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ORIGINAL ARTICLE

Expression of LC3, LAMP2, KEAP1 and NRF2 in Salivary Adenoid Cystic Carcinoma

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Abstract Salivary Adenoid Cystic Carcinoma (SACC) is a tumor characterized by inevitable local progression and terminal hematogenous metastasis. This study aimed to investigate the expression of LC3, LAMP2, KEAP1 and NRF2 in SACC. Human salivary gland tissue microarray which contains 74 SACC, 12 pleomorphic adenoma and 18 normal salivary gland specimens. High expression of LC3, LAMP2, KEAP1 and NRF2 were found in SACC patients, and LC3, LAMP2, KEAP1 and NRF2 expression were significantly higher in SACC than as compared with pleomorphic adenoma and (or) normal salivary gland. The expression of NRF2 was correlated with pathological type of human SACC ($P < 0.05$). Moreover, the high-expression of KEAP1 had significant correlations with LC3 ($P < 0.001$, R = 0.3195), and LAMP2 ($P < 0.001$, R = 0.3346) and NRF2 ($P < 0.05$, R = 0.2246) by using the Pearson correlation coefficient test. Our findings demonstrated that up-regulation of LC3, LAMP2, KEAP1 and NRF2 were associated with carcinogenesis and progression of SACC patients, suggesting that they may be useful molecular targets in salivary adenoid cystic carcinoma.

Keywords LC3 · LAMP2 · KEAP1 · NRF2 · Salivary adenoid cystic carcinoma

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Salivary Adenoid Cystic Carcinoma (SACC) is one of the most common subtypes of salivary gland cancer in the head and neck region. Due to the strong invasion to peripheral nerves, high rate of local recurrence and distant metastasis, patients with SACC have a poor prognosis [1, 2]. Three histologic patterns are generally described in SACC, it is assumed that the less aggressive cribriform and tubular phenotypes progress toward the more aggressive solid subtype. To date, little is known about the actual molecular events underlying the process of increasing malignancy in SACC. Thus, there is an urgent need to identify the key regulatory molecules in the cancer initiation and progression of SACC and for rationalize treatment decisions.

Autophagy is a lysosomal pathway involved in the turnover of cellular macromolecules and organelles, it plays a key role as a self-eating system to produce energy for adaptation and survival under starvation, and is known to protect cells against stresses [3, 4]. Microtubule-associated protein 1 light chain 3 (LC3), which exists in two forms, is essential for autophagosome formation and is indicative of autophagic activity [5]. Lysosome associated membrane protein 2 (LAMP2) is an important protein in the chaperone mediated autophagy pathway, it is a selective autophagy that is responsible for the lysosomal degradation of approximately 30 % of the modified and oxidatively damaged soluble cytosolic proteins that contain the recognizable peptide sequence motif [6]. Expression of LC3 and LAMP2 has been studied in many kinds of cancer, and over-expressed LC3 and LAMP2 were correlated with clinicopathologic characteristics [6–9]. As for the role of autophagy in cancer, it is still very controversial whether it promotes carcinogenesis and development or suppresses tumor

growth by preventing the accumulation of damaged proteins and organelles. Recently, increasing evidence has demonstrated that autophagy inhibition may be exploited to prevent resistance to cancer treatment [10]. Accordingly, modulation of autophagy has a great potential in cancer prevention and treatment.

Oxidative stress was present in various cancer cells compared with normal cell, which was associated with cancer initiation and cancer development uniquely by induction of genomic instability [11]. The KEAP1–NRF2 [Kelch-like ECH-associated protein 1–nuclear factor (erythroid-derived 2)-like 2] system plays an important role in the protection of cells against oxidative and xenobiotic stresses [12]. KEAP1 is essential for the regulation of NRF2 activity. Under normal conditions, NRF2 is constantly ubiquitinated through KEAP1 in the cytoplasm and degraded in the proteasome. Upon exposure to reactive oxygen species (ROS), KEAP1 is inactivated and NRF2 is stabilized. Intriguingly, levels of NRF2 was demonstrated to be increased in a plenty of cancers, cancer cells acquired malignant properties after hijacking the KEAP1–NRF2 system [13, 14]. Both of autophagy and oxidative stress are involved in cancer initiation and progression. Nonetheless, the specific mechanism between autophagy, oxidative stress and SACC is unclear, and the association and roles of these proteins in SACC have not been clarified.

