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Original Article

Low expression of occludin: a predictor of poor prognosis in esophageal squamous cell carcinoma

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Abstract: Occludin is transmembrane protein and a key constituent of tight junction, and might participate in barrier function and fence function of epithelia and endothelia. It has been shown to be aberrantly expressed in malignant tumors and plays a role in carcinogenesis and tumor progression. The prognostic significance of Occludin expression has been implicated in various human cancers. However, the prognostic significance of Occludin expression in esophageal squamous cell carcinoma (ESCC) has not been established. In this study, we screened the tight junction genes aberrantly expressed based on two published gene microarray datasets (GSE20347 and GSE23400), and examined 95 esophageal cancer cases to assess immunohistochemical expression patterns of Occludin based on tissue array. Down-regulation of Occludin expression was shown in ESCC as compared with adjacent non-neoplastic specimens (P = 0.003). Decreased expression of Occludin was correlated with high histological grade (P = 0.017). Decreased expression of Occludin was also correlated with short overall survival (P = 0.014). The results indicated Loss of Occludin expression was associated with poor prognosis in ESCC, and Occludin expression was potentially a good predictor of prognosis in ESCC.

Keywords: Occludin, prognosis, ESCC

Introduction

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death globally [1, 2]. Squamous cell carcinoma (SCC) is the main histological type of esophageal cancer. The so-called Asian-belt encompassing China has a high incidence of esophageal SCC with more than 100/100,000 each year. The prognosis for esophageal SCC is much poorer because of diagnosis at an advanced stage and a high incidence of metastasis and recurrence post-treatment [3]. The overall 5-year survival ranges only around 10% [4]. The TNM staging is the predominant standard for predicting recurrence and metastasis of esophageal SCC, however, lacks sensitivity and accuracy.

Tight junctions are important components contributing to a constitutive barrier of epithelial and endothelial cells [5]. They have two classical functions-barrier function which regulates the passage of various ions and molecules between cells, and fence function which maintains cellular polarity [6]. However, tight junction proteins are also recognized as having functions beyond “barrier and fence”. They also involve in signal transduction mechanisms and regulation of innate immunity in association with carcinogenesis [6]. A diversity of cancerous tissues showed a differential expression of tight junction proteins in human beings. For example, Cldn1 protein was up-regulated in human cervical adenocarcinoma [7] and colorectal cancer tissues [8]. Moreover, altered expression and distribution of tight junction proteins have also been reported in other human malignancies, including pancreas, lung, ovarian, thyroid, prostate, esophagus and breast [9, 10]. In turn, tight junction proteins modulated by signaling pathways, cytokines and growth factors, could influence biological behaviors of malignancies [11]. The loss of cell junctional sealing could involve in proliferation, transformation, infiltration of cancer cells and epithelial-mesenchymal transition in association with metastasis [12, 13]. Breast cancer with bone metastasis had significantly lower occludin expression in comparison with those without bone metastasis [14].

TNM staging system is a classical evaluation system for recurrent status and prognosis of malignancies. However, it also can't evaluate recurrence and prognosis of some cancers accurately. The correlation of tight junction protein with patients' clinicopathological characteristics could be used to evaluate prognosis of malignancies. Occludin, as a constituent of tight junction, is aberrantly expressed in certain types of cancers. Some studies reported Occludin expression was associated with degree of differentiation, TNM staging, metastasis and patents' survival in gallbladder adenocarcinoma, gastric and breast cancers [15-17]. However, correlation of Occludin expression with recurrence and prognosis of ESCC has not been cleared. In this study, tissue microarray was performed to analyze Occludin expression in 95 ESCC in order to elucidate whether Occludin expression is correlated with clinicopathological characteristics and clinical outcomes.

Materials and methods

Gene datasets and analysis

Two published studies were selected involving gene expression profiling in human ESCC [18, 19]. The raw data were accessible through the GEO datasets available on PubMed (GSE20347 and GSE23400). These two datasets were generated using cDNA array. Various numbers of tissue samples were used in these two studies:

1. The study by Hu N, et al utilized Affymetrix HG-U133A 2.0 gene expression arrays to analyze tissue samples of 17 ESCC patients from a high risk region of China. Normal adjacent and ESCC tissues were obtained from each patient with confirmation of histology [18].

