

# Expression of $\alpha$ B-crystallin and its potential anti-apoptotic role in oral verrucous carcinoma

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**Abstract.**  $\alpha$ B-crystallin, a member of the small heat shock protein family, acts as a molecular chaperone.  $\alpha$ B-crystallin has been found to be overexpressed in a number of cancer tissues, including head and neck cancers. Overexpression of  $\alpha$ B-crystallin in cancer tissue may be related to its anti-apoptotic properties; however, its mechanism of action remains unclear. The aim of this study was to investigate the anti-apoptotic role of  $\alpha$ B-crystallin in oral verrucous carcinoma (OVC). Since oral squamous cell carcinoma (OSCC) is the most common tumor of the oral cavity, we selected OSCC as a control group. Immunohistochemical staining was used to evaluate the expression levels. The results showed that the expression of  $\alpha$ B-crystallin was detected in OVC, OSCC and normal oral mucosa (NM). The expression in OVC was higher compared to that of NM, but lower compared to OSCC, indicating that OVC was less aggressive than OSCC with respect to malignancy potential. Furthermore, we found that in OVC, the increased expression of  $\alpha$ B-crystallin coincided with the decreased expression of activated caspase-3. The results indicated that  $\alpha$ B-crystallin may play an anti-apoptotic role via inhibition of the activation of caspase-3 in OVC.

## Introduction

Oral verrucous carcinoma (OVC) is considered a rare, low-grade and well-differentiated carcinoma, with less potential for lymph node metastasis than other oral carcinomas. OVC is also called 'Ackerman's tumor' or 'verrucous carcinoma of Ackerman' since it was first reported and described by Ackerman in 1948 (1). Besides the oral cavity, OVC is

known to occur in the larynx, pyriform sinus, nasal cavity, paranasal sinuses, skin and esophagus, but the oral cavity is the most common site (2). Within the oral cavity, the buccal mucosa and gingival are the most preferred sites.

OVC, which accounts for 2.2-20% of all oral cancer, is mainly found in elderly males, particularly in tobacco smokers (3). OVC is regarded as a variant of squamous cell carcinoma with specific clinical, pathological and cytokinetic features, which also renders it different from squamous cell carcinoma. Concerning the etiology of OVC, tobacco use is believed to be markedly correlated with OVC, including inhaled and smokeless tobacco (4). Opportunist viral activity associated with human papillomavirus (HPV) has also been shown to have a close correlation, which includes HPV-2, 18, 20, 27, 57 and 62 (5,6). In southern or southeastern Asia, betel nut chewing is considered to be another significant cause of OVC (7). Nonetheless, the etiopathogenesis of OVC remains unclear, particularly its molecular mechanisms.

$\alpha$ B-crystallin is a member of the family of small heat shock proteins and acts as a molecular chaperone. The essential biological function of  $\alpha$ B-crystallin is reflected by the conservation of its structure, from bacteria to humans (8). As with Hsp27,  $\alpha$ B-crystallin is also stress inducible. By binding non-naïve, non-host proteins or specific apoptosis-related proteins during cellular stress,  $\alpha$ B-crystallin protects cells from thermal shock, osmotic shock, oxidative insults, ischemia or exposure to heavy metals, and sustains cell homeostasis (9,10).  $\alpha$ B-crystallin may play a significant role in cancer, as its overexpression has been reported in renal, hepatocellular and gastric carcinoma, glioma, and lung and breast cancers (11-14). In cancer,  $\alpha$ B-crystallin has demonstrated anti-apoptotic properties and is even considered to be a potential oncoprotein in a number of types of human cancer (15). Furthermore, it was found that  $\alpha$ B-crystallin suppresses cell apoptosis by inhibiting the activation of caspase-3, but this has not been confirmed in human cancer (16).

Although overexpression of  $\alpha$ B-crystallin has been reported in head and neck cancers, this study, to the best of our knowledge, is the first attempt to characterize expression patterns of  $\alpha$ B-crystallin in OVC and to explore the potential role of  $\alpha$ B-crystallin in oral cancer.

