HHS Public Access

Author manuscript

Anticancer Res. Author manuscript; available in PMC 2015 September 21.

Published in final edited form as:

Anticancer Res. 2015 April ; 35(4): 1861–1866.

Clinical Significance of Kallikrein-related-peptidase-4 in Oral Cancer

PETROS PAPAGERAKIS[1,2], **GIUSEPPE PANNONE**[3], **LI ZHENG**[1,4], **MARIA ATHANASSIOU-PAPAEFTHYMIOU**[1,4], **YASHUO YAMAKOSHI**[5], **HOWARD STAN McGUFF**[6], **OMAR SHKEIR**[3], **KONSTANTINOS GHIRTIS**[1,3], and **SILVANA PAPAGERAKIS**[2]

[1]Department of Pediatric Dentistry and Orthodontics, School of Dentistry, University of Michigan; Ann Arbor, USA

[2]Department of Computational Medicine and Bioinformatics, Medical School, University of Michigan, Ann Arbor, USA

[3]Departments of Clinical and Experimental Medicine, Section of Anatomic Pathology, University of Foggia, Italy

[4]Department of Otolaryngology - Head & Neck Surgery, Medical School, University of Michigan, Ann Arbor, USA

[5]Department of Biomaterials Sciences, School of Dentistry, University of Michigan; Ann Arbor, USA

[6]Departments of Pathology, School of Medicine, University of Texas Health Science Center at San Antonio, Texas, USA

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 cancers. Very little is known in regard with 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 indicates that KLK4 is differentially expressed in oral carcinomas. OSCC cell lines with high invasive and metastatic potential show the highest levels of

Correspondence to: Silvana Papagerakis, Director, Laboratory of Head and Neck Cancer Invasion and Metastasis, 1150 W. Medical Center Drive Room 5434 Med Sci I Ann Arbor, MI 48109-5616; USA. Tel: +1 7346157085, Fax: +1 7347640014, silvanap@umich.edu.

Request for Reprints: please contact corresponding author

Disclosure/conflicts of interest

The authors declare no conflict of interest.

KLK4 expression. KLK4 mRNA and protein expression is 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 the 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 oral cancer patients.

Keywords

Oral squamous cell carcinoma (OSCC); cell lines; Kallikrein-related-peptidase-4 (KLK4); clinical outcome; invasion; metastasis

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 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).

Human tissue kallikrein (KLK)-related peptidase family of serine proteases have been reported to play important roles in physiological processes from development to tissue remodeling (6–8). More recently KLKs appear to also play important roles in neoplastic growth and metastatic spread. Over-expression of KLK4 was associated with poor prognosis, particularly in prostate and ovarian cancers (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*, decreases 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 (16). Data was 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 done in SAS version 9.2 (SAS Institute, Carey, NC, US). A two-tailed $p \leq 0.05$ was considered statistically significant. KLK4 expression was stronger in OSCC primary tumors with a poor clinical outcome, particularly those that developed metastases. There was a trend towards worse patient survival of KLK4 high-expressing cases (p = N.S., Figure 2B). Univariate analysis indicated that OSCC tumors 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 cancers that are contributing to tumor progression and metastatic spread. KLK4 expression was associated with aggressive outcomes and poor survival, particularly in prostate and ovarian cancers (8–12, 27). KLK4 is thought to be involved in bone metastasis of prostate cancer by mediating cross-talk between cancer 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, KLK4 implications 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 with 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 oral cancer patients. 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 size cohort (80 cases, 34) whose patients with KLK4 intense 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.

Acknowledgments

This work was supported by funds from the Department of Orthodontics and Pediatric Dentistry and the University of Michigan Undergraduate Research Opportunity Program (UROP). SP was supported by the Multidisciplinary Clinical Research Career Development Program as a Clinical Scholar on the CTSA Grant (UL1RR025767), University of Texas Health Science Center at San Antonio (UTHSCSA).

