

Expression of aldehyde dehydrogenase family 1 member A1 and high mobility group box 1 in oropharyngeal squamous cell carcinoma in association with survival time

XU QIAN^{1,2}, ANNEKATRIN COORDES¹, ANDREAS M. KAUFMANN³ and ANDREAS E. ALBERS¹

¹Department of Otolaryngology, Head and Neck Surgery, Charité - Medical University of Berlin, Benjamin Franklin Campus, D-12200 Berlin, Germany; ²School of Basic Medical Science, Zhejiang Provincial Key Laboratory of Medical Genetics, Wenzhou Medical University, Wenzhou, Zhejiang 325035, P.R. China; ³Clinic for Gynecology, Charité - Medical University of Berlin, Benjamin Franklin Campus, D-12200 Berlin, Germany

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Abstract. Despite the development of novel multimodal treatment combinations in advanced oropharyngeal squamous cell carcinoma (OSCC), outcomes remain poor. The identification of specifically validated biomarkers is required to understand the underlying molecular mechanisms, to evaluate treatment efficiency and to develop novel therapeutic targets. The present study, therefore, examined the presence of aldehyde dehydrogenase family 1 member A1 (ALDH1A1) and high mobility group box 1 (HMGB1) expression in primary OSCC and analyzed the impact on survival time. In 59 patients with OSCC, the expression of ALDH1A1, p16 and HMGB1, and their clinicopathological data were analyzed. HMGB1 positivity was significantly increased in patients with T1-2 stage disease compared with T3-4 stage disease ($P < 0.001$), whereas ALDH1A1 positivity was not. ALDH1A1⁺ tumors showed significantly lower differentiation than ALDH1A1⁻ tumors ($P = 0.018$). Multivariate analysis showed that ALDH1A1 positivity ($P = 0.041$) and nodal status (N2-3) ($P = 0.036$) predicted a poor prognosis. In this patient cohort, ALDH1A1 and nodal status were identified as independent predictors of a shorter overall survival time. The study results, therefore, provide evidence of the prognostic value of ALDH1A1 as a marker for cancer stem cells and nodal status in OSCC patients.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with an annual incidence of 500,000 new cases and 200,000 mortalities (1,2). Oropharyngeal squamous cell carcinoma (OSCC) is a subtype of HNSCC arising from the oropharynx, which includes anatomically the base-of-the tongue, the tonsils, the soft palate and the side and back wall of the throat. The incidence rates for cancer sites in the oropharynx, such as the tonsils and the base of the tongue, are associated with a rise in human papilloma virus (HPV) infections, but a decreased number of tobacco- and alcohol-related cancers (3). In recent decades, there have been a number of important advances in the standard treatment of HNSCC, including surgical innovations for early-stage tumor patients and novel multimodal treatment combinations for more advanced-stage tumors, such as surgery followed by adjuvant radiotherapy (RT) or chemoradiotherapy (CRT), or CRT alone (4). However, outcomes remain poor in advanced tumor stages due to frequent local and regional metastases, and resistance to therapy (5,6). Therefore, identification of specific validated biomarkers is required to understand the underlying molecular mechanisms, evaluate treatment efficiency and develop novel therapeutic targets.

High mobility group box 1 (HMGB1) is a nuclear, non-histone, chromatin-binding protein (7) that has been implicated in the activities of various cell type, including enterocytes, cardiomyocytes, pituicytes, macrophages and monocytes (8-11). HMGB1 is defined as one of the damage-associated molecular pattern molecules that interact with a variety of pattern recognition receptors in the microenvironment of damaged or necrotic tissues. A number of studies have demonstrated the multiple functions of HMGB1 in tumorigenesis, angiogenesis, lymphangiogenesis and metastasis in various malignancies (12-15). In the tumor microenvironment, cancer cells and inflammatory cells have the ability to release HMGB1 (16). In turn, extracellular HMGB1 can accelerate inflammatory responses and may lead to tumor formation and metastases (16). Receptors for advanced glycosylation end products (RAGE) (17) and Toll-like receptor (TLR)-4 (18,19)

