Epigenetic Loss of Mucosa-Associated Lymphoid Tissue 1 Expression in Patients with Oral Carcinomas

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Abstract
Mucosa-associated lymphoid tissue 1 (MALT1), which is located in a genomic region that encodes unknown tumor suppressor gene(s), activates nuclear factor-κB in lymphocyte lineages. However, its expression and role in the pathology of malignant tumors of epithelial origin is not known. In the present study, we examined MALT1 expression and its implications for the pathology of oral carcinomas. Immunostaining localized MALT1 in the nucleus of normal oral epithelial cells, but the expression was absent in 45.0% of carcinomas (49 of 109 cases) especially at the invasive front. The loss of expression was correlated with tumor recurrence (P = 0.007) and poor patient survival (P < 0.001), and it was an independent prognostic determinant (P < 0.001). MALT1-negative carcinomas exhibited microsatellite instability at the MALT1 locus and a specific cytosine methylation positioned at –256 from the gene, and the expression was recovered by demethylation treatment. In contrast to lymphocyte lineages, carcinoma cells showed MALT1 located at the nucleus independent of its domain structures, and its loss of expression induced the epithelial-mesenchymal transition. These results show that MALT1 is expressed in the nucleus of oral epithelial cells and that its expression is epigenetically inactivated during tumor progression, suggesting that the detection of MALT1 expression is a useful predictive and prognostic determinant in the clinical management of oral carcinomas. [Cancer Res 2009;69(18):7216–23]

Introduction
Worldwide, the annual incidence of new cases of oral carcinoma is estimated at 350,000 to 400,000 and is predicted to increase in the next few decades. In the United States, 30,000 new oral carcinoma cases are diagnosed annually and 11,000 people die of the disease each year. Notably, the 5-year survival rate of patients has not been sufficiently improved. Regardless of the therapeutic approaches used and the location and stage of the diseases, >50% of patients experience a relapse (1). Treatment failure can be attributed to multiple factors, but no reliable molecular marker is currently available. The tailoring of individual treatment strategies to aggressively treat those carcinomas at greatest risk of causing patient death would likely improve long-term survival. There is an urgent need to identify characteristics of the primary tumor that might predict aggressive tumors.

Materials and Methods
Cell lines, plasmids, and short interfering RNA. Oral carcinoma cell lines were used as previously described (11, 12). The full-length wild-type MALT1 (wtMALT1; 1-824 amino acids), the NH2 terminus (1–322 amino acids) of MALT1 (nMALT1), and the NH2 terminus–deleted dominant-negative form (333–824 amino acids) of MALT1 (AMALT1) cDNAs (13) were cloned into pCMV with a FLAG-tag (pCMV-Tag 2A; Stratagene) or pcDNA4-HisMaxB (Invitrogen). An oral carcinoma cell line, HSC2, was transfected with the plasmids, and cells stably expressing wtMALT1 (wtMALT1HSC2 cells), AMALT1 (AMALT1HSC2 cells), or vector alone (mockHSC2 cells) were selected with 100 μg/mL of G418 (14).

Antibodies. A rabbit polyclonal anti-MALT1 antibody against A26GATLNRLREPLLRR40 was purified by an affinity column coupled with synthetic peptides. The antibody preparations were screened by ELISA using the peptide. Antibodies specific to histone H3, IKKα (Cell Signaling), involucrin, β-actin, FLAG (Sigma), cytoketin 10 (Progen Bioteknik GmbH), or E-cadherin (R&D Systems) were used for immunoblotting.

Patient population. A total of 109 individual oral squamous cell carcinomas were taken at Kanazawa University Hospital during incisional or excisional biopsy. The median age of the study patients was 64 y (range, 37–93 y) at the time of diagnosis. Patients underwent surgery (n = 93) or radiation and surgery (n = 16). The details of the pretreatment clinical and
Table 1. Clinicopathologic parameters and MALT1 expression in 109 primary oral carcinomas

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* MALT1 staining was graded by the ratio of immunoreactive carcinoma cells, where 0 is no staining, 1+ is ≤10%, 2+ is 11–40%, and 3+ is ≥41% staining of cells.
† Probability of statistical difference was analyzed by Mann-Whitney U test.
‡ Patients were categorized by tumor size (T stage) and clinical stage according to the UICC WHO grading system and by the stage of lymph node metastasis (N stage).
¶ Probability of statistical difference was analyzed by analysis of covariance.

