

## Clinical Significance of Kallikrein-related Peptidase-4 in Oral Cancer

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**Abstract.** *Kallikrein-related-peptidase-4 (KLK4), a serine protease originally discovered in developing tooth with broad target sequence specificity, serves vital functions in dental enamel formation. KLK4 is involved in degradation of extracellular matrix proteins and it is thought that this proteolytic activity could also promote tumor invasion and metastasis. Recent studies have associated KLK4 expression with tumor progression and clinical outcome, particularly in prostate and ovarian cancer. Very little is known in regard KLK4 involvement in oral squamous cell carcinomas (OSCCs). Our objective was to investigate KLK4 expression in OSCC pathogenesis and disease progression. KLK4 expression was evaluated by immunohistochemistry, western blots and zymograms in OSCC lines. Invasion assays using high versus low/undetectable KLK4-expressing OSCC cell lines were performed jointly with KLK4 siRNA inhibition. A large collection of OSCC specimens was evaluated for KLK4 expression and correlation with patients' characteristics and outcomes were determined. Our data indicate that KLK4 is differentially expressed in oral carcinomas. OSCC cell lines with high invasive and metastatic potential have the highest levels of KLK4 expression. KLK4 mRNA and protein expression correlated with enzyme activity detected by*

*zymograms. Inhibition of KLK4 expression results in diminished invasive potential in OSCC cell lines. Consistently, KLK4 expression is stronger in primary tumors that later either recurred or developed metastases, suggesting that its preferential expression in OSCC might contribute to individual tumor biology. Therefore, this study provides supportive evidence in favor of a prognostic value for KLK4 in OSCC and suggests that KLK4 could serve as a potential therapeutic target in patients with oral cancer.*

Oral squamous cell carcinoma (OSCC) continues to be a disfiguring and deadly disease, which displays a wide range of biologic behavior that cannot be predicted by tumor size, standard histology or even individual gene or protein expression/activity (1). Advanced stage OSCC represents one of the most difficult challenges in head and neck oncology. Despite advances in treatment, the survival of patients with advanced OSCC has not improved significantly over the last 30 years and remains one of the lowest survival rates among the major cancer types (2). Tumor metastasis is a critical factor in patient survival, being responsible for over 90% of cancer-associated mortality in these patients (3). Younger (<40 years) patients with oral cancer have a higher rate of distant failure (4). Accurate prediction of metastasis in OSCC would have an immediate clinical impact through individualized therapeutic strategies. Therapeutic options for metastatic OSCC are limited and often unsuccessful and thus, new treatment approaches are needed (5).

The human tissue kallikrein (KLK)-related peptidase family of serine proteases has been reported to play important roles in physiological processes from development to tissue remodeling (6-8). More recently, KLKs appear to play important roles in neoplastic growth and metastatic spread.

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**Key Words:** Oral squamous cell carcinoma (OSCC), cell lines, Kallikrein-related-peptidase-4 (KLK4), clinical outcome, invasion, metastasis.

