

# Proteomic identification of keratin alterations with enhanced proliferation of oral carcinoma cells by loss of mucosa-associated lymphoid tissue 1 expression

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**Abstract.** Progression of oral carcinomas associates with aberrant activation and inactivation of molecules that work in established or unknown pathways. Although mucosa-associated lymphoid tissue 1 (MALT1) expressed in normal oral epithelium is inactivated in the aggressive subset of carcinomas with worse prognosis, phenotypic changes of carcinoma cells upon the loss of expression is unknown. We performed a proteomic analysis to identify MALT1-regulated proteins in oral carcinoma cells. Four different keratins were included in the ten most abundantly changed proteins. K8/18 were upregulated in MALT1 stably-expressing carcinoma cells and K5/14 in MALT1-marginal control cells. K8/18 upregulation and K5/14 downregulation were MALT1 dose-dependent and observed in a series of oral carcinoma cells. MALT1 suppressed cell proliferation (0.52-fold,  $P < 0.01$ ) and its dominant-negative form stimulated it (1.33-fold,  $P < 0.01$ ). The decreased proliferation associated with reduction of cyclin D1, which was recovered by the short interfering RNA against *MALT1*. Taken together, loss of MALT1 expression alters keratin expression and enhances proliferation of carcinoma cells, and may progress oral carcinomas into the advanced state.

## Introduction

Squamous cell carcinoma is a most common malignant neoplasm of the oral cavity and the patient prognosis is still worse than that of all other cancers combined. The annual

incidence of new cases is predicted to increase in the next few decades (1). Aberrant expression of endogenous and exogenous factors cause phenotypic alterations of carcinoma cells and select aggressive clones to advanced states of carcinoma progression (2). Unveiling molecular pathways of carcinoma progression is prerequisite for the improvement of patient prognosis.

Stratified squamous epithelial cells develop keratin intermediate filaments within them. Epithelial keratins are classified into two groups according to the Mr and pI; type I (K9-K28) and type II (K1-K8, K71-K80). Type I and II keratins are expressed in pairs and constitute keratin filaments in a 1:1 molar ratio. They switch the species according to a cell-type and differentiation and functional states (3). In general, keratinized squamous epithelium expresses K5 and K14 in the basal cells and K1 and K10 in the suprabasal cells. However, keratins alter their species and distribution under various pathological conditions, especially in parallel with differentiation and proliferation states of carcinoma cells (4-6). In addition, emerging evidence highlight that its alterations do not only reflect cellular conditions but also cause phenotypic changes including proliferation, apoptosis, cell growth, protein synthesis and membrane trafficking (7-11).

Our previous studies demonstrated that aggressive oral carcinomas inactivate expression of mucosa-associated lymphoid tissue 1 (*MALT1*) by promoter methylation, and that the patients with the loss of expression had the worst prognoses (12). MALT1 consists of three types of domains: a death domain, Ig-like domains and a caspase-like domain. B cell and T cell receptor antigen signals oligomerize MALT1 with BCL10 and CARMA1/3 into a CBM complex. MALT1 interacts with BCL10 through its Ig-like domains and induces I $\kappa$ B-kinase catalytic activity, resulting in nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation (13,14). In contrast to the established action in lymphocyte lineages, its role in epithelial cells and carcinoma cells is unknown. Unveiling alterations of protein expression in response to MALT1 contributes to understand the progression process. We examined the alterations by proteomic analysis and proliferation of oral carcinoma cells.

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## Materials and methods

**Cell lines.** Oral carcinoma cell lines derived from different sites were used: oral floor, HSC2, KOSC2 and Holu1; tongue, HSC3 and OSC19; and gingiva, TSU and Ca9.22. They were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan) or RIKEN BRC Cell Bank (Tsukuba, Japan). Cells were cultured in RPMI-1640 or DMEM supplemented with 10% fetal bovine serum and 100 U/ml of penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO). HSC2 cells, which marginally express *MALT1*, stably expressing full-length wild-type *MALT1* (*wtMALT1*, *wtMALT1*HSC2 cells), the NH<sub>2</sub> terminal death and Ig-like domains-deleted dominant-negative *MALT1* ( $\Delta$ *MALT1*,  $\Delta$ *MALT1*HSC2 cells) or vector alone (*mock*HSC2 cells) were previously established (12). The *wtMALT1* and  $\Delta$ *MALT1* cDNA cloned into pEBMulti-Hyg with a FLAG-tag (Wako Pure Chemical Industries Ltd., Osaka, Japan) or vector alone were transiently transfected into oral carcinoma cells by electroporation. Cells were harvested 24 h after the transfection and used for analyses. The short interfering RNA (siRNA) targeting *MALT1* (#18601 siRNA) and a negative control siRNA (Silencer Negative Control #1 siRNA; Ambion, Austin, TX) were used.

**Protein extraction and gel electrophoresis.** The *wtMALT1*HSC2 and *mock*HSC2 cells scraped from the culture dish were centrifuged at 1,500 rpm for 7 min at 4°C. The supernatant was discarded and the cell pellets were washed with ice-cold PBS three times. The pellets were solubilized in lysis buffer containing 0.1% NP-40 on ice and sonicated for 30 sec three times. The lysate was centrifuged at 15,000 rpm at 4°C for 15 min to remove insoluble materials. The resultant supernatant was collected and the protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). Protein extracts (30  $\mu$ g) were applied to a one dimensional (1-D) SDS-PAGE gel (10% total acrylamide) and stained with Coomassie Brilliant Blue R-250. All experiments were repeated three times from independent experiments. The gels were put on a flatbed scanner, and protein bands were compared using the Image J 1.46r (15). Protein bands of interest that were electrophoresed at different positions or with different intensities were subjected to MALDI-TOF mass spectrometry (MS) analysis.

