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Histopathological predictors of early stage oral tongue cancer

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ACADEMIC DISSERTATION

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This work is dedicated to people who suffer from tongue cancer. I hope the present research will help to improve the management of this malignancy.

List of original publications

This doctoral thesis is based on the following publications, which are referred to in the text by Roman numerals (I-IV):

- I. **Almangush A**, Bello IO, Keski-Säntti H, Mäkinen LK, Kauppila JH, Pukkila M, Hagström J, Laranne J, Tommola S, Nieminen O, Soini Y, Kosma VM, Koivunen P, Grénman R, Leivo I, Salo T. Depth of invasion, tumor budding, and worst pattern of invasion: Prognostic indicators in early-stage oral tongue cancer. *Head Neck*. 2014 Jun; 36(6):811-8.
- II. **Almangush A**, Bello IO, Coletta RD, Mäkitie A, Mäkinen LK, Kauppila JH, Pukkila M, Hagström J, Laranne J, Soini Y, Kosma VM, Koivunen P, Kelner N, Kowalski LP, Grénman R, Leivo I, Läärä E, Salo T. For early-stage oral tongue cancer, depth of invasion and worst pattern of invasion are the strongest pathological predictors for locoregional recurrence and mortality. *Virchows Arch*. 2015 Jul; 467(1):39-46.
- III. **Almangush A**, Salo T, Hagström J, Leivo I. Tumour budding in head and neck squamous cell carcinoma - A systematic review. *Histopathology*. 2014 Nov; 65(5):587-94.
- IV. **Almangush A**, Coletta RD, Bello IO, Bitu C, Keski-Säntti H, Mäkinen LK, Kauppila JH, Pukkila M, Hagström J, Laranne J, Tommola S, Soini Y, Kosma VM, Koivunen P, Kowalski LP, Nieminen P, Grénman R, Leivo I, Salo T. A simple novel prognostic model for early stage oral tongue cancer. *Int J Oral Maxillofac Surg*. 2015 Feb; 44(2):143-50.

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Abbreviations

BD	Budding and depth of invasion
CAF	Cancer associated fibroblast
CI	Confidence interval
cTNM	Clinical status of tumor size, lymph node, and metastasis
DFS	Disease-free survival
DOI	Depth of invasion
DSS	Disease-specific survival
ECS	Extra-capsular spread
EMT	Epithelial mesenchymal transition
END	Elective neck dissection
HPF	High power field
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HR	Hazard ratio
HRS	Histologic risk score
IF	Invasive front
IPGS	Invasive pattern grading score
LHR	Lymphocytic host response
LRR	Locoregional recurrence
LSCC	Laryngeal squamous cell carcinoma
NPC	Nasopharyngeal carcinoma
OR	Odds ratio
OSCC	Oral squamous cell carcinoma
OS	Overall survival
OTSCC	Oral tongue squamous cell carcinoma
PNI	Perineural invasion

SEER	Surveillance, epidemiology and end results
TB	Tumor budding
TSCC	Tongue squamous cell carcinoma
WPOI	Worst pattern of invasion
α -SMA	α -smooth muscle actin

Abstract

Oral tongue cancer constitutes the majority of malignancies of the oral cavity. In Finland during 2013, the age-adjusted incidence rates of oral tongue cancer were 1.7 per 100,000 in males and 0.8 per 100,000 in females. Staging of the clinical status of tumor size, lymph node, and metastasis (cTNM staging) is a widely accepted system for classification of many cancers including oral squamous cell carcinoma (OSCC). However, this staging system failed to prognosticate the outcome of early stage oral tongue squamous cell carcinoma (OTSCC). Numerous studies designed to introduce prognostic parameters and/or models to complement the insufficiency of cTNM staging and to predict the patient outcome have been carried out. Many of these prognostic models (or systems) were based on histopathologic parameters. However, all of the previously introduced models showed little or no predictive power in early stage (cT1-2N0) OTSCC. Thus, the identification of markers that predict the outcome of patients with early stage OTSCC is still necessary in order to apply effective individualized treatment.

In this international collaborative study, we have examined the prognostic impact of several predictive factors in large patient cohorts from 7 institutions located in 3 geographic regions (Finland, Brazil, and USA). Moreover, we have introduced a new simple predictive model (BD score) for histopathologic classification and prognostication of early OTSCC. We suggest that this model could possibly be easily applied during the surgical resection, so patients with aggressive OTSCC could benefit from elective neck dissection (END) during the same procedure. This model could offer a step forward toward the personalized management of OTSCC. However, additional validation in different patient cohorts is still required.

1 Introduction

The anterior two thirds of the tongue (known as the oral tongue or the mobile tongue) is a subsite belonging to the oral cavity, while the posterior third of the tongue (base of the tongue) is a subsite of the oropharynx. Squamous cell carcinoma (SCC) arising in the oral tongue (OTSCC) (Fig. 1) is reported to have a worse prognosis than SCC arising in the other oral cavity subsites (Rusthoven et al. 2008). The reasons for this poorer prognosis of OTSCC are not known. However, some authors have attributed it to the dense muscle content of the oral tongue combined with a well-developed lymphatic drainage of this part of the oral cavity (Bello, Soini and Salo 2010; Sano and Myers 2007).

The clinical staging system (cTNM) is still the cornerstone for risk stratification in oral cancer as well as in other cancers (Hubert Low et al. 2015). However, in the early stage of oral cancer (especially in early OTSCC), cTNM staging has shown very limited prognostic ability (Piazza et al. 2014b; Po Wing Yuen et al. 2002). Indeed, the cTNM staging cannot assess the biologic behavior of the tumor. This shortcoming of the cTNM staging system may result in inappropriate management and may subject the patient to ineffective treatment or unnecessary overtreatment. Thus, a patient with an aggressive tumor might receive a surgical approach only, or a patient with a non-aggressive tumor might receive a multimodality treatment approach.

In early stage OTSCC, the management is usually a single-modality treatment consisting of surgical resection of the lesion. However, intralesional irradiation, also known as brachytherapy, has been used successfully to treat early OTSCC (Bourgier et al. 2005; Goineau et al. 2015).

The high incidence of occult metastases, recurrences and cancer specific mortality among cases with early OTSCC make it reasonable to apply multi-modality therapy for such cases in some instances. Although hundreds of molecular markers have been suggested over the years as prognosticators in oral squamous cell carcinoma (OSCC), only a few have been repeatedly validated (Soland and Brusevold 2013). Currently, there are no reliable markers available in clinical

practice to select those patients with early OTSCC who might benefit from multi-modality treatment. However, the morphological evaluation of the histopathologic features is still the main guide for pathologists during the assessment of prognosis of OTSCC specimens. Interestingly, the mode and depth of invasion has been shown to be the most important histologic parameter when evaluating the prognosis (Bundgaard et al. 2002).

Since most of the previously published data on prognostic factors of OTSCC (from Finland and from other countries) were based on small cohorts of cases, were from a single institution only, included SCC cases from all oral cavity subsites, or included early and late stages of cancer in the analysis, the current study aimed to evaluate the prognostic value of several predictive parameters in a large, international, multi-institution study with a patient cohort including only early stage cancers (cT1-2N0) from the anterior two thirds of the tongue (oral tongue or mobile tongue).

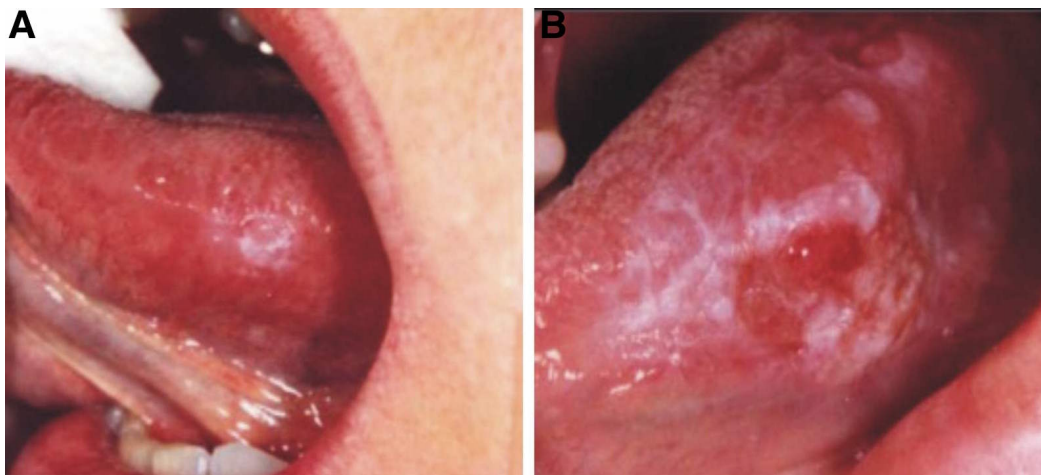


Figure 1: Oral tongue squamous cell carcinoma (OTSCC): Early stage (A); and late stage (B). This Figure is a kind gift from Professor Tuula Salo.

2 Literature review: Oral tongue squamous cell carcinoma

2.1 Incidence

Globally, there were 300,400 new cases of oral cavity cancer (including lip cancer) and 145,400 deaths in 2012 (Torre et al. 2015). Tongue cancer constitutes a high percentage of the oral and oropharyngeal cancers. According to the American Cancer Society, 14,320 new cases of tongue cancer, and 2,190 deaths due to tongue cancer are projected to occur during 2015 in the USA (Siegel, Miller and Jemal 2015). Cancers of the oral tongue and the base of the tongue are usually grouped together, so it is sometimes difficult to find the exact number and incidence of OTSCC. The Finnish Cancer Society (www.cancerregistry.fi) reported 126 new cases of oral tongue cancer in 2013 with 44 deaths as a consequence of the disease.

OTSCC has been shown to possess unique epidemiologic and biologic characteristics that differ from cancers of other subsites of the oral cavity (Rusthoven et al. 2008). In Finland, the incidence of oral tongue cancer has increased slightly during last decades, and this increase was slightly higher in men (www.cancerregistry.fi). In a recent analysis of the Surveillance, Epidemiology, and End Results (SEER), an increase in OTSCC incidence was reported in young white women (Joseph et al. 2015). The analysis of national cancer registries in the Nordic countries, however, has shown an increase in the incidence of tongue cancer (including base of tongue) in both genders (Annertz et al. 2012). Notably, none of the known risk factors (see below) which are implicated in the occurrence of oral cancer, appear to provide a clear explanation for the increasing incidence of tongue cancer (Annertz et al. 2012).

2.2 Risk factors

OTSCC is a multifactorial disease, with use of tobacco (various forms) and alcohol, and areca nut (betel quid) chewing being the most significant risk factors for OTSCC as well as for cancers of other subsites of the head and neck region (Agnihotri and Gaur 2014; Al-Amad, Awad and Nimri

2014; Scully 2011). The reduced risk for OTSCC among never users of tobacco and alcohol was documented (Marks et al. 2014). It has been speculated that alcohol consumption is an important risk factor in Western populations but not in Asian countries (where tobacco related habits are the most important risk factor) (Jayalekshmi et al. 2011). However, other potential risk factors have also been reported for the occurrence of OSCC. These factors include: oral potentially malignant lesions (e.g. oral lichen planus and oral lichenoid lesions) (Casparis et al. 2015; Sun et al. 2013), infection with oncogenic viruses (e.g. human papilloma virus) (Jalouli et al. 2012; Zheng et al. 2010), dietary factors (Meurman 2010) (e.g. low consumption of vegetables and fruits), poor oral hygiene (Oji and Chukwuneke 2012), periodontal disease (Yao et al. 2014), candidiasis (Sanjaya et al. 2011), dental trauma (caused by sharp edge of broken tooth or dental prosthesis) (Bektas-Kayhan et al. 2014; Manoharan, Nagaraja and Eslick 2014), allergy to dental restorations (Weber et al. 2012), and genetic susceptibility (Hillbertz et al. 2012). Combinations of such risk factors may increase the susceptibility for OSCC development. Of note, a recent review showed that HPV is not associated with the recent surge in the incidence of biologically aggressive oral cavity cancer in young patients (Salem 2010).

2.3 Diagnosis

Early diagnosis of tongue lesions (potentially malignant or malignant) enables the surgeon to limit the resected area which diminishes comorbidities. Early diagnosis is also a key to reducing cancer-specific mortality. The concept of “early” in this context is defined as early in the carcinogenetic process, and in the sense of small in size at the time of diagnosis, and with a short time interval between the appearance of symptoms and the date of diagnosis (van der Waal et al. 2011). Early oral cancers are usually asymptomatic and differ from advanced cancers in terms of clinical presentation and outcome (Mashberg and Samit 1995; Seoane et al. 2015). However, a biopsy is still the gold standard for the diagnosis of oral cancer, but this approach is limited in screening programs to lesions already suspected of malignancy (Brinkmann et al. 2011). Interestingly, cancer-

related changes of specific salivary biomarkers (e.g. salivary adenosine deaminase) have recently been suggested as an effective tool for early diagnosis of tongue cancer (Rai et al. 2011). In addition, optical imaging systems and exfoliative cytology (oral brush biopsy) have also been suggested to facilitate early diagnosis of oral cancer (Guner and Epstein 2014). DNA content flow cytometry has been shown to be of great value in detecting oral potentially malignant lesions that have a high risk for malignant transformation (Giaretti et al. 2013; Ma et al. 2014; Pentenero et al. 2009).

