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ORIGINAL ARTICLE

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Combined loss of expression of involucrin and cytokeratin 13 is associated with poor prognosis in squamous cell carcinoma of mobile tongue

Sushma Pandey MDS[1] | **Tine Merete Søland PhD**[1,2] | **Inger Heidi Bjerkli PhD**[3,4] | **Lars Peter Sand PhD**[1] | **Fernanda Cristina Petersen PhD**[1] | **Daniela Elena Costea PhD**[5,6,7] | **Burcu Senguven PhD**[1,8] | **Dipak Sapkota PhD**[1]

[1]Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway

[2]Department of Pathology, Oslo University Hospital, Rikshospitalet, Oslo, Norway

[3]Department of Otorhinolaryngology, University Hospital of North Norway, Tromsø, Norway

[4]Department of Tromsø Medical Biology, Faculty of Health Sciences, UiT – The Arctic University of Norway, Tromsø, Norway

[5]The Gade Laboratory for Pathology, Department of Clinical Medicine, Haukeland University Hospital, University of Bergen, Bergen, Norway

[6]Department of Pathology, Haukeland University Hospital, Bergen, Norway

[7]Centre for Cancer Biomarkers (CCBIO), Faculty of Medicine and Dentistry, University of Bergen, Bergen, Norway

[8]Department of Oral Pathology, Faculty of Dentistry, Gazi University, Ankara, Turkey

Correspondence
Dipak Sapkota, Department of Oral Biology, Faculty of Dentistry, University of Oslo, Sognsvannsveien 10, Domus Odontologica, 0372 Oslo, Norway.
Email: dipak.sapkota@odont.uio.no

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Abstract

Background: This study aimed to evaluate the prognostic significance of expression levels of involucrin (IVL), cytokeratin (CK)-10 and -13 at different intratumor sites (tumor center and invading area) of oral tongue squamous cell carcinoma (OTSCC).

Methods: IVL, CK13 and CK10 expression levels were examined in a multicenter cohort of 146 OTSCCs using immunohistochemistry. External mRNA datasets were used for expression analysis and/or to validate survival associations.

Results: External transcriptomic datasets showed downregulation of *IVL* and *KRT13* in oral malignancies including OTSCC as compared to normal controls. The combined loss of IVL and CK13 expression at the invading core but not at the center core was significantly associated with poor differentiation and reduced 5-year overall survival. Multivariate Cox analysis confirmed the loss of CK13 and IVL expression to be an independent prognostic factor. Transcriptomic dataset corroborated immunohistochemistry results.

Conclusions: Combined expression levlels of IVL and CK13 might be useful as prognostic biomarkers in OTSCC.

KEYWORDS

combination biomarkers, cytokeratin, involucrin, mobile tongue, oral squamous cell carcinoma

1 | INTRODUCTION

Squamous cell carcinoma (SCC) of the tongue is one of the most common malignancies of the oral cavity, representing up to 50% of oral SCC.[1,2] The incidence of tongue SCC has been steadily increasing, whereas the incidence of oral SCC (OSCC) in other anatomical subsites has been decreasing.[3] Tongue SCC has an aggressive clinical behavior and is frequently associated with lymph node involvement and poor prognosis. Although the precise mechanism for aggressive tongue SCC phenotype is not fully understood, the presence of a rich lymphatic network and a highly muscularized structure has been suggested to contribute to the increased incidence of invasion and metastasis in tongue SCC.[4–6]

Topographically, tongue SCC can be divided into lesions arising from the mobile/oral tongue (part of oral cavity) and base of the tongue (part of oropharynx). SCC arising from mobile/oral tongue is called oral tongue SCC (OTSCC). Etiopathogenesis and prognosis of OTSCC are believed to be different than that of SCC in the base of the tongue.[7] Tobacco and alcohol are the main risk factors for OTSCC, whereas the majority of SCC in the base of the tongue are related to human papillomavirus (HPV) infection.[7,8] Furthermore, the prognosis of OTSCC is worse than that of SCC in the base of the tongue.[9] At the molecular level, tobacco-associated OSCC, including OTSCC, is associated with a significantly higher number of mutational events probably attributed to tobacco and alcohol use as compared to HPV-associated SCC in the base of the tongue.[10] These observations underscore the importance of stratification of OTSCC from SCC at the base of the tongue, not only for biomarker studies but also for management purpose.

Among the various molecular changes occurring in carcinomas in the head and neck region, including OTSCC, mutations in genes such as *TP53*, *FAT1*, *NOTCH1*, and deregulation of signaling pathways related to keratinocyte differentiation represent key molecular events.[11,12] Accordingly, downregulation/loss of expression of differentiation protein markers such as involucrin (IVL, encoded by *IVL* gene) and members of the cytokeratin (CK) protein family are frequent observations in OSCC.[13–15] They have been linked to poor prognosis in OSCC/head and neck SCC.[16,17] Nevertheless, the expression patterns of CK10 (encoded by *KRT10* gene), CK13 (encoded by *KRT13* gene), and IVL at different intratumor sites (tumor center [more differentiated area] vs. invading front [less differentiated area]) of OTSCC are currently unknown. In addition, the prognostic significance of these proteins, either alone or in combination, has not been previously examined in a multicenter, HPV-negative homogenous cohort of OTSCC.

This study aimed at investigating the expression of CK10, CK13, and IVL as putative biomarkers for prognosis of OTSCC using immunohistochemistry on a well-characterized multicenter retrospective patient cohort. The results showed that the combined loss of expression of IVL and CK13 at the deep/invading tumor areas was associated with poor tumor differentiation and reduced 5-year survival of patients with OTSCC. These findings were further validated on mRNA data for OTSCC from The Cancer Genome Atlas (TCGA).

