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RESEARCH

Overexpression of Autophagy-Related 16-Like 1 in Patients with Oral Squamous Cell Carcinoma

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Abstract Dyregulation of autophagy has been reported in various human cancers including oral squamous cell carcinoma (OSCC). The objective of this study was to link expression of autophagy-related 16-like 1 (ATG16L1), a protein essential for autophagosome formation, to clinical outcome in a cohort of 90 OSCC patients. Expression level of ATG16L1 was assessed by immunohistochemistry and an immunoreactivity score (IRS), ranging from 0 to 9, was assigned to each case. The results were correlated with clinicopathological parameters and outcome of patients. Twenty-seven patients (30 %) exhibited ATG16L1 overexpression as indicated by an IRS of 9. Overexpression of ATG16L1 was significantly associated with disease stage (p=0.001), size (p=0.031) of the tumor, lymph node metastasis (p=0.004), and histological grade (p=0.038). ATG16L1 overexpression significantly affected the overall survival (p=0.020) and time to recurrence (p=0.031) of OSCC patients in Kaplan-Meier analysis. The present study suggested that ATG16L1 may be used as a biomarker for selecting OSCC patients with a more aggressive phenotype.

Keywords Autophagy · Oral cancer · Immunohistochemistry · Autophagy-related 16-like 1

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Abbreviations

OSCC	Oral squamous cell carcinoma
ATG16L1	Autophagy-related 16-like 1
IRS	Immunoreactivity score
ROC	Receiver operating characteristics
OS	Overall survival
TTR	Time to recurrence

Introduction

Oral cancer is a major global public health problem. In 2008, it is estimated that 263,900 new cases and 128,000 deaths (from oral cancer) were reported worldwide [1]. Oral cancer also has one of the lowest 5-years survival rates of all major cancers, in large part due to the lack of effective treatment and failure to diagnose many lesions in the initial

stages. The high frequency of oral cancer can be attributed to ethnic and geographic factors, and the popularity of addictive habits. Although several factors like tobacco, alcohol, betel consumption, and genetic predisposition have been identified to be possible risk factors [2–7], the cause of oral cancer remains unclear.

Autophagy is an intracellular degradation system carried out by eukaryotic cells for the disposition of old components. In this process, cytoplasmic constituents are sequestered into double-membraned autophagosomes, and subsequently delivered to the lysosome for degradation and recycling [8]. This tightly regulated catabolic process is essential for cell growth, development, and homeostasis [9]. Autophagy is dysregulated in a wide spectrum of human cancers. The role of autophagic degradation in cancer cells is not unique as it may promote or suppress tumorigenesis [10].

Autophagy-related 16-like 1 (ATG16L1) is a component of a large protein complex (APG12-APG5-APG16L) essential for the formation of autophagosomes [11]. To date, the significance of ATG16L in cancer including oral squamous cell carcinoma (OSCC) has not been completed elucidated. We therefore investigated the expression of ATG16L in tumor tissues derived from patients with OSCC and correlated the findings with clinical and pathological characteristics of patients.

Material and Methods

Specimens

Ninety consecutive patients who presented at the Department of Oral and Maxillofacial Surgery, Kaohsiung Medical University Hospital between 2005 and 2009 with histologically confirmed previously untreated primary OSCC were included. Tumor samples were fixed in formalin, embedded in paraffin and stored in the archives of the Department of Pathology, Kaohsiung Medical University Hospital. Clinical data and follow-up were available for all patients. This study was approved by the Kaohsiung Medical University Institutional Review Board and all patients gave written informed consent.

Immunohistochemistry

Tumor tissue blocks were cut into 4-μm sections, deparaffinized, and rehydrated in xylene and ethanol. Slides were then heated in 0.1 M citrate buffer (pH 6.0) for 10 min for antigen retrieval. Endogenous peroxidase activities were inactivated in 3 % hydrogen peroxide 10 min at room temperature. Tissues were incubated in the rabbit polyclonal ATG16L1 antibody (Novus Biologicals, Littleton, CO), at a dilution of 1:250 for 30 min at room temperature. Immunostaining was performed using the DAKO REAL Envision Detection System, Peroxidase/DAB, Rabbit/Mouse (DAKO, Denmark).

