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Oral cancer stem cells drive tumourigenesis through activation of stromal fibroblasts

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Abstract

Background: Cancer stem cells are responsible for tumour progression and chemoresistance. Fibroblasts surrounding a tumour also promote progression and fibroblast "activation" is an independent prognostic marker in oral cancer. Cancer stem cells may therefore promote tumourigenesis through communication with stromal fibroblasts.

Methods: Cancer stem cells were isolated from oral cancer cell lines by adherence to fibronectin or cisplatin resistance. Fibroblasts were exposed to conditioned medium from these cells, and the activation markers, alpha smooth muscle actin and interleukin-6, were assessed using qPCR and immunofluorescence. Stem cell markers and smooth muscle actin were examined in oral cancer tissue using immunohistochemistry.

Results: Adherent and chemoresistant cells expressed increased levels of stem cell markers CD24, CD44 and CD29 compared with unsorted cells. Adherent cells exhibited lower growth rate, higher colony forming efficiency and increased cisplatin resistance than unsorted cells. Smooth muscle actin and Interleukin-6 expression were increased in fibroblasts exposed to conditioned medium. In oral cancer tissue, there was a positive correlation between expression of αSMA and stem cell markers.

Conclusions: Adherence to fibronectin and chemoresistance isolates stem-like cells that can activate fibroblasts, which together with a correlation between markers of both in vivo, provides a mechanism by which such cells drive tumourigenesis.

KEYWORDS

cancer stem cell, fibroblast, oral cancer

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the 8th most common cancer worldwide (Amit et al., 2013). The incidence has been rising in recent years but despite advancements in treatment and detection strategies, the 5-year survival rate remains stubbornly around 50% (Amit et al., 2013). Cancer stem cells (CSCs) are a small population of cells within tumours which retain some of the features of normal stem cells such as slow growth rate and self-renewal but are thought to be those that persist, drive tumour progression and are resistant to therapy (Vermeulen, de Sousa e Melo, Richel, & Medema, 2012). It has become clear that interactions of the tumour cells with the surrounding stroma are important in progression. Indeed, markers

of stromal fibroblast "activation" such as alpha smooth muscle actin (αSMA) have been shown to have greater prognostic significance in oral cancer than features of the tumour itself (Marsh et al., 2011).

CSCs can be isolated using cell sorting utilising differential expression of a number of cell surface markers or through functional assays such as clonogenic potential, xenografting, efflux assays or aldehyde dehydrogenase activity (Islam, Gopalan, Smith, & Lam, 2015). CSCs have been shown to be present in OSCC cell lines and have been identified through colony morphology or rapid adherence to collagen IV (Liang et al., 2014; Locke, Heywood, Fawell, & Mackenzie, 2005). In this study, we have used two functional assays, rapid adherence to fibronectin and resistance to cisplatin, to isolate stem-like cells from two OSCC cells lines to determine whether such methods isolate distinct populations. Furthermore, we hypothesise these CSCs may be responsible for aberrant signalling to fibroblasts which may be central to tumour progression.

2 | MATERIALS AND METHODS

All reagents were from Sigma, UK unless otherwise stated.

2.1 | Cell lines and culture

Authenticated cells lines were obtained from ECACC, Salisbury, UK. H357 cells were cultured in media consisting of: Dulbecco's modified eagles medium (DMEM) and HAMS-F12 medium (3:1), 10% foetal bovine serum (FBS), Penicillin (100 IU/ml) and Streptomycin (100 mg/ml), Amphotericin B (2.5 μg/ml), all from Invitrogen, UK, L-Glutamine (2 mM), Adenine (0.18 mM), Hydrocortisone (5 μg/ml), Cholera toxin (1 nM), Insulin (5 μg/ml) and Epidermal growth factor (10 ng/ml). SCC4 cells were maintained in DMEM and HAMS-F12 medium (1:1) with 10% FBS, and antimicrobials as above. Primary normal oral fibroblasts (NOFs) were derived from gingival tissue obtained with informed, written consent from patients at the Charles Clifford Dental Hospital, Sheffield, UK, under the ethical approval (number 15/LO/0116) granted by the Sheffield Research Ethics Committee. NOFs were cultured in DMEM supplemented with 4.5 g/L D-glucose, 10% (v/v) FBS and antimicrobials as above.

2.2 | Adhesion assay

96-well tissue culture plates (Greiner Bio-One Ltd, UK) were coated with plasma fibronectin diluted in PBS (75 μg/ml) for 1 hr at 37°C and then blocked for a further hour with 1% bovine serum albumin (BSA) in DMEM. 40,000 cells in 100 μl were added and the plate incubated for 10 min at 37°C. Unattached cells were removed by inversion, and the wells were washed twice with 100 μl DMEM. Then, 100 μl DMEM was added to each well followed by 20 μl of MTS solution (CellTiter 96®, Promega, UK) and the plate incubated at 37°C in the dark. The absorbance was measured at 492 nm using an Infinite® M200 spectrophotometer and analysed using Magellan Software (Tecan UK Ltd).

2.3 | Chemoresistance assay

Cells were seeded in a 96-well plate at a density of 100,000 cells per well in their growth medium and incubated for 24 hr at 37°C. Wells were washed with DMEM before adding 15 μM cisplatin (*cis*-Diammineplatinum (II) dichloride) to triplicate wells and incubating for 24 hr at 37°C. Then, wells were washed in DMEM and 100 μl of growth medium was added to each well and incubated for 5 days at 37°C. Wells were washed twice followed by the addition of 100 μl DMEM and 20 μl of MTS solution for 1 hr at 37°C. The absorbance was then measured at 492 nm as above.

