

**Keywords:** oral squamous cell carcinoma; Serpine; smooth muscle actin; extracapsular spread; metastasis; prognosis; magnetic resonance imaging

# SERPINE1 and SMA expression at the invasive front predict extracapsular spread and survival in oral squamous cell carcinoma

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**Background:** Extracapsular spread (ECS) in cervical lymph nodes is the single-most prognostic clinical variable in oral squamous cell carcinoma (OSCC), but diagnosis is possible only after histopathological examination. A promising biomarker in the primary tumour, alpha smooth muscle actin (SMA) has been shown to be highly prognostic, however, validated biomarkers to predict ECS prior to primary treatment are not yet available.

**Methods:** In 102 OSCC cases, conventional imaging was compared with pTNM staging. SERPINE1, identified from expression microarray of primary tumours as a potential biomarker for ECS, was validated through mRNA expression, and by immunohistochemistry (IHC) on a tissue microarray from the same cohort. Similarly, expression of SMA was also compared with its association with ECS and survival. Expression was analysed separately in the tumour centre and advancing front; and prognostic capability determined using Kaplan–Meier survival analysis.

**Results:** Immunohistochemistry indicated that both SERPINE1 and SMA expression at the tumour-advancing front were significantly associated with ECS ( $P < 0.001$ ). ECS was associated with expression of either or both proteins in all cases. SMA+/SERPINE1+ expression in combination was highly significantly associated with poor survival ( $P < 0.001$ ). MRI showed poor sensitivity for detection of nodal metastasis (56%) and ECS (7%). Both separately, and in combination, SERPINE1 and SMA were superior to MRI for the detection of ECS (sensitivity: SERPINE1: 95%; SMA: 82%; combination: 81%).

**Conclusion:** A combination of SMA and SERPINE1 IHC offer potential as prognostic biomarkers in OSCC. Our findings suggest that biomarkers at the invasive front are likely to be necessary in prediction of ECS or in therapeutic stratification.

Head and neck squamous cell carcinoma (HNSCC) is the sixth commonest cancer worldwide and recent studies have shown an increasing incidence (Argiris *et al*, 2008). Oral squamous cell carcinoma (OSCC) is the commonest form of HNSCC and currently treatment is relatively uniform, comprising primary surgery, often with the addition of post-operative radiotherapy (PORT) (or chemoradiotherapy) for high-risk cases (Shaw *et al*,

2011). Unlike oropharyngeal SCC (OPSCC) (Schache *et al*, 2011), OSCC is only rarely mediated by human papillomavirus (HPV) (Lopes *et al*, 2011) and also known to be highly molecularly heterogenous, hence the development of clinically validated biomarkers has been slow (Leemans *et al*, 2011).

Extracapsular spread (ECS) in metastatic cervical lymph nodes is the single-most prognostic clinical variable for recurrence and

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death in OSCC (Alvi and Johnson, 1996; Myers *et al*, 2001; Greenberg *et al*, 2003; Shaw *et al*, 2010). In our recent large cohort, the overall 5-year survival for patients with ECS was 23%, compared with 60% for patients without ECS (Shaw *et al*, 2010). Poor outcomes are concentrated in those patients with ECS for whom trials of intensification or novel therapies might be justified. On the other hand, for cases that demonstrate lower biological aggression or a reduced tendency for metastasis, it may be possible to de-escalate therapy (Barry *et al*, 2013). Although the clinical significance of ECS is well established, its diagnosis currently requires histological examination of lymph nodes and therefore is made only following definitive primary surgery involving neck dissection. There is little in the published literature exploiting ECS as a clinical variable for biological investigation, and likewise, there is limited investigation of its molecular determinants (Zhou *et al*, 2006). Molecular biomarkers identifying high risk of ECS, or poor prognosis, prior to definitive therapy could direct novel therapies or treatment intensification.

An important observation related to the presence of ECS (ECS+) is that tumour recurrences occur most frequently at the primary site, although regional and distant failures do also occur more frequently than in non-ECS (ECS-) cases (Shaw *et al*, 2010). This suggests that the biological determinants of ECS will likely be evident in the primary tumour and may even be identified in diagnostic biopsy specimens (Califano *et al*, 1996). Indeed, molecular fingerprints for metastatic tendencies have been found in primary sites of cancers other than OSCC (Ramaswamy *et al*, 2003).

