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Expression of Epsin3 and its interaction with Notch signalling in oral epithelial dysplasia and oral squamous cell carcinoma

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Abstract

Background: Most oral squamous cell carcinoma patients present with late-stage disease. Early detection of the disease is considered to be the most effective way of improving patient outcomes. Several biomarkers have been identified as indicators of oral cancer development and progression; however, none have been translated into clinical practice. In this study, we have investigated the role of Epsin3, an endocytic adaptor protein, and Notch1, a transmembrane signalling protein, in oral carcinogenesis with a view to explore their potential as biomarkers.

Methods: Oral cancer cell lines and a normal oral keratinocyte cell line were used together with tissue samples of normal oral mucosa ($n = 21$), oral epithelial dysplasia ($n = 74$) and early stage (Stages I and II) oral squamous cell carcinoma ($n = 31$). Immunocytochemical staining, immunoblotting and real-time quantitative polymerase chain reaction (PCR) were performed to assess protein as well as gene expression levels.

Results: The expression levels of Epsin3 and Notch1 mRNA and protein are variable across different oral squamous cell carcinoma derived cell lines. Epsin3 was upregulated in oral epithelial dysplasia and oral squamous cell carcinoma tissues compared with normal epithelium. Overexpression of Epsin3 resulted in a significant reduction of Notch1 expression in oral squamous cell carcinoma. Notch1 was generally downregulated in the dysplasia and oral squamous cell carcinoma samples.

Conclusion: Epsin3 is upregulated in oral epithelial dysplasia and oral squamous cell carcinoma and has the potential to be used as a biomarker for oral epithelial dysplasia. Notch signalling is downregulated in oral squamous cell carcinoma, possibly through an Epsin3-induced de-activation pathway.

KEYWORDS
endocytosis, Epsin3, Notch signalling, oral epithelial dysplasia, oral squamous cell carcinoma

1 | INTRODUCTION

Oral cancer is the 8th and 13th most frequent malignancy in the world for males and females, respectively. The most common type is oral squamous cell carcinoma (OSCC) which constitutes more than 90% of all cases.[1] Despite recent therapeutic advances, the prognosis has not improved over the last decades, and the overall 5-year survival rate remains at around 45%,[1] mainly due to late diagnosis. Early detection of the disease is considered to be the most effective way of improving patient outcomes. Biopsy of potentially malignant lesions of the oral cavity and oral epithelial dysplasia (OED) grading is the most common method for risk assessment; however, this method lacks sensitivity and cannot accurately predict which lesion will progress to cancer.[1] Henceforth, there is a need to develop new tools for early diagnosis and patient stratification. Several molecular biomarkers have been identified; however, these methods have yet to be translated into clinical practice[2]

Epsins are endocytic adaptor proteins that promote the internalisation of transmembrane proteins during clathrin-mediated endocytosis events, possibly through a ubiquitin- and actin-dependent mechanism.[3] It is widely accepted that endocytosis is one of the physiological processes that is modified in cancer progression.[4] A dysregulation of endocytosis could result in intracellular signalling defects, as it was demonstrated for the endocytic adaptor proteins Epsins in endothelial cells.[4] Epsin family in mammals consists of three members: Epsin1 and 2, which are ubiquitously expressed but highly expressed in neuronal cells, and Epsin3 which is enriched in keratinocytes.[5,6] Epsins have multiple ubiquitin interacting motifs (UIM), which are responsible for epsin ubiquitylation.[7] It had been suggested that Epsins utilise these UIM motifs to target ubiquitylated membrane proteins in order to recruit these cargos for clathrin-mediated endocytosis.[7] Example of these ubiquitylated membrane proteins are the Notch ligands Delta, Serrate and LAG-2 (DSL), which bind Notch receptors,[8] activating Notch signalling. Genetic, molecular and bio-physical studies point to a mechanical action of the Notch ligand on its receptor, so that critical proteolytic sites are uncovered for constitutive activation.[8] Although the molecular machinery is not fully characterised, genetic evidence in vertebrates and invertebrates supports clathrin-mediated endocytosis, triggered by DSL ubiquitylation, as the key mechanism to exert the pulling action of the Notch ligand on the Notch receptor.[8]

In the adult, Notch signalling regulates growth homeostasis, while its dysregulation tightly associates with cancer. Notch signalling is reported to have both oncogenic and tumour suppressor functions in cancer, depending on the cellular context. More specifically, in the cells in which Notch is involved in stem cell self-renewal or in cell fate decisions, it acts as an oncogene. Conversely, Notch is a tumour suppressor in those few tissues, like skin and surface epithelia, in which it promotes cell differentiation.[9] Notch signalling is reported to be dysregulated in the head and neck squamous cell carcinoma (HNSCC) as well as in OSCC, as a result of mutations in Notch pathway components, especially Notch1.[9]

In the present study, we analysed, for the first time, the expression profile of Epsin3 and Notch1 in OSCC cell lines and tissues from normal oral epithelia, OED and OSCC.

