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High HMGA2 Expression Correlates with Reduced Recurrence-free Survival and Poor Overall Survival in Oral Squamous Cell Carcinoma

CHIH-YEU FANG[1,2], PHUI-LY LIEW[2,3], CHI-LONG CHEN[2,4], YUN-HO LIN[4,5], CHIA-LANG FANG[1,2] and WEI-YU CHEN[1,2]

[1]*Department of Pathology, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan, R.O.C.;*
[2]*Department of Pathology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, R.O.C.;*
[3]*Department of Pathology, Shuang Ho Hospital, Taipei Medical University, New Taipei City, Taiwan, R.O.C.;*
[4]*Department of Pathology, Taipei Medical University Hospital, Taipei Medical University, Taipei, Taiwan, R.O.C.;*
[5]*School of Dentistry, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan, R.O.C.*

Abstract. *Background/Aim: High-mobility group AT-hook 2 (HMGA2) is an architectural transcription factor that is expressed in many human neoplasms. Oral squamous cell carcinoma (OSCC) is one of the leading cancers in the world, particularly in Southeast Asia. In this study, the expression level of HMGA2 was determined on tissue microarray of OSCC and its correlation with prognosis of patients was studied. Materials and Methods: Immunohistochemistry of HMGA2 was analyzed on resection samples from 148 patients with OSCC. The expression level of HMGA2 was determined by ImmunoRatio. Results: High expression of HMGA2 in OSCC was found to be associated with tumor recurrence (p=0.026). Cox model analysis revealed that high expression of HMGA2 was significantly associated with poor survival in patients with OSCC. The Kaplan–Meier analysis also showed decreased survival in patients with high HMGA2 expression. By combining HMGA2 immunostaining and clinicopathological characteristics as analyzing factors, high HMGA2 expression was specifically correlated with poor survival in patients with perineural invasion and lymph node metastasis of OSCC. Additionally, high expression of HMGA2 was found to be a tumor-stage independent prognostic factor associated with high incidence of tumor recurrence and shortened recurrence-free survival. Conclusion: HMGA2 is not only a biomarker for predicting patients with tumor recurrence and poor survival, but when combined with clinicopathological factors, can categorize patients into different risk groups for better clinical management of OSCC.*

Correspondence to: Dr. Wei-Yu Chen, Department of Pathology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, R.O.C. Tel: +886 227361661 ext. 3132. Fax: +886 223770054. e-mail: wychen.patho@gmail.com.

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Oral cancer, a typical type of head and neck cancer, is one of the leading types of cancers around the world, particularly in Southeast Asia (1). Oral cancer resulted in 135,000 deaths in 2013, which is a 60% increase compared to 84,000 in 1990 (2). Oral squamous cell carcinoma (OSCC) is the most common neoplasm of the head and neck. OSCC is principally associated with consumption of tobacco, alcohol, and, in certain areas, betel areca quid (3, 4). Other important risk factors include poor oral hygiene (5), chronic irritation or inflammation (6), and viral infection (7). Standard treatment for OSCC is surgery, radiation, or both, and is sometimes combined with chemotherapy in advanced disease. The 5-year survival rate for patients with OSCC remains around 50-60% (8), and this rate has not improved appreciably in the past decades despite the introduction of new diagnostic and therapeutic strategies. The mortality of OSCC remains persistently high because of tumor recurrence and advanced stage. Therefore, a better understanding of development of aggressive OSCC is obligatory in order to identify relevant targets for developing effective therapeutic strategies for this dreadful malignancy.

High mobility group AT-hook 2 (HMGA2, formerly known as HMGI-C) is a non-histone architectural transcription factor belonging to the high-mobility group protein family (9). HMGA2 contains structural DNA-binding domains, which have been named AT hooks, which are responsible for binding to the AT-rich DNA sequences (10). HMGA2 is an essential component of the enhanceosome that assembles at

the enhancer region of the genome and regulates expression of target genes (11, 12). HMGA2 protein is mainly expressed during embryogenesis in undifferentiated mesenchymal cells, and its expression becomes very low in or completely absent from differentiated adult tissues (13, 14). Re-expression of HMGA2 was reported in many human neoplasms, including benign and malignant tumors (13, 15). In addition, HMGA2 has been revealed to be associated with the epithelial–mesenchymal transition (EMT) in cancer cells (16), and its expression is correlated with poor prognosis of several cancer types (17-20). Overexpression of HMGA2 was shown to correlate with cancer metastasis (17), but the exact mechanism for its contribution to the development of cancer is not fully understood.