2 | MATERIALS AND METHODS

2.1 | External microarray and TCGA datasets

A microarray dataset (GSE30784)[18] consisting of 45 normal oral mucosa, 17 oral dysplasia, and 167 OSCC cases and TCGA/The Genotype tissue expression (GTEx) datasets[19] were used to compare mRNA expression levels of *KRT10*, *KRT13* and *IVL* in OSCC/head and neck SCC with normal controls. The "box plot" tool in Gene Expression Profiling Interactive Analysis (GEPIA)[20] was used for TCGA/GTEx datasets. Further, mRNA expression levels of *IVL* and *KRT13* from 523 cases of head and neck SCC in the TCGA dataset were exported through cBioPortal.[21,22] Of the 316 cases of OSCC, 114 HPV-negative specimens of OTSCC were used to examine association between the combined mRNA expression levels of *KRT13* and *IVL* with clinicopathological variables and patient survival.

2.2 | Patient cohort

The study included 146 patients with diagnosed OTSCC (all HPV-negative[23]). This study is a part of large patient cohort in Norwegian Oral Cancer (NOROC) study, including all consecutive patients, diagnosed and treated at the three major hospitals treating head and neck cancer (Oslo, Bergen, and Tromsø) in Norway from January 1, 2005 till December 31, 2009 (Figure 1). Of the 146 cases, 128 were primary and 18 were second primary OTSCC. Tissue microarrays (TMA) using tissue cores of 2 mm

diameter from both invading front (the most deepest part of an invasive tumor, henceforth called "invading core") and superficial/center areas (henceforth called "center core") were constructed using formalin-fixed paraffin embedded (FFPE) specimens as described previously.[23] Except for 18, the center and corresponding invading cores were available for all of the cases. For 75 cases from Oslo, duplicate cores were available for both center and invading areas. The study was approved by the Northern Norwegian Regional Committee for Medical Research Ethics (REK nord, ref: 2015/1381). Tumors specimens were classified following the 5th edition of the TNM classification.[24] The following clinicopathological variables were documented: age, sex, date of birth, date of diagnosis, habit history (smoking, alcohol), primary tumor site, tumor size, node and metastasis, disease stage (both pathological and clinical), tumor differentiation, treatment received, and recurrence history of the disease. In addition, date of death and cause of death were also retrieved from patients' hospital record or from the Statistics of Norway, Cause of Death Registry. Study adhered to REMARKS guidelines[25] where appropriate.

2.3 | Immunohistochemistry

IHC was performed at the Department of Oral Biology, University of Oslo. IHC was performed for CK10, CK13 and IVL. The following primary antibodies were used: rabbit monoclonal anti-CK 13 antibody (HPA030877, Atlas antibodies, Bromma, Sweden) at 1:250 dilution; mouse monoclonal anti-IVL (Clone SY5, I9018, Sigma, MO) antibody at 1:4000 dilution; and mouse monoclonal anti-CK10 antibody (sc-53 252, Santa-Cruz Biotechnology, Dallas, TX) at 1:200 dilution. IHC was performed on 4-μm thick FFPE TMA sections following standard procedures using DakoEnVision + Dual Link System-HRP and visualized using 3, 3′ diaminobenzidine (DAB) solution (Dako, Glostrup, Denmark). Briefly, the sections were deparaffinized by placing in an incubator at 56°C overnight, followed by two changes of xylene for 5 min each. Rehydration was performed by two changes of absolute ethanol, 96%, and 70% ethanol for 3 min each. Antigen retrieval for all three proteins was performed by treating the slides with Tris-EDTA (pH 9.0) in a pressure cooker at 100°C for 15 min and 90°C for 10 s. The sections were blocked with 10% normal goat serum (X907, Dako, Glostrup, Denmark) in 3% bovine serum albumin in Tris-buffered saline +0.1% Tween (TBST), and incubated with corresponding primary antibodies for an hour at room temperature. EnVision HRP-mouse (K400111-2 Dako, Glostrup, Denmark) for CK10 and IVL, and -rabbit (K400311-2, Dako, Glostrup, Denmark) for CK13 secondary antibodies were thereafter applied for 30 min at room temperature. Sections were visualized using DAB chromogen for 4 min and counterstained with hematoxylin. Slides were then dehydrated, immersed in xylene and mounted. Lining epithelium (normal and/or dysplastic) adjacent to OTSCC sections served as positive tissue controls, while TBST instead of primary antibody served as a negative control for the secondary antibody. In addition, rabbit immunoglobulin fraction, X0903 (Dako, Glostrup, Denmark) for CK13 and IgG1 M9269 (Sigma, St. Louis, MO) for CK10 and IVL were used as isotype-matched controls to control for nonspecific binding of the primary antibodies. The presence of a red-brown cytoplasmic staining was considered positive, regardless of the intensity.

2.4 | Digital image acquisition and evaluation of immunohistochemistry

TMA-slides were scanned at 40× using Pannoramic MIDI scanner (3DHISTECH, Budapest, Hungary). Afterward, QuPath software version 0.2.0-m8[26] was used for visualization and subsequent analysis of IHC. Lost/destroyed tissue cores during IHC and cores with insufficient tumor cells or with poor image quality were excluded from the analysis. As

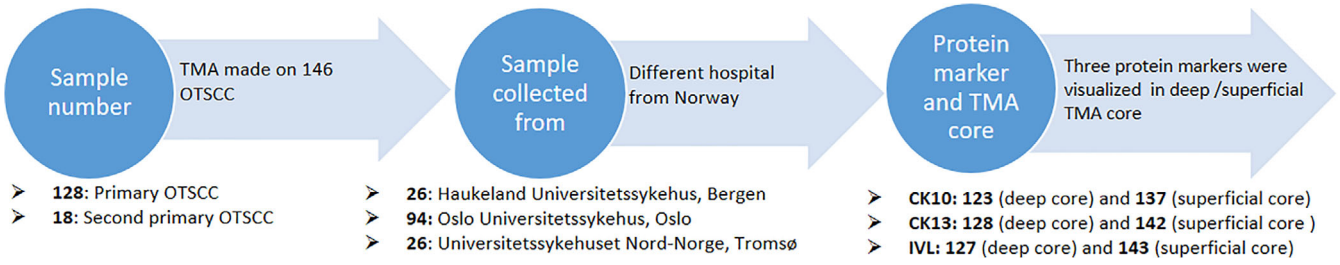

FIGURE 1 Flowchart illustrating the origin and number of specimens (center and invading cores) used for IHC. One hundred and forty-six OTSCC specimens were used to construct TMA. After exclusion of lost/destroyed tissue cores during IHC and cores with insufficient tumor cells or with poor image quality, the numbers of specimens used for statistical analyses were different for different proteins as shown in the flowchart [Color figure can be viewed at wileyonlinelibrary.com]

shown in Figure 1, the total number of cores available for analysis of IVL/CK13/CK10 was 143/142/137 at tumor center and 127/128/123 at the invading area, respectively.

