T-lymphocyte maturation-associated protein gene as a candidate metastasis suppressor for head and neck squamous cell carcinomas

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Previous gene expression profiles revealed the T-lymphocyte maturation-associated protein (*MAL*) gene as being frequently downregulated in head and neck cancer. To define the relationship between the *MAL* gene and the metastatic process, we evaluated the expression status of the gene in matched primary and metastatic tumors of head and neck cancer by semiquantitative reverse transcription–polymerase chain reaction. Furthermore, we aimed to identify potential genetic and epigenetic mechanisms associated with downregulation of MAL, including loss of heterozygosity (LOH), mutation, and hypermethylation. Thirty-five cell lines of University of Turko squamous cell carcinoma (UT-SCC) series derived from head and neck cancer, including nine pairs from matched primary and metastatic tumors, and 30 pairs of matched primary and metastatic tumor samples were analyzed. Twenty out of 35 (57%) cell lines showed downregulation of MAL expression, whereas no expression was found in 10 cell lines (29%). Considering matched primary and metastatic tumor-derived cell-line pairs, four pairs showed decreased expression only in metastasis-derived cells compared with their primary counterparts. Expression analysis of 21 tissue samples demonstrated decreased or no expression of MAL mRNA in 43% of metastatic tumors compared with matched primary tumors. Relating to mechanisms of downregulation, LOH was observed in 30% of primary tumors and 38% of their metastatic counterparts by a MAL-specific microsatellite marker. Furthermore, we found restoration of MAL mRNA after treatment with demethylating agent (5-aza-2′-deoxycytidine) in 9 (45%) out of 20 cell lines. No mutation was found in UT-SCC cell lines. In conclusion, our findings indicate selective downregulation of MAL expression in metastatic cells, suggesting the *MAL* gene as a new metastasis-suppressor candidate for head and neck cancer. LOH and hypermethylation appeared to be important mechanisms for inactivation of MAL function. (*Cancer Sci* 2009; 100: 873–880)

The general prognosis of patients with head and neck squamous cell carcinoma (HNSCC) has not improved significantly, despite major advances having been obtained in terms of early detection, surgical resection, and chemoradiation protocols.[1] The poor outcome has mainly been attributed to local and distant lymph node metastasis as well as recurrence. The presence of cervical lymph node metastases is a common and adverse event in HNSCC and decreases the survival of patients by approximately 50%.[2]

The ability to assess or predict the presence of metastasis has significant prognostic relevance and treatment implications in the management of HNSCC. In order to decrease morbidity and mortality from HNSCC, it is necessary to gain a greater understanding of metastasis and define the molecular factors that contribute to this process. Recently, a set of molecules has been discovered called metastasis suppressors, and loss of their expression could enable cancer cells to acquire metastatic competency.[3] This loss of gene expression may be due to loss of heterozygosity (LOH), somatic mutation, or epigenetic mechanisms such as promoter hypermethylation. It is hypothesized that metastasis-suppressor proteins function through restoring normal homeostatic signaling mechanisms, which inhibit the acquisition of several novel phenotypes by tumor cells that are necessary for metastasis.

Discovery of HNSCC-related metastasis-suppressor genes and their mechanisms of action are important for the development of novel strategies in the prevention and treatment of metastatic tumors. To identify the potential suppressors of tumor metastasis in HNSCC, we first reviewed previous studies on gene expression profiles of HNSCC patients using cDNA microarrays.[4–7] We defined the T-lymphocyte maturation-associated protein (*MAL*) gene as one of the most frequently downregulated genes in HNSCC.

The *MAL* gene encodes a membrane proteolipid with several hydrophobic domains. It was initially identified as a component of the protein machinery for apical transport in epithelial polarized cells.[8,9] Furthermore, recent reports have suggested additional functions for MAL–glycosphingolipid complexes in signaling, cell differentiation, and membrane trafficking processes in epithelial cells;[10,11] however, there was no previous information of a relationship between MAL and metastasis.

In the present study, to define the role of the *MAL* gene in the metastatic process of HNSCC, we assessed the expression status of the gene both in metastatic tumors and cell lines derived from metastatic tumor tissues of HNSCC compared to their primary tumor counterparts. Furthermore, we also evaluated potential genetic and epigenetic mechanisms, including LOH, mutation, and hypermethylation related to the downregulation of MAL expression.

Materials and Methods

Cell lines. In total, 35 cell lines derived from human HNSCC with different sites of origin were used in this study. Almost all cell lines belonged to the University of Turko squamous cell carcinoma (UT-SCC) series established at the University of Turku, Finland, whereas only OKK-TK was developed at Wakayama Medical University. The cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum in a 5%

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Table 1. Clinicopathological characteristics of tumor cell lines derived from head and neck squamous cell carcinoma