For cost-effectiveness, education of the population in self-examination to aid the early diagnosis of oral lesions is strongly recommended (Sarode, Sarode and Karmarkar 2012). Although the oral tongue has the advantage of being easily accessible for clinical examination, about 40-60 % of OTSCC cases are diagnosed at advanced stages (III, IV) (Chen et al. 2008; Ling, Mijiti and Moming 2013; Thiagarajan et al. 2014). The delay in diagnosis and management of OTSCC and other cancers could be subdivided into primary, secondary and tertiary delays. Primary delay (caused by patients themselves) is the duration from the onset of symptoms to seeking medical help from a general physician; secondary delay (caused by the general physician) is defined as the duration from seeking medical help to referral to the treating hospital (tertiary care center); tertiary delay (caused by the treating hospital) indicates the duration from seeking medical advice at a tertiary care center to the beginning of the definitive treatment (Joshi et al. 2014). In a Finnish study, patient delay was associated with increased comorbidity (Teppo and Alho 2009). It is generally recommended that treatment should be carried out within 30 days of diagnosis (Fortin et al. 2002).

According to evidence-based guidelines, during the screening for oral cancer, a practitioner should carefully evaluate any signs of potentially malignant or malignant oral lesions especially in people at high risk for such lesions (e.g. smokers) (Rai et al. 2011). Patients who present with oral lesions (e.g. ulcer, swelling, red or white patch), which last for more than two weeks, should be referred to

a specialist (Wolff, Follmann and Nast 2012). Examination should also include clinical inspection and palpation of the neck to evaluate the status of cervical lymph nodes. In case of an established diagnosis of OTSCC, imaging techniques (ultrasonography, magnetic resonance imaging, computed tomography, positron emission tomography) should be used to confirm the presence or absence of cervical metastasis. However, it is common that metastases (occult metastases) cannot be detected without surgical exploration of neck lymph nodes.

2.4 Histopathology

The WHO grading system (Barnes et al. 2005) classifies OSCCs based on histologic resemblance to tissue of origin into well differentiated, moderately differentiated and poorly differentiated tumors (Fig. 2) as follows:

- I. *Well-differentiated tumor:*** Tumor tissue (and individual tumor cells) looks similar to squamous epithelial cells of the normal oral mucosa. The tumor contains foci of extra- or intracellular keratinization. The cells have few mitotic figures and rarely show nuclear pleomorphism or variation in cell size and shape. Atypical mitoses are rare or absent.
- II. *Moderately-differentiated tumor:*** Tumor cells are less keratinized and mitotic figures are present. There is more nuclear pleomorphism or variation of cell size and shape. A few atypical mitotic figures may be seen.
- III. *Poorly-differentiated tumor:*** Tumor cells are rarely keratinized and they have frequent mitotic figures. Nuclear pleomorphism and variation in cell size and shape are commonly seen. Atypical mitoses are common.

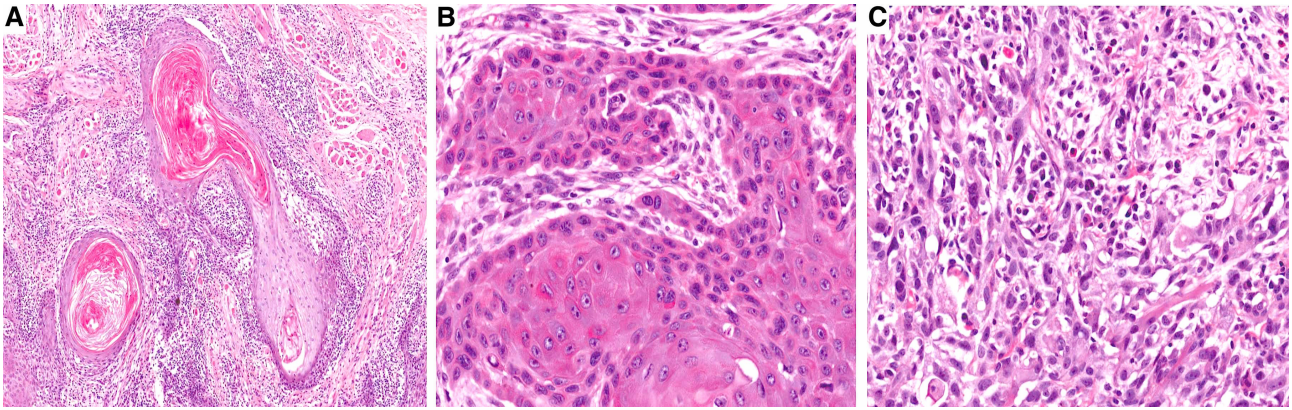


Figure 2: Histopathological appearance of OTSCC. A: Well differentiated tumor; B: Moderately differentiated tumor; C: Poorly differentiated tumor.

There are 6 variants of SCC that might occur in the oral cavity, and these were described by WHO (Barnes et al. 2005) as follows:

Verrucous carcinoma: composed of well differentiated squamous cell carcinoma with marked keratinization and rare mitoses. Verrucous carcinoma has a pushing border; and lymphocytic host response is commonly seen.

Basaloid SCC: consists of two components, basaloid cells and squamous cells. Basaloid cells have hyperchromatic nuclei without nucleoli. Basaloid SCC is characterized by small cystic spaces that contain PAS- and Alcian blue-positive material.

Spindle cell carcinomas: consist of a squamous cell carcinoma component and a spindle cell component. The latter forms the bulk of the tumor. The squamous component might present as in-situ carcinoma or as invasive carcinoma.

Papillary squamous cell carcinoma: characterized by a papillary growth pattern. Keratosis is minimal, and there are foci of necrosis. Papillary SCC invades the stroma as single or multiple nests of tumor cells, and there is dense lymphoplasmacytic inflammation at the tumor stroma interface.

Acantholytic squamous cell carcinoma: characterized by the presence of foci of acantholysis in tumor nests, which create a false appearance of glandular differentiation.

Adenosquamous carcinoma: consists of two components, SCC (in-situ or invasive SCC) and adenocarcinoma (tubular structures) with mucin production.

2.5 Management

An interdisciplinary cancer team should determine the management of oral cancer patients on a case-by-case basis (Wolff, Follmann and Nast 2012). At early stages of HNSCC, cases are usually treated using a single treatment modality (i.e. surgery or radiotherapy) (Langendijk et al. 2010). In general, the basic management of OSCC is surgical resection with the exception of very large tumors. However, a multimodality treatment (surgical resection, elective neck dissection, radiotherapy, and/or chemotherapy) may be required in some cases. For example, in OTSCC cases with an aggressive growth pattern or locoregional spread and in advanced cases it is recommended to combine surgery and other modalities (e.g. irradiation and neck dissection) (Makitie et al. 2007). However, due to the high incidence of occult metastases (23-40%) in early OTSCC (cT1-2N0M0), presently there is no agreement on the optimal treatment. Therefore, elective neck dissection (END) has been suggested as a routine prophylactic management for early stage OTSCC patients with clinically negative nodes (cN0) (Huang et al. 2008). However, in retrospective studies close follow-up and elective neck dissection have given similar results (D'Cruz et al. 2009; Kelner et al. 2014) as was the case in a prospective study as well (Yuen et al. 2009). Although END is associated with morbidity, the policy of observation is not always safe. A delayed diagnosis of nodal metastases may lead to an increase in the risk of extra-capsular spread and involvement of a larger number of nodes by the time of surgery (Kelner et al. 2014). A recent report on early OTSCC has recommended a watchful waiting policy only for T1 cases cooperative for close follow-up, while END should be considered in T2 cases (Tsang et al. 2011). The sentinel lymph node technique has been advocated as an alternative to END in management of early OSCC (Flach et al. 2014; Pedersen et al. 2015) and early OTSCC (Fan et al. 2014).

Postoperative radiotherapy is advised for OTSCC cases with an advanced T stage (T3, T4), positive surgical margins, perineural invasion (PNI), positive lymph nodes, or extracapsular spread (ECS) (Shim et al. 2010). In early OTSCC, cases with recurrence might benefit from postoperative

radiotherapy (Gontarz et al. 2014). A recent study has shown that postoperative brachytherapy is associated with better control and a higher rate of survival compared to surgery alone (Goineau et al. 2015). However, postoperative treatment should be discussed in an interdisciplinary meeting (Wolff, Follmann and Nast 2012).

2.6 Prognosis

For HNSCC, the survival rates vary from one center to another. The five-year survival rates continue to be around 50-65% of cases according to SEER (Pulte and Brenner 2010; Roh et al. 2015). Of note, OTSCC is an aggressive HNSCC, and reported to have a poor prognosis compared with SCC of other subsites of the oral cavity (Rusthoven et al. 2008). For clinically early stage (cT1-2N0) OTSCC, the prognosis is unpredictable with a potential of aggressive invasion, regional metastases and cancer mortality. The presence of metastasis is a sign of a grave prognosis in OTSCC as well as in many other tumors. However, early OTSCC could be considered as a distinct tumor entity with regard to metastases due to the high rate of occult nodal metastases in clinically negative nodes.

2.7 Prevention

About three quarters of oral cavity cancers could be prevented by interfering with exposure to the main risk factors such as alcohol intake and the use of tobacco products (Mangalath et al. 2014; Scully 2011). It is not known exactly how much time is required following cessation of these habits before the risk of oral cancer is decreased to the level of the never-exposed population. However, the risk of head and neck squamous cell cancer (HNSCC) has been reported to decrease in persons who have stopped smoking 1-4 years before (Marron et al. 2010). Importantly, recent reports have shown that HNSCCs occurring in never drinkers and never smokers commonly arise in the subsite of the oral tongue (i.e. OTSCC) (Dahlstrom et al. 2008), and such cases could not be prevented before the recognition of predisposing factors.

2.8 Prognostic factors for OTSCC

2.8.1 Demographic prognostic factors for OTSCC

Age at diagnosis

Young patients with tongue SCC have a worse prognosis than old patients in some reports recently (Hilly et al. 2013; Park et al. 2010). The main weakness of such studies was the small number of young patients and/or small number of events (recurrence, and/or deaths from cancer) in young patients. However, the effect of age on the prognosis of tongue cancer remains somewhat controversial due to inconsistent findings (Hilly et al. 2013; Knopf et al. 2015).

Many authors have reported an aggressive OSCC in pediatric patients (≤ 20 years) (Ribeiro et al. 2011; Sidell et al. 2009; Stolk-Liefferink et al. 2008). However, equivalent survival for pediatric and older patients with OSCC was reported by Morris et al. (Morris and Ganly 2010). Moreover, in another report by Morris and colleagues (Morris et al. 2010), a comparison between a series of 10 patients with OTSCC ages 15 to 20 years with a series of 40 adult patients showed the pediatric patients to have similar or better survival outcomes when compared with adult patients and the authors suggest the same treatment approach for both groups.

Gender

An increased incidence of OTSCC has been reported in white women (especially at a young age) (Patel et al. 2011; Saba et al. 2011). However, the reasons for this trend are unclear, although the increase of smoking among women may be one explanation (Saba et al. 2011). In oral cancer, however, gender was not widely recognized as a significant prognostic factor (Girod et al. 2009). Only a few reports in the literature have elaborated on the role of gender in the prognosis of all stages of OTSCC. Similar survival rates in men and women have been reported by Garavello et al. (Garavello et al. 2008), but an aggressive behavior with a worse prognosis in young women was

reported by Vargas et al. (Vargas et al. 2000). Interestingly, Benowitz et al. (Benowitz et al. 2006) reported that women have a faster metabolism of nicotine than men; and a similar amount of smoking was reported to cause more oral cancer in women than in men (Zavras et al. 2001).

Socioeconomic status

Socioeconomic status has been reported to correlate with survival of oral cancer patients. One explanation may be the occurrence of more comorbidities in oral cancer cases in low socioeconomic groups (Lee et al. 2012). On the other hand, patients with a high socioeconomic status have more opportunities to improve their health status by financial means, knowledge and social connections (Lee et al. 2012). Notably, a limited access to medical services affects the outcome of patients. As an example, a study from the University of Pittsburgh Medical Center (USA) showed that uninsured HNSCC patients were at an advanced stage of cancer at the time of diagnosis, and subsequently at high risk of death compared with patients who had private insurance (Kwok et al. 2010). In contrast, the availability of medical services could improve the survival. As an interesting example, regular visits to a dental clinic were reported to aid in the diagnosis of oral cancer at an earlier stage (Langevin et al. 2012), which subsequently could improve the prognosis. Although information on the effect of socioeconomic status on the prognosis of OTSCC is lacking, Yang et al. (Yang et al. 2010) reported that higher socioeconomic status was strongly associated with higher quality of life for tongue cancer patients after surgery. Such improvement in quality of life would improve the survival rates.

Tobacco use, betel quid chewing and alcohol consumption

Tobacco use and alcohol consumption have been reported as adverse prognostic factors in OSCC (Girod et al. 2009; Jerjes et al. 2012). A recent study from Brazil has shown an impact on the

prognosis of OTSCC for smoking but not for alcohol consumption (Rodrigues et al. 2014). OTSCC in non-smokers and non-drinkers has been speculated to represent a special entity with aggressive behavior and poor prognosis (Sebastian et al. 2014), but, the debate is still ongoing due to conflicting findings. A study by Bachar et al. (Bachar et al. 2011) reported similar outcomes of OTSCC cases with or without risk factors, but their analysis of a subgroup of young patients (less than 40 years) showed a poor prognosis in young patients without risk factors. Moreover, Durr et al. (Durr et al. 2013) reported decreased survival of OTSCC patients who were never-smokers, but the survival difference was not statistically significant.

