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The prognostic role of combining Krüppel-like factor 4 score and grade of inflammation in a Norwegian cohort of oral tongue squamous cell carcinomas

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Abstract

Krüppel-like factor 4 (KLF4) is a zinc-finger transcription factor involved in inflammation, cancer development, and progression. However, the relationship between KLF4, inflammation, and prognosis in oral cancer is not fully understood. KLF4 expression levels were examined in a multicenter cohort of 128 oral squamous cell carcinoma (OSCC) specimens from the tongue (OTSCC) using immunohistochemistry. In two external *KLF4* mRNA datasets (The Cancer Genome Atlas/The Genotype-Tissue Expression Portal), lower *KLF4* mRNA expression was found in OSCC and head and neck squamous cell carcinomas (HNSCC) than in control oral epithelium. These data indicate that down-regulation of *KLF4* mRNA is linked to OSCC/HNSCC progression. Using Cox-multivariate analysis, a significantly favorable 5-year disease-specific survival rate was observed for a subgroup of patients with a combination of high levels of KLF4 expression and inflammation. OSCC cell lines exposed to IFN-γ showed a significant upregulation of nuclear KLF4 expression, indicating a link between inflammation and KLF4 expression in OSCC. Overall, the current data suggest a functional link between KLF4 and inflammation. The combination of high KLF4 nuclear expression and marked/moderate stromal inflammation might be useful as a favorable prognostic marker for a subgroup of OTSCC patients.

KEYWORDS
cancer of the tongue, KLF4, survival, stromal cells

INTRODUCTION

Despite the tremendous efforts towards improvements in diagnosis and management, the survival of oral squamous cell carcinoma (OSCC) patients has not improved significantly [1]. An average 5-year overall survival rate of about 64% underscores the need for a better understanding of OSCC pathogenesis and biology in order to identify molecular markers for prognostic and therapeutic applications [2]. Currently, the TNM-system/clinical staging is the most widely used tool for OSCC prognostication and treatment planning [3].

Krüppel-like factor 4 (KLF4) is a zinc-finger transcription factor that is mainly localized in the nucleus, although cytoplasmic localization has also been reported [4,5]. However, the function of cytoplasmic KLF4 is unclear. KLF4 is highly expressed in differentiated cells and tissues and is involved in normal physiological processes such as cell cycle regulation, differentiation, and migration [6–10]. KLF4 is also involved in diseases such as inflammation and cancer, and has been suggested to both stimulate and suppress the inflammatory process [11,12]. In addition, the expression of KLF4 has been demonstrated to be regulated by inflammatory cytokines [13,14]. Compared with KLF4 expression in normal tissues, an altered expression pattern is reported in malignant tumors of several tissue origins [15–18]. Functionally, both oncogenic and tumor suppressor roles have been described for KLF4 [19–21].

Squamous cell carcinoma of the tongue (OTSCC) is one of the most common malignancies of the oral cavity, representing approximately 50% of OSCC [2,22]. A limited number of studies have been published on the expression and prognostic roles of KLF4 in OSCC. Most of these are from Asian populations [23–27], and include OSCC from different oral sites. Li et al. showed a significantly decreased expression of KLF4 in OSCC as compared with normal oral mucosa [25]. High intratumor KLF4 expression was found in non-recurrent OSCCs and a loss of KLF4 has been reported in poorly differentiated OSCC [23,25]. The low KLF4 expression in poorly differentiated OSCC, generally regarded to have a poor prognostic outcome, is in contrast to the findings from Yoshihama et al. [24], where high KLF4 expression was associated with reduced disease-free survival [24]. In the same study, there was only a trend seen for high KLF4 and reduced disease-specific survival. Considering possible differences regarding etiological factors in the development of OSCC in Asia versus Europe, studies of KLF4 in Western European OSCC populations are of interest [28,29]. To the best of our knowledge, no such study has been reported. Furthermore, investigation of the possible association between KLF4 expression in OTSCC and the grade of stromal lymphocytic infiltrate is of interest due to the role of KLF4 in inflammation, and the influence of stromal inflammation on OSCC prognosis [11,30,31].

The current work represents a substudy of a joint initiative (Norwegian Oral Cancer (NOROC) multicenter study) [22]. Here, 128 primary OTSCC cases diagnosed in Norway from 2005 until 2010 are included. Due to the suggestion of molecular heterogeneity in OSCC in different oral anatomic sub-locations, and OTSCC being the most common malignancy of the oral cavity, only OTSCC are included [32,33]. The aim of the study was to evaluate whether KLF4 tumor expression, alone or in combination with histopathological parameters such as tumor differentiation and inflammation, are independent markers of disease-specific survival in Norwegian OTSCC specimens.