Blinded for clinicopathological information, IHC evaluation was performed by S.P. and D.S. Prior to evaluation, observers (S.P., D.S., and B.T.) had agreed upon calibration and interpretation of immunostaining. All tumor cells, both at the center and invading cores, were used for IHC evaluation. Keratin pearls, stromal components or background artifacts in tumor area were excluded from IHC evaluation. Positive tumor cells were presented as percentages of total number of tumor cells. For cases with duplicate cores, an average of the percentage of positive cells was calculated. The same scoring system was applied for all three protein markers.

2.5 | Statistical analysis

Data are expressed as mean ± standard error of the mean. Wilcoxon matched-pairs signed rank test was used to compare immunoexpression in center versus corresponding invading cores. Mann–Whitney test was used to compare immunoexpression of each protein between primary and second primary OTSCC and to examine the immunoexpression with respect to the degree of tumor differentiation. Analysis of variance (ANOVA) with Tukey's post hoc tests was used to examine mRNA expression levels of *KRT10*, *KRT13* and *IVL* in microarray dataset. OTSCC cases were stratified into high- and low-expression groups using mean expression values of the respective protein markers as cutoff points. Association between the expression of protein markers and clinicopathological parameters was examined using chi-square/Fischer exact test. Kaplan–Meier survival analysis with log-rank tests was performed to examine the association between the expression status of proteins and patient survival. Univariate and multivariate Cox-regression models were used to study the effect of protein expression and other covariates on patient survival. STATA version 16/GraphPad prism version 9.0.0/R studio version 1.3.959 was used for statistical analysis. p-Value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | mRNA expression levels of *IVL* and *KRT13* were significantly downregulated in OSCC/head and neck SCC as compared to normal controls in microarray and TCGA datasets

The mRNA expression levels of *IVL* and *KRT13* were found to be down-regulated in OSCC/head and neck SCC specimens than in normal controls (Figure 2(A,B)). The *KRT10* mRNA expression was higher in cancer lesions as compared to normal control both in OSCC and head and neck SCC. Of interest, the expression levels of *IVL* and *KRT13* was similar between normal controls and oral dysplasia, while *KRT10* mRNA expression was significantly upregulated in oral dysplasia as compared to normal controls (Figure 2(A)).

3.2 | Expression levels of IVL, CK13, and CK10 were similar between primary and second primary OTSCC

Expression levels of IVL, CK13, and CK10 were similar between primary and second primary lesions, both at center and the corresponding invading cores (Figure S1). Hence, in the subsequent analysis, the second primary lesions were analyzed together with primary lesions.

3.3 | Expression levels of IVL and CK13 were downregulated at invading cores as compared to corresponding center cores in OTSCC

Except for occasional stromal and inflammatory cells, IVL, CK13 and CK10 were predominantly expressed by the tumor cells. All protein markers showed mostly cytoplasmic expression (Figure 3(A–F)). Percentage of positive tumor cells was found to be highest for IVL both at center (mean = 63.5% ± 2.2) and invading cores (mean = 56% ± 2.7) (Figure 3(G)) as compared to that of CK13 (24% ± 2.2 at center and 18% ± 2.3 at invading cores) (Figure 3(H)) and CK10 (10% ± 1.5 at center and 9% ± 1.4 at invading cores) (Figure 3(I)). Expression levels of IVL ($p = 0.008$) and CK13 ($p = 0.017$) at the invading cores were significantly downregulated as compared to the corresponding center cores (Figure 3 (G,H)). Expression of CK10 was found to be similar at both the center and corresponding invading cores (Figure 3(I)).

3.4 | Loss of CK13 and IVL expression at the invading cores was associated with poor tumor differentiation and poor 5-year overall survival of patients with OTSCC

The IVL and CK13 expression and clinicopathological characteristics of the cohort are summarized in Tables S1 and S2, Supporting Information. Briefly, the median age

