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Deregulation of Cell Growth and Caspase-3 Expression in Mucoepidermoid Carcinoma of the Salivary Glands

Kuo-Chou Chou[1], Shu-Hui Li[1], Huan-Ching Su[1], Hsing-Wen Hung[2], Long-Chang Chang[1,2], and Yi-Shing Shieh[1,2*]

[1]Department of Oral Diagnosis & Pathology, Tri-service General Hospital, National Defense Medical Center, Taipei, [2]School of Dentistry, National Defense Medical Center, Taipei, Taiwan, Republic of China

Tumor growth is dependent on the balance between proliferation and apoptosis of tumor cells and caspase-3 is one of the primary executioners of apoptosis. However, limited information of these biomarkers is available for mucoepidermoid carcinoma (MEC) of the salivary glands. This study investigates cell proliferation and apoptosis, and caspase-3 expression in MEC and their association with the clinicopathological features of MEC. Ki-67 and caspase-3 immunohistochemical staining and terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) were evaluated in tissues from 41 MEC patients. The Ki-67 staining showed 54% cases with a low proliferation index (PI) and 46% a high PI. The TUNEL staining showed 51% cases with a low apoptosis index (AI), and 49% cases with a high AI. Low and high PI/AI ratios were observed in 49% and 51% of samples, respectively. Significant correlations between PI and tumor grade and lymph node status, and PI/AI ratio with tumor metastasis were noted. Caspase-3 expression was correlated significantly with tumor stage: 44% of samples were caspase-3 positive and 56% negative. Furthermore, caspase-3 expression inversely correlated with AI. Our data suggest that the PI, PI/AI ratio, and caspase-3 expression are potentially useful markers of progression in patients with MEC.

Key words: apoptosis, caspase-3, mucoepidermoid carcinoma, proliferation

INTRODUCTION

Mucoepidermoid carcinoma (MEC) is the most common malignant tumor of the salivary glands[1]. Several studies have attempted to identify clinical and pathological features with prognostic relevance for MEC and have shown that the histopathological appearance seems to have a significant relationship with clinical behavior[2,3]. However, clinical behavior unrelated to histological morphology has also been reported[4]. With this controversy regarding the histological grading of MEC, many investigators have proposed various subclassifications and histopathological grading criteria. However, the complexity of morphological features and histological grades make it difficult to predict the clinical behavior and outcome of MEC[4]. Thus, it is important to identify biological markers to predict the tumor behavior in patients with MEC.

Many biomarkers have been evaluated to determine their relationship with carcinogenesis, cancer progression, and in particular, those critical processes common to the carcinogenic cascade in a vast array of tissues: proliferation and apoptosis[5]. The key aspect of cancer is uncontrolled tumor growth and the balance between proliferation and apoptosis is crucial in determining the overall growth or regression of a tumor[5]. Any disruption in this balance may allow the rate of cancer cell proliferation to exceed that of cell death, resulting in the net continuous accumulation of malignant cells. Examining proliferation and apoptosis and their regulation would help delineate the biology of individual tumors at the molecular and biochemical level, which might provide a clinical advantage.

Induction of apoptosis involves the activation of specific death-signaling pathways, including the activation of proteases termed caspases[6]. The caspases are a family of cysteine proteases that play an essential role in the initiation, regulation, and execution of the downstream proteolytic events that occur during apoptosis[6]. Caspase-3 is one of the primary executioners of apoptosis and is needed for the cleavage of many proteins and for apoptosis-associated chromatin margination, DNA fragmentation, and nuclear

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*Corresponding author: Yi-Shing Shieh, School of Dentistry, National Defense Medical Center. P.O. Box 90048-503, Taipei, Taiwan. Tel: +886-2-8792-3148 Fax: +886-2-8791-9276 or +886-2-8792-3149; E-mail: ndmcyss@nhri.org.tw (YS Shieh)

collapse during apoptosis[7]. The detection of caspase-3 might be a valuable and specific tool for identifying apoptotic cells in tissue sections, even before the appearance of apoptotic morphological features. Little information is available about the expression of caspases in tumor development and progression in MEC.

We investigated cell proliferation activity by Ki-67 immunostaining, and the frequency of apoptosis by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) and caspase-3 expression in 41 cases of salivary gland MEC. We also studied whether clinicopathological factors were correlated with these measures of tumor cell proliferation and apoptosis.