MATERIAL AND METHODS

External transcriptomic data

Values of mRNA expression for *KLF4* from 519 head and neck squamous cell carcinomas (HNSCC) and 44 controls (paratumor epithelium) retrieved from The Cancer Genome Atlas/The Genotype-Tissue Expression Portal (TCGA/GTEx) datasets [43] were plotted using Gene Expression Profiling Interactive Analysis software (GEPIA) [44]. In addition, *KLF4* mRNA expression levels in OSCC ($n = 167$) and normal oral epithelium ($n = 45$) were examined in a microarray dataset [45]. Expression data from the microarray dataset were imported to GraphPad Prism (https://www.graphpad.com/) software and used for the statistical analysis.

Clinical material of oral tongue squamous cell carcinoma

The study was approved by the Northern Norwegian Regional Committee for Medical Research Ethics (REK Nord; 2013/1786 and 2015/1381). In this Norwegian multicenter study, all OSCC cases diagnosed between 1 January 1 2005 through 31 December 31 2009 were retrospectively identified. Using ICD-10 codes (C02-C06) except for codes C05.1 and C05.2 (oropharyngeal sites, and cancer of the external upper or lower lip/vermilion), 128 primary HPV-negative OTSCC were included in the present study (for details of the exclusion criteria, see fig. 1 from Søland et al. [34]). A general description of the OSCC-material is found in Bjerkli et al. [22]. Relevant patient data, ICD-10 diagnosis, TNM classification, treatment received, and minimum of 5-year follow-up (last follow-up date 1 June 2015) were registered in a web-based Case Report Form (CRF). The patients were classified according to the TNM 5th Edition 2005 UICC, the TNM edition at the time of diagnosis [35].

Tissue microarray generation

Tissue microarrays were constructed from formalin-fixed, paraffin-embedded tissue blocks in a fully automated tissue microarray machine (TMA Master II; 3DHISTECH). Two to four tissue cores (both from the invasive front and from more superficial parts of the tumors) with a diameter of 2 mm were arrayed on the tumor paraffin blocks. The stained tissue microarray sections were scanned (Pannoramic MIDI scanner; Thermo Fisher Scientific) and evaluated using the Case-Viewer software (3dhistech.com). For scanned images with inadequate focus, the original glass slides were examined on a Leitz Aristoplan microscope.

Scoring of histopathological parameters

Tumor budding, pattern of invasion, and degree of lymphocytic infiltrate were scored on hematoxylin-eosin-stained whole sections by calibrated and experienced pathologists either independently or in pairs. All seven pathologists were blinded for the patients' clinical outcomes. The scoring of the tumor budding was based on the grading systems as described in Bjerkli et al. [36–40] (Supporting information SF1). The worst pattern of invasion was graded into five categories (Supporting information SF2). The lymphocyte infiltration was scored as 1 for marked/continuous band of lymphocytes, 2 for moderate/large patches of lymphocytes, and 3 for little or no infiltration of lymphocytes, according to Brandwein-Gensler [31].

KLF4 immunohistochemistry of oral tongue squamous cell carcinoma

Immunohistochemistry was performed as described recently [41] (Supporting information SF3). Four micron thick sections of formalin fixed paraffin embedded multi tissue arrays were deparaffinized, hydrated, and quenched in 0.3% hydrogen peroxide in methanol for 30 min prior to heat-induced epitope retrieval in Tris-EDTA buffer at pH 9. Sections were blocked with 5% goat serum and incubated overnight at 4°C with rabbit anti-KLF4 at 167 ng/ml (RRID AB_1852541; Atlas Antibodies), while rabbit Ig (Dako) served as a negative control. The signal was amplified by biotinylated goat anti-rabbit IgG antibody and horseradish peroxidase conjugated ABC reagent (Vectorlabs) utilizing 3,3'-diaminobenzidine as the substrate followed by 0.5% copper (II) sulfate solution as intensifier. Nuclei were counterstained with hematoxylin and blued in phosphate-buffered saline (PBS). Sera and antibodies used for immunocytochemistry and immunohistochemistry were diluted in PBS containing 1% bovine serum albumin (BSA). Pictures were taken with a Nikon E90i microscope equipped with a DS-Ri1 camera using the NIS-elements software and the final images were composed using Adobe Photoshop CS5.

Blinded for the clinicopathological data, the scanned KLF4-stained tissue microarray sections were scored by three of the authors (TMS, DS, and MBS). Brown staining in the cytoplasm and nuclei of OTSCC cells was scored as KLF4 positive. As an internal positive reference, the KLF4 reactivity was evaluated in normal appearing surface epithelium, when present in the tissue cores. For each OTSCC case, both the central/superficial and the corresponding deep/invading cores (single or duplicate cores for each area) were evaluated. The number of tumor cells with KLF4 immunopositivity (nuclear and cytoplasmic, separately) was semi-quantitatively classified into five groups: group 1, <10%; group 2, 10%–

25%; group 3, 25%–50%; group 4, 50%–75%; and group 5, 75%–100% positive tumor cells. The nuclear score is shown in Table 1.

