Expression of Mesenchyme-Specific Gene HMGA2 in Squamous Cell Carcinomas of the Oral Cavity

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ABSTRACT

Carcinoma cells of epithelial origin are predisposed to acquire a fibroblastic feature during progression of neoplasm referred to as the epithelial-mesenchymal transition. HMGA2 is an architectural transcriptional factor that is expressed in the undifferentiated mesenchyme and initiates mesenchymal tumor formation. However, the biological consequence of the expression in the pathology of epithelial-type carcinomas is controversial. The present study was conducted to dissect the expression pattern in oral squamous cell carcinomas. HMGA2 was detected exclusively in carcinoma cell lines and tissues, but not in normal keratinocytes and gingival, by conventional reverse transcription-PCR. Quantitative real-time reverse transcription-PCR demonstrated 160-fold more HMGA2 expression in carcinoma tissues than in normal gingiva and 11-fold more HMGA2 expression in carcinoma cell lines than in normal keratinocytes. HMGA2 expression was observed by immunohistochemistry in 73.8% of 42 carcinomas and localized to the invasive front, where the cells exhibit the epithelial-mesenchymal transition. Fourteen patients who had been classified into a group without lymph node metastasis were positive for HMGA2 staining, and the disease recurred. Furthermore, carcinomas from all 23 patients who died of tumor recurrence stained for HMGA2, and HMGA2 staining was correlated to long-term survival of patients (P < 0.01). Multivariate risk factor analysis demonstrated that HMGA2 expression was an independent prognostic value for disease-specific overall survival (P < 0.01). These results suggest that HMGA2 contributes to the aggressiveness of carcinoma and that detection of HMGA2 expression is a useful predictive and prognostic tool in clinical management of oral carcinomas.

INTRODUCTION

Oral squamous cell carcinoma is the most common neoplasm of the head and neck. Worldwide, the annual incidence of new cases exceeds 300,000. The disease causes great morbidity, and the 5-year survival rate has not improved in more than two decades (1, 2). With few exceptions, carcinomas are derived from single somatic cells and their progeny. The accuracy of established prognostic methods is frequently questionable and is not sufficient for the design of individual treatment strategies. Indeed, clinical staging at presentation remains the most powerful toolkit for estimating the course of the disease. Carcinoma cells in the emerging neoplastic clone accumulate within them a series of genetic and/or epigenetic changes that lead to changes in gene activity and hence to altered phenotypes that are subjected to selection for tumor progression (3). Multiple epigenetic alterations have been characterized during the progression. Loss of epithelial morphology and acquisition of mesenchymal characteristics, often referred to as the epithelial-mesenchymal transition (EMT), are typical for carcinoma cells and predispose tumors to a more advanced state of progression (4–6). The genetic instabilities may trigger alterations in regulatory sequences of correct gene expression and may accelerate the accumulation of EMTs in carcinomas from a standpoint of tumor progression. Identifying the genes that are involved in EMTs and progression of carcinoma toward fatal disease provides insights into understanding the mechanism of tumor progression and development of a novel strategy predicting tumor malignancy and may contribute to long-term survival of patients (6).

The HMGA (HMGI) family consists of three members, HMGA2 (HMGIC), HMGA1a (HMGI), and HMGA1b (HMGY), and the latter two members are encoded by an alternatively spliced mRNA from the HMGA1 (HMGY) gene. A prominent feature of the HMGA family is the three DNA-binding domains, termed AT-hooks, that bind to AT-rich DNA in the minor groove. They have no transcriptional activity per se, but through binding with other transcription factors, they organize the framework of the nucleoprotein-DNA transcriptional complex and enhance the transcription of several genes (7, 8). This is attained by their ability to change the conformation of DNA, and these proteins are therefore termed architectural factors (9).

HMGA2 is expressed in undifferentiated mesenchymal cells, but expression ceases on differentiation (10). An Hmga2 gene-targeting mouse model shows a dwarf phenotype that results from suppression of mesenchymal cell growth (11). The high incidence of chromosomal translocations of the HMGA2 locus, resulting in misexpression, leads to the development of mesenchymal benign tumors (10, 12–15). Because HMGA2 is predominantly expressed in undifferentiated cells during development, it has been hypothesized that it is the inappropriate activation of the HMGA2 gene in a terminally differentiated mesenchymal cell that initiates the tumorigenic pathway and leads to a mesenchymal tumor (16, 17).