2 | MATERIALS AND METHODS

2.1 | Cell lines and tissue samples

Five established oral cancer cell lines were used in this study.[10,11] Cells (excluding immortalised normal oral keratinocyte cell line [OKF6]) were maintained in Dulbecco's modified Eagle's medium (DMEM/F12 1:1 mixture, with 15 mM Hepes and 0.6 μg/mL l-glutamine) (Lonza UK) supplemented with 10% (v/v) foetal calf serum (Sigma), 0.5ug/mL hydrocortisone 21-hemisuccinate sodium salt and 10 mL penicillin streptomycin (200 units/mL penicillin and 200 μg/mL streptomycin, Sigma). The OKF6 cells were cultured in a defined media composed of keratinocyte serum-free media with 0.6 μg/mL l-glutamine, supplemented with 0.2 ng/mL human recombinant epidermal growth factor, 20 μg/mL bovine pituitary extract and 5 mL penicillin-streptomycin (200 units/mL penicillin and 200 μg/mL streptomycin).

Formalin-fixed paraffin-embedded (FFPE) blocks of normal oral mucosa ($n = 21$), OED ($n = 74$) and early-stage (Stages I and II) oral squamous carcinoma ($n = 31$) were available for the study and were used according to a favourable ethics opinion (evaluation of the prognostic potential and functional significance of biomarkers in oral cancer 11/NE/0118 NRES Committee North East—Sunderland).

2.2 | Quantitative reverse transcription PCR

Total RNA was extracted using an RNA extraction kit (PureLink RNA Mini Kit [Ambion Life Technologies]). All reverse transcription were performed using the high-capacity cDNA reverse transcription kit. (Primers specific for human Epsin3 were designed using software from NCBI [USA] and supplied by IDT [UK].) Primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as the reference gene, were from previously published work.[12] All PCRs were carried in triplicate. PCR was performed using Taq polymerase (New England Biolabs) with the following Epsin3 primers: Forward^{1255}GTCCACCATCAGCGGGAC^{1272}, Reverse^{1425}CGGCCTAAAACCTGGGATGT^{1406} (numbered according to the sequence deposited under GenBank accession number NM-017957.2) and GAPDH (IDT, UK). Quantitative PCR was performed (DNA Engine Opticon 2 continuous fluorescence Detector, MJ Research, USA) and QuantiFast SYBR Green Master Mix (Qiagen). C_t values for the target and reference transcripts were converted to relative levels of target transcripts using the 2^{DDCt} method.[13]

2.3 | Immunoblotting

Total proteins were extracted using lysis buffer (RIPA, Sigma). Quantification was carried out by Bradford assay. Total protein 20 μg was subjected to SDS-PAGE on a 12.5% acrylamide gel. Proteins were transferred onto polyvinylidene difluoride membrane (Hybond P, Amersham Pharmacia Biotech) and incubated with rabbit monoclonal anti-Notch1 antibody (1:1000, Cell Signaling), rabbit polyclonal anti-

Epsin3 antibody (1:50, Abcam (ab91225) EPN3 antibody) or anti-rabbit alpha-tubulin (dilution 1:10 000, Abcam) in 1 × PBS, pH 7.4, containing 0.01% Tween 20 and 5% non-fat milk powder overnight at 4°C followed by incubation for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000 dilution) (Sigma). Bound secondary antibodies were visualised using a luminol-based enhanced chemiluminescence kit (Thermo Scientific). Band intensities were quantified by densitometry using GeneTools (SynGene).

