Laboratory Investigation (2013) 93, 1331–1338

Stem cell self-renewal factors Bmi1 and HMGA2 in head and neck squamous cell carcinoma: clues for diagnosis

Hiroshi Yamazaki[1,6], Taisuke Mori[2,6], Masaki Yazawa[3], Akiko M Maeshima[4], Fumihiko Matsumoto[5], Seiichi Yoshimoto[5], Yoshihide Ota[1], Akihiro Kaneko[1], Hitoshi Tsuda[4] and Yae Kanai[2]

Head and neck squamous cell carcinoma (HNSCC) includes both morphological and functional cellular heterogeneity, as would be expected if it arose from dysregulated stem or progenitor cells as opposed to the simple clonal expansion of a mutated cell; however, stemness molecule expression levels and distribution in HNSCC remain unclear. To clarify this, stemness molecule expressions were determined in HNSCC, as well as their properties and prognosis. Two proto-oncogenic chromatin regulators, Bmi-1 and high-mobility-group A2 (Hmga2), were identified in 12 pair cases of HNSCC tumor regions by comparison with their non-cancerous background tissues using cDNA microarray. Both Bmi-1 and Hmga2 are known to promote stem cell self-renewal by negatively regulating the expressions of Ink4a and Arf tumor suppressors. Despite similar targets, Bmi-1 protein was expressed in an early cancerous region and HMGA2 protein was expressed in a region showing more progression. Similarly, Bmi1 expression had no significance with regard to overall survival ($P = 0.67$), whereas HMGA2 expression was associated with decreased overall survival ($P = 0.05$). Quantitative real-time reverse transcription polymerase chain reaction analyses also correlated with protein levels. These findings suggest that Bmi-1 is an early detection marker to distinguish cancerous from non-cancerous regions, whereas HMGA2 is presumed to be a tumor prognosis marker. Among our HNSCC analyses, these stemness molecules expressed fewer primitive rare cells in the tumor than all other cells in the tumor. HNSCC cells with high expression of stemness molecules partly behave like stem cells.

Laboratory Investigation (2013) **93,** 1331–1338; doi:10.1038/labinvest.2013.120; published online 21 October 2013

KEYWORDS: Bmi1; HMGA2; head and neck; squamous cell carcinoma

Head and neck squamous cell carcinoma (HNSCC) is typically associated with virus infection, persistent chronic inflammation, and tobacco and alcohol use. Survival rates have changed in the last 40 years because of reductions in these factors;[1] however, the mortality rate remains high because advanced and recurrent locoregional control is difficult in many cases. Better understanding of the biology of HNSCC is required to define relevant targets and to develop novel therapeutic approaches. HNSCCs have both morphological and functional cellular heterogeneity, as would be expected if they arose from dysregulated stem or progenitor cells as opposed to the simple clonal expansion of a mutated cell.[2] Recently, numerous reports have shown support for the 'cancer stem cell' theory in many types of tumor, including HNSCC.[3,4,5] Acute and chronic myeloid leukemias follow the cancer stem cell model. Both tumors show robustly hematopoietic hierarchical organization in the tumor cells;[6,7] however, for solid cancers such as squamous cell carcinoma, it is not clear how generalizable the cancer stem cell model is. Not all cancer cells have the same capacity to proliferate.[8] In some cancers, most cancer cells appear to have limited ability to proliferate, while in the same tumors, stochastic minority populations of stemness molecules highly expressed in cancer as a 'stem-like cancer' retain the capacity to proliferate indefinitely. Normal stem cells and 'stem-like' cancer cells share various significant properties and have similar characteristics.[9] We previously reported that the combination of Bmi-1 and TERT gene induction immortalized bone marrow stromal cells that

[1]Department of Oral Surgery, School of Medicine, Tokai University, Kanagawa, Japan; [2]Division of Molecular Pathology, National Cancer Center Hospital, Tokyo, Japan; [3]Department of Plastic Surgery, Keio University School of Medicine, Tokyo, Japan; [4]Departments of Pathology and Clinical Laboratories, National Cancer Center Hospital, Tokyo, Japan and [5]Department of Head and Neck Oncology, National Cancer Center Hospital, Tokyo, Japan
Correspondence: Dr T Mori, DMD, PhD, Division of Molecular Pathology, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuou, Tokyo 104-0045, Japan.
E-mail: tamori@ncc.go.jp or taisuke@a8.keio.jp
[6]These authors contributed equally to this work.

Received 12 June 2013; revised 13 September 2013; accepted 14 September 2013

proliferated slowly, but continuously, over 600 days.[10] Moreover, we reported that Bmi-1 is highly expressed in early-stage hepatocellular carcinoma (HCC).[11] Bmi-1, as one of the polycomb group genes, is required for the self-renewal of hematopoietic stem cells (HSCs) and neural stem cells.[12] Here, we identified two proto-oncogenic chromatin regulators, Bmi-1 and high-mobility-group A2 (Hmga2), in 12 pairs of cases in HNSCC tumor lesions and their non-cancerous background by cDNA microarray. To clarify the clinical significance of the expressions of these molecules in HNSCC, we focused on the correlation with pathological factors as well as prognosis.

MATERIALS AND METHODS

Tissue Samples for Oligo-Nucleotide Microarray and Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

HNSCCs and corresponding non-cancerous squamous epithelium tissues were obtained from patients who underwent sequential surgical resection at Tokai University Hospital, Japan, between 2006 and 2008 (38 HNSCC cases: 18 tongue, 13 gingiva, 3 oral floor, 3 buccal mucosa, and 1 palate). Our patients' ages at onset were 34–91 years old (mean 68.5). We macroscopically separated SCCs and non-cancerous lesions. Histological diagnosis was made according to the WHO criteria. Informed consent was obtained from the patients and the Ethics Committee of Tokai University School of Medicine approved the procedures (Approval #06 R-087).