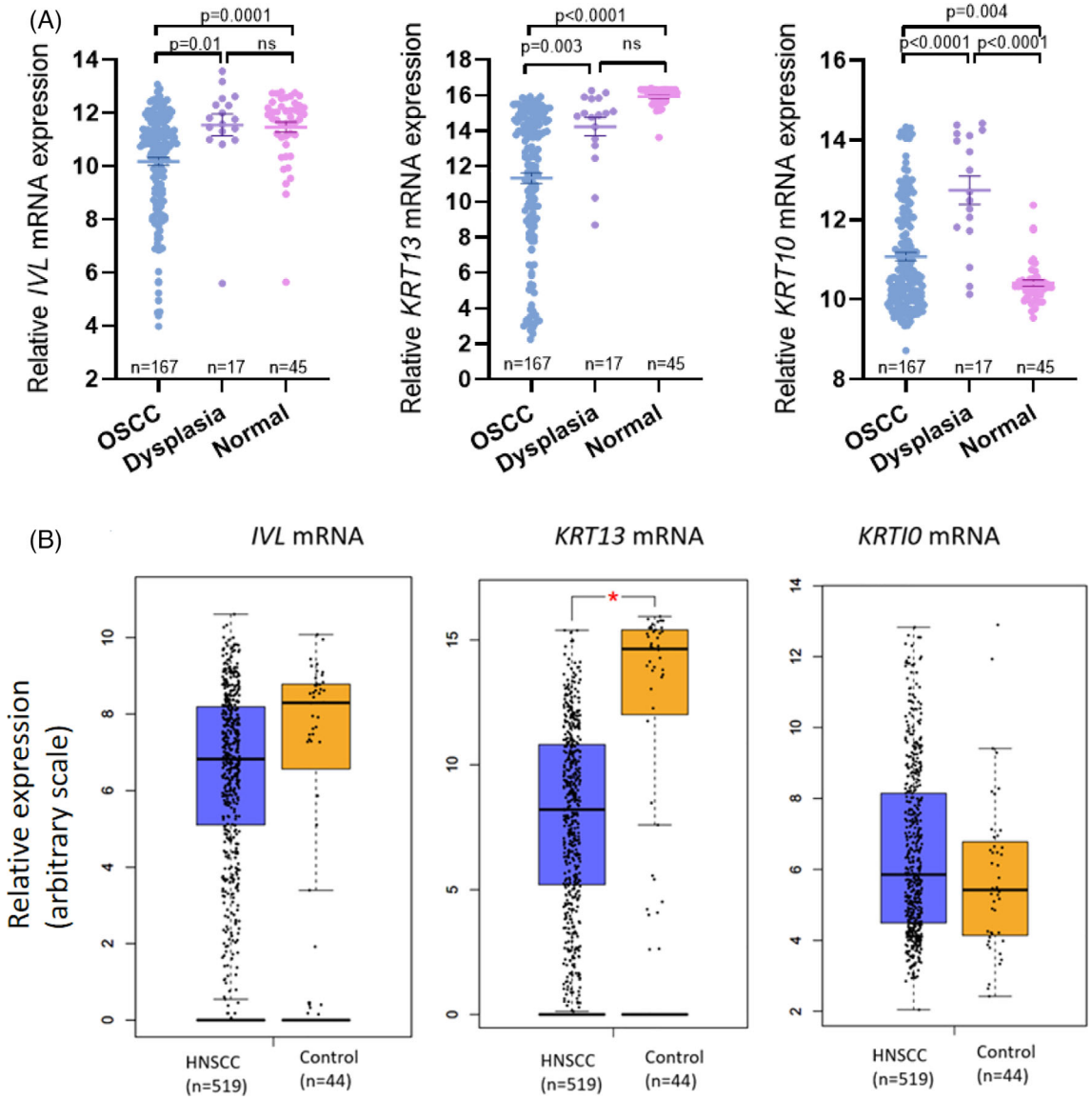

FIGURE 2 In microarray transcriptome dataset (A), mRNA expression levels of *IVL* and *KRT13* were significantly downregulated in OSCC as compared to normal oral mucosa/oral dysplasia. However, *KRT10* was slightly upregulated in OSCC as compared to the normal oral mucosa. In TCGA dataset (B), mRNA expression levels of *IVL* and *KRT13* were downregulated, whereas *KRT10* was slightly upregulated in head and neck SCC as compared to the normal controls [Color figure can be viewed at wileyonlinelibrary.com]

of OTSCC cases was 66 years and the male to female ratio was 1.6:1. Using chi-square test, OTSCC with lower expression of IVL ($p = 0.001$) or CK13 ($p = 0.004$) at the invading core were found to be significantly associated with poor tumor differentiation (Tables S1 and S2). In parallel with these results, with Mann–Whitney test, the expression levels of both IVL and CK13 at the invading cores were found to be significantly higher in well/moderately differentiated lesions as compared to the poorly differentiated tumors (Figure 4(A,B)). IVL expression at center core was similar between well/moderately and poorly differentiated OTSCC, while CK13 was significantly downregulated in poorly differentiated OTSCC (Figure S2). CK10 expression levels at both center and invading cores were not significantly different with respect to differentiation status of OTSCC (Figure S3). As the expression of CK10 was not significantly different with respect to location (center vs. invading) (Figure 3(C)) and differentiation status (well/moderate vs. poor differentiation) (Figure S3), only the expression status of IVL and CK13 at invading cores was considered in the further analysis.

Kaplan–Meier plot showed a reduced 5-year overall (log-rank test, $p = 0.04$) and disease-specific (log-rank, $p = 0.03$) survival probabilities for patients with low IVL expression at the invading cores (Figures S4 and S5). Despite similar trends, expression of CK13 at the

