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Original Article

Expression of caveolin-1, MMP-2 and β-catenin in esophageal squamous cell carcinoma and their clinicopathologic significance

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Abstract: In recent years, many studies have reported on role of Caveolin-1 in tumor, but little attention has been paid on its function in esophageal squamous cell carcinoma (ESCC). In this study, we attempt to provide molecular biomarkers for early diagnosis of ESCC through examining the expression of caveolin-1 and the relationship between Caveolin-1 and MMP-2, β-catenin in ESCC. ESCC tissues (110 cases) and normal esophageal mucosa tissues (15 cases) were collected from 110 patients with ESCC, who underwent the surgical removal of a tumor at thoracic surgery. Immunohistochemical method was used to detect the expressions of Caveolin-1, MMP-2 and β-catenin in ESCC tissues and normal esophageal mucosa tissues, respectively, and the correlations between these three proteins several clinical pathological parameters of ESCC were analyzed. High expression of caveolin-1, MMP-2 and β-catenin were observed in ESCC tissues ($P < 0.05$), and the expression of these proteins were associated with the differentiation, TNM stage, lymph node metastasis and liver metastasis of ESCC ($P < 0.05$). A significant positive correlation was found between Caveolin-1 expression and MMP-2 expression in ESCC ($r = 0.429$, $P = 0.429$), there also was a significant positive correlation between Caveolin-1 expression and β-catenin expression in ESCC ($r = 0.545$, $P = 0.000$). In conclusion, Caveolin-1 is over expressed in ESCC tissues and significantly associates with the differentiation, TNM stage, lymph node metastasis and liver metastasis of ESCC. There are positive correlations among caveolin-1, MMP-2 and β-catenin. This study suggests that caveolin-1 is closely related to the occurrence, development, invasion and metastasis of ESCC.

Keywords: Esophageal squamous cell carcinoma, caveolin-1, MMP-2, β-catenin, immunohistochemistry, correlation analysis

Introduction

Esophageal squamous cell carcinomas (ESCC) is one of the common digestive tract malignant tumor in China and leading cause of cancer death [1]. Treatment on ESCC is promising and curative if the cancer diagnosed at early stage, but many patients show up at later stage which is usually treated with surgery. Therefore, basic studies about ESCC are the key work for cancer therapy and early diagnostic biomarkers would be ideal intervention that facilitates the complicated process of ESCC treatment. Occurrence, invasion and metastasis of tumor are complex process involved in multiple factors, the abnormal proliferation and differentiation of tumor cells are closely related to the up-regulation of many oncogenes and down-regulation of diverse tumor suppressor genes. At present, immunohistochemistry (IHC) is typically used to detect the protein expression of tumor markers for monitoring the tumor recurrence, infiltration and metastasis.

Caveolin-1 mainly distributed in terminal differentiated cells (such as fibroblasts, type I lung cells, endothelial cells and fat cells) [2], it could specifically interact with a variety of cell signaling proteins and suppressed the corresponding activity and functions, playing an important