Cell line	Age (years)	Sex	Primary site	TNM†	Specimen site	Grade
UT-SCC-7‡	67	Male	Temporal region skin	$T_1N_0M_0$	Neck metastasis	moderate
UT-SCC-9	81	Male	Larynx-glottic	$T_2N_1M_0$	Neck metastasis	low
UT-SCC-17‡	65	Male	Larynx-supraglottic	$T_2N_0M_0$	Sternum metastasis	high
UT-SCC-26A	60	Male	Hypopharynx	$T_1N_2M_0$	Neck metastasis	moderate
UT-SCC-42B	43	Male	Larynx-supraglottic	$T_4N_3M_0$	Neck metastasis	high
UT-SCC-58	63	Male	Larynx-transglottic	$T_4N_1M_0$	Neck metastasis	low
UT-SCC-59A‡	81	Male	Temporal region skin	$T_1N_3M_0$	Neck metastasis	high
UT-SCC-61‡	90	Female	Lower lip	$T_1N_0M_0$	Neck metastasis	low
UT-SCC-62‡	56	Male	Hypopharynx	$T_4N_0M_0$	Neck metastasis	moderate
UT-SCC-64	66	Male	Unknown	$T_xN_3M_0$	Neck metastasis	high
UT-SCC-71‡	79	Female	Gingivae	$T_2N_0M_0$	Neck metastasis	low
UT-SCC-77‡	50	Male	Tongue	$T_1N_0M_0$	Neck metastasis	moderate
UT-SCC-79A‡	80	Female	Facial skin	$T_2N_0M_0$	Parotis metastasis	moderate
UT-SCC-84‡	89	Female	Tongue	$T_1N_0M_0$	Neck metastasis	moderate
UT-SCC-90§	35	Male	Tongue	$T_1N_0M_0$	Local recurrence	moderate
UT-SCC-104	80	Male	Larynx-ventricle	$T1N_{2A}M_0$	Neck metastasis	moderate
UT-SCC-115‡	92	Female	Auricula skin	$T_2N_{2A}M_0$	Neck metastasis	moderate

Cell line pairs derived from matched primary and metastatic tumors

	P¶	M††	Age (years)	Sex	Primary site	TNM	Metastatic site	P¶	M††
UT-SCC-6	A§	B	51	Female	Supraglottic larynx	$T_2N_1M_0$	Neck metastasis	low	low
UT-SCC-12	A	B‡	81	Female	Nasal skin	$T_2N_0M_0$	Neck metastasis	low	moderate
UT-SCC-16	A	B‡	77	Female	Tongue	$T_3N_0M_0$	Neck metastasis	high	high
UT-SCC-24	A	B‡	41	Male	Tongue	$T_2N_0M_0$	Neck metastasis	moderate	moderate
UT-SCC-54	A	C‡	58	Female	Buccal mucosa	$T_2N_0M_0$	Neck metastasis	low	NA
UT-SCC-60	A	B	59	Male	Tonsil	$T_4N_1M_0$	Neck metastasis	low	low
UT-SCC-74	A	B	31	Male	Tongue	$T_3N_1M_0$	Neck metastasis	low	moderate
UT-SCC-110	A§	B‡	37	Male	Maxillary sinus	$T_4N_0M_0$	Neck metastasis	NA	NA
OKK-TK	P	M		Male	Maxillary sinus		Neck metastasis	NA	NA

†According to the International Union Against Cancer 1997 TNM classification system; ‡cell lines derived from neck recurrence; §cell lines derived from local recurrence; ¶cell lines derived from primary tumor; ††cell lines derived from metastatic tumor; NA: nor available.

CO_2 incubator at 37°C. The clinicopathological details of the cell lines are shown in Table 1.

Patients and tissue samples. Primary tumor and metastatic tumor samples together with their matched normal counterparts were obtained from 30 patients with HNSCC between 1994 and 2000 at the Department of Otolaryngology Okayama University Hospital (Okayama, Japan) with acquisition of written informed consent from each patient. All tissue samples were frozen in liquid nitrogen immediately after surgery and stored at –80°C until DNA extraction. Patients included 26 men and four women with a mean age of 66.1 years (range, 47–81 years). The clinicopathological details of the patients are shown in Table 2. All of the tumor and normal tissues of each pair were examined by hematoxylin–eosin staining, which revealed that all tumor samples were squamous cell carcinoma, and normal tissues were confirmed for their normal histology. The bioethics committee of the institution approved the study.

RNA isolation, cDNA preparation, and reverse transcription–polymerase chain reaction analysis. Total RNA was prepared by using a modified acid guanidinium phenol–chloroform method (Isogen; Nippon Gene Co., Tokyo, Japan). Total RNA was reverse-transcribed with the SuperScript First-Strand Synthesis system (Invitrogen, Tokyo, Japan) starting with 5 μg total RNA from each sample, according to the procedures provided by the supplier. Expression of MAL mRNA in paired primary and metastatic tumor tissues was examined by semiquantitative reverse transcription (RT)–polymerase chain reaction (PCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as a control. One μL of each RT reaction was amplified in 50 μL mixture containing 5 μL 10× PCR buffer, 4 μM dNTP mixture containing 2.5 mM of each deoxynucleotide triphosphate, 10 pmol of each primer, and 1.25 U r*Taq* DNA polymerase (Takara Bio, Shiga, Japan). Thirty-five PCR cycles for the MAL primers RT-S and RT-AS, and 25 cycles for the GAPDH primers S1 and AS1 (Table 3) were used for amplification (primers designed using Genetyx-Win 5.0.0; Software Development Co., Tokyo, Japan). An initial denaturation step at 94°C for 3 min was followed by 35 cycles of a denaturation step at 94°C for 30 s, an annealing step at 60°C for 1 min, and an extension step at 72°C for 1 min. A final extension step at 72°C for 7 min was added. Reproducibility was confirmed by processing all samples twice.