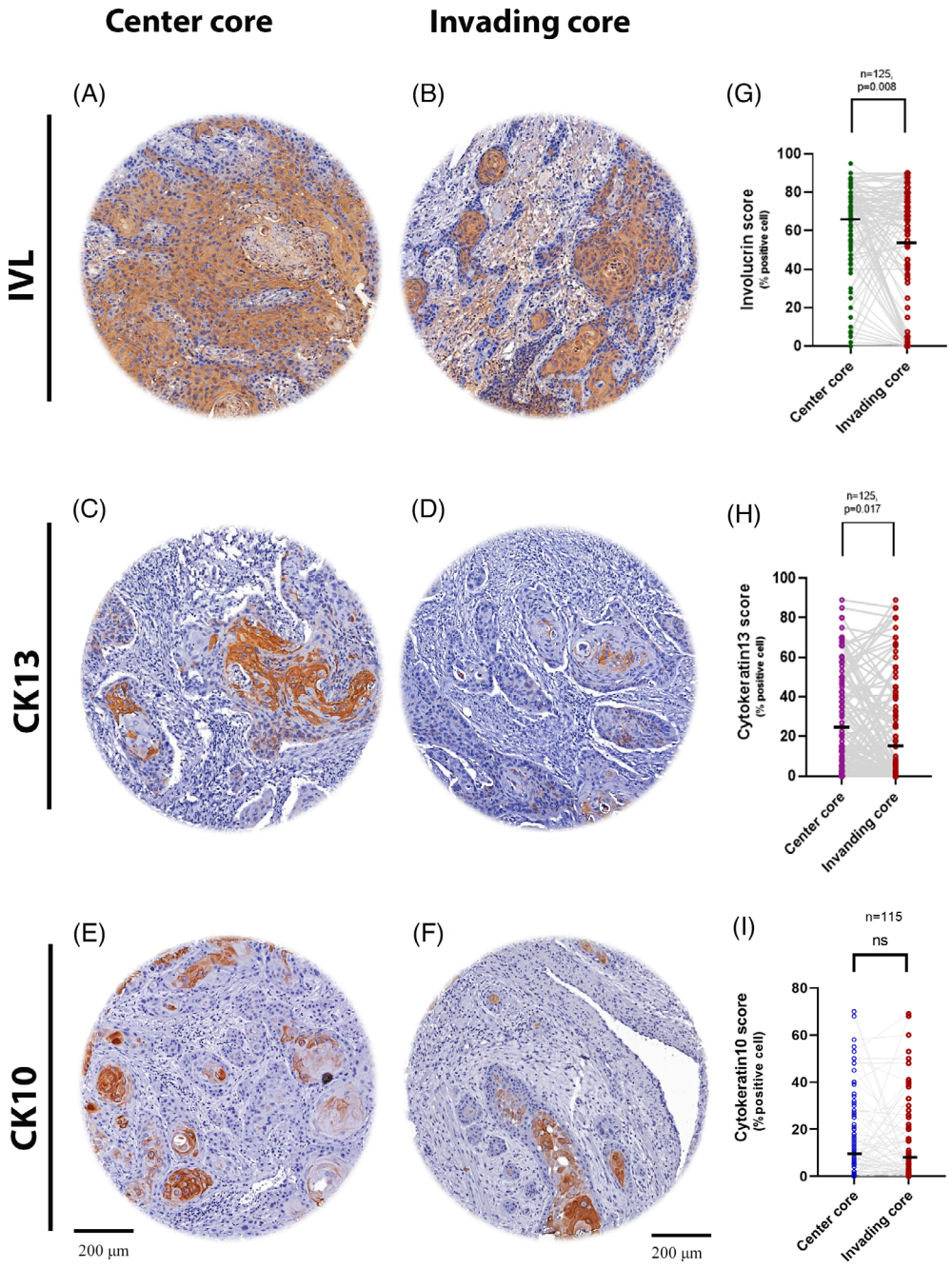

FIGURE 3 Representative images showing expression patterns of IVL (A, B), CK13 (C, D), and CK10 (E, F) at the center and corresponding invading cores. All protein markers showed predominantly cytoplasmic expression in the tumor cells. Quantification of immunostaining showed significantly fewer positive cells at the invading core for IVL (G, $p = 0.008$) and CK13 (H, $p = 0.017$) as compared to the corresponding center cores. Expression of CK10 (I, $p > 0.05$) was similar both at the tumor center and the corresponding invading core. Wilcoxon matched-pairs signed rank test was used for statistical analysis in (G–I). Horizontal bars represent means of expression values. *ns*; nonsignificant. IHC images were exported from QuPath software at 60x [Color figure can be viewed at wileyonlinelibrary.com]

invading core was not statistically associated either with 5-year overall (log-rank, $p = 0.12$) or with disease-specific survivals ($p = 0.21$) (Figures S6 and S7). No association was found between expression of IVL or CK13 at the invading cores and recurrence-free survival (Figure S8).

We next examined the performance of combined expression of IVL and CK13 at invading cores in predicting survival of patients with OTSCC. OTSCC cases were stratified into high combined expression group (high expression of both IVL and CK13, referred to as $IVL^{high} + CK13^{high}$) and low combined expression group (low expression of both IVL and CK13, referred to as $IVL^{low} + CK13^{low}$) by using corresponding mean expression scores as cut-off points. $IVL^{low} + CK13^{low}$, $IVL^{high} + CK13^{high}$, and mixed group either $IVL^{low} + CK13^{high}$ or $IVL^{high} + CK13^{low}$ consisted of 41, 38 and 48 OTSCC cases, respectively. Kaplan–Meier curves for the three groups showed that $IVL^{low} + CK13^{low}$ OTSCC cases had the lowest survival probabilities than for the mixed and high groups (Figure 4(E)) (log-rank, $p = 0.12$). However, when only high and low groups were analyzed, $IVL^{high} + CK13^{high}$ cases were found to have significantly higher (log-rank test, $p = 0.03$) survival probabilities than that of $IVL^{low} + CK13^{low}$ group (Figure 4(F)).

Of note, the expression levels of IVL and CK13 either alone or in combination at the center cores were not statistically associated with patient survival.

