Received: 2 November 2019 | Revised: 23 January 2020 | Accepted: 5 March 2020

DOI: 10.1002/hed.26140

WILEY

ORIGINAL ARTICLE

Loss of S100A14 expression at the tumor-invading front correlates with poor differentiation and worse prognosis in oral squamous cell carcinoma

Sushma Pandey MDS[1] | Tarig A. Osman PhD[2] | Sunita Sharma PhD[3] | Evan M. Vallenari MSC[1] | Aboulghassem Shahdadfar PhD[4] | Chin B. Pun MD[5] | Dej K. Gautam MD[6] | Lars Uhlin-Hansen PhD[7,8] | Oddveig Rikardsen PhD[9] | Anne C. Johannessen PhD[2,10] | Daniela E. Costea PhD[2,10,11] | Dipak Sapkota PhD[1]

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

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

[3]Department of Clinical Dentistry, Centre for Clinical Dental Research, University of Bergen, Bergen, Norway

[4]Centre for Eye Research, Department of Ophthalmology, Oslo University Hospital, Ullevål, Oslo, Norway

[5]Department of Pathology, B.P. Koirala Memorial Cancer Hospital, Bharatpur, Nepal

[6]Department of Surgical Oncology, B.P. Koirala Memorial Cancer Hospital, Bharatpur, Nepal

[7]Department of Clinical Pathology, University Hospital of North Norway, Tromsø, Norway

[8]Department of Medical Biology—Tumor Biology Research Group, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø, Norway

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

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

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

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

Funding information
North Norway Regional Health Authority, Grant/Award Number: SFP1276-16; Norwegian Centre for International Cooperation in Education, Grant/Award Number: CPEA-LT-2016/10106; Norwegian Centre of Excellence grant, Grant/Award Number: 223250; post-doctoral fund (UiB) and starting grant at UiO for DS; Western Norway Regional Health Authority, Grant/Award Number: 912260

Abstract

Background: We previously showed a tumor-suppressive function of S100A14 in oral squamous cell carcinoma (OSCC). This study aimed to examine the prognostic significance and differentiation-related function of S100A14 in OSCC.

Methods: S100A14 expression was examined in 170 OSCCs from Norwegian and Nepalese populations using immunohistochemistry. Pro-differentiation function was investigated by overexpressing and silencing S100A14 expression in OSCC-derived cells. External transcriptomic datasets were used to validate association between S100A14 and differentiation markers in OSCC.

Result: Loss of S100A14 expression at the invading tumor fronts significantly correlated with poor differentiation and reduced 10-years survival of OSCC-patients. Multivariate Cox analysis identified S100A14 to be an independent prognostic factor. Modulation of S100A14 expression in OSCC-derived cells

positively correlated with the expression of differentiation markers. Analysis of external datasets supported the pro-differentiation function of S100A14.
Conclusion: These results indicate that S100A14 is a pro-differentiation protein and its expression might be useful as a prognostic marker in OSCC.

KEYWORDS
Nepal, Norway, oral cancer, prognosis, S100

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of oral cancer (OC) cases. Combined with lip and pharyngeal cancers, OC is the seventh most common cancer type in the world.[1,2] In spite of the recent improvements in diagnostic and treatment methods, the 5-year survival (approximately 60%) of OSCC patients has not improved significantly.[3] This emphasizes the need for better understating of OSCC biology, which might lead the development of biomarker-based tools for earlier diagnosis and targeted therapy of OSCC.

S100A14 protein is one of the members of a large multifunctional group of calcium binding proteins.[4] Differential expression of S100A14 seems to be a common molecular change in human malignancies[4-7] and its expression pattern has been shown to predict disease aggressiveness and patient prognosis in breast,[6,8] gastric,[9] ovarian[10,11] and other malignancies.[5,12] Functionally, S100A14 has been implicated to have a key role in molecular pathways related to cell proliferation, differentiation and apoptosis, as well as migration and invasion in several cancer types.[6,9,13] The role of S100A14 in cellular differentiation is especially important as genes encoding several of the S100 protein members including S100A14 are clustered in the epidermal differentiation complex on chromosome 1q21,[4,14,15] and many of the S100 members have been shown to have a key role in cellular differentiation and differentiation-related pathologies.[16,17] Indeed, S100A14 has been shown to promote differentiation of esophageal squamous cell carcinoma[5] and gastric cancer cells.[9]

Previously, we reported progressive downregulation of S100A14 during the progression of OSCC both *in vivo* and *in vitro*.[18,19] Further work demonstrated that S100A14 might function as a tumor suppressor protein in OSCC by inhibiting cell proliferation and invasion, possibly through the modulation of p53, p21, MMP1, and MMP9 expression.[18,19] S100A14 was further found to interact with and regulate the expression of S100A16, another member of S100 protein family found to suppress OSCC progression by promoting OSCC cell differentiation.[20,21] Nevertheless, the role of S100A14 in OSCC cell differentiation and its prognostic role in OSCC is currently unknown. In the current study, using a large cohort of OSCC, we show that lower expression of S100A14 at the invading fronts/islands in OSCC specimens is associated with poor tumor differentiation and worse prognosis. Experimentally, retroviral mediated overexpression and knockdown of S100A14 led to respective up- and down-regulation of differentiation markers in OSCC cells.