An immunoreactivity score (IRS), ranging from 0 to 9, was assigned to each slide. IRS was obtained by multiplying the intensity and percentage scores. In terms of intensity, negative cytoplasmic staining was scored as 0, weakly positive as 1, moderately positive as 2, and strongly positive as 3. For the percentage score, no immunoreactivity was scored as 0, 1–10 % as 1, 11–50 % as 2 and >50 % as 3. Human cervical adenocarcinoma cells were used as positive control for immunohistochemical stains. Negative control was obtained by replacing the primary antibody with non-immune serum.

Statistical Analysis

Receiver operating characteristics (ROC) curve analysis was used to calculate the expression cut-off value predicting survival for ATG16L1. Evaluation of association between ATG16L1 and clinicopathological variables was assessed by the chi-square test and Fisher's exact test where appropriate. Overall survival (OS) was defined as the period from initial diagnosis to date of death or last follow-up. Time to recurrence (TTR) was defined as the length of time from initial diagnosis to disease recurrence. Patients without evidence of recurrence were censored at last followed-up. Survival curves were drawn according to Kaplan-Meier method and compared with the log-rank test. The Cox proportional hazard model was used for multivariate analysis, with tumor grade, size, and lymph node metastasis as categorical variables. Tests were considered significant when p values were$\leq$0.05.

Results

Distribution of the intensity and percentage scores of ATG16L1 immunohistochemical staining of the 90 OSCC samples is shown in Table 1. A total score was obtained by multiplying the percentage and intensity scores for each sample. An IRS of 9 was determined by ROC curve analysis to be the cutoff value for ATG16L1 expression. Overexpression of ATG16L1 was thus defined as an IRS of 9. The results showed

Table 1 Distribution of intensity and percentage scores of ATG16L1 immunohistochemical staining of the 90 oral squamous cell carcinoma samples

		Percentage score			N
		1	2	3	
Intensity score	1	0	7	7	14
	2	0	18	23	41
	3	1	7	27	35
N		1	32	57	90

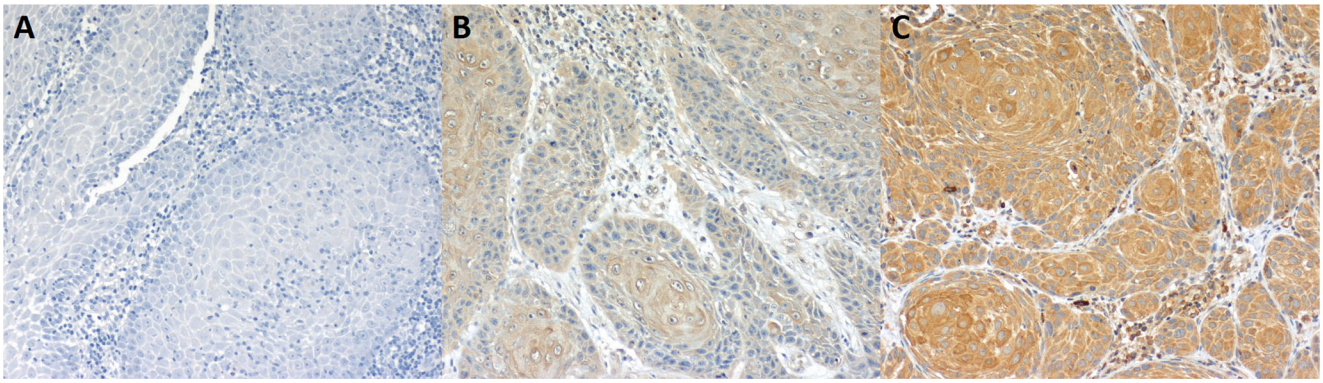

Fig. 1 Representative immunostaining of ATG16L1 protein on human oral squamous cell carcinomas (magnification ×200). Samples were scored as described in Materials and methods and indicated by IRS. **a** IRS=0; **b** IRS=4; **c** IRS=9 (overexpression)

