Expression of beclin 1 in primary salivary adenoid cystic carcinoma and its relation to Bcl-2 and p53 and prognosis

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Abstract

Beclin 1 plays a critical role in autophagy and functions as a haploinsufficient tumor suppressor. The expression and prognostic significance of beclin 1 in head and neck adenoid cystic carcinoma (ACC) are largely unexplored. Therefore, we investigated the expression of beclin 1, Bcl-2, and p53 in head and neck ACC tissue. Tissue samples from 35 cases (15 females, 20 males) of head and neck ACC were utilized for immunohistochemistry. Beclin 1 expression was observed in 32 cases (91.4%) and considered to be high in 15 cases (42.9%) and low in 20 cases (57.1%). Beclin 1 expression was significantly correlated with a histological growth pattern (P = 0.046) and histological grade (P = 0.037). Beclin 1 expression was inversely correlated with Bcl-2 expression (P = 0.013) and significantly associated with overall survival (P = 0.006). Bcl-2 and p53 expression were observed in 21 cases (60.0%) and 16 cases (45.7%). Bcl-2 expression was significantly correlated with perineural invasion (P = 0.041) and not associated with overall survival (P = 0.053). p53 expression was directly correlated with beclin 1 expression (P = 0.044). Our results indicated that beclin 1 may be a novel, promising prognostic factor for clinical outcome in head and neck ACC patients and may play a part in the development of head and neck ACC by interacting with Bcl-2 and p53.

Key words: Adenoid cystic carcinoma; Autophagy; Beclin 1; Bcl-2; p53

Introduction

Adenoid cystic carcinoma is one of the most frequent malignant salivary gland tumors in the head and neck region. The clinical and pathological characteristics include slow growth, perineural invasion, distant metastasis, and potential local recurrence (1,2). Although complete surgical resection and additional radiotherapy have been shown to improve long-term survival, the prognosis of adenoid cystic carcinoma remains poor (3,4). Accordingly, it is very important to objectively predict the assessment of prognosis for resected patients and the development of metastasis.

Autophagy is an evolutionarily conserved catabolic process where cellular cytoplasmic contents are self-digested (literally “eats itself”) (5). In eukaryotic cells, autophagy is a primary mechanism for long-lived protein degradation and organelle turnover, serving as a critical adaptive response that recycles energy and nutrients during starvation or stress (6). Autophagy is regulated by a set of highly conserved genes called ATGs (for AuTophaGy gene) (7). Beclin 1 gene is identified as the mammalian ortholog of yeast Atg6 and plays a pivotal role in mammalian autophagy.

The human beclin 1 gene is located on chromosome 17q21 (8). The role of autophagy as a tumor suppressor was first broached through genetic studies of beclin 1, and beclin 1 was mapped to a tumor susceptibility locus monoallelically deleted and with a high proportion of human breast, intrahepatic biliary duct, ovarian, and brain cancers. In addition, analysis of human tissue samples revealed that there was a significant loss of beclin 1 protein expression in human breast carcinomas compared to normal breast tissue (9-13). Ectopic beclin 1 expression decreased cancer cell proliferation in vitro and reduced tumorigenic potential in vivo, further illustrating a role for autophagy in tumor suppression. In support of this concept, two groups illustrated that heterozygous disruption of beclin 1 promoted tumorigenesis in mice, albeit over an extended latency (14,15). These findings demonstrate that...
beclin 1/atg6 functions as a haploinsufficient tumor suppressor.

Bcl-2, the first protein found to interact with beclin 1, was shown to possess an anti-autophagy function in addition to its well-established role in apoptosis inhibition (16). Because these anti-apoptotic proteins are usually overexpressed in cancer cells, the Bcl-2 protein may have an additive effect on oncogenic properties through inhibition of autophagy (17).

p53, as a tumor suppressor gene, downregulates Bcl-2 transcription activity and plays a duplicate role in autophagy regulation, depending on its subcellular location, and the mutation of p53 is one of the most common genetic events described in human cancers. Immunohistochemical staining for p53 usually shows mutant types of p53 facilitating malignant transformation (18,19). Furthermore, immunohistochemistry of p53 proteins in tumor cells may serve as an additional method to evaluate the status of other important tumor genes (20). Expression of beclin 1 and Bcl-2, an association with p53 expression, as well as any correlation between beclin 1 expression and clinicopathological factors or outcomes in adenoid cystic carcinoma, have not been well characterized.

