Ibrahim O. Bello

TIGHT JUNCTION PROTEINS AND CANCER-ASSOCIATED FIBROBLASTS IN AMELO-BLASTOMA, 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, α-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 α-SMA was able to predict ameloblastic carcinoma when found in the epithelial islands. In conclusion, staining for α-SMA and claudin 7 seems to be beneficial for prognostication in tongue cancer, while α-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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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
cia-9 carbonic anhydrase-9
CAR coxsackie 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-mesenchymal transition
FA focal adhesion
FAP fibroblast-activated protein
FCM flow cytometry
FNAC fine needle aspiration cytology
FOS 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 mammary serine protease inhibitor
MET mesenchymal-epithelial transition
MF myofibroblast
MMP matrix metalloproteinase
MRI magnetic resonance imaging
MVD microvascular density
NG2 neuron-glial 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 sulphone polyacrylamide gel electrophoresis
SFRP1 secreted frizzled-related protein 1
Snail zinc finger phosphoprotein
SPARC secreted protein, acidic, rich in cysteine (osteonectin)
SPSS Statistical Package for the Social Sciences
TGF-β 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
α-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


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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 (Roosaaar 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, nitrosopyrrollodine and nitrosodimethylamine. TSNs have been suggested to act locally on keratinocytes stem cells and systemically by being absorbed and producing DNA adducts, such as 06 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 health care 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 OTSCC (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.

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Proliferative cell markers* (?)</td>
<td>Davies et al. 2006, Bova et al. 1999, Silva et al. 2008a, Wangsa et al. 2008</td>
</tr>
<tr>
<td>Stromal myofibroblasts* (+?)</td>
<td>Keilermann et al. 2007</td>
</tr>
<tr>
<td>Inflammatory response (?)</td>
<td>Sarogiou et al. 1994</td>
</tr>
<tr>
<td>5. Hypoxia markers: Hypoxia-inducible factor (HIF-1 and - 2), carbonic anhydrase (CA)-9, glucose transporter (GLUT)-1, erythropoietin receptor. (?)</td>
<td>Kim et al. 2007, Roh et al. 2009</td>
</tr>
</tbody>
</table>

* 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-XL*, *Bcl-w*, *Bfl-1*, *brag 1*, *A1* and *Mcl-1* inhibit apoptosis while *Bax*, *Bad*, *Bcl-Xs*, *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 αvβ3 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 microenvironment 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 α-SMA, fibroblast-activated protein (FAP), fibroblasts-specific protein-1 (FSP1/S100A4), neuron-glial antigen (NG2) and PDGF-β are presently used in defining CAFs (Ostman & Augsten 2009). These markers stain other cell types apart from fibroblasts (Kalluri & Zeisberg 2006). Production of α-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 α-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 α-SMA positive cell can be regarded as MF if it is positive for at least three other markers mentioned above (e.g. α-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 coxscakie 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).](image)

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-β 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 $TNFRIA$ 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-α, 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-µm 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.

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

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.

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

*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-µm-thick sections. The primary antibodies used in the immunostainings in this study are listed in Table 4.

In brief, the procedure involves deparaffinization in xylenes 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 H2O. The sections were thereafter incubated with the primary antibody for 60 min at room temperature and then overlaid with a biotinylated secondary antibody and Histostatin 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.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody type</th>
<th>Clone</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin 1</td>
<td>Polyclonal rabbit</td>
<td>JAY.8</td>
<td>1:50</td>
<td>Zymed Laboratories, San Francisco, CA, USA</td>
</tr>
<tr>
<td>Claudin 4</td>
<td>Monoclonal mouse</td>
<td>3E2C1</td>
<td>1:50</td>
<td>Zymed Laboratories, San Francisco, CA, USA</td>
</tr>
<tr>
<td>Claudin 5</td>
<td>Monoclonal mouse</td>
<td>4C3C2</td>
<td>1:50</td>
<td>Zymed Laboratories, San Francisco, CA, USA</td>
</tr>
<tr>
<td>Claudin 7</td>
<td>Polyclonal rabbit</td>
<td>ZMD.241</td>
<td>1:50</td>
<td>Zymed Laboratories, San Francisco, CA, USA</td>
</tr>
<tr>
<td>Ocludin</td>
<td>Polyclonal rabbit</td>
<td>ZMD.481</td>
<td>1:50</td>
<td>Zymed Laboratories, San Francisco, CA, USA</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Monoclonal mouse</td>
<td>MM1</td>
<td>1:100</td>
<td>Novocastra Laboratories, Newcastle, UK</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Monoclonal mouse</td>
<td>1A1</td>
<td>1:1000</td>
<td>Dako A/S, Denmark</td>
</tr>
<tr>
<td>EMA</td>
<td>Monoclonal mouse</td>
<td>E29</td>
<td>1:500</td>
<td>Dako A/S, Denmark</td>
</tr>
<tr>
<td>Calponin B</td>
<td>Monoclonal mouse</td>
<td>26A11</td>
<td>1:50</td>
<td>Novocastra Laboratories, Newcastle, UK</td>
</tr>
<tr>
<td>P63</td>
<td>Monoclonal mouse</td>
<td>7JUL</td>
<td>1:25</td>
<td>Novocastra Laboratories, Newcastle, UK</td>
</tr>
<tr>
<td>Maspin</td>
<td>Monoclonal mouse</td>
<td>EAW24</td>
<td>1:50</td>
<td>Novocastra Laboratories, Newcastle, UK</td>
</tr>
</tbody>
</table>
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, α-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-µm 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-µm-thick sections slides (without undergoing enzymatic disintegration and filtration) were also stained directly using modified Fuelgen 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 mm² dishes using the Trizol® method (Invitrogen, Carlsbad, CA, USA). Protein samples of 7 µg 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 α-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, α-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 α-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.](image)

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 α-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. α-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 α-SMA may be beneficial for prognostication in OTSCC.
References


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Original publications


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