2.4 | Proliferation assay

Cells were seeded in triplicate wells of 4 separate 96-well plates at a density of 10,000 cells in 100 μl of their growth medium per well and were incubated at 37°C. At 24-hr intervals, one of the plates was removed, wells washed twice with DMEM followed by the addition of 100 μl DMEM and 20 μl MTS solution and the plate was incubated for 1 hr at 37°C. The absorbance of each well was measured as above.

2.5 | Colony forming assay

Early adherent cells or unsorted cells were plated at 500 cells/ml growth medium and incubated for 14 days at 37°C with a medium changed every 3 days. Wells were then washed with PBS twice and colonies fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Colonies were stained with methylene blue (5 mg/ml) in distilled water for 1 min. Excess stain was washed away and the plate left to dry. Images were captured, and numbers of colonies were counted for each well.

2.6 | Flow cytometry

Cells were detached from flasks using Accutase (BioLegend), re-suspended in flow buffer (PBS containing 10% FCS), adjusted to a density of 1×10^6 cells/ml and kept on ice. FITC conjugated mouse monoclonal anti-human CD24 (BD Pharmingen™, UK, 1/5 dilution), FITC conjugated mouse monoclonal anti-human CD29 (integrin-β1) (Life Technologies Ltd., 1/20 dilution) and PE conjugated mouse mono-clonal anti-human CD44 antibody (BD Pharmingen™, 1/5 dilution) were added for 1 hr on ice in the dark with the absence of antibody serving as a negative control. Cells were then washed, and the pellet re-suspended in cold flow buffer on ice. Finally, median fluorescence expression of CD24, CD44 and CD29 was quantified using a FACS Calibur machine and CellQuest software (BD Biosciences).

2.7 | Quantitative PCR

RNA was extracted using RNeasy mini kits (Qiagen) and genomic DNA digested using RNase free DNase (27KU/sample). cDNA was synthesised using a high capacity cDNA reverse transcription kit (Thermo Fisher). Tubes were then placed into a thermal cycler (BIO-RAD) and run at 25°C for 10 min, 37°C for 2 hr and 85°C for 5 min. All cDNA samples were stored at −20°C.

The following genes were targeted using SYBR green primers from Sigma: CD24 (Forward 5′ ACAGCCAGTCTCTTCGTGGT 3′ Reverse 5′ CCTGTTTTTCCTTGCCACAT 3′), CD44 (Forward 5′ CTGCCGCTTT GCAGGTGTA 3′ Reverse 5′ CATTGTGGGCAAGGTGCTATT 3′) and CD29 (Forward 5′ AATGAATGCCAAATGGGACACGGG 3′ Reverse 5′ TTCAGTGTTGTGGGATTTGCACGG 3′ for isolated and unsorted cells of oral cancer cell lines and α-SMA (Forward 5′ GAAGAAGAGGACAGCACTG 3′ Reverse 5′ TCCCATTCCCACCATCAA 3′) for fibroblasts, the U6 gene (Forward 5′ CTCGCTTCGGCAGCACA 3′ Reverse 5′ AACGTTCACGAATTTGCGT 3′) was used as an endogenous control. Sequences of these primers are shown below. IL-6 expression was quantified using Taqman probes (Hs00985639, Thermo Fisher) with B2M as an endogenous control (Hs00982282). Assays were performed in triplicate on a Rotor-Gene Q real-time PCR machine (QIAGEN). The thermal cycle consisted of 3 stages: 95°C for 10 s, 60°C for 15 s and 72°C for 20 s for 40 cycles. Rotor-Gene 2.1.0.9 software programme (QIAGEN) was used to analyse cycle threshold (Ct) values for all samples, and the qPCR data were quantified using the ΔΔCt method.

2.8 | Immunohistochemical staining

A total of 10 cases of moderate grade oral squamous cell carcinoma (Table 1) were obtained from the School of Clinical Dentistry under the ethical approval number 07/H1309/150 (Sheffield Research Ethics Committee). Sections (4 μm) were de-waxed in xylene followed by 100% ethanol. Slides were incubated for 20 min in 3% (v/v) hydrogen peroxide in 100% methanol (all Fisher Scientific) and antigen retrieval performed by microwaving at full power (Panasonic NN-E252W) in 0.01 M sodium citrate for 8 min. Slides were washed and cooled in PBS and blocked in 100% horse serum at room temperature for 30 min. The serum was removed and primary mouse monoclonal antibodies in horse serum (1/100 v/v) added in a humidified atmosphere overnight at 4°C. The antibodies used in this study were: anti-CD24 antibody (2 μg/ml, ab31622), anti-CD44 antibody (0.5 μg/ml, ab9524), anti-α-SMA antibody (10 μg/ml, ab7817, all Abcam). Slides were washed, and mouse biotinylated secondary antibody and Avidin Biotinylated enzyme Complex (VECTASTAIN® Elite, Vector Laboratories) was prepared and added. DAB (3,3'-diaminobenzidine tetrahydrochloride, Vector) was the added until a dark brown precipitate appeared. The slides were transferred to distilled water, counterstained with haematoxylin and mounted using DPX (Fisher).

Slides were digitally scanned using Aperio ScanScop (Leica Biosystems) and were archived and viewed using e.slide manager Digital Pathology software. Six images at the same magnification were captured for regions of interest (ROI) of the tumour invasive front regions in each tumour section slide using the Aperio ImageScope software (Leica) and the free hand tool of Image J software was used to select and analyse the specific ROI to determine the percentage of antibody staining.

