Inhibition of TGF-β and EGF pathway gene expression and migration of oral carcinoma cells by mucosa-associated lymphoid tissue 1

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Background: Expression of mucosa-associated lymphoid tissue 1 (MALT1) is inactivated in oral carcinoma patients with worse prognosis. However, the role in carcinoma progression is unknown. Unveiling genes under the control of MALT1 is necessary to understand the pathology of carcinomas.

Methods: Gene data set differentially transcribed in MALT1-stably expressing and -marginally expressing oral carcinoma cells was profiled by the microarray analysis and subjected to the pathway analysis. Migratory abilities of cells in response to MALT1 were determined by wound-healing assay and time-lapse analysis.

Results: Totally, 2933 genes upregulated or downregulated in MALT1-expressing cells were identified. The subsequent pathway analysis implicated the inhibition of epidermal growth factor and transforming growth factor-β signalling gene expression, and highlighted the involvement in the cellular movement. Wound closure was suppressed by wild-type MALT1 (66.4%) and accelerated by dominant-negative MALT1 (218.6%), and the velocities of cell migration were increased 0.2-fold and 3.0-fold by wild-type and dominant-negative MALT1, respectively.

Conclusion: These observations demonstrate that MALT1 represses genes activating the aggressive phenotype of carcinoma cells, and suggest that MALT1 acts as a tumour suppressor and that the loss of expression stimulates oral carcinoma progression.

Squamous cell carcinoma is a most common malignant neoplasm of the oral cavity. The patient prognosis is still worse than that of all cancers combined, and the annual incidence of new cases is predicted to increase in the next few decades (Choi and Myers, 2008; Siegel et al, 2012). Phenotypic alterations of carcinoma cells result from the aberrations of endogenous and exogenous factors, and select aggressive clones to progress carcinomas to the more advanced states. Understanding molecular mechanisms of carcinoma progression and developing the new therapeutic approach are prerequisite for the improvement of prognosis.

Oral carcinoma cells frequently inactivate tumour-suppressive proteins during the disease progression (Choi and Myers, 2008). Our previous study demonstrated that mucosa-associated lymphoid tissue 1 (MALT1) is expressed in the nucleus of oral epithelial cells (Chiba et al, 2009) and substitutes keratins depending on its expression (Kawamoto et al, 2013). The advanced carcinomas inactivate MALT1 expression by the promoter methylation, and the loss of expression worse the patient prognosis (Chiba et al, 2009). However, nothing is known about the role of loss of expression in the carcinoma progression at present. The MALT1 is made up of three types of domain: a death domain, Ig-like domains, and a caspase-like domain. The B-cell or T-cell receptor antigen signals oligomerise MALT1 with BCL10 and CARMA1/3 into a CBM complex. The MALT1 interacts with BCL10 through its Ig-like domains and induces IκB-kinase catalytic activity, resulting in nuclear factor-κB (NF-κB) activation in lymphocyte lineages (Thome, 2008; McAllister-Lucas et al, 2011). Unveiling gene data sets that are regulated by MALT1
substantiates the role of loss of expression in the carcinoma progression. In this study, we investigated the gene data sets and pathways by microarray analysis and determined the effect on carcinoma cell phenotype by migration assays.

**MATERIALS AND METHODS**

**Cell lines.** HSC2 oral carcinoma cells, which marginally express MALT1, were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). HSC2 cells stably expressing FLAG-tagged full-length wild-type MALT1 (MALT1HSC2 cells) and the NH2 terminal death and Ig-like domains-deleted dominant-negative MALT1 (ΔMALT1HSC2 cells; Che et al, 2004) and transfected vector alone (mockHSC2 cells) were established previously (Chiba et al, 2009). They were maintained in 10% fetal bovine serum and 100 units per ml of penicillin/streptomycin-containing DMEM (Sigma-Aldrich, St. Louis, MO, USA) in a conventional 5% CO2 incubator.