2. The study by Su H, et al utilized Affymetrix HG-U133B 2.0 gene expression arrays to analyze 53 matched samples of ESCC and normal adjacent tissues. Histological diagnosis was obtained from experienced pathologists [19].

In order to determine individual gene differentially expressed between ESCC and normal adjacent tissues, Significance analysis of microarrays (SAM) was utilized to analyze the raw, normalized datasets. Delta values were adjusted until the FDR was less than 1%. An arbitrary one and a half-fold cut-off threshold were then used to the list of significant genes from SAM analysis. Tight junction genes differentially expressed were selected from each of the datasets. These genes were designated overlap gene list. Differential expression of specific gene was confirmed by tissue array.

Patients and follow-up

In this research, ninety-five ESCC patients who underwent radical esophagectomy were included between July 2006 and December 2008. The tissue samples were stored at the biobank center in National Engineering Center for Biochip at Shanghai. All the tissue samples were obtained with informed written consent, and ethical approval was obtained from the ethnical committee of biobank center related hospital. Formalin-fixed and paraffin-embedded (FFPE) tissue samples and clinicopathological data were collected. Clinical diagnosis and tumor differentiation assessment were according to WHO grading criteria [20]. Pathological staging was determined according to NCCN Esophageal Cancer Guideline [21].

The patients after surgery were followed up ranging from 5.8 years to 7.8 years (median 6.9 years). Tumor relapse was monitored by enhancement thoracic and abdominal CT every 3 months for the first and second years, and 6 months thereafter. Cerebral CT and radioisotope bone scanner were performed if necessary. Any new masses in the related organs were considered as recurrence. The follow-up interruption and death other than tumor relapse was considered as a censoring event.

Construction of tissue microarray (TMA)

Representative cancerous region and its adjacent non-malignant specimen were selected by two separate pathologists from each tissue block. A cylinder-shaped hole were created by a tissue arraying instrument (Beecher Instruments, Sun Prarie, WI) in a square recipient paraffin block. The tissue cores from the donor blocks of cancerous and adjacent tissue samples were removed by a hollow needle with an inner diameter of 1.5 mm. The cores from the donor blocks were inserted into a recipient paraffin block in a precisely spaced, array pattern. A series of 5-μm sections were prepared with a microtome, and a perfect piece of which was placed on polylysine-coated slides. Sections were stained with H&E to confirm the presence

Table 1. Differential expression of tight junction genes in ESCC

	GSE23400		GSE20347	
	Gene symbol	Fold change	Gene symbol	Fold change
Up-regulated	Cldn17	2.84		
	Cldn4	2.48		
	Cldn7	2.58		
	TJP3	4.39		
	TJP1	3.48		
Down-regulated	**Occludin**	**0.42**	**Occludin**	**0.77**
	Cldn6	0.34	Cldn5	0.90
			Cldn7	0.84
			Cldn4	0.87
			TJP1	0.71
			TJP2	0.87
			TJP3	0.83

Bold black indicated the overlapped tight junction genes both in GSE23400 and GSE20347.

Table 2. Expression of Occludin protein within ESCC and adjacent normal tissues based on TMA by IRS

	Mean ± Std. Deviation	*P* Value	Z Value
ESCC	3.596 ± 3.039	0.003	-2.944
Adjacent normal	4.438 ± 2.739		

of tumor within each core, and immunostaining of Occludin was performed as described below.

Immunostaining and evaluation

The TMA slides were heated at 60°C for 20 min, deparaffinized and rehydrated in an ethanol gradient, washed with Tris-buffered saline (TBS). Antigen retrieval was performed for 15 min at water boiling point under high pressure in sodium citrate buffer (PH = 6), followed by 3% hydrogen peroxide in phosphate buffer saline (PBS), followed by PBS for 5 min three times. Upon quenching endogenous peroxidase activity and blocking non-specific binding, anti-occludin polyclonal antibody (PA5-30230, Invitrogen, Camarrilo, CA) was added at a dilution of 1:800. The slides were incubated with primary antibody overnight in a humid chamber at 4°C. The corresponding secondary biotinylated antibody was used for 30 min at room temperature. After further washing with TBS, sections were incubated with StrepABC complex/horseradish peroxidase (1:100, DAKO) for 30 min at room temperature. 3,3-diaminobenzidine terahydrochloride at a concentration of 0.05% was used for chromogenic immunolocalization. Sections were counterstained with hematoxylin before dehydration and mounting. The cores containing breast carcinoma served as positive control.

