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ORIGINAL ARTICLE

Cortactin expression in a Norwegian cohort of human papilloma virus negative oral squamous cell carcinomas of the mobile tongue

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Abstract

Oral squamous cell carcinoma of the tongue (OTSCC) is the most common malignancy among oral squamous cell carcinomas and is frequently associated with an unfavorable prognosis. Local spread and distant metastasis are important causes of poor prognosis in OTSCC. Cortactin amplification and overexpression, a common molecular alteration in oral squamous cell carcinomas, have been linked to invasion and metastasis of tumor cells. However, the intra-tumor expression pattern and prognostic significance of cortactin in human papillomavirus (HPV) negative OTSCC is not fully investigated. Immunohistochemical analysis using tissue microarray consisting of formalin-fixed and paraffin-embedded HPV negative OTSCC (n = 123) specimens showed overexpression of cortactin at tissue cores from invading fronts as compared to the corresponding center cores. High overall cortactin expression was found to be associated with advanced (larger) tumor size and the occurrence of distance metastasis. Kaplan-Meier survival analysis showed that patients with high overall cortactin expression were associated with reduced 5-year survival. Multivariate Cox regression analysis identified high cortactin expression to be an independent prognostic factor in OTSCC. Additionally, siRNA-mediated silencing of cortactin was found to suppress the proliferative and invasive abilities of OTSCC cells in an organotypic co-culture model. Overexpression of cortactin is a promising prognostic marker in HPV-negative OTSCC.

KEYWORDS
invasion, organotypic culture, tumor budding

INTRODUCTION

Squamous cell carcinoma, arising from the oral epithelium is the most common variant of oral cancer [1, 2]. The most significant prognostic factor in oral squamous cell carcinoma (OSCC) is cervical lymph node involvement, whereas tumor size is considered the biggest risk factor for cervical lymph node metastasis [1]. Even though the oral cavity is not a difficult region for visual examination, more than 50% of OSCC patients are diagnosed at stages III or IV [3], leading to a low 5-year overall survival rate (around 60%) [3, 4]. The histological assessment of depth of invasion has been adjoined into the staging of OSCC since previous studies have shown that the thickness of the tumor is related to the risk of cervical lymph node involvement and patient prognosis [4]. Recently in the 8th edition of the American Joint Committee on Cancer

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TNM classification, major modifications of the TNM system were introduced. Here, depth of invasion was included in the T staging of oral squamous cell carcinoma. Therefore, its association with protein biomarkers is worth investigating [5, 6].

Among OSCC patients, those with carcinoma of the oral (anterior 2/3rd) tongue (OTSCC) are more likely to have a higher mortality rate than those with lesions in the base (posterior 1/3rd) of the tongue, retromolar region, or upper or lower gingiva [2]. The management of OTSCC is further challenging due to its anatomical complexity, and the functional and aesthetic concerns that have to be taken into consideration, and thereby requires a multidisciplinary treatment approach [7]. Although they occur in the same organ, OTSCC and squamous cell carcinoma in the base of the tongue differ from each other with respect to etiologic factors, molecular alterations, treatment options, and prognosis [8]. For example, smoking and alcohol use are the main risk factors for OTSCC, whereas human papilloma virus (HPV) is considered the main etiological factor for squamous cell carcinoma in the base of the tongue [9, 10]. At the molecular level, OTSCC are suggested to have a high mutational burden as compared to HPV-driven squamous cell carcinoma in the base of the tongue [11, 12]. Hence, it is important to stratify OTSCC from squamous cell carcinoma of the base of the tongue in biomarker studies. Clinical stage and histopathological tumor features (such as differentiation, perineural invasion) have historically been used as predictive and prognostic guides in the treatment of OTSCC patients. Furthermore, biomarkers have been investigated as potential candidates to predict the clinical outcomes in OTSCC, however none of them are regarded as established prognostic markers in clinical practice [6].

The protein cortactin has been linked with progression and nodal involvement in head and neck squamous carcinoma [13]. Cortactin, encoded by the *CTTN* gene located in the 11q13 chromosomal band, is a cytoskeletal protein that supports actin polymerization [14]. In addition to regulating filamentous actin by binding to and polymerizing it, cortactin stabilizes and organizes branched actin networks by increasing polymerization and reticular assembly of actin monomers [15]. The region 11q13 is amplified in different types of cancer, including head and neck carcinoma [16]. In OSCC an amplification rate of up to 57% has been reported [14]. Overexpression of cortactin due to *CTTN* amplification is related to increased cell motility [17, 18], invasiveness, and tumor growth [16]. Higher cortactin expression has been reported to be associated with cervical node involvement, larger tumor size, higher tumor stage and reduced patient survival in OSCC [14, 19]. siRNA-mediated silencing of cortactin has been shown to result in less migration and invasion in HPV negative head and neck squamous cell carcinoma cell lines [16]. However, the intra-tumor expression pattern (tumor invading front versus corresponding superficial/central areas) and prognostic significance of cortactin in HPV-negative OTSCC specimens is not fully investigated.