Previous studies have indicated that HMGA2 is overexpressed in oral cancer (17, 21). It has been shown that the expression of HMGA2 was correlated with poor prognosis of this disease (17, 21). Here we examined the expression of HMGA2 by immunohistochemical assay of samples from 148 patients with OSCC. The prognosis of these patients was further analyzed by combination of HMGA2 expression and clinicopathological factors.

Materials and Methods

Patients and tissue samples. A total of 148 patients with OSCC who underwent tumor resection at Taipei Medical University Wan Fang Hospital from 1997 to 2010 were retrospectively examined. Patients who received preoperative chemotherapy, radiotherapy, or incomplete surgical resection were excluded. The pathological diagnoses and staging of these cases were reconfirmed by pathologists (W.Y. Chen, C.L. Chen, C.L. Fang and Y.H. Lin), based on the American Joint Committee on Cancer staging system (sixth edition) (22). Postoperative surveillance and treatment were principally performed according to the National Comprehensive Cancer Network guidelines for head and neck cancer. Tissue samples and review of the clinical records were used according to protocols approved by the Taipei Medical University-Institutional Review Board (approval no. WFH-IRB-99049). Representative 1.5 mm-diameter cores of each tumor from the formalin-fixed paraffin-embedded tissue were selected by typical morphology of the diagnosis for tissue microarray construction. Triplicate cancer tissue cores and one non-cancer tissue core were constructed for each case. Sections measuring 2 μm in thickness were cut from the tissue microarray. Postoperative follow-up information including tumor recurrence and survival was obtained from the clinical records. Overall survival (OS) time was defined as time the patient stayed alive from the date of OSCC diagnosis to the end of the study or to death. Disease-free survival (DFS) time was defined as time from the date of diagnosis to the date of local recurrence or newly diagnosed metastasis.

HMGA2 immunohistochemistry. The tissue microarray sections were deparaffinized, rehydrated, and blocked with 3% hydrogen peroxide. Heat-induced antigen retrieval was performed in citric acid buffer (pH 6.0) at 121˚C for 10 min using decloaking chamber (Biocare Medical, Concord, CA, USA). The sections were incubated with rabbit anti-HMGA2 antibodies (HMGI-C, sc-30223, 1: 500; Santa Cruz Biotechnology, Dallas, TX, USA) at 4˚C overnight. Sections were then incubated with a biotin-conjugated secondary antibody (Starr Trek Universal HRP Detection system; Biocare Medical) at room temperature for 30 min, followed by pre-diluted streptavidin-horseradish peroxidase complex at room temperature for 10 min. The immunoreactivity was revealed by adding 3,3'-diaminobenzidine. The sections were counterstained with hematoxylin, dehydrated, and mounted.

Evaluation of HMGA2 expression. The tissue microarray sections stained with HMGA2 were scanned and digitalized using Aperio CS2 slide scanner (Leica Microsystem Inc., Buffalo Grove, IL,

Table I. *Association between clinicopathological characteristics and expression of high-mobility group AT-hook 2 (HMGA2) in patients with oral squamous cell carcinoma.*

Characteristic	HMGA2 expression		*p*-Value
	Low n=90 (61%)	High n=58 (39%)	
Gender			0.199
Male	81 (90%)	48 (83%)	
Female	9 (10%)	10 (17%)	
Mean± SD age (years)	52.7±12.6	57.5±14.4	0.034
Grading of SCC			0.951
Well and moderately differentiated	71 (79%)	46 (79%)	
Poorly differentiated	19 (21%)	12 (21%)	
Tumor T status			0.282
T1+T2	66 (73%)	47 (81%)	
T3+T4	24 (27%)	11 (19%)	
Lymph node metastasis*			0.479
No	61 (69%)	35 (60%)	
Yes	28 (31%)	23 (40%)	
Distant metastasis			0.325
No	89 (99%)	56 (97%)	
Yes	1 (1%)	2 (3%)	
Stage (AJCC 6th Ed)*			0.296
I	29 (33%)	26 (45%)	
II	17 (19%)	6 (10%)	
III	11 (13%)	9 (16%)	
IV	31 (35%)	17 (29%)	
Perineural invasion			0.920
No	47 (52%)	29 (50%)	
Yes	43 (48%)	29 (50%)	
Lymphovascular invasion			0.751
No	64 (71%)	39 (67%)	
Yes	26 (29%)	19 (33%)	
Recurrence*			0.026
No	52 (62%)	22 (42%)	
Yes	32 (38%)	30 (58%)	
Died of disease*			0.078
No	60 (67%)	29 (53%)	
Yes	29 (33%)	26 (47%)	

SD, Standard deviation; SCC, squamous cell carcinoma; AJCC, American Joint Committee on Cancer (22); *Missing data for some patients.