Recent studies have also shown that the presence of activated fibroblasts, otherwise known as myofibroblasts, in the tumour-associated stroma are of high prognostic value in OSCC (Kellermann *et al*, 2007; Vered *et al*, 2010; Marsh *et al*, 2011). Myofibroblasts are usually demonstrated by the presence of alpha smooth muscle actin (SMA) expression, and their presence has also been shown to predict for disease recurrence across a number of tumour types (Surowiak *et al*, 2007; Tsujino *et al*, 2007). Given the prognostic association of both ECS and SMA, significant overlap of cases demonstrating both features would be expected.

Here, using a well-annotated cohort of OSCC patients, we aim to validate candidate biomarkers within primary tumour samples, using quantitative reverse transcription-PCR (qRT-PCR) and protein expression with immunohistochemistry (IHC). Further, we aim to compare the diagnostic accuracy of conventional imaging with these biomarkers in the diagnosis of ECS, and also their prognostic accuracy.

## PATIENTS AND METHODS

**Clinical cohort and imaging.** Ethical approval was obtained (South Sefton EC 47.01 and REC No 10/H1002/53) and 102 patients were included that were treated at the Aintree University Hospital between June 2003 and June 2010. Inclusion criteria for selection were cases with a new histologically confirmed diagnosis of OSCC and treatment with primary surgery. A discovery cohort of 55 cases from June 2003 to November 2008 (pN staging: 17 pN0; 11pN + ECS-; 27pN + ECS+) and a validation cohort of 47 cases from December 2008 to June 2010 (pN staging: 21 pN0; 11pN + ECS-; 15pN + ECS+) were identified. All cases had both snap-frozen and formalin-fixed paraffin-embedded (FFPE) tumour/normal tissue available and were distinct from previously published cohorts from the same regional unit (Field *et al*, 1995; Shaw *et al*, 2006; Rogers *et al*, 2009; Shaw *et al*, 2010; Lwin *et al*, 2012; Shaw *et al*, 2013).

The demographic features, pathological staging and outcomes of the cohort were collected, with nodal and ECS status were

determined using standardised protocols (Woolgar and Triantafyllou, 2009). The data for routine imaging using MRI (magnetic resonance imaging) scans was included, as this represents the current standard of care for pre-treatment staging and diagnosis of ECS. Magnetic resonance imaging scans were reassessed, blinded to pathological neck staging and outcomes, by a single radiologist (RH). Standard radiological criteria for nodal status were used, with ECS suspected when there was high signal change or oedema surrounding the nodes or the fat suppressed on STIR (short T1 inversion recovery) images, when the margins of the nodal masses were indistinct, or when the nodal masses invaded the overlying sternocleidomastoid muscle (van den Brekel *et al*, 1990; Lwin *et al*, 2012).

**RNA extraction.** Tumour tissue was collected at the time of surgical resection from a superficial, non-necrotic, central aspect of the lesions and was immediately stored at -80 °C. RNA was extracted from 3-mm<sup>3</sup> tissue using the AllPrep DNA/RNA/protein mini kit (Qiagen, Valencia, CA, USA) with an on-column RNase-free DNase (Qiagen) treatment to avoid DNA carryover in subsequent RNA preparation. We have previously quantified tumour and stromal proportions in comparable HNSCC specimens, demonstrating tumour proportions >50% in all samples and 80% in two-thirds of samples; microdissection was not undertaken in this series (Schache *et al*, 2011). Total RNA was quantified spectrophotometrically with the Nanodrop 1000 Spectrophotometer (Wilmington, DA, USA). RNA quality and integrity were determined using the Agilent 2100 Bioanalyser (Agilent Technologies, Waldbron, Germany). RNA inclusion criteria for the single-gene assays were >1.8 260/230 ratio, >1.8 260/280 ratio and an RNA integrity number >6 (Hoffman *et al*, 2004). Total RNA (500 ng) was used for cDNA synthesis using the SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and used as follows.

**HPV testing.** As some of the tumours encroached on oral cavity/oropharynx site borders, it was deemed prudent to determine HPV status in all samples to clarify the underlying molecular aetiology of the tumour. In brief, real-time PCR reactions of HPV16 E7 were normalised with  $\beta$ -actin and compared with a standard curve generated from a serial dilution of the HPV16-positive cell line SiHa (ATCC-LGC-HTB-35, Manassas, VA, USA) (Schache *et al*, 2011).