The aim of this study was to explore the prognostic role of cortactin expression in a homogenous, multi-center cohort of HPV-negative OTSCC specimens; and further to investigate the invasion and proliferation-related functions of cortactin using an organotypic co-culture model for OTSCC.

MATERIAL AND METHODS

Patient cohort

The specimens used in the current study are a part of the Norwegian multi-center study NOROC (Norwegian Oral Cancer). Clinical details of the OTSCC specimens used in the current study have described in previous studies [20]. One hundred and twenty-eight patients with primary OTSCC were diagnosed and treated at Rikshospitalet, Oslo University Hospital, Oslo, Haukeland University Hospital, Bergen, and The University Hospital of North Norway, Tromsø in Norway between January 1, 2005 and December 31, 2009. All patients were followed up for at least 60 months.

Clinical data, such as age, gender, date of diagnosis, tumor size, lymph node involvement, distant metastasis, histological grade, recurrence, and date of death and cause were documented. All tumors were HPV negative [8]. The study was approved by the Northern Norwegian Regional Committee for Medical Research Ethics (REK Nord, ref: 2015/1381).

The cases were classified using the current TNM edition (TNM 5th Edition UICC) [21] at the time of diagnosis. The lymphocytic infiltration was scored as described in Brandwein-Gensler [22].

Immunohistochemistry (IHC)

Tissue microarrays were constructed as described previously [8]. More than one core was available for both the center and invading front areas (invading cores) for 75 OTSCC cases from Oslo. Five primary OTSCCs lacking corresponding center and invading cores were excluded from the analysis.

Immunohistochemical procedures and evaluations were carried out at the Department of Oral Biology, University of Oslo. Four-micron thick paraffin sections of the tissue microarray blocks were dried for 2 h at 60˚C, deparaffinized in two changes of xylene for 5 min each and rehydrated in two changes of absolute ethanol, 96% and 70% ethanol for 3 min each. Heat-induced antigen retrieval was performed by placing the slides in a Hellendahl jar filled with citrate buffer (pH 6, S2369; Dako) using a pressure cooker

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(decloaking chamber; Biocare Medical) at 100˚C for 15 min and 90˚C for 10 s. Sections were allowed to cool down for 10 min on ice, rinsed with tap-water, equilibrated in Tris-buffered saline and blocked with 10% normal goat serum (X907; Dako) in 3% bovine serum albumin in Tris-buffered saline + 0.1% Tween (TBST). Sections were incubated with rabbit polyclonal anti-cortactin antibody (HPA057242; Atlas antibodies) at 1:75 dilutions for 1 h at room temperature, followed by incubation with peroxidase labeled polymeric Dako EnVision+ System. Sections were visualized using 3′ diaminobenzidine (DAB) solution chromogen for 8 min and counterstained with Mayer's hematoxylin (Dako). Oral squamous cell carcinoma sections were used as positive control tissue, and striated muscles served as an internal positive control. TBST instead of the primary antibody was used as a negative control for the secondary antibody. Rabbit Ig (X0903; Dako) was used as an isotype-matched control at a similar concentration as the primary antibody.

Organotypic co-cultures ($n = 12$) were stained with mouse monoclonal anti-Ki67 (clone MIB1; Dako) and rabbit polyclonal anti-cortactin antibody (HPA057242; Atlas antibodies), both at 1/200 dilution, overnight at 4˚C following the same procedure as described above.

Immunohistochemical evaluation

All slides were scanned at 40× using a Panoramic MIDI scanner (3DHISTECH) and analyzed using the QUPATH SOFTWARE VERSION 0.2.0-M826. Tumor cells with red-brown cytoplasmic staining were considered positive. Interpretation of the immunostaining was calibrated between the observers (BT and DS) before final blinded evaluation (BT). To score, we used a combination of staining intensity and the percentage of positive tumor cells, as described previously [16]. The cytoplasmic staining intensity was scored on a four-point scale: 1 (no stain), 2 (weak stain), 3 (intermediate stain), and 4 (strong stain). The percentage of positive tumor cells was likewise scored on a four-point scale: 1 (none), 2 (<25%), 3 (25%–50%), and 4 (>50%) [15]. The final score was calculated by multiplying the staining intensity scores and percentage of positive scores, which ranged from 1 to 16 for each tissue microarray core (Figure 1A–H). A score of 8 (median score) was used as a cut-off value to stratify OTSCC cases int the low- (0–8 scores) and high-expression (9–16 score) groups [16, 23, 24]. The invading front and superficial areas were evaluated separately, and the average score of these cores gave us the overall cortactin expression for each case [25].

