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, and HMGA2 staining was correlated to long-term survival of patients (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 frequently questionable and is not sufficient for the design of individual treatment strategies. Therefore, determination of the expression pattern of HMGA2 in squamous carcinomas was performed. In the present study, we examined expression of HMGA2 in 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, and HMGA2 staining was correlated to long-term survival of patients (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.
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 HMG2A exon 5 primer (5’-CTCTAAAAAGATCCACTGCTGAGG-3’). After the reverse transcription, PCR was performed with HMG2A exon 5 primer, exon 2 primer (5’-CCGGTGAGCCCTCTCTAAAGACCC-3’) and Taq DNA polymerase (Life Technologies, Inc.). After 5 min of denaturation at 96°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 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min) with a specific primer set (5’-GTCAGTGTTGAGCAGACCT-3’ and 5’-AGGGGAGATTCAGTGTGG-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’-CAAAACAGAGGTTCCCTCCTAACGAC-3’) and exon 5 primer.

Real-Time Quantitative RT-PCR. For quantitative analysis of HMG2A, HMG2Aa, and GAPDH, 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. The computer-assisted program was used for primer design (Primer Express version 1.5a; Applied Biosystems). Nucleotide sequences of the primers were as follows: for HMG2A, 5’-CAGCAAGCAAAACCCAGC-3’ (forward) and 5’-TGTTGGGCTATTCCATGTG-3’ (reverse); for HMG2Aa, 5’-CAGCGCCGTGAGGCTGCA-3’ (forward) and 5’-TTTCTTCCTGGGATTGTTGGGTG-3’ (reverse); for HMG2B, 5’-CAAGCAGCTCCTCGGAAAG-3’ (forward) and 5’-CCGGCTCTGGCAGACG-3’ (reverse); and for GAPDH, 5’-CGCTGTCGTTACGAGGGT-3’ (forward) and 5’-CGTCTGTTACGAGCTTCCAG-3’ (reverse). Samples with high starting copy number of the target gene show an increase in fluorescence early in the PCR reaction process, resulting in a low threshold cycle (Ct) number when standardized with the internal control GAPDH Ct (ΔCt). Carcinoma ΔCt subtracted from normalized tissue or keratinocyte ΔCt was used to give the relative amount of target mRNA expression (2-ΔΔCt; 21).

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 gradation and staging were assessed according to the 1987 International Union against Cancer (UICC) WHO grading system and by the presence or absence of lymph node metastasis (N status).

Clinical stageb. Carcinoma cells with strong nuclear labeling were determined as positive, and does not cross-react with HMGA1 (23). Alexa Fluor 488 goat antimouse (PC10; 0.4 μg/ml; Sigma-Aldrich) was used as secondary antibody. 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. HMGA2 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, KOS2, 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-HMGA2 antibody (dilution, ×600) 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 HMGA2 immunostaining at the invasive front with different clinicopathological parameters recorded in this study was analyzed by χ2 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 HMGA2 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 HMGA2 in Oral Squamous Carcinoma Cells. Whereas conventional RT-PCR did not detect HMGA2 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 examined exhibited significantly high expression levels of HMGA2 (11.2 ± 6.9; P < 0.05). Immunoblotting analysis of HMGA2 protein expression in nuclear extracts confirmed RNA expression data. Size-fractionated nuclear extracts from two high mRNA-expressing cell lines (KOS2 and OSC19) and two low mRNA-expressing cell lines (TSU and HOC313) were reacted to HMGA2-specific antibody, and a single 19-kDa band developed in the former two (Fig. 1B).