Tissue Samples for Immunohistochemical Analysis

We collected formalin-fixed paraffin-embedded specimens from the 91 cases (33 well-differentiated, 31 moderately differentiated, and 27 poorly differentiated SCCs) of HNSCC. For the overall survival experiment, 64 resected cases (without chemotherapy and radiotherapy) were used in which the tongue squamous cell carcinoma tumor was <4 cm and there was no metastasis (T1, and T2, N0, M0). The tumor tissues were surgically resected between 1998 and 2006 at the National Cancer Center Hospital in Tokyo. Informed consent was obtained from the patients and the Ethics Committee of the National Cancer Center Hospital Tokyo, Japan, approved the procedures (Approval #2010–075).

Tissue Microarray for Immunohistochemical Analysis

The tissue microarray was set up as described previously.[11] Immunohistochemical staining was performed with the following primary antibodies: Bmi1 (1:400; Cell Signaling, Boston, MA, USA, D20B7 XP), HMGA2 (1:400; Biocheck, Foster City, CA, USA). For staining, we used an automated stainer (Dako) according to the manufacturer's protocol. Appropriate positive and negative controls were used for each antibody.

Real-Time Quantitative RT-PCR

qRT-PCR analysis was performed as reported previously[11] three times, including a no-template negative control. The primer sets are shown below. For Bmi-1: 5′-GAGGGTACTT CATTGATGCCACAAC-3′ (forward), 5′-GCTGGTCTCCAGG TAACGAACAATA-3′ (reverse); for HMGA2: 5′-AAGTTGTT CAGAAGAAGCCTGCTCA-3′ (forward), 5′-TGGAAAGACC ATGGCAATACAGAAT-3′ (reverse); and for GAPDH: 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward), 5′-ATGGT GGTGAAGACGCCAGT-3′ (reverse).

Table 1 Relative expression levels of selected stemness genes in 12 paired HNSCC cases by Genechip analysis

Symbol	Description	Max.	Min.	Positive fold-change cases (%)	Mean Ts *vs* Ns	*P*-value	Genbank	Systematic
Bmi1	BMI1 polycomb ring finger oncogene	3.7	0.4	7/12 (58%)	1.22 *vs* 1.09	0.61	NM_005180	A_24_P303989
HMGA2	High mobility group AT-hook 2, transcript variant 1	297.2	0.41	11/12 (92%)	7.79 *vs* 0.68	<0.01	NM_003483	A_23_P95930
GNL3L	Guanine nucleotide-binding protein-like 3 (nucleolar)-like	6.82	1.04	12/12 (100%)	1.23 *vs* 0.83	0.03	NM_019067	A_23_P22499
CD44	CD44 molecule (Indian blood group), transcript variant 1	4.69	0.8	10/12 (83%)	1.30 *vs* 0.86	0.03	NM_000610	A_23_P24870
YBX1	Y-box-binding protein 1	2.15	0.99	10/12 (83%)	1.23 *vs* 0.90	<0.01	NM_004559	A_32_P218989
Epcam	Epithelial cell adhesion molecules	17.58	0.06	6/12 (50%)	3.30 *vs* 1.38	0.29	NM_002354	A_23_P91081
Prom1	Prominin 1 (CD133)	39.31	0.08	3/12 (25%)	0.99 *vs* 1.38	0.76	NM_006017	A_23_P258463
SHH	Sonic hedgehog homolog	4.65	0.11	3/12 (25%)	1.14 *vs* 1.37	0.54	NM_000193	A_23_P111657
Notch1	Notch homolog 1, translocation-associated	2.64	0.19	4/12 (33%)	0.97 *vs* 1.22	0.33	NM_017617	A_23_P60387

Abbreviations: Max.: maximum fold-change; Min.: minimum fold-change; Mean Ts *vs* Ns: average expression levels in tumor lesions *vs* non-cancerous lesions. Positive fold-change cases: number of cases in which the gene was expressed more highly in the tumor than in the control (non-cancerous lesion) of each of the 12 cases.

cDNA Microarray/eArray Analysis

Profiling of mRNA was carried out according to the manufacturer's instructions. All 24 labeled samples were hybridized to the Agilent 60-mer oligo microarray with an $8 \times 15\,000$ probe format (eArray Group: Design ID: 021445 Agilent Technologies eArray website: http://earray.chem.agilent.com).

Statistical Analysis

Data are expressed as the mean ± s.e. The relative mRNA expression levels of Bmi1 or HMGA2 were compared using the unpaired *t*-test. The χ^2 or Fisher's exact probability test was used when appropriate to determine the correlation between clinicopathological variables and Bmi1 or HMGA2 expression. Survival rates were calculated by the Kaplan–Meier method, and the log-rank test was applied to compare survival between different groups. All statistical analyses were performed using Statcel software (OSM, Tokyo, Japan) and SPSS statistical software (SPSS).

RESULTS

Microarray Analysis of Stem Cell-Related Gene mRNA Profiling in 12 HNSCC Cases

As a first step, we examined whether commonly reported stem cell-related genes are expressed in HNSCC. Twelve paired cases of HNSCC tumor regions (Ts) and their non-cancerous background tissues (Ns) were analyzed using Agilent eArray for screening. To identify 'cancer/stem cell' markers, we searched in public databases and selected stemness molecules from among genes highly expressed in human embryonal stem (ES) cells, human HSCs, and also more highly expressed genes, that is, >1.2 times the level in Ts, in our HNSCC microarray data; in addition, we also focused on those described in many reports as 'cancer/stem cell' markers: Prom1, Sonic-hedgehog (SHH), and Notch1. Finally, we selected the following stemness molecules in this study: Bmi1, HMGA2, guanine nucleotide-binding protein-like 3 (GNL3L), CD44, Y-box-binding protein 1 (YBX1), epithelial cell adhesion molecules (EpCAMs), CD133/Prominin 1, SHH, and Notch 1.