Obesity

The effect of increased weight on the prognosis of cancers has been reported previously (Calle et al. 2003). Notably, a recent study found a significant association between obesity and poor survival (both DSS and DFS) in early stage OTSCC (Iyengar et al. 2014). Although there is no specific explanation of how obesity contributes to progression and prognosis of OTSCC, some recommended interventions include exercise, reduction of weight and the use of anti-inflammatory drugs (Iyengar et al. 2014). However, more evidence is required before obese patients with early OTSCC can be considered at high risk of poor prognosis with a need for more aggressive treatment.

2.8.2 Clinical prognostic factors for OTSCC

Clinical staging (cTNM)

cTNM staging is based on the measurement of tumor diameter (T), lymph node status (N) and distant metastases (M). Worldwide, it is still the cornerstone of clinical staging during risk stratification and treatment planning of oral carcinomas. However, cTNM staging does not assess the biological behavior of the tumor. In addition, the cTNM system shows a low or no prognostic

ability in early OTSCC (Po Wing Yuen et al. 2002; Yanamoto et al. 2013). Many recent reports suggested improving it by the incorporation of other parameters that were reported as good predictors for OTSCC. Of these suggested parameters, the depth of invasion or tumor thickness has been widely reported for this purpose (Piazza et al. 2014a).

Clinical appearance of the tumor

There is very little information on the impact of the clinical appearance of the tumor on the prognosis of OTSCC. However, an endophytic appearance of OTSCC at the time of presentation is associated with a poorer prognosis compared to cases with an exophytic (proliferative) appearance (Sharma et al. 2013). In addition, the presence of oral lichen planus (currently or previously) was also reported as a sign of poor prognosis in early OTSCC (Bonnardot et al. 2011). Other factors related to clinical appearance such as a more posteriorly located OTSCC (i.e. tumors located in the posterior part of the oral tongue) confer an adverse prognosis (Bonnardot et al. 2011). It might be speculated that some of these posteriorly located cancers could have arisen at the base of the tongue (where the epithelium originated from the endoderm and where HPV is highly implicated in carcinogenesis), but it has been recognized clinically as carcinoma of the oral tongue (where the epithelium originated from the ectoderm and factors other than HPV were involved). However, such speculation is not supported by evidence.

Midline involvement

Extension of the primary tumor to the midline is an adverse prognostic feature in OTSCC, and it predicts the pattern of LN metastasis (Lloyd et al. 2012; Mallet et al. 2009). A similar finding was also reported in cohorts of OSCC (Capote-Moreno et al. 2010; Kowalski et al. 1999). Importantly, in OTSCC, contralateral LN metastasis (considered as a sign of a very poor prognosis) occurs if the

cancer involves the tongue midline (clinically or subclinically) or if the cancer is located close to the midline where small lymphatic vessels may cross to the other side (Lloyd et al. 2012).

Recurrence

The return of cancer after treatment is defined as recurrence, although the length of time to consider the lesion as recurrence (but not a second primary) is not well defined. The presence of recurrent disease (local, regional or both) is the most common cause of treatment failure and has been documented to imply poor prognosis in OTSCC (Peng et al. 2014; Yanamoto et al. 2013). The cause of cancer recurrence is usually attributed to incomplete surgical resection of the tumor. However, in early OTSCC, which is characterized by the small size of the lesion and easy surgical access, other causes such as aggressive behavior of the tumor may also be considered.

Second primary tumor

A second malignancy that presents in a certain tract simultaneously (i.e. synchronously) or after (i.e. metachronously) the diagnosis of an index tumor is known as a second primary tumor (van der Waal and de Bree 2010). Both the primary tumor and the second primary must be malignant, must be distinct, and the probability of metastasis must be excluded (i.e. the second primary is not a metastasis from the primary tumor) (Warren and Gates 1932). Although the latter two criteria are challenging, analysis of genetic background would, indeed, fulfill such criteria. Patients with oral cancer have an estimated risk of 10-35% for occurrence of second primary malignancy in the upper aerodigestive tract (van Oijen and Slootweg 2000). However, the risk varies among the age groups, with young patients having the highest risk (Bosetti et al. 2011). In tongue cancer, 9% of cases were reported with a second primary tumor (Li et al. 2011). Most of the second primary malignancies in

early OTSCC patients occur in the oral cavity and the oropharynx; and the rate of second primary malignancies was associated with smoking and alcohol consumption (Koo et al. 2015).

The concept of field cancerization indicating foci of invisible genetic alterations in tissues surrounding an OSCC (or in oral precancerous fields) has been described in oral cancer patients (Angadi et al. 2012; Bremmer et al. 2008). These field alterations could be considered as a cancer risk factor (Jaiswal et al. 2013). Based on this concept, it could be speculated that the surgeon has performed a successful surgical resection of early OTSCC with safe margins, but the treatment failure may depend on genetic alterations in the surrounding tissues due to long term exposure to carcinogens. Therefore, the identification of genotypically abnormal cells at histologically clear margins could be useful in prediction of cases at high risk of local failure (Jaiswal et al. 2013).

Metastasis (Nodal or Distant)

Metastasis is a complex process in which cancer cells from the primary tumor disseminate to other organs. The biochemical determinants of OTSCC metastasis are not well understood (Sano and Myers 2007). During the diagnosis of OTSCC (irrespective of tumor size), the presence of positive lymph nodes in the neck (cN+) is a sign of poor prognosis, and indicates a need for intensive treatment of the neck including END and/or radiotherapy. However, in clinically and radiographically negative lymph nodes (cN0), almost one third of the patients were reported to have occult nodal metastasis on histopathological examination of dissected nodes (Thiagarajan et al. 2014), and the corresponding percentage is higher in stage II. As an example, An and co-investigators (An et al. 2008) reported occult nodal metastasis in 15.4% of stage I cases, and 42.9% in stage II. The presence of occult metastasis is recognized as an independent predictor of poor survival in early OTSCC (Ganly, Patel and Shah 2012).

Notably, the navigation of neck lymph nodes through sentinel lymph node biopsy has shown a predictive power superior to END for the prognostication of LN metastasis in early OTSCC (Fan et

al. 2014). The use of this novel surgical approach has been widely suggested recently (Fan et al. 2014; Pedersen et al. 2015). The published studies have shown excellent intraoperative assessment of cervical LNs (Yamauchi et al. 2015). However, arguments against this approach have been raised including the time consuming and labor-intensive process in the pathology laboratory (Liu et al. 2011). Thus, it might be still necessary to use traditional prognostic markers that could help select the patients who would benefit from this navigation surgery. Interestingly, indocyanine green fluorescence has been used recently to guide the sentinel node biopsy, which has improved this technique (Nakamura et al. 2015; van den Berg et al. 2012).

In advanced stages of OTSCC, distant metastases are reported to occur in about 6% of cases (Thiagarajan et al. 2014). Metastasis of OTSCC to distant sites is a sign of very poor prognosis (Goodman et al. 2009); and the most affected sites are the lungs and the bones (Sano and Myers 2007).

Comorbidity

Comorbidity is defined as the presence of other medical conditions (e.g. hepatic, respiratory, cardiovascular, gastrointestinal, or renal diseases) in addition to the primary tumor (but not caused by the latter) (Datema et al. 2010). Comorbidities have been reported as indicators of a worse prognosis in patients with OTSCC (Okuyemi, Piccirillo and Spitznagel 2014) and HNSCC in general (Datema et al. 2010).

2.8.3 Histopathologic prognostic factors for OTSCC

The daily practice of a pathologist includes the evaluation of many histopathologic prognostic features to assign proper histological stratification to guide the clinician towards a suitable

treatment modality (i.e. to avoid undertreatment or overtreatment). Importantly, researchers have a keen interest in cancer cells at the invasive front (IF), and the IF area is usually evaluated by pathologists because these cells have aggressive behavior that differs from that of cells in other parts of the tumor (Bronsert et al. 2014; Bryne et al. 1992; Christofori 2006; Jensen et al. 2015a). In addition, critical biological events such as epithelial mesenchymal transition (EMT) are speculated to occur at the IF (Jensen et al. 2015a; Liang 2011).

Histopathologic grading systems

Histopathologic grading systems were introduced many decades ago for the classification and prognostication of oral cancers. Such models have been proposed by Broders (Broders 1920; Broders 1941), Jakobsson et al. (Jakobsson et al. 1973), Anneroth et al. (Anneroth, Batsakis and Luna 1987), Bryne et al. (Bryne et al. 1992; Bryne et al. 1989), and most recently by Brandwein-Gensler et al. (Brandwein-Gensler et al. 2005). Since their introduction, numerous reports by other researchers have addressed the predictive ability of such grading systems in OSCC. However, none of these models have shown prognostic power in early stage OTSCC.

A. Tumor grade

The first quantitative grading system was introduced by Broders (Broders 1920), and this grading has been widely used for the histopathologic staging of SCC in many locations. Based on the degree of tumor cell differentiation (Fig. 2), Broders divided SCCs into 3 grades: Low grade (Well-differentiated tumor), intermediate grade (Moderately-differentiated tumor) and high grade (Poorly-differentiated tumor) tumors. This system is explained in more detail in section 2.4.

According to this system, high-grade tumors have been suggested to behave aggressively and have a poor prognosis. This grading system might be criticized as it does not evaluate the tumor-host

interface, but it is still widely included in pathology reports of OSCC. However, there are reports that have shown a prognostic power of this system in OSCC (Thomas, Stedman and Davies 2014), while others have reported a weak predictive ability in early OTSCC (Ganly et al. 2013).

B. Grading of Malignancy

In 1973, Jakobsson and colleagues (Jakobsson et al. 1973) introduced a system for grading of malignancy that was applied first in a patient cohort with laryngeal cancer and later was applied to OSCC. Jakobsson's system is based on evaluation of the following parameters: tumor structure, degree of keratinization, nuclear pleomorphism, mitoses, mode of invasion, stage of invasion, vascular invasion and cellular response (Table 1).

Table 1: Histopathologic parameters and their scoring in the Jakobsson grading system (1973).

Morphologic characteristics	Points			
	1	2	3	4
Grading of tumor cell population				
Structure	Papillary	Strands	Small cords and groups of cells	Marked dissociation of cells
Differentiation	Highly keratinized	Moderately keratinized	Poorly keratinized	No keratinization
Nuclear pleomorphism	Few enlarged nuclei (EN)	Moderate number of EN	Numerous irregular EN	Anaplastic immature EN
Mitoses	Single	Moderate number	Great number	Numerous
Grading of tumor host relationship				
Mode of invasion	Well marked borderline	Cords (less marked borderline)	No distinct borderline	Diffuse growth
Stage of invasion	Possibly	Microcarcinoma	Nodular into connective tissue	Massive
Vascular invasion	None	Possibly	Few	Numerous
Lymphoplasmacytic infiltration (cellular response)	Marked	Moderate	Slight	None

Risk stratification:

Low risk: < 15 points; Intermediate risk: 15-20 points; High risk: > 20 points.

C. Multifactorial grading system

In 1987, Anneroth et al. (Anneroth, Batsakis and Luna 1987) reviewed the literature and recommended a new system of malignancy grading in oral squamous cell carcinomas (Table 2) based on the evaluation of parameters related to both tumor cell population (degree of keratinization, nuclear pleomorphism, number of mitoses) and tumor-host relationship (pattern of invasion, depth of invasion, inflammatory cell infiltration).

Table 2: Histopathologic features of the malignancy grading system introduced by Anneroth et al. (1987)

Morphologic characteristics	Points			
	1	2	3	4
Grading of tumor cell population				
Degree of keratinization	> 50% of cells	20-50% of cells	5-20% of cells	0-5% of cells
Nuclear pleomorphism (NP)	Little NP	Moderate NP	Abundant NP	Extreme NP
Number of mitoses/High power field	0-1	2-3	4-5	> 5
Grading of tumor host relationship				
Pattern of invasion (POI)	Pushing borders	Infiltrating, solid cords, bands and/or strands	Small groups or cords of infiltrating cells	Marked cellular dissociation in small groups and/or in single cells
Stage of invasion (Depth)	Carcinoma-in-situ	Distinct invasion involving lamina propria only	Invasion below lamina propria adjacent to muscles and salivary gland tissues	Extensive and deep invasion
Lymphoplasmacytic infiltration	Marked	Moderate	Slight	None

Malignancy grading: Grade I: 6-12 points; grade II: 13-18 points; grade III: 19-24 points. Higher grade is proposed to have poorer prognosis.

