

CD73 as a novel marker for poor prognosis of oral squamous cell carcinoma

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Abstract. Ecto-5'-nucleotidase [cluster of differentiation (CD)73] has important functions in several types of cancer, however, its expression in squamous cell carcinoma (SCC) remains unknown. The present study was designed to investigate CD73 expression in SCC. CD73 expression was assessed by immunohistochemistry in 113 patients with oral SCC (OSCC). The association between CD73 expression and clinicopathological features, overall survival (OS) and disease-free survival (DFS) times of patients were statistically analyzed. CD73 expression was detected in 58.4% (66/113) of OSCC patients, with the immunostaining predominantly localized in the cytomembrane and a little in the cytoplasm. Statistical analysis revealed that CD73 expression was more frequently detected in patients with larger tumors ($P=0.021$). The overexpression of CD73 was significantly associated with clinical stage ($P=0.047$). Furthermore, immunohistochemical staining showed that overexpression of CD73 was inversely correlated with DFS ($P=0.002$) and OS ($P=0.002$) times. Multivariate Cox regression analysis revealed that CD73 expression was an independent prognostic factor for poor DFS ($P=0.018$) and OS ($P=0.021$). The current study is the first to evaluate the clinical significance and prognostic value of CD73 in patients with OSCC. The findings suggest that CD73 is a potential prognostic marker for OSCC.

Introduction

Despite improvements in treatment strategies for oral squamous cell carcinoma (OSCC), it remains one of the most devastating malignancies. OSCC is the sixth most common

type of cancer in the world and accounts for almost 3% of all cancer cases (1-3). Although significant advancements have been made in the prevention, diagnostics and treatment strategies for OSCC, only modest progress has been made in improving the survival time in patients with progression or metastatic disease over the last 20 years (4,5). In 2012, ~300,400 new cases of oral cavity cancer, including lip cancer, and ~145,400 associated mortalities occurred worldwide (6). Promising markers are the basis for improving the early detection and accurate survival evaluation of patients with OSCC.

Cluster of differentiation (CD)73, also known as 5'-nucleotidase (5'-NT) or ecto-5'-NT, is a 70-kD, glycosyl-phosphatidylinositol anchored cell surface enzyme that is encoded by the 5'-nucleotidase ecto gene. CD73 was originally defined as a lymphocyte differentiation antigen, and has both enzymatic and non-enzymatic functions (7). CD73 catalyzes the dephosphorylation of extracellular adenosine monophosphate to adenosine, promoting its suppressive effects on the immune system in the tumor microenvironment, invasion and metastasis of cancer (8). In addition to its enzymatic function, CD73 is also an adhesive and signaling molecule that mediates cancer invasion and metastasis (9). Thus, both the enzymatic and non-enzymatic functions of CD73 are involved in the processes of cancer occurrence and development.

Increased expression of CD73 expression has been observed in several types of malignancy (10,11), such as breast cancer, prostate cancer, bladder cancer and malignant melanoma. In addition, it has prognostic value for patients with colon cancer and has been suggested as a diagnostic factor in papillary thyroid carcinoma (12). However, there is almost no information available regarding the survival influence of CD73 expression on tumor cells in patients with SCC. The current study analyzed the association between CD73 expression and clinicopathological characteristics, including disease-free survival (DFS) and overall survival (OS) time, in patients with OSCC.

Materials and methods

Enrolled patients. Patients with OSCC who underwent surgery (combined primary tumor resection and neck dissection/reconstruction) at the Department of Oral and Maxillofacial-Head and Neck Oncology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China) were

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included in the present study. The inclusion criteria were as follows: i) Patients with OSCC (preoperative pathological diagnosis); ii) patients that underwent primary therapy in the Department of Oral and Maxillofacial-Head and Neck Oncology; iii) patients that did not receive preoperative chemotherapy, radiation therapy or any other treatment prior to surgery; and iv) patients diagnosed between January 2007 and December 2008. Cancerous and adjacent tumor samples were collected immediately after surgery. The distance between the tumor and the adjacent samples was >2 cm. According to the aforementioned criteria, 113 patients were included in the present study. The study was approved by the Ethics Committee of the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. Informed consent was obtained from each patient.