**In-gel digestion and MALDI-TOF MS analysis.** Protein bands differentially detected between the cells were excised from the gel. The gel bands were digested by proteomics grade trypsin (Roche Diagnostics GmbH, Mannheim, Germany), and tryptic peptides were extracted from gels with 1% trifluoroacetic acid in 50% acetonitrile and dried using a vacuum pump. The digest was analyzed by MALDI-TOF MS on a Voyager DE-PRO MALDI-TOF (Applied Biosystems, Foster city, CA) with a nitrogen laser (337 nm). The analyte mixture (1  $\mu$ l) was mixed with 1  $\mu$ l of saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid, and spotted on a MALDI target plate and dried at room temperature. Ion acceleration was set at 20 kV. Mass spectra were obtained by averaging 200 laser shots. Calibration of the spectra was externally calibrated with peptide mass standards (Applied Biosystems). Row data were analyzed using the computer software provided by the manufacturer as monoisotopic masses.

Average of proteins from MALDI-TOF MS spectra was achieved using the MASCOT database (MASCOT ver. 2.0, Matrix Science Inc., Boston, MA) (16). Monoisotopic peptide mass spectra were matched against the SWISS-PROT or NCBI non-redundant databases set at  $\pm$ 1.2 kDa peptide tolerance, limited to the Homo Sapiens proteins.

**Quantitative real-time PCR.** Total RNA extracted from *wtMALT1*HSC2 and *mock*HSC2 cells was reverse transcribed to cDNA by MultiScribe Reverse Transcriptase (Applied Biosystems) and subjected to real-time PCR using the StepOne Real-time PCR system (Applied Biosystems). PCR conditions were 95°C for 20 sec followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec. The TaqMan probes (Applied Biosystems) specific for *KRT8* (Hs01595539\_g1), *KRT18* (Hs02827483\_g1), *KRT5* (Hs00361185\_m1) and *KRT14* (Hs00265033\_m1) were used. Expression levels (n=4) were normalized against *GAPDH* (Hs02758991\_m1). Levels of gene expression ( $2^{-\Delta\Delta C_t}$ ) were determined by the standard curve method (17).

**Immunoblot analysis.** Total cell lysates were immunoblotted with a standard protocol. The lysates in the SDS sample buffer containing 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail (Roche Diagnostics GmbH) were size-fractionated by SDS-PAGE gels under reducing conditions and electrotransferred to PVDF membranes. The membrane was probed with antibodies specific to K5 (clone PRB-160P, Covance, Princeton, NJ), K8 (clone Ks 8.7, Progen Biotechnik GmbH, Heidelberg, Germany), K14 (clone LL002, Cell Marque, Rocklin, CA), K18 (clone DC 10, Dako, Glostrup, Denmark), cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), FLAG-M2 or  $\beta$ -actin (Sigma-Aldrich).

**Immunostaining.** Normal oral epithelium covering the oral floor and the side edge of tongue taken from patients who underwent surgery for non-tumorous diseases or positioned at the far distal site from carcinomas that were histologically considered normal by a pathologist (HY) were used for controls. All tissues were obtained with a written consent of the patient and with approval by institutional review boards of the Nippon Dental University. Unstained formalin-fixed and paraffin-embedded sections were treated with microwave (500 W) in 0.01 M sodium citrate buffer, pH 6.0, and incubated with antibodies against K5, K8, K14 or K18 followed by biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). After treatment with avidin-biotin complexes (Vector Laboratories), the color was developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich).

**Proliferation assay.** The real-time cell electronic sensing assay based on electrical impedance readings in cell monolayers plated in wells containing built-in gold electrodes was performed. We have used the analyzer (xCELLigence RTCA-DP), 16-well E-plates and the integrated software (Roche Diagnostics GmbH). The RTCA-DP system works by measuring the electronic impedance at the cell-sensor electrode interface integrated on the bottom of E-plates. The *wtMALT1*HSC2 cells,  $\Delta$ *MALT1*HSC2 cells and *mock*HSC2 cells were plated at a density of  $1 \times 10^4$  cells/well installed on the analyzer. The analyzer and the installed plates were placed in a standard

cell culture incubator, at 37°C in a humidified atmosphere of 5% carbon dioxide and air. Cells were allowed to adhere to plates overnight and subjected to the analysis.

**Statistical analysis.** Doubling time of  $wtMALT1$ HSC2 cells,  $\Delta MALT1$ HSC2 cells and  $mock$ HSC2 cells were statistically analyzed by Kruskal-Wallis test using JMP 7.0.1 (SAS Institute Inc., Cary, NC).

## Results

**Identification of proteins differentially expressed in  $mock$ HSC2 cells and  $wtMALT1$ HSC2 cells.** Protein bands of  $mock$ HSC2 and  $wtMALT1$ HSC2 cell lysates were compared by 1-D SDS-PAGE. Sixteen bands were differentially detected between them (9 in  $mock$ HSC2 cells and 7 in  $wtMALT1$ HSC2 cells, Fig. 1), and subjected to the MALDI-TOF MS analysis. Six bands were not specified because of the non-satisfactory spectrum or the insufficient confidence of database screening to yield unambiguous results. Finally, 6 and 4 proteins were specified in  $mock$ HSC2 and  $wtMALT1$ HSC2 cells, respectively (Table I). It includes K5 and K14 in  $mock$ HSC2 cells and K8 and K18 in  $wtMALT1$ HSC2 cells. Since alterations of keratin expression associate with phenotypic changes of carcinoma cells (5,6), we focused on the keratin regulation by MALT1 in this study.

**Validation of keratin expression.** The differential keratin expression was validated for its protein and gene expressions. K5 and K14 proteins were predominantly expressed in  $mock$ HSC2 cells and negligibly in  $wtMALT1$ HSC2 cells (Fig. 2A). Suppression of K5 and K14 expression by MALT1 was supported by the increased expression in  $\Delta MALT1$ HSC2 cells. K8 was not detected in  $mock$ HSC2 and  $\Delta MALT1$ HSC2 cells but abundant in  $wtMALT1$ HSC2 cells. K18 was also strongly detected in  $wtMALT1$ HSC2 cells. The differential keratin expression was confirmed at the mRNA level (Fig. 2B). When compared to  $mock$ HSC2 cells, genes encoded by K5 (*KRT5*) and K14 (*KRT14*) were downregulated and upregulated in  $wtMALT1$ HSC2 cells and  $\Delta MALT1$ HSC2 cells, respectively. The  $wtMALT1$ HSC2 cells upregulated K8 (*KRT8*) and K18 (*KRT18*) expression and  $\Delta MALT1$ HSC2 cells downregulated them.