**Gene selection and validation.** Microarray analysis of the discovery cohort had revealed an eight-gene signature for the presence of ECS (unpublished data, not shown), of which the most promising single genes, HEXIM1 and SERPINE1 were selected for validation on the basis of highest area under the curve on receiver operating curve (ROC) analysis. Quantitative RT-PCR was carried out in duplicate using a multiplexed assay with the target probe FAM-labelled (Hs01126604\_m1 and Hs00538918\_s1) and a VIC-labelled endogenous control (GAPDH assay, Paisley, UK: Hs02758991\_g1) on an Applied Biosystems (Paisley, UK) 7500 FAST thermal cycler. This facilitated internal normalisation and determination of relative quantitation was done using the comparative DdCt method ( $2^{-\Delta\Delta C_t}$ ) as described previously (Livak and Schmittgen, 2001). Relative efficiency was determined using a standard curve generated from a serial dilution of total cDNA (derived from cell lines), which was also used as an internal calibrator between plates.

**Tissue microarray (TMA) construction.** TMAs were constructed using cores selected from FFPE blocks of primary OSCC tissue, using a manual tissue arrayer (MTA-1, Beecher Instruments, Sun Prairie, WI, USA) as previously described (Parsons and Grabsch, 2009). Areas of the primary tumour centre, tumour-advancing front (tumour-stroma interface) and adjacent tumour-free mucosa were identified and marked on archival haematoxylin

and eosin-stained (H&E) sections by an oral and maxillofacial pathologist (AT). Triplicate cores, with a 4-mm depth, were obtained from the marked areas and transferred to recipient array blocks in a randomised distribution, with each replicate located on a different array block. Diameter cores (0.6 mm) were obtained from the tumour centre and tumour-free mucosa; 1-mm diameter cores were obtained from the tumour-advancing front. H&E sections of the TMAs were prepared and examined to confirm the accuracy of tissue sampling.

**Immunohistochemistry.** Immunohistochemistry was carried out by standard methods on a DAKO Autostainer (Dako, Ely, UK), using its proprietary kit, the DAKO Envision FLEX/HRP Detection System. In brief, a high-temperature antigen retrieval method was employed using the DAKO PT-Link system (Dako) on 4- $\mu$ m sections of the TMAs and final dilutions of 1:600 of SMA antibody (Clone 1A4, Dako), 1:300 of SERPINE1 antibody (3785, American Diagnostica, Stamford, CT, USA) and 1:800 anti-HEXIM1 ChIP grade (ab25388, Abcam, Cambridge, UK). Primary antibody was omitted from negative controls.

Immunohistochemistry-stained TMA blocks were analysed (AT blinded) and validated (JD) using a semi-quantitative method for scoring; SMA expression that reflected the number/density of myofibroblasts at the tumour centre and advancing front conveyed as intensity of immunoreactivity; and graded as high (florid), intermediate or low (sparse or absent). SERPINE1 and HEXIM1 scoring was similarly graded for intensity of immunoreactivity at the tumour centre with a separate score at the tumour–stroma interface for both tumour cells and stromal cells. Concordance was >95% with remaining cases re-analysed and a consensus score agreed.

**Data analysis.** Statistical analysis was performed using the Software Package for Statistical Analysis, (SPSS) Version 20 (IBM, Armonk, NY, USA). Kaplan–Meier (KM) survival analysis was carried out with a log-rank (Mantel–Cox) test for comparison between curves for overall survival (OS), which was defined by death from any cause. Survival was determined by attendance at the most recent follow-up appointment indicating alive or registered death from case-note review. Gene-expression values between the ECS – and ECS + groups were compared by non-parametric testing (Wilcoxon test) for determination of significance. The classification power of the individual gene expression was assessed using a ROC curve analysis with calculation of the area under curve. Significance of differences between IHC scores with pathological staining and ECS were determined using  $\chi^2$ -analysis.

## RESULTS

The clinical and pathological characteristics of the cohort are shown in Table 1. Tongue and floor of mouth were the commonest sites with the majority of tumours pT2 or pT4. The presence of ECS was highly significant for OS (estimated 5-year survival: pN + ECS 10%; pN + ECS – 70%; pN0 62%  $P < 0.001$ ) with median follow-up 19 months (range 1–87 months). There was a male preponderance in the pN0 cases and a female preponderance in the pN + ECS + cases ( $P < 0.007$ ). Other than this unexpected gender association, this relationship between ECS and survival and other features were consistent with our previous larger clinical cohort (6).

