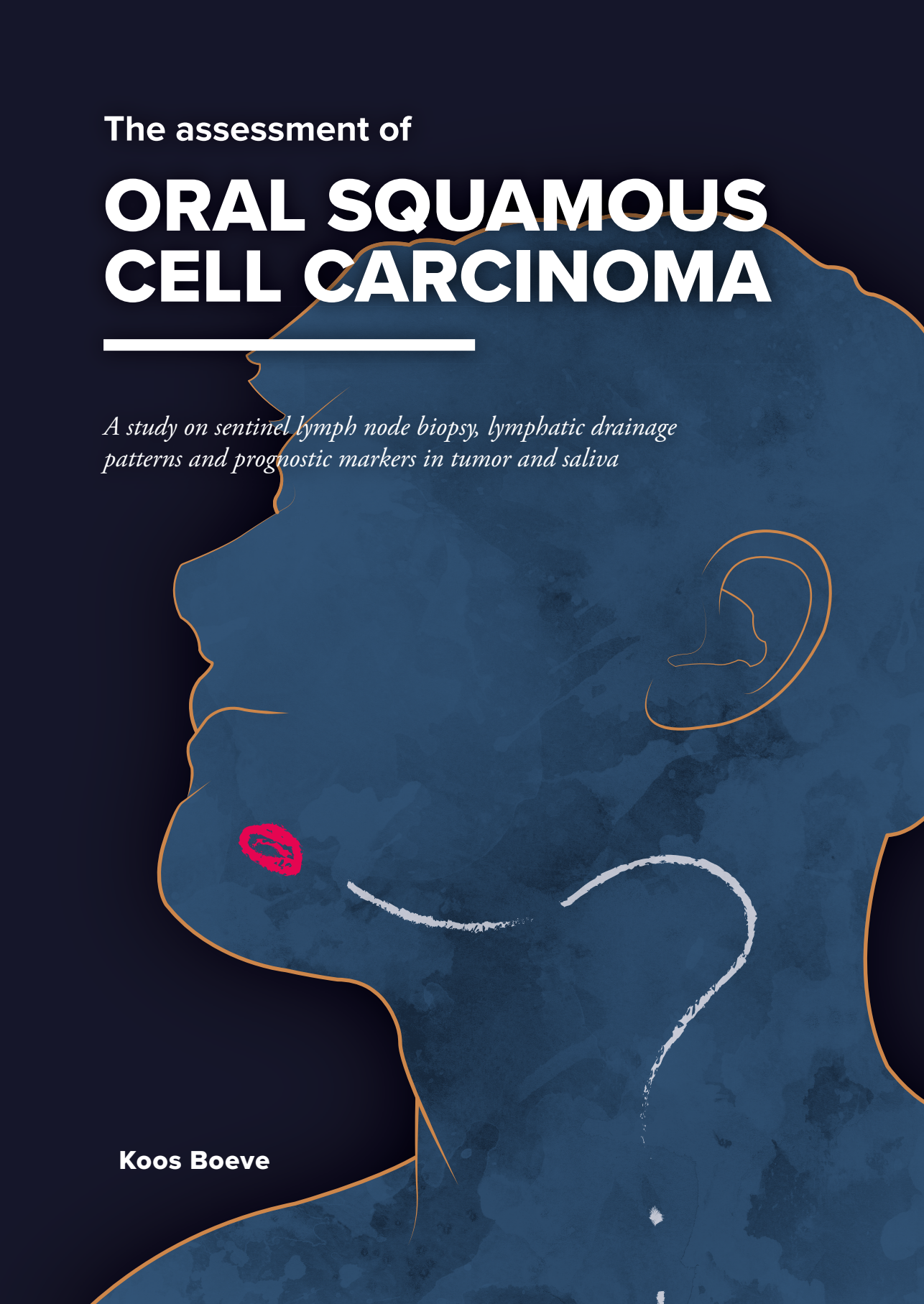
The assessment of

ORAL SQUAMOUS CELL CARCINOMA

A study on sentinel lymph node biopsy, lymphatic drainage patterns and prognostic markers in tumor and saliva



Koos Boeve

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COLOPHON

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A study on sentinel lymph node biopsy, lymphatic drainage patterns and prognostic markers in tumor and saliva

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CHAPTER 1

General introduction and scope of this thesis

GENERAL INTRODUCTION

Cancer of the head and neck

Head and neck cancer is the seventh most common cancer worldwide with an incidence of 600.000 cases per year [1]. These head and neck tumours mainly arise from the epithelial layers of the upper aerodigestive tract resulting in more than 90% squamous cell carcinomas (HNSCC) [1]. Other malignant tumours like adenocarcinomas, melanomas and lymphomas are less common in the head and neck area [2]. The upper aerodigestive tract includes the anatomical locations of the oral cavity, pharynx, larynx and the mucosa of the lip (Figure 1A). HNSCC is provoked by random (epi)genetic aberrations that, in the majority of the cases, are caused by smoking or heavy alcohol consumption. Tobacco and alcohol use have a synergetic effect, i.e. the combination of smoking and alcohol consumption resulted in a higher risk of developing a HNSCC than the sum of the individual effects [3]. Other etiological factors for HNSCC are human papilloma virus infection, which is almost completely restricted to base of tongue and tonsil tumours [4], Epstein-Barr virus infection in nasopharynx tumours [5] and ultraviolet light exposure (sunlight) for the lower lip tumours [6]. Betel nut can induce carcinogenesis and is especially an important etiological factor for oral cancer in Asian cultures, where chewing betel quid is popular [7]. A higher incidence of HNSCC is also seen in the elderly [8]. The role of chronic inflammation, such as oral lichen planus, in HNSCC is not completely understood, but data suggest that patients with these chronic diseases might have a higher risk of malignant transformation of involved epithelium [9]. Treatment protocols differ between anatomical locations of HNSCC, e.g. oral cavity tumours are primarily treated by surgical resection of the tumour, while pharyngeal tumours have radiotherapy as primary treatment. The different anatomical locations, etiological factors and treatment protocols demonstrate the heterogeneity of HNSCC.

Squamous cell carcinoma of the oral cavity

Oral squamous cell carcinoma (OSCC) is the most frequently diagnosed subtype of HNSCC [2,10]. In 2018, the incidence in the Netherlands was 967 new cases for OSCC [11]. Most affected oral cavity side is the lateral tongue, followed by the floor of the mouth. In general, OSCCs metastasize first to lymph nodes in the cervical neck levels I-III (Figure 1B), thereby following the lymphatic drainage patterns, before metastasizing to lymph nodes in other neck levels or further down in the body [12] or haematologically to other organs as lung, skin and liver [13]. Metastasis to cervical neck levels is known as regional metastasis, while spread of tumour cells to other parts of the body is known as distant metastasis. Primary treatment with curative intent of OSCCs consists of surgical resection of the tumour. In case of a clinically positive lymph node or high chance of lymph node involvement (defined as >4 mm tumour infiltration depth), tumour resection is combined with a neck dissection.

Histopathological assessment of the tumour resection specimen enables patient selection for adjuvant treatment in cases with unfavourable pathological features. Surgery is often followed by radiotherapy in patients with an intermediate risk for recurrences defined as lymphovascular or perineural invasion, close surgical resection margins (1-5 mm), pT3-T4 staged tumours or a ≥pN1 lymph node status [14,15]. Postoperative radiotherapy is combined with chemotherapy in cases with a high risk for local, regional or distant recurrence what is defined as positive surgical resection margins (less than 1 mm tumour free margin), multiple positive lymph nodes or extranodal extension [14,15]. Surgical re-resection is an option in cases with close or positive tumour resection margins for local control with curative intent. Despite surgical resection and adjuvant therapy based on the pathological features, curative treatment is still a challenge and reflected in the overall survival (OS) that only improved six percent in the Netherlands from 56% in 1989 to a 62% five-year OS in 2012 for OSCC in general [11,16]. Two important challenges affecting the survival in OSCC are studied in this thesis: first, the detection of occult metastasis using the sentinel lymph node biopsy or molecular tumour biomarkers in early stage OSCC. And secondly, the detection of local recurrences and second primary tumours using molecular tumour biomarkers in saliva.

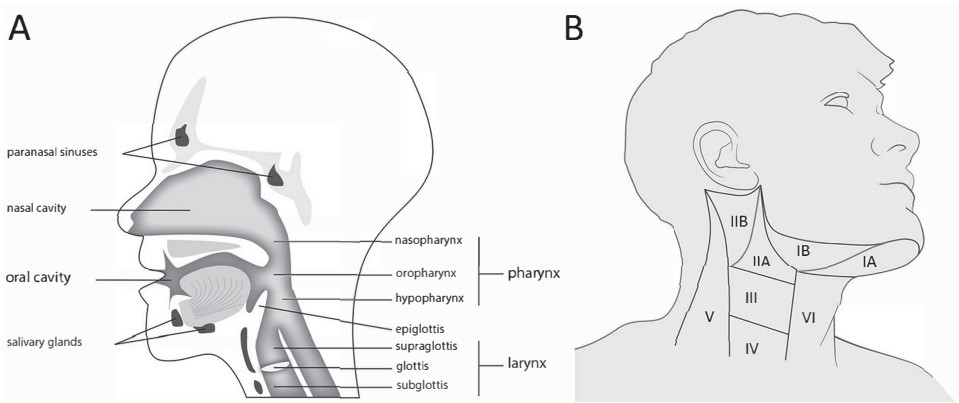

Figure 1. Head and neck locations. The main anatomical locations of head and neck cancer (1A) and the six cervical neck levels with four sublevels (1B). Oral cavity tumours metastasize mainly first to lymph nodes located in level I-III.

[A: Adapted from Gibcus 2008 with permission, B: Copyright © Koos Boeve, UMCG, 2019 Groningen].

CHALLENGE 1: DETECTION OF OCCULT METASTASIS IN EARLY STAGE (cT1-2N0) OSCC

Clinical neck staging has been extended in the last decades from mere physical examination by palpation to imaging of the neck by Computed Tomography (CT), Magnetic Resonance Imaging (MRI) and followed by Ultrasound guided Fine Needle Aspiration Cytology (USgFNAC) in case of suspicion for lymph node metastasis [17]. Despite this evolution in neck staging, still 23-37% of the early stage (cT1-2N0) OSCC patients are diagnosed with occult metastases [18-20]. Occult metastasis means that these metastases were not detected clinically, and thus defined as 'clinically negative neck', but postoperatively by histopathological examination or present as late metastasis after treatment of the primary tumour has been completed. Conventionally two strategies were available for patients with a clinically negative neck: frequent clinical examination of the neck (known as watchful waiting) or an elective neck dissection (END). In the eighties of the last century, neck levels I-V (Figure 1B) were dissected during an END, which was later restricted to levels I-III. A level I-III END is also known as a 'selective neck dissection' (SND) [12]. With the END, 63-77% of the patients are overtreated and risk postoperative morbidities such as loss of shoulder function or lymph oedema [16]. Using watchful waiting as neck strategy will result in occult metastasis detection at a more unfavourable stage [21]. Overtreatment with ENDs and late detection in case of watchful waiting are major limitations for these two conventional neck strategies and were reasons to search for individual selection for a neck dissection. Tumour infiltration depth is one of these well-studied predictive variables for lymph node status and survival and was incorporated with a 4 mm cut-off in treatment protocols to select patients for an END instead of watchful waiting [22,23]. The predictive value of tumour infiltration depth resulted in incorporation in the 8th edition of the pTNM classification with 5 mm and 10 mm cut-offs (pT1 ≤5 mm, pT2 5-10 mm, pT3 <10 mm, Table 1).

Table 1. Differences between the 7th and 8th AJCC pathological T-classification

T category	7th TNM: tumour diameter	8th TNM: tumour infiltration depth added
T1	≤2 cm	≤5 mm
T2	>2 and ≤4 cm	>5 and ≤10 mm
T3	>4 cm	>10 mm
T4	Moderately and very advanced	Extrinsic tongue muscle infiltration is now deleted

Moderately advanced local disease: tumour invades adjacent structures only. Very advanced local disease: tumour invades masticator space, pterygoid plates, or skull base or encases the internal carotid artery. Tumour diameter was the only criterion for both clinical and pathological T1-3 staged tumours in the 7th T classification of the American Joint Committee on cancer (AJCC). In the 8th edition pathological T classification tumour infiltration depth was added for T1-3 tumours and extrinsic tongue muscle infiltration was deleted for the T4 category. In the 8th edition pT1-3 tumours are staged by both tumour diameter and tumour infiltration depth. [SOURCE: American Joint Committee on Cancer TNM cancer staging [24,25]]

Sentinel lymph node biopsy procedure in early stage (cT1-2N0) OSCC

Now the sentinel lymph node biopsy (SLNB) procedure is extensively used in breast cancer and melanomas, it was also introduced in head and neck cancer as a less invasive procedure for neck staging compared to the END [26]. The SLNB procedure enables detection and harvesting of the sentinel lymph nodes (SLNs) (Figure 2).

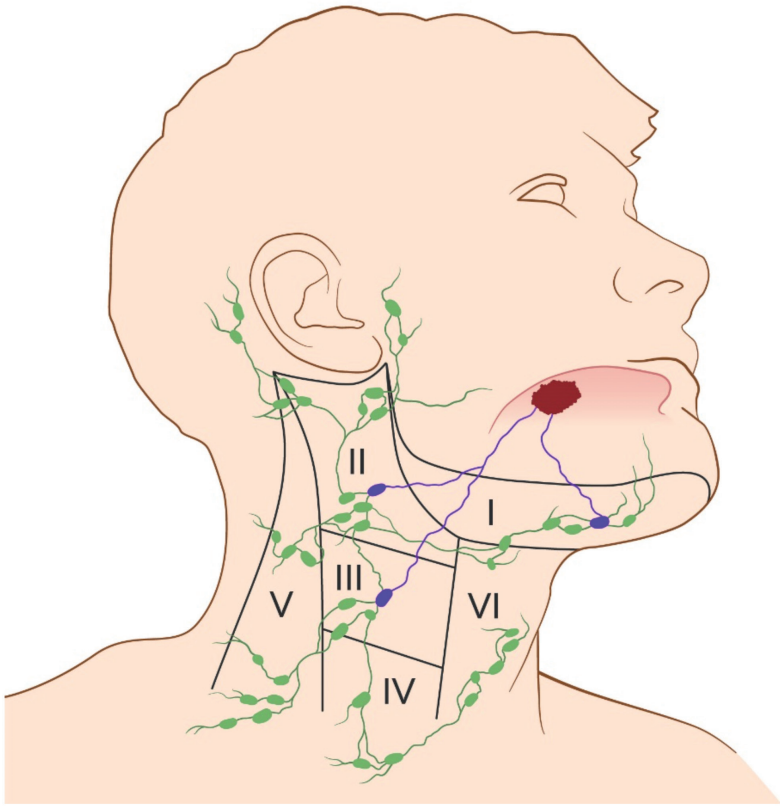

Figure 2. Lymphatic drainage patterns in oral cancer. Oral cancer (marked in RED) metastasizes first by lymphatic drainage patterns to cervical located lymph nodes (marked in GREEN and PURPLE). The first lymph nodes in such lymphatic drainage patterns are called sentinel lymph nodes (SLN, marked in PURPLE). These SLNs are normally the first locations positive for metastasis.

Since distant metastasis is reported in 6% to 12% of the OSCC cases [13,27,28], especially in those with advanced lymph node involvement (i.e. extranodal extension, ≥pN2), the SLN status indicates whether or not the tumour has metastasized regionally or to distant

locations. The SLNB procedure (Figure 3) consists of a peritumoral injection of a radioactive tracer one day before surgery followed by imaging of the tracer using lymphoscintigraphy on the same day as the injection [18,26]. During surgery, the SLN is detected and harvested with a small incision and a handheld gamma-probe. Postoperatively, the SLN is assessed by a pathologist for the presence of lymph node metastasis. The small number of lymph nodes in a SLNB specimen (~2) compared to the high number of lymph nodes in an END (~20) [29], allows an extensive pathological work-flow with step-serial-sectioning and an immunohistochemical keratin staining of all slides in addition to the conventional hematoxylin-eosin (HE) staining (Figure 3). Step-serial-sectioning and keratin staining are not part of the END pathological work-flow.

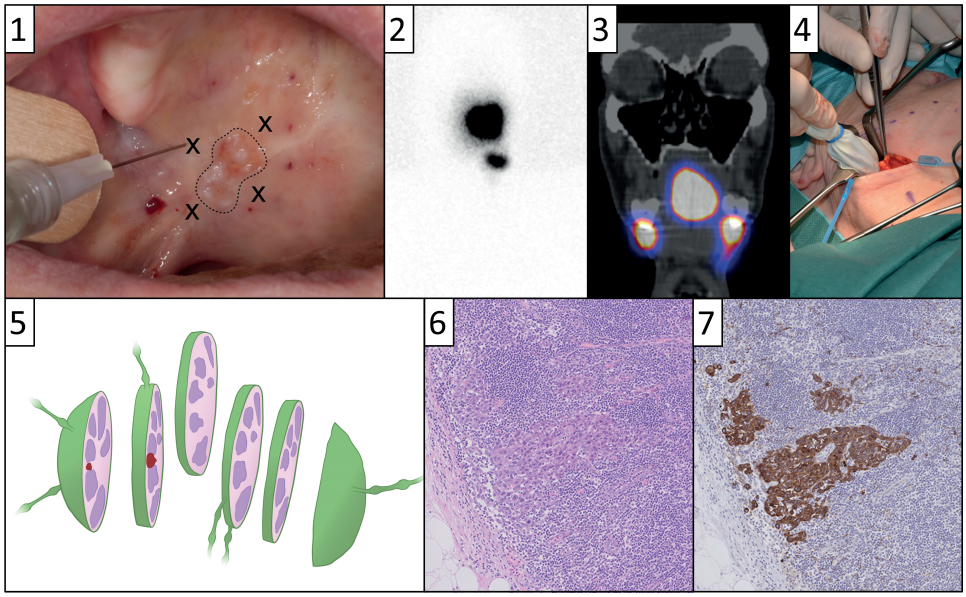

Figure 3: The sentinel lymph node biopsy procedure. The sentinel lymph node biopsy (SLNB) procedure consists of a preoperative peritumoral injection of a radioactive tracer (1, crosses indicate injection sites around the tumour which is marked by a dotted line), visualisation of lymphatic drainage patterns and sentinel lymph node (SLN) location by static and dynamic lymphoscintigraphy (2) and SPECT-CT scanning (3). Intra -operatively, SLNs are identified and harvested using a handheld gamma-probe and a small incision (4). The assessment of only a few lymph nodes in a SLNB specimen enables extensive histopathological examination with step-serial-sectioning of six slides (5), conventional hematoxylin-eosin (6) and additional keratin immunohistochemistry (7) that contributes to the detection of small metastasis with a size of isolated tumour cells (<0.2 mm).

In early stage OSCC the SLNB procedure has been reported to be accurate in detecting occult metastasis with a pooled sensitivity of 87% and a pooled negative predictive value (NPV) of 94% in a meta-analysis using 66 studies [30]. Moreover, the SLNB revealed individual lymphatic drainage patterns and detected occult metastasis with a size of just individual metastasis cells [31]. After the introduction in OSCC the SLNB procedure was modified several times [26]. Preoperatively the single photon emission computed tomography (SPECT)-CT scan was added to the SLNB imaging protocol and resulted in detection of additional SLNs in 22% of the patients [32]. Blue dye was part of the SLNB procedure as intra-operatively injected tracer and visualised lymphatic drainage patterns by blue staining [26].This tracer was discontinued in several Dutch centres because blue dye deteriorated the demarcation of surgical resection margins and had only a limited additional value to the preoperative imaging using a radioactive tracer [26]. Step-serial-sectioning with additional keratin staining were added to the pathological assessment protocol to increase the sensitivity of detecting small metastases.

Although the high accuracy in detection of occult metastases [30], many of the reported studies consisted of small cohorts and differed in reference treatment for the SLNB negative neck (i.e. END or clinical follow-up), SLNB procedure (e.g. use of a gamma probe, blue dye or SPECT-CT) and pathological work-up (with or without additional keratin staining or step-serial-sectioning). Furthermore, several studies provided incomplete clinico-pathological information. This heterogeneity and lack of complete data underlined the need for studies using complete and homogeneous cohorts. Additionally, the SLNB has some limitations: First, the SLNB procedure seems to be less accurate in patients with a floor of mouth (FOM) tumour what might be caused by the shine-through phenomenon (Figure 4) and resulting in a lower detecting rate of SLNs located in level IA [33]. Secondly, in case of a metastasis positive SLN a complete neck dissection of cervical levels I-V, known as a (modified) radical neck dissection, needs to be done in a second operation. The modified radical neck dissection might be more challenging as a result of fibrosis induced by the SLNB procedure. Finally, although the SLNB is minor surgery compared to the END, it is still an invasive technique for neck staging for early stage OSCC patients of which the majority has no lymph node involvement. Histopathological and (epi)genetic tumour profiling using the biopsy specimen of the primary tumor might be helpful to define patients preoperatively for a more optimal neck strategy with a watchful waiting, SLNB or a neck dissection [34].

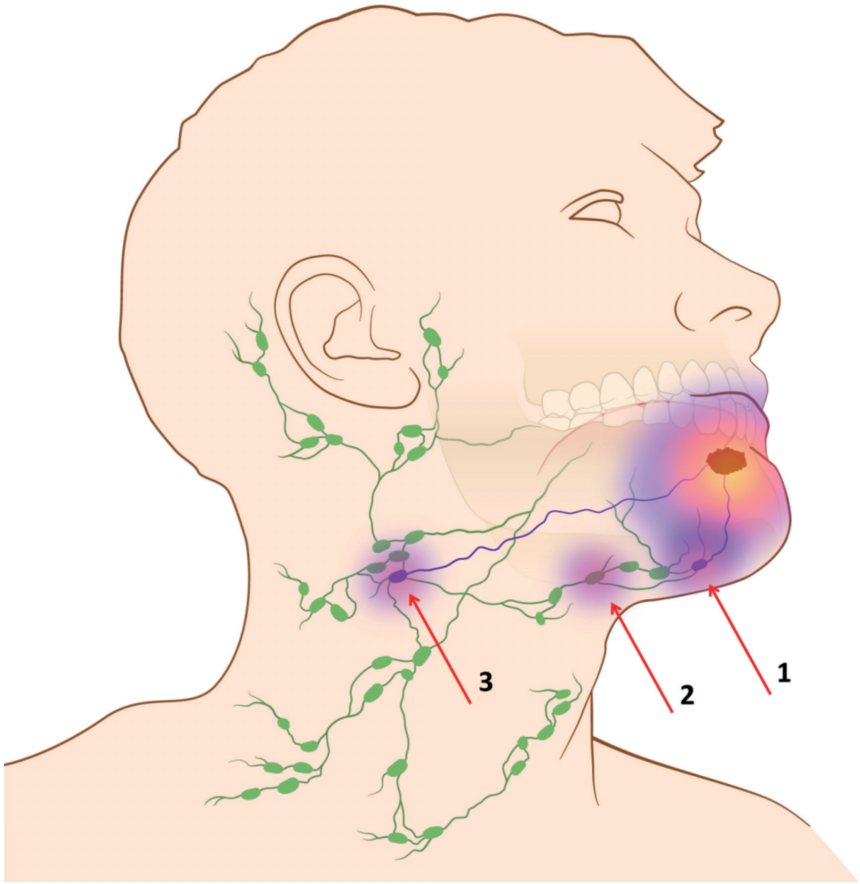

Figure 4. Shine-through phenomenon in oral cancer. Sentinel lymph nodes (marked in PURPLE) located in level I (arrow 1) are not always separately visual from the primary tumour with lymphoscintigraphy and SPECT-CT imaging caused by the location of that sentinel lymph node within the radioactive tracer hotspot of the tumour, known as shine-through phenomenon. Second lymph nodes in the drainage pattern might be wrongly suggested and harvested as SLN (arrow 2) or only SLNs in other neck levels (arrow 3) are harvested [33].

Histopathological and molecular tumour biomarkers predicting lymph node status in OSCC

Tissue from tumour biopsy or surgical resection material enables to associate tumour characteristics with lymph node status in OSCC. Histopathological tumour characteristics such as tumour infiltration depth, pT status, perineural invasion, lymphovascular invasion, degree of differentiation and pattern of invasion have been associated with lymph node status for decades [35-39]. Of these histopathological characteristics, tumour infiltration depth was reported as an independent predictive marker with the highest predictive value and has been used in clinical practice for risk assessment of lymph node status [21,22,35]. Although some of the other histopathological tumour characteristics (lymphovascular or perineural invasion, close surgical resection margins or a pT3-T4 staged tumour) are used as adverse features to select patients for adjuvant radiotherapy, these characteristics are not used as risk assessment to select patients for a neck dissection. Lack of clear validations and large intra- and interobserver variability might be reasons why these markers are not introduced in the clinical setting for predicting lymph node status [40]. For example, tumour pattern of invasion was associated with lymph node status [41]. However, an analysis of five different scoring methods for tumour invasion pattern showed just a moderate reproducibility [40].

More recently, molecular tumour biomarkers have been studied widely for their association with lymph node status. Many different cellular processes are involved in metastasis of tumour cells, such as cell adhesion (detachment of the primary tumour), cell mobility (movement to vascular structures), cell remodelling (passing vascular walls), resistance to blood flow (adhesion to vasculature), direct exposure to immune system, homing and cell division (metastasis formation in the lymph node) [42-44]. Some of these cellular processes are (de-)regulated by increased expression of proto-oncogenes and the inactivation of tumour suppressor genes caused by (epi)genetic alterations [45,46]. For example, amplification of the 11q13 chromosome is such a genetic alteration and one of the most frequently (36%) detected alterations in head and neck cancer [47]. *CTTN*, *CCND1* and *FADD* are three genes located in the commonly amplified region at chromosome 11q13 and overexpression of their proteins is associated with shorter survival and positive lymph node status in head and neck cancer [48-50]. *CTTN* encodes for cortactin, a protein with multiple binding domains such as F-actin, Src and Erk [51]. Cortactin is involved in cytoskeleton formation, cell morphology and cell migration which are important processes to enable a cell to metastasize [51]. Expression of cortactin results in migration in vitro [52] in agreement with the observed association with lymph node status in patient biopsies of the 11q13 amplification [53]. The *CCND1* gene encodes for the cyclin D1 protein, which is especially known for promoting cell cycle progression during G1 [54], but also plays a

key function in cell migration control, DNA repair and mitochondrial activity modulation [54]. Fas Associated Death Domein (FADD) is the protein encoded by the *FADD* gene and plays an important role in the apoptotic signalling, but has been related to cell cycle progression, innate immunity and autophagy more recently [48]. Recently, a study showed an increased rate of lymph node metastasis in head and neck squamous cell carcinoma patients with a high FADD expression [55]. Despite a clear association of expression of these genes such as *CTTN/*cortactin, *CCDN1/*cyclin D1 and *FADD* with lymph node status, and exploration of the underlying cellular processes involved in metastasis, none of these is currently implemented as a diagnostic tumour biomarker for lymph node status in early stage OSCC. One of the reasons is the lower predictive values of these tumour biomarkers for lymph node status compared to the SLNB procedure [30,56]. These lower predictive values might be due to the multistep character of metastasis with involvement of many different cellular processes (see above) [42]. Therefore predicting lymph node status using a single tumour marker only, most likely will not result in clinical suitable predictive values. Recently introduced laboratory techniques, such as DNA microarrays, enable the selection of genes and panels of genes using genome wide (epi)genetic approaches [56-58]. Despite that a gene-signature of 852 genes showed a high sensitivity of 86% and a NPV of 89% for detecting lymph node metastasis in a validation with early stage OSCC [56], such signatures for assessment of lymph node metastasis are not implemented to the clinical setting caused by the high costs, unfeasibility to use with formalin-fixed paraffin-embedded tissue and the availability of the high accurate SLNB procedure [59]. Moreover, comparing different signatures revealed hardly any similarity in selected genes and proteins between these expression signatures [60-63], that might display the heterogeneity in expression signatures among these tumours and the challenge of selecting genes or signatures with clinical applicable predictive values [57,63].

A promising method to select predictive molecular biomarkers for lymph node status is the analysis of the DNA hypermethylation status of certain cancer related genes. DNA methylation is an epigenetic process that regulates DNA transcription by adding a methyl group to a cytosine that precedes a guanine nucleotide, referred to as CpG sites [46]. A high density of these CpG sites are referred to as CpG islands and commonly observed within the regulatory regions of gene promotors [45]. Methylated CpG islands are associated with a lower gene expression (Figure 5) [45,64]. During cancer progression, gene promotor DNA methylation gradually increases (referred to as hypermethylation), while DNA methylation of repetitive sequences decreases (referred to as hypomethylation) (Figure 5) [65]. Abnormal expression of cancer-relevant genes by methylation has been reported to be at least as common as affecting transcription by genetic DNA alterations such as DNA amplification and mutations [66]. Because of hypermethylation is an independent mechanism of transcriptional regulation related to DNA sequence alterations, these mechanisms might

be complementary in regulating tumour processes such as metastasis [45]. Moreover, hypermethylation is more common earlier in the carcinogenesis compared to DNA mutations [45]. In addition, hypermethylation of certain genes *(MGMT, DAPK1*) has been reported to be associated with lymph node status [67]. Recently, using a global genome-wide screening approach, three new markers were identified to be differently methylated comparing OSCC with and without lymph node metastasis (*WISP1*, *RAB25* and *S100A9*) [68,69] [Clausen, S100A9, in prep]. Because of the clear association with lymph node status, a panel of these methylation markers might contribute to the detection of lymph node metastasis, however the accuracy in detecting occult metastasis in early stage OSCC has not been validated yet.

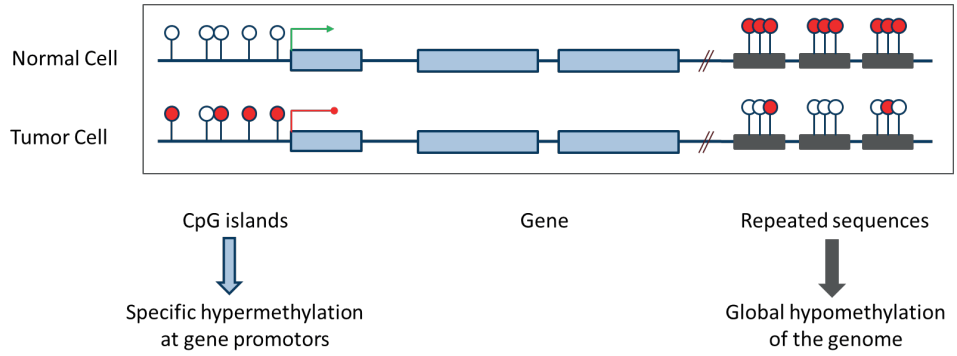

Figure 5. DNA methylation in cancer. Methylation of CpG sites (red lollipops) in the promotor region of a cancer-related gene induces silencing of gene expression and might contribute to carcinogenesis [45]. Methylation of CpG sites throughout the genome outside the coding domains of genes (GRAY boxes) decreases (hypomethylation) in cancer [45].

[Adapted from the Atlas of genetics and cytogenetics in oncology and haematology in 2013[70]].

CHALLENGE 2: DETECTION OF LOCAL RECURRENCES AND SECOND PRIMARY TUMOURS

Local recurrences and second primary tumours are reported in up to 10-30% percent of the cases in OSCC [71]. Local recurrence is defined as tumour growth within the same area (maximal distance of 20 mm) and within three years after diagnosis of the initial tumour, while second primary disease is defined as intra-oral tumour growth not fitting to one of the criteria of local recurrence. Causes for local recurrences and second primary tumors of OSCC are residual tumor cells after treatment and field cancerization of the oral mucosa (Figure 6) [42].

Residual tumour cells are isolated cells after treatment of the first primary tumour which have the potential to develop as local recurrence. Field cancerization is the presence of precancerous epithelium with or without clinical manifestation (Figure 6) [42]. In general, DNA of epithelial cells undergoes several changes before they turn into a malignant tumour cell [42]. First, a stem cell in the basal layer of the epithelium will undergo (epi)genetic alterations and change into a preneoplastic stem cell. After proliferation this preneoplastic stem cell might cause a preneoplastic field of oral epithelium [72]. The ongoing exposure to the etiological factor (e.g. tobacco smoke, alcohol and betel nuts) might encounter secondary DNA alterations and turn one of these preneoplastic cells into a neoplastic cell and finally into a tumour with invasive growth surrounded by a field of preneoplastic cells (Figure 6) [42]. Because most etiological factors affects the total mucosa of the oral cavity, field cancerization is not restricted to the area of the first primary tumour and second primaries could arise from such other fields located in the oral cavity as well as in other locations of the upper aerodigestive tract (Figure 6) [42].

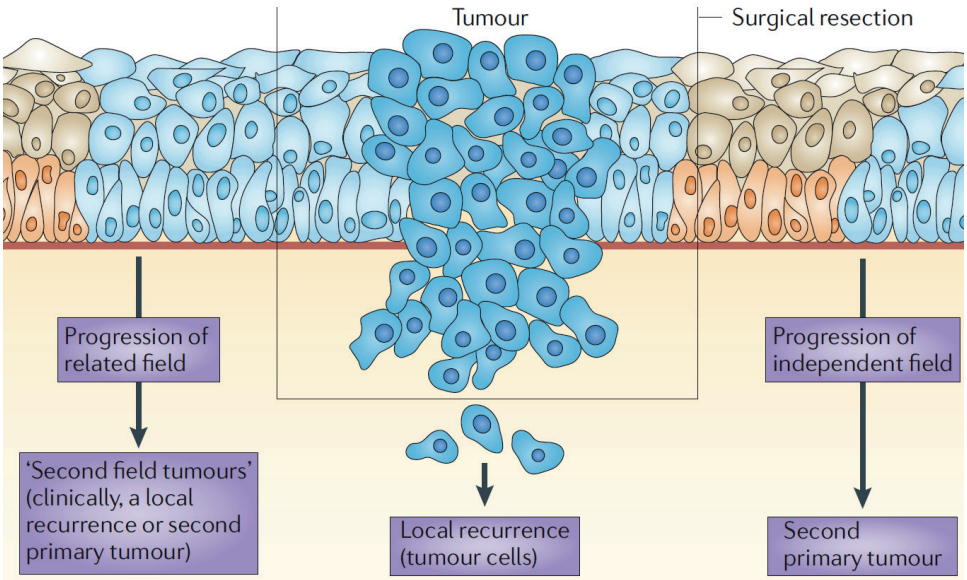

Figure 6. Field cancerization and residual tumour cells. Schematic model of the mucosa of OSCC cases with the different causes for local recurrences and second primary tumours as a result of field cancerization and residual tumour cells.

[Source: Leemans CR, Braakhuis BJ, Brakenhoff RH, Nat Rev Cancer, 2011 [42], with permission].

Early detection and treatment of local recurrences and second primaries is challenging. Since clinical manifestation might be lacking, field cancerization and residual cells are not recognized during surgery, clinical examination or histopathological assessment and consequently detection is delayed until conversion into a local recurrence or second primary tumour. In some cases there is clinical manifestation of field cancerization (e.g. leukoplakia), and in this particular cases therapy is limited by the extensiveness of the field cancerization. The intra-oral clinical examination, during follow-up after treatment of the first primary tumour, might be limited by postoperative fibrosis induced by surgery or irradiation [73] and as a result of reconstruction of the resection area of the first primary with tissue from extra-oral donor sites.

To prevent patients for local recurrences or second primary tumours, histopathological predictive characteristics have been studied. Close (1-5 mm) or involved (<1 mm) resection margins of the tumour, perineural and lymphovascular invasion, grade of differentiation, tumour infiltration depth and tumour pattern of invasion have been reported as predictive for local recurrence [74-76]. Even in cases with free resection margins (>5 mm) [75,77], a local recurrence rate of 8% to 11% is reported [77,78]. Other studies analysed the detection of minimal residual cancer or preneoplastic fields using molecular markers in surgical resection margins [76,79]. Although some promising results of molecular tumour biomarkers with 100% positive predictive values for local recurrences [79], robust validation in several trials with clearly defined margins and sufficient power is lacking [80].

Currently, patients with involved or close resection margins, or T3-T4 staged tumours are treated with adjuvant radiotherapy, chemotherapy or a surgical re-resection to lower the risk for a local recurrence [81]. Moreover, preneoplastic regions located adjacent to the resection area could be removed using laser therapy [82]. Due to the clinical invisibility, removal of the total preneoplastic field is uncertain. For example, oral leukoplakia is a clinically visible type of field cancerization with an annual malignant transformation risk of 1% [83]. Even if these visible leukoplakia fields are removed completely with carbon dioxide laser surgery, a local recurrence risk of 3-40% for oral leukoplakia was reported in a systematic review [83]. Therefore, after treatment of the first primary tumour, all OSCC patients are clinically assessed according to a strict follow-up scheme starting with a 6 week interval followed by three and six months intervals in the Netherlands.

Biomarkers for diagnosis and monitoring of recurrent disease in OSCC using liquid biopsies

Although field cancerization and residual tumours cells are often clinically invisible, their already existing (epi)genetic alterations might be detectable and used as biomarker to monitor OSCC patients in order to diagnose local recurrences in an early stage [46].

Tumour cells and tumour DNA are often released in plasma and referred to as respectively circulating tumour cells (CTC) and circulating tumour DNA (ctDNA) [84]. Plasma collected via minimal-invasive blood collection method is referred to as liquid biopsy, and has great promise as a source for predictive testing and monitoring of treatment response [85]. The precise mechanism behind the release of ctDNA in plasma is not totally clarified. Probably that apoptotic neoplastic corpuscles are released into the bloodstream whether or not after phagocytation by white blood cells and necrotic tumour cell debris including DNA [85]. Another hypothesis is that viable tumour cells migrate into lymphatic or blood vessels and become necrotic after missing the opportunity to form a metastasis [85]. In addition to plasma, other body fluids can service as a source for CTCs and ctDNA including urine, cerebrospinal fluid and saliva [84]. Tumour DNA and tumour cells in saliva are more likely from apoptotic and necrotic cells which detach from the tumour surface in the oral cavity. The relative easy way to collect plasma (minimal-invasive) and especially saliva (non-invasive) makes it very promising sources for diagnosing (local) recurrences. ctDNA in saliva and plasma might also be used for monitoring of therapy response in patients with advanced disease and treated by radio-, chemo- or targeted therapy if complete resection by surgery is not possible, such as could be the case in for example oropharyngeal tumours [85]. Several types of biomarkers have been studied: DNA, RNA, methylation and protein based markers [85,86]. Some of these studies reported sensitivities and negative predictive values of more than 80% in detecting OSCC associated biomarkers in saliva [87] and serum [85,88]. Moreover, the marker concentration levels in serum were associated with overall survival and were independent prognostic markers [88]. Despite these promising results, none of the liquid biopsies is implemented clinically nowadays for OSCC.

Biomarkers for diagnosing OSCC using liquid biopsies are facing challenges before being used clinically. Besides the release of ctDNA or CTCs into the bloodstream or saliva, also often large amount of DNA derived from healthy cells and in saliva also from other organisms (bacteria, viruses, archaea and fungi) are present in these fluids [89]. In general, the amount of ctDNA is mostly a very low fraction of the total amount of DNA extracted from plasma or saliva referred to as cell free DNA from plasma (cfDNA) [85]. Therefore, a ctDNA marker needs to be very specific for OSCC in order to detect ctDNA in a background of total cfDNA. For this purpose, OSCC-specific methylation markers might also be useful in saliva to detect progression of disease or monitor treatment using a non (saliva) or very minimal-invasive (blood) approach to collect appropriate material. Recently, a review [85] included ten studies which reported using methylation markers in saliva from OSCC patients showing sensitivities from 34% to 93% and specificities from 72% to 93% for detecting OSCC in saliva. Although these promising results, validation with large and independent data and all tumour stages is not available. Also no other markers to identify ctDNA in saliva with appropriate accuracies up to 90% and clear validations are reported [85].

SCOPE OF THIS THESIS

The aim of this thesis was to analyse the prognostic or predictive value of clinical, histopathological and molecular tumour markers which are associated with (sentinel) lymph node status or with the detection of cancer in saliva of oral squamous cell carcinoma patients.

Tumour infiltration depth has been a well-studied tumour marker for predicting lymph node status and survival in OSCC [22]. Also extranodal extension has been proven as predictive marker for OSCC. Recently, these histopathological markers were incorporated in the 8th edition TNM classification [24]. In **chapter 2** the potential impact of the changes within the 8th edition pTNM classification on the prognosis and treatment strategy of oral squamous cell carcinoma compared the use of the "old" 7th edition pTNM classification in a series of 211 pT1-T2 patients with a long-term follow-up was evaluated.

The sentinel lymph node biopsy procedure (SLNB) was introduced in early stage OSCC for detecting occult metastasis. A meta-analysis on SLNB procedure accuracy showed heterogeneity in the existing studies for reference standards, imaging techniques and pathological examination [30]. In **chapter 3** the sensitivity and negative predictive value of the SLNB procedure in detecting occult metastases in cT1-2N0 OSCC was assessed. For this purpose, a well-defined cohort was used with clinical-follow up as reference standard for the SLN negative patients, SPECT-CT part of the imaging protocol and step-serial-sectioning and additional keratin staining as standard histopathological examination.

Despite the relative common local recurrences and second primary tumours in OSCC, only one study with 22 patients reported on the SLNB procedure in patients with a previously treated neck [90]. The SLNB procedure also provides information about the individual lymphatic drainage patterns, that might be helpful in these previously treated patients with altered lymphatic drainage patterns. In **chapter 4** the accuracy of SLNB procedure was assessed and lymphatic drainage patterns evaluated using a multicentre consecutive cohort of cT1-2N0 patients with a previously treated neck in three Dutch head and neck cancer centres.

Maxillary tumours are relatively rare and evidence on drainage patterns of these specific locations is lacking. Conventionally, the opinion was that these tumours rarely metastasize or only to retropharyngeal located lymph nodes [91]. In **chapter 5** we retrospectively determined lymphatic drainage patterns of 11 patients with maxillary tumours who had neck staging with the SLNB procedure.

Previously an association was observed between lymph node metastasis and disease specific survival and between lymph node metastasis and the expression of cortactin, cyclin D1, and FADD, three genes located in the chromosome 11q13 region and amplified in 13 to 29% of the HNSCC [47,55]. In **chapter 6,** both 11q13 chromosome amplification and expression of cortactin, cyclin D1 and FADD were associated with occult metastasis using a large multicentre cohort of 313 early stage OSCC patients collected from the databases of UMCU and UMCG.

Although the SLNB procedure is minor surgery compared to an END, it is still an invasive procedure in ~75% of the early stage OSCC patients who eventually had no lymph node involvement. In **chapter 7** molecular tumour biomarkers (cortactin, cyclin D1, FADD, RAB25, S100A9) previously reported as associated with lymph node status in OSCC, were analysed for their clinical value to select patients with a low risk for lymph node metastasis for a watchful waiting instead of a SLNB procedure. All included early stage OSCC patients had neck staging using the SLNB procedure which provide detailed lymph node involvement information. Expression levels of the selected molecular markers were associated with lymph node status using tissue micro arrays constructed from tumour biopsy and tumour resection tissues.

In **chapter 8** we describe the selection of new OSCC-specific methylation markers using an OSCC-methylome based on genome wide methylation screening approach. Selected methylation markers were validated in a feasibility study with saliva of ten OSCC patients and ten healthy controls using a quantitative methylation specific PCR (QMSP).

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CHAPTER 2

Addition of tumour infiltration depth and extranodal extension improves the prognostic value of the pathological TNM classification for early-stage oral squamous cell carcinoma

8th OSCC TNM classification prognostic value

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ABSTRACT

Aims: In the 8th edition of the American Joint Committee on Cancer TNM staging manual, tumour infiltration depth and extranodal extension are added to the pathological classification for oral squamous cell carcinoma. The currently available 8th TNM validation studies lack patients with conservative neck treatment, and changes in the classification especially affect patients with small tumours. The aim of this study was to determine the potential impact of the changes in the 8th edition pTNM classification on the prognosis and treatment strategy for oral squamous cell carcinoma in a well-defined series of pT1–T2 patients with long-term follow-up.

Methods and results: Two hundred and eleven first primary pT1–T2 oral squamous cell carcinoma patients, with surgical resection as primary treatment, were analysed retrospectively. One hundred and seventy-three patients underwent a neck dissection, and 38 patients had frequent clinical neck assessments. Long-term follow-up (median 64 months) and reassessed tumour infiltration depth were available. Classification according to the 8th edition criteria resulted in 36% total upstaging with the T classification and 16% total upstaging with the N classification. T3-restaged patients (n = 30, 14%) had lower 5-year disease-specific survival rates than T2-staged patients (81% versus 67%, p = 0.042). Postoperative (chemo)radiotherapy could have been considered in another seven (3%) patients on the basis of the 8th edition criteria.

Conclusions: Addition of tumour infiltration depth and extranodal extension in the 8th TNM classification leads to the identification of oral squamous cell carcinoma patients with a worse prognosis who might benefit from an improved postoperative treatment strategy.

INTRODUCTION

In 2016, the 8th edition of the American Joint Committee on Cancer (AJCC) TNM staging manual was released [1]. As compared with the 7th edition, tumour infiltration depth and extranodal extension (ENE) were incorporated into the pathological TNM classification for oral squamous cell carcinoma (OSCC) [1,2]. On the basis of the 8th edition criteria, 7th edition pT1 patients with a tumour infiltration depth between 5 and 10 mm are restaged as pT2 and all pT1 and pT2 patients with a tumour infiltration depth of >10 mm are restaged as pT3. Following the pN classification in the 8th edition, cases with a single positive lymph node <30 mm in diameter with ENE are restaged from pN1 to pN2b, and all other ENE-positive patients are restaged as pN3b.

The incorporation of tumour infiltration depth and ENE in the pathological TNM classification was based on data from both the International Consortium for Outcome Research in Head and Neck Cancer (ICOR) (n = 3149) and the National Cancer Data Base (n = 7264) [2,3]. The 8th edition has been validated in various independent databases: the pT and pN classifications by Lydiatt et al. (n = 1792) [2] and Matos et al. (n = 298) [4], and the pN classification by Garcia et al. (n = 1137) [5]. These studies confirmed a better prediction of survival per stratification with the 8th pTNM classification edition, whereby patients who had been upstaged because of the incorporation of tumour infiltration depth and ENE generally had lower survival rates.