For the organotypic co-cultures, a proliferation index (PI) was calculated to reflect the proportion of Ki67-positive nuclei of all tumor cells [26].

Cell culture

The tongue squamous cell carcinoma-derived, HPV-negative cell-line SCC25 [27–29] was cultured as described previously [26]. SCC25 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12 Ham 1:1 medium, supplemented with 10% v/v fetal bovine serum, 10 ng/mL epidermal growth factor, 0.4 µg/mL hydrocortisone, 0.5× insulin-transferrin-Selenium-G (Gibco), 50 µg/mL ascorbic acid and 4 mM L-glutamine.

Primary cell cultures were established from oral mucosal tissue obtained after third molar extractions as described recently [30]. In brief, the epithelial sheet and the connective tissue were separated by tissue digestion, then cut into small pieces prior to cell isolation through enzymatic treatment. Fibroblasts were cultured in DMEM supplemented with 10% v/v fetal bovine serum and 4 mM l-glutamine.

All mono-cultures were routinely passaged by trypsinization at approximately 80% confluence.

Tissue engineering of organotypic co-cultures

The organotypic co-cultures were obtained by growing SCC25 on top of a collagen biomatrix supplemented with fibroblasts, as described previously [31]. In brief, 12 matrices containing collagen type I (Corning), Dulbecco's Modified Eagle's Medium, 10% v/v fetal bovine serum and 250,000 primary fibroblasts/matrix were prepared in 24-well plates. The next day, approximately 400,000 SCC25 cells were seeded on top of each matrix, of which six matrices received SCC25 cells silenced for *CTTN*. After 24 h of co-culture, the matrices were transferred to a pre-wetted curved metal grid covered by a piece of lens paper, placed in 6-well plates with OT medium. This medium is identical to the medium used for the SCC25 cell line, with the exception of fetal bovine serum which was replaced with albumin (0.1% v/v). The fluid level was adjusted and monitored daily, keeping the matrix into the air–liquid interface, while half of the volume was replaced with fresh medium every second day. After 7 days on grids, the organotypic cultures were cut in half and one half was fixed in 10% neutral buffered formalin for 2 h at room temperature.

Both mono- and co-cultures were grown in culture medium containing antibiotic-antimycotic solution, in a humidified atmosphere of 5% CO_2 in air at 37˚C. Non-specified cell culture media and solutions were obtained from Sigma (Merck).

siRNA-mediated silencing of *CTTN* gene

siRNAs targeting *CTTN* (with sequences: 5′-GGAUGGAUAAGAAUGCGUCAACCTT −3′, 3′-AGCCUACCUAUUCUUACGCAGUUGGAA-5′) were designed and custom made

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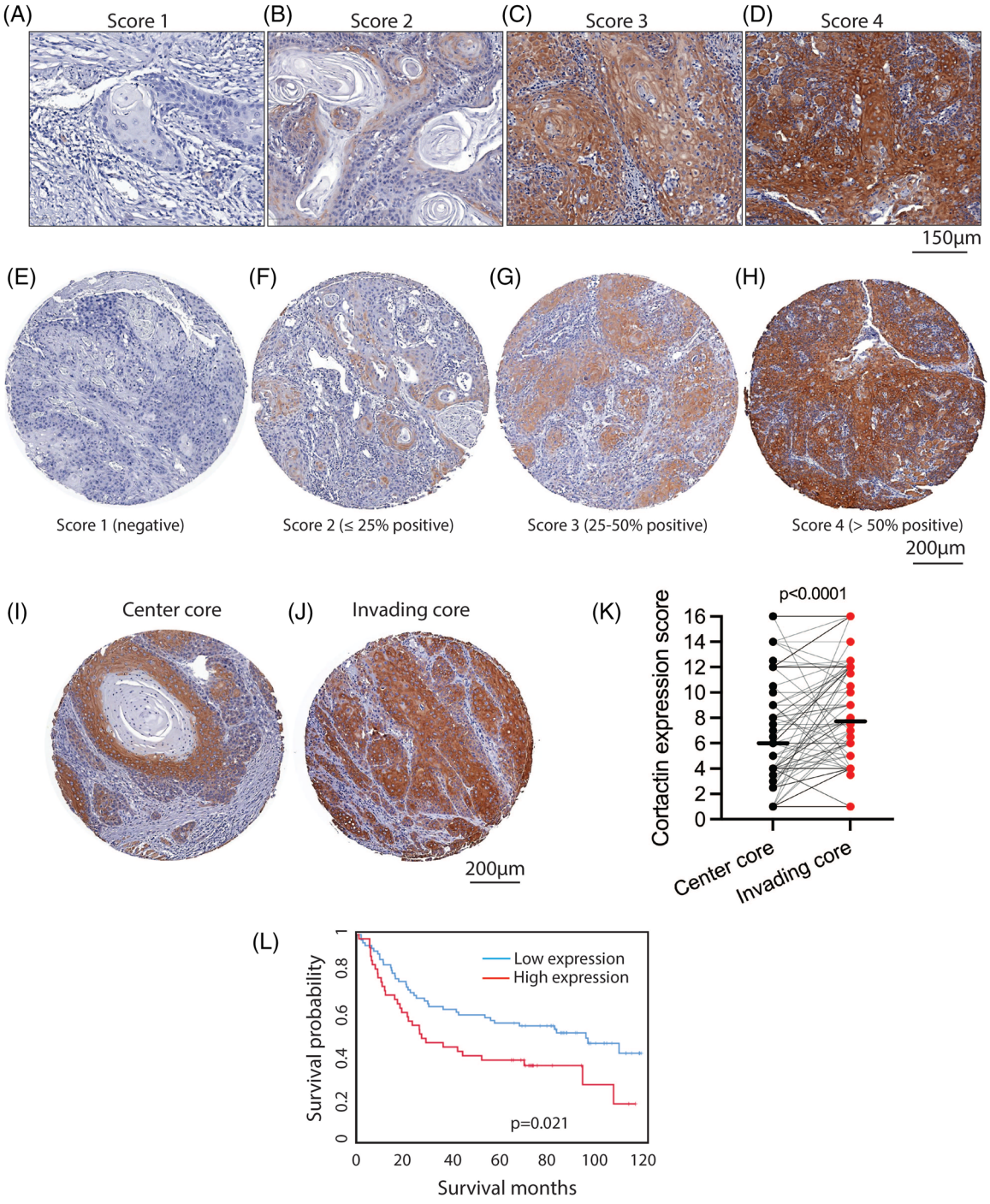