**MALT1-dependency of keratin alterations.** To exclude the possibility of long-term effect of MALT1 expression on the keratin expression, *MALT1* cDNA was transiently transfected into the parental HSC2 cells (Fig. 3). It dose-dependently downregulated K5 and K14 and upregulated K8 and K18. Since there is a possibility that the keratin alterations are specific to HSC2 cells, we transiently transfected *wtMALT1* and  *$\Delta MALT1$*  cDNA in a set of oral carcinoma cells (Fig. 4). In contrast to the predominant reduction of K5 and K14 expression by *wtMALT1* in most of carcinoma cells, *wtMALT1* upregulated K8/18, especially in oral floor carcinoma cells.

**Keratin expression in normal oral epithelium.** HSC2 cells and KOSC2 cells were established from carcinomas of the non-keratinized oral floor epithelial origin (18,19). Since the oral cavity is covered by non-keratinized and keratinized epithelium, keratin expression in non-keratinized oral floor and keratinized tongue epithelium were verified by the immunostaining. K8 and

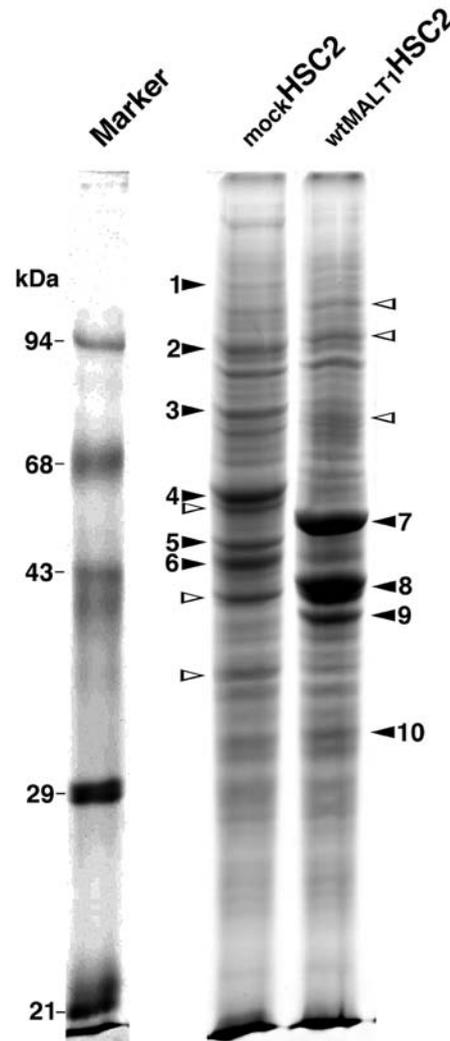


Figure 1. Protein expression in  $mock$ HSC2 cells and  $wtMALT1$ HSC2 cells. Total cell lysates of  $mock$ HSC2 cells and  $wtMALT1$ HSC2 cells were loaded on a 1-D SDS-PAGE gel under reducing conditions. Protein bands differentially detected between the cells indicated by arrowheads were subjected to MS analysis. Numbered closed arrowheads are specified proteins and open arrowheads are unambiguous proteins. The molecular marker proteins are phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and soya-bean trypsin inhibitor (21 kDa).

K18 were stained at the basal cells of oral floor epithelium but not the tongue epithelium, and K5 and K14 vice versa (Fig. 5). The sublingual gland staining confirmed a previous study (20); serous cells, intercalated and striated ducts were strongly positive for K8 and K18 and faintly for K5, and excretory ducts for K8, K5 and K14. Mucous cells were negative for these keratins.

**Reduction of cell proliferation by MALT1.** Since keratin substitution closely associates with proliferation status of squamous epithelial cells (21), effects of MALT1 on cell proliferation were examined using the real-time sensing RTCA-DP system. Fig. 6A illustrates the remarkable enhancement of  $\Delta MALT1$ HSC2 cell and suppression of  $wtMALT1$ HSC2 cell proliferation compared to the  $mock$ HSC2 cells. Doubling time of  $mock$ HSC2 cells was  $11.1 \pm 0.3$  h, and  $\Delta MALT1$ HSC2 cells and  $wtMALT1$ HSC2

Table I. Lists of identified proteins differentially expressed in  $w_t$ MALT1 HSC2 and  $mock$  HSC2 cells.

Protein no.	Accession no. <sup>a</sup>	Annotation	Mr (kDa)		Coverage (%) <sup>d</sup>	MS score <sup>e</sup>	Authors/(Refs.) <sup>f</sup>
			Theor. <sup>b</sup>	Obs. <sup>c</sup>			
<i>mock</i> HSC2 cells							
1	Q8N1G0	Zinc finger protein 687	115.6	129.5	16	27	Malovannaya <i>et al</i> (38)
2	Q8NB90	Spermatogenesis associated 5	97.7	97.9	25	32	Heallen <i>et al</i> (39)
3	P11021	GRP78 precursor	72.1	72.3	38	85	Dong <i>et al</i> (40)
4	P13647	Keratin 5	62.4	62.4	27	64	
5	P02533	Keratin 14	44.7	51.6	23	38	
6	43502	RAD51 homolog C	42.6	42.2	23	47	Clague <i>et al</i> (41)
$w_t$ MALT1 HSC2 cells							
7	Q6GMYO	Keratin 8	53.4	53.7	34	61	
8	PO5783	Keratin 18	47.3	48.1	38	52	
9	P38159	RNA-binding motif protein	42.3	44.3	43	40	Tsuei <i>et al</i> (42)
10	Q02978	Solute carrier family 25	34.1	34.1	26	28	Zhong <i>et al</i> (43)

<sup>a</sup>Uniprot (www.uniprot.org) accession number. <sup>b</sup>Theoretical Mr (kDa). <sup>c</sup>Observed Mr (kDa) in 1-D SDS-PAGE. <sup>d</sup>Number of sequence coverage in MALDI-TOF MS. <sup>e</sup>MASCOT score. <sup>f</sup>References except for keratins.