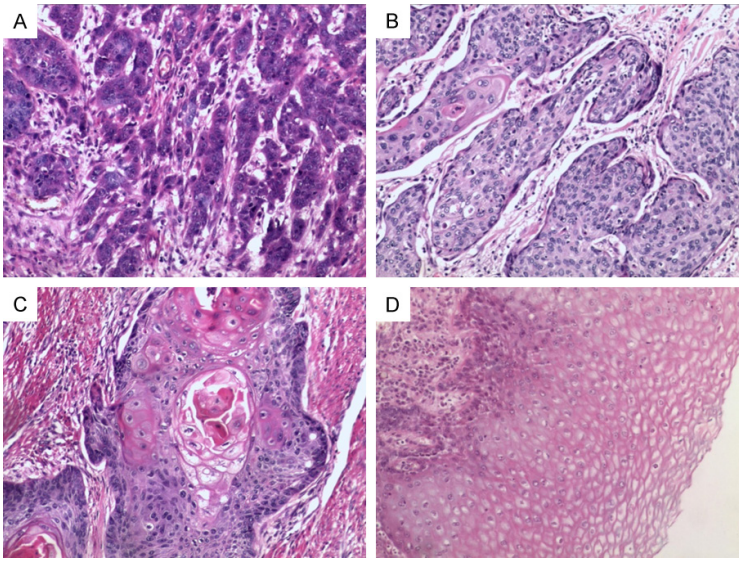

Figure 1. HE staining results of ESCC tissues and normal esophageal mucosa tissues. A: Poor differentiated squamous cell carcinoma (×400); B: Moderate differentiated squamous cell carcinoma (×400); C: Well differentiated squamous cell carcinoma (×400); D: Normal esophageal mucosa tissue (×400).

Table 1. Statistical results about ESCC staging

Classification	Differentiated degree			Lymph node metastasis		Liver metastasis		TNM stage	
	G1	G2	G3	Yes	No	Yes	No	I + II	III + IV
Number	21	55	34	78	32	21	89	63	47

G1, well differentiated; G2, moderate differentiated; G3, poor differentiated.

regulatory role in cell proliferation, differentiation, migration, apoptosis and secretion [3-5]. Researches showed that Caveolin-1 was over expressed in esophageal cancer, pancreatic cancer, bladder cancer, prostate cancer and other cancers; meanwhile, its high expression is closely related to the differentiation, growth, invasion and metastasis of malignant tumor cells and poor prognosis [6-10].

Matrix metalloproteinases (MMPs) are a type of proteolytic enzymes closely related with tumor cell invasion and metastasis, mainly synthesized and secreted by endothelial cells, granulocytes and tumor cells [11]. MMP-2 is belong to the MMP family that most closely associate with malignant tumors, has a strong positive expression in ESCC tissues and almost no expression in normal tissues [12]. In addition, several studies had confirmed that MMP-2 expression was enhanced in many malignant tumor tissues, cultured tumor cells and cells transformed with oncogenes [13, 14]. Thus, MMP-2 was widely used in the monitoring of tumor invasion, metastasis and poor prognosis.

β-catenin is an important cell adhesion molecule and one component of cytoskeleton, highly conserved and can interact with a variety of proteins specifically [15]. The β-catenin widely exists in various cells (fibroblasts, endothelial cells and so on), involved in mediating cell adhesion and controlling growth and differentiation of multiple tissues [16]. β-catenin presents in the cytoplasm and keeps a dynamic balance between free and combination form to regulates the adhesion, proliferation and other signaling. Previous studies have found an abnormal accumulation of β-catenin in cytoplasm of diverse cancers [17-19].

In this study, ESCC tissues were selected as the research object to explore the distributions and expressions of Caveolin-1, MMP-2 and β-catenin in ESCC tissues. At the same time, correlation analyses were conducted between Caveolin-1, MMP-2, β-catenin and clinicopathological parameters of ESCC, elucidating the relationships among these three proteins. The aim of this study was to assess the role of Caveolin-1 in the progression of ESCC cell infiltration and hopefully find molecular marker for early diagnosis and prognosis monitoring, providing basis information for diagnosis and treatment of ESCC.

Materials and methods

Study population

110 ESCC tissues and 15 normal esophageal mucosa tissues from 110 ESCC patients were studied, who underwent surgical resection in the first affiliated hospital of Hebei North University (Hebei, China) from September 2013 to September 2014. ESCC tissues were collected from the center of cancer nests; normal