that ATG16L1 was overexpressed in 30 % (n=27) of the 90 OSCC samples tested. Representative immunostaining are shown in Fig. 1. Table 2 depicts the relationship between ATG16L1 expression and clinicopathologic factors in OSCC as determined by categorical analyses. Overexpression of ATG16L1 was significantly associated with stage (p=0.001), size (p=0.031), and grade (p=0.038) of the tumor. ATG16L1 was also significantly overexpressed in OSCCs with lymph node metastasis (p=0.004). No significant link was established between the expression of ATG16L1 and factors such as age, tobacco, alcohol, and betel consumption. The Kaplan-Meier plots showed that ATG16L1 overexpression, in contrast to without ATG16L1 overexpression, had a negative impact on OS (p=0.020) (Fig. 2a). This was also confirmed for the TTR

Table 2 Association of ATG16L1 expression with clinicopathologic variables in 90 oral squamous cell carcinoma patients

	ATG16L1 overexpression				n	p-value
	No (n=63)		Yes (n=27)			
Age, years, mean (SD)	56.1	(11.0)	54.6	(11.3)	90	0.553
Alcohol						
Yes	46	(73.0 %)	20	(74.1 %)	66	0.917
No	17	(27.0 %)	7	(25.9 %)	24	
Smoker						
Yes	53	(84.1 %)	22	(81.5 %)	75	0.758
No	10	(15.9 %)	5	(18.5 %)	15	
Betel chewer						
Yes	58	(92.1 %)	24	(88.9 %)	82	0.692
No	5	(7.9 %)	3	(11.1 %)	8	
Tumor stage						
I	13	(20.6 %)	3	(11.1 %)	16	0.001
II	21	(33.3 %)	2	(7.4 %)	23	
III	17	(27.0 %)	6	(22.2 %)	23	
IV	12	(19.1 %)	16	(59.3 %)	28	
Tumor size						
≤20 mm	14	(22.2 %)	4	(14.8 %)	18	0.031
>20 mm, ≤40 mm	28	(44.4 %)	6	(22.2 %)	34	
>40 mm	21	(33.3 %)	17	(63.0 %)	38	
Lymph node metastasis						
–	35	(55.6 %)	6	(22.2 %)	41	0.004
+	28	(44.4 %)	21	(77.8 %)	49	
Tumor grade						
I	41	(71.9 %)	11	(44.0 %)	52	0.038
II	15	(26.3 %)	13	(52.0 %)	28	
III	1	(1.8 %)	1	(4.0 %)	2	

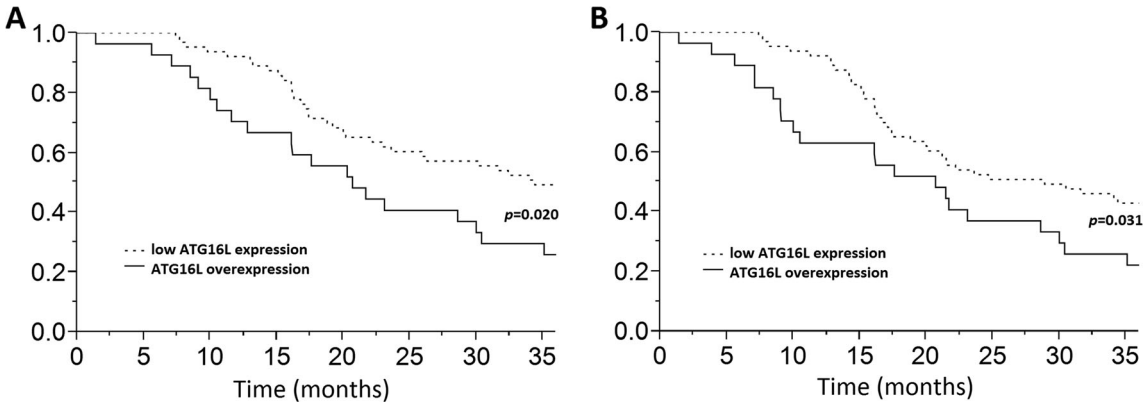

Fig. 2 Overall survival (**a**) and time to recurrence (**b**) plots by the Kaplan-Meier method. Oral squamous cell carcinoma patients with ATG16L1 overexpression had worse outcomes