2 | MATERIALS AND METHODS

2.1 | Formalin-fixed paraffin embedded (FFPE) human tissue specimens

The tissue samples used in the current work consisted of FFPE archival OSCC specimens from Norway (Bergen and Tromsø) and Nepal (Chitwan). Tissue samples from Bergen, Tromsø, and Chitwan were collected respectively from Haukeland University Hospital (between 1998 and 2012), University Hospital of North Norway (between 1998 and 2002) and B.P. Koirala Memorial Cancer Hospital (between 2011 and 2014). Informed consent was obtained from the participating patients. Collection of specimens from Bergen was approved by the Committee for Medical and Health Research Ethics in West Norway (2010/481 REK vest), from Tromsø by Committee for Medical and Health Research Ethics in North Norway (2015/1383 REK nord) and from Nepal by the Committee for Medical and Health Research Ethics in West Norway (2011/1244 REK vest) and Nepal Health Research Council (ref 526/2012). A total number of 183 FFPE OSCCs from Norway (Bergen: 64 and Tromsø: 46) and Nepal (73) were used for S100A14 immunohistochemistry (IHC). Protocols used for the tissue collection, processing (formalin fixation and paraffin embedding) and storage were similar at Bergen and Tromsø. A similar protocol was also established (by DS) and followed at the B.P. Koirala Memorial Cancer Hospital. Out of 183 specimens stained, 8 specimens were excluded from the final analysis because they either consisted of very little tumor tissue or very few invading areas. Out of the remaining175 specimens (Bergen: 63, Tromsø: 43, and Chitwan: 69), five cases (all from Norway) were found to be human papilloma virus (HPV) positive and

were excluded from the final statistical analysis. All OSCC patients included in the study were newly diagnosed cases, and had no history of chemo- or radiotherapy prior to surgery. Details of the clinicopathological information of these OSCC cases are reported in Table 1. REMARK guidelines[22] were followed where appropriate.

2.2 | S100A14 IHC

Using rabbit polyclonal anti-human S100A14 primary antibody (10489-1-AP, Proteintech, Chicago, Illinois, 1:1000 dilutions), IHC was performed on FFPE OSCC specimens as described previously.[18,20] The negative controls consisted of sections incubated with 3% bovine serum albumin (BSA) instead of the primary antibody.

2.3 | S100A14 IHC evaluation

The stained slides were scanned at ×40 using Nano Zoomer XR digital scanner, Hamamatsu) and analyzed manually at ×40 using Aperio ImageScope (version 12.3.2.8013) software, Leica Biosystems. Blinded for the clinical information and after being calibrated to minimize the interobservation variation, the IHC evaluation was done by two of the authors (DS and SS). The semiquantitative evaluation of S100A14 expression was done both at the tumor center and at the invading front following a composite scoring system as described previously for S100A16 protein.[21] Briefly, three consecutive fields (>500 cells/field, whenever possible) both at the tumor center and the invading front (the deepest part of an invasive tumor, >3 to 4 cell layers thick) were used for evaluation. In cases where clear invasive fronts were not possible to identify, deepest invading tumor islands consisting of >50 cells were used for evaluation. A composite percentage, localization and intensity (PLI) scoring system combining the number of S100A14 positive cells (P score), cellular localization (membranous or cytoplasmic or both, L score) and intensity (I score) was used for S100A14 scoring as described previously.[21]

2.4 | p16 IHC for HPV

HPV status of OSCCs was investigated by using IHC for p16 protein in the FFPE tissues. The procedures for antigen retrieval, blocking, and visualization of the antigen were similar as described for S100A14 IHC. The primary and the secondary antibodies were mouse monoclonal anti-human p16 primary antibody (G175-405 clone, BD Pharminogen, 1:1000 dilutions) and anti-mouse secondary antibody conjugated with horseradish peroxidase labeled polymer (K400111-2 EnVision System, DAKO), respectively. Known HPV positive FFPE specimens of oropharyngeal squamous cell carcinoma were used as positive controls. The negative controls consisted of sections incubated with 3% BSA instead of the primary antibody. OSCC cases with more than 70% p16 positive cancer cells (both nuclear and cytoplasmic) were considered HPV positive.

TABLE 1 S100A14 expression (PLI score) and clinicopathological variables of the OSCC patients from Norway and Nepal

	PLI score at invading fronts/islands[a]		
Variables	**Low, n (%)**	**High, n (%)**	***P*-value**
Age[b] (years)			
≤60	41 (50.0)	41 (50.0)	1.00
>60	44 (50.0)	44 (50.0)	
Gender			
Female	27 (47.4)	30 (52.6)	.626
Male	58 (51.3)	55 (48.7)	
Smoking			
No	26 (42.6)	35 (57.4)	***.036***
Yes	49 (50.5)	48 (49.5)	
Unknown	10 (83.3)	2 (16.7)	
Alcohol			
No	19 (30.2)	44 (69.8)	***.000***
Yes	38 (59.4)	26 (40.6)	
Unknown	28 (65.1)	15 (34.9)	
Location			
Tongue	39 (54.9)	32 (45.1)	.654
Gingiva and buccal mucosa	32 (45.1)	39 (54.9)	
Floor of the mouth	11 (47.8)	12 (52.2)	
Palate, lip and oropharynx	3 (60.0)	2 (40.0)	
Differentiation			
Poor and moderate	62 (78.5)	17 (21.5)	***.000***
Well	23 (25.3)	68 (74.7)	
Lymph node involvement[c]			
Negative (N0)	50 (49.5)	51 (50.1)	.950
Positive (N1 and N2)	34 (50.0)	34 (50.0)	
Tumor size			
T1 and T2	46 (58.2)	33 (41.8)	***.046***
T3 and T4	39 (42.9)	52 (57.1)	
Recurrence[d]			

(Continues)

TABLE 1 (Continued)

Variables	PLI score at invading fronts/islands[a] Low, n (%)	High, n (%)	*P*-value
No	26 (60.5)	17 (39.5)	.761
Yes	11 (64.7)	6 (35.3)	
Tumor stage[c]			
Early (I and II)	32 (56.1)	25 (43.9)	.233
Late (III and IV)	52 (46.4)	60 (53.6)	

Note: The bold italic values were used to indicate significant $p < .05$.
Abbreviation: OSCC, oral squamous cell carcinoma, PLI, percentage, localization and intensity.
[a]OSCCs were stratified into high and low S100A14 expression groups by using median S100A14 PLI score as a cut-off.
[b]Patients were categorized into low- and high-age groups based on the median age.
[c]Data on node status and stage were missing for one OSCC case from Tromsø.
[d]Data on recurrence were not available for all OSCC cases from Tromsø and Nepal.