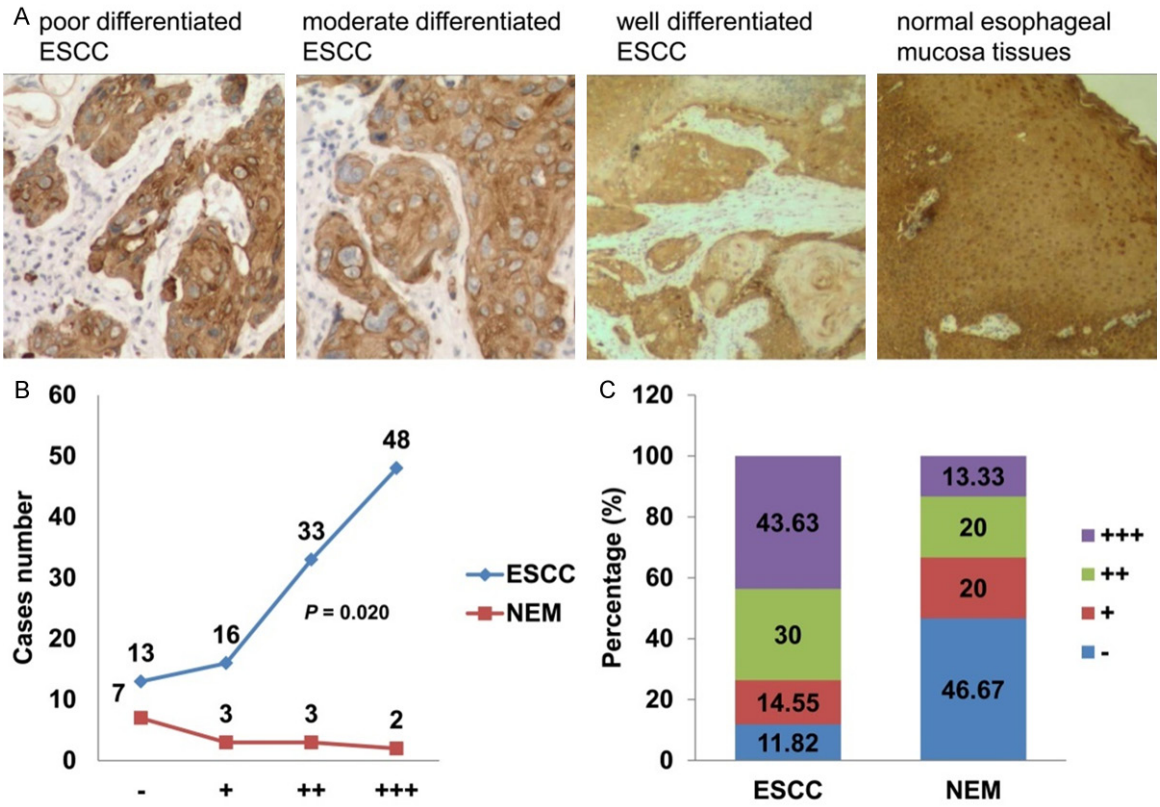

Figure 2. Expression of Caveolin-1 in ESCC tissues and normal esophageal mucosa tissues. A: Immunohistochemical results of Caveolin-1 expression in ESCC tissues; B: Cases numbers of positive expression (++, +++) and negative expression (-, +) in ESCC tissues and normal esophageal mucosa tissues ($\chi^2 = 5.398$, $P = 0.020$); C: Percentage of positive expression (++, +++) and negative expression (–, +) in ESCC tissues and normal esophageal mucosa tissues (NEM).

esophageal mucosa tissues were from the cut edges (at least 10 cm away from the tumor edges) and were confirmed by pathological examination. All tumor tissues were primary tumor and no preoperative treatment with neoadjuvant chemo-radiotherapy, immunotherapy or biological therapy. ESCC tissues were from 69 males and 41 females aged from 34 to 74 (51 ± 4.6), normal esophageal mucosa tissues were from 9 males and 6 females aged from 37 to 71 (53 ± 2.0). This study was approved by the ethics review board of Hebei North University Medical School and written informed consents were obtained from all patients.

Histological grade and clinical stage

Histological grade and clinical stage were evaluated by two experienced pathologists, and clinical staging according to the TNM system of esophageal squamous carcinoma enacted by American Joint Committee on Cancer (AJCC) in 2009 [20]. Differentiation of ESCC cells were observed with hematoxylin-eosin staining. The tissue samples were cut into 4-μm thick sections after dehydration then were stained with hematoxylin and eosin after deparaffinage.