However, little is known about HMGA2 expression in carcinomas of epithelial origin. HMGA2 is ectopically expressed in human invasive carcinomas of the breast (18). Antisense strand inhibition of HMGA2 in rat thyroid cells eliminates transformation by v-mos and v-ras-Ki in vitro (19). These studies suggest a role of HMGA2 in epithelial carcinoma formation and progression. The functionality of ectopic expression in carcinoma progression and pathogenesis remains to be elucidated. Chromosomal translocations disrupting the HMGA2 locus (12q13–15) have never been reported within oral squamous carcinomas. In the present study, we examined expression of HMGA2 by real-time quantitative reverse transcription-PCR (RT-PCR), tissue localization of HMGA2 in oral squamous cell carcinomas, and correlation of HMGA2 expression to long-term survival of patients.

MATERIALS AND METHODS

Cell Lines and Tissue Samples. Immortalized cell lines derived from oral squamous carcinoma (Ca9-22, Ho1u1, HOC313, HSC3, KOS2, OSC19, SCCKN, SCCFT, and TSU) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan), Health Science Research Resources Bank (Osaka, Japan), or RIKEN Cell Bank (Tsukuba, Japan). Two independent normal human gingival keratinocytes were primary cultured and maintained in defined keratinocyte-SFM (Gibco BRL, Gaithersburg, Germany). A total of five oral squamous cell carcinomas (two well-differentiated carcinomas and three moderately differentiated carcinomas) and three normal oral tissues without history...
of oral carcinoma were obtained from patients undergoing operation for carcinoma resection or dental surgery (20).

**Reverse Transcription-PCR.** Total RNA isolated from cell lines, oral squamous cell carcinomas, or normal oral tissues was reverse transcribed by SuperScript II (Life Technologies, Inc.) with HMG2exon5 primer (5'-CTTACAAAGATACCTCGTCTGGAGG-3'). After the reverse transcription, PCR was performed with HMG2exon 5 primer, exon 2 primer (5'-CCGGTGACGCTCTCTAAAGGACC-3') and Taq DNA polymerase (Life Technologies, Inc.). After 5 min of denaturation at 94°C, 35 cycles of reaction (94°C for 30 s, 65°C for 30 s, and 72°C for 1 min) were performed, followed by a 7 min final extension at 72°C. For GAPDH, random primed reverse transcription samples were subjected to PCR (30 cycles of reaction for 30 s, 52°C for 30 s, and 72°C for 1 min) with a specific primer set (5'-GTCAGTGTTGGAGGACGACCT-3' and 5'-AGGGGAGATCTGTGTTG TG-3').

For tissue samples, reaction solution of 20-cycle PCR using exon 2 and exon 5 primers as described above were subjected to the nested PCR reaction with exon 3 primer (5'-CAAAACAGAAGTCCCTCAAAGCAGC-3') and exon 5 primer.

**Real-Time Quantitative RT-PCR.** For quantitative analysis of HMG2, HMGA1a, HMGA1b, and GAPDH expression levels, total RNA extracted was subjected to real-time RT-PCR using the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA) in duplicate. A computer-assisted program was used for primer design (Primer Express ver. 2.0). For the nested PCR reaction, the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA) in duplicate. The expression levels of HMG2, HMGA1a, and HMGA1b were determined by real-time PCR according to the manufacturer’s instructions. The primers used were as follows: for HMG2, 5'-CAGCACGAAACCAACC-3' (forward) and 5'-TGGTGGCGCATTCCGTAGT-3' (reverse); for HMGA1a, 5'-CACGGCTGATGAGGTCG-3' (forward) and 5'-TTCCTCTCCGAGTTGTTG-3' (reverse); for HMGA1b, 5'-CAAGCAGCTCAGGAAAGA-3' (forward) and 5'-CCGGCTGGCTTGGACGAC-3' (reverse); and for GAPDH, 5'-GAAGGCGAGGGGAAGGACG-3' (forward) and 5'-CGTCGTACGTACACTTCAGG-3' (reverse). Samples with high starting copy number of HMG2, HMGA1a, and HMGA1b were subjected to the nested PCR reaction (94°C for 30 s, 52°C for 30 s, and 72°C for 1 min). In brief, the reactions were as follows: the reaction mixture contained 10 ng of total RNA, 0.5 units of Taq DNA polymerase (Promega, Madison, WI), 200 μM of each dNTP, 1× PCR buffer, and 100 μM of each primer. The PCR conditions were as follows: 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min (30 cycles). The expression level was determined by the comparative Ct method (21). The Ct values were calculated using the following formula:

\[
\text{Ct}_{	ext{sample}} = \frac{\text{Ct}_{	ext{sample}} - \text{Ct}_{\text{GAPDH}}}{\Delta \text{Ct}} 
\]

where \( \text{Ct}_{\text{sample}} \) is the cycle at which the sample fluorescence is detected, \( \text{Ct}_{\text{GAPDH}} \) is the cycle at which the GAPDH fluorescence is detected, and \( \Delta \text{Ct} \) is the difference in cycle number between the sample and the control. The expression level was calculated as the fold difference in expression between sample and control.

**Immunohistochemistry.** A total of 42 human oral squamous cell carcinoma tissues were taken at Kanazawa University Hospital during incisional or excisional biopsy with informed consent of patients between October 1987 and September 2000. The median age of the study patients was 65 years (range, 37–92 year) at the time of diagnosis, and mean follow-up was 107 months (range, 43–171 months). Most patients were treated with preoperative chemotherapy, and all patients underwent radical surgery. Details of the pretreatment characteristics are displayed in Table 1. Histological grade and staging were assessed according to the 1987 International Union against Cancer (UICC) tumor-node-metastasis (TNM) classification (22). Control normal tissues (n = 5) were also obtained at operation or autopsy from tongue, buccal mucosa, gingiva, and lip of patients without a history of head and neck cancers. Formalin-fixed and paraffin-embedded tissue sections (4 μm) were deparaffinized and rehydrated followed by microwave treatment in 0.01 M sodium citrate buffer (pH 6.0 (500 W)), 0.1% trypsin (Sigma-Aldrich, St. Louis, MO), or proteinase K (DAKO, Glostrup, Denmark). After incubation with normal serum, sections were incubated with rabbit anti-HMG2 antibody (dilution, 1:100; Ref. 23), mouse anti-E-cadherin (HECD-1; 20 μg/ml; R&D System, Minneapolis, MN), or mouse anti-proliferating cell nuclear antigen (PCNA (PC10; 0.4 μg/ml; Sigma-Aldrich)]. Anti-HMG2 antibody used is specific for HMG2 and does not cross-react with HMGA1 (23). Alexa Fluor 488 goat antirabbit IgG or 546 antirabbit IgG (Molecular Probes, Eugene, OR) were used as secondary antibodies. To clarify the specificity of antibody reactivity, incubation with nonimmune mouse or rabbit IgG (20 μg/ml) instead of primary antibodies was performed. For the avidin-biotin complex detection system, biotinylated antirabbit IgG (DAKO) was used as secondary antibody, and color was developed with 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich).

Carcinoma cells with strong nuclear labeling were determined as positive reactions, but cells with weak nuclear staining and/or diffuse cytoplasmic staining were not counted as positive. HMG2 staining in the nucleus of carcinoma cells at the invasive front was verified with light microscopy by two independent observers (J. M. and K. I.). Borderline cases between positive and negative were jointly reviewed, and a consensus was sought.

**Immunoblotting.** Nuclear extracts of TSU, HOC313, KOS2C, and OSC19 cells were prepared by methods described elsewhere (24, 25). The extracts (35 μg) were size-fractionated by a SDS-PAGE gel (14% total acrylamide) under reducing conditions and electrotransferred onto nitrocellulose membranes. The membrane was probed with anti-HMG2 antibody (dilution, 1:1000) and then probed with biotinylated antirabbit IgG at a concentration of 1:2000, and color was developed with 3,3’-diaminobenzidine tetrahydrochloride.