All patients received primary surgery aiming for pathological resection margins of 5 mm and with dissection of appropriate cervical lymph node levels. The distribution of adjuvant treatment received by the cohort was PORT (50–66 Gy) in 63 cases (62%), post-operative concomitant cisplatin (POCRT) (75 mg m<sup>-2</sup> in 3 cycles) chemotherapy in 7 cases (7%), no adjuvant treatment in

**Table 1. Clinical and pathological characteristics of the study cohort (n = 102)**

	pN0	pN + ECS –	pN + ECS +
<b>Age (years)</b>			
Median	59.5	60	63
Range	29–89	48–75	51–85
Mean	60	59	65
<b>Gender</b>			
Male	26	6	13
Female	12	13	32
<b>Tumour site</b>			
Tongue	11	9	17
Floor of mouth (FOM)	16	6	13
Buccal	1	0	3
Lower alveolus	5	0	6
Other	5	4	6
<b>Pathological T-stage</b>			
T1	5	0	1
T2	17	16	23
T3	3	1	7
T4	13	2	14
<b>Pathological N-stage</b>			
N0	38	0	0
N1	0	10	8
N2a	0	0	2
N2b	0	6	26
N2c	0	3	7
N3	0	0	2
MRI (% available)	31 (82%)	16 (84%)	36 (80%)
RNA (% available)	35 (92%)	17 (89%)	37 (82%)
Survival (est. Kaplan–Meier)	62%	70%	10%
Total	n = 38	n = 19	n = 45

Abbreviations: ECS = extracapsular spread; MRI = magnetic resonance imaging.

31 cases (31%) and data not available in 1 case (1%). Within the pN0 group, 24 (63%) did not receive adjuvant therapy, whereas 14 (37%) had PORT for close or involved margins. Within the pN + ECS-ve group, 4 (21%) did not receive adjuvant therapy, whereas 15 (79%) had PORT. Within the ECS group, 3 (7%) had no adjuvant, 34 (77%) had PORT and 7 (16%) POCRT. The reason for some patients not receiving POCRT were advanced age (>70 years), significant contraindicating co-morbidity or that their date of treatment preceded the published evidence supporting POCRT. In addition, for a group of five patients prolonged surgical and medical complications, or patient preference resulted in them not receiving the prescribed adjuvant therapy.

After quality-control assessment, 89 out of 102 RNA samples were suitable for qRT-PCR (Table 1), MRI scans were available for 83 cases and all 102 cases were utilised for TMA construction and subsequent IHC. Four out of 89 cases were identified as HPV positive, all of which were used in the single-gene analysis; only one of these cases was potentially overlapping the oropharynx. As none of the four HPV-positive cases were associated with ECS, they were included in the cohort for biomarker studies because the most important concern regarding known prognostic bias was excluded.

Technical validation of the microarray findings by qRT-PCR showed a moderately positive correlation between the two methods for SERPINE1 expression ( $r = 0.56$ ,  $P = 0.77$ ), but a poorer correlation for HEXIM1 ( $r = 0.22$ ,  $P = 0.15$ ). Area under curve analysis of the ROC curves based on ECS status using single-gene analysis for SERPINE1 was 0.68 and HEXIM1 was 0.67 (Supplementary Table 1). Immunohistochemistry showed that HEXIM1 was ubiquitously expressed with widespread

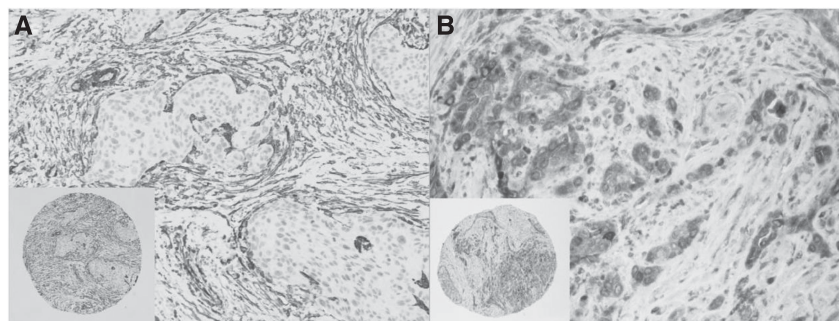


Figure 1. Examples of (A) SMA and (B) SERPINE1 IHC demonstrating distribution of expression at the tumour-advancing front. ( $\times 5$  and  $\times 20$  objective). A full colour version of this figure is available at the *British Journal Of Cancer* journal online.

