Intra-operative Sentinel Lymph Node Evaluation: Implications of Cytokeratin 19 expression for the adoption of OSNA in Oral Squamous Cell Carcinoma

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Complete List of Authors:	Shaw, Richard; University of Liverpool, Mersey Head & Neck Oncology Group, Dept Molecular Clinical Cancer Medicine Christensen, Anders; Copenhagen University Hospital, Rigshospitalet, Dept. of ORL, H&N Surgery and Audiology Java, Kapil; University of Liverpool, Mersey Head & Neck Oncology Group, Dept Molecular Clinical Cancer Medicine El Maddani, Rehab; University of Liverpool, Mersey Head & Neck Oncology Group, Dept Molecular Clinical Cancer Medicine Liloglou, Triantafillos; University of Liverpool, Molecular & Clinical Cancer Medicine Asterios, Triantafyllou; Aintree University Hospitals NHS Foundation Trust, Cellular Pathology von Buchwald, Christian; Rigshospitalet, Copenhagen University Hospital, Department of Otolaryngology, Head & Neck Surgery and Audiology Wessel, Irene; Copenhagen University Hospital, Rigshospitalet, Dept. of ORL, H&N Surgery and Audiology Kiss, Katalin; Rigshospitalet, Copenhagen University Hospital, Department of Pathology Kjaer, Andreas; Rigshospitalet, Copenhagen University Hospital, Department of Clinical Physiology, Nuclear Medicine & PET and Cluster for Molecular Imaging Lelkaitis, Giedrius; Rigshospitalet, Copenhagen University Hospital, Department of Pathology long, Anna; Newcastle upon Tyne Hospitals NHS Foundation Trust , Cellular Pathology Risk, Janet; University of Liverpool, Mersey Head & Neck Oncology Group, Dept Molecular Clinical Cancer Medicine Robinson, Max; Newcastle University, Centre for Oral Health Research

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Background : Intra-operative analysis of sentinel lymph nodes would enhance the care of early stage oral squamous cell carcinoma (OSCC). We aim to determine the frequency and extent of cytokeratin 19 (CK19) expression in OSCC primary tumours and surrounding tissues to explore the feasibility of a 'clinic-ready' intra-operative diagnostic test (One Step Nucleic Acid Amplification - OSNA, Sysmex).

Methods: Two cohorts were assembled: cohort 1, OSCC with stage and site that closely match cases suitable for sentinel lymph node biopsy (SLNB): cohort 2, HNSCC with sufficient fresh tumour tissue available for the OSNA assay (>50mg). CK19 assays included qRT-PCR, RNA in-situ hybridisation (ISH) and immunohistochemistry (IHC), as well as OSNA.

Results: CK19 mRNA expression was detected with variable sensitivity, depending on method, in 60-80% of primary OSCC tumours, while protein expression was observed in only 50% of tumours. Discordance between different techniques indicated that OSNA was more sensitive than qRT-PCR or RNA-ISH, which in turn were more sensitive than IHC. OSNA results showed CK19 expression in 80% of primary cases, so if used for diagnosis of lymph node metastasis would lead to a false negative result in 20% of patients with cervical lymph node metastases.

Conclusions: OSNA in is current form is not suitable for use in OSCC SLNB owing to inadequate expression of the CK19 target in all case. However, the same assay technology would likely be very promising if applied using a more ubiquitous squamous epithelial target.

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Synopsis: The expression of cytokeratin 19 (CK19) expression in oral cancer (OSCC) was 60-80%. OSNA (One Step Nucleic Acid Amplification) was more sensitive than other RNA assays or immunohistochemistry. CK19 OSNA would lead to unacceptable (20%) false negative results for sentinel lymph node biopsy.

Introduction

Renewed interest in sentinel lymph node biopsy (SLNB) for early stage oral squamous cell carcinoma (OSCC) has resulted from reassuring data with 95% negative predictive value (NPV)[1,2], and also recent trials reinforcing the survival benefit of surgical neck staging[3]. A significant drawback of SLNB is that, in the event of a positive lymph node, a costly (and more morbid) second surgical episode is necessitated. This delay mandated by serial examination of SLN, delays the commencement of adjuvant therapy and creates additional patient distress. SLNB in OSCC would be facilitated by intra-operative staging however frozen section analysis has been found to be somewhat insensitive[4,5], certainly highly operator dependent, and has not found general acceptance[6]. PCR-based techniques have been reported for head and neck squamous cell carcinoma (HNSCC) [7,8] but lack a 'clinic-ready' platform.

One-step nucleic acid amplification (OSNA) uses loop-mediated isothermal amplification (LAMP)[9], amplifying RNA with high sensitivity, specificity, efficiency and rapidity under isothermal conditions. OSNA employs six specially designed primers at eight sequences within *CK19* mRNA subtending high sensitivity and specificity. In breast cancer, OSNA has been validated to at least 96%[10] concordance with histopathology, has been widely adopted and approved in UK NICE guidelines[11]. OSNA necessitates an additional 30-40 minutes operative time, but avoids second surgeries and accelerates commencement of adjuvant therapies from 8.4 to 6.2 weeks [12].