We investigated the expression of beclin 1, Bcl-2, and p53 in head and neck adenoid cystic carcinoma tissue via immunohistochemical analysis in relation to survival and other prognostic factors.

Material and Methods

Patients and specimens

All of the tissues analyzed were obtained from 35 consecutive patients (15 females, 20 males) with head and neck adenoid cystic carcinoma who underwent a primary resection between December 2002 and March 2010 at Shandong Provincial Hospital, affiliated with Shandong University of China. All patients gave written informed consent to participate in the study. Two independent observers reviewed all of the original hematoxylin and eosin-stained sections and chose the most representative slide from each case to perform immunohistochemical staining. Two or three representative cores, 3 mm in diameter, were obtained from a representative area of each paraffin-embedded tumor tissue. The median age of the patients was 47.8 years (range, 18-72 years), and tumor size ranged from 0.7 to 4 cm (median size, 1.8 cm). According to the American Joint Committee on Cancer staging criteria (tumor-node-metastasis: TNM), 12 patients (34.3%) had Stage I and II disease, and 23 patients (65.7%) had Stage III and IV disease. Three histological grades were established: Grade I, tumors with tubular and cribriform areas but without solid components; Grade II, cribriform tumors that were either pure or mixed with less than 30% of solid areas; and Grade III, tumors with a predominantly solid pattern (21).

Immunohistochemical staining

Sections (4 µm thick) of formalin-fixed, paraffin-embedded tissue were deparaffinized, dehydrated via a graded alcohol series, and subjected to blocking with hydrogen peroxide. An antigen retrieval procedure was performed by microwave in 10 mM citrate buffer, pH 6.0. Sections were incubated for 15 min at room temperature with primary monoclonal antibodies for beclin 1 (1:50, Abcam, UK) and Bcl-2 (1:200, Abcam), and monoclonal antibodies for p53 (1:300 Abcam) using a biotin-free polymeric horseradish peroxidase linker antibody conjugate system, and nuclei were counterstained with hematoxylin in a BenchMark ULTRA advanced staining system (Ventana Medical Systems, USA). The positive controls were supplied by Abcam, and negative controls were obtained by omitting primary antibody replaced by PBS.

Evaluation of immunohistochemical staining

The expression of beclin 1 ascertained by immunohistochemical staining emerged as fine granular and diffuse staining in the plasma membrane, cytoplasm, and/or nucleus of tumor cells. Bcl-2 expression was discerned in the cytoplasm and p53 was seen in the cell nucleus of adenoid cystic carcinoma. Immunohistochemical staining for beclin 1 was evaluated in compliance with intensity and proportion. The intensities were scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The percentage of staining area was classified as 0 (<5% of tumor cells), 1 (6-25% of tumor cells), 2 (26-50% of tumor cells), 3 (51-70% of tumor cells), and 4 (>70% of tumor cells) (22). Scores for intensity and proportion were multiplied to give a total score. The staining pattern of the slides was defined as follows: 0 to 3 (low beclin 1 expression) and 4 to 7 (high beclin 1 expression) (23). Tumor cells were considered immunopositive for Bcl-2 and p53 when stained in 30% or more of the cells (22,24). All slides were evaluated independently by two observers without clinicopathological information.

Statistical methods

Statistical analysis was performed using the SPSS software package (version 16.0; SPSS, USA). A P value of less than 0.05 was considered to be significant. Association was evaluated according to the Fisher exact test between beclin 1, Bcl-2, and p53 expression and several clinicopathological variables. The Kaplan-Meier method was applied to determine the probability of survival, and data were analyzed by applying the log-rank test. Overall survival was defined as date of surgery to date of death due to cancer.

Results

Beclin 1 expression and its association with clinicopathological variables

Normal head and neck glands showed moderate to strong beclin 1 expression (Figure 1). Beclin 1 expression
was also found in the plasma membrane, cytoplasm, and/or nucleus of tumor cells. Beclin 1 expression was present in 32 cases (91.4%; see Figure 2) and considered to be high in 15 cases (42.9%) and low in 20 cases (57.1%). The correlations between beclin 1 and clinical variables are listed in Table 1. Beclin 1 expression was significantly correlated with histological growth patterns (P = 0.046) and histological grade (P = 0.037). Beclin 1 expression was not significantly associated with age (P = 0.741), gender (P = 1.000), primary site (P = 1.000), TNM stage (P = 1.000), lymph node involvement (P = 0.365), tumor size (P = 0.430), perineural invasion (P = 0.738), perivascular invasion (P = 1.000), or lymphatic invasion (P = 1.000).