Definition of OS and DFS. OS time was calculated from the date of initial surgery to the date mortality from OSCC. If the patient did not succumb to OSCC, the end point was defined as mortality from any cause or the last date of follow-up. DFS was defined as the time from the date of initial surgery to the date of local or distant progression. If progression did not occur, the end point was defined as mortality from any cause or as the fifth year after surgery.

Immunohistochemical analysis. Sections (4 μ m) were cut from formalin-fixed (Mingsheng Disinfectant Co., Ltd., Chengdu, China) and paraffin-embedded (Shanghai Hualing Recovery Appliance Factory, Shanghai, China) tissues blocks of OSCC, and placed on positively charged glass slides (Citotest Labware Manufacturing Co., Ltd., Haimen, China). Following paraffin removal with xylene (Sangon Biotech Co., Ltd., Shanghai, China) and dehydration with ethanol absolute (Ling Feng Chemical Reagent Co., Ltd., Shanghai, China), the slides were steamed with 10 mmol/l citrate buffer (pH 6.0; DakoCytomation, Carpinteria, CA, USA) for 20 min for antigen retrieval. After cooling to room temperature, the sections were incubated overnight at 4°C (15 h) with primary mouse monoclonal anti-CD73 antibody (1:50 dilution; cat no. sc-32299; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After several washes with phosphate-buffered saline, the sections were incubated with horseradish peroxidase-labeled goat anti-mouse or anti-rabbit secondary antibody (cat no. GK500705; Gene Tech, Shanghai, China) for 45 min at 37°C, prior to adding diaminobenzidine (Dako, Glostrup, Denmark) for 3.5 min at room temperature.

Using an Axio Scope.A1 microscope (Carl Zeiss AG, Oberkochen, Germany), CD73 staining was independently evaluated by an expert pathologist who was blinded to the clinical information. The evaluation was analyzed according to the intensity of staining and the percentage of stained cells. Scoring estimations were stratified into four categories, as follows: +++, 20-49% moderately to intensely stained cells or \geq 50% positively stained cells; ++, 10-19% intensively stained cells or 20-49% weakly stained cells; +, 10-19% weakly to moderately stained cells; \pm , <10% positively stained cells; and -, 0% positively stained cells. For statistical analysis, + and above were recorded as positive; - and \pm were ranked as negative (13).

Statistical analysis. Comparisons of variables between the groups were based on the χ^2 goodness-of-fit test or

Table I. Characteristics of the patients enrolled in this study (n=113).

Characteristic	Value
Age, years	
Median (range)	59.5 (26-83)
Gender, n (%)	
Male	71 (62.8)
Female	42 (37.2)
T stage, n (%)	
T1	44 (38.9)
T2	45 (39.8)
T3	15 (13.3)
T4	9 (8.0)
Clinical stage, n (%)	
I	23 (20.4)
II	23 (20.4)
III	31 (27.4)
IV	36 (31.8)
Differentiation, n (%)	
Well	24 (21.2)
Moderate	80 (70.8)
Poor	9 (8.0)
CD73 cytology, n (%)	
Positive	66 (58.4)
Negative	47 (41.6)
Metastatic lymph nodes, n (%)	
Positive	36 (31.9)
Negative	77 (68.1)

Fisher's exact test. DFS and OS times were estimated by the Kaplan-Meier life-table method. Univariate and multivariate parameters [gender, age, smoking, alcohol consumption, tumor stage (T stage) (14), lymph node metastasis, clinical stage (15), degree of differentiation] were analyzed with respect to DFS and OS using the Cox regression hazard model. Each experiment was repeated 3 times. All analyses were performed using SPSS software (version 13; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CD73 expression in primary OSCCs and its correlation with clinicopathological characteristics. The characteristics of enrolled patients are indicated in Table I. Positive staining was noted in 58.4% (66/113) of OSCC samples. CD73 staining was predominantly localized in the cytomembrane with a small amount of staining in the cytoplasm. The OSCC samples showed marked overexpression of CD73 compared with matched adjacent noncancerous mucosa (Fig. 1). The association between CD73 expression and clinicopathological variables are indicated in Table II. Statistical analysis of the association between CD73 expression and clinicopathological

Table II. Association between clinicopathological characteristics and CD73 expression in 113 patients with oral squamous cell carcinoma.