2.9 | Immunofluorescence

Cells were seeded at a density of 5,000 cells on glass coverslips (Fisher Scientific) in 1 ml of their growth medium in a 24-well plate for 48 hr at 37°C. Coverslips were washed with PBS, fixed with 100% methanol (Fisher) for 15 min, permeabilised with 4 mM sodium deoxycholate in PBS for 15 min and incubated with blocking buffer (25 μg/ml BSA in PBS) for 30 min at room temperature. 0.2 ml of FITC conjugated anti-alpha smooth muscle actin (α-SMA) antibody (1:100 dilution, Clone A.4) was added for 1 hr at room

TABLE 1 Clinical and histopathological data of patients and tumours in the selected study sample

Case	Age & gender	Sizemm	Depthmm	Front	Host response.	Peri-neural	Vascular	Margin	Mets	ECS
1	88 F	34	10	Disc	Mod	No	No	Close	Yes	Yes
2	68 M	18	5.5	Coh	Poor	No	No	Clear	Yes	No
3	63 M	30	23	Disc	Mod	No	Yes	Invol	Yes	No
4	79 M	30	17	Disc	Poor	Yes	No	Close	Yes	No
5	55 F	12	1.5	Coh	Poor	No	No	Invol	Yes	No
6	42 F	30	12.5	Disc	Poor	Yes	No	Close	Yes	Yes
7	70 F	49	11.5	Disc	Mod	No	Yes	Close	Yes	Yes
8	71 M	62	20	Coh	Mod	No	No	Invol	Yes	Yes
9	91 M	14	6	Disc	Strong	No	No	Clear	Yes	Yes
10	72 F	50	8	Disc	Mod	Yes	No	Close	Yes	Yes

Note: All tumours were of moderate grade.

Abbreviations: Coh, cohesive; Disc, discohesive; ECS, extra capsular spread; F, female; Invol, involved; M, male; Mod, moderate.

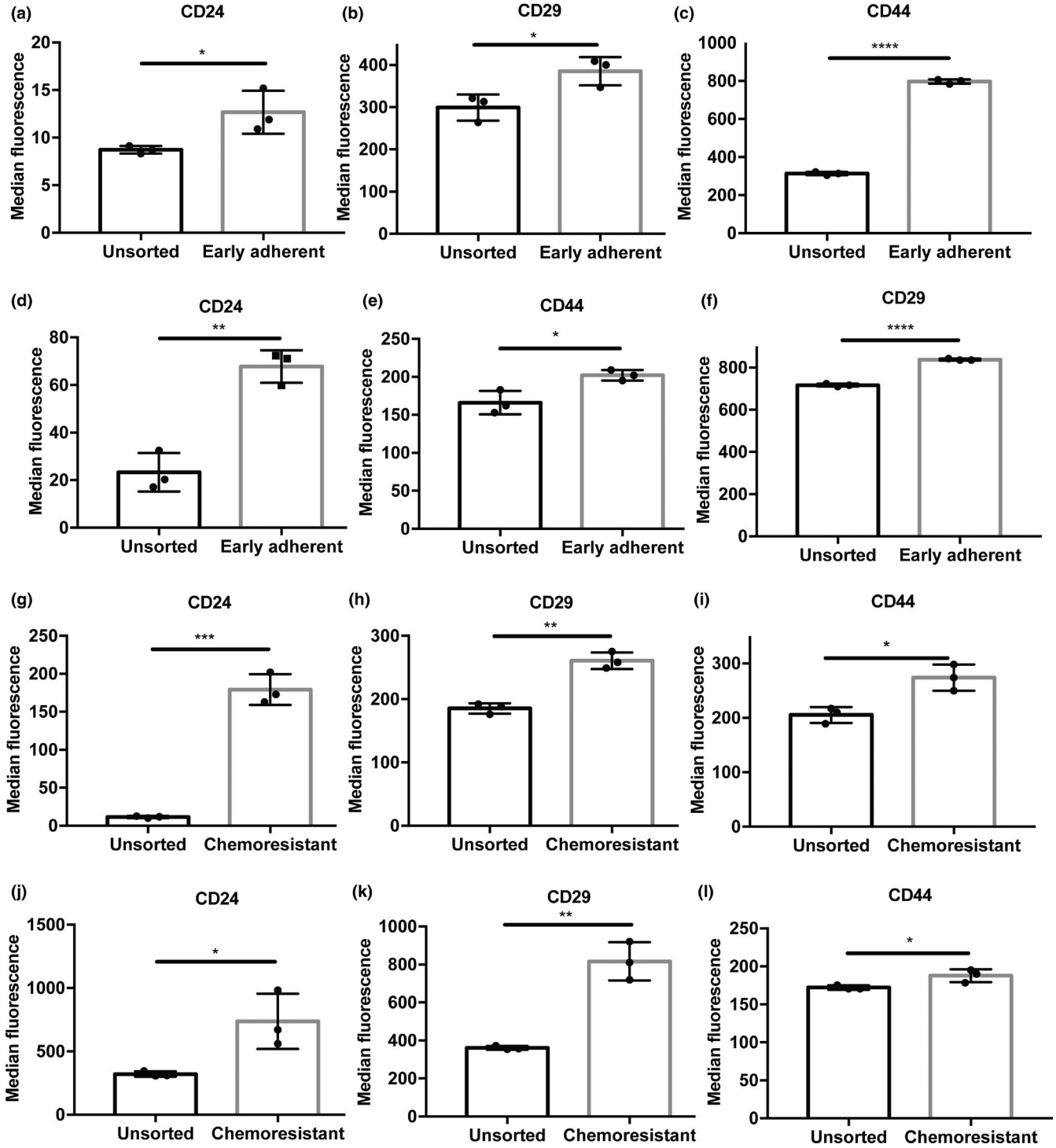

FIGURE 1 Expression of stem cells markers by unsorted OSCC cells and those sorted using early adherence to fibronectin or chemoresistance to cisplatin determined using flow cytometry. Cell surface expression of CD24, CD44 and CD29 by early adherent H357 (a-c) and SCC4 cells (d-f). Cell surface expression of CD24, CD44 and CD29 by chemoresistant H357 (g-i) and SCC4 cells (j-l). $n = 3$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

temperature. Coverslips were washed and mounted with Prolong™ Diamond mountant with DAPI (Thermo Fisher). Slides were viewed and images captured using a fluorescent microscope (Axioplan 2 and image-ProPlus 7.0.1 software, Zeiss). The level of fluorescent staining was quantified using Image J software (version 1.52, NIH, USA).

