Expression of Osteopontin Protein in Esophageal Squamous Cell Carcinoma

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Abstract

This study aimed to examine (1) the protein expression of osteopontin (OPN) in esophageal squamous cell carcinoma (ESCC) tissue and (2) whether OPN could be used to predict ESCC patients' disease severity and prognosis. In total, 54 newly-diagnosed ESCC patients who received eophagectomy were studied. OPN protein expression was detected by immunohistochemistry method. The information of the patients' clinico-pathologic characteristics was obtained by chart review. OPN protein overexpression was present in 37.0% (20/54) of tumor tissues and 13.0% (7/54) of normal tissues. It was obviously stronger in cancer part than the corresponding normal part in 35 pairs (64.8%) of ESCC tissues. There were no significant associations between OPN protein expressions and patient's cancer stage or survival. Our findings indicated that OPN was associated with the development of ESCC although it cannot predict patients' survival. (J Intern Med Taiwan 2010; 21: 419-426)

Key words: Osteopontin, Esophageal squamous cell carcinoma, Prognosis

Introduction

Esophageal cancer is the 9th leading cause of cancer deaths in Taiwan and the 6th one among men[1]. The age-adjusted mortality rate was 4.85 per 100,000 people[1] and the incidence of esophageal cancer increased by more than 70% from 1990 to 1999[2]. More than 95% of the cell type is squamous cell carcinoma and its prognosis is very poor (5-year survival rate below 10%)[3-5]. Therefore, early diagnosis using new tumor markers and advanced image study is crucial to improve the treatment outcome.

Osteopontin (OPN) is a secreted adhesive

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glycoprotein; it is not only expressed in bone and epithelium, but also plays an important role in the process of cancer formation[6-8]. Overexpression of OPN protein or transcript has been revealed in several cancer types, such as cancers of breast, stomach, colon and lung, suggesting a role in tumorigenesis[9-12]. Recently, cumulative evidence has indicated that OPN is a candidate marker of tumor prognosis and survival in patients with breast cancer[13], non-small-cell lung cancer[14] and nasopharyngeal carcinoma[15].

Few studies have been conducted to examine the effect of OPN expression on the clinical significance of ESCC, and the results remain inconsistent[16,17]. Thus, the aim of this study was to elucidate the correlation of OPN protein expression with tumor formation, progression, and prognosis of ESCC in Taiwan.

Materials and Methods

Patients and Specimens

Fifty-four newly-diagnosed, histologically-confirmed ESCC patients were recruited from the Departments of Gastroenterology and Thoracic Surgery at Kaohsiung Medical University Hospital in Kaohsiung, Taiwan, between 1997 and 2003[18]. All of them underwent esophagectomy without any previous cancer treatment. They were followed up till the date of data analysis in January 2007. Information about the demographics, disease characteristics (tumor location and tumor-node-metastasis, TNM, stage), course of treatment, and vital and recurrence status was obtained from chart review.

Tumor Staging and Treatment Modality

The extent of the tumor was evaluated in each patient by physical examination, chest radiography, abdominal ultrasonography, gastroendoscopy and computed tomography of the chest[5]. Pulmonary function test and evaluation of heart function were performed to access the patients' operation risk. Bronchoscopy was performed when indicated by symptoms, the location of the tumor, or chest radiography. The presence of tumor metastasis to the lung, regional lymph nodes and the liver was evaluated by computed tomography. Isotope bone scans were occasionally performed if indicated.

The treatment decisions in our hospital were based mainly on the initial TNM system and the presence of organ insufficiency. In patients with resectable disease and normal organ function, radical esophagectomy with lymph node dissection was strongly recommended. If the primary tumor was marginally resectable (T3 or T4), surgery with adjuvant chemoradiation or concurrent chemoradiation therapy (CCRT) were performed. In those of clearly un-resectable disease (stage IVb), definitive chemoradiation was indicated[5]. In this study, we only recruited those who underwent esophagectomy and had a disease stage before IVa for further evaluation of OPN expression.

The tissues used in this study were obtained from the following two locations of the paraffin blocks: (a) tumor; and (b) uninvolved esophageal tissue taken from the maximum distance to the tumor (mean distance from the tumor was 8.3cm). Sections were pooled for analysis from areas estimated by the pathologists (Drs. Yang SF and Wu CC) to have at least 75% malignant cells. This study was approved by the Review Board of the Kaohsiung Medical University Hospital.