FIGURE 1 Representative images showing variable staining intensity (from score 1 to 4, A–D) and the percentage of positive cells (from negative to >50%, E–H). Representative images showing immunohistochemical expression of cortactin at the center (I) and corresponding invading core (J). Images were exported from QuPath software, scale bar = 200 µm. Quantification of immunostaining showed significantly higher positive cells at the invading core ($p < 0.0001$) as compared to the corresponding center cores. Horizontal bars represent mean (±SEM) expression of cortactin. Paired *t*-test was used in K (K). Kaplan-Meier curves showing reduced overall 5-year survival for patients with overexpression of cortactin (Log rank test, $p = 0.021$) (L).

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by IDT (Integrated DNA Technologies), and resuspended at a concentration of 10 μM. SCC25 cells were transfected with 10 nM of non-targeting or *CTTN* siRNA, utilizing 4 μL of INTERFERin (Polyplus) transfection reagent. Optimal concentration of siRNA and type of transfection reagent was determined experimentally. After 2 days, cells were used for organotypic co-culture and the silencing effect on protein level was analyzed by Western blot.

Western blot

Whole-cell lysates were prepared by cell lysis in PBS containing 0.5% NP40 and protease inhibitors (Pierce). Lysates were denatured by heating at 95˚C for 5 min before 30 μg total protein was loaded onto a 4%–20% gradient gel (Criterion TGX; Bio-Rad) and run at 120V for 1.5 h. Protein was transferred to a PVDF membrane by semi-dry transfer. The blots were blocked with 5% bovine serum albumin prior overnight incubation with antibody for cortactin (Atlas Antibodies, HPA057242), and β-Actin (sc-47,778; Santa Cruz Biotechnology) was used as loading control. Bound antibody was amplified with horseradish peroxidase conjugated secondary antibody followed by Clarity Western ECL substrate (Bio-Rad). Signals were visualized on an iBright FL1500 system (Thermo) and images were analyzed with FIJI SOFTWARE (IMAGEJ, https://imagej.nih.gov/ij/).

Analysis of tumor cell invasion and epithelial thickness in organotypic co-cultures

The formalin fixed co-cultures were dehydrated through graded ethanol, cleared in xylene and embedded in paraffin. Four μm thick sections were dried, deparaffinized and hydrated as describe above, and standard hematoxylin-eosin staining was performed. Stained slides were scanned at 40× and analyzed with QUPATH SOFTWARE as described earlier.

For analysis, the sagittal section of each organotypic co-culture was divided into five equal parts, of which non-uniform thick or tapered ends were excluded. A 200 μm^2 grid was used to standardize the assessment of cell invasion and tumor budding [31, 32].

For cell invasion, an invasion index was calculated using the measurements depicted in Figure S1A. First, a horizontal line along the non-invading cells was drawn, following the imaginary basal membrane. Then, epithelial thickness was measured from the deepest point of invading tumor cells to the epithelial surface, as well as to the imaginary basal membrane. The invasion index was calculated as the ratio of invasion thickness/total thickness. Budding was defined as one single cell, or small clusters of less than five invading cells at the invasive front. The budding score was calculated as the number of buddings per 200 μm^2 [33].