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In HNSCC the clinical potential of OSNA is unproven, and careful validation is required. Although gene signatures for OSCC or epithelial tissue have been developed with sensitive RT-PCR using other target cytokeratins[8], or PVA/EPCAM[7], the opportunity around *CK19* is the availability of a 'clinic-ready' diagnostic test with stringent quality assurance. Several reports have shown that CK19 is a component of the cytoskeleton of HNSCC[13,14] and qRT-PCR for cytokeratins appear sensitive and specific in detecting cervical lymph node metastasis in HNSCC[8,15]. *CK19* OSNA has recently been validated for lymph node staging in colorectal[16,17] and stomach[18,19] adenocarcinoma. The extent of CK19 expression in HNSCC, and therefore whether OSNA could have clinical utility, remains unproven.

Goda et al[20] analysed 213 HNSCC lymph nodes with CK19 OSNA, suggested an overall accuracy of 94% per node and 94% per patient. Matsuzuka et al[21] found a NPV of 95.9% in HNSCC. Suzuki examined CK19 expression in HSNCC, finding a lower rate of expression and suggesting that clinical use of OSNA only in a selected subset of HNSCC known to be CK19 positive[22]. All three studies were undertaken in a Japanese population with a variety of stages and sites of HNSCC, for example Goda et al[20] report on cT1-4 and N0-3 OSCC and Matsuaka et al.[21] report on a combination of HNSCC sites and also include advanced stages. As SLNB is routinely offered only to cT1-2N0 OSCC, these reports do not ideally reflect the target clinical population in question. It remains uncertain if the expression of CK19 is sufficiently high and uniform to make the CK19 OSNA suitable for use in OSCC SLNB.

The aim of this study is to establish the frequency and extent of CK19 expression in primary OSCC and surrounding, potentially contaminating, tissues. We aim to establish expression of *CK19* mRNA using both OSNA and other techniques, as well as protein expression. In the event that CK19 expression is <95% we will pilot assays to be used on diagnostic biopsies of primary tumours in order to stratify them as suitable, or not, for OSNA analysis of SLNB.

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Lastly we aim to test the concordance between matched primary tumour and metastatic lymph node in CK19 expression.

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Methods

Tissue

A clinical cohort was assembled from the tissue banks of the Universities of Liverpool and Copenhagen with appropriate ethical approvals and consent. Clinicopathological characteristics and results are summarised in Supplemental table S1.

Cohort 1 (43 cases from Liverpool) met the criteria: OSCC, clinical stage cT1N0 and cT2N0, formalin-fixed paraffin-embedded (FFPE) and fresh frozen tumour tissue available. The OSNA assay interrogates fresh (or frozen) sentinel lymph nodes >50mg, preferably in their entirety. This presented an ethical and logistic barrier as the lymph nodes are required for histopathological staging, and banked primary tumour samples were exclusively <50mg in T1/T2 OSCC. We therefore elected to analyse primary tumour in order to establish CK19 expression using a number of assays, *excluding* OSNA. 34/43 had available matched FFPE lymph nodes.

Cohort 2 (87 cases, 44 Liverpool and 43 Copenhagen) met the criteria: OSCC, >50mg snap frozen primary tumour tissue, most of these were cT3/4 cases.

Tissue preparation & handling:

Cohort 1: Immunohistochemistry (**IHC):** 4µm sections were stained for CK19 protein by two methods: a mouse monoclonal antibody (clone b170, Leica Biosystems) on a Ventana Benchmark Ultra Autostainer (Ventana Medical Systems Inc.) at a dilution of 1:100 using standard retrieval conditions (MMC1) and the detection polymer Ultraview (Ventana Medical Systems Inc): a mouse monoclonal antibody (clone RCK 108, Dako) diluted 1:100 and the EnVision FLEX system on an Autostainer Link 48 instrument (Dako) using high pH antigen retrieval. Negative controls omitted addition of the primary antibody.

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***in situ* hybridization (ISH):** *CK19* RNA ISH was carried out on 4µm FFPE sections using proprietary reagents (RNAscope, Advanced Cell Diagnostics Inc.). Sections were deparaffinised and pre-treated with heat and protease before hybridisation with target-specific probes: *CK19*, *PPIB* (constitutively expressed endogenous gene; positive control) and *dapB* (bacterial mRNA; negative control) in a dedicated hybridization oven (HybEZ oven, Advanced Cell Diagnostics Inc.). Probe hybridization was detected using the chromogen 3,3'-diaminobenzidine (DAB).

Both IHC and ISH techniques were optimized using known positive (breast ductal carcinoma) and negative tissue (lymph node). Tissue cores from controls constituted a 'control block', sections of which were mounted on each test slide to quality assure the staining methods. The tests were scored by two pathologists (MR & AT). Staining was assessed by assigning an intensity score (0, no staining; 1, weak; 2, moderate; 3, strong) and percentage of malignant cells stained. These were used to calculate an H score (product of intensity and percentage), but also classified in a binary fashion (positive vs. negative).

RNA was prepared from fresh frozen tissue of primary tumours using an miRNeasy kit (Qiagen), and following reverse transcription (cDNA kit, Applied Biosystems), a CK19 qRT-PCR assay was prerformed with the following primers/probe; Fwd: 5'CACTACTACACGACCATCCAGGAC 3', Rev: 5' CGGAAGTCATCTGCAGCCA 3', Probe: 5' TAMRA-ACGGGCATTGTCGATCTGCAGGAC-BHQ2. The qPCR reaction utilised the Universal Master Mix II (Applied Biosystems) , the thermal profile: 50^{o}C for 2 min, 95 ^{o}C for 10 min, 45 cycles of 95 ^{o}C for 15sec and 60 ^{o}C for 1 min, using a 7500 FAST instrument (Applied Biosystems). The relative quantification (RQ) value was calculated as: $RQ = 2^{-\Delta\Delta Ct}$, where Ct is the cycle threshold for each target.

