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Expression of KITENIN and its association with tumor progression in oral squamous cell carcinoma

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ABSTRACT

Objective: KAI1 COOH-terminal interacting tetraspanin (KITENIN) contributes to tumor invasion and metastasis in various cancers. The aim of this study was to investigate expression of KITENIN in patients with oral cavity squamous cell carcinoma (SCC) and to determine whether KITENIN affects tumor cell behavior in oral cavity SCC cell line.
Methods: Western blotting and immunohistochemistry was used to assess alteration of KITENIN expression in human oral cavity SCC and normal oral cavity mucosa. To evaluate the impact of KITENIN knockdown, the cell invasion assay and cell migration assay using small-interfering RNA were performed.
Results: KITENIN protein expression was significantly increased in human oral cavity SCC tissues than in normal oral cavity mucosa by Western blotting. KITENIN immunoreactivity was strongly identified in human oral cavity SCC relative to adjacent normal tissue. Knockdown of KITENIN resulted in significantly reduced cell invasion in human oral cavity SCC cells ($p = 0.001$). Cell migration showed a marked decrease in KITENIN knockdown oral cavity SCC cells compared to the negative control oral cavity SCC cells ($p = 0.01$).
Conclusion: KITENIN is associated with tumor invasiveness and metastasis in human oral cavity SCC.

1. Introduction

Head and neck cancers are a biologically similar group of epithelial cancers that occur throughout the upper aerodigestive tract. Squamous cell carcinoma (SCC) arising from mucosa of the oral cavity, pharynx, and larynx is the most common type and accounts for 90% of cases. Oral cavity SCC is a relatively common malignant tumor of the head and neck region. The primary therapeutic modality for oral cavity SCC is surgery. Although recent advances in surgical techniques and chemoradiation modalities have improved locoregional control, wide surgical resection of oral cavity SCC causes inevitably various oral dysfuctions. Thus, new treatment modalities as the molecular target therapy are clearly needed.

KAI1/CD82 encodes a transmembrane glycoprotein of the tetraspanin family and was initially identified as a gene located on human chromosome 11p11.2, responsible for the specific inhibition of tumor metastasis in a rat prostate cancer model [1–3]. The evidence suggests that down-regulation or loss of KAI1/CD82 expression occurs with invasive and metastatic disease, and is an important step in the progression of many types of human malignancy, leading to the proposal that KAI1/CD82 might be a metastasis suppressor [4–12]. The metastasis suppressor function was reported to be decreased in a spliced variant of KAI1 at the COOH-terminal region [13]. Subsequently, via a yeast two-hybrid system, a protein that interacted with the COOH-terminal cytoplasm domain of KAI1 was identified [14]. This protein was found to be the Vang-like 1 (VANGL1), whose function has not fully understood in carcinogenesis and renamed as KAI1 C-terminal interacting tetraspanin (KITENIN) [14,15]. In contrast to KAI1/CD82, KITENIN increases migration and invasiveness of colon cancer cells and specific knockdown of KITENIN inhibits tumor metastasis in a mouse model of colon cancer [13,14,16,17]. In

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bladder cancer cell lines, KITENIN-positive cancer cells with loss of KAI1/CD82 are associated with increased in vitro invasive ability [18]. KITENIN promotes tumor growth and pulmonary metastasis in a syngeneic mouse SCC model [19]. These findings have raised interest in KITENIN as a potential molecular therapeutic target. However, with regard to humans, there is no information on the expression and potential role of KITENIN in oral cavity SCC.

We evaluated the expression of KITENIN in human oral cavity SCC specimens. We also attempted to determine whether KITENIN affects tumor cell invasion and migration in a human oral cavity SCC cell line. This study is the first to demonstrate KITENIN expression and its association with tumor progression in human oral cavity SCC and may provide the basis for targeting of KITENIN for molecular therapy in oral cavity SCCs.

2. Materials and methods

2.1. Patients and tumor specimens

Fresh oral cavity SCC tissues and paired normal mucosa tissues were collected from six patients who underwent definitive surgery for oral cavity SCC; two tongue SCC T1N0, one tongue SCC T3N0, two floor of mouth SCC T1N0, and one floor of mouth SCC T2N1. The oral cavity SCC tissues and paired normal mucosa tissues were compared for KITENIN protein expression by Western blotting.

Paraffin tissue sections were collected from 18 patients who underwent definitive surgery for oral cavity SCC. The oral cavity SCC and adjacent normal tissue were compared for KITENIN protein expression by immunohistochemistry.