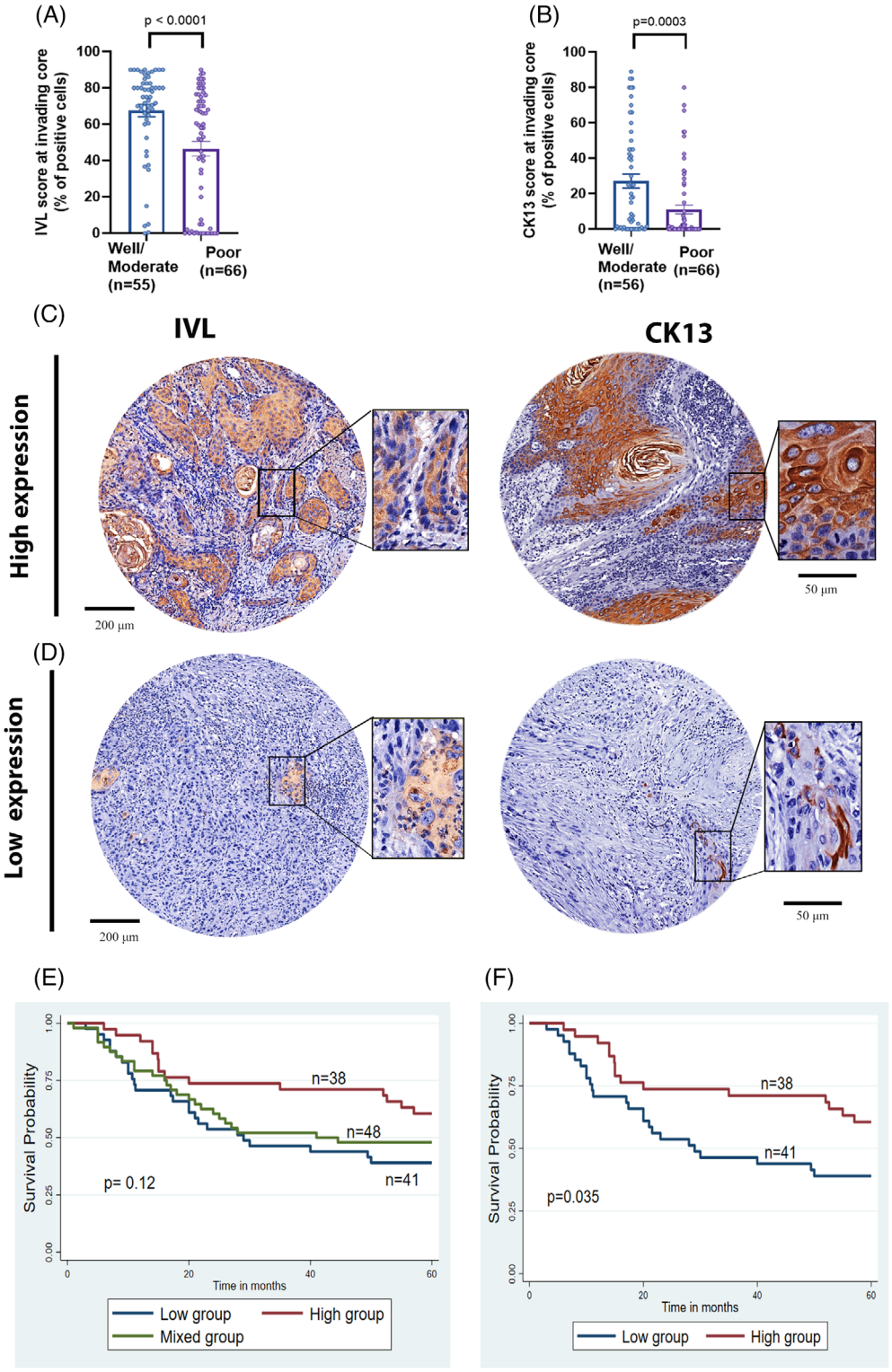

FIGURE 4 A significantly greater number of positive cells for IVL (A, $p < 0.0001$) and CK13 (B, $p = 0.0003$) were found at the invading core in well/moderately differentiated OTSCC as compared to the poorly differentiated lesions. Mann–Whitney test was used for statistical analysis. Horizontal bars represent the means of expression values. The error bars represent SEM. Representative images showing OTSCC cases with high (C) and low (D) combined expression of IVL and CK13. IHC images were exported from QuPath software at 60x. (E) Kaplan–Meier plot demonstrating 5-year overall survival curves for high ($IVL^{high} + CK13^{high}$), low ($IVL^{low} + CK13^{low}$), and mixed group ($IVL^{low} + CK13^{high}$) or ($IVL^{high} + CK13^{low}$) (log-rank test, $p = 0.12$). (F) Kaplan–Meier plot showed a significantly higher (log-rank test, $p = 0.035$) 5-year overall survival for high ($IVL^{high} + CK13^{high}$) group as compared to the low ($IVL^{low} + CK13^{low}$) group [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | Combination of CK13 and IVL expression as an independent prognostic factor to predict survival of patients with OTSCC in Cox proportional hazards regression analysis

Univariate Cox analysis showed low combined expression of IVL and CK13 at invading cores, increased tumor size (clinical), node involvement (clinical), and late clinical stage to be significant risk factors for reduced 5-year overall survival (Table 1). Multivariate Cox regression analysis using variables as described in Table 1 demonstrated combined CK13 and IVL expression at invading core to be an independent prognostic factor for 5-year survival of patients with OTSCC (HR = 0.38, CI = 0.17–0.85, $p = 0.02$) (Table 1). The test of proportional-hazards

TABLE 1 Univariate and multivariate Cox proportional hazards regression analysis for combined expression of IVL and CK13 and clinicopathological covariates

	Univariate Cox			Multivariate Cox		
Variables	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
IVL + CK13 expression[a]						
Low	1.00			1.00		
High	0.50	0.26–0.96	**0.04**	0.38	0.17–0.85	**0.02**
Age[b]						
≤66	1.00			1.00		
>66	1.8	0.96–3.38	0.06	1.30	0.64–2.66	0.73
Differentiation[c]						
Well	1.00			1.00		
Moderate and poor	1.20	0.63–2.30	0.56	0.75	0.36–1.60	0.46
Clinical stage[d]						
Early (1 and 2)	1.00			1.00		
Late (3 and 4)	3.24	1.68–6.24	**<0.00**	2.00	0.33–11.90	0.45
Clinical tumor size[e]						
Smaller	1.00			1.00		
Larger	3.66	1.66–8.10	**0.001**	1.8	0.52–6.80	0.34
Node status[f]						
Negative	1.00			1.00		
Positive	2.66	1.28–5.25	**0.008**	1.80	0.34–9.31	0.48

Note: The values marked in bold indicate that they were less than the level of significance (0.05) used for the statistical analysis.

Abbreviations: CI, confidence interval; HR, hazard ratio.

[a]OTSCCs were stratified into high (IVL^{high} + $CK13^{high}$) and low (IVL^{low} + $CK13^{low}$) expression groups by using corresponding mean expressions as cut-off points.