Despite the validation with big data, the clinical impact for small tumours (pT1-2) is not really clear. As mentioned by Matos et al. and the ICOR study, their populations were limited to patients undergoing neck dissections. Patients with a clinically negative neck and not treated with selective neck dissections - also known as watchful waiting - were not included [3,4]. This point is important because incorporation of infiltration depth in the pT classification could also influence prognosis and as a result change the treatment strategy for these early-stage patients. Therefore, our aim was to study the clinical impact of the 8th edition pTNM classification on the survival of 7th edition pT1-T2 patients treated with surgical resection of the tumour combined with neck dissection or a watchful waiting strategy. We selected pathologically staged T1-T2 OSCC patients from our large and homogeneous database with extensive clinicopathological and long-term follow-up data [6,7].

MATERIAL AND METHODS

Patients

This cohort with reassessed tumour infiltration depth has been previously described [6,7]. Briefly, 246 consecutive patients with pT1-T2 OSCC according to the 7th edition, diagnosed

between 1997 and 2008 with a first primary tumour and treated with surgical resection of the tumor at the University Medical Centre Groningen, were selected from our database. Thirty-five patients were excluded because of multiple head and neck tumours (n = 3), irretrievable haematoxylin and eosin (HE) slides (n = 13), or unreliable assessment of infiltration depth because of missing epithelial surfaces and tangential tissue cutting (n = 19), resulting in 211 patients being available for tumour infiltration depth reassessment. Thirty-eight patients (18%) with a pT1 tumour did not undergo a neck dissection, but were followed closely (watchful waiting). This strategy was common in the era before the awareness that an infiltration depth of 4 mm implied a high chance of tumour spread to lymph nodes [6]. The 38 patients - with watchful waiting - had a median tumour infiltration depth of 3.2 mm (IQR 2.1-5.6 mm). In total, 211 patients were used for analysis and 173 of these were treated with neck dissection. The clinical and histopathological characteristics of the study group are shown in Table 1. In total, 72 patients received postoperative radiotherapy, but none of the watchful waiting patients were postoperatively irradiated. The median follow-up time was 64 months (range 0-193 months). Thirteen patients (6%) were diagnosed with local recurrence and 26 (12%) with regional recurrence. Of the 38 watchful waiting patients, two patients were diagnosed with a local recurrence and seven patients with regional recurrences during their follow-up. Sixty-eight patients (32%) died in the first 5 years after treatment, 57% because of the OSCC. OSCC related death (median 63 years; IQR 54-70 years) occurred at a significantly younger age than OSCC unrelated death (median 71 years; IQR 62-79) (p = 0.010).

Data collection

Clinical and pathological data were collected retrospectively from the patient files. Tumour H&E-stained slides were revised by one dedicated head and neck pathologist, and tumour infiltration depth was reassessed by the use of digital microscopy and computerised measurements (Research Assistant 6; RVC; Soest, The Netherlands). Tumour infiltration depth was measured from the mucosal surface or from the reconstructed mucosal surface in cases of ulcerated or exophytic tumours [7], this differs from the AJCC manual in using the mucosal surface instead of the mucosal basement membrane [2]. ENE was defined as an extension of tumour cells beyond the nodal capsule and forms part of the standard pathology report in our centre. Cases with no convincing extension beyond the nodal capsule (i.e. no stromal reaction) were scored as negative. We revised the pathological tumour and pathological nodal classification according to the 8th edition. Five-year disease specific survival (DSS) was defined as the time from first treatment until disease specific death or the last follow-up, with a maximum of 5-years. Three-year disease-free survival

(DFS) was defined as the time until local, regional or distant recurrence or the last follow-up within 3 years after the start of the initial treatment. Death was censored and did not count as a DFS event.

Table 1. Population characteristics

Variables		**n**	**(%)**
Total patients		**211**	**(100)**
Gender	Male	118	(56)
	Female	93	(44)
Age at diagnosis	Mean *(SD)*	62	(13)
(years)	Range	25 to 94	
Site	Tongue	108	(51)
	Gum	14	(7)
	Floor of mouth	64	(30)
	Cheek mucosa	7	(3)
	Retromolar area	12	(6)
	Other	6	(3)
cT status (7^{th})	1-2	189	(90)
	3-4	22	(10)
cN status (7^{th})	cN+	50	(24)
	cN0	161	(76)
Histopathological characteristics	Tumour thickness (mm) *Median (IQR)*	6.00	(3.3 to 9.0)
	Range	0.1 to 20.0	
	Perineural invasion	35	(17)
	Lymphovascular invasion	19	(9)
	Involved margins (<1 mm)	32	(15)
PO(C)RT		72	(34)
Follow-up (months)	Median (IQR)	64	(30 to 99)
	Range	0 to 193	
Recurrences	Locoregional recurrence	13	(6)
	Regional recurrence	26	(12)
	Distant metastasis	6	(3)
Death	Due to disease	36	(17)
	Overall	68	(32)

Abbreviations: OSCC, oral squamous cell carcinoma; SD, standard deviation; IQR, interquartile range; PO(C)RT, postoperative chemo or radiation therapy.

Ethical justification

As this study used retrospectively evaluated data from patients treated according to the Dutch national guidelines for oral cavity cancer, approval from the hospital research ethics board was not necessary according to the Dutch ethical regulations [8].

Statistics

Categorical data are presented as number and percentage, normally distributed data are presented as mean with standard deviation (SD), and skewed data are presented as median with IQR. Fisher's exact or chi-squared tests were used to test the associations between categorical data. The log-rank test was used to analyse differences between the Kaplan-Meier curves. DSS is reported as a percentage of survival after 5 years and DFS is reported as a percentage of survival after 3 years. The STATA statistical software (*Release 15.1)* was used to determine the 95% confidence intervals of the DSS and DFS survival percentages (Stata Corp., College Station, TX, USA). All other statistical analyses were performed with IBM SPSS STATISTICS 23 (SPSS, Chicago, IL, USA). *P*-values of <0.05 were considered to be significant for all of the statistical analyses.

RESULTS

Tumour staging

In total, 211 patients with a median tumour infiltration depth of 6.0 mm (IQR: 3.3 to 9.9 mm) were used for the pT classification analysis. Tumour restaging according to the 8th edition resulted in upstaging of 75 (36%) of the 211 patients: 12 (6%) patients on the basis of both tumour infiltration depth and ENE, and 63 (30%) patients on the basis of tumour infiltration depth only. Figure 1A shows the differences between the 7th and 8th pT editions. Fifty-four pT1 patients (44%) and 21 pT2 patients (24%) were restaged according to the 8th edition criteria. Patients with tongue tumours were significantly more often restaged to pT2 (31%) or pT3 (19%) than patients with tumours in the other anatomical locations, for whom restaging occurred in 12% (pT2) and 9% (pT3) ($p < 0.001$). Of the 38 watchful waiting patients, 11 (29%) were restaged to pT2. These patients had significantly shorter DSS ($p = 0.016$) and DFS ($p = 0.033$) than the other 27 patients (Figure 2A, B). Within the watchful waiting group, three (11%) of the 27 non-restaged patients and four (36%) of the 11 restaged patients were diagnosed with regional recurrences during follow-up (not-significant). Sixteen of the 45 patients (35%) restaged from pT1 to pT2 had undergone postoperative radiotherapy after surgical resection of the tumour. Twenty-three of the 30 patients restaged as pT3 had been postoperatively irradiated. The 8th edition pT classification showed a good stratification with significantly shorter DSS for the pT1-T2 patients upstaged to pT3 than for non-restaged pT2 patients (81% versus 66%, $p = 0.048$, Figure 3B and Table 2).

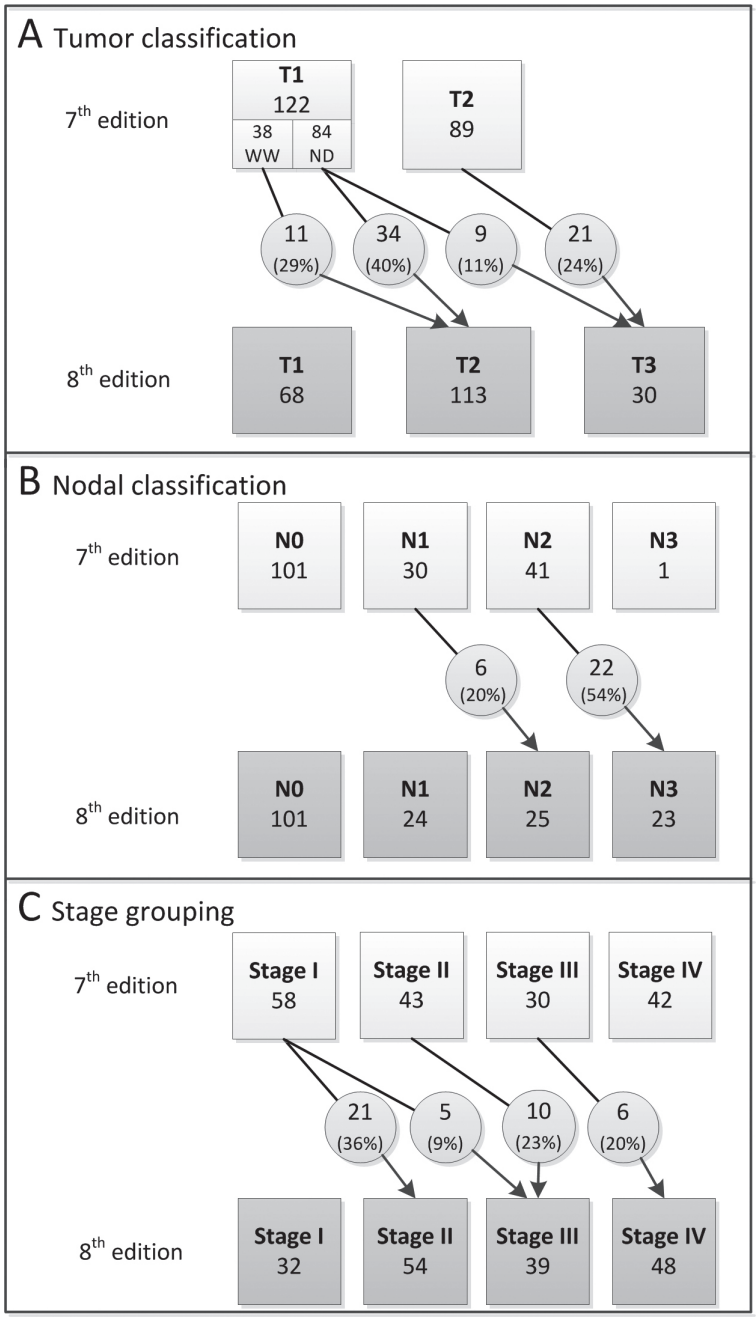


Figure 1. Differences in tumour, nodal and stage grouping between the 7th and 8th editions of the AJCC TNM classification.

Abbreviations: T, tumour; N, nodal; WW, watchful waiting; ND, neck dissection; AJCC, American Joint Committee on Cancer.

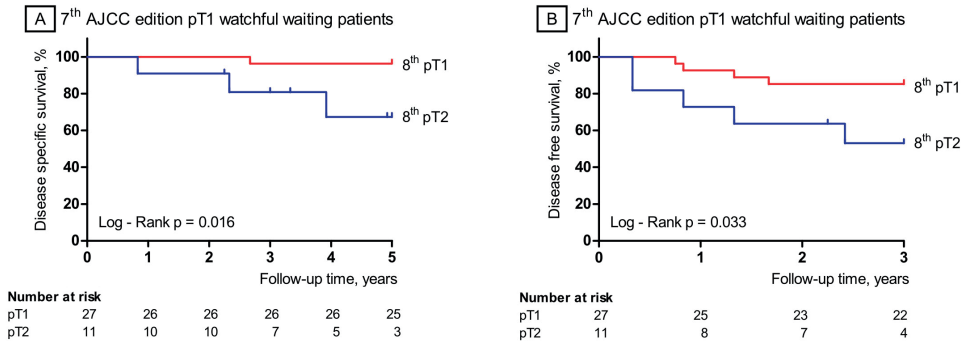


Figure 2. Differences in disease specific survival (A) and disease free survival (B) for 7th pT1-classified patients with a watchful waiting strategy of the neck which were staged using the 8th edition pT criteria.

Nodal Staging

Of the 173 neck dissection patients, 72 (42%) were diagnosed with nodal metastasis. Twenty-eight (16%) of these 173 patients were restaged with the 8th edition criteria because of ENEs (Figure 1B). No significant differences were seen in N-status restaging between anatomical locations. Twenty-six of the 28 restaged patients had been treated postoperatively with radiotherapy, which was combined with chemotherapy in one patient. The 8th edition pN classification showed good stratification, with an 89% 5-year survival rate for the watchful waiting patients and pN3 staged patients having the shortest survival rates, although the difference in DSS between pN2 staged and pN3 staged patients was not significant (69% versus 48%, p = 0.072, Figure 3D and Table 2).

Stage Grouping

In total, 42 (20%) patients were restaged with the 8th edition criteria. Differences in stage grouping per category between the 7th and 8th editions are shown in Figure 1C. Restaging with the 8th edition resulted in a smaller difference in DSS between stage I and stage II: 11% versus 3% difference in the 5-year DSS between the 7th and 8th stage I and stage II editions respectively. After restaging with the 8th edition criteria, the difference in DSS was larger between stage II and stage III, being 12% with the 7th edition (85% and 73%, not significant) versus 20% with the 8th edition (94% and 74%, p = 0.007) (Figure 3F and Table 2).

Table 2. Disease specific survival rates , 95% confidence intervals and log-rank test of all stages

		Survival		Log-rank test	
Edition	**Category**	**5yr, %**	**95% CI**	**Compared groups**	**p-value**
7th pT class	T1	89	82 to 93	T1 vs T2	**0.002**
	T2	72	61 to 81		
8th pT class	T1	91	81 to 96	T1 vs T2	0.077
	T2	81	72 to 87	T2 vs T3	**0.048**
	T3	66	45 to 80	T1 vs T3	**0.001**
7th pN class	WW	89	73 to 86	WW vs N0	0.734
	N0	92	84 to 96	N0 vs N1	**0.005**
	N1	73	54 to 86	N1 vs N2	0.264
	N2	59	42 to 73	N2 vs N3	0.402
	N3	0	NA		
8th pN class	WW	89	73 to 96	WW vs N0	0.734
	N0	92	84 to 96	N0 vs N1	**0.016**
	N1	75	53 to 88	N1 vs N2	0.793
	N2	69	46 to 84	N2 vs N3	0.072
	N3	48	26 to 67		
7th SG class	WW	89	73 to 96	WW vs Stage I	0.195
	Stage I	96	86 to 99	Stage I vs Stage II	0.056
	Stage II	85	70 to 93	Stage II vs Stage III	0.184
	Stage III	73	54 to 86	Stage III vs Stage IV	0.220
	Stage IV	58	40 to 72		
8th SG class	WW	89	73 to 96	WW vs Stage I	0.270
	Stage I	97	78 to 99	Stage I vs Stage II	0.594
	Stage II	94	83 to 98	Stage II vs Stage III	**0.007**
	Stage III	74	58 to 85	Stage III vs Stage IV	0.167
	Stage IV	59	43 to 72		

Abbreviations: CI, confidence interval; N, nodal; T, tumour; NA, not applicable; SG, stage grouping; WW, watchful waiting.

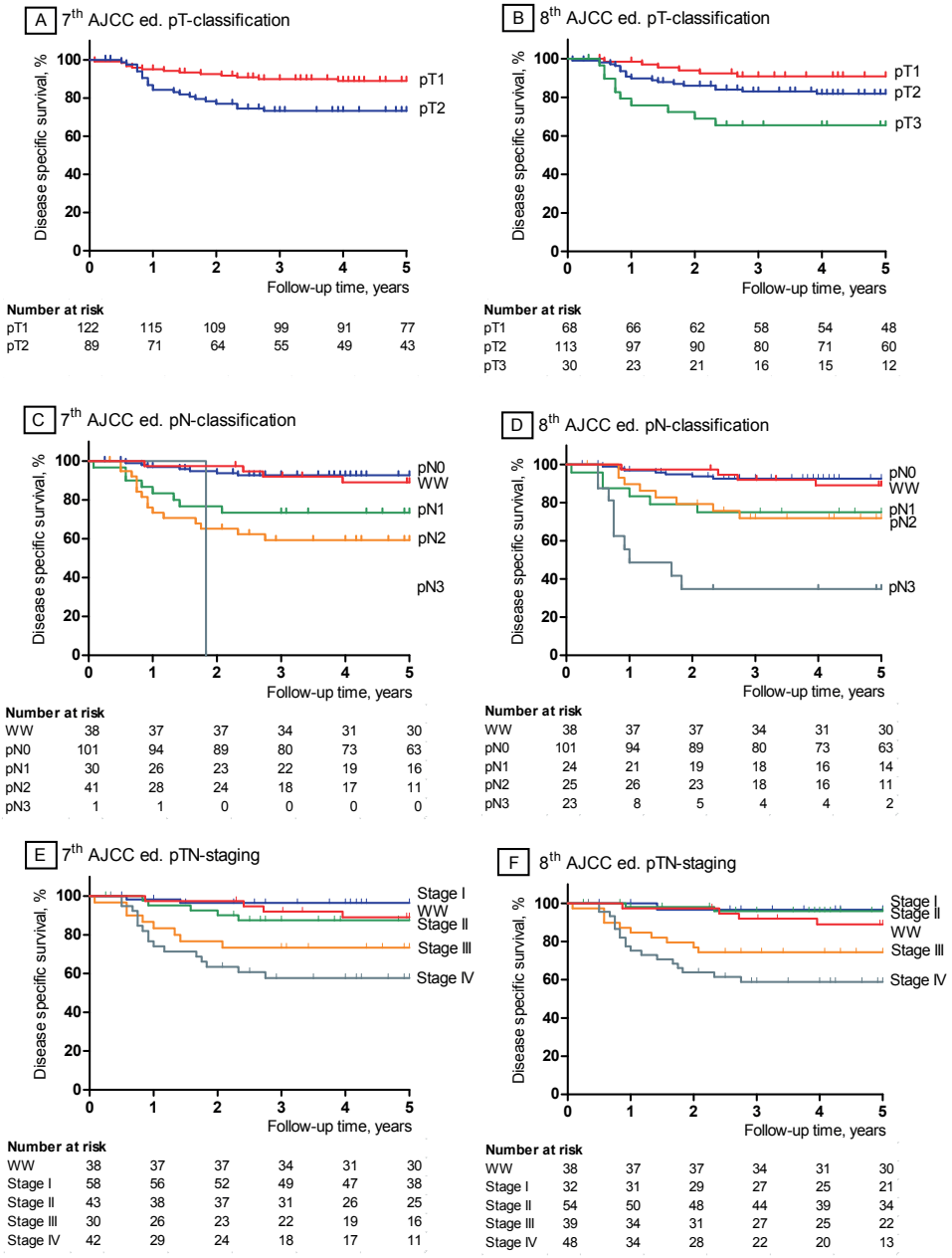


Figure 3. Disease specific survival Kaplan – Meier curves for the 7th edition (A+C+E) and 8th edition (B+D+F) TNM classifications. Five-year survival rates with their 95% confidence intervals for each disease specific survival curve and log-rank test are given in Table 2.

Abbreviations: AJCC, American Joint Committee on Cancer; ed., edition; DSS, disease specific survival; N, nodal; T, tumour; WW, watchful waiting

DISCUSSION

The aim of this study was to determine the clinical impact of the addition of tumour infiltration depth and ENE in the 8th edition of the pathological TNM classification on survival and potential alterations in treatment strategy for pT1-T2 OSCC patients who had been treated on the basis of the 7th edition. In this study, 36% and 16% of all of the patients were restaged with the 8th edition criteria according to the pT and pN classifications, respectively. Patients restaged as pT3 showed significantly shorter DSS than the 8th edition pT1-T2 staged patients. Another seven (3%) patients who were restaged as pT3 could possibly have benefited from postoperative radiotherapy.

This study used a well-defined 7th edition pT1-T2 cohort with extensive clinical data to add to the current evidence validating the 8th edition TNM classification [2,4,5]. Patients with a watchful waiting strategy of the neck were also included, which was not the case in the large ICOR study and the validation study by Matos et al. [3,4]. Recently, two other studies investigated the differences between the 7th edition and 8th edition TNM staging by using early-stage OSCC patients [9,10]. These studies differed from the current study by using sentinel lymph node biopsy (SLNB)-staged patients or by analysing only the pT categories and not the pN categories. This study confirms the previously mentioned validation study findings regarding the shorter survival rate of patients restaged as pT3 and pN3 with the 8th edition criteria [2,4,5]. However, the number of restaged patients differs between studies. In this study 44% of the 7th edition pT1 patients were restaged, versus 44% and 61% in other studies [3,4] and 24% of the pT2 patients were restaged, versus 62% and 47% in other studies [3,4]. Remarkably, one of the other studies did not restage any of the 7th edition pT1 patients to pT3 [3]. Differences in restaging rates might be explained by differences in clinical care between the countries. In The Netherlands, people visit their general dental practitioner once a year or more, whereas one of the validation studies stated in the discussion that the restaging rates could have been limited by a high rate of advanced disease which is a reality in emerging countries [4].

Restaging to a higher classification level with the 8th edition criteria is only possible for 7th edition pT1-T2 patients. Consequently, the 8th edition is clinically most relevant for these patients. This is why we used a cohort of 7th edition pT1-T2 patients to obtain an unadulterated view of the differences in prognosis. The inclusion of only pT1-T2 patients resulted in a relatively small number of 8th edition pT3 patients as compared with other studies. Also, the ENE rate in this study is lower than in the other 8th edition TNM validation studies: 39% versus 51% and 53% respectively [4,5]. The inclusion of only pT1-T2 patients could explain the lower ENE rate than in studies that also included more advanced disease.

We previously stated that a tumour infiltration depth of 4 mm could serve as an optimal cut-off between elective and therapeutic neck dissections, on the basis of results obtained with the same cohort [6]. Therefore, it is not surprising that the 8th edition pT2 patients (tumour infiltration depth of 5-10 mm) showed shorter survival in this study. Furthermore, another study suggested using a 4 mm tumour infiltration depth as a cut-off for pT3 tumours instead of the 8th edition AJCC pT cut-offs [9]. Twelve patients in this cohort had a watchful waiting of the neck and an infiltration depth of >4 mm because they were treated before the introduction of the 4 mm cut-off in our centre. Exclusion of these 12 patients resulted in a 100% 5-year survival for the remaining watchful waiting patients and similar survival stratifications for the 7th and 8th pT and pN categories (Supplementary data 1 and 2).

The benefit of this cohort was the availability of long-term follow-up because no adjustments were made for OSCC in the 7th pTNM classification edition when it was released in 2009, as compared with the 6th edition [11].

Additions to the pTNM classification are useful if they can be measured robustly and have a clinical impact. The national guidelines in The Netherlands support postoperative radiotherapy of T3-T4 tumours, even those with clear margins [12]. If the patients in this cohort had been staged with the 8th edition and treated accordingly, another 3% of the patients would have received postoperative radiotherapy. Although the patients who were restaged according to the 8th edition pT classification criteria showed lower DSS, prospective studies are needed to confirm that radiotherapy is beneficial for these patients. Besides the adjuvant therapy, SLNB is currently used as staging technique for cT1-2N0 patients in our centre [13]. This study shows that the 30 (15%) patients who were restaged as T3 would not have had an indication for a SLNB according to the 8th edition criteria. Den Toom et al. stated that 8th edition pT3 patients with tumours ≤40 mm in diameter probably benefit from staging of the neck with the SLNB procedure [10]. However, further data are needed to verify whether the SLNB is still a reliable neck staging technique for patients restaged from 7th edition pT1-T2 to 8th edition pT3. In our centre, pN3 patients are treated postoperatively with concomitant chemoradiotherapy according to the current guidelines [12]. Despite the better prognostic value of the 8th edition pN classification, pN staging with the 8th edition would not alter postoperative treatment strategies in our centre.

The growth of OSCCs can occur in an exophytic, an ulcerative or a superficial manner [2,6]. These differences in surface growth have resulted in various methods of assessment of tumour infiltration depth and thickness in the past[2]. To prevent underestimation (ulcerative growth) or overestimation (exophytic growth) of the prognosis, for the 8th pT classification tumour infiltration needs to be measured vertically from the reconstructed mucosa by use of the adjacent mucosal basement membrane of the normal epithelium [2].

In this study, the mucosal surface was used instead of the basement membrane. Healthy epithelial thicknesses are approximately 216 µm (SD 59 µm) for the tongue and 99 µm (SD 22 µm) for the mucosa of the anterior floor of the mouth [14]. Because of these small differences between healthy mucosal surfaces and basement membranes, it is improbable that tumour infiltration depth assessment by use of the basement membrane would have a large impact on our data. This was confirmed by an earlier study reporting an extremely high correlation between both methods (3.7% pT category difference) [4]. Another study reported a 5.7% difference in pT category when it compared both methods without correcting for exophytic growth [15]. In cases of metastasis in lymph nodes, all cases with extension of the metastasis through the fibrous capsule into the surrounding tissue should be scored as ENE positive [2]. To study the effect of ENE size in the future, Lydiatt et al. advocate to divide ENE positive lymph nodes with minor ENE (<2 mm) and major ENE (>2 mm and metastasis without recognisable lymph node) [2].

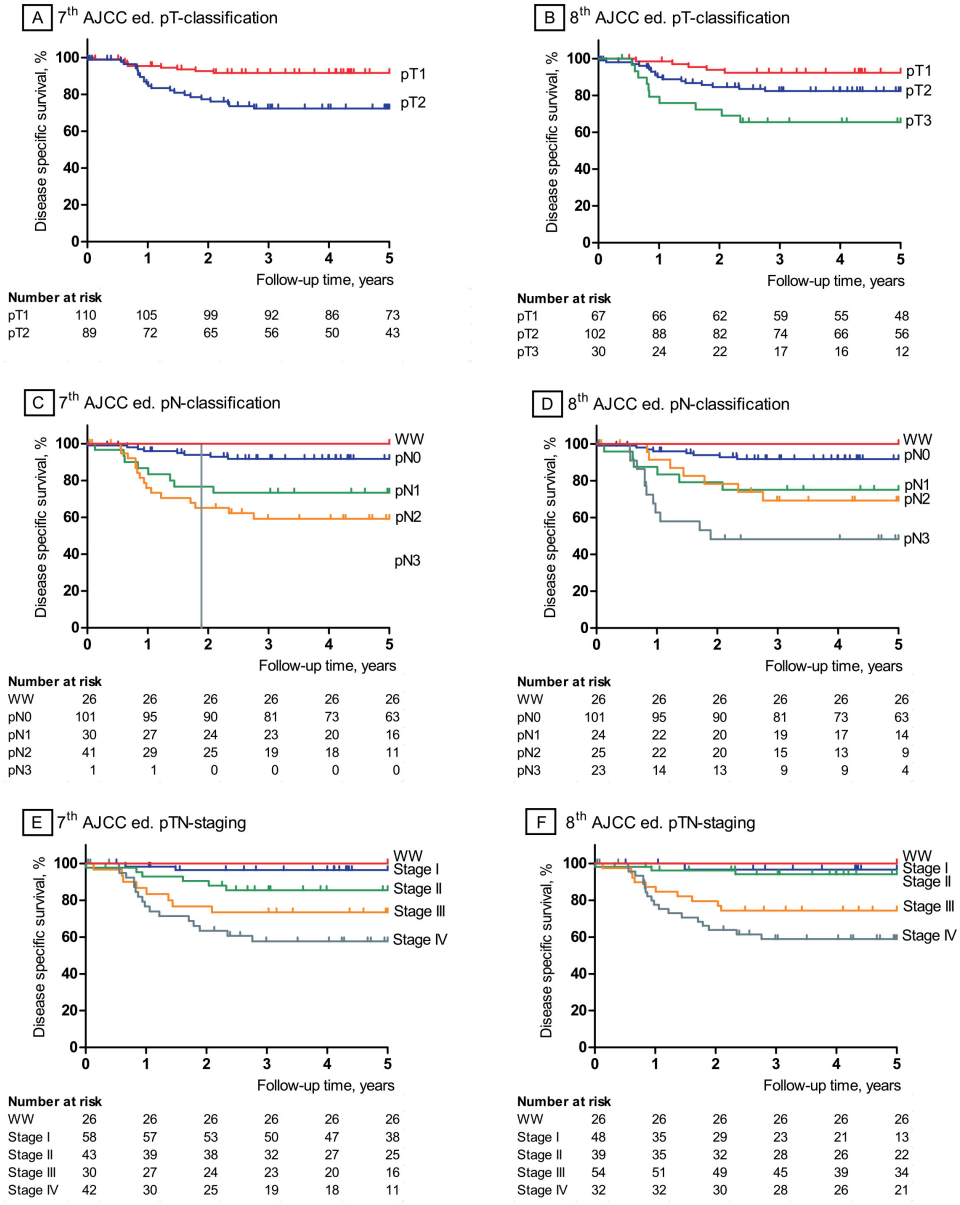
This study demonstrates, in a well-defined retrospective cohort of 211 pT1-T2 (7th edition) OSCC patients, that the addition of tumour infiltration depth and ENE, as used in the 8th edition of the AJCC pathological TNM classification, identifies a group of restaged patients with a worse prognosis.

SUPPLEMENTARY DATA

Supplementary data 1. Disease specific survival rates , 95% confidence intervals and log-rank test of all stages after excluding watchful waiting patients with a tumour infiltration depth > 4 mm

Edition	Category	Disease Specific Survival		Log-Rank test	
		5 yr %	95% CI	Compared stages	p-value
7th pT	T1	92	85 to 96	T1 vs T2	**0.001**
	T2	72	61 to 81		
8th pT	T1	92	83 to 97	T1 vs T2	0.066
	T2	82	73 to 89	T2 vs T3	**0.042**
	T3	66	45 to 80	T1 vs T3	**0.001**
7th pN	WW	100	NA	WW vs N0	0.135
	N0	92	84 to 96	N0 vs N1	**0.005**
	N1	73	54 to 86	N1 vs N2	0.264
	N2	59	42 to 73	N2 vs N3	0.402
	N3	0	NA	NA	
8th pN	WW	100	NA	WW vs N0	0.135
	N0	92	84 to 96	N0 vs N1	**0.016**
	N1	75	53 to 88	N1 vs N2	0.793
	N2	69	46 to 84	N2 vs N3	0.072
	N3	48	26 to 67		
7th SG	WW	100	NA	WW vs Stage I	0.333
	Stage I	96	86 to 99	Stage I vs Stage II	0.056
	Stage II	85	70 to 93	Stage II vs Stage III	0.184
	Stage III	73	54 to 86	Stage III vs Stage IV	0.220
	Stage IV	58	40 to 72		
8th SG	WW	100	NA	WW vs Stage I	0.352
	Stage I	97	79 to 100	Stage I vs Stage II	0.594
	Stage II	94	83 to 98	Stage II vs Stage III	**0.007**
	Stage III	74	58 to 85	Stage III vs Stage IV	0.167
	Stage IV	59	43 to 72		

Abbreviations: CI, confidence interval; N, nodal; T, tumor; NA, not applicable; SG, stage grouping; WW, watchful waiting



Supplementary data 2. Disease specific survival Kaplan – Meier curves for the 7th edition (A+C+E) and 8th edition (B+D+F) TNM classifications after excluding watchful waiting patients with a tumour infiltration depth >4 mm. Five-year survival rates with their 95% confidence intervals for each disease specific survival curve and log-rank test are given in supplementary data 1.

Abbreviations: AJCC, American Joint Committee on Cancer; ed., edition; DSS, disease specific survival; N, nodal; T, tumor; WW, watchful waiting

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CHAPTER 3

High sensitivity and negative predictive value of sentinel lymph node biopsy in a retrospective early stage oral cavity cancer cohort in the Northern Netherlands

SLNB in early stage oral cavity cancer

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ABSTRACT

Objectives: In cT1-2N0 oral squamous cell carcinoma (OSCC) occult metastases are detected in 23-37% of cases. Sentinel lymph node biopsy (SLNB) was introduced in head and neck cancer as a minimally invasive alternative for an elective neck dissection in neck staging. Meta-analyses of SLNB accuracy show heterogeneity in the existing studies for reference standards, imaging techniques and pathological examination. The aim of this study was to assess the sensitivity and negative predictive value (NPV) of the SLNB in detecting occult metastases in cT1-2N0 OSCC in a well-defined cohort.

Design: Retrospective study. The SLNB procedure consisted of lymphoscintigraphy, SPECT-CT scanning and gamma probe detection. Routine follow-up was the reference standard for the SLNB negative neck. Histopathological examination of sentinel lymph nodes (SLN) consisted of step serial sectioning, haematoxylin-eosin and cytokeratin AE1/3 staining.

Setting: Two comprehensive oncology centres.

Participants: A total of 91 consecutive patients with primary cT1-2N0 OSCC treated by primary resection and neck staging by SLNB procedure between 2008 and 2016.

Main outcome measures: Sensitivity and negative predictive value.

Results: In all cases, SLNs were harvested. A total of 25 (27%) patients had tumour-positive SLNs. The median follow-up was 32 months (range 2-104). Four patients were diagnosed with an isolated regional recurrence in the SLNB negative neck side resulting in an 85% sensitivity and a 94% NPV.

Conclusion: In our cohort, the SLNB detected occult metastases in early OSCC with 85% sensitivity and 94% NPV. This supports that SLNB is a reliable procedure for surgical staging of the neck in case of oral cT1-2N0 SCC.

INTRODUCTION

Regional metastases occur in 23-37% of the early stage (cT1-2N0) oral squamous cell carcinomas (OSCC) [1-3]. Lymph node status is an important prognostic factor for outcome and treatment decision making of head and neck cancer [1-8]. However, not all metastases are clinically detectable with the current diagnostic modalities [9-11]. Occult metastases are conventionally treated by removal of the lymph nodes by elective neck dissection (END) after research showed higher rates of overall and disease specific survival compared to a watchful waiting strategy [12]. However, an END has disadvantages: it leads to overtreatment in 63-77% of the cases and has a risk of postoperative morbidity (e.g. shoulder pain, reduced limb movement) [13]. Therefore, there is a need for a better neck staging modality.

The sentinel lymph node biopsy (SLNB) was introduced in oral cavity cancer as a less invasive lymph node staging technique after successful implementation in melanoma and breast cancer [5]. The limited number of lymph nodes (LN) with the SLNB enables a more meticulous pathological examination incorporating step serial sectioning (SSS) and additional immunohistochemistry (IHC) [14]. Recently, Liu and Wang reported a meta-analysis of 3566 early stage OSCC patients from 66 studies with a pooled sensitivity of 87% and negative predictive value (NPV) of 94% for SLNB in detecting occult metastasis [15]. However, many of these studies consist of small cohorts and differ in reference treatment, SLNB localisation technique (e.g. use of gamma-probe, blue dye or single photon emission CT (SPECT-CT)) and pathological work-up (with or without IHC or SSS). Furthermore, several studies provide incomplete clinico-pathological information. This heterogeneity and lack of complete data underline the need for more studies using complete and homogeneous cohorts. The aim of this study was to determine the sensitivity and NPV of the SLNB in detecting occult metastases in a large, well-defined cohort. For this purpose, we used a retrospective cT1-2N0 OSCC cohort of 91 patients all treated by primary surgical resection, neck staging with the SLNB procedure and routine follow-up as reference standard for the SLNB negative neck.

PATIENTS AND METHODS

Ethical consideration

Sentinel lymph node biopsy was part of standard treatment and data were retrospectively gathered from existing data sources; therefore no approval from the hospital research ethics board was required according to the Dutch ethical regulations [16,17]. Five patients were

also included in a Dutch multicenter SNLB validation trial before the SLNB was incorporated in the Dutch guidelines. Written informed consent was obtained from each of these five individual patients after study approval from the ethical board of the UMCG [7].

Patients and setting

Patients treated at the Oral & Maxillofacial Surgery or Otorhinolaryngology / Head & Neck Surgery departments of the University Medical Center Groningen (UMCG) (n = 91) or the Oral & Maxillofacial Surgery department of the Medical Center Leeuwarden (MCL) (n = 12) between October 2008 and September 2016 were included. Inclusion criteria: clinically T1-2N0 OSCC; primary treatment by surgical resection; neck staging by SLNB. Twelve patients were excluded because of pT3-4 tumours (n = 2), multiple primary head and neck squamous cell carcinoma at diagnosis (n = 5), incomplete SLNB protocol (n = 3) and, multifocal tumours without free surgical resection margins and uncertainty of clear injection around the tumour (n = 2). Clinico-pathological data of the 91 (100%) patients were retrospectively collected from the digital patients files (Table 1). Clinical neck staging was performed by extensive palpation and CT or MRI (UMCG) or by ^{18}F-FDG positron emission tomography (PET)-CT (MCL) scanning and in both centers followed by US-guided with fine needle aspiration cytology in case of enlarged (>1 cm) of otherwise suspicious nodes. Cases with a positive SNLB underwent a modified radical neck dissection (MRND) during a second surgery. Routine follow-up of the neck was used as reference standard in the SLNB negative patients. In total seven (8%) patients received adjuvant radiotherapy for irradical tumour resection of the deep margin (n = 4), pN2 neck stage (n = 3) and/or extranodal extension (n = 1).

Sentinel lymph node biopsy procedure

One day before surgery ^{99m}Tc-nannocolloid (median 100 MBq, IQR 95-102, data available for 90 patients) (GE Healthcare, The Netherlands) was injected around the tumour. Dynamic visualization by lymphoscintigraphy followed immediately after injection for 20 minutes in anterior or oblique views (20x60s s, 128x128 matrix) and also immediately static images (300 s, 256x256m matrix) in anterior and lateral direction were generated (Ecam or Symbia S (MCL), or Symbia T (UMCG), Siemens, Knoxville, TN, USA). The static visualization was repeated after 2-4 h. Thereafter visualization by SPECT-CT scanning of the head and neck using a two-headed gamma camera equipped with parallel-hole ultra-high resolution collimators and a 2-slice CT scanner (32 views of 20 s, 128x128 matrix; mAs 30, kV 110, 3.0 mm slice) was performed, only in the UMCG. SPECT-CT scanning was added to the protocol after treatment of the first five patients. The position of the SLN was marked on the overlaying skin with a Cobalt-57 point-source-marker and a gamma-probe (Europrobe, EuroMedical Instruments, France (MCL) and Neoprobe, Mammotome, Cincinatti, Ohio (UMCG)). The first lymph nodes in a lymphatic path from the tumour were marked as SLNs.

Table 1. Patient and tumour demographic characteristics

Demographic Characteristics	Overall, n (%)	Histopathological status of SLNB		p-value
		pN0, n (%)	pN+ (%)	
Total	91 (100)	66 (100)	25 (100)	NA
Sex				
Male	43 (48)	32 (48)	11 (44)	0.815
Female	48 (52)	34 (52)	14 (56)	
Age at first treatment, y				
median (IQR)	62 (56-70)	61 (56-71)	64 (57-69)	0.996
Tumour location *n(%)*				
Tongue	52 (57)	35 (53)	17 (68)	0.111*
FOM	27 (30)	23 (35)	4 (16)	
Cheek mucosa	8 (9)	5 (8)	3 (12)	
Upper gum	3 (3)	2 (3)	1 (4)	
Lower gum	1 (1)	1 (2)	0 (0)	
cT classification				
cT1	66 (73)	51 (77)	15 (60)	0.119
cT2	25 (27)	15 (23)	10 (40)	
pT classification				
pT1	73 (80)	57 (86)	16 (64)	**0.036**
pT2	18 (20)	9 (14)	9 (36)	
SLNB side				
Ipsilateral	57 (63)	40 (61)	17 (68)	0.701
Contralateral	1 (1)	1 (2)	0 (0)	
Both sides	33 (36)	25 (38)	8 (32)	
Number of SLNs per patient				
Median (IQR)	3 (2-4)	3 (2-4)	3 (2-4)	0.585
Tumour infiltration depth				
<4.59 mm	59 (65)	46 (70)	13 (52)	0.142
≥4.59 mm	32 (35)	20 (30)	12 (48)	
Perineural invasion				
Yes	6 (7)	3 (5)	3 (12)	0.340
No	85 (93)	63 (95)	22 (88)	
Lymphovascular invasion				
Yes	9 (10)	6 (9)	3 (12)	0.702
No	82 (90)	60 (91)	22 (88)	

Table 1. Continued

Demographic Characteristics	Overall, n (%)	Histopathological status of SLNB		p-value
		pN0, n (%)	pN+ (%)	
Tumour border configuration				
Pushing	54 (59)	45 (68)	9 (36)	**0.008**
Infiltrative	37 (41)	21 (32)	16 (64)	
Differentiation grade				
Well	29 (32)	21 (32)	8 (32)	1.000
Moderate	62 (68)	45 (68)	17 (68)	
Follow up data				
Time in months *median (IQR)*	32 (21-47)	37 (22-49)	25 (19-33)	**0.014**
total range	2-104			
Recurrence				
Local / 2nd primary	9 (10)	5 (8)	4 (16)	**
Isolated regional rec.	5 (5)	2 (3)	3 (12)	
Dead				
Dead of disease	3 (3)	0 (0)	3 (12)	**
Dead not of disease	7 (8)	6 (9)	1 (4)	

Abbreviations: SLNB: sentinel lymph node biopsy. pN0: SLNs negative for metastases. pN+: SLNs positive for metastases. NA: not applicable. IQR: interquartile range. FOM: floor of mouth. Rec: recurrence

*: Tongue vs floor of mouth, other subgroups too small to analyse. **: Group too small to analyse

Surgical procedure

SLNs were harvested with a small incision in the neck after gamma probe assisted localization. The neck side of the SLNB was indicated by the results of the lymphoscintigraphy. In 28 (31%) patients, non-SLNs were harvested due to location of the SLN in a conglomerate of lymph nodes or the impossibility to remove the SLNs without harvesting these non-SLNs. SLNs were separated from the non-SLNs ex vivo using the gamma-probe in the operation theatre. Finally, the neck background radioactivity was checked with the gamma-probe to make sure that no SLN was left behind. Blue-dye was used intra-operatively in fourteen patients but not on regular base in both hospitals.

Histopathological procedure

SLNs were histopathologically examinated by step serial sectioning of the entire SLNs with an interval of 500 µm, conventional staining with hematoxyline-eosine (H&E) and an additional pan-cytokeratin antibody (AE 1/3) immunohistochemistry staining. The non-SLNs were

examinated according to the SLN protocol in four patients and using standard H&E without IHC or step serial sectioning in 24 patients. MRND lymph nodes were examined using the routine protocol. Histology of all SLNs and primary tumours was revised by a head and neck pathologist (BvdV). Lymph node metastases were classified according to Hermanek; ITC's <0.2 mm, micrometastasis 0.2-2 mm and macrometastasis >2 mm [18]. Infiltrative tumour border configuration was defined according to the classification of Heerema: small groups or cords of infiltration cells, widespread cellular dissociation in small groups of cells or in single cells and tumor satellites or any size ≥1 mm away from main tumour [19]. 4.59 mm was used as tumour infiltration depth cut-off according to Melchers [20].

Statistical analysis

IBM SPSS Statistics 23 (Statistical Package for the Social Sciences, Inc., Chicago, IL, USA) was used for analysis. Categorical data are presented as number (n) and their percentages (%). Associations between categorical data were tested with the Fisher's exact or Chi-squared test. Continuous data were tested using the Student's *t* test or the Mann-Whitney U test for normally or skewed distributed data, respectively. False negative SLNB patients were defined as patients with isolated regional recurrence in the SLNB negative neck side and were used to calculate the sensitivity and negative predictive value. Significant differences were defined as a p-value ≤ 0.05.

RESULTS

Sentinel lymph nodes were identified in all 91 cases (100%). In total 274 SLNs were harvested with a median of 3 (range 1-11) per patient. The results of the SLN procedures are summarised in Table 1. In all patients, at least one SLN was intraoperatively detected. However, in 4 patients (4%) additional hotspots were noticed besides the harvested SLNs on the SPECT-CT without intraoperative detectable radioactive LNs. In 1 of these 4 patients, the harvested SLN was positive and the neck was treated by MRND in a second operation. The other 3 patients were isolated regional recurrence (IRR) free after 10, 11 and 47 months of routine follow-up. In one patient with a ventral floor of mouth tumour, only a contralateral SLN was identified. The other patients had ipsilateral (n = 57, 63%) or bilateral (n = 33, 36%) located SLNs.

Positive SLNs were found in 25 (27%) patients. In 1 patient with a 1 mm metastasis in the SLN routine follow-up was chosen instead of a MRND. This patient was still recurrence free after 23 months. In none of the patients with micrometastases or ITCs in the SLN additional metastases were found in the MRND specimen (Figure 1, Table 2, p = 0.024). Also, none of the 57 non-SLNs harvested during the SLNB were positive. Finally, skip metastases were

not seen: all patients with positive SLNs had at least one positive SLN in level I-III. Infiltrative tumour border configuration (p = 0.008) and pT2 tumour stage (p = 0.036) showed an association with lymph node status (Table 1).