Immunohistochemical staining

Each tissue specimen obtained for esophagectomy was routinely embedded in paraffin wax after 10% formalin fixation, and cut into several 3-μm-thick sections for conventional H&E staining and OPN immunostaining by using a polyclonal antibody anti-OPN (AA25-40 United States Biological, USA, dilution at 1:500) with avidin-biotin-peroxidase complex method (DAKO Cytomation LSAB 2 System-HRP, DAKO

Sytomation Inc, USA) following the manufacturer's instructions. The immunostaining was done manually at room temperature. The sections, mounted on glass slides, were deparaffinized through serial baths in xylene and rehydrated in a graded series of alcohol and water. To remove any endogenous peroxidase activity and nonspecific background staining, the sections were soaked in absolute methanol containing 0.3% hydrogen peroxide for 10 minutes at room temperature. After being washed with phosphate buffered saline (PBS) for 5 minutes, slides were incubated with the anti-OPN primary antibody for 60 minutes at room temperature. After rinsing with PBS twice for 5 minutes, sections were subsequently incubated with biotin-conjugated goat anti-mouse IgG antibody for 30 minutes. After being washed with PBS twice for 5 minutes, slides were incubated with avidin-biotin-peroxidase complex for 30 minutes and washed again with PBS twice. Finally, the sections were incubated with 0.05% 3,3V-diaminobenzidine tetrahydrochloride (DAKO Cytomaion liquid DAB + Substrate chromogen System, USA) and then rinsed in distilled water. All slides were lightly counterstained with Mayer's hematoxylin for 30 seconds, washed in running water, dehydrated, and mounted with Canadian balsam. We used the tumor part of an ESCC male patient as the positive control when performing IHC staining.

Evaluation of immunohistochemical staining

The results of OPN staining were read by a qualified pathologist (Dr. Yang SF) who was blinded to the clinical statuses of the patients. During reading, the pathologist also confirmed the quality of the staining and whether enough viable cancer tissue (>70%) was present. According to the modified method of Ito *et al.*[16], the OPN immunoactivity was evaluated in five different areas of each slide to classify into three groups by OPN expression intensity: negative or trace positive for - or +, moderate or focal strong positive for ++, and strong positive for +++.

Statistical Analysis

OPN protein overexpression was defined as ++ or +++. The difference in selected clinicopathologic variables between tumor specimens with and without protein overexpression (yes *vs.* no) was analyzed by chi-square or Fisher's exact test. The significant variables ($p < 0.05$) in the univariate analysis were evaluated in a multiple logistic regression model. Survival curves of stage (I-II, III, IVa) and overexpression of OPN protein (yes *vs.* no) were estimated according to the Kaplan-Meier method from the date of primary tumor surgery to the time of death due to tumor progression. The difference in survival curves was examined by means of the log-rank test. Cox's proportional hazards modeling of factors that were significant in univariate analysis was performed to identify which factors might have a significant influence on survival. The data were analyzed using the SAS statistical package. All *p*-values were two-sided and statistical significance was defined as *p*-value < 0.05.

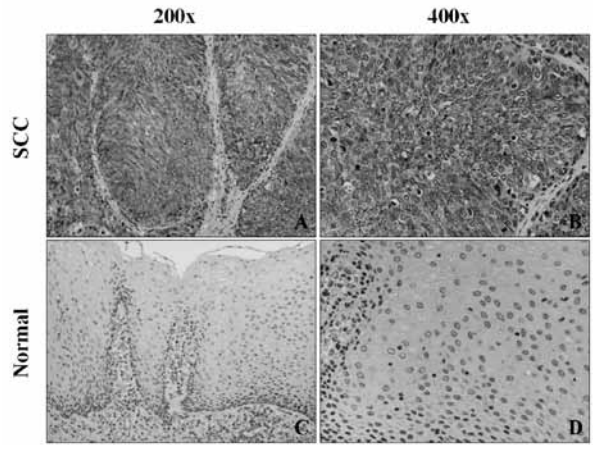

Fig. 1 Osteopontin (OPN) immunostaining in squamous cell carcinoma (SCC) and the adjacent non-cancerous squamous epithelium of esophagus. The cancer cells showed a diffuse cytoplasmic expression of OPN (A and B), while the adjacent non-cancerous squamous epithelium (C and D) showed weakly or no OPN staining. (original magnification, A and C, ×200 , B and D, × 400).

Results

OPN protein expression

OPN was intensively positive in the perinuclear cytoplasm of cancer cells (Fig. 1). The staining intensity was stronger in cancer part than the corresponding normal part in 35 pairs (64.8%) of ESCC tissue. 37.0% (20/54) of the tumor tissues and 13.0% (7/54) of the normal tissues expressed strong positive staining (++, +++) and were defined as presence of OPN protein overexpression. As shown in Table 1, there were no significant associations between OPN protein expression and cancer stage or patients' prognosis. Since cigarette smoking, alcohol drinking and betel quid chewing are three major risk factors for ESCC in Taiwan, we also examined the relationship of those substances use and the expression of OPN protein. However, users did not reveal significantly higher OPN level than non-users (Table 1).