2.10 | Fibroblast activation

OSCC cells were seeded at a density of 200,000 cells per well and incubated at 37°C for 24 hr. Cells were washed twice and 1 ml 0.5% FCS in DMEM: F12 (3:1) was added and incubated at 37°C for 48 hr. Normal oral fibroblasts (NOFs) were plated onto sterile coverslips

in growth medium at 5,000 cells/well for immunofluorescence and 100,000 cells/well for qPCR. Conditioned medium from OSCC cells was centrifuged at 300 g for 10 min to remove any cells. Medium was removed from the NOFs, and 1 ml conditioned medium from isolated and unsorted OSCC cells was added. In addition, medium containing 10 ng/ml human recombinant TGF-β1 (R&D Systems) was used as a positive control and medium alone as a negative control. The plates were then incubated at 37°C for 48 hr.

2.11 | Statistical analysis

Unless otherwise stated, all experiments were performed three times in triplicate ($n = 3$). GraphPad Prism, version 7 software was used for analysis and comparisons made using unpaired two tailed Student's t test with a p value <.05 considered significant. Simple linear regression was used to test the statistical significance of data correlations.

3 | RESULTS

H357 and SCC4 cells isolated by adherence to fibronectin for 10 min exhibited significantly higher surface protein levels of the stem cell markers CD24, CD44 and CD29 compared with unsorted cells (Figure 1a-f). This was also the case for cells that were resistant to cisplatin (Figure 1g-l). Expression of these stem cell marker genes using qPCR was similarly significantly increased in early adherent and chemoresistant cells from both lines (Figure 2a-d). Isolated cell populations also displayed functional characteristics of stem cells with reduced growth rate (Figure S1) and an enhanced ability to form colonies (Figure 3a,b).

Our two functional methods of separating stem cells appeared to be isolating a similar population as rapidly adherent cells were more resistant to cisplatin than unsorted cells (Figure 3c,d), and chemoresistant cells were more adherent to fibronectin (Figure 3e,f).

Conditioned media from early adherent and chemoresistant cells from both cells lines resulted in an upregulation of both αSMA and IL-6 gene expression in normal oral fibroblasts to a similar degree (Figure 4). The effect on αSMA expression was however not as large as that of the well described effects of TGF-β1 on this gene (Figure 4a-d). In this study, treatment with TGF-β1 did not result in an increase in IL-6 gene or protein expression (Figure 4e-h). Protein expression of αSMA was similarly affected in fibroblasts treated with the conditioned medium from early adherent and chemoresistant cells with strong fluorescent intracellular stress fibre staining visible in these but not cells treated with unsorted cell conditioned medium (Figure 5a-d). Quantification of the fluorescence intensity revealed that for bot cell lines conditioned medium from isolated cell was able to elicite a response similar to that of TGF-β1 (Figure 5g,j).

Immunohistochemical analysis of CD24 and CD44 was performed in OSCC tissue and showed expression of both throughout tumour epithelium and islands (Figure 6a,c). Image analysis revealed expression of both stem cell markers correlated closely with expression of αSMA in the tumour stroma (Figure 6b,d,e) as well as with each other (Figure 6f).

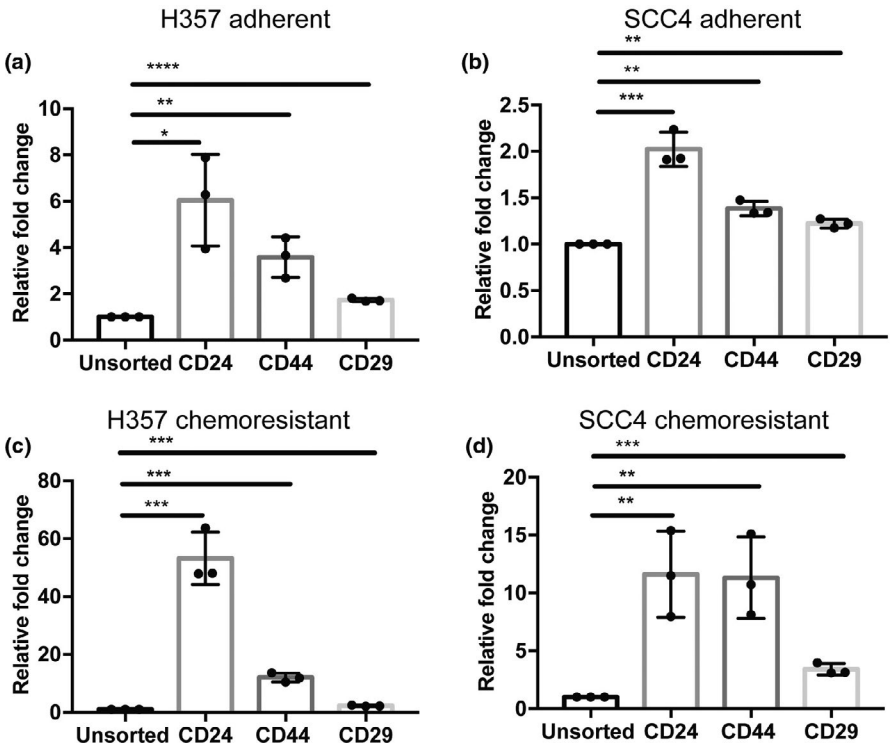

FIGURE 2 Gene expression of stem cell markers by OSCC cells sorted using early adherence to fibronectin or chemoresistance to cisplatin determined using quantitative PCR. Fold change relative to gene expression in unsorted cells shown for early adherent H357 (a) and SCC4 cells (b) and chemoresistant H357 (c) and SCC4 cells (d). $n = 3$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$