In the present study, we are interesting to research the expression LC3, LAMP2, KEAP1 and NRF2 in SACC specimens, and compared to pleomorphic adenoma and normal salivary gland through tissue microarray in order to determine their role in the carcinogenesis of SACC. We also focused our attention on the association between the expression of LC3, LAMP2, KEAP1 and NRF2 protein of SACC patients.

Materials and Methods

Patient Samples

All tissue samples from patients were obtained from patients surgically treated in the department of Oral Maxillofacial-Head Neck Oncology, School and Hospital of Stomatology Wuhan University using formalin-fixed, paraffin-embedded (FFPE). For tissue microarray (T12-412-TMA1), we constructed a cohort including 74 SACC (cribriform pattern: 28, tubular pattern 26, solid pattern 20, 50 patient overlap with previous described), 12 pleomorphic adenoma and 18 normal salivary gland in collaboration with Shanghai Biochip Company, Ltd., Shanghai, China. All tumors were histology analyzed and classified according to the 2005 Word Health Organization classification system. This study was approved by the Medical Ethics Committee of Hospital of Stomatology Wuhan University (PI: Zhi-Jun Sun) and was performed according to the Institutional Guidelines. Written informed consent was obtained from all participants.

Immunohistochemical Staining

Immunohistochemical studies of the human SACC tissue microarrays were done using the following antibodies: monoclonal mouse anti-human LC3, LAMP2 (dilution 1:300 and 1:200) from Abcam Biotechnology Inc., (Cambridge, UK); polyclonal rabbit anti-human NRF2 (1:200) from Abcam Biotechnology Inc., (Cambridge, UK); and monoclonal mouse anti-human KEAP1 (dilution 1:300) from Proteintech Group Inc., (Chicago, USA). Immunohistochemical staining was performed using a peroxidase-labeled streptavidinbiotin technique. Briefly, tissue sections were deparaffinized and rehydrated. Next, sections were incubated in 3 % hydrogen peroxide and treated with 10 % normal goat serum. Then sections were incubated overnight within primary antibody, followed by second antibody and an avidin-biotin-peroxidase reagent. Diaminobenzidine as well as a counterstaining with haematoxylin resulted in the visualization of the immunostaining.

Scoring of Immunohistochemistry Results

Automated image acquisition using an Aperio ScanScope CS scanner (Vista, CA, USA) has been described previously [14]. In brief, monochromatic, high-resolution images which were obtained of each histospot were evaluated on a computer screen for intensity of staining, the images were driven by custom program. The signal intensity of LC3, LAMP2, KEAP1 and NRF2 from pixels were measured on scales by Aperio Quantification software (Version 9.1). Histoscore of membrane and nuclear staining was calculated as a percentage of different positive cells using the formula $(3+) \times 3 + (2+) \times 2 + (1+) \times 1$. Moreover, the biomarker levels were categorized as low or high expression groups) for outcome analyses by using the histoscores distribution..

Hierarchical Clustering

In Microsoft excel, the staining scores were converted into scaled values centered on zero, then the Cluster 3.0 with average linkage based on Pearson's correlation coefficient was used to achieve the hierarchical analysis, and the results were visualized using the Java TreeView 1.0.5. Finally, the clustered data were arranged with markers on the horizontal axis and tissue samples on the vertical axis. Biomarkers with a close relationship are located next to each other.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5.03 (GraphPad Software, Inc., La Jolla, CA) statistical packages. One-way ANOVA followed by the post-Tukey or Bonferroni multiple comparison tests was used to evaluate the difference in protein levels among each group. The correlation of these markers was analyzed by two-tailed Pearson statistics with Gaussian distribution. The level of significance was set to $P < 0.05$. All P values reported were based on two-sided tests.