2.5 | External databases

mRNA expression levels of *S100A14* and differentiation related molecules (*IVL*, *KRT13*, *KRT4*, *TGM1*, and *FLG*) were obtained from TCGA[23] and two external microarray datasets[24,25] for OSCC/head and neck squamous cell carcinoma. These data were used for correlation analyses. Correlation analysis for TCGA dataset was carried out using open access cBioPortal for Cancer Genomics tool.[26,27]

2.6 | Cell culture, construction of expression, and shRNA vectors and transfection

The OSCC-derived CaLH3[28] and VB6[29] cell-lines were cultured in humidified environment at 37°C with 5% CO_2 as described previously.[18] S100A14 expression vector was constructed as described previously.[18,20] Briefly, human cDNA encoding *S100A14* was subcloned into the pRetroX-IRES-ZsGreen1 retroviral expression vector (cat-no: 632520, Clontech) for the construction of S100A14 expression vector. For shRNA, oligonucleotides targeting *S100A14* mRNA were annealed and inserted in the RNAi-Ready pSIREN-RetroQ-DsRed-Express expression vector (cat. no: 632487, Clonetech). For details of the expression and shRNA vector construction, see Supplementary methods. CaLH3 and VB6 cells infected with retrovirus with S100A14 insert were referred to as "S100A14 CaLH3" and "S100A14 VB6", whereas the cells infected with and retrovirus without S100A14 insert were called and "control CaLH3 and control VB6," respectively. Similarly, CaLH3 cells infected with retrovirus with shRNA targeting *S100A14* and shRNA targeting *LacZ* gene were referred to as "S100A14 shRNA CaLH3" and "LacZ shRNA CaLH3," respectively.

2.7 | Immunoblotting

Twenty to thirty μg of reduced cell lysates were resolved in 4% to 20% Criterion TGX Precast Midi Protein Gel (Cat no: 5671093, Biorad) and immunoblotted with antibodies as described in Table S1.

2.8 | Statistics

SPSS 25 and/or GraphPad prism version 8.0.1 for Windows (www.graphpad.com) were used for statistical analysis. Difference between the means of two groups was analyzed by using paired/unpaired *t* tests. Pearson correlation analysis was used to examine correlation between mRNA levels of *S100A14* and keratinocyte differentiation markers in external microarray datasets. OSCC cases were categorized into high- and low-S100A14 expression groups using the median PLI scores both at the tumor center and at the invading front/island as cut-off values. Because of the fewer number of OSCC cases (5.9%) with poor differentiation, these cases were merged together with OSCCs with moderate differentiation. Hence, only two groups (well vs moderately-poorly differentiated OSCCs) were considered for statistical analysis. Association between the S100A14 expression and other binary variables was examined using chi-square test. Survival analysis was performed using the Kaplan-Meier analysis (log-rank test). Clinicopathological variables with significant association with 10-year overall survival in univariate Cox analysis were used to create a model for multivariate Cox proportional hazard analysis. Specimens from Nepal were excluded from the survival analysis, as the survival data were not available for them. Level of significance was set at 5%.

3 | RESULTS

3.1 | HPV status

Five OSCC cases (four from tongue and one from lip) from Norwegian cohort were positive for p16 (more than 70% p16 positive cancer cells, both nuclear and cytoplasmic) suggesting positivity for HPV infection. None of the samples from Nepal tested positive for HPV. Only the HPV negative cases were used for statistical analysis.