Oral squamous cell carcinoma cell lines

The oral squamous cell carcinoma-derived cell line CaLH3 [42] was grown in Dulbecco's Modified Eagle Medium (DMEM) with Nutrient mixture F12 1:1 (Gibco) supplemented with 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, 0.5X Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A) (Gibco), 50 μg/ml sodium L-ascorbate, 2 % L-Glutamine and 1x Antibiotic Antimycotic Solution (Sigma). PE/CA-PJ49 clone E10 (ECACC), established from a tongue squamous cell carcinoma in a 57-year-old male patient, was grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1x Antibiotic Antimycotic Solution.

Cell treatments and immunocytochemistry

Oral cancer cells (CaLH3 and PE/CA-PJ49 clone E10) were seeded on coverslips with a density of 3×10^5 cells per well in a 24 well plate one day prior to 24 h exposure to the following cytokines: 10 ng/ml of IL-1β, 50 ng/ml of IL-6, 10 ng/ml of IFN-γ, 50 ng/ml of TNF-α (all from Peprotech), or 0.2 ng/ml TGF-β1 (RnD). Concentrations were determined by earlier experiments by our group (not published). After a quick rinse with PBS, the cells were fixed with 4% paraformaldehyde for 10 min at 37°C, washed and blocked overnight with 1% BSA in PBS at 4°C. The cell membranes were permeabilized in a solution containing 0.1% Triton-X100 and 0.1% sodium citrate for 10 min at 4°C, washed with PBS and blocked with 5% normal goat serum prior to incubation overnight with 0.2 μg/ml rabbit anti-KLF4 antibody at 4°C. After washing, the slides were incubated with biotinylated goat anti-rabbit IgG (RRID AB_2313606; Vectorlabs) followed by Cy2-labeled streptavidin (GE Healthcare). The nuclei were stained with DAPI (ThermoFisher), before the coverslips were washed and mounted with polyvinyl alcohol mounting medium containing 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma-Aldrich). Sera and antibodies used for immunocytochemistry were diluted in PBS containing 1% BSA. Pictures were taken with a Nikon E90i microscope equipped with a DS-Ri1 camera using the NIS-elements software and the final images were composed using Adobe Photoshop CS5.

Quantification of KLF4

For the quantification of KLF4 staining in the CalH3 and E10 cancer cell lines before and after treatment with IFN-γ,

TABLE 1 Number and percent of cases of oral tongue squamous cell carcinomas according to the percentage of tumor cells with nuclear KLF4 staining

Tumor area	N	Percentage of tumor cells with nuclear KLF4 staining				
		<10%	10%–25%	25%–50%	50%–75%	≥75%
KLF4 central/ superficial[*]	117	41 (35%)	13 (11%)	10 (9%)	13 (11%)	40 (34%)
KLF4 deepest[**]	105	45 (43%)	23 (22%)	6 (6%)	8 (8%)	23 (22%)
KLF4 whole tumor[***]	121	73 (60%) Low				48 (40%) High

Because of rounding, percentages may not total 100.

*11, ** 23, and *** 7 cases were not evaluated due to lack of tissue core or appropriate/representative tumor tissue.

pictures of five randomly chosen microscopic fields were taken at 20× for each of the six technical replicates. The images in lossless TIFF format without compression were imported into the ImageJ software (https://imagej.nih.gov/ij/) and both KLF4 positive areas and intensity were quantified. Since the KLF4 staining was almost exclusively limited to the nuclei, the KLF positive area was quantified by dividing the area of KLF4 positive staining by the total area of nuclear staining (DAPI). For KLF4 staining intensity, mean pixel intensity above threshold was used. For both types of KLF4 quantifications, the mean score of the five microscopic fields for each stained coverslip was used for statistical analyses.

Statistical analysis

In the external transcriptomic data, the box plot tool in the GEPIA software was used to compare the expression of *KLF4* mRNA in HNSCC and control oral epithelium. Differences observed between OSCC and controls (normal oral epithelium) were statistically evaluated by two-tailed t-tests for independent samples (Student's t-test). A p-value of <0.05 was considered statistically significant.

The association between KLF4 expression and clinicopathological variables was examined using chi-square tests. Survival analysis was performed using 5-year disease-specific survival as the end-point. Survival analysis was based on Kaplan–Meier plots and log-rank tests for comparison of the five subgroups of OTSCC patients stratified based on the nuclear score of KLF4 (Table 1). Based on the survival plots, subgroups 0–4 were pooled into a KLF4^{low} group while group 5 represented the KLF4^{high} group. Thereby, the cut-off value for the KLF4^{low} tumors was <75%, and 75%–100% for the KLF4^{high} group.

Univariate and multivariate Cox-regression models were used to study the effect of protein expression and other covariates on patient disease-specific survival. Variables from the univariate analysis with a p-value of <0.05 were used for the multivariable Cox regression analysis to investigate their independent contributions. To evaluate the effect of IFN-γ on

the nuclear expression of KLF4 in cell culture, independent-samples Mann–Whitney U tests were run on the mean KLF4 positive fraction of the total nuclear area, as well as on mean pixel intensity values. The analysis was performed using IBM SPSS V28 (IBM). A p-value below 0.05 was considered statistically significant.