**Statistical Analysis.** One-way ANOVA followed by contrast statements (Scheffe’s F-test and Fisher’s Protected Least Significant Difference) was performed to compare the relative amount of mRNA (2^(-ΔΔCt)). Distribution of HMG2 immunostaining at the invasive front with different clinicopathological parameters recorded in this study was analyzed by chi-square test for independence or by the Mann-Whitney U test. For survival analysis, the Kaplan-Meier method was used, and the statistical difference was analyzed by the log-rank test. To determine whether the prognostic levels of HMG2 staining are independent of clinicopathological parameters, the influence of these factors on patient survival was analyzed by the multivariate Cox proportional hazards method.

**RESULTS**

**Expression of HMG2 in Oral Squamous Carcinoma Cells.** Whereas conventional RT-PCR did not detect HMG2 in normal keratinocytes, four of nine carcinoma cell lines showed expression of the gene, and three cell lines showed weak expression (Fig. 1A). In contrast to marginal expression in normal keratinocytes (mean ± SD, 1.9 ± 1.0), real-time RT-PCR demonstrated that nine carcinoma cell lines exhibited significantly high expression levels of HMG2 (11.2 ± 6.9; P < 0.05). Immunoblotting analysis of HMG2 protein expression in nuclear extracts confirmed RNA expression data. Size-fractionated nuclear extracts from two high mRNA-expressing cell lines (KOS2C and OSC19) and two low mRNA-expressing cell lines (TSU and HOC313) were reacted to HMG2-specific antibody, and a single 19-kDa band developed in the former two (Fig. 1B).

The expression and role of HMG2 in carcinomas of epithelial origin are poorly understood. We examined the expression pattern of

| Table 1 Clinicopathological parameters and HMG2 expression in 42 primary oral carcinomas |
|---------------------------------|------------------|------------------|------------------|
| Parameter                        | n    | Positive (%) | Negative (%) | P*       |
| Age (yrs)                        |      |               |               |          |
| <50                              | 9    | 6 (67)        | 3 (33)        | 0.5824   |
| >50                              | 33   | 25 (76)       | 8 (24)        |          |
| Sex                              |      |               |               |          |
| Female                           | 21   | 14 (67)       | 7 (33)        |          |
| Male                             | 21   | 17 (81)       | 4 (19)        | 0.2924   |
| T stage b                        |      |               |               |          |
| T1                               | 7    | 4 (57)        | 3 (43)        |          |
| T2                               | 21   | 16 (76)       | 5 (24)        |          |
| T3                               | 7    | 4 (57)        | 3 (43)        |          |
| T4                               | 7    | 7 (100)       | 0 (0)         | 0.2072   |
| N status b                       |      |               |               |          |
| N−                               | 29   | 18 (62)       | 11 (38)       | 0.0097   |
| N+                               | 13   | 13 (100)      | 0 (0)         |          |
| Clinical stage b                 |      |               |               |          |
| Stage 1                          |      |               |               |          |
| Well                             | 28   | 21 (75)       | 7 (25)        | 0.0500   |
| Moderate                         | 10   | 7 (70)        | 3 (30)        |          |
| Poor                             | 4    | 3 (75)        | 1 (25)        | 0.9519   |

* *= Probability of statistical difference (P) was analyzed by χ2 test for independence. Patients were categorized by tumor size (T stage) and clinical stages according to the International Union against Cancer (UIICC) WHO grading system and by the presence or absence of lymph node metastasis (N status).
of tumor as indicated in Fig. 2A). Therefore, detailed analyses focusing on the invasive front were undertaken in this study. Squamous carcinomas consist of irregular masses of tumor cells, carcinoma cell nests, that proliferate and penetrate downward into connective tissues. Carcinoma cells could retain the properties of epithelial phenotypes. Cellular differentiation is a characteristic most discernable as the presence or absence of keratinization. Keratinization takes place in the form of keratin pearls and is gradually increased toward the center of carcinoma cell nests. Thus, carcinoma cells in the center often show a more differentiated phenotype than the edges of the nests (29). However, we could not find preferential localization of HMGA2 in the nests. Normal oral tissues did not react to HMGA2 staining (Fig. 2E). The immunoreactivity of carcinoma cells that metastasized to the lymph nodes (n = 5) was indistinguishable from that in the corresponding primary sites (data not shown).