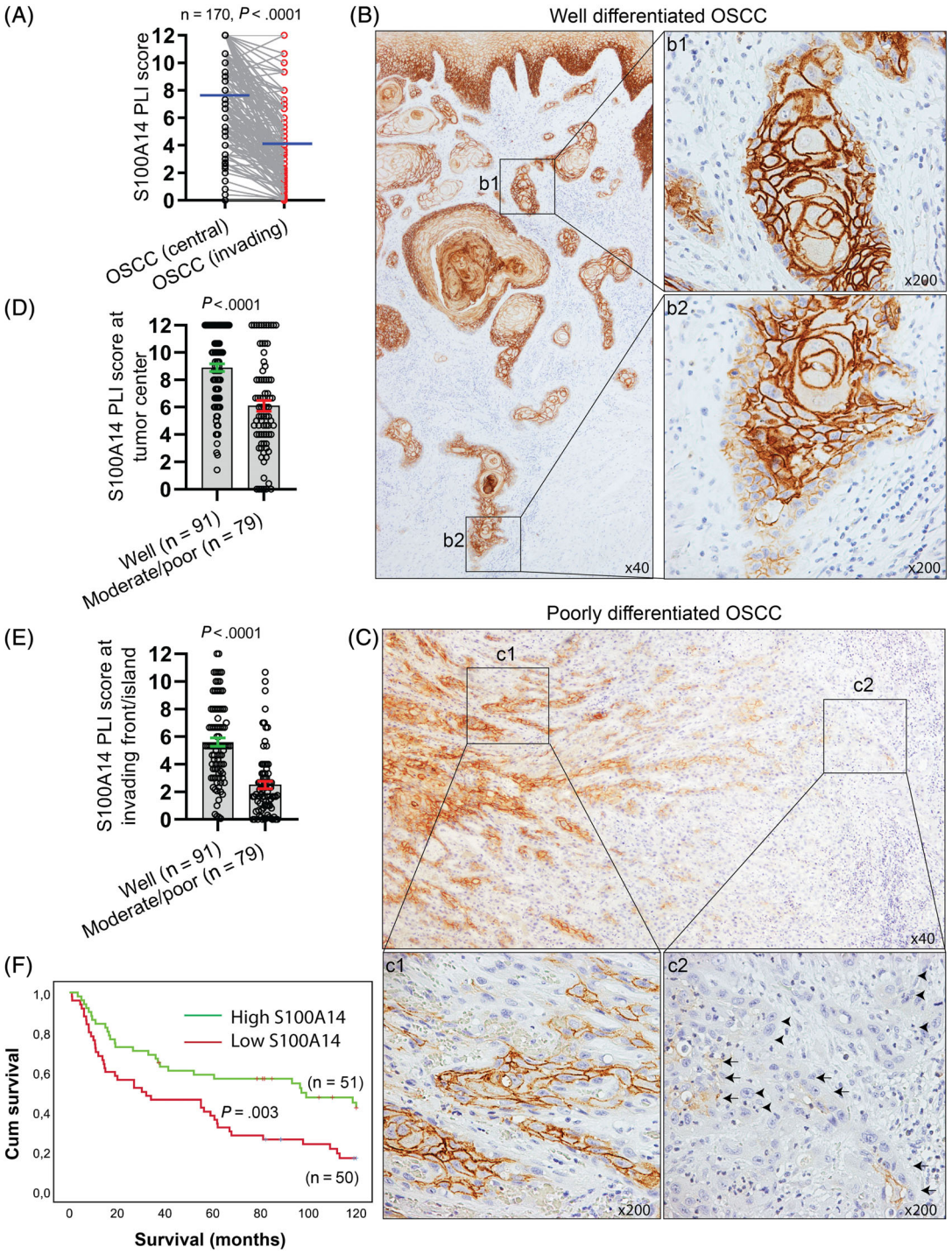

FIGURE 1 S100A14 protein was downregulated at the invading front/island of majority of moderately-poorly differentiated OSCC and low S100A14 protein expression correlated with poor OSCC prognosis. A, Graphic illustration of S100A14 PLI score demonstrated downregulation of S100A14 at the invading front/island of OSCC as compared to the corresponding tumor center of OSCC lesions. Paired *t* tests test was used for the statistical analysis. The horizontal bars indicate mean PLI scores. B, Representative well-differentiated OSCC lesion demonstrating strong, mostly membranous S100A14 staining at both the tumor center (b1) and the invading front area (b2). C, Representative images of poorly differentiated OSCC lesion showing a gradient of S100A14 expression: central area (c1) showed a relatively strong, membranous staining in contrast to almost undetectable staining in the invading front area (c2, arrowheads indicated tumor cells). S100A14 PLI score was found to be significantly higher both at the tumor center, D, and at the invading fronts/islands, E, in well-differentiated OSCCs as compared to that of moderately-poorly differentiated lesions. Unpaired *t* tests test was used for the statistical analysis in D and E. The error bars represent SEM. F, Kaplan-Meier curves showing reduced 10-year survival probabilities for patients with low S100A14 PLI score at the invading front/island. Log-Rank test was used for statistical analysis. OSCC, oral squamous cell carcinoma [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | S100A14 expression was downregulated at the invading fronts/islands of OSCC

Almost all of the OSCC specimens expressed S100A14 in cancer cells and/or the adjacent normal/dysplastic epithelium (if present). However, S100A14 expression pattern was highly variable across the different locations of the same OSCC lesion. Overall, the expression was weaker at the invading fronts/islands (poorly differentiated regions) as compared to central/superficial areas (more differentiated regions). Indeed, semiquantitative evaluation showed that S100A14 PLI score was significantly lower ($P < .0001$) at the invading front/island as compared to the central location of corresponding OSCCs (Figure 1A). Majority of the well differentiated OSCC specimens demonstrated a moderate to strong membranous S100A14 expression both at the superficial/central areas as well as at the invading front/islands (Figure 1B). Most of the moderately-poorly differentiated OSCCs, however, expressed moderately strong, membranous S100A14 staining at the tumor center, whereas the staining was very weak or negative at the invading front/island of tumor cells (Figure 1C). Additionally, although weak, the S100A14 expression appeared more cytoplasmic in the invading front/islands in the moderately-poorly differentiated OSCCs (Figure 1C and c2). Indeed, when stratified with respect to the differentiation status, S100A14 PLI score for well differentiated OSCC lesions was significantly higher both at the tumor center (Figure 1D) as well as at the invading front/island (Figure 1E) as compared to the corresponding areas of moderately and poorly differentiated lesions. Except occasional inflammatory cells, none of the structures in the stroma expressed S100A14.

3.3 | Loss of S100A14 expression at the invading front/islands of OSCC correlated negatively with tumor differentiation and 10-years overall survival

Examination of possible correlation between S100A14 expression and clinicopathological variables showed that low S100A14 PLI score at the invading front/island was significantly associated with moderate-poorly differentiated OSCCs ($P < .0001$), alcohol drinking habit ($P < .0001$), smoking ($P = .036$) and tumor size ($P = .046$) (Table 1). Norwegian and Nepalese specimens when analyzed separately, no significant correlation was observed between the S100A14 PLI score at the invading front/island and clinical parameters, except for the tumor differentiation (Norwegian cases, $P < .0001$); Nepalese cases, $P = .002$) (Tables S2 and S3).