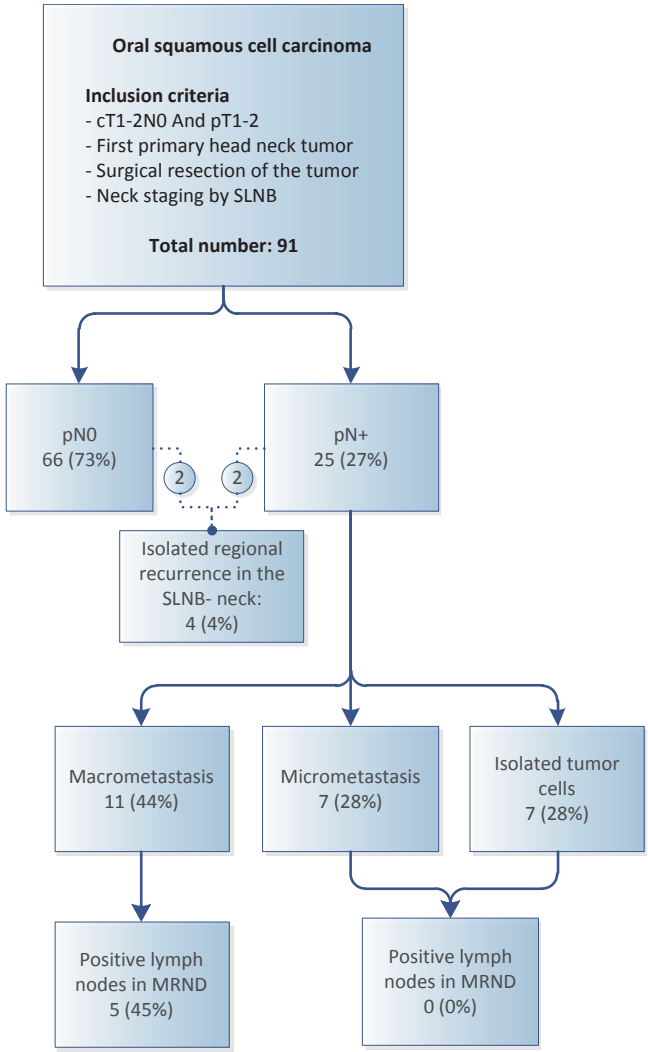

Figure 1. Flowchart of the UMCG and MCL cT1-2N0 oral squamous cell carcinoma cohort. In total 91 patients were used for analysis. A total of 25 patients had metastasis positive SLNB. Two patients with pN0 and two patients with pN+ SLNB neck status were diagnosed with isolated regional recurrence in the SLNB negative neck side.

Abbreviations: pN0: All SLNs negative for metastasis. pN+: at least one SLN positive for metastasis. MRND: modified radical neck dissection. SLNB: sentinel lymph node biopsy

Table 2. Association between SLN metastasis size and additional metastases in modified radical neck dissection lymph nodes

		MRND lymph node status		
SLN status	**n (%)**	**pN0, n (%)**	**pN+, n (%)**	**p-value**
Isolated Tumour Cells	7 (29)	7 (37)	0 (0)	
Micrometastases	6 (25)	6 (32)	0 (0)	**0.024**
Macrometastases	11 (46)	6 (32)	5 (100)	

Abbreviations: SLN: sentinel lymph node. MRND: modified radical neck dissection. pN0: LNs negative for metastases. pN+: LNs positive for metastases.

Follow-up and regional recurrence

Overall the median FU was 32 months (IQR 21-47, Range 2-104, Table 1). All patients with a follow-up <10 months died. In total, 8 (9%) patients of this cohort died. Three patients died of disease, two 10 months and one 21 months after the initial treatment.

Local recurrence and second primary tumours, with or without regional recurrence, were seen in 9 (10%) cases. Isolated regional recurrence was detected in 5 (5%) patients. One of these patients had IRR after a positive SLN and subsequent neck dissection at that neck side. The other 4 patients were diagnosed with IRR after 4, 6, 9 and 19 months. Their tumour, treatment and recurrence characteristics are shown in Table 3. The first patient had a positive ipsilateral SLN and was 4 months later diagnosed with level I and level II IRRs at the contralateral side. Revision of the SPECT-CT images and the conventional CT images of the IRR did not reveal new insights. The second patient had ipsilateral negative SLNs and was diagnosed with level Ib and level IV IRRs after 9 months. Revision of the SPECT-CT images of this patient showed a lymph node with a diameter of 7 mm without radioactivity just at the inside of the mandibular angle in level Ib. This lymph node was most likely not resected during the SLNB procedure and could be the same as the IRR lymph node. The third patient had a positive contralateral SLN. IRR occurred on the ipsilateral side, which was SLNB negative and was therefore not treated by MRND. Revision of the lymphoscintigraphy images revealed a low signal in level Ib at the ipsilateral side, what might be a missed SLN. The fourth patient had a negative SLN in level II and was diagnosed with IRR in level Ib, both ipsilateral. Revision of the SPECT-CT scan showed a LN within the radioactive hotspot of the floor of mouth tumour of this patient. Most likely, this is the same LN in which the IRR was diagnosed (Figure 2).

Due to the four IRRs, the SLNB detected occult metastases with 85% sensitivity and 94% NPV.

Table 3. Characteristics of the four patients with isolated regional recurrence

	Patients with isolated regional recurrence			
Variables	**1**	**2**	**3**	**4**
Tumour	Tongue	Cheek mucosa	Tongue	FOM
pT classification	1	1	2	2
Infiltration depth (mm)	8	5.0	3.7	2.7
Border growth	Pushing	Infiltrative	Infiltrative	Infiltrative
Resection margins	Free	Free	Free	Free
Perineural growth or Lympho- / angioinvasion	Yes, both	No	No	No
Differentation grade	Good	Moderate	Moderate	Moderate
Reresection	Yes	No	No	No
Postoperative radiotherapy	Tumour & Neck	No	No	No
SLNB side	Ipsilateral	Ipsilateral	Both	Both
Positive SLN side	Ipsilateral	NA	Contralateral	NA
MRND side	Ipsilateral	NA	Contralateral	NA
Regional recurrence side	Contralateral	Ipsilateral	Ipsilateral	Ipsilateral
Number of SLNs recurrence side	NA	3	1	1
Number of positive SLNs recurrence side	NA	0	0	0
SLN level recurrence side	NA	Level II	Level II	Level II
Recurrence level	Level I + II	Level Ib + IV	Level Ib, II, IV	Level Ib
Number of LNs (positive / total)	2/44 (ENE+)	6/41	4/46	NA †
Maximum diameter regional recurrence metastasis (mm)	25	12	15	13 †
Time between 1st treatment and rec. (months)	4	9.2	5.5	19
Total follow-up (months)	27	36	9	25
Dead of disease	NA	NA	Yes	NA

Abbreviations: SLNB: sentinel lymph node biopsy. MRND: modified radical neck dissection. NA: not applicable. ENE: extranodal extension.

† The isolated regional recurrence of patient 4 was not operatively removed, therefore only clinical data was available.

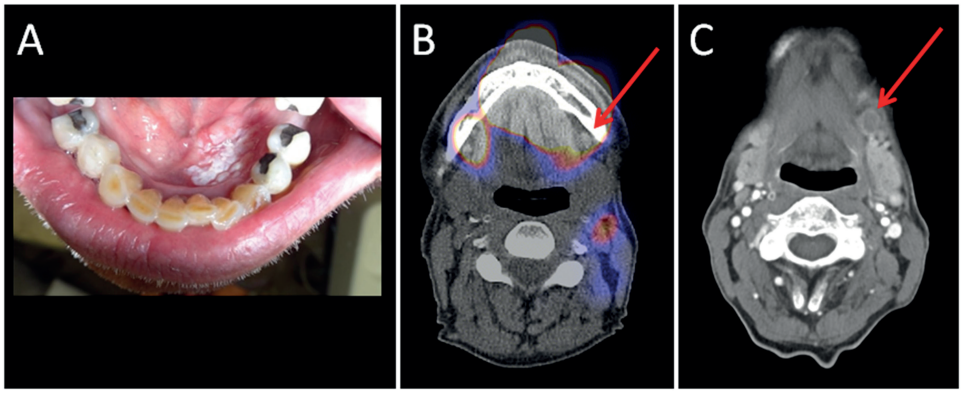

Figure 2. Shine-through phenomenon example. Patient with a floor of mouth tumour on the left side close the midline (A), with a lymph node within the tumour hotspot (B) and an isolated regional recurrence after 19 months (C).

DISCUSSION

Synopsis of key findings

In our retrospective cohort of 91 patients treated for cT1-2N0 OSCC, 4 patients developed isolated regional recurrence on the side of a negative SLNB. This resulted in 85% sensitivity and 94% negative predictive value.

Comparison to previous studies

The sensitivity and NPV are in agreement with the results of other studies with routine follow up as a reference: sensitivity range 80-94% and NPV range 88-97.5% (number of patients 59-415) [1,6-8,21]. A recent meta-analysis also showed comparable results: sensitivity 87%, NPV 94% [15]. The slightly higher NPV of this cohort compared to these meta-analyses can be explained by the relative short follow-up of some patients in our cohort. Two of the 66 patients (3%) with routine follow up after a negative SLNB were diagnosed with IRR. This percentage is much lower than the conventional 20% change of having IRR from Weis et al, which is generally used in literature as threshold to choose between watchful waiting and END [22]. The low percentage IRR indicates the accurate selection of cT1-2N0 patients for neck dissection or routine follow up by performing a SLNB.

False negativity was defined as patients with IRR in an earlier SLNB negative neck side, regardless of a positive SLNB on the other side of the neck. Four (4%) patients in our cohort were diagnosed with IRR in a SLNB negative side of the neck, which is comparable with other studies [6,8]. Retrospectively, the reason for missing these regional metastases remains unclear; shine-through phenomenon and aberrant lymphatic drainage due to metastatic tumour in the SLNs might be involved. Another possible explanation might be micrometastases in lymph nodes, other than the SLN (skip metastases).

Other studies reported a lower sensitivity of the SLNB procedure in FOM tumours compared to other oral cavity subsites due to the shine-through phenomenon [7,8,23,24]. One patient in this study had a FOM with an IRR resulting in an 80% sensitivity and a 96% NPV for FOM tumours. Retrospectively, this SLNB was overlooked because of this shine-through phenomenon (Figure 2). To overcome shine-through and subsequent regional recurrences, Stoeckli et al. proposed a surgical technique with dissection of all the LNs in level I irrespective of the location of the SLNs [25]. Van den Berg et al., combined the SLNB procedure with radio- and fluorescence guidance and found this combination especially helpful in detecting SLNs located close to the primary tumour [26]. Our data support the findings of the previously mentioned studies [25,26], that patients with primary tumours adjacent to level I could benefit from additional techniques besides the SLNB procedure alone.

The upstaging rate in this study (27%) is in agreement with the literature; 23-37% [1,2,7,8]. We found no additional metastasis in the MRND lymph nodes after a SLNB positive for ITCs or micrometastases. Recently, den Toom et al. reported that the ratio of positive versus negative SLNs and the size of the tumour in the SLN possibly could be predictive factors for non-SLN metastasis in SLN positive patients. However, their analysis was underpowered due to the use of the ITC, micro- and macrometastasis classification in just a few SLNB studies [27]. No additional metastasis in ITC or micrometastasis SLN positive patients, could be the reason why Liu and Wang et al. concluded in their meta-analysis that SSS is not necessary for SLN assessment [15]. Despite the lack of impact of the SSS on the IRR rate, in agreement with den Toom and our data presented in this paper, SLN metastasis size might be used to select patients for routine follow-up instead of MRND [8]. Besides the SSS itself, also the step interval size could be discussed. After the second international conference on SLNB, intervals of 150 µm were recommended [28]. As was reported earlier for breast cancer, Jefferson et al. suggested that SSS intervals of 2 mm are thin enough to detect micrometastasis [29,30]. In this study intervals of 500 µm were used, because our head and neck SCC protocol was adapted from our vulvar SCC SLNB protocol. This is a protocol we have much experience with and has shown to provide accurate staging of vulvar SCC in our centre [31-33]. Besides this, the accuracy we found is comparable to that of most head and

neck SLNB studies [15]. Moreover, the ITC, micro- and macrometastasis ratio is comparable with other studies, indicating that we did not miss ITCs using this protocol. We therefore assume that this protocol has not influenced our results. However, we propose to continue SSS and classification of SLN metastasis size according to Hermanek, until well powered studies have defined the clinical impact of the SLN metastasis size [18]. Afterwards, further research is needed to reach consensus about minimal interval thickness for SSS to detect these metastases with clinical impact.

Thirty-three patients had SLNs on both sides of the neck, also in cases with lateralised border of tongue tumours. Moreover, 1 patient did not show ipsilateral lymphatic drainage patterns, but instead showed a negative contralateral SLN. This patient did not develop IRR at either side within 34 months follow-up. These 34 (37%) patients showed the advantage of detecting unexpected drainage patterns with the SLNB procedure and were thereby prevented from undertreatment.

Despite the good accuracy of the SLNB procedure, improvements might be made for the clinical negative neck. For example, in our centres the use of blue dye has been abandoned, because it blurred surgical tumour resection margins intra-operatively. A disadvantage of the SLNB procedure is the second operation for the MRND after a positive SLNB. Especially in frail elderly or patients with multiple comorbidities, a second operation with general anaesthesia is undesirable due to a higher complication and mortality chance [34]. Moreover in all positive cases, scar tissue makes the neck dissection surgery more challenging in the SLN levels. To avoid repeat surgery, the possibility of intraoperatively staging of SLNs with frozen sections has been studied [35]. However, frozen sections have a substantial false negative rate; therefore frozen sections of the SLNs are not applied in our centres. Also a substantial amount of the SLN is lost for the FFPE sections and thereby increasing the risk of missing ITCs and micrometastases [35].

In an ideal situation, patients at high risk of lymph node metastases are preoperatively selected for MRND or watchful waiting. In the current study, an infiltrative tumour border configuration or a pT2 tumour was significantly associated with more regional metastases. Our research group reported earlier infiltration depth and lymphovascular invasion as independent predictors for nodal status in pT1-2N0 and N-status determination by routine HKD and watchful waiting [20]. These markers are not associated with positive lymph nodes in this study. The lack of significance could be explained by the difference in patient selection between the mentioned study by Melchers (cN0 and cN+) and this study (cN0) [20]. Therefore, the SLNB procedure is still more accurate in detecting occult metastasis in

cT1-2N0 OSCC than the current clinical and pathological markers. In addition, it would be interesting to study the prognostic value of OSCC lymph node status associated biological markers such as *WISP1*, *RAB25* or *EpCAM* in cT1-2N0 OSCC SLNB staged patients [36-38].

Study limitations

Limitation of this study is that the SLNB procedure was not part of the standard workflow for cT1-2N0 OSCC patients in the first years after introduction. If we analyse the accuracy without the 6 patients from this period, the sensitivity and NPV are still 85% and 94% respectively.

CONCLUSION

In this retrospective well-defined cohort consisting of 91 patients we showed that the sentinel lymph node biopsy is an accurate diagnostic technique in detecting occult metastases in cT1-2N0 OSCC and is a save and reliable alternative to an END or watchful waiting.

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CHAPTER 4

High rate of unexpected lymphatic drainage patterns and a high accuracy of the sentinel lymph node biopsy in oral cancer after previous neck treatment

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ABSTRACT

Rationale: This study evaluates the lymphatic drainage patterns and determines the accuracy of the sentinel lymph node biopsy (SLNB) in patients diagnosed with a cT1-2N0 OSCC and a history of neck surgery or radiotherapy in three Dutch head and neck centers.

Materials and Methods: Retrospective analysis of 53 cT1-2N0 OSCC patients, who underwent SLNB between 2007 and 2016, after a history of neck surgery or radiotherapy. Ten patients had previous treatment of the neck only contralateral from the current tumour. These ten patients were not used for the analysis of lymphatic drainage patterns. The 43 patients with previous ipsilateral or bilateral treatment of the neck had a history of ipsilateral SLN extirpation (n = 9; 21%), neck dissection (n = 16; 37%), radiotherapy (n = 10; 23%), or combined neck dissection and radiotherapy (n = 8; 19%).

Results: SLNs were detected in 45 patients, resulting in an identification rate of 85% (45/53). Three patients (7%) had at least one positive SLN. One patient (1/45; 2%) was diagnosed with regional recurrence during the follow-up after a negative SLNB (sensitivity 75%, negative predictive value 98%). The first SLN was detected in level I-III in 58% of the patients, unexpected drainage patterns were observed in 30% (first SLN level IV 9% and level V 5% and contralateral neck in well-lateralized tumours 16%). In 12% no lymphatic drainage pattern was visible.

Conclusions: SLNB seems to be a reliable procedure for neck staging of cT1-2N0 OSCC patients with a previously treated neck. SLNB determines the individual lymphatic drainage patterns, enabling visualization of unexpected drainage pattern variability in 30% of these patients.

INTRODUCTION

Presence of lymphatic metastases in the neck is consistently observed as main prognostic factor in patients with oral squamous cell carcinoma (OSCC) [1-3]. Sentinel lymph node biopsy (SLNB) proved to be reliable as diagnostic staging modality for detection of occult lymph node metastases: in a large recent meta-analysis a pooled sensitivity of 87% (95% CI 85-89%), a negative predictive value of 94% (95% CI 93-95%) and an AUC of 0.98 (95% CI 0.97-0.99%) were found [4]. These meta-analysis results are based on patients with primary OSCC and a previously untreated neck. Despite the relatively common local recurrences and second primary tumours in head and neck cancer, only one study of Flach et al. reported about the accuracy of the SLNB in 22 patients with a previously treated neck [5].

It is well known that patients with OSCC suffer a high risk for local recurrences (10-30%) and an annual risk of 3-4% for developing second primary tumours [3,6-8]. Previous treatment of the neck most likely alters lymphatic drainage patterns. Current evidence about the drainage patterns in previously treated OSCC patients using SLNB is limited to a study by Flach et al. (n = 22) and a feasibility study by Pitman et al. (n = 5) [5,9]. Experience of alteration in lymphatic drainage patterns after previous treatment has also been reported in breast cancer and melanoma [10-15]. While gaining more and more experience with SLNB in our institutions during the last years, SLNB has been used increasingly as staging method in patients with a previously treated neck. Moreover, SLNB is valuable in assessment of the individual lymphatic drainage patterns, compensating for potential variabilities as a result of previous treatment which were reported in 67% of the cases by Flach et al [5].

However, since the study of SLNB in OSCC patients with a previously treated neck consisted of only 22 patients, more research had to be performed to confirm the findings of that study [5]. The aim of this study was to assess the accuracy of SLNB and secondly, to evaluate the lymphatic drainage patterns in a consecutive cohort of cT1-2N0 patients with a previously treated neck in three Dutch head and neck cancer centers.

METHODS

In three Dutch head and neck centers 53 patients diagnosed between 2007 and 2016 met the inclusion criteria and were retrospectively analyzed. Patients with early stage local recurrent disease or second (or even third) primary squamous cell carcinoma of the oral cavity or oropharynx with a clinically negative neck and surgical resection of the tumour combined with SLNB staging of the neck were included (cT1-2N0, following the 7th TNM staging classification, Table 1). In their history, all patients had received prior treatment

Table 1. Patient characteristics

Characteristics	No.	(%)
Total number of patients	53	(100)
Gender		
Male	29	(55)
Female	24	(45)
Age *y mean (SD)*	65	(55-75)
(range)		(44-88)
pT status (7th TNM)		
1	44	(83)
2	9	(17)
Tumour locations		
Tongue	31	(58)
FOM	9	(17)
Buccal mucosa	5	(9)
Inferior alveolar process	4	(8)
Other	4	(8)
Previous treatment or surgery ipsilateral neck		
No	10	(19)
RT alone	8	(15)
ND alone	16	(30)
ND + RT	8	(15)
CRT	2	(4)
SLNB	9	(17)
Previous treatment or surgery contralateral neck		
No	25	(47)
RT alone	9	(17)
ND alone	6	(11)
ND + RT	6	(11)
CRT	2	(4)
SLNB	5	(9)
Follow-up		
Follow-up time months, *median (IQR)*	26	(13-42)
Regional recurrence	1	(1)
Death	13	(25)
Death of local recurrence or second primary	4	(8)

Ipsilateral and contralateral side of the neck is related to the side of the local recurrence or the second primary. Abbreviations: FOM, floor of mouth; RT, radiotherapy; ND, neck dissection; CRT, chemoradiation; SLNB, sentinel lymph node biopsy

of the neck with SLNB, neck dissection, (chemo)radiotherapy or a combination of these modalities (Supplementary data 1). Twelve patients were previously included in the study by Flach et al., their follow-up was updated [5].

The SLNB procedure was described extensively before [16,17]. Briefly, patients received preoperatively injections with ^{99m}Tc-nannocolloid followed by dynamic and static lymphoscintigraphy and SPECT-CT scanning one day before surgery, intra-operatively gamma probe detection and postoperative step serial sectioning of the sentinel lymph node with additional immunohistochemical keratin staining.

As visualized in our study design (Figure 1) all 53 patients were used for analysis regarding the accuracy of the procedure and 43 patients were included for the drainage pattern analysis. Earlier studies showed the potential of bilateral drainage patterns in well-lateralized patients. Because of this potential bilateral drainage also 10 patients were included with a history of only contralateral treatment of the neck (their first tumour was contralateral of the second) whom might affect the SLNB accuracy [16,18].

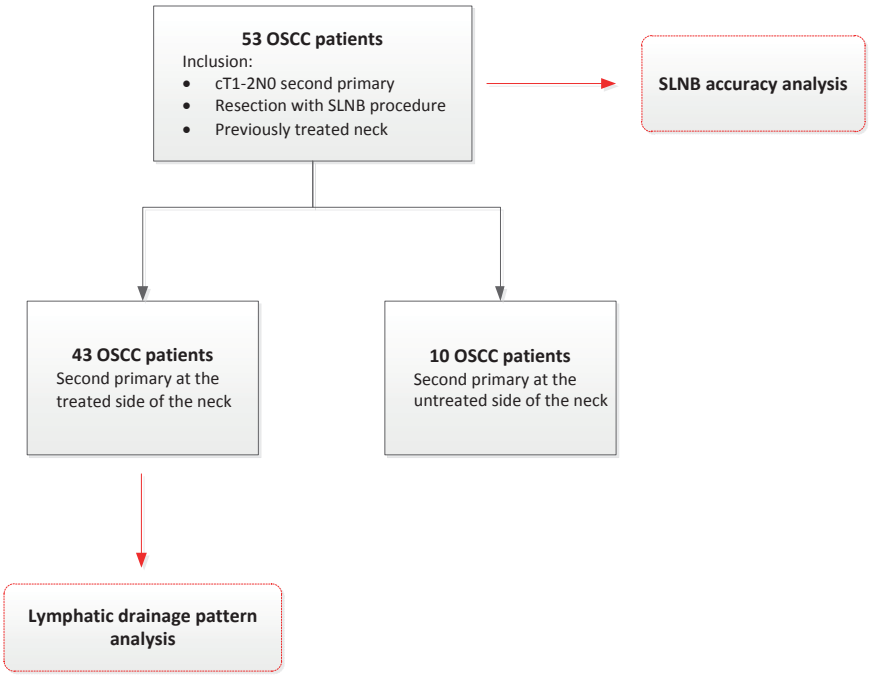


Figure 1: Study design. All 53 patients were used for the SLNB accuracy analysis, only the 43 patients with a history of neck treatment at the ipsi- or bilateral side were used for the analysis of altered lymphatic drainage patterns.

In OSCC lymphatic drainage is at least expected in level I-III at the ipsilateral side of the neck [18]. With the second aim to detect unexpected drainage patterns, only 43 patients with previous treatment of the ipsilateral side of the neck were used for lymphatic drainage pattern analysis.

In this study, definition of lateralization of the neck is related to the site of the local recurrence or second primary tumour.

Ethical consideration

Due to the retrospective design no approval was required from the hospital research ethics board of our centers according to the Dutch ethical regulations. SLNB was part of the standard management of these patients and patient information regarding clinical and pathological characteristics and follow-up was retrospectively collected from electronic patient files.

RESULTS

The data of 53 patients, 29 male (55%) and 24 female (45%) were used for analysis. Mean age was 65 years. Tongue was the most affected tumour location (59%), followed by floor of mouth. Forty-four patients (83%) were diagnosed with a pathologically T1 tumour and 9 patients (17%) with a T2 tumour. These and other characteristics are summarized in Table 1. Characteristics per patient are given in Supplementary data 1.

SLNB accuracy

Fifty-three patients were used for the SLNB accuracy analysis. Neck dissection, with or without postoperative radiotherapy, was seen most as previous treatment in both the ipsilateral and contralateral neck compared to the local recurrence or second primary side (Table 1). Thirteen patients (25%) died during follow-up of which four (8%) died as a result of the local recurrence or second primary tumour in the oral cavity (disease specific death: median 26 months, IQR 13-42 months).

No SLNs were visualized by lymphoscintigraphy in 7 of these 53 patients resulting in an 87% imaging detection rate. In one patient no SLNs were detected intraoperatively, despite preoperative visualization. In two patients with bilateral drainage on lymphoscintigraphy the SLNs were not detected in one neck side intraoperatively, but were harvested in the other side of the neck, resulting in a surgical detection rate of 93% (43/46, Supplementary data 1). In total, at least one SLN was harvested in 85% of the patients (45/53). Three patients had a positive SLN, respectively in the ipsilateral neck with a history of a SLNB, in the

ipsilateral neck without a history of pretreatment and in the ipsilateral neck with a history of chemoradiation therapy. In the first two patients, no additional metastases were detected after harvesting respectively 21 and 17 lymph nodes in the completed neck dissection specimens. Because of the history of chemoradiation and the metastasis size (ITC), the last patient received watchful waiting instead of a neck dissection. These 3 patients did not show regional disease during follow-up.

One patient (2%) was diagnosed with regional recurrence without local disease in level II at the ipsilateral side of the neck after 7 months of follow-up. This patient had a second primary tumour located in the buccal mucosa and only negative SLNs were found in level I at the contralateral side. This patient was previously treated with a MRND at the ipsilateral side of the neck for the first primary tumour, followed by postoperative chemoradiation at both sides of the neck. This patient was still alive after 19 months of follow-up after the regional recurrence was surgically removed and postoperatively irradiated.

One regional recurrence resulted in a 75% sensitivity with a 95% CI of 22-98% (3 of 4 true positive) and 98% NPV with a 95% CI of 88-100% (42 of 43 true negative) of the SLNB in patients with a previously treated neck.

If we restrict the accuracy analysis to patients with a history of neck dissection and/or radiotherapy in the ipsilateral neck, one out of 34 patients showed a positive SLN and one patient showed regional recurrence after a negative SLNB, resulting in a 50% sensitivity (1 of 2 true positive) with a 95% CI of 3-97% and a NPV of 97% (32 of 33 true negative) with a 95% CI of 82-100%.

Lymphatic drainage patterns

In 38 of the 43 patients with a second primary or local recurrence at the previously treated neck side SLNs were detected, resulting in an 88% identification rate. The five patients without detectable SLNs had in common a history of radiotherapy of the neck (Supplementary data 1). Since lymphatic drainage is expected generally in levels I-III for OSCC, in 30% (13/43) patients unexpected drainage was found. Of these 13 patients, four patients showed SLNs located ipsilaterally in level IV as closest located SLN, in two patients this closest location was ipsilaterally in level V. Seven patients had only SLNs located contralateral from the side of the well-lateralized local recurrence or second primary tumour (Supplementary data 1). Besides a lower identification rate, unexpected drainage was more common in patients with a history of neck irradiation compared to patients with a history of a SLNB and comparable to patients with a previous neck dissection, respectively 40% versus 11% and 38%. However the highest unexpected drainage was found after a history of neck dissection combined with postoperative radiotherapy (88%). Localization of harvested SLNs per patient and

per different prior treatment are given in Supplementary data 2. Some SLNs were found in earlier dissected neck levels. For example, eight of the 13 patients with a history of a selective supraomohyoid neck dissection had SLNs located in level I-III, also three of the seven patients with a history of a MRND had SLNs located in level II-IV (Supplementary data 2).

If we restrict the drainage pattern analysis to patients with a history of treatment of the ipsilateral neck, unexpected drainage patterns were found in 12 (35%) of the 34 patients and no drainage to any side of the neck was found in 5 patients (12%).

DISCUSSION

This study demonstrates that SLNB in a previously treated neck can be performed with a high accuracy (sensitivity 75%, NPV 98%). In this study unexpected lymphatic drainage patterns were found in 30% of the patients and no drainage was found in 12% of the patients.

SLNB in early stage OSCC has been frequently described in literature during the last decade with high sensitivity rates and negative predictive values [4]. SLNB was initially implemented in our institutions for patients with primary OSCC without previous treatment of the neck. However, after gaining more experience with SLNB, this staging technique was also extended to patients with a previously treated neck [5]. As a result of the previous treatment, lymphatic drainage patterns could be disrupted resulting in aberrant drainage patterns compared to primary OSCC. Lack of knowledge about these aberrant drainage patterns resulted in missing a standard neck staging and standard elective neck dissection in previously treated patients. Flach et al. showed in a study of 22 patients that the SLNB could be useful in previously treated patients with a high sensitivity and negative predictive value for neck staging and especially for assessment of the individual lymphatic drainage patterns after previous treatment [5].

As mentioned in the introduction, only one feasibility study and the above mentioned study of Flach et al. are published for SLNB in patients with a pretreated neck [5,9]. However, interesting studies in a variety of tumour types have been published regarding SLNB in recurrent or second primary tumours. In a recent meta-analysis of aberrant lymphatic drainage in recurrent breast cancer an 59.6% intraoperatively SLN identification rate was found [10]. The authors concluded that SLNB in these patients avoided unnecessary axillary lymph node dissection and provide targeted localized surgery [10]. Similarly, in recurrent vulvar cancer the SLNB procedure seemed feasible, although the authors stated that the

procedure appears technically more challenging compared to initial surgery. In a cohort of 27 patients, SLNs were found in two groins at unpredicted localizations and four lateral tumours showed bilateral SLNs [19]. Beasley et al. reported about the feasibility of SLNB in recurrent melanoma (107 patients) and also found in 24% of the patients additional sites of SLNs compared to the first SLNB procedure [15].

Although it is difficult to compare different tumour types, a trend towards a lower identification rate of SLNs compared to untreated patients was observed in present and all above mentioned studies. The most common explanation is the damage of lymphatic pathways due to prior treatment and a more difficult technical procedure to harvest SLNs in previously treated nodal basins. In untreated OSCC identification rates of 97-98% have been reported, while in this study a rate of 85% was found [16,17,20,21]. All patients without harvested SLNs had radiotherapy in history, sometimes combined with surgery. This lower identification rate was not observed in patients with a prior SLNB procedure, possibly reflecting that SLNB ensures less damage to lymphatic vessels compared to radiotherapy. Furthermore, despite the lower identification rate in previously treated patients no lower NPV of the SLNB for neck staging was found in this study. This might indicate that lymphatic drainage patterns in these patients are not only aberrant, but may even be absent. Nonetheless, this study included only three patients with positive SLNs and one patient with a regional recurrence after a negative SLNB procedure. Due to the low number of SLN positive patients and regional recurrences, it might be prematurely to conclude that SLNB is a reliable procedure in previously treated patients. This is also reflected in a sensitivity rate with a wide 95% CI. However, the high NPV of 98% with a 95% CI of 88-100% strongly suggest that SLNB is a promising procedure for these pretreated patients, but its reliability needs further investigation.

Although surgery of the lymphatic drainage patterns is part of the SLNB procedure, the procedure is strictly not part of the treatment but belongs to the diagnostic modalities for neck staging. Therefore subanalysis of patients with a history of neck treatment (neck dissection and/or radiotherapy) are presented in the results regarding the accuracy of the SLNB procedure and lymphatic drainage patterns. These figures indicate that in OSCC patients who had undergone more extensive treatment of the neck (i.e. neck dissection and/or radiotherapy) lymphatic drainage follow more frequently an unexpected pattern or was absent (35% vs 30%). Due to the low number of lymph node metastases (2 and 3) the sensitivity of SLNB (50% and 75%) could not sensibly be compared.

Unexpected drainage pathways are generally reported in all tumour types, including our study. These findings strengthen the value of SLNB in assessing the individual lymphatic drainage pattern. In patients who received already prior treatment (e.g. radiotherapy) it

is perhaps even more important to select the actual lymph nodes at risk for metastasis, considering the fact that treatment options are limited due to their prior therapy. In this study an overall unexpected drainage pattern was found in 30% of the patients, which was most frequently found after prior radiotherapy (40%) and especially when this was preceded by a neck dissection (88%). In early stage OSCC patients with an untreated neck unexpected drainage patterns were reported in up to 16% in a large multicenter trial [22].

Even though it is well possible to determine individual drainage patterns with the SLNB, one of the disadvantages is to perform an additional neck dissection during a second surgical procedure in case of a positive SLNB procedure. Although improvements a recent review concluded that still no other modality (e.g., ultrasound, CT, MRI and PET-CT) is accurate enough to detect occult metastasis preoperatively in a clinically negative neck reliably [23]. Moreover, posttreatment effects and the high rate of unexpected drainage in pretreated patients might affect the sensitivity of these modalities in detecting occult metastasis.

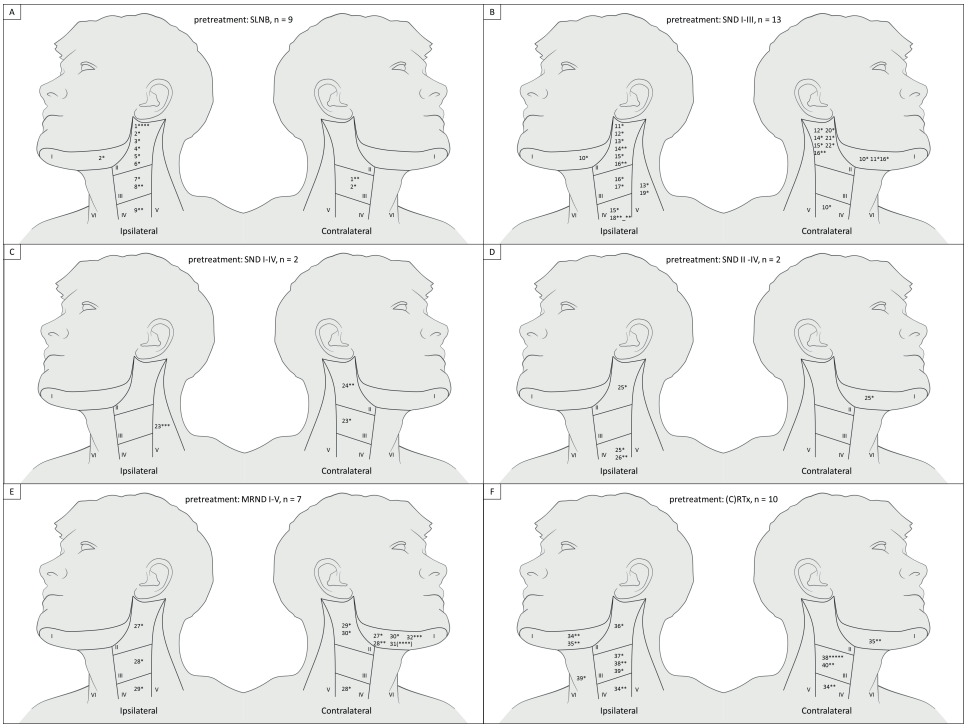
A limitation of the accuracy analysis is the low number of metastasis and regional recurrences in our cohort. A possible explanation for these low numbers compared to untreated patients (with an often reported risk of nodal metastases of approximately 25-30%) could be our close follow-up scheme after treatment of their first tumour. Patients in follow-up are potentially earlier diagnosed with recurrent or second primary OSCC, which might cause a relatively high number of early T1 tumours in this cohort. Despite these limitations, this study showed that metastasis appear in early stage local recurrences and second primary tumours. Currently, no guidelines about neck treatment are available for cT1-2N0 OSCC patients with a previously treated neck. In untreated OSCC prognosis was better after an elective neck dissection (of the standard lymph node levels at risk for metastasis) compared to a 'wait and see' policy [24]. Because of the aberrant drainage patterns, we advocate to use the SLNB also in patients with early stage second primaries or local recurrences to select patients who might benefit from treatment of the neck . However, more extensive research is needed to confirm that this strategy actually improves the prognosis of these patients.

CONCLUSION

SLNB seems to be a reliable procedure for neck staging of cT1-2N0 OSCC patients with a previously treated neck. Moreover, SLNB determines the individual lymphatic drainage patterns, enabling visualization of drainage pattern variability in 30% of these patients.

SUPPLEMENTARY DATA

Supplementary data 1 is available on the following pages.



Supplementary data 2. Locations of the SLNs divided by history of the neck.
_: in total 12 removed LNs in one conglomerate (Figure B, ipsilateral level IV).
(****): four LNs removed without activity on gamma probe (figure E, contralateral level I)
Supplementary data 2 is in a higher resolution available at: https://www.sciencedirect.com/science/article/pii/S1368837519301423?via%3Dihub

Supplementary data 1. Individual patient characteristics with their previous treatment.

Number	Sex	Age	cT (7th)	cN (7th)	pT (7th)	pN (7th)	Tumor location	Tumor side	History ipsilateral
1	Male	67	1	0	1	0	Tongue	Left	SLNB
2	Female	61	1	0	1	0	Floor of mouth	Midline	SLNB
3	Female	71	1	0	1	0	Tongue	Right	SLNB
4	Female	49	1	0	1	0	Tongue	Left	SLNB
5	Female	44	1	0	1	0	Tongue	Right	SLNB
6	Male	55	1	0	1	1	Tongue	Right	SLNB
7	Female	71	1	0	1	0	Floor of mouth	Right	SLNB
8	Female	59	1	0	1	0	Tongue	Left	SLNB
9	Male	73	1	0	1	0	Tongue	Left	SLNB
10	Male	59	1	0	2	0	Tongue	Left	Selective ND I-III
11	Male	50	1	0	1	0	Tongue	Left	Selective ND I-III
12	Female	88	2	0	2	0	Hard palate	Midline	Selective ND I-III
13	Female	49	1	0	1	0	Tongue	Left	Selective ND I-III
14	Female	82	1	0	1	0	Buccal mucosa	Left	Selective ND I-III
15	Male	75	1	0	1	0	Buccal mucosa	Left	Selective ND I-III
16	Female	76	1	0	2	0	Inferior alveolar process	Left	Selective ND I-III
17	Male	74	1	0	1	0	Tongue	Left	Selective ND I-III
18	Male	71	1	0	1	0	Tongue	Right	Selective ND I-III
19	Female	51	1	0	1	0	Buccal mucosa	Right	Selective ND I-III
20	Male	78	1	0	2	0	Tongue	Right	Selective ND I-III
21	Male	72	1	0	2	0	Floor of mouth	Left	Selective ND I-III + RT
22	Female	63	2	0	1	0	Tongue	Right	Selective ND I-III + RT
23	Female	51	2	0	2	0	Tongue	Left	Selective ND I-IV
24	Female	68	1	0	1	0	Tongue	Left	Selective ND I-IV
25	Male	51	1	0	1	0	Tongue	Left	Selective ND II-IV
26	Male	57	1	0	1	0	Floor of mouth	Right	Selective ND II-IV
27	Female	73	2	0	1	0	Inferior alveolar process	Left	MRND
28	Male	60	1	0	1	0	Tongue	Left	MRND + RT
29	Female	59	1	0	1	0	Tongue	Right	MRND + RT
30	Male	68	1	0	1	0	Tongue	Right	MRND + RT
31	Female	67	2	0	2	0	Buccal mucosa	Right	MRND + CRT
32	Male	71	2	0	1	0	Tongue	Right	MRND + RT
33	Male	66	1	0	1	0	Tongue	Left	MRND + RT
34	Male	58	1	0	1	0	Floor of mouth	Left	RT alone
35	Female	69	1	0	1	0	Floor of mouth	Right	RT alone
36	Female	60	1	0	1	0	Tongue	Left	RT alone
37	Male	74	1	0	1	0	Tongue	Right	RT alone
38	Female	62	1	0	1	0	Uvula	Left	RT alone
39	Male	80	1	0	1	1	Tongue	Right	CRT
40	Female	60	1	0	1	0	Tongue	Right	RT alone
41	Male	69	1	0	1	0	Floor of mouth	Paramedian	RT alone
42	Male	58	1	0	1	0	Buccal mucosa	Right	RT alone
43	Male	57	1	0	1	0	Floor of mouth	Right	CRT
44	Male	76	1	0	1	0	Tongue	Right	No treatment
45	Male	61	2	0	1	1	Floor of mouth	Left	No treatment
46	Male	70	2	0	2	0	Retromolar trigone	Left	No treatment
47	Male	81	1	0	1	0	Tongue	Right	No treatment
48	Male	49	1	0	1	0	Tongue	Left	No treatment
49	Female	66	1	0	1	0	Tongue	Left	No treatment
50	Male	57	1	0	1	0	Tongue	Left	No treatment
51	Male	52	1	0	1	0	Pharyngeal arch	Right	No treatment
52	Female	60	1	0	1	0	Inferior alveolar process	Left	No treatment
53	Female	71	1	0	2	0	Inferior alveolar process	Right	No treatment

Abbreviations: 7th, 7th TNM classification; RT, radiotherapy; CRT, chemoradiotherapy; ND, neck dissection; MRND, modified radical neck dissection; SLNB, sentinel lymph node biopsy; FoM, floor of mouth; MFH, Malignant Fibrous Histiocytoma; CIS, Carcinoma in situ.

History contralateral	Lymphatic drainage patterns	SLNB postitive	Head neck oncology history
No treatment	Ipsi and contralateral	No	T1N0 Tongue
No treatment	Ipsi and contralateral	No	T1N0 FoM
SLNB	Ipsilateral	No	TT2N0 Tongue
No treatment	Ipsilateral	No	T1N0 FoM
No treatment	Ipsilateral	No	T1N0 Tongue
MRND	Ipsilateral	Yes, micro	T1N1 FoM
SLNB	Ipsilateral	No	T1N0 FoM
SLNB	Ipsilateral	No	T2N1 Tongue
No treatment	Ipsilateral	No	T1N0 Tongue
No treatment	Ipsi and contralateral	No	(1) T1N0 Buccal mucosa, (2) T1N0 FoM
No treatment	Ipsi and contralateral	No	T1N0 Tongue
No treatment	Ipsi and contralateral	No	T4N0 Superior alveolar process
No treatment	Ipsilateral	No	T1N2b Tongue
No treatment	Ipsi and contralateral	No	(1) T2N0 Inferior alveolar process, (2) T1Nx Buccal mucosa
No treatment	Ipsi and contralateral	No	T2N0 Buccal mucosa
No treatment	Ipsi and contralateral	No	T1N0 Inferior alveolar process
No treatment	Ipsilateral	No	T1N0 Tongue
No treatment	Ipsi and contralateral	No	T1N0 Tongue
Selective ND I-III	Ipsilateral	No	(1) T4aN0 Buccal mocusa, (2) T1N0 Tongue
No treatment	Contralateral	No	T1N0 Tongue
No treatment	Contralateral	No	(1) Retromolar trigone, (2) T2N0 Buccal mucosa
No treatment	Contralateral	No	T2N1 Tongue
No treatment	Ipsi and contralateral	No	T2N1 Tongue
No treatment	Contralateral	No	T1N2b Tongue
Selective II-IV	Ipsi and contralateral	No	(1) MFH grade 2, (2) T2N2b Hypopharynx
MRND	Ipsilateral	No	T2N0 Tonsil
No treatment	Ipsi and contralateral	No	Tongue
No treatment	Ipsi and contralateral	No	T2N0 Tongue
RT alone	Ipsi and contralateral	No	T2N2b Tongue
No treatment	Contralateral	No	T2N2b Hypopharynx
CRT	Contralateral	No	(1) T1N0 Buccal mucosa, (2) T2N0 Inferior alveolar process
Selective ND I-IV + RT	Contralateral	No	Larynx
Selective ND I-IV + RT	No	NA	T4N1 Larynx
RT alone	Ipsi and contralateral	No	T1N1 Supraglottic larynx
RT alone	Ipsi and contralateral	No	T2N0 Supraglotic larynx
RT alone	Ipsilateral	No	T2N0 Glottic larynx
RT alone	Ipsilateral	No	T4N0 Larynx
RT alone	Ipsi and contralateral	No	T3N0 Uvula
No treatment	Ipsilateral	Yes, itc	(1) T4N1 Tongue, (2) T1Nx Tongue
No treatment	Contralateral	No	T1N0 Oropharynx
RT alone	No	NA	T2N1 Tongue
No treatment	No	NA	Myxofibrosarcoma maxillary sinus
CRT	No	NA	(1) T3N0 Supraglottic larynx, (2)T1N0 FoM
MRND	Ipsilateral	No	T1N0 Tongue
RT alone	Ipsi and contralateral	Yes, macro	T4aN1 Larynx
RT alone	Ipsilateral	No	(1) T2N0 Soft palate, (2) CIS FoM
SLNB	Ipsilateral	No	T2N0 Tongue
SLNB	Ipsilateral	No	T1N0 Tongue
MRND + RT	No	NA	T2N2b Tongue
MRND + RT	No	NA	T2N0 Tongue
MRND + RT	Ipsilateral	No	T2N3 Tonsil
MRND	Contralateral	No	T1N1 Inferior alveolar process
Selective ND I-III + RT	No	NA	T2N0 Tongue

Supplementary data 1 is a higher resolution available at: https://www.sciencedirect.com/science/article/pii/S1368837519301423?via%3Dihub

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CHAPTER 5

Lymphatic drainage patterns of oral maxillary tumors: Approachable locations of sentinel lymph nodes mainly at the cervical neck level

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ABSTRACT

Background: There is debate if the lymphatic drainage pattern of oral maxillary cancer is to the retropharyngeal lymph nodes or to the cervical lymph nodes. Insight in drainage patterns is important for the indication for neck treatment. The purpose of this study was to identify the lymphatic drainage pattern of oral maxillary cancer via preoperative lymphoscintigraphy.

Methods: Eleven consecutive patients with oral maxillary cancer treated in our center between December 01, 2012 and April 22, 2016 were studied. Sentinel lymph nodes identified by preoperative lymphoscintigraphy after injection of ^{99m}Tc-nanocolloid and by intraoperative detection using a γ-probe, were surgically removed and histopathologically examined.

Results: In 10 patients, sentinel lymph nodes were detected and harvested at cervical levels I, II or III in the neck. In 2 patients a parapharyngeal sentinel lymph node was detected. One of the harvested sentinel lymph nodes (1/19) was tumor positive.

Conclusion: This study suggests the likelihood of 73% of exclusively cervical level I-III SLNs in oral maxillary cancer.

INTRODUCTION

Carcinomas of the oral cavity metastasize predominantly by the route of the lymphatic vessels to the lymph nodes in the neck. Removal of the lymph nodes by neck dissection is an important part of the treatment of oral cavity cancer and improves disease outcome [1]. The risk of having metastasis from oral squamous cell carcinoma (OSCC) is influenced by tumor characteristics such as localization, infiltration depth and stage [2,3].