Clinicopathological feature	No.	CD73 expression, %		χ^2	P-value
		Negative	Positive		
Gender				0.999	0.331
Male	71	27	44		
Female	42	20	22		
Age, years				2.004	0.184
<60	56	27	29		
≥60	57	20	37		
Smoking				0.065	0.844
Yes	40	16	24		
No	73	31	42		
Drinking				0.656	0.522
Yes	31	11	20		
No	82	36	46		
T stage				4.358	0.021
T3/T4	24	5	19		
T1/T2	89	42	47		
Lymph node metastasis				0.159	0.838
+	36	14	22		
-	77	33	44		
Clinical stage				3.941	0.047
III/IV	46	14	32		
I/II	67	33	34		
Histological type				0.778	0.378
Poor	9	5	4		
Well/moderate	104	42	62		

CD73, cluster of differentiation 73; T stage, tumor stage.

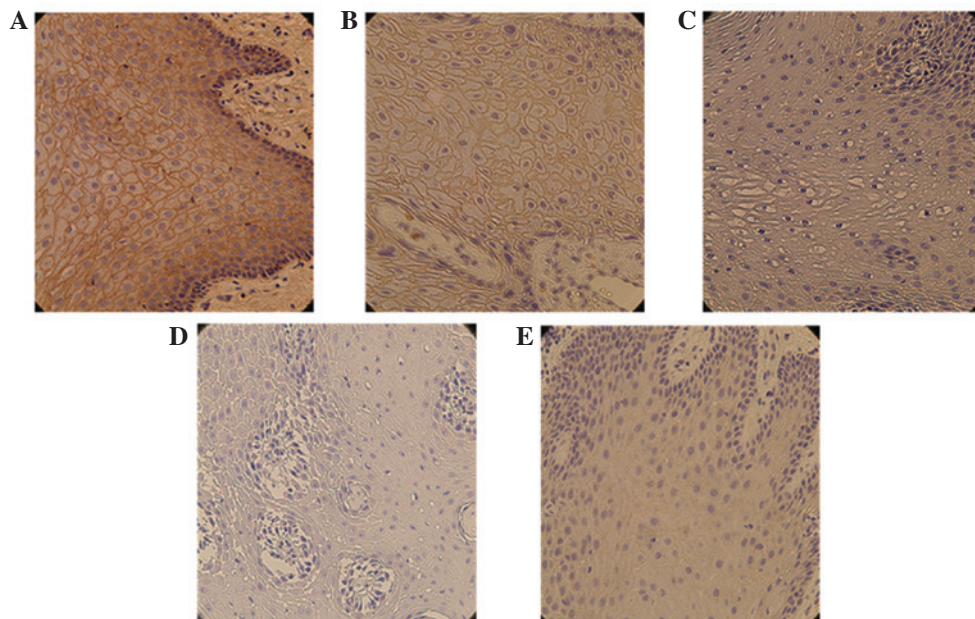


Figure 1. Association between CD73 expression in primary oral squamous cell carcinoma by immunohistochemical staining. (A) +++, (B) ++, (C) + and (D) - scoring in OSCC. (E) - scoring in adjacent non-cancerous mucosa. Magnification, x400.

Table III. Statistical analysis of clinicopathological variables associated with DFS and OS in patients with oral squamous cell carcinoma using the univariate Cox proportional hazards model.

Variable	DFS			OS		
	HR	95% CI	P-value	HR	95% CI	P-value
CD73 expression			0.003			0.003
Positive vs. negative	2.926	1.447-5.917		2.936	1.452-5.936	
Gender			0.584			0.567
Male vs. female	1.189	0.640-2.210		1.199	0.645-2.228	
Age, years			0.060			0.057
<60 vs. ≥60	1.027	0.999-1.055		1.027	0.999-1.056	
Smoking			0.751			0.751
Yes vs. no	0.904	0.487-1.681		0.904	0.487-1.681	
Drinking			0.905			0.913
Yes vs. no	0.961	0.496-1.860		0.964	0.498-1.866	
T stage			0.000			0.000
T3/T4 vs. T1/T2	1.797	1.342-2.408		1.816	1.356-2.432	
Lymph node metastasis			0.000			0.000
+ vs. -	1.897	1.344-2.687		1.915	1.358-2.701	
Clinical stage			0.000			0.000
III/IV vs. I/II	1.677	1.282-2.194		1.698	1.298-2.220	
Histological type			0.008			0.009
Poor vs. well-moderate	2.174	1.227-3.853		2.134	1.213-3.754	

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; CD73, cluster of differentiation 73; T stage, tumor stage.