Survival analysis showed a significant correlation between the reduced expression of S100A14 at the invading front/island (Log-Rank test, $P = .003$), higher age (Log-Rank test, $P = .027$), poorer tumor differentiation (Log-Rank test, $P = .01$), node positive status (Log-Rank test, $P = .009$) or higher pathological stage (Log-Rank test, $P = .001$) with reduced 10-years overall survival of OSCC

TABLE 2 Results of a multivariate Cox regression analysis for predicting the overall survival of OSCC cases

	Univariate Cox			Multivariate Cox		
Variables	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
S100A14						
Low	0.49	0.30-0.79	***.004***	0.55	0.32-0.96	***.035***
High						
Age						
≤63	1.70	1.05-2.73	***.029***	1.77	1.07-2.94	***.025***
>63						
Differentiation						
Well	1.86	1.14-3.03	***.012***	1.51	0.86-2.66	.148
Mod. and poor						
Node status						
N0	1.89	1.16-2.07	***.01***	1.29	0.72-2.30	.385
N1 and N2						
Clinical stage						
Early (I and II)	2.28	1.38-3.76	***.001***	1.88	1.02-3.47	***.043***
Late (III and IV)						

Abbreviations: CI, confidence interval; HR, hazard ratio; Mod., moderate; OSCC, oral squamous cell carcinoma.

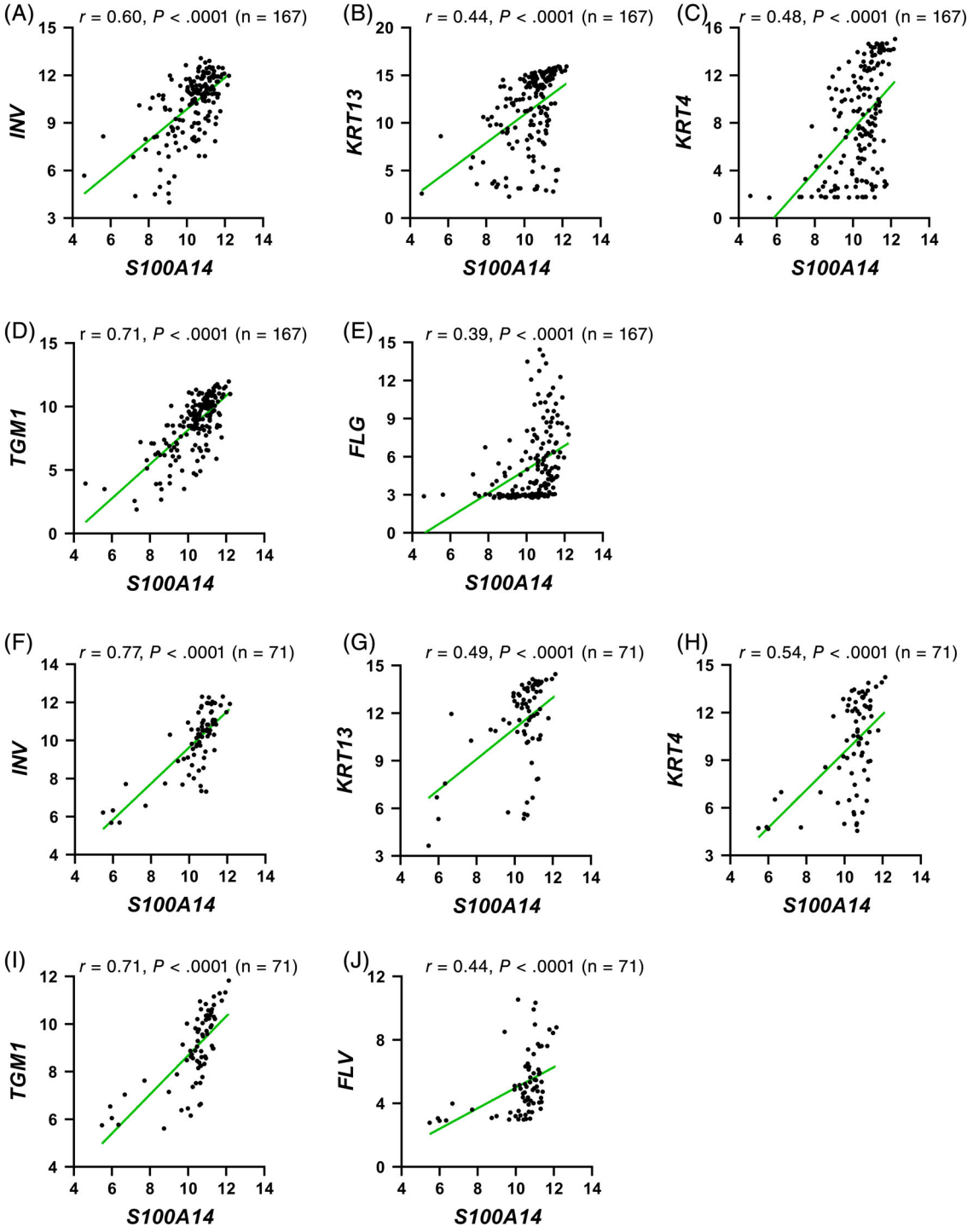

FIGURE 2 *S100A14* mRNA expression was positively correlated with differentiation markers in OSCC specimens from external microarray datasets. mRNA expression levels of *S100A14, IVL, KRT13, KRT4, TGM1*, and *FLG* were obtained from external microarray dataset (A-E, Chen et al, 2008; F-J, Thurlow et al, 2010) and their correlation was examined using Pearson analysis. mRNA expression levels of *S100A14* and the selected keratinocyte differentiation-related makers were found to be significantly correlated. The *x*-axis represented the relative mRNA level (arbitrary value) of *S100A14*, whereas the *y*-axis represented mRNA levels for differentiation markers. OSCC, oral squamous cell carcinoma [Color figure can be viewed at wileyonlinelibrary.com]

patients. Univariate Cox analysis showed S100A14 expression, age, differentiation, node status and clinical stage to be significantly associated with 10-year overall survival. Multivariate Cox regression analysis demonstrated that