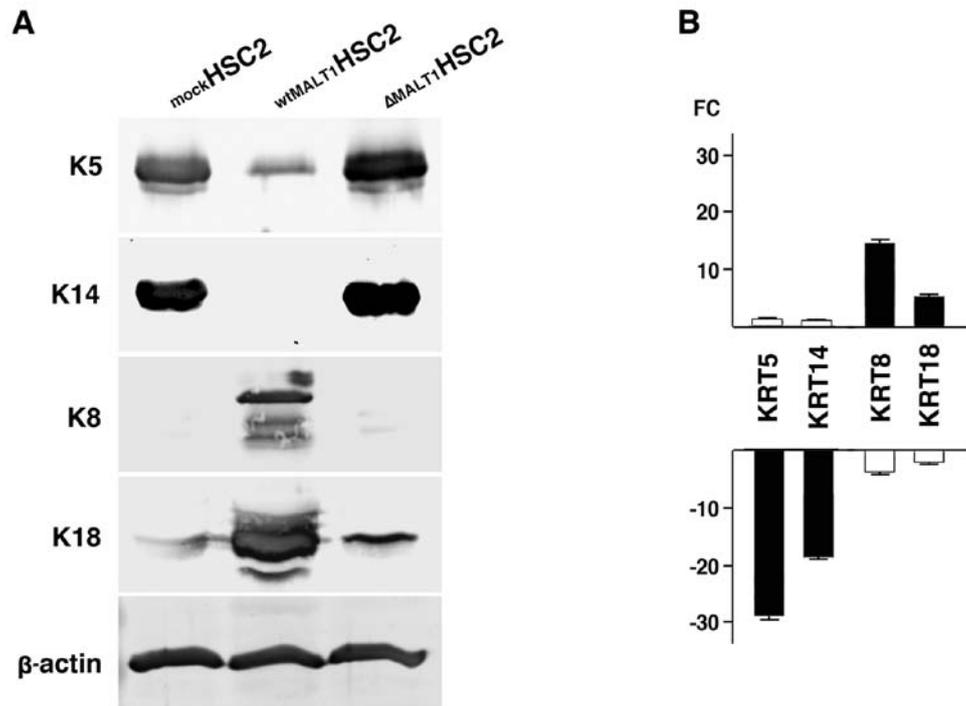


Figure 2. Validation of keratin expression in HSC2 cells. (A) Expression of K5, K14, K8 and K18 in  $mock$  HSC2 cells,  $w_t$ MALT1 HSC2 cells and  $\Delta$ MALT1 HSC2 cells were validated by immunoblotting.  $\beta$ -actin was used for an internal control. (B) Relative fold of expression of genes encoding K5 (*KRT5*), K14 (*KRT14*), K8 (*KRT8*) and K18 (*KRT18*) in  $w_t$ MALT1 HSC2 cells (closed bars) and  $\Delta$ MALT1 HSC2 cells (open bars) were compared with  $mock$  HSC2 cells by quantitative real-time PCR (n=4). The expression was standardized by expression level of *GAPDH* in each sample.

were  $8.4 \pm 0.5$  and  $21.3 \pm 2.1$  h, respectively ( $P < 0.01$ ). Decreased proliferation of  $w_t$ MALT1 HSC2 cells was not due to the cell death because they did not increase the TUNEL reactivity (Fig. 6B)

and the trypan blue staining (data not shown). The  $w_t$ MALT1 HSC2 cells ceased cyclin D1 expression but restored it by the siRNA against MALT1 in a dose-dependent manner (Fig. 6C and D).

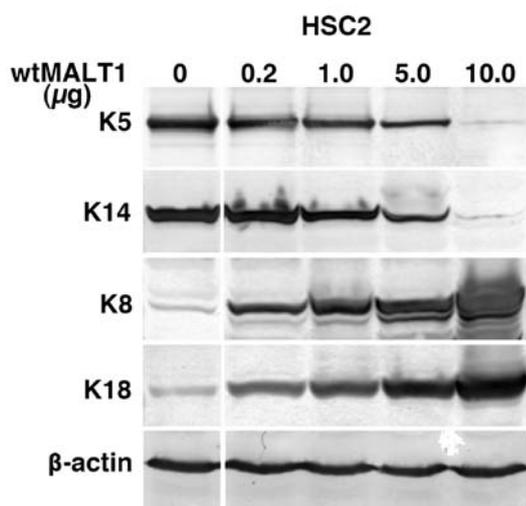


Figure 3. Dose-dependent expression of keratins. HSC2 cells transiently transfected with different doses of *wtMALT1* cDNA were examined for keratin expression by immunoblot analysis.  $\beta$ -actin was used for an internal control.

The  $\Delta$ MALT1 HSC2 cells and *mock* HSC2 cells did not respond to the siRNA because of the lack of siRNA-binding site in a  $\Delta$ MALT1 gene construct and the marginal expression of endogenous *MALT1*, respectively.

## Discussion

MALT1 is expressed in normal epithelial cells of the oral cavity and the loss of expression closely associates with carcinoma progression in an unknown mechanism (12). Identifying protein expression and cellular phenotype under the control of MALT1 contributes to the understanding of its role. We report that the loss of expression stimulates K5/14 expression and proliferation of oral carcinoma cells and decreases K8/18 expression.