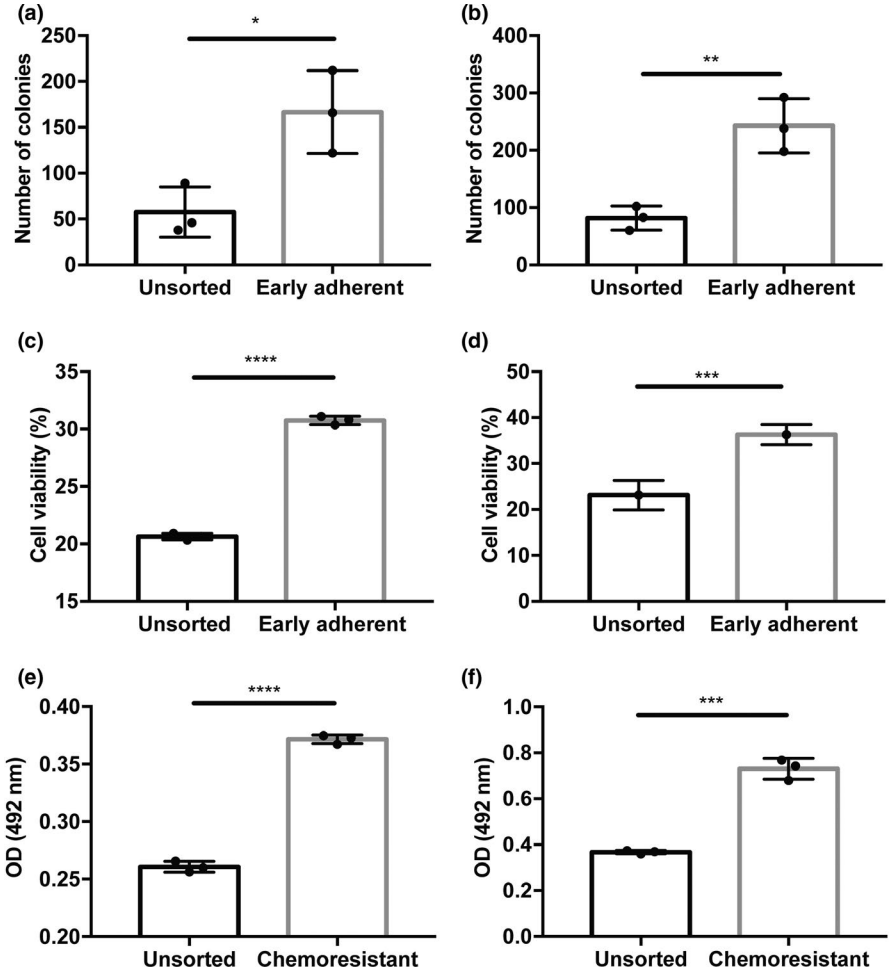

FIGURE 3 Colony forming ability of early adherent H357 (a) and SCC4 cells (b) compared with unsorted cells. Cell viability of early adherent H357 (c) and SCC4 cells (d) following exposure to cisplatin compared with unsorted cells. Early adhesion of chemoresistant H357 (e) and SCC4 cells (f) to fibronectin compared with unsorted cells. $n = 3$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$

4 | DISCUSSION

Our data showed rapid adhesion to fibronectin and resistance to cisplatin in a sub population of cells from the OSCC lines H357 and SCC4. We chose these assays based on the fact that they are both functional in nature and do not purely relate to expression of stem cell markers. Previous studies have predicted that the percentage of stem cells is less than 10% of the total unfractionated keratinocytes (Jones, Harper, Watt, & Le, 1995; Potten & Morris, 1988) which is in accordance with our study. In data not shown, we have demonstrated that both these cell lines are much more adherent to collagen I which is agreement with previous work suggesting that this matrix protein has limited use in isolating stem cells as most basal layer keratinocytes rapidly adhere (Kaur & Li, 2000). Isolated populations of cells (early adherent and chemoresistant) from the both oral cancer cell lines tested expressed significantly higher levels of integrin-β1 (CD29) compared with unsorted cells which, as this is a receptor for fibronectin, may explain their enhanced adherence.

To characterise the isolated population of cells, we selected three of the most common head and neck squamous cell carcinoma stem cell surface markers CD44 (hyaluronate receptor), CD24 (specific ligand of P-selectin receptor) and CD29 (integrin-β1). Our selection of multiple markers was motivated by the lack of a universal or single specific marker for CSCs, and there is variation in the most appropriate markers to best distinguish CSCs from non-CSCs populations in different tumour type (Tirino et al., 2013). Our findings from FACS and gene expression analysis showed that the isolated cells expressed significantly increased levels of all investigated stem cell surface markers (CD24, CD44 and CD29) compared with that of unsorted cells. This is consistent with studies by Han, Fujisawa, Husain, & Puri (2014) which showed a population of CD24+/CD44+ head and neck squamous cell carcinoma cells expressed higher levels of CD29 and possessed "stemness" characteristics. In addition, low proliferation rate but enhanced colony forming ability is considered important in vitro functional assays for characterising of CSCs (Franken, Rodermond, Stap, Haveman, & Van Bree, 2006; Roesch et al., 2010). Our results indicated a reduced growth rate of sorted cell subpopulations compared with unsorted cells in both OSCC cell lines. Furthermore, early adherent cells showed a significantly higher clonogenic potential compared with unsorted cells, which is in agreement with previous studies on HNSCC adhesion to collagen IV (Liang et al., 2014).