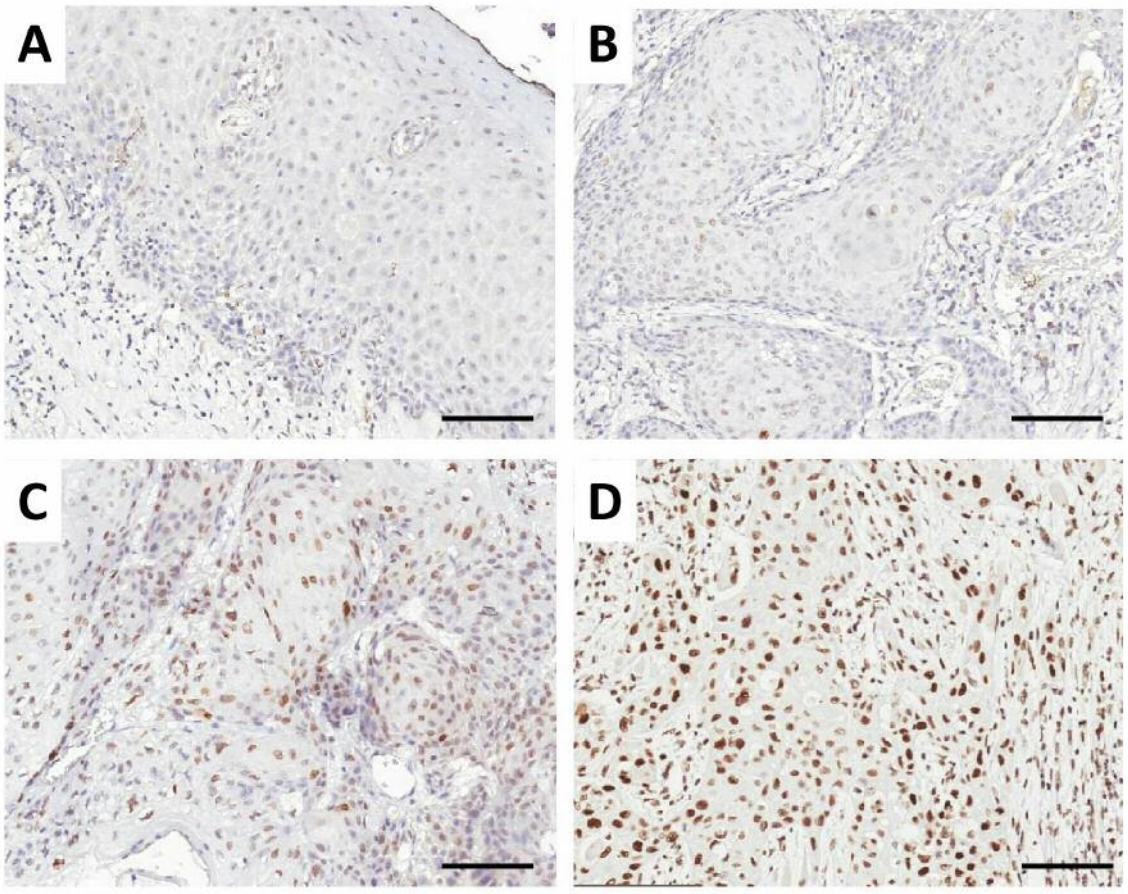


Figure 1. *Expression of high-mobility group AT-hook 2 (HMGA2) protein in oral squamous cell carcinoma (OSCC) tissues. Expression of HMGA2 was absent from normal oral epithelium (A). In OSCC tissues, the expression of HMGA2 was found to range from none (B), to low (C), and high (D). Scale bar: 100 μm.*

USA). The percentage of epithelial cells with nuclear HMGA2 expression was analyzed using ImmunoRatio, a web application for automatic quantitative image analysis (http://153.1.200.58:8080/immunoratio/; Institute of Biomedical Technology, University of Tampere, Tampere, Finland) (23). For expression evaluation, the average percentage of nuclear HMGA2 expression among the three tumor cores for each case was obtained accordingly. The expression of HMGA2 in patients was determined according to the following criteria: low HMGA2 expression: fewer than 30% of tumor cells stained with HMGA2; and high HMGA2 expression: 30% or more of tumor cells stained with HMGA2.

Statistical analysis. The relationships between clinicopathological characteristics and HMGA2 expression were analyzed using the chi-square test for categorical data and Student *t*-test for continuous variables. DFS and OS curves were calculated using the Kaplan–Meier method, and the difference between low and high HMGA2 expression groups was evaluated by log-rank test. Univariate and multivariate analyses of Cox proportional-hazards model was used to determine the significant prognostic factors of DFS and OS. A value of $p<0.05$ was considered statistically significant.