METHODS

Samples

Formalin-fixed, paraffin-embedded tissue blocks were obtained from 41 patients with MEC diagnosed between January 1980 and March 2006. Specimens were obtained from either an incision biopsy or total surgical excision of the tumors. Clinical information was obtained from 35 patients with MEC, none of whom had received any form of tumor-specific therapy before diagnosis. Six patients were excluded from the correlation analysis because their clinical information was not available. Histological grading and clinical staging were determined by methods described previously[8].

Immunohistochemical Staining and In Situ Apoptotic Cell Detection

All specimens for immunohistochemical staining were fixed in 10% neutral formalin, embedded in paraffin, and cut in 5μm serial sections. Immunohistochemical staining was performed with the DAKO EnVision stain system (Dako, Copenhagen, Denmark). Briefly, tissue sections were deparaffinized, rehydrated, and then heated in a plastic slide holder containing 10 mM citrate buffer (pH 6.0) in a microwave oven for 30 min to retrieve antigenicity. Endogenous peroxidase activity was blocked by immersing the sections in 3% H_2O_2 in methanol for 10 min. After washing in 10 mM Tris-buffered saline (pH 7.4), sections were incubated with 10% normal goat serum to block nonspecific binding. Sections were then incubated with primary antibodies—monoclonal mouse anti-human Ki-67 antibody (250μg/mL, 1/200 dilution, Dako) or polyclonal rabbit anti-human cleaved caspase-3 antibody (Dako, Carpinteria, CA)— for 60 min at room temperature, followed by incubation with the peroxidase labeled polymer for 30 min. Diaminobenzidine hydrochloride (DAB) containing 0.03% H_2O_2 was used as the chromogen to visualize the peroxidase activity. Sections were counterstained with hematoxylin and cover-slipped. For the negative control, primary antibodies were omitted or replaced with IgG from the same species, and all other steps followed.

To identify apoptotic cells, TUNEL was performed using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, Temecula, CA) according to the manufacturer's instructions.

Determination of Proliferation Index and Apoptotic Index

To quantify cell proliferation and apoptosis, stained tissue sections were initially screened at low power (100×) to identify the hot spot areas. The proliferation index (PI) was calculated as the percentage of Ki-67-positive cells and the apoptotic index (AI) was calculated as the percentage of TUNEL positive cells relative to the total number of counted tumor cells. These ratios were calculated from observations of > 1000 cells in each section, as described previously[9]. The balance between cell proliferation and apoptosis was quantified by dividing the PI by the AI, giving a PI/AI ratio. For cutoff point analysis, the median values of PI, AI, and PI/AI were used to categorize the tumor into high and low groups.

Assessment of Caspase-3 Expression

Caspase-3 immunoreactivity was assessed as described previously, with some modification[10,11]. Briefly, the degree of immunostaining was evaluated as follows: (— to ±) no staining or faint staining intensity, (+) moderate staining intensity, (++) fair staining intensity, and (+++) strong staining intensity in the cytoplasm and/or nucleus. For further analysis, carcinomas displaying the staining patterns graded ++ and +++ were regarded as positive for staining, and the other two patterns (— to ± and +) were grouped together and classified as negative for staining.

Statistical Analysis

The proportions of high or low PI, AI, and PI/AI ratio and positive or negative caspase-3 expression were analyzed using the χ^2 test to compare these with clinicopathological features. Statistical significance was set at $P < 0.05$.

RESULTS

PI and AI in Normal and Cancer Tissues

To examine the proliferation and apoptosis of cells in tissues, we stained sections with anti-Ki-67 and TUNEL. In the epithelium, nuclear Ki-67 reactivity was confined to