**Microarray analysis.** Gene-expression profile was measured with RNA extracted from wtMALT1HSC2 cells and mockHSC2 cells and processed for aRNA synthesis, labelling, and hybridisation by Mitsubishi Chemical Mediscience, Inc. (Tokyo, Japan). First-strand cDNA synthesis with 100 ng of total RNA, synthesis of bioinn-labelled aRNA, and clean up were carried out using GeneChip 3’IVT Express Kit (Affymetrix Japan, Tokyo, Japan). For hybridisation, 15 μg of fragmented aRNA was incubated with GeneChip Human Genome U133A 2.0 in 250 μl of hybridisation solution in GeneChip Hybridization Oven 640 (Affymetrix Japan) at 45 °C for 16 h. GeneChips were then washed and stained with GeneChip Fluidics Station 450 (Affymetrix Japan) according to manufacturer’s instruction. Microarrays were scanned with GeneChip Scanner 3000 (Affymetrix Japan), and the signals were processed using GeneChip Command Console Software (AGCC, Affymetrix Japan). The scanned images were first assessed by visual inspection, then analysed to generate raw data files saved as CEL files using the default setting of AGCC. Robust Multichip Analysis was used to normalise the different arrays, and further analysis was carried out with GeneSpring GX10 (version 11.5.1, Agilent Technologies, Inc., Tokyo, Japan). Three replicates were used for microarray analysis and statistics. To find differentially expressed genes, we used Aspin-Welch’s paired t-test and considered the significance when the P-value was below 0.05. The Benjamini–Hochberg false discovery rate (FDR) method was used to correct for multiple testing effect. Probe sets were considered to have changed qualitatively in a specific comparison if an adjusted P-value of 0.05 was obtained. Their normalised data were used to generate a list of differentially expressed genes between wtMALT1HSC2 cells and mockHSC2 cells when they had an absolute change of ≥2-fold. Gene-expression changes that met these criteria were termed as ‘significant RNA state change’ and had directional qualities of ‘up’ or ‘down’ (i.e., they can be upregulated or downregulated in wtMALT1HSC2 cells). The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (Edgar et al, 2002) and are accessible through GEO Series accession number GSE42335 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42335).

**Functional and pathway analyses of differentially expressed genes.** The differentially expressed gene list was ranked according to their fold changes and compared with sets of genes that were annotated according to the gene ontology (GO) for molecular function, cellular component, and biological process (Ashburner et al, 2000). Results with P-value ≤0.05 and FDR were used to identify canonical pathways associated with the differentially expressed genes by uploading the list into the Ingenuity Pathway Analysis (IPA) website (version 9.0, http://www.ingenuity.com). Briefly, the Ingenuity Knowledge base contains information from scientific publications regarding direct and indirect relationships between genes and proteins. Each identifier was mapped to its corresponding gene object in the Ingenuity knowledge base. These genes, called focus genes, were overlaid onto a global molecular network in the Ingenuity knowledge base. Networks of these focus genes were then algorithmically generated based on the information available in the IPA web site. All of the differentially expressed genes were included in the analysis, and ranked according to P-value (Mori et al, 2009). In figures represented in this study, the upregulated genes were marked with red and the downregulated genes with green with the intensity of the colour being an indicator of the fold of expression.

**Real-time PCR.** Total RNA extracted from cell lines was reverse transcribed to cDNA by MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) and subjected to real-time PCR using the StepOne Real-time PCR system (Applied Biosystems). PCR conditions were 95 °C for 20 s followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The TaqMan probes (Applied Biosystems) specific for LNX (Hs00220138_m1), TFF1 (Hs00907239_m1), GATA3 (Hs00231122_m1), TP53 (Hs01034249_m1), CDH1 (Hs00174360_m1), FABP4 (Hs01086177_m1), FABP6 (Hs01031183_m1), VEGFC (Hs00153458_m1), SNAIL2 (Hs00950344_m1), HMGA2 (Hs00171569_m1), IL6ST (Hs00174360_m1), CDH2 (Hs00983062_m1), PXN (Hs01104424_m1), and IL11 (Hs01058413_g1) were used. Expression levels were normalised against ACTB (TaqMan Endogenous Control Human ACTB; Applied Biosystems). Levels of gene expression (2−DDCt) were determined by the standard curve method (Schmittgen and Livak, 2008).