Results

Association of expression levels of HMGA2 with clinicopathological characteristics of OSCC. The expression of HMGA2 was absent in normal oral epithelium (Figure 1A). HMGA2 was often up-regulated in certain oral cancer samples, with distinctive nuclear staining pattern (Figure 1, B-D). In this study, HMGA2 expression was dichotomized into low and high expression groups, based on the percentage of positive-stained cells (see Materials and Methods).

The associations of HMGA2 expression with clinicopathological features and patient characteristics are listed in Table I. High expression of HMGA2 was observed in 58 (39%) out of 148 OSCC cases. The level of HMGA2 did not differ with regard to gender, tumor grading, stage, metastasis, and invasion. High HMGA2 expression was noted in patients of older age in this cohort (57.5±14.4 years, mean±SD, $p=0.034$). Recurrence of disease was significantly higher in patients with high HMGA2 expression than in those with

Table II. *Univariate and multivariate analyses of the overall survival and disease-free survival in patients with oral squamous cell carcinoma.*

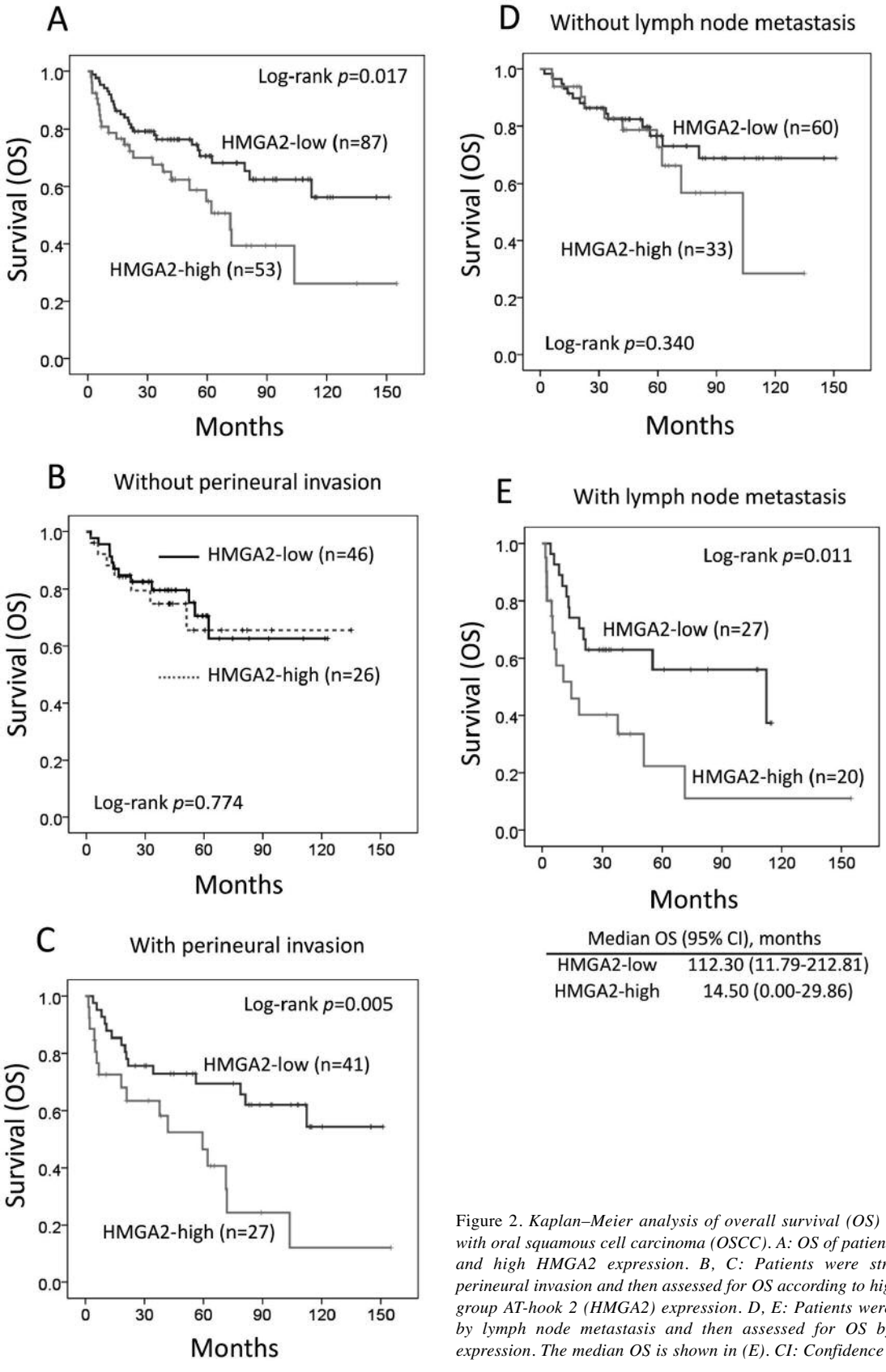
Variables	Univariate analysis				Multivariate analysis			
	Overall survival		Disease-free survival		Overall survival		Disease-free survival	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Gender								
Male	1		1		1		1	
Female	0.81 (0.32-2.04)	0.652	1.11 (0.55-2.25)	0.764	0.86 (0.29-2.61)	0.794	1.15 (0.53-2.52)	0.726
Age								
<65 Years	1		1		1		1	
≥65 Years	1.18 (0.59-2.37)	0.638	0.73 (0.36-1.47)	0.374	0.88 (0.37-2.12)	0.780	0.609	0.270
Differentiation								
Well to moderate	1		1		1		1	
Poor	1.57 (0.87-2.95)	0.136	1.02 (0.57-1.83)	0.950	0.79 (0.37-1.66)	0.528	0.79 (0.39-1.58)	0.506
Tumor T status								
T1+T2	1		1		1		1	
T3+T4	5.24 (2.98-9.21)	<0.001	2.76 (1.62-4.72)	<0.001	4.83 (1.91-12.19)	0.001	5.41 (2.28-12.81)	<0.001
Lymph node metastasis								
No	1		1		1		1	
Yes	1.94 (1.42-2.64)	<0.001	1.63 (1.24-2.14)	0.001	1.81 (1.06-3.08)	0.030	2.45 (1.42-4.23)	0.001
Distant metastasis								
No	1		1		1		1	
Yes	2.43 (0.33-17.79)	0.381	1.42 (0.20-10.24)	0.731	1.11 (0.14-9.06)	0.926	0.39 (0.05-3.04)	0.368
AJCC stage								
I + II	1		1		1		1	
III + IV	4.75 (2.57-8.77)	<0.001	2.05 (1.26-3.34)	0.004	1.31 (0.38-4.56)	0.670	0.33 (0.10-1.10)	0.071
Perineural invasion								
No	1		1		1		1	
Yes	1.72 (0.96-3.10)	0.069	1.60 (0.97-2.61)	0.065	1.27 (0.57-2.85)	0.562	1.34 (0.70-2.56)	0.381
Lymphovascular invasion								
No	1		1		1		1	
Yes	1.54 (0.86-2.74)	0.144	1.62 (0.98-2.68)	0.060	1.05 (0.44-2.47)	0.920	1.06 (0.53-2.12)	0.881
HMGA2								
Low	1		1		1		1	
High	2.05 (1.14-3.70)	0.016	2.01 (1.22-3.30)	0.006	2.53 (1.30-4.93)	0.006	2.34 (1.37-3.99)	0.002

