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...
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 (Ramawamy 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 (Surwia 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/novel 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-mm² 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 therefore 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 Bioanalyzer (Agilent Technologies, Waldbronn, 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 β-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 ΔΔCt method (2−ΔΔCt) 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-μm sections of the TMAs and final dilutions of 1:600 of SMA antibody (Clone IA4, Dako), 1:300 of SERPINE1 antibody (3785, American Diagnostica, Stamford, CT, USA) and 1:800 anti-HEXIM 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. Immunohistochemistry scoring was similarly graded for intensity of immunoreactivity at 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 registered death from case-note review. Gene-expression values were calculated using a ROC curve analysis with calculation of the area under curve (AUC). The classification power of the individual gene expression was assessed using a ROC curve analysis with calculation of the accuracy of tissue sampling.

**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⁻² in 3 cycles) chemotherapy in 7 cases (7%), no adjuvant treatment in 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 = 0.07), 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

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

| Pathological T-stage | pN0 | pN+ ECS – | pN+ ECS + |
|----------------------|----|----------|----------|
| Age (years)          | Mean | 59.5 | 60 | 63 |
|                      | Range | 29–89 | 48–75 | 59 |

| Gender              | Male | Female |
|---------------------|------|--------|
|                     | 26   | 6      |
|                     | 12   | 13     |
|                     | 13   | 32     |

| Tumour site      | Tongue | Floor of mouth (FOM) | Buccal | Lower alveolus | Other |
|------------------|--------|----------------------|--------|---------------|-------|
|                  | 11     | 16                   | 1      | 5             | 5     |
|                  | 9      | 6                    | 0      | 0             | 4     |
|                  | 17     | 6                    | 3      | 5             | 5     |
|                  | 16     | 6                    | 3      | 1             | 3     |
|                  | 15     | 7                    | 2      | 1             | 2     |
|                  | 14     | 6                    | 2      | 1             | 2     |

| Pathological N-stage | N0 | N1 | N2a | N2b | N2c | N3 | MRI (% available) | RNA (% available) | Survival (est. Kaplan–Meier) |
|----------------------|----|----|-----|-----|-----|----|------------------|-------------------|------------------------|
|                      | 38 | 0  | 0   | 0   | 0   | 0  | 31 (82%)         | 35 (92%)          | 62%                    |
|                      | 0  | 10 | 2   | 2   | 3   | 0  | 16 (84%)         | 17 (89%)          | 70%                    |
|                      | 3  | 1  | 7   | 26  | 0   | 0  | 36 (80%)         | 37 (82%)          | 10%                    |
|                      | 13 | 2  | 14  | 0   | 0   | 0  | n = 38           | n = 19            | n = 45                  |

Abbreviations: ECS = extracapsular spread, MRI = magnetic resonance imaging.
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. SERPINE1 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 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).
confirm that preoperative assessment of patients with MRI has protein expression in the primary tumour offers promise in this selection for clinical trials with novel therapies. The present clinical decision-making regarding escalation of therapy as well as surgery. Identification of an appropriate biomarker would aid existing techniques are inadequate for its detection prior to The presence of ECS in OSCC is of high prognostic value and yet 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's 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; Coella 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 (Severino et al., 2008). 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; Dobroossy, 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).

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.

This 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).
SERPINE1 and SMA expression in OSCC

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.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.
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