Correspondence to: Dr Andreas E. Albers, Department of Otolaryngology, Head and Neck Surgery, Charité - Medical University of Berlin, Benjamin Franklin Campus, Hindenburgdamm 30, D-12200 Berlin, Germany
E-mail: andreas.albers@charite.de

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are also involved in HMGB1-mediated tumorigenesis. A study in HNSCC cell lines showed that the interaction between HMGB1 and its receptor RAGE resulted in the development of metastasis (17). Wild *et al* observed that HMGB1 may enhance tumor-infiltrating regulatory T cell (Treg) immunosuppression by acting as a chemoattractant on the Tregs, which express RAGE and TLR4 receptors (20). A correlation with a poor prognosis was also found in laryngeal squamous cell carcinoma with a high serum HMGB1 level (21) and in HNSCC with a high HMGB1 protein expression level (22). To date, however, the expression pattern of HMGB1 and its impact on survival is not known in oropharyngeal squamous cell carcinoma (OSCC).

Another important hypothesis in generating and maintaining malignancy and driving metastasis is concerning cancer stem (-like) cells (CSCs) (6). As shown in our previous studies, aldehyde dehydrogenase 1A1 (ALDH1A1)-positive CSCs exhibit characteristics (23,24) that include self-renewal, invasion and epithelial-mesenchymal-transition traits. The prognostic relevance of ALDH1A1 has also been identified in patients with HNSCC (25,26). Therefore, the identification of ALDH1A1 expression and the assessment of its correlation with HMGB1 expression and clinicopathological parameters may aid in further elucidating the biology of HNSCC from a CSC perspective and its relevance for prognosis.

The present study investigated HMGB1 and ALDH1A1 expression in patients with OSCC with the aim of assessing whether this expression was correlated with clinicopathological factors and to investigate its association with overall survival (OS) time.

Materials and methods

Patients. A total of 59 OSCC patients with no prior history of malignancies and treatment were included in this study. The main clinical and pathological data were collected from the Institute of Pathology (Charité - Medical University of Berlin, Benjamin Franklin Campus, Berlin, Germany) database and patient charts, as illustrated in Table I. Tissue biopsies were obtained during panendoscopy to confirm histologically suspected HNSCC. Residual material was used for the present study. OS time was calculated from the date of diagnosis of OSCC to the date of mortality or last follow-up. This study was approved by the Institutional Review Board of Charité - Medical University of Berlin (Berlin, Germany).

Histology and immunohistochemistry. Sections (2- μ m thick) from formalin-fixed, paraffin-embedded specimens were collected from the Institute of Pathology. An immunohistochemical staining method [EnVision System-horseradish peroxidase (HRP) mouse/rabbit; Dako, Hamburg, Germany] was used following deparaffinization in xylene and rehydration. Primary antibodies used included mouse monoclonal antibody specific for p16 (1:100 dilution; clone DCS-50; catalog no. sc-65476; Neomarkers, Fremont, CA, USA), ALDH1A1 (1:100 dilution; clone 44; catalog no. 611195; BD Biosciences, San Jose, CA, USA) and HMGB1 (1:200 dilution; catalog no. ab18256; Abcam, Cambridge, MA, USA). Antigens were

retrieved by steam heating for 20 min in a 0.01 M trisodium citrate buffer (pH 6.0). ChemMate Peroxidase-Blocking Solution (Dako) was used to block endogenous peroxidase activity for 10 min at room temperature. The slides were incubated with antibodies for 2 h, followed by the addition of HRP-labeled anti-mouse antibody at room temperature. Immunoreactive proteins were visualized with 3,3-diaminobenzidine and counterstained with Mayer's hematoxylin (Dako). The sections were dehydrated and mounted. Positive and negative controls were included in each run for the quality control of immunoreactivity. Normal tonsillar tissue served as the positive control and an isotype control (Dako) was used to replace the primary antibody as a negative control.

Three experienced, independent observers (including a pathologist) performed semiquantitative evaluation of the slides, as described in our previous study (25). The evaluators were blinded to the clinical data. The grading system for p16, ALDH1A1 and HMGB1 was used, as previously published for OSCC (26,27).