Our findings that early adherent cells of both cancer cell lines were more resistant to cisplatin in comparison with unsorted cells and vice versa suggests that we have isolated a similar population of cells. Previous studies found enhanced cisplatin chemoresistance in cancer cells with increased adhesion to fibronectin (Hartmann,

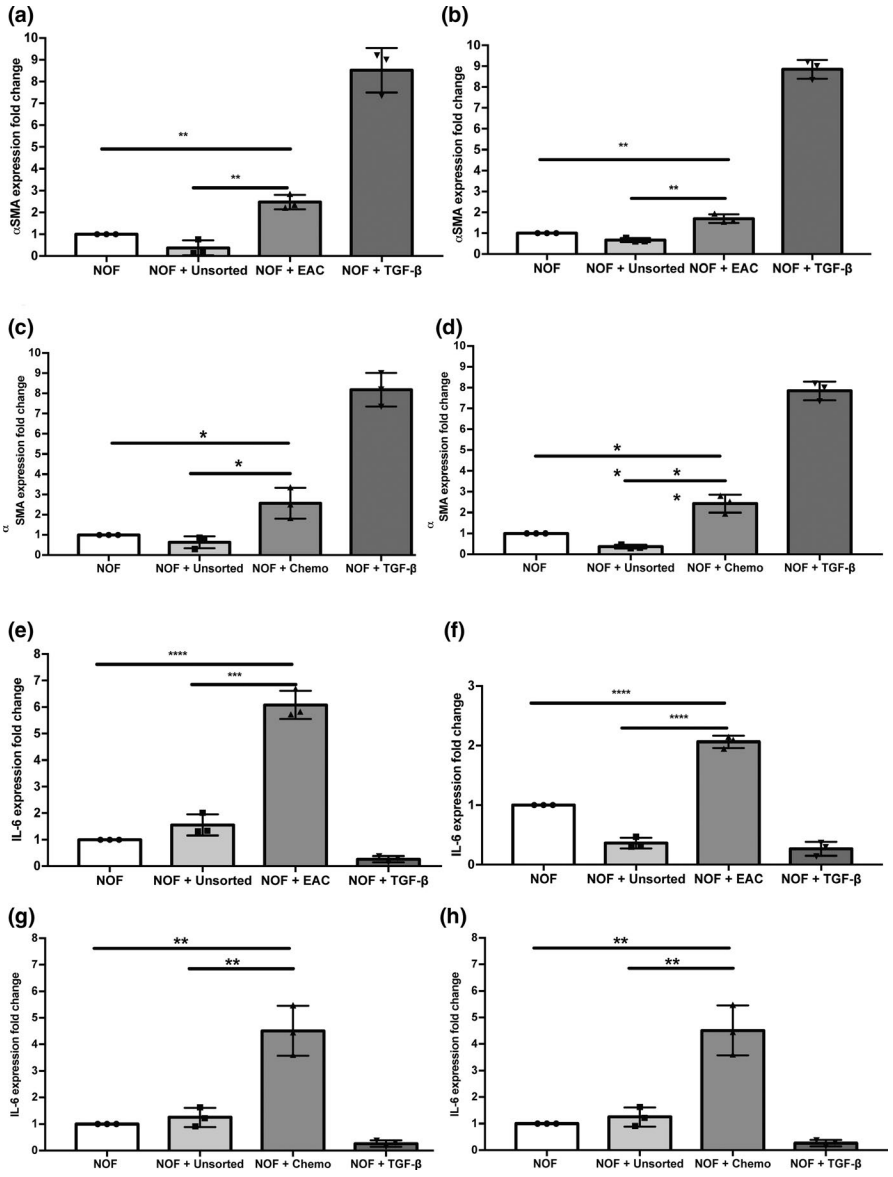

FIGURE 4 Fold change in α-smooth muscle actin gene expression determined by quantitative PCR in normal human oral fibroblasts (NOFs) following exposure to conditioned media from unsorted and early adherent H357 (a) and SCC4 cells (b) and chemoresistant H357 (c) and SCC4 cells (d) along with TGF-β1 (10 ng/ml). Fold change in interleukin-6 gene expression determined by quantitative PCR in normal human oral fibroblasts (NOFs) following exposure to conditioned media from unsorted and early adherent H357 (e) and SCC4 cells (f) and chemoresistant H357 (g) and SCC4 cells (h). $n = 3$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$

Burger, Glodek, Fujii, & Burger, 2005; Nakahara et al., 2003), possibly mediated by α5β1 integrin survival signals inhibiting apoptosis. Vallo et al. (2017) reported upregulated levels of β1 integrin in cisplatin-resistant urethral cancer cell lines.

Cancer cells secrete cytokines and growth factors that mediate fibroblast activation into cancer-associated fibroblasts (CAFs), which is considered an early and important event in tumourgenesis (Beacham & Cukierman, 2005; Liotta & Kohn, 2001). To investigate whether the isolated CSCs signal differently to fibroblasts, we analysed expression of two genes commonly upregulated in CAFs: α-SMA and IL-6 (Bolt et al., 2018; Kunz-Schughart & Knuechel, 2002) upon exposure to condition media from isolated cells. Our results revealed upregulation of both markers (α-SMA and IL-6) when fibroblasts were incubated with conditioned media collected from isolated cells (early adherent and chemoresistant) for the both examined oral cancer cell lines (H357 and SCC4) in comparison with conditioned media of unsorted cells. To our knowledge, this observation has not been previously reported.

