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ORIGINAL ARTICLE

ATG9A overexpression is associated with disease recurrence and poor survival in patients with oral squamous cell carcinoma

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Abstract ATG9A is an integral membrane protein required for autophagosome formation and a membrane carrier in the autophagy pathways. The present study was designed to investigate the expression of ATG9A in oral squamous cell carcinoma (OSCC). Clinically annotated tumor specimens from 90 patients with OSCC were subjected to immunohistochemistry using an antibody against ATG9A and immunoreactivity was scored using an immunoreactivity score (IRS). Scores were compared with clinical and pathologic data to assess association with outcome. Overexpression of ATG9A was defined as an IRS of ≥9 by receiver operating characteristics curve analysis and was identified in 25 (28 %) of 90 cases. ATG9A overexpression was associated with disease recurrence and overall survival (OS) in both univariate ($p=0.030$ and 0.025, respectively) and multivariate ($p=0.026$ and 0.038, respectively) Cox analyses. Kaplan–Meier plots also showed that patients with ATG9A overexpression had shorter 3-year OS ($p=0.017$) and time to recurrence ($p=0.021$) than those with low ATG9A expression. These results suggest that the presence of ATG9A in the cytoplasm of tumor cells may be an independent biomarker for disease recurrence and survival in patients with OSCC.

Keywords Autophagy · Oral cancer · Immunohistochemistry · ATG9A

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Introduction

Oral squamous cell carcinoma (OSCC), the most common malignancy of the oral cavity and the head and neck, is a major public health issue in Taiwan and Southeast Asia. Modern management of OSCC including chemotherapy and radiotherapy has produced very limited survival benefit. In fact, the 5-year survival rate of patients with OSCC has remained unchanged for the past few decades [1, 2]. This highlights the need for the identification of biomarkers in OSCC that can help in early disease diagnosis, improve the prognosis, and/or aid in choosing optimal therapy.

Autophagy is a eukaryotic degradative mechanism which maintains cellular homeostasis in environmental stress [3]. It is generally activated by metabolic stresses including hypoxia, nutrient deprivation, and an increase in proliferation [4]. During this process, bulk cytoplasm is sequestered within double-membrane vesicles called autophagosomes and delivered to the lysosome for subsequent degradation and recycling [5]. ATG9 is an integral membrane protein localized in the phagophore/pre-autophagosomal structure (PAS), the origin of the autophagosomal membranes [6–8]. ATG9 is required for both the formation and the expansion of the autophagosomes [9, 10].

Autophagy is associated with cancer, both in suppressing tumor growth as well as potentially contributing to the survival of cancer cells [11–13]. The complex role of autophagy and its regulation in cancer cells continue to emerge, it is clear that autophagy plays a role in cancer cell metabolism and in cell-death pathways in cancer cells [14]. Expression of the ATG9A protein has not been studied in human cancers including OSCC. The aims of the present study were to examine the expression of ATG9A in a cohort of patients with primary OSCCs by immunohistochemistry and to determine its clinical relevance.

Materials and methods

Patients and specimens

We identified 90 consecutive patients with newly diagnosed OSCC who had undergone surgical resections between 2005 and 2009 at Kaohsiung Medical University Hospital, Kaohsiung, Taiwan. Patients had provided written informed consent and given permission to use clinical data and their unused stored tumor specimens for research. Clinical data including date of diagnosis and recurrence, age at diagnosis, TNM stage, survival time, tumor grade, tobacco, as well as betel and alcohol consumption were collected with identifiers removed in accordance with a protocol approved by the Kaohsiung Medical University Hospital Institutional Review Board.