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Cohort 2: **OSNA:** OSCC biopsies from cohort 2 with mass between 50 and 600 mg were snap frozen and stored at -80°C until shipment to Sysmex on dry ice. Samples were processed according to manufacturer's instructions (Sysmex, Kobe, Japan) using a designated instrument (RD-100*i*) and reagent system (LYNOAMP & LYNORHAG). Individual tumour samples were placed in 4 ml of homogenizing buffer LYNORHAG (0.2 M glycine-HCl pH 3.5, 5% Brij35 and 20% DMSO), and homogenised for 60 s at 10 000 rpm with a Polytron System PT1300D (Kinematica AG, Switzerland) and LYNOPREP blades to prepare a homogeneous lysate. 1 ml of lysate was centrifuged to remove cell debris and then further diluted 1:10 and 1:100 with LYNORHAG. The diluted lysates were used directly for amplification without RNA extraction or purification. Isothermal amplification reactions were performed at 65°C. The rise time required for precipitation of magnesium pyrophosphate to reach a turbidity of 0.1 OD at 465 nm was obtained for each sample and the number of *CK19* mRNA copies determined using a calibration curve. OSNA was classified as following: (-) = < 250 copies; (-L) = < 250 copies; (+) = > 250 & < 5000 copies, (++) = > 5000 copies; (++) or (+) were positive results, while (-) or (-L) were negative

RNA quality was analysed for negative (- or –L) samples in order to exclude false negatives. OSNA lysates were processed with the Qiagen RNeasy kit (Qiagen, Venlo, Netherlands). Total RNA was quantified spectrophotometrically (260/280 nm ratio). RNA integrity was assessed using RNA Integrity Number (RIN) with a Bioanalyser (Agilent, Santa Clara, CA, USA).

RNA was prepared from unused OSNA lysates and from a separate aliquot of fresh frozen tissue from the same tumours, reverse transcribed and subject to *CK19* qRT-PCR assay as described above.

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Inter-plate qRT-PCR variation was reduced by using the ΔΔCt method to normalise expression with respect to two tumours that had previously been shown to highly express CK19. A technical threshold of 0.005 x the mean ΔCt of the reference tumours was observed in two experiments and was adopted to distinguish positive from negative *CK19* expression in all qRT-PCR experiments.

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Results

Cohort 1. Of 43 primary cT1/T2N0 tumours tested with CK19 IHC, 21 (48.8%) were positive. 29 of 39 primary tumours evaluable in *CK19* RNA ISH tests were *CK19* positive (74.4%), 4 failed quality assurance checks. CK19 IHC was concordant with the *CK19* RNA ISH in 26 of 39 cases (66.7%). For both tests, the staining was generally weak and heterogeneous (Figures 1 & 2) with positive cases having H scores between 5 to 200. Discordant cases (n=13) had lower H scores (Mean 42.7, Range 5-160). Of the 13 discordant results, 9 were positive in RNA ISH and negative in IHC, reflecting higher sensitivity of RNA ISH. Stage I/II OSCC was less likely to be positive by IHC than stage III/IV ($P<0.01$). No such discrepancy was observed for RNA ISH.

CK19 IHC and *CK19* RNA ISH were concordant in 6 of 8 cases with corresponding lymph node metastases, omitting two failed tests (Table 1). In one case, the primary tumour was positive for both tests, but the corresponding lymph node metastasis was negative (patient 3392: Figure 2, Table 1). Lymph nodes with no evidence of metastatic carcinoma (n=26) did not contain any CK19 positive cells. There were no epithelial lymph node inclusions (salivary or thyroid), however, in one case CK19 positive perinodal salivary gland tissue was included in the section, but this might have been dissected free prior to analysis in an SLNB protocol.

18 of 26 (69%) primary cT1/T2N0 tumour tissues were positive for *CK19* mRNA by qRT-PCR. *CK19* qRT-PCR was concordant with IHC in 16 of 26 cases (62%) and with *CK19* RNA ISH in 13 of 22 cases (59%)(Table 2). Discordant IHC cases tended to be positive by qRT-PCR (7/10). By contrast, discordant ISH cases were equally likely to be positive or negative (4/9 positive by qRT-PCR), however, the ISH positives had lower H scores (mean 33.0, range 5-160).

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Cohort 2. Of 87 primary tumour samples analysed by OSNA, 7 were excluded owing to compromised RNA integrity (low RIN). Examination of representative, H&E stained sections from the 43 Danish samples identified 5 that did not contain tumour tissue by pathological examination, one with compromised RNA integrity. The remaining 4 were OSNA positive: 2 contained oral epithelium and 2 contained salivary tissue. Of 76 tumour samples, 61 (80%) were *CK19* mRNA positive by OSNA, with no correlation for either tumour stage or site (Supplementary table S2).