2.4 | Overexpression of Epsin3 in H103 and BICR31 OSCC cell lines

A commercially available mammalian expression vector containing the human Epsin3 sequence (Lenti ORF clone of Human Epsin3, Myc-DDK-tagged, 10 µg OriGene) was transformed into a Top10 chemically competent DH5α *Escherichia coli* (*E. coli*) cells in order to generate a high concentration of vector to be used in the transfection experiments. H103 OSCC cells were seeded in six-well plates at a density of 4 × 105 cells/well. Twenty-four hours post-seeding approximately 90% confluency cells were transfected either with human Epsin3 plasmid or p3XFLAG-CMV-10 (Sigma Aldrich) with a ratio of DNA to GeneJammer 1 µg:2 µL. Twenty-four hours post-transfection, RNA and protein were prepared from cells.

2.5 | Preparation of cell blocks

To examine Epsin3 and Notch1 expression in the OSCC cell lines, FFPE cell pellets were prepared. The cells were harvested using trypsin, the detached cells were resuspended in 6 mL of growth medium and pelleted by centrifugation. Without disturbing the pellet, 4 mL of 10% (v/v) neutral buffered formalin (NBF) was added and left overnight at room temperature to fix. Following fixation, the NBF was removed and discarded. The fixed cell pellets were placed on a filter paper, which were folded and placed in a standard plastic processing cassette. The pellets were processed and embedded in paraffin wax blocks.

2.6 | Immunohistochemistry

Four micrometres sections of FFPE cell blocks or tissue were mounted on adhesive slides (Superfrost Plus, Thermo Fisher Scientific, UK). Immunohistochemistry (IHC) was carried out on a Ventana Benchmark Ultra autostainer (Ventana Medical Systems Inc, USA). The primary antibodies were: anti-Epsin3 antibody (mouse monoclonal 1:25 dilution), against the mouse Epsin3 sequence (KQNGMKEPEALDLGVLGEAL),[14] Notch1 (Cell Signaling, rabbit monoclonal, 1:200) and Notch1 (Abcam, rabbit monoclonal, 1:400). The antibodies were optimised using normal tonsil, breast cancer and prostate cancer specimens.

2.7 | Image analysis

Slides were scanned using an Aperio Scanscope platform (×400 magnification). Files were analysed using the Aperio Spectrum image analysis system (Spectrum Version 11.1.0.751, Aperio Technologies, Inc.). Representative areas were annotated and analysed using the Aperio cellular algorithm.

2.8 | Statistical analysis

Statistical analysis was performed using SPSS 23.0 software (IBM, Portsmouth, UK). All data are presented as the mean ± SD from three independent experiments. Kaplan–Meier survival curves were generated using Log Rank Mantel–Cox test. For OED, malignant transformation was designated as the event. For early-stage OSCC, death from any cause was designated as the event.

3 | RESULTS

3.1 | Expression levels of Epsin3 and Notch1 in OSCC cell lines

To test the hypothesis that Epsin3 and Notch signalling could be involved in oral carcinogenesis, mRNA expression of Epsin3 and Notch1 was examined in nine human OSCC-derived cell lines and in the OKF6. Quantitative reverse transcription PCR (RT-qPCR) analysis showed variable Epsin3 expression between the cell lines, with distinct differences in the mRNA expression levels of Epsin3 between the OSCC cell lines and OKF6. The H357 cells had the highest Epsin3 mRNA expression ($p < 0.001$) compared with normal OKF6 cells. Notch1 mRNA showed different levels of expression in the OSCC cell lines analysed, with the BICR31 cell line expressing the highest level compared with all other cell lines. OKF6 cell line showed the lowest level of expression for both Epsin2 and Notch1 (Figure 1).

We further investigated the expression profile of Epsin3 and Notch1 protein in the OSCC cell lines using IHC. Epsin3 protein was present and detected in all cell samples, with different levels of expression in the different OSCC cell lines (Figure 2A). The staining for each cell line was subject to image analysis, and the percentage of Epsin3 positive cells was calculated. Between the OSCC-derived cell lines, there was a significant difference: we confirmed the RT-qPCR results, finding that H357 had the highest Epsin3 protein expression compared to H103, H376 and BICR56 ($p < 0.001$) and BICR31 ($p < 0.05$) OSCC cells and normal OKF6 ($p < 0.05$) (Figure 2A). The expression levels of Notch1 were different between the OSCC cell lines (Figure 2B). The percentage of positive cells was calculated and analysed. We confirmed the RT-qPCR findings, showing that BICR31 had significantly more Notch1 positive cells than OKF6, H103, H357, H376 and BICR56 cell lines ($p < 0.001$) (Figure 2B).