HMGA2, High-mobility group AT-hook 2; HR, hazard ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer.

low expression (58% *vs.* 38%, $p=0.026$). Tumor-specific death resulting from OSCC was found to be higher in the high-HMGA2 group (47%) compared to the low-HMGA2 group (33%), although the difference is not statistically significant ($p=0.078$).

HMGA2 is an unfavorable prognostic factor for patients with OSCC. The survival of patients with OSCC was analyzed by the Cox proportional-hazards regression analysis for prognostic factors. The univariate analysis revealed that tumor stage, T status, lymph mode metastasis, and HMGA2 expression were significant predictors for poor prognosis in OSCC (Table II, $p<0.05$). A 2.0-fold risk increase in patients with high HMGA2 expression for both OS and DFS was observed ($p=0.016$ and $p=0.006$, respectively). In the multivariate analysis, tumor T status, lymph mode metastasis, and HMGA2 expression were found to be significant predictors for poor outcome ($p<0.05$). A 2.5-fold risk increase of death in OS [95% confidence interval (CI)=1.30-4.93; $p=0.006$] and 2.3-fold risk increase of recurrence in DFS (95% CI=1.37-3.99; $p=0.002$) was observed in patients with high HMGA2 expression. These results indicate that high expression of HMGA2 is not only a maker for adverse outcome (endpoint OS), but also predicted poor recurrence-free survival (DFS) in patients with OSCC.