Each region of the oral cavity has a specific lymphatic drainage pattern that is used by surgeons to plan the patient specific treatment [4]. Oral maxillary cancer is relatively rare compared with other anatomic subsites of oral cavity cancer (e.g. tongue and floor of mouth) and is rarely included in studies on oral lymphatic drainage patterns [4-6]. Lack of evidence on the lymphatic drainage patterns of oral maxillary cancer has led to a variety of guidelines on the treatment of the clinical negative (cN0) neck; watchful waiting, elective neck dissection (END) or radiotherapy. ENDs are recommended if the probability of a lymph node metastasis exceeds 20% [7]. Traditionally, neck dissections were reported to be uncommon in oral maxillary cancer, which has been based on a low regional failure rate in a few studies with different definitions of oral maxillary cancer from the previous century [8,9]. Another reason for restraining neck dissections were a few reports about parapharyngeal regional failure in a study with oral maxillary and sinus maxillary cancer [10]. Surgical treatment of the parapharyngeal space is technically challenging and mostly radiotherapy is given. More recent studies have investigated maxillary OSCC specifically and found a regional lymph node metastasis rate at least as high as other regions of the oral cavity, especially in the cervical lymph nodes; overall incidence range regional failure 14 -38% [11-18]. Because of these new insights in regional metastases rates, several authors recommend an END in case of cT3-4N0 tumors and also to consider an END in cT1-2N0 maxillary OSCC [11-14,16-19].

Because ENDs are indicated in maxillary OSCC, these patients might possibly benefit from a sentinel lymph node biopsy (SLNB), which is accepted as a minimal invasive alternative for an END in early stage oral cavity cancer [20]. However, as far as we know, no literature is available for the location of the sentinel lymph node in maxillary OSCC. Better insight into the lymphatic drainage pattern, especially in the location of the sentinel lymph node, is needed to make a clear decision in treatment of the cN0 neck in maxillary OSCC [18]. We hypothesized that the sentinel lymph node in patients with maxillary OSCC is located at cervical levels because retrospective studies have shown that late metastasis of maxillary OSCC occur in these cervical levels. The purpose of this study was to identify the lymphatic drainage pattern of oral maxillary cancers via preoperative lymphoscintigraphy which was executed as part of the SLNB procedure.

MATERIAL AND METHODS

Patient selection

All consecutive patients diagnosed with oral maxillary cancer at the Department of Oral & Maxillofacial Surgery of the University Medical Center Groningen between December 01, 2012 and April 22, 2016, were included in the analysis if they had a primary oral tumor located at the hard palate or alveolar bone. Patient characteristics are given in Table 1. Information regarding patient characteristics, clinical and pathological tumor characteristics and diagnostic imaging data was collected retrospectively. All patients were treated according to the Dutch National guidelines for oral cavity cancer [21]. Because SLNB is part of this guideline, no approval was required from the hospital research ethics board according to the Dutch ethical regulations [22].

Table 1. Patient and tumor characteristics

Patient #	Sex	Age at treatment	Tumor morphology	Location Tumor	cTN	pTN
1	Female	75	OSCC	Upper gum right side	T1N0	T2N2b
2	Female	64	OSCC	Upper gum left side	T1N0	T1N0
3	Female	90	OSCC	Upper gum right side	T4aN0	T4aN0
4	Female	73	OSCC	Hard palate right side, close to midline	T4aN0	T4aN0
5	Female	60	OSCC	Upper gum left side, close to midline	T4N2b/c	T4N0
6	Male	77	OSCC	Upper gum left side, close to midline	T4N0	T4N0
7	Female	54	Melanoma	Hard palate right side, close to midline	T4N1	T4N2b
8	Male	70	OSCC	Upper gum left side, close to midline	T4aN0	T4aN2b
9	Female	88	OSCC	Hard palate left side, close to midline	T2N0	T2N0
10	Female	65	OSCC	Upper gum, midline and right side	T1N0	T2N0
11	Male	66	OSCC	Upper gum right side	T4N0	T2Nx*

Abbreviations: cTN; clinical TN classification, pTN; pathological TN classification, OSCC; oral squamous cell carcinoma.

* Patient #11 had no detectable SLNs at the lymphoscintigraphy

Sentinel lymph node procedure

The SLNB procedure was performed as described earlier [23]. Briefly, 1 day before operation ^{99m}Tc-nanocolloid was injected peritumorally at 4 locations; median 100 MBq (range 60-100 MBq). Slow infiltration of the tracer is required to inject successfully in the oral maxilla without leakage. Injection was immediately followed by dynamic lymphoscintigraphy for 20 minutes in anterior or oblique views (20 x 60 s, 128 x 128 matrix) and static images (300 s, 256 x 256 matrix) in 2 directions; anterior and lateral. The static images were repeated after 2 to 4 hours, followed by a Single Photon Emission Computed Tomography (SPECT)-CT scan of the head and neck using a 2-headed gamma camera equipped with parallel-hole ultra-high resolution collimators and a 2-slice CT scanner (32 views of 20 s, 128 x 128 matrix; mAs 30, kV 110, 3.0 mm slice; Siemens, Knoxville, TN). After these images, the position of the sentinel lymph node was marked on the overlying skin by using a ^{57}Cobalt point-source-marker and a γ-probe. The first focus on lymphoscintigraphy, in any direction of the tumor, was considered as the sentinel lymph node. All the lymph nodes with their own lymphatic track directly from the tumor were marked as sentinel lymph node. Neither number nor neck level were restricted for the sentinel lymph nodes. The lymphoscintigraphy images used in this study were all revised by a senior nuclear medicine physician (A.H.B.).

Surgical procedure

Patients were operated within 24 hours after the lymphoscintigraphy with resection of the tumor and staging of the neck by SLNB. In 5 patients, this was combined with a neck dissection. The SLNB side depended on the location and size of the tumor and prior neck treatment. In case of a small tumor, a tumor close to the midline, or a tumor crossing the midline, respectively, an ipsilateral, contralateral, or bilateral SLNB was indicated (Table 2). All lymph nodes with a high signal on the γ-probe at the marked position on the skin were harvested and marked as sentinel lymph nodes. Because of the conglomeration of lymph nodes, it is not always possible to separate 1 lymph node with a high signal during an operation. In that case, more harvested lymph nodes were marked as SLNs at one location. In several patients, a few additional non-radioactive lymph nodes close to the sentinel lymph node were also harvested to ensure sentinel lymph collection. These additional lymph nodes were separated ex vivo from the sentinel lymph nodes by using the γ-probe. Blue dye was not used intra-operatively in our cohort.

Histopathological examination

The histopathological examination of the sentinel lymph node is also described earlier [23]. Briefly, step-serial-sectioning of the entire sentinel lymph node was performed in our center with an interval of 500 µm. All levels were stained with hematoxyline-eosine and for immunohistochemistry with pan-cytokeratin antibody (AE 1/3). The additionally harvested

non-sentinel lymph nodes were investigated by routine histopathological examination (standard H&E staining, without step-serial-sectioning or additional immunohistochemistry), or in some cases using step-serial-sectioning and both stainings. The sentinel lymph node slides were revised by a dedicated head & neck pathologist (B.v.d.V.).

Table 2. Sentinel lymph node biopsy information

Patient #	SLNB side	Ipsilateral neck surgery	Parapharyngeal SLN*	No. of harvested SLNs†	No. of harvested non-SLNs
1	Ipsilateral	SLNB	None	3	1
2	Ipsilateral	SLNB	Yes, ipsilateral	1	0
3	Contralateral	None‡	None	2	2
4	Contralateral	END	None	1	0
5	Contralateral	END	None	1	0
6	Contralateral	END	None	1	1
7	Contralateral	MRND	Yes, contralateral	4	2
8	Contralateral	MRND	None	1	0
9	Bilateral	SLNB	None	2	1
10	Bilateral	SLNB	None	3	0
11§	NA	NA	None	0	0

Abbreviations: SLNB; sentinel lymph node biopsy, END; elective neck dissection, MRND; modified radical neck dissection, SLN; sentinel lymph node, No.; number.
* Parapharyngeal SLN detected with lymphoscintigraphy.
† Number of harvested lymph nodes marked as SLNs
‡ Ipsilateral treated by END and radiotherapy in the past
§ Patient #11 had no detectable SLNs at the lymphoscintigraphy

RESULTS

Patients

In total, 11 patients with oral maxillary cancer were analyzed: 10 patients with primary maxillary OSCC and 1 patient with a primary maxillary mucosal melanoma (Table 1), 3 men and 8 women aged between 54 and 90 years at the time of treatment. Two patients had previous OSCC treated by resection, neck dissection and postoperative radiotherapy at the same side (patient #3) and the other side (patient #9) as the maxillary OSCC tumor. Patient #11 had previous treatment with neck dissection and radiotherapy because of a pN3 OSCC metastasis by unknown primary.

Sentinel lymph node locations

All SLNBs were performed without complications and all cervical located sentinel lymph nodes were intra-operatively identified and removed. In 10 patients, the sentinel lymph nodes were visible on lymphoscintigraphy at cervical level I, II or III. In Figure 1, the locations are given of these cervical sentinel lymph nodes on lymphoscintigraphy at the side planned for SLNB. Only in 2 patients a parapharyngeal sentinel lymph nodes was visible in addition to other sentinel lymph nodes at cervical level II (Figure 2). In patient #2, the sentinel lymph node was located retromaxillary at the ipsilateral side, and in patient #7 the sentinel lymph node was located retropharyngeally at the contralateral side. In the other 9 patients no parapharyngeal sentinel lymph node was detected (Table 2). During surgery, 19 lymph nodes with a high radioactive signal on the γ-probe were harvested at the marked positions on the skin (Table 2). In patients 1, 3, 6, 7 and 9, additional non-radioactive harvested lymph nodes were separated from the SLNs using the γ-probe. The SLNB was performed bilaterally in 2 patients. Two patients had a SLNB at the ipsilateral side and in 6 patients, the SLNB was performed at the contralateral side in the neck compared to the tumor side (Table 2). In patient #11, no sentinel lymph node was visible at the lymphoscintigraphy after injection of 100 MBq ^{99m}Tc-nanocolloid. The tumors ranged in pathological T classification from T1 to T4.

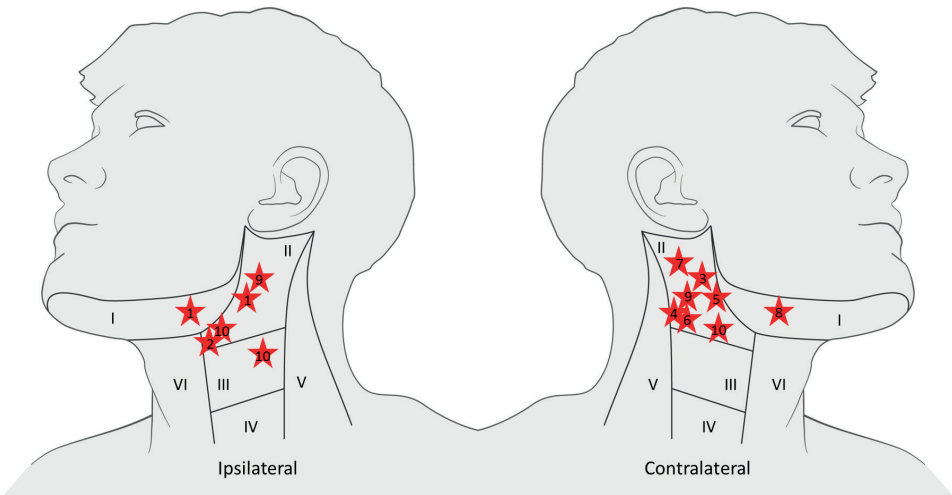

Figure 1. Positions of sentinel lymph nodes (SLNs) on the lymphoscintigraphy separated for the ipsilateral and contralateral sides compared to the tumor. Numbers in the red stars correspond with the patients in the tables and each star is one hot spot on the lymphoscintigraphy. More contralateral SLNs were imaged because only the SLNs on the side that was planned for the sentinel lymph node biopsy are shown (see also Table 2). Patient #11 had no detectable SLNs at the lymphoscintigraphy.

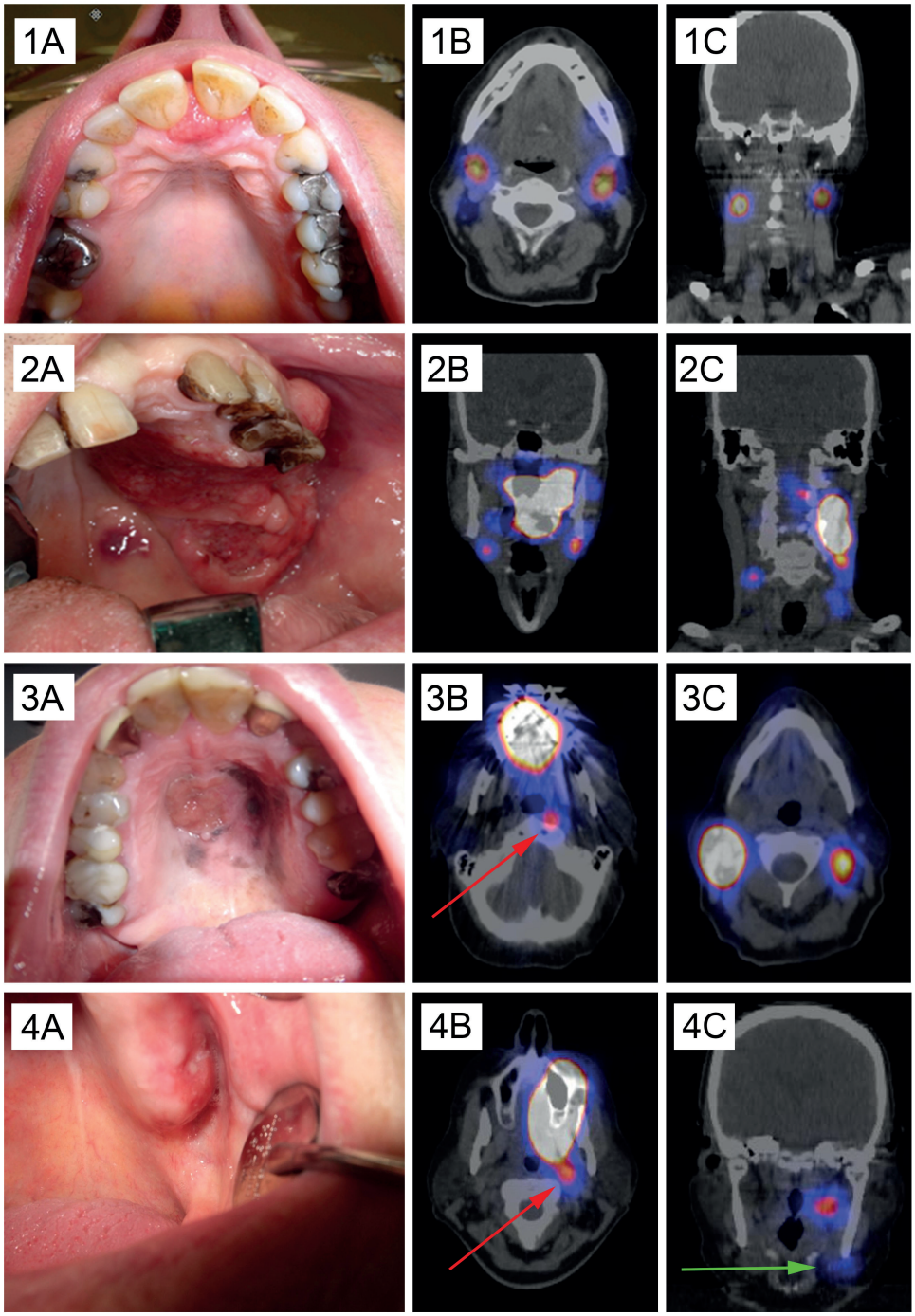
1A
1B
1C
2A
2B
2C
3A
3B
3C
4A
4B
4C

Figure 2. Four examples of lymphatic drainage patterns in oral maxillary cancer. To illustrate our cohort, 4 of the 8 included patients with oral maxillary cancer are shown. (1A–1C) Patient #10 with a small tumor in the midline was planned for a bilateral sentinel lymph node biopsy (SLNB). On both the transversal (B) and coronal (C) slides of the single photon emission CT (SPECT)-CT scan, are the sentinel lymph nodes (SLNs) visible in the cervical levels. (2A–2C) Patient #8, planned for a contralateral SLNB, with a tumor close to the midline (big radioactive spot on the coronal slides B and C) and bilateral SLNs in the cervical neck levels at the SPECT-CT scan. (3A–3C) Patient #7 with a melanoma in the hard palate close to the midline and planned for a contralateral SLNB. This is the patient in whom a retropharyngeal SLN was visible on the SPECT-CT scan (3B, red arrow), besides the SLNs at the cervical level (3C). (4A–4C) Patient #2 with an upper gum tumor on the left side. This is the patient with a retromaxillary SLN (4B, red arrow), besides a cervical SLN at level II (4C, green arrow; hardly visible at this slide).

In patient #1, 1 of the 3 resected sentinel lymph nodes at level II was tumor positive by histopathological examination with a metastasis size of 6.6 mm in diameter and without extranodal growth. All additional harvested lymph nodes were negative for regional metastasis.

DISCUSSION

Because of ongoing debate on the route of lymphatic drainage of maxillary OSCC, we studied the lymphatic drainage pattern of oral maxillary cancer via preoperative lymphoscintigraphy. Insights in lymphatic drainage patterns of oral maxillary cancer are important to determine if an END is adequate treatment of the neck in maxillary OSCC. In 10 patients, sentinel lymph nodes were detected by lymphoscintygraphy at cervical level and these sentinel lymph nodes could be harvested during surgery on the ipsilateral or contralateral side at cervical levels I, II or III. Eight patients (73%) had exclusively cervical located sentinel lymph nodes and only in 2 patients (18%) parapharyngeal sentinel lymph nodes were detected on lymphoscintigraphy, in combination with cervical sentinel lymph nodes. Therefore, the common location of the sentinel lymph nodes of oral maxillary cancer at the cervical neck levels, indicates the potential use of the SLNB procedure in maxillary OSCC.

Because of the retrospective study design, lymphoscintigraphy information from both neck sides was not available for all patients. When a (elective) neck dissection was indicated at the ipsilateral side compared to the tumor, only the contralateral lymphoscintigraphy information was available. Three patients (numbers 3, 9 and 11) were not excluded despite of previous neck treatment by neck dissection. A prospective study design with lymphoscintigraphy of both neck sides and without previously treated patients would be

recommended to study the accuracy of the SLNB in oral maxillary cancer. However, in this study they were not excluded because the still highly valuable information for clinicians; still existing drainage patterns to sentinel lymph nodes at cervical level in previously treated patients. Patient #11 had no visible sentinel lymph nodes at the lymphoscintigraphy more than 20 years after previous treatment with neck dissection and radiotherapy. Absence of detectable sentinel lymph nodes could be explained by the previous treatment of the neck, however other studies shows also a few patients without detectable sentinel lymph nodes, even when they had no previous neck treatment [20,24].

For a long time, a low metastasis rate of oral maxillary cancer has been the general view, probably driven by earlier results of low regional failure in oral maxillary cancer combined with a possible parapharyngeal lymphatic drainage pattern, there was no indication for an END [10]. However, more recent studies reported the cervical metastasis rate for maxillary OSCC is at least as high as OSCC originating from other anatomic regions. Also, in case of watchful waiting, a lower survival rate was reported when patients developed late metastasis [11,14,16-19]. The higher metastasis rate and the shorter survival of maxillary OSCC with regional metastasis have indicated the need for ENDs in maxillary OSCC, especially in pT3-4 but also in pT1-2 tumors [11-17]. Although ENDs are effective in preventing regional recurrences at the cervical level, it still leads to 70% overtreatment and possible morbidity (e.g. reduced shoulder movement, pain or lymph edema) [7,25,26]. To reduce overtreatment of the neck and to lower the complication rate, SLNB has been introduced in OSCC [23]. Recently, Den Toom et al. have shown that SLNB in OSCC adequately selects patients with cT1-T2N0 OSCC for additional neck dissection or follow-up (sensitivity of 93% and a negative predictive value of 97%). In that study, the sentinel lymph node identification rate by preoperative lymphoscintigraphy was 98% [20]. However, patients with maxillary OSCC were not included in that study.

In this single-center retrospective study only 11 patients, with different tumor pathology and tumor status, could be analyzed because of the relative rarity of this tumor location. Based on our results it seems that oral tumors located at the maxilla also preferentially and frequently drain to the cervical neck levels and not so much parapharyngeally. We hypothesize that when the SLNB procedure shows a sentinel lymph node either in the neck or parapharyngeally, a personalized strategy should be applied. If accessible, the sentinel lymph node could be surgically removed and if the sentinel lymph node is located parapharyngeally, radiotherapy could be considered, depending on the risk of metastasis. The results of this small heterogeneous cohort support the need for further studies to assess lymphatic drainage routes and the possible diagnostic value of the SLNB in maxillary OSCC.

CONCLUSION

This study suggests the likelihood of 73% of exclusively cervical level I to III located SLNs in oral maxillary cancer, with up to 18% of cases with a combination of parapharyngeal and cervical located SLNs. Therefore, it seems feasible to perform a SLNB in case of maxillary OSCC. However, this study only describes the lymphatic drainage patterns and not the SLNB accuracy in oral maxillary cancer.

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CHAPTER 6

Amplification and protein overexpression of cyclin D1: Predictor of occult nodal metastasis in early oral cancer

Cyclin D1 in early oral cancer

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ABSTRACT

Background: Accurate nodal staging is pivotal for treatment planning in early (Stage I-II) oral cancer. Unfortunately, current imaging modalities lack sensitivity to detect occult nodal metastases. Chromosomal region 11q13, including genes *CCND1*, Fas-associated death domain (*FADD)* and *CTTN*, is often amplified in oral cancer with nodal metastases. However, evidence in predicting occult nodal metastases is limited.

Methods: In 158 patients with early tongue and floor of mouth (FOM) squamous cell carcinomas both *CCND1* amplification and cyclin D1, FADD and cortactin protein expression were correlated with occult nodal metastases.

Results: *CCND1* amplification and cyclin D1 expression correlated with occult nodal metastases. Cyclin D1 expression was validated in an independent multicenter cohort, confirming the correlation with occult nodal metastases in early FOM cancers.

Conclusion: Cyclin D1 is a predictive biomarker for occult nodal metastases in early FOM cancers. Prospective research on biopsy material should confirm these results before implementing its use in routine clinical practice.

INTRODUCTION

Oral cavity squamous cell carcinomas (SCCs) have the tendency to metastasize to regional lymph nodes in the neck. Determination of the nodal status at the time of diagnosis of the primary tumor is crucial for both prognosis and treatment planning. Even optimal imaging with magnetic resonance imaging (MRI), computed tomography (CT), Positron emission tomography–computed tomography (PET-CT) or ultrasound eventually combined with fine needle aspiration cytology (FNAC) has insufficient sensitivity to detect metastatic disease in the neck [1]. This results in a 30% to 40% occult (i.e. clinically and by imaging undetectable) lymph node metastases in early (stage I-II) oral cavity SCC [2]. If the probability of occult cervical metastasis exceeds 20%, literature recommends a selective neck dissection over watchful waiting supported with ultrasound [3,4]. Some clinicians even prefer to decrease this risk below 10%. However, this policy leads to overtreatment of 60% to 70% of the cN0 patients, who are exposed to the potential morbidity of general anesthesia and surgery of the neck such as shoulder dysfunction, paralysis of the lower lip, lymph edema or an altered neck contour [2,5]. There is a need for better diagnostics that are more effective in predicting lymph node metastasis.

Two upcoming diagnostic modalities with promising results that overcome this clinical problem are the sentinel node biopsy (SNB) and tumor profiling with biomarkers [1,2]. Although SNB is also an intervention under general anesthesia, it is minor surgery with a lower complication rate as compared to a selective neck dissection [6]. The advantage of tumor profiling on preoperative biopsies over the use of SNB is its non-invasive nature. In 2005, the first gene expression profile to predict nodal metastasis was developed and recently validated in a Dutch multicenter study with a negative predictive value (NPV) of 89% (95% confidence interval 74% to 96%) [2]. This gene expression profile is expensive and its positive predictive value (PPV) was only 37%, which would still result in a substantial amount of unnecessary neck dissections. Therefore, the gene expression profile is not yet the ideal diagnostic modality that could lower overtreatment of the true cN0 neck in early oral cavity SCC [2,7]. Nevertheless, a combination of both tumor profiling and SNB could further improve the diagnostic accuracy of staging the neck [8].

In head and neck squamous cell carcinoma (HNSCC), amplification of the 11q13.3 chromosome region occurs frequently (36%) [9] and has been correlated with aggressive tumor growth, lymph node metastasis, decreased locoregional control and overall survival (OS) [9-12]. In a recent study investigating gene copy number aberrations of 36 common oncogenes and tumor suppressor genes, we identified gain of region 11q13, containing oncogenes *CCND1, CTTN, FGF4* and Fass-associated death domain (*FADD*), as a potential predictor for nodal metastasis in early oral cavity SCC, with a NPV of 81% and

positive predictive value of 46% [13]. In HNSCC, the commonly amplified region contains 9 genes that are overexpressed when amplified including *FADD, CCND1, TPCN2, PPFIA1, FLJ42258, CTTN1, FGF19, ORAOV1* and *ANO1* [11]. At least 3 of these oncogenes on this region (*CCND1*, *CTTN* and *FADD)* play key roles in cellular migration of epithelial cells and are, therefore, potential biomarkers for metastases in oral cancer [9,12,14,15]. Furthermore, immunohistochemical (IHC) expression of cyclin D1, FADD and cortactin have been described as potential predictors for increased disease-related mortality, for lymph node metastasis and poor prognosis in oropharyngeal carcinomas [10-12]. Until now, only 1 study investigated *CCND1* amplification and expression in early oral cavity SCC, in a relatively small cohort of 45 patients [16].

To validate the value of *CCND1* as a predictive biomarker for the detection of occult nodal metastasis, we correlated gene amplification of *CCND1* and protein overexpression of 3 major oncogenes (cyclin D1, FADD and cortactin) with nodal status in a large consecutive and well-documented cohort of early oral cavity SCC. Furthermore, intra-tumor heterogeneity of protein expression of these biomarkers was analyzed to see if a biopsy could represent the whole tumor for these potential biomarkers. The correlation between expression of cyclin D1 and lymph node metastasis was subsequently validated in an independent multicenter cohort of oral cavity SCC.

MATERIALS AND METHODS

Cohort

We enrolled a consecutive cohort of 158 patients with cT1-2 cN0 tongue and floor of mouth (FOM) cancers, primarily treated by surgery between January 2004 and December 2010 at the University Medical Center Utrecht as described earlier [13,17]. All cases were clinically lymph node negative, based on extensive imaging with both CT or MRI and ultrasound with FNAC, in case of a suspicious lymph node. Patients with a medical history of HNSCC or a synchronous primary tumor were excluded from this study. Demographic, clinical, histologic and treatment data were retrieved from electronic medical records (see Table 1).

For validation, 2 independent cohorts of early tongue and FOM, SCCs primarily treated by surgery at the University Medical Center Utrecht (1996-2003, n = 73) and the University Medical Center Groningen (1997-2008, n = 82) were used [18,19]. For both validation cohorts, tissue microarrays (TMAs) were available.

Table 1. Baseline characteristics of initial and validation cohorts

	Initial (158 tumors)	Validation (155 tumors)
Center		
UMC Utrecht	158 (100%)	73 (47%)
UMC Groningen	0 (0%)	82 (53%)
Age (mean, range in years)	62, 23-90	62, 25-94
Sex		
male	97 (61%)	87 (56%)
female	61 (39%)	68 (44%)
Smoking		
no	75 (47%)	NA
yes	83 (53%)	
Alcohol		
no	76 (48%)	NA
yes	82 (52%)	
Location		
FOM	65 (41%)	68 (44%)
tongue	93 (59%)	87 (56%)
Clinical T-classification		
T1	77 (49%)	51 (33%)
T2	81 (51%)	104 (67%)
Treatment		
Surgery	122 (77%)	89 (57%)
Surgery + PO(Ch)RT	36 (23%)	66 (43%)
Neck dissection		
No*	41 (26%)	0 (0%)
yes	117 (74%)	155 (100%)
Infiltration depth		
0-4 mm	54 (34%)	NA
>4 mm	104 (66%)	
Perineural growth		
no	115 (73%)	NA
yes	43 (27%)	
Vascular invasive growth		
no	144 (91%)	NA
yes	14 (9%)	

Table 1. Continued

	Initial (158 tumors)	Validation (155 tumors)
Tumor front		
cohesive	55 (35%)	NA
non-cohesive	102 (65%)	
missing	1 (1%)	
Extracapsular spread		
no	156 (99%)	NA
yes	2 (1%)	

* histological status of patients without neck dissection was based on follow-up of at least 2 years.
Abbreviations: FOM, floor of mouth; NA, data not available; PO(Ch)RT, postoperative (chemo)radiotherapy.

Tissue microarray

From 158 tumors, sufficient formaldehyde-fixed paraffin-embedded tissue was available for incorporation in a TMA. From each tumor block, 3 tissue cylinders with a diameter of 0.6 mm were punched out, avoiding areas of necrosis, and arrayed in a recipient paraffin block. The TMAs contain normal tonsillar epithelium as control tissue to ensure similarity of staining quality and intensity between the different blocks.

Fluorescence in-situ hybridization

Fluorescence in situ hybridization (FISH) was performed on fresh sectioned, 4 micrometer thick paraffin TMA sections. Slides were deparaffinized and pretreated with sodium citrate and protease buffers. Afterward, the slides were dehydrated and hybridized with 15 µL Vysis CCND1 / CEP11 FISH probe (Abbott Molecular Diagnostics, The Netherlands) in a ThermoBrite (Abbott Laboratories, Chicago, IL) at 37°C overnight. The next day, they were washed in saline-sodium citrate buffers, counterstained with diamidino-phenylindole, dehydrated and mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). One-hundred tumor cell nuclei per tumor were analyzed for the *CCND1* gene and CEP11 probe signals at X100 magnification on a Leica DM5500 B microscope system using Application Suite Advanced Fluorescence software (Leica Microsystems, Rijswijk, The Netherlands). The CCND1/CEP11 ratio was calculated to correct for centromere signals. A ratio >1.25 until 2.00 was defined as low-level and a ratio ≥2.00 as high-level amplification.

Immunohistochemistry

IHC staining for cortactin and FADD was performed manually. For cyclin D1, the Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, AZ) automatically staining procedure

was used. In short, 4 µm thick paraffin sections were deparaffinized with xylene and rehydrated. Endogenous peroxidase activity was blocked using a 0.3% hydrogen peroxide phosphate-citrate buffer for 15 minutes. Next, the slides were washed in water and subsequently subjected to antigen retrieval by boiling in EDTA buffer, pH 9.0 (cyclin D1 and FADD) or citrate buffer, pH 6.0 (cortactin) for 20 minutes. After cooling down and washing with phosphate buffered saline (PBS) for 5 minutes, tissue slides were incubated with the primary antibody cyclin D1 (clone SP4, USA; dilution 1:100; Cellmarque, Rocklin, CA), primary antibody FADD (556402, dilution 1:100; BD Pharmingen™, San Jose, CA, USA) or primary antibody Cortactin (610049, dilution 1:200; BD Transduction Laboratories™, San Jose, CA, USA) for 60 minutes. After washing with PBS (3 times), the slides were incubated with poly-horseradish peroxidase goat goat anti-mouse/rabbit/rat (Bright Vision, Imunologic, Duiven, The Netherlands, ready to use) for 30 minutes followed by washing with PBS (3 times). Slides were then developed with diaminobenzidine for 10 minutes and hematoxylin was used for counterstaining. Oral cancer with known amplification of the 11q13 has been used as positive control (with antibody) and as a negative test (without antibody) control in each test.

IHC staining of tumor cells was scored by a dedicated head and neck pathologist (S.M.W.). A core was considered inadequate/lost when the core contained <5% tumor tissue or when more than >95% of the core contained no tissue. For cyclin D1, the percentage of nuclear staining and for both FADD and cortactin intensity of cytoplasmatic staining (0, none; 1, weak; 2, moderate; 3, strong) was scored semi-quantitative. During validation as biomarker, Cyclin D1 expression was also scored by an independent head and neck cancer researcher (K.B.) to assess interobserver agreement.

Statistical Analysis

To investigate the consistency of IHC staining of cyclin D1, FADD and cortactin within the tumor, we analyzed the intraclass correlation coefficient (ICC) among the 3 scored cores. The ICC is a descriptive statistic that describes how strongly different quantitative measures resemble each other, in this case multiple cores of the same tumor. An ICC <0 reflects 'poor' , 0 to 0.20 reflects 'slight', 0.21 to 0.4 reflects 'fair', 0.41 to 0.60 reflects 'moderate', 0.61 to 0.8 reflects 'substantial', and above 0.81 reflects 'almost perfect' reliability of the measurement. Any measurement should have an ICC of at least 0.6 to be useful with regard to reliability of the result [20]. Correlation between *CCND1* copy number results and nuclear cyclin D1 expression was analyzed using the Kruskal-Wallis test. For correlation with occult nodal metastasis, protein expression results were dichotomized. For cyclin D1 protein expression, ROC-curve analysis was used to determine cut-off levels for prediction of occult nodal metastasis. For both *CCND1* gene amplification and protein expression the Pearson chi-

square test (or Fisher's exact when appropriate) was used. Binary logistic regression analysis was used to evaluate the value of multiple variables in predicting occult nodal metastases. For interobserver agreement of cyclin D1 expression during the validation phase, the ICC between both observers (S.M.W. and K.B.) was analyzed. If not mentioned otherwise, a 2-sided p-value < 0.05 was considered significant. All statistical analyses were performed using SPSS 21.0 Statistical Software (IBM, New York, USA).

Ethical justification

Because the remaining tissue after the clinical diagnostic process was used, no ethical approval was required according to Dutch National Ethical Guidelines (www.federa.org). Anonymous or coded use of leftover tissue for scientific purposes is part of the standard treatment agreement with patients in our center [21].

RESULTS

Descriptive Analysis

Table 2 shows descriptive IHC and FISH results. For cyclin D1, FADD and cortactin, protein expression could be scored in at least 1 core in 96%, 97% and 96%, respectively, of the tumors. In case of multiple scored cores, mean nuclear staining (%) for cyclin D1 and maximum cytoplasmic intensity for FADD and cortactin was used as overall protein expression score. Examples of IHC staining patterns, including staining pattern of normal tonsil epithelium are illustrated in Figure 1. Normal tissue showed weak/moderate staining for FADD and cortactin and some nuclear stained cells for cyclin D1 near the basal layer. For FADD and cortactin, strong positive staining was considered as overexpression. Tumors with a nuclear cyclin D1 in at least 15% of tumor cells are considered as overexpressed cyclin D1, based on ROC curve analysis. Of the scored tumors, 39% showed overexpression of cyclin D1, 19% of FADD and 15% of cortactin. To address the possibility of tumor heterogeneity, the ICC was determined for tumors with three scored cores. This revealed a very good consistency of expression of these three proteins within the tumor; cyclin D1 (0.89), FADD (0.89) and cortactin (0.90). Examples of FISH images of *CCND1* are illustrated in Figure 2. FISH results were available for 88% of the tumors, 19 tumors were excluded because of lack of fluorescence signal or insufficient tumor cells.

Correlation CCND1 copy number and cyclin D1 protein expression

For 139 tumors, both *CCND1* copy number analysis by FISH and cyclin D1 protein expression by IHC were scored. Overall, *CCND1* copy number results are significantly correlated with increased nuclear cyclin D1 expression, see Supplementary Figure 1. This correlation was

mainly significant between normal copy number and high-level amplification, with adjusted p-value < 0.001. Normal copy number versus low-level amplification and low-level versus high-level amplification showed no significant differences in nuclear cyclin D1 expression, with adjusted p-values of 0.277 and 0.051, respectively.

Table 2. Descriptive analysis

		Cyclin D1	FADD	Cortactin
Immunohistochemistry	Cores per tumor (%)			
	0	6 (4%)	4 (2%)	6 (4%)
	1	10 (6%)	8 (5%)	10 (6%)
	2	43 (27%)	25 (16%)	22 (14%)
	3	99 (63%)	121 (77%)	120 (76%)
	Intratumor Heterogeneity ICC (95% CI)	0.89 (0.84-0.92)	0.89 (0.85-0.92)	0.90 (0.86-0.92)
	Expression (%)			
	normal	90 (57%)	124 (79%)	128 (81%)
	overexpression	62 (39%)	30 (19%)	24 (15%)
	missing	6 (4%)	4 (3%)	6 (4%)
FISH	Tumors (%)			
	Normal copy number	97 (62%)		
	low-level amplification	18 (11%)		
	high-level amplification	24 (15%)		
	missing	19 (12%)		

Abbreviations: FISH: fluorescence in situ hybridization, ICC: intraclass correlation coefficient

Biomarker for (occult) nodal metastasis

To address the value of *CCND1* amplification and expression of cyclin D1, FADD and cortactin as potential biomarkers for occult nodal metastasis, we correlated amplification or overexpression with histologically proven nodal metastases. In early oral cancer, the NPV (i.e. true negative outcome in case of negative test result) varied between 79% and 85% among different biomarkers and techniques (see Table 3). Combination of protein expression of the three 11q13 oncogenes (cyclin D1, FADD and cortactin) slightly improved the NPV comparable with the NPV of cyclin D1 expression alone, 85% versus 84%.

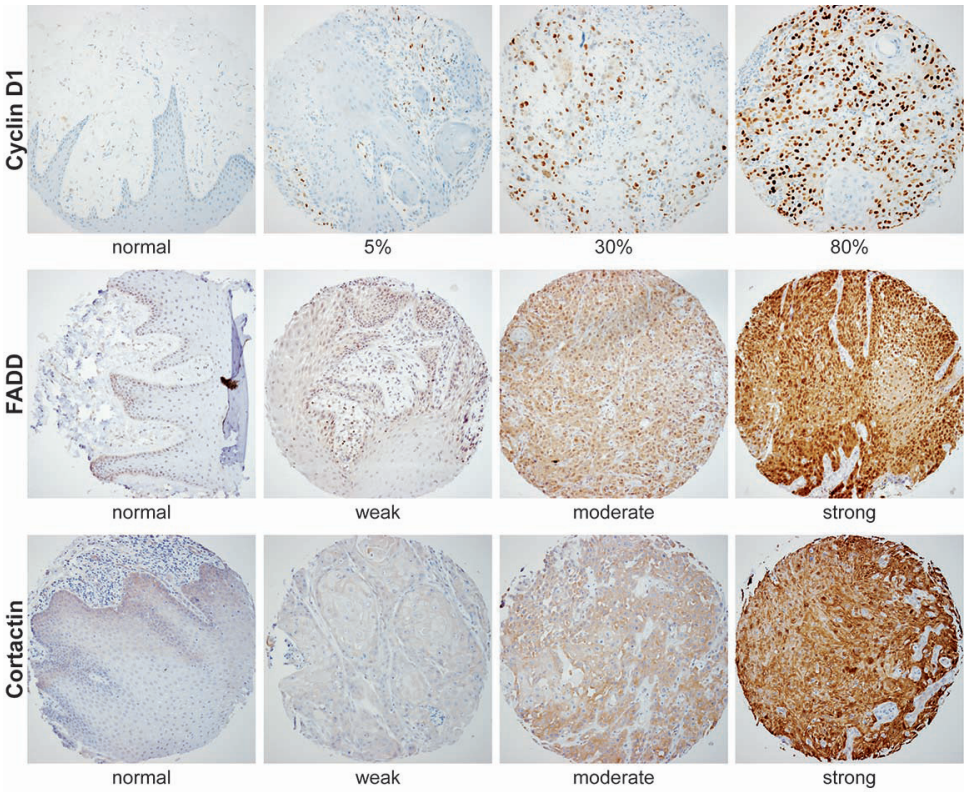


Figure 1. Immunohistochemical staining of cyclin D1 (% of nuclear staining), FADD (intensity of cytoplasmic staining) and cortactin (intensity of cytoplasmic staining) on oral cancer and normal oral mucosa (first column).

Separate analysis per subsite, showed that in early FOM oral cavity SCC the most significant biomarkers (*CCND1* amplification and cyclin D1 overexpression) have a higher NPV of 95% ($p = 0.021$) for cyclin D1 normal expression and 97% ($p = 0.067$) for *CCND1* normal copy number by FISH, compared with a NPV of 76% for both techniques in early tongue oral cavity SCC. Although *CCND1* normal copy number FOM OSCC shows the highest NPV, the correlation is not significant (see Table 4).

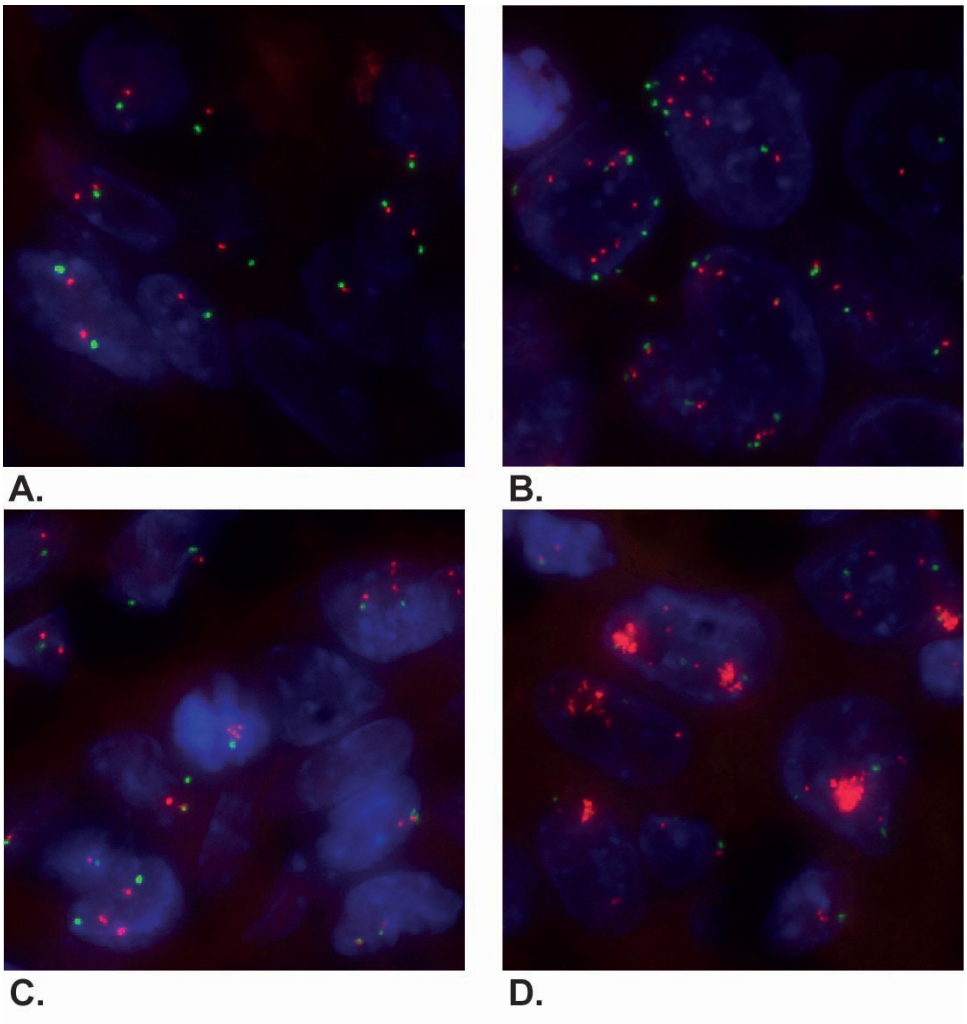


Figure 2. Fluorescence in-situ hybridization of *CCND1* in oral cancer. Signals: DAPI, nucleus; green, centromere chromosome 11; red, *CCND1* gene. A. normal copy number. B. polysomy chromosome 11. C. low-level amplification. D. high-level amplification.

Table 3. Correlation of copy number and protein expression results with occult nodal metastasis

N-classification*	N0	N+	p-value
CCND1 copy number			**0.004**
Normal	**80 (83%)**	17 (17%)	
Low-level amplification	13 (72%)	5 (28%)	
High-level amplification	12 (50%)	12 (50%)	
Cyclin D1			**0.001**
Normal expression	**76 (84%)**	14 (16%)	
Overexpression	38 (61%)	24 (39%)	
FADD			**0.001**
Normal expression	**101 (81%)**	23 (19%)	
Overexpression	16 (53%)	14 (47%)	
Cortactin			**0.008**
Normal expression	**102 (80%)**	26 (20%)	
Overexpression	13 (54%)	11 (46%)	
Cyclin D1 / FADD / Cortactin			**0.001**
All normal expression	**67 (85%)**	12 (15%)	
Mixed expression	38 (72%)	15 (28%)	
All overexpression	7 (41%)	10 (59%)	

*Final N-classification is based on either histological confirmation after neck dissection or follow-up of at least 2 years. In bold: the negative predictive value (NPV) in early OSCC.

Table 4. Correlation of CCND1 by FISH and cyclin D1 by IHC with occult nodal metastasis

	Tongue (cT1-2cN0)			FOM (cT1-2cN0)		
N-classification*	**N0**	**N+**	**p-value**	**N0**	**N+**	**p-value**
CCND1 by FISH			0.004			0.067
Normal	**51 (76%)**	16 (24%)		**29 (97%)**	1 (3%)	
Low of high-level amplification	10 (43%)	13 (57%)		15 (79%)	4 (21%)	
Cyclin D1 by IHC			0.033			**0.021**
Normal expression	**37 (76%)**	12 (24%)		**39 (95%)**	2 (5%)	
Overexpression	21 (54%)	18 (46%)		17 (74%)	6 (26%)	

*Final N-classification is based on either histological confirmation after neck dissection or follow-up of at least 2 years. In bold: the negative predictive value (NPV) in early OSCC. *Abbreviations:* FOM, floor of mouth; FISH, fluorescence in-situ hybridization.