To investigate the possible effect of an increased Epsin3 expression on Notch signalling in OSCC, we transiently transfected the

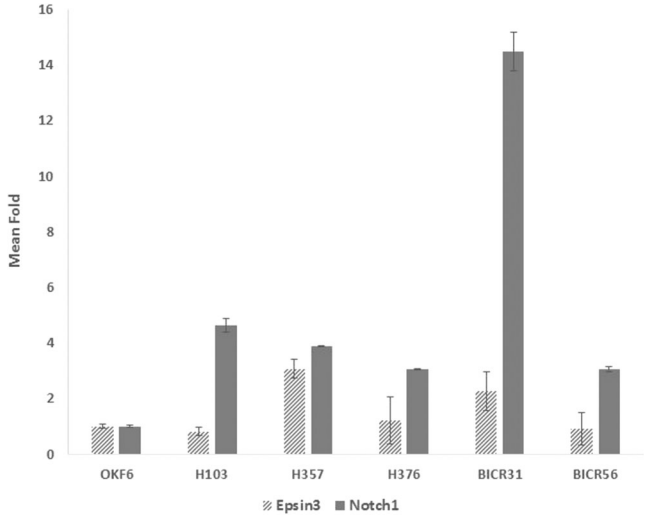

FIGURE 1 Epsin3 and Notch1 gene expression in oral squamous cell carcinoma (OSCC)-derived cell lines. Quantitative analysis of Epsin3 and Notch1 mRNA levels in normal oral keratinocytes (OKF6) and OSCC-derived cell lines by RT-qPCR. The values are relative to OKF6 cells and expressed as the mean ± SD of three independent experiments.

H103 cell line, which displays medium levels of endogenous Notch1, with an Epsin3 plasmid construct. Transfected cells displayed significantly higher levels of Epsin3 mRNA ($p < 0.001$) and protein ($p < 0.001$), compared with cells transfected with the empty vector (p3XFLAG-CMV-10 vector) (Figure 3A,B). Levels of Notch1 expression at both the mRNA and protein levels were significantly reduced in cells over expressing Epsin3 compared with the transfected control cells (Figure 3A,B).

3.2 | Expression of Epsin3 and Notch1 in normal oral mucosa, OED and OSCC

IHC was carried out to examine Epsin3 and Notch1 expression profiles in tissue from a well-characterised cohort of patients with OED ($n = 74$) and OSCC ($n = 40$ of which 9 were dysplastic and underwent malignant transformation); normal oral mucosa was used as a control ($n = 21$). OED samples were graded using a binary classification into high-grade ($n = 40$) and low-grade lesions ($n = 33$).[15] OSCC were staged using the 'Tumour, Node, Metastasis' (TNM) classification developed by the International Union Against Cancer, only patients with early disease (Stages I and II) were included in this study. Epsin3 protein expression was measured using an *H* score (the product of the staining intensity [0–3] and the proportion of cells stained [0%–100%]; range 0–300). Normal oral epithelium showed weak to moderate expression of Epsin3. However, OED and OSCC showed variable Epsin3 expression ranging from weak to strong (Figure 4A). The number of cases which showed weak Epsin3 expression was 11 in high-grade and 15 in low-grade OED, respectively. The number of OSCC cases that showed weak Epsin3 expression was 16.