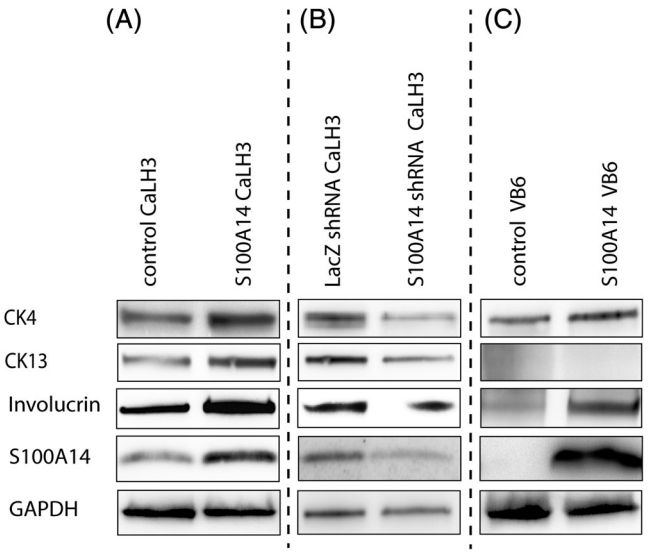

FIGURE 3 Retroviral mediated modulation of S100A14 positively regulated the expression of differentiation-related markers in OSCC cell-lines. S100A14 was overexpressed and knocked-down in CaLH3 cells and concomitant modulation of differentiation markers was examined. Western blot analysis showed upregulation of differentiation markers (cytokeratin 13, cytokeratin 4 and involucrin) in CaLH3, A, and of cytokeratin 4 and involucrin in VB6 cells, C, with S100A14 overexpression. Supporting these results, S100A14 knockdown resulted in downregulation of the corresponding differentiation related proteins in CaLH3, B. OSCC, oral squamous cell carcinoma

S100A14 expression at the invading front/island was an independent prognostic factor for the survival of OSCC patients (HR = 0.55, CI = 0.32-0.96, $P = .035$) (Table 2).

Low PLI score at the tumor center correlated with moderate-poorly differentiated OSCCs ($P < .0001$). Nevertheless, despite a trend for a positive correlation, the PLI score at the tumor center was not significantly correlated with 10-year overall survival (Log-Rank test, $P = .056$) (data not shown).

3.4 | mRNA expression levels of S100A14 and keratinocyte differentiation markers correlated significantly in external datasets of OSCC

A significant positive correlation between the S100A14 PLI score and OSCC differentiation status prompted us to test this at the molecular level in other cohorts of OSCCs. Indeed, *S100A14* mRNA levels were positively correlated with mRNA levels of keratinocyte differentiation markers (*IVL*, *KRT13*, *KRT4*, *TGM1*, and *FLG*) in two OSCC microarray[24,25] (Figure 2) and TCGA[23] datasets (Figure S2).

3.5 | S100A14 overexpression led to induced expression of differentiation-related markers in OSCC-derived cells

Having observed a significant positive association between the expression status of S100A14 and more differentiated phenotype in OSCC specimens, we next investigated whether S100A14 could modulate the expression of differentiation-related markers in OSCC-derived cell-lines. Retroviral mediated overexpression and knockdown of S100A14 respectively led to up- and down-regulation of involucrin, cytokeratin 13 and cytokeratin 4 in CaLH3 cells (Figure 3A,B). Similarly, overexpression of S100A14 was associated with upregulation of involucrin and cytokeratin 4 in VB6 cells (Figure 3C).

4 | DISCUSSION

We previously reported a loss of S100A14 expression at the invading front/island (poorly differentiated areas) of OSCC as compared to the tumor surface/tumor center (more differentiated areas), suggesting a possible functional link between S100A14 and cellular differentiation.[18] In the current study, using S100A14 IHC in a large cohort of OSCCs, we demonstrated that S100A14 expression positively correlated with tumor differentiation and better patient survival. Further, the use of external OSCC datasets and *in vitro* experiments provided evidence for pro-differentiation function of S100A14 in OSCC cells.

Malignancies with more differentiated phenotype often behave less aggressively and are associated with better clinical outcomes.[30,31] Accordingly, molecular regulators with pro-differentiation function are suggested to have tumor suppressive functions and they might serve as potential biomarkers in OSCC management.[32,33] Several observations in the current study provide evidence for a pro-differentiation function for S100A14 in OSCC. First, the expression of S100A14 was significantly downregulated at the invading front/island (poorly differentiated area of a tumor) as compared to the corresponding central area (better differentiated area) of OSCC (Figure 1A). Second, when stratified with respect to the differentiation status, the well differentiated OSCC lesions expressed significantly higher S100A14 both at the invading front/island and tumor center as compared to the moderately and poorly differentiated lesions (Figure 1B-E). Similar observations have been reported in esophageal squamous cell carcinoma[5] and gastric cancer.[9] Importantly, clinicopathological analysis showed a significant correlation between low S100A14 protein (at the invading front/island) levels and reduced 10-year overall survival probabilities for OSCC patients (Figure 1F). These

data suggest that S100A14 expression at the invading front/island can serve as an independent prognostic factor for OSCC. Third, S100A14 overexpression or knockdown mediated concomitant modulation of expression of differentiation markers provided direct evidence for a differentiation promoting function for S100A14 in OSCC cells (Figure 3). Corroborating these findings, mRNA expression levels of *S100A14* was significantly correlated with mRNA levels of a number of differentiation markers such as *INV*, *KRT13*, *KRT4*, *TGM1*, and *FLG* in OSCC lesions in three independent OSCC datasets (Figure 2 and Figure S2). Differentiation promoting function of S100A14 in OSCC is in line with previous studies in esophageal squamous cell carcinoma[5] and gastric cancer cells.[9]

Keratinocyte differentiation is a multistep process involving a complex-interplay between extracellular and intracellular signals, transcription factors, structural proteins, enzymes and metallic ions such as calcium.[34,35] A previous study suggested involvement of JunB (a component of AP-1 transcription factor) in S100A14 mediated differentiation of esophageal squamous cell carcinoma cells.[5] However, the signaling molecules and pathways for prodifferentiation function of S100A14 are not fully understood. We previously showed that S100A14 positively regulated the expression and function of tumor suppressor protein p53 and its downstream signaling molecule, the p21, leading to a G1-cell cycle arrest and reduced OSCC cell proliferation.[19] Given the key role of p53[36,37] and p21[38] in cell-cycle arrest and keratinocyte differentiation, a possible involvement of these proteins in S100A14 mediated prodifferentiation function cannot be excluded in OSCC. Additionally, cooperation between S100A14 and S100A16 represents a likely mechanism in keratinocyte differentiation as S100A14 has been found to interact with and regulate the expression of S100A16, which in turn promotes keratinocyte differentiation.[21] Nevertheless, these suggestions warrant further in-depth investigation.