Tumor characteristics and cyclin D1 expression

Cyclin D1 overexpression is correlated with increased infiltration depth (>4 mm, p = 0.001). Cyclin D1 expression was not correlated with unfavorable growth patterns in the primary tumor such as vascular invasive growth, perineural growth, noncohesive growth

or extracapsular spread in the metastasis. A logistic regression model revealed cyclin D1 expression as a most robust predictor for occult nodal metastasis (p = 0.005), together with noncohesive tumor front (p = 0.015) and perineural growth (p = 0.033).

Validation of cyclin D1 on independent cohort

Baseline characteristics of the independent multicenter cohort of 155 early tongue and FOM carcinomas, are given in Table 1. Cyclin D1 expression could be scored in 147 tumors (95%). The interobserver agreement between both observers (S.M.W. and K.B.), blinded for each other scores, had an ICC of 0.94. In the whole cohort cyclin D1 expression was significantly correlated with occult nodal metastasis (p = 0.033). When tumor sites were analyzed separately, cyclin D1 correlated only with occult nodal metastasis in early FOM oral cavity SCC, with a NPV of 79% (p = 0.020; Table 5).

Table 5. Correlation of cyclin D1 by IHC with occult nodal metastasis in validation cohort

	pN0 (90 tumors)	pN+ (47 tumors)	p-value
Whole cohort of OSCC			**0.033**
Normal cyclin D1 expression	**55 (76%)**	17 (24%)	
Cyclin D1 overexpression	45 (60%)	30 (40%)	
Tongue			0.449
Normal cyclin D1 expression	**29 (74%)**	10 (26%)	
Cyclin D1 overexpression	28 (67%)	14 (33%)	
FOM			**0.020**
Normal cyclin D1 expression	**26 (79%)**	7 (21%)	
Cyclin D1 overexpression	17 (51%)	16 (49%)	

Abbreviations: IHC, immunohistochemistry; OSCC, oral squamous cell carcinoma; FOM, floor of mouth; pN, histological N-classification based on elective neck dissection. In bold: the negative predictive value (NPV) in early OSCC.

DISCUSSION

Adequate determination of the nodal status is pivotal for appropriate treatment planning in early oral cavity SCC. Unfortunately, even optimal imaging with CT or MRI, PET-CT and ultrasound with FNAC lacks high sensitivity for the detection of nodal metastasis. As a result, an elective neck dissection or SNB still are the preferred staging techniques of the neck in clinically early oral cavity SCC (cT1-2cN0) [1]. However, this policy leads to an overtreatment of the neck in 60% to 70% of the patients, which urges the need for predictive biomarkers in early oral cancer [2]. In earlier research, copy number gain in region 11q13 was identified as a potential biomarker in early oral cancer with a NPV of 79% [13]. A review with meta-analysis revealed a correlation with nodal metastasis in oral cavity SCC of both amplification

of *CCND1* and overexpression of its encoded protein cyclin D1. However, only one small study was performed in early oral cavity SCC to establish its value in the detection of occult nodal metastasis [14].

In this largest study so far in early oral cavity SCC, both *CCND1* copy number and nuclear cyclin D1 expression are significantly correlated with occult nodal metastasis with a NPV of respectively, 83% and 84% in clinically early oral cancer. These results are in line with a NPV of 83% found by Myo et al,[22] which is the only other study investigating the correlation between *CCND1* amplification and occult nodal metastasis in early oral cancer. Protein expression of both FADD and cortactin had a slightly lower NPV compared to cyclin D1. As expected, combined expression of these 3 proteins did not improve the NPV for occult nodal metastasis significantly, because the genes encoding for these proteins are situated on the same chromosomal region (11q13.3), which is often amplified as a whole in HNSCC [9]. Subsite analysis reveals a higher NPV of *CCND1* amplification and cyclin D1 expression in the FOM compared with tongue tumors, 95%, 97% and 76% respectively. As a consequence, the cyclin D1 biomarker may have a complementary role to the SNB procedure, as this procedure lacks accuracy in FOM tumors (NPV of 88% instead of 98% in other subsites) because of the close relationship between the primary tumor and first draining nodes, known as 'shine-through' phenomenon [23,24]. Multicenter validation in the described Utrecht and Groningen cohorts, including a total of 155 early tongue and FOM tumors, confirmed the predictive value for occult nodal metastasis of cyclin D1 expression in FOM tumors but not in tongue tumors. However, the NPV was lower than in the initial cohort (79% versus 95%). This might be explained by the composition of the validation cohorts; only patients with an elective neck dissection were included in these cohorts, which lead to selection bias.

For the clinical application of a biomarker predicting occult nodal metastasis, it is pivotal that a biopsy represents the whole tumor, i.e. expression of a biomarker is consistent in the biopsy as well as the resection specimen. Because the phenomenon of intratumor heterogeneity is common in head and neck cancer, consistency of biomarkers must be checked [25]. A well-known method to analyze intratumor heterogeneity is by establishing the ICC among multiple samples, in this study multiple cores, of the same tumor [26]. IHC expression of all 3 studied proteins (cyclin D1, FADD and cortactin) showed high concordance with an ICC between 0.89 and 0.90, which indicates almost perfect agreement between the cores [20]. Therefore, IHC expression of these proteins in a biopsy is representative for expression in the whole tumor in early oral cavity SCC. Furthermore, the high interobserver agreement of cyclin D1 expression (ICC = 0.94) showed the high reproducibility of this biomarker.

Cyclin D1 expression did not correlate with unfavorable growth patterns, which is in line with other studies, although some studies found a correlation with differentiation grade in oral cancer [27,28]. However, it must be realized, that the benefit of the biomarker lies in its preoperative application on the incisional biopsy: to decide whether or not to perform a neck dissection at the same time when resecting the primary tumor. Reliable acquisition of the histological tumor characteristics can only take place after the ablative surgery [29].

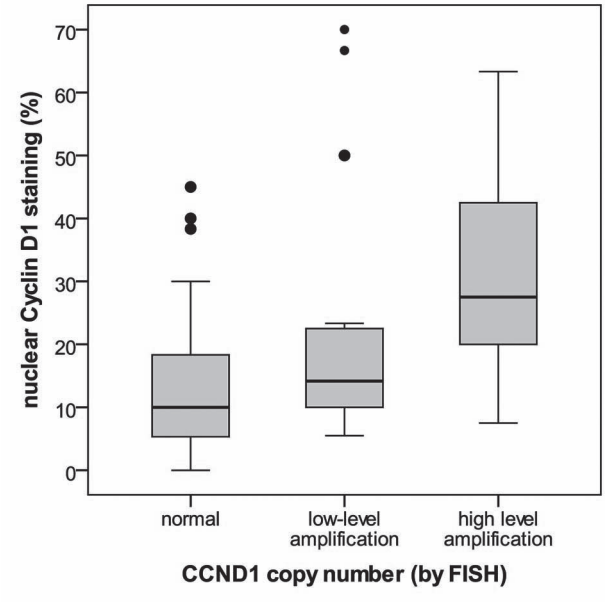
CCND1 high-level amplification was significantly correlated with higher nuclear cyclin D1, although not all amplified tumors showed high cyclin D1 expression and some tumors showed high nuclear cyclin D1 staining without amplified *CCND1*. These inconsistencies between genomic alterations and protein expression levels are in line with earlier reports in breast and head and neck cancer and could be explained by regulation of transcription, translation and protein stability [30,31].

This study has been performed in a clinically relevant large consecutive cohort of early oral cavity SCC. However, some limitations have to be mentioned. First, both IHC and FISH analysis have been performed on resection specimens. Although this allowed us to investigate intratumoral heterogeneity, which is essential for potential biomarkers, it is relevant to validate these findings for incisional biopsies as well, to confirm its diagnostic value in daily clinical practice. Second, not all included patients underwent the same treatment of the neck. The majority received an elective neck dissection, in which micrometastasis could be missed [32]. In twenty-three percent of our cases, definitive status of the neck was established by follow-up of at least two years. All nodal metastases during follow-up have been confirmed by ultrasound with FNAC or histopathological examination of the resection specimen after a therapeutic neck dissection. Although one patient with watchful waiting in our group received postoperative irradiation of the primary tumor, we believe the amount of bias this caused is minimal. Third, as already mentioned, the cohorts used for validation are prone for selection bias as only patients treated with a neck dissection were included.

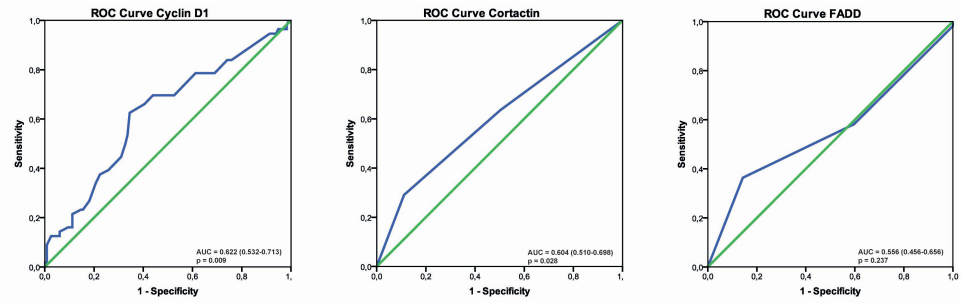
In conclusion, this study identified cyclin D1 expression as a highly sensitive biomarker for occult nodal metastasis in early FOM oral cavity SCC with a NPV of 95%, which seems to be at least as accurate as the SNB is at this site of the oral cavity. As the intratumoral heterogeneity of this biomarker is minimal, this should make cyclin D1 expression in an incisional biopsy representative for the complete tumor. Furthermore, reproducibility of the cyclin D1 expression outcome is shown by the high interobserver agreement. Although the correlation with occult nodal metastasis in FOM tumors was still significant in our validation cohort, the NPV lowered to 79%, potentially because of selection bias. For this reason, its value as a diagnostic biomarker should be validated in a prospective study on incisional

biopsies before its incorporation in clinical care. In early tongue oral cavity SCC, the NPV of cyclin D1 expression was only 76%, which is too low for a watchful waiting policy. Therefore, we advocate for SNB or selective neck dissection as long as more sensitive diagnostic biomarkers for occult nodal disease in early oral cavity SCC other than the FOM are lacking.

SUPPLEMENTARY DATA



Supplementary Figure 1. Correlation *CCND1* copy number (by fluorescence in situ hybridization; FISH) and nuclear cyclin D1 expression (by immunohistochemistry staining). Overall Kruskal-Wallis test p < 0.001. Adjusted p-values pairwise comparisons: normal vs low-level, p = 0.277; normal vs high-level, p < 0.001; low-level vs high-level, p = 0.051.



Supplementary Figure 2. ROC curves of cyclin D1, FADD and cortactin with area under the curve (AUC) and 95% confidence interval.

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CHAPTER 7

In T1N0 staged oral squamous cell carcinomas with a tumor infiltration depth below 4 mm cortactin expression is associated with lymph node status

A study based on sentinel lymph node biopsy status

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ABSTRACT

Background: Compared to the elective neck dissection, sentinel lymph node biopsy (SLNB) is minor surgery. However, 70% of the patients with early stage (cT1-2N0) oral squamous cell carcinomas (OSCC) have no lymph node involvement making a SLNB an unnecessary procedure. This study aimed to evaluate reported molecular tumor biomarkers associated with lymph node metastasis, in early stage OSCC to improve neck strategy selection criteria.

Material and Methods: Expression of cortactin, cyclin D1, FADD, RAB25 and S100A9 was tested on primary tumor material from a homogeneous, well-described cohort of 87 OSCC patients who underwent SLNB neck staging, and associated with lymph node status.

Results: Cortactin was associated with lymph node status in patients with a pT1 tumor and infiltration depth <4 mm (OR 16.0, 95% CI 2.0-127.9) with an negative predictive value of 92%.

Conclusions: Expression of cortactin is a promising tumor marker to select early stage OSCC patients for a watchful waiting strategy instead of a SLNB.

INTRODUCTION

A positive lymph node status (N-status) is one of the most important prognostic factors for shorter disease specific and disease-free survival in oral squamous cell carcinoma (OSCC) [1]. In OSCC, metastatic cells will drain to regional lymph nodes located in cervical neck levels [2]. Despite a clinically negative neck, early stage OSCC (cT1-2N0) patients have a reported 23% to 37% risk for occult (preoperatively undetected) lymph node metastasis [3-5].

The sentinel lymph node biopsy (SLNB) has been introduced in the last decade as diagnostic technique for the detection of occult metastasis, in order to select early stage OSCC patients for a neck dissection or a clinical follow-up strategy [1]. In the Netherlands, patients with a metastasis-positive sentinel lymph node (SLN) currently receive a modified radical neck dissection (MRND) in a second surgical procedure [6,7]. A meta-analysis reported a high pooled sensitivity (87%) and negative predictive value (94%) for detecting occult metastasis using the SLNB procedure [8]. Advantages of the SLNB are individual drainage pattern assessment [9] and more detailed lymph node information with the possibility to detect micrometastasis (0.2-2 mm) and isolated tumor cells (ITCs, metastasis size <0.2 mm) [7]. Despite these advantages, the SLNB is still an invasive staging procedure. Preoperative selection of patients at risk for occult metastasis using molecular tumor profiling might lead to a more individualized neck strategy with a watchful waiting strategy for low risk patients [10], a SLNB for intermediate risk patients and a neck dissection for patients with a high risk [11]. The additional and more detailed information on individual drainage patterns, micrometastasis and ITCs provided by the SLNB might have impact on the clinical value of molecular biomarkers that have previously been associated with N-status [12]. Currently, only few studies reported the value of clinico-pathological markers to predict N-status in a cohort of early stage OSCC patients who underwent neck staging using the SLNB procedure [12-14] with clinical follow-up as reference for the SLNB negative patients. Only one study validated the clinical value of CD44 as a molecular tumor biomarker, but did not find an association with N-status [11].

Before the introduction of the SLNB procedure, patients with a pT1cN0 tumor and a tumor infiltration depth <4 mm were selected for a watchful waiting strategy instead of an elective neck dissection [15]. Tumor profiling using molecular biomarkers to predict N-status in OSCC has been studied before. One such a well-studied genetic alteration in head and neck squamous cell carcinomas that has been associated with N-status is amplification of chromosome 11q13 [16-19]. Three genes (*CTTN, CCND1* and *FADD*) located in this commonly amplified region have previously been validated for N-status in a well-defined early stage OSCC cohort with neck staging by END. A negative predictive value (NPV) of 80-84% was

reported [17]. Other promising markers associated with N-status in OSCC were *RAB25* and *S100A9* hypermethylation and expression levels, markers selected using a genome wide methylation assay [20][Clausen, S100A9, manuscript in prep].

These molecular biomarkers (cortactin, cyclin D1, FADD, RAB25 and S100A9) have been reported as potential prognostic and predictive tumor markers in early stage OSCC, but have not been studied in relation to SLNB staged patients. The aim of this study was to analyze the additional clinical value of combining classical prognostic factors (tumor stage and tumor infiltration depth) with molecular tumor biomarkers in order to select patients for a watchful waiting or SLNB procedure as neck strategy using preoperative tumor biopsies of early stage OSCC patients who underwent neck staging using the SLNB procedure.

MATERIAL AND METHODS

Patients and treatment

Patients diagnosed with cT1-2N0 and pT1-2 OSCC (7th TNM classification), treated by primary surgical resection and neck staging with SLNB between 2008 and 2017 were selected for analysis. These patients were treated in the University Medical Center Groningen (UMCG) (n = 101) and the Medical Center Leeuwarden (MCL) (n = 12). Most of these patients (n = 91) were part of a clinical study assessing the accuracy of the SLNB procedure [7]. Clinical and histopathological data were retrospectively collected from the electronical patient files (Table 1). Pathological reports are standardized in our centers and contain data on tumor infiltration depth, tumor pattern of invasion, extranodal extension, perineural invasion, lymphovascular invasion and N-status. In case of missing histopathological data, tumors or lymph nodes were reassessed by a pathologist (BvdV). Eighty-seven of the 113 tumors were suitable for biomarker analysis (see tissue microarray construction below). Twenty-six (30%) of these 87 patients had a positive SLN and received a modified radical neck dissection in a second operation. SLNB negative patients (70%) had a clinical follow-up of the neck. Four SLN negative patients were diagnosed with a regional recurrence without local disease during follow-up (false negatives) after 8, 9, 18 and 22 months. Ten patients received postoperative radiotherapy, which was combined with chemotherapy in two patients indicated by involved surgical resection margins, multiple positive lymph nodes or extranodal extension. The median follow-up time was 35 months (IQR 20-49 months). Thirteen (15%) patients deceased during the follow-up of which two died as a result of the OSCC.

Table 1. Patient and tumor characteristics and true N-status

	True N negative		True N positive		
	n	**(%)**	**n**	**(%)**	**p-value**
Total	57	(66)	30	(34)	
Gender					
male	30	(53)	12	(40)	0.367
female	27	(47)	18	(60)	
Age at first treatment					
median (IQR)	64	(57 to 71)	66	(59 to 75)	0.302
Site					
tongue	31	(54)	21	(70)	0.209
floor of mouth	19	(33)	4	(13)	
cheek	3	(5)	4	(13)	
others	4	(7)	1	(3)	
pT status (7^{th} TNM)					
1	50	(88)	19	(63)	**0.012**
2	7	(12)	11	(37)	
Tumor infiltration depth (mm)					
median (IQR)	3.5	(2.0 to 5.0)	5.0	(3.6 to 7.0)	**0.007**
Tumor infiltration depth					
<4.0 mm	31	(54)	9	(30)	**0.042**
≥4.0 mm	26	(46)	21	(70)	
Perineural invasion					
no	55	(97)	24	(80)	**0.018**
yes	2	(3)	6	(20)	
Lymphovascular invasion					
no	53	(93)	25	(83)	0.265
Yes	4	(7)	5	(17)	
Tumor grade					
well	19	(33)	6	(20)	0.222
moderate	38	(67)	24	(80)	
Tumor pattern of invasion					
pushing	42	(64)	11	(37)	**0.001**
infiltrative	15	(25)	19	(60)	
Cortactin					
low expression	43	(75)	18	(60)	0.148

Table 1. Continued

	True N negative		True N positive		
	n	(%)	n	(%)	p-value
high expression	14	(25)	12	(40)	
Cyclin D1					
low expression	26	(46)	11	(37)	0.497
high expression	31	(54)	19	(63)	
FADD					
low expression	39	(70)	15	(50)	0.101
high expression	17	(30)	15	(50)	
RAB25					
low expression	23	(40)	8	(27)	0.245
high expression	34	(60)	22	(73)	
S100A9 nuclear					
low expression	17	(30)	10	(33)	0.809
high expression	40	(70)	20	(67)	
S100A9 cytoplasmic					
low expression	23	(40)	10	(33)	0.643
high expression	34	(60)	20	(67)	

True N-status is determined by the combination of postoperative pathological lymph node status (pN) combined with regional recurrence (false negatives). Four patients with a negative SLNB (pN0) were diagnosed with a regional recurrence and counted as true N positives. Molecular expression was dichotomized for cortactin, cyclin D1, RAB25 and S100A9 using a ROC-analysis. FADD was semi-quantitatively scored.
Abbreviations: IQR, interquartile range; TNM, American Joint Committee of Cancer TNM classification.

The SLNB protocol used was described before [7]. Briefly, one day before surgery a radioactive tracer (^{99m}Tc-nannocolloid) was peritumorally injected, followed by dynamic and static lymphoscintigraphy and Single Photon Emission Computed Tomography (SPECT)-CT scanning. The next day during surgery, SLNs were located and harvested using a handheld gamma-probe. Postoperatively, the SLNs were histopathological assessed with step-serial-sectioning and slides from each level were stained for hematoxylin and eosin (HE) and additional cytokeratin (CK AE1-AE3, clone AE1/AE3, Ventana Medical Systems, Tucson, AZ).

Tissue microarray construction

All HE slides of the primary tumor were revised by a dedicated head and neck pathologist to assess tumor cell presence. Twenty-six of the 113 tumors were too small or had no redundant tissue available for TMA construction. Five other tumors were not suitable for inclusion in the TMA as a result of limited tumor size, but could be assessed using whole

tumor slide sections. Finally, 87 cases were available for analysis (82 tumors on two TMA blocks and five whole tumor slide sections). The TMA construction procedure in our center has been described earlier [21]. Briefly, tumors were marked on the HE slides by a pathologist, after which three 0.6 mm cores were taken from the corresponding donor FFPE blocks and added to the recipient TMA FFPE block using a Manual Tissue Arrayer I (Beecher Instruments, Sun Prairie, WI). Both TMA blocks had a unique lay-out with normal tissue samples of liver, testis, cervix, colon and placenta incorporated into the TMAs as negative and positive control tissues. 3 µm thick sections were cut from the TMA blocks for the immunohistochemical staining of the markers. For IHC staining, the first and last sections were HE stained to confirm presence of tumor in the cores of TMA sections.

Immunohistochemistry

The staining procedures and antibodies have been described before [20,22,23] and are summarized in Table 2. Cyclin D1 was automatically stained using the Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, AZ, US). For the other markers: after deparaffinization in xylene and rehydration in a graded alcohol series, sections were treated for antigen retrieval followed by endogenous peroxidase blockage by incubating in 0.3% peroxide solution. Slides were then incubated for one hour with the primary Mouse anti human antibody, followed by 30 minutes incubation of a Rabbit anti Mouse (RAM_{po}) secondary antibody and 30 minutes incubation of a Goat anti Rabbit tertiary antibody (GAR_{po}). All antibodies were Horseradish Peroxidase conjugated and diluted in 1% BSA – PBS serum (primary antibody) or diluted in 1:100 1% BSA – PBS – 1% AB serum (RAM_{po} and GAR_{po}). Slides were developed with 3,3-di-amniobenzidine (DAB) chromogen solution (DAKO, Glostrup, Denmark) and counterstained with hematoxylin. HE, cyclin D1 and FADD stained glass slides were digitized using the NanoZoomer 2.0 HT with a 40x magnification lens and the NDP. view 2 Viewing software U12388-01 (Hamamatsu Photonics, Hamamatsu City, Shizuoku, Japan). Cortactin, RAB25 and S100A9 slides were digitized using the IntelliSite Ultra Fast Scanner with the IntelliSite Image Management System software (Phillips Electronics, Best, The Netherlands).

All molecular biomarkers were scored as described earlier [17,20,22,23][Clausen S100A9 in prep]. Cortactin and cyclin D1 were scored as the percentage of tumor cells with a higher cytoplasmic expression (cortactin) or nuclear expression (cyclin D1) compared to normal epithelium or surrounding tissue cells. FADD was scored semi-quantitatively as tumor cells with negative (0), weakly positive (+), positive (++) or strong positive (+++) cytoplasmic expression. RAB25 was scored as percentage of tumor cells with negative (-), moderate positive (+) or strong positive (++) cytoplasmic expression. S100A9 was scored for both percentage of tumor cells with any nuclear (-/+) expression or percentage of any

cytoplasmic (-/+) expression. Markers were independently scored by two observers (KB with MM or LSM). Discordances were discussed with a dedicated head and neck pathologist (BvdV) until consensus was reached. Except for FADD, optimal cut-offs between high and low expression of the markers and N-status were determined using a ROC curve and the average expression of the cores per tumor. The cut-offs were 46% of cytoplasmic expression for cortactin, 73% of nuclear expression for cyclin D1, 4% of any expression (+, ++) of RAB25 and 17% of nuclear expression and 55% of cytoplasmic expression for S100A9. For FADD, cases with at least one core scored as ++ or +++ were defined as high expression. Representative examples of low and high expression detected by immunohistochemical staining are shown in Figure 1.

Table 2. Antibody information

Antibody	Clone	Company	Catalog No.	Website	Dilution
Cortactin	30	BD BIOSCIENCES	610050	http://www.bdbiosciences.com/us/reagents/research/antibodies-buffers/cell-biology-reagents/cell-biology-antibodies/purified-mouse-anti-cortactin-30cortactin/p/610050	1:1000
Cyclin D1	SP4-R	Roche	790-4508	http://reagent-catalog.roche.com/product/1530?type=1990	
FADD	A66-2	BD Biosciences	556402	http://www.bdbiosciences.com/us/applications/research/apoptosis/purified-antibodies/purified-mouse-anti-human-fadd-a66-2/p/556402	1:100
RAB25	3F12F3	Santa Cruz Biotechnoly	Sc-65978	https://www.scbt.com/scbt/product/rab-25-antibody-3f12f3	1:50
S100A9	S36.48	BMA biomedicals	T1026	http://www.bma.ch/en/products/t-1026	1:100

Ethical considerations

This study used retrospective data and leftover tumor tissue which was collected during treatment according to the national guidelines for oral cavity cancer. Therefore, this research was not a clinical study with human subjects as meant in the Medical Research Involving Human Subjects Act as was concluded by the local Medical Ethics Review Board of the University Medical Center Groningen (M18.225755) and no further approval was required.

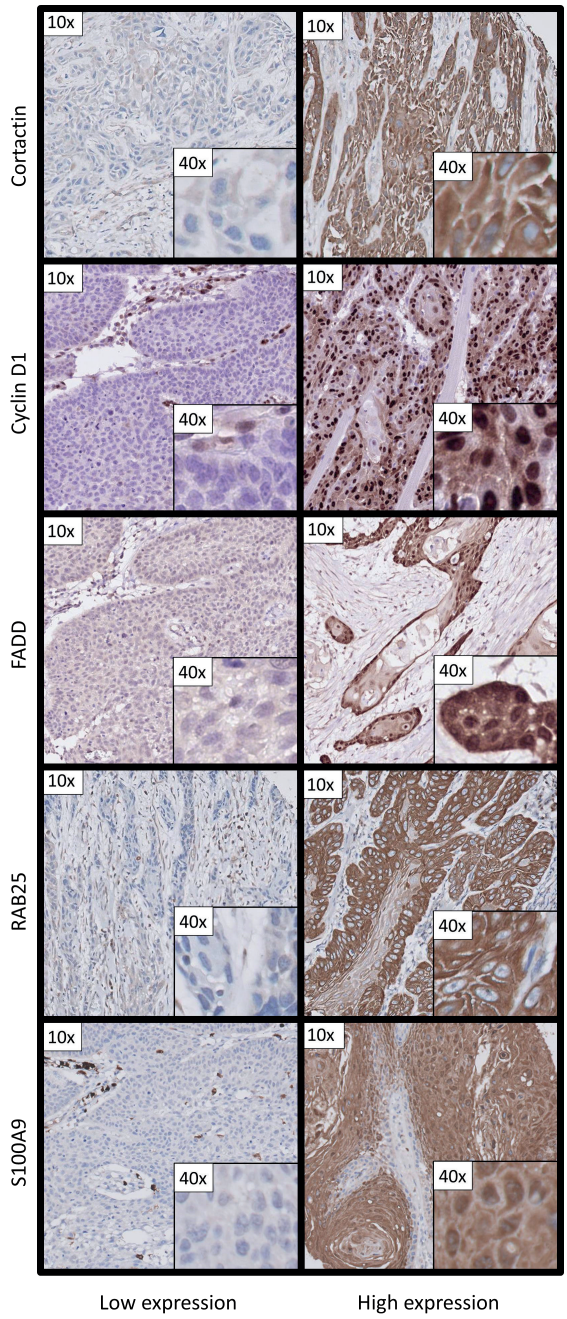

Figure 1. Representative examples of low and high expression on tissue microarryas constructed of OSCC primary tumor tissue for the biomarkers used in this study. Examples are given with a 10x and 40x magnification.

Statistics

Numbers (n) with corresponding percentages, mean with standard deviation (SD) and median with interquartile range (IQR) are given for respectively categorical data, normally distributed data and skewed distributed data. Associations between categorical data were tested using the Fisher's exact test or Chi-square test. The Student's *t* test (normally distribution) or Mann-Whitney *U* test (skewed distribution) were used for the associations between groups with continuous data. True N-status positive (N+) was defined as patients with histological proven metastasis in their SLN (pN+) Also the four patients with a negative SLNB (pN0) and a regional recurrence without local disease during their follow-up were considered as true N-status positive. Associations with true N-status were multivariable tested using a stepwise binary logistic regression model in which variables with a p-value ≤ 0.1 from an univariable analysis were included. The homogeneity of the tumor markers was tested by calculating the intraclass correlation coefficient (ICC) using only cases with three scorable TMA cores. An ICC of 0.61 (substantial correlation) or higher was used as cut-off between a homogeneous or heterogeneous expression of the marker [17,24]. In case of homogeneity (ICC > 0.61) also cases with one of two cores were included. In 88% (FADD) or more (other biomarkers) of the tumors on the TMAs were 2 or 3 cores available (supplementary data 1). The ICC analysis using cases with three available cores showed the lowest ICC of 0.66 for RAB25 and the highest of 0.86 for FADD, representing respectively a substantial and almost perfect reproducibility of measurements (Supplementary data 1). Therefore, all molecular tumor markers were considered as homogeneous and cases with one or two cores on the TMA were included for further analyses. The threshold for significant differences was a p-value ≤ 0.05. All statistical analyses were performed using IBM SPSS Statistics 23 (Statistical Package for the Social Sciences, Inc., Chicago, IL, USA).

RESULTS

Twenty-six patients had a positive SLN. Four patients with a negative SLNB were diagnosed with a regional recurrence during follow-up. These 30 (34%) patients were considered as true N positives. The other 57 (66%) were considered as true N negatives (Table 1).

None of the molecular tumor biomarkers was associated with true N-status in the total cohort of cT1-2N0 OSCC patients with SLNB neck staging. An univariable analysis of associations between true N-status and clinico-pathological and molecular tumor biomarkers showed significant associations for pT status (p = 0.012), tumor infiltration depth (p = 0.007), perineural invasion (p = 0.018) and infiltrative tumor pattern of invasion (p = 0.001) (Table 1). In a multivariable analysis pT status (OR 3.6, 95% CI 1.1-11.3) and infiltrative tumor pattern of invasion (OR 4.4, 95% CI 1.7-11.7) were found to be independent factors for true N-status.

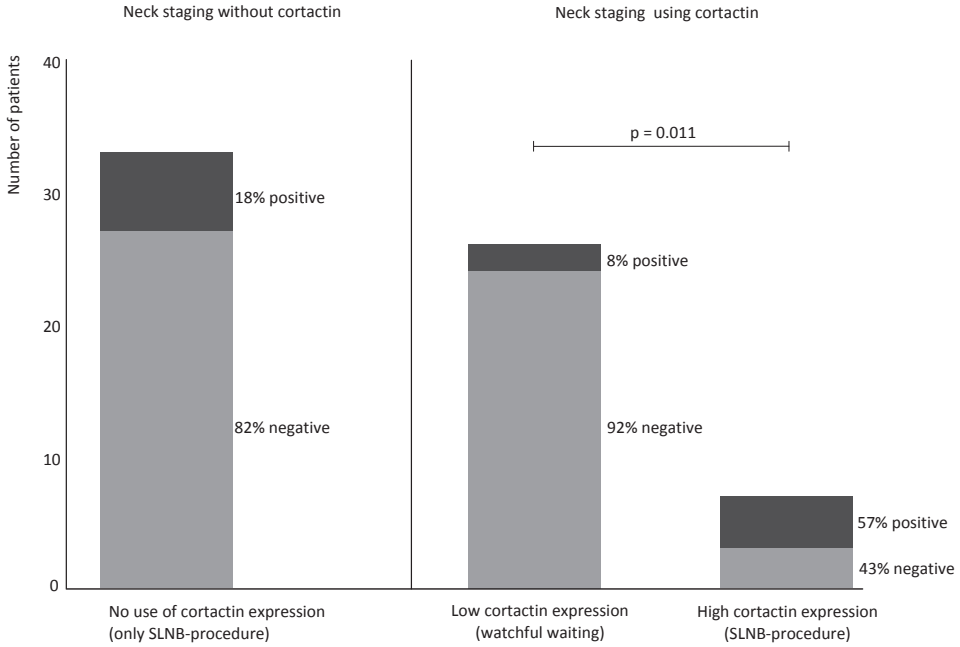

Figure 2. Differences between neck staging using only the sentinel lymph node biopsy procedure or combined with cortactin expression levels. The differences in selection of neck staging procedures in 33 patients with a pT1cN0 OSCC and a tumor infiltration depth <4 mm with and without using cortactin expression. These 33 patients had neck staging using SLNB while 82% had no neck lymph node involvement (negative true N-status). If these patients were selected for a watchful waiting procedure using cortactin expression, 8% out of 26 patients with a low cortactin expression would have been false negatives (positive true N-status) and 43% out of 7 patients with a high cortactin expression and without lymph node involvement (negative true N-status) would have had neck staging using SLNB procedure.

To investigate whether patients with a low risk of occult metastasis could be identified for a watchful waiting strategy of the neck instead of a SLNB, we analyzed the additional value of these same molecular biomarkers in a subgroup of 33 patients with a pT1cN0 OSCC and a tumor infiltration depth <4 mm. Of these 33 low risk patients, 18% had a positive true N-status whereas 44% positive true N-status cases were observed within the 54 patients with pT1 or pT2 tumors and a tumor infiltration depth ≥4 mm ($p = 0.019$). In the 33 low risk patients, cortactin (OR 16.0, 95% CI 2.0-127.9) and FADD (OR 8.8, 95% CI 1.2-62.2) expression were strongly associated with true N-status. Cortactin and FADD showed overexpression in the same four patients with a positive true N-status. Cortactin expression as predictive marker for true N-status in these 33 low risk patients resulted in a sensitivity of 67% and NPV of 92% (Table 3). If these 33 pT1cN0 patients with tumor infiltration depth <4 mm

were selected for watchful waiting without using cortactin, six (18%) would have had an incorrect neck staging strategy (Figure 2). If cortactin was used as predictive marker for N-status, 26 (79%) patients would have been selected for a watchful waiting strategy based on a low cortactin expression and only two (8%) patients would have a positive true N-status whereas 24/26 (92%) indeed would be true negative (Figure 2).

None of the clinico-pathological characteristics or biomarkers were associated with true N-status in the remaining 54 patients with a pT1 or pT2 staged tumor and a tumor infiltration depth ≥4 mm.

Table 3. Molecular biomarkers in pT1cN0 staged OSCC with a tumor infiltration depth <4 mm

	True N negative		True N positive		
	n	**(%)**	**n**	**(%)**	**p-value**
Cortactin					
low expression	24	(89)	2	(33)	**0.011**
high expression	3	(11)	4	(67)	
Cyclin D1					
low expression	13	(48)	2	(33)	0.665
high expression	14	(52)	4	(67)	
FADD					
low expression	22	(82)	2	(43)	**0.034**
high expression	5	(18)	4	(57)	
RAB25					
low expression	15	(56)	4	(67)	1.000
high expression	12	(44)	2	(33)	
S100A9 nuclear					
low expression	18	(67)	4	(67)	1.000
high expression	9	(33)	2	(33)	
S100A9 cytoplasmic					
low expression	14	(52)	4	(67)	0.665
high expression	13	(48)	2	(33)	

True N-status is determined by the combination of postoperative pathological lymph node status (pN) combined with regional recurrence (false negatives). Patients with a negative SLNB (pN0) and diagnosed with a regional recurrence were counted as true N positives. Cortactin and FADD expression were significantly associated with true N-status in pT1cN0 staged OSCC and a tumor infiltration depth <4 mm with the highest sensitivity of 67% (4/(4+2)) and negative predictive value of 92% (24/(24+2)) for cortactin.

DISCUSSION

SLNB is nowadays used as neck staging strategy in early stage (cT1-2N0) OSCC. Although the SLNB procedure is less invasive compared to the conventional END, it is still an invasive staging technique while in ~70% of these early stage OSCCs no lymph node involvement is observed. In this study we analyzed the additional clinical value of molecular tumor biomarkers to select early stage OSCC patients with a low risk of occult metastasis for a watchful waiting strategy instead of performing a SLNB procedure. For that purpose, we selected patients with a pT1cN0 OSCC and a tumor infiltration depth <4 mm who were conventionally selected for a watchful waiting strategy instead of an END [15] and found that low cortactin expression selected true N-status negative patients with a 92% NPV. No association was found between any of the molecular markers and the true N-status in the total cohort or between the molecular markers and pT1 or pT2 tumors with a tumor infiltration depth ≥4 mm.

In 2012, a diagnostic algorithm for early stage OSCC using gene-expression profiling of tumor biopsy specimens to select low risk patients for a watchful waiting strategy instead of a SLNB procedure has been proposed [10]. An 89% NPV for selecting patients for a watchful waiting strategy instead of SLNB was observed. In that study, 11% false negatives in patients selected as N-status negative by gene-expression profile but with lymph node involvement was found [10]. S100A9 was the only molecular biomarker of the current study which was also part of that gene-expression profile. Another study reported in 92 early stage OSCC patients staged by SLNB that tumor infiltration depth ≤2 mm might be used to select patients for watchful waiting, 2-5 mm for a SLNB and >5 mm for an END [11]. In a large cohort with data of 199 early stage OSCC patients with SLNB neck staging from two Dutch head and neck centers, a cut-off of 3.4 mm was reported as most optimal (sensitivity 83%, specificity 47%) between tumor infiltration depth and N-status based on a ROC-curve analysis [12]. Because a 15% risk for metastasis below the cutoff, the authors stated that tumor infiltration depth could not serve as an optimal predictive marker to select patients for a watchful waiting strategy instead of a SLNB procedure [12]. The difference with our study is that we used cortactin in combination with a tumor infiltration depth cut-off of 4 mm defined in a large cohort of OSCC to predict N-status [15]. Without cortactin, the risk for positive N-status is 18% in pT1cN0 and with cortactin that risk lowered to 8%. If we used the 3.4 mm cut-off as reported by den Toom [12] in combination with or without cortactin in our pT1cN0 patients, the metastasis risk lowered from 18% to 9% (data not shown). These data show that infiltration depth between 3 mm and 5 mm did not significantly affect the risk showing the potential additional value of cortactin to select patients with a low metastases risk for a watchful waiting strategy instead of a SLNB.

Cortactin and FADD were both significantly associated with true N-status in pT1 patients with a tumor infiltration depth <4 mm and both genes are located at the 11q13 chromosome which is frequently amplified in HNSCC [25,26]. Cortactin had the highest predictive value in this study and is a candidate driver gene of the 11q13 amplification [23]. Cortactin has many binding sites and interactions with other proteins which play an important role in metastasis as was reviewed in 2019 [16]. Cortactin plays a key-role in the regulation of protrusive structures such as invadopodia and lamellipodia that enable respectively the invasion and migration of tumor cells by changing the actin cytoskeleton [16,27-29]. Also in-vitro studies showed that cell migration of OSCC cell lines was regulated by cortactin expression [29-31]. The pooled data of nine studies with OSCC patients showed an association between N-status and amplification or expression of *CTTN/*cortactin with an OR 2.78 (95% CI 1.68-4.60) (reviewed in [32]). The authors stated that cortactin could assist in the selection of patients for a watchful waiting strategy, but that further research is needed to define optimal patient selection [32]. In the current study, we have shown that cortactin expression has additional clinical value in pT1cN0 patients by better selection of watchful waiting with lower risk for lymph node metastases with a NPV of 92%. This is similar to the pooled NPV of 94% for the SLNB procedure in a meta-analysis [8]. Our study is based on a relatively small cohort, but our findings warrant further research with large prospective data to validate the selection of low risk patients for a watchful waiting strategy using cortactin expression in tumor biopsy specimens of OSCC tumors.

This is one of the first studies using SLNB data for the validation of molecular tumor biomarkers associated with lymph node status. Although this study is limited by a relatively small number of patients compared to non-SLNB OSCC studies [15,33,34], it shows that cortactin expression might have additional value in preoperative neck strategy decisions in a subgroup of early stage OSCC patients.

CONCLUSION

This study showed the association between cortactin and lymph node status in pT1cN0 OSCC patients with a tumor infiltration depth <4 mm and SLNB neck staging. A combination of a tumor size smaller than 20 mm, a tumor infiltration depth smaller than 4 mm and a low cortactin expression might have clinical value to select patients for a watchful waiting strategy instead of subjecting patients to a SLNB procedure. Further prospective research is needed to confirm the clinical value of cortactin as a predictive marker next to the SLNB procedure in one neck staging protocol in early stage OSCC.

SUPPLEMENTARY DATA

Supplementary data 1. Number of cores per tumor for each marker and corresponding intraclass correlation coefficient

	Cortactin	Cyclin D1	FADD	RAB25	S100A9 nucleus	S100A9 cyto†
1 core	4	8	9	4	5	5
2 cores	22	22	18	26	27	27
3 cores	56	52	54	52	50	50
ICC	0.71	0.70	0.86	0.66†	0.72	0.81
(95% CI)	(0.58 to 0.81)	(0.57 to 0.80)	(0.79 to 0.91)	(0.53 to 0.77)	(0.53 to 0.85)	(0.72 to 0.93)

ICC is based on cases with 3 cores.

† ICC based on the average percentage of any (moderate and strong) staining per core

ICC: <0 "poor," 0 to 0.20 "slight," 0.21 to 0.4 "fair," 0.41 to 0.60 "moderate", 0.61 to 0.80 "substantial", >0.81 "almost perfect"

Abbreviations: ICC, intraclass correlation coefficient; cyto, cytoplasmic; CI, confidence interval.

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CHAPTER 8

Detection of tumor cells in saliva from patients with oral squamous cell carcinoma using DNA hypermethylation of *KCNA5* and *TIMP3*

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ABSTRACT

Background: The high local recurrence and/or second primary tumor rate of 20-30% in patients with oral squamous cell carcinoma (OSCC) is partly caused by residual tumor cells of the first primary tumor and the presence of precancerous epithelium that has not (yet) clinically manifested. Since OSCC cells are shed into the oral cavity, the detection of tumor-specific DNA methylation markers in saliva could be a tool for the early detection of local recurrences or second primary tumors of OSCC. The aim of this explorative study was to identify and validate new methylation markers to detect OSCC cells in saliva. For that purpose, we investigated molecular biomarkers methylated in OSCC and not in normal cells from a genome-wide methylation screening analysis using MethylCap-Seq analysis of 12 OSCCs compared to controls. In addition, we selected 4 markers reported to be methylated in saliva by others (*EDNRB, HOXA9, NID2* and *TIMP3).*

Results: Using our OSCC methylome, seven genomic locations representing six genes (*C11orf85*, *CMTM2*, *FERMT3*, *KCNA5*, *SIPA1* and *TBX4*) were identified that were significantly hypermethylated in tissues of OSCC compared to DNA from controls. QMSP analysis using saliva from OSCC patients compared to non-cancer controls of similar age or younger age, revealed only a difference for *KCNA5* methylation (respectively $p = 0.003$ and $p = 0.001$). Moreover, when *KCNA5* was combined with other markers, the combination with *TIMP3* revealed a 100% diagnostic potential in detecting OSCC patients compared to non-cancer controls of similar age using saliva.

Conclusions: In this explorative study we identified several new OSCC-specific methylation markers with a high sensitivity and high negative predictive value for the detection of OSCC in saliva. Two methylation markers (*KCNA5* and *TIMP3*) might be useful for early detection of local recurrence or second primary tumors in saliva cells of OSCC patients. A larger prospective study should be done to confirm the clinical relevance of these two markers.

BACKGROUND

Oral Squamous Cell Carcinoma (OSCC) is the most common subtype of head and neck cancer. It is the sixth most common cancer worldwide, accounting for 650,000 new cases and 350,000 related deaths annually [1]. Over the last 30 years, the incidence of OSCC has almost doubled, while the 5-year survival increased by 10% [2], reaching a 5-year survival of only 48% [3]. Risk factors for recurrence of OSCC are locally residual cancer after treatment of the first primary tumor or field cancerization of the oral mucosa [4,5].

Residual tumor cells are isolated cells of the first primary tumor which can remain after treatment and have the potential to develop into a local recurrence. Due to the small size of isolated cells and often submerged location, these residual tumor cells are often discovered late by regular clinical examination or imaging [5].

Due to the long-term exposure to tobacco and alcohol the epithelium of the upper aerodigestive tract might harbor areas with accumulation of pre-cancerous (epi)genetic changes [6,7], with or without clinical manifestation which is known as field cancerization [5]. These (epi)genetic changes drive carcinogenesis (6) and therefore areas with field cancerization are at risk of developing a new malignant tumor [8].

Besides the difficulty in detecting residual tumor cells/precancerous epithelial cells and the challenge of detecting the conversion of clinical visible precancerous fields (e.g. leukoplakia and erythroplakia) into new tumors as early as possible, the detection of local recurrences at an early stage is complicated by the consequences of earlier treatment. The resection area of the first primary tumor might be reconstructed with tissue from extra-oral donor sites and fibrosis is induced by surgery and irradiation [9]. Although local recurrences and new primary tumors are clinically difficult to detect at an early stage, (epi)genetic alterations in DNA from residual primary tumor cells or field cancerization cells released into saliva might be detectable before clinical manifestation of recurrent disease [10]. Using (epi)genetic alterations to detect tumor DNA in saliva is therefore a promising new non-invasive strategy for the early detection of local recurrences.

Alteration in DNA methylation status is one of the epigenetic aberrations that drives tumor genesis in OSCC [11]. Changes in DNA methylation are associated with etiological factors such as cigarette smoking and alcohol consumption [6,7] through regulation of DNA methyltransferases (DNMT) [8,12,13]. Changes in DNMT expression might result in genome-wide hypermethylation associated with one of the hallmarks of cancer, chromosomal instability [14] as well as the downregulation of tumor suppressor genes [14]. Moreover, DNA methylation changes occurs early in tumorigenesis [14]. Therefore, DNA methylation

markers might also be useful for the early detection of tumor cells or be detectable in shed DNA fragments in liquid biopsies such as plasma and sputum [10] and has been reported in lung [15], breast [16], colorectal [16] and hepatocellular cancer [17]. The detection of tumor cells in saliva of patients with head and neck SCC has been reported as well [18-20] and requires markers with high sensitivity and high specificity. In patients with OSCC, only few markers that are methylated in tumor tissue but not in normal epithelium, have been reported [10]. To identify new methylation markers in patients with OSCC that are associated with lymph node status, we recently used a genome-wide methylation screening method based on MethylCap-Seq analysis [21] and reported a methylome of several OSCC cases and numerous new differentially methylated tumor markers [22,23].