**Immunoblot.** Total cell lysates were used for the immunoblot with a standard protocol. The lysates in the SDS sample buffer containing 1 mM phenylmethylsulphonyl fluoride and a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) were size fractionated by SDS–PAGE gels under reducing conditions and electrotransferred onto PVDF membranes. The membrane was probed with antibodies specific to TFF1, GATA3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), LNX (R&D Systems, Minneapolis, MN, USA), FABP4, FABP6, and β-actin (Sigma-Aldrich).

**Wound-healing and migration assays.** Subconfluent cell monolayer (80–90% confluency) with or without MALT1 short-interfering RNA (siRNA) transfection (50 nM #18601 siRNA; Ambion, Austin, TX, USA) was maintained in 1% fetal bovine serum-containing culture medium. Silencer Negative Control 1 siRNA (Ambion) was used as a negative control. Wounds were created by the scratch using a pipettman tip and cultured up to 48 h. The wound closure was evaluated by measuring the width of the remaining wound (Sossey-Alaoui et al, 2005). 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 System works by measuring the electronic impedance at the cell-sensor electrode interface integrated on the bottom of e-plates (Yu et al, 2006). For time-lapse microscopy, wtMALT1HSC2 cells, ΔMALT1HSC2 cells, and mockHSC2 cells were labelled with CellTracker Green Fluorescent Probe (Lonza, Walkersvill, MD USA) and seed onto 35-mm plastic dishes. After 10 h, the single cell migration was monitored using a laser scanning microscope (LSM 700; Carl Zeiss, Oberkochen, Germany) equipped with a transparent environmental chamber (Climabox, Carl Zeiss) under 5% CO2 in air at 37 °C. The microscope was driven by the IMARIS (Carl Zeiss) every 10 min
during 16 h \( (n=4) \). Cell migration was characterised and quantified using an interactive tracking method (Zahm et al, 1997).

**Statistical analysis.** Statistical analyses on the data manipulation for microarray analysis were indicated above. Percentage of wound closure and migration velocities was analysed by Wilcoxon test using JMP 7.0.1 (SAS Institute Inc., Cary, NC, USA).

### RESULTS

**Genes regulated by MALT1.** Genes expressed in \( \text{wtMALT1HSC2} \) cells and \( \text{mockHSC2} \) cells were initially compared by the microarray analysis, and 2933 genes with a fold change of \( \geq 2.0 \) and with the \( P<0.05 \) were specified (Figure 1A). They included 1433 upregulated and 1500 downregulated genes in \( \text{wtMALT1HSC2} \) cells. Strict filtering of normalised intensity value with a fold change of \( \geq 10.0 \) (415 probe sets) showed that the upregulated genes \( (n=328) \) fold change, \( 120.5 \pm 580.2 \), mean \( \pm \) s.d. \) were more robustly changed than the downregulated genes \( (n=87, 21.2 \pm 16.0, P=0.002; \) Figure 1B). Top 10 for each was shown in Table 1 (complete list was shown in Supplementary Table 1).

**Validation of microarray data.** Quantitative real-time PCR was done to validate the genes expressed at various levels, and confirmed the expression in an almost same pattern of the microarray analysis (Figure 1C). The differential protein expression encoded by representative genes that suppress \( (\text{LNX, TFF1, and GATA3}) \) and stimulate \( (\text{FABP4 and FABP6}) \) carcinoma progression \( \text{(Ohmachi et al, 2006; Kouro} \text{s-Mehr et al, 2008; Li et al, 2011; Nieman et al, 2011; Sou} \text{tto et al, 2011)} \) was substantiated at the protein level (Figure 1D). Although expression of other genes was not validated in this study, many of downregulated genes associate with oral carcinoma progression such as \( \text{TWIST1, LMO4, ZEB2, WNT3, and MMP14 (Shimada et al, 2000; Mizunuma et al, 2003; Uraguchi et al, 2004; Maeda et al, 2005; Okamura et al, 2009)} \).