Immunohistochemistry

Immunohistochemical staining was carried out on 4-μm paraffin OSCC tissue sections. Briefly, sections soaked in 0.1 M

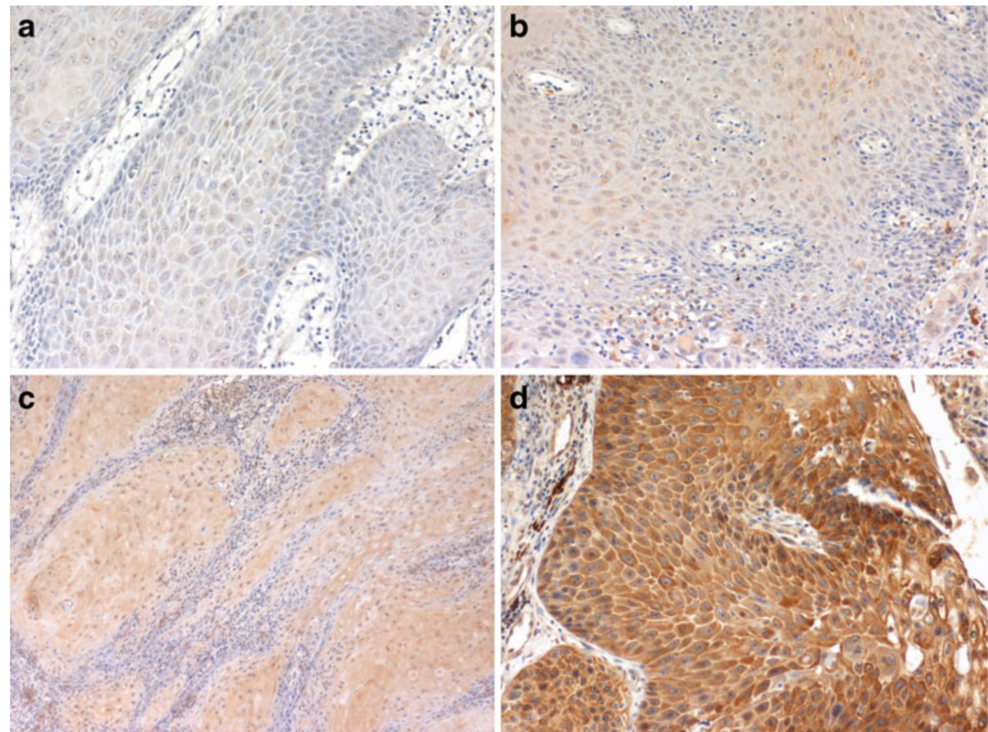

Fig. 1 Representative images of expression of ATG9A protein by immunohistochemical staining in primary OSCCs. **a** Low ATG9A expression (IRS=2). **b** Low ATG9A expression (IRS=4). **c** Low ATG9A expression (IRS=6). **d** ATG9A overexpression (IRS=9)

citrate buffer (pH 6.0) were placed in an oven at 121 °C for 10 min after deparaffinization and hydration. Endogenous peroxidase was then blocked using 3 % hydrogen peroxide in methanol at room temperature. After washing with Tris buffer solution (TBS), the sections were incubated with primary antibody against ATG9A (1:400 dilution; Novus Biologicals, Littleton, CO). DAKO REAL Envision Detection System (DAKO, Denmark) was used with visualization of peroxidase reactivity using 3′,3-diaminobenzidine. Finally, the slides were counterstained with hematoxylin. Positive controls consisted of human placenta tissue and negative controls consisted of replacement of the primary antibody with non-immune serum. ATG9A reactivity was evaluated using an immunoreactivity score (IRS), which takes into account both the percentage of positive cells and the intensity of immunoreactivity at high magnification. For each slide, randomly selected five non-overlapping fields were counted at 200× until a total count of 1,000 cells was reached. The percentage of tumor cells showing positive cytoplasmic staining was recorded as follows: 0=staining in <1 %; 1=staining in 1–10 %; 2=staining in 11–50 %; and 3=staining in >50 % of tumor cells. The intensity was estimated as 0, 1, 2, and 3 for negative, weak, moderate, and strong reactivity, respectively. We then multiplied the two scores to obtain an IRS value.