High expression of HMGA2 is correlated with poor prognosis in patients with perineural invasion and lymph node metastasis from OSCC. Kaplan–Meier analysis and the log-rank test were used to evaluate the prognostic significance of HMGA2 in patients with OSCC (Figure 2).



Median OS (95% CI), months	
HMGA2-low	112.30 (11.79-212.81)
HMGA2-high	14.50 (0.00-29.86)

Figure 2. *Kaplan–Meier analysis of overall survival (OS) in patients with oral squamous cell carcinoma (OSCC). A: OS of patients with low and high HMGA2 expression. B, C: Patients were stratified by perineural invasion and then assessed for OS according to high-mobility group AT-hook 2 (HMGA2) expression. D, E: Patients were stratified by lymph node metastasis and then assessed for OS by HMGA2 expression. The median OS is shown in (E). CI: Confidence interval.*

High expression of HMGA2 in patients was found to be correlated with low OS (p=0.017, Figure 2A), corroborating the Cox model result (Table II). Interestingly, the stratification of patients by perineural invasion revealed that in patients without perineural invasion, HMGA2 expression was not a significant factor affecting OS (p=0.774, Figure 2B). It was found that the survival curves were almost identical for the low- and high-HMGA2 groups in patients without perineural invasion (Figure 2B). However, in patients with perineural invasion, high expression of HMGA2 predicted markedly poor survival in patients with OSCC (p=0.005, Figure 2C). Additionally, the stratification according to lymph node metastasis also showed that in patients without lymph node metastasis, HMGA2 expression was not a significant factor affecting OS (p=0.340, Figure 2D). It was found that the survival curves for the initial 60 months of follow-up were almost identical for the low- and high-HMGA2 groups (Figure 2D), and only after 60 months did the curve of high-HMGA2 group turn further down, albeit no statistical difference was observed overall. However, in patients with lymph node metastasis, high expression of HMGA2 predicted significantly poor survival in patients with OSCC (p=0.011, Figure 2E). In patients with lymph node metastasis, the median OS was 112.3 months (95% CI=11.79-212.81 months) for the low-HMGA2 group and 14.5 months (95% CI=0.00-29.86 months) for the high-HMGA2 group, which represents a great difference in survival between these two groups. These results indicate that high HMGA2 expression is correlated with poor prognosis of OSCC, particularly in patients with perineural invasion and lymph node metastasis.

High expression of HMGA2 is correlated with high incidence of OSCC recurrence and short recurrence-free time. Compared to OS, DFS documented the time period from the date of diagnosis to the date of recurrence or metastasis. Hence, DFS may reflect the success rate of response to initial clinical treatments. Our study indicates that recurrence of OSCC is correlated with high HMGA2 expression (Table I). To better understand the association between HMGA2 expression and tumor recurrence, a recurrence analysis based on DFS was performed (Figure 3). It was found that high expression of HMGA2 in patients was correlated with high incidence of tumor recurrence rate and low DFS (p=0.005, Figure 3A). Even when stratified by tumor stage, HMGA2 expression remained a consistent factor affecting DFS in patients with OSCC (p<0.05, Figure 3B and C). In patients with either early-stage (I and II) or late stage (III+IV) OSCC, DFS was significantly different between the low- and high-HMGA2 groups (p=0.038 and p=0.008, respectively). In patients with early-stage disease, the incidence of recurrence was significantly higher in the high-HMGA2 group than in low-HMGA2 group (Figure 3B). In patients with late-stage disease (Figure 3C), the median DFS was 84.67 months (95% CI=1.36-167.97 months) for the low-HMGA2 group and 7.83 months (95% CI=5.14-10.52 months) for the high-HMGA2 group, which is a great disparity in recurrence-free time even in patients with advanced disease. Interestingly, the median DFS for patients with early-stage tumor with high HMGA2 expression was 61.80 months (95% CI=23.91-99.69 months, Figure 2B), while it was 84.67 months (95% CI=1.36-167.97 months) for those with late-stage disease with low-HMGA2 expression (Figure 2C), indicating that the effect of high HMGA2 expression is as powerful as advanced tumor stage in affecting the recurrence-free survival of patients with OSCC. These results also indicate that HMGA2 is a significant, tumor stage-independent prognostic factor for DFS. Our results indicate that high expression of HMGA2 is a predictor of high recurrence rate and poor DFS in patients with OSCC.