Positive immunostaining of occludin was defined mainly in the location of cytoplasm. It was graded by the intensity and percentage of cells with positive staining. The immunoreactivity was assessed by two pathologists simultaneously, and a consensus was reached for each core. The staining intensity of occludin was scored from 0 to 3 (0 = negative; 1 = weak; 2 = moderate; 3 = strong). The percentage of positive cells was also graded into 6 categories: 0 (negative), 1 (1%-20%), 2 (21%-40%), 3 (41%-60%), 4 (61%-80%), 5 (81%-100%). In the cases with a discrepancy between duplicated cores, the average score from the duplicated cores was taken as the final score. The whole level of occludin expression in carcinoma was evaluated by immunoreactive score (IRS), which was calculated by multiplying the scores of staining intensity and the percentage of positive cells. Based on IRS, staining pattern was defined as low expression (IRS≤6) and high expression (IRS>6).

Statistical analysis

The differential expression of occludin between cancerous and adjacent tissues was determined by Wilcoxon signed rank test. Correlation analysis between clinicopathological and molecular parameters was analyzed by Spearman's rank analysis. Survival curves were performed with the Kaplan-Meier analysis and compared by the log-rank test. A multivariate analysis was applied by the Cox regression model to evaluate independent prognostic factors for ESCC. A two-tailed *P* value of less than 0.05 was considered as statistical significance. All the statistical analysis was performed with SPSS 19.0 version (SPSS Inc, Chicago, IL).

Results

Differential expression of tight junction genes in ESCC

GSE23400 data showed 5 tight junction genes were over-expressed, and the expression of 2 tight junction genes was down-regulated.

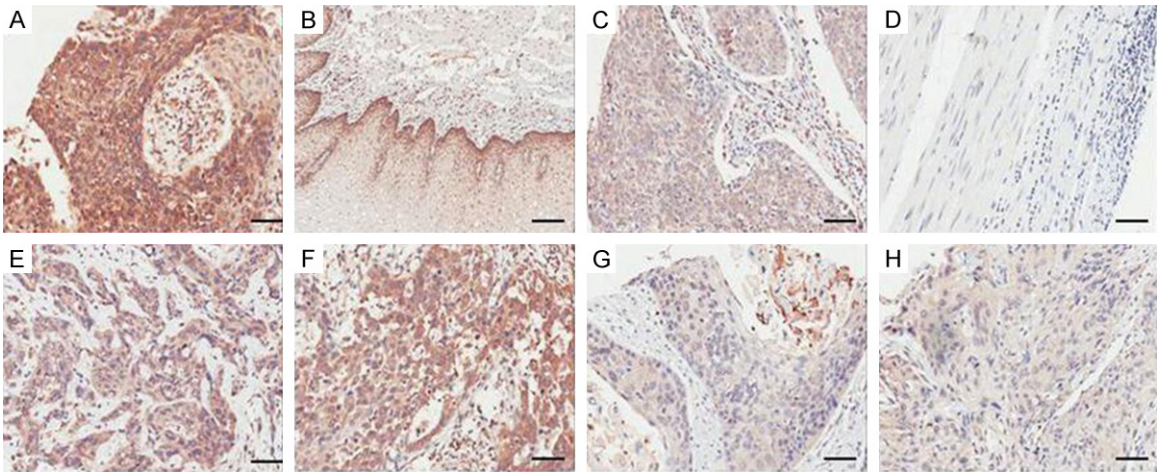

Figure 1. Immunohistochemical expression patterns of Occludin. A. The strong staining intensity of Occludin. B. The moderate staining intensity of Occludin. C. The weak staining intensity of Occludin. D. The negative staining intensity of Occludin. E, F. The high expression of Occludin in ESCC. G, H. The low expression of Occludin in ESCC. The scale bar represented 50 μm.