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## Materials and methods

**Tissue specimens.** A total of 17 OVCs, 15 oral squamous cell carcinomas (OSCCs) and 15 samples of healthy oral mucosa were obtained at Xiangya Hospital, Central South University, between 2002 and 2009. All patients gave written consent under the protocol reviewed, and the study was approved by the institutional review board of the Xiangya Hospital, Central South University. The OVC group, comprised 14 males and 3 females, with a mean age of 51.18 years (range 23-79). The most frequent site of OVC was the gingiva, observed in 9 patients (52.94%), followed by the buccal mucosa in 4 patients (23.52%). The OVC cases had no neck lymph node or distant metastasis (Table I). In the OSCC group, there were 9 males and 6 females. There were 4 patients with lymph node metastasis and none with distant metastasis (Table I).

OVC was only diagnosed when the characteristic clinical and pathological features were present (2). OSCC was diagnosed by the 1997 WHO criteria, and their clinical stage (TNM) was determined in terms of the 2002 UICC criteria (17,18). Normal oral mucosa (NM) specimens were obtained from healthy volunteers. All tissue specimens were fixed in 4% buffered formalin solution and paraffin-embedded sections were made for routine pathological diagnosis and immunohistochemical staining.

**Immunohistochemical staining for  $\alpha$ B-crystallin.** Immunohistochemical staining was performed on 4  $\mu$ m serial sections following routine procedures of deparaffinization and dehydration. The slides were incubated in 3% H<sub>2</sub>O<sub>2</sub> for 20 min to block endogenous peroxidase activity, and were then boiled under pressure in citrate buffer (pH 6.0, for 5 min) for antigen retrieval. The tissues were then incubated with the  $\alpha$ B-crystallin antibody (1:100 dilution, sc-101437, mouse anti- $\alpha$ B-crystallin monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or activated-caspase-3 (p17) antibody [1:50 dilution, BS7004, rabbit anti-caspase-3 (p17) monoclonal antibody, Bioworld Technology, St. Louis Park, MN, USA] at 4°C in a moist chamber overnight. Slides were then washed with Tris-buffered saline (TBS) and incubated with Polymer Helper and polyperoxidase-anti-mouse/rabbit IgG successively for approximately 20 min, respectively. The color was developed with DAB and counterstained with hematoxylin (Polymer Detection System for immunohistological staining, PV-9000, Zhongshan, Godbridge, China). Negative controls were incubated with phosphate-buffered saline as a replacement for the primary antibody.

**Evaluation of staining.** The expression level was scored based on the percentage of positively stained cells and the intensity of staining. A mean percentage of positive tumor cells was determined by the examination of 200 cells in at least 10 areas, at a magnification of x400. Five categories were presented according to the percentage of positive cells (PP): i) 0, <5%; ii) 1, 5-24%; iii) 2, 25-49%; iv) 3, 50-75%; or v) 4, >75%. The intensity of staining (SI) was scored as follows: i) 0, no -; ii) 1, weak +; iii) 2, moderate ++; and iv) 3, intense +++. The final immunoreactive score (IRS): IRS= SI x PP, was: i) -, 0; ii) +, 1-2; iii) ++, 4-6; iv) +++, 8-12. The evaluation was performed by two observers independently.

Table I. Lymph node metastasis and stage category of OVC and OSCC.

	Lymph node metastasis		Stage category	
	Positive No. (%)	Negative No. (%)	I-II No. (%)	III-IV No. (%)
OVC	0 (0)	17 (100)	15 (88.2)	2 (11.8)
OSCC	4 (26.7)	11 (73.3)	9 (60.0)	6 (40)

OVC, oral verrucous carcinoma; oral squamous cell carcinoma (OSCC).