We analyzed proteins that were differentially expressed in *wtMALT1* HSC2 cells and *mock* HSC2 cells by the MS analysis, and detected keratins and other proteins involved in gene transcription, and proliferation, chemo-resistance and development of tumors (Table I). It is noteworthy that 4 different keratins

were included. Since K8/18 and K5/14 are primary pairs, it is reasonable to hypothesize that MALT1 affects keratin filament organization. Although K8 and K18 are known as simple epithelial keratins and not expressed in keratinized epithelium (3), non-keratinized squamous epithelium of the esophagus expresses them in the basal cells (22). Oral floor epithelium shares its histological characteristics with the esophagus (23). Keratin expression in non-keratinized oral floor epithelium is largely different from that in keratinized oral epithelium (24), and HSC2 cells were established from an oral floor carcinoma (18). K8/18 and K5/14 expression and localization in oral floor epithelium has not been clearly documented, and K5 and K14 expression in non-keratinized epithelium is a controversial issue. We immunostained them in oral floor epithelium and tongue epithelium that is juxtaposed to oral floor. K8 and K18 were positively stained in the basal cells of oral floor epithelium and K5 and K14 at that of tongue epithelium. The staining patterns in the sublingual gland strictly confirmed a previous study (20), indicating specific reactions of the staining. These data show that K8/18 and K5/14 expression in non-keratinized and keratinized oral epithelium are largely different.

K5 and K14 reduction was a MALT1 dose-dependent in HSC2 cells and observed in most of the carcinoma cell lines, indicating that K5 and K14 repression by MALT1 is a prevalent feature in oral carcinoma cells. They are expressed in mitotically active basal cells of oral epithelium (4), upregulated in oral carcinomas (25), and downregulated upon differentiation of carcinoma cells (21). The K14 knockdown initiates epithelial differentiation marker expression including involucrin and K1 and suppresses carcinoma cell proliferation and tumorigenicity (21). We previously showed that MALT1 upregulates involucrin and K10, a primary partner of K1, and downregulates vimentin, a mesenchymal cell-type intermediate filament (12). The present study demonstrated the reduction of proliferation by MALT1; 1.92-fold increase and 1.33-fold decrease of doubling time of *wtMALT1* HSC2 cells and  $\Delta$ MALT1 HSC2 cells, respectively. Decreased proliferation of *wtMALT1* HSC2 cells associated with the dramatic reduction of cyclin D1 expression that was recovered by transfection of *MALT1*-siRNA in a dose-dependent manner. These facts underscore an involvement of loss of MALT1 expression in K5 and K14 upregulation that stimulates de-differentiation and proliferation of oral carcinoma cells.

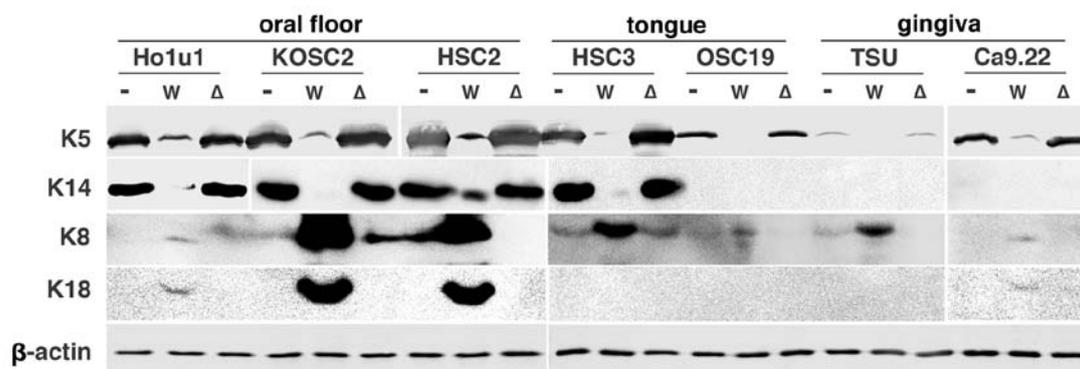


Figure 4. Effect of MALT1 on keratin expression in various oral carcinoma cells. Seven different oral carcinoma cell lines were transiently transfected vector alone (-),  $\Delta$ MALT1 ( $\Delta$ ) or *wtMALT1* cDNA (w, 10  $\mu$ g) and subjected to immunoblotting for K5, K14, K8 and K18.  $\beta$ -actin was used for an internal control.