39 of the tumours from Liverpool had sufficient tissue to allow extraction of mRNA from a separate portion of the tumour. Of these, 23 (59%) were *CK19* positive. qRT-PCR data was concordant with OSNA data in 29/37 (78%) of cases, with OSNA proving the most sensitive test in all 8 discordant cases. In order to investigate this more fully, RNA from the OSNA tissue lysates from all 87 samples was subject to qRT-PCR. 4 samples were excluded based on the low RNA levels (GAPDH amplification). 56/83 (67%) of these samples were positive for *CK19* expression, showing concordance with OSNA data in 70/81 (85%) of cases. All discordant cases demonstrated positivity by OSNA but were negative by qRT-PCR.

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Conclusions

CK19 expression is detectable in between 50 and 80% of OSCC, depending upon the assay used. RNA-ISH and qRT-PCR are more sensitive than IHC, while OSNA appears to be the most sensitive method. The prevalence of *CK19* expression by OSNA is still, at 80%, insufficient to suggest that OSNA could be used without prior screening of biopsy tissue for CK19, as it could result in 20% of positive lymph nodes being called as false negative. CK19 expression in OSCC has been previously reported to range between 53% and 91%[13,14] and our results confirm that CK19 can be detected only in a subset of primary tumours. In this regard, OSCC differs from breast, colorectal & stomach sites, all adenocarcinomas, where CK19 OSNA has been clinically validated. Although the chemistry and platform available through OSNA appear to be well suited to clinical use in being highly reliable, sensitive and specific, the gene target CK19 appears to offer insufficient expression in OSCC for clinical application. Should a more appropriate gene target (perhaps CK5 or 14) be available, it may be that this would be suitable, subject to the appropriate and necessary clinical validations.

Although, theoretically, OSNA might be used on a fresh biopsy sample to select CK19 positive tumours suitable for OSNA assay in SLNB, concern remains that surrounding oral mucosa or salivary gland could be included leading to a false positive. *CK19* mRNA ISH carried out on an existing FFPE diagnostic biopsy, might be more convenient and provide histological context, avoiding false positives. However, our results show that CK*19* mRNA ISH expression was usually low and heterogeneous, limiting diagnostic confidence and making the assay vulnerable to inter-observer variability. Consequently, we could not suggest a reliable assay to stratify which tumours are suitable for OSNA assay in SLNB.

In one case, the primary tumour was positive and the matched lymph node metastasis was negative by both CK19 ISH and IHC. Contamination in the neck structures with ectopic

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salivary (0.9%)[23] or thyroid tissue (1.5%)[24] have been reported either within or immediately surrounding lymph nodes and could produce false positives in any methodology that uses solid specimens. It may be that careful dissection of single SLNB would eliminate this, but again a validation study would be helpful.

Our data successfully incorporated a new assay (*CK19* mRNA ISH) and shows potential clinical avenues in OSCC for molecular diagnostics. We have *CK19* data on 123 OSCC which effectively rules out the need for potentially burdensome, and clinically risky, validation studies. The international collaboration between two academic head and neck cancer centres and industry augers well should a more suitable assay become available. Such an assay might additionally be applicable to cutaneous SCC and anogenital SCC which would increase the test's commercial viability. It is encouraging that OSNA assays with differing gene targets, most recently with MMP7[25] (matrix-metalloproteinase 7) are available.

The concept of intra-operative diagnostics in OSCC remains attractive, but awaits a suitable assay. At present SLNB analysis is based on evaluation of stepped serial sections from only a proportion of the sentinel node, thus a rapid technique examining the entire sentinel node for tumour deposits may provide more accurate staging. An automated intra-operative method would also avoid the substantial additional workload for the pathology team performing serial SLNB examination. In head & neck oncology, intra-operative diagnostics appear even more attractive than in melanoma and breast, as OSCC remains largely a surgically treated disease and completing all surgery in one operation would facilitate the wider acceptance of SLNB.

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Supplemental Table S1 Summary of characteristics of tumours and tabulated results for cohort 1 and 2

Supplemental Table S2. Distribution of *CK19* expression by OSNA and stage

Table 1 Test results for primary tumours with corresponding lymph node metastases.

Table 2 qRT-PCR concordance with IHC and ISH staining

Table 3 Distribution of *CK19* mRNA expression by OSNA

Figure 1 A primary tumour that shows weak, heterogeneous CK19 positivity and a corresponding sub-capsular lymph node metastasis with stronger CK19 staining. This case illustrates the difficulty, with either IHC or ISH, to offer a confident diagnostic test to identify cases from diagnostic biopsy suitable for CK19 OSNA.

Figure 2 A primary tumour that shows weak, heterogeneous CK19 positivity and a corresponding lymph node metastasis with no CK19 staining. If this case had undergone SLNB analysis using CK19 OSNA, even with the apparent security of a 'positive' primary tumour, it is likely that a false negative result would be returned with consequent under-treatment and neck recurrence.