**Prognostic Significance of HMGA2 Expression in Primary Oral Carcinomas.** Immunostaining data showed ectopic expression of HMGA2 at the invasive front. HMGA2 expression was found to correlate with N status; positive HMGA2 staining was observed in 18 of 29 patients (62.1%) without lymph node metastasis and 13 of 13 patients (100%) with metastasis (P = 0.0097; Table 1). However, other clinicopathological parameters including age of patients at diagnosis, sex, T stage, clinical stage, and histological tumor differentiation did not correlate with HMGA2 staining. Thus, HMGA2 staining is not simply associated with disease progression.

Twenty-three of 42 patients died of disease recurrence (Dc), and carcinomas from all 23 of these patients stained positively for HMGA2 (Table 3). Nine of these patients had been diagnosed with lymph node metastasis (N+), but more importantly, 14 patients who

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**Table 2 Relative fold of HMGA2 expression in oral carcinoma tissues compared with normal counterparts**

<table>
<thead>
<tr>
<th>Cases</th>
<th>HMGA2</th>
<th>HMGA1a</th>
<th>HMGA1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>168.9</td>
<td>5.7</td>
<td>18.4</td>
</tr>
<tr>
<td>2</td>
<td>84.4</td>
<td>4.9</td>
<td>17.1</td>
</tr>
<tr>
<td>3</td>
<td>111.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>137.2</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>315.2</td>
<td>2.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Mean ± SD 163.4 ± 90.4× 2.8 ± 2.5 7.3 ± 9.5

*P < 0.05.*
Table 3 Association between numbers of HMGA2-positive carcinomas and lymph node metastasis and/or tumor recurrence

Data were represented as percentage of HMGA2-positive cases in each parameter, and case number is represented as number of HMGA2-positive cases per cases examined in parentheses.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>N+</th>
<th>N−</th>
<th>Dc</th>
<th>Ao</th>
</tr>
</thead>
<tbody>
<tr>
<td>N+/Dc</td>
<td>100 (13/13)</td>
<td>62.1 (18/29)</td>
<td>100 (23/23)</td>
<td>42.1 (8/19)</td>
</tr>
<tr>
<td>N−/Dc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N+/Ao</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N−/Ao</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N+/Dc</td>
<td>100 (14/14)</td>
<td>26.7 (4/15)</td>
<td>100 (9/9)</td>
<td>100 (4/4)</td>
</tr>
</tbody>
</table>

*p*, cases with lymph node metastasis; *N−*, cases without lymph node metastasis; Dc, cases died of disease recurrence; Ao, cases free from disease recurrence. Total number examined = 42.

had been classified as lymph node metastasis negative (N−) were positive for HMGA2, and the disease recurred. Of equal significance, the remaining 11 patients who did not express HMGA2 did not have a tumor recurrence (Ao) during the follow-up period. Among the 23 HMGA2-positive cases, 8 cases did not experience disease recurrence. Four of the eight cases were N+ (N+/Ao). Another four cases without lymph node metastasis (N−/Ao) also stained HMGA2 positive but were rather small in size (two T1 and two T2) when compared with HMGA2-positive N−/Dc tumors (two T1, five T2, four T3, and three T4 tumors; *P* = 0.0315 by Mann-Whitney U test). These data suggest the possibility that HMGA2-positive patients free from tumor recurrence were in the early stage of tumor progression and tumors were successfully removed by radical surgery. Univariate analysis by Kaplan-Meier curve of overall survival of patients with HMGA2-positive versus -negative carcinomas demonstrated that patients with HMGA2-positive staining exhibited a lower disease-specific survival rate (*P* = 0.0006, log-rank test; Fig. 3).

To identify the significant prognostic factors associated with oral carcinoma-specific death, multivariate risk factor analysis was performed using the Cox proportional hazards regression model (Table 4). T stage (*P* = 0.0030) and HMGA2 staining (*P* = 0.0075) were found to be significant independent predictors of death from carcinomas. For overall survival, positive HMGA2 staining was an independent predictor of reduced survival with a hazard ratio of 3.482 and a 95% confidence interval ranging from 1.395 to 8.691. No other variables including age, sex, N status, clinical stage, and histological tumor differentiation were associated with survival according to the multivariate analysis.