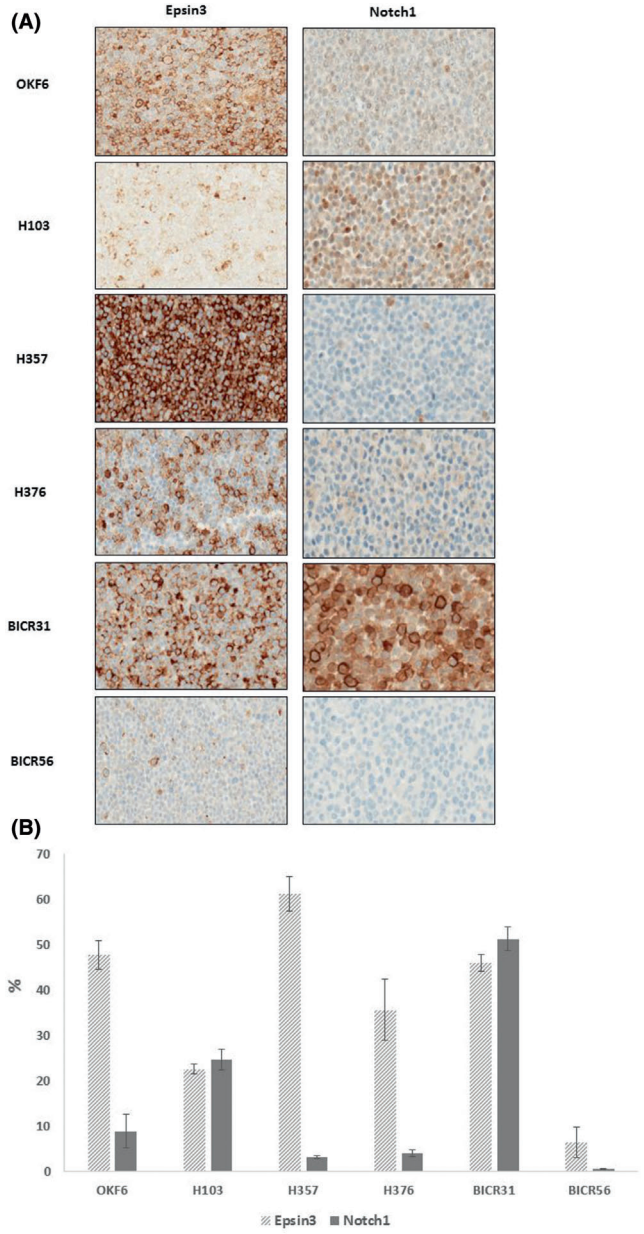

FIGURE 2 Epsin3 and Notch1 protein expression in OSCC-derived cell lines. Epsin3 and Notch1 protein levels in normal oral keratinocytes (OKF6) and OSCC-derived cell lines by immunocytochemistry (A; ×200 magnification). Image analysis was used to measure the percentage of cells with any staining; the values are the mean ± SD of three randomly selected fields (B).

Epsin3 protein expression was significantly higher in dysplastic epithelium relative to the normal epithelium ($p < 0.001$). Cases of high-grade epithelial dysplasia showed significantly higher expression levels of Epsin3 compared with the low-grade dysplasia ($p < 0.05$). The expression was also significantly higher in dysplastic tissues that underwent malignant transformation relative to cases that did not transform into cancer ($p < 0.001$), and OSCC had significantly higher Epsin3 expression compared with the normal tissues ($p < 0.0001$) (Figure 4B).