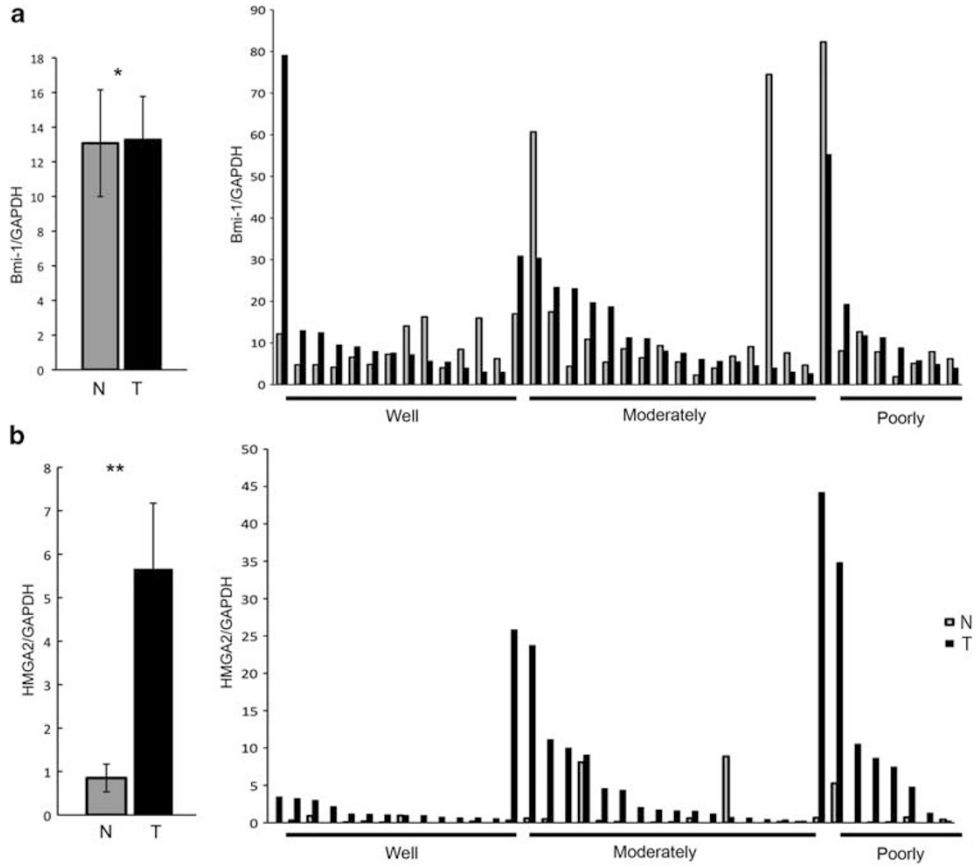

Figure 1 Bmi-1 and HMGA2 expressions in head and neck squamous cell carcinoma (HNSCC) clinical samples. mRNA expression levels in 38 cases of HNSCC. The relative mRNA expression levels in tumor tissues (T: dark gray/black bar) and their corresponding non-cancerous background tissues (N: gray bar) in each of the 38 cases are shown in the right panel. Samples 1–13 are from well-differentiated HNSCCs; 14–30 from moderately differentiated HNSCCs; 31–38 from poorly differentiated HNSCCs. (**a**) Bmi-1 and (**b**) HMGA2. The average expression level of Bmi-1 was slightly higher in tumor tissues than in non-cancerous background liver tissues (13.27 *vs* 13.07; $^*P = 0.96$; left panel). The average expression level of HMGA2 was significantly higher in tumor tissues than in non-cancerous background squamous epithelium (6.05 *vs* 0.77; $^{**}P < 0.01$; left panel).

The average HMGA2, GNL3L, CD44, and YBX1 expressions in Ts were significantly higher than in Ns (mean T *vs* N; 7.79 *vs* 0.68, $P=0.02$; 1.23 *vs* 0.83, $P<0.01$; 1.30 *vs* 0.86, $P=0.03$; 1.23 *vs* 0.90, $P<0.01$, respectively). Bmi-1 and EpCAM in Ts were also higher than in Ns, but the differences were not statistically significant (1.22 *vs* 1.09, $P=0.61$; 3.30 *vs* 1.38, $P=0.29$, respectively). Meanwhile, Notch1 and CD133/Prominin 1 mean expressions in Ts were lower than in Ns (mean 0.97 *vs* 1.22, $P=0.33$; 0.99 *vs* 1.38, $P=0.76$, respectively). However, each individual case exhibited markedly higher expression levels in T. From among the screening array data, two candidate molecules, Bmi-1 and HMGA2, were identified as diagnostic markers. These molecules are proto-oncogenic chromatin regulators that promote stem cell self-renewal by negatively regulating the expression of Ink4a and Arf tumor suppressors. The relative expression and details of the selected stemness genes are summarized in Table 1.

Bmi-1 and HMGA2 Expression in HNSCC Clinical Samples

As a second step, Bmi-1 mRNA expression levels were investigated in 38 tumors (13 well-differentiated, 17 moderately differentiated, and 8 poorly differentiated HNSCCs) and their background non-cancerous squamous epithelium using qRT-PCR. Of these, 8 (61.6%) cases of well-differentiated, 10 (58.8%) cases of moderately differentiated, and 4 of 8 (50.0%) cases of poorly differentiated tumors showed higher expression than the corresponding non-cancerous background (Figure 1a, left). These results implied that relatively high Bmi-1 expression was observed in the well-differentiated HNSCCs and declined with progression. The average Bmi-1 expression was higher in tumors than in the non-cancerous background squamous epithelium, but the difference was not statistically significant (13.27 *vs* 13.07; $P=0.96$; Figure 1a, right).

