005). More than 90% of cases occur in people who are older than 40 years, the average age being 60 years (Silverman 1998). In India, the peak age is at least one decade earlier than that reported for Western countries (Parkin *et al.* 1993). Moreover, there has been a relatively steep increase in the number of younger subjects affected by the disease in the USA and UK in recent decades (Llewellyn *et al.* 2003, Schantz & Yu 2002).

#### **2.1.1 Incidence of oral (mobile) tongue squamous cell carcinoma (OTSCC)**

The most common site affected by SCC is the tongue, representing between 25–40% of intraoral carcinomas (Regezi *et al.* 2008). Most of tongue SCCs are found

in the anterior two thirds (mobile or oral tongue) where they display a great propensity for metastasis even at the early (T1-T2) stages, accounting for the relatively high rate of treatment failures (Silver & Moisa 1991). OTSCC exhibits neck node metastasis more than any other carcinoma, and at the time of presentation, approximately 40% of patients have neck metastasis and 40% of stage 2 lesions show occult metastasis (Byers *et al.* 1997, Leipzig *et al.* 1982). The Finnish Cancer Registry reported the age adjusted-incidence of tongue cancer in the period 1999–2003 of 1.5 per 100,000 person-years in men and 0.8 per 100,000 person-years in women in Finland (Finnish Cancer Registry 2007a). The corresponding numbers in 2007 were 1.6 per 100,000 person-years for men and 1.0 per 100,000 person-years for women, suggesting a slight shift in incidence towards women (Finnish Cancer Registry 2007b)

### **2.1.2 Etiology**

OTSCC appears to share the same risk factors as all oral cancers. The two most well-documented factors are tobacco use and alcohol, which together account for about three-fourths of all cases in Europe, America and Japan (Barnes *et al.* 2005). Although both are now considered to be independent risk factors, they display a highly synergistic effect when used together over a long period (Blot *et al.* 1988, Lewin *et al.* 1998).

The most common form of tobacco use is cigarette smoking. There is a strong dose-response relationship between smoking and development of oral cancer (Lewin *et al.* 1998). Pipes and cigars have also been associated with oral cancers (Franceschi *et al.* 1990), although some earlier studies have suggested that these practices carry a lesser risk compared to cigarette smoking (Wynder *et al.* 1977). Smokeless tobacco used in the form of moist or dry snuff or chewing tobacco is used in South-East Asia. In Scandinavia and the USA, smokeless tobacco is mostly used as snuff where studies have shown that it does not increase the incidence of oral cancer (Bouquot & Meckstroth 1998, Rosenquist 2005, Schildt *et al.* 1998). This latter finding has been disputed by other investigators who showed that smokeless tobacco is associated with increased risk of developing oral cancers in users (Roosaar *et al.* 2008, Winn *et al.* 1981). In a recent systematic review of the effect of smokeless tobacco and oral cancer by Colilla (2009), it was pointed out that the conflicting result by different workers is a reflection of the problems in the study designs. In the hospital case control studies, results are generally not applicable to the general population and

adjustment for concurrent alcohol intake may not be made; in population-based studies, the samples are either too small or adjustment for use of alcohol and tobacco made through proxy reports; and in the cohort studies various flaws were identified, including assessment of smokeless tobacco use at baseline only and that subjects chosen may not truly reflect the health status of the general population (Colilla 2009).

In parts of Asia, Papua New Guinea, the Indian subcontinent and parts of North Africa, smokeless tobacco is packaged mixed with other ingredients such as lime, areca nut, betel leaf and slaked lime, ash, and sodium bicarbonate to form chewable preparations known by different names in the localities where they are prepared (Gupta *et al.* 1996). A recent study has confirmed that chewing tobacco of this type carries a far greater risk than smoking tobacco alone without these additives, although the risk increases even more when both practices are done together (Muwonge *et al.* 2008).

Many carcinogens have been identified in tobacco smoke or the water soluble components dissolved in the saliva, but the most studied of these are the polycyclic aromatic hydrocarbon benz-pyrenes present in tars and tobacco-specific nitrosamines (TSNs): nitrosonornicotine, nitrosopyrrolodine and nitrosodimethylamine. TSNs have been suggested to act locally on keratinocytes stem cells and systemically by being absorbed and producing DNA adducts, such as O<sup>6</sup> methyl guanine, which cause damage to replicating cells (Hoffmann & Hecht 1985).

Since the early 1970s, alcohol has been suggested to be an even more important risk factor in intraoral cancer in younger male subjects than tobacco (Hindle *et al.* 2000). An independent carcinogenic effect of alcohol in cancers of the upper aerodigestive tract has been reported since 1961 (Reviewed by Boffetta & Hashibe 2006). Since then many studies have confirmed that in non-smokers who use large quantities of alcohol, the risk of developing oral cancer becomes elevated (Fioretti *et al.* 1999, Ng *et al.* 1993). Various mechanisms of action have been suggested for the carcinogenesis of alcohol in oral cancers, including DNA damage by acetaldehyde (the primary metabolite of alcohol), acting as a solvent, and increasing the permeability to carcinogens e.g. from tobacco at mucosal sites (Wight & Ogden 1998). It can also act as a harbinger of nutritional deficiencies because of its high caloric content and suppression of appetite (Harris *et al.* 1997). The high risk sites for intraoral alcohol carcinogenesis have been suggested to be the mobile tongue and hypopharynx (IARC 1988). Other investigators have suggested that the floor of the mouth carries a higher risk in

those who smoke tobacco in addition to heavy drinking (Franceschi *et al.* 1992, Jovanovic *et al.* 1993).

Some other risk factors have been mentioned in the past, although the evidence supporting them has not been consistent. Dietary intake of food rich in vegetables, fruit, vitamins and fiber has been shown to have a protective effect against oral cancer (Block *et al.* 1992, De Stefani *et al.* 2005). Anti-oxidants contained in fruits and vegetables such as beta-carotene, beta-cryptoxanthin, and vitamins A, C and E, are scavengers for free radicals from damaged cells and are said to offer some protective effect against oral and pharyngeal cancer (Boeing *et al.* 2006, De Stefani *et al.* 2000, Zheng *et al.* 1993). This reduction has been suggested to be more evident in the tongue, mouth and pharynx (McLaughlin *et al.* 1988). Intake of vegetables and fruits has also been found to be beneficial to patients who already have oral cancer as it reduces recurrence and improves survival (Sandoval *et al.* 2009).

Poor oral hygiene has been associated with oral cancer, usually with the caveat that most of the subjects in this category have other risk factors (Zheng *et al.* 1990). In heavy drinkers, poor oral hygiene may contribute a two-fold increase to acetaldehyde production from ethanol in the saliva (Homann *et al.* 2001). A recent study seemed to suggest that poor oral hygiene may be considered an independent risk factor (Conway 2009).

The relationship of trauma to oral cancer has also been explored. Irritation of the mouth, such as from unsatisfactory dental prosthesis and oral mouthwashes containing relatively large amounts of alcohol, has been suggested as a risk factor in tongue and oral cancer (Conway 2009, Velly *et al.* 1998). Dental prostheses do not increase the risk except when causing chronic ulceration or when associated with other risk factors (Velly *et al.* 1998).

There is some evidence that human papilloma virus (HPV) may play some role in tongue cancer (Dahlgren *et al.* 2004, Mork *et al.* 2001). It has also been suggested as a probable cause for the increased incidence and onset of head and neck cancer in younger population (Scully 2002). However, the role of HPV in OTSCC has been disputed by a recent study (Liang *et al.* 2008). The high-risk types are HPV 16 and 18. The main mechanism of action of these viruses is by inserting specific DNA fragments into the host cellular genome, leading to the inactivation of cellular tumor suppressor proteins, retinoblastoma (Rb) and p53, thereby removing the checkpoint that controls the cell cycle by arresting cells in G0–G1 and allowing cells to proliferate indefinitely (Talbot & Crawford 2004). However, HPVs are more commonly found in the base of tongue lesions than

mobile tongue where they are even suggested to improve patient survival (Dahlgren *et al.* 2004, Pintos *et al.* 2008). Their role in oral carcinogenesis remains questionable and detection methods will need to be improved (Campisi & Giovannelli 2009, Liang *et al.* 2008).

## **2.2 Prognostic factors in OTSCC**

The most important negative prognostic factor for OTSCC is the high incidence of neck nodal metastasis (Chen *et al.* 2008). At the time of diagnosis, more than 40% of patients already have regional spread of disease (CDC 1998). Multiple cervical micrometastases are common even in the early-stage tongue cancers, with cT1NO and cT2NO tumors showing figures of 36% and 58% respectively (Yoshida *et al.* 2005, Yuen *et al.* 1999). Recurrence (local and regional) is also very common in treated patients, the majority of them occurring within a year after treatment (Franceschi *et al.* 1993).

### **2.2.1 Clinical prognostic factors**

#### *Socio- demographic factors*

Socio-demographic factors are generally thought to be of weak prognostic value in all types of oral cancers (Woolgar 2006). Moreover, the studies on these factors are often contradictory in their conclusions. There is no agreement in literature about the prognostic value of age in patients with OTSCC. Matched-pair analysis of patients older or younger than 40 years showed that younger patients have increased frequency of tumor recurrence, distant metastases and cancer-related deaths compared with older patients (Garavello *et al.* 2007, Hyam *et al.* 2003, Liao *et al.* 2006). Patients who present at an age over 60 years tend to be associated with poorer prognosis than those who are younger (Kantola *et al.* 2000). Several other studies have also confirmed that younger age is associated with better survival (Annertz *et al.* 2002, Atula *et al.* 1996, Davidson *et al.* 2001). Some investigators found no difference between the young and the older age groups in terms of prognosis (Pitman *et al.* 2000, Siegelmann-Danieli *et al.* 1998, Veness *et al.* 2003). One study suggested that there are two distinct patterns in young patients: an indolent form with freedom from disease for over 15 years and

an aggressive type associated with up to 40% mortality within 2 years (Popovtzer *et al.* 2004).

Some studies have shown that relative survival rates in men are lower than in women with OTSCC (Berrino & Gatta 1998, Dickman *et al.* 1999, Zheng *et al.* 1999), while some others have found no such association (Mathew Iype *et al.* 2001). Shiboski and co-workers reported significant mortality in the black (African-American) adult population compared with whites, mainly because they had a higher proportion of tongue cancer and presented more with late-stage disease than whites (Shiboski *et al.* 2007). It was suggested that whites have better access to and utilization of healthcare facilities. Nichols & Bhattacharyya (2007) in the USA found that blacks with OTSCC have slightly lower mean overall and disease-specific survival when compared with matched white population with OTSCC, in addition to having significantly higher T stage and N stage at the time of presentation. However, there was no statistically significant difference in either overall or disease-specific survival.

In people under 65 years old, survival rates fell from 47% to 39% between 1968 and 1987 in Scotland, with the highest increase recorded among subjects from the more socially deprived areas (Macfarlane *et al.* 1996). Although more important in buccal mucosa and gingiva than the tongue, betel quid use has also been associated with decreased survival (Lo *et al.* 2003). Smoking and chewing tobacco was found to have a significant adverse effect on survival in a population where alcohol use is relatively uncommon (El-Husseiny *et al.* 2000). Alcohol usage was also found to be significantly associated with decreased survival in patients with stage III-IV tongue carcinomas (Kantola *et al.* 2000)

### *Clinical stage*

The TNM staging of tumors has been used for many decades in the prognostication of cancers of the oral cavity, including tongue cancers, and has recently been updated (Sobin & Wittekind 2002). It seems particularly useful in prediction of prognosis of later-stage cancers (Kantola *et al.* 2000, Silveira *et al.* 2007). However, it is known that early-stage tongue cancers have a high propensity for occult locoregional metastases in which TNM staging may not accurately predict prognosis (Yoshida *et al.* 2005). In order to improve the sensitivity of clinical staging, fine-needle aspiration cytology (FNAC), computed tomography (CT), magnetic resonance imaging (MRI), ultrasonography and positron emission tomography (PET) are continually being used to help in

detection of cervical lymph node metastasis (Sano & Myers 2007). Despite all these advances in imaging techniques, almost a quarter of micrometastases would still go undetected (van den Brekel *et al.* 1998). More recently, many workers have used genetically based methods such as molecular (gene expression profiling) signatures to predict cervical lymph node metastasis in OSCC and HNSCC (Colella *et al.* 2008, Nguyen *et al.* 2007, O'Donnell *et al.* 2005, Roepman *et al.* 2005). Some have reported the effectiveness of these methods to be superior to conventional diagnostic methods (Roepman *et al.* 2005). These methods have not been widely used because they have not yet been validated by large multicenter studies. Successful primary treatment does not exclude the appearance of cervical nodal metastases, either (Nakagawa *et al.* 2003).

### *Tumor size*

Tumor size includes diameter, width, area, volume and depth. The TNM system takes the tumor diameter into consideration in staging of the tumor. However, many studies have consistently confirmed that of all these parameters in tumor size measurements, tumor depth seems to be the only independent prognostic factor that adversely affects lymph node metastasis, local recurrence and survival rate in OSCC (Asakage *et al.* 1998, Brown *et al.* 1989, Jung *et al.* 2009; Yuen *et al.* 2002). However, there is no agreement yet on the standard value of depth that predicts poor prognosis for the patient between these studies or a preoperative study to measure tumor invasion directly (Jung *et al.* 2009). The value of tumor depth as a guide to treatment is particularly important in T1/T2 tumors, and a more aggressive treatment may be advocated in cases where the depth has reached a certain cut-off value (Asakage *et al.* 1998). Preoperative documentation of tumor thickness is almost impossible unless done during surgical operation when surgical block is prepared and therefore a decision on the management of the neck will need to wait for the surgical pathology report. Jung *et al.* (2009) have advocated the use of MRI for determining the tumor depth preoperatively.

### **2.2.2 Histopathologic prognostic factors**

Various histopathologic parameters are routinely considered as potential prognostic factors in mobile tongue cancer. They include tumor grade, lymphovascular invasion, perineural invasion, tumor angiogenesis, malignancy score and apoptosis.

**Table 1. A list of some well-known histopathologic and molecular markers as prognostic factors in OTSCC.**

Prognostic factors	References
Tumor grade* (?)	Al-Rajhi <i>et al.</i> 2000, O-charoenrat <i>et al.</i> 2003, Okamoto <i>et al.</i> 2002, Woolgar, 2006
Malignancy grading (score)* (+)	Anneroth <i>et al.</i> 1987, Bryne <i>et al.</i> 1989, Högmo <i>et al.</i> 1999, Kantola <i>et al.</i> 2000, Kurokawa <i>et al.</i> 2005, Odell <i>et al.</i> 1994, Silveira <i>et al.</i> 2007, Woolgar & Scott 1995, Weijers <i>et al.</i> 2009, Yuen <i>et al.</i> 2002
Lymphovascular invasion* (+)	Brown <i>et al.</i> 1989, Chen <i>et al.</i> 2008, Hosal <i>et al.</i> 1998, Myers <i>et al.</i> 2000, Silva <i>et al.</i> 2008b, Woolgar & Scott 1995
Perineural invasion* (++)	Brown <i>et al.</i> 1989, Chen <i>et al.</i> 2008, Hosal <i>et al.</i> 1998, Myers <i>et al.</i> 2000, Silva <i>et al.</i> 2008b, Sparano <i>et al.</i> 2004, Woolgar & Scott 1995
Apoptosis* (?)	Atula <i>et al.</i> 1996, de Vicente <i>et al.</i> 2006, Xie <i>et al.</i> 2004, Yao <i>et al.</i> 1999
Tumor angiogenesis* (?)	Cho <i>et al.</i> 2007, Chuang <i>et al.</i> 2006, Faustino <i>et al.</i> 2008, Fernandez <i>et al.</i> 2007, Högmo <i>et al.</i> 1999, Kim <i>et al.</i> 2006, Mineta <i>et al.</i> 2002, Shpitzer <i>et al.</i> 1996
Proliferative cell markers* (?)	Davies <i>et al.</i> 2006, Bova <i>et al.</i> 1999, Silva <i>et al.</i> 2008a, Wangsa <i>et al.</i> 2008
Stromal myofibroblasts* (+?)	Kellermann <i>et al.</i> 2007
Tight junction proteins* (?)	
Nuclear DNA content* (?)	Cooke <i>et al.</i> 1994, Hemmer & Kreidler 1990, Högmo <i>et al.</i> 1999, Saito <i>et al.</i> 1994, Wangsa <i>et al.</i> 2008
Inflammatory response (?)	Sarioglu <i>et al.</i> 1994
Other molecular markers	
1. EGFR family, cyclins B1, D1, ErbB-2 (HER-2), (+?)	Fujii <i>et al.</i> 2001, Goto <i>et al.</i> 2002, Harada <i>et al.</i> 2006, Katoh <i>et al.</i> 2002, Kwong <i>et al.</i> 2005, Lim <i>et al.</i> 2004, Mineta <i>et al.</i> 2000, Nagler <i>et al.</i> 2002, Ryott <i>et al.</i> 2009, Silva <i>et al.</i> 2008a, Ulanovski <i>et al.</i> 2004
2. Tumor suppression markers: TP53, p16 <sup>INK4A</sup> , p14 <sup>ARF</sup> , p21, pRb, p27, maspin* (+)	Bova <i>et al.</i> 1999, Cho <i>et al.</i> 2007, Goto <i>et al.</i> 2005, Keum <i>et al.</i> 2006, Kwong <i>et al.</i> 2005, Mineta <i>et al.</i> 1999, Xie <i>et al.</i> 2002, Yasumatsu <i>et al.</i> 2001, Yuen <i>et al.</i> 2001
3. Matrix metalloproteinases (MMPs) (+)	Kawano & Yanagisawa 2006, Kim <i>et al.</i> 2006, Korpi <i>et al.</i> 2008, Kosunen <i>et al.</i> 2007, Nyberg <i>et al.</i> 2002, Yoshizaki <i>et al.</i> 2001
4. Adhesion-related factors: E-cadherin, CD44, versican, hyaluronan (HA) and catenins (+)	Chang <i>et al.</i> 2002, Chow <i>et al.</i> 2001, Kosunen <i>et al.</i> 2007, Kosunen <i>et al.</i> 2004, Li <i>et al.</i> 2009, Lim <i>et al.</i> 2004, Menezes <i>et al.</i> 2007, Narkio-Makela <i>et al.</i> 2009, Okamoto <i>et al.</i> 2002, Pukkila <i>et al.</i> 2007
5. Hypoxia markers: Hypoxia-inducible factor (HIF-1 and -2), carbonic anhydrase (CA)-9, glucose transporter (GLUT)-1, erythropoietin receptor. (?)	Kim <i>et al.</i> 2007, Roh <i>et al.</i> 2009

\* The variable is reviewed in this section. ? Role in prognosis is questionable or yet to be proven in OTSCC. + Some or unequivocal role in prognosis. ++ Relatively important role in prognosis.