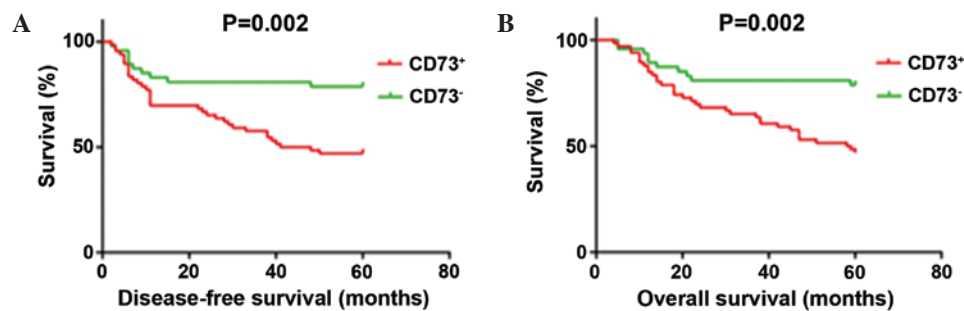


Figure 2. Survival analysis according to the expression status of CD73 for patients with oral squamous cell carcinoma. Kaplan-Meier survival curves for (A) disease-free survival and (B) overall survival in the study cohort (n=113). CD73, cluster of differentiation 73.

characteristics identified that CD73 has a direct association with T stage ($P=0.021$) and clinical stage ($P=0.047$). CD73 expression was not significantly associated with other characteristics.

Association between CD73 expression, and DFS and OS times among patients with OSCC. Using follow-up data from the 113 patients with OSCC, the present study analyzed whether CD73 expression affected DFS and OS. Kaplan-Meier survival curves showed that CD73-positive expression cases had significantly poorer DFS ($P=0.002$) and OS ($P=0.002$) times compared with CD73-negative cases. The 5-year DFS rate for positive CD73 expression was 47.0 compared with

78.7% for patients that were negative for CD73; similarly, the 5-year OS rates for CD73-positive and -negative expression were 50.0 vs. 78.7%, respectively (Fig. 2). After patients were stratified by T stage, cases with positive CD73 expression showed significantly poorer DFS and OS compared with CD73-negative cases among those classified as T1/T2 or clinical stage I/II. This trend was not observed in T3/T4 and clinical stage III/IV patients with OSCC (Fig. 3).

CD73 is an independent poor prognostic marker for DFS and OS times among patients with OSCC. Univariate Cox regression analysis showed that CD73 expression was significantly associated with poor DFS (HR, 2.926; 95% CI, 1.447-5.917;

Table IV. Statistical analysis of clinicopathological variables associated with DFS and OS in patients with oral squamous cell carcinoma using the multivariate Cox proportional hazards model.

Variable	DFS			OS		
	HR	95% CI	P-value	HR	95% CI	P-value
CD73 expression			0.018			0.021
Positive vs. negative	2.417	1.162-5.028		2.355	1.137-4.878	
Gender			0.947			0.897
Male vs. female	1.025	0.486-2.165		1.050	0.498-2.214	
Age, years			0.087			0.070
<60 vs. ≥60	1.028	0.996-1.061		1.030	0.998-1.063	
Smoking			0.757			0.715
Yes vs. no	0.852	0.308-2.353		0.823	0.289-2.343	
Drinking			0.681			0.715
Yes vs. no	0.795	0.267-2.369		0.810	0.262-2.505	
T stage			0.002			0.003
T3/T4 vs. T1/T2	2.735	1.428-5.236		2.598	1.369-4.932	
Lymph node metastasis			0.002			0.003
+ vs. -	2.775	1.443-5.337		2.633	1.376-5.039	
Clinical stage			0.040			0.060
I/II vs. III/IV	0.464	0.223-0.964		0.500	0.243-1.030	
Histological type			0.025			0.045
Poor vs. well/moderate	2.569	1.127-5.857		2.269	1.019-5.049	

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; CD73, cluster of differentiation 73; T stage, tumor stage.