**GO and pathway analyses.** To analyse the function of MALT1-responsive genes, all data sets of genes were first evaluated by the GO analysis. It suggested that the genes are involved in inter- and intra-cellular signalling, cellular organisation, and developmental processes (Table 2). The IPA top three bio-functions were 'Cancer', 'Cellular Movement', and 'Gastrointestinal Disease', and canonical pathways were 'HER2-Signalling in Breast Cancer', 'Glioma Invasiveness Signalling', and 'Role of Tissue Factor in Cancer' (Figure 2). The list with \( P<0.05 \) and threshold value of \( –\log (P\text{-value}), 0.05 \), were summarised in Supplementary Table 2. Other classification of our interests that were not included in the GO analysis but closely correlated with carcinoma progression including extracellular matrix-degrading enzymes, collagens, laminins, integrins, WNT pathways, and cancer and/or epidermal stem cell markers were listed in Supplementary Table 3.

The pathway analysis was performed on all genes identified with an absolute change of \( \geq 2\text{-fold} \). Since the IPA showed a most close association of the gene data sets to 'Cancer', we overviewed 'Molecular Mechanisms of Cancer' to identify the signalling pathway network affected by MALT1. Ras and SMAD pathways occupied central positions in the network (Supplementary Figure 1).

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**Figure 1.** Microarray analysis and its validation by real-time PCR and immunoblot. Log2 fold-change (FC) and their corresponding –log10(P-value) of all genes in the microarray were taken for construction of the volcano plot. Genes upregulated and downregulated >2.0-fold change with a \( P\text{-value} <0.05 \) were depicted in light red and green, respectively. All other genes that were not significantly altered were in black (A). Normalised intensity value of gene data set in \( \text{mockHSC2} \) cells (m) and \( \text{wtMALT1HSC2} \) cells (w) with different FCs (i, \( \geq 2.0 \); ii, \( \geq 4.0 \); iii, \( \geq 10.0 \)) was illustrated. Genes upregulated and downregulated in \( \text{wtMALT1HSC2} \) cells were depicted in blue and red, respectively (B). Expression of genes of interests was analysed by quantitative real-time PCR \( (n=3) \). FC of genes of interests in \( \text{wtMALT1HSC2} \) cells relative to \( \text{mockHSC2} \) cells were standardised by the expression of \( \text{ACTB} \) (Q). Protein expression was examined by the immunoblot. \( \beta\text{-actin} \) was used as an internal control (D).
Enhanced migration by loss of MALT1 expression. The IPA biofunction analysis suggested a close association of MALT1 in 'Cellular Movement', and enhanced migration is a representative phenomenon of the aggressive behaviours of carcinoma cells (Hannah and Weinberg, 2011). Therefore, migration of \textit{wtMALT1HSC2} and \textit{amMALT1HSC2} cells was compared with that of \textit{mockHSC2} cells by several sets of experiments. The conventional monolayered wound-healing assay on slide glasses showed 80.5% reduction in wound closure by \textit{wtMALT1HSC2} cells and the 185.0% enhancement by \textit{amMALT1HSC2} cells compared with the \textit{mockHSC2} cells (Figure 4A and B). The siRNA against \textit{MALT1} facilitated the \textit{wtMALT1HSC2} cell wound closure (\textit{P}=0.003; Figure 4C; Supplementary Table 4). The \textit{amMALT1HSC2} cells did not respond to the siRNA because of the lack of binding site in its gene construct. To quantify it more accurately and continuously, the real-time wound-healing assay using the RTCA-DP system was performed. As shown in Figure 4D, the enhanced wound coverage by \textit{amMALT1HSC2} cells (218.6%) and the reduction by \textit{wtMALT1HSC2} cells (66.4%) were confirmed. Since the wound closure reflects many aspects of cells (Yilmaz and Christofori, 2010), single cell migration was evaluated by the time-lapse analysis (Figure 4E). Migration velocities of \textit{mockHSC2} cells were 1.01 ± 0.05 μm h⁻¹, while \textit{wtMALT1HSC2} cells (218.6%) and the reduction by \textit{amMALT1HSC2} cells (66.4%) were confirmed. Since the wound closure reflects many aspects of cells (Yilmaz and Christofori, 2010), single cell migration was evaluated by the time-lapse analysis (Figure 4E). Migration velocities of \textit{mockHSC2} cells were 1.01 ± 0.05 μm h⁻¹, and \textit{wtMALT1HSC2} cells almost unmoved for 16 h (0.21 ± 0.01 μm h⁻¹; \textit{P}=0.021).