## *Tumor grade*

Histological grading of oral SCC has been based on the WHO classification of tumors which utilizes the degree of keratinization, cellular and nuclear pleomorphism and mitotic activity in dividing them into 3 categories viz. (Pindborg *et al.* 1997):

Grade 1 (Well-differentiated): Histological and cytological features bear a close resemblance to normal squamous epithelial lining of the oral mucosa with keratinization of cells common, few mitotic figures with absent or rare atypical mitosis. Nuclear and cellular pleomorphism or multinucleated epithelial cells are rarely seen.

Grade 2 (Moderately differentiated): Less keratinization, more mitotic figures with a few of them showing atypical mitosis, more nuclear and cellular pleomorphism and less distinct intercellular bridges compared to well-differentiated tumors.

Grade 3 (Poorly differentiated): Keratinization is rarely seen, frequent mitotic figures and atypical mitoses are common, obvious nuclear and cellular pleomorphism, occasional multinucleated cells and absent intercellular bridges.

Grades 1 and 2 are considered low grade while grade 3 is high grade. In tumors showing different grades, the higher grade determines the final categorization. It is still widely used as a prognostic variable in most studies, but most often confirmed to be of little value in prognostication (Al-Rajhi *et al.* 2000, O-charoenrat *et al.* 2003, Okamoto *et al.* 2002, Woolgar 2006). The reasons given for this include the subjective nature of the assessment, inadequate sampling from tumors showing histological heterogeneity, assessment based on structural features of tumor cells rather than functional features, and evaluation based on the tumor cells alone with no regard for the tumor micro-environment (Pindborg *et al.* 1997).

## *Malignancy grading system (malignancy score)*

Subsequent to observing that the tumor grade system was a rather poor prognostic indicator in OSCC, investigators began to suggest new grading systems. This has been reviewed by Anneroth *et al.* (1987). The initial suggestion by Jakobsson *et al.* (1973) for laryngeal cancers (and applied to all HNSCC) involves grading the tumor using criteria including tumor structure, degree of keratinization, nuclear pleomorphism, mitoses, mode of invasion, stage of invasion, vascular invasion

and cellular response. The system was refined by Anneroth *et al.* (1987), who proposed making the grading from the less differentiated part of the tumor. This classification was further refined for OSCC by Bryne *et al.* (1989) who advocated using only the deepest invasive margin of the tumor for grading based on five criteria: degree of keratinization, nuclear pleomorphism, number of mitoses, pattern of invasion and inflammatory cell infiltration. The introduction of these new grading systems was followed by studies which proved their prognostic value in OTSCC (Högmo *et al.* 1999, Kantola *et al.* 2000, Kurokawa *et al.* 2005, Odell *et al.* 1994, Woolgar & Scott 1995). Despite these promising results, many recent studies have shown that the prognostic significance of these systems in OTSCC is questionable (Silveira *et al.* 2007, Weijers *et al.* 2009; Yuen *et al.* 2002).

### *Lymphovascular invasion*

Lymphovascular invasion has been associated with poor prognosis in OTSCC either because it is closely associated with cervical nodal metastasis or locoregional recurrence or both (Brown *et al.* 1989, Chen *et al.* 2008, Silva *et al.* 2008b). Using a series comprising OTSCC and SCC of the floor of the mouth, Brown *et al.* (1989) showed that lymphovascular invasion correlated with the development of regional disease but not with survival. A similar finding was reported by Hosal *et al.* (1998). A study carried out on patients younger than 40 years with OTSCC showed that lymphovascular invasion was associated with decreased survival (Myers *et al.* 2000). As pointed out by Woolgar (2006), a drawback of using this parameter by pathologists is that it is difficult to define and recognize with certainty.

### *Perineural invasion*

Most of the studies that found statistical association between lymphovascular invasion and poor prognosis in OTSCC also had similar finding for perineural invasion (Brown *et al.* 1989, Chen *et al.* 2008, Myers *et al.* 2000, Silva *et al.* 2008b). In some of these studies, there seemed to be a stronger association of perineural invasion with poor outcome than with lymphovascular invasion. Sparano *et al.* (2004) reported that perineural invasion was an independent factor for occult nodal metastasis on multivariate analysis while lymphovascular invasion was not in a series of 45 clinically negative neck (N0) patients with early

OTSCC (T1/T2). Identifying perineural invasion is a tedious task involving a careful review of all tumor slides (Brown *et al.* 1989). A study on OSCC showed that identification was increased by more than 50% after careful reviewing of slides and staining with S-100 (Kurtz *et al.* 2005).

### *Apoptosis*

Apoptosis is a genetically regulated process involved in programmed cell death (marked by an absence of injuries to neighboring cells and absence of inflammation) that occurs in many physiologic and sometimes pathologic conditions. It is generally recognized that failure of physiologic apoptosis is one of the causes of tumor growth and proliferation. At the molecular level, a key event in apoptosis is the release of cytochrome c, which forms a complex with apoptosis-inducing factor, ultimately leading to activation of caspases, which cleaves DNA to cause cell death. This process is regulated by the BCL2 family of proteins. *Bcl-2*, *Bcl-X<sub>L</sub>*, *Bcl-w*, *Bfl-1*, *brag 1*, *AI* and *Mcl-1* inhibit apoptosis while *Bax*, *Bad*, *Bcl-X<sub>s</sub>*, *Bid*, *Bik* and *Hrk* promote apoptosis (Reviewed by Soini *et al.* 1998). The extent of apoptosis in histological tumor sections is determined by the apoptotic index (AI), which is usually defined as the percentage of apoptotic cells and bodies in tumor cell population. Low AI score and low expression of *Bax* has been correlated with poor prognosis in OTSCC while low expression of *Bcl-2* was associated with better clinical outcome (Xie *et al.* 1999). The same study also showed that high *Bcl-2/Bax* ratio was associated with a poor prognosis. Bag-1, a Bcl-2 binding protein which enhances the antiapoptotic properties of the latter, and also represents a link between growth factor receptors and antiapoptotic mechanism, has also been correlated with poor prognosis in OTSCC when highly expressed (Xie *et al.* 2004).

However, in a study of 23 patients with early-stage OTSCC (T1N0M0), a higher AI score was found to be associated with significantly increased nodal metastasis (Naresh *et al.* 2001). The authors hypothesized that in early-stage OTSCC, the tumor requires a greater number of tumor cell multiplications to arrive at a given size or volume compared to those with lower AI values. Since acquisition of genetic aberrations is directly related to the number of duplications, tumor cells with enhanced metastatic potentials are more likely in those tumors with high AI. According to the authors, high AI in low-volume tumors may therefore be a marker for poor prognosis (Naresh *et al.* 2001).

## *Tumor angiogenesis*

No tumor can grow to a clinically detectable size or ensure its sustenance unless it is vascularized. For continuous growth to occur a tumor needs neovascularization, which permits the cell to maintain contact with its host vascular bed. Tumor angiogenesis is determined morphologically by evaluating its microvascular density (MVD). This is made easier by staining the section with markers such as CD31, factor VIII-related antigen (von Willebrand factor) and  $\alpha_v\beta_3$  integrin (Fernandez *et al.* 2007, Pazouki *et al.* 1997). Recently, a more specific marker of ongoing tumor angiogenesis (CD105) has been used (Chuang *et al.* 2006). MVD is usually evaluated by identifying areas of greater vascular density in the tumor mass (hot spots) under high power. High MVD has been associated with poor prognosis in early OTSCC (Chuang *et al.* 2006, Shpitzer *et al.* 1996). However, the majority of the studies on OTSCC have not been able to validate this finding (Fernandez *et al.* 2007, Högmo *et al.* 1999, Kantola *et al.* 2000, Leedy *et al.* 1994). Fernandez *et al.* (2007) have identified that the reasons for these conflicting results include major differences in study design, such as different reagents, different microscopic fields or field sampling techniques and the technique of selecting hot spots. Nevertheless, all studies have confirmed OTSCC to be well vascularized.

At the molecular level, the two most important angiogenic factors are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). To circumvent the inconsistencies of results obtained for MVD, these two have also been studied in OTSCC. VEGF was found to be associated with a poor prognosis (Chuang *et al.* 2006, Kim *et al.* 2006, Mineta *et al.* 2002). One study has identified the expression of VEGF-C, a member of the VEGF family, with increased nodal metastasis and poor prognosis in comparison with VEGF-C negative tumors (Tanigaki *et al.* 2004). This has been contradicted by a more recent study by Faustino *et al.* (2008) in 83 patients with OTSCC and SCC of the floor of the mouth, which found no relationship between VEGF-C expression and nodal metastasis. Cho *et al.* (2007) also found no correlation between VEGF expression and tumor recurrence or survival of OTSCC patients. The intensity of bFGF was found not to be directly related to growth pattern of OTSCC or to the intensity of its neoangiogenesis, suggesting that because of its ubiquitous presence in the tumor, it may not be a good marker for prognosis in OTSCC (Forootan *et al.* 2000).

### *Markers of cell proliferative activities*

Cell cycle phase/cell proliferation markers, such as Ki-67 and proliferating cell nuclear antigen (PCNA), are an important adjunct to histologically-based tumor classification, and serve as useful indicators of tumor behavior and response to treatment. Ki-67 is a monoclonal antibody that binds to a protein (Ki-67 nuclear antigen) that is expressed during the active phases of cell cycle (G1, S, G2 and mitosis), but not in the resting phase (G0). High tumor positivity for Ki-67 has been associated with high degree of recurrence and poor survival in OTSCC patients (Silva *et al.* 2008a). This was similar to another report in stage I OTSCC, although it (high Ki-67 positivity) was not a factor in the overall survival of the patients (Wangsa *et al.* 2008). Davies *et al.* (2006) found no relationship between locoregional recurrence and increased expression of Ki-67 in OTSCC, and found that *decreased* Ki-67 expression at the invasive front is associated with a 6-fold increase in recurrence within 18 months (Davies *et al.* 2006). A larger study comprising 148 OTSCC patients also found no association between Ki-67 expression and prognosis (Bova *et al.* 1999).

### *Cancer-associated fibroblasts*

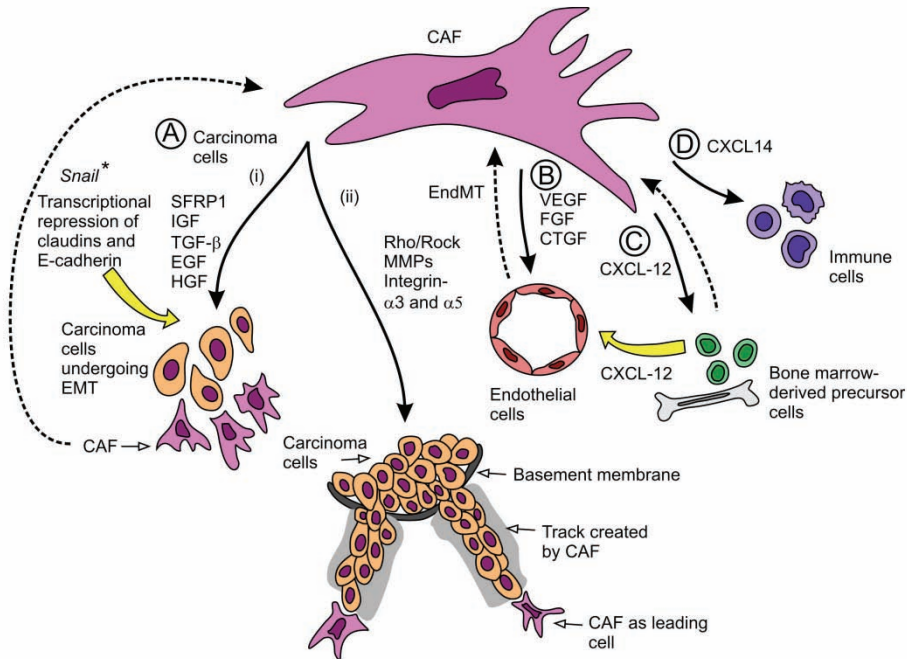
Within the last decade, emerging evidence has indicated that the tumor micro-environment is critical to the initiation and progression of tumors (Almholt & Johnsen 2003, Muller *et al.* 2000, Santin 2000). The tumor microenvironment is made up of distinct cell types including endothelial cells, immunocytes, antigen-presenting cells, such as macrophages and dendritic cells, fibroblasts, including a subset known as cancer-associated fibroblasts (CAFs), pericytes and myofibroblasts.

CAFs (also known as tumor-associated fibroblasts, peritumoral fibroblasts or reactive stroma) are believed to play important roles in most processes that are essential to tumor initiation and survival by directly being sources of pro-tumorigenic signals, by production of factors that contribute to tumor angiogenesis and recruitment of pro-tumorigenic inflammatory cells (Kalluri & Zeisberg 2006, Orimo *et al.* 2005, Orimo & Weinberg 2006, Ostman & Augsten, 2009) (Figure 1). CAFs are biologically different from fibroblasts obtained outside of tumor masses (Orimo *et al.* 2005). The origin of these cells is still unknown. Based on different studies, CAFs are presently thought to originate from four sources: local fibroblasts or fibroblast precursors, bone marrow-derived

precursor cells, malignant or normal epithelial cells undergoing epithelial-mesenchymal transition (EMT) and endothelial cells (Ostman & Augsten 2009).

There is presently no specific marker for CAFs. Morphologic characteristics and expression of markers such as  $\alpha$ -SMA, fibroblast-activated protein (FAP), fibroblasts-specific protein-1 (FSP1/S100A4), neuron-gial antigen (NG2) and PDGF- $\beta$  are presently used in defining CAFs (Ostman & Augsten 2009). These markers stain other cell types apart from fibroblasts (Kalluri & Zeisberg 2006). Production of  $\alpha$ -SMA (which is also seen in vascular smooth muscle cells, pericytes and myoepithelial cells) has therefore been widely used to characterize these cells (De Wever *et al.* 2008, Orimo & Weinberg 2006). In fact, De Wever *et al.* (2008) set the criterion for defining stromal MF as positivity to  $\alpha$ -SMA, and to at least three other markers from a list of positive markers such as paladin 4Ig, podoplanin, vimentin/desmin, endosialin, cadherin 11, prolyl-4 hydroxylase (P4H) as well as negative markers such as cytokeratin, CD14, CD31, CD34 and smoothelin. Therefore, an  $\alpha$ -SMA positive cell can be regarded as MF if it is positive for at least three other markers mentioned above (e.g.  $\alpha$ -SMA + vimentin + P4H and negative for cytokeratin). It is regarded as CAF if this criterion is not met. Some workers have referred to CAF as MF despite its not meeting the criterion set by De Wever for classification as MF (Orimo & Weinberg 2006, Kellermann *et al.* 2006, Vered *et al.* 2009b).

Presence of high density of CAF or its associated proteins such as FAP and SPARC has been associated with poor outcome in breast, colorectal, pancreatic and oral cancers (Cohen *et al.* 2008, Infante *et al.* 2007, Kellermann *et al.* 2007, Tsujino *et al.* 2007, Yazhou *et al.* 2004). A recent study by Vered *et al.* (2009b) reported that increased amount of CAFs in the stroma of OTSCC is an independent predictor of local recurrence of the tumor.

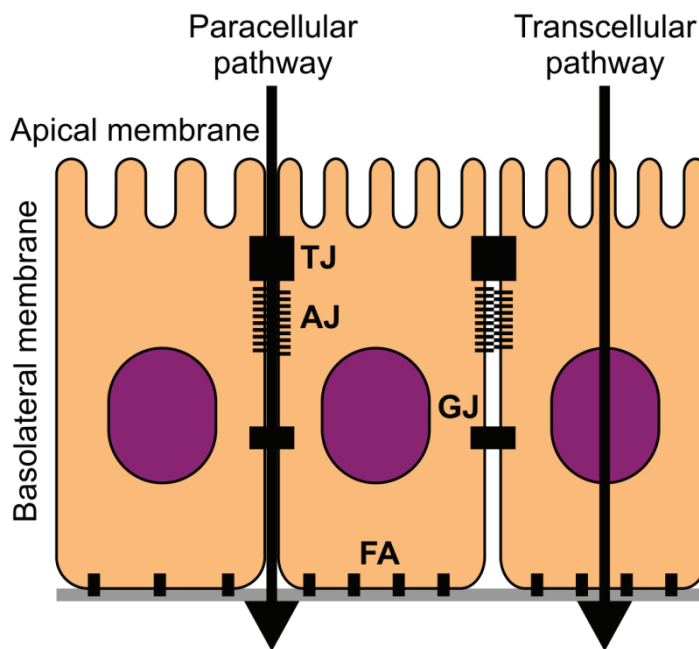


**Fig. 1. Protumorigenic signals of CAFs. (A):** on carcinoma cells (i) they produce classical growth factors which aid metastasis or/and epithelial-mesenchymal transition (EMT) and, (ii) they create the pathway through the extracellular matrix by force-mediated and protease-mediated remodeling for cancer cells which do not undergo EMT. **(B):** On endothelial cells they produce factors that stimulate angiogenesis. **(C and D):** On bone marrow and immune cells, they produce chemokines that aid in recruiting bone marrow-derived precursor cells and immune cells into the growing tumor. Bone marrow cells also secrete chemokines that aid angiogenesis (yellow arrow). The broken arrows indicate that the products of these processes may actually be the origin of CAFs or add to the CAF population within the tumor microenvironment. The proposed linkage between transcriptional repressors (such as *Snail*) of claudins and E-cadherin and this network is also shown (yellow arrow\*). EndMT indicates endothelial-mesenchymal transition. Modified from Gaggioli *et al.* (2007), Östman & Augsten (2009) and Kalluri & Zeisberg (2006).

### *Tight junction proteins*

Fluid compartments are separated by epithelia and endothelia. This is achieved by a set of specialized junctions from the apical to the basolateral portions of the cells: tight junctions (TJ; zonula occludens), adherens junctions (zonula

adherens), gap junctions and focal adhesions. The TJ, being the most apical serves two functions (Figure 2): it controls the movement of molecules between epithelial or endothelial cells through the paracellular route (gate function) and maintains cell polarity between the apical and basolateral membranes of individual cells (fence function) (Balkovetz 2006). TJs are essentially made up of transmembrane proteins: claudins, occludin, and tricellulin, which have four transmembrane domains; and junctional adhesion molecules (JAMs) and the coxsackie virus and adenovirus receptor (CAR), which are single transmembrane proteins. The complex of TJ-associated proteins found inside the cell includes ZO-1, ZO-2, ZO-3, cingulin, 7H6, symplekin, and ZA-1 (Hossain & Hirata 2008). CAR functions as a primary receptor for coxsackie B and adenovirus.



**Fig. 2. Specialized cellular junctions.** The paracellular (fence function) and transcellular (gate function) routes of the TJs are shown with solid arrows. TJ: tight junction, AJ: adherens junction, GJ: gap junction, FA: focal adhesion. Modified according to Balkovetz (2006).

Claudins are essential for the barrier function of epithelia and endothelia. In humans, up to 24 isoforms are currently known, with molecular weights between 20–27 kDA (Gonzalez-Mariscal *et al.* 2003). The role of occludin (molecular

weight approximately 64 kDa) is still not well understood. Tricellulin (approximately 70 kDa), the most recently discovered transmembrane protein, is known to be concentrated in the tricellular TJs at positions where three cells meet (Ikenouchi *et al.* 2005). It is assumed to play roles in the formation of bicellular and tricellular TJs. JAMs have been shown to co-precipitate with ZO-1, suggesting that the former may be indirectly involved in the recruitment of occludin to the TJ via ZO-1 (Hossain & Hirata 2008).