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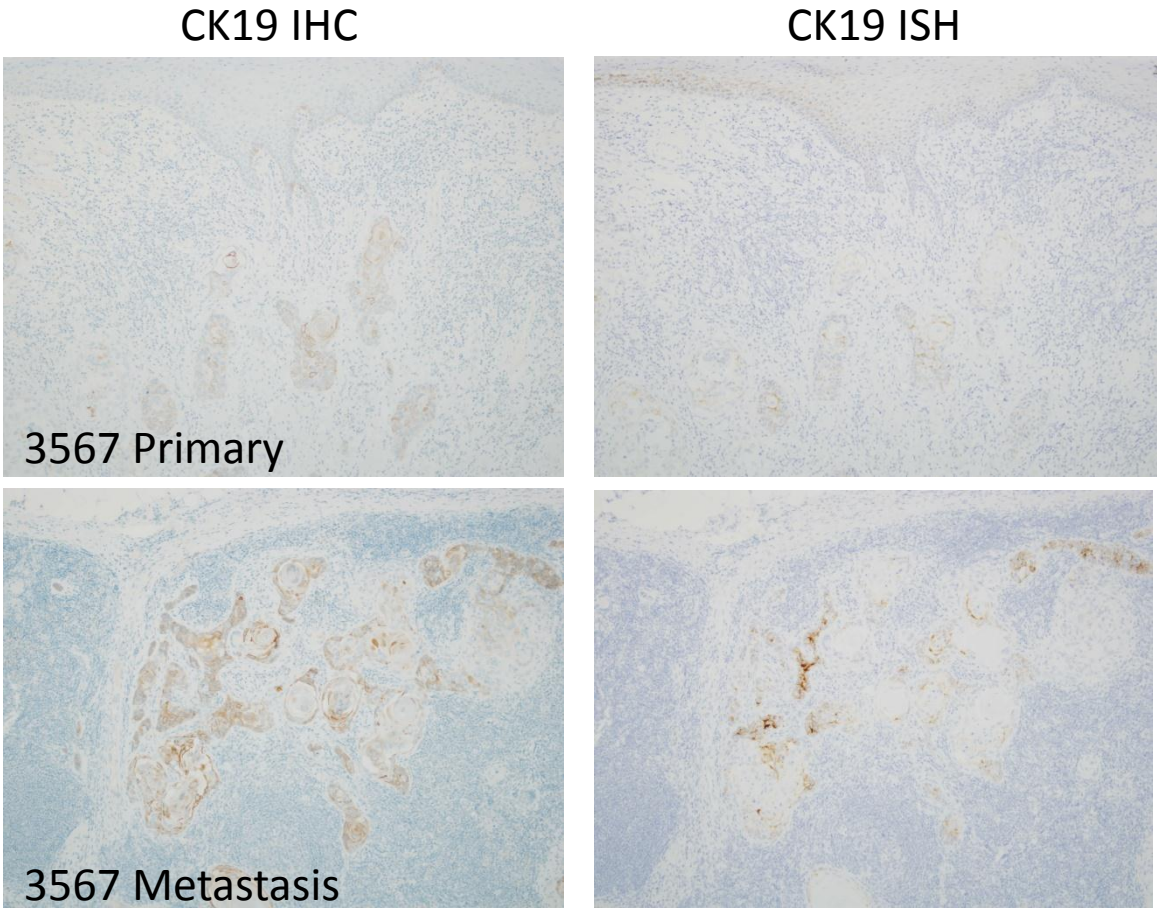


Figure 1. A primary tumour that shows weak, heterogeneous CK19 positivity and a corresponding sub-capsular lymph node metastasis with stronger CK19 staining.