in which ATG16L1 overexpression was associated with shorter TTR (p=0.031) (Fig. 2b). In the univariate Cox regression analysis, ATG16L1 overexpression (p=0.027), tumor grade≥ II (p<0.001), tumor size>40 mm (p<0.001), and lymph node metastasis (p<0.005) all had a statistically significant association with OS. However, in the subsequent multivariate analysis, only tumor size retained significant association with OS (p=0.007) (Table 3).

Discussion

Cancer of the oral cavity is one of the most devastating and disfiguring of all malignancies and is a major health issue, especially in the developing countries. The pathogenesis of oral cancer is complex and multifactorial. Previous reports have suggested that dysregulation of autophagy, which alters the metabolic and degradation process of the cells, have profound consequences and is associated with many types of human cancer, including OSCC [10, 12–15].

Altered expression of ATGs has been reported in various human malignancies [16]. The dysregulation of ATG proteins have also been shown to correlate with clinicopathological variables and disease outcomes in cancer patients. These findings highlighted the pivotal role of autophagy in tumorigenesis.

ATG16L1 is one of the least characterized ATG proteins in the context of human carcinogenesis. We report here overexpression of ATG16L1 protein in 30 % (27/90) of human OSCC cases tested. In categorical tests, ATG16L1 overexpression was significantly linked to disease stage, tumor size, lymph node metastasis, and histological grade. The Kaplan-Meier method also showed that ATG16L1 overexpression was associated with poor outcome in terms of shorter OS and TTR. To further determine whether ATG16L1 overexpression was associated with survival in patients with OSCC, we performed Cox regression analysis that included tumor grade, size, and lymph node metastasis as covariates. The ATG16L1 overexpression phenotype and all these covariates were all significantly associated with shorter OS in the univariate anlysis. In the multivariate analysis, only tumor size was indicated to be an independent prognostic variable for OS. ATG16L1 overexpression did not retain statistical significance, possibly due to the limited number of cases and/or its close association with other covariates.

The findings of the current study are in agreement with those of Nomura et al. who showed that ATG16L1 overexpression was linked to lymph node metastasis in patients with OSCC [12]. This study further illustrated the role that ATG16L1 overexpression has in the pathogenesis of OSCC by negatively impacting the survival and recurrence of patients of OSCC. The most important limitation of the present study lies in the fact that the number of patients was limited. Further work with larger sample sizes needs to be done to achieve more statistical power and provide more definitive evidence regarding the impact of ATG16L1 overexpression on the prognosis of patients with OSCC.

Table 3 Cox regression analysis of overall survival in patients with oral squamous cell carcinoma

	Univariate			Multivariate		
	HR	95 % CI	p	HR	95 % CI	p
ATG16L1 overexpression	1.92	1.08–3.33	0.027	1.47	0.82–2.60	0.195
Tumor grade ≥ II	2.97	1.65–5.36	< 0.001	0.99	0.97–1.02	0.685
Tumor > 40 mm	3.34	1.92–5.94	< 0.001	2.45	1.28–4.81	0.007
Lymph node metastasis	2.21	1.26–4.00	0.00	1.73	0.87–3.59	0.119

Taken together, the present study provided additional evidence with respect to dysregulation of autophagy in human OSCC. These data suggested that ATG16L1 overexpression may have prognostic significance in human OSCC and raised the need to exploit autophagy in the target treatment of human OSCC.

Conflict of Interest We declare that we have no conflict of interest.

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