Using a 200 μm × 200 μm grid on digital images (400× magnification) of organotypic co-cultures, 200 μm long horizontal line was drawn across the region of interest in the epithelial layer and a polygon was drown following the epithelial cells in the imaginary basal layer and the most superficial layer (excluding the surface keratin) around the drawn line (Figure 2H,I). The average thickness of the epithelium was calculated by dividing the surface area of the drawn polygon by 200 μm (length of the region of interest). At least four regions of interest were measured for each organotypic co-culture.

Statistical analysis

Statistical analyses were carried out using SPSS STATISTICS VER. 26.0 (IBM). A p-value of <0.05 was considered to indicate statistical significance. Paired student's t-test was used to examine differences in the expression of cortactin between the invading and the corresponding superficial cores. Unpaired student's t-test was used to examine differences in the number of buddings and epithelial thickness between the *CTTN* knock-down and control organotypic co-culture models. Pearson Chi-Square test was used to assess the association between cortactin scores and clinical/histological covariates. Kaplan-Meier curves and long-rank test were used for survival distributions. Multivariate Cox regression analysis was used to investigate the effect of clinicopathological covariates on patient survival.

RESULTS

The clinicopathological variables and cortactin expression in 123 primary OTSCC are summarized in Table 1. The gender distribution of the OTSCC were found to be 74 (60.2%) males and 49 (39.8%) females. At the time of diagnosis, the median age of the patient cohort was 65 years (range: 25−90 years). Patients were categorized into low- and high-age groups based on the median age.

Of 123 cases expressing cortactin, 75 (61%) cases showed low expression and 48 (39%) cases showed high expression (Table S1).

Cortactin expression at the invading front- and corresponding center-cores in OTSCC

The tumor cells as well as striated muscle cells and endothelial cells of the stroma predominantly showed cytoplasmic

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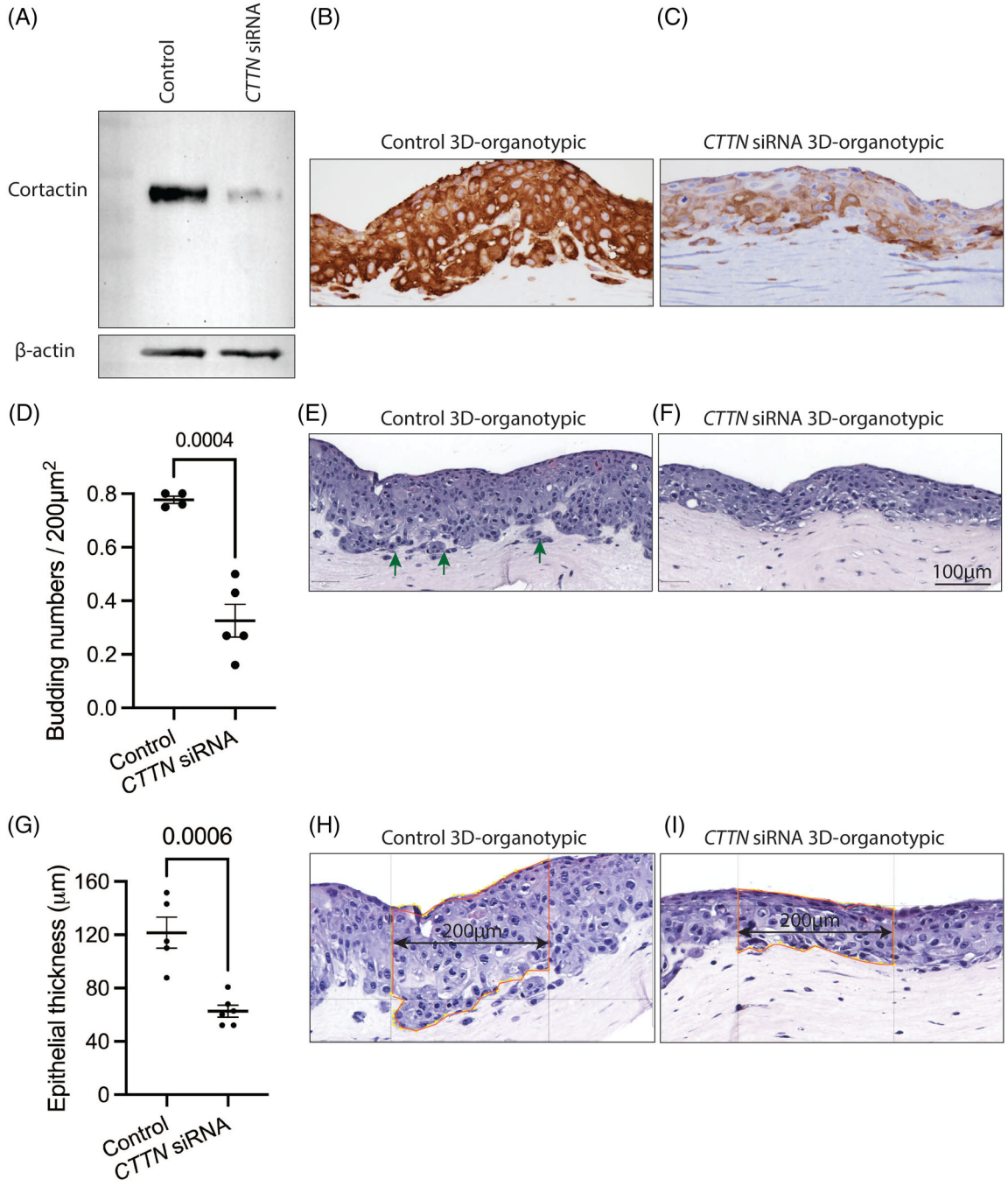