D. Malignancy grading system according to invasive front

Bryne and colleagues (Bryne et al. 1989; Bryne, Thrane and Dabelsteen 1991) suggested that cellular differentiation of the upper part of the tumor does not have any prognostic value in OSCC, while the invasive cells (i.e. cancer cells at the IF) of the same specimen have a significant prognostic value. So they proposed a grading system based on the following 5 parameters: degree of keratinization, nuclear pleomorphism, number of mitoses, pattern of invasion and inflammatory cell

infiltration. At the invasive front of the OSCC, they (Bryne et al. 1992) evaluated the same parameters used previously by Anneroth et al. (Anneroth, Batsakis and Luna 1987). Interestingly, they eliminated the stage of invasion; and also suggested omitting the number of mitoses as the latter is a time-consuming parameter affecting the reproducibility of the malignancy grading (Bryne et al. 1992). According to this system, malignancy grading ranged from 4 to 16 points divided into grade I (4-8 points), grade II (9-12 points), and grade III (13-16 points). A higher grade predicted a poorer prognosis.

E. Histologic risk assessment model

Brandwein-Gensler et al. (Brandwein-Gensler et al. 2005) proposed the histologic risk score (HRS) model based on evaluation of three parameters: worst pattern of invasion (WPOI), lymphocytic host response (LHR), and perineural invasion (PNI) (Table 3).

Table 3: HRS of Brandwein-Gensler et al. (2005)

Parameter	Type	Definition	score assigned
Worst pattern of invasion (WPOI)	Type 1	Pushing border	0
	Type 2	Finger-like growth	0
	Type 3	Large tumor islands (> 15 cancer cells)	0
	Type 4	Small tumor islands (\leq 15 cancer cells)	1
	Type 5	Tumor satellites which lie 1 mm or more away from the main tumor mass or the nearest satellite (under x 20 magnification)	3
Lymphocytic host response (LHR)	Type 1	Strong	0
	Type 2	Intermediate	1
	Type 3	Weak	3
Perineural invasion (PNI)	None	No PNI	0
	Small	Tumor wrapping around small nerve (< 1 mm in diameter)	1
	Large	Tumor wrapping around large nerve (\geq 1 mm in diameter)	3

Risk stratification:

Low risk: score 0; intermediate risk: score 1 or 2; and high risk: score 3 or higher.

Margin status

Close margins (<5 mm) are widely used as a predictor of worse prognosis and subsequently an indication for adjuvant therapy in OSCC (Ch'ng et al. 2013). In early stage OTSCC, the margin status of a glossectomy specimen was reported to be a predictor of local control (Chang et al. 2013). However, wide surgical margins in all directions (lateral, medial, anterior, posterior, mucosal and deep) have been recommended in early OTSCC (Iseli et al. 2012). The use of margin status as a prognostic marker is still an issue of debate due to factors related to the method of specimen processing, variations in the amount of shrinkage of the specimens and the use of different cutoff points for defining adequate margins (El-Fol et al. 2015; Mistry, Qureshi and Kumaran 2005). Such factors should not be neglected. In addition, the lack of compartmentalized anatomy in the tongue causes difficulties in the assessment of margin status of OTSCC (Montero et al. 2014). It has also been reported that surgery alone (without any postoperative adjuvant treatment) will provide acceptable control in OSCC cases where close (but uninvolved) surgical margins were the only adverse feature (Ch'ng et al. 2013). However, in clinical practice most of the cases with positive margins are stratified as high-risk cases and are eligible for postoperative chemo-radiotherapy, irrespective of the adequacy of such margins (Montero et al. 2014).

Dysplasia at margins

In oral leukoplakia, the presence of epithelial dysplasia has been recognized as a risk for poor prognosis (i.e. malignant transformation) (Weijers et al. 2002). Similarly, the presence of moderate or severe dysplasia at the resection margins of early OTSCC has been recognized as an adverse prognostic feature (Sharma et al. 2013; Sopka et al. 2013). From a surgical point of view, further excision of such dysplastic tissues should be performed in the same operation, and may reduce the risk of local recurrence.

Perineural invasion (PNI)

The invasion of cancer cells around and/or into a nerve has been reported as an adverse predictive factor in OTSCC (Goodman et al. 2009; Shen et al. 2014; Tai et al. 2012), and it is one of the parameters often included in pathology reports of many cancers. In addition, PNI has been one of the parameters in a proposed histologic staging model of early OSCC (Li et al. 2013). Surprisingly, a recent study on early OSCC (cT1-2N0) reported that PNI correlated significantly with neck recurrence and with a worse survival in T1 (n=146), but not in T2 (n=161) cases (Tai et al. 2013). The mechanism of perineural growth in oral cancer is still unclear. In early stage OTSCC, Kolokythas and colleagues (Kolokythas et al. 2010) suggested that both nerve growth factor (NGF) and its high-affinity receptor tyrosine kinase A (TrkA) participate in the mechanism of PNI. In addition, it was postulated that tumor cells interact with the microenvironment of the nerve, and that tumor cells in this new environment behave differently, and subsequently more aggressively (Roh et al. 2015). However, the difficulties in evaluation of PNI require improvements in the method of examination (e.g. use of nerve-specific staining) (Marchesi et al. 2010).

Lymphovascular invasion (LVI)

Lymphovascular invasion (LVI) is defined as the presence of cancer cells in the wall or in the lumen of lymphatic or blood vessels (Jardim et al. 2015). It has been documented as a useful prognosticator in OTSCC (Michikawa et al. 2012; Okuyemi, Piccirillo and Spitznagel 2014). Such an invasion of cancer cells into these vessels provides an avenue for nodal and distant metastases. However, the assessment of LVI is difficult using H-E staining, while it is easy to identify in Elastica van Gieson (EVG) staining (Michikawa et al. 2012).

Muscle invasion

The tongue is a muscular organ, and muscle invasion is quite common in OTSCC (Sharma et al. 2013). Recent studies have reported that invasion of intrinsic muscles has prognostic power for recurrence and/or occult lymph node metastasis in OTSCC (An et al. 2008; Chandler et al. 2011; Sharma et al. 2013). In cases of cT1N0 OTSCC, An et al (An et al. 2008) recommend performing END when involvement of tongue muscles is present.

Pattern of invasion

In many cancers (including early OTSCC), the pattern of tumor invasion (POI) has been shown to be a predictive histopathologic feature (Aita et al. 2015; Horn et al. 2012; Li et al. 2013; Soland et al. 2008; Yanamoto et al. 2013). The POI (Fig. 3) has been described as a “cohesive” POI (in which the tumor invades as large pushing borders or as finger-like growths) or as an “infiltrative” POI (in which the tumor invades as small islands or strands of ≤ 15 cancer cells). Recently, Brandwein-Gensler and colleagues (Brandwein-Gensler et al. 2005) have described tumor satellites as a part of an infiltrative POI. A tumor satellite is defined as the presence of isolated tumor islands 1 mm or more away from the main tumor mass or from the closest other tumor satellites. Tumor satellites are reported to be a predictive feature of occult metastasis in early OTSCC (Yang et al. 2011). In a further development, Chang et al. (Chang et al. 2010) have introduced the invasive pattern grading score (IPGS) as a modification of POI in OSCC.

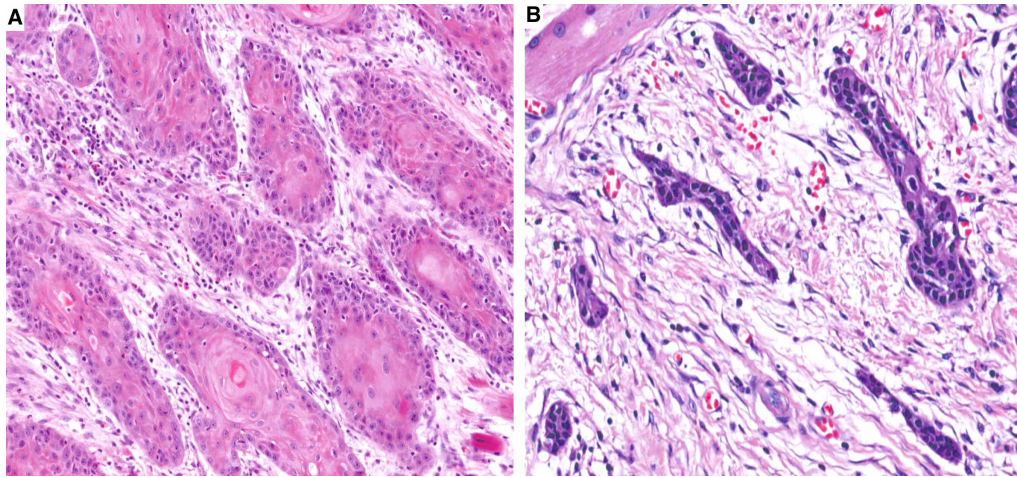


Figure 3: Large tumor islands as an example of cohesive POI (A); and small tumor islands as an example of infiltrative POI (B).

Tumor budding (TB)

The presence of single cancer cells or small clusters of less than five cells at the IF (Fig. 4) is defined as tumor budding (TB), and it has been widely recognized as a prognostic feature in many cancers including tongue cancer (Karamitopoulou 2013; van Wyk et al. 2014; Wang et al. 2011). According to Mitrovic et al. (Mitrovic et al. 2012), tumor budding (sprouting) was first described by Imai in the 1950s. Later on, numerous studies have evaluated TB in different cancers. The biological background of TB is still not fully understood. However, many researchers have found a strong correlation between TB and markers related to progression of cancer. For example, TB correlated with emergence of CAFs and laminin-5 gamma 2 in OSCC (Marangon Junior et al. 2014); and TB has also been correlated with other markers in colorectal cancer (Dawson et al. 2014; Guzinska-Ustymowicz 2006). In addition, TB correlated with EMT in tongue carcinoma and other malignancies (Karamitopoulou 2012; Liang et al. 2013; Wang et al. 2011; Yusra, Semba and Yokozaki 2012). EMT is well known as a key player in cancer progression. In OTSCC, it has been demonstrated that markers of EMT are common in both primary and metastatic OTSCC (Vered et al. 2010). Importantly, a recent study has reported TB as a prognosticator for occult lymph node metastasis and poor survival in early tongue cancer (Xie et al. 2014). The intensity of TB at the IF is

usually determined by counting of the buds in a high power field using a magnification of $\times 20$ (e.g. equal to 1.3 mm square). However, other magnifications and areas were also used.

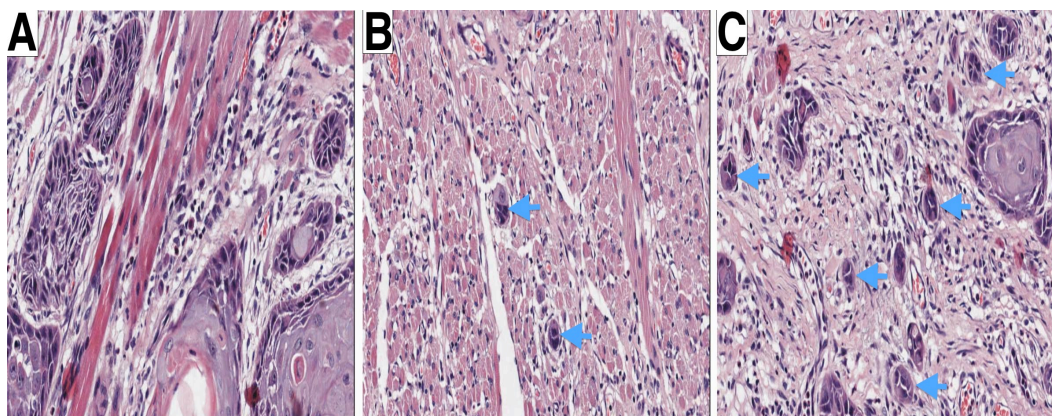


Figure 4: Intensity of tumor budding at IF of early OTSCC. A and B: Low intensity (< 5 buds); C: High intensity (≥ 5 buds).

Depth of invasion (DOI)

The depth of invasion or tumor thickness (these terms are often used as synonyms) has been widely recognized as a significant prognosticator in oral cancer. There is wide agreement from numerous researchers about the predictive role of DOI in OTSCC. On the other hand, there is disagreement about the optimal cutoff for risk stratification in relation to DOI, where cutoff points such as 2mm (Ganly, Patel and Shah 2012), 4mm (Ganly et al. 2013; Han et al. 2015), 5mm (P et al. 2003), and other DOI values (Huang et al. 2009; Po Wing Yuen et al. 2002) have been suggested. However, a recent meta-analysis suggested 4 mm as the optimal cutoff point in OSCC (Huang et al. 2009). The accuracy of non-invasive preoperative measurement of DOI by the use of magnetic resonance imaging (Iwai et al. 2002; Jung et al. 2009; Okura et al. 2008; Preda et al. 2006) and/or ultrasonography (Natori et al. 2008) may increase the importance of this parameter in prognostication. Notably, modification of the cTNM staging system by the incorporation of DOI has been recommended (Hubert Low et al. 2015; Piazza et al. 2014a).

Pathologic lymph node status (pN status)

The occurrence of occult metastasis in negative (clinically and radiographically) LN of the neck ranges between 23-40% (Feng et al. 2014; Peng et al. 2014) in early OTSCC cases. Although the pathologic staging of cervical LNs provides valuable prognostication in such cases (Ganly, Patel and Shah 2012), it cannot be applied before neck dissection which is associated with morbidity.

Extra-capsular spread (ECS)

The spread of metastatic tumor cells beyond the capsule of a lymph node is an indicator of more aggressive behavior in HNSCC (Puri, Fan and Hanna 2003). In OTSCC, early recurrence and subsequent poor prognosis was strongly associated with the presence of ECS (Greenberg et al. 2003; Okuyemi, Piccirillo and Spitznagel 2014). Similar to pN status, the main argument against ECS is that it can be examined only after neck dissection.