Quantification of the RT-PCR products. PCR products were separated through a 2% agarose gel and stained with ethidium bromide. The intensity of ethidium bromide staining of each band was measured by a CCD image sensor (Gel Print 2000/VGA; Toyobo, Osaka, Japan) and analyzed by a computer program for band quantification (Quantity One; Toyobo). The value of metastasis-specific MAL expression was determined by calculating the ratio of the expression levels in the tumor and in the matched primary tumor sample, each of which was normalized for the corresponding GAPDH expression level (M, MAL/GAPDH expression ratio in metastatic tumor samples; T, MAL/GAPDH expression ratio in matched primary tumor samples; M/T ratio, relative MAL expression in metastatic tumor samples compared with their matched primary tumors after normalization). Decreased and increased expression levels were determined as classes L and H when this ratio was less than 0.6 and greater than 1.4, respectively, as reported previously.[12] Class N (normal expression) was a ratio value between 0.6 and 1.4.

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Table 2. mRNA expression and loss of heterozygosity (LOH) analysis of T-lymphocyte maturation-associated protein (MAL) and clinical characteristics of patients

Case	MAL exp†		MAL-MS1		Age (years)	Sex	Localization	TNM‡
			Primary	Metastasis				
1	0.02	L	ND	ND	65	M	Oral cavity	T3N2M0
2	ND		NI	NI	70	F	Oral cavity	T1N1M0
3	6.73	H	Ret	Ret	70	M	Oropharynx	T3N1M0
4	4.42	H	NI	NI	55	M	Hypopharynx	T1N2M0
5	1.58	H	Ret	Ret	53	F	Oral cavity	T4N1M0
6	ND		LOH	LOH	81	M	Larynx	T3N2M0
7	1.37	N	Ret	ND	73	M	Larynx	T4N2M0
8	ND		LOH	LOH	75	F	Maxilla	T4N2M0
9	ND		LOH	LOH	47	M	Oral cavity	T2N3M0
10	0.69	N	Ret	Ret	65	M	Oropharynx	T1N2M0
11	ND		Ret	Ret	69	M	Larynx	T3N2 cM0
12	6.71	H	Ret	Ret	58	M	Hypopharynx	T3N1M0
13	0.01	L	Ret	Ret	79	M	Larynx	T4N2 cM0
14	4.21	H	NI	NI	53	M	Hypopharynx	T3N1M0
15	0.01	L	Ret	Ret	68	M	Hypopharynx	T4N3M0
16	3.92	H	LOH	LOH	57	M	Hypopharynx	T4N2M0
17	0.19	L	Ret	LOH	69	M	Hypopharynx	T3N2M0
18	ND		Ret	Ret	68	F	Oral cavity	T4N2M0
19	0.35	L	NI	NI	59	M	Larynx	T3N2aM0
20	1.73	H	Ret	Ret	57	M	Oropharynx	T4N1M0
21	2.26	H	Ret	Ret	72	M	Oral cavity	T3N1M0
22	ND		Ret	LOH	67	M	Oral cavity	T3T2 bM0
23	ND		NI	NI	71	M	Oropharynx	T3N1M0
24	0.90	N	LOH	ND	66	M	Hypopharynx	T4N2M0
25	ND		ND	Ret	76	M	Oropharynx	T2N2M0
26	0.54	L	LOH	LOH	68	M	Oropharynx	T2N3M0
27	0.04	L	Ret	Ret	73	M	Oral cavity	T4N2 bM0
28	0.26	L	Ret	Ret	66	M	Larynx	T2N2 bM0
29	0.17	L	Ret	ND	63	M	Hypopharynx	T3N2 cM0
30	1.56	H	LOH	LOH	71	M	Hypopharynx	T2N2 bM0

†Ratio of expression levels was described in Materials and Methods.
‡According to the International Union Against Cancer 1997 TNM classification system.
F, female; H, high mrna expression; L, low mrna expression; M, male; N, normal mrna expression; ND, not done; NI, not informative; Ret, retention of allele.

Quantitative real-time RT-PCR. To confirm the validity of the semiquantitative RT-PCR expression data, the MAL mRNA levels in HNSCC cell lines were assessed by quantitative real-time RT-PCR carried out on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The real-time PCR was carried out in a final volume of 20 μL for each microtube containing 1 μL cDNA sample, 10 μL of 2× TaqMan Universal Master Mix with AmpErase uracil-*N*-glycosylase, and 600 nM primers and MAL probe. For primers and GAPDH probe (endogenous control gene), 1 μL of 20× TaqMan Gene Expression Assay (Applied Biosystems) was used. The thermocycle program was set at 50°C for an initial hold for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C, and extension at 60°C for 1 min. All samples were run in triplicate. RQ Study Software (Applied Biosystems, Foster City, CA, USA) analyzed the amplification results by a comparative method (ΔΔCt) to define the relative quantification of MAL as a fold change. The primer and probe sets used for MAL amplification were as follows: forward primer, 5′-CCT GCC CAG TGG CTT CTC; reverse primer, 5′-GGA GGA GGC CAC CAG GAT; and TaqMan probe (VIC) 5′-CCC GAC TTG CTC TTC ATC TTT GAG TTT AT-(TAMRA) as reported by Tracey *et al.*[13] For GAPDH (endogenous control), the primer and probe set of the TaqMan Gene Expression Assay (Hs99999905_m1; Applied Biosystems) was used.