Table 1 Correlation of ATG9A expression with clinical and pathologic parameters in OSCC

Parameters	ATG9A overexpression		*N*	*p* value
	No (*N*=65)	Yes (*N*=25)		
Age, years, mean (SD)	54.9 (10.7)	55.5 (12.4)	90	0.816
Sex				
Male	61 (93.9 %)	23 (92.0 %)	84	0.668
Female	4 (6.2 %)	2 (8.0 %)	6	
Alcohol				
Yes	50 (76.9 %)	16 (64.0 %)	66	0.214
No	15 (23.1 %)	9 (36.0 %)	24	
Tobacco				
Yes	55 (84.6 %)	20 (80.0 %)	75	0.599
No	10 (15.4 %)	5 (20.0 %)	15	
Betel				
Yes	60 (92.3 %)	22 (88.0 %)	82	0.680
No	5 (7.7 %)	3 (12.0 %)	8	
Grade				
I	42 (70.0 %)	10 (45.5 %)	52	0.060
II	16 (26.7 %)	12 (54.6 %)	28	
III	2 (3.3 %)	0 (0.0 %)	2	
Stage				
I	15 (23.1 %)	1 (4.0 %)	16	0.002
II	21 (32.3 %)	2 (8.0 %)	23	
III	14 (21.5 %)	9 (36.0 %)	23	
IV	15 (23.1 %)	13 (52.0 %)	28	
T-primary tumour				
≤20 mm	15 (23.1 %)	3 (12.0 %)	18	0.041
>20 mm, ≤40 mm	28 (43.1 %)	6 (24.0 %)	34	
>40 mm	22 (33.9 %)	16 (64.0 %)	38	
N-regional lymph node				
N0	36 (55.4 %)	5 (20.0 %)	41	0.002
N1	18 (27.7 %)	17 (68.0 %)	35	
N2	11 (16.9 %)	3 (12.0 %)	14	

Statistical analysis

Scoring of immunoreactivity was compared with clinical data to assess correlation with clinical outcome. Receiver operating characteristics (ROC) curve analysis was first used to obtain an expression cut-off IRS value predicting survival for ATG9A. Time to recurrence (TTR) was defined as the time from diagnosis to the time of first recurrence. Patients without evidence of recurrence were censored at last followed-up.

Table 2 Univariate and multivariate Cox regression analysis of disease recurrence in relation to clinical and pathologic features

	Univariate			Multivariate		
	HR	95 % CI	*p* value	HR	95 % CI	*p* value
ATG9A overexpression	1.90	1.07–3.26	0.030	2.13	1.10–4.07	0.026
	1			1		
Sex						
Female	1			1		
Male	1.37	0.51–5.64	0.575	1.66	0.50–8.10	0.444
Age	1.00	0.97–1.03	0.990	1	0.97–1.03	0.978
T-primary tumour						
≤20 mm	1			1		
>20 mm, ≤40 mm	1.23	0.55–3.00	0.621	1.18	0.50–3.02	0.714
>40 mm	2.91	1.39–6.82	0.004	2.16	0.87–5.70	0.098
N-regional lymph node						
N0	1			1		
N1	1.71	0.94–3.13	0.076	1.17	0.57–2.45	0.671
N2	3.47	1.67–6.93	0.001	2.32	0.91–5.96	0.077
Alcohol						
No	1			1		
Yes	1.04	0.59–1.94	0.893	0.97	0.48–2.04	0.934
Tobacco						
No	1			1		
Yes	1.26	0.63–2.88	0.532	1.69	0.66–5.13	0.288
Betel						
No	1			1		
Yes	0.79	0.35–2.28	0.633	0.41	0.12–1.64	0.197

Table 3 Univariate and multivariate Cox regression analysis of overall survival in relation to clinical and pathologic features