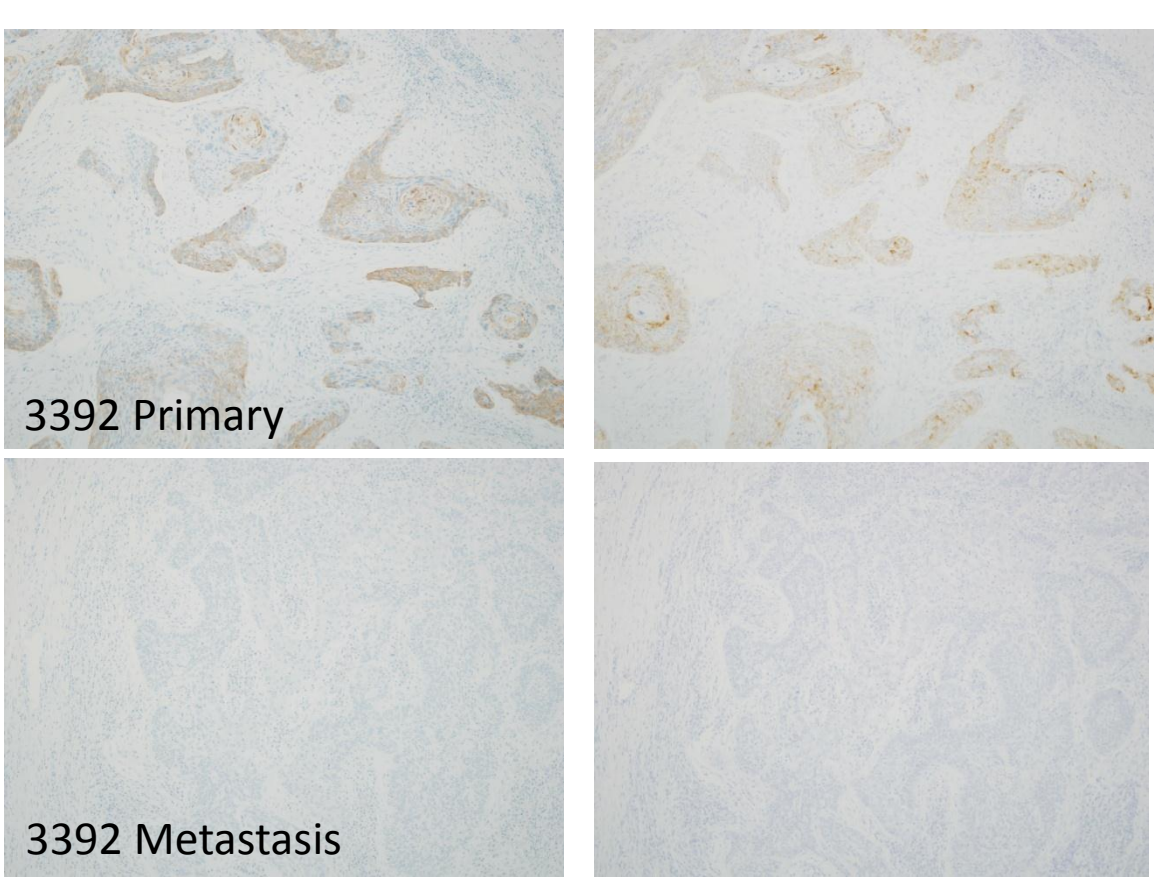
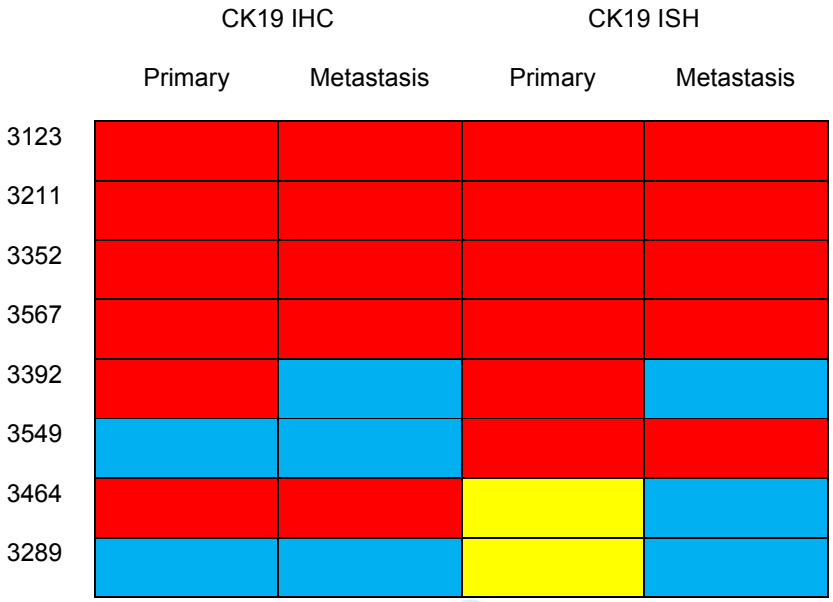


Figure 2. A primary tumour that shows weak, heterogeneous CK19 positivity and a corresponding lymph node metastasis with no CK19 staining

Table 1. Test results for primary tumours with corresponding lymph node metastases.



Red = positive

Blue = negative

Yellow = not available, test failed quality assurance checks.

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Table 2 qRT-PCR concordance with IHC and ISH staining

		IHC		ISH	
		+	-	+	-
qRT-PCR	+	11	7	11	4
qRT-PCR	-	3	5	5	2

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Table 3 Distribution of *CK19* mRNA expression by OSNA

OSNA	Number of samples (%)	
(++)	45 (56%)	65 (81%)
(+)	20 (25%)	
(-)	12 (15%)	15 (19%)
(-L)	3 (4%)	
Total	80 (100%)	

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			CK19 IHC					*CK19* mRNA ISH						*CK19* qRT-PCR	Clinicopathological data		
pt no	Primary vs Met	Tumour presence	tumour score	node score	Intensity	%	H score	tumour score.	node score.	Intensity.	%.	H score.	Comments	pos/neg	site	stage	pathology
3123	Primary	SCC	1		1	10	10	1		1	10	10			tongue (ventral)	4	pT2N2c
3123	LN	SCC		1	1	10	10		1	1	50	50					
3149	Primary	SCC	0		0	0	0	0		0	0	0			tongue (lateral)	1	pT1N1
3153	Primary	SCC	0		0	0	0	1		2	70	140			FOM	2	pT2N0
3165	Primary	SCC	1		1	50	50	1		1	20	20		1	tongue (lateral)	2	pT2N0
3165	LN	No tumour															
3172	Primary	SCC	0		0	0	0	0		0	0	0		1	FOM	2	pT2N0
3172	LN	No tumour															
3188	Primary	SCC	0		0	0	0	0		0	0	0		0	FOM	2	pT2N0
3188	LN	No tumour															
3211	Primary	SCC	1		1	50	50	1		1	20	20		**1**	buccal	4	pT2N2b
3211	LN	SCC		1	1	70	70		1	2	25	50					
3212	Primary	SCC	0		0	0	0	1		1	20	20			FOM	1	pT1N0
3212	LN	No tumour															
3229	Primary	SCC	0		0	0	0	1		1	50	50		1	tongue (anterior)	2	pT2N0
3232	Primary	SCC	1		2	20	40	1		1	10	10		1	FOM	1	pT1N0
3232	LN	No tumour											CK19 ISH background				
3258	Primary	SCC	1		1	70	70	1		1	50	50		1	FOM	2	pT2N0
3258	LN	No tumour															
3274	LN	No tumour															
3274	Primary	SCC	1		1	90	90	1		1	20	20		0	tongue (ventral)	3	pT2N1
3288	LN	No tumour															
3288	Primary	SCC	1		1	20	20	1		2	50	100		1	tongue (ventral)	1	pT1N0
3289	Primary	SCC	0		0	0	0						CK19 ISH internal control negative	1	FOM	3	pT2N1
3289	LN	SCC		0	0	0	0		0	0	0	0					
3333	Primary	SCC	0		0	0	0	0		0	0	0	CK19 ISH internal control weak		tongue (ventral)	2	pT2N0
3333	LN	No tumour							0	0	0	0	CK19 ISH internal control weak				
3340	Primary	SCC	1		1	100	100	1		1	5	5			tongue (ventral)	2	pT2N0
3340	LN	No tumour															
3341	Primary	SCC	0		0	0	0	1		2	10	20			tongue (ventral)	2	pT2N0
3352	LN	SCC		1	2	100	200		1	2	70	140					
3352	Primary	Adenosq. ca.	1		2	100	200	1		2	100	200			FOM	4	pT2N2b
3355	Primary	SCC	0		0	0	0	1		1	50	50			tongue	1	pT1N0
3355	LN	No tumour															
3364	Primary	SCC	0		0	0	0	1		2	50	100			tongue	2	pT2N0
3361	Primary	SCC	1		1	20	20	0		0	0	0			tongue	2	pT2N0
3361	LN	No tumour															
3379	Primary	SCC	1		1	5	5	1		1	60	60			buccal	2	pT2N0
3379	LN	No tumour															
3392	Primary	SCC	1		2	70	140	1		2	80	160		0	FOM	3	pT2N1
3392	LN	SCC		0	0	0	0		0	0	0	0					
3395	Primary	SCC	1		1	80	160	0		0	0	0			FOM	2	pT2N0
3395	LN	No tumour															
3394	Primary	SCC	0		0	0	0	0		0	0	0	CK19 ISH internal control weak	1	tongue	1	pT1N0