Immunohistochemistry

SP (streptavidin-peroxidase) method was used to detect caveolin-1, MMP-2 and β-catenin in this study. Tissue samples were cut into 3-μm thick sections and baked in 70°C oven for 2 h to prevent falling. The sections were dewaxed in graded xylene followed by antigen repairing with microwave and inactivating endogenous peroxidase with 43% H_2O_2. Sections were incubated with primary antibodies (anti-Caveolin-1 antibody, anti-MMP-2 antibody, anti-β-catenin antibody; ZSGB-BIO, Beijing, China) overnight at 4°C then incubated with secondary antibody anti-IgG (ZSGB-BIO, Beijing, China) for 20 min at 37°C. Those antibodies were used according to the specifications and PBS displaced primary antibody as negative criteria. Immunoreac-

Table 2. Relationship between the expression of Caveolin-1 in ESCC and the clinicopathological characteristics

Group	n	Positive (n)	Positive (%)	χ^2	P
Gender				1.614	0.204
Male	61	42	68.85		
Female	49	39	79.59		
Age				0.4551	0.499
< 60	51	36	70.59		
≥ 60	59	45	76.27		
Tumor size				2.0347	0.154
< 5 cm	58	46	79.31		
≥ 5 cm	52	35	67.31		
Differentiation				14.879	0.000
Well	21	10	47.62		
Moderate	55	39	70.91		
Poor	34	32	94.11		
Depth of invasion				0.2283	0.633
T1-T2	42	32	76.19		
T3-T4	68	49	72.06		
Lymph node metastasis				16.648	0.000
Yes	78	66	84.62		
No	32	15	46.88		
TNM stage				7.816	0.005
I-II	63	40	63.49		
III-IV	47	41	87.23		
Liver metastasis				6.239	0.013
Yes	21	20	95.24		
No	89	61	68.54		

tion products were visualized with DAB detection kit (Sigma, St Louis, USA) followed by counterstaining with hematoxylin for 5 sec. The protein expressions of cells were observed and counted using a BX-51 microscope (Olympus Corporation, Tokyo, Japan) under 400×.

Determination of results

Caveolin-1 protein mainly located in cytoplasm/cytomembrane, where would display brownish yellow granules in positive cells; those with canary yellow to brownish yellow granules in the cytoplasm of cells were MMP-2 protein positive; β-catenin protein was presented as brownish yellow or tawny particles which was ectopic expression and located in the cytoplasm and nucleus. Immunohistochemical results were comprehensively analyzed according to the intensity of positive staining and positive cell percentage [16, 21]. These were separately done by two professors from the pathology department and research section of Hebei North University and then checked the results; two experts observed together and determined if there were inconsistent results. Scoring standard was established based on positive cell percentage: 0: ≤ 5%, 1: 6%-25%, 2: 26%-50%, 3: 51%-75%, 4: > 75%; scoring standard according to staining intensity: 0: no staining, 1: canary yellow, 2: yellow, 3: brownish yellow. The product of these two scores stood for the immunohistochemical results: 0: (-), 1-4: (+), 5~8: (++), 9~12: (+++); '-' and '+' was accounted as negative, '++' and '+++' as positive.

Statistical analysis

Integrated all kinds of data were used to statistical analysis with SPSS 11.0 software package. Chi-square test was used for count data and Spearman was used for correlation analysis, $P < 0.05$ was considered as statistical significance.

Results

TNM stage

ESCC tissues of 110 cases were evaluated by two experienced pathologists in accordance with the TNM system. The results are shown in **Figure 1** and **Table 1**, in total of 110 ESCC tissues, 21 cases were well differentiated, 55 cases were moderate differentiated, 34 cases were poor differentiated; the numbers of cases with lymphatic metastases or hepatic metastases were 78 and 21, respectively. Therefore, there were total 63 clinical staging I-II cases and 47 clinical staging III-IV cases.