Normal lung fibroblasts incubated with conditioned media from cancer cells treated with cisplatin showed an increased activation of these fibroblasts into CAFs (Shintani et al., 2016). This is similar to our findings that normal oral fibroblasts incubated with conditioned media collected from chemoresistant cells were activated and expressed intense α-SMA staining as well as upregulated CAF markers. Integrin pathways (α5β1 and αvβ6) also regulate and induce TGF-β1 in oral and dermal fibroblasts (Lygoe, Wall, Stephens, & Lewis, 2007). Therefore, the activation of fibroblasts incubated with condition media of isolated cells could be attributed to stimulation of integrin-β1 receptors that may result in higher TGF-β1 which in turn increases activation of normal oral fibroblasts into CAFs. Integrin-β1 receptors may also mediate costimulatory signals that required for inflammatory cytokine gene expression and subsequent production of inflammatory cytokines (Miyake et al., 1993). Several studies reported that some inflammatory cytokines are secreted by cancer cells induce the activation of stromal fibroblasts into CAFs (Giannoni

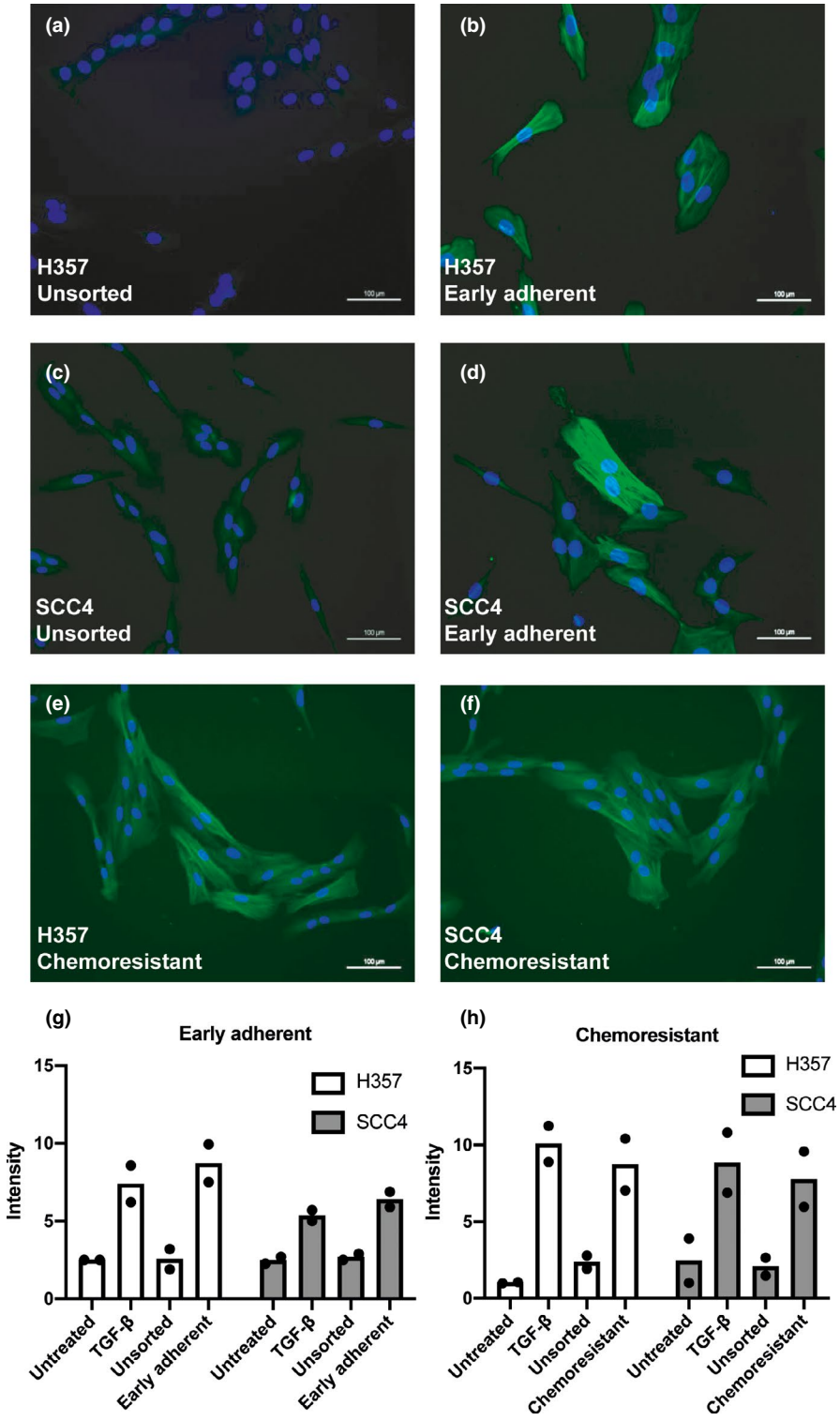

FIGURE 5 Expression of α-smooth muscle actin by fluorescence immunocytochemistry in unsorted H357 (a) and SCC4 (c) cells compared with early adherent H357 (b) and SCC4 cells (d) and chemoresistant H357 (e) and SCC4 cells (f). Representative images of $n = 2$ shown, bar = 100 μm. Quantification of fluorescence intensity in early adherent (g) and chemoresistant (h) H357 (open squares) and SCC4 cells (filled squares). Expression in media treated fibroblasts (negative control) is compared with those exposed to TGF- β1 (10 ng/ml) (positive control) and conditioned media from unsorted and sorted cells ($n = 2$)

et al., 2010; Paland et al., 2009). Therefore, it may be that activation of NOFs exposed to conditioned media from isolated cells is due to pro-inflammatory cytokines such as IL-6, IL-8 and IL-1β (Doldi et al., 2015).