Statistics. Statistical analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Categorical variables are expressed as percentages and frequencies, and numerical variables are represented as the mean $\pm$ standard deviation. Qualitative data was compared using the χ^2 or Fisher's exact test as appropriate. OSCC-free survival was determined using the Kaplan-Meier method. The Cox multivariate regression model was applied to evaluate hazard ratios (HRs) and P-values. $P < 0.05$ was regarded to indicate a statistically significant difference.

Results

Patient characteristics and HPV status. The median age of the 59 patients with OSCC was 58.0 years (range, 42-82 years), with a male to female ratio of 6.4:1. Of the 59 lesions, 13 (22%) were located in the tongue, 39 (66%) in the tonsil and 7 (12%) at another oropharynx site. The tumor-node-metastasis staging of each patient was collected from the Institute of Pathology data and clinical charts (28). At the time of the last follow-up, 30 (51%) patients had succumbed.

Due to the scarcity of material available from the specimens, which was insufficient for HPV-DNA testing, p16 was used as a surrogate HPV marker (29). In total, of the 59 primary tumors, 14 specimens were p16⁺ (24%) (Table I). There were no statistically significant correlations between p16 positivity and any clinicopathological parameters.

HMGB1 and ALDH1A1 expression, and the risk of OSCC. The study investigated the association between HMGB1, ALDH1A1, p16 and clinicopathological variables. The expression of HMGB1 was above the cutoff in 59% (35/59) of primary tumors, while the expression of ALDH1A1 was above the cutoff in 41% (24/59) (24). HMGB1 positivity was significantly higher in patients with T1-2 stage than T3-4 stage disease ($P < 0.001$), whereas ALDH1A1 positivity was not. ALDH1A1⁺ tumors displayed significantly lower differentiation compared with ALDH1A1⁻ tumors ($P = 0.018$). There was no correlation between ALDH1A1 positivity and age, gender, tobacco or alcohol consumption, p16 status or HMGB1

Table I. Correlation between clinicopathological characteristics and examined variables in 59 patients with oropharyngeal squamous cell carcinoma.

Characteristic	Total, n	p16 expression, n (%)		P-value	HMGB1 expression, n (%)		P-value	ALDH1A1 expression, n (%)		P-value
		+	-		+	-		+	-	
Gender				0.422			0.564			0.564
Female	8	1 (12.5)	7 (87.5)		4 (50.0)	4 (50.0)		4 (50.0)	4 (50.0)	
Male	51	13 (25.5)	38 (74.5)		31 (60.8)	20 (39.2)		20 (39.2)	31 (60.8)	
Age, years				0.259			0.169			0.400
<60	33	6 (18.1)	27 (81.9)		17 (51.5)	16 (48.5)		15 (45.5)	18 (54.5)	
≥60	26	8 (30.8)	18 (69.2)		18 (69.2)	8 (30.8)		9 (34.6)	17 (65.4)	
Primary tumor site				0.270			0.586			0.267
Tongue	13	6 (46.1)	7 (53.9)		5 (38.5)	8 (61.5)		7 (53.9)	6 (46.1)	
Tonsil	39	7 (17.9)	32 (82.1)		27 (69.2)	12 (30.8)		16 (41.0)	23 (59.0)	
Others	7	1 (14.3)	6 (85.7)		3 (42.9)	4 (57.1)		1 (14.3)	6 (85.7)	
Smoking				0.908			0.324			0.324
Never	9	2 (22.2)	7 (77.8)		4 (44.4)	5 (55.6)		5 (55.6)	4 (44.4)	
Past and present	50	12 (24.0)	38 (76.0)		31 (62.0)	19 (38.0)		19 (38.0)	31 (62.0)	
Alcohol intake-consumption				0.516			0.056			0.056
Never	21	6 (28.6)	15 (71.4)		9 (42.9)	12 (57.1)		12 (57.1)	9 (42.9)	
Past and present	38	8 (21.0)	30 (79.0)		26 (68.4)	12 (31.6)		12 (31.6)	26 (68.4)	
Tumor differentiation				0.917			0.400			0.018
Moderate	33	8 (24.2)	25 (75.8)		18 (54.5)	15 (45.5)		9 (27.2)	24 (72.8)	
Poor	26	6 (23.0)	20 (77.0)		17 (65.4)	9 (34.6)		15 (57.7)	11 (42.3)	
Tumor stage				0.942			<0.001			0.691
T1-2	30	7 (23.3)	23 (76.7)		22 (73.3)	8 (26.7)		15 (50.0)	15 (50.0)	
T3-4	29	7 (24.1)	22 (75.9)		13 (44.8)	16 (55.2)		9 (31.0)	20 (69.0)	
Lymph node metastasis				0.753			0.603			0.285
N0 and N1	22	3 (13.6)	19 (86.4)		14 (63.6)	8 (36.4)		7 (31.8)	15 (68.2)	
N2 and N3	37	11 (29.7)	26 (70.3)		21 (56.8)	16 (43.2)		17 (45.9)	20 (54.1)	