[b]Patients were categorized into low- and high-age groups based on the median age.

[c]OTSCC were categorized into well/moderate and poor differentiated groups.

[d]Patients were categorized into early (stage I-II) and late (stage III-IV) using clinical TNM staging.

[e]Tumors were categorized into smaller (T1 and T2) and larger (T3 and T4) tumors using clinical tumor size.

[f]Tumors were categorized into node positive (N1 and N2) and negative (N0) groups using clinical node status.

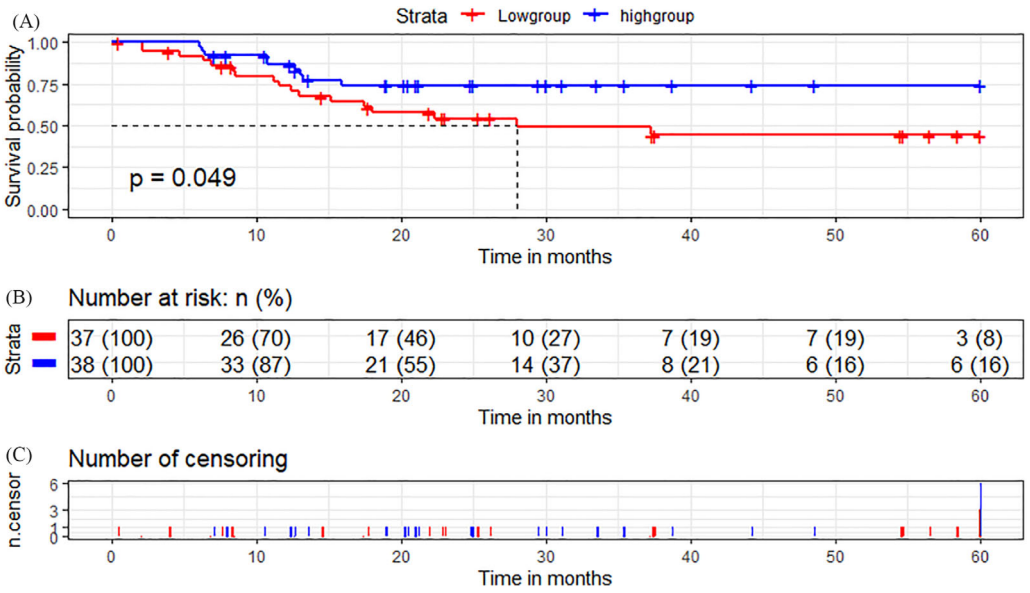

FIGURE 5 (A) Kaplan–Meier curves showing reduced 5-year overall survival for patients with low combined mRNA expression of *IVL* and *KRT13* (log-rank test, $p = 0.049$). The risk table B showed absolute number of cases at risk for both combined high and low expression groups at different time points, and C showed number of censored subjects at different months [Color figure can be viewed at wileyonlinelibrary.com]

assumption using global test showed that there was no deviation from proportionality ($p > 0.05$) (Table S3).

3.6 | Low combined mRNA expression of *IVL* and *KRT13* was associated with poor tumor differentiation and reduced 5-year overall survival of OTSCC cases in TCGA datasets

HPV-negative OTSCC cases from TCGA datasets were stratified into high and low expression groups by using median expression values of *IVL* or *KRT13* as cut-off points. Median values were used due to significant scatter of *IVL* or *KRT13* mRNA levels. The number of cases with high and low combined expression was 38 and 37 (Figure 5). Parallel to the IHC results, the low combined group was significantly associated ($p = 0.002$) with poor tumor differentiation (Table 2). In addition, the Kaplan–Meier survival analysis showed that low combined mRNA expression of *IVL* and *KRT13* was significantly associated with reduced overall 5-year survival as compared to the combined high expression group (log-rank test, $p = 0.049$) (Figure 5).

4 | DISCUSSION

During the normal differentiation process of oral epithelium, keratinocytes undergo a series of biochemical and morphological changes leading to accumulation of an array of filamentous proteins (differentiation markers) such as CKs and IVL.[27,28] The control of epithelial differentiation is deregulated in human epithelial malignancies and accordingly altered expression patterns of a number of differentiation markers are frequently observed in OSCC.[11] However, the prognostic significance of altered expression patterns of CK10, CK13 and IVL, either alone or in combination, at different intratumor sites (tumor center and invading area) in a homogenous cohort of OTSCC is not known. The current study, using IHC for CK10, CK13 and IVL in TMA on a large OTSCC cohort, revealed that the reduced combined expression of IVL and CK13 at invading cores was associated with poor tumor differentiation and poor prognosis. These findings were further validated by analyzing mRNA expression levels of *IVL* and *KRT13* genes in TCGA dataset for OTSCC.

Using microarray and TCGA transcriptomic datasets, mRNA expression levels of *IVL* and *KRT13* were found to

TABLE 2 Association between the combined expression *IVL* and *KRT13* mRNA and clinicopathological variables of the patients with OTSCC in TCGA dataset

	Combined mRNA expression of *IVL* and *KRT13*[a]		
Variables	**Low, *n* (%)**	**High, *n* (%)**	***p*-value**
Age[b] (years)			
≤60	21 (52.0)	19 (47.5)	0.558
>60	16 (45.7)	19 (54.2)	
Sex			
Female	16 (48.4)	17 (48.4)	0.89
Male	21 (50.0)	21 (50.0)	
Differentiation (WHO classification, whole tumor)[c]			
Well	01 (00.7)	12 (92.3)	**0.002**
Moderate	27 (55.0)	22 (45.0)	
Poor	09 (69.2)	04 (30.7)	
Lymph node involvement[d]			
Negative	15 (41.6)	21 (58.4)	0.20
Positive	22 (56.5)	17 (43.5)	
Tumor size[e]			
Smaller (1 and 2)	18 (47.3)	20 (52.7)	0.73
Larger (3 and 4)		19 (51.3)	18 (48.7)

Note: The values marked in bold indicate that they were less than the level of significance (0.05) used for the statistical analysis.