In the current study, to identify new biomarkers associated with OSCC and not with other tissue, we assessed the available methylome of a series of OSCC cases generated by MethylCap-Seq analysis [23] with the methylome of 80 control tissues. We describe the identification of several new markers which are significantly hypermethylated in OSCC and not in non-cancer control samples. We validated the performance of these OSCC specific DNA hypermethylation markers using quantitative methylation specific PCR (QMSP) in a proof of principle pilot study with saliva of OSCC patients and non-cancer controls. In addition, we included five DNA methylation markers previously reported to be associated with OSCC[19,20,24]. The aim of this study was to identify methylation markers with a high sensitivity and a high negative predictive value (NPV) for the detection of tumor cells in saliva from patients with OSCC.

MATERIALS AND METHODS

Identification of novel methylation markers using MethylCap-seq analysis

The strategy of methylation marker selection is summarized in Figure 1. To identify genomic loci hypermethylated in OSCC and not in normal tissue, *in silico* analysis was performed of MethylCap-Seq data [25] as reported previously [21,26]. In summary, 12 OSCC samples and two pools of leukocytes of 500 ng DNA each were fragmented using Covaris S2 (Covaris, Woburn, MA, USA). Subsequently, methylated DNA fragments were separated from unmethylated fragments by enrichment with the MethylCap kit (Diagenode, Belgium), paired-end sequenced using the Illumina Genome Analyzer II and mapped to the human reference genome (NCBI build 37.3). For further analysis, only pair-end sequenced fragments (reads) were included that could be mapped to unique specific loci, and summarized using an in house generated . "Map of the Human Methylome" for MethylCap-seq data [27].

For further analyses only the MCs that are located either in a promoter region, between 2000 bp upstream to 500 bp downstream of the Transcription Start Site (TSS) or in the first exon of an Ensemble (v65), gene were selected and statistically compared using R with R-package Bayseq [28]. The most equally methylated MCs amongst all 12 OSCC were ranked according the likelihood of equal methylation. Additionally, an approximate false discovery rate (FDR) was calculated. The 5000 most equally methylated MCs with the lowest FDR were used for further analysis. These highest ranked 5000 MCs in OSCC were compared to the 2276 MCs available in the MethylCap-Seq data of the two leukocyte pools, by the Mann-Whitney U test (wilcox.test function in R). All MCs with a p-value <0.05 were selected for further analyses (n = 335, Supplementary data 1). In the next step, all MCs were selected with a 100% positive and negative predictive value defined by ≤2 reads in both leukocytes pools as well as ≥3 reads in all 12 OSCC (Supplementary data 1). Finally, the MCs were compared to the semi-quantitative methylation data of the "Map of the Human Methylome" (non-OSCC primary tumor samples (n = 32), non-OSCC cancer stem cells (n = 11), normal tissue (n = 22), stem cells (n = 6) and normal cell lines (n = 9)) [27] to select MCs without methylation detected in the average methylome.

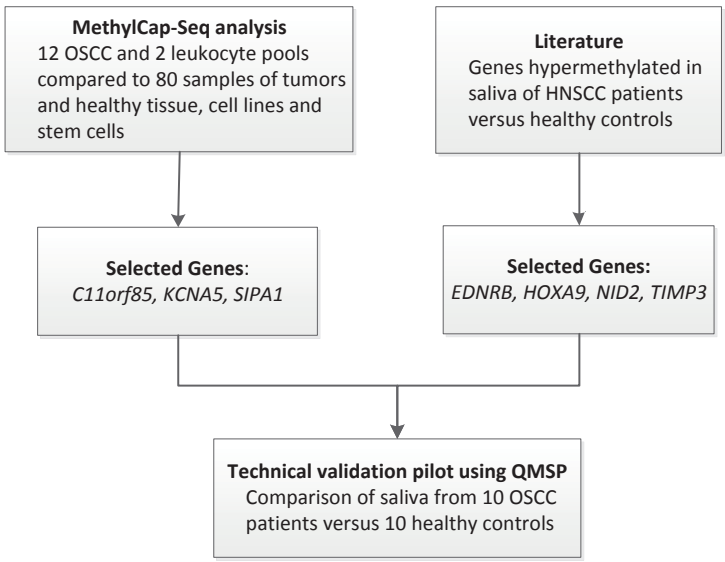


Figure 1. Study design. Methylation markers were selected using a MethylCap-Seq protocol. Selected genes were technically validated in a pilot study with saliva from 10 OSCC patients and 10 healthy controls (five younger and age-matched controls) and compared to methylation markers associated with OSCC and selected from literature.

Abbreviations: OSCC, oral squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; QMSP, quantitative methylation-specific PCR.

Technical validation of OSCC-methylation markers

For the validation, saliva from in total 10 OSCC patients were collected: seven males and three females with a median age of 63 years and with pT1-2 (n = 7) and pT3-4 (n = 3) staged tumors. For methylation status in the original tumor tissue, six fresh frozen (FF) tumor biopsies and nine formalin fixed paraffin embedded (FFPE) tumor resection tissues were available for DNA isolation. Saliva samples were collected from healthy (non-cancer) controls. Five patients were planned to undergo benign corrective jaw surgery (median age 45 years, significant younger than the OSCC patients p = 0.050) and five patients were scheduled to receive dental implants (median age 67 years, age-matched with the OSCC patients). Characteristics of the patients and controls are summarized in Table 1. All patients and controls had no prior history of HNSCC or immunological diseases such as Sjögren's syndrome and no apparent infections in the oral cavity during saliva collection. Saliva was collected preoperatively on the day of surgery between 07:00 and 10:00 AM to exclude variation due to circadian rhythm. Patients and controls had at least 90 min without stimulation of the salivary glands by drinking, smoking or eating. Patients and controls deposited 2 ml whole saliva into a 15 ml falcon tube without a time limit. Samples were recoded for lab processing.

Ethics approval and consent to participate

Written approval and informed consent of all twenty patients and controls included in the validation study was obtained. Because of the non-invasive character of saliva sample collection, this research was not a clinical study with human subjects as meant in the Medical Research Involving Human Subjects Act as was concluded by the local Medical Ethics Review Board of the University Medical Center Groningen (M12.116657) and no further approval was required.

DNA isolation

Saliva DNA integrity was preserved by adding 2.5 ml of 1 tablet Roche Complete mini Protease Inhibitor Cocktail (pro. #. 04693159001) dissolved in 10 ml filtered (4°C) PBS. The saliva PBS mixture was equally divided in three 1.5 ml Eppendorf Tubes and centrifuged at 14000 rpm for 10 min at 4°C. The pellets were incubated in 600 µl 1% SDS-proteinase K. Both the pellet and the supernatant were separately stored at -80°C.

Tumor DNA was isolated as follows. Approximately eight 10 µm thick sections were cut from the FFPE blocks. For quality control, the first and last section (3 µm thick) were HE-stained to check for tumor load. A dedicated head and neck pathologist marked areas with >60% neoplastic cells. The 10 µm FFPE sections were deparaffinized using xylene and

neoplastic-enriched areas were macrodissected and used for DNA extraction. From the FF tissues, approximately four 10 µm thick sections were cut. Both, the FF and FFPE sections were incubated overnight at 60°C in 300 µl 1% SDS-proteinase K solution.

Table 1. Clinical characteristics of all included subjects

Patient characteristics	OSCC Patients (n)	Non age-matched Controls (n)	Age-matched Controls (n)
Total	10	5	5
Age (years) *			
Median (IQR)	63 (58 to 74)	45 (30 to 62)	67 (57 to 70)
Gender **			
Male	7	2	4
Female	3	3	1
Saliva DNA yield (µg)***			
Median (range)	64 (6 to 140)	32 (16 to 75)	32 (20 to 57)
FFPE tumor tissue	9	NA	NA
FF tumor tissue	6	NA	NA
Tumor localization		NA	NA
Tongue	4	NA	NA
Gum	2	NA	NA
Floor-of-mouth	3	NA	NA
Cheek	1	NA	NA
pT		NA	NA
1-2	7	NA	NA
3-4	3	NA	NA
pN		NA	NA
0	5	NA	NA
+	2	NA	NA
X	3	NA	NA
Infiltration depth (mm)		NA	NA
Median (range)	3 (1 to 23)	NA	NA
Tumor diameter (mm)		NA	NA
Median (range)	22 (7 to 52)	NA	NA

* OSCC versus orthognatic, p = 0.050; no significant differences between the other groups . ** No significant differences between patient and control groups. *** No significant differences between patient and control groups. Abbreviations: OSCC, oral squamous cell carcinoma; IQR, interquartile range; ug, microgram; mm, millimeter; NA, not applicable; FFPE, formalin fixed, paraffin embedded; FF, fresh frozen.

DNA was extracted from sections and saliva cell pellets by phenol-chloroform extraction and ethanol precipitation as described previously [29]. Samples were dissolved in TE-4 buffer (50 µl for FFPE and FF, 300 µl for saliva) and stored at 4°C. DNA quality and quantity was assessed using the Nanodrop and Biomed II PCR protocol [30].

Bisulfite treatment and Quantitative Methylation Specific PCR (qMSP)

Isolated DNA was treated with bisulfite for methylation-specific-PCR (MSP) as previously described [23,29]. Briefly, bisulfite treated DNA (bisDNA) was acquired using the EZ DNA methylation kit (Zymogen, BaseClear, Leiden, The Netherlands), according to the manufacturer's protocol. Methylation-specific-PCR (MSP) was performed on 20 ng bisDNA as follows: 10 min 95°C, 40 cycli (1 min 95°C, 1 min $T_{annealing}$, 1 min 72°C), followed 10 min 72°C and ∞ 4°C. Primer sequences and $T_{annealing}$ are summarized in Table 2. As controls in each qMSP, leukocyte DNA from healthy individuals (as a control for endogenous methylation), leukocyte DNA that was *in vitro* methylated (I.V.) by SssI methyltransferase (New England BioLabs Inc., Bioké, Leiden, The Netherlands) (as a control for methylated DNA) and leukocyte DNA that was amplified according to manufacturer's protocol using whole genome amplification with the Illustra Ready-To-Go GenomiPhi HY DNA Amplification Kit (GE Healthcare, Little Chalfont, UK) (as a control for hypomethylation). Cytosine conversion by bisulfite treatment was checked with primers specific for bisulfite treated Beta-Actin (ACTB) and DAPK as described earlier [31,32]. After MSP, PCR products were separated and visualized by custom Ethidium Bromide staining.

QMSP was performed as previously described with an internal dual-labeled hybridization probe (IDT, Coralville, IA) [29,31]. For *CMTM2* and *FERMT3* no specific primers and probes within 250 bp of the methyl core region could be designed. For four genes (*C11orf85*, *KCNA5*, *SIPA1* and *TBX4*), QMSP primers and probes were designed by Methyl Primer Express TM Software v1.0 (Thermo Fisher Scientific, Applied Biosystems, Leiden, The Netherlands) and checked using Clone Manager software (Sci-Ed software, Denver, USA) (Table 2). Serial dilutions of I.V. DNA were used to calculate standard curves for each primer-probe set, resulting in suitable conditions for the detection of methylation of *C11orf85, KCNA5* and *SIPA1*. For *TBX4* no optimal condition was found and therefore *TBX4* was excluded for further analysis. The amount of bisulfite treated DNA input of each sample was determined by qMSP for ACTB (Table 2) as reported previously [31]. Fluorescence was measured in triplicates for 50 cycles using the following mixture: 7.5 µl of 2* LightCycler 480 Probes Master mix (Roche Diagnostics GmbH, Mannheim), 300 nM of forward and reverse primers (IDT, Coralville, IA), 200 nM of probe (IDT) and 2.5 µl bisulfite-modified DNA (~25 ng). Each sample was analyzed by LightCycler 480 (Roche Diagnostics GmbH, Mannheim). Relative methylation

Table 2. The sequences of the primers and probes for all genes used for methylation detection by QMSP and MSP

Gene name	Method	QMSP forward 5′-3′	QMSP reverse 5′-3′	QMSP probe 6-FAM 5′-3′TAMRA	Amplicon length	$T_{annealing}$	Reference
ACTB	QMSP	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	ACCACCACCCAACACACAATAACAAACACA	133	60	NA
C11orf85	QMSP	GAAATGCGTACGCGTAGATC	CAACTTCGAAACTCGTACCG	TGGGAAGCGTATTTGCGCGTGC	118	60	NA, MethylCap-Seq
EDNRB	QMSP	GGGAGTTGTAGTTTAGTTAGTTAGGGAGTAG	CCCGCGATTAAACTCGAAAA	TTTTTATTCGTCGGGAGGAG	75	60	Demokan et al.[19]
HOXA9	QMSP	AATAAATTTTATCGTAGAGCGGTAC	CATATAACAACTTAATAACACCGAA	GCGCCCCCATTAACCGTACGCGT	226	60	Guerrero-Preston et al.[24]
NID2	QMSP	GCGGTTTTTAAGGAGTTTTATTTTC	CTACGAAATTCCCTTTACGCT	ACGCCGCTACCCCAAACCTTACGA	99	62	Guerrero-Preston et al.[24]
KCNA5	QMSP	TTTTTTGACGTTAGGGTTAAGC	GAACGCCTAACGTCAAACTC	AGAGGGGTCGGTCGATCGTTGG	103	60	NA, MethylCap-Seq
SIPA1	QMSP	TTCGAGTCGAGGTTAGTTC	CAAATCGACTAACCTCTTCG	CGTAGCGGTAGCGATGTAGGC	124	60	NA, MethylCap-Seq
TBX4	QMSP	TTCGTTTTTAGTTCGAGTTGC	CTACGCTCTCAATCCTACGC	CGGCGTTAGTGGACGCGG	99	60	NA, MethylCap-Seq
TIMP3	QMSP	GCGTCGGAGGTTAAGGTTGTT	CTCTCCAAAATTACCGTACGCG	AACTCGCTCGCCCGCCGAA	95	62	Sun et al.(19)
ACTB	MSP	TAGGGAGTATATAGGTTGGGGAAGTT	AACACACAATAACAAACACAAATTCAC		103	57	Melchers et al.[29]
DAPK meth	MSP	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA		98	60	Melchers et al.[29]
DAPK unmeth	MSP	GGAGGATAGTTGGATTGAGTTAATGTT	CCCTCCCAAACACCAACC		101	60	Melchers et al.[29]

Abbreviations: NA, not applicable; (Q)MSP, (quantitative) methylation specific pcr; (un)meth, (un)methylated.

levels for each sample were calculated as ratios using absolute measurements: the average DNA quantity of the gene of interest divided by the average DNA quantity of *ACTB* and then multiplied by 10,000.

The PubMed electronical database was searched for DNA methylation biomarkers that were reported to be hypermethylated in saliva of head and neck SCC patients compared to saliva of healthy controls. This search revealed four genes, *EDNRB* [19], *HOXA9* [24], *NID2* [24] and *TIMP3* [20] which were used as a reference. QMSP primers and probes were selected from literature for *EDNRB, HOXA9, NID2* and *TIMP3* [19,20,24] (Table 2).

Statistical analysis

The Mann-Whitney U test was used for comparing MethylCap-Seq read counts of OSCC and leukocytes and was also used for comparing methylation levels between saliva of patients and controls. Optimal cut-offs were determined by ROC-curves. The diagnostic potential of the biomarkers in detecting OSCC in saliva was determined by the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Differences in methylation levels between the tumors and saliva within the OSCC patients were compared using the related Wilcoxon signed-rank test. All tests were performed two-tailed. Results were considered significant when $p < 0.05$ or FDR < 0.05. Statistical analysis was performed with IBM SPSS Statistics 23 (Statistical Package for the Social Sciences, Inc., Chicago, IL, USA).

RESULTS

Selection of OSCC specific methylation markers

With the MethylCap-Seq analysis, a total of 11.6 to 22.3 x 10^6 reads were sequenced per sample. Approximately 6.91 to 14.6 x 10^6 unique reads could be mapped back to the genome per sample [22,23]. Statistical analysis of reads around the transcription start site resulted in a ranking list of the 5000 most significant equally methylated regions among the 12 OSCCs. In total 335 methylation cores (MCs) representing 319 genes were significantly differentially methylated between the 12 OSCC samples and the two leukocyte pools (Supplementary data 1). Of these 335 MCs, 53 MCs were not hypermethylated in the leukocytes (≤2 reads). Seven of these MCs were hypermethylated (≥3 reads) in all OSCC and had a 100% positive and negative predictive value for hypermethylation in OSCC and leukocytes. These seven MCs were associated with six genes: *C11orf85*, *CMTM2*, *FERMT3*, *KCNA5*, *SIPA1* and *TBX4*. Semi-quantitative comparison with the methylation data in the Map of the Human Methylome showed no methylation in a panel of 80 reference samples that were considered as samples not associated with OSCC (thus considered as negative controls). For *TBX4*, *CMTM2* and *FERMT3* no suitable QMSP primers/probes could be designed or (Q)MSP did not pass the

technical validation. The design and technical validation of *C11orf85*, *KCNA5* and *SIPA1* QMSP was optimal for further analysis. In addition, based on literature search we included *EDNRB* [19], *HOXA9* [24], *NID2* [24] and *TIMP3* [20], as these genes were reported to be associated with OSCC in saliva.

Technical validation of OSCC-specific methylation markers to detect tumor cells in saliva from patients with OSCC

To select methylation markers with high sensitivity and high specificity, we collected a total of 2 ml of saliva from 10 patients with OSCC and from 10 non-cancer controls (five orthognathic and five dental implant patients) referred to as controls in this study (Table 1). Median amount of isolated DNA from saliva was 64 µg (range: 6 to 140 µg) among the OSCC patients, 32 µg (range: 16 to 75 µg) among the orthognathic patients and 32 µg (range: 20 to 57 µg) among the dental implant patients (Table 1). There were no significant differences in DNA yield from the pellets between the OSCC, orthognathic and dental implant patients.

QMSP analysis of the seven selected methylation markers on bisulfite-treated DNA from saliva cells from 10 OSCC patients and 10 controls, revealed significant differences in methylation levels of *EDNRB* ($p = 0.016$) and *KCNA5* ($p < 0.001$) (Figure 2). In fact, methylation of *C11orf85*, *HOXA9, NID2* and *SIPA1* was detected in all controls and methylation of *EDNRB* in 50% of the controls (not associated with age) (Figure 2). Five control patients were significantly younger than the OSCC patients (Table 1). Comparing QMSP data from saliva from OSCC with either controls of similar or younger age, revealed a difference for only *KCNA5* methylation (both OSCC-patients vs younger or older controls $p = 0.001$) and *EDNRB* methylation (only OSCC vs younger controls $p = 0.003$) (data not shown). Age-matched analysis did not affect the results of the other methylation markers.

One explanation for the fact that not all markers were hypermethylated in saliva cells of OSCC patients compared to saliva cells of controls could be that the original tumor is not methylated for each of these methylation markers. To evaluate the effect on the sensitivity and NPV of detecting tumor cells in saliva in patients with methylated tumor tissues, the methylation status of these seven markers was tested in available tumor tissues of these same 10 OSCC patients. Methylation of four markers (*EDNRB, C11orf85, KCNA5* and *SIPA1)* was detected in all 10 tumor tissues (Supplementary data 2). Methylation was detected in nine (*HOXA9*) and seven (*NID2* and *TIMP3*) of the 10 tumor tissues (Supplementary data 2). When performing the analysis with OSCC cases showing methylation of tumor tissue of *HOXA9, NID2* or *TIMP3,* no differences were found in methylation levels in saliva between OSCC cases and controls (data not shown).

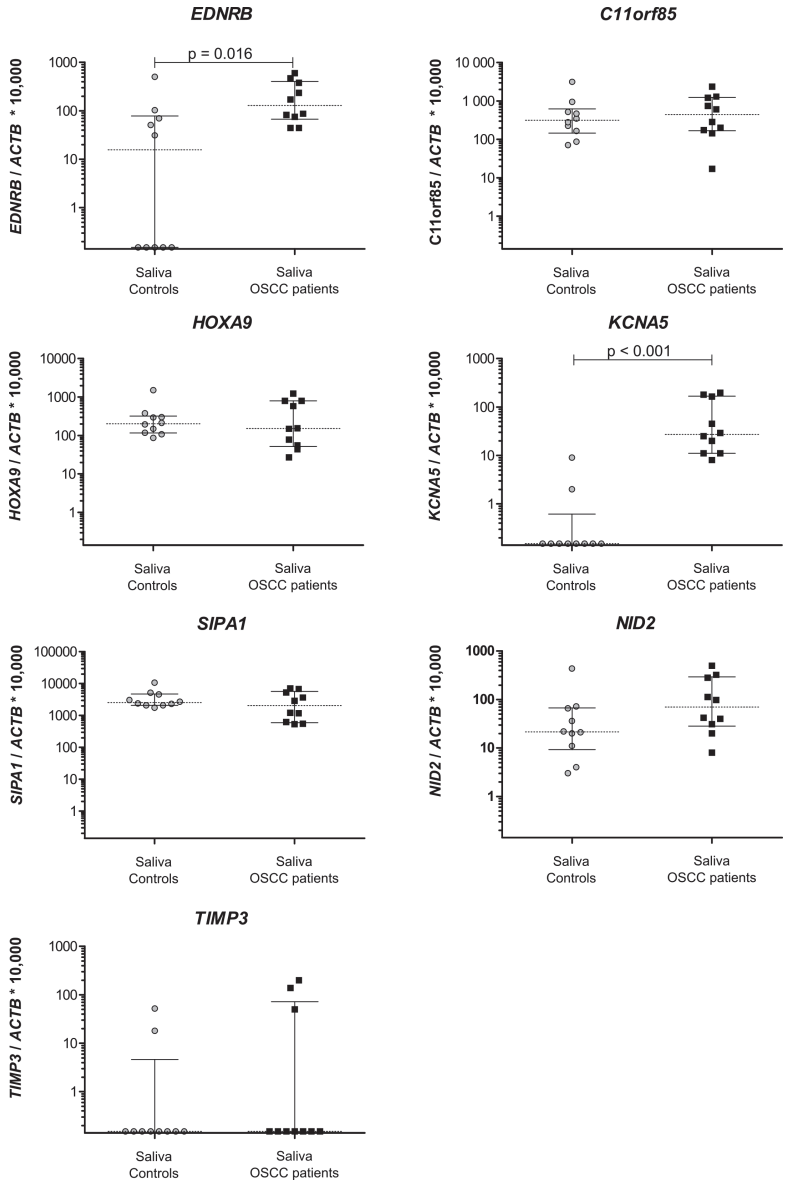


Figure 2. DNA methylation levels of seven OSCC-specific markers in saliva cells of OSCC patients and healthy controls. QMSP analysis of seven methylation markers using DNA extracted from cells in saliva collected from 10 OSCC patients (saliva OSCC patients) and as healthy control saliva from five younger and five age-matched controls (saliva controls). Methylation levels on the x-axis are defined as the average DNA quantity of the gene of interest divided by the average DNA quantity of *ACTB* and then multiplied by 10,000. Dotted and continuous line represents median with interquartile range. Only statistically significant differences ($p < 0.050$, using the Mann-Whitney-U test) between saliva of 10 controls and 10 OSCC samples are shown.

Table 3. OSCC diagnostic potential in saliva of the selected methylation markers

	A. OSCC patients (n = 10) vs all controls (n = 10)						B. OSCC patients (n = 10) vs age-matched controls (n = 5)					
Gene name	**AUC**	**Optimal cut-off**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**AUC**	**Optimal cut-off**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
C11orf85	0.56	559	50	80	71	62	0.44	533	50	60	71	38
EDNRB	0.82	38	100	60	71	100	0.68	22	100	40	77	100
HOXA9	0.44	479	40	90	80	60	0.39	479	40	80	80	40
KCNA5	0.99	5	100	80	83	100	0.98	10	90	100	100	83
NID2	0.72	27	80	60	67	75	0.66	38	70	80	88	57
SIPA1	0.40	5239	20	90	67	53	0.32	5239	30	80	75	36
TIMP3	0.57	34	30	90	75	56	0.65	25	30	100	100	42

A ROC analysis of methylation in saliva between 10 OSCC patients and 10 control patients (A) and an age-matched analysis of saliva between 10 OSCC patients and five dental implant control patients (B) for the optimal cut-off points to detect OSCC in saliva. *KCNA5* combined with *TIMP3* could detect OSCC with a 100% sensitivity, specificity, PPV and NPV in an age-matched analysis**.**
Abbreviations: AUC, area under the curve; PPV, positive predictive value; negative predictive value; OSCC, oral squamous cell carcinoma.

To evaluate the possible clinical relevance for the detection of tumor cells in saliva independent on methylation status in the original OSCC tissue, we determined the optimal cut-off to discriminate between OSCC and non-cancer control DNA in saliva cells for each marker. ROC analysis among all 20 patients (10 OSCC patients versus 10 controls), revealed a high area under the curve (AUC) with a 100% sensitivity and 100% NPV of *EDNRB* (AUC 0.82) and *KCNA5* (AUC 0.99) for detecting patients with OSCC using saliva cells (Table 3A). The other five markers showed a lower sensitivity and NPV when using the most optimal cut-off. The analysis on the age-matched patients (10 OSCC versus five aged-matched controls) resulting in other optimal cut-offs also showed a 100% sensitivity and 100% NPV for *EDNRB* (AUC 0.68) (Table 3B). For *KCNA5* (AUC 0.98) both the sensitivity (90%) and NPV (83%) decreased slightly, but interestingly with the highest specificity (100%) and positive predictive value (PPV 100%) (Table 3B). As none of the markers had a 100% diagnostic potential, we combined one or more methylation markers in age matched samples. This analysis revealed that *KCNA5* combined with *TIMP3* had the highest diagnostic potential (100% for this limited dataset) in detecting saliva cells in patients with OSCC (data not shown).

DISCUSSION

DNA methylation of OSCC specific tumor markers might be useful as biomarkers for early detection of new primaries or local recurrences in OSCC patients, preferably prior to clinical manifestation. In this study we used the methylome of tissue biopsies of 12 patients with OSCC generated using genome-wide methylation screening by MethCapSeq analysis [23] to identify DNA methylation biomarkers with a high diagnostic potential for the detection of OSCC. Seven new OSCC-specific biomarkers representing six genes were identified by selection of equally methylated markers between all 12 OSCC and not methylated in two pools with leukocytes from four different individuals. Moreover, the acquired highest ranking methylated candidate markers were compared to a vast methylome database of over 80 different samples considered as negative control samples. For the validation of these markers using QMSP, we could design optimal primers/probes assays for three markers (*C11orf85, KCNA5* and *SIPA1*). To evaluate biomarkers with the highest performance, DNA was isolated from saliva cells acquired from 10 OSCC patients and their corresponding tumor tissues. Saliva cells from five younger controls and five age-matched controls planned to undergo benign surgery served as healthy (non-cancer) controls. *KCNA5* was the best marker (independent of age) as it was significantly hypermethylated in OSCC saliva cells in comparison to control saliva. The possible clinical relevance of *KCNA5* is further illustrated by the very high sensitivity (90%), NPV (83%), specificity (100%) and PPV (100%), the highest of all markers tested in this study (Table 3B). Moreover, a panel of *KCNA5* and *TIMP3* could

further improve the diagnostic potential of detecting OSCC in saliva cells (100%) in an age matched analysis. Due to the limited size of our pilot group, the diagnostic potential of these biomarkers must be validated on a larger independent and prospective cohort. Similarly, a saliva database containing samples of 5-year-follow-up, pre- and post-operative as well as pre-malignant cases should be constructed for prospective studies and to assess the background methylation caused by non-tumor cells.

The use of molecular markers for the early detection and monitoring treatment response and disease progression using body fluids like saliva, sputum, plasma, cerebrospinal fluid and urine [10,33] has limited clinical utility today [34] but has great promise to contribute to improved clinical care by early detection of OSCC or monitoring the treatment response. Since DNA methylation is important in carcinogenesis, occurs early in tumorigenesis and is detectable in patient saliva [35], DNA methylation markers could contribute to the early detection of local recurrences of OSCC. Additionally, aberrations in DNA methylation arise early in tumorigenesis [14]. Therefore, our results warrants further analysis in larger independent cohorts.

Several methylation markers for the detection of cells in saliva of patients with OSCC were reported previous (*EDNRB, HOXA9, NID2* and *TIMP3*) [19,20,24]. As a comparison to our new markers, we analyzed these markers in parallel on the same samples using QMSP. In our cohort, methylation of *HOXA9* and *NID2* was detected in all saliva cells of health individuals. Methylation of *EDNRB* was observed in 50% of these saliva, but the difference between saliva of OSCC patients and of age-matched controls was not significant. An explanation for the frequent methylation in normal control, especially in the saliva of the "older" age-matched "healthy" (non-cancer) saliva cells is that methylation of many genomic sequences has been reported to increase with age [36]. Therefore, methylation of these reported genes are not suitable as methylation markers in the "older" age-matched OSCC cohort.

Note that the four markers selected from literature showed significant methylation in our age-matched samples from normal saliva, which can also be an explanation why these markers were not present in our selected list of 2276 highest ranking methylation cores from the MethylCap-seq analysis of OSCC tissue samples.

With the genome-wide methylation analysis, within the methylome of millions of methylated DNA fragments in 12 OSCC samples, we eventually identified and validated three of the six new candidate markers for OSCC *(C11orf85, KCNA5* and *SIPA1)*. The pathophysiology of the novel genes related to OSCC or other types of cancer is not yet fully clarified. *KCNA5* is a member of the voltage-gated potassium (K_V) channel subfamily A [37]. In Ewing sarcoma cells methylation of the *KCNA5* promoter region is correlated with cell survival and

proliferation [38]. Signal-induced proliferation associated protein 1 (*SIPA1*) is located at the 11q13 chromosome close to *CCND1* (cyclin D1) and is known for influencing growth factors and cytokines by regulating *RAP1* in hematopoietic cells [39,40]. Loss of *SIPA1* resulted in myeloproliferative disorders in mice [40]. The interaction between *SIPA1* and *RAP1* is also associated with metastasis in breast and prostate cancer by mediating cell adhesion signaling and metastasis suppressor gene signaling [40]. Recently, *SIPA1* was found to be overexpressed in OSCC and correlated to lymph node metastasis [41]. *C11orf85* also called *MAJIN* (membrane anchored junction protein) plays a role in telomere attachment to the inner nuclear membrane during meiosis [42,43]. *C11orf85/MAJIN* is related to cancer as one of the genes in a 92-gene signature that is prognostic for overall survival in multiple myeloma patients [44]. Currently, no studies are available that report the exact role of *C11orf85/MAJIN* in oncogenesis. The biological significance of these three methylated genes in OSCC has not been elucidated in great detail and needs further investigation in future.

CONCLUSIONS

In conclusion, using the methylome of 12 OSCC tissue samples based on a genome-wide methylation screening approach, we have identified several novel biomarkers commonly methylated in OSCC. With one of these methylation markers (*KCNA5*) cells in saliva that are associated with OSCC patients could be detected with a high diagnostic potential. Moreover, it is of interest to perform a larger scale evaluation for *KCNA5* combined with *TIMP3, given the* 100% diagnostic potential found for detecting OSCC cells in saliva. Irrespective of the small study size, our findings demonstrate the high sensitivity of Quantitative Methylation Specific PCR for detecting methylation on saliva cell DNA. DNA methylation detection using saliva has potential as an easy, low-cost, non-invasive and accurate diagnostic tool to improve the early detection of local recurrences or second primary tumors in OSCC. Our findings warrant evaluation of the clinical relevance of these methylation markers in larger cohorts.

SUPPLEMENTARY DATA

Supplementary data 1. OSCC hypermethylation markers selected with MethylCap-Seq data. All 335 Methylation Cores (MCs) with a p-value < 0.05 between the 5000 highest ranked MCs in OSCC compared to the 2276 MCs available in the MethylCap-Seq data of the two leukocyte pools by Mann-Whitney-U using R and the wilcox.test function. The final seven MCs representing six genes with a 100% positive and negative predictive value defined by ≤2 reads in both leukocytes pools as well as ≥3 reads in all 12 OSCC are highlighted in bold and underlined.

					Number of reads													
Gene name	**Chromosome locus**	**Gene regio start (GSE42409)**	**Gene regio end (GSE42409)**	**p-value (Mann-Whitney U test)**	**OSCC patient 1**	**OSCC patient 2**	**OSCC patient 3**	**OSCC patient 4**	**OSCC patient 5**	**OSCC patient 6**	**OSCC patient 7**	**OSCC patient 8**	**OSCC patient 9**	**OSCC patient 10**	**OSCC patient 11**	**OSCC patient 12**	**Leukocyte pool 1**	**Leukocyte pool 2**
NUDT14	14	105649604	105650313	0.000	7	9	10	8	8	5	6	9	11	6	10	9	14	14
AC012074.3	2	25595094	25595647	0.000	9	9	9	5	9	10	6	13	9	8	10	10	14	14
PIGQ	16	614601	615239	0.000	5	9	9	11	2	4	8	5	9	9	14	7	15	15
AC021021.1	2	6635378	6635815	0.000	8	6	7	8	6	6	14	9	10	9	12	8	14	14
KCNA5	12	5153088	5153505	0.000	6	4	11	6	5	10	12	11	7	6	10	3	1	1
RAPSN	11	47470539	47471210	0.000	7	7	8	12	7	10	8	14	7	10	11	8	14	14
RP11-56M3.1	10	92913015	92913355	0.000	9	10	7	10	5	7	7	13	9	8	10	9	13	13
KIF22	16	29800874	29801501	0.000	3	8	5	8	6	9	11	5	6	10	9	7	12	12
AC007272.7	2	201963822	201964264	0.000	4	7	7	6	6	4	7	9	6	6	10	6	10	10
AL359844.1	10	70782804	70783197	0.000	5	12	10	6	4	6	9	13	9	6	11	8	14	14
NAT12	14	57855731	57856072	0.000	8	9	9	6	5	6	7	12	8	10	8	6	4	4
EIF2S2	20	32702015	32702335	0.000	6	10	8	6	9	9	14	15	8	9	11	4	3	3
ACTBP11	1	224052444	224052660	0.000	6	10	9	4	5	11	9	13	12	4	14	1	17	16
PTPRS	19	5341427	5341949	0.000	7	9	5	7	5	5	14	5	7	7	8	8	12	12
AC017104.4	2	232254143	232254467	0.000	5	12	9	10	3	11	12	11	10	5	13	7	15	15
TBX4	17	59531961	59532563	0.000	5	13	7	5	4	9	13	9	11	4	12	4	1	0
PABPCP2	2	147344801	147345449	0.000	8	13	9	10	9	4	13	15	8	9	10	7	15	15
AC113607.1	2	905373	905826	0.000	3	10	17	10	10	5	11	18	16	3	10	10	20	19
TH	11	2192576	2193202	0.000	5	11	6	12	8	2	7	15	4	5	13	5	15	15
SIPA1	11	65408027	65408751	0.000	6	9	13	7	4	10	16	9	14	3	9	7	2	1
GPR39	2	133174634	133175088	0.000	9	14	8	5	4	5	17	5	9	4	17	5	17	17
ING5	2	242665670	242666172	0.000	3	10	2	9	6	8	13	9	7	5	13	5	14	15
C11orf85	11	64739412	64739716	0.000	9	8	9	6	4	7	15	11	8	4	11	3	2	2
AC011530.1	19	46318069	46318747	0.000	6	10	12	9	9	3	13	11	10	3	11	5	3	3
AL139130.1	1	156357691	156358065	0.000	11	8	8	3	1	8	16	10	13	4	8	4	1	0
MUC2	11	1075204	1076015	0.000	4	13	7	7	7	10	8	17	7	8	17	10	16	16
SLC22A20	11	64981227	64981938	0.000	4	8	10	9	8	9	17	12	7	5	13	4	3	3
AP001476.3	21	47457079	47457365	0.000	3	10	6	9	5	7	13	11	7	6	16	4	14	15
C3orf24	3	10149532	10150224	0.000	5	10	7	8	6	11	10	10	15	6	10	7	13	13
AL031296.2	1	12587915	12588225	0.000	7	11	7	5	5	5	12	11	4	3	11	6	12	12
CENPB	20	3765976	3766968	0.000	5	13	7	12	5	12	6	14	9	9	15	4	15	15
CMTM2	16	66613072	66613394	0.000	6	12	5	3	4	10	13	8	10	4	12	4	1	2
ODF3	11	195013	195431	0.000	5	12	8	7	7	7	10	9	9	7	10	6	11	11

Supplementary data 1. Continued

Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	Number of reads													
					OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
TBX4	17	59532564	59532803	0.000	6	12	3	7	4	8	15	8	7	5	8	5	1	2
AL512362.1	14	104917026	104917422	0.000	7	9	7	10	7	7	11	10	7	7	11	5	15	16
GSC	14	95237544	95237981	0.000	5	5	9	4	7	12	6	8	14	12	11	6	2	3
PATZ1	22	31740363	31741170	0.000	7	7	9	5	7	9	16	10	9	3	9	6	2	3
TBC1D3	17	36282803	36283142	0.000	5	11	4	10	4	13	16	8	5	8	11	8	15	14
AL355075.1	14	20903481	20903844	0.000	7	6	8	7	7	6	11	11	6	6	12	6	13	14
SNED1	2	241936268	241936700	0.001	6	8	3	9	9	6	6	13	5	5	9	7	13	12
AP001476.3	21	47456602	47457078	0.001	4	10	6	9	5	7	7	10	6	10	9	6	14	13
AC068993.1	12	79187669	79188167	0.001	4	7	4	8	5	7	9	10	6	5	11	5	10	10
PCK2	14	24562608	24563016	0.001	4	11	2	10	4	6	9	13	8	6	7	6	13	12
AC007189.1	2	49142926	49143500	0.001	8	12	13	11	7	6	21	15	14	8	18	10	19	18
DIP2C	10	737199	737975	0.001	11	17	10	7	6	4	16	11	8	10	10	5	16	15
C20orf197	20	58629936	58630496	0.001	1	10	4	9	7	13	8	10	10	3	9	13	3	2
5_8S_rRNA	16	33964201	33964553	0.001	8	12	8	9	3	5	6	16	11	5	9	7	13	13
AC021016.2	2	219218778	219219347	0.001	8	9	7	6	5	8	10	11	9	6	13	7	13	14
AC010928.2	18	58329757	58330190	0.001	9	13	6	7	3	4	12	6	12	3	7	7	12	12
FERMT3	11	63974229	63974772	0.001	5	5	5	7	5	9	11	6	8	5	10	7	1	2
CHST6	16	75529780	75530072	0.001	2	15	8	4	5	6	6	13	4	5	15	5	14	13
ATL3	11	63439439	63440134	0.001	5	11	7	8	5	5	9	12	7	7	11	6	11	11
RP11-165M6.1	13	107078138	107078692	0.001	12	6	5	6	7	5	12	11	7	7	13	5	12	12
RPL13AP3	14	56233075	56233429	0.001	7	13	10	7	9	8	9	13	12	6	12	9	15	14
OR1M1	19	9204180	9204744	0.001	6	11	7	7	6	4	11	11	10	7	8	4	12	13
AC131097.1	2	242845825	242846198	0.001	7	12	8	3	6	4	7	12	8	5	11	9	12	13
U3	17	42380910	42381319	0.001	6	10	4	6	7	4	8	6	12	6	7	7	4	4
WFIKKN1	16	678644	679024	0.001	7	12	3	9	8	10	12	13	10	7	11	14	15	14
RPS10L	20	820108	820639	0.001	7	9	5	8	4	9	5	9	10	6	10	6	10	10
AL135798.1	1	117284700	117285259	0.001	7	17	11	16	12	9	14	14	15	9	17	8	17	18
AL356957.13	1	149287471	149287898	0.001	10	5	6	6	5	9	9	11	6	5	10	6	3	2
AC008069.2	2	17036367	17036607	0.001	8	11	7	6	7	9	13	12	6	10	13	4	13	14
AL008723.1	22	32665325	32665766	0.001	6	14	9	8	4	11	7	14	7	8	11	6	13	14
ZNF547	19	57873156	57873573	0.001	4	11	11	4	5	5	8	11	10	8	13	3	12	12
IGHA1	14	106174533	106175030	0.001	8	14	6	13	13	7	10	10	16	11	15	7	15	15
BX322557.4	21	46772281	46773134	0.001	6	12	3	11	6	4	9	18	8	7	8	6	13	14
HES5	1	2463378	2464184	0.001	8	13	5	10	9	10	6	14	6	7	9	11	14	13
AP001466.1	21	15308923	15309360	0.001	5	10	4	5	9	9	10	12	7	6	10	3	11	11
C2orf85	2	242812123	242812826	0.001	7	14	7	6	7	9	10	10	14	10	13	7	13	13
AL451069.4	10	134243532	134244554	0.002	9	11	6	7	8	11	7	12	12	5	14	6	13	14
FCN3	1	27702197	27702709	0.002	6	10	6	5	6	6	9	8	6	4	9	7	9	9
AL139188.2	13	30438274	30438770	0.002	6	17	8	7	3	6	8	18	10	9	15	9	15	16
TMEM132C	12	128899599	128900228	0.002	5	13	5	8	4	12	10	13	12	4	10	9	14	13
AC074212.1	19	46236309	46236955	0.002	7	11	10	12	6	6	16	17	11	11	15	7	20	22

Supplementary data 1. Continued

					Number of reads													
Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
C21orf77	21	33948372	33948737	0.002	7	11	6	8	5	9	13	9	4	6	10	10	12	13
ACAP3	1	1246319	1246922	0.002	6	18	10	8	15	9	16	17	14	11	15	8	17	17
ADAD2	16	84224448	84224795	0.002	6	11	10	11	5	5	12	16	10	5	15	9	14	14
KIAA0323	14	24897965	24898530	0.002	4	8	5	9	4	6	6	13	7	7	9	7	10	10
C2CD2	21	43374507	43375097	0.002	4	14	10	9	11	7	10	16	16	8	14	3	15	16
TIMM13	19	2428888	2429827	0.002	10	10	12	10	6	4	16	15	9	9	12	8	14	14
AC110299.1	2	242456556	242457232	0.002	4	8	8	8	7	8	8	9	11	5	9	6	12	13
U1	1	146550207	146550837	0.002	7	18	7	4	4	9	22	15	8	5	8	5	2	3
AP005380.2	18	5132955	5133396	0.002	3	11	6	9	7	3	11	12	10	6	13	4	12	13
SETDB1	1	150896594	150896961	0.002	8	6	3	9	6	4	12	10	8	4	8	4	10	10
AC103563.10	2	95638175	95638602	0.002	3	9	8	6	6	4	6	10	6	9	12	6	10	10
CALM2	2	47405104	47405186	0.002	0	1	1	0	1	0	0	1	0	1	1	1	0	0
MSMB	10	51548149	51548150	0.002	1	0	1	0	1	0	1	0	1	1	1	0	0	0
GLS2	12	56881692	56881936	0.002	0	0	1	0	1	1	1	1	1	0	1	0	0	0
HSD11B2	16	67463366	67463367	0.002	0	0	1	1	1	0	1	0	1	0	1	1	0	0
ETV2	19	36132506	36132707	0.002	0	1	0	1	1	0	1	1	0	1	1	0	0	0
FCRLB	1	161689631	161689644	0.002	0	1	1	1	0	0	1	1	0	1	1	0	0	0
LIPA	10	91175701	91176201	0.002	3	10	7	5	4	8	10	10	7	7	10	5	10	10
SYT14	1	210111479	210111996	0.003	9	4	3	3	4	12	16	12	8	5	4	2	2	1
SKI	1	2157600	2158529	0.003	8	8	4	7	7	8	6	13	6	5	9	7	12	11
ELF5	11	34534937	34535481	0.003	7	13	7	6	4	7	7	15	4	7	16	9	13	13
AC133919.5	16	90160195	90160692	0.003	6	14	8	6	8	6	10	11	11	4	7	7	5	5
AC012075.1	2	81694490	81694825	0.003	5	13	6	9	4	8	8	17	8	6	14	3	14	13
SFRS6	20	42084031	42084724	0.003	7	8	3	5	8	7	5	10	6	9	8	6	9	9
AC006269.1	17	53638377	53639063	0.003	6	16	10	6	7	7	17	15	14	8	13	7	15	16
AC018804.7	2	130986044	130986416	0.003	10	9	7	7	6	4	13	16	10	10	12	5	13	14
AC025279.1	16	29300194	29301153	0.003	5	9	6	8	9	9	4	9	12	12	12	7	13	12
hsa-mir-410	14	101532662	101533132	0.003	3	11	5	12	7	7	5	13	13	6	10	5	12	13
WDR24	16	738987	739624	0.003	19	15	12	14	8	8	15	15	18	8	19	10	18	18
LRRC30	18	7231085	7231660	0.003	6	19	5	10	4	6	13	13	12	8	11	4	15	14
C16orf81	16	89225822	89226056	0.003	5	13	5	10	8	9	7	12	9	3	12	8	12	13
C17orf62	17	80409641	80409982	0.003	7	9	7	4	7	6	9	9	6	7	10	4	5	5
AC080112.1	17	38523323	38523956	0.003	6	9	6	8	7	4	5	13	7	7	10	7	10	10
F2	11	46741219	46741868	0.003	9	15	7	8	8	12	7	15	8	7	11	7	14	13
AL359457.2	13	20134785	20135212	0.003	5	12	8	6	5	6	6	16	10	8	11	7	13	12
AP001266.1	11	65546062	65546392	0.003	9	13	10	9	6	8	13	12	16	12	14	9	14	14
AL356961.2	13	112760878	112761112	0.003	8	4	8	4	1	9	17	11	7	1	9	2	2	1
SYT8	11	1846938	1847982	0.004	8	18	12	17	10	14	10	28	6	9	21	11	20	21
GPR25	1	200842396	200842812	0.004	7	7	8	7	9	14	19	10	7	2	11	10	5	4
C2orf65	2	74875016	74875561	0.004	7	9	6	6	8	8	7	12	7	4	12	5	5	5
CASP7	10	115478883	115479141	0.004	6	9	6	7	7	6	15	8	12	8	9	6	4	5