The current project benefited from the inclusion of OSCC specimens from two different countries (Norway and Nepal) with differing race, lifestyle, ethnicity and etiological factors. Despite these differences, previous studies have suggested involvement of common biological pathways during OSCC development in western and developing countries.[39,40] In parallel, S100A14 was found to be significantly downregulated at the invading fronts/islands in poorly differentiated OSCC as compared to the well differentiated ones in both countries (Figure S1), indicating that deregulation of S100A14 might be a common molecular alteration in OSCC irrespective of the associated risk factors and genetic background of the individuals.

In conclusion, our results suggest that S100A14 is a differentiation promoting protein in OSCC. Together with our previous findings indicating a tumor suppressive function (inhibition of OSCC cell proliferation and invasion) for S100A14,[18,19] it can be suggested that S100A14 is involved in multiple biological processes relevant for tumor suppression in OSCC. Further studies are necessary to understand S100A14-mediated signaling pathways and to establish potential use of S100A14 as a prognostic marker in OSCC.

ACKNOWLEDGMENTS

The authors would like to thank Gunnvor Øijordsbakken (Department of Clinical Medicine, The Gade Laboratory for Pathology, University of Bergen) for assistance with immunohistochemistry. This study was funded by postdoctoral fund (UiB) and starting grant at UiO for DS; the North Norway Regional Health Authority (Helse Nord project no. SFP1276-16), the Norwegian Centre of Excellence grant (ID 223250), the Western Norway Regional Health Authority (Helse Vest project no. 912260), and the Norwegian Centre for International Cooperation in Education (project no. CPEA-LT-2016/10106).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Dipak Sapkota, Anne C. Johannessen, and Daniela E. Costea conceived and designed research; Dipak Sapkota, Anne C. Johannessen, Daniela E. Costea, Aboulghassem Shahdadfar, Chin B. Pun, Dej K. Gautam, Lars Uhlin-Hansen, and Oddveig Rikardsen contributed with specimens and reagents; Sushma Pandey, Dipak Sapkota, Tarig A. Osman, Sunita Sharma, Evan M. Vallenari, Daniela E. Costea, and Anne C. Johannessen performed the experiments, analyzed the data and reviewed the manuscript. Dipak Sapkota and Daniela Elena Costea supervised the work. All authors read and approved the manuscript.

ORCID

Daniela E. Costea https://orcid.org/0000-0001-7673-0358
Dipak Sapkota https://orcid.org/0000-0003-0061-825X

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015;136(5):E359-E386.
2. Shield KD, Ferlay J, Jemal A, et al. The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012. *CA Cancer J Clin*. 2017;67(1):51-64.

3. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin*. 2010;60(5):277-300.
4. Pietas A, Schlüns K, Marenholz I, Schäfer BW, Heizmann CW, Petersen I. Molecular cloning and characterization of the human S100A14 gene encoding a novel member of the S100 family. *Genomics*. 2002;79(4):513-522.
5. Chen H, Ma J, Sunkel B, et al. S100A14: novel modulator of terminal differentiation in esophageal cancer. *Mol Cancer Res*. 2013;11(12):1542-1553.
6. Tanaka M, Ichikawa-Tomikawa N, Shishito N, et al. Co-expression of S100A14 and S100A16 correlates with a poor prognosis in human breast cancer and promotes cancer cell invasion. *BMC Cancer*. 2015;15(1):53.
7. Basnet S, Sharma S, Costea DE, Sapkota D. Expression profile and functional role of S100A14 in human cancer. *Oncotarget*. 2019;10(31):2996-3012.
8. Sidse E, Tykgaard HL, Martin B, Charlotte B-A, Ditzel HJ, Rikke L-L. S100A14 is a novel independent prognostic biomarker in the triple-negative breast cancer subtype. *Int J Cancer*. 2015;137(9):2093-2103.
9. Zhu M, Wang H, Cui J, et al. Calcium-binding protein S100A14 induces differentiation and suppresses metastasis in gastric cancer. *Cell Death Dis*. 2017;8:e2938.
10. Zhao H, Guo E, Hu T, et al. KCNN4 and S100A14 act as predictors of recurrence in optimally debulked patients with serous ovarian cancer. *Oncotarget*. 2016;7(28):43924-43938.
11. Cho H, Shin HY, Kim S, et al. The role of S100A14 in epithelial ovarian tumors. *Oncotarget*. 2014;5(11):3482-3496.
12. Wang X, Yang J, Qian J, Liu Z, Chen H, Cui Z. S100A14, a mediator of epithelial-mesenchymal transition, regulates proliferation, migration and invasion of human cervical cancer cells. *Am J Cancer Res*. 2015;5(4):1484-1495.
13. Qe J, Chen H, Luo A, Ding F, Liu Z. S100A14 stimulates cell proliferation and induces cell apoptosis at different concentrations via receptor for advanced glycation end products (RAGE). *PLoS One*. 2011;6(4):e19375.
14. Schäfer BW, Wicki R, Engelkamp D, Mattei MG, Heizmann CW. Isolation of a YAC clone covering a cluster of nine S100 genes on human chromosome 1q21: rationale for a new nomenclature of the S100 calcium-binding protein family. *Genomics*. 1995;25(3):638-643.
15. Volz A, Korge BP, Compton JG, Ziegler A, Steinert PM, Mischke D. Physical mapping of a functional cluster of epidermal differentiation genes on chromosome 1q21. *Genomics*. 1993;18(1):92-99.
16. Martinsson H, Yhr M, Enerbäck C. Expression patterns of S100A7 (psoriasin) and S100A9 (calgranulin-B) in keratinocyte differentiation. *Exp Dermatol*. 2005;14(3):161-168.
17. Wolf R, Lewerenz V, Büchau AS, Walz M, Ruzicka T. Human S100A15 splice variants are differentially expressed in inflammatory skin diseases and regulated through Th1 cytokines and calcium. *Exp Dermatol*. 2007;16(8):685-691.
18. Sapkota D, Bruland O, Costea DE, Haugen H, Vasstrand EN, Ibrahim SO. S100A14 regulates the invasive potential of oral squamous cell carcinoma derived cell-lines in vitro by modulating expression of matrix metalloproteinases, MMP1 and MMP9. *Eur J Cancer*. 2011;47(4):600-610.
19. Sapkota D, Costea DE, Blø M, et al. S100A14 inhibits proliferation of oral carcinoma derived cells through G1-arrest. *Oral Oncol*. 2012;48(3):219-225.
20. Sapkota D, Costea DE, Ibrahim SO, Johannessen AC, Bruland O. S100A14 interacts with S100A16 and regulates its expression in human cancer cells. *PLoS One*. 2013;8(9):e76058.
21. Sapkota D, Bruland O, Parajuli H, et al. S100A16 promotes differentiation and contributes to a less aggressive tumor phenotype in oral squamous cell carcinoma. *BMC Cancer*. 2015;15(1):631.
22. Altman DG, McShane LM, Sauerbrei W, Taube SE. Reporting recommendations for tumor marker prognostic studies (REMARK): explanation and elaboration. *PLoS Med*. 2012;9(5):e1001216.
23. Lawrence MS, Sougnez C, Lichtenstein L, et al. Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature*. 2015;517(7536):576-582.
24. Thurlow JK, Peña Murillo CL, Hunter KD, et al. Spectral clustering of microarray data elucidates the roles of microenvironment remodeling and immune responses in survival of head and neck squamous cell carcinoma. *J Clin Oncol*. 2010;28(17):2881-2888.
25. Chen C, Méndez E, Houck J, et al. Gene expression profiling identifies genes predictive of oral squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2008;17(8):2152-2162.
26. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012;2(5):401-404.
27. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6(269):pl1.
28. Harper LJ, Piper K, Common J, Fortune F, Mackenzie IC. Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma. *J Oral Pathol Med*. 2007;36(10):594-603.
29. Thomas GJ, Lewis MP, Whawell SA, et al. Expression of the αvβ6 integrin promotes migration and invasion in squamous carcinoma cells. *J Invest Dermatol*. 2001;117(1):67-73.
30. Kademani D, Bell RB, Bagheri S, et al. Prognostic factors in intraoral squamous cell carcinoma: the influence of histologic grade. *J Oral Maxillofac Surg*. 2005;63(11):1599-1605.
31. Arduino PG, Carrozzo M, Chiecchio A, et al. Clinical and histopathologic independent prognostic factors in oral squamous cell carcinoma: a retrospective study of 334 cases. *J Oral Maxillofac Surg*. 2008;66(8):1570-1579.
32. Wu X, Cao W, Wang X, et al. TGM3, a candidate tumor suppressor gene, contributes to human head and neck cancer. *Mol Cancer*. 2013;12(1):151.
33. Botti E, Spallone G, Moretti F, et al. Developmental factor IRF6 exhibits tumor suppressor activity in squamous cell carcinomas. *Proc Natl Acad Sci U S A*. 2011;108(33):13710-13715.
34. Eckert RL, Rorke EA. Molecular biology of keratinocyte differentiation. *Environ Health Perspect*. 1989;80:109-116.
35. Presland RB, Dale BA. Epithelial structural proteins of the skin and oral cavity: function in health and disease. *Crit Rev Oral Biol Med*. 2000;11(4):383-408.
36. Guinea-Viniegra J, Zenz R, Scheuch H, et al. Differentiation-

induced skin cancer suppression by FOS, p53, and TACE/ADAM17. *J Clin Invest*. 2012;122(8):2898-2910.
37. Woodworth CD, Wang H, Simpson S, Alvarez-Salas LM, Notario V. Overexpression of wild-type p53 alters growth and differentiation of normal human keratinocytes but not human papillomavirus-expressing cell lines. *Cell Growth Differ*. 1993;4 (5):367-376.
38. Weinberg WC, Denning MF. p21WAF1 control of epithelial cell cycle and cell fate. *Crit Rev Oral Biol Med*. 2002;13(6): 453-464.
39. Dysvik B, Vasstrand EN, Lovlie R, et al. Gene expression profiles of head and neck carcinomas from Sudanese and Norwegian patients reveal common biological pathways regardless of race and lifestyle. *Clin Cancer Res*. 2006;12(4): 1109-1120.
40. Roman E, Meza-Zepeda LA, Kresse SH, Myklebost O, Vasstrand EN, Ibrahim SO. Chromosomal aberrations in head and neck squamous cell carcinomas in Norwegian and Sudanese populations by array comparative genomic hybridization. *Oncol Rep*. 2008;20(4):825-843.

SUPPORTING INFORMATION

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

How to cite this article: Pandey S, Osman TA, Sharma S, et al. Loss of S100A14 expression at the tumor-invading front correlates with poor differentiation and worse prognosis in oral squamous cell carcinoma. *Head & Neck*. 2020;1–11. https://doi.org/10.1002/hed.26140