Expression of claudins in tissues varies. In a study of normal rat liver, pancreas, stomach, and small and large intestines, using claudins 2, 3, 4 and 5, striking differences were obtained in the expression levels and patterns of each claudin within the same tissue or different tissues (Rahner *et al.* 2001). Similar differences have also been reported in the kidney and bladder of rat, mouse and rabbits (Acharya *et al.* 2004, Kiuchi-Saishin *et al.* 2002, Reyes *et al.* 2002). In malignant epithelial tumors involving different tissues, loss or gain of expression of claudins has been associated with biologic behavior and prognosis (Lanigan *et al.* 2009, Martin *et al.* 2008, Nakanishi *et al.* 2008, Ohtani *et al.* 2009, Soini *et al.* 2006). In esophageal SCC, reduced expression of claudin 1 and 7 has been associated with poor prognosis (Miyamoto *et al.* 2008, Usami *et al.* 2006). Claudin 7 was found to be down-regulated in HNSCC in comparison to normal epithelium (Al Moustafa *et al.* 2002). No previous study has related tight junction proteins to OTSCC until the present study.

### *Maspin*

First isolated in human breast epithelial cells, maspin (*mammary serine protease inhibitor*) – a 42 kDa protein member of the serpin superfamily of protease inhibitors – was found to be expressed in normal breast epithelial cells but reduced or absent in breast carcinomas (Zou *et al.* 1994). It was later found to be expressed in many other tissues including epithelial lesions of the tongue (Vered *et al.* 2009a). It has been shown to have tumor suppressor properties, including inhibition of tumor angiogenesis, tumor cell motility, invasion and metastasis and promotion of apoptosis (Bailey *et al.* 2006, Sheng *et al.* 1996). In stage I and II OTSCC, the absence of maspin expression was associated with cervical lymph node metastasis (Yasumatsu *et al.* 2001). Cho *et al.* (2007) found no correlation between maspin expression with clinical stage or tumor recurrence. In studies done on OSCC, high maspin expression was found to be associated with improved survival (Iezzi *et al.* 2007, Xia *et al.* 2000)

### *Nuclear DNA content*

DNA cytometry is used for gross estimation of nuclear DNA content (DNA ploidy). It involves the staining of cell nuclei using a stoichiometric DNA binding stain and measuring the amount of staining obtained (van Diest *et al.* 1998). Two different methods are used: image (static) cytometry and flow cytometry. In flow cytometry, nuclei in a cell suspension are stained with a fluorescent dye, sucked into the flow cytometer where the fluorescence is excited and measured by means of a photomultiplier system. Image cytometry is done by applying an absorption stain to cells on a glass slide and measuring the optical density by image analysis (van Diest *et al.* 1998). In about 80% of cases, the DNA histograms produced by both methods are relatively identical (Falkmer *et al.* 1990). A large number of cells can be studied in a short time in flow cytometry, while static cytometry is interactive and the operator usually selects the cells from the image obtained from the microscope in addition to being able to select normal diploid cells as an internal control. This is one of the reasons for occasionally obtaining different histograms from the same specimen using both methods. Other reasons are related to the use of flow cytometry, including the presence of excessive cell debris or large numbers of DNA diploid cells (such as lymphocytes), which may mask small aneuploid peaks; lack of reliable internal control that works in deparaffinized samples; and also cytometrists have no criteria of histogram classification for flow cytometry. In OTSCC, conflicting results have been obtained with regard to prognosis. Some investigators have found aneuploidy to be related to poor prognosis (Hemmer & Kreidler 1990, Saito *et al.* 1994). Some other investigators have found out that it did not produce any additional information with regard to prognosis (Cooke *et al.* 1994, Högmo *et al.* 1999, Wangsa *et al.* 2008).

### **2.3 Epithelial-mesenchymal transition (EMT): Complimentary roles for TJ destruction and CAF recruitment favouring cancer progression?**

EMT is defined as the conversion of epithelial cells to migratory fibroblastoid cells (Usami *et al.* 2008). EMT is known to underlie a variety of tissue remodeling that occurs during embryonic development, such as mesoderm and neural crest formation, and has also been associated with tumor invasion and metastasis (Ikenouchi *et al.* 2003, Thiery 2003, Yang & Weinberg 2008). It is also

known to occur during wound healing and fibrosis (Thiery 2003). EMT is a biologic process involving loss of cell-cell adhesion, reorganization of actin skeleton and redistribution of organelles (Thiery 2003). EMT is regulated at the molecular level in both development and disease by several mechanisms, such as TGF- $\beta$  and other tyrosine kinase receptors' signaling, Wnt signaling, the notch pathway, proteolytic digestion of extracellular matrix by MMPs and transcriptional repression of E-cadherin and claudins (Baum *et al.* 2008, Chang *et al.* 2007, Thiery 2003, Yang & Weinberg 2008). In regard to transcriptional repression of E-cadherin and claudins, this has been a constant finding during EMT in which TJs disappear. This independent transcriptional repression has been attributed to the zinc-finger transcription factor, *Snail*, which plays a central role in EMT (Nieto 2002). Ikenouchi *et al.* (2003) demonstrated that when *Snail* was overexpressed in cultured mouse epithelial cells, EMT was induced and concomitant repression of claudin and occludin was observed. They also showed that *Snail* binds directly to the E-boxes of the promoters of claudin/occludin genes, resulting in complete repression of their promoter activity. In human esophageal SCC, nuclear expression of *Snail* at the invasive front has been associated with reduced expression of E-cadherin, and claudins 1 and 7 in addition to increased lymphovascular invasion, clinicopathological tumor stage and nodal metastasis (Usami *et al.* 2008).

EMT has been adduced as essential for tumor invasion and metastasis, and also as one of the mechanisms by which epithelial cells are recruited as CAFs. It is possible to speculate that the activities of *Snail* may be central to the complementary role played by claudins and E-cadherin in EMT and subsequent recruitment to CAFs in the tumor process (Figure 1). A corresponding reverse process known as mesenchymal to epithelial transition (MET) also occurs in development and disease, and seems to account for viability of micrometastasis and transformation to clinically significant metastasis (Chaffer 2007, Thiery 2002).

## **2.4 Ameloblastoma and ameloblastic carcinoma**

### **2.4.1 Incidence**

Ameloblastomas are benign, slow-growing, locally invasive neoplasms of odontogenic origin with a strong tendency to recur after treatment. The tumor

consists of epithelial neoplastic islands or strands made up of peripheral columnar or cuboidal cells resembling ameloblasts or preameloblasts of the dental germ enclosing a central core of loosely arranged angular or stellate cells which closely resembles the stellate reticulum of the dental germ. Ameloblastomas are classified into three types: solid/multicystic, unicystic and peripheral (Barnes *et al.* 2005). All references made here are in relation to the solid/multicystic variant. Ameloblastomas are the most common odontogenic tumors if odontomas (which are generally regarded as hamartomas or developmental anomalies) are not considered. They constitute approximately 1% of all oral tumors and about 12% of odontogenic tumors (Buchner *et al.* 2006). In Africans, they are estimated to account for between 11 and 24% of all oral and tumor-like lesions (Adebayo *et al.* 2005, Arotiba *et al.* 1997). The prevalence could rise to between 40 and 73% within odontogenic tumors when odontomas are not considered or in centers where odontomas are not commonly diagnosed (Adebayo *et al.* 2005, Buchner *et al.* 2006, Jing *et al.* 2007, Okada *et al.* 2007). Ameloblastomas are seen in all age groups, although most cases are found between 30 and 60 years of age (Adebayo *et al.* 2005, Buchner *et al.* 2006). A number of reports gave a slightly lower peak incidence in the second and third decades (Adeline *et al.* 2008, Arotiba *et al.* 1997).

The tumor seems not to have gender predilection (Adeline *et al.* 2008, Okada *et al.* 2007). Most studies show a fairly even distribution between both genders, although prevalence may be slightly skewed to either of the two (Adebayo *et al.* 2005, Buchner *et al.* 2006, Jing *et al.* 2007, Olgac *et al.* 2006). Most cases occur in the mandible, especially the molar and angle regions (Adebayo *et al.* 2005, Okada *et al.* 2007, Olgac *et al.* 2006). Some reports showed that anterior mandibular predilection may be very common in Africans and Chinese (MacDonald-Jankowski *et al.* 2004, Reichart *et al.* 1995). Other reports have suggested a racial predilection (Ajagbe & Daramola 1982, Shear & Singh 1978). Using the mixed population of South Africa, Shear & Singh (1978) showed that the tumor occurs more commonly in African Bantus than in Caucasians. This postulation was disputed by some workers as being due to selection biases (Sawyer *et al.* 1985).

Ameloblastic carcinoma is a rare odontogenic malignancy exhibiting typical features of benign ameloblastoma in addition to histological features of malignancy, such as cellular or nuclear pleomorphism, high mitotic count, and perineural invasion irrespective of whether or not there is evidence of metastasis. The WHO currently describes two types: primary and secondary

(dedifferentiated) (Barnes *et al.* 2005). The incidence of ameloblastic carcinoma is unknown. As of 2008, 67 cases have been reported worldwide (Angiero *et al.* 2008). Approximately two-thirds of cases occur in the mandible and the rest in the maxilla (Akrish *et al.* 2007, Benlyazid *et al.* 2007). Men are affected slightly more than women (Benlyazid *et al.* 2007). The median age of reported cases is 44 years, although occurring in a wide age range (4–84 years) (Benlyazid *et al.* 2007). There appears to be no racial predilection. The majority of cases with metastases were to the lungs.

### **2.4.2 Etiology**

The etiology of ameloblastoma is unknown (Namin *et al.* 2003). Abnormalities in expression of several genes involved in normal tooth development such as *FOS* and *TNFR1A* have been suggested as having a role in the histogenesis of ameloblastoma (Heikinheimo *et al.* 2002). The tumor is believed to be derived from odontogenic epithelium with potential sources including the enamel organ, odontogenic rests (rests of Malassez and Serres), reduced enamel epithelium and the epithelial lining of odontogenic cysts, especially dentigerous cysts (Regezi *et al.* 2008).

The origin of ameloblastic carcinoma is also unknown. Primary ameloblastic carcinoma arises *de novo* while secondary (dedifferentiated) ameloblastic carcinomas arise in a pre-existing benign ameloblastoma (Barnes *et al.* 2005).

## **2.5 Prognostic factors in ameloblastomas and ameloblastic carcinoma**

Despite being locally invasive, ameloblastoma can be effectively controlled with adequate surgical treatment. Recurrence rates are very high with inadequate or conservative treatment (Ghandhi *et al.* 2006). Ameloblastic carcinomas are associated with tumor recurrence and poor prognosis in more than one third of cases (Dhir *et al.* 2003). Clinical factors appear to play a prominent role in prognosis.

### **2.5.1 Clinical prognostic factors**

Clinical factors that are important in the prognosis of ameloblastoma include the jaw that is affected, involvement of surrounding soft tissues and the treatment

modality used. When conservative treatment such as curettage and enucleation are used, the recurrence rate could rise as high as 90% in mandibular and 100% in maxillary tumors (Sehdev *et al.* 1974). The architectural pattern of ameloblastoma is such that the border of the tumor within the cancellous bone lies beyond the apparent macroscopic surface and radiographic boundaries of the tumor (Ghandhi *et al.* 2006). Radical surgery is usually associated with good results, in which case recurrence rate could be as low as 0% (Ghandhi *et al.* 2006). Maxillary ameloblastoma is usually associated with a poorer prognosis compared to mandibular ameloblastoma (Zwahlen & Gratz 2002). The former is usually associated with a lack of early symptoms, with patients typically consulting the physician when the tumor has spread beyond the maxilla. Medullary bone, which the tumor actively invades, is in abundance in the maxilla, while cortical bone, which the tumor is only able to erode rather than invade, is rare in the maxilla. In addition, the majority of ameloblastomas affecting the maxilla are located posterior to the canine tooth, giving them close intimacy with the nasal cavity, paranasal sinuses, orbits, pterygomaxillary fossa and vital structures at the base of the skull (Jackson *et al.* 1996). Tumor spread to these areas is relatively easy and makes definitive treatment difficult (Feinberg & Steinberg 1996). Inadequate resection of maxillary ameloblastoma was associated with a 5-year survival rate of 16% (Bredenkamp *et al.* 1989). Infiltration of the surrounding soft tissue by ameloblastoma is also associated with a high rate of treatment failure. This results from the difficulty in identifying the tumor boundary (Ghandhi *et al.* 2006).

In comparison to ameloblastomas, ameloblastic carcinomas have a more rapid growth rate, are more likely to perforate the cortex and are more frequently associated with pain and sensory disturbance (Akrish *et al.* 2007). The main prognostic factors for ameloblastic carcinoma are the appearance of recurrent tumor and metastatic deposits particularly to distant sites (Dhir *et al.* 2003). Recurrence occurs frequently in ameloblastic carcinoma, which justifies a long follow-up. Most occur within a period of 1.5 years (Akrish *et al.* 2007). A high rate of distant metastatic spread (preferentially hematogenous) appears to be the single most important prognostic factor. This is in contrast to squamous cell carcinoma, which spreads preferentially by the lymphatic pathway (Benlyazid *et al.* 2007). The adequacy of surgical resection does not seem to influence the metastatic spread of ameloblastic carcinoma (Akrish *et al.* 2007).

### **2.5.2 Histopathologic and molecular markers as prognostic factors**

Not much is known about the histopathologic and molecular prognostic factors in ameloblastoma or ameloblastic carcinoma as very few studies on survival analysis have been done. Most studies have concentrated on indirect linkage of proteins expressed in the tumor to biologic behavior. Tumor growth and invasive behavior are thought to be associated with increased activity of matrix metalloproteinases (MMPs), especially MMP 2 and 9 (Pinheiro *et al.* 2004). Other markers which may be involved in growth and invasiveness of the tumor include TNF- $\alpha$ , anti-apoptotic proteins (Bcl-2, Bcl-xL), integrins (alpha5beta1 integrin) and interface proteins (FGF) (Regezi *et al.* 2008, Souza Andrade *et al.* 2007).



### **3 Aims of the study**

Ameloblastoma and OSCC are probably the most common clinically significant odontogenic tumor and soft tissue malignancy, respectively, affecting the oral cavity. Ameloblastoma in its benign form is locally aggressive compared to other odontogenic tumors, while in the malignant form it is associated with poor prognosis. Despite advances in its diagnosis and management, OTSCC, which represents the largest percentage of oral cancers, has not been associated with a substantially improved prognosis for decades. Clinicians continually rely on clinical presentation to predict the prognosis of these lesions because no molecular marker has been found to predict prognosis unequivocally. The aims of this study include:

1. To study the expression of TJ proteins: claudins (1, 4, 5, 7) and occludin, and carcinoma-associated fibroblasts in ameloblastomas and OTSCC
2. To investigate the relationship between these markers and prognosis in OTSCC.
3. To compare the effectiveness of CAF density with an epithelial proliferative marker, Ki-67; a tumor suppressor marker, maspin; and the gross DNA content (DNA ploidy) as measured from static and flow cytometry in prognostication in OTSCC.
4. To investigate the usefulness of Ki-67, epithelial membrane antigen (EMA), DNA ploidy and CAF density in differentiating between ameloblastoma and ameloblastic carcinoma.



## **4 Materials and methods**

### **4.1 Tissue specimens, patients and follow-up information**

#### **4.1.1 Ameloblastoma, ameloblastic carcinoma and dental germ (I and III)**

All cases of ameloblastoma and ameloblastic carcinoma detected between 1987 and 2005 were retrieved from the archives of the Department of Diagnostics and Oral Medicine, University of Oulu. Additional cases were kindly provided by Professor PJ Slootweg of the Department of Pathology, Radboud University, Nijmegen, the Netherlands. All the tissues had been previously fixed in 10% formalin and embedded in paraffin. For morphological analysis, 5- $\mu$ m sections were obtained from the paraffin-embedded samples and stained with hematoxylin-eosin. After re-evaluation, only the solid-multicystic types of benign ameloblastoma were selected, resulting in the final samples of 25 cases of benign ameloblastoma and 4 cases of ameloblastic carcinoma (study I). Loss of tissue in some of the samples reduced the case number to 18 cases of benign ameloblastoma and 3 cases of ameloblastic carcinoma in the later study (study III). Two developing teeth in their late bell stages were obtained from the lower jaw of a legally aborted fetus at Oulu University Hospital. These had been used with ethical approval in a previous study (Väänänen *et al.* 2004). Patients' demographic characteristics are shown in Table 2.

**Table 2. Demographic characteristics of patients with ameloblastoma and ameloblastic carcinoma.**

Patients	All (n = 29) (%)	Ameloblastoma (n = 25) (%)	Ameloblastic carcinoma (n = 4) (%)
Sex			
Male	17 (59)	15 (60)	2 (50)
Female	12 (41)	10 (40)	2 (50)
Age at the time of diagnosis			
Median (years)	52	52	85
Range (years)	16–89	16–85	40–89
0–39 years	8 (28)	8 (32)	0
40–59 years	9 (31)	8 (32)	1 (25)
60+ years	12 (41)	9 (36)	3 (75)
Site of primary tumors			
Maxilla	7 (24)	4 (16)	3 (75)
Mandible	22 (76)	21 (84)	1 (25)
Histologic type of tumor			
Plexiform		12 (48)	
Follicular		7 (28)	
Acanthomatous		2 (8)	
Mixed		4 (16)	

#### **4.1.2 Squamous cell carcinoma of mobile tongue cases (II and IV)**

All cases of mobile tongue cancers between 1983 and 2005 were retrieved from the archives of the Department of Diagnostics and Oral Medicine and the Department of Pathology, University of Oulu. The paraffin-embedded tissues were stained with hematoxylin-eosin and reviewed. 97 cases that met the inclusion criteria of having enough histological material, sufficient clinical data and resection margins greater than 5mm were included in this study. Loss of tissue samples in some of the material, however, resulted in fewer cases for study IV (77 cases) although additional material (51 cases seen between 1981 and 2006) was obtained from Dr M. Vered from the Department of Oral Medicine and Pathology, University of Tel Aviv, Israel. Patients' clinicopathologic characteristics are shown in Table 3.

The tongue cancers were histologically graded and staged using the current UICC and WHO-based classifications (Barnes *et al.* 2005; Sobin & Wittekind 2002). Invasive front grading described by Bryne *et al.* (1992) was also done.