References

1. Shah, J.; Johnson, N. Oral Cancer. London: Martin Dunitz Press; 2003.
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61:69–90. [PubMed: 21296855]
3. de Bree R, Haigentz M Jr, Silver CE, Paccagnella D, Hamoir M, Hartl DM, Machiels JP, Paleri V, Rinaldo A, Shaha AR, Takes RP, Leemans CR, Ferlito A. Distant metastases from head and neck squamous cell carcinoma. Oral Oncol. 2012; 48:780–786. [PubMed: 22520053]
4. Liao CT, Wang HM, Hsieh LL, Chang JT, Ng SH, Hsueh C, Lee LY, Lin CH, Chen IH, Kang CJ, Huang SF, Yen TC. Higher distant failure in young age tongue cancer patients. Oral Oncol. 2006; 42:718–725. [PubMed: 16529977]
5. Papagerakis S, Pannone G, Zheng L, About I, Taqi N, Nguyen NP, Matossian M, McAlpin B, Santoro A, McHugh J, Prince ME, Papagerakis P. Oral epithelial stem cells - implications in normal development and cancer metastasis. Exp Cell Res. 2014; 325:111–129. [PubMed: 24803391]
6. Borgono CA, Diamandis EP. The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer. 2004; 4:876–890. [PubMed: 15516960]
7. Paliouras M, Borgono C, Diamandis EP. Human tissue kallikreins: the cancer biomarker family. Cancer Lett. 2007; 249:61–79. [PubMed: 17275179]
8. Clements JA, Willemsen NM, Myers SA, Dong Y. The tissue kallikrein family of serine proteases: functional roles in human disease and potential as clinical biomarkers. Crit Rev Clin Lab Sci. 2004; 41:265–312. [PubMed: 15307634]
9. Dong Y, Kaushal A, Bui L, Chu S, Fuller PJ, Nicklin J, Samaratunga H, Clements JA. Human kallikrein 4 (KLK4) is highly expressed in serous ovarian carcinomas. Clin Cancer Res. 2001; 7:2363–2371. [PubMed: 11489814]
10. Klokk TI, Kilander A, Xi Z, Waehere H, Risberg B, Danielsen HE, Saatcioglu F. Kallikrein 4 is a proliferative factor that is overexpressed in prostate cancer. Cancer Res. 2007; 67:5221–5230. [PubMed: 17545602]
11. Obiezu CV, Scorilas A, Katsaros D, Massobrio M, Yousef GM, Fracchioloi S, Diamandis EP. Higher human kallikrein gene 4 (KLK4) expression indicates poor prognosis of ovarian cancer patients. Clin Cancer Res. 2001; 7:2380–2386. [PubMed: 11489816]
12. Xi Z, Klokk TI, Korkmaz K, Kurys P, Elbi C, Risberg B, Danielsen H, Loda M, Saatcioglu F. Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer. Cancer Res. 2004; 64:2365–2370. [PubMed: 15059887]
13. Swedberg JE, Nigon LV, Reid JC, de Veer SJ, Walpole CM, Stephens CR, Walsh TP, Takayama TK, Hooper JD, Clements JA, Buckle AM, Harris JM. Substrate-guided design of a potent and selective kallikrein-related peptidase inhibitor for kallikrein 4. Chem Biol. 2009; 16:633–643. [PubMed: 19549601]
14. Jin Y, Qu S, Tesikova M, Wang L, Kristian A, Mælandsmo GM, Kong H, Zhang T, Jerónimo C, Teixeira MR, Yuca E, Tekedereli I, Gorgulu K, Alpay N, Sood AK, Lopez-Berestein G, Danielsen HE, Ozpolat B, Saatcioglu F. Molecular circuit involving KLK4 integrates androgen and mTOR signaling in prostate cancer. Proc Natl Acad Sci U S A. 2013; 110:E2572–2581. [PubMed: 23798432]
15. Lu Y, Papagerakis P, Yamakoshi Y, Hu JC-C, Bartlett JD, Simmer JP. Functions of KLK4 and MMP-20 in dental enamel formation: Minireview. J Biol Chem. 2008; 389:695–700.
16. Shkeir O, Athanassiou-Papaefthymiou M, Lapadatescu M, Papagerakis P, Czerwinski MJ, Bradford CR, Carey TE, Prince ME, Wolf GT, Papagerakis S. In vitro cytokine release profile: predictive value for metastatic potential in head and neck squamous cell carcinomas. Head Neck. 2013; 35:1542–1550. [PubMed: 23322448]
17. Papagerakis P, Pannone G, Shabana AH, Depondt J, Santoro A, Ghirtis K, Berdal A, Papagerakis S. Aberrant beta-catenin and LEF1 expression may predict the clinical outcome for patients with oropharyngeal cancer. Int J Immunopathol Pharmacol. 2012; 25:135–146. [PubMed: 22507326]
18. Davis SJ, Divi V, Owen JH, Bradford CR, Carey TE, Papagerakis S, Prince ME. Metastatic potential of cancer stem cells in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg. 2010; 136:1260–1266. [PubMed: 21173377]