Results

Expression of LC3, LAMP2 in the Normal Salivary Gland, Pleomorphic Adenoma and SACC Tissues

To characterize the expression of LC3, LAMP2 in human adenoid cystic carcinoma. Immunohistochemical analysis revealed that LC3, LAMP2 were highly expressed in SACC. Pathologically, SACC is characterized by basaloid tumor consisting of epithelial and myoepithelial cells in variable morphologic configurations, including cribriform, tubular, and solid histological patterns. The positive expression of LC3 and LAMP2 was mainly located in cytoplasm of epithelial and myoepithelial cells in cribriform SACC around cylindromatous microcystic spaces (Fig.1). In the tubular form, well-formed ducts and tubules with central lumina are lined by inner epithelial and outer myoepithelial cells. Similar immunoreactivity of LC3 and LAMP2 were identified in both inner and outer cells (Fig.1). The solid type SACC is formed of sheets of uniform basaloid cells lacking tubular or microcystic formation. The LC3 and LAMP2 expressed throughout the sheets and nests of basaloid cells. For pleomorphic adenoma (PMA), which composed of benign epithelial component with ductal structures and a mesenchymal myxoid component. The expression of LC3 and LAMP2 are rarely observed in epithelial or mesenchymal component of PMA as well as normal salivary gland.

Although it is assumed that the less aggressive cribriform and tubular phenotypes progress toward the more aggressive solid subtype, our results showed that the expression of LC3 were not correlated with cribriform, tubular, and solid histological patterns of SACC ($P > 0.05$, Fig.2). We also detected that the expression of LC3 was negative in the ductal epithelial cells of normal salivary gland, and negative or weak in myoepithelial cells of pleomorphic adenoma. The expression of LC3 in SACC was significantly higher when compared with normal salivary gland and pleomorphic adenoma ($P < 0.001$, $P < 0.001$, respectively, Fig.2). In addition, moderate and strong LAMP2 expressions were observed in SACC, which was significantly higher when compared with normal salivary gland and pleomorphic adenoma ($P < 0.001$, $P < 0.001$, respectively, Fig.2). LAMP2 expressions were observed in cytomembrane and/or cytoplasm of epithelial and myoepithelial cells in SACC (Fig.1). There was no significant difference of LAMP2 expressions among the cribriform, tubular, and solid histological patterns of human adenoid cystic carcinoma ($P > 0.05$, Fig.2).

Expression of KEAP1 and NRF2 in the Normal Salivary Gland, Pleomorphic Adenoma and SACC Tissues

The KEAP1- NRF2 system plays an important role in tumor development, but little is known about its correlation with carcinogenesis of SACC. The positive expression of KEAP1 was mainly located in cytoplasm and/or nucleus of epithelial and myoepithelial cells in partial sheets of SACC with a diffuse expression pattern (Fig.3). There are no difference of KEAP1 expression in inner or outer epithelial cell in tubular or cribriform SACC. We also detected that the expression of

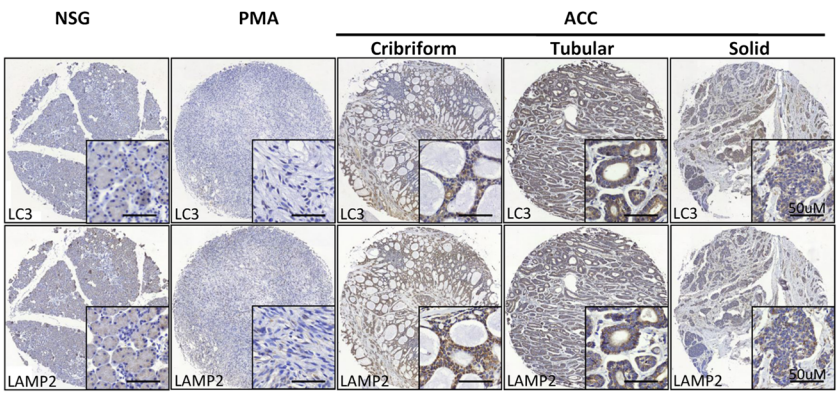

Fig. 1 Quantatitive of histoscore of LC3, LAMP2 expression in SACC, pleomorphic adenoma and normal salivary gland. We also detected that the expression of LC3 was negative or weak LC3 expressions in normal salivary gland, and weak in pleomorphic adenoma, the positive expression of LC3 was mainly located in cytoplasm and/or nucleus of tumor cells in cribriform, tubular, and solid histological patterns in human adenoid cystic carcinoma. (Scale bars =50um)