Previous studies have reported that cells with co-expression of CD24 and CD44 were confirmed as CSCs in different types of cancer, including head and neck squamous cell carcinoma (Han et al., 2014). Our results revealed a significant positive correlation between the expression of CD24 and CD44 markers in oral squamous cell carcinoma. Our findings demonstrated a positive correlation between the expression of both CSC markers CD24 and CD44 in the tumour and the expression of α-SMA in stroma. This correlation has not been previously reported and suggests that our findings in vitro may also be of relevance in vivo. Studies conducted on OSCC indicate that epithelial cancer cells induced the activation of stroma and increased expression of stromal

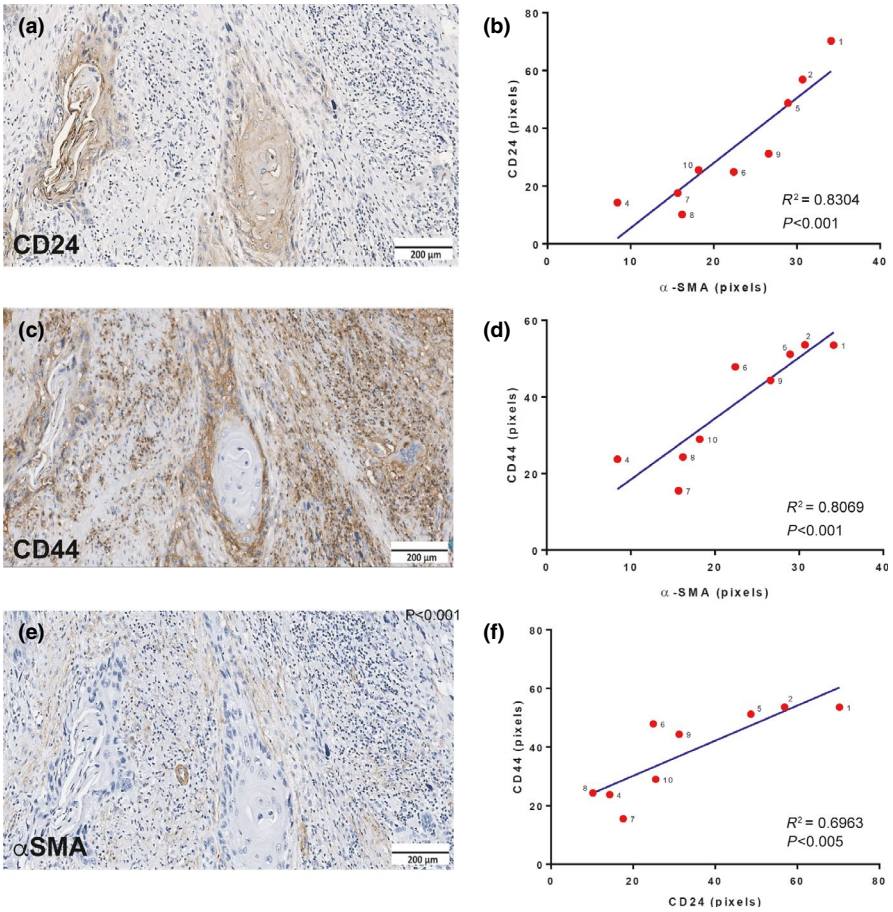

FIGURE 6 Immunohistochemical expression of CD24 (a), CD44 (c) and αSMA (e) in a representative OSCC tissue sample along with the correlation of expression analysis in all 10 cases of OSCC for CD24 and αSMA (b), CD44 and αSMA (d) and CD24 and CD44 (f). Bar = 200 µm

α-SMA (Etemad-Moghadam, Khalili, Tirgary, & Alaeddini, 2009; Prasad et al., 2016). Furthermore, there is a significant positive link between α-SMA expression in the tumour stroma and the increasing invasiveness and subsequent poor prognosis of OSCC (Dourado, Guerra, Salo, Lambert, & Coletta, 2018; Kellermann et al., 2007). Similarly, many studies correlated the elevated expression of CD24 and CD44 stem cell markers in oral squamous cell carcinoma with an increasing in tumour progression, aggressiveness, invasion and metastasis. Han et al. (2009) reported CD24 and CD44 expression as important diagnostic markers for OSCC. Expression of CD44, CD24 and CD29 correlates with the increased growth rate and resistance to radiotherapy in head and neck squamous cell carcinoma (Koukourakis, Giatromanolaki, Tsakmaki, Danielidis, & Sivridis, 2012; Oliveira et al., 2011) and poor overall survival outcomes in patients with OSCC (de Moraes et al., 2017). Abdulmajeed, Dalley, and Farah (2013) considered increased expression of CD24 a potent diagnostic marker for detection of oral epithelial dysplasia and OSCC.

We have demonstrated that a population of cells expressing stem cell markers and functional properties can be isolated for oral cancer cell lines using either rapid adherence to fibronectin or resistance to cisplatin. Secreted factors from these cells can activate normal oral fibroblasts as demonstrated by upregulated αSMA and IL-6 expression. It may therefore be the case that cancer stem cells drive tumour progression through effects on the surrounding tumour stroma.

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CONFLICT OF INTEREST

The authors confirm they have no conflict of interest.

AUTHOR CONTRIBUTION

Mohanad JN Al-Magsoosi: Investigation; Methodology; Project administration; Writing-original draft. **Daniel W Lambert:** Conceptualization; Supervision; Writing-review & editing. **Syed A Khurram:** Conceptualization; Supervision; Writing-review & editing. **Simon Whawell:** Conceptualization; Data curation; Formal analysis; Project administration; Supervision; Writing-original draft; Writing-review & editing.

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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