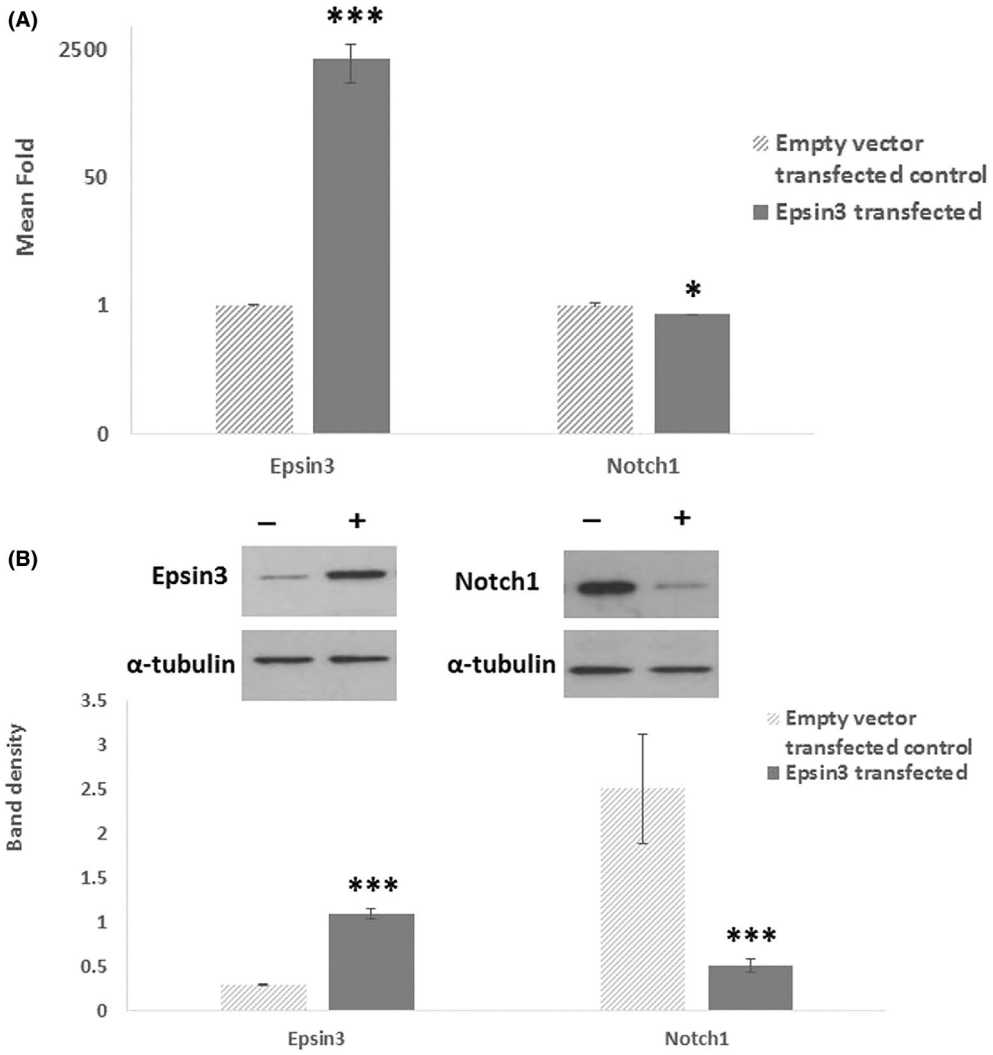

FIGURE 3 Overexpression of Epsin3 in H103 cells. H103 cells transfected with human Epsin3 had significantly higher levels of Epsin3 mRNA than the empty vector transfected controls (A). The Epsin3 transfected cells had slightly lower Notch1 mRNA than cells transfected with the empty vector, the reduction was statistically significant ($p < 0.05$) (A). Western blotting confirmed that Epsin3 transfected cells had higher levels of Epsin3 protein than the empty vector transfected controls. Notch1 protein expression was lower in the Epsin3 transfected cells compared with empty vector transfected controls. The western blots were analysed using densitometry and demonstrated a statistically significant difference; the values are the mean band density ± SD of three separate western blots (B).

Notch1 showed weak to moderate expression in the basal keratinocytes of normal epithelium. However, OED and OSCC showed variable Notch1 expression, with some samples showing similar staining intensity as the normal epithelium, whereas in other samples, there was no staining (Figure 4A). Notch1 expression was categorised as either positive (some staining observed) or negative (no staining). Notch1 was expressed in the majority of normal samples (92%), but in a reduced number of dysplasia samples (58%) and OSCC (42%), with a statistically significant difference ($p < 0.01$ and $p < 0.001$, respectively). Kaplan–Meier survival time analysis showed that cases with loss of Notch expression have a severely reduced lifespan ($p < 0.001$; Log Rank Mantel–Cox test) (Figure 4C).

4 | DISCUSSION

Head and neck cancer is a major global healthcare problem with late diagnosis and formation of secondary tumours being the main reason for poor survival rates.[16] Prevention, by elimination of risk factors, and early detection, by the identification of potentially malignant disorders and diagnosis of early-stage OSCC, are the keys to improving outcomes for patients.[17] The present study sought to examine the functional significance of Epsin3 and Notch1 in OSCC and its precursor, OED. Our data show that Epsin3 expression is variable between OSCC cell lines in vitro and H357 OSCC cell line had consistently higher Epsin3 expression levels when compared with a normal oral keratinocyte cell line. Upregulation of Epsin3 has been associated with the invasiveness of tumour cells in other cancers.[5,18,19] To investigate the expression profile of Epsin3 in vivo, we performed IHC using OED and OSCC tissue samples. Our data show higher levels of Epsin3 in the oral dysplasia and OSCC compared with the normal oral tissues. The expression was also higher in cases with high-grade epithelial dysplasia compared with low-grade dysplasia. Relevant to our results, considering upregulation of Epsin3 in epithelial tissues that undergo pathological changes (i.e., dysplasia and OSCC), upregulation of Epsin3 has also been documented in skin keratinocytes during wound healing. Epsin3 was expressed mainly in the migrating keratinocyte cells but was absent in differentiating cells. It was found to be downregulated in intact skin.[6] Taken together, these

results are suggestive for a crucial function of Epsin3 in cell proliferation and migration.