The expression and role of HMGA2 in carcinomas of epithelial origin are poorly understood. We examined the expression pattern of

<table>
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<th>Negative (%)</th>
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<tr>
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<td>3 (75)</td>
<td>1 (25)</td>
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Table 1: Clinicopathological parameters and HMGA2 expression in 42 primary oral carcinomas
immunoblotting for HMGA2 as described in reverse transcription of the OSC19 RNA sample. Lane 3, KOSC2, and OSC19 were subjected to HOC313, Lane 1, normal keratinocytes, and Lanes 1 and 3, HSC3; KOSC2; in carcinoma cell lines (Lane 3, A). HMGA2 gene to size 216 bp (data not shown), indicating specific detection of HMGA2 expression. PCR and standardized the level by normal oral tissue with the lowest expression (2^\Delta\Delta C_t). Normal tissues showed negligible expression (1.3 \pm 0.4), but carcinoma tissues ectopically expressed the HMGA2 gene at levels 84–315-fold greater than that of normal tissues (163.4 \pm 90.4; P < 0.05).

The HMGA2 gene in carcinoma tissues. Normal gingiva from patients without a history of head and neck tumor did not express the HMGA2 gene. In contrast, a single 259-bp band was augmented by conventional RT-PCR using an exon 2 and 5 primer set in all five carcinoma tissues. Subsequent nested PCR with exon 3 and 5 primers reduced the size to 216 bp (data not shown), indicating specific detection of HMGA2 transcripts by RT-PCR. To quantify the expression levels in normal and carcinoma tissues, we subjected RNA to real-time RT-PCR and standardized the level by normal oral tissue with the lowest expression (2^\Delta\Delta C_t). Table 2. Normal tissues showed negligible expression (1.3 \pm 0.4), but carcinoma tissues ectopically expressed the HMGA2 gene at levels 84–315-fold greater than that of normal tissues (163.4 \pm 90.4; P < 0.05).

**Immunolocalization of HMGA2 in Oral Carcinoma Tissues.**

Because the above data provided evidence of ectopic expression of HMGA2 in oral carcinoma cells, we wished to determine whether HMGA2 expression in primary carcinoma tissues correlates with clinicopathological parameters in patients with oral carcinomas. Samples of 42 individual primary carcinomas were analyzed by immunohistochemistry for nuclear staining with an antibody specific for HMGA2. HMGA2 was detected in 31 of 42 carcinomas (73.8%). HMGA2 staining was negligible in the central area of carcinoma tissues (Fig. 2, A and B). However, HMGA2 staining was seen in most of the invasive front (Fig. 2, A and B), where carcinoma cells proliferate downward into the connective tissue (Refs. 26–28; deep margin 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...
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 \( (P = 0.0006, \text{log-rank test}; \text{Fig. 3}) \).

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 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−/Dc</th>
<th>N+/Ao</th>
<th>N−/Dc</th>
<th>N+/Ao</th>
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<tr>
<td>N−</td>
<td>72.4</td>
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<tr>
<td>N+/Dc</td>
<td>27.6</td>
<td>27.6</td>
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\( ^{a} \) N+, 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.

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

<table>
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<th>Parameters</th>
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<th>( P )</th>
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<td>Age</td>
<td>0.694</td>
<td>0.295–1.237</td>
<td>0.1678</td>
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<tr>
<td>Sex</td>
<td>1.156</td>
<td>0.653–2.045</td>
<td>0.6183</td>
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<td>1.427–5.728</td>
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<td>0.553–1.932</td>
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<td>Clinical stage</td>
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<td>0.891–4.090</td>
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<td>Histological differentiation</td>
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<td>0.588–4.395</td>
<td>0.6514</td>
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<td>HMGA2 staining</td>
<td>3.482</td>
<td>1.395–8.691</td>
<td>0.0075</td>
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\( ^{a} \) CI, confidence interval.

\( ^{b} \) Cox proportional hazards method.

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Fig. 3. Disease-specific survival in 42 oral carcinoma patients based on the expression of HMGA2. The graph summarizes Kaplan-Meier survival analysis for patients with positive or negative HMGA2 staining. Statistically significant differences were examined between negative and positive HMGA2 staining \( (P = 0.0006) \).

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 \( \mu m \) (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 Hmg2a 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 carcinomas 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 Hmg2a-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 HMGI-C 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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