**Table 3. Clinicopathologic characteristics of all patients with OTSCC.**

	All patients (n = 148)	Oulu (n = 97)	Tel Aviv (n = 51)
<b>Sex</b>			
Male	72 (49)	46 (48)	26 (51)
Female	76 (51)	51 (52)	25 (49)
<b>Age (years)</b>			
0–39	13 (9)	7 (7)	6 (12)
40–59	45 (30)	31 (32)	14 (27)
60–99	90 (61)	59 (61)	31 (61)
Range	20–99	26–99	20–80
Median	65	65	62
<b>Grade*</b>			
I	36 (37)	36 (37)	
II	50 (52)	50 (52)	
III	11 (11)	11 (11)	
<b>Stage</b>			
I / II	85 (57)	51 (53)	34 (67)
III / IV	60 (41)	43 (44)	17 (33)
Unknown	3 (2)	3 (3)	0 (0)
<b>Invasive front grading*</b>			
Low (5 - 10)	32 (33)	32 (33)	
High (11–20)	61 (63)	61 (63)	
Missing	4 (4)	4 (4)	
<b>Neck Metastasis</b>			
Yes	40 (27)	25 (26)	15 (29)
No	94 (63)	58 (60)	36 (71)
Missing	14 (10)	14 (14)	0 (0)
<b>Recurrence</b>			
Yes	53 (36)	33 (34)	20 (61)
No	81 (55)	50 (52)	31 (39)
Unknown	14 (9)	14 (14)	0 (0)
<b>Primary treatment</b>			
Surgery	71 (48)	55 (57)	16 (31)
Surgery and radiotherapy	52 (35)	26 (27)	26 (51)
Surgery, radio- and chemotherapy	10 (7)	2 (2)	8 (16)
Surgery and chemotherapy	1 (1)	0 (0)	1 (2)
Missing	14 (9)	14 (14)	0 (0)
<b>Follow-up</b>			
Median time (range) (months)	36 (1–267)	36 (1–267)	34 (1–230)
Death due to cancer	38	26	12
Death due to other causes	28	23	5
Death due to unknown cause	1	1	0

\*Data from Tel Aviv (Israel) was not available and was not used.

## 4.2 Immunohistochemistry for paraffin sections (I–IV)

For immunohistochemical analysis, selected blocks were cut into 5- $\mu$ m-thick sections. The primary antibodies used in the immunostainings in this study are listed in Table 4.

In brief, the procedure involves deparaffinization in xylene and rehydration in graded ethanol. Antigen retrieval was done by heating the sections in a microwave oven in 10mmol/L citrate buffer, pH 6.0, for 10 minutes. Endogenous peroxidase activity was quenched in 0.3% hydrogen peroxide diluted in H<sub>2</sub>O. The sections were thereafter incubated with the primary antibody for 60 min at room temperature and then overlaid with a biotinylated secondary antibody and Histostat SP kit (Zymed, San Francisco, CA, USA) for antibody detection. Color was developed in diaminobenzidine solution (DAKO A/S Denmark) or AEC substrate chromagen staining kit (Zymed, San Francisco, CA, USA). Counterstaining was done with Mayer's hematoxylin and the slides were rehydrated (only those with color developed by diaminobenzidine) and mounted. All steps were accompanied with washes by phosphate buffered saline (PBS). Negative controls were obtained by substituting the primary antibody with non-immune rabbit or mouse serum and PBS, and positive controls were obtained from non-neoplastic tissue samples from kidney, breast, skin and liver.

**Table 4. Antigens and respective antibodies used in the studies.**

Antigen	Antibody type	Clone	Dilution	Manufacrer
Claudin 1	Polyclonal rabbit	JAY.8	1:50	Zymed Laboratories, San Francisco, CA, USA
Claudin 4	Monoclonal mouse	3E2C1	1:50	Zymed Laboratories, San Francisco, CA, USA
Claudin 5	Monoclonal mouse	4C3C2	1:50	Zymed Laboratories, San Francisco, CA, USA
Claudin 7	Polyclonal rabbit	ZMD.241	1:50	Zymed Laboratories, San Francisco, CA, USA
Occludin	Polyclonal rabbit	ZMD.481	1:50	Zymed Laboratories, San Francisco, CA, USA
Ki-67	Monoclonal mouse	MM1	1:100	Novocastra Laboratories, Newcastle, UK
$\alpha$ -SMA	Monoclonal mouse	1A4	1:1000	Dako A/S, Denmark
EMA	Monoclonal mouse	E29	1:500	Dako A/S, Denmark
Calponin B	Monoclonal mouse	26A11	1:50	Novocastra Laboratories, Newcastle, UK
P63	Monoclonal mouse	7JUL	1:25	Novocastra Laboratories, Newcastle, UK
Maspin	Monoclonal mouse	EAW24	1:50	Novocastra Laboratories, Newcastle, UK

#### **4.2.1 Assessment of immunohistological staining (I–IV)**

The full details of immunohistological staining evaluation are given in the individual studies. Briefly, the immunohistochemical staining was assessed for intensity and semi-quantitatively. For assessment of intensity (claudins 1, 4, 5, 7, occludin,  $\alpha$ -SMA, EMA, and maspin), the stained slides were graded as +, weak; ++, medium; and +++, strong. The overlying normal-appearing epithelium was used as the internal control in study II because claudins and occludin stain epithelia.

Quantitative immunostaining (for the markers mentioned above) was assessed as follows: -, no immunostaining present; +, < 25% of cells positive; ++, 25–75% of cells positive; +++, more than 75% of cells positive. The whole areas of the sections were screened. Ki-67 was assessed by choosing five representative fields (x 400 magnification) and taking photomicrographs. The labeling index was calculated as the number of positive cells divided by the total number of tumor cells expressed as a percentage. In order to ensure easy reproducibility by the investigators taking part in the assessments, the parameters to be assessed were clearly defined, as were the areas of the sections from which assessments were to be made. Where photomicrographs were used, the same photomicrographs were assessed by the investigators. All assessments were done by at least two investigators and a final assessment was then done jointly.

#### **4.3 Image cytometry (III and IV)**

Fifty-micrometer-thick sections were cut from paraffin-embedded tissue blocks, deparaffinized twice in 3 ml HistoClear (National Diagnostics, Atlanta, GA, USA) and rehydrated in decreasing concentrations of ethanol and washed twice in phosphate buffered saline. Enzymatic disintegration was done in 3 ml freshly prepared 0.05% pronase solution (Sigma, St Louis, MO, USA) for 30 minutes at 37°C in a shaking water bath with intermittent vortex mixing. The nuclei were then filtered with a nylon mesh with a 30- $\mu$ m pore size, spun on glass slides at 1,250g for 15 minutes and air dried at room temperature. Fuelgen staining was performed using acid hydrolysis in 5M HCl at room temperature for one hour, after which the sections were washed in distilled water, stained with Schiff's reagent for 165 minutes at room temperature, rinsed in distilled water and treated three times for 10 minutes in fresh sodium thiosulphate and rinsed for 5 minutes. The sections were dehydrated in graded alcohol and xylene and mounted. A

second set of 8- $\mu$ m-thick sections slides (without undergoing enzymatic disintegration and filtration) were also stained directly using modified Fielgen staining.

#### **4.3.1 DNA measurement**

The measurements were made with a densitometric device, the Ahrens Cytometry Analysis system, (Institut für Meß-Technik, Hamburg, Germany) comprising an Olympus BH2 microscope (40x objective) and a CCD camera with a green filter (550nm). Lymphocytes or granulocytes were measured within each section as an internal control to assess the position of the normal diploid 2c value (c = haploid genome equivalent). The DNA content of approximately 200–300 cells was then measured. The histogram obtained was classified as DNA diploid if the mean ploidy value was  $\leq 2.5c$ , aneuploid if the mean ploidy value was  $2.5 < c \leq 3.5$  or  $> 4.5c$ , and tetraploid if the value was  $3.5 < c \leq 4.5$ . Ploidy is the mean value of the G1 fraction position of measured cells on the DNA scale.

#### **4.4 Flow cytometry (III)**

Nuclei were obtained as for image cytometry (section 4.3), stained with propidium iodide and flow cytometry was carried out with a FacStar flow cytometer (Becton Dickinson, CA, USA). For each histogram 20,000 particles were measured. The DNA index was calculated by dividing the modal channel number of the aneuploid peak by the modal channel number of the diploid peak which was considered to be the G0/G1 peak with least fluorescence.

#### **4.5 Western blot (II)**

Western blot analysis was undertaken to assess the presence of claudins and occludin *in vitro* in a tongue cancer cell line and also to determine if the antibodies used are effective under *in vitro* conditions. Highly invasive tongue cancer cell line HSC-3 (JRCB Cell Bank 0623, National Institute of Health Sciences, Osaka, Japan) was used.

For the procedure, total proteins were extracted from the subconfluent cultures of HSC-3 cells cultured in 250 mm<sup>2</sup> dishes using the Trizol® method (Invitrogen, Carlsbad, CA, USA). Protein samples of 7  $\mu$ g were separated on 11% SDS-PAGE. Proteins were electrotransferred to a nitrocellulose membrane

(Bioscience, Dassel, Germany). The membranes were incubated with primary antibodies: claudin-1, -4, -5, -7 and occludin (dilution 1:500). After washing, the membranes were incubated with the secondary peroxidase-conjugated anti-mouse IgG antibody and peroxidase-conjugated anti-rabbit IgG antibody (dilution 1:800) (Amersham Pharmacia Biotech, Buckinghamshire, England). An ECL Western blotting detection kit (Amersham Pharmacia Biotech, Buckinghamshire, England) was used to visualize the proteins as described by the manufacturer.

#### **4.6 Statistical analysis**

In all statistical analyses, the software package SPSS® for Windows®, (SPSS Inc. Chicago, IL.) was used. For categorical variables, Fisher's exact test was used to test for association between groups. Mann-Whitney U test was used to compare association between non-parametric variables. The statistical association between the variables studied and patient survival was done using Mantel-Cox log rank test and Kaplan Meier survival plots. Cox's proportional hazards multiple regression model was then applied by adding known additional clinicopathologic prognostic variables that are believed to affect prognosis. The proportional hazard assumption was verified by comparing estimated log (-log) (LML) survival plots of the different categories used. When cumulative mortality curves were used, the computations were performed and graphs produced using the tools in the cmprsk package of the R environment (R Development Core Team 2009). In all cases, *P* values (2-sided) of less than 0.05 were considered statistically significant.

#### **4.7 Ethical considerations**

The study protocol was approved by the Ethical Committee of the Northern Ostrobothnia Hospital District. The approval for tongue cancer patients including access to clinical data was granted in March 2003 (18/2003 NOHD), and November 2005 (57/2005 NOHD). The ethical approval for the ameloblastoma studies was granted in June 2005 (33/2005 NOHD).



## **5 Results**

### **5.1 Claudins 1, 4, 5, 7 and occludin in ameloblastoma/ameloblastic carcinoma/dental germ and OTSCC (I)**

#### **5.1.1 Pattern of staining in dental germ, ameloblastoma, ameloblastic carcinoma and clinical significance**

Antibodies to claudins 1, 4, 5, 7 and occludin stained the epithelial cells in the sections, and the staining pattern was membranous. There was a striking similarity in the staining pattern of claudins for ameloblastomas and the developing dental tissue. The general tendency in both tissues was to display more intense staining for claudin 1 and 7 in the centrally located cells than in the peripheral cells. The dental germs displayed intense staining of the inner and outer enamel epithelium, stratum intermedium, stellate reticulum, ameloblasts and the newly formed enamel by claudins 1 and 7. The staining reaction was negative in the dental papilla and odontoblasts (claudin 7) or relatively weak (claudin 1), and completely negative for newly formed dentine. Staining for claudin 4 was essentially only seen in the central cells of ameloblastoma, and in outer enamel epithelium and stellate reticulum of the developing teeth. Claudin 5 preferentially stained the vascular structures. Occludin immunoreactivity was very weak or negative. In ameloblastic carcinoma, the staining pattern was generally similar but stronger than in benign ameloblastoma.

There was no difference in the expression of claudins in ameloblastoma and ameloblastic carcinoma, although ameloblastic carcinoma displayed a stronger staining pattern. Claudins 1 and 4 showed significantly more cases, displaying stronger staining in central than peripheral cells in benign ameloblastomas alone, and also when assessed together with malignant ameloblastomas ( $P < 0.05$ ).

#### **5.1.2 Pattern of staining in OTSCC and relationship to prognosis (II)**

In both the superficial and invasive fronts of OTSCC, immunoreactivity for claudins 1 and 7 was strong in intensity compared to the overlying epithelium, claudin 4 was moderate and claudin 5 relatively weak, although there were some individual differences from this general trend. Occludin was generally very weak or negative in immunoreactivity.

The staining pattern was compared with OTSCC-specific death by using log rank test. In the superficial part of the tumors, univariate analysis showed that there was no evidence of statistical association between the staining intensity or quantity of the claudins and the disease-specific survival of the patients. At the invasive front of the tumors, staining intensity of claudin 7 showed a statistically significant association with disease-specific patient survival. When staining was less or more intense than the adjacent normal epithelium, there was an association with poor disease-specific survival (HR 3.42, 95% CI 1.16–10.10,  $P = 0.023$  and HR 3.16, 95% CI 1.20–8.31,  $P = 0.02$  respectively). The quantity of tumour cells stained by claudin 7 also showed an association with cancer-specific survival. Low quantitative staining was associated with decreased survival (HR 4.87, 95% CI 1.44–16.44;  $P = 0.01$ ).

Multivariate analysis that included TNM stage, gender and patient's age category at diagnosis (< 70 years vs. > 70 years) as additional prognostic variables was applied to verify these associations. The only independent variable that seems to be associated with decreased patient survival was age above 70 years at diagnosis, although TNM stage was also an independent factor for poor prognosis in the claudin 7 quantitative multivariate model. The association between claudin 7 intensity and quantity and disease-specific survival was reduced when these additional factors were included in the model.

### *Western blot analysis (II)*

Western blot analysis of the total protein extract of cultured tongue cancer cell line HSC-3 cells showed several bands for claudin 1 between 18 and 37 kDa; weak single bands of 22.1 kDa and 23 kDa for claudin 4 and 5, respectively. Antibodies against claudin 7 and occludin revealed a strong band of 22.3 kDa for claudin 7 and double bands at 54 and 64 kDa for occludin.

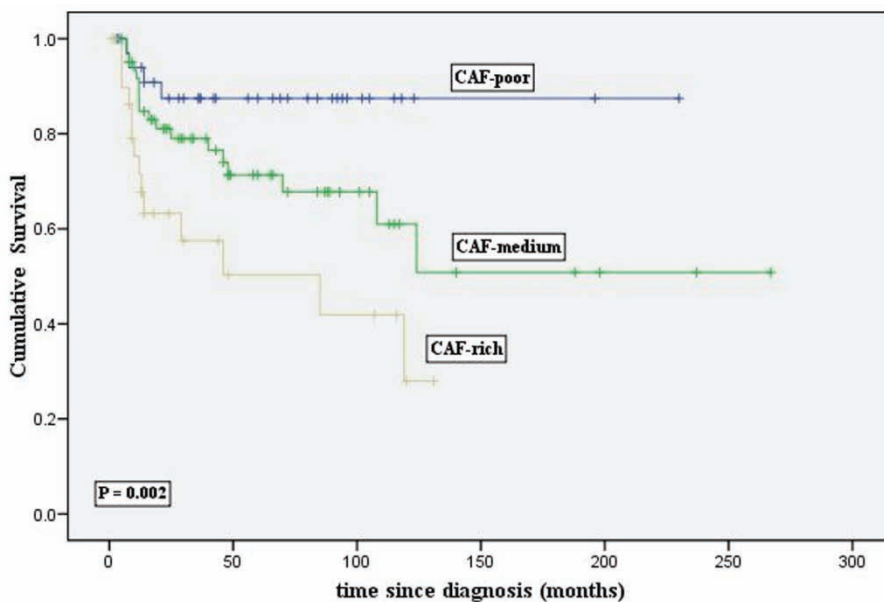
## **5.2 Cancer-associated fibroblasts in ameloblastoma, ameloblastic carcinoma and OTSCC (III and IV)**

### **5.2.1 Pattern of staining and prognosis**

In the ameloblastomas, strong immunoreactivity to  $\alpha$ -SMA was found in the stroma surrounding the tumors. In general, the benign tumors had less

immunoreactivity compared to the malignant tumors. In ameloblastic carcinoma,  $\alpha$ -SMA immunoreactivity was also found within the epithelial islands. CAF density was not significantly different between ameloblastoma and ameloblastic carcinoma, although in the latter the quantity had higher scores and they were haphazard in arrangement.

OTSCC displayed variable staining for  $\alpha$ -SMA but it was generally noted that they were more numerous at the invasive front than at the superficial parts of the tumor and that the quantity stained decreased when there was increased inflammatory cell infiltration in the area around the tumor. Increased CAF density was associated with cancer-specific mortality but not with mortality due to other causes (Figure 3).



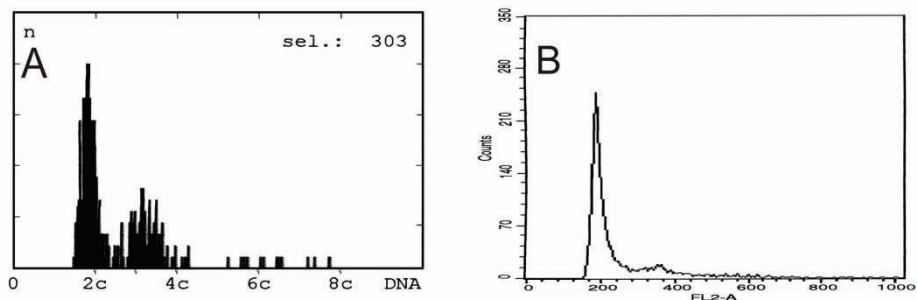
**Fig. 3. Disease-specific survival in OTSCC patients in relation to the tumor CAF density. Increased CAF density was strongly associated with death due to OTSCC.**

When adjusted for age and neck metastasis, increasing CAF density was an independent factor in increased mortality of patients with OTSCC. There was a stepwise increase in mortality as CAF density increased. Using the lowest scores (0 to 1) as the reference, the HRs for deaths from OTSCC adjusted for age and neck metastasis were 1.94 (95% CI 0.64 - 5.83;  $P = 0.239$ ) for CAF-medium, and 3.91, (95% CI 1.25 - 12.2;  $P = 0.019$ ) for CAF-rich.

### 5.3 DNA content by image (static) and flow cytometry (III and IV)

In cases where ICM (after using cell disintegration and filtration) was successfully done, all the ameloblastomas were diploid except in one case of ameloblastic carcinoma that was aneuploid. Direct ICM using prepared slides from the paraffin blocks (without cell disintegration and filtration) was also attempted for all ameloblastomas and ameloblastic carcinomas, but there was severe overlapping of nuclei in all the samples, making assessment of individual nuclei impossible. Therefore no result was obtained from direct ICM.

Flow cytometric analysis results obtained showed that 16 of the samples of ameloblastomas had interpretable histograms. The ameloblastic carcinoma that was aneuploid in ICM was diploid by FCM, although it showed a high s-phase fraction, an indication that it was abnormal (Figure 4). Other histograms were similar to those obtained from ICM in cases where both methods yielded good histograms.