3394	LN	No tumour															
3399	Primary	SCC	0		0	0	0	1		1	50	50			tongue	2	pT2N0
3399	LN	No tumour															
3401	Primary	SCC	1		1	20	20	1		2	70	140		1	tongue (ventral)	1	pT1N0
3401	LN	No tumour															
3408	Primary	SCC	0		0	0	0	0		0	0	0		0	tongue (ventral)	2	pT2N0
3423	Primary	SCC	1		2	80	160	1		2	60	120		1	FOM	2	pT2N0
3423	LN	No tumour															
3441	Primary	SCC	1		1	10	10	1		2	60	120		1	tongue (lateral)	2	pT2N0
3441	LN	No tumour															
3458	Primary	SCC	1		2	80	160	1		2	90	180		1	FOM	2	pT2N0
3460	Primary	SCC	1		1	10	10	1		1	10	10		0	tongue	1	pT1N0
3460	LN	No tumour															
3462	Primary	SCC	0		0	0	0						CK19 ISH tumour cut out	1	FOM	2	pT2N0
3464	Primary	SCC	1		1	60	60						CK19 ISH internal control negative	1	tongue	4	pT2N2b
3464	LN	SCC		1	2	60	120		0	0	0	0					
3549	Primary	SCC	0		0	0	0	1		1	<5	<5	Focal minimal	0	tongue	3	pT2N1
3549	LN	SCC		0	0	0	0		1	1	<5	<5	Focal minimal				
3496	Primary	SCC	0		0	0	0	1		1	10	10			hard palate	2	pT2N0
3496	LN	No tumour															
3500	Primary	SCC	0		0	0	0						CK19 ISH external negative contol posi	0	tongue	2	pT2N0
3500	LN	No tumour															
3449	Primary	SCC	0		0	0	0	0		0	0	0		1	tongue (lateral)	1	pT1N0
3449	LN	No tumour															
3545	Primary	SCC	0		0	0	0	1		2	50	100		0	tongue	2	pT2N0
3545	LN	No tumour															
3551	Primary	SCC	1		2	100	200	1		2	50	100		NR	FOM	2	pT2N0
3551	LN	No tumour															
3556	Primary	SCC	0		0	0	0	1		1	10	10		NR	tongue (ventral)	2	pT2N0
3556	LN	No tumour															
3567	Primary	SCC	1		1	20	20	1		1	70	70		1	tongue	4	pT2N2b
3567	LN	SCC		1	2	70	140		1	1	70	140					
3571	Primary	SCC	0		0	0	0	0		0	0	0		1	buccal		pT2N0
3571	LN	No tumour															
			21	5				29	5					18			
			43	8				39	9					26			