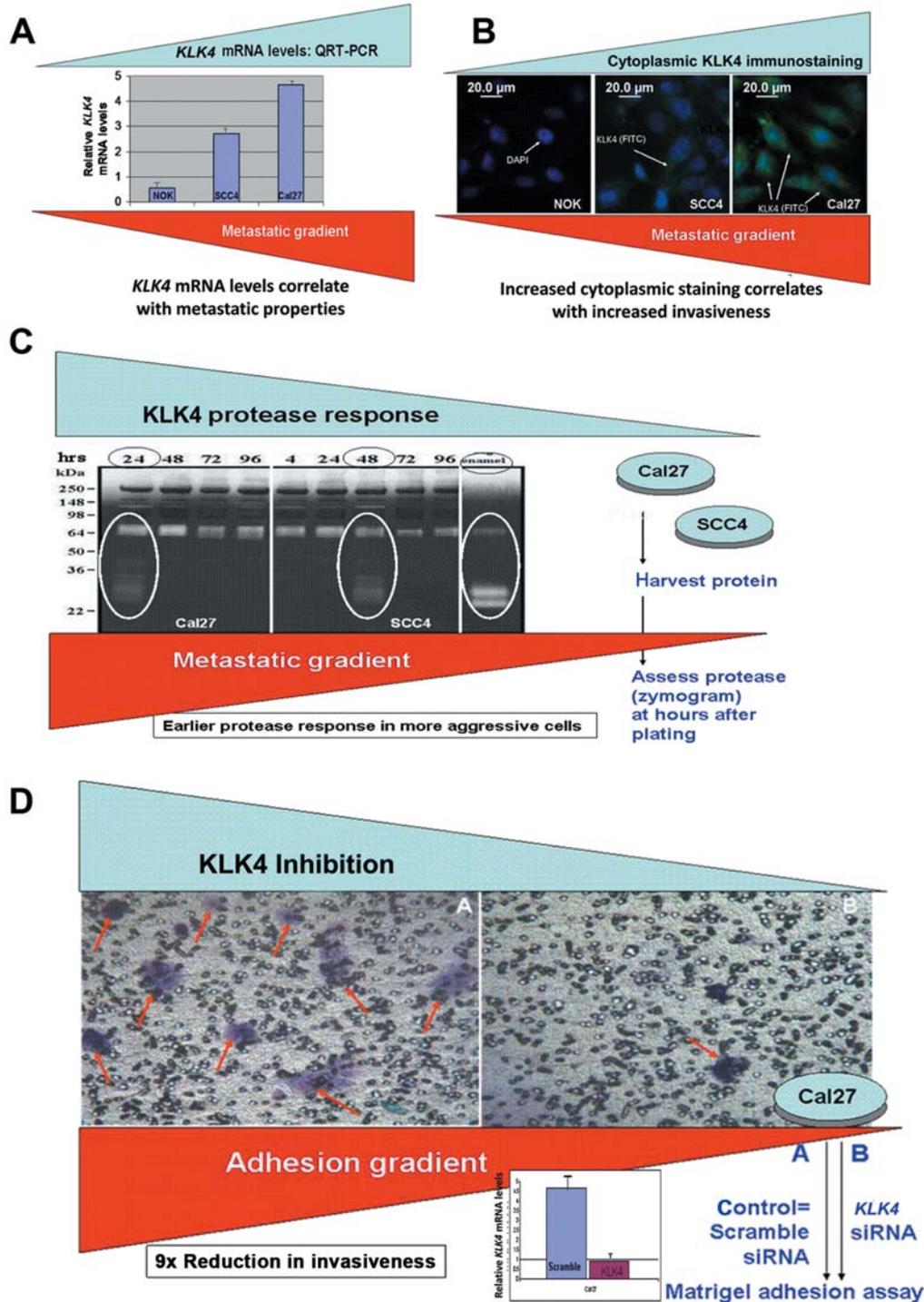


Figure 1. Kallikrein-related peptidase-4 (KLK4) detection in oral squamous cell carcinoma lines (SCC4 and Cal 27 comparatively) at RNA (A; quantitative real-time PCR) and protein (B; immunofluorescence) levels. KLK4 RNA and protein expression was correlated with enzyme activity detected by zymograms (C; dental enamel was used as control). Inhibition of *in vitro* invasiveness of Cal27 cells with KLK4 specific small interfering RNA (siRNA, D). Quantification of cell invasion indicated that KLK4 expression was linked to oral squamous cell carcinoma (OSCC) propensity for invasion, whereas inhibition of KLK4 expression resulted in diminished invasive potential in OSCC cell lines. Transfections with control siRNA (Ambion, Grand Island, NY, USA) or KLK4 siRNA (SantaCruz Biotechnology, Dallas, TX, USA) was carried out in triplicate with Lipofectamine RNAi Max (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Twenty-four hours post-transfection with siRNA, invasion assay was carried out for another 24 hours using medium with 10% fetal bovine serum and epithelial growth factor (Lonza Basel, Switzerland) as chemo-attractants. Invading cells were fixed and stained with Diff-Quick stain. Representative phase micrograph of Cal27 cells invading the Matrigel matrix. Scale bar=100  $\mu$ m.