2.2. Immunohistochemistry

An horseradish peroxidase (HRP)/3,3′-diaminobenzidine (DAB) immunohistochemical staining method was used on formalin-fixed paraffin-embedded tissues. 5-μm tissue sections were cut from each paraffin block, and then mounted and dried on glass slides. Tissues were deparaffinized using xylene, then rehydrated in graded alcohol solutions and retrieved with retrieval buffer. Endogenous peroxidase activity was blocked with peroxidase-blocking solution (Dako, Carpinteria, CA, USA), followed by incubation with polyclonal rabbit anti-human KITENIN antibody in primary diluents solution (Invitrogen, Carlsbad, CA, USA) overnight at 4 °C. After washing in TBS-Tween 20 buffer (TBST), tissues were stained using the Dako Real™ Envision HRP/DAB detection system (Dako). Tissues were counterstained with hematoxylin and mounted with coverslips. Stained tissues were viewed and photographed using a light microscope.

Assessment of staining was interpreted by two independent observers who had no knowledge of the clinical findings. Depending on the strength of the staining, they were classified as negative (−), one plus positive (1+), and two plus positive (2+).

2.3. Cell culture and transfection

The PCI 50 human oral cavity SCC cell line was kindly provided by Dr. M.W. Sung (Seoul National University, Seoul, South Korea). Cell lines were cultured in RPMI1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 50 units/ml penicillin, and 50 ug/ml streptomycin (Gibco, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. For transfection, cells were prepared and maintained in culture dishes with medium and cells were seeded on 6-well plates at 2×10^5 cells per well at the time of transfection. The synthesized human KITENIN-specific small interfering RNA (siRNA) (5′-GCUUGGACUUCAGCCUCGUAGUCAA-3′) and negative control siRNA (Qiagen, Valencia, CA, USA) were transfected with Lipofectamine™ 2000 (Invitrogen) into the cells. KITENIN knock-down was identified by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting.

2.4. Cell invasion assay

Cell invasion was measured using a Transwell invasion apparatus (Costar, Cambridge, UK). Transwell filters (8.0 μm pores) were coated with 1% gelatin solution on both the top and bottom surfaces overnight and dried at room temperature. Cells transfected with each siRNA (KITENIN siRNA, negative control siRNA) were seeded at 2×10^5 cells in 120 μl of 0.2% bovine serum albumin (BSA) in the upper chamber. Subsequently, 400 μl 0.2% BSA containing 7 μg/ml fibronectin (Calbiochem, La Jolla, CA, USA) as the chemoattractant were loaded into the lower chamber. The apparatus was incubated for 24 h at 37 °C. Cells on the top surface of the filter were wiped with cotton balls. Cells that had migrated to the bottom surface of the Transwell were stained with Diff Quik solution (Sysmex, Kobe, Japan) and counted in five random squares in the microscopic field of view. Results are expressed as the mean ± standard error of the number of cells/field.

2.5. Cell migration assay (wound healing assay)

Cells were seeded in a 6-well plate (2×10^7 cells/well) and, the next day, were transfected with KITENIN siRNA and negative control siRNA. A uniform wound gap was made by an insert (Ibidi, Bonn, Germany). After incubation for 24 h, the insert was detached and the progression of cell migration was photographed at 0, 8, 24, and 48 h using an inverted microscope. The distance between gaps was normalized to 1 cm after capture of six random sites.

2.6. Protein isolation and Western blot analysis

Cells and tissues were lysed in RIPA buffer (1 M Tris–HCl, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA) with 1 mM phenylmethanesulfonyl fluoride (PMSF), Halt™ phosphatase inhibitor, and Halt™ protease inhibitor cocktail (Thermo, Rockford, IL, USA). Resolved proteins (10–20 μg) were subjected to SDS-polyacrylamide gels and then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% BSA in Tris buffered saline Tween (TBST) at room temperature for 1 h, membranes were sequentially blotted with primary antibody: polyclonal anti-human KITENIN, anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-human actin (I-19; Santa Cruz Biotechnology) to control for loading variations at 4 °C overnight. After rinsing in TBST, each membrane was incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 h. Blots were detected with chemiluminescence (ECL) HRP substrate (Millipore) and recorded using Ras-4000 image reader (Fujifilm, Tokyo, Japan). The density of each Western blot was measured, and then each KITENIN level was normalized with respect to the corresponding actin level.

2.7. RNA isolation and RT-PCR

The RNA from cells was isolated using Trizol reagent (Invitrogen), reverse transcribed, and amplified using specific primers for KITENIN and GAPDH. Primer sequences were as follows: KITENIN 5′-AAG CCT TCA TCC ACA TCC AGC-3′/5′-GAG AGC CAT CGA TCC TTG TCA-3′; GAPDH 5′-ACC ACA GTC CAT GCC ATC AC-3′/5′-TCC ACC ACC CTG TTG CTG TA-3′. For cDNA synthesis, 1 μg mRNA was mixed with 50 ng/ul oligo-dT (Promega, Madison, WI, USA), MMLV reverse transcriptase (Invitrogen), and RNAsin (Takara, Otsu, Shiga,