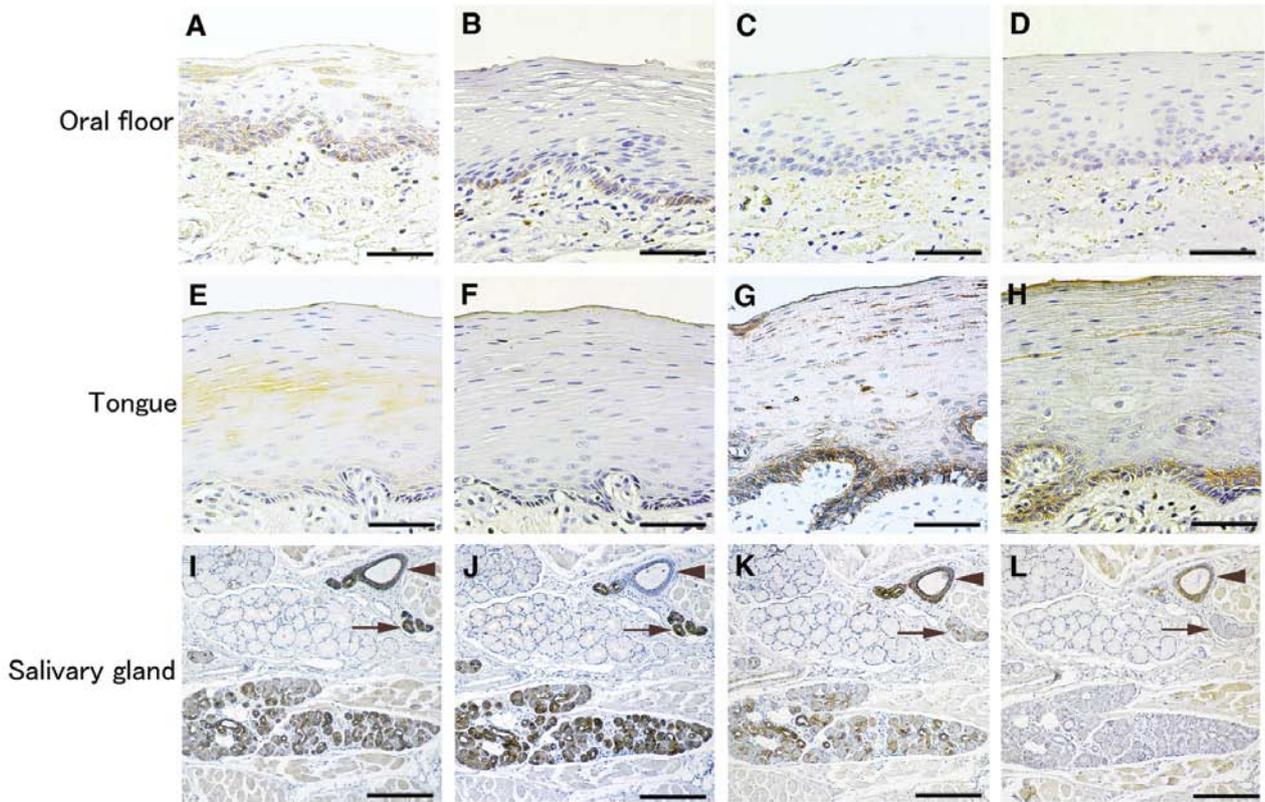


Figure 5. Expression of keratins in normal oral floor and gingival epithelium. Oral epithelium covering the oral floor (non-keratinized epithelium), the tongue edge (keratinized epithelium) and sublingual gland were immunostained for K8 (A, E, I), K18 (B, F, J), K5 (C, G, K) and K14 (D, H, L). K8 and K18 were localized at basal cells of oral floor and K5 and K14 at that of tongue. In the sublingual gland, keratin localization confirmed a previous study (Azevedo *et al* 20). Arrows and arrowheads point to excretory ducts and striated ducts, respectively. Bar, 35 μm (A-H) and 70 μm (I-L).

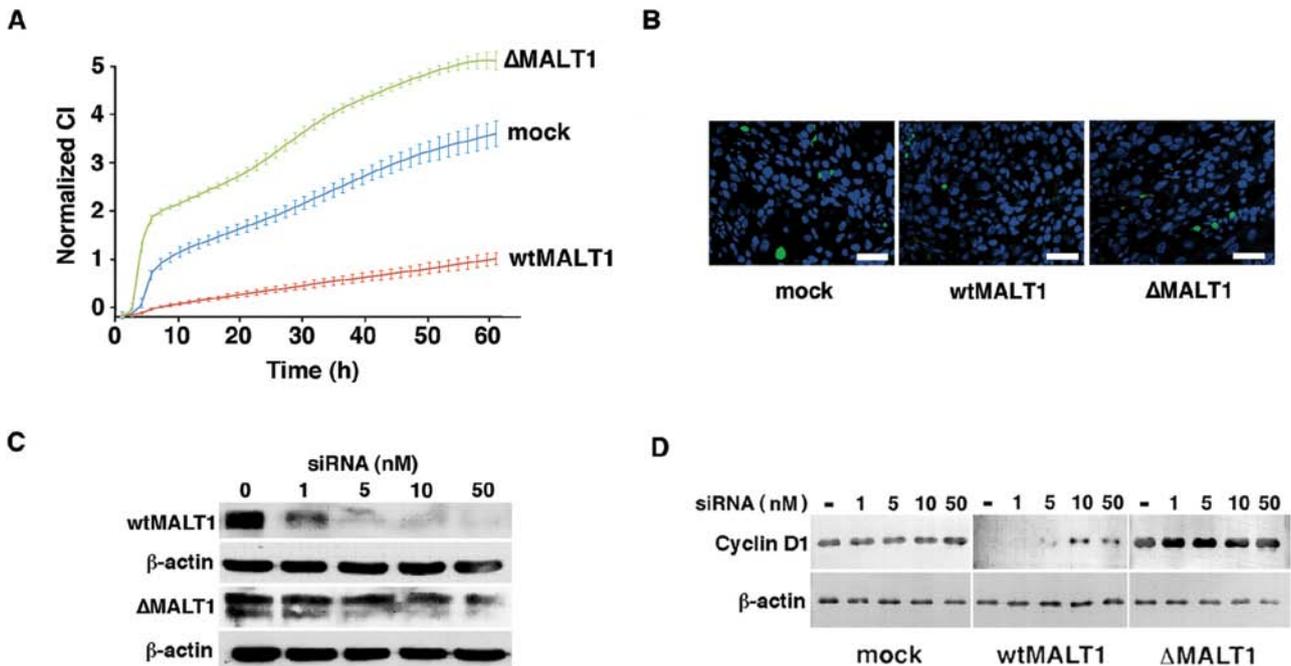


Figure 6. Proliferation of HSC2 cells stably expressing the *MALT1* constructs. (A) Real-time monitoring of proliferation of  $_{\text{mock}}$ HSC2 cells,  $_{\text{wtMALT1}}$ HSC2 cells and  $_{\Delta\text{MALT1}}$ HSC2 cells. Normalized cell index (CI) indicated electrical impedance readings between E-plates and attached cells. Mean value for each cell (n=5) is presented as SD at every 10 min. (B) Apoptosis analysis of  $_{\text{mock}}$ HSC2 cells,  $_{\text{wtMALT1}}$ HSC2 cells and  $_{\Delta\text{MALT1}}$ HSC2 cells by TUNEL assay. (C) Knockdown of MALT1 protein expression in  $_{\text{wtMALT1}}$ HSC2 cells and  $_{\Delta\text{MALT1}}$ HSC2 cells by the siRNA. The *wtMALT1* and  $\Delta$ MALT1 protein expression was probed by immunoblot analysis with an anti-FLAG antibody.  $\beta$ -actin is shown as the internal control. (D) Cyclin D1 expression in  $_{\text{mock}}$ HSC2 cells,  $_{\text{wtMALT1}}$ HSC2 cells and  $_{\Delta\text{MALT1}}$ HSC2 cells. Cyclin D1 in response to MALT1 siRNA dose-dependency was analyzed by immunoblotting.  $\beta$ -actin indicates an internal control.