**DISSCUSSION**

A well-established role of NF-κB activation by MALT1 in lymphocytes and enhanced NF-κB activity in advanced oral carcinomas intimate that MALT1 promotes the carcinoma progression (Molinolo \textit{et al}, 2009; McAllister-Lucas \textit{et al}, 2011). However, MALT1 is expressed in normal oral epithelial cells and the loss of expression closely associates with the worse prognosis of carcinoma patients (Chiba \textit{et al}, 2009). Then, we hypothesised that MALT1 has the differential role between epithelial cells and lymphocyte lineages, and analysed gene data sets that were affected for 16 h (0.21 ± 0.01 μm h⁻¹; \textit{P}=0.021).
Comparative analysis using IPA demonstrated that the genes downregulated by MALT1 affect EGF and TGF-β pathways and cellular movement, suggesting the stimulation of oral carcinoma aggressiveness by loss of MALT1 expression.

EGFR encoding EGFR (ErbB1, HER-1), a most predominant EGF receptor in head and neck carcinomas (Be et al, 2004), was downregulated. Although other EGF receptors (ErbB2–4) were upregulated, their expression and pathological role in head and neck carcinomas are a controversial issue (Schoppmann et al, 2010; Bussu et al, 2012; Zanaruddin et al, 2013). The EGFR ligands, AREG, TGFα, and EREG downregulated by MALT1, are overexpressed in oral carcinomas and stimulate proliferation of carcinoma cells (Rubin Grandis et al, 1996; Tsai et al, 2006; Shigeishi et al, 2008). Mucosa-associated lymphoid tissue 1 also downregulated EGF signalling molecules, RAS (HRAS and NRAS) and PKC (PKCα, PKCβ, and PKCζ). The amplification of EGFR loci per se attributes to oral carcinoma development and progression (Sheu et al, 2009), and EGFR-mediated pathway is strongly activated in an aggressive subset of oral carcinoma with worse prognosis (Ang et al, 2002). Although the frequency of EGFR active mutation in oral carcinomas is controversial (Hsieh et al, 2011; McIntyre et al, 2012), MALT1 may negatively regulate the pathway by the suppression of gene expression regardless of the presence or absence of mutation. These data suggest the liberation of EGF-EGFR pathway from the suppressor upon loss of MALT1 expression.

In parallel with the suppressive role for EGF signalling gene expression, MALT1 downregulated TGFB2, TGFB2, SMAD2, SMAD3, and SMAD4. A protein complex made of these SMADs is crucial for the TGF-β1-TGFB2 signal transduction (Roberts and Wakefield, 2003). Although TGF-β acts as a potent tumour suppressor at the early stage of carcinoma progression, it stimulates cell proliferation, invasion, metastasis, and angiogenesis at the late stage (Roberts and Wakefield, 2003). Loss of MALT1 expression occurs at the late stage of oral carcinoma progression (Chiba et al, 2009). Mucosa-associated lymphoid tissue 1 also downregulated genes encoding gp130 (IL6ST) and IL-11 (IL11), which stimulate carcinoma progression under the TGF-β pathway (Calon et al, 2012). These data imply that the loss of expression facilitates oral carcinomas to activate TGF-β signalling and pathway at the late stage of progression.