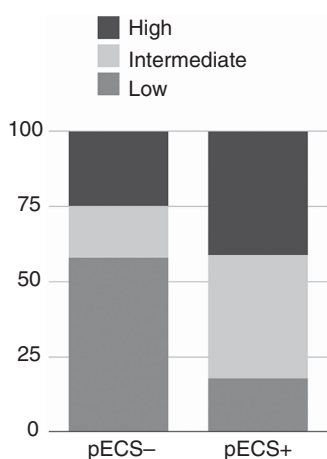


Figure 2. SMA-advancing front stroma vs ECS.

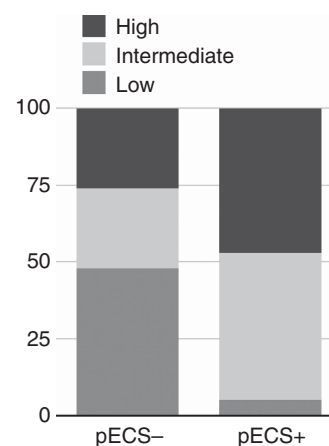


Figure 3. SERPINE1-advancing front tumour vs ECS.

intranuclear high intensity in most cores and therefore non-discriminatory for any pathological features and is not discussed further.

Low-intensity SMA expression was always associated with a focal distribution, whereas intermediate or high-intensity SMA expression was associated with multifocal or diffuse distribution. Significant associations were observed between SMA expression in myofibroblasts at the tumour-advancing front (Figure 1A) and the nodal status (pN0 or pN+) ( $P < 0.001$ ), N-stage ( $P = 0.007$ ) and ECS status ( $P < 0.001$ ) of the tumour (Figure 2). However, differences in SMA expression at the tumour centre did not significantly correlate with any of these variables ( $P = 0.09$ ,  $P = 0.52$  and  $P = 0.4$ , respectively).

SERPINE1 expression in the tumour cells at the tumour-advancing front (Figure 1B) was positively correlated with nodal status, N-stage and ECS (each  $P < 0.001$ ) (Figure 3), but not T-stage. SERPINE 1 expression in stromal cells at the tumour-advancing front did not show these associations. No significant differences in SERPINE1 expression were observed in the tumour centre in relation to T-stage and ECS, but correlations with nodal status and N-stage were highly significant ( $P = 0.002$  and  $P = 0.007$ , respectively).

Analysis of the distribution of SERPINE1 and SMA expression among the whole cohort in relation to ECS status indicate that low positivity for SMA and SERPINE1 effectively excludes ECS-positive tumours (Figure 4).

Neither singular SERPINE1 ( $P = 0.06$ ) nor SMA expression ( $P = 0.042$ ) at the tumour-advancing front matched the prognostic ability of ECS ( $P < 0.001$ ) to predict OS (Shaw *et al*, 2010)

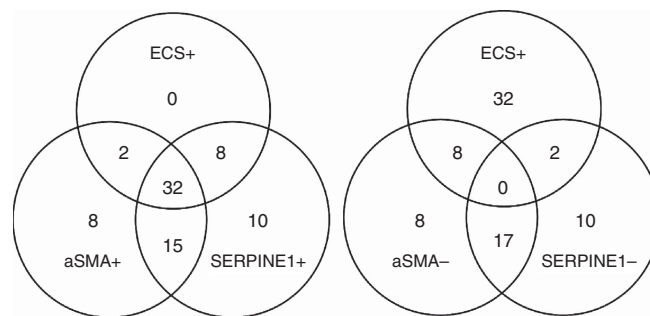


Figure 4. Venn diagrams showing SMA and SERPINE1 expression vs ECS.

(Figure 5A and B). It became evident that high and intermediate expressions cluster in their prognostic association, so they were considered jointly for further analyses. The combination where both proteins are positively expressed is highly prognostic ( $P < 0.001$ ) (Figure 5C) and highly informative for this cohort, being seen in 32 out of 42 (76%) of ECS cases.