Tissues (n = 3) were snap-frozen in TRIzol reagent (Invitrogen) and reverse transcribed to a single-stranded cDNA with random hexamer (Invitrogen), followed by reverse transcription-PCR (RT-PCR) by running 30 cycles (94°C for 40 s, 54°C for 40 s for GAPDH, and 56°C for 40 s for MALT1; 72°C for 1 min) using gene-specific primer sets for MALT1 and GAPDH for MALT1 (Supplementary Table S1). For the quantitative analysis of MALT1 expression, carcinoma cells cultured for 4 d in the presence or absence of 5 μm/L 5-aza-2′-deoxycytidine (5-aza) were harvested for isolation of total RNA. The cDNA reverse transcribed from RNA by MultiScribe Reverse Transcriptase (Applied Biosystems) was subjected to real-time PCR using the StepOne Real-Time PCR system (Applied Biosystems) in triplicate. 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 MALT1-specific TaqMan probe was synthesized from sequence at the boundary of exons 15 and 16 (Assay-on-Demand Gene Expression system, Hs01012005-m1; Applied Biosystems). Expression levels were normalized against β-actin (TaqMan Endogenous Control Human ACTB; Applied Biosystems). Levels of gene expression (2^−ΔΔCt) were determined by the standard curve method (12).

DNA extraction. Microdissection and DNA extraction from 8-μm-thick paraffin-embedded tissue sections (n = 47) were carried out using a laser capture microdissection apparatus (Leica Microsystems). Tumor cells and adjacent normal cells were separately microdissected, and genomic DNA was extracted by a standard protocol (16) and quantified by a spectrophotometer.

Microsatellite markers and PCR. Genomic DNA was subjected to microsatellite analysis using an established marker (D18S1117) positioned at 151.2 kb downstream of MALT1. Because there are no established microsatellite markers within the MALT1 locus, three intragenic markers were designed (MALT1e1 at intron 2, MALT1e2 at intron 4, and MALT1e3 at intron 13) spanning (CA)n, (TG)n, and (CA)n, respectively (Supplementary Table S1). Genomic DNA (20 ng) was amplified in a volume of 20 μL by 35 cycles of touchdown PCR (denaturation for 45 s at 94°C, annealing for 1 min and elongation for 1 min at 72°C). The annealing temperature began at 66°C and was lowered 1°C per cycle. About 15 μL of the amplified products were electrophoresed through acrylamide gels stained by Vistra Green (Amersham Bioscience) and were scanned by a Typhoon 9410 Image Analyzer (Amersham Bioscience).

Genetic instability and allelic imbalance. The allelic profile of each carcinoma was initially scored for microsatellite instability (MSI) according to a criterion for alterations in allelic length (17). Sometimes it was not possible to distinguish between MSI and the loss of heterozygosity (LOH), especially in cases with intimately close heterozygous allelic bands. A signal reduction in one of two alleles but not of allelic loss but also from allelic shift attributable to comigration with an adjacent allele. Thus, if multiple bands or novel allelic bands that were not observed in normal DNA were found in the corresponding tumor DNA, they were interpreted as MSI. The LOH of tumor DNA was scored as relative allelic ratio, which was calculated by dividing the tumor allelic ratio by the normal allelic ratio (18).

Bisulfite-modified sequencing of MALT1 promoter region. The 5' proximal promoter region of MALT1 was analyzed on the computer program CpG Island Finder4 to find CpG islands. Genomic DNA isolated from carcinoma cell lines was treated with sodium bisulfite (18) and applied for direct sequencing using a PCR primer set from −48 to −341 (Supplementary Table S1).

Methylation-specific PCR. Genomic DNA isolated from carcinoma cell lines or carcinoma cells of tissue sections was modified by sodium bisulfite treatment. Methylation-specific PCR (MSP) was carried out on the modified DNA to detect cytosine methylation positioned at −256 (from the transcription start site) using primer sets recognizing methylated 5-azaC (methyl primers) and unmethylated 5-azaC (unmethyl primers), which amplify the MALT1 CpG island from −139 to −240 (Supplementary Table S1). PCR amplification using AccuPrime Taq DNA polymerase (Invitrogen) was performed by running 48 cycles as follows: denaturing for 30 s at 94°C, annealing for 30 s (at 59°C for first 4 cycles, 57°C for next 4 cycles, and 55°C for 30 cycles) and extension for 1 min. The PCR products were electrophoresed through 3% agarose gels stained by Vistra Green (Amersham Bioscience) and were scanned by a Typhoon 9410 Image Analyzer (Amersham Bioscience).