Table 3. Clinicopathological characteristics of ESCC patients

Characteristic	Sample number	Percent
Gender		
Male	73	76.8
Female	22	23.2
Age (years)		
≤65	49	51.6
>65	46	48.4
Histological grade		
I	7	7.4
II	64	67.4
III	24	25.2
Tumor size (cm)		
≤5	58	61.1
>5	28	29.5
Unknown	9	9.4
T stage		
T1-2	17	17.9
T3-4	74	77.9
Unknown	4	10.2
Lymph nodes metastasis		
N0	45	47.4
N1-3	49	51.6
Unknown	1	1
TNM stage		
TNM1-2	47	49.5
TNM3-4	44	46.3
Unknown	4	4.2

GSE20347 data included 7 tight junction genes down-regulated (**Table 1**). It was observed only the expression of Occludin was down-regulated in both datasets.

The expression of occludin was down regulated in ESCC specimens comparing with the corresponding adjacent specimens

The expression of Occludin was down-regulated in ESCC in comparison with that in adjacent tissues based on 2 datasets (**Table 1**). Furthermore, TMA was used to investigate Occludin protein expression in ESCC and adjacent tissues. The specific staining was semi-quantitatively scored by IRS. In this study, the specific staining for Occludin within ESCC and adjacent tissues was primarily located in the cytoplasm. The expression of Occludin protein in ESCC was down-regulated as comparison with that in adjacent tissues (**Table 2**). The representative images of immunostaining were shown in **Figure 1**.

Correlation analysis between occludin protein expression and clinicopathological characteristics

The clinicopathological characteristics for ESCC patients were presented in **Table 3**. TMA in combination with immunostaining was used to investigate the correlation of Occludin protein expression with clinicopathological characteristics. The expression of Occludin protein within

Table 4. Correlation analysis between Occludin expression and clinicopathological characteristics

	Occludin expression in ESCC		
	Correlation coefficient	Sig. (2-tailed)	Sample number
Occludin expression in adjacent	**0.573**	**0.000**	**76**
Gender	0.033	0.755	93
Age	-0.006	0.951	93
Tumor size	0.034	0.759	84
Histological grade	**-0.248**	**0.017**	**93**
T stage	0.009	0.933	89
Lymph nodes metastasis	0.113	0.284	92
TNM stage	0.046	0.671	89

Bold black indicated the significance of correlation analysis.

ESCC was significantly associated with that in adjacent tissues (r = 0.573, P = 0.00) and histological grade (r = -0.248, P = 0.017). There were no statistical differences among Occludin protein expression, gender, age, tumor size, T stage, lymph node metastasis and TNM staging (**Table 4**).

Univariate survival analysis and multivariate cox regression analysis

Univariate analysis was performed by Kaplan-Meier survival curve and log-rank test. It was demonstrated that the overall survival (OS) of ESCC with low expression of Occludin was inferior to those with high expression (P = 0.014), whereas the OS of adjacent tissues with low expression of Occludin was no statistical difference comparing with that with high expression (P = 0.081; **Figure 2**). Moreover, inferior OS was also observed in patients with male (P = 0.019), larger tumor size (P = 0.012), lymph node metastasis (P = 0.001), advanced TNM stage (P = 0.001) and the advanced depth of tumor invasion (P = 0.025).

In addition, we also performed multivariate analysis. All the significant factors in the univariate analysis were included in further multivariate analysis. It was indicated that expression of Occludin in ESCC tissues was an independent factor to predict OS of the patients (**Table 5**).

Discussion

Occludin, together with claudins, functions as a selective "gate and fence" in epithelial and endothelial cell layers. Besides, a number of studies illustrated Occludin was also important for carcinogenesis and cancer invasion. The purpose of this study was to figure out the relations between Occludin expression and clinicopathological findings in ESCC. We demonstrated that Occludin expression was down-regulated in ESCC as compared with adjacent normal tissues and Loss of Occludin was associated with poor prognosis.