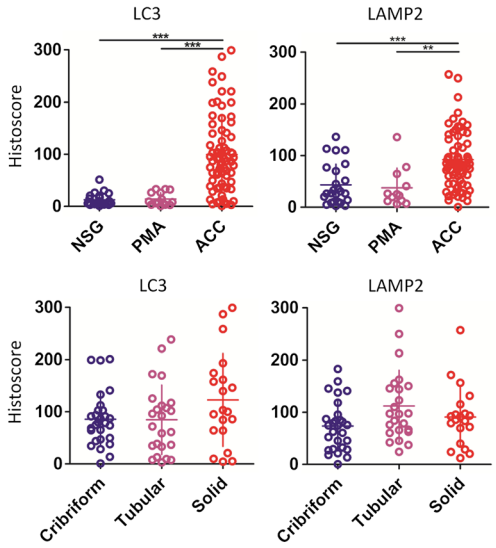

Fig. 2 LC3 levels in SACC was significant higher when compared with normal salivary gland or pleomorphic adenoma, and LAMP2 levels in SACC was significant higher when compared with normal salivary gland or pleomorphic adenoma (Mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; One-way ANOVA); The expression of LC3, LAMP2 were not correlated with pathological grade of human SACC (Quantification using Aperio nuclear quantification software, and statistics using Graph Pad Prism 5. Mean ± SEM; *, $P < 0.05$; Mann–Whitney U test).

KEAP1 was weak in myoepithelial cells of pleomorphic adenoma. Negative KEAP1 expression was observed in acinic cells, while weak positive nuclear expressed in myoepithelia or ductal epithelial cells of normal salivary gland. Our results showed that the expression of KEAP1 was not correlated with cribriform, tubular, and solid histological patterns of SACC ($P > 0.05$, Fig.4). In normal salivary gland, the expression of NRF2 was found weak NRF2 expressions were observed in cytomembrane and/or cytoplasm of epithelial and myoepithelial cells in SACC (Fig.3). In addition, moderate and strong NRF2 expressions were observed in myoepithelial component of pleomorphic adenoma, which was significantly higher when compared with normal salivary gland and SACC ($P < 0.001$, $P < 0.001$, respectively, Fig.4). In addition, we analyzed whether KEAP1 and NRF2 expression in human adenoid cystic carcinoma tissues correlated with patient clinicopathologic characteristics. The results showed that NRF2 expressions was correlated with pathological type of SACC, the expression of NRF2 in tubular SACC was significantly higher than in cribriform SACC ($P < 0.05$, Fig.4).

The Relationships Among Expression of LC3, LAMP2, KEAP1 and NRF2 in SACC

To authenticate the mechanism of autophagy and oxidative stress in human adenoid cystic carcinoma, we studied the association of autophagy markers LC3, LAMP2 with the immunohistochemical protein expression of oxidative stress markers KEAP1 and NRF2, the Spearman rank correlation coefficient test and linear tendency test was used (Fig.5). Of interest, we found that the expression of higher cytoplasmic KEAP1 was statistically associated with cytoplasmic LC3 ($P < 0.001$, $R = 0.3195$), and membrane LAMP2 ($P < 0.001$, $R = 0.3346$) and nuclear NRF2 ($P < 0.05$, $R = 0.2246$). By hierarchical clustering, the expression of tumor associated KEAP1 and NRF2, autophagy marker LAMP2 is more close to expression of LC3 (Fig.5).

Discussion

LC3 is best characterized as a marker of autophagy and has been found to be associated with carcinogenesis in cancer. In the present study, our results showed that the positive expression of LC3 in SACC was significantly higher when compared with normal salivary gland and pleomorphic adenoma. In human oral squamous cell carcinoma [9], elevated LC3 expression was found to be significantly associated with clinical and pathological characteristics of patients. LC3 was involved in the development and progression of prostate cancer and benign prostatic hyperplasia, indicating that autophagy may be a useful way to reduce the risk of prostate cancer [15]. In addition, LC3 expression was significantly correlated with lymph node