We also analyzed HMGA2 mRNA levels in the same clinical cases. HMGA2 mRNA expression in well-differentiated

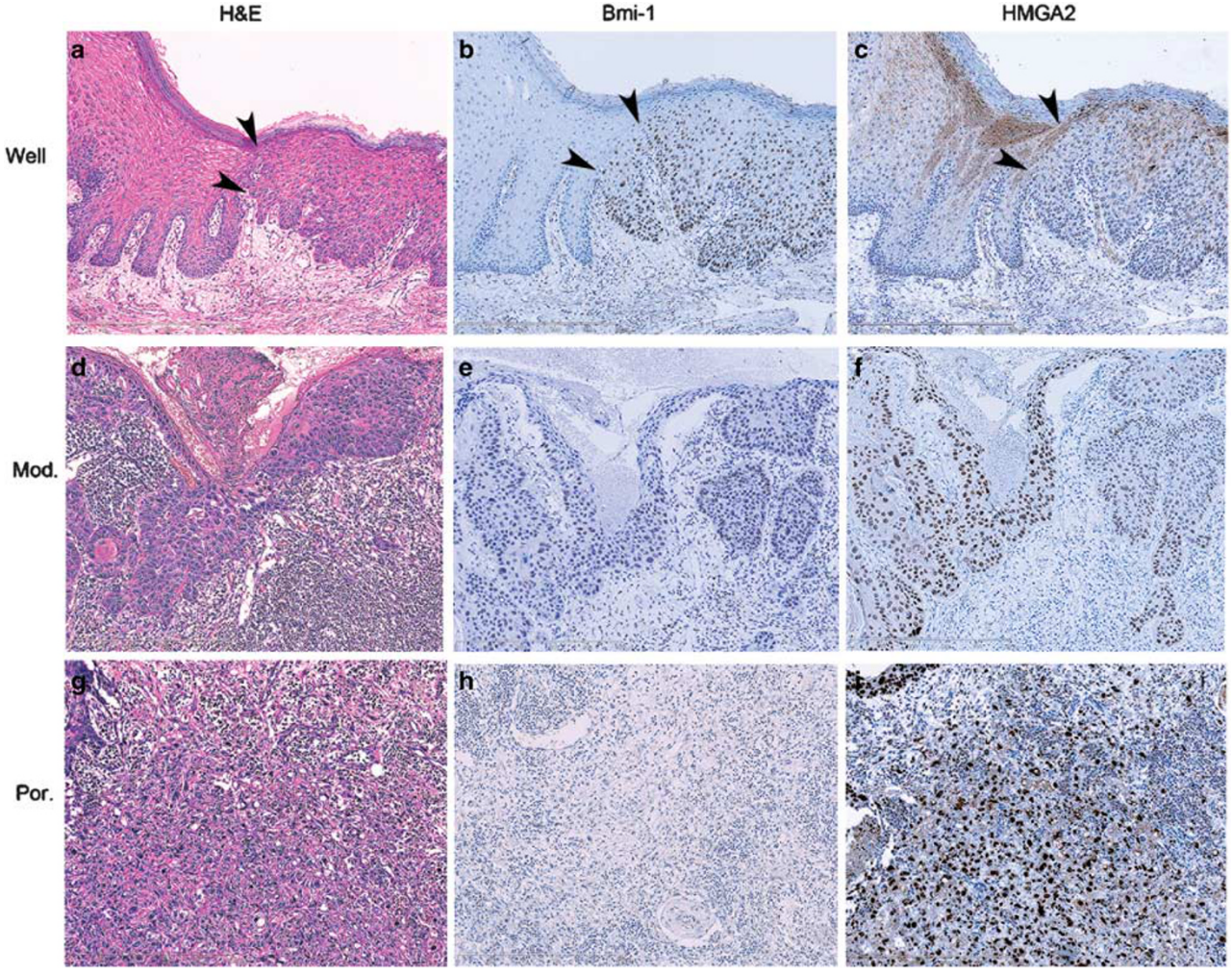

Figure 2 Bmi-1 and HMGA2 expression in well, moderately, and poorly differentiated HNSCC. Clear nuclear staining of Bmi-1 (scored as 2+) and an absence of HMGA2 (scored as negative) staining were observed in well-differentiated HNSCC (arrowheads). Bmi-1 expression appeared weaker (scored as 1+), and also a weaker staining pattern of HMGA2 (scored as 1+) was seen in moderately differentiated HNSCC. Absence of Bmi-1 (scored as negative) and strong staining of HMGA2 (scored as 2+) were observed in poorly differentiated HNSCC (**a**, **d**, **g**: H&E stain; **b**, **e**, **h**: corresponding Bmi-1 staining of each serial section; and **c**, **f**, **i**: corresponding HMGA2 staining of each serial section). (**a–c**) Well-differentiated HNSCC. (**d–f**) Moderately differentiated HNSCC. (**g–h**) Poorly differentiated HNSCC. Arrowheads outline the border between non-cancerous and tumor regions.