FIGURE 2 SCC25 cell lines were treated with siRNA targeting CCTN and the cell lysate was subjected to Western blot analysis. Western blot showed a significantly decreased cortactin protein level in SCC25 cells treated with siRNA targeting CCTN. β-actin was used as a loading control (A). Representative images for immunohistochemical expression of cortactin in control (B) and CTTN knock-down organotypic co-cultures (C). The number of buddings was found to be significantly lower in CTTN knocked-down organotypic co-culture models as compared to the control co-culture models ($p = 0.0004$). The student's t-test was performed for statistical analysis. Horizontal bars represent the mean (±SEM) of buddings (D). Representative images of H&E stained sections demonstrate higher numbers of tumor buddings in control (E) as compared to the CTTN knock-down organotypic co-culture models (F). The thickness of epithelium was found to be significantly reduced in CTTN knock-down organotypic co-culture models as compared to the control co-culture models ($p = 0.0006$). The student's t-test was performed for statistical analysis. Horizontal bars represent the mean (±SEM) of epithelium thickness (G). Representative images of H&E stained sections demonstrate larger epithelial thickness in control (H) as compared to the CTTN knock-down organotypic co-culture models (I).

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TABLE 1 Association between overall cortactin expression and clinicopathological variables of OTSCC patients.

Variables	Overall cortactin score		
	Low, *n* (%)	High, *n* (%)	*P*
Age (years)			0.185
≤65	42 (56)	21 (43.8)	
>65	33 (44)	27 (56.3)	
Gender			0.277
Female	27 (36)	22(54.2)	
Male	48 (64)	26 (45.8)	
Differentiation			0.305
Well	16 (22.5)	7 (14.9)	
Moderate and poor	55 (77.5)	40 (85.1)	
Lymph node involvement			0.711
Negative (N0)	51 (69.9)	32 (12.3)	
Positive (N1 & N2)	22 (30.1)	16 (33.3)	
Tumor size			**0.005**
Small (T1 & T2)	64 (87.7)	32 (12.3)	
Advanced (large) (T3 & T4)	9 (66.7)	16 (33.3)	
Stage			0.144
Early (I & II)	46 (61.3)	23 (47.9)	
Late (III & IV)	29 (38.7)	25 (52.1)	
Recurrence			0.966
Yes	18 (25.4)	11 (75)	
No	53 (74.6)	33 (25)	
Distant metastasis			**0.048**
Yes (M1)	8 (12.1)	11 (26.8)	
No (M0)	58 (87.9)	30 (73.2)	
Inflammation			0.415
Low	47 (65.3)	26 (57.8)	
High	25 (36)	19 (42.2)	

OTSCC cases were stratified into high and low expression groups using overall expression score of 8 as a cut off value.

cortactin expression. There was no expression in inflammatory cells or normal/paratumoral keratinocytes (Figure S1B).

Expression levels of cortactin at the invading front cores were significantly upregulated as compared to that of the corresponding central cores ($p < 0.001$) (Figure 1I–K).

Association between overall cortactin expression, tumor size and distant metastases in OTSCC

Out of 123 cases, 25 (20.4%) had tumors larger than 4 cm. The Chi-square test showed that high overall expression of cortactin ($n = 48$) was significantly associated with advanced (larger) tumor size in OTSCC patients ($p = 0.005$) (Chi-square test). 19 (17.8%) were found to develop distant metastasis in the follow-up period. We found a significant association between high overall cortactin expression and distant metastasis ($p = 0.048$) (Table 1).

Association between high overall cortactin expression and 5-year overall survival in OTSCC

In total, 61 patients died within the 5-year follow-up period. Kaplan-Meier survival analysis showed that patients with high overall cortactin expression had lower 5-year overall survival probabilities (Log-rank test, $p = 0.037$) (Figure 1L). Similarly, higher cortactin expression at the tumor center was significantly associated with poor 5-year overall survival ($p = 0.021$). A high cortactin expression at the invading front was not statistically significantly associated with patient survival ($p = 0.243$), although a trend was seen. A higher age, poor tumor differentiation, larger tumor sizes, higher stage, lymph node involvement, presence of distant metastasis, and local recurrence were all associated with poor 5-year overall survival.