In the present study, we examined the expression levels of Notch1 mRNA in OSCC cell lines. Our data are consistent with previous in vitro studies, which reported that Notch1 mRNA is upregulated in OSCC.[20] However, IHC showed differential expression levels of Notch1 protein across the OSCC cell lines. Notch1 protein was very low in two cell types, H357 and BICR56, which align with previously reported mutations within the Notch1 gene in these cell lines, which lead to the production of a truncated protein,[21] possibly not detected by our current Notch1 antibody, or may have resulted in a direct reduction in protein expression. Loss-of-function mutation in Notch pathway members has already been observed in HNSCC,[22] cutaneous SCC, lung SCC and oesophageal SCC.[23,24] Most of these mutations occur in the Notch receptor domain resulting in formation of non-functioning truncated proteins suggesting a defect in Notch signalling.[9] Our IHC study revealed lower expression levels of Notch1 protein in dysplasia and OSCC compared with normal tissues. Furthermore, we found that cases with reduction of Notch1 expression are more likely to undergo malignant transformation than cases with high Notch1 expression. Observations from a previous study demonstrated the downregulation of Notch1 in high-grade oral precancerous lesions and OSCC compared with normal epithelium.[25,26] Collectively, our results support the hypothesis that Notch receptor expression has a tumour-suppressive function in OSCC. Elevated Notch1 in oral cancer has been suggested previously[20] and upregulation of Notch1 expression was found to be correlated with the invasiveness of OSCC,[27] potentially indicating the oncogenic role of Notch signalling in OSCC. Nonetheless, observations that revealed overexpression of Notch signalling in the HNSCC were obtained from in vitro studies using human HNSCC cells, which cannot accurately delineate the processes that arise during development of the tumour in vivo.[9]

Our observations possibly correlate the expression level of Epsin3 with Notch function. Epsin3 gene was overexpressed in H103 OSCC cell line. The expression of Notch1 was measured at both the RNA and protein levels after transfection. We found that overexpression of Epsin3 was associated with a significant reduction of Notch1 expression in OSCC cell line. This result was comparable with our immunohistochemical analysis of oral cancer tissues, which showed higher levels of Epsin3 and lower levels of Notch1 with significant correlation between the expression levels of both proteins in these tissue samples. Henceforth, as a putative model system, we propose that elevated levels of Epsin3 might lead to enhanced internalisation of