Table 1. Demographic and clincopathologic characteristics and overexpression of OPN protein

	OPN expression		
Variables	Yes	No	p-value
Age (years)			0.33[1]
≤ 65	17 (85)	24 (71)	
>65	3 (15)	10 (29)	
Gender			1.00[1]
Male	18 (90)	31 (91)	
Female	2 (10)	3 (9)	
Smoke			
No	5 (25)	10 (29)	0.73
Yes	15 (75)	24 (71)	
Alcohol			0.36[1]
No	4 (20)	12 (35)	
Yes	16 (80)	22 (65)	
Betel			0.72
No	11 (55)	17 (50)	
Yes	9 (45)	17 (50)	
Tumor differentiation			0.91
Well	5 (25)	9 (26)	
Moderate & poor	15 (75)	25 (74)	
Tumor size			0.60
T1-2	6 (30)	8 (24)	
T3-4	14 (70)	26 (76)	
Nodal status			0.68
N0	10 (50)	19 (56)	
N1-2	10 (50)	15 (44)	
Stage			0.73[1]
I-II	9 (45)	19 (56)	
III	18 (40)	11 (32)	
IVa	3 (15)	4 (12)	
Adjuvant therapy			0.78
No	14 (70)	25 (74)	
Yes	6 (30)	9 (26)	

[1]Fischer exact test.

Survival curve

The median survive of those 54 subjects was 8 months (range = 0-60). In univariate analysis, cancer stage was significantly associated with patients' survival period (Fig. 2A). Although patients with OPN over-expression had shorter life spans after operation than those without, the difference was not significant enough to predict the patients' survival period (p = 0.984, Fig. 2B). After considering for other covariates, only clinical stage can predict the patients' survival period (adjusted hazard ratio = 1.72, 95% confidence interval = 1.01-2.49; p = 0.047).

Discussion

In this study, we identified the increased expression of OPN protein in the 54 paired ESCC tissues. Meanwhile, 13.0% (7/54) of the normal esophageal tissue of ESCC patients were stained positive for OPN protein. OPN plays a role in a variety of physiological cellular functions, including inflammation, apoptosis[19] and the process of tumor formation[6]. However, compared to the corresponding normal tissue, the expression of OPN was even higher in many human cancers, including cancers of breast, stomach, colon, and lung[9-12]. Few studies have examined the expression