P=0.003) and OS (HR, 2.936; 95% CI, 1.452-5.936; P=0.003) times of patients with OSCC (Table III). Multivariate analysis using the Cox regression hazard model confirmed that CD73 expression was an independent prognostic factor for poor DFS (HR, 2.417; 95% CI, 1.162-5.028; P=0.018) and OS (HR, 2.355; 95% CI, 1.137-4.878; P=0.021; Table IV) among patients with OSCC. Multivariate analysis also indicated that T stage, clinical stage, degree of differentiation and lymph node metastasis have independent prognostic value in OSCC (Table IV).

Discussion

To the best of our knowledge, the current study is the first to evaluate the expression status of CD73 in patients with OSCC. The associations between CD73 expression and clinicopathological characteristics were evaluated in patients with OSCC. The findings indicated that positive CD73 expression may be a novel prognosticator of adverse clinical outcome in patients with OSCC. In addition, the present results identified that CD73 expression status was statistically associated with the T stage, clinical stage, degree of differentiation and lymph node metastasis. According to these findings, we propose that CD73 may be a novel molecular prognostic marker in the evaluation of OSCC patient survival. The current findings may be the beginning of a new era of research into the role of CD73 in SCC.

Overexpression of CD73 has been observed in broad types of cancer (16) and a growing body of literature has revealed the

function of CD73 in cancer progression. For example, overexpression of CD73 was significantly associated with a worse prognosis in patients with triple negative breast cancer (17). By contrast, a number of retrospective studies reported that overexpression of CD73 was strongly correlated with improved clinical outcome in patients with breast cancer (18,19). Furthermore, Lu *et al* (20) investigated the expression status of CD73 in gastric cancer, and showed that CD73 expression was positively associated with cancer stage, depth of invasion and metastasis, with low OS observed in the patients with overexpression of CD73. In addition, numerous studies have revealed that high levels of CD73 are statistically associated with a poor prognosis in colorectal cancer and gallbladder cancer (21,22). Zhao *et al* (23) reported the CD73 status in various leukemia subtypes, and found the CD73 status was correlated with leukemia subtype and differentiation. In addition, a retrospective study reported that CD73 overexpression was positively correlated with lymph node metastasis in prostate cancer, and was more frequently observed in epithelial ovarian cancer patients with better prognosis, lower stage and better differentiation (19,24). Similar results were observed in malignant melanoma (25). Taken together, the aforementioned findings indicate that CD73 is a significant molecular prognosticator in various types of cancer. However, there is almost no information regarding CD73 expression in the tumor cells of patients with SCC. Therefore, the current study aimed to conduct research in this field.