ALDH1A1, aldehyde dehydrogenase family 1 member A1; HMGB1, high mobility group box 1.

positivity (Table I). There was also no significant correlation between HMGB1, ALDH1A1 and p16 expression (data not shown).

Kaplan-Meier survival estimations indicated that patients with ALDH1A1 positivity in the primary tumor experienced significantly reduced OS times ($P=0.047$) (Fig. 1). Patients with N0-1 stage disease experienced better survival than patients with N2-3 stage ($P=0.014$) (Fig. 1). However, HMGB1 positivity and a negative HPV status were not significantly associated with outcome in the patient cohort studied (data not shown). In addition, the clinicopathological parameters, and ALDH1A1, HMGB1 and p16 positivity were analyzed by the Cox proportional hazards model (Table II). On univariate and multivariate analysis, ALDH1A1 positivity and N2-3 stage exhibited a significant effect on OS. These data suggest that ALDH1A1 positivity and nodal status are independent prognostic factors in OSCC. No other parameters were associated with outcome.

Discussion

Increasing evidence has demonstrated the multiple functions of HMGB1 in cancer progression, including apoptosis, angiogenesis, inflammatory process, invasion and metastasis, indicating the significance of HMGB1 as a potential therapeutic target in human malignancies. The present study investigated the correlation of HMGB1 expression with clinicopathological characteristics and prognosis, as assessed by immunohistochemical assay in patients with OSCC. It was demonstrated that the expression of HMGB1 in OSCC patients with stage T1-2 was higher than that in those with stage T3-4. This observation is different to the observation reported by Liu *et al*, which showed a stronger expression of HMGB1 in patients with stage 3-4 in a study cohort of patients with HNSCC mainly located in the supraglottic and glottic regions (22). In addition to the report of expression on the tissue level, another study found that the serum HMGB1 level in laryngeal squamous cell carcinoma patients was significantly increased in patients with T3-4 stage

Table II. Univariate and multivariate analysis for factors possibly influencing overall survival in 59 patients with oropharyngeal squamous cell carcinoma.

Factor	Univariate		Multivariate	
	Hazard ratio	P-value	Hazard ratio	P-value
History of smoking (yes vs. no)	1.867	0.260	1.710	0.398
Alcohol intake (yes vs. no)	1.411	0.380	1.781	0.216
Tumor differentiation (high-grade vs. intermediate-grade)	1.519	0.286	0.887	0.781
Tumor stage (pT3-4 vs. pT1-2)	0.658	0.280	0.580	0.230
Lymph node metastasis (pN2-3 vs. pN0-1)	1.354	0.026	1.346	0.036
p16 (positive vs. negative)	0.535	0.149	0.619	0.316
ALDH1A1 (positive vs. negative)	1.516	0.039	1.650	0.041
HMGB1 (positive vs. negative)	0.337	0.051	0.348	0.052

ALDH1A1, aldehyde dehydrogenase family 1 member A1; HMGB1, high mobility group box 1.