Table 3. Primers used for expression and mutation analysis

Gene	Exon	Site	Sequence (5′–3′)
MAL	1	F	GAGCCAGCGAGAGGTCTG
		R	TTCCCCTCATTCTGTTGGTC
	2	F	ATGCCTGCCCTGTTCTCTTTG
		R	CTCACCTGGCACTGGAAAAGC
	3	F	CCCCACAGCAGTGAAGTGAGA
		R	CTAGGCAGCCTCCACACACAC
	4	F	GATGCAGTGCAGACGCTGTG
		R	TCCACCATCAAGGGCATTTCT
MAL		RT-S	CAGTGGCTTCTCGGTCTTCAC
		RT-AS	GTAAACACAGCACCCACGAGC
Glyceraldehyde-3-phosphate dehydrogenase		S1	AGACCACAGTCCATGCCA TCAC
		AS1	GGTCCACCACCCTGTTGCTGT
MAL-MS1		F	CCCAGCACGTAACTCCTCTT
		R	CACTGTGGGTGAAGCTGATG
MAL-MS2		F	TACCCAGCACAGAAACTCTC
		R	CGTGAGCTTTCCTCTGACTC

MAL, T-lymphocyte maturation-associated protein; F, forward primer; R, reverse primer; RT-S, sense primer for complementary DAN; RT-AS, anti-sense primer for complementary DNA; S1, sense1; AS1, anti-sense primer.

Immunofluorescence analysis. Cells were seeded at 5×10^4 per well in eight-well chambered coverslips and cultured overnight. Sections were fixed in acetone for 10 min at 4°C. After blocking in 1% bovine serum albumin, the cells were incubated with primary antibody (MAL [H-70], 1:70; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The secondary antibody was Texas Red-conjugated goat-anti rabbit IgG (1:100; Santa Cruz Biotechnology). Sections were mounted with Ultracruz Mounting Medium with 4′,6-diamidino-2-phenylindole (Santa Cruz Biotechnology) to stain cell nuclei. All experiments were carried out in the absence of primary, secondary, or both antibodies as negative controls. Immunofluorescence images were captured using an Eclipse E800 microscope (Nikon, Tokyo, Japan). Identical acquisition methods were used for all samples to allow direct comparison of the resulting images. Quantification of immunofluorescence signal was conducted using Image-Pro plus 4.0 software (Media Cybernetics, Silver Springs, MD, USA). Measurements were made for the total intensity of immunofluorescent staining using 10 images of areas within each slide and separate measurements were made for each cell to achieve the average intensity per 100 cells.

5-Aza-2′-deoxycytidine treatment. To examine MAL expression in response to treatment with 5-aza-2′-deoxycytidine (5-aza-CdR) (Sigma, St Louis, MO, USA), cell lines were incubated for 72 h with 4 μM 5-aza-CdR, and then harvested for RNA extraction and RT-PCR.

DNA isolation. Genomic DNA was isolated from frozen tissue samples and cell line cultures by sodium dodecylsulfate–proteinase K treatment, phenol–chloroform extraction, and ethanol precipitation. Although tissue samples were not microdissected, hematoxylin–eosin staining during initial diagnosis revealed that normal tissue from each pair did not contain tumor cells, and that the tumor cell ratio in each tumor tissue sample was over 70%.

Microsatellite analysis. LOH analysis was carried out with the MAL-specific microsatellite marker MAL-MS (Table 3). The mapping information and sequences were obtained from recent genomic information at http://www.ncbi.nlm.nih.gov/genome/guide/human. The heterozygosity and repeat numbers of the tandem nucleotide repeats for the design of microsatellite markers were acquired from the information site http://www.gramene.org/db/searches/ssrtool. The primers were designed based on the contiguous genomic sequence (NT_026970) using Genetyx-Win 5.0.0. The procedure for analysis has been described previously.[14] Briefly, after sense primers were labeled with 5-iodoacatamidefluorescein, PCR was carried out in 20 μL reaction mixture containing 10 pmol of each primer, 100 ng genomic DNA, 1× PCR buffer, 200 μM of each deoxynucleoside triphosphate, and 0.5 U *Taq* DNA polymerase (Takara, Kyoto, Japan). The PCR products were applied to an ABI Prism 3100 DNA sequencer (Applied Biosystems) and analyzed using Genescan analysis software version 3.7 (Applied Biosystems).

Mutation analysis of *MAL*. Four coding exons of the *MAL* gene were amplified with intron-spanning primers designed using Genetyx-Win 5.0.0 software. The primers are listed in Table 3. PCR was carried out as described above. PCR products were purified using ExoSAP-IT (USB, Cleveland, OH, USA) prior to sequence-specific PCR. Purified PCR products were reamplified with a BigDye terminator sequencing kit (v1.1 cycle sequencing kit; Applied Biosystems), ethanol precipitated, and direct sequenced on an automated capillary sequencer using the primers above (ABI Prism 310). Any detected nucleotide change was confirmed by independent PCR amplification and sequencing.

Results

Expression analysis of MAL mRNA in HNSCC cell lines. We evaluated the expression level of MAL mRNA in 35 HNSCC cell lines, including 26 nodal metastasis-derived cell lines. Furthermore, nine cell line pairs established from matched primary and metastatic tumors of the same patients were also included. Semiquantitative RT-PCR was carried out using primers designed to encompass the exon–intron junctions on the cDNA in order to eliminate the potential contamination of genomic DNA. Out of 26 metastatic cell lines, 17 (65%) showed downregulation of MAL expression, whereas only four out of nine primary tumor-derived cell lines displayed downregulation. Moreover, no expression was found in 10 cell lines (29%), including eight metastasis-derived cell lines. Considering primary and metastatic tumor-derived cell line pairs, four cell line pairs showed decreased or no expression in both, whereas four cell line pairs showed decreased expression only in the metastatic cell lines compared with their primary counterparts (Fig. 1a).