	Univariate			Multivariate		
	HR	95 % CI	*p* value	HR	95 % CI	*p* value
ATG9A overexpression	1.98	1.09–3.47	0.025	2.05	1.04–3.94	0.038
	1			1		
Sex						
Female	1			1		
Male	1.21	0.44–4.96	0.747	1.62	0.48–8.03	0.470
Age	1.01	0.98–1.04	0.430	1.00	0.97–1.04	0.779
T-primary tumour						
≤20 mm	1			1		
>20 mm, ≤40 mm	1.40	0.57–3.93	0.477	1.33	0.51–3.94	0.570
>40 mm	4.21	1.87–11.25	0.0002	3.04	1.16–8.91	0.023
N-regional lymph node						
N0	1			1		
N1	1.83	0.98–3.46	0.056	1.27	0.62–2.67	0.510
N2	3.59	1.68–7.40	0.001	2.41	0.95–6.11	0.063
Alcohol						
No	1			1		
Yes	0.85	0.47–1.59	0.588	0.80	0.39–1.68	0.543
Tobacco						
No	1			1		
Yes	1.13	0.56–2.60	0.739	1.69	0.65–5.32	0.299
Betel						
No	1			1		
Yes	0.65	0.29–1.88	0.392	0.37	0.10–1.53	0.163

Overall survival (OS) was defined as the time from diagnosis to the time of death or last follow-up. The length of follow-up ranged from 2 to 95 months, with a mean of 36 months. Survival curves were compared using Kaplan–Meier models. The correlation of clinical and pathologic data with outcome was also determined using Cox models to control for known prognostic factors. A p value <0.05 was considered statistically significant.

Results

Ninety OSCC patients were included in the analysis. ATG9A expression in tumor cells assessed by immunohistochemistry was cytoplasmic. Figure 1 shows the representative ATG9A immunohistochemical staining patterns and IRS scores. Using an ROC curve analysis, an IRS of 9 was determined to be the cutoff value for ATG9A overexpression. Twenty-five cases (28 %) were classified into the ATG9A overexpression group. Pearson's χ^2 test revealed that ATG9A overexpression was significantly associated with larger tumor volume (p=0.041), lymph node metastasis (p=0.002), and more advanced stage (p=0.002) in OSCC (Table 1). To determine whether ATG9A overexpression was associated with OS and TTR in patients with OSCC, we performed Cox regression including sex, age, tumor size, regional lymph node metastasis, alcohol, tobacco, and betel consumption as covariates. In univariate analysis, ATG9A overexpression (p=0.030, hazard ratio [HR]=1.90, 95 % confidence interval [CI]=1.07–3.26), tumor size >40 mm (p=0.004, HR=2.91, 95 % CI=1.39–6.82) and regional lymph node N2 (p=0.001, HR=3.47, 95 % CI=1.67–6.93) were statistically significant associated with disease recurrence (Table 2). In the subsequent multivariate analysis, only ATG9A overexpression (p=0.026, HR=2.13, 95 % CI=1.10–4.07) remained as significant and independent predictive variable. Similarly, unadjusted Cox estimates for OS revealed that ATG9A overexpression (p=0.025, HR=1.98, 95 % CI=1.09–3.47), tumor size >40 mm (p=0.0002, HR=4.21, 95 % CI=1.87–11.25) and N2 (p=0.001, HR=3.59, 95 % CI=1.68–7.40) were predicted mortality (Table 3). After adjustment, the predictive value for OS of ATG9A overexpression (p=0.038, HR=2.05, 95 % CI=1.04–3.94) and tumor size >40 mm (p=0.023,