RESULTS

KLF4 mRNA levels in OSCC and HNSCC

Analysis of the external mRNA datasets showed a trend for down-regulation of KLF4 mRNA levels in HNSCC as compared with the controls (Figure 1A). Similarly, KLF4 mRNA levels were significantly down-regulated in OSCC as compared with the controls ($p = 0.015$) (Figure 1B).
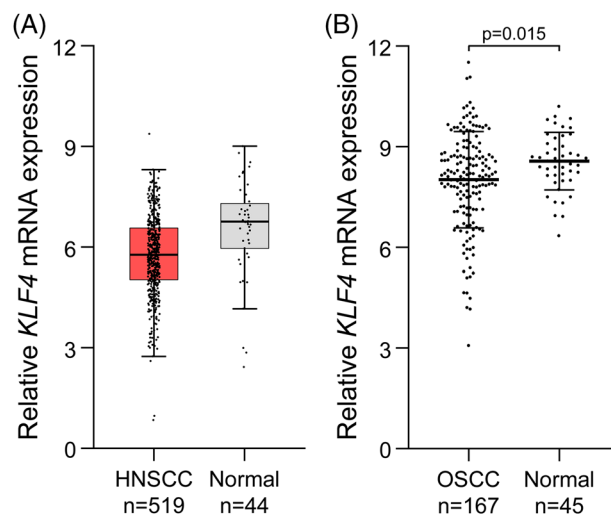

FIGURE 1 *KLF4* mRNA levels in head and neck squamous cell carcinoma (HNSCC) and oral squamous cell carcinoma (OSCC). (A) *KLF4* mRNA levels in HNSCC and paratumor control epithelium. GEPIA box plot analysis tool [44]. (B) *KLF4* mRNA expression in OSCC and normal control epithelium (Student's t-test)

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FIGURE 2 Immunohistochemical staining of oral tongue squamous cell carcinoma (OTSCC). A high (A) and low (B) nuclear KLF4 expression in representative OTSCC. Immunopositivity is seen in brown. Hematoxylin counterstain. Scalebar $= 50\mu$m

KLF4 protein expression in primary OTSCC

KLF4 expression was scored both at the superficial/central cores and at the corresponding invasive tumor front cores. However, the intratumoral location of KLF4 staining was of no significance as indicated by chi-square tests when combined with histological parameters and 5-year disease-specific survival (data not shown). Thus, the following results are based on the most abundant KLF4 score in each OTSCC specimen, irrespective of the intratumor location.

One hundred and twenty-eight primary OTSCC were included in the current study. After excluding missing cores or cores with no tumor cells, 121 OTSCC lesions were examined for the expression of KLF4 (Table 1). Forty percent of the OTSCC demonstrated nuclear KLF4 staining in 75%–100% of the tumor cells (KLF4^{high}), while 60% of the tumors demonstrated nuclear KLF4 score in <75% (KLF4^{low}) of the tumor cells (Figure 2A,B). Cytoplasmic KLF4 expression was absent in the majority of OTSCC ($n = 99$). Of the 22 samples expressing cytoplasmic KLF4, all demonstrated nuclear staining as well. Fifteen tumors expressed cytoplasmic staining in less than 10% of the tumor cells, while the remaining 7 cases expressed cytoplasmic staining in >10% of tumor cells. Due to the low number of OTSCC expressing cytoplasmic KLF4, the current study focused on the nuclear and not the cytoplasmic expression of KLF4 for statistical analyses.

Factors associated with nuclear KLF4 protein in OTSCC

Among the clinical and histopathological parameters (Table S1), there was a significant association between high (75%–100%) nuclear KLF4 expression in tumor cells and age <65 years and a well differentiated phenotype of

OTSCC ($p = 0.043$, $p = 0.035$, respectively) by chi-square test. No significant association was found for nuclear KLF4 expression and gender, T-stage, N-stage, clinical stage, pattern of invasion, or tumor budding. A borderline significant association between high nuclear KLF4 expression and a marked stromal inflammation was demonstrated ($p = 0.052$).

Nuclear KLF4 expression in OTSCC and 5-year disease-specific survival

The evaluation of the numbers of KLF4 positive cells and disease-free survival was performed by use of Kaplan–Meier plots and log-rank tests as described in the Material and Methods section. A trend for favorable 5-year disease-specific survival was seen for patients with high KLF4 expression as compared to those with low expression ($p = 0.064$). In fact, 71% of patients with OTSCC expressing a nuclear KLF4 score of 75%–100% were alive 5 years after diagnosis. In contrast, only 49% of patients with OTSCC expressing nuclear KLF4 in <75% of the tumor cells were alive 5 years after diagnosis (Figure 3A).