19. Debela M, Beaufort N, Magdolen V, Schechter NM, Craik CS, Schmitt M, Bode W, Goettig P. Structures and specificity of the human kallikrein-related peptidases KLK 4, 5, 6, and 7. Biol Chem. 2008; 389:623–632. [PubMed: 18627343]
20. Pampalakis G, Sotiropoulou G. Tissue kallikrein proteolytic cascade pathways in normal physiology and cancer. Biochimica et Biophysica Acta. 2007; 1776:22–31. [PubMed: 17629406]
21. Yousef GM, Diamandis EP. Tissue kallikreins: new players in normal and abnormal cell growth? Thromb Haemost. 2003; 90:7–16. [PubMed: 12876620]
22. Simmer JP, Fukae M, Tanabe T, Yamakoshi Y, Uchida T, Xhu J, Margolis HC, Hu JC-C, Bartlett JD. Purification, characterization, and cloning of enamel matrix serine proteinase 1. J Dent Res. 1998; 77:377–386. [PubMed: 9465170]
23. Simmer JP, Bartlett JD. Kallikrein 4 is a secreted protein. Cancer Res. 2004; 64:8481–8483. [PubMed: 15548723]
24. Hu JC, Zhang C, Sun X, Yang Y, Cao X, Ryu O, Simmer JP. Characterization of the mouse and human PRSS17 genes, their relationship to other serine proteases, and the expression of PRSS17 in developing mouse incisors. Gene. 2000; 251:1–8. [PubMed: 10863090]
25. Ryu OH, Hu JC-C, Yamakoshi Y, Villemain JL, Cao X, Zhang CH, Barlett JD, Simmer JP. Porcine kallikrein-4 activation, glycosylation, activity, and expression in prokaryotic and eukaryotic hosts. Eur J Oral Sci. 2002; 110:358–365. [PubMed: 12664466]
26. Ramsay AJ, Dong Y, Hunt ML, Linn M, Samaratunga H, Clements JA, Hooper JD. Kallikrein-related peptidase 4 (KLK4) initiates intracellular signaling via protease-activated receptors (PARs). KLK4 and PAR-2 are co-expressed during prostate cancer progression. J Biol Chem. 2008; 283:12293–12304. [PubMed: 18308730]
27. Takayama TK, McMullen BA, Nelson PS, Matsumura M, Fujikawa K. Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase. Biochemistry. 2001; 40:15341–15348. [PubMed: 11735417]
28. Gao J, Collard RL, Bui L, Herington AC, Nicol DL, Clements JA. Kallikrein 4 is a potential mediator of cellular interactions between cancer cells and osteoblasts in metastatic prostate cancer. Prostate. 2007; 67:348–360. [PubMed: 17221837]
29. Veveris-Lowe TL, Lawrence MG, Collard RL, Bui L, Herington AC, Nicol DL, Clements JA. Kallikrein 4 (hK4) and prostate-specific antigen (PSA) are associated with the loss of E-cadherin and an epithelial-mesenchymal transition (EMT)-like effect in prostate cancer cells. Endocr Relat Cancer. 2005; 12:631–633. [PubMed: 16172196]
30. Pannone G, Santoro A, Feola A, Bufo P, Papagerakis P, Lo Muzio L, Staibano S, Ionna F, Longo F, Franco R, Aquino G, Contaldo M, De Maria S, Serpico R, De Rosa A, Rubini C, Papagerakis S, Giovane A, Tombolini V, Giordano A, Caraglia M, Di Domenico M. The role of E-Cadherin down-regulation in oral cancer: Cdh1 gene expression and epigenetic blockage. Curr Cancer Drug Targets. 2014; 14:115–127. [PubMed: 24274398]
31. Matsumura M, Bhatt AS, Andress D, Clegg N, Takayama TK, Craik CS, Nelson PS. Substrates of the prostase-specific serine protease prostase/klk4 defined by positional-scanning peptide libraries. Prostate. 2005; 62:1–13. [PubMed: 15389820]
32. Mize GJ, Wang W, Takayama TK. Prostate-specific kallikreins-2 and -4 enhance the proliferation of DU-145 prostate cancer cells through protease-activated receptors-1 and -2. Mol Cancer Res. 2008; 6:1043–1051. [PubMed: 18567807]
33. Pettus JR, Johnson JJ, Shi Z, Davis JW, Koblinski J, Ghosh S, Liu Y, Ravosa MJ, Frazier S, Stack MS. Multiple kallikrein (KLK 5, 7, 8, and 10) expression in squamous cell carcinoma of the oral cavity. Histol Histopathol. 2009; 24:197–207. [PubMed: 19085836]
34. Zhao H, Dong Y, Quan J, Smith R, Lam A, Weinstein S, Clements J, Johnson NW, Gao J. Correlation of the expression of human kallikrein-related peptidases 4 and 7 with the prognosis in oral squamous cell carcinoma. Head Neck. 2011; 33:566–572. [PubMed: 20665732]
35. Obiezu CV, Shan SJC, Soosaipillai A, Luo L-Y, Grass L, Sotiropoulou G, Petraki CD, Papanastasiou PA, Levesque MA, Diamandis EP. Human kallikrein 4: quantitative study in tissues and evidence for its secretion into biological fluids. Clinical Chemistry. 2005; 51:1432–1442. [PubMed: 15961548]