Since EGF and TGF-β signalling frequently interact each other and co-regulate gene expression that enhance aggressive phenotypes of carcinoma cells (Kretzschmar et al, 1997; Wendt et al, 2010; Deharvengt et al, 2012), it is difficult to discern which signalling has a principal effect on the target gene expression in MALT1HSC2 cells. Of note, a mesenchyme-specific transcriptional factor, HMGA2, that is upregulated by these signalling induces epithelial–mesenchymal transition (EMT) in carcinoma cells (Li et al, 1997; Thuault et al, 2006). Induction of the EMT gains carcinomas invasive and chemoresistant properties (Yilmaz and Christofori, 2010; Hanahan and Weinberg, 2011). HMGA2 is predominantly expressed at invasive front oral carcinoma cells showing the EMT and in the patients with worse prognosis (Miyazawa et al, 2004), and MALT1 expression rapidly declines at the invasive front carcinoma cells (Chiba et al, 2009). HMGA2 represses CDH1 expression through SNAI2, ZEB2, and so on.
and TWIST1 (Li et al., 1997; Thuault et al., 2008), which were downregulated in wtMALT1HSC2 cells. As numbers of previous studies established a fact that inactivation of E-cadherin (CDH1) expression is a critical determinant for aggressive carcinomas (Hanahan and Weinberg, 2011; Hashimoto et al., 2012), expression status of MALT1 may have a great impact on oral carcinoma progression.

Mucosa-associated lymphoid tissue 1 also suppressed other EMT inducers (WNT3, FGFs, PDGFs, IL6R, and FGFRs) and...
MALT1 suppresses aggressive carcinoma phenotypes

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Figure 4. Migration of carcinoma cells. (A) Knock-down of MALT1 protein expression in wtMALT1HSC2 cells and MALT1HSC2 cells by the siRNA against wtMALT1. The protein expression was probed by the immunoblotting using an anti-FLAG antibody. (B) Migration of cells 24 h after injury in the presence or absence of 50 nM MALT1 siRNA. White broken lines represent the original wound edges. Scale bar = 1 mm. (C) Percentage of wound closure at 24 h. The graph indicates means ± s.d. of wound closure at 24 h (n = 5). *P < 0.001 (compared with mock + siRNA) and **P < 0.005 (compared with wtMALT1 without siRNA). (D) Real-time sensing wound-healing assay. The coverage of wounds was monitored by the xCELLigence system until 14 h after injury. (E) Representative examples of wtMALT1HSC2 (wtMALT1), MALT1HSC2 (MALT1), and mockHSC2 (mock) cell migration for 16 h using time-lapse microscopy (n = 4). Scale bar = 50 μm.

related genes encoding FAK signalling (integrins and paxillin), extracellular matrix-degrading enzymes (MMPs, ADAMs, plasminogen activators, kallikreins and cathepsins), CD44, SPARC, collagens, laminins, Jagged2, and podoplanin (Supplementary Table 1). It appears likely that loss of MALT1 expression activate the EMT machinery.

Enhanced migration is one of most representative characteristics of the EMT (Yilmaz and Christofori, 2010; Hanahan and Weinberg, 2011). Strong suppression of the migration by MALT1 supports our hypothesis that MALT1 has a distinct role in oral carcinoma cells from lymphoma cells. Since TGF-β and EGF pathways synergistically accelerate the EMT and migration of carcinoma cells, their liberation from the suppression by MALT1 may have a key readout circuitry in oral carcinoma progression. Detailed analysis for the role of MALT1 on the pathways should contribute to understand the pathology of oral carcinomas and to develop novel therapeutic strategies for the carcinoma patients.

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