Inflammation and differentiation in OTSCC and 5-year disease-specific survival

Cases of OTSCC with a marked/moderate inflammation in the tumor stroma had a significantly better 5-year disease-specific survival as compared to patients with slight or no inflammation ($p = 0.026$) (Figure 3B). The 5-year disease-specific survival rate was 68% in the marked/moderate group versus 45% in the slight/no inflammation group. There was no significant association between OTSCC differentiation and 5-year disease-specific survival ($p = 0.265$).
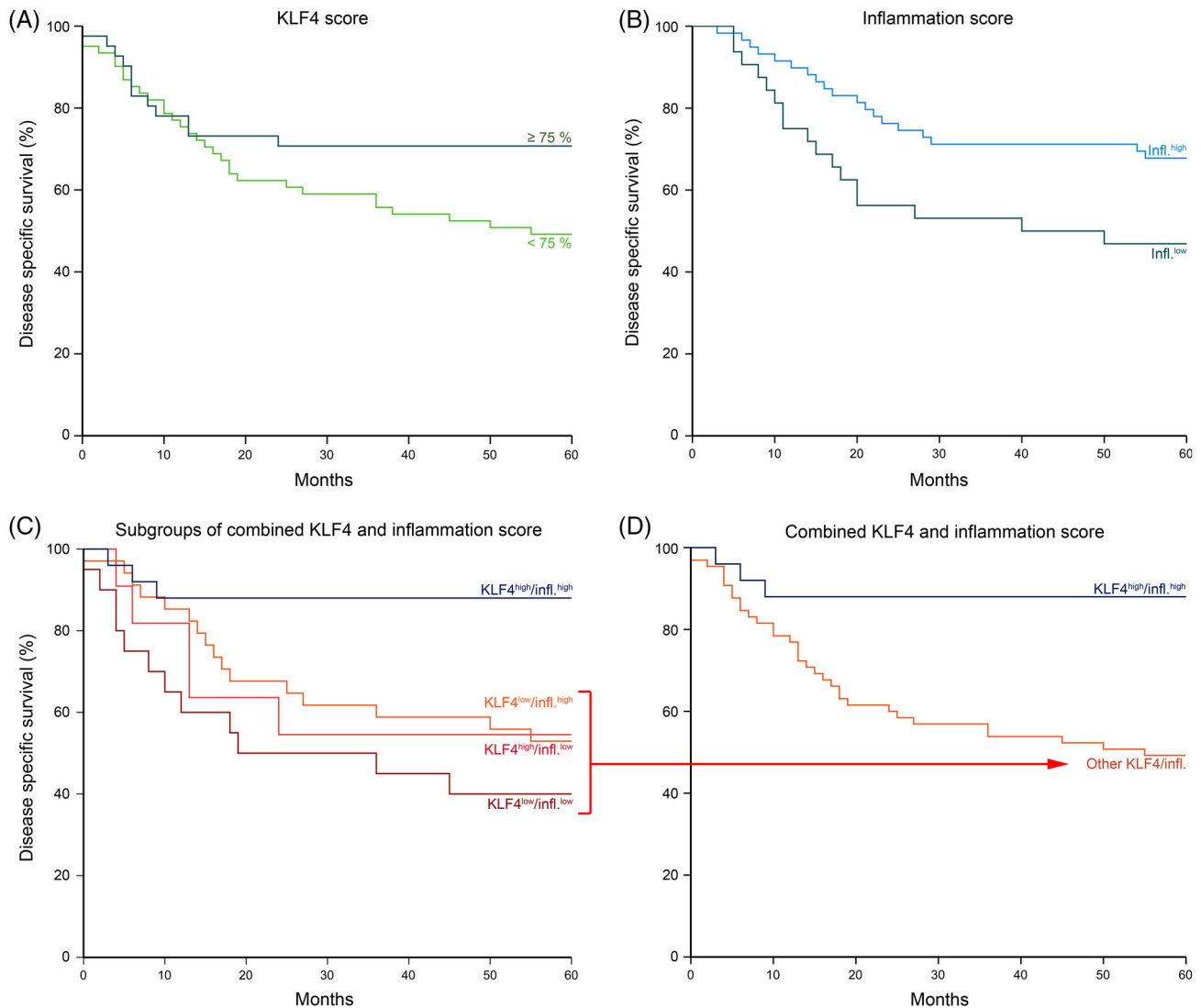
FIGURE 3 Kaplan–Meier plots (log rank test) of 5-year disease-specific survival in oral tongue squamous cell carcinomas with regard to the presence of KLF4 and inflammation. (A) A trend was seen for high KLF4 expression and favorable 5-year disease-specific survival ($p = 0.064$). (B) Patients with marked/moderate inflammation in the tumor stroma had a significantly better disease-specific survival as compared with patients with slight or no inflammation ($p = 0.026$), (all tumors $n = 128$). (C,D) The combination of different KLF4 and inflammation score identified 105 patients of which survival data were available for 91. Here, a subgroup of patients with a high nuclear KLF4 expression (KLF4^{high}) and a marked/moderate stromal inflammation (inflammation[high]) demonstrated a significantly favorable disease-specific survival as compared to patients with other combinations of KLF4/inflammation ($p = 0.006$)

