Oral Oncology 47 (2011) 365–370

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Expression of Cyr61 in primary salivary adenoid cystic carcinoma and its relation to Ki-67 and prognosis

Qiong-lan Tang [a,1], Song Fan [b,1], Hai-gang Li [a], Wei-liang Chen [b,*], Xi-ming Shen [a], Xiao-ping Yuan [c], Shao-hai Chang [b], Yang Song [d]

[a] Department of Pathology, The Sun Yet-sen Memorial Hospital (Second Affiliated Hospital) of Sun Yat-sen University, Guangzhou, Guangdong 510120, China
[b] Department of Oral and Maxillofacial Surgery, The Sun Yet-sen Memorial Hospital (Second Affiliated Hospital) of Sun Yat-sen University, Guangzhou, Guangdong 510120, China
[c] Department of Radiology, The Sun Yet-sen Memorial Hospital (Second Affiliated Hospital) of Sun Yat-sen University, Guangzhou, Guangdong 510120, China
[d] Department of Oral and Maxillofacial Surgery, Guanghua College of Stomatology, Institute of Stomatological Research, Sun Yat-sen University, Guangzhou, Guangdong 510055, China

ARTICLE INFO

Article history:
Received 3 December 2010
Received in revised form 7 February 2011
Accepted 27 February 2011
Available online 24 March 2011

Keywords:
Salivary adenoid cystic carcinoma
Clinicopathological features
Cyr61
Ki-67
Prognosis

SUMMARY

Cysteine-rich protein 61 (Cyr61) selectively binds heparin and insulin-like growth factors and mediates a variety of biological actions, including cell adhesion, differentiation, proliferation, migration, angiogenesis, and tumorigenesis. Cyr61 is also a prognostic factor for tumor progression and survival of individuals with various types of tumors. This study investigated the relationship between the expression level of Cyr61 and clinicopathological features, as well as the prognostic significance of Cyr61 expression in human salivary adenoid cystic carcinoma (SACC). The expression of Cyr61 and Ki-67, a cell-proliferation marker, was examined immunohistochemically in paraffin embedded tissue specimens from 60 SACC patients who underwent radical surgery between 1995 and 2004. A chi-square test was used to investigate the relationship between Cyr61 and Ki-67 expression and clinicopathological features. Survival analysis was performed to determine the prognostic significance of Cyr61 expression. Cyr61 expression was observed in 39 cases (39/60, 65%) of SACC, and Cyr61 expression was positively correlated with Ki-67 expression ($P = 0.002$). A high expression of Cyr61 was significantly associated with solid subtype, perineural invasion, vascular invasion or cancer embolus, advanced stage, recurrence, and metastasis ($P < 0.05$). The survival rate of patients with high expression of Cyr61 or Ki67 was significantly lower than that of patients with low expression. Multivariate Cox's proportional hazards analysis showed that vascular invasion, TNM stage, recurrence, distant metastasis, Ki-67 expression, and Cyr61 expression were independent prognostic factors of overall survival ($P < 0.05$). Cyr61 expression is significantly correlated with Ki-67 expression and may have potential value in screening high-risk cases for recurrence and metastasis, as well as identifying poor prognosis in SACC patients.

Introduction

Salivary adenoid cystic carcinoma (SACC) is one of the most common salivary gland malignant tumors. It is characterized by slow growth, perineural and perivascular invasion, and the potential to produce late locoregional recurrence and distant metastasis.[1] The rate of distant metastasis in SACC, mainly to the lungs and bones, is the highest among maxillofacial-region malignant neoplasms. Its peculiar biological behaviors always cause great difficulty in treatment and prognosis. Thus, investigations of the biological characteristics of SACC infiltration and metastasis are very important.[2]

Cysteine-rich protein 61 (Cyr61) belongs to the family of CCN matricellular proteins that comprises Cyr61, connective tissue growth factor (CTGF), nephroblastoma overexpressed (Nov), Wnt-induced secreted protein 1 (WISP-1), WISP-2, and WISP-3.[3] Cyr61 selectively binds heparin and insulin-like growth factors and mediates a variety of biological actions, including cell adhesion, differentiation, proliferation, migration, and angiogenesis.[3,4] Previous studies have demonstrated that the expression level of Cyr61 is correlated with tumor growth and metastasis in breast cancers, gliomas, extranodal NK/T-cell lymphoma, and esophageal carcinoma, and is also a prognostic factor for tumor progression and survival of individuals with these tumors.[5–8] Ki-67 is considered to be a more accurate marker of the proliferative stage of tumor cells than proliferating-cell nuclear antigens (PCNA),[9] and Ki-67 immunoreactivity has been reported to be correlated with the prognosis of SACC.[10]

* Corresponding author. Address: Department of Oral and Maxillofacial Surgery, Sun Yet-sen Memorial Hospital (Second Affiliated Hospital) of Sun Yat-sen University, 107 Yan-jiang Road, 510120 Guangzhou, China. Tel.: +86 020 81332429; fax: +86 020 81332853.
E-mail address: drchen@vip.163.com (W.-l. Chen).
[1] These authors contributed equally to this work.

1368-8375/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.oraloncology.2011.02.022

However, the utility of Cyr61 in SACC prognosis has not been evaluated. We reviewed SACC medical data in our hospital and Cyr61 expression was detected in tumor tissues from 60 SACC cases. We then investigated the relationship between the expression level of Cyr61 and clinicopathological features of SACC, as well as patient clinical outcomes.