Expression analysis of MAL mRNA in primary and metastatic tumor tissues. We analyzed the expression level of MAL mRNA in 21 metastatic tumor samples, comparing them with their paired primary tumor tissues by semiquantitative RT-PCR (Fig. 1b; Table 2). Expression analysis demonstrated decreased expression of MAL mRNA in 9 out of 21 metastatic tumors (43%), including two samples with no detectable expression compared with those of matched primary tumor samples. Three of the samples (14%) showed a similar level of expression in primary and metastatic tumors, whereas nine of the samples (43%) appeared to have increased expression of MAL in metastatic tumor tissues.

Real-time RT-PCR also confirmed the results of semiquantitative RT-PCR in the pairs of primary and metastatic tumor-derived cell lines. Out of eight cell line pairs, four displayed remarkable decreases of MAL expression in the metastasis-derived cell lines compared with their primary counterparts (Fig. 2a).

Restoration of MAL mRNA expression after 5-aza-CdR treatment. Potential CpG islands in the MAL promoter were detected according to the CpGplot algorithm (http://www.ebi.ac.uk/emboss/cpgplot/). To determine whether methylation of MAL is associated with transcriptional silencing, we examined and compared the expression of MAL mRNA before and after treatment with 5-aza-CdR, a demethylating agent, by RT-PCR in 20 cell lines (Fig. 1c). We found restoration of MAL mRNA after 5-aza-CdR treatment in 9 (UT-SCC-24A, 26A, 58, 59A, 60B, 64, 74A-B, and 104) of 20 (45%) demethylated cell lines. An increase in MAL mRNA expression was not detected in 11 cell lines (UT-SCC-6A, 9, 12A, 24B, 54C, 61, 62, 71, 77, 90, and 110A), including five cell lines with no MAL expression.

Real-time RT-PCR validated the results of semiquantitative RT-PCR concerning restoration of MAL mRNA after demethylation. We analyzed the expression status of MAL in 12 cell lines before and after treatment with 5-aza-CdR and 58% (7/12) showed more than two-fold upregulation of expression related with demethylation (Fig. 2b).

Immunofluorescence analysis of MAL protein. To validate the expression of MAL protein, we carried out immunofluorescence analysis in two cell line pairs: UT-SCC-16A with UT-SCC-16B, and OKKP with OKKM. The metastasis-originated cell lines UT-SCC-16B and OKKM demonstrated 55 and 40% decreases of the immunofluorescence signal, respectively, compared to their primary counterparts (Fig. 3).

Mutation analysis of MAL. We also examined the mutation status of exons 1–4 of MAL in our 24 head and neck cancer cell lines. Exon–intron boundaries were established from the National Center for Biotechnology Information sequence database. PCR amplicons were designed to span all four coding exons of MAL by Genetyx software. All coding exons and exon–intron junctions of MAL were screened for mutation by PCR amplification and subsequent direct sequencing. Sequence analysis of each cell line demonstrated no nucleotide substitution in exons 1–4 of MAL.

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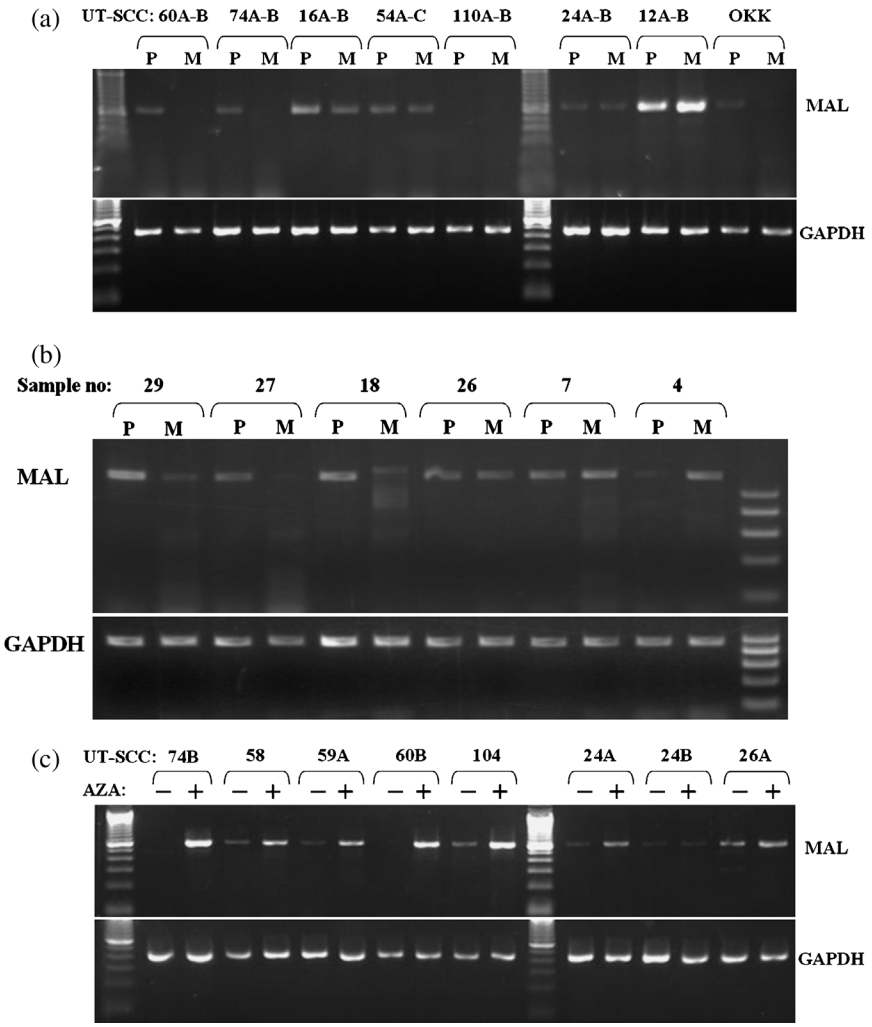