Proliferation and Differentiation and HMGA2 Localization. Because the biological role of HMGA2 in oral carcinomas is not known, we compared tissue localization of HMGA2 and that of E-cadherin or PCNA by double immunofluorescent microscopy. An epithelial differentiation marker, E-cadherin, mediates homophilic intercellular adhesion of epithelial cells, and loss of E-cadherin expression is frequently observed at the invasive front and initiates tumor dedifferentiation (5, 30–33). In fact, oral carcinomas also reduced immunoreactivity of E-cadherin at the invasive front as reported previously (34). HMGA2 was predominantly observed in E-cadherin-negative carcinoma cells (Fig. 4A). PCNA is a DNA polymerase-associated protein synthesized in late G1 to S phase of the cell cycle. Nuclear PCNA staining provides a molecular marker of cell proliferation and frequently detects carcinoma cells at the invasive front (28, 35). PCNA is also expressed in myofibroblasts juxtaposing to carcinoma cells at the invasive front (36). In the present study, both HMGA2 and PCNA stained carcinoma cells and surrounding fibroblast-like stromal cells at the invasive front. However, only a small portion of these stainings overlapped each other, *i.e.*, double-labeled carcinoma or fibroblast-like cells were identified in 2–3% of HMGA2-positive and 4–8% of PCNA-positive populations (Fig. 4B). The HMGA2-reactive fibroblast-like cells exhibited extensive cytoplasm and large and rounded nuclei, suggesting that the cells could be myofibroblasts. Myofibroblasts are frequently observed in tissue reaction in various pathological conditions and considered to be activated fibroblasts (36, 37). Inflammatory myofibroblastic tumor cells aberrantly express HMGA2 by chromosomal rearrangement (38). It is reasonable that oral carcinoma tissues also associate with HMGA2-positive myofibroblasts at the invasive front.

Table 4 Contribution of various potential prognostic factors to disease-free survival in oral carcinoma patients (n = 42)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Risk ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.694</td>
<td>0.295–1.237</td>
<td>0.1678</td>
</tr>
<tr>
<td>Sex</td>
<td>1.156</td>
<td>0.653–2.045</td>
<td>0.6183</td>
</tr>
<tr>
<td>T stage</td>
<td>2.860</td>
<td>1.427–5.728</td>
<td>0.0030</td>
</tr>
<tr>
<td>N status</td>
<td>1.033</td>
<td>0.553–1.932</td>
<td>0.9181</td>
</tr>
<tr>
<td>Clinical stage</td>
<td>1.909</td>
<td>0.891–4.090</td>
<td>0.2424</td>
</tr>
<tr>
<td>Histological differentiation</td>
<td>1.608</td>
<td>0.588–4.395</td>
<td>0.6514</td>
</tr>
<tr>
<td>HMGA2 staining</td>
<td>3.482</td>
<td>1.395–8.691</td>
<td>0.0075</td>
</tr>
</tbody>
</table>

*a*, CI confidence interval.  
b, Cox proportional hazards method.

Fig. 4. Double immunostaining of HMGA2 and E-cadherin or PCNA. A, at the invasion front, HMGA2 (red) was localized to carcinoma cells and fibroblast-like cells, but E-cadherin (green) expression was restricted to a few carcinoma cells at the center of carcinoma nests. B, HMGA2 (red) was independently localized with PCNA (green)-positive carcinoma cells (arrowheads) and surrounding mesenchymal cells (arrows). C, Double immunostaining on normal oral epithelium tissue specimens was performed using antibodies to HMGA2 (red) and PCNA (green). C, broken lines indicate margins of carcinoma nests or basal borders of normal oral epithelium. Bar, 25 (B) and 100 μm (A and C).
DISCUSSION