Combination of nuclear KLF4 expression and stromal inflammation as an independent prognostic factor in OTSCC

We next examined the association between 5-year disease-specific survival probability of OTSCC patients and the combination of KLF4 expression and degree of stromal inflammation in the tumor tissue ($n = 105$). Survival data were available for 91 OTSCC cases. In a univariate Cox regression analysis, a subgroup of 25 OTSCC with a significantly favorable 5-year disease-specific survival

($p = 0.006$) was identified (Table 2). In this prognostically favorable group, both high nuclear KLF4 expression and marked/moderate stromal inflammation (KLF[high] + stromal inflammation[high]) were present. The 5-year disease-specific survival was 88% (22 out of 25) in this group versus 49% (33 out of 65) in the rest of the OTSCC patient group with varying combined scores of KLF4 and inflammation (Figure 3C,D). Furthermore, KLF4^{high}/inflammation[high] was found to be an independent prognostic marker ($p = 0.013$) in contrast to clinical stage, T-stage, and N-stage in a Cox multivariate analysis of 5-year disease-specific survival (Table 2).

TABLE 2 Results of univariable (crude HR estimates) and multivariable (adjusted HR estimates) Cox regression analysis of the 5-year disease specific survival among 105 OTSCC cases

Variable	Level	Crude estimates			Adjusted estimates		
		HR	95% CI	*p*-value	HR	95% CI	*p*-value
Gender	Male	1.00	-	-			
	Female	0.85	0.4–1.1	0.654			
Age[a]	<65 years	1.00	-	-			
	≥65 years	1.40	0.7–2.7	0.291			
T-value	T1 and T2	1.00	-	-	1	-	-
	T3 and T4	2.40	1.0–5.6	0.040	2.07	0.7–6.2	0.192
N-value	Node negative	1.00	-	-	1	-	-
	Node positive	2.20	1.1–4.4	0.020	3.55	0.4–29.6	0.240
Clinical stage[b]	Early	1.00	-	-	1	-	-
	Late	2.43	1.3-4.7	0.009	0.44	0.5–4.4	0.490
Differentiation[c]	Well	1.00	-	-			
	Moderate or Poor	1.96	0.6–6.4	0.265			
Combined KLF4/inflammation	KLF4^{high}/inflammation$^{marked-moderate}$	1.00	-	-	1	-	-
	Other KLF4/inflammation	5.18	1.6–16.9	0.006	4.51	1.4–14.9	0.013

Other KLF4/inflammation = KLF^{high} + inflammation^{low}, KLF4^{low} + inflammation$^{marked/moderate}$, and KLF4^{low} + inflammation^{low}

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

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

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

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

Interferon-gamma and nuclear KLF4 expression in OSCC cell lines

The influence of several inflammatory cytokines on KLF4 expression was evaluated in two different OSCC cell lines. In the cell line PE/CA-PJ49 clone E10, weak nuclear expression of KLF4 was observed in scattered cells, whereas the CaLH3 cell line revealed a (slightly) higher fraction of weak to moderate KLF4 positive nuclei (Figure 4A,B). Following 24 h exposure to IFN-γ, a statistically significant increase in nuclear expression of KLF4 (quantity and intensity) was found for both cell lines (Figure 4C–F) (SF4). Other cytokines revealed a less obvious increase in KLF4 expression. Cytoplasmic localization of KLF4 was detected only in a few single cells, regardless of type of the treatment or cell line.

DISCUSSION

In the present study, the expression of the transcription factor KLF4 was evaluated in a retrospective cohort of OTSCC from a Norwegian oral cancer multicenter study. Here, a subgroup of patients with a combined high KLF4 expression and dense stromal lymphocytic infiltrate were identified to have a favorable prognosis.

In normal oral mucosa, KLF4 is expressed in the nuclei of epithelial cells throughout the epithelium [25]. To examine a possible link between a deregulated expression of *KLF4* mRNA and HNSCC/OSCC progression, we examined the expression of *KLF4* mRNA in HNSCC/OSCC as compared to normal controls. A decreased *KLF4* mRNA expression level in HNSCC and OSCC versus control oral epithelium was seen in mRNA datasets examined in the current study and indicate a possible functional implication of KLF4 in OSCC. The results from the mRNA datasets are in line with the reports on a general decreased KLF4 protein expression in OSCC as well as in colon cancer specimens when compared with their healthy counterparts [23,46]. In the present study, there was an association between high KLF4 protein expression and well-differentiated OTSCC. This suggests that OSCC with KLF4 retention might have a less malignant phenotype. This is in line with Li et al. [25] who suggested a role of KLF4 as a tumor suppressor in OSCC.