Table 2 Characteristics of 91 squamous cell carcinomas on the basis of immunostaining

	Bmi-1 expression		
Characteristics	2+/1+	−	*P*-value
No. of tumors	48	43	NA
Mean age (years)	61.1	63.4	NA
Gender			0.64
Female	19	15	
Male	29	28	
Differentiation			0.26
Well-differentiated SCC ($n=33$)	19	14	
Moderately differentiated SCC ($n=31$)	13	18	
Poorly differentiated SCC ($n=27$)	16	11	
Stage			<0.001
Stages I and II	48	30	
Stages III and IV	0	13	

	HMGA2 expression		
Characteristics	2+/1+	−	*P*-value
No. of tumors	66	25	NA
Mean age (years)	61.2	64.9	NA
Gender			0.51
Female	26	8	
Male	40	17	
Differentiation			<0.001
Well-differentiated SCC ($n=33$)	14	19	
Moderately differentiated SCC ($n=31$)	26	5	
Poorly differentiated SCC ($n=27$)	26	1	
Stage			0.04
Stages I and II	60	18	
Stages III and IV	6	7	

cases was significantly lower than in moderately and poorly differentiated cases. HMGA2 mRNA expression increased during HNSCC progression; however, almost all expressions were higher in tumors than in the corresponding non-cancerous background tissues. Of these, 13 (100%) were from well-differentiated, 16 (94.1%) were from moderately differentiated, and 8 (100.0%) were from poorly differentiated (Figure 1b, left) tumors. The average HMGA2 expression in all tumors was also significantly higher than in non-cancerous background tissues (6.05 *vs* 0.77, $P<0.01$; Figure 1b, right). All analyses were repeated three times.

Next, Bmi-1 and HMGA2 protein expression and histopathological factors were investigated in 91 clinical cases (33 well-differentiated, 31 moderately differentiated, and 27 poorly differentiated HNSCCs). The criteria for HNSCC

Table 3 Immunohistochemical analysis of Bmi-1 and HMGA2 expression in squamous cell carcinoma

	Bmi-1 staining score		
Histology	2+	1+	−
Well-differentiated SCC ($n=33$)	6 (18.2%)	13 (39.4%)	14 (42.4%)
Moderately differentiated SCC ($n=31$)	2 (6.5%)	11 (35.5%)	18 (58.1%)
Poorly differentiated SCC ($n=27$)	3 (11.1%)	13 (48.1%)	11 (40.7%)
	HMGA2 staining score		
	2+	1+	−
Well-differentiated SCC ($n=33$)	0 (0%)	14 (42.4%)	19 (57.6%)
Moderately differentiated SCC ($n=31$)	13 (41.9%)	13 (41.9%)	5 (16.1%)
Poorly differentiated SCC ($n=27$)	24 (88.9%)	2 (7.4%)	1 (3.7%)

evaluation were determined by immunohistochemical analysis of Bmi1 expression: clear staining of the nucleus was scored as a positive expression of 2+, weak staining inside the nucleus was scored as 1+, and no staining was considered negative. A score of 2+ was observed in 6 of 33 (18.2%) and 1+ in 13 of 33 (39.4%) well-differentiated HNSCCs, a score of 2+ was observed in 2 of 31 (6.5%) and 1+ in 11 of 31 (35.5%) moderately differentiated HNSCCs, and a score of 2+ was observed in 3 of 27 (11.1%) and 1+ in 13 of 33 (48.1%) poorly differentiated HNSCCs. In contrast, negativity was observed in 14 of 33 (42.4%) well-differentiated HNSCCs, 18 of 31 (35.5%) moderately differentiated HNSCCs, and 11 of 27 (40.7%) poorly differentiated HNSCCs. These results suggest that Bmi-1 expression did not show significant behavior in any HNSCC; however, Bmi-1 clearly distinguished a non-cancerous lesion at the boundary of the early-stage carcinoma/carcinoma in situ/CIS (Figures 2a and b), and 19 of 33 cases were positive (scored as 2+ or 1+) for Bmi1 protein. Meanwhile, 14 of 33 (42%) well-differentiated cases did not show Bmi1 protein expression, of which five cases were diagnosed as verrucous carcinoma and the other cases as hyper-keratinizing-type SCC (data not shown). Thus, Bmi-1 protein expression was found in early-stage well-differentiated HNSCCs. Similarly, Bmi-1 protein levels tended to decline with hyper-keratinizing and/or poorly differentiated HNSCC (Tables 2 and 3).

HMGA2 expression was also localized to the nucleus, which was evaluated by immunostaining with the same criteria for Bmi1 evaluation. A score of 2+ was observed in 0 of 33 (0%) and 1+ in 14 of 33 (42.4%) well-differentiated HNSCCs, a score of 2+ was observed in 13 of 31 (41.9%) and 1+ in 13 of 31 (41.9%) moderately differentiated HNSCCs, and a score of 2+ was observed in 24 of 27 (88.9%) and 1+ in 2 of 27 (7.4%) poorly differentiated HNSCCs. In contrast, it was observed as negative in 19 of 27 (57.6%) well-differentiated HNSCCs, 5 of 31 (16.1%) moderately differentiated HNSCCs,