4 http://cpgdlands.usc.edu/
for the rest), and extension for 30 s at 68 °C (19). Distilled water, instead of sodium bisulfite, was used as a negative control.

**Statistical analyses.** For the analysis of probabilities of postoperative survival of patients (n = 104), except those who died of other diseases, we divided patients into two groups, a negative group (grade 0 and 1) and a positive group (grade 2 and 3), using the Kaplan-Meier method and analyzing the statistical difference by the log-rank test. The postoperative period was measured from the date of surgery to the date of the last follow-up or death. The influence of clinicopathologic parameters and MALT1 immunoreactivity on patient survival was analyzed by the multivariate Cox proportional hazards method, which included variables of pathologic stage, patient age, and MALT1 immunostaining status. The Mann-Whitney U test and analysis of covariance were used for all other comparisons. Differences with *P* values of <0.05 were considered statistically significant.

**Results**

**Loss of MALT1 expression in progressive carcinomas.** To investigate the expression and localization of MALT1, we generated an antibody specific for the NH2 terminus of MALT1 (Supplementary Fig. S1). MALT1 localizes in the nuclei of basal and suprabasal cells of normal gingival epithelium, but not in their cytoplasm (Fig. 1A). We immunostained 109 individual primary oral carcinomas and graded them into four categories (0–3+) according to the percentage of carcinoma cells with nuclear staining. MALT1 was not detected in 45.0% of the cases, and in the remainder, its level of expression declined in conjunction with the dedifferentiation of carcinomas (*P* = 0.001) and the progression of clinical stage (*P* = 0.029; Table 1). In contrast to the central area of carcinoma tissues, carcinoma cells at the invasive front, where they proliferate downward into the connective tissue and exhibit epithelial-mesenchymal transition (EMT; ref. 12), showed weaker staining (Fig. 1A).

A high-power view of the staining at the center or invasive front is shown in c (inset c in L) or f (inset f in L), respectively. An arrowhead shows cytoplasmic staining in a fibroblast and an arrow shows nuclear staining in a carcinoma cell in an inset in c. Scale bars, 125 μm (L), 50 μm (nor, wel, po, neg, c, and f), and 15 μm (insets). B, MALT1 immunoreactivity of carcinoma cells was compared between groups with clinical stages (left: S III/IV, stages I and II; S III/IV, stages III and IV; *P* = 0.012), tumor differentiation (center: W, well-differentiated tumor; M/P, moderately and poorly differentiated tumors; *P* = 0.002), or tumor recurrence (right: Ao, cases free of tumor recurrence; Dc, cases that died of tumor recurrence; *P* = 0.007). Columns, mean; bars, SD. C, long-term survival of MALT1-negative and MALT1-positive patients (n = 104; *P* < 0.001; log-rank test).
Table 2. Contribution of clinicopathologic risk factors to disease-specific survival of oral carcinoma patients (n = 104)

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<td>MALT1 staining (negative vs positive)</td>
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<td>1.220–2.683</td>
<td>&lt;0.001</td>
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*Confidence interval.
†Cox proportional hazards method.
‡N−, cases without lymph node metastasis; N+, cases with metastasis.
§Negative, groups 0 and 1; positive, groups 3 and 4.