Bcl-2 expression and its association with clinicopathological variables

Bcl-2 expression of adenoid cystic carcinoma was observed in 21 cases (60.0%; see Figure 1). Correlations between Bcl-2 and clinical variables are listed in Table 1. Bcl-2 expression in head and neck adenoid cystic carcinoma was significantly correlated with perineural invasion (P = 0.041; Table 1). Bcl-2 expression was not significantly associated with age (P = 1.000), gender (P = 1.000), primary site (P = 0.282), TNM stage (P = 1.000), lymph node involvement (P = 1.000), tumor size (P = 1.000), histological growth pattern (P = 0.089), histological grade (P = 0.296), perivascular invasion (P = 0.635), or lymphatic invasion (P = 0.369).

p53 expression

As shown in Table 1, positive p53 expression of adenoid cystic carcinoma was observed in 16 cases (45.7%; Figure 1). In the 20 tumor tissues with low beclin 1 expression, 6 were positive and 14 were negative for p53 expression. In contrast, in the 15 tumor tissues with high beclin 1 expression, 10 were positive and 5 were negative for p53 expression. p53 expression was directly
Table 1. Correlation between beclin 1 expression and Bcl-2 expression and clinicopathologic variables in 35 cases of head and neck adenoid cystic carcinoma.

| Variables                         | Beclin-1 expression | P    | Bcl-2 expression | P    |
|----------------------------------|---------------------|------|------------------|------|
|                                  | Low expression      |      | Bcl-2 expression |      |
|                                 | High expression     |      |                  |      |
| Age (years)                      |                     |      |                  |      |
| <48                              | 8 (22.9%)           | 0.741| 6 (17.1%)        | 9 (25.7%) | 1.000|
| ≥48                              | 12 (34.3%)          |      | 8 (22.9%)        |      |
| Gender                           |                     |      |                  |      |
| Female                           | 9 (25.7%)           | 1.000| 6 (17.1%)        | 9 (25.7%) | 1.000|
| Male                             | 11 (31.4%)          |      | 8 (22.9%)        |      |
| Primary site                     |                     |      |                  |      |
| Major salivary gland\textsuperscript{a} | 13 (37.1%)       | 1.000| 11 (31.4%)       | 12 (34.3%) | 0.282|
| Minor salivary gland\textsuperscript{b} | 7 (20.0%)        |      | 3 (8.6%)         | 9 (25.7%) |
| TNM stage                        |                     |      |                  |      |
| I, II                            | 7 (20.0%)           | 1.000| 5 (14.3%)        | 7 (20.0%) | 1.000|
| III, IV                          | 13 (37.1%)          |      | 9 (25.7%)        | 14 (40.0%) |
| Lymph node involvement           |                     |      |                  |      |
| Absent                           | 16 (45.7%)          | 0.365| 12 (34.3%)       | 18 (51.4%) | 1.000|
| Present                          | 4 (11.4%)           |      | 2 (5.7%)         | 3 (8.6%) |
| Tumor size (cm)                  |                     |      |                  |      |
| ≤3 cm                            | 17 (48.6%)          | 0.430| 11 (31.4%)       | 17 (48.6%) | 1.000|
| >3 cm                            | 3 (8.6%)            |      | 4 (11.4%)        |      |
| Histological growth pattern      |                     |      |                  |      |
| Tubular, cribriform              | 9 (25.7%)           | 0.046| 11 (31.4%)       | 10 (28.6%) | 0.089|
| Solid                            | 11 (31.4%)          |      | 3 (8.6%)         | 11 (31.4%) |
| Histological grade               |                     |      |                  |      |
| Well, moderately                 | 8 (22.9%)           | 0.037| 10 (28.6%)       | 10 (28.6%) | 0.296|
| Poorly                           | 12 (34.3%)          |      | 4 (11.4%)        | 11 (31.4%) |
| Perineural invasion              |                     |      |                  |      |
| Absent                           | 9 (25.7%)           | 0.738| 10 (28.6%)       | 7 (20.0%) | 0.041|
| Present                          | 11 (31.4%)          |      | 4 (11.4%)        | 14 (40.0%) |
| Perivascular invasion            |                     |      |                  |      |
| Absent                           | 18 (51.4%)          | 1.000| 13 (37.1%)       | 18 (51.4%) | 0.635|
| Present                          | 2 (5.7%)            |      | 1 (2.9%)         | 3 (8.6%) |
| Lymphatic invasion               |                     |      |                  |      |
| Absent                           | 17 (48.6%)          | 1.000| 11 (31.4%)       | 19 (54.3%) | 0.369|
| Present                          | 3 (8.6%)            |      | 3 (8.6%)         | 2 (5.7%) |
| p53 expression                   |                     |      |                  |      |
| Positive                         | 6 (17.1%)           | 0.044| 6 (17.1%)        | 10 (28.6%) | 1.000|
| Negative                         | 14 (40.0%)          |      | 8 (22.9%)        | 11 (31.4%) |