Table 2 indicates that MRI offers very poor sensitivity, although it shows good specificity for overall nodal status and presence of ECS. Either SERPINE1 or SMA expression at the advancing front offer significantly better sensitivity at the cost of specificity for both nodal and ECS status compared with MRI, whereas high or intermediate expression of both SERPINE1 and SMA was sensitive for the diagnosis of ECS, but again lacked specificity. In combination, however, negative SMA and SERPINE1 expressions were able to exclude the presence of ECS (100% specificity) (Table 2).

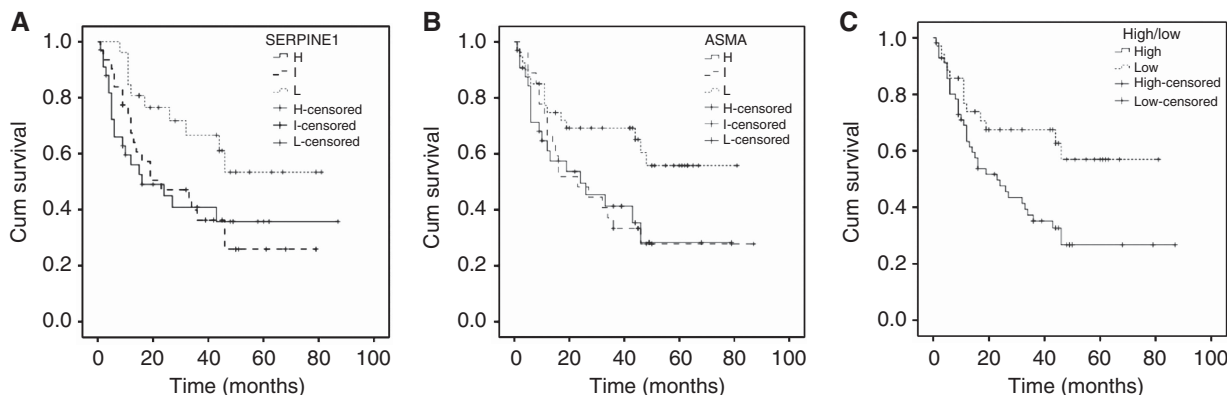


Figure 5. Kaplan–Meier survival curves (OS) for (A) SERPINE1 expression in tumour cells at the tumour-advancing front, (B) SMA expression in the stromal cells at the tumour-advancing front and (C) combined SERPINE1 + and SMA + .

**Table 2. Comparison of diagnostic accuracy of MRI vs SMA and/or SERPINE1 expression at the advancing front for nodal status (pN + vs pN0) and ECS**

Technique	Nodal state	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
MRI	N +	56	84	86	50
	ECS +	7	100	100	60
SMA +	N +	76	67	80	62
	ECS +	82	56	59	80
SERPINE1 +	N +	78	45	75	51
	ECS +	95	50	61	92
SMA + SERPINE1 +	N +	67	48	71	42
	ECS +	81	54	56	77
SMA-SERPINE1 –	N –	28	87	52	69
	ECS –	34	100	100	56

Abbreviations: ECS = extracapsular spread; MRI = magnetic resonance imaging; SMA = alpha smooth muscle actin.

**DISCUSSION**

The presence of ECS in OSCC is of high prognostic value and yet existing techniques are inadequate for its detection prior to surgery. Identification of an appropriate biomarker would aid clinical decision-making regarding escalation of therapy as well as selection for clinical trials with novel therapies. The present findings suggest that a combination of SMA and SERPINE1 protein expression in the primary tumour offers promise in this and also shows a significant association with survival. They also confirm that preoperative assessment of patients with MRI has very poor sensitivity for the detection of ECS (Shaw *et al*, 2010; Liao *et al*, 2012; Lwin *et al*, 2012). The advantages of our study are that detailed clinical and outcome data were obtained, together with MRI data re-assessed by a single H&N radiologist with a special interest, so a direct comparison of prognostic variables with prediction of survival could be analysed.

Despite the relatively modest number of cases examined, this is the largest validation study using ECS as a clinical correlate. Tumour heterogeneity is an important feature of HNSCC and is reflected in both clinical and molecular characteristics of the disease (Severino *et al*, 2008). For example, OSCC has a much greater propensity for spread with poor outcomes compared with OPSCC (Franceschi *et al*, 1993; Dobrossy, 2005; Timar *et al*, 2005; Yu *et al*, 2008). This site specificity is also reflected by the presence of HPV, which is known to have a distinct gene expression profile (Ragin *et al*, 2006; Slebos *et al*, 2006; Schlecht *et al*, 2007; Shaw *et al*, 2010; van Hooff *et al*, 2012b). We established the HPV status of the cohort using a gold standard test and identified that the presence of HPV was unlikely to be a confounding variable in our analysis.