Figure 2. MSI at the MALT1 locus. A, expression of MALT1 mRNA in normal gingiva and carcinoma tissues. MALT1 expression in normal gingival tissues (normal tissues 1–3) and carcinoma tissues (patient no. 2–54) was examined by RT-PCR. – RT, negative control without reverse transcription of RNA isolated from a normal tissue number 3 sample. B, MSI and LOH patterns in representative carcinoma patients. Retained microsatellite markers (white), LOH (black), and MSI (gray) are shown. C, representative gel electrophoresis of microsatellite PCR. Gray arrowheads, MSI; black arrowheads, allelic imbalance. Genomic DNA of carcinoma cells (T) and distant normal cells (N) were independently isolated from the same tissue section in each patient by laser-captured microdissection. D, distributions of MSI at the MALT1 locus and tumor differentiation and MALT1 staining. The presence of MSI was determined as 1 and microsatellite stability (MSS) as 0. Well, well-differentiated tumors; Mod, moderately differentiated tumors; and Po, poorly differentiated tumors (left; P = 0.041). MALT1 immunoreactivity of carcinoma cells was compared with genetic instability (right; P < 0.001). Columns, mean; bars, SD.
have tumor recurrence (Ao) during the follow-up period (1.91 ± 1.10; P = 0.007; Fig. 1B). The MALT1-negative group (grades 0 and 1) exhibited significantly lower disease-specific survival than the positive group (grades 2 and 3; P < 0.001, log-rank test; Fig. 1C). The progression of T stage and clinical stage, the presence of lymph node metastasis, and carcinoma dedifferentiation also lowered survival (Supplementary Fig. S2). Univariate risk factor analysis found that the MALT1-negative group increased the risk ratio of death (2.734; P < 0.001; Supplementary Table S2). A multivariate model was then used to adjust for the potential influence of confounding factors. MALT1 staining was a significant predictor of disease-specific death independent from other risk factors (P < 0.001; Table 2). Loss of MALT1 staining (grades 0 and 1) reduced patient survival with a hazard ratio of 1.809 and a confidence interval ranging from 1.220 to 2.683. These data indicate a close association of loss of MALT1 expression with oral carcinoma progression.

Genetic instability and promoter methylation. MALT1 gene expression was detected by RT-PCR in all normal gingival tissues but was frequently undetectable in carcinoma tissues (Fig. 2A). The absence of expression did not seem to result from the presence of a premature termination codon, because the expression pattern was identical when a primer set for exon 1 was used (data not shown). Then, allelic imbalance and MSI at the MALT1 locus in 47 randomly selected carcinomas was analyzed. In 51.1% of these carcinomas, the electrophoretic mobility of the band was shifted relative to their normal counterparts, reflecting changes of dinucleotide repeats (Supplementary Table S3). Fourteen cases exhibited two or three MSI (29.8%), and 10 cases showed one MSI (21.3%). Allelic loss was limited in four cases, and homozygous deletion in one. The other 18 cases (38.3%) exhibited microsatellite stability. The frequency of MSI increased in less differentiated carcinomas (71.4%; 20 of 28 cases) and MALT1-negative carcinomas (83.3%; 20 of 24 cases), and the frequency of microsatellite stability increased in well-differentiated carcinomas (71.4%; 10 of 14 cases) and MALT1-positive carcinomas (77.8%; 14 of 18 cases; Fig. 2D). An additional microsatellite marker in the vicinity (D18S1117) had MSI limited to two cases, indicating that the genetic instability at the MALT1 locus is selective.

Because MSI is closely associated with gene silencing through promoter methylation (19, 21), the methylation status of the MALT1 promoter CpG island (−13 to −341 from the transcription start site) was investigated in carcinoma cell lines with different MALT1 expression levels (Fig. 3A). Although the CpG island contains 37 methylation-susceptible cytosines, bisulfite-modified genomic sequencing detected methylation specifically at −256C and not at other cytosines (Supplementary Fig. S3). Then, MSP was performed on carcinoma cells isolated from tissue sections (n = 20). The methylation was preferentially detected in MALT1-negative and MSI carcinomas (Fig. 3B). MSP analyses of carcinoma cell lines confirmed the correlation between −256C methylation and MALT1 expression (Fig. 3C). The 5-aza-2-deoxycytidine demethylation treatment of MALT1 promoter-methylated cell lines (HSC2 cells and SCCKN cells) up-regulated MALT1 expression, but unmethylated cell lines (Ho1N1 cells and KOS2 cell) did not respond to the treatment (Fig. 3D). These data indicate that the specific cytosine methylation at −256 is responsible for the loss of MALT1 expression.