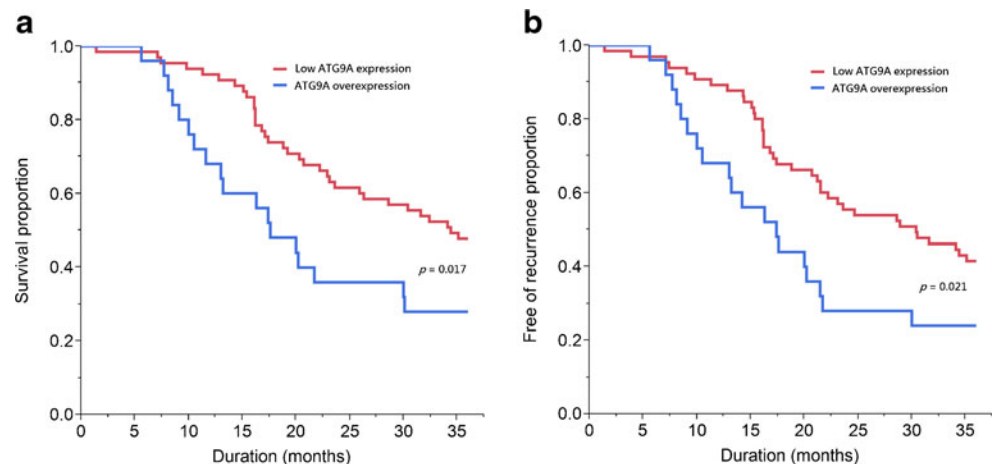

Fig. 2 Kaplan–Meier plots for time to recurrence (**a**) and overall survival (**b**) of OSCC patients with overexpression relative to low expression of ATG9A protein

HR=3.04, 95 % CI=1.16–8.91) maintained significance. Kaplan–Meier analysis demonstrated that patients with tumors overexpressing ATG9A had a significantly decreased 3-year OS and TTR (p=0.017 and 0.021, respectively; Fig. 2) compared with those with low ATG9A expression.

Discussion

Autophagy, a catabolic process of bulk cytoplasmic contents through an autophagosomic–lysosomal pathway, is involved in the preservation of cellular nutrients under stress or starvation conditions as well as the normal turnover of cytosolic components. Autophagy is important for cellular homeostasis and may be defective in tumor cells. The role of autophagy in cancer remains a topic of intense debate. Autophagy has been shown to act potently to both promote and inhibit carcinogenesis [14, 15]. The role of autophagy in the regulation of cancer development and progression in the context of human OSCC is not clear. In the present study, we examined the expression and clinical relevance of ATG9A, a multi-spanning membrane protein required for autophagosome formation in the autophagy pathways [9, 10, 16], in human OSCC.

Our results show that expression of ATG9A protein is increased in a subset of human OSCC. Among the clinical and pathological variables examined, a significant correlation was observed between ATG9A overexpression and larger tumor volume (p=0.041), regional lymph node involvement (p=0.002), and more advanced stage (p=0.002). Notably, 88 % (22/25) of this cohort of patients exhibiting the ATG9A overexpression phenotype were of stage III/IV OSCC. Because these characteristics are well-known important prognostic parameters in OSCC, it is reasonable to suggest that ATG9A overexpression, linked with elevated autophagy activity, contributes to tumor development in human OSCC. Overexpression of ATG9A, demonstrated by immunohistochemistry, is significantly correlated with poor 3-year OS in patients with OSCC. Moreover, patients with ATG9A overexpression had shorter TTR in the first 3 postoperative years compared with patients with low ATG9A expression. In multivariate regression analysis adjusted for sex, age, tumor size, lymph node metastasis, and known risk factors for oral cancer such as alcohol, tobacco, and betel use, ATG9A overexpression was identified as an independent prognostic factor for disease recurrence of OSCC and patient survival.

This is the first report describing the expression and clinical relevance of ATG9A in OSCC. ATG9A expression as determined by immunohistochemistry might have prognostic value in human OSCC. Stratification based on ATG9A protein expression level revealed that overexpression of this protein might be used as an indicator of poor outcome. Pharmacological inhibition of autophagy has shown to effectively enhance tumor cell death in preclinical models [17–19]. Previous studies also provided evidence that autophagy protects cancer cells from the pro-apoptotic effects of various cancer drugs and a rationale for the use of autophagy inhibitors in combination with therapies designed to induce apoptosis in human cancers [11, 20–22]. This system thus might represent a novel way to explore effective treatment modalities for OSCC.