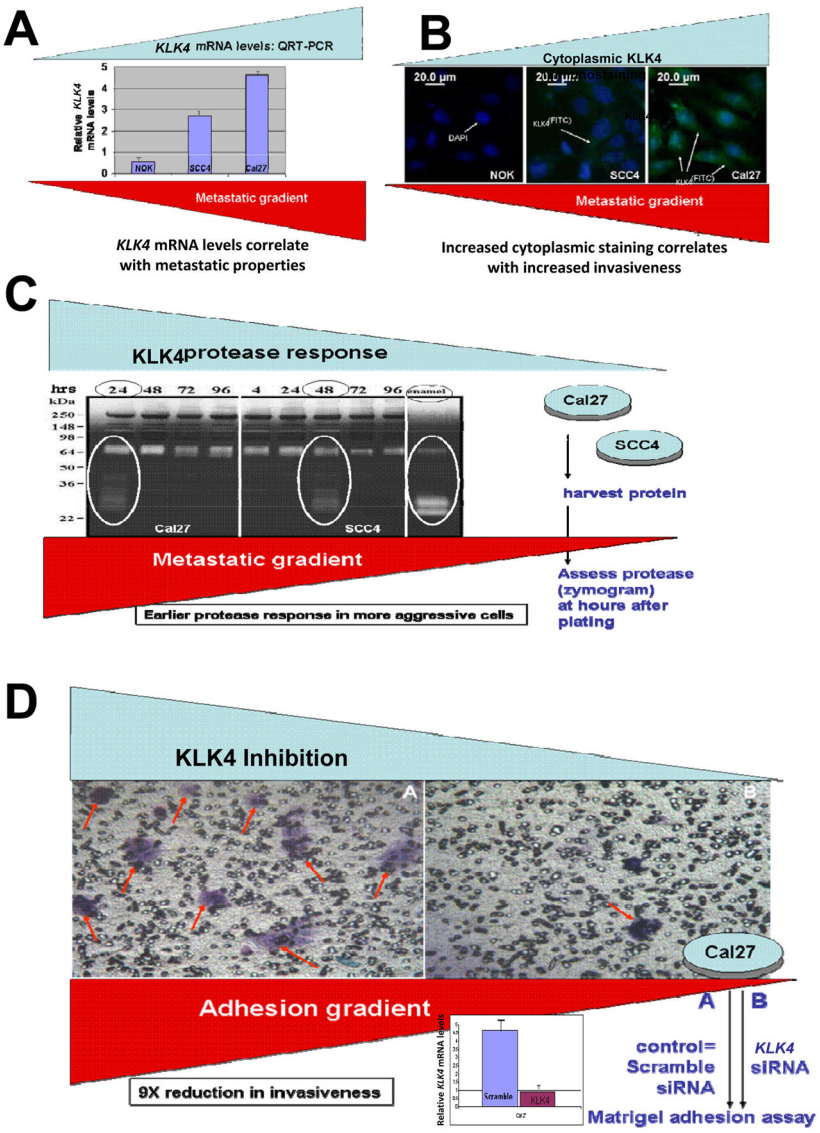

Figure 1. KLK4 detection in oral squamous cell carcinomas lines (SCC4 and Cal 27 comparatively) at RNA (A; quantitative real time PCR) and protein (B; immunofluorescence) levels. KLK4 RNA and protein expression was correlated with enzyme activity detected by zymograms (C; dental enamel was used as control). Inhibition of in vitro invasiveness of Cal27 cells with KLK4 specific small interfering RNA (siRNA, D)

Quantification of the cell invasion indicated that KLK4 expression was linked to OSCC propensity for invasion, whereas inhibition of KLK4 expression resulted in diminished invasive potential in OSCC cell lines. Transfections with control siRNA (Ambion, Grant Island, NY, USA) or *KLK4* siRNA (SantaCruz Biotechnology, Dallas, TX, USA) was