The expression pattern of tight junction proteins was able to distinguish from different types of carcinoma, which was potential for diagnosis. Loss of Occludin expression might be one phenotypic feature distinguishing diffuse-type gastric carcinoma from intestinal-type gastric carcinoma [22]. Cldn1 expression was reported to be stronger in premalignant stages (e.g., carcinoma *in situ* of breast and cervical intraepithelial neoplasia), while a significant decrease was found in invasive carcinoma (e.g., invasive breast and cervical cancer) [23, 24]. In addition, the expression pattern of tight junction proteins could be used to differentiate carcinoma from non-neoplasia, Several studies reported Cldn3 and Cldn4 were over-expressed in ovarian and uterine serous papillary carcinoma as compared with normal ovarian cells and endometrium, which showed potential diagnostic markers for neoplasia [25, 26].

Over-expression of tight junction proteins would be expected to increase cell adhesion and consequently suppress invasion and motility of tumor cells. However, in certain cancers, overexpression of tight junction proteins might be related to carcinogenesis. The expression of Occludin was significantly increased in hepatocellular carcinoma and urothelial carcinoma as compared with non-neoplastic tissues and normal controls [27, 28]. Cldn1 protein was found to be increased in colon carcinoma, which was identified as a potential target for β-catenin/Tcf signaling [29, 30]. Cldn3 and Cldn4 were overexpressed in ovarian carcinoma [26]. In uterine serous papillary carcinoma, the mRNA levels for Cldn3 and Cldn4 were higher in carcinoma as compared with normal endometria [25]. Several additional reports confirmed these two Occludins were also increased in other can-

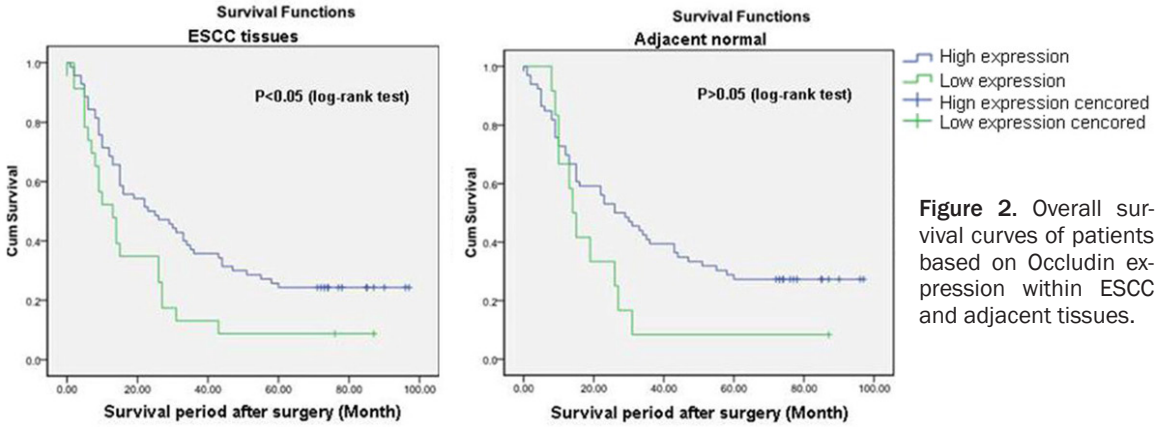

Figure 2. Overall survival curves of patients based on Occludin expression within ESCC and adjacent tissues.

Table 5. Multivariate Cox regression analysis predicting survival within ESCC patients

	RC	SE	Wald	DF	*P* value	RR	95% CL of RR	
							LL	UL
Low expression of Occludin in ESCC	**0.537**	**0.267**	**4.066**	**1**	**0.044**	**1.763**	**0.214**	**2.886**
T stage	0.209	0.392	0.286	1	0.593	1.233	0.572	2.658
Lymph nodes metastasis	-0.180	0.633	0.081	1	0.777	0.836	0.242	2.890
TNM stage	0.88	0.668	1.735	1	0.188	2.412	0.651	8.941

RC: Regression coefficient; SE: Standard error; DF: Degree of freedom; RR: Relative risk; CL: Confidence level; LL: lower limit; UL: upper limit. Bold black indicated the significance of the factor for predicting prognosis of ESCC patients.

cers, such as breast, prostate and pancreatic cancers [9]. These results suggested tight junction proteins including Occludin might involve in regulation of signaling pathway, which were not associated with the formation of tight junctions [31].