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

References

1. Landis SH, Murray T, Bolden S, Wingo PA (1999) Cancer statistics, 1999. CA Cancer J Clin 49(8–31):31
2. Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. CA Cancer J Clin 55:74–108
3. Marino G, Lopez-Otin C (2004) Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. Cellular and molecular life sciences : CMLS 61:1439–1454
4. Levine B, Klionsky DJ (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell 6:463–477
5. Mizushima N (2007) Autophagy: process and function. Gene Dev 21:2861–2873
6. Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, Ohsumi Y (2001) The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. EMBO J 20:5971–5981
7. Krick R, Muehe Y, Prick T, Bremer S, Schlotterhose P, Eskelinen EL, Millen J, Goldfarb DS, Thumm M (2008) Piecemeal microautophagy of the nucleus requires the core macroautophagy genes. Mol Biol Cell 19:4492–4505
8. Sekito T, Kawamata T, Ichikawa R, Suzuki K, Ohsumi Y (2009) Atg17 recruits Atg9 to organize the pre-autophagosomal structure. Gene Cell: Devoted Mol Cell Mech 14:525–538
9. Young AR, Chan EY, Hu XW, Kochl R, Crawshaw SG, High S, Hailey DW, Lippincott-Schwartz J, Tooze SA (2006) Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. J Cell Sci 119:3888–3900
10. Yamada T, Carson AR, Caniggia I, Umebayashi K, Yoshimori T, Nakabayashi K, Scherer SW (2005) Endothelial nitric-oxide synthase antisense (NOS3AS) gene encodes an autophagy-related protein (APG9-like2) highly expressed in trophoblast. J Biol Chem 280: 18283–18290
11. Yang ZJ, Chee CE, Huang S, Sinicrope FA (2011) The role of autophagy in cancer: therapeutic implications. Mol Canc Therapeut 10:1533–1541
12. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gelinas C, Fan Y, Nelson DA, Jin S, White E (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Canc Cell 10:51–64
13. Gozuacik D, Kimchi A (2004) Autophagy as a cell death and tumor suppressor mechanism. Oncogene 23:2891–2906
14. Mathew R, Karantza-Wadsworth V, White E (2007) Role of autophagy in cancer. Nature reviews. Cancer 7:961–967
15. Kimmelman AC (2011) The dynamic nature of autophagy in cancer. Gene Dev 25:1999–2010
16. Webber JL, Young AR, Tooze SA (2007) Atg9 trafficking in mammalian cells. Autophagy 3:54–56

17. White E, DiPaola RS (2009) The double-edged sword of autophagy modulation in cancer. Clin Cancer Res: Off J Am Assoc Cancer Res 15:5308–5316
18. Katayama M, Kawaguchi T, Berger MS, Pieper RO (2007) DNA damaging agent-induced autophagy produces a cytoprotective adenosine triphosphate surge in malignant glioma cells. Cell Death Differ 14:548–558
19. Carew JS, Nawrocki ST, Kahue CN, Zhang H, Yang C, Chung L, Houghton JA, Huang P, Giles FJ, Cleveland JL (2007) Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. Blood 110:313–322
20. Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB (2007) Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Investig 117:326–336
21. Li X, Fan Z (2010) The epidermal growth factor receptor antibody cetuximab induces autophagy in cancer cells by downregulating HIF-1alpha and Bcl-2 and activating the beclin 1/hVps34 complex. Canc Res 70:5942–5952
22. Han J, Hou W, Goldstein LA, Lu C, Stolz DB, Yin XM, Rabinowich H (2008) Involvement of protective autophagy in TRAIL resistance of apoptosis-defective tumor cells. J Biol Chem 283:19665–19677