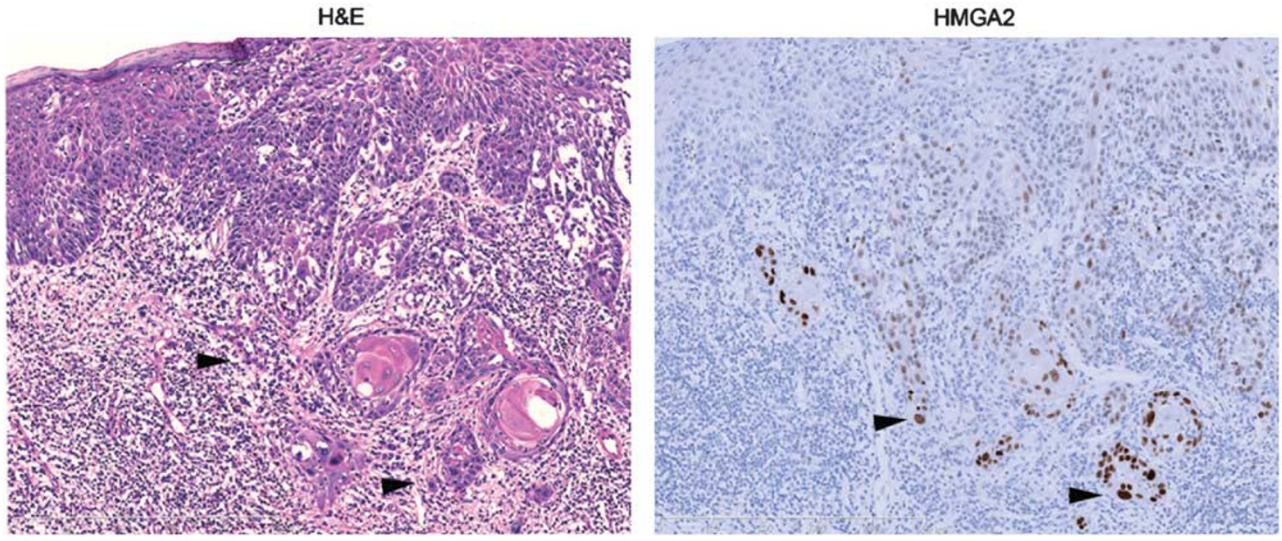

Figure 3 HMGA2 expression correlates with invasion and poor histopathological differentiation. HMGA2-expressing nuclei showed graded staining in invasive HNSCC. Typically, cells strongly expressing HMGA2 were observed at the tip of infiltrating single cells (arrowheads). In contrast, the tumor surface showed weak HMGA2 expression (**a**) H&E staining; (**b**) HMGA2.

and 1 of 27 (3.7%) poorly differentiated HNSCCs (Table 2). Almost all poorly differentiated cases were (2+) positive for HMGA2 protein expression. In contrast, there were no scores of 2+ in well-differentiated HNSCCs. These results suggest that HMGA2 expression correlates with invasion and poor histopathological differentiation. Typically, cells with strong HMGA2 expression were observed at the tip of infiltrating single cells (arrowheads in Figure 3), although at the same tumor site, very weak expression appeared on the surface of tumor cells. Thus, HMGA2 staining intensity was graded from the surface to the infiltrative region (Figure 3).

Clinical Significance of Bmi-1 and HMGA2 Protein Expression in Resected Case of Tongue Squamous Cell Carcinoma

The median age of the 91 patients (57 men and 34 women), who had a tumor of <4 cm in diameter (classified as T2, T1, and Tis, N0, M0) in the surgery-alone group, was 62 years (range, 29–91 years). During a median follow-up of 2777 days (range, 219–3956 days), the overall 5-year survival rate was 65.7%, with a median survival of 2718 days (95% confidence interval (CI), 2325–3112 days). Of the 91 tongue SCCs, 48 (52.7%) were positive and 43 (47.3%) were negative for Bmi-1 staining. χ^2 And log-rank analyses revealed no significant difference between Bmi-1-positive tumors (median survival of 2651 days; 95% CI, 2137–3165 days) and negative tumors (median survival of 2611 days; 95% CI, 2043–3179 days; $P=0.69$, Figure 4a and Table 2). Meanwhile, of the 91 tongue SCCs, 66 (72.5%) were positive and 25 (27.5%) were negative for HMGA2 staining. χ^2 Analysis revealed that histopathological differentiation was significantly associated with HMGA2 expression ($P=<0.001$; Table 2). Similarly, HMGA2 expression indicated decreased overall survival, with a median of 2187 days (95% CI, 1545–2829 days) compared with HMGA2-negative tumors with a median survival of 2611 days (95% CI, 2533–3455 days; $P=0.05$, Figure 4b).

DISCUSSION

In recent years, numerous reports have described 'cancer stem cell' markers, for example, adhesion molecules: EpCAMs, CD44, CD133, WNT, and SHH signal pathways, and chromatin regulators.[13,14] In this study, we analyzed for the first time whether stem cell-related genes are upregulated in HNSCCs compared with their corresponding non-cancerous tissue using gene expression profiling. As a result, chromatin regulators Bmi1 and HMGA2 demonstrated a tendency to be highly expressed in tumors. In addition, CD44,[15] GNL3L/nucleostemin,[16] and YBX1[17] showed higher average expression in tumors. By contrast, EpCAM, CD133 (Prominin 1),[18] and SHH expression levels showed great variability among individual cases. These molecules are expressed in tumor regions at relatively high mean levels; however, the stemness molecule expression pattern did not show a similar tendency among the cases. In contrast, Notch pathways showed a tendency toward lower expression in tumors compared with the level in non-cancerous tissue. From these results, we focused on Bmi-1 and HMGA2 as proto-oncogenic chromatin regulators known to be crucial for the promotion of stem cell self-renewal by negatively regulating the expression of Ink4a and Arf.[19–21] Bmi-1, one of the polycomb group (PcG) family members, was identified as an Myc-cooperating gene[22] and is essential for the generation of self-renewing adult murine HSCs and neural stem cells.[23] A notable point is that Bmi1-positive lingual keratinized epithelial cells work to maintain and regenerate stem cells.[24]