**Fig. 4. Histograms from ICM (A) and FCM (B) from the same case of ameloblastic carcinoma. The aneuploid clone that was visible in A is not seen in B, although the S-phase fraction in B was high (18.7%). The aneuploid clone has probably been masked by excessive cell debris or large amounts of DNA diploid cells (e.g. lymphocytes) in B.**

In OTSCC, direct ICM was used and this was found not to be related to the clinicopathologic factors and cancer-specific mortality of the patients. Using diploid tumors as the reference group, there was no association between aneuploidy and cancer-specific mortality (HR 1.0, 95% CI 0.33–3.09;  $P = 0.994$ ) or tetraploidy and cancer-specific mortality (HR 1.08, 95% CI 0.27–4.32;  $P = 0.917$ )

#### **5.4 Ki-67 staining and labelling index (LI) (III and IV)**

In ameloblastomas, all the tissue sections demonstrated variable amounts of positively stained nuclei by Ki-67 of both peripheral and central cells. There was no clear-cut difference in the staining patterns of the different histologic types of ameloblastoma. However, the pattern seemed to be more orderly in the follicular type, with the peripheral or suprabasal cells showing more staining than the more centrally located cells. The mean percentage LI for the benign ameloblastomas was 6.4% and for the ameloblastic carcinomas 18.2%. There was a statistically significant difference in Ki-67 LI median values between the two groups ( $P = 0.01$ ). There was no significant statistical difference in Ki-67 LI between follicular and plexiform ameloblastoma ( $P = 0.38$ ).

In the mobile tongue cancers, variable staining of the nuclei of the tumor cells was also observed. However, the staining did not seem to be associated with clinicopathologic factors ( $P > 0.05$ ) and mortality due to OTSCC in the patients when low staining was compared to medium (HR 0.58; 95% CI 0.19–1.78;  $P = 0.342$ ), and high (HR 0.85; 95% CI 0.29–2.45;  $P = 0.760$ ).

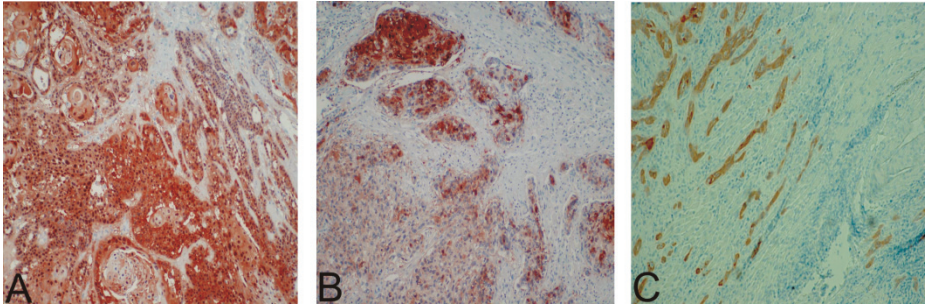
#### **5.5 EMA, Calponin and p63 (III)**

EMA did not stain the ameloblastoma and ameloblastic carcinomas strongly. It also did not show any differential staining between the two tumors. Calponin and p63 were used in staining all ameloblastic carcinomas and two randomly chosen benign ameloblastomas to assess myoepithelial differentiation. p63 stained the epithelial cells while calponin only stained a few cells in one of the ameloblastic carcinomas. They were both negative in the stroma.

#### **5.6 Maspin staining (IV)**

All OTSCC showed variable degrees of maspin immunoreactivity, which was generally more intense than the overlying epithelium. Cells more centrally placed within the tumor masses displayed greater intensity than those that were peripheral. In addition, the pattern of invasion appears to influence the immunoreactivity: large pushing invading masses showed higher intensity than those invading as smaller strands or cords or widely dissociated cells (Figure 5). Maspin immunoreactivity showed some evidence of association with OTSCC-specific mortality with regard to the medium score compared to the low score in

the adjusted model using neck metastasis and age as additional factors, but this was not found when the high score was compared to the low score. The adjusted model had HR 3.26 (95% CI 1.15–9.26;  $P = 0.027$ ) for medium score and HR 1.55 (95% CI 0.49–4.88;  $P = 0.452$ ) for high score when both were compared to low score. The wide overlap in CI value obviously showed some association between the maspin score and the prognosis of the patients, but the statistical evidence was rather weak.



**Fig. 5. Maspin staining in OTSCC. Strong staining in most cells (A), in less than half of the cells (B) invading as solid masses, and weak staining in most cells (C) invading as dissociated cords or cells.**

## 6 Discussion

### 6.1 Claudins and Occludin in ameloblastoma, ameloblastic carcinoma and the tooth germ

This study found no significant difference in the expression of claudins and occludin between ameloblastoma and ameloblastic carcinoma. No previous study of TJ proteins in ameloblastoma was found in the literature. Based on this study, TJ proteins may have a limited role in the development and progression of these tumors. However, this would need further clarification. Claudin and occludin expression has been studied in many epithelial tumors (Lanigan *et al.* 2009, Nakanishi *et al.* 2008, Nemeth *et al.* 2009, Pan *et al.* 2007, Soini *et al.* 2006). Both increased expression and down-regulation of these proteins has been implicated in the various tumors studied. This is to be expected, as claudins are a multigene family comprising up to 24 different members, with each showing a unique tissue expression pattern. Even in the same organ, certain cell types co-express multiple claudins whose combination and proportion vary (Furuse & Tsukita 2006).

The study also showed that the TJ proteins' expression in the ameloblastomas is similar to that found in the developing tooth. TJ proteins have been studied in the developing teeth of rats and mice (Hoshino *et al.* 2008, Ohazama & Sharpe 2007). These studies concluded that TJs of the epithelial cells of the odontogenic apparatus show specific expression of claudins and occludin and that this may play a role in the differentiation of the epithelial cells. Moreover, it is noteworthy that ameloblastomas are derived from the odontogenic epithelium, with potential sources including the enamel organ, odontogenic rests (rests of Malassez and Serres), reduced enamel epithelium and epithelial lining of odontogenic cysts. The need for maintaining cell-cell attachment may also be a reason for the overexpression of claudins in the central stellate reticulum-like cells, an area where microcysts usually develop in ameloblastoma. However, changes in the central cells of ameloblastoma have not been linked with clinical tumor behavior, and the same pattern of staining was also observed in the normal developing tooth.

## 6.2 Claudins and Occludin in OTSCC

The present study showed that increased or decreased expression of claudin 7 relative to the normal epithelium overlying the tumor or epithelium adjacent to the ulcerated superficial portion of the tumor (in terms of intensity and quantity) was associated with decreased cancer-related survival. TJ proteins have not been studied in OTSCC, but previous reports for head and neck regions have shown that claudin 7 expression was down-regulated in HNSCC (Al Moustafa *et al.* 2002). Most of the studies reported have been done on esophageal SCC (Lioni *et al.* 2007, Miyamoto *et al.* 2008, Takala *et al.* 2007, Usami *et al.* 2006). The results have been conflicting, although the strongest body of evidence seemed to favor the decreased expression of claudin 7 especially in terms of increasing the invasive capacity of the tumor cells. Lioni *et al.* (2007) have shown that mislocalization of claudin 7 occurs in esophageal keratinocytes during malignant transformation, and this leads to loss of E-cadherin expression and increased invasion in esophageal SCC. No similar study has been done in tongue cancer. However, loss of E-cadherin expression has also been found to be related to poor prognosis in mobile tongue cancer (Chang *et al.* 2002).

Changes at the invasive front of oral SCC have been thought of as being of prognostic importance. The invasive front grading suggested by Bryne *et al.* (1992) is based on this principle. Although we did not find any association between invasive front grading and cancer-specific survival, we did find that derangement in claudin 7 expression at this site is predictive of poor prognosis.

It is thought that reduced expression of claudin 7 leads to dismantling of the TJs and progression of the tumor. The finding that overexpression of claudin 7 is associated with poor prognosis is explained by the fact that the resultant protein may also lead to disrupted TJ function by mechanisms such as increasing the activity of matrix metalloproteinase 2 or affecting cell signaling pathways by interacting with ZO-1 or by some unknown mechanisms (Agarwal *et al.* 2005, Furuse *et al.* 2001, Oku *et al.* 2006). Overexpression of claudin 7 may actually be of non-functional claudin 7 which promotes tumorigenesis. Although claudins 1, 4 and 5 showed varying staining patterns in the tumors, they were not found to be related to the prognosis of OTSCC. Occludin showed weak or absent staining in most tumors and was not found to be of any prognostic significance.

### **6.3 Cancer-associated fibroblasts in ameloblastoma, ameloblastic carcinoma and OTSCC**

CAFs were found in ameloblastoma and ameloblastic carcinoma. Ameloblastic carcinoma of the maxilla is associated with particularly poor prognosis and high rate of pulmonary metastasis (Dhir *et al.* 2003). It would therefore serve as a good model for assessing poor prognosis in ameloblastomas. The presence of CAFs in the stroma in relation to benign ameloblastoma may be one explanation for its relatively aggressive behavior as a benign tumor. CAFs have not been extensively studied in odontogenic tumors. The present study seemed to be in congruence with a previous study which showed that ameloblastoma and keratocystic odontogenic tumor (KOT) had high CAF density which was not significantly different from that found in OSCC (Vered *et al.* 2005). Solid/multicystic ameloblastoma and KOT are well recognized as the most aggressive benign odontogenic tumors in terms of invasive capacity.

Ameloblastic carcinomas in this study had abundant CAFs but in addition, also showed the presence of  $\alpha$ -SMA positive cells within the epithelial islands. This was an incidental finding which is not readily explained. Moreover, this was not restricted to the peripheral cells but also to the central cells. A plausible explanation for this is that the cells may have acquired a myofibroblastic phenotype, a necessary prelude to EMT.

In OTSCC, the density of CAF was significantly associated with increased disease-specific mortality and still remained an independent prognostic factor even after adjusting for other factors that affected prognosis in this study, such as pathologically diagnosed nodal metastasis and age at diagnosis. Locoregional recurrence was also a very important indicator of mortality but was not included in the adjusted model because it was not a baseline variable. This study showed a stepwise association with mortality increasing in OTSCC as the density of CAFs increases. The relative aggressiveness of mobile tongue cancers may therefore be contributed in part by the increased CAFs acting on mechanisms already discussed above in relation to EMT/MET. Other mechanisms reported in the literature include: a) production of many known tumor promoting factors including growth factors, chemokines, cell surface proteins and extracellular matrix proteins that greatly increase the metastatic and invasive potential of the tumor cells (Kalluri & Zeisberg 2006, Karnoub *et al.* 2007); b) expression of pathways that are complimentary to tumor cell growth and invasion, e.g. CAF cells express metabolic pathways that buffer and recycle the acidic products

generated by anaerobic metabolism of tumor cells (Koukourakis *et al.* 2006); c) tumor cells sometimes do not need to undergo extensive EMT but are led through the ECM by force-mediated and protease-mediated remodeling of the ECM by CAF cells (Gaggioli *et al.* 2007). d) CAF cells are also known to play a role in modulating the sensitivity of the tumor cells to anti-cancer therapy (Micke & Ostman 2004). A recent study done on OTSCC reported that abundant CAFs (reported by authors as stromal MFs) was associated with local recurrence and decreased survival, and is an independent predictor of tumor recurrence (Vered *et al.* 2009b).

#### **6.4 DNA content in ameloblastoma, ameloblastic carcinoma and OTSCC**

DNA ploidy analysis has traditionally been done using flow cytometry or image (static) cytometry. As expected, DNA aneuploidy/non-diploidy is more likely to be found in a carcinoma than in its corresponding benign tumor (Muller *et al.* 1993). The current study on ameloblastoma also demonstrated nuclear non-diploidy to be more associated with ameloblastic carcinoma. Like most studies involving ameloblastic carcinoma including that of Muller *et al.* (1993), there is the drawback that the cases are too few to make useful comparisons with ameloblastoma. As already noted, ameloblastic carcinoma is a very rare tumor.

In most studies that involve the use of both flow and image cytometry, it has always been an interesting exercise to compare the concordance rate of the histograms obtained in both methods. Both methods have their advantages and disadvantages (Alanen *et al.* 1998, van Diest *et al.* 1998). Concordance rate has been said to be in the region of 80%, although some investigators have obtained rates as high as 100% (Alanen *et al.* 1998, Baretton *et al.* 1995, Muller *et al.* 1993). Excluding tumors with uninterpretable histograms from either of the two methods, our rate was 92%, which falls within the usual values. It was also noted that where histograms are not in concord, the S-phase fraction value may be helpful in giving an indication that a diploid histogram may actually be abnormal.

In OTSCC, direct image cytometry was used in all the cases. The present results showed that DNA content was not related to prognosis of the patients. The majority of the tumors exhibited aneuploidy despite being associated with variable clinical outcome. Previous studies on OTSCC and other oral cancers have arrived at similar conclusions (Baretton *et al.* 1995, Cooke *et al.* 1994, Wangsa *et al.* 2008). In fact, in one of the studies, the aneuploidy rate in OTSCC

was 97% compared to 3% diploidy which ultimately precluded any attempt to compare the clinical outcomes in both groups of patients (Wangsa *et al.* 2008). The current impression is that genomic instability is very high in OTSCC and measuring the gross DNA content would not be helpful in prognostication. Some other investigators who have worked with all oral cancers (not specifically only on the tongue) have found DNA aneuploidy to be associated with poor prognosis or at least associated with increased cervical nodal metastasis (Balsara *et al.* 1994, Hemmer *et al.* 1999).

### **6.5 Tumor cell proliferative activity in ameloblastoma, ameloblastic carcinoma and OTSCC**

In ameloblastomas, there was a significant difference in the median Ki-67 LI between benign and malignant ameloblastoma. The ameloblastic carcinomas had a higher mean LI. However, two individual cases in the benign ameloblastomas showed high Ki-67 values that were comparable to those of malignant ameloblastoma. However, these cases seemed to be outliers when compared to the rest in the series. The significance of this finding is that Ki-67 index may be useful in comparing the two tumors, but benign ameloblastoma may sometimes have a high proliferative activity.

In OTSCC, a high Ki-67 LI has been associated with poor prognosis or increased locoregional recurrence (Silva *et al.* 2008a, Wangsa *et al.* 2008). The latter study found no association between Ki-67 expression and patient survival, however. Davies *et al.* (2006) similarly found no association between high Ki-67 index and recurrence. In fact, in their study they reported that low Ki-67 index was associated with a 6-fold increase in recurrence within 18 months. In this study, an association between Ki-67 index and patient mortality in OTSCC was not evident.

### **6.6 Maspin and OTSCC**

Maspin is known to have tumor suppressor properties. Yasumatsu *et al.* (2001) reported improved patient survival in patients with increased expression of maspin in early stage OTSCC. A similar finding was reported by Iezzi *et al.* (2007) for patients with OSCC. However, other investigators have found no association between patient prognosis and increased maspin expression in

OTSCC (Cho *et al.* 2007). This study did not find a strong statistical association between maspin and patients' prognosis.

## 7 Conclusions

In the present study, the contribution of tight junction proteins (claudins 1, 4, 5 and 7, and occludin) and cancer-associated fibroblasts as prognostic indicators was studied in ameloblastomas, ameloblastic carcinomas and mobile (oral) tongue carcinoma, OTSCC. Additional markers such as DNA content and Ki-67 (proliferation marker) were also studied in these lesions. It is shown here that claudin 7 and CAFs may play significant roles in poor prognosis in OTSCC and that the appearance of cell with myofibroblastic phenotype in epithelial areas of ameloblastic carcinoma may be an important factor for differentiation between ameloblastoma and ameloblastic carcinoma.

The specific conclusions of this study are as follows:

1. The staining patterns for TJ proteins do not seem to differ significantly between benign ameloblastoma and ameloblastic carcinoma.
2. The presence of cells with a myofibroblastic phenotype within ameloblastic carcinoma seems to be important in differentiating this tumor from benign ameloblastoma.
3.  $\alpha$ -SMA is more useful in differentiating ameloblastic carcinoma from ameloblastoma compared with Ki-67, EMA and DNA content of tumor cells.
4. Derangement in claudin 7 expression is associated with a poor disease-specific survival in OTSCC.
5. Claudins 1, 4, 5 and occludin expression patterns do not seem to be associated with disease-specific survival in OTSCC.
6. Abundance or increasing density of CAFs in the stroma of OTSCC is a strong marker of poor disease-specific survival in OTSCC. It is also a better predictor of prognosis in OTSCC compared with Ki-67, maspin and DNA content.
7. Routine staining for claudin 7 and  $\alpha$ -SMA may be beneficial for prognostication in OTSCC.



## References

- Acharya P, Beckel J, Ruiz WG, Wang E, Rojas R, Birder L & Apodaca G (2004) Distribution of the tight junction proteins ZO-1, occludin, and claudin-4, -8, and -12 in bladder epithelium. *Am J Physiol Renal Physiol* 287: F305-F318.
- Adebayo ET, Ajike SO & Adekeye EO (2005) A review of 318 odontogenic tumors in Kaduna, Nigeria. *J Oral Maxillofac Surg* 63: 811–819.
- Adeline VL, Dimba EA, Wakoli KA, Njiru AK, Awange DO, Onyango JF & Chindia ML (2008) Clinicopathologic features of ameloblastoma in Kenya: a 10-year audit. *J Craniofac Surg* 19: 1589–1593.
- Agarwal R, D'Souza T & Morin PJ (2005) Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. *Cancer Res* 65: 7378–7385.
- Ajagbe HA & Daramola JO (1982) Primary tumors of the jaw in Nigerian children. *J Natl Med Assoc* 74: 157–161.
- Akrish S, Buchner A, Shoshani Y, Vered M & Dayan D (2007) Ameloblastic carcinoma: report of a new case, literature review, and comparison to ameloblastoma. *J Oral Maxillofac Surg* 65: 777–783.
- Al Moustafa AE, Alaoui-Jamali MA, Batist G, Hernandez-Perez M, Serruya C, Alpert L, Black MJ, Sladek R & Foulkes WD (2002) Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells. *Oncogene* 21: 2634–2640.
- Alanen KA, Lintu M & Joensuu H (1998) Image cytometry of breast carcinomas that are DNA diploid by flow cytometry: time to revise the concept of DNA diploidy? *Anal Quant Cytol Histol* 20: 178–186.
- Almholt K & Johnsen M (2003) Stromal cell involvement in cancer. *Recent Results Cancer Res* 162: 31–42.
- Al-Rajhi N, Khafaga Y, El-Husseiny J, Saleem M, Mourad W, Al-Otieschan A & Al-Amro A (2000) Early stage carcinoma of oral tongue: prognostic factors for local control and survival. *Oral Oncol* 36: 508–514.
- Angiero F, Borloni R, Macchi M & Stefani M (2008) Ameloblastic carcinoma of the maxillary sinus. *Anticancer Res* 28: 3847–3854.
- Annertz K, Anderson H, Biorklund A, Moller T, Kantola S, Mork J, Olsen JH & Wennerberg J (2002) Incidence and survival of squamous cell carcinoma of the tongue in Scandinavia, with special reference to young adults. *Int J Cancer* 101: 95–99.
- Arotiba JT, Ogunbiyi JO & Obiechina AE (1997) Odontogenic tumours: a 15-year review from Ibadan, Nigeria. *Br J Oral Maxillofac Surg* 35: 363–367.
- Asakage T, Yokose T, Mukai K, Tsugane S, Tsubono Y, Asai M & Ebihara S (1998) Tumor thickness predicts cervical metastasis in patients with stage I/II carcinoma of the tongue. *Cancer* 82: 1443–1448.