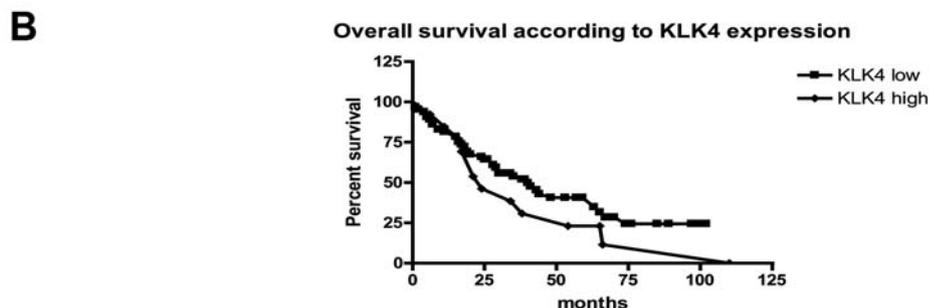
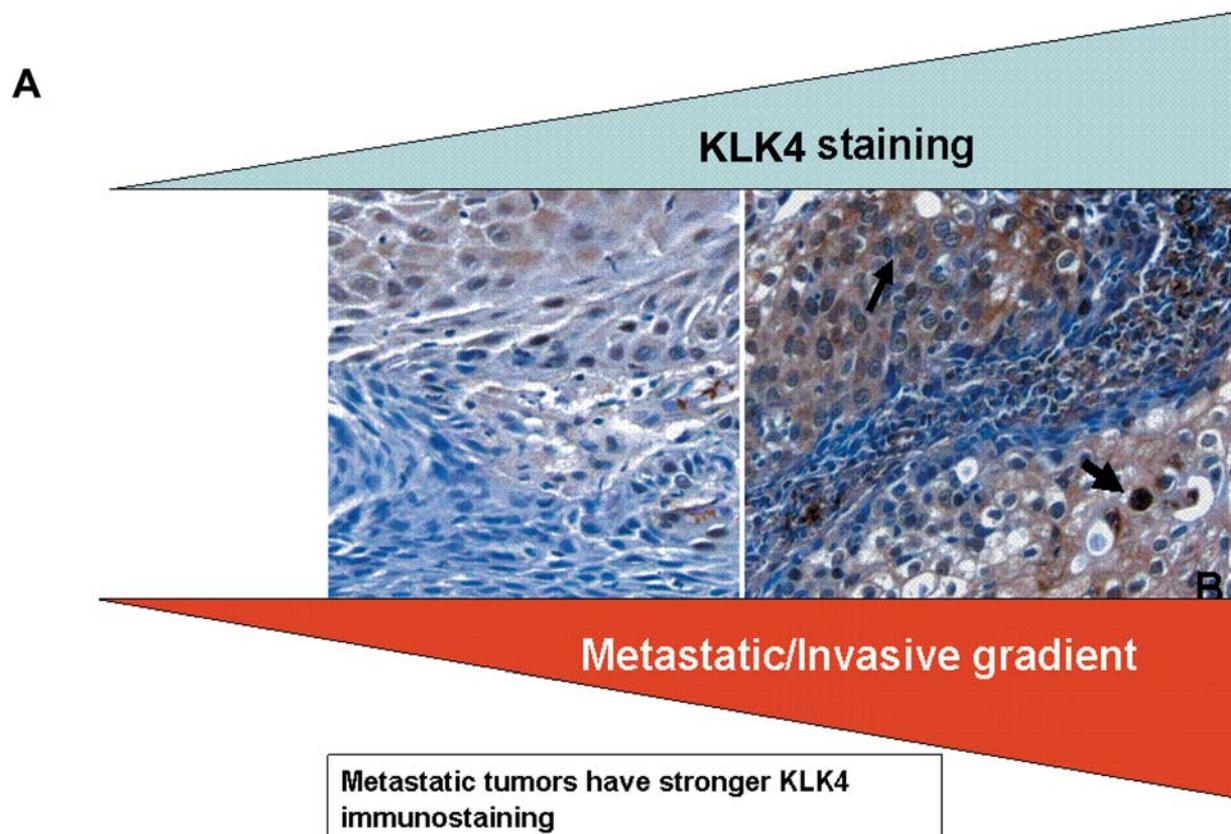