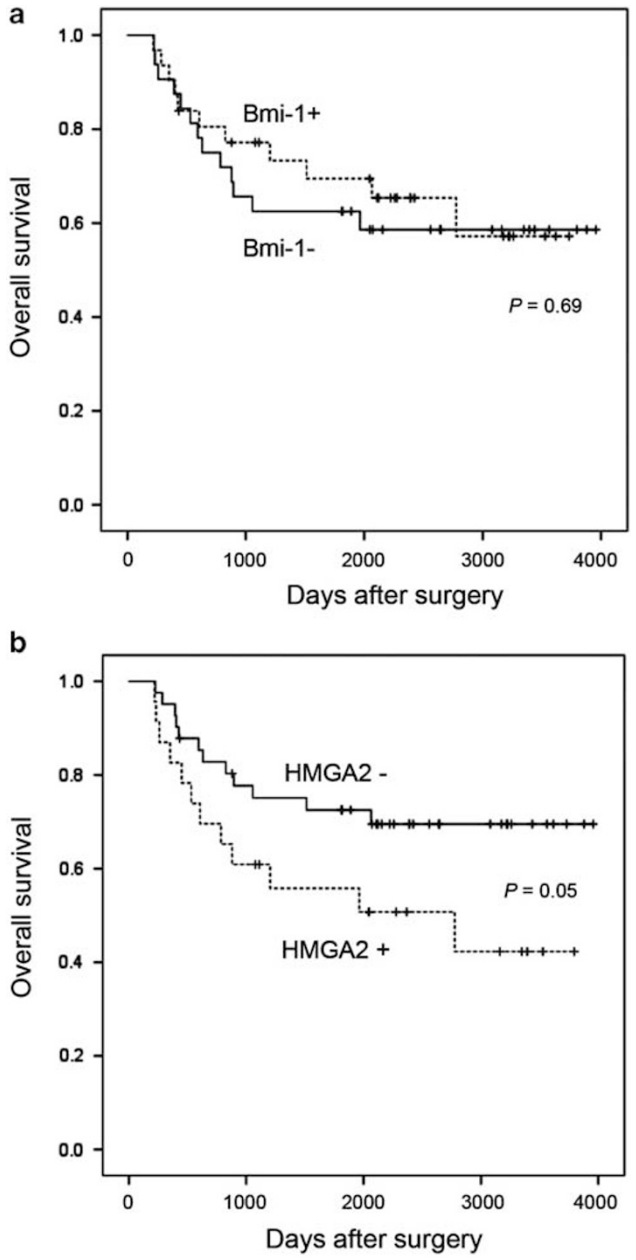

Figure 4 Comparison of Kaplan–Meier survival curves according to Bmi-1 and HMGA2 immunostaining in tongue squamous cell carcinoma. Overall survival curve for the patient group with HNSCC with Bmi-1 expression did not differ significantly from that for the group with HNSCC-negative Bmi1 expression ($P = 0.69$) (**a**). In contrast, the patient group with HNSCC and HMGA2 expression had poor prognosis compared with the patient group with HNSCC-negative HMGA2 expression ($P = 0.05$) (**b**). The patients with HNSCC with Bmi-1 or HMGA2 expression (scored as 2+ and 1+) are shown by a dotted line and the patients with HNSCC but no Bmi-1 or HMGA2 expression (scored as negative) are shown by a solid line.

HMGA2 is one of the high-mobility group-A family members known to be widely expressed during embryogenesis, whereas their expression is low in adult tissues. HMGA family proteins bind to the minor groove of AT-rich DNA sequences, modify chromatin conformation, and enhance the affinity of transcription factors.[25] Generally, both PcG and HMGA family molecules are highly expressed in developmental-stage cells or limited premature/stem cells in adulthood. Imbalance of the expressions of these molecules causes cancer because of self-renewing and progression capabilities.[26,27] Bmi-1 mRNAs and proteins tend to be expressed in well-differentiated/early and lose expression in poorly differentiated/progressed HNSCC (Tables 1–3). Subsequent identification of Bmi-1 protein expression could distinguish early lesions of cancerous tissue from non-cancerous tissues in HNSCCs (Figure 2). This occurred in the same manner as in HCCs, as we previously reported.[11]

On the other hand, HMGA2 mRNAs and proteins tended to be expressed in poorly differentiated HNSCC and weakly or not expressed in well-differentiated/early HNSCC (Figure 2). Interestingly, these results showed that, despite having the same target proto-oncogenic chromatin regulator, Bmi-1 and HMGA2 expression patterns clearly contrasted. Several recent studies have implied that HMGA2 expression correlated with lymph node metastases and tumor progression in several solid tumors.[28–30] HMGA2 expression in oral squamous cell carcinoma has also been reported.[31] Our results are consistent with previous reports. Although tongue SCC at stage 'T2' is known to have excellent prognosis, HMGA2-expressing tumors showed significantly decreased overall survival. In particular, HMGA2 protein was most highly expressed at the invasive front of single cancer cells (Figure 3).

From the standpoint of developmental and stem cell biology, previous work has established that Bmi1 is expressed in postnatal HSCs, but not in prenatal proliferative HSCs.[32] Thus, we speculate that Bmi1 expression arises in the cancer-developing stage of early tumors with high plasticity. The long half-life of the cancer 'cell of origin' allows the accumulation of multiple mutations and epigenetic changes required for multi-step evolution toward progression. These progressed cancer cells showed decreased Bmi1 expression and gained proliferative activity instead of loss of plasticity. Similarly, very weak HMGA2 expression in the cancer-developing stage occurred in early tumors with high plasticity. HMGA2 expression subsequently increased as proliferative activity was obtained. It was also reported that HMGA2 expression was observed in the developmental proliferative stage but not in the mature stage.

In this study, we demonstrated that stemness molecule expression patterns do not show a similar tendency among cases and fewer primitive rare cells are expressed in the tumor than almost all other cells in HNSCC cases. Bmi1 is expressed in early and well-differentiated HNSCCs, whereas HMGA2 is expressed in the invasive front and/or poorly differentiated HNSCCs. These results showed that Bmi1 immunostaining could be a novel marker to distinguish early cancer from

non-cancerous tissue, whereas HMGA2 immunostaining could be a poor prognostic and invasive marker of HNSCCs.