**Statistical analysis.** Statistical significance was evaluated by the Kruskal-Wallis test among the three groups of OVCs, OSCCs and NM; the Mann-Whitney U test for comparison of every two groups of OVCs, OSCCs and NM; the  $\chi^2$  analysis and Fisher's exact test for analyzing the correlations with clinicopathological parameters. The Spearman's rank correlation test was used to analyze the expression of  $\alpha$ B-crystallin and activated caspase-3 in tumor cells. P<0.05 was considered to be statistically significant and data analysis was performed using SPSS software, version 16.0.

## Results

**Protein expression of  $\alpha$ B-crystallin.**  $\alpha$ B-crystallin was expressed predominantly in the cytoplasm of tumor cells. In 17 OVC cases, there were 2 with negative staining, 7 with weak staining, 6 with moderate staining (Fig. 1B) and 2 with strong staining. In all 15 OSCC cases,  $\alpha$ B-crystallin was expressed and the intensity of staining was demonstrated as moderate (10) or strong (5), respectively (Fig. 1C), whereas in NM, there were 5 cases with negative staining (Fig. 1A) and 10 with weak staining. Statistically significant differences were shown between every two groups (Table II).

Using the  $\chi^2$  test analysis,  $\alpha$ B-crystallin expression in OVC was compared with clinicopathological parameters. No statistically significant correlation was found with age, gender, site and clinical stage. However in OSCC, the expression of  $\alpha$ B-crystallin was correlated significantly with the clinical stage of tumors [intensity of staining in stage I-II was weak (3) or moderate (6), while in stage III-IV staining was moderate (1) and strong (5),  $\chi^2=10.557$ ,  $p=0.032$ ], but was not significantly correlated with age, gender, site, differentiation and nodal status.

**Protein expression of activated caspase-3.** Expression of caspase-3 was detected in the cytoplasm of specimens. Compared to NM, expression of activated caspase-3 was significantly reduced in OVC and OSCC (Table III, Fig. 2). In the OVC and OSCC cases, however, there was no statistically significant difference for their expression (Table III).

Using the  $\chi^2$  test analysis, no significant difference was found for the expression of activated caspase-3 and clinicopathological variables in OVCs and OSCCs samples ( $p>0.05$ ).

**Correlation of two protein expressions.** In OVCs, the overexpression of  $\alpha$ B-crystallin was significantly correlated to

Table II. Expression of  $\alpha$ B-crystallin in OVC, OSCC and NM.

Group	$\alpha$ B-crystallin				Total	Z	P-value
	Negative	Weak	Moderate	Strong			
OVC	2	7	6	2	17	-2.118 <sup>a</sup>	0.044 <sup>a</sup>
OSCC	0	0	10	5	15	-2.792 <sup>b</sup>	0.010 <sup>b</sup>
NM	5	10	0	0	15	4.268 <sup>c</sup>	0.000 <sup>c</sup>
Total	7	17	16	7	47		

<sup>a</sup>Comparison between OVC and OSCC, <sup>b</sup>comparison between OVC and NM, and <sup>c</sup>comparison between OSCC and NM. OVC, oral verrucous carcinoma; OSCC, oral squamous cell carcinoma; NM, normal oral mucosa.

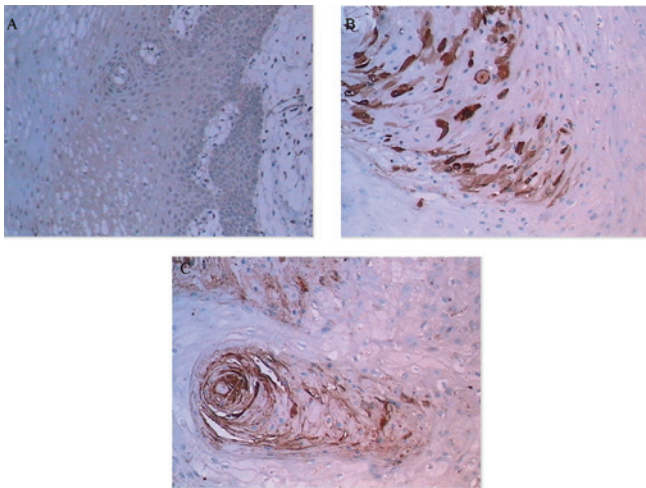


Figure 1. Immunohistochemical staining of  $\alpha$ B-crystallin in NM, OVC and OSCC tissue. (A) NM exhibited negative  $\alpha$ B-crystallin expression. (B) In OVC tissue,  $\alpha$ B-crystallin was moderately expressed. (C) In OSCC tissue,  $\alpha$ B-crystallin was markedly expressed.  $\alpha$ B-crystallin was mainly expressed in cytoplasm (B,C). Original magnification, x100.