- Atula S, Grenman R, Laippala P & Syrjanen S (1996) Cancer of the tongue in patients younger than 40 years. A distinct entity? *Arch Otolaryngol Head Neck Surg* 122: 1313–1319.
- Bailey CM, Khalkhali-Ellis Z, Seftor EA & Hendrix MJ (2006) Biological functions of maspin. *J Cell Physiol* 209: 617–624.
- Balkovetz DF (2006) Claudins at the gate: determinants of renal epithelial tight junction paracellular permeability. *Am J Physiol Renal Physiol* 290: F572-F579.
- Balsara BR, Borges AM, Pradhan SA, Rajpal RM & Bhisey AN (1994) Flow cytometric DNA analysis of squamous cell carcinomas of the oral cavity: correlation with clinical and histopathological features. *Eur J Cancer B Oral Oncol* 30B: 98–101.
- Baretton G, Li X, Stoll C, Fischer-Brandies E, Schmidt M & Lohrs U (1995) Prognostic significance of DNA ploidy in oral squamous cell carcinomas. A retrospective flow and image cytometric study with comparison of DNA ploidy in excisional biopsy specimens and resection specimens, primary, tumors, and lymph node metastases. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 79: 68–76.
- Barnes L, Eveson JW, Reichart P & Sidransky D (2005) World Health Organization Classification of Tumours. Pathology and Genetics of Head and Neck Tumours. IARC Press: Lyon.
- Baum B, Settleman J & Quinlan MP (2008) Transitions between epithelial and mesenchymal states in development and disease. *Semin Cell Dev Biol* 19: 294–308.
- Benlyazid A, Lacroix-Triki M, Aziza R, Gomez-Brouchet A, Guichard M & Sarini J (2007) Ameloblastic carcinoma of the maxilla: case report and review of the literature. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 104: e17-e24.
- Berrino F & Gatta G (1998) Variation in survival of patients with head and neck cancer in Europe by the site of origin of the tumours. EUROCORE Working Group. *Eur J Cancer* 34: 2154–2161.
- Block G, Patterson B & Subar A (1992) Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer* 18: 1–29.
- Blot WJ, McLaughlin JK, Winn DM, Austin DF, Greenberg RS, Preston-Martin S, Bernstein L, Schoenberg JB, Stemhagen A & Fraumeni JF Jr (1988) Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res* 48: 3282–3287.
- Boeing H, Dietrich T, Hoffmann K, Pischon T, Ferrari P, Lahmann PH, Boutron-Ruault MC, Clavel-Chapelon F, Allen N, Key T, Skeie G, Lund E, Olsen A, Tjønneland A, Overvad K, Jensen MK, Rohrmann S, Linseisen J, Trichopoulou A, Bamia C, Psaltopoulou T, Weinehall L, Johansson I, Sanchez MJ, Jakszyn P, Ardanaz E, Amiano P, Chirlaque MD, Quiros JR, Wirfalt E, Berglund G, Peeters PH, van Gils CH, Bueno-de-Mesquita HB, Buchner FL, Berrino F, Palli D, Sacerdote C, Tumino R, Panico S, Bingham S, Khaw KT, Slimani N, Norat T, Jenab M & Riboli E (2006) Intake of fruits and vegetables and risk of cancer of the upper aero-digestive tract: the prospective EPIC-study. *Cancer Causes Control* 17: 957–969.
- Boffetta P & Hashibe M (2006) Alcohol and cancer. *Lancet Oncol* 7: 149–156.

- Bouquot JE & Meckstroth RL (1998) Oral cancer in a tobacco-chewing US population—no apparent increased incidence or mortality. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 86: 697–706.
- Bova RJ, Quinn DI, Nankervis JS, Cole IE, Sheridan BF, Jensen MJ, Morgan GJ, Hughes CJ & Sutherland RL (1999) Cyclin D1 and p16INK4A expression predict reduced survival in carcinoma of the anterior tongue. *Clin Cancer Res* 5: 2810–2819.
- Bredenkamp JK, Zimmerman MC & Mickel RA (1989) Maxillary ameloblastoma. A potentially lethal neoplasm. *Arch Otolaryngol Head Neck Surg* 115: 99–104.
- Brown B, Barnes L, Mazariegos J, Taylor F, Johnson J & Wagner RL (1989) Prognostic factors in mobile tongue and floor of mouth carcinoma. *Cancer* 64: 1195–1202.
- Bryne M, Koppang HS, Lilleng R, Stene T, Bang G & Dabelsteen E (1989) New malignancy grading is a better prognostic indicator than Broders' grading in oral squamous cell carcinomas. *J Oral Pathol Med* 18: 432–437.
- Buchner A, Merrell PW & Carpenter WM (2006) Relative frequency of central odontogenic tumors: a study of 1,088 cases from Northern California and comparison to studies from other parts of the world. *J Oral Maxillofac Surg* 64: 1343–1352.
- Byers RM, Weber RS, Andrews T, McGill D, Kare R & Wolf P (1997) Frequency and therapeutic implications of "skip metastases" in the neck from squamous carcinoma of the oral tongue. *Head Neck* 19: 14–19.
- Campisi G & Giovannelli L (2009) Controversies surrounding Human Papilloma Virus infection, head & neck vs oral cancer, implications for prophylaxis and treatment. *Head Neck Oncol* 1: 8.
- CDC (Center for Disease Control) (1998) Preventing and controlling oral and pharyngeal cancer. Recommendations from a National Strategic Planning Conference. *MMWR Recomm Rep* 47: 1–12.
- Chaffer CL, Thompson EW & Williams ED (2007) Mesenchymal to epithelial transition in development and disease. *Cells Tissues Organs* 185: 7–19.
- Chang HW, Chow V, Lam KY, Wei WI & Yuen A (2002) Loss of E-cadherin expression resulting from promoter hypermethylation in oral tongue carcinoma and its prognostic significance. *Cancer* 94: 386–392.
- Chang JY, Wright JM & Svoboda KK (2007) Signal transduction pathways involved in epithelial-mesenchymal transition in oral cancer compared with other cancers. *Cells Tissues Organs* 185: 40–47.
- Chen YW, Yu EH, Wu TH, Lo WL, Li WY & Kao SY (2008) Histopathological factors affecting nodal metastasis in tongue cancer: analysis of 94 patients in Taiwan. *Int J Oral Maxillofac Surg* 37: 912–916.
- Cho JH, Kim HS, Park CS, Kim JK, Jung KY, Shin BK & Kim HK (2007) Maspin expression in early oral tongue cancer and its relation to expression of mutant-type p53 and vascular endothelial growth factor (VEGF). *Oral Oncol* 43: 272–277.
- Chow V, Yuen AP, Lam KY, Tsao GS, Ho WK & Wei WI (2001) A comparative study of the clinicopathological significance of E-cadherin and catenins (alpha, beta, gamma) expression in the surgical management of oral tongue carcinoma. *J Cancer Res Clin Oncol* 127: 59–63.

- Chuang HC, Su CY, Huang HY, Chien CY, Chen CM & Huang CC (2006) High expression of CD105 as a prognostic predictor of early tongue cancer. *Laryngoscope* 116: 1175–1179.
- Cohen SJ, Alpaugh RK, Palazzo I, Meropol NJ, Rogatko A, Xu Z, Hoffman JP, Weiner LM & Cheng JD (2008) Fibroblast activation protein and its relationship to clinical outcome in pancreatic adenocarcinoma. *Pancreas* 37: 154–158.
- Colella S, Richards KL, Bachinski LL, Baggerly KA, Tsavachidis S, Lang JC, Schuller DE & Krahe R (2008) Molecular signatures of metastasis in head and neck cancer. *Head Neck* 30: 1273–1283.
- Colilla SA (2009) An epidemiologic review of smokeless tobacco health effects and harm reduction potential. *Regul Toxicol Pharmacol* Sep 29. (In press).
- Conway D (2009) Oral health, mouthwashes and cancer—what is the story? *Evid Based Dent* 10: 6–7.
- Cooke LD, Cooke TG, Forster G, MacDonald DG, Robertson AG & Soutar DS (1994) Flow cytometric analysis of DNA content in squamous carcinoma of the tongue: the relationship to host and tumour factors and survival. *Clin Otolaryngol Allied Sci* 19: 131–134.
- Dahlgren L, Dahlstrand HM, Lindquist D, Hogmo A, Bjornestal L, Lindholm J, Lundberg B, Dalianis T & Munck-Wikland E (2004) Human papillomavirus is more common in base of tongue than in mobile tongue cancer and is a favorable prognostic factor in base of tongue cancer patients. *Int J Cancer* 112: 1015–1019.
- Davidson BJ, Root WA & Trock BJ (2001) Age and survival from squamous cell carcinoma of the oral tongue. *Head Neck* 23: 273–279.
- Davies L, Hardin NJ & Beatty BG (2006) Can Ki-67 predict recurrence of NO squamous cell carcinoma of the tongue? *Ann Otol Rhinol Laryngol* 115: 12–17.
- De Stefani E, Boffetta P, Ronco AL, Correa P, Oreggia F, Deneo-Pellegrini H, Mendilaharsu M & Leiva J (2005) Dietary patterns and risk of cancer of the oral cavity and pharynx in Uruguay. *Nutr Cancer* 51: 132–139.
- De Stefani E, Brennan P, Boffetta P, Ronco AL, Mendilaharsu M & Deneo-Pellegrini H (2000). Vegetables, fruits, related dietary antioxidants, and risk of squamous cell carcinoma of the esophagus: a case-control study in Uruguay. *Nutr Cancer* 38: 23–29.
- de Vicente JC, Olay S, Lequerica-Fernandez P, Sanchez-Mayoral J, Junquera LM & Fresno MF (2006) Expression of Bcl-2 but not Bax has a prognostic significance in tongue carcinoma. *J Oral Pathol Med* 35: 140–5.
- De Wever O, Demetter P, Mareel M & Bracke M (2008) Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* 123: 2229–2238.
- Dhir K, Sciubba J & Tufano RP (2003) Ameloblastic carcinoma of the maxilla. *Oral Oncol* 39: 736–741.
- Dickman PW, Hakulinen T, Luostarinen T, Pukkala E, Sankila R, Soderman B & Teppo L (1999) Survival of cancer patients in Finland 1955–1994. *Acta Oncol* 38 (Suppl 12): 1–103.

- El-Husseiny G, Kandil A, Jamshed A, Khafaga Y, Saleem M, Allam A, Al-Rajhi N, Al-Amro A, Rostom AY, Abuzeid M, Otieschan A & Flores AD (2000) Squamous cell carcinoma of the oral tongue: an analysis of prognostic factors. *Br J Oral Maxillofac Surg* 38: 193–199.
- Falkmer UG, Hagmar T & Auer G (1990) Efficacy of combined image and flow cytometric DNA assessments in human breast cancer - a methodological study based on a routine histopathological material of 2024 excised tumor specimens. *Anal Cell Pathol* 2: 297–312.
- Faustino SE, Oliveira DT, Nonogaki S, Landman G, Carvalho AL & Kowalski LP (2008) Expression of vascular endothelial growth factor-C does not predict occult lymph-node metastasis in early oral squamous cell carcinoma. *Int J Oral Maxillofac Surg* 37: 372–378.
- Fernberg SE & Steinberg B (1996) Surgical management of ameloblastoma. Current status of the literature. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 81: 383–388.
- Fernandez MM, Garcia-Rozado A & Parente PL (2007) Is microvascular density an independent prognostic factor in squamous cell carcinoma of the tongue? *Acta Otorrinolaringol Esp* 58: 341–346.
- Finnish Cancer Registry (2007a) Cancer in Finland 2004 and 2005. Cancer Society of Finland Publication No. 72.
- Finnish Cancer Registry (2007b) Cancer statistics: Age-adjusted incidence rates of cancer per 100,000 person-years in 1961–2007, by primary site and period. URI: <http://www.cancerregistry.fi/eng/statistics/AID122.html>. Cited 2009/10/25.
- Fioretti F, Bosetti C, Tavani A, Franceschi S & La Vecchia C (1999) Risk factors for oral and pharyngeal cancer in never smokers. *Oral Oncol* 35: 375–378.
- Forootan SS, Ke Y, Jones AS & Helliwell TR (2000) Basic fibroblast growth factor and angiogenesis in squamous carcinoma of the tongue. *Oral Oncol* 36: 437–443.
- Franceschi D, Gupta R, Spiro RH & Shah JP (1993). Improved survival in the treatment of squamous carcinoma of the oral tongue. *Am J Surg* 166: 360–365.
- Franceschi S, Barra S, La Vecchia C, Bidoli E, Negri E & Talamini R (1992) Risk factors for cancer of the tongue and the mouth. A case-control study from northern Italy. *Cancer* 70: 2227–2233.
- Franceschi S, Talamini R, Barra S, Baron AE, Negri E, Bidoli E, Serraino D & La Vecchia C (1990) Smoking and drinking in relation to cancers of the oral cavity, pharynx, larynx, and esophagus in northern Italy. *Cancer Res* 50: 6502–6507.
- Fujii M, Ishiguro R, Yamashita T & Tashiro M (2001) Cyclin D1 amplification correlates with early recurrence of squamous cell carcinoma of the tongue. *Cancer Lett* 172: 187–192.
- Furuse M, Furuse K, Sasaki H & Tsukita S (2001) Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *J Cell Biol* 153: 263–272.
- Furuse M & Tsukita S (2006) Claudins in occluding junctions of humans and flies. *Trends Cell Biol* 16: 181–8.

- Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K & Sahai E (2007). Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol* 9: 1392–1400.
- Garavello W, Spreafico R & Gaini RM (2007) Oral tongue cancer in young patients: a matched analysis. *Oral Oncol* 43: 894–7.
- Ghandhi D, Ayoub AF, Pogrel MA, MacDonald G, Brocklebank LM & Moos KF (2006) Ameloblastoma: a surgeon's dilemma. *J Oral Maxillofac Surg* 64: 1010–1014.
- Gonzalez-Mariscal L, Betanzos A, Nava P & Jaramillo BE (2003) Tight junction proteins. *Prog Biophys Mol Biol* 81: 1–44.
- Goto H, Kawano K, Kobayashi I, Sakai H & Yanagisawa S (2002) Expression of cyclin D1 and GSK-3beta and their predictive value of prognosis in squamous cell carcinomas of the tongue. *Oral Oncol* 38: 549–556.
- Goto M, Tsukamoto T, Inada K, Mizoshita T, Ogawa T, Terada A, Hyodo I, Shimozato K, Hasegawa Y & Tatematsu M (2005) Loss of p21WAF1/CIP1 expression in invasive fronts of oral tongue squamous cell carcinomas is correlated with tumor progression and poor prognosis. *Oncol Rep* 14: 837–846.
- Gupta PC, Murti PR & Bhonsle RB (1996) Epidemiology of cancer by tobacco products and the significance of TSNA. *Crit Rev Toxicol* 26: 183–198.
- Harada H, Omura K, Nakajima Y, Hasegawa S & Mogi S (2006) Cyclin B1 is useful to predict occult cervical lymph node metastases in tongue carcinoma. *J Exp Clin Cancer Res* 25: 351–356.
- Harris C, Warnakulasuriya KA, Gelbier S, Johnson NW & Peters TJ (1997) Oral and dental health in alcohol misusing patients. *Alcohol Clin Exp Res* 21: 1707–1709.
- Heikinheimo K, Jee KJ, Niini T, Aalto Y, Happonen RP, Leivo I & Knuutila S (2002) Gene expression profiling of ameloblastoma and human tooth germ by means of a cDNA microarray. *J Dent Res* 81: 525–530.
- Hemmer J & Kreidler J (1990) Flow cytometric DNA ploidy analysis of squamous cell carcinoma of the oral cavity. Comparison with clinical staging and histologic grading. *Cancer* 66: 317–320.
- Hemmer J, Nagel E & Kraft K (1999) DNA aneuploidy by flow cytometry is an independent prognostic factor in squamous cell carcinoma of the oral cavity. *Anticancer Res* 19: 1419–1422.
- Hindle I, Downer MC, Moles DR & Speight PM (2000) Is alcohol responsible for more intra-oral cancer? *Oral Oncol* 36: 328–333.
- Hoffmann D & Hecht SS (1985) Nicotine-derived N-nitrosamines and tobacco-related cancer: current status and future directions. *Cancer Res* 45: 935–944.
- Högmo A, Kuylenskierna R, Lindholm J & Munck-Wikland E (1999) Predictive value of malignancy grading systems, DNA content, p53, and angiogenesis for stage I tongue carcinomas. *J Clin Pathol* 52: 35–40.
- Homann N, Tillonen J, Rintamaki H, Salaspuro M, Lindqvist C & Meurman JH (2001) Poor dental status increases acetaldehyde production from ethanol in saliva: a possible link to increased oral cancer risk among heavy drinkers. *Oral Oncol* 37: 153–158.

- Hosal AS, Unal OF & Ayhan A (1998) Possible prognostic value of histopathologic parameters in patients with carcinoma of the oral tongue. *Eur Arch Otorhinolaryngol* 255: 216–219.
- Hoshino M, Hashimoto S, Muramatsu T, Matsuki M, Ogiuchi H & Shimono M (2008) Claudin rather than occludin is essential for differentiation in rat incisor odontoblasts. *Oral Dis* 14: 606–612.
- Hossain Z & Hirata T (2008) Molecular mechanism of intestinal permeability: interaction at tight junctions. *Mol Biosyst* 4: 1181–1185.
- Hyam DM, Conway RC, Sathiyaseelan Y, Gebiski V, Morgan GJ, Walker DM & Veness MJ (2003) Tongue cancer: do patients younger than 40 do worse? *Aust Dent J* 48: 50–54.
- IARC. (1988). International Agency for Research on Cancer, Alcohol drinking. IARC monographs on the evaluation of carcinogenic risks to humans, Vol 44: Lyon.
- Iezzi G, Piattelli A, Rubini C, Goteri G, Artese L, Perrotti V & Carinci F (2007) Maspin expression in oral squamous cell carcinoma. *J Craniofac Surg* 18: 1039–1043.
- Ikenouchi J, Matsuda M, Furuse M & Tsukita S (2003) Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. *J Cell Sci* 116: 1959–1967.
- Infante JR, Matsubayashi H, Sato N, Tonascia J, Klein AP, Riall TA, Yeo C, Iacobuzio-Donahue C & Goggins M (2007) Peritumoral fibroblast SPARC expression and patient outcome with resectable pancreatic adenocarcinoma. *J Clin Oncol* 25: 319–325.
- Jackson IT, Callan PP & Forte RA (1996) An anatomical classification of maxillary ameloblastoma as an aid to surgical treatment. *J Craniomaxillofac Surg* 24: 230–236.
- Jakobsson PA, Eneroth CM, Killander D, Moberger G & Mårtensson B (1973) Histologic classification and grading of malignancy in carcinoma of the larynx. *Acta Radiol Ther Phys Biol* 12: 1–8.
- Jing W, Xuan M, Lin Y, Wu L, Liu L, Zheng X, Tang W, Qiao J & Tian W (2007) Odontogenic tumours: a retrospective study of 1642 cases in a Chinese population. *Int J Oral Maxillofac Surg* 36: 20–25.
- Jovanovic A, Schulten EA, Kostense PJ, Snow GB & van der Waal I (1993) Tobacco and alcohol related to the anatomical site of oral squamous cell carcinoma. *J Oral Pathol Med*, 22: 459–462.
- Jung J, Cho NH, Kim J, Choi EC, Lee SY, Byeon HK, Park YM, Yang WS & Kim SH (2009) Significant invasion depth of early oral tongue cancer originated from the lateral border to predict regional metastases and prognosis. *Int J Oral Maxillofac Surg* 38: 653–660
- Kalluri R & Zeisberg M (2006) Fibroblasts in cancer. *Nat Rev Cancer* 6: 392–401.
- Kantola, S, Parikka M, Jokinen K, Hyrynkans K, Soini Y, Alho OP & Salo T (2000) Prognostic factors in tongue cancer - relative importance of demographic, clinical and histopathological factors. *Br J Cancer* 83: 614–619.

- Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R & Weinberg RA (2007) Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 449: 557–563.
- Katoh K, Nakanishi Y, Akimoto S, Yoshimura K, Takagi M, Sakamoto M & Hirohashi S (2002) Correlation between laminin-5 gamma2 chain expression and epidermal growth factor receptor expression and its clinicopathological significance in squamous cell carcinoma of the tongue. *Oncology* 62: 318–326.
- Kawano K & Yanagisawa S (2006) Predictive value of laminin-5 and membrane type 1-matrix metalloproteinase expression for cervical lymph node metastasis in T1 and T2 squamous cell carcinomas of the tongue and floor of the mouth. *Head Neck* 28: 525–533.
- Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, Nishimoto I, Kowalski LP & Coletta RD (2007) Myofibroblasts in the stroma of oral squamous cell carcinoma are associated with poor prognosis. *Histopathology* 51: 849–853.
- Keum KC, Chung EJ, Koom WS, Cho JH, Cho SH, Choi EC, Lee CG, Suh CO & Kim GE (2006) Predictive value of p53 and PCNA expression for occult neck metastases in patients with clinically node-negative oral tongue cancer. *Otolaryngol Head Neck Surg* 135: 858–864.
- Kim SH, Cho NH, Kim K, Lee JS, Koo BS, Kim JH, Chang JH & Choi EC (2006) Correlations of oral tongue cancer invasion with matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) expression. *J Surg Oncol* 93: 330–337.
- Kim SJ, Shin HJ, Jung KY, Baek SK, Shin BK, Choi J, Kim BS, Shin SW, Kim YH, Kim JS & Oosterwijk E (2007) Prognostic value of carbonic anhydrase IX and Ki-67 expression in squamous cell carcinoma of the tongue. *Jpn J Clin Oncol* 37: 812–819.
- Kiuchi-Saishin Y, Gotoh S, Furuse M, Takasuga A, Tano Y & Tsukita S (2002) Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. *J Am Soc Nephrol* 13: 875–886.
- Korpi JT, Kervinen V, Maklin H, Vaananen A, Lahtinen M, Laara E, Ristimaki A, Thomas G, Ylipalosaari M, Astrom P, Lopez-Otin C, Sorsa T, Kantola S, Pirila E & Salo T (2008) Collagenase-2 (matrix metalloproteinase-8) plays a protective role in tongue cancer. *Br J Cancer* 98: 766–775.
- Kosunen A, Pirinen R, Ropponen K, Pukkila M, Kellokoski J, Virtaniemi J, Sironen R, Juhola M, Kumpulainen E, Johansson R, Nuutinen J & Kosma VM (2007) CD44 expression and its relationship with MMP-9, clinicopathological factors and survival in oral squamous cell carcinoma. *Oral Oncol* 43: 51–59.
- Kosunen A, Ropponen K, Kellokoski J, Pukkila M, Virtaniemi J, Valtonen H, Kumpulainen E, Johansson R, Tammi R, Tammi M, Nuutinen J & Kosma VM (2004). Reduced expression of hyaluronan is a strong indicator of poor survival in oral squamous cell carcinoma. *Oral Oncol* 40: 257–263.
- Koukourakis MI, Giatromanolaki A, Harris AL & Sivridis E (2006) Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. *Cancer Res* 66: 632–637.

- Kurokawa H, Zhang M, Matsumoto S, Yamashita Y, Tomoyose T, Tanaka T, Fukuyama H & Takahashi T (2005) The high prognostic value of the histologic grade at the deep invasive front of tongue squamous cell carcinoma. *J Oral Pathol Med* 34: 329–333.
- Kurtz KA, Hoffman HT, Zimmerman B & Robinson RA (2005) Perineural and vascular invasion in oral cavity squamous carcinoma - Increased incidence on re-review of slides and by using immunohistochemical enhancement. *Arch Pathol Lab Med* 129: 354–359.
- Kwong RA, Kalish LH, Nguyen TV, Kench JG, Bova RJ, Cole IE, Musgrove EA & Sutherland RL (2005) p14ARF protein expression is a predictor of both relapse and survival in squamous cell carcinoma of the anterior tongue. *Clin Cancer Res* 11: 4107–4116.
- Lanigan F, McKiernan E, Brennan DJ, Hegarty S, Millikan RC, McBryan J, Jirstrom K, Landberg G, Martin F, Duffy MJ & Gallagher WM (2009) Increased claudin-4 expression is associated with poor prognosis and high tumour grade in breast cancer. *Int J Cancer* 124: 2088–2097.
- Leedy DA, Trune DR, Kronz JD, Weidner N & Cohen JI (1994) Tumor angiogenesis, the P53 antigen, and cervical metastasis in squamous cell carcinoma of the tongue. *Otolaryngol Head Neck Surg* 111: 417–422.
- Leipzig B, Cummings CW, Chung CT, Johnson JT & Sagerman RH (1982) Carcinoma of the anterior tongue. *Ann Otol Rhinol Laryngol* 91: 94–97.
- Lewin F, Norell SE, Johansson H, Gustavsson P, Wennerberg J, Biorklund A & Rutqvist LE (1998) Smoking tobacco, oral snuff, and alcohol in the etiology of squamous cell carcinoma of the head and neck: a population-based case-referent study in Sweden. *Cancer* 82: 1367–1375.
- Li S, Jiao J, Lu Z & Zhang M (2009) An essential role for N-cadherin and beta-catenin for progression in tongue squamous cell carcinoma and their effect on invasion and metastasis of Tca8113 tongue cancer cells. *Oncol Rep* 21: 1223–1233.
- Liang XH, Lewis J, Foote R, Smith D & Kademani D (2008) Prevalence and significance of human papillomavirus in oral tongue cancer: the Mayo Clinic experience. *J Oral Maxillofac Surg* 66: 1875–1880.
- Liao CT, Wang HM, Hsieh LL, Chang JT, Ng SH, Hsueh C, Lee LY, Lin CH, Chen IH, Kang CJ, Huang SF & Yen TC (2006) Higher distant failure in young age tongue cancer patients. *Oral Oncol* 42: 718–725.
- Lim SC, Zhang S, Ishii G, Endoh Y, Kodama K, Miyamoto S, Hayashi R, Ebihara S, Cho JS & Ochiai A (2004) Predictive markers for late cervical metastasis in stage I and II invasive squamous cell carcinoma of the oral tongue. *Clin Cancer Res* 10: 166–172.
- Lioni M, Brafford P, Andl C, Rustgi A, El-Deiry W, Herlyn M & Smalley KS (2007) Dysregulation of claudin-7 leads to loss of E-cadherin expression and the increased invasion of esophageal squamous cell carcinoma cells. *Am J Pathol* 170: 709–721.
- Llewellyn CD, Linklater K, Bell J, Johnson NW & Warnakulasuriya KA (2003) Squamous cell carcinoma of the oral cavity in patients aged 45 years and under: a descriptive analysis of 116 cases diagnosed in the South East of England from 1990 to 1997. *Oral Oncol* 39: 106–114.

- Lo WL, Kao SY, Chi LY, Wong YK & Chang RC (2003) Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: factors affecting survival. *J Oral Maxillofac Surg* 61: 751–758.
- MacDonald-Jankowski DS, Yeung R, Lee KM & Li TK (2004) Ameloblastoma in the Hong Kong Chinese. Part 1: systematic review and clinical presentation. *Dentomaxillofac Radiol* 33: 71–82.
- Macfarlane GJ, Sharp L, Porter S & Franceschi S (1996) Trends in survival from cancers of the oral cavity and pharynx in Scotland: a clue as to why the disease is becoming more common? *Br J Cancer* 73: 805–808.
- Martin TA, Harrison GM, Watkins G & Jiang WG (2008) Claudin-16 reduces the aggressive behavior of human breast cancer cells. *J Cell Biochem* 105: 41–52.
- Mathew Iype E, Pandey M, Mathew A, Thomas G, Sebastian P & Krishnan Nair M (2001) Squamous cell carcinoma of the tongue among young Indian adults. *Neoplasia* 3: 273–277.
- McLaughlin JK, Gridley G, Block G, Winn DM, Preston-Martin S, Schoenberg JB, Greenberg RS, Stemhagen A, Austin DF & Ershow AG (1988) Dietary factors in oral and pharyngeal cancer. *J Natl Cancer Inst* 80: 1237–1243.
- Menezes MB, Lehn CN & Goncatves AJ (2007) Epidemiological and histopathological data and E-cadherin-like prognostic factors in early carcinomas of the tongue and floor of mouth. *Oral Oncol* 43: 656–661.
- Micke P & Ostman A (2004) Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? *Lung Cancer* 45 (Suppl 2): S163-S175.
- Mineta H, Miura K, Ogino T, Takebayashi S, Misawa K & Ueda Y (2002) Vascular endothelial growth factor (VEGF) expression correlates with p53 and ki-67 expressions in tongue squamous cell carcinoma. *Anticancer Res* 22: 1039–1044.
- Mineta H, Miura K, Suzuki I, Takebayashi S, Amano H, Araki K, Harada H, Ichimura K, Wennerberg JP & Dictor MR (1999) Low p27 expression correlates with poor prognosis for patients with oral tongue squamous cell carcinoma. *Cancer* 85: 1011–1017.
- Mineta H, Miura K, Takebayashi S, Ueda Y, Misawa K, Harada H, Wennerberg J & Dictor M (2000) Cyclin D1 overexpression correlates with poor prognosis in patients with tongue squamous cell carcinoma. *Oral Oncol* 36: 194–198.
- Miyamoto K, Kusumi T, Sato F, Kawasaki H, Shibata S, Ohashi M, Hakamada K, Sasaki M & Kijima H (2008) Decreased expression of claudin-1 is correlated with recurrence status in esophageal squamous cell carcinoma. *Biomed Res* 29: 71–76.
- Mork J, Lie AK, Glatte E, Hallmans G, Jellum E, Koskela P, Moller B, Pukkala E, Schiller JT, Youngman L, Lehtinen M & Dillner J (2001). Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. *N Engl J Med* 344: 1125–1131.
- Muller B, Fischer B & Kreutz W (2000) An acidic microenvironment impairs the generation of non-major histocompatibility complex-restricted killer cells. *Immunology* 99: 375–384.

- Muller S, DeRose PB & Cohen C (1993) DNA ploidy of ameloblastoma and ameloblastic carcinoma of the jaws. Analysis by image and flow cytometry. *Arch Pathol Lab Med* 117: 1126–1131.
- Muwonge R, Ramadas K, Sankila R, Thara S, Thomas G, Vinoda J & Sankaranarayanan R (2008) Role of tobacco smoking, chewing and alcohol drinking in the risk of oral cancer in Trivandrum, India: a nested case-control design using incident cancer cases. *Oral Oncol* 44: 446–454.
- Myers JN, Elkins T, Roberts D & Byers RM (2000) Squamous cell carcinoma of the tongue in young adults: Increasing incidence and factors that predict treatment outcomes. *Otolaryngol Head Neck Surg* 122: 44–51.
- Nagler RM, Kerner H, Laufer D, Ben-Eliezer S, Minkov I & Ben-Itzhak O (2002) Squamous cell carcinoma of the tongue: the prevalence and prognostic roles of p53, Bcl-2, c-erbB-2 and apoptotic rate as related to clinical and pathological characteristics in a retrospective study. *Cancer Lett* 186: 137–150.
- Nakagawa T, Shibuya H, Yoshimura R, Miura M, Okada N, Kishimoto S, Amagasa M & Omura K (2003) Neck node metastasis after successful brachytherapy for early stage tongue carcinoma. *Radiother Oncol* 68: 129–135.
- Nakanishi K, Ogata S, Hiroi S, Tominaga S, Aida S & Kawai T (2008) Expression of occludin and claudins 1, 3, 4, and 7 in urothelial carcinoma of the upper urinary tract. *Am J Clin Pathol* 130: 43–49.
- Namin AK, Azad TM, Eslami B, Sarkarat F, Shahrokhi M & Kashanian F (2003) A study of the relationship between ameloblastoma and human papilloma virus. *J Oral Maxillofac Surg* 61: 467–470.
- Naresh KN, Lakshminarayanan K, Pai SA & Borges AM (2001) Apoptosis index is a predictor of metastatic phenotype in patients with early stage squamous carcinoma of the tongue - A hypothesis to support this paradoxical association. *Cancer* 91: 578–584.
- Narkio-Makela M, Pukkila M, Lagerstedt E, Virtaniemi J, Pirinen R, Johansson R, Kosunen A, Lappalainen K, Hamalainen K & Kosma VM (2009) Reduced gamma-catenin expression and poor survival in oral squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 135: 1035–1040.
- Nemeth Z, Szasz AM, Tatrai P, Nemeth J, Gyorffy H, Somoracz A, Szijarto A, Kupcsulik P, Kiss A & Schaff Z (2009) Claudin-1, -2, -3, -4, -7, -8, and -10 protein expression in biliary tract cancers. *J Histochem Cytochem* 57: 113–121.
- Ng SK, Kabat GC & Wynder EL (1993). Oral cavity cancer in non-users of tobacco. *J Natl Cancer Inst* 85: 743–745.
- Nguyen ST, Hasegawa S, Tsuda H, Tomioka H, Ushijima M, Noda M, Omura K & Miki Y (2007) Identification of a predictive gene expression signature of cervical lymph node metastasis in oral squamous cell carcinoma. *Cancer Sci* 98: 740–746.
- Nichols AC & Bhattacharyya N (2007) Racial differences in stage and survival in head and neck squamous cell carcinoma. *Laryngoscope* 117: 770–775.
- Nieto MA (2002) The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* 3: 155–166.

- Nyberg P, Moilanen M, Paju A, Sarin A, Stenman UH, Sorsa T & Salo T (2002) MMP-9 activation by tumor trypsin-2 enhances in vivo invasion of human tongue carcinoma cells. *J Dent Res* 81: 831–835.
- O-charoenrat P, Pillai G, Patel S, Fisher C, Archer D, Eccles S & Rhys-Evans P (2003) Tumour thickness predicts cervical nodal metastases and survival in early oral tongue cancer. *Oral Oncol* 39: 386–390.
- Odell EW, Jani P, Sherriff M, Ahluwalia SM, Hibbert J, Levison DA & Morgan PR (1994) The prognostic value of individual histologic grading parameters in small lingual squamous cell carcinomas. The importance of the pattern of invasion. *Cancer* 74: 789–794.
- O'Donnell RK, Kupferman M, Wei SJ, Singhal S, Weber R, O'Malley B, Cheng Y, Putt M, Feldman M, Ziober B & Muschel RJ (2005) Gene expression signature predicts lymphatic metastasis in squamous cell carcinoma of the oral cavity. *Oncogene* 24: 1244–1251.
- Ohazama A & Sharpe PT (2007) Expression of claudins in murine tooth development. *Dev Dyn* 236: 290–294.
- Ohtani S, Terashima M, Satoh J, Soeta N, Saze Z, Kashimura S, Ohsuka F, Hoshino Y, Kogure M & Gotoh M (2009) Expression of tight-junction-associated proteins in human gastric cancer: downregulation of claudin-4 correlates with tumor aggressiveness and survival. *Gastric Cancer* 12: 43–51.
- Okada H, Yamamoto H & Tilakaratne WM (2007). Odontogenic tumors in Sri Lanka: analysis of 226 cases. *J Oral Maxillofac Surg* 65: 875–882.
- Okamoto M, Nishimine M, Kishi M, Kirita T, Sugimura M, Nakamura M & Konishi N (2002) Prediction of delayed neck metastasis in patients with stage I/II squamous cell carcinoma of the tongue. *J Oral Pathol Med* 31: 227–233.
- Oku N, Sasabe E, Ueta E, Yamamoto T & Osaki T (2006) Tight junction protein claudin-1 enhances the invasive activity of oral squamous cell carcinoma cells by promoting cleavage of laminin-5 gamma2 chain via matrix metalloproteinase (MMP)-2 and membrane-type MMP-1. *Cancer Res* 66: 5251–5257.
- Olgac V, Koseoglu BG & Aksakalli N (2006) Odontogenic tumours in Istanbul: 527 cases. *Br J Oral Maxillofac Surg* 44: 386–388.
- Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL & Weinberg RA (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 121: 335–348.
- Orimo A & Weinberg RA (2006) Stromal fibroblasts in cancer: a novel tumor-promoting cell type. *Cell Cycle* 5: 1597–1601.
- Ostman A & Augsten M (2009) Cancer-associated fibroblasts and tumor growth—bystanders turning into key players. *Curr Opin Genet Dev* 19: 67–73.
- Pan XY, Wang B, Che YC, Weng ZP, Dai HY & Peng W (2007). Expression of claudin-3 and claudin-4 in normal, hyperplastic, and malignant endometrial tissue. *Int J Gynecol Cancer* 17: 233–241.