ACKNOWLEDGMENTS

We are grateful to Ms Mari Fujiwara, Ms Michiko Suzuki, Ms Sachiko Miura, and Ms Chizu Kina for their technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (C) to TM and a Grant-in-Aid for Scientific Research (C) to HY from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Termine N, Panzarella V, Falaschini S, *et al.* HPV in oral squamous cell carcinoma vs head and neck squamous cell carcinoma biopsies: a meta-analysis (1988–2007). Ann Oncol 2008;19:1681–1690.
2. Shackleton M, Quintana E, Fearon ER, *et al.* Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell 2009;138:822–829.
3. Al-Hajj M, Wicha MS, Benito-Hernandez A, *et al.* Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100:3983–3988.
4. Joshua B, Kaplan MJ, Doweck I, *et al.* Frequency of cells expressing CD44, a head and neck cancer stem cell marker: correlation with tumor aggressiveness. Head Neck 2012;34:42–49.
5. Zhang Z, Filho MS, Nor JE. The biology of head and neck cancer stem cells. Oral Oncol 2011;48:1–9.
6. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730–737.
7. Reya T, Morrison SJ, Clarke MF, *et al.* Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–111.
8. Umezawa A, Gorham JD. Dueling models in head and neck tumor formation. Lab Invest 2010;11:1546–1548.
9. Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 2003;3:895–902.
10. Mori T, Kiyono T, Imabayashi H, *et al.* Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential. Mol Cell Biol 2005;25:5183–5195.
11. Effendi K, Mori T, Komuta M, *et al.* Bmi-1 gene is upregulated in early-stage hepatocellular carcinoma and correlates with ATP-binding cassette transporter B1 expression. Cancer Sci 2010;101:666–672.
12. Ohtomo R, Mori T, Shibata S, *et al.* SOX10 is a novel marker of acinus and intercalated duct differentiation in salivary gland tumors: a clue to the histogenesis for tumor diagnosis. Mod Pathol 2013;26: 1041–1050.
13. Chaves-Perez A, Mack B, Maetzel D, *et al.* EpCAM regulates cell cycle progression via control of cyclin D1 expression. Oncogene 2012;32: 641–650, e-published.
14. Song LB, Li J, Liao WT, *et al.* The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells. J Clin Invest 2009;119:3626–3636.
15. Munz M, Fellinger K, Hofmann T, *et al.* Glycosylation is crucial for stability of tumour and cancer stem cell antigen EpCAM. Front Biosci 2008;13:5195–5201.
16. Okamoto N, Yasukawa M, Nguyen C, *et al.* Maintenance of tumor initiating cells of defined genetic composition by nucleostemin. Proc Natl Acad Sci USA 2011;108:20388–20393.
17. To K, Fotovati A, Reipas KM, *et al.* Y-box binding protein-1 induces the expression of CD44 and CD49f leading to enhanced self-renewal, mammosphere growth, and drug resistance. Cancer Res 2010;70: 2840–2851.
18. Felthaus O, Ettl T, Gosau M, *et al.* Cancer stem cell-like cells from a single cell of oral squamous carcinoma cell lines. Biochem Biophys Res Commun 2011;407:28–33.
19. Smith KS, Chanda SK, Lingbeek M, *et al.* Bmi-1 regulation of INK4A-ARF is a downstream requirement for transformation of hematopoietic progenitors by E2a-Pbx1. Mol Cell 2003;12:393–400.
20. Nishino J, Kim I, Chada K, *et al.* Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf expression. Cell 2008;135:227–239.
21. Bracken AP, Kleine-Kohlbrecher D, Dietrich N, *et al.* The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev 2007;21:525–530.
22. Park IK, Qian D, Kiel M, *et al.* Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 2003;423:302–305.
23. Jacobs JJ, Scheijen B, Voncken JW, *et al.* Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ ARF. Genes Dev 1999;13:2678–2690.
24. Tanaka T, Komai Y, Tokuyama Y, *et al.* Identification of stem cell that maintain and regenerate lingual keratinized epithelial cells. Nat Cell Biol 2013511–518.
25. Fusco A, Fedele M. Roles of HMGA proteins in cancer. Nat Rev Cancer 2007;7:899–910.
26. Pardal R, Molofsky AV, He S, *et al.* Stem cell self-renewal and cancer cell proliferation are regulated by common networks that balance the activation of proto-oncogenes and tumor suppressors. Cold Spring Harb Symp Quant Biol 2005;70:177–185.
27. Chiba T, Miyagi S, Saraya A, *et al.* The polycomb gene product BMI1 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma. Cancer Res 2008;68:7742–7749.
28. Meyer B, Loeschke S, Schultze A, *et al.* HMGA2 overexpression in non-small cell lung cancer. Mol Carcinog 2007;46:503–511.
29. Hetland TE, Holth A, Kaern J, *et al.* HMGA2 protein expression in ovarian serous carcinoma effusions, primary tumors, and solid metastases. Virchows Arch 2012;460:505–513.
30. Hristov AC, Cope L, Reyes MD, *et al.* HMGA2 protein expression correlates with lymph node metastasis and increased tumor grade in pancreatic ductal adenocarcinoma. Mod Pathol 2009;22:43–49.
31. Miyazawa J, Mitoro A, Kawashiri S, *et al.* Expression of mesenchyme-specific gene HMGA2 in squamous cell carcinomas of the oral cavity. Cancer Res 2004;64:2024–2029.
32. Jang YY, Sharkis SJ. Fetal to adult stem cell transition: knocking Sox17 off. Cell 2007;130:403–404.