This study demonstrated that 73.8% of oral squamous carcinoma occurs with ectopic expression of the developmentally regulated and mesenchymal architectural transcription factor HMGA2. HMGA2 is expressed primarily in the developing mesenchyme of a variety of organs but is absent in the adult (11, 39). This expression pattern is also documented oral anlage that the mesenchyme of the maxilla and mandible exhibit high Hmga2 expression (39). Reactivation of developmentally regulated genes, including transcription factors, chromatin-regulatory proteins, and transcriptional regulators, is associated and predicted to have a role in the pathology of many tumor types (40). Aberrant activation of expression of tumor-associated genes is generally restricted to within a cell type corresponding to where tumor comes from (41). However, expression of HMGA2 represented in this study indicates that oral carcinoma cells of epithelial origin ectopically express a mesenchyme-specific transcription factor at the invasive front. The expression pattern of HMGA2 in carcinoma tissues was evaluated by immunostaining, and its possible prognostic significance was analyzed in comparison with clinicopathological parameters and patient survival. The 5-year survival rate of patients with oral carcinoma has improved only marginally over the past decade (2). Treatment failures can be attributed to multiple factors but remain difficult to predict because no reliable molecular marker is currently available in early detection or as an indicator of prognosis. The tailoring of individual treatment strategies to aggressively treat those carcinomas at greatest risk of 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. In this study, we assessed the clinical implications of HMGA2 immunostaining with prognosis. HMGA2 staining was closely associated with tumor recurrence and patient survival. This is highlighted by the fact that 100% of patients who died of tumor recurrence stained HMGA2 positive, and every HMGA2-positive N− patient died of tumor recurrence. Furthermore, staining was closely associated with the long-term patient survival rate independent of other risk factors. Treatment of clinically N− patients with chemotherapy or radiotherapy with neck dissection is a controversial issue (1). Survival of clinically N− patients free from disease recurrence was limited to 51.7% (15 of 29 cases). However, 100% of HMGA2-negative patients (11 of 11 cases) survive without tumor recurrence. Our data indicate that HMGA2 is a novel superior marker for tumor recurrence and strongly suggest a possibility that HMGA2 staining predicts tumor aggressiveness and stratifies patients into risk groups.

A histologically normal Hmga2-null mutant mouse shows a dwarf phenotype resulting from suppression of mesenchymal cell growth (11). Nuclear staining of PCNA is considered to be a hallmark of cell proliferation (28, 35). However, in the present study, double immunostaining of HMGA2 and PCNA showed that only a small fraction of cells exhibits colocalization and did not correlate HMGA2 expression and cell proliferation in oral carcinomas. Similar results were reported; HMGA1 protein staining is increased in severe dysplastic adenoma and carcinomas of the colon, whereas nonneoplastic polyps, in which there is increased proliferation of epithelial cells but not cellular atypia, do not stain for HMGA1 protein (42). Therefore, these data imply a role of HMGA2 in the biological state of the cells rather than a function in proliferation.

Carcinoma cells at the invasive front can lose their epithelial characteristics and express a set of genes typified in mesenchymal cells (4−6, 43, 44). Gain of fibroblast-like phenotype in carcinoma cells, as referred to EMTs, directly enhances invasion of collagen matrices (45). Compulsive induction of EMTs in squamous carcinoma cells drives tumor progression through enhancement of invasive and metastatic features (46, 47). Predominant staining of HMGA2 at the invasive front presented in this study confirmed expression in invasive carcinomas of the breast (18). Forced expression of the HMGA1 gene in breast carcinoma cells up-regulates a panel of mesenchymal genes that associate with EMTs and facilitate tumor invasion (48). HMGA2 overexpression in nontumorigenic fibroblasts develops distant metastases when injected into athymic mice (49). Predominant staining of HMGA2 at the invasive front, wherein carcinoma cells lose expression of an epithelial marker, E-cadherin, has led us to speculate that HMGCIC expression triggers the pathway of EMTs and contributes to tumor invasion and metastasis. Future analysis will be required to identify direct target genes and understand the role of HMGA2 in the pathology of oral carcinomas.

The present study demonstrated for the first time that HMGA2 is ectopically expressed at the invasive front of oral carcinomas and has a significant impact on tumor progression and patient survival. HMGA2 immunostaining could be a prognostic determinant stratifying patients into risk groups. Even if the majority of the tumor is clinically low grade, would the portion of the tumor that is HMGA2 positive be considered by a pathologist to be of a higher grade? Future avenues of research may give evidence of a role of HMGA2 in the pathology of oral carcinomas and provide a novel strategy for cancer therapy.

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HMGA2 IN ORAL SQUAMOUS CELL CARCINOMAS

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