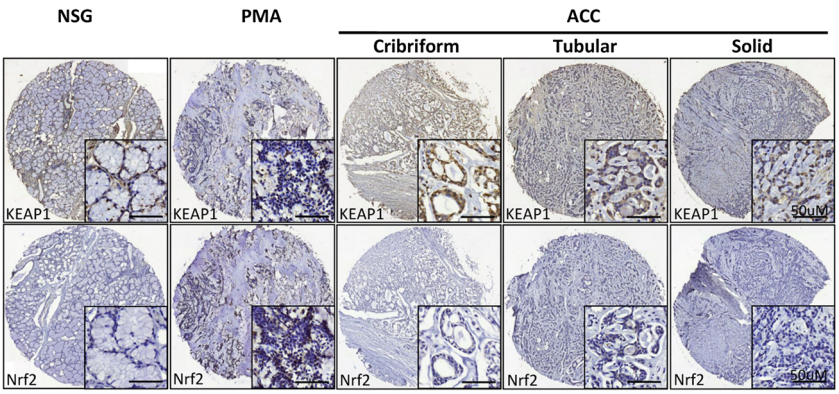

Fig. 3 Quantatitive of histoscore of KEAP1 and NRF2 expression in SACC, pleomorphic adenoma and normal salivary gland. In normal salivary gland, the expression of NRF2 was found weak, NRF2 expressions were observed in cytomembrane and/or cytoplasm in adenoid cystic carcinoma. In addition, moderate and strong NRF2 expressions of were observed in pleomorphic adenoma. (Scale bars = 50 um)

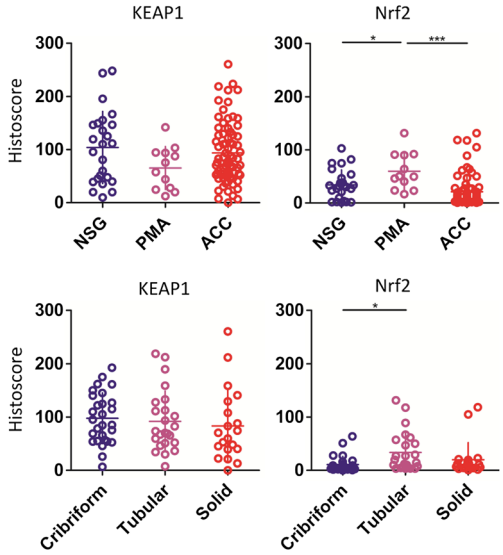

Fig. 4 KEAP1 levels in SACC was not significant higher when compared with normal salivary gland or pleomorphic adenoma, and NRF2 levels in SACC was significant higher when compared with normal salivary gland or pleomorphic adenoma (Mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; One-way ANOVA); The expression of NRF2 was correlated with pathological grade of human SACC (Quantification using Aperio nuclear quantification software, and statistics using Graph Pad Prism 5. Mean ± SEM; *, $P < 0.05$; Mann–Whitney U test).

involvement in SACC [16], which suggest that LC3 may play an important role in the caricinogenesis of SACC.

LAMP2, the best characterized lysosomal membrane protein, is found in later endocytic compartments of animal cell, being used as a marker of lysosome-associated membranes [17], which is little known about its correlation with carcinogenesis of human carcinoma including SACC. Our present study showed that LAMP2 expressions were observed in SACC, which was significantly higher when compared with normal salivary gland and pleomorphic adenoma. Furthermore, elevated expression of LAMP2 was observed in breast tumor tissues of all patients under investigation, suggesting a survival mechanism via chaperone-mediated autophagy and LAMP2 [6].

The KEAP1–NRF2 pathway plays a central role in the protection of cells against oxidative and xenobiotic stresses, which has been reported to promote cancer development and resistance to chemotherapeutic drugs [12, 18]. In our study, positive NRF2 expressions were observed in pleomorphic adenoma and SACC. Concomitantly, NRF2 expression was correlated with pathological type of SACC. This finding is consistent with those of previous studies on solid tumors [19, 20], which have shown that KEAP1–NRF2 pathway is significantly involved in cancer initiation and progression of SACC.