MALT1 strongly upregulated K8 and K18 in oral floor carcinoma cells and faintly in keratinized oral epithelium-derived carcinoma cells. Forced expression of K18 in breast carcinoma cells, which are originated from the most representative K8/18-positive simple epithelial cells, reduces proliferation and aggressive behavior of carcinoma cells *in vitro* and in mice (11,26,27). Reduction of K8/18 expression actively renders the aggressive properties to carcinoma cells (11). Although the pathological contribution of K8/18 to oral carcinomas is controversial (28,29), this study demonstrated the K8/18 upregulation and the K5/14 downregulation by MALT1 that is expressed at the early stage of carcinomas and inactivated at the late stage (12).

K5/14-positive breast carcinomas exhibit worse pathological grades, and patient survival than the K8/18-positive carcinomas (30). Expression of K5/14 is an independent risk factor for worse prognosis of oral carcinomas (31). Although a molecular mechanism for MALT1-dependent keratin alteration is uncertain, our recent microarray analysis indicated that MALT1 preferentially downregulates EGF and TGF- $\beta$  pathway gene expression (32). EGF and TGF- $\beta$  signaling suppress K8/18 expression and stimulate K5/14 expression, provoking proliferative and aggressive behavior of carcinoma cells (33-37). Loss of MALT1 expression initiates the K8/18-to-K5/14 alteration with enhanced proliferation and may prompt aggressive behavior of oral carcinomas toward worse prognosis. Future studies on the molecular action of MALT1 is required to extend the understanding of pathophysiology of oral carcinoma progression.

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### References

- Choi S and Myers JN: Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. *J Dent Res* 87: 14-32, 2008.
- Hanahan D and Weinberg RA: Hallmarks of cancer: the next generation. *Cell* 144: 646-674, 2011.
- Schweizer J, Bowden PE, Coulombe PA, Langbein L, Lane EB, Magin TM, Taltais L, Omary MB, Parry DA, Rogers MA and Wright MW: New consensus nomenclature for mammalian keratins. *J Cell Biol* 174: 169-174, 2006.
- Bosh FX, Ouhayoun JP, Bader BL, Collin C, Grund C, Lee I and Franke WW: Extensive changes in cytokeratin expression patterns in pathologically affected human gingiva. *Virchows Archiv B Cell Pathol* 58: 59-77, 1989.
- Karantza V: Keratins in health and cancer: more than mere epithelial markers. *Oncogene* 30: 127-138, 2011.
- Kurokawa I, Takahashi K, Moll I and Moll R: Expression of keratins in cutaneous epithelial tumors and related disorders - distribution and clinical significance. *Exp Dermatol* 20: 217-228, 2011.
- Coulombe PA and Omary MB: 'Hand' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr Opin Cell Biol* 14: 110-122, 2002.
- Oshima RG: Apoptosis and keratin intermediate filaments. *Cell Death Differ* 9: 486-492, 2002.
- Pan X, Hobbs RP and Coulombe PA: The expanding significance of keratin intermediate filaments in normal and disease epithelia. *Curr Opin Cell Biol* 25: 47-56, 2013.
- Kim S, Wong P and Coulombe PA: A cytoskeletal protein regulates protein synthesis and epithelial cell growth. *Nature* 441: 362-365, 2006.
- Fortier AM, Asselin E and Cardin M: Keratin 8 and 18 loss in epithelial cancer cells increases collective cell migration and cisplatin sensitivity through claudin 1 up-regulation. *J Biol Chem* 288: 11555-11571, 2013.
- Chiba T, Maeda G, Kawashiri S, Kato K and Imai K: Epigenetic loss of mucosa-associated lymphoid tissue 1 expression in patients with oral carcinomas. *Cancer Res* 69: 7216-7223, 2009.
- Thome M: Multifunctional roles of MALT1 in T-cell activation. *Nat Rev Immunol* 8: 495-508, 2008.
- McAllister-Lucas LM, Baens M and Lucas PC: MALT1 protease: a new therapeutic target in B lymphoma. *Clin Cancer Res* 17: 6623-6631, 2011.
- Schneider CA, Rasband WS and Eliceiri KW: NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9: 671-675, 2012.
- Perkins DN, Pappin DJC, Creasy DM and Cottrell JS: Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20: 3351-3367, 1999.
- Schmittgen DD and Livak KJ: Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3: 1101-1108, 2008.
- Momose F, Araida T, Negishi A, Ichijo H, Shioda S and Sasaki S: Variant sublines with different metastatic potentials selected in nude mice from human oral squamous cell carcinomas. *J Oral Pathol Med* 18: 391-395, 1989.
- Inagaki T, Matsuware S, Takahashi R, Shimada K, Fujie K and Maeda S: Establishment of human oral-cancer cell lines (KOSC-2 and -3) carrying p53 and c-myc abnormalities by genetic treatment. *Int J Cancer* 56: 301-308, 1994.
- Azevedo RS, De Almeida OP, Kowalski LP and Pires FR: Comparative cytokeratin expression in the different cell types of salivary gland mucoepidermoid carcinoma. *Head Neck Pathol* 2: 257-264, 2008.
- Alam H, Sehgal, Kundu ST, Dalal S and Vaidya MM: Novel function of keratins 5 and 14 in proliferation and differentiation of stratified epithelial cells. *Mol Biol Cell* 22: 4068-4078, 2011.
- Bosh FX, Leube RE, Achtstatter T, Moll R and Franke WW: Expression of simple epithelial type cytokeratins in stratified epithelia as detected by immunolocalization and hybridization *in situ*. *J Cell Biol* 106: 1635-1648, 1998.
- Squier CA and Kremer MJ: Biology of oral mucosa and esophagus. *J Natl Cancer Inst Monogr* 29: 7-15, 2001.
- Clausen H, Moe D, Buschard K and Dabelsteen E: Keratin proteins in human oral mucosa. *J Oral Pathol* 15: 36-42, 1986.
- Thiel UJE, Feltens R, Adryan B, Gieringer R, Brochhausen C, Schyon R, Fillies T, Grus F, Mann WJ and Brieger J: Analysis of differentially expressed proteins in oral squamous cell carcinoma by MALDI-TOF MS. *J Oral Pathol Med* 40: 369-379, 2011.
- Iyer SV, Dange PP, Alam H, Sawant SS, Ingle AD, Borges AM, Shirsat NV, Dalal SN and Vaidya MM: Understanding the role of keratins 8 and 18 in neoplastic potential of breast cancer derived cell lines. *PLoS One* 8: e53532, 2013.
- Buhler H and Schaller G: Transfection of keratin 18 gene in human breast cancer cells causes induction of adherent proteins and dramatic regression of malignancy *in vitro* and *in vivo*. *Mol Cancer Res* 3: 365-371, 2005.
- Matthias C, Mack B, Berghaus A and Gires O: Keratin 8 expression in head and neck epithelia. *BMC Cancer* 8: 267, 2008.
- Imai K, Kumagai S, Nakagawa K, Yamamoto E, Nakanishi I and Okada Y: Immunolocalization of desmoglein and intermediate filaments in human oral squamous cell carcinomas. *Head Neck* 17: 204-212, 1995.
- Abd El-Rehim DM, Pinder SE, Paish CE, Bell J, Blamey RW, Robertson JFR, Nicholson R and Ellis IO: Expression of luminal and basal cytokeratins in human breast carcinoma. *J Pathol* 203: 661-671, 2004.
- Garrel R, Dromard M, Costes V, Barbotte E, Comte F, Gardiner Q, Cartier C, Makeieff M, Crampette L, Guerrier B and Boule N: The diagnostic accuracy of reverse-transcription PCR quantification of cytokeratin mRNA in sentinel lymph node invasion in oral and oropharyngeal squamous cell carcinoma: a comparison with immunohistochemistry. *Clin Cancer Res* 12: 2498-2505, 2006.
- Ohyama Y, Kawamoto Y, Chiba T, Yagishita H, Sakashita H and Imai K: Inhibition of TGF- $\beta$  and EGF pathway gene expression and migration of oral carcinoma cells by mucosa-associated lymphoid tissue 1. *BrJ Cancer* (In press).