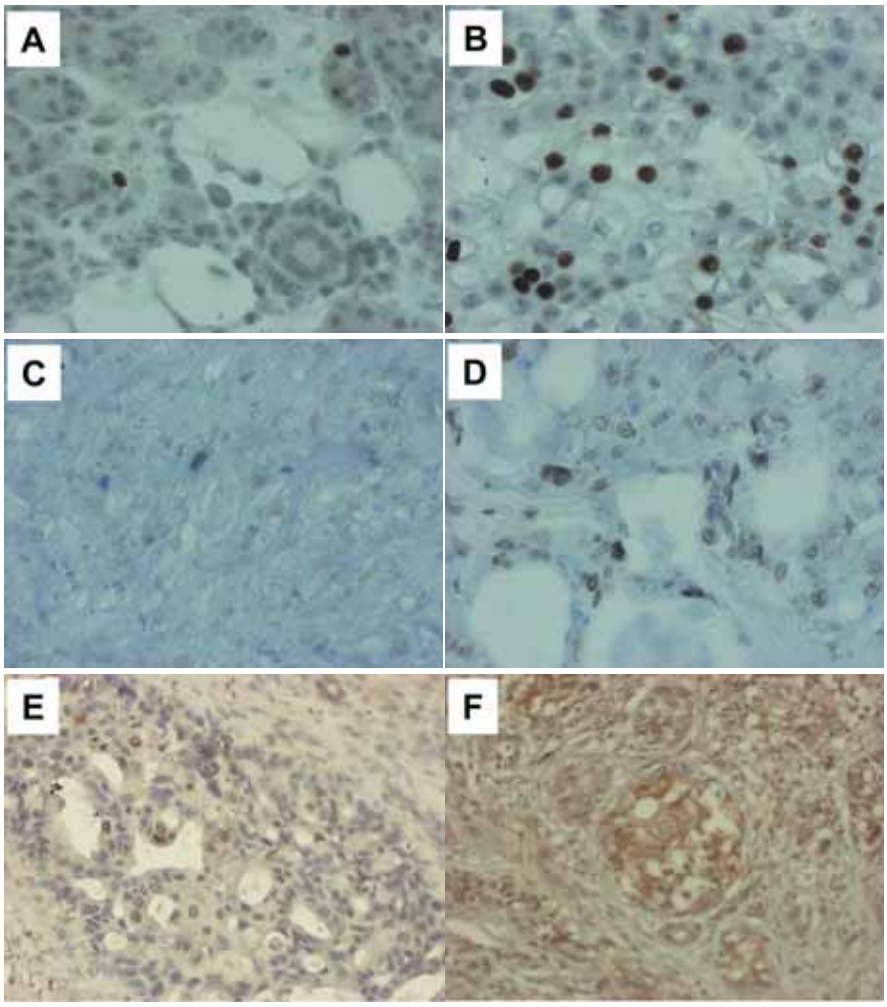

Fig. 1 Representative sections of PI and AI with low (A, C) and high (B, D) index, and negative (E) and positive (F) caspases-3 expression in MEC. (A) Few Ki-67 positive cells were observed in MEC tumor low PI index. (B) In the high PI index tumor, the Ki-67 stained positive tumor cells were observed frequently in the nuclei of intermediate and epidermoid cells. (C) In low AI tumor, scarcity of TUNEL positive cells was noted. (D) Increased apoptotic bodies in the tumor cell nuclei were found in the tumor with high AI index. (E) Occasional caspase-3 staining was observed in the tumor with negative staining. (F) Increased caspase-3 staining intensity and percentage of caspase-3 positive cells was noted in the positive staining tumor. (Original magnification × 200).

the basal and suprabasal layers, whereas in the tumors, Ki-67 staining was observed in the nuclei of intermediate and epidermoid cells. A low PI was seen in 54% of cases and a high PI in 46% (Fig. 1A and 1B). Little TUNEL staining was noted in the normal tissues. In the cancerous tissues, apoptotic cells were noted frequently, and 51% of cases had a low AI and 49% a high AI (Fig. 1C and 1D). Low and high PI/AI ratios were observed in 49% and 51% of samples respectively. Table 1 presents the correlations between the PI, AI, and the PI/AI ratio and the clinicopathological features. The PI was significantly correlated with lymph node status ($P = 0.010$) and tumor grade ($P = 0.013$), and the PI/AI ratio correlated significantly with tumor metastasis ($P = 0.045$).

Expression of Caspase-3 in Normal and Cancer Tissues

Germinal center lymphocytes of the lymphoid tissue in the salivary gland tissue and plasma cells showed intense caspase-3 immunostaining, which severed as an internal positive control. In normal epithelium, negative to faint staining was noted. The intensity of the caspase-3 staining of cancerous cells varied and showed a heterogeneous pattern (Fig. 1E and 1F). In MEC samples, caspase-3 immunoreactivity was localized in the cytoplasm, although nuclear staining was frequently noted. Positive and negative caspase-3 immunohistochemical expression was observed in 18 of 41 (44%) and 23 of 41 (56%) of tumor samples, respectively. Table 1 shows the correlations between caspase-3 protein expression and clinicopathological features. Caspase-3 protein expression was significantly correlated with the clinical stage ($P = 0.002$).

In the final set of experiments, we examined whether the expression of caspase-3 correlated with biological variables in the tumors. As shown in Table 2, caspase-3 expression correlated significantly with AI ($P = 0.019$).