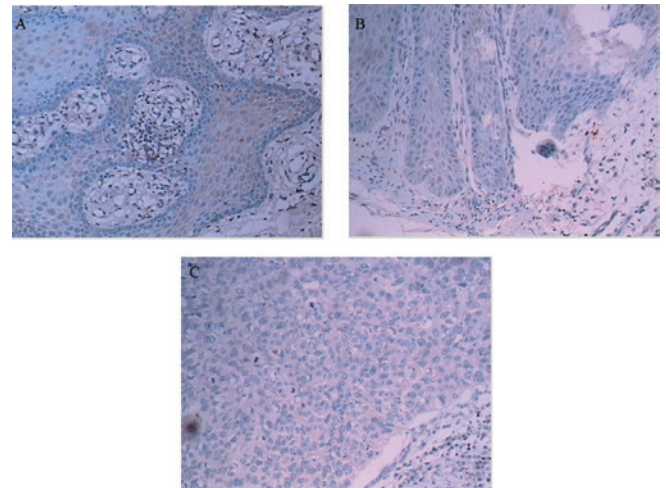


Figure 2. Immunohistochemical staining of activated caspase-3 in NM, OVC and OSCC tissue. (A) NM exhibited weak activated caspase-3 expression. (B,C) OVC and OSCC tissue showed negative activated caspase-3 expression. Original magnification, x100.

Table III. Expression of activated caspase-3 in OVC, OSCC and NM.

Group	Activated caspase-3				Total	Z	P-value
	Negative	Weak	Moderate	Strong			
OVC	11	6	0	0	17	-1.448 <sup>a</sup>	0.202
OSCC	7	8	0	0	15	-3.934 <sup>b</sup>	0.000
NM	1	7	7	0	15	-2.983 <sup>c</sup>	0.040
Total	19	21	7	0	47		

<sup>a</sup>Comparison between OVC and OSCC, <sup>b</sup>comparison between OVC and NM, and <sup>c</sup>comparison between OSCC and NM. OVC, oral verrucous carcinoma; OSCC, oral squamous cell carcinoma; NM, normal oral mucosa.

the repression of activated caspase-3 statistically ( $p < 0.05$ , Table IV). In OSCCs, while no significant correlation between the two proteins was found, a trend towards increasing  $\alpha$ B-crystallin expression with decreasing activated caspase-3 expression was noted (Table V).

## Discussion

$\alpha$ B-crystallin was originally found in the human lens, which contributed to the transparency of the lens as a component of crystallin. Later, it was gradually found to exist in

Table IV. Correlation analysis between  $\alpha$ B-crystallin and activated caspase-3 in OVC.

	$\alpha$ B-crystallin				r	P-value
	Negative	Weak	Moderate	Strong		
Activated caspase-3					-0.547	0.023
Negative	0	4	5	2		
Weak	2	3	1	0		
Moderate	0	0	0	0		
Strong	0	0	0	0		
Total	2	7	6	2		

OVC, oral verrucous carcinoma.

Table V. Correlation analysis between  $\alpha$ B-crystallin and activated caspase-3 in OSCC.