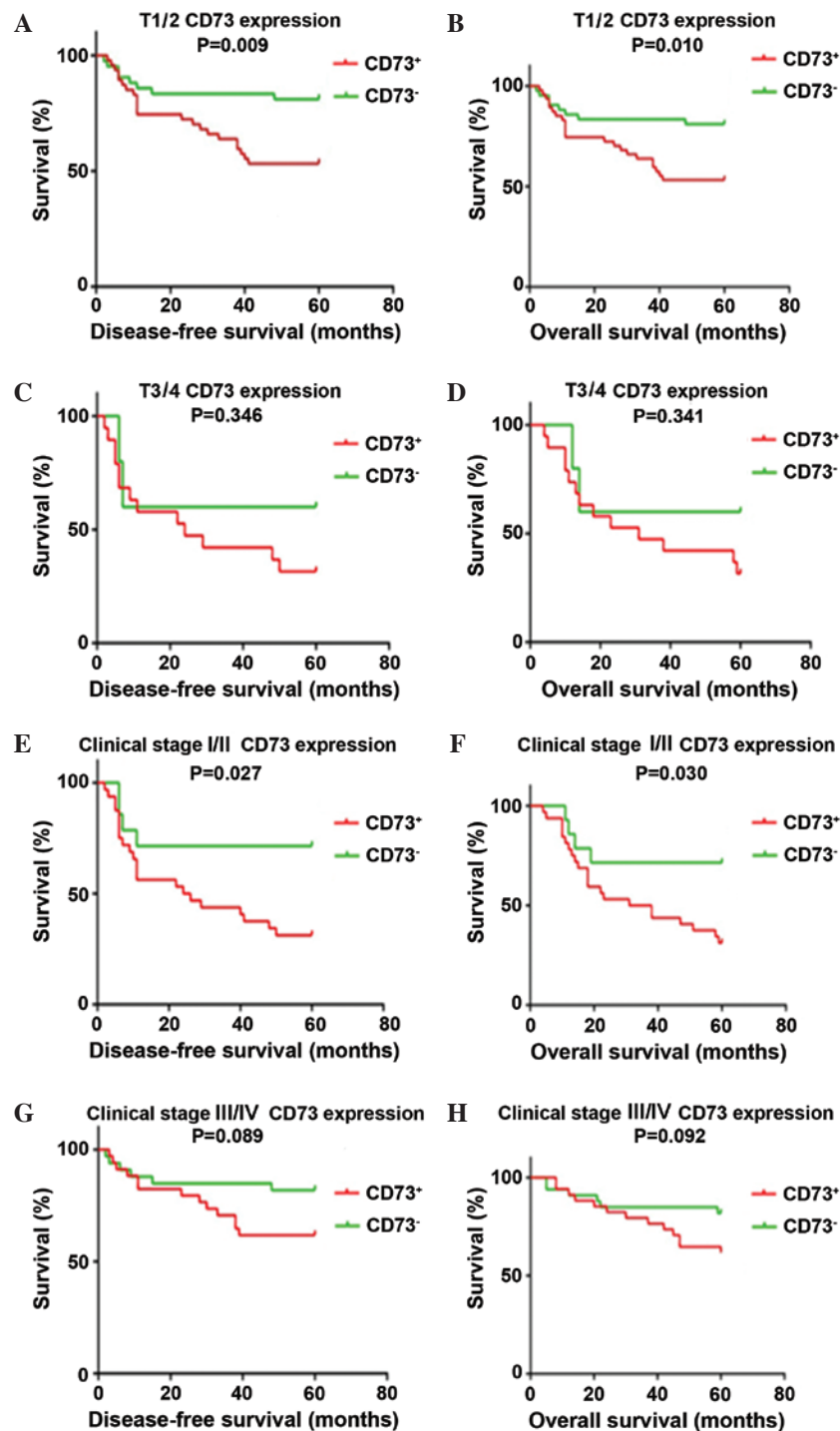


Figure 3. Kaplan-Meier survival curves showing disease-free survival (DFS) and overall survival (OS) in patients with oral squamous cell carcinoma (OSCC) according to T stage or clinical stage. CD73 expression is associated with (A) DFS or (B) OS among OSCC patients with T1/T2 tumors but is not significantly associated with (C) DFS and (D) OS among patients with T3/T4 tumors. CD73 expression is not associated with (E) DFS or (F) OS among OSCC patients in clinical stage I-II but is not significantly associated with DFS (G) and OS (H) among patients in clinical stage III-IV. T stage, tumor stage; CD73, cluster of differentiation 73.

T stage and clinical stage are the most important prognostic markers in the evaluation of OSCC patient survival (26,27). The current findings confirmed the validity of the T stage and clinical stage at the molecular level. Furthermore, CD73 may be a crucial molecular prognostic marker in early T stage (T1/T2) or early clinical stage (I/II) OSCC.

The limitations of the present study include the retrospective nature of the study and the limited number of enrolled

patients. The findings of the study should be replicated in a randomized prospective study that includes a large sample size. Additional molecular studies are required to confirm whether CD73 has an important role in OSCC progression. The current authors are currently performing a further molecular study to confirm whether CD73 has an important role in OSCC.

In conclusion, the results of the present study revealed upregulation of CD73 in clinical OSCC tissues. In addition, it

was identified that the grading of CD73 expression by immunohistochemical staining was able to significantly predict OSCC prognosis in a multivariate analysis. Furthermore, the CD73 expression status was significantly associated with T stage and clinical stage, and overexpression of CD73 was inversely correlated with the DFS and OS times of patients with OSCC. Thus, the results of the present study consistently suggest that CD73 is likely to be a novel prognostic marker of OSCC and holds promise for future tailored treatment strategies.

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