Fig. 1. Expression analysis of T-lymphocyte maturation-associated protein (MAL) mRNA in head and neck squamous cell carcinoma (HNSCC) cell lines and tumor tissues by reverse transcription–polymerase chain reaction (RT-PCR). (a) Expression of the *MAL* gene in eight representative HNSCC cell line pairs derived from matched primary (P) and metastatic (M) tumors. The UT-SCC-60, 74, 16, and OKK-TK metastasis-derived cell lines had no or substantially reduced expression compared to their primary tumor-derived cell line counterparts. Only the UT-SCC-12 cell line pair displayed strong bands in both cell lines, whereas the other cell line pairs showed absent or significantly reduced bands in both cell lines. (b) Expression of the *MAL* gene in matched primary tumor (P) and metastatic tumor (M) of the lymph node: samples 29, 27, 18, and 26 showed decreased expression of MAL in metastatic tissues compared with primary tumors. Upper numbers show sample numbers. Lower panels represent expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (c) Restoration of MAL expression by demethylation. UT-SCC cells were left untreated (–) or treated (+) with 4 μM 5-aza-2′-deoxycytidine (AZA) for 72 h and were analyzed by RT-PCR: UT-SCC-74B, 58, 59A, 60B, 104, and 24A showed restoration of MAL expression.

Loss of heterozygosity status of the *MAL* locus. We examined the LOH status of the *MAL* locus using the highly polymorphic microsatellite marker MAL-MS in 29 matched normal and HNSCC tissues. The MAL-MS marker is located approximately 165 kb centromeric to the *MAL* gene (contiguous sequence NT_026970 and Human Genome Resources; http://www.ncbi.nlm.nih.gov/genome/guide/human). LOH status was analyzed in primary tumors and their metastatic counterparts separately. Microsatellite analysis showed 30% of LOH frequency in primary tumors and 38% in metastatic counterparts. Considering the close localization of the microsatellite with MAL and the high LOH percentage of each marker, these results suggest that LOH is an important genetic mechanism in loss of function for MAL in metastatic tumors. Representative samples of informative cases and those with LOH are shown in Figure 4.

Discussion

Tumor masses consist of heterogenic subpopulations of cancer cells. According to cancer stem cell theory, only a specific subpopulation of these cells has the ability to sustain cancer growth and metastatic activity, whereas all of the other cancer cells have only a limited growth potential or no growth potential at all. Based on this concept, to define the aggressiveness of a tumor, it is mandatory to analyze the molecular characteristics of metastatic cell populations as an appropriate representative of cancer stem cells.

In the present study, we found downregulation of *MAL* gene expression both in cell lines derived from lymph node metastasis

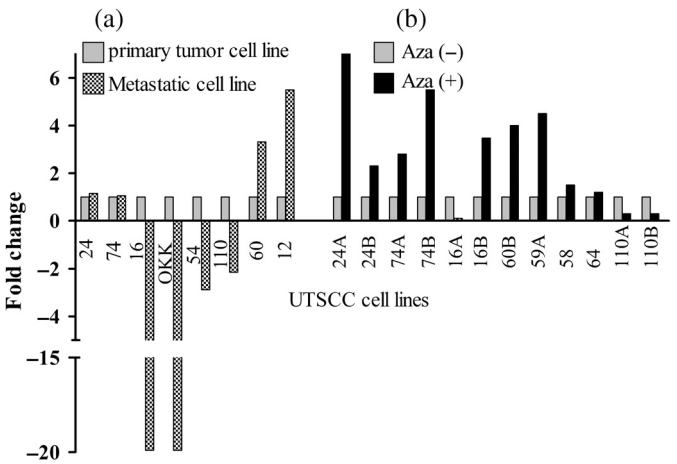

Fig. 2. Confirmation of the expression levels of T-lymphocyte maturation-associated protein (MAL) mRNA by real-time polymerase chain reaction. (a) Comparison of MAL expression levels between primary tumor-originated and metastasis-originated UT-SCC cell lines. (b) Comparison of MAL expression levels before (Aza–) and after demethylating agent treatment (Aza+) of UT-SCC cell lines.