\textsuperscript{a}Submandibular gland, parotid gland, sublingual gland; \textsuperscript{b}maxillary sinus, nasal cavity, base of tongue, floor of the mouth, external acoustic canal. *P<0.05, high compared to low beclin 1 expression and positive compared to negative Bcl-2 expression ($\chi^2$ test).

correlated with beclin 1 expression (P = 0.044), but not significantly with Bcl-2 (P = 1.000).

**Relationship between beclin 1 and Bcl-2 expression**

As shown in Table 2, in the 20 tumor tissues with low beclin 1 expression, 4 were negative and 16 were positive for Bcl-2 expression. By contrast, in the 15 tumor tissues with high beclin 1 expression, 10 were negative and 5 were positive for Bcl-2 expression. Beclin 1 expression was inversely correlated with Bcl-2 expression in head and neck adenoid cystic carcinoma (P = 0.013).

**Results of the overall survival analysis**

The mean follow-up period was 38 months (range, 5-107 months), and, for 35 patients, 15 died of disease, 18 were alive on the day of the study, and 2 were lost during the follow-up period. Based on the use of the Kaplan-Meier method, overall survival in the high beclin 1 expression group tended to be better than in the low beclin 1 expression group (P = 0.006; see Figure 2). Bcl-2 expression showed no significant difference for the overall survival rate in patients with adenoid cystic carcinoma (P = 0.053; see Figure 3). As shown in Table 3, the absent
lymph node involvement group survived better than the group with lymph nodes present \((P=0.017)\), the group with a solid histological growth pattern had worse overall survival than the group with tubular and cribriform histological growth patterns \((P=0.000)\), and the well and moderate histological grade group had better overall survival than the poor histological grade group \((P=0.000)\). Overall survival was not significantly associated with age \((P=0.185)\), gender \((P=0.793)\), primary site \((P=0.524)\), TNM stage \((P=0.606)\), tumor size \((P=0.457)\), perineural invasion \((P=0.081)\), perivascular invasion \((P=0.665)\), lymphatic invasion \((P=0.569)\), or p53 expression \((P=0.135)\).

**Discussion**

In this study, we demonstrated that 91.4% of patients expressed the beclin 1 protein in head and neck adenoid cystic carcinoma tissues. This phenomenon also appeared in human gastric and colorectal cancers in which the expression of beclin 1 was 83 and 95%, respectively, but was different from human breast cancer, intrahepatic cholangiocellular carcinoma, chondrosarcoma, melanomas, and glioblastoma multiform tissue in which there was a significant loss of beclin 1 protein expression \((10,13,24-27)\). In our data, beclin 1 expression was used to divide patients into two groups: one with high beclin 1 expression and one with low beclin 1 expression. Beclin 1 expression showed a statistically significant correlation with histological growth pattern and histological grade, and clinical characteristics were good in patients with high beclin 1 expression and poor in patients with low beclin 1 expression. The cumulative Kaplan-Meier survival curve showed that survival in the high beclin 1 expression group tended to be better than in the low beclin 1 expression group, demonstrating that beclin 1 could be a good indicator of prognosis in head and neck adenoid cystic carcinoma. Autophagy is activated in cancer cells accommodating to multiple stresses and has been demonstrated to facilitate tumor cell survival \((6)\). However, genetic evidence supports that autophagy functions as a tumor suppressor mechanism \((28)\).