Furthermore, it was also shown that the relationship between autophagy and oxidative stress is complicated and has not been elucidated. We found that expression of higher KEAP1 was statistically associated with LC3, and LAMP2 and NRF2, indicating that oxidative is closely associated with autophagy. Recently, a study showed that an enhanced accumulation of vacuoles carrying the marker LC3 after inactivation of LAMP2 by RNA interference, yet a decreased colocalization of LC3 and lysosomes [21]. Previous researches show that LC3 is regulated by Atg7 [22], in Atg7-

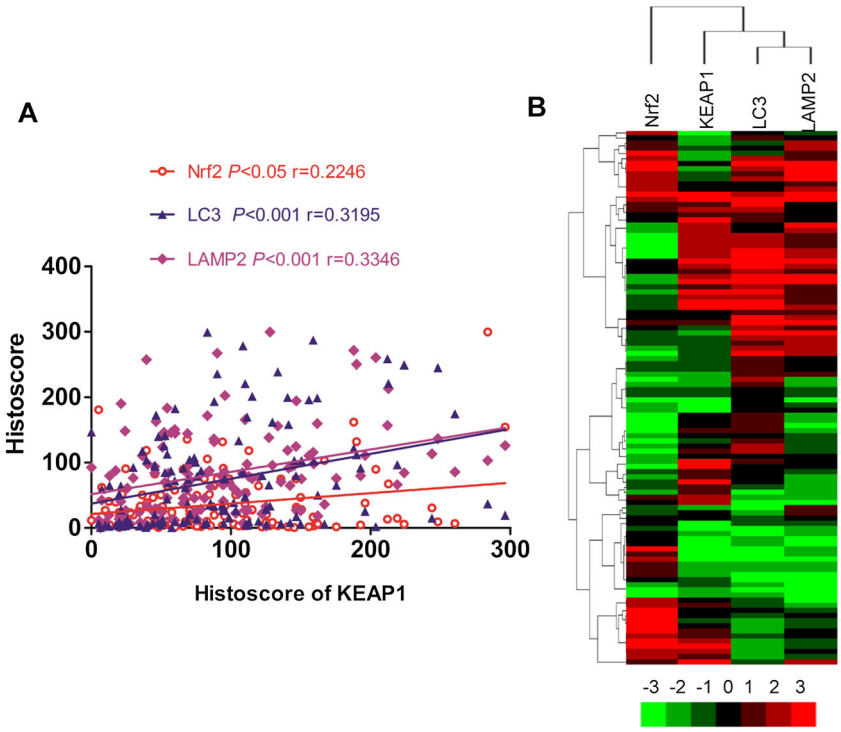

Fig. 5 Correlation of LC3, LAMP2, KEAP1 and NRF2 in human SACC tissue array: A) KEAP1 was found to be closely associated the LC3 ($P < 0.001, r = 0.3195$), LAMP2 ($P < 0.001, r = 3346$) and NRF2 ($P < 0.05, r = 0.2246$, Fig. 3 **a** in human SACC tissue array; **b** Hierarchical Clustering of LC3, LAMP2, KEAP1 and NRF2 in human SACC tissue array. Histoscore based on quantification using Aperio quantification software and statistics with Graph Pad Prism5. Mean ± SEM; 2-tailed Pearson correlation statistics

deficient cells, redox-sensitive KEAP1 degradation was decreased, downregulation of KEAP1 decreased autophagy levels, increased NRF2 activation, suggesting a feedback loop between ROS-regulated KEAP1–NRF2 and Atg7-regulated autophagy [23]. Recent studies suggest that both of autophagy and KEAP1–NRF2 pathway are significantly involved in cancer initiation and progression [23, 24], and the signaling are entwined in ways that are not yet fully understood. Through a better understanding of how oxidative stress regulate autophagy there is an opportunity to develop cancer treatment strategies in which oxidative stress and autophagy are either induced or inhibited depending on the molecular context of the individual cancer and its microenvironment. Therefore, both must be considered in the activation of carcinogenesis and development of cancer therapeutics.

In conclusion, our study has demonstrated that LC3, LAMP2 and KEAP1–NRF2 pathway are over-expressed in human adenoid cystic carcinoma tissues, indicating increased level of autophagy activity and oxidative stress. Our results indicate that that LC3, LAMP2 and KEAP1–NRF2 pathway could play an important role in carcinogenesis and of development SACC, and further investigation are warranted to clarify the mechanisms, which may provide novel therapeutic possibilities of SACC.

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Conflict of Interest Statement The authors have declared that no competing interests exist.

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