	$\alpha$ B-crystallin				r	P-value
	Negative	Weak	Moderate	Strong		
Activated caspase-3					-0.363	0.183
Negative	0	0	4	2		
Weak	0	2	3	3		
Moderate	0	1	0	0		
Strong	0	0	0	0		
Total	0	3	7	5		

OSCC, oral squamous cell carcinoma.

various other tissues including the heart, brain, kidney and muscle, but the expression in non-lens tissue was low (19). Overexpression of  $\alpha$ B-crystallin has been detected in a number of types of human cancer, including hepatocellular carcinoma, glioma, lung, gastric and breast cancer. The expression in cancer has been found to be correlated with lymph node metastasis, low rates of survival and poor prognosis of patients, and  $\alpha$ B-crystallin was even considered a novel oncoprotein in basal-like breast carcinoma (11-15). In the current study of OVC and OSCC, we also found the overexpression of  $\alpha$ B-crystallin. The intensity of immunohistochemistry staining increases from NM to OVC and OSCC (Fig. 1). The expression of  $\alpha$ B-crystallin in stage III-IV was found to be significantly higher than in stage I-II, and the stronger staining site in OVC was in the pushing border, which may reflect the maximum proliferative activities of this tumor. These findings indicated that  $\alpha$ B-crystallin may play a significant role in tumor progression.

When comparing the OVC cases with OSCC cases, we found that the staining of  $\alpha$ B-crystallin in OVC was significantly lower than in OSCC. This may partially show that OVC behaves in a gentler manner than OSCC (20). In this study, there were no patients with lymph node metastasis in the OVC group, whereas there were 4 patients (26.7%) in the OSCC group. In the OVC group, only 2 patients (11.8%) were stage III-IV, but in the OSCC group, 40% of patients were

stage III-IV. The results indicated that OVC is different from OSCC and there may be different pathological mechanisms between the two tumors (20,21).

In tumors, the overexpression of  $\alpha$ B-crystallin may play a significant role in preventing tumor cell apoptosis. For cell apoptosis, there are two main pathways: mitochondrial and death receptor pathways. In the initial stages of the two pathways, the cell responds to a variety of stimuli, exogenous and endogenous, by releasing apoptosis-related factors, which then activate/recruit procaspase-8 and -9, respectively. Then by multiple steps, active caspase-8 and -9 initiate the proteolytic activation of procaspase-3: first, caspase-8 or -9 cleaves procaspase-3 at an aspartate residue between its large and small subunits to generate a p24 intermediate (the prodomain and the large subunit) and the p12 small subunit; second, the p24 intermediate generates the p20 and p17 forms of the large subunit by autoproteolysis. Two p17/p12s form the active caspase-3 as heterodimers, and then active caspase-3 induces apoptosis of the cell by proteolyzing key cell targets (22). Caspase-3 is thus the central molecule in the two pathways. Kamradt *et al* found that  $\alpha$ B-crystallin is capable of inhibiting the autoproteolyzing maturation of caspase-3 by directly binding to the p24 intermediate, resulting in the negative regulation of cytochrome-C and caspase-8-dependent apoptosis (23). These authors also observed a similar course in myogenic differentiation. In the study by Dimberg *et al*, it was found that the



activated caspase-3 expression was significantly elevated when the authors knocked down the  $\alpha$ B-crystallin gene (24).

In this study, we used the specific antibody of p17 generated in the activation of caspase-3 to detect the activated caspase-3. Similarly to Dimberg *et al*, we also found that in OVC the increase in expression of  $\alpha$ B-crystallin coincides with the decrease in expression of activated caspase-3, and we then confirmed that the expression of  $\alpha$ B-crystallin was significantly reversely correlated with the expression of activated caspase-3 statistically. This result indicates that in OVC,  $\alpha$ B-crystallin may function as an anti-apoptosis protein by inhibiting the activation of caspase-3, and may play a significant role in the carcinogenesis of OVC.

Although in OSCC, the statistical significance of the two protein expressions was not found, we observed that with the increasing expression of  $\alpha$ B-crystallin, the expression of activated caspase-3 was reduced (Table V). This indicates that more samples need to be accumulated for further study.

In conclusion,  $\alpha$ B-crystallin is overexpressed in OVC and may play a role in anti-apoptosis via inhibition of the activation of caspase-3 in OVC.

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