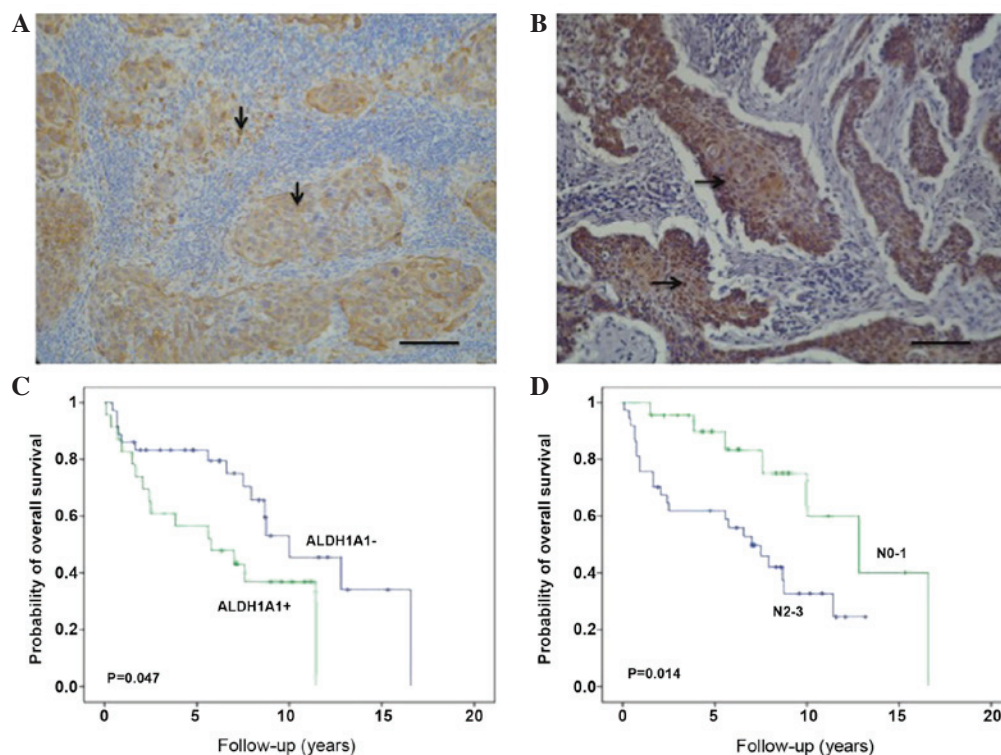


Figure 1. Expression of ALDH1A1 and HMGB1 in OSCC. Representative examples of (A) ALDH1A1 and (B) HMGB1 immunostaining in OSCC at x200 magnification. ALDH1A1⁺ tumors (vertical arrows); HMGB1⁺ tumors (horizontal arrows). (C) Kaplan-Meier curve for probability of OS by ALDH1A1 negativity and positivity in patients with OSCC. (D) Kaplan-Meier curve for OS by lymph node metastasis N0-1 and N2-3 in patients with OSCC. Scale bar, 100 μ m. ALDH1A1, aldehyde dehydrogenase family 1 member A1; HMGB1, high mobility group box 1; OSCC, oropharyngeal squamous cell carcinoma; OS, overall survival.

disease when compared with patients with T1-2 stage (21). However, a recent case control study in patients staged N0 with early tongue carcinoma (T1/2N0M0) showed that HMGB1 expression cannot predict occult neck metastasis (27). The present study found that there was also no correlation between HMGB1 expression and the presence of nodal metastasis. These clinicopathological observations from the aforementioned studies presents a more complicated pattern of HMGB1 expression in tumors derived from different head and neck regions,

and disease states, suggesting currently unknown reasons for the distinct biological behavior other than anatomical reasons. In the present analysis, no correlation between the expression of p16 and HMGB1 was evident, indicating mechanisms independent of HPV/p16-induced disease. In addition to the role of HMGB1 in promoting carcinogenesis, recent studies in cancer cell lines and animal models have suggested an antitumor role of intracellular HMGB1. Zuo *et al* described an anti-metastatic effect of HMGB1 in a cell line derived from human lung cancer

(A549 cells) and tumor models (30). After knockdown of HMGB1, β -actin polymerization, cellular skeleton formation, cancer cell migration and invasion were significantly increased. HMGB1 has also been described with functions as a tumor suppressor and radiosensitizer in breast cancer (31). Therefore, further studies investigating this aspect of the underlying role of HMGB1 in tumor biology would be noteworthy.