Multivariate Cox regression analysis using variables in Table 2 showed that a high overall cortactin expression was an independent prognostic factor for 5-year overall survival of OTSCC (HR = 1.77, 95.0% CI = 1.0–3.0, $p = 0.034$) (Table 2).

Influence of siRNA-mediated knockdown of cortactin on epithelial thickness and invasive abilities of SCC-25 cells in organotypic co-cultures

To investigate the role of cortactin in proliferation and invasion, knockdown of *CTTN* was performed in the SCC-25 cell line. siRNA targeting *CCTN* resulted in significantly decreased cortactin protein level (up to 80% reduction), as confirmed by Western blot analysis (Figure 2A). Immunostaining of organotypic co-culture showed cortactin was downregulated in *CTTN* siRNA group as compared to the control organotypic co-cultures (Figure 2B,C).

Tumor invasion was investigated by evaluating the tumor buddings and invasion index. The number of tumor buddings were lower in the *CTTN* knock-down group ($p = 0.0004$) than in the controls (Figure 2D–F). A similar trend was observed with respect to the invasion index, although the difference was not statistically significant ($p = 0.097$) (Figure S1C).

The effect of cortactin knockdown on cell proliferation was evaluated by morphometric analysis of the thickness of epithelium and by counting the Ki67 stained SCC-25 cells in organotypic co-cultures. The epithelial thickness in

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TABLE 2 Univariate and multivariate cox regression analyses for overall cortactin expression and other clinicopathological covariates for predicting the overall survival of OTSCC cases.

	Univariate cox			Multivariate cox		
Variables	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Cortactin expression[a]	1.75	1.0–2.8	**0.021**	1.77	1.0–3.0	**0.034**
Age[b]	2.22	1.3–3.6	**0.001**	2.98	1.7–5.0	**0.000**
Differentiation[c]	1.58	0.8–3.1	0.182	0.95	0.4–1.9	0.090
Tumor size[d]	3.11	1.8–5.2	**0.000**	1.50	0.7–3.0	0.259
Node status[e]	2.86	1.7–4.6	**0.000**	3.24	1.3–7.6	**0.007**
Stage[f]	3.62	2.1–5.9	**0.000**	1.30	0.4–3.4	0.593

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

[a]OTSCCs were stratified into high and low cortactin expression groups by using the cut-off value of eight.

[b]Patients were categorized into low- and high-age groups based on the median age (65).

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

[d]OTSCC were categorized into smaller (T1 & T2) and advanced (larger) (T3 & T4) tumors using clinical tumor size.

[e]OTSCC were categorized into node positive (N1&N2) and negative (N0) using clinical node status.

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

CTTN knock-down organotypic co-cultures was significantly reduced as compared to the control organotypic co-cultures ($p = 0.0006$) (Figure 2G–I). In line with this observation, the PI index was moderately but non-significantly ($p = 0.201$) reduced in organotypic co-cultures with *CTTN* knock-down (52.3%) as compared to the mock siRNA organotypic co-cultures (58.3%) (Figure S1D).

DISCUSSION

Overexpression of cortactin has been linked to increased invasive potential and poor prognosis in several cancers including head and neck squamous cell carcinomas [1, 2]. Although it has been suggested that cortactin expression is highly enhanced in the most invading tumor cells as compared to the other parts of OSCC [15], the possible intra-tumor heterogeneity of cortactin expression in OTSCC is not fully understood. In the current study, cortactin expression was found to be significantly upregulated in tumor cells at the invading front as compared to the cells at the tumor center. This is in line with the reported invasion promoting function of cortactin in OSCC [15, 16].

Several observations in the current study indicated a link between cortactin and its invasion promoting function. Firstly, the expression of cortactin was found to be significantly higher in the tissue core from the invasive front as compared to the corresponding center core. Secondly, increased overall expression of cortactin was found to be associated with higher incidence of distant metastasis in the clinical samples. Both of these findings were further confirmed by analyzing the invasive ability of OSCC in organotypic co-cultures. Here, siRNA-mediated down-regulation of *CTTN* led to significant reduction of tumor buddings as compared to the control model without *CTTN* knock-down. This indicates that the expression of cortactin might be related to OTSCC invasion. These observations are in line with previous studies where cortactin expression was associated with increased invasive ability of OSCC cells and node status [15, 34, 35]. For example, siRNA-mediated knock-down of *CTTN* was associated with reduced invasion ability of OSCC cells with concomitant modulation of epithelial and decrease in mesenchymal phenotype markers [15, 34].