Supplementary data 1. Continued

Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	Number of reads													
					OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
AL591848.2	1	246954217	246954457	0.004	8	13	6	4	6	7	11	9	8	6	11	4	12	11
hsa-mir-381	14	101512070	101512894	0.004	6	14	5	6	6	7	9	14	13	7	15	9	14	13
PYY2	17	26553901	26554658	0.005	7	10	9	5	9	6	13	14	10	5	11	5	12	13
AP000345.1	22	23909004	23909277	0.005	5	11	8	5	4	7	14	11	8	5	10	6	11	11
CTA-299D3.1	22	48943316	48944061	0.005	3	13	6	10	6	6	12	9	10	9	6	5	11	11
AL122127.9	14	106351596	106351950	0.005	2	8	11	9	5	7	6	10	9	10	8	6	5	5
CHRM4	11	46407278	46407882	0.005	12	16	9	10	11	9	14	11	9	10	13	7	15	14
AP001623.1	21	43720930	43721824	0.005	6	8	8	8	6	7	13	9	7	4	10	5	10	10
BSND	1	55464529	55465160	0.005	6	9	7	5	6	6	7	13	4	7	8	5	11	10
ZNF570	19	37959287	37959750	0.005	7	15	8	6	4	8	11	13	10	4	7	10	12	13
MAFB	20	39319652	39320415	0.005	8	12	11	6	6	7	17	26	5	7	9	4	4	3
C13orf36	13	37247969	37248446	0.005	11	2	6	5	3	10	2	16	3	4	8	5	2	2
PPPDE1	1	244814626	244815003	0.006	10	7	3	6	8	4	10	9	3	7	11	9	10	10
ZNF583	19	56916205	56916724	0.006	7	10	5	9	12	6	11	8	10	7	12	7	11	11
AP001931.1	11	57520088	57520392	0.006	9	10	11	8	7	10	14	15	12	11	7	5	7	7
TUBGCP2	10	135125479	135125859	0.006	4	9	6	9	6	5	6	8	11	5	11	4	11	10
AQP5	12	50355718	50356251	0.006	3	16	6	9	6	6	9	10	11	9	12	6	13	12
CCDC79	16	66835674	66836220	0.006	7	11	10	8	8	6	8	13	9	6	14	8	13	12
DLGAP1	18	3879613	3879881	0.006	6	26	8	11	7	4	14	21	14	8	12	9	18	18
MASP2	1	11108391	11108763	0.006	7	7	3	9	6	6	8	12	5	8	8	7	5	5
SSU_rRNA_5	21	9826641	9826839	0.006	28	10	16	76	35	5	12	37	12	30	49	25	8	9
FAM38A	16	88804843	88805299	0.006	8	13	7	9	6	12	7	18	9	4	9	11	13	13
CCDC79	16	66835180	66835673	0.007	8	18	13	8	8	7	13	14	11	8	13	7	14	15
AL691429.2	10	134778953	134779462	0.007	6	10	7	9	3	7	7	11	13	6	12	4	12	11
C15orf60	15	73735188	73735531	0.007	6	13	5	10	8	4	12	15	4	10	10	6	12	13
CCL15	17	34330963	34330964	0.007	1	1	0	0	0	1	1	0	1	1	0	0	0	0
RAB22A	20	56884284	56884425	0.007	1	0	1	1	0	0	0	0	1	0	1	1	0	0
RP11-529I10.1	10	103329231	103329589	0.007	0	0	1	1	1	0	0	1	1	1	0	0	0	0
HSD17B12	11	43702084	43702130	0.007	1	0	1	0	0	1	1	0	0	1	1	0	0	0
GYLTL1B	11	45944422	45944514	0.007	1	1	1	0	0	0	1	0	1	1	0	0	0	0
AP003108.2	11	61276076	61276077	0.007	0	0	1	1	0	0	0	1	1	1	1	0	0	0
BCAT1	12	25101998	25102064	0.007	0	0	1	1	0	1	0	0	1	1	1	0	0	0
AC084398.1	12	102323488	102323489	0.007	1	1	0	0	1	0	1	0	0	1	0	1	0	0
AB019437.11	14	107150907	107150953	0.007	0	1	0	1	0	0	0	1	1	1	1	0	0	0
ARNT2	15	80696362	80696410	0.007	1	0	0	1	1	0	0	0	1	1	1	0	0	0
PLD6	17	17109465	17109466	0.007	1	0	0	1	1	0	1	0	0	1	0	1	0	0
MFAP4	17	19290689	19290762	0.007	1	0	0	1	0	1	0	1	0	1	1	0	0	0
SECTM1	17	80291235	80291459	0.007	1	0	1	1	0	0	0	1	1	0	1	0	0	0
MYO1F	19	8644031	8644155	0.007	1	1	0	1	0	0	1	0	1	1	0	0	0	0
C1orf113	1	36772133	36772221	0.007	0	0	1	0	0	1	1	1	0	1	1	0	0	0
CACNA1S	1	201082700	201082731	0.007	0	0	1	1	1	0	1	0	0	0	1	1	0	0

Supplementary data 1. Continued

					Number of reads													
Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
TMEM18	2	678865	678866	0.007	1	0	0	1	1	0	0	1	0	1	1	0	0	0
ZFAND6	15	80350338	80350339	0.007	0	1	1	0	0	1	0	1	0	0	1	1	1	1
U6	1	22314468	22314516	0.007	0	1	0	1	0	0	1	0	1	1	1	0	1	1
SPI1	11	47400930	47401026	0.007	1	0	0	1	0	1	0	0	1	0	1	1	1	1
SLC15A3	11	60720092	60720229	0.007	0	0	0	1	1	0	1	1	0	0	1	1	1	1
AP000770.1	11	116510340	116510341	0.007	1	0	0	1	1	0	0	1	1	0	0	1	1	1
SCARNA11	12	8748456	8748579	0.007	1	1	0	1	0	0	0	0	1	0	1	1	1	1
COMP	19	18903207	18903230	0.007	0	0	1	1	0	0	1	1	0	0	1	1	1	1
INSM1	20	20348794	20348956	0.007	0	0	1	1	1	0	1	1	0	0	1	0	1	1
C16orf81	16	89226057	89226378	0.007	7	12	6	12	8	15	9	11	10	5	15	9	13	14
AC012652.1	15	41521764	41522036	0.007	8	8	2	9	11	5	7	10	5	7	11	7	10	10
CNIH	14	54910018	54910240	0.007	7	13	7	10	3	6	11	13	12	7	15	4	15	17
SPO11	20	55904448	55905206	0.008	6	16	8	9	8	18	7	15	6	11	14	6	18	16
AC018755.9	19	52101660	52102180	0.008	6	15	8	11	11	6	11	22	8	12	14	8	16	15
AC008271.1	2	15830701	15831610	0.008	10	8	7	9	7	13	17	13	11	14	14	9	8	8
TACR2	10	71175640	71176127	0.008	8	8	7	5	6	12	17	12	9	8	8	7	12	13
5S_rRNA	1	228770930	228771604	0.008	14	29	7	6	2	8	32	30	7	16	23	17	31	27
CDKN3	14	54861108	54861777	0.008	8	9	7	5	8	9	10	12	14	6	9	5	11	11
AL356957.13	1	149287899	149288411	0.008	8	9	6	7	4	5	10	9	7	3	15	3	4	4
AC118470.1	1	247802955	247803176	0.009	12	7	5	4	4	7	20	7	6	6	5	2	3	2
AL139161.2	1	236136540	236137128	0.009	6	6	7	5	6	6	9	7	8	7	9	5	8	8
TMEM85	15	34515638	34515959	0.009	3	10	6	7	7	6	7	7	7	9	11	7	10	11
MRPL28	16	422537	423002	0.009	9	10	5	5	9	6	8	11	19	6	10	5	13	12
Y_RNA	14	100048449	100049145	0.009	6	14	6	13	8	5	14	13	15	8	7	8	13	14
AL928742.3	14	106004966	106005349	0.010	5	10	7	7	6	13	4	10	16	9	21	5	14	14
CALML5	10	5540692	5541293	0.010	4	15	7	8	8	4	9	15	4	8	16	11	13	13
INHA	2	220431911	220432323	0.010	8	8	4	12	6	10	11	9	11	5	17	4	13	12
GPS1	17	80008024	80008393	0.010	5	10	7	13	9	7	15	13	14	8	13	6	13	14
AC011491.1	19	6378815	6379216	0.011	9	14	7	6	6	5	13	11	7	6	10	4	12	11
AC104841.1	2	242165415	242165901	0.011	7	12	8	8	8	6	14	13	12	8	8	8	7	7
AL391244.1	1	1354451	1355097	0.011	10	14	11	13	8	12	8	17	14	11	18	6	15	15
ESPNP	1	17046419	17046814	0.011	8	15	10	7	6	7	11	10	8	6	19	10	17	15
RAB1B	11	66033857	66034662	0.011	11	9	4	10	8	8	11	19	13	7	9	7	17	15
TMEM79	1	156251158	156251235	0.012	0	1	1	0	1	0	1	1	0	0	2	0	0	0
GGT5	22	24642529	24642530	0.012	0	1	0	1	1	0	0	2	0	1	1	0	0	0
ALKBH7	19	6369985	6370742	0.012	6	7	7	7	6	6	11	8	7	7	11	4	9	9
BAIAP2	17	79006444	79007013	0.012	11	19	12	6	7	10	17	18	17	8	14	7	16	17
AL136038.2	14	64061697	64062069	0.012	11	22	8	9	10	5	13	17	19	13	16	5	17	17
AL358176.2	1	240799808	240800561	0.012	3	12	5	8	5	9	14	15	7	10	11	5	12	12
TBX4	17	59533693	59534236	0.012	6	11	9	6	5	7	15	11	10	6	13	5	3	1
GZMM	19	543219	543909	0.012	5	10	4	5	6	6	13	7	7	6	8	5	9	9

Supplementary data 1. Continued

Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	Number of reads													
					OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
CATSPER1	11	65793385	65793796	0.013	8	11	8	10	9	17	14	9	11	10	12	6	14	13
BACH1	21	30670042	30670853	0.013	8	8	4	9	8	5	5	14	10	7	7	8	10	10
P4HA3	11	74022781	74023219	0.013	3	5	6	7	9	8	10	9	9	5	11	4	5	5
MSGN1	2	17997725	17998458	0.014	6	8	5	5	11	9	8	10	10	6	7	6	10	11
BTBD6	14	105713084	105713865	0.014	8	17	10	9	7	7	7	14	16	8	11	6	14	13
AL359737.3	13	19173660	19174402	0.014	5	8	5	10	5	6	10	11	10	7	6	3	5	5
PLEKHN1	1	900206	900744	0.015	8	12	7	6	7	8	11	11	6	10	11	7	12	11
GDF2	10	48416585	48416977	0.015	2	11	10	12	5	5	10	10	7	7	11	3	13	15
RRP15	1	218457065	218457480	0.015	9	6	7	6	7	6	15	10	7	3	9	4	11	10
RASGRF1	15	79382595	79382766	0.015	8	1	0	2	2	9	11	11	1	1	3	2	1	0
AC104024.2	17	16884183	16885006	0.015	3	7	6	7	5	8	9	9	5	8	9	4	10	9
FAM108A6	22	22471677	22472380	0.015	6	10	3	9	5	11	17	6	9	7	13	4	5	5
NKX2-2	20	21496275	21496756	0.016	0	1	2	6	4	10	1	12	0	6	10	2	1	1
GBP5	1	89739002	89739591	0.016	2	13	10	9	6	4	13	15	11	4	8	9	12	12
RASGRP1	15	38857242	38857791	0.016	7	6	3	11	8	8	10	14	13	5	9	7	12	11
ATHL1	11	289630	290144	0.016	6	14	7	15	7	12	5	14	15	9	18	6	7	7
AL451043.2	1	147716091	147716663	0.016	13	21	13	11	13	10	29	16	18	16	25	15	21	22
RP4-697K14.1	20	62199420	62200049	0.016	10	13	11	6	5	12	14	14	9	4	13	7	3	5
RUSC1	1	155290535	155291063	0.016	5	1	6	6	3	12	1	7	12	5	13	3	3	2
AC124861.4	2	241196681	241197295	0.017	7	16	9	7	7	14	12	17	13	8	13	10	14	14
USP18	22	18631319	18631711	0.017	9	14	6	12	7	9	12	10	7	8	9	8	7	6
AC009237.7	2	96190971	96191608	0.017	8	18	11	11	5	9	15	13	13	9	19	7	15	15
FAM3B	21	42675620	42676210	0.017	9	13	6	12	3	4	21	16	6	10	9	3	14	16
C11orf85	11	64739717	64740014	0.017	5	9	9	6	5	8	16	10	6	5	8	4	4	5
MAD2L2	1	11751298	11751523	0.017	0	0	1	1	0	1	1	0	0	1	1	1	1	1
WFIKKN1	16	680974	681202	0.017	1	0	1	0	0	1	1	0	0	1	1	1	1	1
IRX6	16	55357787	55358034	0.017	1	0	1	1	0	0	1	0	1	1	0	1	1	1
FAM71E1	19	50978981	50980006	0.017	6	7	10	9	9	5	13	8	7	9	15	4	12	11
FKBP4	12	2901927	2902637	0.017	8	11	7	8	5	8	7	11	7	7	14	6	6	5
C16orf81	16	89225495	89225821	0.017	2	13	6	9	8	12	5	18	15	2	16	7	16	14
FAM92A2	15	41455529	41456044	0.018	5	10	6	10	3	6	9	10	8	5	8	4	9	9
RPL12L3	20	19804150	19804670	0.018	3	11	8	7	3	14	4	17	4	7	8	7	12	11
AC138969.3	16	16459071	16459683	0.018	0	10	1	15	5	14	5	9	17	6	19	12	14	15
FSCN2	17	79492961	79493634	0.018	5	6	2	9	9	7	7	8	10	8	14	7	10	10
C1orf159	1	1053304	1053617	0.018	4	14	8	7	5	5	6	17	11	8	8	9	13	15
RBP3	10	48389910	48390847	0.018	6	15	7	11	9	12	17	13	11	6	10	6	14	13
SLA2	20	35274515	35274715	0.019	3	3	2	2	0	10	3	14	0	1	8	4	0	1
COX6A2	16	31439306	31439752	0.019	8	5	10	7	8	4	6	10	11	8	11	6	10	11
NACA2	17	59668192	59668741	0.019	6	10	6	10	8	5	14	10	12	8	13	9	7	7
C13orf35	13	113299262	113300283	0.019	15	8	10	8	6	9	15	11	6	6	12	7	12	12
AC015651.1	17	61926521	61927086	0.019	5	12	2	7	6	9	9	14	9	7	14	5	11	12

Supplementary data 1. Continued

					Number of reads													
Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
JMJD4	1	227921399	227921906	0.019	3	6	6	7	7	14	13	9	12	5	12	6	11	12
GALNT13	2	154728002	154728308	0.020	9	1	3	1	3	4	14	7	0	4	2	2	1	1
RNF17	13	25337805	25338421	0.020	5	9	10	7	4	6	8	16	8	5	14	8	12	11
AC112777.1	12	20704358	20704532	0.020	9	14	9	21	5	10	8	13	13	6	33	5	19	18
C21orf33	21	45551474	45551798	0.020	3	10	4	14	6	5	10	14	8	4	13	6	12	11
GP1BA	17	4836017	4836468	0.021	8	9	3	9	7	5	16	11	6	8	13	5	12	11
AL592464.2	1	2729504	2730299	0.021	8	17	8	11	8	8	20	18	13	9	14	4	16	15
SNX32	11	65601265	65601550	0.021	0	2	0	3	2	14	0	13	3	2	7	0	0	0
KAT2A	17	40274881	40275820	0.021	9	10	11	12	7	9	11	14	7	6	14	6	15	17
NNAT	20	36149825	36150208	0.021	7	12	7	7	3	11	11	20	10	8	8	5	5	6
KIAA0562	1	3774998	3775624	0.021	6	15	3	8	12	8	5	12	5	9	16	10	13	12
AL117692.1	14	50519321	50519571	0.021	6	9	5	5	9	3	7	12	7	5	10	4	10	9
AL109945.1	1	32815223	32815649	0.022	8	7	6	5	11	10	10	10	9	8	8	5	6	5
hsa-mir-380	14	101491469	101492406	0.022	5	15	7	8	9	12	10	15	13	7	16	6	13	14
GRK1	13	114321368	114322096	0.022	6	15	9	8	5	8	10	19	12	8	12	7	13	13
TCL1A	14	96179944	96180575	0.022	10	6	8	5	5	11	9	17	7	8	8	7	13	15
snoU13	17	77685085	77685964	0.022	3	11	4	8	4	10	7	10	9	5	15	6	12	14
AMN	14	103388267	103388745	0.023	5	12	5	5	6	9	3	14	8	7	11	6	10	11
DSCR4	21	39493391	39493628	0.023	6	12	4	8	9	7	12	15	8	9	6	8	11	11
ASPA	17	3375365	3375814	0.023	8	8	6	6	6	8	15	11	7	8	12	4	13	15
NTN5	19	49176094	49176747	0.023	10	8	12	7	12	8	16	17	8	8	16	8	17	15
NAV1	1	201591881	201592262	0.024	10	20	5	11	7	14	18	10	8	4	16	5	6	7
AC004448.7	17	19396521	19397115	0.024	4	7	8	8	6	5	8	5	6	7	10	7	8	8
NEFH	22	29876170	29876886	0.024	9	12	11	8	7	12	21	16	11	5	10	9	8	7
AL158216.1	1	42506718	42507155	0.025	9	10	4	5	6	10	15	8	8	6	8	4	10	11
PAOX	10	135193152	135194112	0.025	3	8	15	18	10	9	10	19	11	6	10	4	14	14
AC068134.5	2	233252919	233253570	0.025	5	13	8	6	9	8	14	14	8	8	13	2	13	15
MBD3	19	1593742	1594594	0.025	7	7	7	10	7	3	16	12	6	5	14	6	11	12
AP002347.1	11	59665177	59665838	0.025	10	14	10	8	5	7	16	10	8	11	9	10	13	12
P4HA3	11	74021586	74022694	0.026	4	5	9	7	7	9	11	8	10	6	15	9	13	15
ACTRT2	1	2936088	2936661	0.026	5	12	5	5	5	11	12	10	8	3	10	6	12	14
EP400NL	12	132567723	132568038	0.026	5	7	7	10	4	3	11	8	5	8	11	5	5	5
SFT2D3	2	128456522	128456875	0.026	7	20	10	6	11	8	8	15	10	7	19	9	14	15
PROKR2	20	5294594	5294876	0.027	5	19	6	9	9	14	13	15	14	7	12	7	14	14
AC018731.1	2	152042525	152042811	0.028	5	11	10	7	4	7	14	6	8	9	8	7	6	6
AC105272.1	1	104112490	104113282	0.028	8	9	5	5	11	10	9	16	8	5	9	10	11	11
AL034420.1	20	50481186	50481798	0.028	6	12	9	6	8	4	12	10	9	9	8	4	13	15
WDR90	16	697261	697875	0.028	9	11	6	8	5	15	4	11	14	8	12	6	15	13
FAM83E	19	49116077	49116544	0.029	4	17	8	7	6	3	7	16	4	8	7	7	11	11
AP001187.6	11	64658499	64658834	0.029	5	16	9	8	7	9	5	15	10	12	8	10	12	12
PROX1	1	214156052	214156507	0.029	5	2	5	6	4	13	20	10	1	3	11	4	3	1

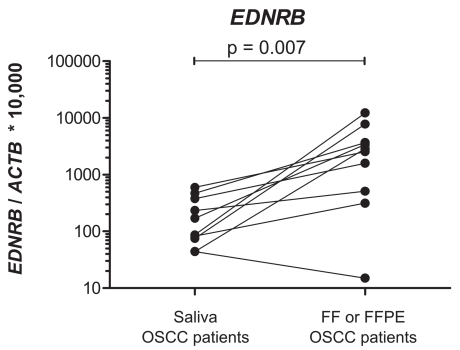
Supplementary data 1. Continued

Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	Number of reads													
					OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
hsa-mir-663	20	26188963	26189097	0.029	75	40	79	140	38	41	26	34	43	68	78	47	21	2
AL049812.1	20	40626799	40628118	0.030	14	25	14	14	14	9	23	19	13	8	19	9	19	19
MBD1	18	47808654	47809093	0.030	5	16	7	11	5	8	10	9	8	4	18	5	12	12
NPAS4	11	66188392	66189250	0.030	4	5	8	8	3	15	14	11	16	5	15	2	5	3
NTSR2	2	11809606	11810729	0.031	11	13	13	10	8	5	14	13	6	8	16	6	16	14
AL035669.3	20	61406620	61407125	0.032	7	9	4	9	3	8	9	11	5	3	9	8	10	9
AP001476.3	21	47455564	47456395	0.032	2	12	10	17	8	5	9	11	10	8	11	9	15	13
KRT85	12	52760680	52761247	0.032	6	12	5	11	7	9	20	14	10	12	17	4	14	14
5S_rRNA	12	34358079	34358737	0.033	5	12	9	5	5	11	14	17	11	7	12	5	13	15
UTS2R	17	80332010	80332560	0.033	7	15	5	15	10	11	16	15	10	9	17	7	15	17
MYEOV	11	69061709	69062020	0.033	1	10	12	4	5	4	5	6	5	6	27	7	13	12
AC010528.1	16	76268977	76269409	0.033	8	11	7	9	7	8	12	13	9	7	13	6	11	12
PSMA8	18	23713594	23714084	0.034	8	15	7	8	6	6	6	16	20	6	11	7	13	13
TUBB6	18	12306268	12306837	0.034	8	11	7	6	6	2	8	11	3	7	14	4	5	4
CEACAM16	19	45199937	45200643	0.035	9	6	10	8	8	10	6	9	8	8	16	8	6	7
AL357712.1	10	8203710	8204202	0.035	6	10	6	7	8	4	10	9	5	9	9	4	10	9
MRPL20	1	1343891	1344780	0.035	4	15	7	6	8	4	15	10	7	11	10	7	11	12
AC093393.1	2	33952332	33952821	0.035	5	11	10	7	6	10	11	12	12	5	10	6	15	13
AL122018.1	1	236273020	236273412	0.036	6	12	5	9	8	7	11	9	10	5	12	7	15	13
RP11-56M3.1	10	92913356	92913775	0.036	9	13	11	9	6	9	15	7	14	8	12	8	13	12
AC008993.3	19	93193	93664	0.036	8	16	6	7	8	8	13	16	11	12	10	1	6	7
hsa-mir-663	20	26188638	26188962	0.036	76	42	78	139	39	40	24	33	41	66	79	44	21	1
AL391244.1	1	1353425	1353858	0.036	2	8	8	7	5	8	9	10	5	7	11	8	9	9
TM7SF2	11	64878569	64879196	0.037	5	19	5	10	7	11	15	16	13	9	13	9	14	14
AL358237.2	20	58662433	58662514	0.037	0	1	0	6	0	0	6	0	1	0	3	0	3	3
AC215219.3	12	94127	94541	0.038	6	15	5	10	11	4	13	9	15	5	18	4	18	15
C10orf139	10	1205273	1205468	0.038	5	12	8	9	6	3	13	7	6	8	17	6	11	11
MSLNL	16	834001	834714	0.038	3	12	9	11	7	5	5	9	10	9	12	4	11	10
AL355376.2	10	29084569	29085115	0.038	7	10	7	6	6	3	10	8	7	5	12	9	14	12
HTR6	1	19991919	19993142	0.038	2	8	4	12	6	11	12	15	7	5	12	5	11	11
RPS6KB2	11	67194641	67195339	0.038	4	12	9	8	5	2	8	12	4	7	11	7	13	11
KRT71	12	52946425	52946854	0.038	5	16	3	8	10	6	15	6	10	7	16	5	12	12
GPHA2	11	64702199	64702982	0.039	6	13	10	10	7	15	13	21	17	10	15	11	7	9
AC012075.1	2	81694040	81694489	0.040	12	17	12	7	6	8	14	15	13	7	20	9	15	17
CDK2AP1	12	123758033	123758565	0.040	6	9	5	7	5	9	8	11	16	5	9	6	10	11
AC007248.2	2	102866968	102867275	0.040	8	12	5	5	6	10	10	16	8	12	17	5	6	7
AGRN	1	953352	954148	0.041	3	9	8	7	6	9	10	9	12	11	8	8	10	11
ZNRF4	19	5455175	5455830	0.041	7	11	10	13	8	4	19	17	11	8	11	5	14	13
PHACTR4	1	28695091	28695513	0.042	4	9	5	7	9	3	10	12	4	5	14	4	5	4
AC022748.1	15	79042124	79042505	0.042	12	17	5	10	5	9	9	15	15	9	11	12	13	14
CIRBP	19	1268296	1268904	0.042	7	11	6	11	6	6	6	9	5	9	19	10	12	14

Supplementary data 1. Continued

					Number of reads													
Gene name	**Chromosome locus**	**Gene regio start (GSE42409)**	**Gene regio end (GSE42409)**	**p-value (Mann-Whitney U test)**	**OSCC patient 1**	**OSCC patient 2**	**OSCC patient 3**	**OSCC patient 4**	**OSCC patient 5**	**OSCC patient 6**	**OSCC patient 7**	**OSCC patient 8**	**OSCC patient 9**	**OSCC patient 10**	**OSCC patient 11**	**OSCC patient 12**	**Leukocyte pool 1**	**Leukocyte pool 2**
MLNR	13	49794460	49795110	0.042	11	3	12	5	6	6	8	19	7	11	6	5	5	3
FSIP2	2	186603232	186604189	0.043	3	9	7	7	6	5	11	11	9	4	13	4	4	2
FGF3	11	69633336	69634068	0.044	2	2	12	5	1	7	9	6	10	7	38	3	2	2
CLSTN3	12	7280735	7280996	0.044	6	10	9	9	11	7	11	11	11	8	17	5	15	13
DHODH	16	72041197	72041688	0.046	5	8	5	9	6	7	10	6	10	8	11	4	10	9
AC022400.1	10	75491360	75491675	0.046	10	15	10	12	7	5	19	12	10	16	17	6	9	8
KRT33A	17	39506596	39507113	0.046	8	13	6	4	10	8	15	9	8	3	14	8	11	12
AL512638.1	1	115826147	115826583	0.047	6	11	7	6	6	6	8	11	7	5	11	7	9	9
AC092810.2	1	209405064	209405472	0.047	5	16	5	7	7	7	8	15	5	4	13	5	3	5
TNNT3	11	1940716	1941338	0.048	9	10	10	6	5	9	6	12	4	10	12	7	14	12
HSF5	17	56565440	56565821	0.048	11	11	9	9	9	4	10	11	13	6	14	4	6	4
AP002748.2	11	66304685	66305454	0.048	6	11	5	7	4	10	10	5	8	9	16	5	6	5
FAM100A	16	4665443	4666047	0.048	3	11	9	15	5	7	6	9	11	12	13	6	12	11
EDARADD	1	236511487	236512106	0.049	4	13	12	7	6	8	18	16	11	7	14	5	13	13
FAM21C	10	46220649	46221042	0.050	3	5	6	9	5	11	8	9	13	8	10	5	11	13

Abbreviations: Chr, chromosome; TSS, transcription start side; FDR, false discovery rate; bp, base pair).
Location of the methylation as extracted from the "Map of the Human Methylome" [http://www.biobix.be/map-of-the-human-methylome/, BIOBIX (Lab of Bioinformatics and Computational Genomics), Ghent, University of Ghent, Belgium 2014]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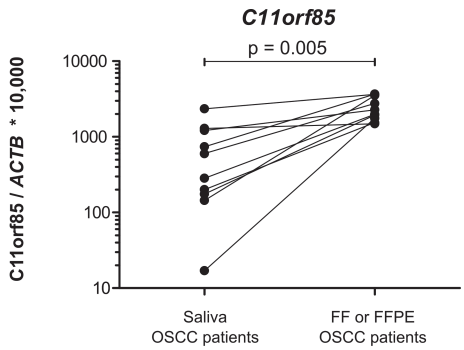
10000
1000
100
10
Saliva
OSCC patients
FF or FFPE
OSCC patients


C11orf85
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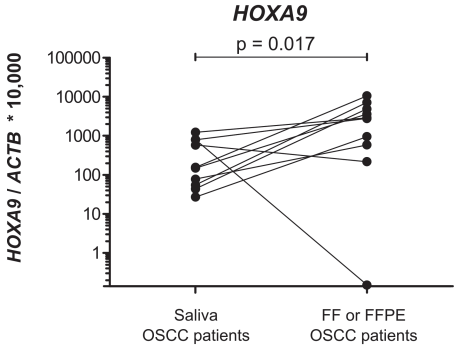
100
10
Saliva
OSCC patients
FF or FFPE
OSCC patients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10
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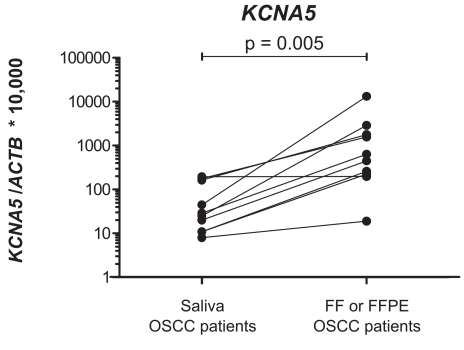
Saliva
OSCC patients
FF or FFPE
OSCC patients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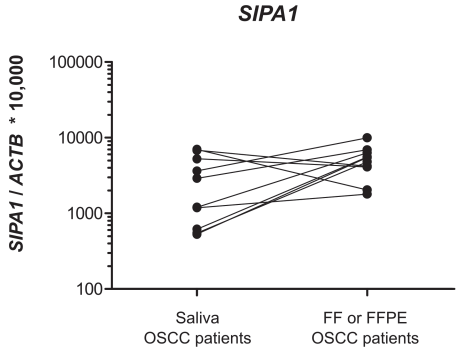
Saliva
OSCC patients
FF or FFPE
OSCC patients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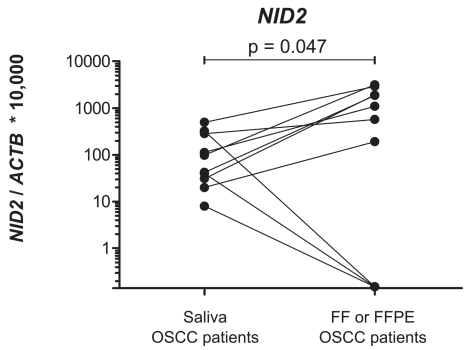
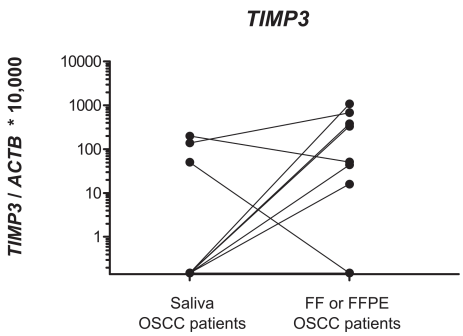
OSCC patients
FF or FFPE
OSCC patients


NID2
p = 0.047
NID2 / ACTB * 10,000
10000
1000
100
10
1
Saliva
OSCC patients
FF or FFPE
OSCC patients


TIMP3
TIMP3 / ACTB * 10,000
10000
1000
100
10
1
Saliva
OSCC patients
FF or FFPE
OSCC patients

Supplementary data 2. DNA methylation levels of seven OSCC specific markers in saliva and tumor tissues of OSCC patients. Differences in methylation level of the markers between DNA isolated of saliva (saliva patients), fresh frozen (FF tissue) and formalin fixed paraffin embedded tissue (FFPE tissue) of oral squamous cell carcinoma (OSCC) patients. Methylation levels on the x-axis are defined as the average DNA quantity of the gene of interest divided by the average DNA quantity of *ACTB* and then multiplied by 10,000. Saliva and tumor samples from the same patient are connected by a continuous line in the figure. Tumors were defined as methylated if methylation was present in FFPE or FF tumor tissue. Differences between saliva, FF or FFPE were compared using the Wilcoxon rank test, only significant differences ($p < 0.050$) are shown.

Abbreviations: FF, fresh frozen; FFPE, formalin fixed paraffin embedded.

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CHAPTER 9

General discussion and future perspectives

GENERAL DISCUSSION

Current evidence and consensus for neck staging in early stage (cT1-2N0) OSCC using the SLNB procedure

A major problem in early stage (cT1-2N0) oral squamous cell carcinomas (OSCC) is that eventually 23-37% of these patients are diagnosed with occult metastases [1]. Conventionally, neck staging was done with an elective neck dissection (END) or a watchful waiting strategy in these patients [2]. In 1994, Weiss et al. proposed that if the risk of occult metastasis was more than 20%, an END was recommended over a watchful waiting [2,3]. That 20% risk was mainly based on T status and anatomical location [4,5]. After multiple studies showed a relationship between tumour infiltration depth and the risk of occult metastasis, tumour infiltration depth with a 4 mm cut-off was added for neck strategy selection [4]. In 2015 a prospective randomized controlled trial analysed 255 watchful waiting and 245 elective neck dissection cases and found a higher five year overall survival and disease free survival for the END (80% and 70% respectively) compared to watchful waiting (68% and 46% respectively) [6] and concluded that END was superior over watchful waiting in early stage OSCC. A major consequence of subjecting all patients to an END is that in the majority (~75%) of the early stage OSCC cases no neck metastasis will be present, while patients are at risk for developing surgery induced morbidities such as shoulder dysfunction [2].

A new era with less invasive neck staging in early stage OSCC started with the introduction of the sentinel lymph node biopsy (SLNB) procedure. Although the SLNB is still an invasive procedure, it is minor surgery compared to the END, which is reflected by a smaller incision (48 mm versus 92 mm [7]), shorter hospital stay (1 versus 3 days [8]) and lower complication rates. In a study with 33 patients staged by SLNB and 29 by END, all 15 postoperative complications appeared in the END group (bleeding n = 5, nerve injury = 8, infection n = 1 and tracheotomy n = 1) and no complications were seen in the SLNB group [7]. Two other studies reported better postoperative shoulder functions and also a smaller scar: 84.2 mm and 73.9 mm for the SLNB group, compared to a scar of 183.3 mm and 171.5 mm in the END group [9,10]. Although the less invasive character is important, correct staging of the neck is the main reason for the use of the SLNB procedure. A meta-analysis using 66 studies in 2017 reported a high pooled sensitivity of 87% and pooled negative predictive value (NPV) of 94% for the SLNB procedure in detecting occult metastasis in early stage OSCC [1]. Limitation of that meta-analysis was the heterogeneity in study protocols with differences in experience of the surgeons, reference standard for the negative SLNB (clinical follow-up or END), preoperative imaging procedures (with or without Single Photon Emission Computed Tomography (SPECT)-CT) and pathological assessment (with or without step-serial-sectioning and additional keratin immunohistochemistry (IHC)) [1]. In **chapter 3** we

retrospectively analysed the sensitivity and NPV of the SLNB in a well-defined cohort using watchful waiting as reference standard for the SLNB negative neck and SPECT-CT scanning and step-serial-sectioning with additional keratin staining were part of the protocol. We confirmed the high sensitivity (85%) and NPV (94%) for the detection of occult metastasis in early stage OSCC [11]. Randomized studies which compare the accuracy of the SLNB procedure and END procedures for detecting occult metastasis in OSCC patients with a clinically negative neck (cN0) are not available yet [12]. However, the reported regional recurrence rate in the pN0 staged neck is 6% in a meta-analysis of the SLNB procedure [1] and comparable to the regional recurrence rate in END studies which is reported between 3% to 10% [12-14]. Thus, the SLNB procedure has an accuracy in the detection of occult metastasis in early stage OSCC at least as high as the END procedure with a lower complication and postoperative morbidity rate.

In 2018, a conference about the SLNB in head and neck cancer was held in London with the aim to reach consensus about the SLNB procedure [15,16]. After that conference, consensus about imaging protocols [15] and surgical procedures were [16] reported and a report about the pathological consensus is expected soon. Neck staging with the SLNB can be used in patients with a clinically negative neck based on preoperative imaging using Computed Tomography (CT), Magnetic Resonance Imaging (MRI) or ultrasound with or without fine needle cytology aspiration (USgFNAC) [15,16]. In a review the sensitivity and specificity of these imaging modalities for the detection of occult metastasis in cN0 patients was reported (reviewed in [17]). Although five studies reported high specificities (92- 100%) for CT, US and USgFNAC, these modalities lack sufficiently high sensitivities in cN0 OSCC patients: CT 49-60%, MRI 55-65%, US 48-66% and USgFNAC 73% [18-21] and should therefore always be followed by a SLNB procedure.

Consensus about the preoperative SLNB imaging protocol consisted of two to four mucosal injections peritumourally of the tracer (^{99m}TC-labelled nanocolloids). The injection activity depends on the one (40-50 MBq) or a two day (70-120 MBq) protocol and should be diluted in a maximum volume of 0.4 to 0.5 mL [15]. The injection is immediately followed by dynamic (0-10 min) lymphoscintigraphy and later on by early static (15 min) and late static (2 hrs) lymphoscintigraphy [15]. The lymphoscintigraphy should be followed by SPECT-CT (>2 hrs) [15]. The use of both the SPECT-CT and the lymphoscintigraphy resulted in the additional detection of SLNs in 22% of the cases in a study with 66 SLNB patients [22]. Moreover, in 8% of the cases were non-sentinel lymph nodes reported as SLNs by using planar lymphoscintigraphy only. A hand-held gamma probe is recommended for the intra-operative detection [15,23].

The recommendations in the surgical consensus guidelines include the imaging modalities for the clinical neck staging (at least CTI, MRI or US), sentinel lymph node definition, optical tracers, lymphatic drainage patterns, tumour infiltration depth, size of a positive SLN and follow-up (frequently examination in the first two year) [16]. During surgery, the sentinel lymph nodes are defined as the lymph nodes with an at least 10 times higher count compared to the background and at least a 10% count of the hottest harvested lymph node measured using a hand-held gamma probe [16,23]. The gamma count is not associated with metastasis, or in other words, the metastasis is not always located in the lymph node with the highest count [16]. In general, 2-3 SLNs per patient are detected and removed [16,23-25].

Although the pathological procedure consensus is not reported yet, many centres use step-serial-sectioning and additional IHC nowadays [17,26], after this method was already successfully implemented in other cancer types such as breast, melanoma and endometrium cancer [27-29]. For example, in breast cancer it was reported that up to 40% of the metastases were missed with the conventional single level haematoxylin and eosin (HE) staining [28]. In OSCC, a study showed detection of metastases in 16% of 80 SLNs of OSCC patients using routine HE staining, 23% with the addition of step-serial-sectioning and in 25% with both step-serial-sectioning and additional cytokeratin immunohistochemistry [30]. Especially, isolated tumour cells (defined as metastasis <0.2 mm) were only found with the addition of step-serial-sectioning [30]. In that study the two metastases with the lowest number of isolated tumour cells were only found by using combined step-serial-sectioning and keratin staining. The SLNB protocol used in **chapters 3, 4** and **5** met the recommendations of the consensus meeting about preoperative imaging (cN0 by CT, MRI or USgFNAC and SLN detection by lymphoscintigraphy and SPECT-CT), intraoperative detection using a hand-held gamma probe, postoperative pathological assessment (step-serial-sectioning and additional IHC) and clinical follow-up as reference standard for negative SLNs. Moreover, a median of 3 SLNs per patient was reported comparable with the number in the consensus guidelines. Therefore, the results in **chapter 3** are in accordance with current evidence and recommended guidelines [11].

As described, the SLNB procedure is superior to the END regarding the extend of surgery, morbidity rate and complication rate [7,9,10]. However, also the cost-effectiveness of the SLNB compared to an END or watchful waiting strategy is important. Two studies reported a cost-effectiveness analysis of the SLNB procedure [31,32]. These analyses were based on measurements of costs and quality of life related to early stage OSCC combined with follow-up data of regional recurrences and mortality. Although in one study the SLNB baseline costs were similar compared to the END (€9180,- versus €9241,-) [32], both studies reported that the SLNB procedure was more effective represented by a small increase in quality-adjusted

life years (QALYs) after five years, respectively 3.63 versus 3.61 [32] and 3.70 versus 3.67 [31], equivalent with ~1 week profit in full health. Combining costs and effectiveness resulted in SLNB as most cost-effective strategy for neck staging in early stage OSCC regarding current known sensitivities and NPVs in both studies compared to the END or watchful waiting [31,32]. The fact that initial costs for a less invasive staging technique (SLNB) are comparably to an END procedure [32] could be explained by the additional use of hospital resources: imaging with lymphoscintigraphy and SPECT-CT, additional IHC and second surgery for the neck dissection in case of a positive SLN.

The high accuracy, the cost-effectiveness and the minimally invasive character with low morbidity rates provided an expanding interest and implementation of the SLNB procedure in several national guidelines worldwide as reported in 2017 [33]. However, not all head and neck oncology centres in these countries use SLNB as standard care for the neck staging in early stage OSCC [8,33]. For those units that are interested in the SLNB procedure, it is important to know that success depends on the experience of the surgeon [34]. Inexperienced surgeons can drop the NPV with 5% [35]. Moreover, a dramatically lower sensitivity can be expected with a surgical experience of less than 5 SLNB procedures, while an increase to a 94% sensitivity was reported with an experience of more than 10 SLNB procedures [34,36]. To reduce the risk of a low accuracy in the first period after implementation, Schilling et al. reported a step-wise training program for the implementation of the SLNB procedure [33]. Besides extensive training before the start of the implementation, also a combination of both the SLNB and END is recommended in the first 10 cases to evaluate the obtained accuracy of the SLNB and to prevent patients for regional recurrences as a result of the low experience [33]. In **chapter 3**, we repeated the accuracy analysis after exclusion of the first patients with SLNB neck staging in our centre. The exclusion of these patients resulted not in a lower sensitivity or NPV in our study what might indicate that the SLNB procedure was implemented after thorough training of the surgeons [11]. Also important to notice is the multidisciplinary approach of the SLNB procedure [33]. Not only head and neck surgeons, also the physicians of the nuclear medicine and molecular imaging department must be trained. Moreover regarding the multidisciplinary character, currently, a single-day protocol (tracer dose of 40-50 MBq) or a two day protocol (tracer dose of 70-120 MBq) are recommended options for the SLNB procedure [15]. Single and two-day refer to the day of imaging and surgery. These single or two day protocols are only effective if imaging, surgery and pathology departments work together in a close cooperation and schedules and resources are aligned to each other as was stated in a 10-year evaluation of a study to the SLNB procedure in melanoma [37].

Summarizing, the SLNB procedure used in **chapters 3, 4** and **5** met the recommendations of the current consensus guidelines for SLNB in early stage OSCC. Therefore, the reported

accuracy in detecting occult metastasis in primary early stage OSCC (**chapter 3**) using the SLNB procedure could be used as reference for other centres. The protocol reported in **chapter 3** is a minor surgery alternative for neck staging with the conventional END regarding complications, postoperative morbidity and cost-effectiveness.

Individual lymphatic drainage pattern assessment in head and neck cancer using the SLNB procedure

Another advantage of the SLNB procedure is the assessment of individual lymphatic drainage patterns. Normally in lateralized OSCC tumours, lymphatic drainage goes to lymph nodes located in the ipsilateral levels I-III [38]. An analysis of 583 OSCC patients with T1-T4 staged tumours, revealed no skip metastasis to level IV or V, or in other words, metastases in level IV or V were always accompanied by at least one metastasis in level I-III [39]. In **chapter 3** [11], we did not find skip metastases at the ipsilateral side, however we reported bilateral drainage patterns in 37% of the early stage OSCC cases while also cases with well lateralized tongue tumours were part of this cohort. Moreover, one patient with a well lateralized tongue tumour only had a SLN (negative for metastasis) at the contralateral side of the neck compared to the tumour [11]. Contralateral drainage of lateralized tumours is not uncommon and reported in 10% of the OSCC cases [16,24]. After an END, up to 39% of the regional recurrences are reported in the contralateral neck [40]. The assessment of individual drainage patterns with the detection and harvesting of SLNs with unexpected drainage patterns using the SLNB procedure, might prevent patients for undertreatment. Therefore, if surgical removable, the consensus guidelines recommend harvesting of SLNs located in unexpected locations (i.e. not in ipsilateral levels I-III), because these SLNs might represent an anatomical variation with a direct lymphatic drainage pattern and are therefore potential locations for the first metastasis deposits [16].

The assessment of individual drainage patterns using the SLNB procedure was also helpful in exploring the drainage patterns of patients with a previously treated neck (**chapter 4**) [41,42]. Local or local-regional recurrences are reported in 10-30% of head and neck squamous cell carcinomas (HNSCC) [43]. At the time of diagnosis of a local recurrence or second primary OSCC, many of these patients already underwent a SLNB procedure, neck dissection or radiotherapy for their first primary tumour. Knowledge about how these treatment altered lymphatic drainage patterns was restricted to two studies with a total of 27 patients [44,45]. Therefore, no consensus for the neck strategy of these patients was available in literature. In **chapter 4** [42], we reported on the accuracy of the SLNB in 53 early stage (cT1-2N0) OSCC patients with earlier treatment (dissection or radiotherapy) or surgery (SLNB) of the neck and analysed the lymphatic drainage patterns of these patients. With the low number of events (lymph node metastases 3/45 and regional recurrences 1/45) taken into account, the SLNB procedure seemed accurate in detecting occult metastasis. No drainage patterns

were found for five (12%) patients with a history of radiotherapy. Unexpected drainage (no drainage to ipsilateral levels I-III) was found in 30% of the cases to ipsilateral levels IV and V or to contralateral levels. The altered drainage patterns as a result of the previous neck treatment limit neck staging in cN0 patients with a neck history: which levels needs an END or clinical examination in case of a watchful waiting strategy? Moreover, extensive surgery (neck dissection) in an earlier treated neck is unfavourable because the previous treatment most likely induced fibrosis in the neck. For that reason, the SLNB procedure is currently the most optimal technique for neck staging in previously treated early stage OSCC patients with a clinically negative neck and recommended in the surgical guidelines of the SLNB consensus meeting in 2018 [16].