Figure 3. Methylation of MALT1 promoter cytosine. A, expression of MALT1 mRNA in oral carcinoma cell lines and normal keratinocytes (HaCaT). MALT1 expression was quantitatively examined by real-time PCR. Relative expression was standardized by the expression levels of GAPDH in each sample (n = 3). B, methylation at −256C of MALT1 promoter in carcinomas. MSP using primer sets discriminating methylated (M) and unmethylated (U) at −256C was performed with bisulfite-modified genomic DNA extracted from carcinoma tissue sections. IMS, MALT1 immunostaining positive (+) or negative (−) cases. MSI, presence (+) or absence (−) of MSI at the MALT1 locus. C, methylation at −256C of MALT1 promoter in carcinoma cell lines. Methylation at −256C was examined by MSP. D, MALT1 mRNA expression in carcinoma cell lines with and without demethylation treatment. Total RNA was isolated from cells with (+) and without (−) 5-aza-2-deoxycytidine (5-aza) treatment and was subjected to quantitative real-time PCR. Columns, means of four samples in each cell line; bars, SD. * P < 0.001.
Nuclear localization of MALT1. The abovementioned findings strongly suggest that MALT1 localizes within the nucleus and that the epigenetic loss of expression is associated with the progression of oral carcinomas. Because MALT1 is characterized as a cytoplasmic signaling molecule (19), its domain responsible for nuclear localization in oral carcinoma cells was examined. HSC2 cells were used, which express negligible amounts of endogenous MALT1, and we established wtMALT1HSC2 cells, ΔMALT1HSC2 cells, and mockHSC2 cells. The wtMALT1 and ΔMALT1 proteins were expressed at comparable levels, and they were present in the nuclear fraction but not in the cytoplasmic fraction (Fig. 4A). Transiently transfected wtMALT1, nMALT1, and ΔMALT1 in HSC2 cells were also exclusively present in the nucleus, as previously reported (Fig. 4B; ref. 22), indicating that no specific domain is responsible for the nuclear localization.

Induction of EMT. Because MALT1 expression is prominently lost in carcinoma cells at the invasive front, where carcinoma cells gain the EMT phenotype (12), the expression of keratinocyte differentiation and mesenchymal cell-type markers were examined (Fig. 4C). The expression of differentiation markers (cytokeratin 10, IKKα, E-cadherin, and involucrin) was up-regulated in wtMALT1, nMALT1, and ΔMALT1 in HSC2 cells were also exclusively present in the nucleus, as previously reported (Fig. 4B; ref. 22), indicating that no specific domain is responsible for the nuclear localization.

Discussion

The present study shows that epigenetic loss of MALT1 expression is closely associated with the progression of oral carcinomas. MALT1 staining in the nucleus was lost in 45.0% of carcinomas with advanced clinical stages and less differentiation, as well as in patients who died of disease recurrence. Patients with loss of MALT1 expression showed the worst prognosis, and the loss of expression was an independent risk factor for patient survival. MALT1-negative carcinomas exhibited genetic instability, with the expression epigenetically inactivated by a specific cytosine methylation at C0 in the promoter. The expression of the dominant-negative form of MALT1 (ΔMALT1) induced EMT of carcinoma cells. Because chromosome 18q21.31 encodes the yet unknown tumor suppressor gene (7, 8), the present data suggest that MALT1 negatively regulates oral carcinoma progression.

Although MALT1 is known to activate NF-κB in lymphocyte lineages (9), it is also expressed in other cell types (12, 20), suggesting the possibility that it has unknown roles in different cell types. Unexpectedly, MALT1 was localized in the nucleus but not the cytoplasm of normal oral epithelial and carcinoma cells. The exclusive nuclear localization was confirmed by immunoblotting using HSC2 cells, which stably or transiently express MALT1. Although the mechanism of nuclear localization is not currently known, MALT1 binds to HSC70 (23), which shuttles binding proteins to the nucleus and may associate with keratinocyte differentiation (24, 25). We confirmed a previous study (22), which found that both the NH2 terminus (death and Ig-like domains) and the COOH terminus (caspase-like domain) exist independently in the nucleus. Because MALT1 was localized in the nucleus of normal oral epithelial cells and MALT1-BCL10 complexes were not...
exported into the cytoplasm in oral carcinoma cells, unknown mechanism(s) to retain MALT1 in the nucleus may exist in keratinocyte lineages.