DISCUSSION

In MEC, as in most tumors, the clinical stage and histological morphology at the time of diagnosis are the most practical variables for determining the prognosis and for selecting the treatment protoco[8,12]. However, a considerable proportion of MEC patients, even with early-stage disease, develop recurrence after primary treatment[4], warranting a quest for new prognostic factors in MEC. Cellular proliferation and apoptosis each play a crucial role in

Table 1 Correlation of PI, AI, PI/AI, and caspase-3 expression with clinicopathologic features

	PI		AI		PI/AI		Caspase-3 expression	
Characteristic	Low	High	Low	High	Low	High	Negative	Positive
Gender								
Male (13)	8	5	8	5	5	8	7	6
Female (22)	12	10	10	12	13	9	11	11
Location								
Major (27)	14	13	11	16	14	13	18	9
Minor (14)	8	6	10	4	6	8	5	9
Size								
T1-T2 (27)	17	10	13	14	16	11	16	11
T3-T4 (8)	3	5	5	3	2	6	2	6
LN status								
No (22)	[a]16	6	10	12	12	10	15	7
Yes (19)	6	13	11	8	8	11	8	11
Metastasis								
No (31)	19	12	16	15	[c]18	13	16	15
Yes (4)	1	3	2	2	0	4	2	2
Grade								
Low (15)	[b]11	4	10	5	9	6	8	7
Intermediate (10)	7	3	6	4	6	4	4	6
High (16)	4	12	5	11	5	11	11	5
Staging								
Local (18)	12	6	7	11	11	7	[d]14	4
Advanced (17)	8	9	11	6	7	10	4	13

[a], P = 0.010; [b], P = 0.013; [c], P = 0.045; [d], P = 0.002

Table 2 correlation of caspase-3 expression with PI, AI, PI/AI in MEC

	Caspase-3 expression		
	Negative (n = 23)	Positive (n = 18)	P value
PI			
Low (n = 22)	11	11	0.298
High (n = 19)	12	7	
AI			
Low (n = 21)	8	13	**0.019**
High (n = 20)	15	5	
PI/AI			
Low (n = 20)	11	9	0.570
High (n = 21)	12	9	

tumor development. In MEC, few data are available about the relative contributions of these processes to the pathogenesis of the tumor. We compared biomarkers in samples obtained from patients with MEC in different clinical and pathological conditions. Our results show that indices of apoptosis (AI) and proliferation (PI) are higher in tumors than in normal tissues, indicating that the pathogenesis of MEC may result from the deregulation of these factors.

We found a close relationship between the proliferation of cancer cell (Ki-67 labeling index) and histological grade. The number of Ki-67 positive nuclei in mucous cells was significantly lower than in epidermoid and intermediated cells. These results are consistent with previous studies that showed that Ki-67 antigen labeling was correlated to tumor proliferation and clinical outcome in a wide variety of malignancies[13]. Notably, apoptosis of tumor cells alone did not correlate with the clinicopathological features in the present study. Thus, we examined the ratio of cell proliferation to apoptosis (PI/AI) and our results showed that PI/AI is significantly associated with increased tumor size and advanced stage. This finding suggests that proliferation but not apoptosis may be the determining factor in tumor progression in MEC.

Caspases are cysteine proteases that are required for the execution of the cell death process. At least 14 members of the caspase family have been identified, most of which can induce apoptosis when overexpressed in mammalian cells[14]. Caspase-3 is believed to be most commonly involved in the execution of apoptosis in various cell types[15]. Interestingly, we found an inverse relationship between caspase-3 expression and the apoptosis index in our patients and a significant correlation of the intensity of caspase-3 immunostaining with tumor progression, that is, a high proportion of caspase-3 positive cases were advanced tumors. Our data are consistent with a study of pancreatic tumors[11] and suggests that the expression of caspase-3 in tumor cells may reflect aggressive biological features in certain tumors. Recent reports may explain these results[16, 17]. For example, Kanada et al. demonstrated that during apoptosis progression, active caspase-3 is translocated from the cytoplasm into the nucleus, which is necessary for the proteolytic activation of caspase-3[17]. Thus, translocation of active caspase-3 to other subcellular compartments may play an important role in the apoptotic process. If cytoplasmic caspase-3 fails to be activated, it cannot participate in apoptosis and contribute to the aggressive behavior of tumor. Therefore, investigating the regulation of caspase-3 activation could be helpful for clarifying the molecules that inhibit caspase-3 activation.

In conclusion, we have demonstrated that an imbalance between cell survival and death and the expression of caspase-3 may be valuable markers of disease progression in patients with MEC. Application of such markers may provide useful information about the expected biological behavior of tumor cells in salivary gland carcinomas. Such tumor markers have potential in therapeutic strategies aimed to regulate cell survival and death in MEC by inducing apoptosis or by inhibiting proliferation.

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