Currently, conflicting results are reported on KLF4 expression and prognostic outcome in OSCC. In the present study, a trend was found for a high KLF4 expression and a favorable prognostic outcome. This is in line with the favorable overall survival in OSCC patients with retained nuclear KLF4 reported by Chen et al. [27]. Furthermore, a high intratumor KLF4 score has also been shown in non-recurrent OSCC. In addition, a tumor suppressive function for the *Klf4* gene has been reported in tongue epithelium. Here, deletion of the *Klf4* gene demonstrated acceleration of the carcinogenic process in mice [47]. In contrast, high KLF4 expression has also been
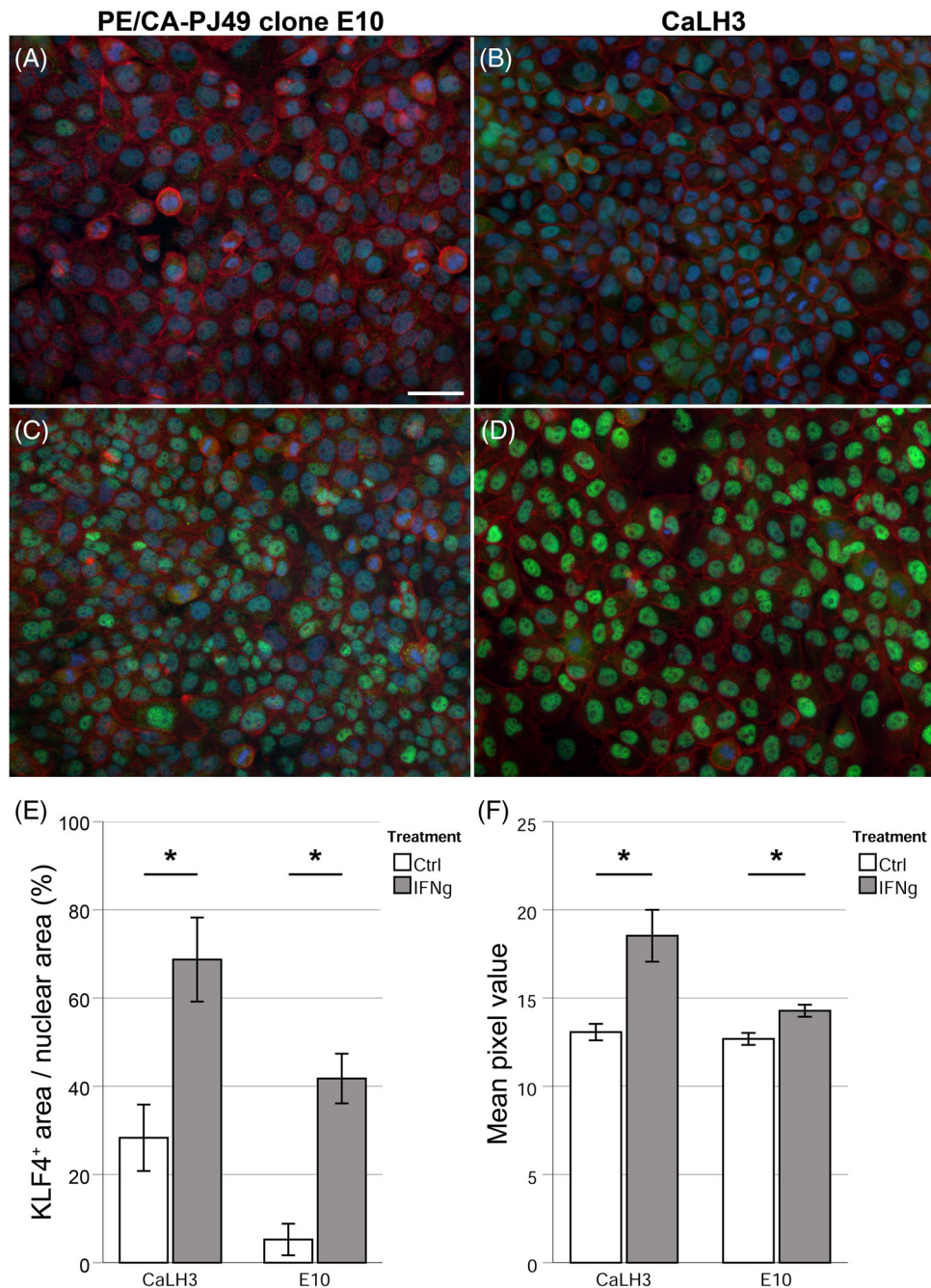
PE/CA-PJ49 clone E10　　　　**CaLH3**