The expression pattern of MALT1 in carcinoma tissues was evaluated by immunostaining. The loss of staining was closely associated with clinical stage, tumor recurrence, and long-term patient survival rate independent of other risk factors. The loss of staining was observed in carcinoma cells at the invasive front. Carcinoma cells at the invasive front can lose their epithelial characteristics and express a gene set of types found in mesenchymal cells (11, 12, 26, 27). In this study, keratinocyte differentiation markers (cytokeratin 10, IKKα, E-cadherin, and involucrin) and mesenchymal cell type markers (vimentin and fibronectin) were up-regulated by wtMALT1 and ΔMALT1, respectively, indicating that loss of MALT1 expression induces EMT. Induction of EMT in squamous carcinoma cells drives tumor progression through the enhancement of migratory, invasive, and metastatic features (28, 29). These data strongly suggest that MALT1 staining predicts tumor aggressiveness and stratifies patients into risk groups and that the loss of expression results in EMT induction, which accounts for the transition to a more advanced stage of oral carcinoma.

Immunostaining data also indicate that the loss of expression does not occur at an early stage of carcinogenesis. This was examined with the perspective of genetic instability and promoter methylation, and it was found that MALT1-negative carcinomas frequently associate with genetic instability at the locus and 5-az-C methylation in its promoter. MSI itself arises through defective DNA mismatch repair genes, which exhibit allelic imbalance in 80% of head and neck carcinomas (30) and are closely associated with gene silencing through promoter methylation (19, 31). Recent studies indicated that MSI and aberrant promoter methylation occur selectively but not randomly at specific susceptible sites, resulting in a functional impact on the progression of tumors (32). Because MSI at the close proximity to the MALT1 locus (D18S1117) was observed at a low frequency (two cases, 4.3%), genetic instability at the MALT1 locus may be a selective phenomenon. Cytosine methylation responsible for the epigenetic gene inactivation is often distributed throughout the CpG islands of tumorsuppressor genes (18). However, up-regulation of MALT1 expression by demethylation treatment in carcinoma cells with 5-az-C methylation indicates that methylation at the specific cytosine results in the loss of MALT1 gene expression, as previously described for other tumor-associated genes (33–35). Although computational algorithms found no known transcription factor-binding sites at this position, it is possible that the methylation disturbs the accessibility of a transcription factor through architectural impediment of transcription unit formation. It is also interesting to note that sustained induction of EMT is responsible for de novo methylation at the CDH1 (E-cadherin gene) promoter (36) and that the loss of MALT1 expression results from this methylation and induces EMT. The mechanism and role of MALT1 expression loss during carcinoma progression may exist in the intricate cellular system.

Although MALT1+/– mice are viable and fertile without any gross abnormalities, the mice are defective with regard to NF-κB activation in T and B cells (37, 38). The lack of NF-κB activation in these cell types was also noted in BCL10 or CARMA1 knockout mice (39, 40), indicating a critical role of the CBM complex in T-cell receptor– or B-cell receptor–mediated pathways. However, genetic ablation of NF-κB p65 and c-REL subunits decreases proliferation of epidermal cells (41), and enhanced activation of NF-κB containing p65 subunit is closely associated with the progression of squamous cell carcinomas and poor patient prognosis (42–44). These data suggest that MALT1 is functionally redundant in development or plays a role other than NF-κB activation in keratinocytes and carcinoma cells. The present study shows that MALT1 is expressed in the nucleus of oral epithelial cells and has epigenetically inactivated its expression in parallel with tumor progression. MALT1 immunostaining could be a prognostic determinant for stratifying patients into risk groups. Understanding the role of the loss of MALT1 expression in the pathology of oral carcinomas may provide a novel strategy for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 3/27/09; revised 7/12/09; accepted 7/14/09; published OnlineFirst 9/8/09.

Grant support: Uehara Memorial Foundation and the Japan Society for the Promotion of Science #18392073 (K. Imai).

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We thank Dr. M. Seto (Aichi Cancer Center) for providing MALT1 and BCL10 cDNAs and Drs. K. Chada (UMDNJ) and J. D’Armiento (Columbia University) for critical reading of the manuscript.

References


MALT1 Expression Loss in Oral Carcinomas


Epigenetic Loss of Mucosa-Associated Lymphoid Tissue 1 Expression in Patients with Oral Carcinomas

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