Figure 2. Kallikrein-related peptidase-4 (KLK4) expression in primary oral squamous cell carcinoma (OSCC) metastatic (A) versus non-metastatic (B) specimens-representative illustrations of the immunohistochemical staining. KLK4 is highly expressed in the metastatic tumor specimen with a cytoplasmic pattern of various intensities within the tumor and stromal cells (A). In contrast, in the non-metastatic tumor specimen, only few areas of tumor cells are positive for KLK4 with a cytoplasmic but less intense distribution (B). Magnification: 40  $\mu$ m (A), 20  $\mu$ m (B). The Kaplan-Meier curve has identified two prognostic subgroups: patients with high KLK4 expressing tumors versus those with low KLK4 expressing tumors. There was a trend towards worse patient survival among those with high KLK4 expressing tumors ( $p>0.05$ ).

Overexpression of KLK4 was associated with poor prognosis, particularly in prostate and ovarian cancer (7, 9-12). It is suggested that KLK4 promotes invasion by assisting degradation of basement membranes and extracellular matrix of connective tissues. In addition, KLK4 can activate many

tumorigenic and metastatic pathways including the protease activated receptors (PARs) (13). KLK4 knockdown results in a significant decline in prostate cancer cell proliferation *in vitro* and *in vivo*, reduces anchorage-independent growth, induces apoptosis and dramatically sensitizes prostate cancer

cells to apoptosis-inducing agents. Furthermore, *in vivo* nanoliposomal *KLK4* siRNA delivery in mice bearing prostate tumors results in profound remission (14).

Mutations in the human *KLK4* gene lead to the autosomal recessive disease called amelogenesis imperfecta characterized by dysregulation of dental epithelium that causes tooth enamel alterations due to lack of *KLK4* proteolytic activity (15). *KLK4* is also reported to be expressed in oral epithelium and its expression was found to be dysregulated in a recent study of OSCC. However, the precise roles of *KLK4* in OSCC and its potential use as anti-cancer target in oral cancer patients remain unclear. Our objective was to investigate *KLK4* expression in OSCC cell lines and tumor specimens from patients afflicted with oral carcinomas to assess its potential as a molecular biomarker and prognostic indicator. We also evaluated the clinical significance of targeting *KLK4* expression in OSCC.

## Methods and Results

*In vitro* characterization of *KLK4* expression in oral cancer. Our analysis included the American Type Culture Collection (Manassas, VA, USA) SCC4 (CRL-1624: tongue, non-metastatic early TNM stage, II) and Cal 27 (CRL-2095: tongue, metastatic advanced TNM stage, IV) cell lines. Human normal oral keratinocytes (NOK; ScienCell Research Laboratories, Carlsbad, CA, USA) served as the control.

Relative quantification of *KLK4* mRNA by real time-polymerase chain reaction (PCR) normalized to the housekeeping gene was performed using the TaqMan PCR kit (Applied Biosystems, Foster City, CA, USA) as reported elsewhere (16). Data were analyzed with Sequence Detector v1.6.3 software (Applied Biosystems). *KLK4* protein levels and enzymatic activity was evaluated by Western blot in supernatants of OSCC cell lines comparatively to NOK (17). *KLK4* RNA (*KLK4* mRNA) and protein (*KLK4*) expression was correlated with enzyme activity detected by zymograms (Figure 1A-C). Invasion assay using Matrigel chambers (BD BioSciences, Bedford, MA, USA) were performed as described (18). We assessed the effects of *KLK4* loss-of-function with specific small interfering RNA inhibition on OSCC invasion.

Our data indicates that *KLK4* is differentially expressed in OSCC cell lines; *KLK4* protein and *KLK4* mRNA were highest in the advanced OSCC line (Cal27), while in SCC4 and NOK *KLK4* was undetectable/low (Figures 1-2). *KLK4* expression was linked to OSCC propensity for invasion, whereas inhibition of *KLK4* expression resulted in diminished invasive potential in OSCC (Figure 1D).