A high overall OTSCC cortactin expression was associated with a reduced 5-year overall survival. Further, a multivariate Cox-regression analysis identified high overall cortactin expression to be an independent prognostic factor in OTSCC. These results suggest that the association between overall cortactin expression with patient survival was not influenced by clinical variables such as tumor size, tumor stage and node status. Moreover, the prognostic potential of overall cortactin expression was less likely to be influenced by the choice of treatment methods (surgery alone, surgery + post-operative radiotherapy or surgery + post-operative radiotherapy + chemotherapy), as the type of treatment did not have a significant association with patient survival in the current study (data not shown).

This is in line with previous findings both in oral and head and neck squamous cell carcinomas [16, 35-37]. Although investigation of the predictive value of cortactin expression was not the focus of the current study, low expression of cortactin in combination with a <2 cm tumor size and a <4 mm tumor infiltration depth has been suggested to have a predictive value to select patients for a watchful waiting strategy instead of subjecting patients to a sentinel lymph node biopsy [38]. Besides, a high combined expression of cortactin and focal adhesion kinase has been suggested to have predictive value in identifying oral epithelial dysplasia that have higher risk for malignant transformation [17].

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While analyzing the expression of cortactin with respect to intra-tumor location, high expression of cortactin only in the center and not in the invading front cores was associated with a significantly poor 5-year survival. This might imply that besides the invasion promoting function in tumor cells at the invasive front, other possible tumor promoting functions of cortactin, such as increased cell proliferation in the tumor center might be important for cortactin-mediated tumor progression and poor prognosis.

Besides having a role in invasion and metastasis, cortactin has been linked to an increased proliferation of cancer cells, including OSCC [39–41]. In the present study, a high expression of cortactin was found to be significantly associated with an advanced (larger) tumor size. Such a positive association between cortactin expression and tumor size was recently demonstrated in head and neck squamous cell carcinomas [13]. In line with these observations, siRNA-mediated *CTTN* knock-down led to significant reduction in epithelial thickness in the present organotypic co-culture as compared to the control co-cultures. In support of these results, the number of Ki67-positive SCC25 cells were reduced, although not significantly, in *CTTN* knock-down organotypic co-cultures as compared to the control samples. These results indicate a functional link between cortactin and OSCC cell proliferation. Although not examined in the current study, the ability of cortactin to promote proliferation has been previously linked to the inhibition of cyclin-dependent kinase inhibitors such as p21, p27, and p57 in head and neck squamous cell carcinoma cells [40]. Besides the proliferation activity, the epithelial thickness also depends on the dynamic interplay between the differentiation status and apoptotic activity of keratinocytes [42]. The association between cortactin expression and tumor differentiation is not fully understood as previous studies have indicated both positive and negative associations between cortactin and tumor differentiation [35, 43]. In the current study, no significant association was observed between the overall cortactin expression and tumor differentiation. Besides differentiation, cortactin expression has also been functionally linked with apoptosis of gastric epithelial cells [44]. However, a role of tumor cell apoptosis and differentiation in the cortactin knock-down mediated reduction of epithelial thickness in the present study cannot be completely ruled out. The potential association between cortactin and cell differentiation and apoptosis warrants further investigation.

There is a considerable molecular and prognostic heterogeneity among OSCC with respect to anatomical sub-location [7, 8]. One of the strengths of the current study is the utilization of only HPV-negative tumors. Furthermore, we have used multi-center specimens representative of the Norwegian population and a relatively large cohort of OSCC from one specific oral site, the anterior part of the tongue. Moreover, the association between the cortactin expression and tumor size/cell invasion in the clinical specimens has been substantiated using organotypic co-cultures. Considering the intra-tumor heterogeneity of tumor cells, the use of tissue cores as compared to the use of whole tissue section can be considered as a limitation of the current study. However, inclusion of both central and invading front cores, with four replicate cores available for 75 of the cases, is expected to minimize the influence of intratumor heterogeneity. Indeed, a high degree of concordance has been shown between the use of whole section and duplicate tissue cores [45–47].

In conclusion, high cortactin expression is associated with clinical features of an aggressive tumor phenotype such as advanced (larger) tumor size and distant metastasis, and represents a potential prognostic marker in OTSCC.

AUTHOR CONTRIBUTIONS

Conceptualization: Burcu Toközlü, Dipak Sapkota, Tine Søland. **Methodology**: Burcu Toközlü, Dipak Sapkota, Tine Søland. **Investigation**: Burcu Toközlü, Dipak Sapkota, Olaf Schreurs, Evan Vallenari. **Formal analysis**: Burcu Toközlü, Dipak Sapkota, Tine Søland. **Writing — original draft preparation**: Burcu Toközlü, Dipak Sapkota. **Writing — review and editing**: Burcu Toközlü, Dipak Sapkota, Tine Søland, Olaf Schreurs, Evan Vallenari.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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