Gene-expression studies have previously shown the ability to predict disease progression and poor survival outcomes in OSCC and are often demonstrated to outperform existing clinical methods (Chung *et al*, 2004; Toruner *et al*, 2004; O’Donnell *et al*, 2005; Roepman *et al*, 2005; Chen *et al*, 2008; Kondoh *et al*, 2008; Rickman *et al*, 2008; Kang *et al*, 2009; Mendez *et al*, 2009). Some studies have also compared expression signatures from primary tissue paired with matched metastatic tissue and showed similarities in the patterns of expression observed (Roepman *et al*, 2006; Mendez *et al*, 2007; Colella *et al*, 2008; Liu *et al*, 2008). This supports our hypothesis that molecular determinants of ECS are present in the primary tumour. In a recent meta-analysis of over 60 studies of differential gene-expression profiling in HNSCC, one study of a limited number of cases predicted ECS by gene-expression analysis from primary site tissue (Zhou *et al*, 2006; Yu *et al*, 2008). It is also evident from many studies that there is a high risk for false discovery, and careful validation is mandated before establishing clinical biomarkers (Mroz and Rocco, 2012; van Hooff *et al*, 2012a).

Our sampling of tissue for gene-expression analysis was superficial, which may be criticised by excluding the more informative tumour-advancing front. The majority of the lesions examined were, however, large pT2 or pT4 tumour, and there was a low likelihood of the tumour-advancing front being included in the samples, even if a deep biopsy were possible. This is a common pathological experience and is reinforced from our previous studies indicating that the stromal component was limited in our research tissues, suggesting the absence of the advancing front (3). The results of the qRT-PCR analysis thereby would be expected to be predominantly of the epithelial compartment. Gene-expression analysis of stroma-rich samples may be more informative in the

identification of biomarkers associated with late stages of disease such as metastasis, but methods of obtaining them need to be developed.

SERPINE1, the gene from our ECS signature, was validated with qRT-PCR and IHC on a TMA. In general, most microarray studies with single-gene validation demonstrate a correlation with microarray techniques (Rickman *et al*, 2008). Mendez *et al* (2009) showed good correlation between qRT-PCR data and microarray findings for SERPINE1, with a correlation coefficient of 0.74. Our finding of a moderately positive correlation is somewhat surprising, given the greater dynamic range of single-gene assays. This may be due to false discovery associated with microarray analysis or low fold-changes of the selected genes, as some microarray validation studies have shown that consistency was not achievable for genes showing a <four-fold difference in expression (Rajeevan *et al*, 2001; Chuaqui *et al*, 2002).

There are several gene-expression studies that have identified SERPINE1 as an important gene associated with metastasis and this has been supported by IHC analysis in OSCC, but those studies did not use a combined approach on the same tumours (Yasuda *et al*, 1997; Schmalbach *et al*, 2004; Roepman *et al*, 2005; Liu *et al*, 2008; Mendez *et al*, 2009). To corroborate and extend our RNA expression analyses, we used IHC to determine differences in expression at the tumour centre and the tumour-advancing front, and to determine which site has the greatest discrimination in relation to pathological variables (Chuaqui *et al*, 2002). We have shown that the intensity of SERPINE1 expression in tumour cells at the tumour-advancing front correlated with N-stage, the presence of nodal metastasis and ECS, while expression in tumour cells at the tumour centre and stromal cells at the tumour-advancing front was not significant. This is in keeping with the biological role of SERPINE1 in invasion and metastasis (Klein *et al*, 2012).

SERPINE1, also known as plasminogen activator inhibitor-1, is a regulator of the urokinase and tissue-type plasminogen activators. These serine proteases in turn activate the pro-enzyme plasminogen to plasmin that promotes invasion by degradation of the extracellular matrix, as well as activation of matrix metalloproteinases (Baker *et al*, 2007). The plasminogen activator/plasmin system can also promote invasion by downregulation of cell-cell adhesion molecules such as E-cadherin (Hayashido *et al*, 2005). The plasminogen activation system in the metastatic cascade also influences proliferation, migration, angiogenesis and extravasation, and it has been implicated in several tumour types including colorectal and breast cancer (Gandolfo *et al*, 1996; Duffy, 2004; Dano *et al*, 2005).