Materials and methods

Reagents

Rabbit anti-human polyclonal antibody for Cyr61 (SC-13100) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse anti-human monoclonal antibody for Ki-67 (clone 7B11) from Zymed (San Francisco, CA, USA), and a general ultrasensitive streptavidin peroxidase immunohistochemistry system from Beijing Zhongshan Golden Bridge Biotechnology Co. (Beijing, China). DAB chromogen solution was from Dako (Carpinteria, CA, USA). All other chemicals used were of analytical reagent grade.

Patients and tumor samples

Sixty-five patients with SACC underwent radical surgery at the Department of Oral and Maxillofacial Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China, between January 1995 and December 2004. Five cases without complete clinicopathological materials were excluded. Thus, the experimental group comprised 60 SACC cases, with 10 specimens of normal salivary gland tissues used as the control group. None of these SACC patients had received radiotherapy, chemotherapy, or other related anti-tumor therapies before surgery. Data gathered included age, gender, site of origin, surgical margin status, histological type, neural invasion, vascular invasion or cancer embolus, TNM classification, recurrence, distant metastasis, treatment, and survival time.

The SACC group comprised 29 males and 31 females. The average age at surgery was 53 years (range: 23–74 years). Twenty-six tumors with ACC of the major salivary glands (10 from the sublingual gland, nine from the submandibular gland and seven from the parotid gland) were identified; 34 showed ACC of the minor salivary glands (11 originated from the palate, nine from the tongue, six from the cheek, six from the mouth floor and two from the retromolar pad). All of the surgical margins were negative. Histopathological diagnosis and histological subtypes were determined according to the guidelines of World Health Organization,[1] with 28 of the cribriform-tubular subtype and 32 of the solid subtype. At the time of surgery, 28 cases of SACC showed nerves surrounded or invaded by tumor cells, six cases had lymph node (LN) metastasis, and 20 cases had vascular invasion or cancer embolus (Fig. 1A–F). Patients were classified according to clinical stage based on the 2010 criteria of the International Union against Cancer:[11] 12 cases were in stage I, eight in stage II, 18 in stage III, and 22 in stage IV. Cases in stages I and II were categorized as the early-stage group and those in stages III and IV as the advanced-stage group. Radiological examination and postoperative biopsy confirmed 15 patients with recurrence and 20 with distant metastasis after surgery. In SACC surgery and treatment, 16 patients underwent extended resection of the primary tumor alone, 14 had extended resection and ipsilateral modified neck dissection, 12 had extended resection and postoperative radiotherapy of 40–60 Gy with or without postoperative chemotherapy (5-fluorouracil + methotrexate + vincristine), and six underwent extended resection and ipsilateral modified neck dissection plus postoperative radiotherapy with or without postoperative chemotherapy.

Tumor specimens were collected after obtaining patient informed consent in accordance with institutional guidelines.

Follow-up data

All durations were measured from the date of surgery to the date of dropout, SACC-related death, or last contact for patients who were still alive when the survival duration was calculated. The follow-up period lasted 8–120 months, ending 31 October 2010.

Immunohistochemical staining

Histological material fixed in 10% formalin and embedded in paraffin was cut into 4-μm sections. Immunostaining was performed using a streptavidin-peroxidase immunostaining kit following the manufacturer's instructions. Sections were deparaffinized in xylene and hydrated using distilled water according to standard methods. Antigens were retrieved by immersing sections in EDTA buffer solution (pH 8.0) and then boiling them in a microwave for 15 min. They were then cooled for 1 h at room temperature and washed in water and phosphate-buffer saline (PBS). Next, 3% hydrogen peroxide was applied to block endogenous peroxide activity, and the sections were incubated with normal goat serum to reduce nonspecific binding. The sections were subsequently incubated with anti-Cyr61 (1:600) or anti-Ki-67 (1:50) at 4 °C overnight. Later, general biotin-linked secondary antibody was added and incubated at 37 °C for 30 min. Streptavidin peroxidase solution was then added and incubated at 37 °C for 30 min. DAB staining was applied for 5 min for chromogenesis. Hematoxylin was used to counterstain nuclei. The sections were then mounted.

For a negative control, preimmune serum was used in place of primary antibody. For a positive control, a known positively expressing tissue (mammary carcinoma) was used.

Evaluation of Cyr61 and Ki-67 immunostaining

Immunostaining staining was evaluated by two independent observers who were blinded to clinical data. Yellow-brown granules in the nucleus or cytoplasm indicated a positive result. Five high-power fields were randomly selected for observation. All of the experiments were repeated three times.

The positive localization of Cyr61 was in the cytoplasm. Under a high-power objective lens, a section was scored according to staining intensity and staining area, as described previously.[12] The scoring for positive cells was set arbitrarily as follows: <10% = 0 points, 10–25% = 1 point, 25–50% = 2 points, and $\geqslant$50% = 3 points. Although there was variability in the total number of cells counted in each specimen, the percentage of positively stained was estimated by counting 300 cells per area from 5 high-power fields. Scoring differences were discussed to reach consensus. Staining strength was scored as follows: weak = 1 point, moderate = 2 points, strong = 3 points. The sum of the two values determined the result: a total of 1 point was negative, and 2–5 points was positive.

The positive localization of Ki-67 was in the nucleus. Nuclei from >500 tumor cells were counted microscopically among 5–8 random fields in each sample, and the average of the two scores were obtained. Ki-67 expression was classified as high at $\geqslant$4.0% and low at <4.0%.[10]

Statistical analysis

All statistical analyses were performed using the SPSS WIN program package 13.0 (SPSS, Inc., Chicago, IL, USA). A chi-square test was used for single-factor analysis to determine significance. The overall survival curves were constructed using the Kaplan–Meier method, and differences were compared by log-rank tests. Cox's multivariate proportional hazard regression model was used to