	CK19 qRT-PCR (sample A)	*CK19* OSNA (sample B)		*CK19* qRT-PCR (sample B)		Clinicopathological data			
sample no	present/ absent	present/ absent.	semi-quantitation	present/ absent,	qRT comments	site	stage	pathology	pathology comment
L1	0	NR		1		tongue (ventral)	4	pT2N2b	
L2	0	0	(-)	0		maxilla	2	pT2N0	
L3	0	1	`+	0		tongue	3	pT3N0	
L4	1	1	`++	1					
L5	1	1	`++	1		maxilla	4a	pT4N0	
L6	0	1	`+	0		FOM	4a	pT4N0	
L8	1	1	`++	1		FOM	4a	pT4N2c	
L9	ND	1	`+	0		buccal	2	pT2N0	
L10	1	1	`++	1		FOM	4a	pT4No	
L11	1	1	`++	1		tongue	3	pT3N1	
L12	1	1	`++	1		tongue	4a	pT4N1	
L13	0	1	`+	0		tongue (lateral)	4	pT2N2C	
L14	1	1	`++	1		tongue (ventral)/FOM	4a	pT4N0	
L15	0	0	(-)	0		mandibular alveolus	4a	pT4N0	
L17	1	1	`++	1		FOM	4a	pT4N0	
L18	0	0	(-)	0		gingiva	4a	T4aN0	verrucous carcinoma carcinoma
L19	1	1	`++	1		tonsil/BOT	4a	pT3N2b	
L20	0	0	(-)	0		FOM	4	pT2N2b	
L21	ND	1	`+	1		Mandible	4a	pT4N0	
L22	1	1	`++	1		alveolus	2	pT2N0	
L23	1	1	`++	1		tongue (lateral)	4a	pT4N1	
L24	ND	0	(-)	0		tongue (lateral)	3	pT3N0	
L25	0	0	(-)	0		mandible	4a	pT4N0	
L26	ND	0	(-)	0		tongue (lateral)	4a	pT3N2b	
L27	0	0	(-)	0		maxilla	4a	pT4aN0	adenoid cystic carcinoma
L28	1	ND		ND		tongue (lateral)	4a	pT2N2b	
L30	1	1	`++	1		retromolar	4a	pT2N2b	
L31	0	1	`++	0		retromolar	3	pT3N0	
L32	1	1	`++	1		RFOM	3	pT3N0	
L34	ND	1	`+	1		mandible	4a	pT4N1	
L35	0	1	`+	0		tongue	4a	pT3N2c	
L36	1	1	`++	1		maxilla	4a	pT4aN0	trnasitional type SCC
L38	1	1	`+	1		tongue (lateral)	4a	pT2N2b	
L40	1	1	`++	1		tongue	4a	pT3N2b	
L41	0	0	(-)	0		maxilla	4b	pT4bN2b	

L42	0	1	`++	0		retromolar	3	pT3N1	
L43	0	1	`+	0		maxilla	4a	pT4aN2b	
L44	1	1	`++	1		tonsil	3	pT1N1	baslaoid SCC IS negative
L45	1	1	`++	1		tonsil	4a	pT2N3	ISH positive
L46	1	1	`++	1		maxilla	4a	pT4aNX	
L47	0	1	`++	1		tongue	3	pT2N1	
L48	1	1	`++	1		tonsil	3	pT2N1	ISH negative
L49	1	1	`++	1		tonsil	3	pT3N0	ISH negative
L50	ND	1	`+	1		tongue	3	pT3N1	
L51	1	1	`+	1		mandible	4a	pT4aN0	
`+ve n=	**23**	**34**		**27**					
n=	39	43		44					

C1		1	(++)	1		Gingiva	4		Salivary tissue - no tumor
C2		1	(++)	1		Tongue	2		
C3		0	(-)L	0		Tongue	3		
C4		0	(-)	0		Tongue/floor of mouth	3		
C5		1	(+)	1		Gingiva	4		
C6		1	(++)	1		Tongue	3		Normal tissue
C7		NR		NR	low GAPDH	Floor of mouth	4		Normal tissue
C8		1	(++)	1		Tongue	3		Salivary tissue - no tumor
C9		NR		NR	low GAPDH	Gingiva + buccal	3		
C10		1	(++)	1		Gingiva	3		
C11		1	(++)	1		FOM	3		no tumor or salivary tissue
C12		NR		NR	low GAPDH	Tongue	3		
C13		1	(++)	1		Cheek/gingiva	4		
C14		1	(++)	1		Tongue	3		
C15		1	(+)	1		Tongue	3		
C16		1	(+)	1		Tongue	3		
C17		1	(+)	1		Gingiva	4		
C18		1	(++)	1		Gingiva	4		
C19		1	(++)	1		Cheek	3		
C20		NR		0	low GAPDH	Regio maxillars	3		Veruccous carcinoma
C21		1	(++)	1		Tongue	3		
C22		1	(++)	1		Gingiva	4		
C23		1	(++)	1		Tongue	2		
C24		0	(-)	0		gingiva	4		
C25		0	(-)	0		Tongue	3		
C26		1	(+)	1		Gingiva	3		
C27		1	(++)	1		Floor of mouth/gingiva	4		Sparce tumor tissue

C28		1	(+)	0		Tongue/ganebue	3		
C29		1	(++)	1		Tongue	3		
C30		1	(+) I	0		FOM	3		
C31		1	(++)	1		Tongue	3		
C32		1	(++)	1		Tongue	3		
C33		1	(++)	1		Tongue/floor of mouth	3		
C35		1	(++)	1		Tongue	3		
C36		0	(-)	0		Gingiva	3		
C37		NR		NR	low GAPDH	Tongue	2		Tumor and necrosis
C39		1	(++)	1		Floor of mouth	3		
C40		1	(+)	1		Gingiva	4		
C41		1	(+)	1		Tongue	3		
C42		1	(++)	1		Hard palate	4		
C43		1	(++)	1		Tongue	3		
C44		0	(-)	0		Hard palate	3		
C45		NR		0		Tongue	3		
	`+ve n=	31		29					
	n=	37		39					

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Supplementary Table 2. Distribution of *CK19* expression by OSNA and stage

	OSNA	
	+	-
Stage II	4	1
Stage III	30	6
Stage IV	30	6

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