2.8.4 Immunohistochemical prognostic indicators in OTSCC

The advance in molecular pathology research has discovered numerous markers associated with cancer progression. Such markers may reveal molecular differences between early stage cancers, and subsequently may help in assessing the prognosis. Using immunohistochemical staining of formalin-fixed sections, researchers have tested hundreds of molecules to evaluate their prognostic ability in oral cancer. Sølund et al. (Solund and Brusevold 2013) recently reviewed the literature for any immunohistochemical markers introduced as prognosticators of OSCC during the period between October 2006 and October 2012. They found a total of 214 immunohistochemical markers in 172 prognostic studies of OSCC. They noted that most of these markers (75%) were reported only once without later validation, and thus the significance could not be analyzed. Only three

frequently used markers could be included in that review: Ki67 (11 studies), p53 (11 studies), and the epidermal growth factor receptor (EGFR) (7 studies) (Soland and Brusevold 2013).

In Table 4, we present a list of immunohistochemical markers tested recently for prognostication of OTSCC.

Table 4: Prognostic immunohistochemical markers for OTSCC evaluated since 2008

Author	Marker	Number of cases	Prognostic role
(Wangsa et al. 2008)	Ki-67	76 cases	Ki-67 is a predictor of loco-regional recurrence in early OTSCC
(Bello et al. 2008)	Claudin 7	97 cases	Claudin 7 is a predictor of disease-specific survival (DSS) in OTSCC
(Hayry et al. 2010)	Bmi-1	73 cases	Bmi-1 is predictor of recurrence in early OTSCC
(Zhang et al. 2011)	Lymphatic vessel density (LVD)	138 cases	LVD is a predictor for lymphatic metastasis in OTSCC
(Mostaan et al. 2011)	CD44	92 cases	Underexpression of CD44 is a significant marker for cervical lymph node metastasis
(Wang et al. 2012)	Snai2	129 cases	Snai2 is an important factor in progression of OTSCC
(Dayan et al. 2012)	CD163+ CD80+ Foxp3+	64 cases	A high score of CD163+ CD80+ Foxp3+ associated with poor survival in OTSCC
(Zhang et al. 2011)	MMP-1 and MMP-2	138 cases	Overexpression of MMP-1 and MMP-2 was a risk factor for lymph node metastasis in OTSCC
(Makinen et al. 2012; Makinen et al. 2014)	MMP-13 and MMP-7	73 cases	MMP-13 and MMP-7 are predictors of poor prognosis in cT1-2N0 OTSCC
(Aparna et al. 2014)	MMP-2 and MMP-9	59 cases	MMP-2 and MMP-9 have strong predictive value for local recurrence, regional metastases, distant metastasis and shorter survival in early OTSCC
(Bitu et al. 2013)	Cathepsin K	121 cases	Cathepsin K has a protective role in the progression of OTSCC and predicts the survival
(Kauppila et al. 2014; Kauppila et al. 2013)	TLR-5 and TLR-9	121 cases	TLR-5 and TLR-9 are predictive markers for survival in OTSCC
(Makinen et al. 2015)	TLR-2, -4, and -9	73 cases	TLR-2, -4, and -9 are important prognosticators in early OTSCC
(Kelner et al. 2015)	Activin A	110 cases	Activin A is a prognosticator of occult lymph node metastasis and overall survival (OS) in early OTSCC
(Han et al. 2015)	E-cadherin and vimentin	95 cases	Expression of EMT (positive vimentin and negative E-cadherin) is an indicator for cancer recurrence and nodal metastasis in OTSCC
(Vered et al. 2015)	Caveolin-1	64 cases	Caveolin-1 in OTSCC predicts recurrence and overall survival
(Imayama et al. 2015)	FOXC2	61 cases	FOXC2 is a predictor of survival in OTSCC

Cancer-associated fibroblasts (CAFs)

Tumor stroma is thought to play critical roles in cancer progression. Cancer-associated fibroblasts (CAFs) with a myofibroblast-like phenotype represent one of the most abundant types of stromal cells in many cancers (Augsten 2014). An early evaluation of the presence of myofibroblasts in oral cancer was carried out by Kellermann et al. (Kellermann et al. 2007) who have used immunohistochemical analysis of α -SMA in 83 cases of tongue SCC. They found that a high frequency of myofibroblasts correlates with lymph node metastasis, extra-capsular spread, and poor overall survival. Furthermore, similar findings were reported by different studies showing that CAFs are predictors of cervical lymph node metastasis (Ding et al. 2014), locoregional recurrence (Dayan et al. 2012), disease specific survival (Bello et al. 2011), and overall survival (Dayan et al. 2012; Ding et al. 2014) in OTSCC. In addition, Ding et al (Ding et al. 2014) found a significant correlation between expression of α -SMA with that of N-cadherin, vimentin, and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1). They suggested that α -SMA-positive myofibroblasts may be associated with EMT and with the microenvironment of nodal metastasis. Moreover, a similarity in the pattern of CAF distribution in primary OTSCC and in metastases in regional lymph nodes has been reported (Vered et al. 2010).

3 Aims of the study

The aims of this study were to identify powerful and easily applicable prognosticators for early stage (cT1-2N0) OTSCC. Specifically, the purposes of this study were:

- A.** To evaluate the prognostic value of a histologic risk model, tumor budding, and depth of invasion in early (cT1-2N0) OTSCC (Study I).
- B.** To test the predictive value of CAFs in a cohort of early (cT1-2N0) OTSCC (Study I).
- C.** To evaluate the impact of several clinicopathologic factors in a large international cohort of early OTSCC cases from three geographic regions (Study II).
- D.** To summarize the findings related to tumor budding in head and neck cancer (Study III).
- E.** To introduce a simple histopathologic prognostic model for early stage OTSCC (Study IV).

4 Material and methods

A total of 479 cases of early OTSCC were included (variants of SCC were not included) in this thesis. Of these, 233 Finnish cases were included in study I, 479 cases (224 Finnish, 109 Brazilian, 146 American) in study II, and 311 cases (224 Finnish, 87 Brazilian) in study IV (Table 5 and 6). A description of the method of evaluation of each prognostic factor is provided below.

Table 5: Clinicopathologic characteristics of patients (modified from Study I, II, and IV).

Characteristics	Number of cases (%)		
	Study I (233 cases)	Study II (479 cases)	Study IV (311 cases)
Age			
≤ 60 years	86 (36.9)	229 (47.8)	129 (41.5)
> 60 years	147 (63.1)	250 (52.2)	182 (58.5)
Gender			
Male	109 (46.8)	262 (54.7)	165 (53.1)
Female	124 (53.2)	217 (45.3)	146 (46.9)
Clinical stage			
cT1N0	113 (48.5)	212 (44.3)	124 (39.9)
cT2N0	120 (51.5)	267 (55.7)	187 (60.1)
Tumor grade			
I (well differentiated)	83 (35.6)	129 (26.9)	105 (33.8)
II (moderately differentiated)	109 (46.8)	258 (53.9)	131 (42.1)
III (poorly differentiated)	41 (17.6)	92 (19.2)	75 (24.1)
Worst pattern of invasion			
Cohesive	53 (22.7)	166 (34.7)	78 (25.1)
Invasive (infiltrative)	180 (77.3)	313 (65.3)	233 (74.9)
Lymphocytic hostresponse			
Strong or intermediate	120 (61.2)	246 (51.4)	169 (54.3)
Little or no response	76 (38.8)	233 (48.6)	142 (45.7)
Perineural invasion			
Absent	198 (85.0)	376 (78.5)	269 (86.5)
Present	35 (15.0)	103 (21.5)	42 (13.5)
Histologic risk score			
Low risk (< 3)	111 (47.6)	192 (40.1)	130 (41.8)
High risk (≥ 3)	122 (52.4)	287 (59.9)	181 (58.2)
Tumor budding			
Low (< 5 buds)	152 (65.2)	-	215 (69.1)
High (≥ 5 buds)	81 (34.8)	-	96 (30.9)
Depth of invasion			
Superficial (< 4 mm)	80 (34.3)	151 (31.5)	116 (37.3)
Deep (≥ 4 mm)	153 (65.7)	321 (67.0)	195 (62.7)
Missing	-	7 (1.5)	-
BD model			
Low risk (0)	-	-	103 (33.1)
Intermediate risk (1)	-	-	125 (40.2)
High risk (2)	-	-	83 (26.7)
Cancer associated fibroblast*			
Low score (0-1)	27 (32.9)	-	-
Medium score (2-3)	40 (48.8)	-	-
High score (4)	15 (18.3)	-	-

* Only samples of 82 patients were available for staining.

Table 6: Clinicopathologic characteristics of 479 patients who have been involved in the analysis of disease-specific survival (DSS) and disease-free survival (DFS). Modified from Study I, II, and IV.

Characteristic	Number of cases (%)			
	Finland* n=224 (%)	USA n=146 (%)	Brazil n=109 (%)	Total n=479 (%)
Age (years)				
10-40	17 (7.6)	13 (8.9)	12 (11.0)	42 (8.8)
41-59	63 (28.1)	65 (44.5)	48 (44.0)	176 (36.7)
60-74	85 (37.9)	52 (35.6)	37 (33.9)	174 (36.3)
75-96	59 (26.3)	16 (11.0)	12 (11.0)	87 (18.2)
Gender				
Male	105 (46.9)	81 (55.5)	76 (69.7)	262 (54.7)
Female	119 (53.1)	65 (44.5)	33 (30.3)	217 (45.3)
Grade				
I (well differentiated)	81 (36.1)	17 (11.6)	31 (28.4)	129 (26.9)
II (moderately differentiated)	103 (46.0)	114 (78.1)	41 (37.6)	258 (53.9)
III (poorly differentiated)	40 (17.9)	15 (10.3)	37 (33.9)	92 (19.2)
Clinical stage				
T1N0	108 (48.2)	86 (58.9)	18 (16.5)	212 (44.3)
T2N0	116 (51.8)	60 (41.1)	91 (83.5)	267 (55.7)
WPOI				
Low	52 (23.2)	74 (50.7)	40 (36.7)	166 (34.7)
High	172 (76.8)	72 (49.3)	69 (63.3)	313 (65.3)
LHR				
Strong or intermediate	144 (64.3)	59 (40.4)	43 (39.4)	246 (51.4)
A little or no response	80 (35.7)	87 (59.6)	66 (60.6)	233 (48.6)
PNI				
Absent	189 (84.4)	89 (61.0)	98 (89.9)	376 (78.5)
Present	35 (15.6)	57 (39.0)	11 (10.1)	103 (21.5)
Histologic risk score				
Low risk (< 3)	108 (48.2)	45 (30.8)	39 (35.8)	192 (40.1)
High risk (≥ 3)	116 (51.8)	101 (69.2)	70 (64.2)	287 (59.9)
Depth of invasion				
< 4mm	79 (35.3)	31 (21.2)	41 (37.6)	151 (31.5)
≥ 4mm	145 (64.7)	115 (78.8)	61 (56.0)	321 (67.0)
Missing	-	-	7 (6.4)	7 (1.5)

* Nine cases were excluded due to insufficient data on recurrence.

4.1 Evaluation of cancer associated fibroblasts (Study I)

Paraffin embedded blocks of 82 cases treated for early stage (cT1N0, n = 31; and cT2N0, n = 51) OTSCC were retrieved from the archives of three Finnish University hospitals located in Helsinki (n = 18), Oulu (n = 37), and Kuopio (n = 27). The immunohistochemical staining of α -smooth muscle actin (α -SMA) was carried out on sections of 4 μ m thickness which were first deparaffinized in xylene and rehydrated through a series of graded ethanols. For antigen retrieval, the sections were heated (10 minutes) in a microwave oven (10mmol/L citrate buffer, pH 6.0). Then

endogenous peroxidase quenched in 0.3% hydrogen peroxide (diluted in H₂O). The sections were incubated at room temperature for 60 minutes with the primary antibody: monoclonal mouse anti- α SMA (diluted 1:1000) manufactured by Dako A/S, Glostrup, Denmark. After that, the slides were overlaid with a biotinylated secondary antibody, and a Histostain SP kit (Zymed, San Francisco, CA, USA) was used for antibody detection. Color was developed in diaminobenzidine solution (DAKO A/S Denmark). Counterstaining was done with Mayer's hematoxylin and the slides were rehydrated and mounted. Between the staining steps, the sections were washed with phosphate buffered saline (PBS).

Each case was scored according to the area with the densest staining. The slides were first scanned with low magnification ($\times 4$ objective), and then the density of CAFs was assessed using a $\times 10$ objective (2.6 mm square). The density of cancer-associated fibroblasts (CAFs) was used to classify the cases into 2 groups: High CAF score (or CAF rich pattern) *vs.* low CAF score (or CAF poor pattern). The cases were also categorized into 3 groups (low, intermediate, and high) to compare with a previous study (Bello et al. 2011).