Discussion

In this study, we demonstrated that high HMGA2 expression was correlated with increase incidence of tumor recurrence (Table I) and is an independent prognostic marker of OSCC (Table II). Our results are concordant with previous studies indicating that a poor prognosis is associated with high-level expression of HMGA2 in OSCC (17, 21, 24-26). These results suggest that HMGA2 is a useful predictive and prognostic biomarker in clinical management of oral carcinomas. In addition, our study has further demonstrated that, when analyzing with other clinicopathological factors such as lymph node metastasis and perineural invasion, the expression level of HMGA2 is a practical indicator that can categorize patients with OSCC into different risk groups (Figure 2 and 3). Such classification could be of particular importance for clinical management of patients with OSCC and in searching for effective target therapies against HMGA2-associated malignancies.

It had been shown that overexpression of HMGA2 promotes metastasis in cancer (16, 19, 27), and down-regulation of HMGA2 inhibits invasion of cancer cell lines (28). HMGA2 was shown to up-regulate SNAI1 expression and induce epithelial–mesenchymal transition, which may promote cell invasion and metastasis (16, 27, 29). In this study, although the expression level of HMGA2 was not directly correlated with the incidence of perineural invasion and lymph node metastasis in OSCC (Table I), it was found that in patients with OSCC with these two pathological features, high expression of HMGA2 always led to poor survival (Figure 2C and E). Conversely, in patients without perineural invasion and lymph node metastasis, the expression level of HMGA2 did not significantly affect their OS (Figure 2B and D). These results may imply that when tumor cells were still confined at the primary site, the