In certain tumors, loss of Occludin was associated with carcinogenesis. A frequent complete loss of Occludin was observed in cutaneous squamous cell carcinoma as compared with the precursor lesions and sun-exposed skin, which might result in a decrease of epithelial cell-cell adhesion and a reduction of susceptibility to apoptosis [32]. Loss of Occludin protein was also observed in gallbladder adenocarcinoma [15], poorly differentiated carcinoma from stomach and colon [16, 33, 34] and cholangiocarcinoma [35] as compared with adjacent normal tissues and specific benign lesions. Tight junction was important for barrier function and cell polarity. Disruption of tight junction manifested by loss of Occuldin lead to destruction of barrier function and loss of cellular polarity, resulting in abnormal diffusion of nutrients and other factors necessary for survival and growth of malignant cells [36-38]. Loss of cell-to-cell adhesion triggered dissociation of cancer cells from primary nests, which was a crucial step for cancer progression and metastasis [9]. It was reported breast cancer with bone metastasis had significantly lower occludin expression as compared with that without metastasis, especially for breast ductal cancer [14]. A study gained the similar result that Occludin mRNA was significantly decreased in metastatic breast cancer as compared with normal breast tissues [17]. Although weak expression of Occludin protein was examined in normal hepatocytes, down-regulation of Occudin mRNA was reported in metastasis as compared with normal hepatic tissues [39]. In our present study, Loss of Occludin protein and down-regulation of Occludin mRNA were markedly observed in ESCC as compared with adjacent normal tissues.

We also reported the potential role of Occludin in clinico-pathological correlation of ESCC. Loss of Occludin was only associated with advanced tumor grade in our study. Other studies also suggested the association of Occludin expression with clinico-pathological parameters. It was reported loss of Occludin had a clear relationship with increased invasion, reduced adhesion and metastasis in breast

cancer, which indicated loss of Occludin lead to complex changes in cellular phenotype of human cancers [14, 17]. In gallbladder carcinoma, loss of Occludin was also associated with advanced tumor grade, a maximal tumor size >2 cm, lymph node metastasis and invasion to regional tissues [15]. Similarly, Occludin expression was significantly decreased in gastric carcinoma with undifferentiated-type [33]. On the one hand, loss of Occludin might lead to loss of cell-cell adhesion, driving the motility of cancer cells and the release of cancer cells from primary nests, conferring invasive and metastatic properties [9]. On the other, loss of Occludin also might lead to poor differentiation of cancer cells by influencing signaling pathways regulating cellular proliferation, differentiation and apoptosis, showing invasive and metastatic features [11]. However, some studies reported no association between Occludin expression and clinicopathological characteristics in cervical adenocarcinoma [40], hepatocellular carcinoma [27], ovarian carcinoma [41] and squamous cell carcinoma of tongue [41]. The discrepancy was not cleared now.

Many patients suffered from advanced ESCC with lymph node metastasis and regional invasion at the time of diagnosis, as it was difficult to detect this lesion at an early stage [42]. Clinicopathological characteristics, e.g., degree of differentiation, mass location, depth of invasion and lymph node status were associated with staging and grading [43], which were crucial parameters for predicting survival and prognosis of tumors [44]. It was also urgent to identify some novel and potential prognostic markers. Occludin might be a potential prognostic factor for ESCC manifested by regulating cell-cell adhesiveness and associating with frontal invasion and metastasis. For example, a negative prognostic effect of Occludin low-expression was reported in malignant gallbladder lesions [15] and brain tumors [45], showing a favorable-prognostic potential of Occludin. However, Occludin expression did not show any prognostic effects in some tumors, e.g., hepatocellular carcinoma [27], gastric carcinoma [33] and squamous cell carcinoma of the tongue [46]. The reasons for differential prognostic effects of Occludin in different cancers were unknown. The discrepancy was related to the possibility that functional implications of Occludin differed among organs.

In conclusion, this study determined the prognostic value of Occludin expression and loss of Occludin was found to be associated with poor prognosis in ESCC. Therefore, we considered Occludin might be a novel and potential indicator of prognosis in ESCC.

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Disclosure of conflict of interest

None.

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