For a long time, lymphatic drainage of maxillary tumours was thought to be to the para- or retropharyngeal located lymph nodes [46]. Moreover, a lower incidence of lymph node metastases compared to other oral cavity tumours was assumed [46]. In **chapter 5** [41], we have shown that SLNs of patients with maxillary tumours are mainly located in cervical neck levels I-III. However, in 2 out of 10 patients drainage patterns to parapharyngeally located lymph nodes were also detected. In 2016, an overview of the literature on the incidence of lymph node involvement in maxillary tumours from patients with an END neck staging or with a regional recurrence was published [47]. In eight studies reported from 2001 till 2013, an incidence rate of 14% to 38% including all stages of maxillary OSCC was reported [47], which is not lower than that of other oral cavity locations, as was assumed before [46]. Another study reported a similar incidence (14%) but mentioned especially the high rate (46%) of contralateral metastases in cN0 maxillary OSCC patients [48]. Remarkably, none of these ten studies [48-57] on metastases of maxillary OSCC, mentioned lymph node involvement of para- or retropharyngeally located lymph nodes. A review from 2019 analysed the involvement of retropharyngeally located lymph nodes in head and neck cancer [58]. Of all 32 included studies, **chapter 5** [41] was the only included study that used the SLNB procedure to analyse lymphatic drainage patterns to retropharyngeally located lymph nodes in head and neck cancer [58]. Four of the 32 studies [59-61] reported about retropharyngeal lymph node involvement in OSCC. Incidence rates of 1% [60] and 7% [59] were reported for retropharyngeal metastases, which were most seen in, but were not restricted to maxillary OSCC. One study analysed the two-year disease specific (DSS) and diseases free (DFS) survival and reported a dramatically lower survival for patients diagnosed with retropharyngeal metastases during follow-up compared to patients diagnosed with retropharyngeal metastases at initial treatment, respectively 20% versus 13% for DSS and 24% versus 10% for DFS [60].

Taken together, this current knowledge of maxillary OSCC (incidence, bilateral drainage, retropharyngeal drainage, impact on survival and the assessment of individual drainage

patterns with the SLNB procedure as reported in **chapter 5**), the SLNB might also be a suitable neck staging technique in cN0 maxillary OSCC and might prevent these patients for undertreatment of retropharyngeal and contralateral located lymph nodes.

Limitations of the SLNB procedure in oral squamous cell carcinoma

Although the high accuracy in detecting occult metastasis and the assessment of individual lymphatic drainage patterns, the SLNB also has several limitations and uncertainties such as: lower accuracy in floor of mouth tumours, a second surgery in SLNB positive patients and the strategy after a positive SLN with isolated tumour cells.

Higher false negative (regional recurrences) rates up to 25% have been reported for the SLNB procedure in floor of mouth (FOM) tumours [16,62] compared to the 6% in the other OSCCs [1]. This lower accuracy is caused by the shine-through phenomenon [11,63]: the hotspot of the SLN is located within the hotspot of the tumour on lymphoscintigraphy and SPECT-CT. As described also in **chapter 3**, a level I dissection was proposed in a study in which 50% of the SLNs in level I of FOM tumours were detected only intra-operatively using the level I dissection and not preoperatively by lymphoscintigraphy or SPECT-CT [63]. Another option is the combination of a conventional radio-guided tracer with a fluorescence tracer indocyanine green (ICG)-(99m)Tc-nanocolloid [64]. Seventy-five percent of the SLNs in five patients with a FOM tumour were solely detected by this hybrid tracer. The surgical consensus guidelines implemented these options for cases with a high potential of shine-through in level I and recommend a low threshold to explore level I [16].

The clinical value of isolated tumour cells (ITCs) and micrometases, defined as a size of < 0.2 mm and 0.2-2 mm [65,66], respectively, in SLNs of OSCC patients is not completely clear. Before a neoplastic cell has disseminated from the primary tumour site and formed a metastasis in a lymph node (or other organ), these tumour cells have to go through different processes (e.g. detachment, invasion, migration, extravasation, cell division, etc.) [67,68]. If one of these processes is not completely fulfilled, these disseminated tumour cells will most likely not result in a metastasis [67]. For example, a cell can reach a lymph node or other organ, but stay there in a dormant state (disseminated tumour cell (DTC) [67,69,70]. Probably, these DTCs are a result of mechanical manipulation during biopsy [67,71] and lack ability to grow into an invading metastasis [67]. ITCs and the micrometastases detected in lymph nodes by the SLNB procedure might be such dormant DTCs [70] and harvesting of these SLNs might be therapeutic and consequently, these patients might not benefit from an additional MRND. In breast cancer, a recurrence rate of 0.4% was reported in cases with micrometastases in their SLNs (ITC information was not available) with five years of follow-up and without a benefit for patients additionally treated with axillary lymph node dissection [72]. This observation resulted for breast cancer in a pN0(i+) classification for SLNs

with a metastasis size <0.2 mm in the 8th AJCC TNM classification [73]. As a result, in the revised Dutch guideline (in 2017 and 2018) for breast cancer adjuvant axillary treatment (dissection or radiotherapy) is not recommended any more for pN0(i+) SLNs [74]. In **chapter 3** [11], 37% of the SLN metastases of OSCC patients had a metastasis <0.2 mm and none of these patients had additional metastases in their neck dissection specimen, hypothesizing that these SLN positive patients might not need a neck dissection at all or only a selective neck dissection, because of the SLN was the only lymph node harbouring metastases. A low rate of additional lymph nodes in the dissection specimen could also be the reason that postoperative pathological assessment with step-serial-sectioning and additional keratin IHC in addition to the conventional HE staining improved the NPV with only 2% (from 94 to 96%) in a prospective trial [35]. In 2017, a study reported about 234 early stage OSCC patients with positive SLNs and reported metastases sizes from 12 other studies [75]. Additional metastases in the non-SLNs from the neck dissection specimen were found in 13% of the patients with ITCs in their SLNs, 20% of the micrometastases and 40% of the macrometastases (>2 mm) [75]. Although metastasis size in the SLNB of OSCC patients might be associated with the presence of metastasis in non-SLNs in the neck dissection specimen, even the incidence of non-SLN metastasis after ITCs in OSCC (13%) is higher compared to the recurrence rate after ITCs and micrometastases in SLNs of breast cancer patients without a lymph node dissection (0.4%) [75,76]. This low recurrence rate in breast cancer could be explained by the adjuvant systemic therapy which is common in breast cancer treatment: nearly all (~96.5%) patients received systemic therapy (chemo- or hormonal therapy) in the trial that compared the SLNB procedure with or without axillary dissection in breast cancer [75,76]. For that reason, the 2018 consensus guideline for SLNB in OSCC patients recommended to consider SLNs with ITCs as positive SLNs and they needs to be followed by a neck dissection [16]. Probably that characterization of genetic and expression profiles of tumour cells of the primary tumor site and of ITCs in lymph nodes might help to select ITCs with or without metastatic potential [67] and contribute to an even more individualised strategy of the cN0 neck in the future.

Summarising, the SLNB procedure has a lower accuracy in OSCC of the FOM as a result of the shine-through phenomenon (**chapter 3**). Therefore the SLNB procedure must be combined with a level I dissection in FOM tumours. Although we reported no additional metastases in cases with an ITC or micrometastases in their SLN, because of the additional metastases rate in other studies, a MRND after a positive SLN with ITCs or a micrometastasis is still recommended.

Neck staging in OSCC using molecular biomarkers: cortactin as important driver of metastasis

As described more extensively in **chapter 1,** many cellular processes, such as cell motility, determine the metastatic potential of a tumour cell [68,77]. Epigenetic or genetic alterations of genes with a key-role in cell migration are reported as prognostic for lymph node status [68,78]. A review and meta-analysis from 2015 analysed 11q13 chromosome amplification as promising molecular tumour marker for lymph node status in OSCC and found that *CCND1* (one of the genes of that amplicon) and overexpression of its corresponding protein cyclin D1 was a prognostic marker [79]. In **chapter 6** [80] we analysed the clinical value of *CCND1* amplification and the overexpression of three proteins from three major oncogenes (*CTTN*/ cortactin, *CCND1*/cyclin D1 and FADD) located at the 11q13 amplicon in a well-defined cohort of early stage tongue and FOM OSCC with neck staging using the END procedure. All these biomarkers showed promising NPVs for the detection of occult metastasis: *CCND1* copy number 83% and expression of cyclin D1 84%, FADD 81% and cortactin 80% [80]. In **chapter 7** we found that cortactin was predictive for SLN status in small and superficial tumours (7Th TNM pT1 and a tumour infiltration depth <4 mm) with a 92% NPV of early stage OSCC patients with neck staging using the SLNB procedure.

Cortactin is the protein encoded by the *CTTN* gene (also known as *EMS1*), which is one of the genes located at the 11q13 chromosome [79,81,82]. 11q13 amplification is detected in 46% (range 13-100%) of the OSCC cases [82] and *CTTN* is a candidate driver for this amplification [81]. Overexpression of cortactin promotes cell migration and lymph node metastasis in OSCC [82] and is therefore a promising molecular marker to predict lymph node status. Many binding sites have been reported for cortactin by which important cellular processes involved in metastasis are regulated [82], such as binding to Arp2/3 complex in cytoplasm and cell periphery to regulate nucleation and stabilization of actin [83], binding to F-actin in cytoplasm and nucleus to regulate cell migration [84], binding to ZO-1 in cell tight junctions affects the connection between cell-cell adhesion and actin cytoskeleton [85].

One of the best studied cellular processes involved in metastasis in which cortactin plays a key-role is the regulation of protrusive structures [86]. Invadopodia and lamellipodia are these protrusive structures and enable the invasion and migration of a tumour cell by regulating the actin cytoskeleton and extracellular matrix (Figure 1).

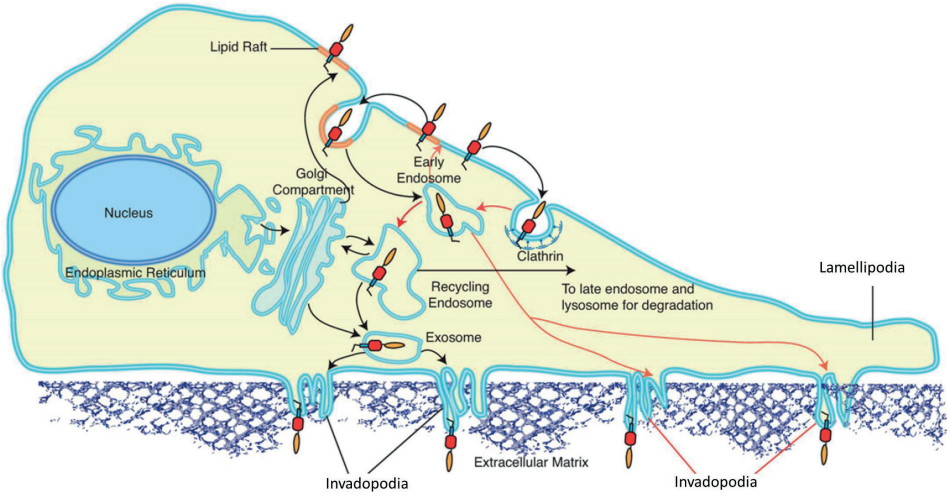

Figure 1. Schematic overview of protrusive structures of a tumour cell mediated by cortactin. This figure shows the protrusive structures invadopodia at the bottom layer and lammellipodia at the front of a tumour cell. This figure shows the delivery to the invadopodia of the membrane type 1 matrix metalloprotease (MT1-MMP, depicted by the green/red/yellow complex) which exposure results in degradation of the extracellular matrix and is one of the cellular processes involved in cell motility regulated by cortactin

[Adapted from Frittoli et al. [87], with permission].

Invadopodia are especially facilitating cell invasion. Formation of invadopodia is reported in a four-stage model by Artym et al. [88] and in the review of Ramos-Garcia et al [82]. During stage I, the invadopia initiation site is characterized by accumulation of cortactin which aggregates with F-actin. During stage II (preinvadopodia), there is more accumulation of the cortactin-F-actin aggregate, resulting in recruitment of membrane type 1 metalloprotease (MT1-MMP). MT1-MMP is an important protein in cell migration by breaking down the extracellular matrix (stage III; mature invadopia) (Figure 1). Cortactin overexpression in stage III promotes also excretion of MT2 and MT3-MMPs into the extracellular matrix and that result in more breakdown [89]. Stage IV (late invadopia) is characterized by the dissolution of cortactin and F-actin while high concentrations of MT1-MMP remain. Lamellipodia are essential for locomotion of cells with an important role for cortactin [82,90]. Protrusion of a lamellipodium starts with actin filament polymerization and elongation which is mediated by cortactin, Arp2/3 complex and the cofilin protein before the other stages of lamellipodium-regulated cell migration take place. In vivo and in vitro studies showed that the expression level of cortactin is related to the cytoplasmic location in squamous cell carcinoma cell lines [82,91,92]. Overexpression of cortactin induces accumulation of cortactin in the cytoskeleton periphery which is essential for the regulation of the protrusive

structures (invadopodia and lamellipodia). Moreover, in vitro studies with squamous cell carcinoma cell lines showed an increase in cell migration with cortactin overexpression [91,93].

A systematic review and meta-analysis from 2019 analysed the clinico-pathological significance of *CTTN*/cortactin alterations in HNSCC [94]. Nine studies (including **chapter 6**) with a low heterogeneity were included for the association with lymph node status in OSCC. Pooled data of these nine studies resulted in a OR 2.78 (95% CI 1.68-4.60) for positive lymph nodes in patients with *CTTN*/cortactin alterations. In that meta-analysis, the authors stated that *CTTN*/cortactin alterations might be helpful to select patients for watchful waiting instead of another neck strategy [94]. However, they recommended further validation of cortactin overexpression with immunohistochemistry (IHC) as preferred approach instead of *CTTN* amplification detection because its simplicity, low cost and routine automatized application in a pathology setting. Moreover, none of nine included studies used data of patients with neck staging using the SLNB procedure. In line with these studies, in **chapter 7** we reported that cortactin overexpression analysed by IHC was associated with SLN status in pT1cN0 OSCCs with a tumour infiltration depth <4 mm.

Morand et al. reported that patients might be selected for their neck strategy preoperatively using tumour infiltration depth [95]. They proposed a model for neck staging wherein patients with a tumour infiltration depth <2 mm receive watchful waiting, 2-5 mm a SLNB and >5 mm an END during first surgery. Leusink et al. proposed a model wherein patients are selected for a watchful waiting strategy instead of a SLNB procedure by a molecular tumour profiling using tumour biopsy specimens [96]. In **chapter 7** we propose that patients with a pT1 staged tumour and a tumour infiltration depth <4 mm and without cortactin overexpression might be selected for a watchful waiting strategy of the neck, while patients with cortactin overexpression or a higher tumour infiltration depth are selected for a SLNB procedure. Larger and prospective studies using tumour biopsy specimens are needed to define and validated the tumour infiltration depth cut-offs for the combination with cortactin.

FUTURE PERSPECTIVES

Detection of occult metastasis

The SLNB procedure is a major step forward in neck staging of early stage OSCC patients compared to the END procedure. However, as long as patients are staged using an invasive procedure and false negatives occur, neck staging needs improvement to reach a more individually successful treatment of early stage patients.

In **chapter 3**, 46% of the SLN positive patients had macrometastasis (>2 mm) which was the case in 40% of the SLN positive patients in nine other studies [75]. The question is, is it possible to detect occult metastases with a higher sensitivity preoperatively? Recently, a review with promising results was published for detecting small metastases using a Combidex-enhanced MRI (CEM) [97]. The CEM is based on the intravenously administration of a solution of ultra-small (20-50 nm) superparamagnetic iron oxides (USPIO) particles (Combidex). CEM was already reported in the nineties, however Combidex was not available for a long time after the manufacturer withdrew Combidex from the registration process in Europe. Recently, the Radboud University Medical Center obtained all rights and manufactured Combidex again. After Combidex is administered, the USPIOs are picked up by macrophages. These macrophages are accumulated in lymph nodes and as a result normal lymph nodes (metastasis negative) lose MR signal (black) while lymph nodes positive for metastases remain white or have a white spot because the macrophages cannot accumulated at the position of the metastases. The difference between black and white lymph nodes enables to distinct positive and negative lymph nodes with a normal size from each other [97]. Moreover, with the CEM it is also possible to detect (pathological) lymph nodes as small as 2 mm [97,98]. Before the withdrawal of Combidex, a very promising 82% sensitivity and 93% NPV for the detection of lymph node metastasis were reported in 375 patients with prostate cancer [99]. In a study with 28 HNSCC patients the sensitivity for the detection of lymph node metastases increased from 52% to 82% after USPIOs were added to the MRI protocol [100]. Although the current evidence with the CEM is mainly from prostate and bladder cancer, Heesakkers et al. stated that this technique might be useable in other cancers as well. With the knowledge that ~25% of the early stage patients are diagnosed with an occult metastasis of which 40% is more than 2 mm in size, it might be that CEM lowers the occult metastasis rate with 10%.

As mentioned earlier, a hybrid tracer with fluorescence is one of the recommendations in the consensus guidelines to prevent false negatives in FOM tumours [16] as a result of the shine-through phenomenon. Besides that hybrid tracer, several experimental techniques are mentioned in the imaging consensus guidelines such as the use of portable gammacameras, freehand SPECT, opto-nuclear probe for acoustic gamma and virtual augmented reality [15]. These techniques are not validated yet, but might be used in the future. Also the use of another radiolabelled colloid tracer was reported [101]. 99mTc-Tilmanocept (Lymphoseek®) has the theoretically advantage of a rapid injection site clearance and a stable binding within lymph nodes as a result of the small particle size (7 nm) and the binding to macrophages by the CD206 receptors [102]. These rapid clearance and stable lymph node accumulation might prevent false negatives caused by shine-through. A phase III trial with 101 included

patients (T1-4, N0) reported promising sensitivity and NPVs which were at least as high as for 99mTc-nannocolloid [101], however a clear validation with both tracers in one study and only early stage OSCC is not available yet.

Detection of local recurrences

In up to 30% of the OSCC, local recurrences or second primary tumours are detected during follow-up [103,104]. As described in **chapter 1**, early detection of local recurrences or second primary tumours is challenging, but essential to improve prognosis. A promising technique to prevent patients for local recurrences is the intra-operative use of image-guided surgery [105] with fluorescently labelled antibodies [106]. Image-guided surgery technique is based on tumour tissue visualisation using a fluorescence tracer conjugated to an antibody [105]. This conjugated antibody binds to an antigen which is upregulated in cancer tissue, resulting in a higher fluorescence signal of tumour tissue compared to surrounding tissues. Using a multispectral fluorescence camera in the operation theatre, this difference in fluorescence can be visualised and used to define surgical resection margins or to visualize residual tumour tissue after initial surgical resection. Currently, clinical trials reported the feasibility and safety of the fluorescently labelled antibodies Panitumumab-IRDye800CW and Cetuximab-IRDye800CW in HNSCC [106,107]. Further studies are needed to confirm that this technique contributes to clear surgical resection margins to prevent patients for re-resections and lower local recurrence rates. As this method is very useful for guiding surgery, this assay might be to insensitive to detect single cells in clear surgical margins. Clear surgical margins are most often defined as tumour cell free margins of at least 5 mm. However, even in patients with clear surgical resection margins, local recurrences are reported in up to 11% of these cases [108,109]. In these particular cases, additional methods might be investigated.

Various molecular methods are available that are able to detect tumour specific mutations at very low levels in a background of normal cells. E.g. the detection of minimal residual cancer with p53 mutations in surgical margins has been reported with promising sensitivities (75% and 85%) but still missed high specificities (67% and 58%) [110]. In **chapter 8** we selected OSCC specific methylation markers with a genome wide approach. Important advantages of hypermethylation markers are the binary state (hypermethylated or not) and DNA hypermethylation appears more often and earlier compared to mutations [77]. These methylation markers might also be useful to detect minimal residual cancer in surgical resection margins that are microscopically tumour cell free.

Another option is to use these methylation markers to detect and monitor circulating tumour cells (CTCs) or circulating tumour DNA (ctDNA) in saliva or plasma of OSCC patients after initial treatment. This might be a useful and non-invasive method to monitor treatment

response after radiotherapy or to an early detection of local or regional recurrences [111]. In **chapter 8** we demonstrated the detection of OSCC cells in saliva using methylation markers. Although some small studies showed already promising results with the detection of CTCs or ctDNA of OSCCs in body fluids such as plasma and saliva, clear validation with large and prospective data is lacking [111].

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CHAPTER 10

English summary & Nederlandse samenvatting

ENGLISH SUMMARY

Oral squamous cell carcinomas (OSCC) almost always primarily metastasize to the cervical lymph nodes; these are known as regional metastases. Lymph node status is an important prognostic factor for outcome and treatment decision making of head and neck cancer. However, not all regional metastases are clinically detectable with the current diagnostic modalities, such as computed tomography (CT), magnetic resonance imaging (MRI) or Ultrasound guided Fine Needle Aspiration Cytology (USgFNAC). Although they are clinically not detectable, regional metastases occur in 23-37% of the early stage (cT1-2N0) OSCC. These clinically undetectable metastases are known as occult metastases. Occult metastases are conventionally assessed by performing an elective neck dissection (END) after research showed higher rates of overall and disease specific survival compared to a watchful waiting strategy. However, an END has disadvantages: it leads to overtreatment in 63-77% of the cases and has a risk of postoperative morbidity (e.g. shoulder pain, reduced limb movement). Therefore, there is a need for a better and less invasive neck staging modality. In this thesis, we studied the predictive and prognostic value of the sentinel lymph node biopsy procedure, histopathological characteristics and molecular markers for the assessment of neck status in OSCC.

Because prior research showed a strong prognostic value for tumour infiltration depth and extranodal extension in OSCC, these histopathological characteristics were incorporated into the 8th edition of the American Joint Committee on Cancer TNM staging manual. However, the currently available 8th TNM validation studies lack patients with conservative neck treatment and changes in the classification especially affect patients with small tumours, which are often treated conservatively. In **chapter 2** we determined the impact of the implementation of the 8th TNM staging criteria. In a retrospective cohort of 211 first primary pT1–T2 OSCC patients with surgery as primary treatment, the old and new TNM staging criteria were compared. One hundred and seventy-three patients underwent a neck dissection and 38 patients had frequent clinical neck assessments. Classification according to the 8th edition criteria resulted in 36% total upstaging with the T classification and 16% total upstaging with the N classification. T3-restaged patients had lower 5-year disease-specific survival rates than T2-staged patients. Postoperative (chemo)radiotherapy could have been considered in another 3% of the patients on the basis of the 8th edition criteria. We concluded that addition of tumour infiltration depth and extranodal extension in the 8th TNM classification leads to better staging of oral squamous cell carcinoma and to a selection of patients who might benefit from an adjuvant treatment like postoperative (chemo)radiotherapy.

The sentinel lymph node biopsy (SLNB) was introduced in head and neck cancer staging as a minimally invasive alternative for an elective neck dissection in detecting occult neck metastases. Meta-analyses of SLNB accuracy showed heterogeneity in the existing studies for reference standards, imaging techniques and pathological examination. In **chapter 3** we determined the accuracy of the SLNB in detecting occult metastasis in 91 consecutive patients with primary cT1-2N0 OSCC treated by primary resection and neck staging by SLNB procedure between 2008 and 2016. The SLNB consisted of lymphoscintigraphy, SPECT-CT-scanning and gamma probe detection. Routine follow-up was the reference standard for the SLNB negative neck. Histopathological examination of sentinel lymph nodes (SLN) consisted of step serial sectioning, haematoxylin-eosin and cytokeratin AE1/3 staining. In all cases SLNs were harvested. A total of 27% patients had tumour-positive SLNs. Four patients were diagnosed with an isolated regional recurrence in the SLNB negative neck side resulting in an 85% sensitivity and a 94% negative predictive value. We concluded that the SLNB is a reliable procedure for surgical staging of the neck in case of oral cT1-2N0 SCC.

It is well known that patients with OSCC suffer a high risk for local recurrences (20-30%) and an annual risk of 3-4% for developing second primary tumours. The treatment of recurrences and second primary tumours is hampered by the previous treatment of the neck due to altered lymphatic drainage patterns. SLNB could be helpful to assess these altered lymphatic drainage patterns. Current evidence about the drainage patterns in previously treated OSCC patients using SLNB is limited to two small studies (n = 22 and n = 5). In **chapter 4** we retrospectively analysed 53 cT1-2N0 OSCC patients from three centres, who underwent SLNB between 2007 and 2016, after a history of neck surgery or radiotherapy. The SLNB procedure was identical to the procedure used in chapter 3. SLNs were detected in 85% of the cases. The SLNB had a sensitivity of 75% and negative predictive value of 98%. Unexpected drainage patterns were observed in 30% of the cases and in 12% no visible lymphatic drainage patterns were observed. We concluded that the SLNB seems to be a reliable procedure for neck staging of cT1-2N0 OSCC patients with a previously treated neck and enables visualization of unexpected drainage pattern variability.

Oral maxillary cancer is relatively rare compared with other anatomic subsites of oral cavity cancer (eg. tongue and floor of mouth) and is rarely included in studies on oral lymphatic drainage patterns. For this location of OSCC, low metastasis rates and drainage patterns to parapharyngeal located lymph nodes have previously been reported. In **chapter 5** we retrospectively studied drainage patterns of 11 patients with oral maxillary cancer that underwent a SLNB procedure. In 10 patients, SLNs were detected and harvested at cervical levels. In two patients, a parapharyngeal SLN was also detected. We concluded that in the majority of the maxillary cancer patients SLNs are located in cervical levels and only in a minority in the parapharyngeal area.

Another technique to assess OSCC patients without clinical and radiological neck involvement is selection using molecular tumour markers. The advantage of such markers is the non-invasive character. Chromosomal region 11q13, including genes *CCND1*, Fas-associated death domain (*FADD)* and *CTTN*, is often amplified in OSCC with nodal metastases. However, evidence in predicting occult nodal metastases is limited. In **chapter 6** we used 158 patients with early tongue and floor of mouth (FOM) squamous cell carcinomas to correlate the molecular markers *CCND1* amplification, cyclin D1, FADD and cortactin protein expression with occult nodal metastases. *CCND1* amplification and cyclin D1 expression correlated with occult nodal metastases. Cyclin D1 expression was validated in an independent multicenter cohort, confirming the correlation with occult nodal metastases in early FOM cancers. Therefore, cyclin D1 is a predictive biomarker for occult nodal metastases in early FOM cancers.

Preoperative selection of patients at risk for occult metastasis using molecular tumour markers might lead to a more individualized neck strategy with a watchful waiting strategy for low risk patients, a SLNB for intermediate risk patients and an elective neck dissection for patients with a high risk. The additional and more detailed information on individual drainage patterns, micrometastasis and isolated tumour cells provided by the SLNB might have impact on the clinical value of molecular biomarkers that have previously been associated with nodal status. In **chapter 7** associations between molecular tumour markers (cortactin, cyclin D1, FADD, RAB25 and S100A9) and lymph node metastasis was investigated in 87 early stage OSCC patients who underwent SLNB neck staging. Cortactin was associated with lymph node status in patients with a pT1 tumour and infiltration depth <4 mm with a negative predictive value of 92%. Therefore, cortactin is a promising tumour marker to select early stage OSCC patients for a watchful waiting strategy instead of a SLNB.

Not only the detection of occult lymph node metastasis is challenging in OSCC, also the early detection of local recurrences and second primary tumours is a major challenge. The high local recurrence rate of 20-30% in patients with oral squamous cell carcinoma (OSCC) is partly caused by residual tumour cells of the first primary tumour and the presence of precancerous epithelium (field cancerization) that has not (yet) clinically manifested. Due to this field cancerization, the risk for a second primary tumour is about 20%. Since OSCC cells are shed into the oral cavity, the detection of tumour-specific DNA methylation markers in saliva could be a tool for the early detection of local recurrences or second primary tumours of OSCC. In **chapter 8** we selected seven genomic locations representing six genes (*C11orf85*, *CMTM2*, *FERMT3*, *KCNA5*, *SIPA1* and *TBX4*) that are methylated in OSCC and not in normal cells from a genome-wide methylation screening analysis using MethylCap-Seq analysis of 12 OSCCs compared to controls. In addition, we selected 4 markers reported to be methylated in saliva of OSCC patients by others (*EDNRB, HOXA9, NID2* and *TIMP3)*.

Quantitative methylation specific PCR analysis using saliva from ten OSCC patients compared to ten non-cancer controls of similar or younger age, revealed only a difference for *KCNA5* methylation. Moreover, when *KCNA5* was combined with other markers, the combination with *TIMP3* revealed a 100% diagnostic potential in detecting OSCC patients compared to non-cancer controls of similar age using saliva. We concluded that this newly discovered *KCNA5* by MethylCap-Seq combined with *TIMP3* might be useful for early detection of local recurrence or second primary tumours in saliva cells of OSCC patients.

NEDERLANDSE SAMENVATTING

Het merendeel van de tumoren in de mondholte zijn orale plaveiselcelcarcinomen (OPCC's), die uitgaan van het mondslijmvlies. Wanneer OPCC's uitzaaien gaat dit vrijwel altijd eerst naar de lymfeklieren in de hals, dit worden regionale metastasen genoemd. In een later stadium kunnen ook metastasen via de bloedbaan "op afstand" ontstaan naar bijvoorbeeld de longen. De aan- of afwezigheid van regionale metastasen is een belangrijke prognostische factor voor de uitkomst van de ziekte en voor de keuze van de behandeling van OPCC's. Echter niet alle regionale metastasen zijn klinisch te detecteren met de huidige diagnostische technieken, zoals de computertomografie (CT) scan, magnetic resonance imaging (MRI) scan of de echogeleide cytologische punctie. Ondanks dat ze met deze relatief gevoelige klinische technieken niet detecteerbaar zijn, blijken er toch regionale metastasen in de lymfklier aanwezig te zijn in 23-37% van de patiënten met een vroeg stadium OPCC (cT1-2N0). Deze klinisch niet te detecteren regionale en afstandsmetastasen worden occulte metastasen genoemd. Occulte regionale metastasen werden in het verleden gediagnosticeerd door de lymfklieren van level I-III van de hals als één blok met de omgevende weke delen te verwijderen, een zogenoemde electieve halsklierdissectie. Een electieve halsklierdissectie kreeg de voorkeur nadat onderzoek aantoonde dat dit voor een betere overleving zorgde in vergelijking met het achterwege laten van een halsklierdissectie en te volstaan met poliklinische controle. Echter met deze electieve halsklierdissectie worden de 63-77% patiënten, die geen occulte metastase blijken te hebben, onnodig geopereerd. Een dergelijke ingreep heeft risico's op postoperatieve morbiditeit, bijvoorbeeld schouderpijn of een bewegingsbeperking van de schouder en de arm. Dit nadeel geeft aan dat er een noodzaak is voor nauwkeurige en minder invasieve methoden om occulte metastasen te kunnen detecteren. In dit proefschrift deden we onderzoek naar de voorspellende waarde voor het aantonen van occulte metastasen door gebruik te maken van de schildwachtklierbiopsie procedure, histopathologische karakteristieken en moleculaire tumormarkers.

Nadat verschillende studies hadden aangetoond dat tumor infiltratiediepte en extranodale groei sterk prognostisch waren voor de overleving na de behandeling van het OPCC, werden deze histopathologische karakteristieken toegevoegd aan de 8e editie van de American Joint Committee on Cancer TNM classificatie. De studies die de basis vormden voor de aanpassing van de TNM classificatie, bevatten echter geen patiënten die een conservatieve behandeling van de hals hebben gehad, terwijl deze toevoegingen aan de TNM classificatie mogelijk vooral invloed hebben op de behandeling van patiënten met een vroeg stadium tumor. In **hoofdstuk 2** bepaalden we de klinische impact van de aanpassingen in de nieuwe TNM classificatie in een retrospectieve database van 211 patiënten met een eerste primaire pT1-2 OPCC die allemaal primair chirurgisch zijn behandeld. Honderddrieënzeventig

van deze patiënten werden behandeld met een halsklierdissectie en 38 werden alleen poliklinisch gecontroleerd op uitgroei van occulte metastasen. Classificatie volgens de 8e TNM editie resulteerde voor 36% van de patiënten in een hogere T stagering en in 16% van de patiënten in een hogere N stagering. De T3 gestageerde patiënten hadden een kortere overleving dan de T2 gestageerde patiënten. Aan de hand van de 8e TNM classificatie had in 3% van de patiënten een aanvullende postoperatieve behandeling met chemo- of radiotherapie overwogen kunnen worden. We concludeerden dat de toevoeging van tumor infiltratiediepte en extranodale groei aan de 8e editie van de TNM classificatie zorgt voor de identificatie van meer OPCC patiënten met een slechtere prognose en van meer patiënten die mogelijk voordeel hebben van een (intensieve) adjuvante behandeling.

De schildwachtklierbiopsie (SWK-biopsie) procedure werd geïntroduceerd voor OPCC's als minder invasieve stageringsmethode voor de hals, in vergelijking met de electieve halsklierdissectie, om te vermijden dat patiënten zonder occulte metastasen een halsklierdissectie ondergaan. Meta-analyses naar de accuratesse van de SWK-biopsie laten veel verschillen tussen studies zien als het gaat om referentie standaarden, beeldvormende technieken en histopathologische beoordelingen. In **hoofdstuk 3** bepaalden we de accuratesse van de SWK-biopsie voor het detecteren van occulte metastasen in 91 patiënten met een primaire cT1-2N0 OPCC, die primair chirurgisch werden behandeld en een SWK-biopsie ondergingen tussen 2008 en 2016. De SWK-biopsie procedure bestond uit SWK detectie met een lymfoscintigrafie scan, SPECT-CT scan en een gamma-probe. Poliklinische controle diende als referentie voor de 63% van de patiënten met een negatieve SWK-biopsie. Histopathologisch onderzoek van de SWK bestond uit het snijden van de schildwachtklier op meerdere niveau's en de detectie van occulte metastasen op basis van standaard histopathologisch onderzoek en een cytokeratine kleuring. In alle 91 patiënten werden SWK's gevonden. In totaal hadden 27% van de patiënten een metastase-positieve SWK. Vier patiënten werden gediagnosticeerd met een regionaal recidief nadat er eerder met de SWK-biopsie geen occulte metastase was aangetroffen. Dat resulteert in een sensitiviteit van 85% en een negatief voorspellende waarde van 94%. We concludeerden dat de SWK-biopsie procedure betrouwbaar is voor het stageren van de hals van cT1-2N0 OPCC patiënten.

Het is bekend dat 20-30% van de patiënten met een OPCC een lokaal recidief krijgen en dat deze patiënten een jaarlijks risico hebben van 3-4% op een tweede primaire tumor. Eerdere behandelingen van de hals kunnen de lymfogene drainagepatronen veranderen. De literatuur over mogelijk afwijkende drainagepatronen in eerder voor een OPCC behandelde patiënten, in kaart gebracht met de SWK-biopsie procedure, is beperkt tot twee kleine studies. In **hoofdstuk 4** onderzochten we retrospectief 53 cT1-2N0 OPCC patiënten uit drie hoofd-hals oncologie centra, die een SWK-biopsie procedure hebben ondergaan tussen

2007 en 2016, nadat ze eerder chirurgische of radiotherapeutische behandeling van de hals hebben gehad. De SWK-biopsie procedure was gelijk aan de procedure zoals beschreven in hoofdstuk 3. SWK's werden in de 85% van de patiënten gedetecteerd. De SWK-biopsie procedure had een sensitiviteit van 75% en een negatief voorspellende waarde van 98%. Drainagepatronen naar onverwachte locaties waren aanwezig in 30% van de patiënten en bij 12% kon helemaal geen drainagepatroon worden aangetoond. We concludeerden dat de SWK-biopsie procedure een betrouwbare techniek lijkt te zijn voor het stageren van de hals van patiënten met een OPCC. Daarnaast maakt de SWK-biopsie procedure het mogelijk om onverwachte lymfogene drainagepatronen te visualiseren in de hals van cT1-2N0 OPCC patiënten die eerder behandeling van de hals hebben ondergaan.

Patiënten met een OPCC uitgaande van de bovenkaak (maxilla) komen minder vaak voor in vergelijking met de andere locaties in de mond, zoals de tong of mondbodem en worden nauwelijks geïncludeerd in onderzoeken naar lymfogene drainagepatronen van dit gebied. In het verleden werden lage metastaseringspercentages verondersteld en daarnaast bestond de indruk dat de drainage vooral naar parafaryngeaal gelegen lymfeklieren plaatsvindt. In **hoofdstuk 5** onderzochten we retrospectief de drainage patronen van 11 patiënten met een OPCC uitgaande van de maxilla aan de hand van de SWK-biopsie procedure. In 10 patiënten werden de SWK's gevonden in cervicale hals levels. In twee patiënten werden daarnaast parafaryngeaal gelokaliseerde lymfeklieren gevonden. We concludeerden dat in de meerderheid van een OPCC uitgaande van de maxilla, op basis van de SWK's, de drainage naar de cervicale lymfeklieren verloopt.

Een andere techniek om te voorkomen dat patiënten zonder occulte metastasen een halsklierdissectie ondergaan, is de selectie aan de hand van prognostische moleculaire markers in de primaire tumor. Het voordeel hiervan is dat dit een niet invasieve methode voor het stageren van de hals is. Chromosoom regio 11q13, waarop onder meer de genen *CCND1*, Fas-associated death domain (*FADD*) en *CTTN* zijn gelegen, is vaak geamplificeerd in OPCC's met regionale metastasen. Echter, het bewijs dat amplificatie van het 11q13 chromosoom of de over-expressie van de daarop gelegen genen, voorspellend is voor de aanwezigheid van occulte metastasen is nog minimaal. In **hoofdstuk 6** gebruikten we 158 patiënten met een vroeg stadium tong of mondbodem PCC om de moleculaire markers *CCND1* amplificatie en de expressie van cycline D1 (*CCND1*), FADD en cortactin (*CTTN*) te correleren met occulte metastasen. *CCND1* amplificatie en cycline D1 expressie correleerden met de aanwezigheid van occulte metastasen. De validatie van cycline D1 in een onafhankelijk multicenter cohort, bevestigde de correlatie met occulte metastasen voor vroeg stadium mondbodem tumoren. We concludeerden dat cycline D1 een voorspellende marker is voor de aanwezigheid van occulte metastasen.

Preoperatieve selectie van patiënten met een hoog risico op occulte metastasen met moleculaire markers kan leiden tot een meer individuele behandelstrategie voor de hals, met poliklinische controle voor laag risico patiënten. Voor patiënten met een gemiddeld tot hoog risico is een SWK-biopsie de eerste keus en een electieve halsklierdissectie in de gevallen waar een SWK-biopsie niet betrouwbaar kan worden verricht. De meer gedetailleerde informatie over drainage patronen, micrometastasen en geïsoleerde tumorcellen die wordt verkregen met de SWK-biopsie kan van invloed zijn op de klinische waarde van moleculaire markers die eerder zijn geassocieerd met lymfeklierstatus. In **hoofdstuk 7** werden markers, eerder beschreven als voorspellend voor lymfeklierstatus (cortactin, cycline D1, FADD, RAB25 en S100A9), geassocieerd met occulte metastasen in 87 patiënten met een vroeg stadium OPCC die een SWK-biopsie hebben ondergaan. Cortactin was geassocieerd met de aanwezigheid van occulte metastasen in patiënten met een pT1 tumor en een tumor infiltratiediepte van minder dan 4 mm met een negatief voorspellende waarde van 92%. We concludeerden dat cortactin een veelbelovende marker is voor de selectie van vroeg stadium OPCC patiënten voor een klinische controle in plaats van een SWK-biopsie.

Naast het detecteren van occulte metastasen is de vroegdetectie van lokale recidieven en tweede primaire tumoren een grote uitdaging in OPCC patiënten. Het aanzienlijke percentage lokale recidieven en tweede primaire tumoren (20-30%) wordt respectievelijk veroorzaakt door residuale tumorcellen van de eerste primaire tumor en/of door de aanwezigheid van premaligne epitheel dat (nog) niet klinisch zichtbaar is. Aangezien PCC cellen die loskomen van het oppervlakte van de tumor terechtkomen in het speeksel, kan het zijn dat de detectie van tumor-specifieke DNA methylatie in DNA uit speekselcellen kan dienen als een techniek voor het detecteren van lokale recidieven of tweede primaire tumoren. In **hoofdstuk 8** selecteerden we genomische gebieden van zes genen (*C11orf85*, *CMTM2*, *FERMT3*, *KCNA5*, *SIPA1* en *TBX4*) die gemethyleerd zijn in OPCC's en niet in normale cellen. Voor de identificatie van deze OPCC-specifieke methylatie markers werd gebruik gemaakt van een screening van het totale methyloom van 12 OPCC's en controle weefsels met behulp van een MethylCap-Seq analyse. Daarnaast selecteerden we vier markers uit de literatuur die werden beschreven als gemethyleerd in speeksel van OPCC patiënten (*EDNRB, HOXA9, NID2* en *TIMP3*). Een kwantitatieve methylatie specifieke PCR analyse van speeksel van tien OPCC patiënten in vergelijking met het speeksel van tien niet-OPCC controles liet alleen een verschil zien voor de methylatie van *KCNA5*. Wanneer *KCNA5* werd gecombineerd met andere markers was een 100% diagnostisch potentieel zichtbaar voor de combinatie met *TIMP3* in het detecteren van OPCC patiënten en niet-OPCC controles van dezelfde leeftijd aan de hand van het speeksel. Op basis van onze pilotstudie concludeerden we

dat *KCNA5* een potentiële nieuwe methylatiemarker is voor de vroegdetectie van lokale recidieven of tweede primaire tumoren in het speeksel van OPCC patiënten en dat grotere studies ter bevestiging van de klinische waarde van deze markers gerechtvaardigd zijn.

CHAPTER 11

List of publications

Dankwoord

Curriculum vitae

Sponsoring

LIST OF PUBLICATIONS

Publications part of this thesis at the moment of printing and ordered by date of publication

1. **Amplification and protein overexpression of cyclin D1: Predictor of occult nodal metastasis in early oral cancer.** Rob Noorlag; Koos Boeve; Max J.H. Witjes; Ron Koole; T.L. Peeters; Ed Schuuring; Stefan M. Willems, Robert J. van Es. *Head Neck. 2017 Feb;39(2):326-333. doi: 10.1002/hed.24584. Epub 2016 Sep 21.*
2. **Lymphatic drainage patterns of oral maxillary tumors: Approachable locations of sentinel lymph nodes mainly at the cervical neck level.** Koos Boeve; Kees-Pieter Schepman; Bert van der Vegt; Ed Schuuring; Jan L. Roodenburg; Adrienne H. Brouwers; Max J. Witjes. *Head Neck. 2017 Mar;39(3):486-491. doi: 10.1002/hed.24628. Epub 2016 Dec 22.*
3. **High sensitivity and negative predictive value of sentinel lymph node biopsy in a retrospective early stage oral cavity cancer cohort in the Northern Netherlands.** Koos Boeve; Kees-Pieter Schepman; Ed Schuuring; Jan L.N. Roodenburg; Gyorgy B. Halmos; Boukje A.C. van Dijk; Rene A.C. Boorsma; Jan G.A.M. de Visscher; Bert van der Vegt; Ed Schuuring; Jan L. Roodenburg; Adrienne H. Brouwers; Max J.H. Witjes. *Clinical Otolaryngology.2018;43:1080–1087. DOI: 10.1111/coa.13107. Epub 2019 Mar 25.*
4. **High rate of unexpected lymphatic drainage patterns and a high accuracy of the sentinel lymph node biopsy in oral cancer after previous neck treatment.** Koos Boeve*; Inne J. den Toom*; Stijn van Weert; Elisabeth Bloemena; Adrienne H. Brouwers; Otto S. Hoekstra; Bart de Keizer; Bert van der Vegt; Stefan M. Willems; C. René Leemans; Max J.H. Witjes; Remco de Bree. *Oral Oncol. 2019 Jul;94:68-72. doi: 10.1016/j.oraloncology.2019.05.007. Epub 2019 May 21. * Both authors contributed equally.*
5. **Addition of tumour infiltration depth and extranodal extension improves the prognostic value of the pathological TNM classification for early-stage oral squamous cell carcinoma.** Koos Boeve; Lieuwe J. Melchers; Ed Schuuring; Jan L.N. Roodenburg; Gyorgy B. Halmos; Boukje A.C. van Dijk; Bert van der Vegt; Max J.H. Witjes. *Histopathology. 2019 Sep;75(3):329-337. doi: 10.1111/his.13886. Epub 2019 Jul 29.*
6. **Elective neck dissection or sentinel lymph node biopsy in early stage oral cavity cancer patients: the Dutch experience.** Koos Boeve*'; Inne J. den Toom*; Daphne Lobeek; Elisabeth Bloemena; Maarten L. Donswijk; Bart de Keizer; W. Martin C. Klop; C. René Leemans; Stefan M. Willems; Robert P. Takes; Max J.H. Witjes; Remco de Bree. *Cancers 2020, 12, 1783; doi:10.3390/cancers12071783, epub 2020 July 3. *Both authors contributed equally.*

DANKWOORD

Het begon allemaal officieel op 1 september 2014. Dat was de datum dat mijn contract inging voor het promotietraject op de afdeling MKA-chirurgie en de afdeling Pathologie & Medische Biologie van het UMCG. Wat volgde waren vijf enerverende jaren waarin ik vrijwel altijd met veel plezier aan dit project heb gewerkt, mooie congressen heb bezocht en inspirerende mensen heb ontmoet. Velen hebben bijgedragen aan deze mooie periode uit mijn carrière. Hieronder wil ik een aantal van hen bedanken, maar mijn dank aan degenen die niet genoemd worden is niet minder groot.

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CURRICULUM VITAE

Jacobus (Koos) Boeve was born on November 10, 1988 in Kampen, the Netherlands. He started his medical education in 2007 at the Rijksuniversiteit in Groningen. In 2014, he received his medical degree and started his PhD project at the Department of Oral and Maxillofacial (OMF) Surgery and the Department of Pathology and Medical Biology at the University Medical Center Groningen (UMCG). The PhD project was combined with dental education at the same university. He presented his projects at different national and international congresses. One presentation was awarded with the Marten Hut award during the Dutch Association for OMF surgery (NVMKA) annual congress and one presentation was awarded with the Best Oral Communication award of the 7th ICHNO congress, both in 2019. During the last year of the PhD project, he worked also as resident not in training at the Department of OMF Surgery at the Scheper hospital in Emmen (1 day/week). He started as resident in training at the UMCG Department of OMF Surgery in September 2019.

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- De Johannahoeve
- DGC de Nieuwe Hanze, www.dgcdenieuwehanze.nl
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