33. Zeineldin R, Rosenberg M, Ortega D, Buhr C, Chavez MG, Stack MS, Kusewitt DF and Hudson LG: Mesenchymal transformation in epithelial ovarian tumor cells expressing epidermal growth factor receptor variant III. *Mol Carcinog* 45: 851-860, 2006.
34. Kinouchi M, Takahashi H, Itoh Y, Ishida-Yamamoto A and Iizuka H: Ultraviolet B irradiation increase keratin 5 and keratin 14 expression through epidermal growth factor receptor of SV40-transformed human keratinocytes. *Arch Dermatol Res* 293: 634-641, 2002.
35. Jiang CK, Tomic-Canic M, Lucas DJ, Simon M and Blumenberg M: TGF beta promotes the basal phenotype of epidermal keratinocytes: transcriptional induction of K#5 and K#14 keratin gene. *Growth Factor* 12: 87-97, 1995.
36. Shukla A, Ho Y, Liu X, Ryscavage A and Glick AB: Cripto-1 alters keratinocyte differentiation via blockade of transforming growth factor-beta 1 signaling: role in skin carcinogenesis. *Mol Cancer Res* 6: 509-516, 2008.
37. Zhang Q, Helfand BT, Jang TL, Zhu LJ, Chen L, Yang XJ, Kozlowski J, Smith N, Kundu SD, Yang G, Raji AA, Javonovic B, Pins M, Lindholm P, Guo Y, Catalona WJ and Lee C: Nuclear factor- $\kappa$ B-mediated transforming growth factor- $\beta$ -induced expression of vimentin is an independent predictor of biochemical recurrence after radical prostatectomy. *Clin Cancer Res* 15: 3557-3567, 2009.
38. Malovannaya A, Lanz RB, Jung SY, Bulyanko Y, Le NT, Chan DW, Ding C, Yucer N, Krenciute G, Kim BJ, Li C, Chen R, Li W, Wang Y, O'Malley BW and Qin J: Analysis of the human endogenous coregulator complexome. *Cell* 145: 787-799, 2011.
39. Heallen TR, Adams HP, Furuta T, Verbrugge KJ and Schumacer JM: An Afg2/Spaf-related Cdc48-like AAA ATPase regulates the stability and activity of the *c. elegans* Aurora B kinase AIR-2. *Dev Cell* 15: 603-616, 2008.
40. Dong D, Stapleton C, Luo B, Xiong S, Ye W, Zhang Y, Jhaveri N, Zhu G, Ye R, Liu Z, Bruhn KW, Craft N, Groshen S, Hofman FM and Lee AS: A critical role of GRP78/BiP in the tumor microenvironment for neovascularization during tumor growth and metastasis. *Cancer Res* 71: 2848-2857, 2011.
41. Clague J, Wilhoite G, Adamson A, Bailis A, Weitzel JN and Neuhausen SL: RAD51C germline mutations in breast and ovarian cancer cases from high-risk families. *PLoS ONE* 6: e25632, 2011.
42. Tsuei DJ, Hsu HC, Lee PH, Jeng YM, Pu YS, Chen CN, Lee YC, Chou WC, Chang CJ, Ni YH and Chang JMH: RBMY, a male germ cell-specific RNA-binding protein, activated in human liver cancers and transforms rodent fibroblasts. *Oncogene* 23: 5815-5822, 2004.
43. Zhong Q, Putt DA, Xu F and Lash LH: Hepatic mitochondrial transport of glutathione: studies in isolated rat liver mitochondria and H4IIE rat hepatoma cells. *Arch Biochem Biophys* 474: 119-127, 2008.