We compared these findings with a documented feature associated with poor outcomes in OSCC, SMA expression in stromal cells. The intense SMA expression at the tumour-advancing front, was significantly associated with the N-stage and presence of nodal metastasis and its patterns are in keeping with previous studies (Kellermann *et al*, 2007; Surowiak *et al*, 2007; Marsh *et al*, 2011; Thode *et al*, 2011). The distribution of SMA immunoreactivity in the tumour stroma varies widely between different tumours and between different areas in the same tumour, and reflects heterogeneity (Vered *et al*, 2010). However, there is a high level of concordance (up to 98%) reported for triplicate TMA cores (as used in the present study) being representative of the full sections (Hoos and Cordon-Cardo, 2001; Gulmann *et al*, 2003; Parsons and Grabsch, 2009). Relatively large cores were selected at the tumour-advancing front (1 mm) to ensure that adequate amounts of both tumour and adjacent stroma were present for scoring. Interestingly, high and intermediate levels of SMA expression were also seen at the tumour centre, although SMA expression would be expected to be the greatest at the advancing front where the tumour lies in proximity to stromal cells. This may reflect areas of myofibroblastic reaction/tumour-associated stroma at chronologically earlier sites, which have subsequently become incorporated within the growing tumour.

Both separately and in combination, SMA and SERPINE1 protein expression were superior to MRI for the detection of ECS, which was associated with expression of either, or both, proteins in all cases. Similarly, a combined approach using both SMA and SERPINE1 expression at the advancing front showed greater ability to predict survival than expression of the individual proteins. The most recent and largest study of prognostic stromal features in OSCC reported a greater prognostic significance for SMA expression in the stroma of OSCC than the presence of ECS (Marsh *et al*, 2011). Our study, however, suggests that ECS is of greater prognostic value, but that SMA plus SERPINE1 expression in combination was highly significantly associated with adverse outcomes.

The prognostic and diagnostic accuracy of SMA and SERPINE1 expression at the invasive front corresponds with observations that histological and molecular determinants at the invasive front are of greater prognostic value than the tumour centre (Bryne *et al*, 1992; Piffko *et al*, 1998; Kellermann *et al*, 2007; Vered *et al*, 2010; Kato *et al*, 2011). Our findings indicate that there are differences in the expression of both proteins at the tumour centre and the advancing front. Accordingly, realisation of their prognostic value would only be feasible if the advancing front is included in the preoperative diagnostic incisional biopsy. This, as discussed above, may prove difficult or impossible to achieve, particularly in large established tumours. Nevertheless, as SERPINE1 and SMA expression were more significantly associated with ECS and nodal status at the tumour-advancing front than the tumour centre, insights into the biological perspectives of ECS would be better explored with approaches focusing on the tumour-stroma interface. Future work validating these data in a larger series and in a prospective cohort with deep biopsy may determine the clinical applicability of this approach in future biomarker discovery and disease stratification in OSCC. The biological importance of SERPINE1 in cellular invasion together with the presence of myofibroblasts can also be further studied in three-dimensional co-culture organotypic models that utilise both epithelial and mesenchymal cells in which these genes can be upregulated or downregulated.

The growing interest in the tumour microenvironment and the effects of myofibroblasts in invasion and metastasis open up new avenues for investigation in both biological understanding and potential therapy in the metastatic cascade. Relationships between the advancing front and nodal metastasis/ECS reiterate the importance of this frontier for detection of appropriate biomarkers. We have demonstrated an informative approach by combining microarray findings that identified a biological determinant for ECS (SERPINE1) with a promising immunohistochemical marker of adverse outcome (SMA) to identify patients with poor survival in OSCC.

## ACKNOWLEDGEMENTS

J Dhanda received funding to conduct this research from the Royal College of Surgeons of England Research Department, Saving Faces—The Facial Surgery Research Foundation, The Faculty of Dental Surgery—The Royal College of Surgeons of England, The British Association of Oral & Maxillofacial Surgeons. DR Sibson and B Lloyd received funding to conduct this research from the Clatterbridge Cancer Research Trust (now North West Cancer Research).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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