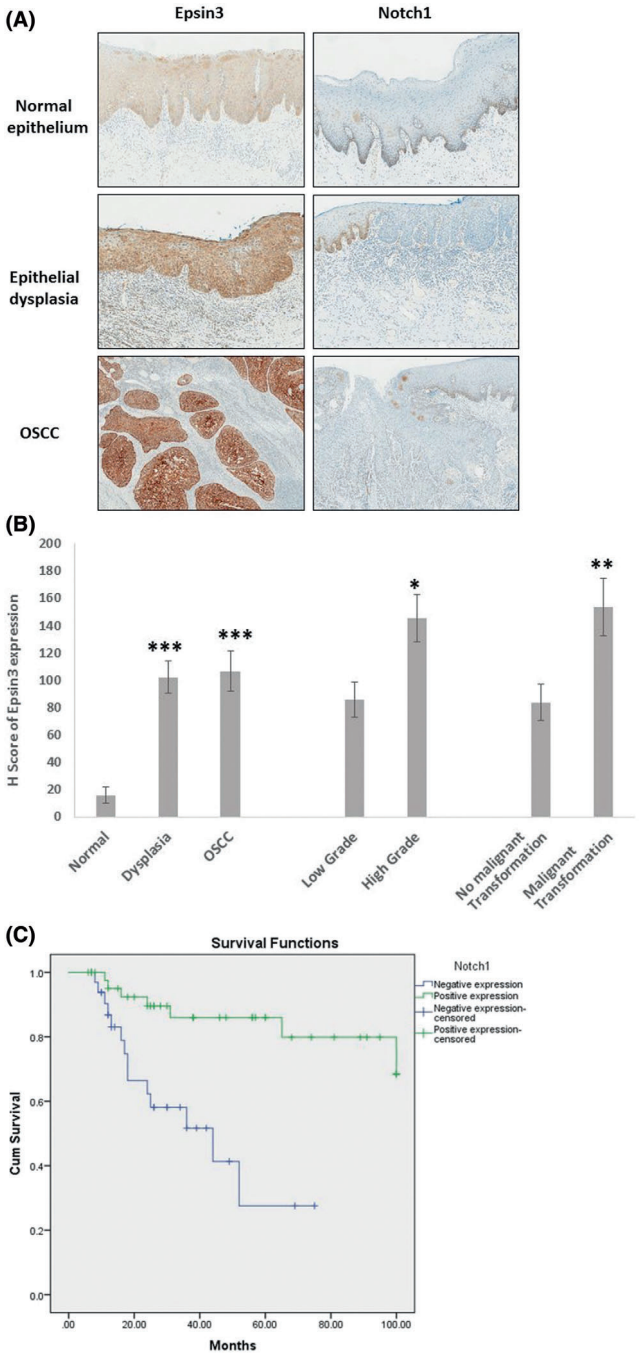

FIGURE 4 Epsin3 and Notch1 expression in oral tissue samples by immunohistochemistry. Normal oral epithelium showed weak cytoplasmic staining of Epsin3. Epithelial dysplasia and oral squamous cell carcinoma (OSCC) showed stronger Epsin3 expression. Normal oral epithelium showed weak to moderate cytoplasmic staining for Notch1 in the basal keratinocytes. Oral epithelial dysplasia and OSCCs showed variable expression ranging from weak staining to no staining (A). Semi-quantitative assessment of immunohistochemical staining using an H score showed that Epsin3 was significantly higher in epithelial dysplasia and OSCCs relative to normal epithelium. Cases with high-grade epithelial dysplasia had a significantly higher Epsin3 expression than the cases with low-grade epithelial dysplasia. Dysplasias that underwent malignant transformation had a higher Epsin3 expression than cases that did not transform. Significance measured by Mann–Whitney *U* test *<0.05; **<0.01; ***<0.001; 50× magnification; B). Kaplan–Meier time to event analysis showed that cases of epithelial dysplasia that showed loss of Notch1 expression (blue line) were more likely to undergo malignant transformation than cases that retained some Notch expression (green line; $p < 0.001$ Log Rank Mantel–Cox test; C).

Notch1 through DSL receptors, which in turn may hamper the biophysics of Notch activation[28] and/or the spreading of Notch signalling inside the cell through intracellular transport.[29] As a consequence, Notch1 downstream targets will be suppressed, leading to loss of control of cell proliferation, apoptosis and migration, which are all hallmarks of this type of cancer. Alternatively, overexpression of Epsin3 may hamper Notch signalling in signal-sending cell where Notch receptor and Notch ligand co-expression results in *cis*-interactions among these molecules, whereas cis-interactions are believed to inhibit Notch signalling. Internalisation of these complexes is an essential step for Notch activation[8] and therefore, disturbance or inhibition of internalisation of ligand-receptor complexes may lead to dysregulation of Notch signalling.

A limitation of the present study is that OED, OSCC and normal mucosa samples were not matched. Moreover, this study has not investigated the effect of Epsin3 knockdown or inhibition on oral cancer cell growth or invasion.

In summary, Epsin3 protein is upregulated in OSCC cell lines, OED tissue samples and OSCC tissue samples, suggesting for the very first time that Epsin3 may be implicated in oral carcinogenesis. In addition, our findings reveal that Notch1 protein was either reduced or completely lost during the development of oral cancer, which may be through direct or indirect responses to increased Epsin3 levels, highlighting the tumour suppressor function of Notch signalling pathway in OSCC. Further work is needed in this area as well as in the field of epsin regulation of Notch signalling in surface epithelia.

AUTHOR CONTRIBUTIONS

Ottavio Cremona and Marco Carrozzo contributed to the study design and writing and reviewing the manuscript. Halah Ahmed contributed to RT-qPCR, immunoblotting immunohistochemistry and transfection experiments and drafting the manuscript. Ian Paterson contributed to Notch signalling experiments. Sadat A. Aziz contributed to transfection experiments. Max Robinson contributed to cell lines and sample enrolment and immunohistochemistry, acquisition of data and statistical analysis and writing and reviewing the manuscript. Ruth A. Valentine contributed to RT-qPCR, immunoblotting and transfection experiments and writing and reviewing the manuscript.

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FUNDING INFORMATION

All authors certify that they have no affiliations with or involvement in any organisation or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

ETHICS STATEMENT

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the NRES Committee North East—Sunderland (Evaluation of the prognostic potential and functional significance of biomarkers in oral cancer 11/NE/0118).

INFORMED CONSENT

Consent to participate was waived by the IRB due to the retrospective nature of the work without therapeutic alterations.

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