(65%) and metastatic tumor tissues (43%) in lymph nodes compared with their primary tumor counterparts of HNSCC. Interestingly, in four cell line pairs out of nine (44%), metastatic tumor-derived cell lines showed decreased or no expression of

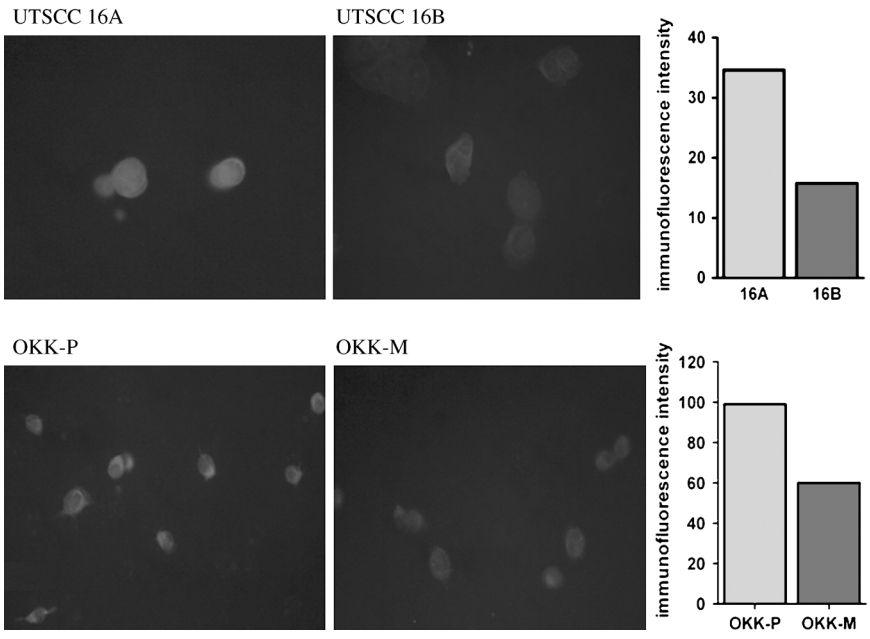

Fig. 3. Representative images of T-lymphocyte maturation-associated protein (MAL) expression as determined by immunofluorescence microscopy (Texas Red). Comparative values of immunofluorescence intensity averages for primary tumor-originated cell lines (16A and OKK-P) and their metastasis-originated counterparts (16B and OKK-M) are given on the right.

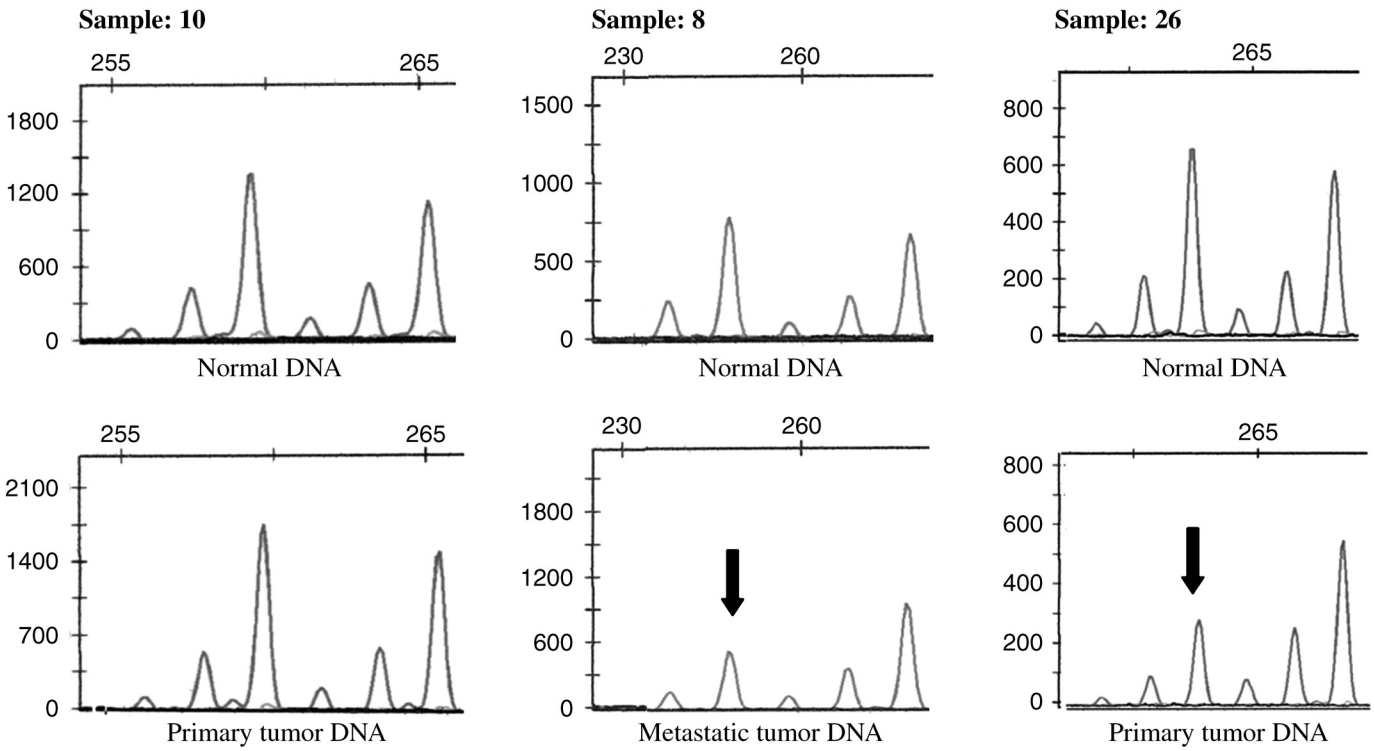

Fig. 4. Representative electropherograms of loss of heterozygosity (LOH) analysis on the T-lymphocyte maturation-associated protein gene locus by microcapillary electrophoresis and data analysis. LOH was scored by comparing the peak heights of tumors and matched normal gene alleles. The arrows mark the lost allele of samples 8 and 26 in primary tumor and metastasis, respectively, whereas sample 10 represents retention.