[a]OTSCCs were stratified into high and low groups using combined mRNA expression levels of *IVL* and *KRT13* by using corresponding median expression values as cut-off points.

[b]Patients were categorized into low- and high-age groups based on the median age.

[c]OTSCC were categorized into well, moderate, and poor differentiated groups.

[d]Tumors were categorized into node positive (N1 and N2) and negative (N0) groups using clinical node status.

[e]Tumors were categorized into smaller (T1 and T2) and larger (T3 and T4) cases using clinical tumor size.

be significantly downregulated in OSCC/head and neck SCC as compared to normal controls (Figure 2(A,B)). Interestingly, their expression levels were similar in normal controls and oral dysplasia (Figure 2(A)), despite some previous studies that have shown a downregulation of CK13 or IVL in dysplastic lesions compared to normal controls.[29–31] This discrepancy could be due to different methods used for expression analysis or due to variation in the grade of dysplasia of the specimens used. In contrast, the upregulation of *CK10* mRNA already in oral dysplasia and OSCC/head and neck SCC (Figure 2(A,B)) suggests that its upregulation might be an early event in OSCC. Overall, in accordance with previous reports,[32] these findings suggest that dysregulation in differentiation is a common event in OSCC.

The current study further corroborates evidence that the expression levels of CK13 and IVL are related to the differentiation status of malignant keratinocytes in OTSCC. First, the expression levels of IVL and CK13 were downregulated at the invading cores, a poorly differentiated area of a tumor[33] as compared to the corresponding center cores in OTSCC (Figure 3(G,H)). Second, lower combined expression of IVL and CK13 was significantly associated with poor tumor differentiation (Table 2). In line with this, the expression levels of CK13 and IVL at invading cores were found to be significantly lower in poorly differentiated OTSCC as compared to the well/moderately differentiated lesions (Figure 4(A,B)). Consistent with our results, previous studies have shown positive associations between the expression of CK13 and IVL with tumor differentiation in SCC, including OSCC.[34–36] Nevertheless, the lack of association of CK10 expression with tumor differentiation is in contrast to a previous report in OSCC.[34,37] This discrepancy could be related to the location of lesions in the oral cavity as the expression of CK10 has been shown to vary with anatomical sites and the degree of keratinization of oral epithelium.[38,39] Besides this, the region of tumor tissue chosen for evaluation of CK10 might be related to this discordance as we have used TMA while Sadafi et al. and Bloor et al. have used whole sections.

OTSCC/OSCC cases with poorly differentiated phenotype have been shown to have poor prognosis.[40,41] In agreement with these observations, OTSCC cases with combined loss of CK13 and IVL expression at invading cores was significantly associated with poorer 5-year overall survival (Figure 4(E,F)). Multivariate Cox regression analysis further indicated that the combined loss of IVL and CK13 at invading core was an independent prognostic factor (HR = 0.38, CI = 0.17–0.85, $p = 0.02$) in OTSCC. Although the prognostic significance of IVL expression alone or in combination with CK13 was not available in literature, in line with our findings, the loss of expression of CK13 has been linked with poor prognosis in tongue SCC.[16,17] The IHC results were paralleled by analysis of TCGA dataset where OTSCC cases with low combined expression of *IVL* and *CK13* mRNA levels were found to have significantly lower 5-year overall survival probabilities (Figure 5).

The strength of this study is the use of multicenter, HPV-negative homogenous specimens of OTSCC representing the Norwegian population. Although often overlooked, a significant molecular and prognostic heterogeneity among carcinomas from different anatomical sites in oral cavity has been described[10,42,43] and this is particularly important to keep in mind for biomarker studies. Moreover, evaluation of both center and invading cores suggested that there is a significant intratumoral heterogeneity with respect to the expression of CK13 and IVL. Taken together, these suggestions emphasize that measures should be taken to minimize the influence of intrasite and intratumoral heterogeneity during biomarker/translational studies. On the other hand, the use of tissue cores representing specific areas rather than the whole tumor section can be considered as a potential selection bias/limitation of the study. Nevertheless, duplicate cores for both center and invading areas were available for more than 50% of the cases. In addition, as the specimens used in the current study being a representative cohort of Norwegian population, the external validity of the results of the study should be verified in population with differing race, lifestyles, and habitual risk factors as compared to that of the Norwegian population.

In conclusion, our results further substantiated the fact that the expression status of IVL and CK13 might be useful as markers of differentiation in malignant oral keratinocytes, as evidenced by the loss of expression at the invading cores and in poorly differentiated lesions. In addition, the combined expression status of IVL and CK13 at the invading cores, but not at the tumor center, might be potentially useful in predicting the prognosis of patients with OTSCC. These findings could have implications in management of and further research initiatives in OTSCC. However, further confirmatory studies using whole tissue sections with a wide panel of differentiation markers and including functional studies will further aid in confirming the relevance of these dysregulated proteins in OTSCC.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHOR CONTRIBUTIONS

Dipak Sapkota, Burcu Senguven, and Daniela Elena Costea conceived and designed research. Dipak Sapkota, Tine Merete Søland, Lars Peter Sand, IngerHeidi Bjerkli, Daniela Elena Costea, and Fernanda Cristina Petersen contributed with specimens and reagents. Sushma Pandey and Burcu Senguven performed the experiments. Sushma Pandey and Burcu Senguven analyzed the data. Sushma Pandey and Dipak Sapkota wrote the manuscript. All coauthors critically reviewed and revised the manuscript. Dipak Sapkota, Daniela Elena Costea, and Fernanda Cristina Petersen supervised the work. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Sushma Pandey https://orcid.org/0000-0002-5483-1577
Tine Merete Søland https://orcid.org/0000-0003-1687-3247
Daniela Elena Costea https://orcid.org/0000-0001-7673-0358
Dipak Sapkota https://orcid.org/0000-0003-0061-825X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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