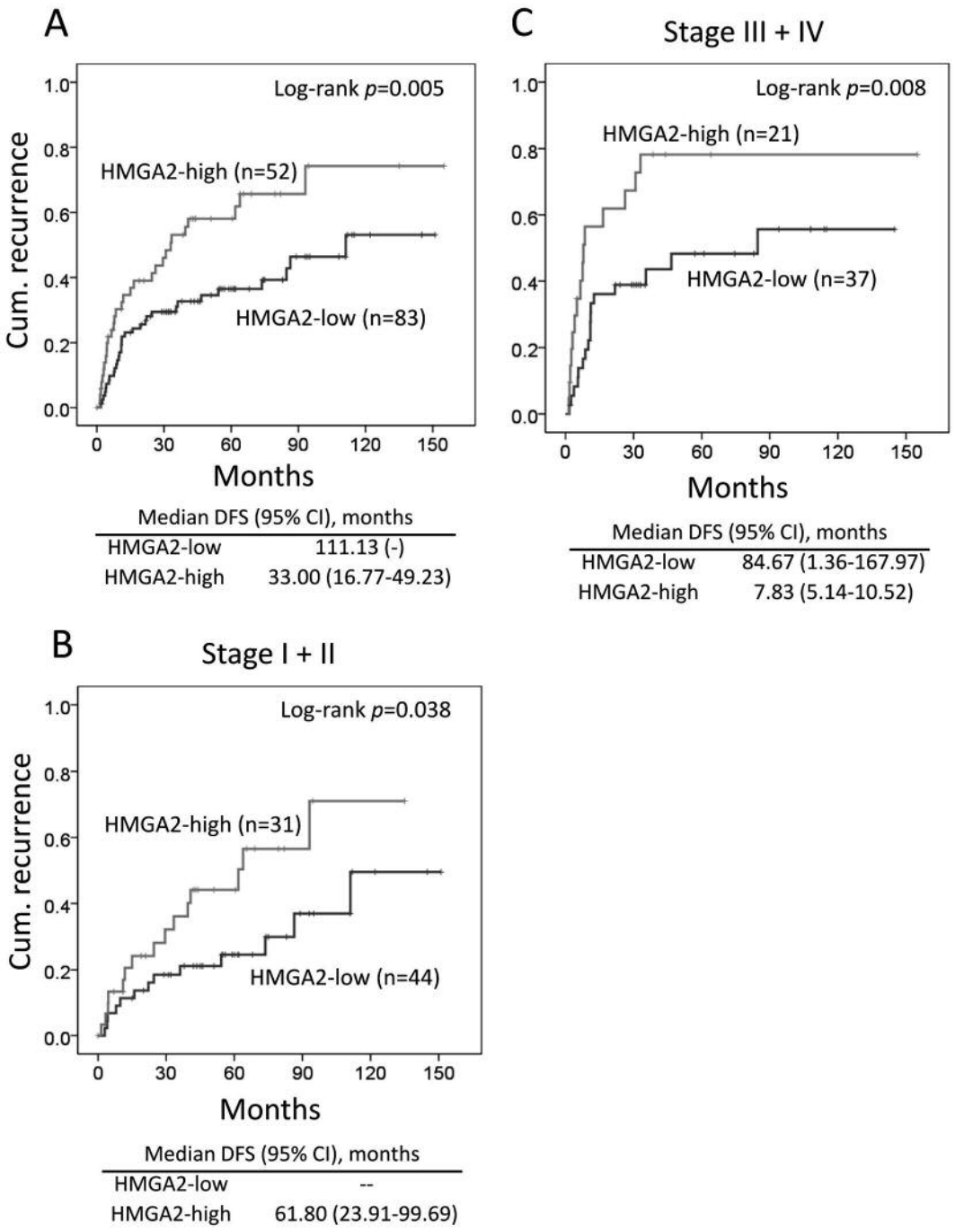


Figure 3. *Analysis of disease-free survival by cumulative tumor recurrence in patients with oral squamous cell carcinoma (OSCC). A: Cumulative tumor recurrence of patients with low and high expression of high-mobility group AT-hook 2 (HMGA2) protein. B, C: Patients were stratified by tumor stage (I+II and III+IV) and then assessed for DFS according to HMGA2 expression. The median DFS is shown where applicable. CI: Confidence interval; Cum: cumulative.*

expression of HMGA2 was not potent enough to affect the clinical outcome of patients with standard treatment protocols (Figure 2B and D). However, when local invasion or lymph node spread of OSCC cells occurred, high HMGA2 expression significantly aggravated the progression of disease and resulted in a very poor survival of patients (Figure 2C and E). Similarly, high expression of HMGA2 was significantly associated with high recurrence rate and short

disease-free time of patients with OSCC, regardless of tumor stage (Figure 3). It was found that the incidence of recurrence was the lowest and the DFS was the highest in patients with early-stage disease and low HMGA2 expression, while the incidence of recurrence was the highest and the DFS was the lowest in patients with late-stage disease and high HMGA2 expression. Even within the late-stage group, the difference in DFS between the low- and high-HMGA2 groups was very prominent (median DFS=84.67 *vs*. 7.83 months, Figure 3C). Therefore, by combining perineural invasion, lymph node metastasis, or tumor stage with HMGA2 expression in analysis, patients with OSCC can be categorized into risk groups with appreciably different outcomes. For high-risk patients, alternative tumor surveillance programs or aggressive treatment protocols may be required to achieve a better disease response and outcome. This categorization could be particularly important for clinical management of OSCC. In addition, classifying patients with high HMGA2 expression may enable them to benefit from therapies targeted specifically against HMGA2 when such treatments are available.

Since HMGA2 is mainly expressed during embryogenesis, it is considered as a tumor-specific target for cancer therapy (30). It was also shown that HMGA2 overexpression increased the efficacy of radiotherapy in patients with colorectal cancer (19), and can sensitize breast cancer cells *in vitro* to double-strand DNA breaks caused by radiation and certain kinds of chemotherapeutic compounds (30). Therefore, patients with HMGA2 expression may benefit from therapies that specifically target HMGA2-associated malignancies. Since our data indicate that HMGA2 overexpression predicts poor outcome in OSCC, particularly in patients with perineural invasion and lymph node metastasis, the treatment strategy for these specific patients might be adjusted and alternative options could be taken into consideration. It was shown that the presence of HMGA2 may render cells resist to certain genotoxicants (31) while sensitizing them to a different class of chemotherapeutic compounds (30, 31). For instance, HMGA2 expression is associated with enhanced selective chemosensitivity towards the topoisomerase II inhibitor, doxorubicin, in breast cancer cells (30). Therefore, the effectiveness of HMGA2-sensitive chemotherapeutic compounds may be evaluated in patients with OSCC with high HMGA2 expression. Similarly, since HMGA2 overexpression was shown to increase the efficacy of radiotherapy (19), the radiotherapy protocol for patients with high HMGA2 expression could be tuned for optimal efficacy. Recurrence of cancer can be attributed to multiple factors and remains difficult to predict since no reliable biomarker is available as an indicator of prognosis. Our data strongly suggest that HMGA2 immunostaining might predict tumor recurrence of OSCC and stratify patients into risk groups with distinct outcome (Figure 3). Although this observation might need to be further verified with larger series of patients, if confirmed this finding will impact on the choice of the most suitable treatment strategy that can maximize the efficacy of radiotherapy and chemotherapy in OSCC.

Taken together, our study demonstrates that high HMGA2 expression is correlated with increased tumor recurrence and is an independent prognostic marker for OSCC. High expression of HMGA2, predominantly in patients with perineural invasion and lymph node metastasis, leads to poor clinical outcome. Based on these observations, a new strategy targeting high HMGA2 expression in patients with OSCC is urgently required. The selective use of effective protocols for patients with high HMGA2 expression could increase the success of therapy and minimize the associated toxicity, thus improving the overall survival of patients with OSCC.

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