**Table 2.** Correlation between beclin 1 expression and Bcl-2 expression in 35 cases of head and neck adenoid cystic carcinoma.

| Bcl-2     | Low expression | High expression | \(\chi^2\) test |
|-----------|----------------|-----------------|----------------|
| Negative  | 4 (11.4%)      | 10 (28.6%)      | 0.013*         |
| Positive  | 16 (45.7%)     | 5 (14.3%)       |                |

* Significantly different by the \(\chi^2\) test.

**Figure 3.** Cumulative Kaplan-Meier survival curve (Bcl-2) showing that the survival in the negative Bcl-2 expression group \((n=14)\) and the positive Bcl-2 group \((n=21)\) was not significantly different.

**Table 3.** Analysis of 12 clinicopathologic variables for overall survival rate in 35 cases of head and neck adenoid cystic carcinoma.

| Variables                               | Overall survival rate (\(P\)) |
|-----------------------------------------|-------------------------------|
| Age (<48/>=48 years)                    | 0.185                         |
| Gender (female/male)                    | 0.793                         |
| Primary site (major/minor)              | 0.524                         |
| TNM stage (I, II/III, IV)               | 0.606                         |
| Lymph node involvement (absent/present) | 0.017*                        |
| Tumor size (<=3 cm/>=3 cm)              | 0.457                         |
| Histological growth pattern (tubular, cribriform/solid) | 0.000*                        |
| Histological grade (well, moderately/poorly) | 0.000*                        |
| Perineural invasion (absent/present)    | 0.081                         |
| Perivascular invasion (absent/present)  | 0.665                         |
| Lymphatic invasion (absent/present)     | 0.569                         |
| p53 expression (positive/negative)      | 0.135                         |

TNM: tumor-node-metastasis. *Significantly different by the Kaplan-Meier test.
hampered, autophagy-incompetent cells are more prone to undergo necrosis in response to metabolic stress, whereas autophagy-competent cells are more resistant to cell death (30). In addition, a recent study demonstrated that autophagy is necessary for the establishment of oncogene-induced senescence, which provides an alternative mechanism for autophagy-mediated tumor suppression (31). Therefore, further research is required to show whether and how autophagy inhibits or promotes head and neck adenoid cystic carcinoma.

Our findings demonstrated that beclin 1 expression was inversely correlated with Bcl-2 expression in head and neck adenoid cystic carcinoma tissue. It appeared likely that Bcl-2 inhibited beclin 1-dependent autophagy. This finding is consistent with those of previous studies on solid tumors, which have shown that Bcl-2 negatively regulates beclin 1-dependent autophagic cell death (9,23,32). Accordingly, it could be assumed that Bcl-2 may be an oncogene that not only inhibits apoptosis but also blocks autophagy (33). In addition to Bcl-2, other anti-apoptotic proteins, bcl-xL, bcl-w, and mcl-1 in the Bcl-2 family are also able to interact with beclin 1 and suppress autophagy (33). Camisasca et al. (34) reported that Bcl-2 expression emerged as an independent marker in survival analysis and the expression of Bcl-2 family members seems to be an important marker of a favorable prognosis in oral squamous cell carcinoma. We found that Bcl-2 expression showed a statistically significant correlation with perineural invasion but survival of negative Bcl-2 was inversely correlated with Bcl-2 expression. Variants of p53 that preferentially localize to the cytoplasm effectively repressed autophagy, whereas p53 mutants that display a prominently nuclear distribution failed to inhibit autophagy in HCT 116 colon carcinoma cells (36). Therefore, further studies are necessary to determine the functions in autophagy of the mutant p53 protein localized in the nucleus of adenoid cystic carcinoma cells.

For overall survival, we found that the absent lymph node involvement group had better survival than the present group, a solid histological growth pattern was worse than a tubular and cribriform histological growth pattern, and well and moderate histological grade had better survival than poor histological grade. This result is consistent with previous findings that solid type is related to a poor prognosis, contrary to the cribriform or tubular types, which had a better prognosis (1,37).

In conclusion, we have characterized the expression of beclin 1, Bcl-2, and p53 in head and neck adenoid cystic carcinoma tissue. Our results indicated that beclin 1 may be a novel, promising prognostic factor for clinical outcomes in head and neck adenoid cystic carcinoma patients. Therefore, it seems reasonable to assume that beclin 1 might play a part in the development of head and neck adenoid cystic carcinoma by interacting with Bcl-2 and p53. Further studies are warranted to confirm our findings and to clarify the involvement of beclin 1 in the development of head and neck adenoid cystic carcinoma.

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