carried out in triplicate with Lipofectamine RNAi Max (Invitrogen, Grant Island, NY) according to the manufacturer's instructions. Twenty-four hours post-transfection with the 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 μm.

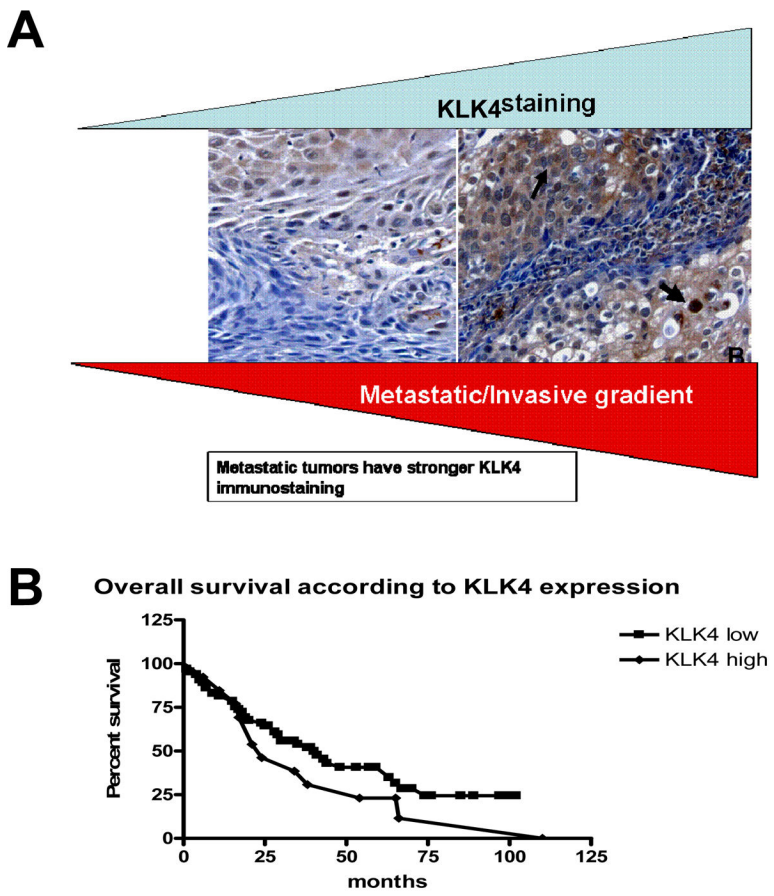

Figure 2. KLK4 expression in primary OSCC metastatic (A) versus non-metastatic (B) specimens – representative illustrations of the immunohistochemical staining

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 μm (A), 20 μm (B). The Kaplan-Meyer curve has identified two prognostic subgroups: patients with KLK4 high-expressing tumors *versus* those with KLK4 low-expressing tumors. There was a trend towards worse patient survival among the KLK4 high-expressing tumors (p = N.S).