4.2 Evaluation of tumor budding, depth of invasion and histologic risk score in early stage OTSCC (Study I)

The hematoxylin and eosin (H-E) stained surgical slides of 233 cases treated for early stage (cT1-2N0) OTSCC at the five Finnish University hospitals (Helsinki, Turku, Tampere, Oulu and Kuopio) were retrieved. The patients' clinicopathological characteristics are shown in Table 5. Tumor budding (TB) and depth of invasion (DOI) were scored as explained previously in other studies (Jerjes et al. 2010; Wang et al. 2011). In brief, TB is defined as the presence of single cancer cells or small clusters of less than five cells at the invasive front (IF) of the tumor (Fig. 4). The IF of all available sections was scanned using a low magnification ($\times 4$) objective, then the field with the highest tumor budding was counted using a high magnification objective ($\times 20$). For risk

stratification based on TB, a cutoff of five buds (low risk <5 buds vs. high risk ≥ 5 buds) was used. DOI was measured from the tumor surface to the point of deepest invasion. A cutoff point of 4 mm was used to classify cases based on DOI (superficial $<4\text{mm}$ vs. deep $\geq 4\text{mm}$), as recommended in a recent meta-analysis (Huang et al. 2009). The parameters of the histologic risk score (HRS), which include worst pattern of invasion (WPOI), lymphocytic host response (LHR), and perineural invasion (PNI), were scored as previously described (Brandwein-Gensler et al. 2005) and explained briefly in Table 3.

4.3 Evaluation of prognostic impact of age in early OTSCC (Study II)

To recruit a large number of young cases, we conducted a collaboration with Dr. Simion I. Chiosea and Dr. Ann Margaret Chang (Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA). The data of 146 cases treated there for early (cT1-2N0) OTSCC were retrieved and included in this study. In addition, the data of 109 cases treated at the A.C. Camargo Hospital, Sao Paulo, Brazil were also included in this analysis forming a total of 479 cases with early stage OTSCC as a cohort for this study (Table 6). To analyze the impact of age on the prognosis of early OTSCC, patients were grouped into 4 age groups (≤ 40 years, 41-59 years, 60-74 years, and 75-96 years).

4.4 Validation of prognostic value of DOI and WPOI in a larger patient cohort from three geographic regions (study II)

The data of 479 cases (Table 6) involved in the age analysis were also used to validate our previous findings about DOI and WPOI reported in study I. The scoring of histopathologic parameters of the cases from Brazil and the USA was performed using the same criteria as in study I. Data on tumor depth were missing in 7 cases from Brazil (1.5%).

4.5 Systematic review of studies of tumor budding in HNSCC (study III)

The search word tumo(u)r budding (both English and American spelling) was employed for the databases of Scopus, PubMed and Web of Science. Our search was limited to literature in the English language only, up to and including February 2014. Later on, the search was updated to include literature up to and including March 2015 (Fig. 5). The search results were then refined using the following terms: head and neck squamous cell carcinoma, oral squamous cell carcinoma, oral tongue squamous cell carcinoma, tongue squamous cell carcinoma, nasopharyngeal carcinoma, laryngeal squamous cell carcinoma, oropharyngeal squamous cell carcinoma, hypopharyngeal squamous cell carcinoma and sinonasal squamous cell carcinoma.

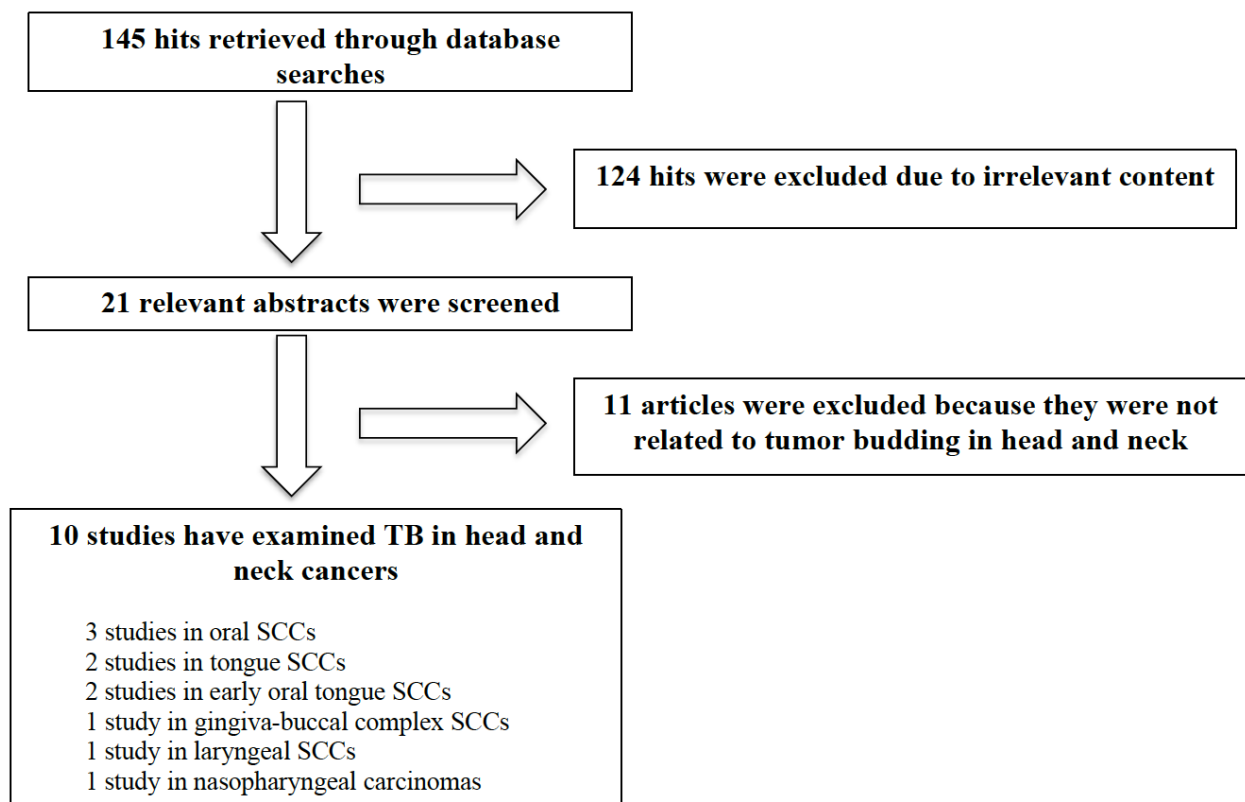


Figure 5: Flowchart of database searches (study III and unpublished data).

4.6 Proposal for new prognostic model in early OTSCC (study IV)

The H-E stained surgical slides of 87 cases treated for early stage (cT1-2N0) OTSCC at the A.C. Camargo Hospital, Sao Paulo, Brazil were retrieved and scored for the presence of TB and

measured for DOI. From the Finnish cohort involved in study I, 9 cases were excluded due to insufficient data for recurrences. Thus a total of 311 cases were eligible for study IV, which comprised the rest of the Finnish cases (224 cases) and the 87 Brazilian cases (Table 5). The cut off points for risk stratification of both tumor budding and depth of invasion were similar to those used in study I. The scores of tumor budding and depth of invasion were assigned in one predictive model (BD model) as follows:

Score 0: Both tumor budding and depth of invasion are less than the cut-off points.

Score 1: Either tumor budding or depth of invasion exceeds the cut-off point.

Score 2: Both tumor budding and depth of invasion exceed the cut-off points.

4.7 Statistical analysis

Statistical analyses were carried out using SPSS software. The impact of the studied factors on prognosis of early OTSCC patients was evaluated in relation to disease-specific survival (study I, II and IV), disease-free survival (study II and IV), and overall survival (study II). Disease-free survival (DFS) was defined as the time from surgery to recurrence (at the site of the primary tumor, in neck LNs, or both) or the last date of follow-up. Kaplan-Meier (KM) plots were constructed to present cumulative survival outcomes and compared using a log rank test. Similarly KM plots were constructed for DFS using recurrence as the end point. The unadjusted (univariate) analysis was used for single parameters. The adjusted (multivariate) analysis was fitted with categorical covariates to assess the strength of each parameter.

4.8 Ethics statement

Use of patient materials and follow-up information were approved by the following authorities: The National Supervisory Authority for Welfare and Health “VALVIRA” (for study I, II, and IV), the Brazilian Human Research Ethics Committee (for study II and IV), and the Total Quality Council of the University of Pittsburgh Medical Center (for study II).

5 Results

The clinicopathologic characteristics of the patients were explained in Table 5 and Table 6. There was significant variation (Pearson Chi-Square, $P < 0.05$) among the cohorts as follows: In the Finnish cohort, there were more old patients (≥ 75 years), more women, and more invasive tumors than in the Brazilian or the US cohorts. The tumors in the Brazilian patients were more poorly differentiated and larger than those in the Finnish or US patients. In the US patients, there were more PNI, and the number of deeper tumors ($\geq 4\text{mm}$) was more than in the Finnish or Brazilian cohorts.

5.1 Cancer associated fibroblasts in early stage OTSCC (study I)

According to the density of CAFs, 27 cases (32.9%) were assigned a low score, 40 cases (48.8%) received a medium score, and 15 cases (18.3%) had a high score. The density of CAFs showed a weak correlation with cancer mortality, and was not a promising predictor of survival when cases were stratified into low and high risk groups based on the density of CAFs ($P = 0.4$). The cumulative survival curves (Fig. 6) showed that the CAF rich pattern was not associated with a higher rate of OTSCC mortality when compared to CAF poor pattern (i.e. low and intermediate scores together).

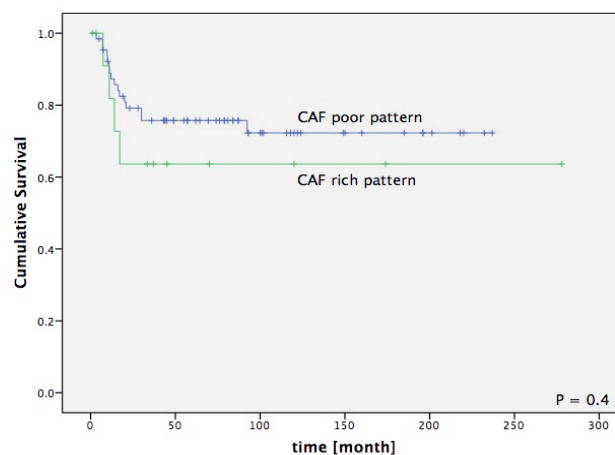


Figure 6: Disease-specific survival of OTSCC patients in relation to density of CAFs.

5.2 Tumor budding in early stage OTSCC (study I)

One hundred and fifty two cases (65.2%) had a low budding score (<5 buds), while 81 cases (34.8%) had a high score (≥ 5 buds). High scores of TB were significantly associated with a higher rate of OTSCC mortality with a HR of 2.04 (95% CI 1.17–3.55), $P = 0.01$.

5.3 Histologic risk score (HRS) in early stage OTSCC (study I and II)

HRS was evaluated in 233 cases of early stage OTSCC (Table 5), and did not show any association with patient survival ($P > 0.05$). Furthermore, we have evaluated separately the prognostic impact of each parameter involved in the HRS (WPOI, LHR, and PNI). Only WPOI was associated strongly with mortality due to OTSCC (HR 4.47; 95% CI 1.59–12.51; $P = 0.004$). Moreover, we re-evaluated the prognostic role of HRS and its parameters (WPOI, LHR, PNI) in a larger cohort (study II) of 479 cases (Table 5). Similarly, HRS did not show a predictive role for survival (unpublished data). In particular, the WPOI turned out to be a prognostic feature for OTSCC mortality (HR 2.34; 95% CI 1.26–4.32). However, the WPOI was not a strong predictor for locoregional recurrence in early OTSCC (HR 1.46; 95% CI 0.95–2.25).

5.4 Depth of invasion (DOI) in early stage OTSCC (study I, II)

In the Finnish cohort (study I), DOI showed a strong predictive power for DSS in early OTSCC (HR 2.55; 95% CI 1.25–5.20, $P = 0.01$). The same result was confirmed in our multicenter study from Finland, Brazil, and the USA (study II) which reported DOI as a prognosticator for DSS (HR 2.44; 95% CI 1.34–4.47), and also for DFS, but with less predictive power for the latter (HR 1.67; 95% CI 1.07–2.60).

5.5 Impact of age on the prognosis of early OTSCC (II)

Forty two patients (8.8%) were ≤ 40 years of age, 176 patients (36.7%) were between 41 to 59, 174 patients (36.3%) were between 60 to 74, and 87 patients (18.2%) were 75 years old or older.

Statistical analysis did not show any evidence for poor prognosis in young patients with early OTSCC. At the same time, the analysis did not allow a definitive result concerning the prognosis of young patients with OTSCC due to the small number of recurrences and cancer-related mortality in this young group. Instead, old patients (≥ 75 years) were shown to have a higher rate of locoregional recurrence (HR 1.74; 95% CI 1.08-2.81), and a higher incidence of cancer mortality (HR 2.39; 95% CI 1.30-4.40).