FIGURE 4 The influence of IFN-γ on KLF4 nuclear expression. Oral cancer cells (CaLH3 and PE/CA-PJ49 clone E10) were immunostained with anti-KLF4 antibody (green) and incubated with DAPI (blue) for nuclear localization (six technical replicates). (A,B) Before IFN-γ exposure. (C,D) Following 24 h exposure to IFN-γ. Scale bar = 50μm. (E,F) In both cell lines, a statistically significant increase in KLF4 expression was found after IFN-γ exposure, as shown by KLF4 positive area/total nuclear area (E), and mean pixel intensity (F)

shown as a predictor of both poor OSCC disease-free survival and a worse disease-specific survival in HNSCC [24,48]. Furthermore, for OSCC, a trend was seen for high KLF4 expression and poor disease-specific survival while scoring KLF4 at the invasive front [24]. The complex interplay between KLF4 and diverse tumor microenvironments is thought to play a key role modulating the function of KLF4 as an oncogene or a tumor suppressor [49]

A possible limitation of the study could be the use of TMA-sections in contrast to the use of whole sections. TMA cores might not reflect the true tumor heterogeneity and can over- or underrepresent subpopulations of tumor cells with different expression of a particular protein. In order to minimize possible bias related to the tumor heterogeneity, tissue cores representing both the invasive front and the more superficial parts of each tumor were included in the TMA block. From

the majority of the OTSCC, four tissue cores were prepared. Two tissue cores were made from the rest of the tumors. Four cores are reported to achieve a high degree of concordance when comparing results from whole sections with those of TMA cores [50]. A high concordance is also reported using triplicate and duplicate TMA cores [51,52].

Due to the role of KLF4 in inflammation, a combined OTSCC KLF4 expression and stromal inflammation score was included in the survival analysis. Here, a 5-year disease-specific survival of about 90% was seen for patients with a combined high KLF4 expression and a dense stromal lymphocytic infiltrate compared with a disease-specific survival of approximately 70% for the separate inclusion of either KLF4 expression or inflammation in the survival analysis. In contrast to the KLF4/inflammatory score, clinical stage was not of prognostic significance in the multivariate analysis. The combination of KLF4 expression and inflammatory score emphasizes the importance of combining possible prognostic parameters in a survival analysis in order to identify subgroups of patients for whom a different prognostic outcome can be recognized. To the best of our knowledge, no other studies have investigated the association between KLF4 expression and the density of the lymphocytic infiltrate in OTSCC with patient prognosis.

Little is known about the influence of inflammatory cytokines on KLF4 expression in cells and tissues [13,14]. However, several studies have suggested KLF4 as a regulator of inflammation [11,12,53]. Here, we demonstrate a statistically significant increase in nuclear KLF4 expression induced by IFN-γ in two oral cancer cell lines. This is in accordance with studies on human colon carcinoma cells and macrophages [13,14]. Further studies are needed to explore the molecular mechanisms for IFN-γ mediated induction of nuclear KLF4 expression and its possible influence on OSCC cell phenotype.

Nuclear KLF4 expression was found in the majority of OTSCC, while cytoplasmic KLF4 was found only in a low number of tumors. The cytoplasmic KLF4 was only present in OTSCC that exhibited nuclear KLF4, and a combination of cytoplasmic and nuclear KLF4 did not add to the total KLF4 score in the clinical specimens. Only nuclear staining was included in the KLF4 score by Yoshihama et al. [24], though cytoplasmic KLF4 protein expression was observed. Of interest, both nuclear and cytoplasmic staining was included in the histoscore presented by Roy et al. [23]. However, the relative influence of nuclear and cytoplasmic scores on the total KLF4 score was not presented.

Our results are based on the highest nuclear KLF4 score in each OTSCC sample irrespective of its tumor tissue location. Neither separate scoring of nuclear KLF4 at the most invasive part nor in more superficial parts of OTSCC showed a significant association of KLF4 with clinical and histological parameters. This is in contrast to Yoshihama et al. [24], who demonstrated a significant association between KLF4 at the invasive edge of OSCCs and clinical stage, as well as Anneroth histological score. The difference might be explained by the selection of cut-off values defining KLF4 high and low tumors. The cut-off was at the median (66.72%) of KLF4 positive nuclei by Yoshihama et al. [24], versus at 75% in the present study.

We conclude that a combined score of high KLF4 nuclear expression and marked/moderate stromal inflammation was an independent favorable prognostic marker for a subgroup of patients in a Norwegian cohort of OTSCC. Such a combination of a histological parameter and protein expression is promising in order to identify subgroups of patients with different prognostic outcomes. We have shown that the expression of KLF4 can be upregulated by IFN-γ, an inflammatory cytokine that can be produced by the lymphocytic infiltrate in OTSCC. Our findings could have implications in management of and further research initiatives in OTSCC. Further confirmatory studies using whole tissue sections including functional studies will further aid in confirming the relevance of combined KLF4 and inflammation score in OTSCC. In the present study, the patients were classified according to the 5th edition of the TNM classification. Thereby, we suggest that the value of KLF4 as a prognostic biomarker should be evaluated in a separate cohort of OTSCC patients by the current TNM classification.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The study was approved by the Northern Norwegian Regional Committee for Medical Research Ethics (REK Nord; 2013/1786 and 2015/1381).

AUTHOR CONTRIBUTIONS

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