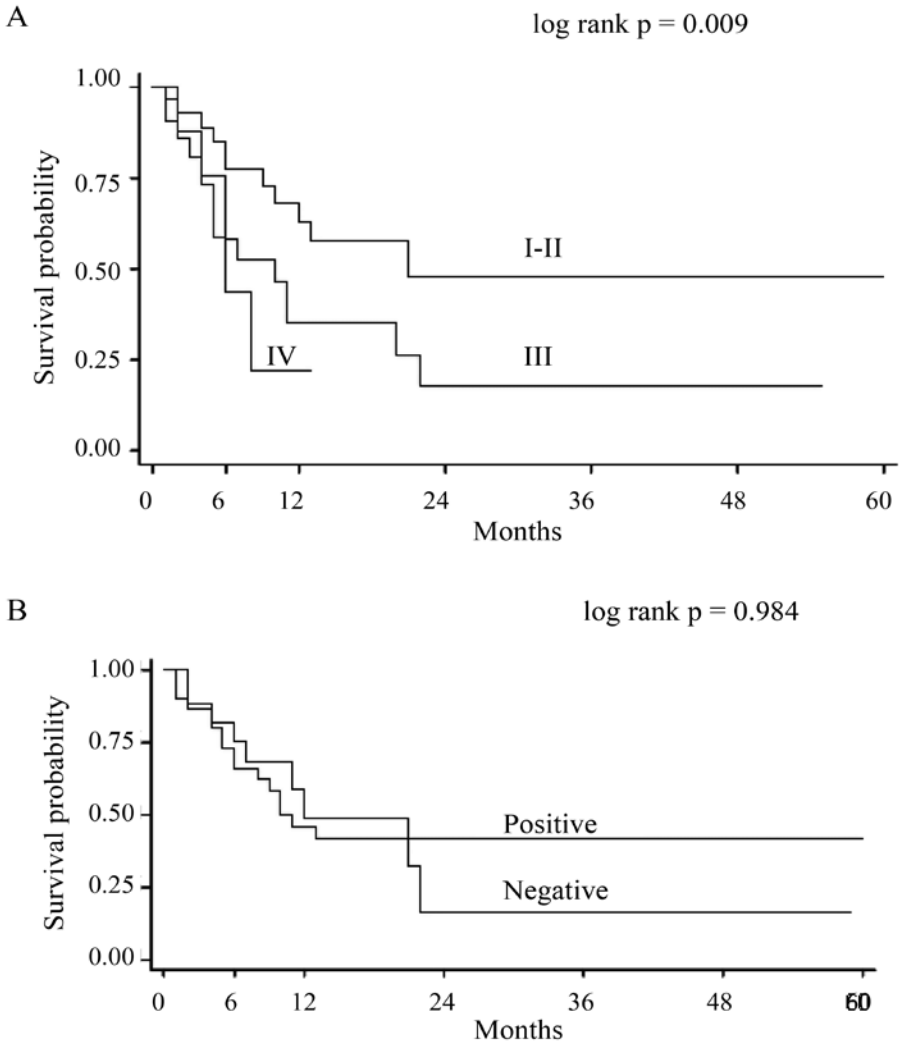

Fig 2. Survival curves for patients stratified by stage (A) and OPN protein levels (B).

of OPN in ESCC. Initially, one study reported an overexpression of OPN mRNA in all 6 squamous cell tumor tissues and 15 out of 19 adenocarcinoma tumor tissues, compared to matched histologically normal esophageal mucosa, by Northern blotting and quantitative densitometry[20]. Coppola *et al.*[8] studied the OPN protein expression in a wide variety of tumors and found 7 out of 10 ESCC tumor had a high cytoplasmic OPN staining. These findings, including ours, suggest OPN play an important role in esophageal tumorigenesis.

OPN level in tumor tissue was also demonstrated to be associated with tumor progression in breast, lung, prostate, and colon cancer[9,11,21,22]. Increased OPN expression was found to be a poor prognostic indicator for survival in patients[9,10,12,23]. Recently, Ito *et al.*[16] reported that OPN protein overexpression revealed by immunohistochemistry was associated with poor prognosis ($p < 0.001$), distant lymph node metastasis ($p = 0.0004$), tumor staging ($p = 0.027$), and histological grade ($p = 0.024$) among 144 clinical tumor specimens from Japanese patients. Cox's proportional hazard model showed OPN protein was the strongest independent prognostic factor, after considering for other factors. In contrast, another article from the same racial population (175 Japanese ESCC patients) found that OPN protein expression was significantly correlated with depth of invasion and lymph node metastasis, but not lymphatic and venous invasion as well as patients' survival (negative vs. positive: Hazard ratio = 1.271, 95% CI = 0.818-1.975, $p = 0.2869$), suggesting the conflicting findings[17]. The consistency and discrepancy between the studies of Ito *et al.*, Kito *et al.*, and ours deserves for the further investigation between OPN levels and clinical staging and prognosis of esophageal cancer.

This study has several limitations. Firstly, the small sample size might introduce type II error. Secondly, we did not have a negative control during IHC staining. Neither did we perform Western blotting to confirm the finding. Simultaneous measurement of plasma OPN level and mRNA expression in esophageal tumor will be helpful to clarify the role of OPN in the development of ESCC.

In summary, we demonstrated the high OPN protein levels in ESCC tissues, compared with the normal part, although it cannot be used to predict the prognosis of ESCC. The role OPN in ESCC is worthy of the further investigation.

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造骨蛋白在食道鱗狀上皮細胞癌的蛋白質表現量

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摘 要

本研究的目的是檢驗：(1) 造骨蛋白 (osteopontin) 在食道鱗狀上皮細胞癌的蛋白質表現，以及 (2) 是否可用造骨蛋白來預測病患的疾病嚴重度及預後。共有 54 位新診斷並接受食道切除術的食道鱗狀上皮細胞癌患者收案。我們以免疫組織染色法偵測組織中造骨蛋白的蛋白質表現量，並經由查閱病歷得到病患的臨床病理資料。造骨蛋白蛋白質的過度表現在 37.0% (20/54) 的腫瘤部位及 13.0% (7/54) 的周邊正常食道組織可偵測得到。相對於其對照的正常部位，造骨蛋白在其中 35 對 (64.8%) 食道癌組織有明顯更強的表現。造骨蛋白蛋白質的表現量與患者的癌症分期或存活時間並無相關。我們的研究發現雖然造骨蛋白不能用來預測病患的預後，但和食道鱗狀上皮細胞癌的發生過程有關。