5.6 Tumor budding (TB) in HNSCC (study III)

The result of database searches until March 2014 retrieved 122 hits, and TB has been evaluated in only five of them (study III). In addition, an update of the database searches revealed another five studies, which have examined TB (not included in our systematic review published earlier). In total, 10 studies (Table 7) examined TB in HNSCC as follows: three studies (Angadi et al. 2015; Marangon Junior et al. 2014; Sawazaki-Calone et al. 2015) in OSCC (different subsites), two in TSCC (Wang et al. 2011; Xie et al. 2014), two in early OTSCC (Almangush et al. 2014; Almangush et al. 2015), one study in the gingival-buccal complex SCC (Manjula et al. 2015), one in laryngeal SCC (Sarioglu et al. 2010) and one in nasopharyngeal carcinoma (Luo et al. 2012). Two studies from our group (Almangush et al. 2015; Sawazaki-Calone et al. 2015) have analyzed TB as a part of the BD predictive model (study IV). Interestingly, the findings of these ten studies indicate that TB has a prominent role in progression and prognosis of HNSCC. Table 7 summarizes these studies.

Table 7: Summary of 10 studies that examined TB in HNSCC.

Author and year	Site	Stage	Cases	Cutoff/Stain	Main findings
(Sarioglu et al. 2010)	Larynx	II–IV	64	2/3 of IF ^a /H-E ^b	TB is a prognosticator for distant metastasis in laryngeal SCC, P = 0.04
(Wang et al. 2011)	Tongue	I–IV	133	5 buds ^c /H-E	TB is a predictor of overall survival, HR 3.02 (95% CI 1.53-5.97), P = 0.001. TB has a strong association with EMT
(Luo et al. 2012)	Nasopharynx	I–IV	105	5 buds/IHC ^d	TB is a predictor of survival, HR 4.17 (95% CI 1.85-9.37), P = 0.001. Budding cells have a higher level of ALDH1, P < 0.001
(Almangush et al. 2014)	Oral tongue	I-II	233	5 buds/H-E	TB is a predictor of DSS, HR 2.04 (95% CI 1.17-3.55), P = 0.01
(Marangon Junior et al. 2014)	Oral cavity	NA	57	5 buds/IHC	High scores of TB associated with high density of stromal myofibroblasts, odds ratio (OR) 6.18 (95 CI 1.49-25.57), P < 0.05. High TB associated with high expression of laminin-5 gamma2, OR 16.04 (95% CI 3.61-71.19), P < 0.05
Update of database searches					
(Xie et al. 2014)	Tongue	I-II	195	5 buds/IHC	TB is a predictor of survival in early TSCC, HR 5.58 (95%CI 1.22-25.38), P = 0.02
(Angadi et al. 2015)	Oral Cavity	I-IV	75	10 buds ^e /H-E	High-TB score is a predictive marker for lymph node metastasis, OR 6.79 (95% CI 2.28-20.18), P = 0.001
(Manjula et al. 2015)	Gingivo-buccal complex	I-IV	33	10 buds ^f /H-E	TB is a predictor of nodal metastases (only in univariate analysis), P = 0.014
(Almangush et al. 2015)	Oral tongue	I-II	311	5 buds/H-E	TB is a parameter of the BD prognostic model (study IV). TB was a predictor of DSS, HR 1.76 (95% CI 1.01–3.06), P = 0.044; and also a predictor of recurrence, HR 1.80 (95% CI 1.10–2.93), P = 0.020
(Sawazaki-Calone et al. 2015)	Oral cavity	I-IV	113	5 buds/H-E	TB is a parameter of the BD prognostic model which is introduced in study IV

a: High score = budding is present in $\geq 2/3$ of the IF area

b: Hematoxylin and eosin

c: Low <5 buds; High ≥ 5 buds

d: Immunohistochemistry (pan-cytokeratin staining)

e: Low: <10 buds; High: ≥ 10 buds

f: Low: ≤ 10 buds; High >10 buds

NA: Not available

5.7 A histopathologic prognostic model (BD model) for early stage OTSCC (IV)

According to BD scores (0-2), 103 cases (33.1%) had score 0 (low risk), 125 cases (40.2%) had score 1 (intermediate risk), and 83 cases (26.7%) had score 2 (high risk). A high BD score (score 2) predicted the locoregional recurrence (HR 2.19, 95% CI 1.20-4.00) as shown in Figure 7A. Similarly, score 2 predicted the cancer-specific mortality (HR 5.11, 95% CI 2.05-12.75) (Fig 7B).

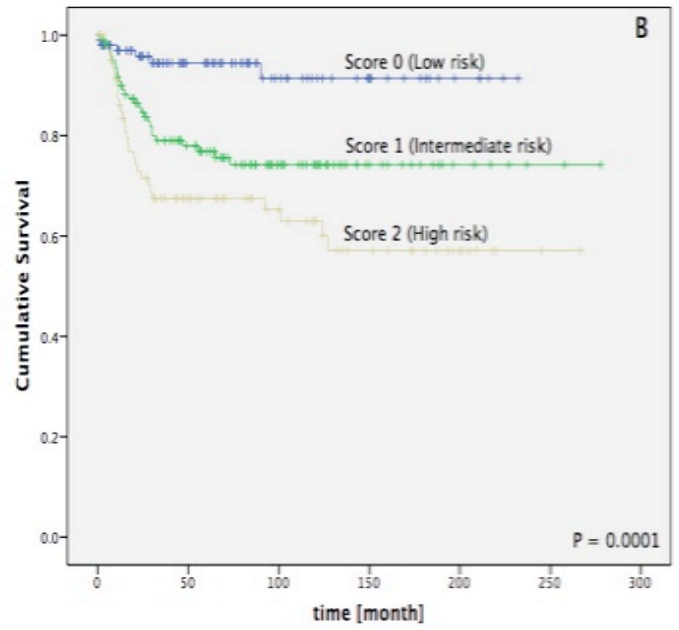
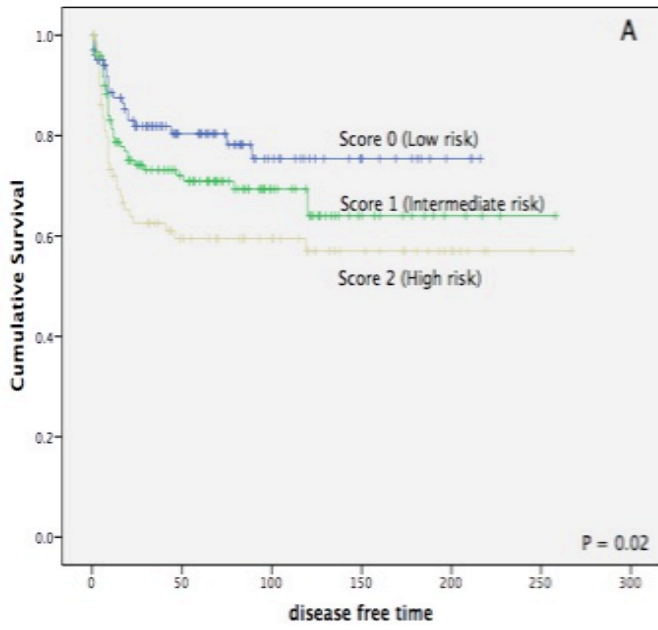


Figure 7: Disease-free survival (A) and disease-specific survival (B) in early OTSCC patients in relation to BD scores.

6 Discussion

At an early stage (cT1-2N0), OTSCCs are characterized by a high incidence of recurrences, occult metastases and cancer-related mortality. This indicates that the risk of poor prognosis has been underestimated by the current cTNM staging, and this subsequently leads to inappropriate management. Numerous previous studies have proposed new prognostic factors for OTSCC. Most of these studies have shown important methodological shortcomings including heterogeneity of tumor site (i.e. including cancers of all oral cavity subsites), heterogeneity of tumor stage (i.e. including early and late stages), a time-consuming or labor-intensive protocol due to special methodology (e.g. immunostaining requirements), complexity of suggested scoring methods in some systems, conclusions based on very small cohorts, or the experience of a single institution only. In this study, we have attempted to overcome such obstacles by introducing prognosticators that could easily be included in pathology reports and would be applicable for survival assessment in early OTSCC. Since there were differences in the percentages (%) of the studied parameters between the cohorts, we have included the centers as a variable in the multivariate analysis of the Finnish study. In addition, the countries were categorized as a variable in the multivariate statistical analysis of the international cohorts. There was no significant difference between the centers or the countries on multivariate analysis. Below, we provide a brief discussion on each prognostic factor examined in this study.

6.1 Cancer associated fibroblasts (CAFs) in early OTSCC

The tumor microenvironment makes a strong contribution to cancer progression, and α -SMA positive myofibroblasts are among the main components of this microenvironment (Thode et al. 2011). Prior research from our group (Bello et al. 2011) and others (Ding et al. 2014; Kellermann et al. 2007) has demonstrated that the density of CAFs is an independent predictor in OTSCC. Notably, most of the previous studies have included early and late stages of OTSCC. According to

the best of our knowledge, no previous study has evaluated CAFs in a homogenous cohort of early OTSCC or early OSCC. However, we found that CAFs have little or no prognostic value in early OTSCC. Similarly, a recent study analyzed CAFs in early OTSCC, and demonstrated that their prognostic value does not have statistical significance (Kelner et al. 2015). Interestingly, a high CAF score was an unusual event in our cohort. We speculated that an increase in density of CAFs in OTSCC might require more time than is available during the early stages. However, larger cohorts than ours are required to reach conclusions on the role of CAFs in early OTSCC.

6.2 Tumor budding (TB) in early OTSCC

TB has been identified as a histopathologic prognostic marker in various cancers (Karamitopoulou et al. 2013; Landau et al. 2014; Lugli, Karamitopoulou and Zlobec 2012). The current study is the first to examine the prognostic power of TB in early OTSCC. Interestingly, a recent study on early tongue cancer has found that a high intensity of TB is a predictor of poor survival. In addition, they found that TB is a prognosticator for occult metastases (Xie et al. 2014). TB has been suggested as analogous to epithelial cells that are undergoing EMT in tongue cancer (Wang et al. 2011), and correlate strongly with EMT in other cancers (Karamitopoulou 2012; Niwa et al. 2014). It was demonstrated that reduced expression of a cell adhesion molecule (E-cadherin) occurs in the budding cells of tongue cancer (Wang et al. 2011) and other cancers (Nakagawa et al. 2013; Yamaguchi et al. 2010). Moreover, a recent genetic study has compared cells from a center of OSCC with budding cells and found that the latter have a particular gene expression signature including factors involved in EMT (Jensen et al. 2015a). This study also noted many genes that indicate an increase in production of extracellular matrix in budding cells.

High TB scores were strongly correlated with increased density of stromal myofibroblasts in OSCC (Marangon Junior et al. 2014). However, Jensen et al. (Jensen et al. 2015b) in their in vivo study have reported that the presence of TB is not dependent on the existence of stromal myofibroblasts.

Therefore, the crosstalk between the invading TB cells and myofibroblasts still requires further research.

In cancers other than OSCC, there is more research on TB, and several markers related to cancer progression have correlated strongly with TB. For example, a cancer stem cell marker, aldehyde dehydrogenase 1, was associated with TB in nasopharyngeal carcinoma (Luo et al. 2012). In colorectal cancer, TB was associated with cathepsin B (Guzinska-Ustymowicz 2006) and the epidermal growth factor receptor (EGFR) (Ljuslinder et al. 2011).

Our systematic review has demonstrated TB as a promising prognostic marker in HNSCC. TB has been studied in patient cohorts of OSCC, gingival-buccal SCC, tongue SCC (all stages), early OTSCC, LSCC and NPC (Table 7). In these studies, tumors were associated with different etiological factors. Moreover, the method of TB assessment was not similar in some studies, and the cutoff point for risk stratification varied. In addition, variation in the size of the field among the optical microscopes is also expected (i.e. square mm area of $\times 20$ objective varies from one microscope to another). In our cohort, we have used an OLYMPUS BH2 which has $\times 20$ objective of 1.3 mm square.

6.3 Depth of invasion (DOI) in early OTSCC

Evaluation of DOI in our cohort of 479 cases (study II) with early OTSCC has revealed that DOI is a significant predictor for both LRR and DSS. The results of various studies have shown disagreement on the optimal cutoff point for risk stratification (Ganly, Patel and Shah 2012; Han et al. 2015; Huang et al. 2009). Such disagreement is one of the main obstacles for using DOI as a guide in management of early OTSCC. However, inclusion of SCC from different subsites of the oral cavity in the same analysis is possibly one of the reasons for the disagreement between previous publications. There is evidence that $\text{DOI} \geq 2$ mm is high risk for cases having a SCC in the floor of the mouth while in tongue SCC, $\text{DOI} \geq 4$ mm cases were at high risk (Balasubramanian et

al. 2014). Notably, DOI has been suggested as a modification for current cTNM staging in OSCC (Ebrahimi et al. 2014; Hubert Low et al. 2015; Piazza et al. 2014a). However, further research is needed to confirm the usefulness of such a modification.

6.4 Histologic risk score (HRS) in early OTSCC

HRS was introduced in 2005 by Brandwein-Gensler and colleagues (Brandwein-Gensler et al. 2005). This prognostic model is based on three histopathologic parameters: WPOI, LHR and PNI. Her group has validated this model twice: First in 2010 (Brandwein-Gensler et al. 2010), and later in 2013 (Li et al. 2013) using a cohort of early OSCC only. However, our current study did not find significant prognostic power for HRS in early OTSCC. To avoid potential bias in scoring of HRS, we have analyzed the prognostic value of HRS separately in 146 cases of early OTSCC scored by Chang et al. (Chang et al. 2013), but also then we did not find significant predictive value for HRS (unpublished data). Similarly, in their recent study Rodrigues and colleagues did not find prognostic power for HRS in a large cohort of 202 cases of OTSCC (all stages). (Rodrigues et al. 2014).