*Correlations of KLK4 expression with OSCC clinicopathologic parameters.* Upon approval by the Institutional Board Review of the University of Texas Health Science Center at San Antonio, we identified consecutive cases (n=78) of previously untreated primary carcinomas from the pathology archival records (1990-2007). Median follow-up was 3 years. Serial sections immunostained for *KLK4* (Abcam, Cambridge, MA USA) using our protocol (17) were analyzed for intensity and localization of *KLK4* protein (Figure 2A). Survival time was defined from diagnosis to death or last follow-up. Statistical analyses were performed in SAS version 9.2 (SAS Institute, Cary, NC, US). A two-tailed  $p \leq 0.05$  was considered

statistically significant. *KLK4* expression was stronger in primary OSCC with a poor clinical outcome, particularly those that developed metastases. There was a trend towards worse patient survival of *KLK4* high-expressing cases ( $p > 0.05$ , Figure 2B). Univariate analysis indicated that OSCCs with a multifocal pattern of growth and with relapses or metastases in the clinical history were characterized by a higher *KLK4* immunohistochemical expression ( $p = 0.020$  and  $0.05$ , respectively).

## Discussion

Tissue-specific proteases are critical to the invasive and metastatic abilities of cancer cells by assisting in the degradation of epithelial basement membranes and extracellular matrix of connective tissues (19-21). *KLK4* is a serine protease first identified in developing tooth, with vital functions in dental enamel formation (15, 22-24). The digestion of enamel proteins *in vivo* demonstrates that *KLK4* is capable of aggressively cleaving peptide bonds in a broad range of amino-acid contexts (25), a feature also proven critical for tumor cells to locally invade and spread (7, 26).

Accumulating evidence suggests that *KLK4* is part of enzymatic cascades critically activated in some types of cancers that contribute to tumor progression and metastatic spread. *KLK4* expression was associated with aggressive outcomes and poor survival, particularly in prostate and ovarian cancer (8-12, 27). *KLK4* is thought to be involved in bone metastasis of prostate cancer by mediating cross-talk between cancerous cells and bone cells (28). There is also evidence that *KLK4* expression is associated with loss of E-cadherin and an epithelial mesenchymal transition-like effect in prostate cancer cells, thus making the tumor cells increasingly undifferentiated and more aggressive (29); E-cadherin involvement in oral carcinogenesis is critical (30). Recombinant *KLK4* has the ability to degrade insulin-like growth factor-binding proteins and to activate various genes with roles in tumor cell proliferation and differentiation, angiogenesis and metastasis (6, 8, 31, 32). Despite its critical role in cancer progression at the primary site and metastatic spread, implications of *KLK4* in head and neck cancer have not been extensively studied (33, 34). Here, we provide evidence that *KLK4* expression may contribute to aggressive progression of oral carcinomas, particularly in regard to their propensity for invasion and metastasis. *KLK4* inhibition resulted in diminished invasive potential of the OSCC cells, suggesting that targeting of *KLK4* might have useful clinical applications in patients with oral cancer. *KLK4* has also been detected in seminal plasma in prostate cancer patients (35), and our study has detected it in OSCC supernatants suggesting that detection of secreted *KLK4* could be used for cancer monitoring. Our analysis indicated preferential *KLK4* expression in OSCC with a multifocal growth pattern and with multiple separate primaries, a clinical situation very difficult to manage. Furthermore, cases that developed either

recurrences or metastases during follow-up expressed higher KLK4 levels and these correlations were proven statistically significant. However, only a trend for worse survival was noted in our cohort (79 cases); these results are in alignment with the findings of the only other OSCC analysis of a similar sized cohort (80 cases, 34) whose patients with intense KLK4 staining had significantly shorter overall survival, but no significant correlations with relapse or metastasis were found. Overall, our data provides novel evidence in support of KLK4 prognostic value in oral cancer patients. Better understanding KLK4 functions will provide opportunities to therapeutically target metastatic spread and improve patient outcomes.

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### Conflicts of interest

The Authors declare no conflict of interest.

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