CSCs take part in the initiation and progression of HNSCC, as well as in predicting prognosis. In the current study cohort, it was found that the overexpression of ALDH1A1 was correlated with poorly-differentiated tumor tissue and reduced survival time in patients with OSCC. This finding was consistent with a previously studied cohort of OSCC obtained from a different region in Germany (26). A recent meta analysis concluded that, in the head and neck region, ALDH1A1 expression was highly correlated with tumor differentiation and lymph node metastasis, but not significantly with T stage (32). A correlation of higher ALDH1A1 expression with decreased OS and disease-free survival time was also presented. Taken together, these data suggested that ALDH1A1 could potentially be used as a prognostic biomarker and predictor for evaluating the risk of OSCC, depending on ALDH1A1 expression. The correlation of ALDH1A1 and HMGB1 expression is also of concern since HMGB1 may also be involved in the progression of HNSCC. Further statistical analyses were therefore performed in the present study, however, no correlation between ALDH1A1 and HMGB1 expression (data not shown) was found. Moreover, the overexpression of HMGB1 did not predict OS time. Therefore, the ability of HMGB1 expression as a biomarker to evaluate the disease state and predict prognosis in OSCC remains under debate. Due to the double-edged function of intracellular and extracellular HMGB1 in carcinogenesis, it will be of future note to study the interaction of HMGB1 with cultured CSCs and bulk tumor cells in the laboratory. As recently reviewed by our group, CSCs are able to escape immunoreaction by inhibiting T-cell proliferation and activation, triggering T-cell apoptosis and inducing regulatory T cells (Treg), and the investigation of this regarding the involvement of HMGB1 will be noteworthy (33). Wild *et al* found an elevated HMGB1 level in the sera of HNSCC (laryngeal, pharyngeal SCC and oral cavity) patients and in tumor tissues (20). These increased HMGB1 levels could act, as it was hypothesized, as a chemoattractant for Treg, promoting the survival of Treg, and promoting the suppressive capacity of Treg in a dose-dependent manner. HMGB1 was monitored for its regulation of tumor growth by increasing microRNA (miR)-21 expression to mediate the enhanced activity of matrix metalloproteinases (MMPs) through MMP inhibitors (34). In our recent study, it was found that upregulated miR-21 could stimulate cancer cell proliferation in HNSCC lines and it was observed that miR-21 expression may act as a marker of progression, with prognostic value in patients with HNSCC (35). Therefore, the biological effects of tumor-derived HMGB1 with a CSC population within the tumor microenvironment on regulating tumor immune responses leaves more questions open than answered.

One important therapeutic strategy for head and neck cancer, particularly OSCC, is the better prognosis of patient subgroups with HPV infection and no history of tobacco abuse (36). In the present study, p16 expression was employed as a surrogate marker in determining HPV status. No significant

correlations between the expression of ALDH1A1 or HMGB1 and p16 were found. Furthermore, p16 did not predict survival time in this study population of patients with OSCC, which had also been recently shown for European populations (37), but was in contrast with studies from the United States of America (38). This is probably due to less cessation of tobacco abuse in European regions.

In summary, the current study results present and confirm the prognostic value of ALDH1A1 expression as a CSC marker in patients with OSCC. HMGB1 may be involved in the pathogenesis of OSCC, but did not predict survival in the studied patient cohort. Cervical lymph node metastasis is also presented as an important prognostic factor in OSCC. Future expansion and inclusion of multiple approaches in understanding the pathogenesis of OSCC would provide opportunities for system-level monitoring of disease and development of individualized cancer therapies.

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