MAL compared to matched cell lines derived from primary tumors, whereas four cell line pairs displayed decreased or no expression both in primary and metastatic-derived cell lines. Previous gene expression profiles revealed that the *MAL* gene is frequently downregulated in primary tumors of HNSCC.[5–7] Moreover, one study on gene expression profiles of oral cavity oropharyngeal carcinomas showed decreased expression of MAL in primary tumors with metastasis.[4] Interestingly, it was found in a recent study that ectopic expression of MAL in esophageal carcinoma cells leads to inhibition of cell motility.[15] These observations together with our present data suggest that a decrease of MAL expression may be an important biomarker to define cancer cell subpopulations with metastatic ability, and the *MAL* gene may function as a metastasis suppressor in HNSCC, although further functional studies are needed to clarify the role of MAL in the metastatic process of HNSCC.

The *MAL* gene encodes a membrane proteolipid that is emphasized as a central component of the integral protein

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machinery for apical transport.[16] The hydrophobicity profile of MAL was found to show remarkable homology to different proteolipids involved in gap junction formation.[8] Puertollano *et al*. proposed that MAL is responsible for the formation and trafficking of apical transport vesicles in polarized epithelial cells.[17] Transportation of newly synthesized proteins designed for the apical or basolateral subdomains is fundamental for the establishment and maintenance of polarity in epithelial cells. Defects in cell polarity are closely related with cancer transformation and metastasis progression linked with loss of E-cadherin expression contrary to polarized epithelial cells with limited ability to move because of cell–cell adhesion.[18] It will be crucial in further studies to clarify the role of the MAL protein in the establishment of cell polarity.

Inactivation of metastasis-suppressor genes may occur via genetic and epigenetic alterations, including LOH, deletion, mutation, and hypermethylation of gene promoters. We found remarkable LOH frequency (30%) in primary tumor samples showing lymph node metastasis. Furthermore, LOH was also confirmed in metastatic tumor samples in lymph nodes. The *MAL* gene is located on chromosome 2 at the q11 locus. Previously, LOH on chromosome 2q has been shown to be correlated with poor prognosis in early stage HNSCC and tumor-node-metastasis (TNM) stage in oral squamous cell carcinoma.[19,20] In lung carcinoma, frequency of LOH on 2q has been found to be significantly higher in brain metastasis than in primary tumors.[21] Interestingly, another study on oral carcinomas displayed an increased LOH frequency in metastatic tumors compared with primary tumors in the 2q12 region (D2S436), which is in close association with the *MAL* locus.[22] All of these studies are in accordance with our findings and support the role of LOH in the 2q11-12 locus relating to development of metastasis.

Loss of heterozygosity in only one allele is usually not enough for biallelic inactivation of the gene except homozygous deletion. As a second silencing event, we analyzed the mutation status; however, no nucleotide change was found in any of the exons of MAL in the UT-SCC cell line series. Similarly, no somatic mutation of *MAL* has been found in cervical cancer;[23] implying that it is an unlikely mechanism in downregulation of MAL expression.

The promoter region of *MAL* includes CpG islands and we demonstrated that methylation of *MAL* was associated with gene silencing in nine HNSCC cell lines by restoration of MAL expression after treatment with a demethylating agent. These results are consistent with earlier reports. Mimori *et al*. observed re-expression of the *MAL* gene in only 3 out of 13 esophageal carcinoma cell lines, whereas simultaneous inhibition of deacetylation and methylation induced MAL expression in 12 out 13 cell lines.[15] Similarly, Lind *et al*. reported hypermethylation in cancer cell lines from various tissues, including breast, kidney, pancreas, uterus, and colon with frequencies of 50–95%, and colon cancer displayed the highest frequency among them.[24] These findings indicate that methylation of the *MAL* promoter is an important and significant inactivation mechanism not only for HNSCC but also for various types of other cancers.

The nature of the relationship between primary and metastatic tumor cells in the formation of metastases is not clearly known. Therefore, different models have been hypothesized to explain the metastatic ability of a primary tumor. It is still a matter of debate whether metastasis depends on only a highly capable minor cell population within the tumor, or whether whole cells of primary tumors have a predisposition for metastasis especially in patients with poor prognosis. The recent study of Kang *et al*. on the genetic properties of breast cancer for bone metastasis revealed that both models may exist together.[25] In that study, microarray analysis defined genetic predisposition in primary tumors for metastatic ability; however, metastatic tumor cells themselves also had different genetic characteristics than their own primary origins. In our study, we showed that metastasis-originated cell lines have different genetic and epigenetic features compared with their primary counterparts on the basis of MAL expression. Perhaps these types of genetic and epigenetic alterations will define the final steps for the formation of metastasis in a predisposed primary tumor background.

In conclusion, our results show that expression of the *MAL* gene was decreased or lost selectively in metastatic tumor cells compared with their primary tumor counterparts in HNSCC, suggesting that the *MAL* gene may be a new candidate metastasis-suppressor gene for HNSCC. LOH and hypermethylation of the promoter region appear to be important mechanisms for inactivation of MAL function. Further investigation including *in vitro* and *in vivo* experiments needs to be conducted to identify the functional role of the *MAL* gene in the metastatic process.

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