According to our experience with HRS, we noted that the model will assign a high risk score (score 3) in cases with little or no host response (Table 3). We argue that in such cases the cancer may show cohesive invasion (i.e. pushing border, finger-like growth or large separate islands) and also no PNI. Similarly for WPOI or PNI, tumor cases will be assigned score 3 (high risk) if only one of these parameters is high irrespective of the other parameters. Therefore, several cases have been assigned high risk (score 3) when they only had one parameter with a high score. Thus, in HRS the assignment of high risk (score 3) can be based on a single parameter instead of a combined score.

Our separate analysis of each of the parameters included in the HRS has shown that only WPOI is an independent prognosticator in early OTSCC. This is also in line with findings from other studies (Li et al. 2013; Yanamoto et al. 2013). It is speculated that anti-tumor effects of the inflammatory cells might play a role in prognostication of patient survival. For example, a high density of

inflammatory infiltration was associated with a favorable survival in OTSCC (Chatzistamou et al. 2010) and TSCC (Lundqvist et al. 2012). However, the analysis of LHR in our cohort did not show any predictive power in early OTSCC. A shortcoming should be acknowledged; in this study LHR was analyzed as a single parameter (on hematoxylin and eosin stained slides) without identifying the different subtypes of lymphocytes.

6.5 Tumor grade (differentiation) in early OTSCC

Our data analysis has shown a weak prognostic power of tumor grade in early OTSCC. On the other hand, Thomas et al. (Thomas, Stedman and Davies 2014) recently analyzed the database of Surveillance, Epidemiology, and End Results (SEER), and reported a strong correlation between poorly differentiated tumors and mortality in early OSCC (in all subsites of the oral cavity). However, Ganly et al. (Ganly et al. 2013) did not find tumor grade to have significant prognostic value for recurrence (local or regional) in a series of 164 cases treated for early OTSCC. The same finding is presented in our current study, which has the largest series of early OTSCCs to date. The current data have enough statistical power to address this issue and to conclude that tumor grade is not a promising prognosticator in early OTSCC.

6.6 Prognostic impact of patient age in early OTSCC

The current study has conducted an international collaboration of seven institutions but the number of young patients (≤ 40 years) is still small (42 cases). However, in this material we did not find evidence for poor survival in young patients compared to other age groups. Conversely, we have noted that the risk of recurrence and cancer-specific mortality increases with age, and is distinctly more common in the oldest age group (≥ 75 years). Based on our results we are not able to say whether young patients have a worse, better or similar survival, because the number of recurrences and deaths among the young were very small. Notably, Knopf and colleagues (Knopf et al. 2015) has compared the outcome of 66 young patients (≤ 45 years) with 210 older ones, and found that

young patients have a significantly better survival. However, Manuel et al. (Manuel et al. 2003) have reported that the prognosis is similar in OTSCC patients younger or older than 45 years. Importantly, our data analysis and review of the literature indicate that caution should be exercised in inferring poor prognosis in the young age group with OTSCC. Limitations in previous studies such as small numbers of young patients and/or small numbers of survival events (Garavello, Spreafico and Gaini 2007; Soudry et al. 2010; Vargas et al. 2000) could have influenced their results. Such limitations also hamper definitive conclusions about the impact of young age on the prognosis of OTSCC. Of note, studies which have analyzed population-based registries, have not reported poor prognosis in young age groups. Conversely, some of these population studies have reported a better prognosis in young patients. For example, Annertz et al. (Annertz et al. 2002) have analyzed data from cancer registries in Scandinavian countries (Denmark, Finland, Norway and Sweden), and reported a total of 5,024 patients with tongue SCC between 1960 and 1994. Of these, 276 patients (5.5%) belonged to the young age group (< 40 years). Interestingly, their study reported a significantly better survival for young patients compared to older ones.

In our data, two pediatric cases (< 20 years) were treated for early OTSCC. These two cases had recurrences and both patients died of OTSCC. However, data on survival of OTSCC in children are still insufficient because it is a very rare clinical entity.

6.7 A proposal for a new histopathologic predictive model in early OTSCC

The main result of this study is the BD predictive model (Fig. 8 and 9), which has the advantages of being simple, easily applicable, and requiring inexpensive staining. The two parameters (Budding and Depth) included in this model have been reported earlier as promising prognosticators in tongue SCC (Ganly et al. 2013; Wang et al. 2011; Xie et al. 2014), as well as in SCCs of other oral subsites (Angadi et al. 2015; Huang et al. 2009).

Initial reports of several prognosticators of OTSCC indicated they had excellent predictive power, but subsequent studies have failed to validate them. In general, new prognosticators of malignancies should be better than the ones already available (Soland and Brusevold 2013). Based on this recommendation, we have (Sawazaki-Calone et al. 2015) compared the prognostic performance of our BD model with that of the WHO grading system of SCC (Barnes et al. 2005), a malignancy grading system of the deep invasive front (Bryne et al. 1992), and the histologic risk score (HRS) model (Brandwein-Gensler et al. 2005) in a new cohort of 113 OSCC patients from Brazil. To avoid possible bias and to evaluate the reproducibility of our model, other observers have performed the scoring of this new cohort of OSCC following the same criteria we have previously introduced for the BD model. The findings of this validation study demonstrated that the BD model has a promising prognostic power superior to that of other examined models. However, the validation study has the disadvantages of including SCC of more than one oral cavity subsite, and also including cancers of both early and late stages. Therefore, further validation of the BD model preferably should be carried out using a homogenous patient cohort similar to that of the current study. Moreover, further studies should compare the different method of scoring of TB similar to that recently conducted on colorectal cancer (Koelzer et al. 2015). Comparing different cutoff points for risk stratification based on TB and DOI could help identification of the ideal cutoff points for the components of this model.

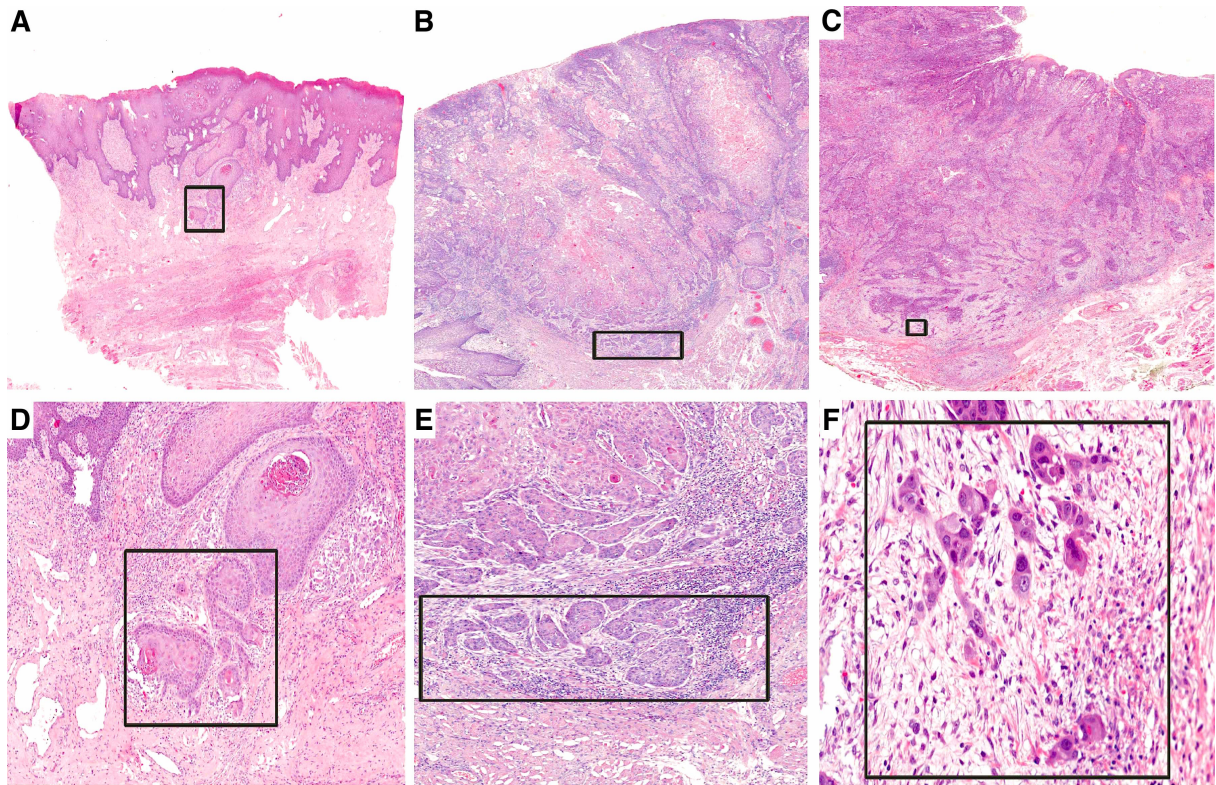


Figure 8: Histopathology of BD model. A: score 0; B: score 1; C: score 2. D, E, and F: magnification of rectangular boxes of A, B and C respectively.

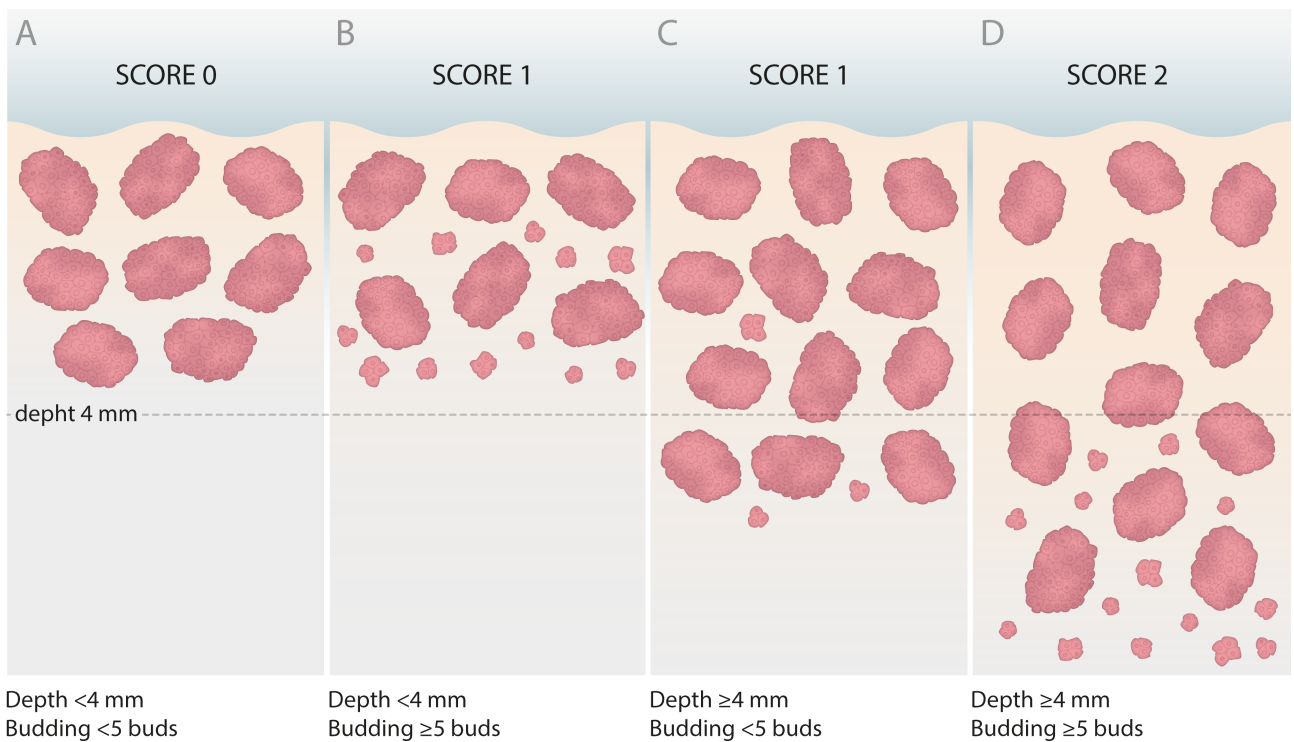


Figure 9: Schematic illustration of BD model. A: score 0; B and C: score 1; D: score 2.

7 Conclusion

In this multicenter international study we have examined many clinicopathologic parameters that have been suggested to play a role in prognostication of oral cancers. Specifically:

- A.** We introduced a novel prognostic model (BD model) for the prediction of patient survival. Our model is based on simple histopathologic parameters, which could be easily evaluated during the operation and allow the surgeon to decide the definitive surgical procedure (i.e. wider surgical margins and SNB or neck dissection) for cases at high risk without interrupting anesthesia. In addition, this model will be useful to select those patients who might need postoperative radiotherapy.
- B.** We evaluated the prognostic power and the significance of many histopathologic parameters in one of the largest patient cohorts of early OTSCC. The examined parameters are usually evaluated by pathologists in daily practice and included in the pathology reports. Only depth of invasion and pattern of invasion (defined as WPOI) have shown a promising predictive value. However, tumor grade has a weak predictive power; while perineural invasion and lymphocytic host response did not show any predictive power.
- C.** We found that the histologic risk model is not a promising prognostic indicator in early OTSCC.

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Alhadi Almangush

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