- Parkin DM, Pisani P & Ferlay J (1993) Estimates of the worldwide incidence of eighteen major cancers in 1985. *Int J Cancer* 54: 594–606.
- Parkin DM, Whelan SL, Ferlay J, Teppo L & Thomas DB (2003) *Cancer Incidence in five continents*. Vol. VIII. IARC Press: Lyon.
- Pazouki S, Chisholm DM, Adi MM, Carmichael G, Farquharson M, Ogden GR, Schor SL & Schor AM (1997) The association between tumour progression and vascularity in the oral mucosa. *J Pathol* 183: 39–43.
- Petersen PE (2003) The World Oral Health Report 2003: continuous improvement of oral health in the 21st century—the approach of the WHO Global Oral Health Programme. *Community Dent Oral Epidemiol* 31 (Suppl 1): 3–23.
- Pindborg JJ, Reichart PA, Smith CJ & van der Waal I (1997) *Histological Typing of cancer and precancer of the oral mucosa*, 2nd Edition. Springer-Verlag: New York.
- Pinheiro JJ, Freitas VM, Moretti AI, Jorge AG & Jaeger RG (2004) Local invasiveness of ameloblastoma. Role played by matrix metalloproteinases and proliferative activity. *Histopathology* 45: 65–72.
- Pintos J, Black MJ, Sadeghi N, Ghadirian P, Zeitouni AG, Viscidi RP, Herrero R, Coutlee F & Franco EL (2008) Human papillomavirus infection and oral cancer: a case-control study in Montreal, Canada. *Oral Oncol* 44: 242–250.
- Pitman KT, Johnson JT, Wagner RL & Myers EN (2000) Cancer of the tongue in patients less than forty. *Head Neck* 22: 297–302.
- Popovtzer A, Shpitzer T, Bahar G, Marshak G, Ulanovski D & Feinmesser R (2004) Squamous cell carcinoma of the oral tongue in young patients. *Laryngoscope* 114: 915–917.
- Pukkila M, Kosunen A, Ropponen K, Virtaniemi J, Kellokoski J, Kumpulainen E, Pirinen R, Nuutinen J, Johansson R & Kosma VM (2007) High stromal versican expression predicts unfavourable outcome in oral squamous cell carcinoma. *J Clin Pathol* 60: 267–272.
- R Development Core Team (RDC) (2009) *R: A language and environment for statistical computing*. Vienna Austria, R Foundation for Statistical Computing. ISBN 3-900051-07-0. URI: <http://www.R-project.org>.
- Rahner C, Mitic LL & Anderson JM (2001) Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. *Gastroenterology* 120: 411–422.
- Regezi JA, Sciubba JJ & Jordan RCK (2008) *Oral pathology: clinical, pathologic correlations*. 5th Ed. Saunders Elsevier: St Louis (MO).
- Reichart PA, Philipsen HP & Sonner S (1995) Ameloblastoma: biological profile of 3677 cases. *Eur J Cancer B Oral Oncol* 31B: 86–99.
- Reyes JL, Lamas M, Martin D, del Carmen Namorado M, Islas S, Luna J, Tauc M & Gonzalez-Mariscal L (2002) The renal segmental distribution of claudins changes with development. *Kidney Int* 62: 476–487.

- Roepman P, Wessels LF, Kettelarij N, Kemmeren P, Miles AJ, Lijnzaad P, Tilanus MG, Koole R, Hordijk GJ, van der Vliet PC, Reinders MJ, Slootweg PJ & Holstege FC (2005) An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nat Genet* 37: 182–186.
- Roh JL, Cho KJ, Kwon GY, Ryu CH, Chang HW, Choi SH, Nam SY & Kim SY (2009) The prognostic value of hypoxia markers in T2-staged oral tongue cancer. *Oral Oncol* 45: 63–68.
- Roosaar A, Johansson AL, Sandborgh-Englund G, Axell T & Nyren O (2008) Cancer and mortality among users and nonusers of snus. *Int J Cancer* 123: 168–173.
- Rosenquist K (2005) Risk factors in oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. *Swed Dent J Suppl* 179: 1–66.
- Ryott M, Wangsa D, Heselmeyer-Haddad K, Lindholm J, Elmberger G, Auer G, Lundqvist EV, Ried T & Munck-Wikland E (2009) EGFR protein overexpression and gene copy number increases in oral tongue squamous cell carcinoma. *Eur J Cancer* 45: 1700–1708.
- Saito T, Sato J, Satoh A, Notani K, Fukuda H, Mizuno S, Shindoh M & Amemiya A (1994) Flow cytometric analysis of nuclear DNA content in tongue squamous cell carcinoma: relation to cervical lymph node metastasis. *Int J Oral Maxillofac Surg* 23: 28–31.
- Sandoval M, Font R, Manos M, Dicenta M, Quintana MJ, Bosch FX & Castellsague X (2009) The role of vegetable and fruit consumption on survival and other habits following the diagnosis of oral cancer: a prospective study in Spain. *Int J Oral Maxillofac Surg* 38: 31–39.
- Sano D & Myers JN (2007) Metastasis of squamous cell carcinoma of the oral tongue. *Cancer Metastasis Rev* 26: 645–662.
- Santin AD (2000) Lymph node metastases: the importance of the microenvironment. *Cancer* 88: 175–179.
- Sarioglu T, Yilmaz T, Sungur A & Gursel B (1994) The effect of lymphocytic infiltration on clinical survival in cancer of the tongue. *Eur Arch Otorhinolaryngol* 251: 366–369.
- Sawyer DR, Mosadomi A, Page DG, Svirsky JA & Kekere-Ekun AT (1985). Racial predilection of ameloblastoma? A probable answer from Lagos (Nigeria) and Richmond, Virginia (U.S.A.). *J Oral Med* 40: 27–31.
- Schantz SP & Yu GP (2002) Head and neck cancer incidence trends in young Americans, 1973–1997 with a special analysis for tongue cancer. *Arch Otolaryngol Head Neck Surg* 128: 268–274.
- Schildt EB, Eriksson M, Hardell L & Magnuson A (1998) Oral infections and dental factors in relation to oral cancer: a Swedish case-control study. *Eur J Cancer Prev* 7: 201–206.
- Scully C (2002) Oral squamous cell carcinoma; from an hypothesis about a virus, to concern about possible sexual transmission. *Oral Oncol* 38: 227–234.
- Sehdev MK, Huvos AG, Strong EW, Gerold FP & Willis GW (1974). Proceedings: Ameloblastoma of maxilla and mandible. *Cancer* 33: 324–333.

- Shear M & Singh S (1978) Age-standardized incidence rates of ameloblastoma and dentigerous cyst on the Witwatersrand, South Africa. *Community Dent Oral Epidemiol* 6: 195–199.
- Sheng S, Carey J, Seftor EA, Dias L, Hendrix MJ & Sager R (1996) Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. *Proc Natl Acad Sci U S A* 93: 11669–11674.
- Shiboski CH, Schmidt BL & Jordan RC (2007) Racial disparity in stage at diagnosis and survival among adults with oral cancer in the US. *Community Dent Oral Epidemiol* 35: 233–240.
- Shpitzer T, Chaimoff M, Gal R, Stern Y, Feinmesser R & Segal K (1996) Tumor angiogenesis as a prognostic factor in early oral tongue cancer. *Arch Otolaryngol Head Neck Surg* 122: 865–868.
- Siegelmann-Danieli N, Hanlon A, Ridge JA, Padmore R, Fein DA & Langer CJ (1998) Oral tongue cancer in patients less than 45 years old: Institutional experience and comparison with older patients. *J Clin Oncol* 16: 745–753.
- Silva SD, Perez DE, Alves FA, Nishimoto IN, Pinto CA, Kowalski LP & Graner E (2008a). ErbB2 and fatty acid synthase (FAS) expression in 102 squamous cell carcinomas of the tongue: correlation with clinical outcomes. *Oral Oncol* 44: 484–490.
- Silva SD, Perez DE, Nishimoto IN, Alves FA, Pinto CA, Kowalski LP & Graner E (2008b) Fatty acid synthase expression in squamous cell carcinoma of the tongue: clinicopathological findings. *Oral Dis* 14: 376–382.
- Silveira EJ, Godoy GP, Lins RD, Arruda Mde L, Ramos CC, Freitas Rde A & Queiroz LM (2007) Correlation of clinical, histological, and cytokeratin profiles of squamous cell carcinoma of the oral tongue with prognosis. *Int J Surg Pathol* 15: 376–383.
- Silver CE & Moisa I (1991) Elective treatment of the neck in cancer of the oral tongue. *Semin Surg Oncol* 7: 14–19.
- Silverman SJ (1998) Oral cancer. American Cancer Society, 1998: Hamilton, Ontario.
- Sobin LH & Wittekind C (2002) TNM: Classification of Malignant Tumors. John Wiley and Sons: New York.
- Soini Y, Paakko P & Lehto VP (1998) Histopathological evaluation of apoptosis in cancer. *Am J Pathol* 153: 1041–1053.
- Soini Y, Tammola S, Helin H & Martikainen P (2006) Claudins 1, 3, 4 and 5 in gastric carcinoma, loss of claudin expression associates with the diffuse subtype. *Virchows Arch* 448: 52–58.
- Souza Andrade ES, da Costa Miguel MC, Pinto LP & de Souza LB (2007) Ameloblastoma and adenomatoid odontogenic tumor: the role of alpha2beta1, alpha3beta1, and alpha5beta1 integrins in local invasiveness and architectural characteristics. *Ann Diagn Pathol* 11: 199–205.
- Sparano A, Weinstein G, Chalian A, Yodul M & Weber R (2004) Multivariate predictors of occult neck metastasis in early oral tongue cancer. *Otolaryngol Head Neck Surg* 131: 472–476.

- Steward BW & Kleihues P (2003) World Cancer Report. WHO International Agency for Research on Cancer: Lyon.
- Takala H, Saarnio J, Wiik H & Soini Y (2007) Claudins 1, 3, 4, 5 and 7 in esophageal cancer: loss of claudin 3 and 4 expression is associated with metastatic behavior. *Apmis* 115: 838–47.
- Talbot SJ & Crawford DH (2004) Viruses and tumours—an update. *Eur J Cancer* 40: 1998–2005.
- Tanigaki Y, Nagashima Y, Kitamura Y, Matsuda H, Mikami Y & Tsukuda M (2004) The expression of vascular endothelial growth factor-A and -C, and receptors 1 and 3: correlation with lymph node metastasis and prognosis in tongue squamous cell carcinoma. *Int J Mol Med* 14: 389–395.
- Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2: 442–454.
- Thiery JP (2003) Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 15: 740–746.
- Tsujino T, Seshimo I, Yamamoto H, Ngan CY, Ezumi K, Takemasa I, Ikeda M, Sekimoto M, Matsuura N & Monden M (2007) Stromal myofibroblasts predict disease recurrence for colorectal cancer. *Clin Cancer Res* 13: 2082–2090.
- Ulanovski D, Stern Y, Roizman P, Shpitzer T, Popovtzer A & Feinmesser R (2004) Expression of EGFR and Cerb-B2 as prognostic factors in cancer of the tongue. *Oral Oncol* 40: 532–537.
- Usami Y, Chiba H, Nakayama F, Ueda J, Matsuda Y, Sawada N, Komori T, Ito A & Yokozaki H (2006) Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus. *Hum Pathol* 37: 569–577.
- Usami Y, Satake S, Nakayama F, Matsumoto M, Ohnuma K, Komori T, Semba S, Ito A & Yokozaki H (2008) Snail-associated epithelial-mesenchymal transition promotes oesophageal squamous cell carcinoma motility and progression. *J Pathol* 215: 330–339.
- Väänänen A, Tjaderhane L, Eklund L, Heljasvaara R, Pihlajaniemi T, Herva R, Ding Y, Bartlett JD & Salo T (2004) Expression of collagen XVIII and MMP-20 in developing teeth and odontogenic tumors. *Matrix Biol* 23: 153–161.
- van den Brekel MWM, Castelijns JA & Snow GB (1998) Diagnostic evaluation of the neck. *Otolaryngol Clin N Am* 31: 601–620.
- van Diest PJ, Brugal G & Baak JP (1998) Proliferation markers in tumours: interpretation and clinical value. *J Clin Pathol* 51: 716–724.
- Velly AM, Franco EL, Schlecht N, Pintos J, Kowalski LP, Oliveira BV & Curado MP (1998) Relationship between dental factors and risk of upper aerodigestive tract cancer. *Oral Oncol* 34: 284–291.
- Veness MJ, Morgan GJ, Sathiyaseelan Y & Gebiski V (2003) Anterior tongue cancer: Age is not a predictor of outcome and should not alter treatment. *Anz J Surg* 73: 899–904.
- Vered M, Allon I & Dayan D (2009a) Maspin, p53, p63, and Ki-67 in epithelial lesions of the tongue: from hyperplasia through dysplasia to carcinoma. *J Oral Pathol Med* 38: 314–320.

- Vered, M, Dobriyan A, Dayan D, Yahalom R, Talmi YP, Bedrin L, Barshack I & Taicher S (2009b) Tumor-host histopathologic variables, stromal myofibroblasts and risk score, are significantly associated with recurrent disease in tongue cancer. *Cancer Sci* Sep 10. (In press).
- Vered M, Shohat I, Buchner A & Dayan D (2005) Myofibroblasts in stroma of odontogenic cysts and tumors can contribute to variations in the biological behavior of lesions. *Oral Oncol* 41: 1028–1033.
- Walker DM, Boey G & McDonald LA (2003) The pathology of oral cancer. *Pathology* 35: 376–383.
- Wangsa D, Ryott M, Avall-Lundqvist E, Petersson F, Elmberger G, Luo J, Ried T, Auer G & Munck-Wikland E (2008) Ki-67 expression predicts locoregional recurrence in stage I oral tongue carcinoma. *Br J Cancer* 99: 1121–1128.
- Weijers M, Snow GB, Dick Bezemer P & van der Waal I (2009) Malignancy grading is no better than conventional histopathological grading in small squamous cell carcinoma of tongue and floor of mouth: retrospective study in 128 patients. *J Oral Pathol Med* 38: 343–347.
- Wight AJ & Ogden GR (1998) Possible mechanisms by which alcohol may influence the development of oral cancer—a review. *Oral Oncol* 34: 441–447.
- Winn DM, Blot WJ, Shy CM, Pickle LW, Toledo A & Fraumeni JF Jr (1981) Snuff dipping and oral cancer among women in the southern United States. *N Engl J Med* 304: 745–749.
- Woolgar JA (2006) Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncology* 42: 229–239.
- Woolgar JA & Scott J (1995) Prediction of cervical lymph node metastasis in squamous cell carcinoma of the tongue/ floor of mouth. *Head Neck* 17: 463–472.
- Wynder EL, Mushinski MH & Spivak JC (1977) Tobacco and alcohol consumption in relation to the development of multiple primary cancers. *Cancer* 40: 1872–1878.
- Xia W, Lau YK, Hu MC, Li L, Johnston DA, Sheng S, El-Naggar A & Hung MC (2000) High tumoral maspin expression is associated with improved survival of patients with oral squamous cell carcinoma. *Oncogene* 19: 2398–2403.
- Xie X, Clausen OP & Boysen M (2004) Bag-1 expression as a prognostic factor in tongue squamous cell carcinomas. *Laryngoscope* 114: 1785–1790.
- Xie X, Clausen OP, De Angelis P & Boysen M (1999) The prognostic value of spontaneous apoptosis, Bax, Bcl-2, and p53 in oral squamous cell carcinoma of the tongue. *Cancer* 86: 913–920.
- Xie X, Clausen OP & Boysen M (2002) Prognostic significance of p21WAF1/CIP1 expression in tongue squamous cell carcinomas. *Arch Otolaryngol Head Neck Surg* 128: 897–902.
- Yang J & Weinberg RA (2008) Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell* 14: 818–829.
- Yao L, Iwai M & Furuta I (1999) Correlations of bcl-2 and p53 expression with the clinicopathological features in tongue squamous cell carcinomas. *Oral Oncol* 35: 56–62.

- Yasumatsu R, Nakashima T, Hirakawa N, Kumamoto Y, Kuratomi Y, Tomita K & Komiyama S (2001) Maspin expression in stage I and II oral tongue squamous cell carcinoma. *Head Neck* 23: 962–966.
- Yazhou C, Wenlv S, Weidong Z & Licun W (2004) Clinicopathological significance of stromal myofibroblasts in invasive ductal carcinoma of the breast. *Tumour Biol* 25: 290–295.
- Yoshida K, Kashima K, Suenaga S, Nomi N, Shuto J & Suzuki M (2005) Immunohistochemical detection of cervical lymph node micrometastases from T2N0 tongue cancer. *Acta Otolaryngol* 125: 654–658.
- Yoshizaki T, Maruyama Y, Sato H & Furukawa M (2001) Expression of tissue inhibitor of matrix metalloproteinase-2 correlates with activation of matrix metalloproteinase-2 and predicts poor prognosis in tongue squamous cell carcinoma. *Int J Cancer* 95: 44–50.
- Yuen AP, Lam KY, Chan AC, Wei WI, Lam LK, Ho WK & Ho CM (1999) Clinicopathological analysis of elective neck dissection for N0 neck of early oral tongue carcinoma. *Am J Surg* 177: 90–92.
- Yuen APW, Lam KY, Lam LK, Ho CM, Wong A, Chow TL, Yuen WF & Wei WI (2002) Prognostic factors of clinically stage I and II oral tongue carcinoma - A comparative study of stage, thickness, shape, growth pattern, invasive front malignancy grading, Martinez-Gimeno score, and pathologic features. *Head Neck* 24: 513–520.
- Yuen PW, Chou V, Choy J, Lam KY, Ho WK & Wei WI (2001) The clinicopathologic significance of p53 and p21 expression in the surgical management of lingual squamous cell carcinoma. *Am J Clin Pathol* 116: 240–245.
- Zheng T, Boyle P, Willett WC, Hu H, Dan J, Evstifeeva TV, Niu S & MacMahon B (1993) A case-control study of oral cancer in Beijing, People's Republic of China. Associations with nutrient intakes, foods and food groups. *Eur J Cancer B Oral Oncol* 29B: 45–55.
- Zheng TZ, Boyle P, Hu HF, Duan J, Jian PJ, Ma DQ, Shui LP, Niu SR, Scully C & MacMahon B (1990) Dentition, oral hygiene, and risk of oral cancer: a case-control study in Beijing, People's Republic of China. *Cancer Causes Control* 1: 235–241.
- Zheng Y, Kirita T, Kurumatani N, Sugimura M & Yonemasu K (1999) Trends in oral cancer mortality in Japan: 1950–1993. *Oral Dis* 5: 3–9.
- Zou Z, Anisowicz A, Hendrix MJ, Thor A, Neveu M, Sheng S, Rafidi K, Seftor E & Sager R (1994) Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science* 263: 526–529.
- Zwahlen RA & Gratz KW (2002). Maxillary ameloblastomas: a review of the literature and of a 15-year database. *J Craniomaxillofac Surg* 30: 273–279.

## Original publications

- I Bello IO, Soini Y, Slootweg PJ & Salo T (2007) Claudins 1, 4, 5, 7 and occludin in ameloblastomas and developing human teeth. *J Oral Pathol Med* 36: 48–54.
- II Bello IO, Vilen S-T, Niinimaa A, Kantola S, Soini Y & Salo T (2008) Expression of claudins 1, 4, 5, 7 and occludin and relationship with prognosis in squamous cell carcinoma of the tongue. *Hum Pathol* 39: 1212–1220.
- III Bello IO, Alanen K, Slootweg PJ & Salo T (2009) Alpha-smooth muscle actin within epithelial islands is predictive of ameloblastic carcinoma. *Oral Oncol* 45: 760–765
- IV Bello IO, Vered M, Dobriyan A, Yahalom R, Alanen K, Nieminen P, Kantola S, Läärä E, Dayan D & Salo T (2009) Increased density of carcinoma-associated fibroblasts strongly predicts poor prognosis in mobile tongue cancer. Manuscript.

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