Expression of caveolin-1 and its relationship with the clinicopathological parameters of ESCC

Caveolin-1 distributed in cytomembrane and cytoplasm, immunohistochemical staining were buff in normal esophageal mucosa epithelial cell and uneven yellow or brownish-yellow granules in ESCC cells (**Figure 2A**). The positive expression rates of Caveolin-1 in ESCC tissues and normal tissues were 73.64% (81/110) and

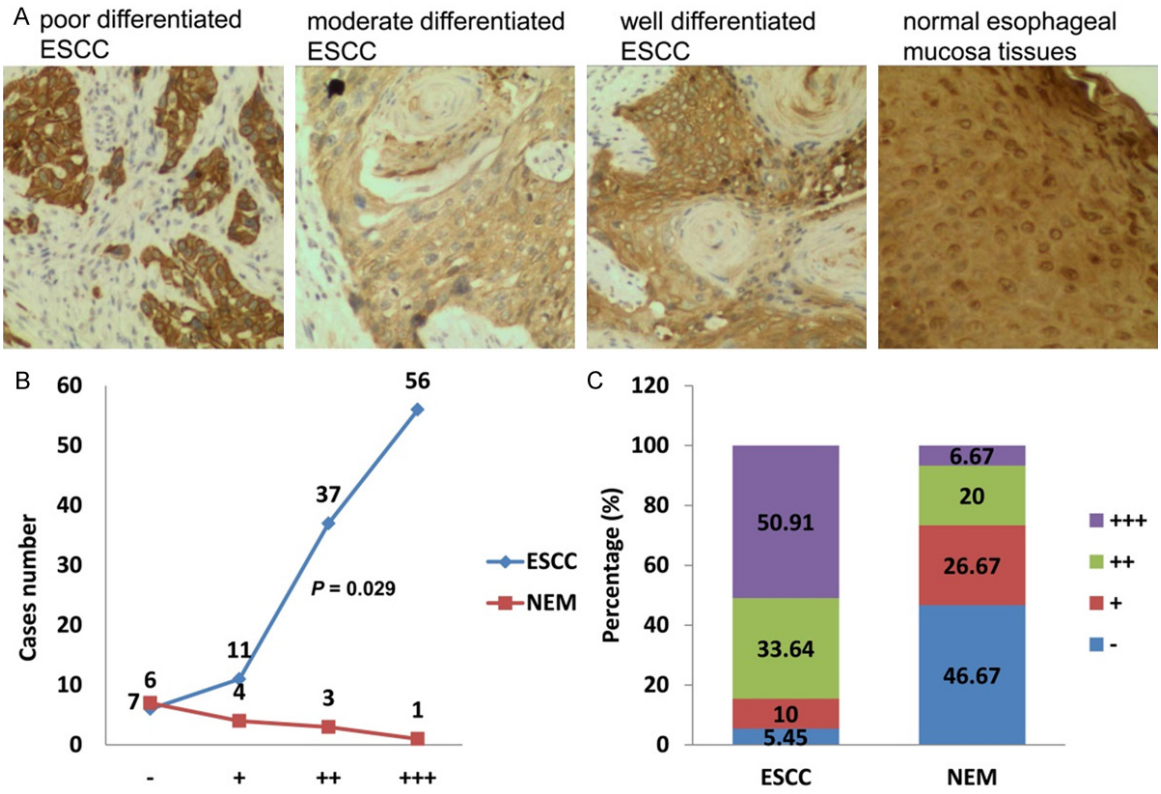

Figure 3. Expression of MMP-2 in ESCC tissues and normal esophageal mucosa tissues. A: Immunohistochemical results of MMP-2 expression in ESCC tissues; B: Cases numbers of positive expression (++, +++) and negative expression (–, +) in ESCC tissues and normal esophageal mucosa tissues (χ^2 = 4.744, *P* = 0.029); C: Percentage of positive expression (++, +++) and negative expression (–, +) in ESCC tissues and normal esophageal mucosa tissues (NEM).

33.33% (5/15) respectively, and that difference had a statistical significance (χ^2 = 5.398, *P* = 0.020) (**Figure 2B** and **2C**). Correlational analyses between Caveolin-1 expression and the clinicopathological parameters of ESCC had done too and the results are shown in **Table 2**, positive expression of Caveolin-1 was correlated with differentiation, lymph node metastasis, liver metastasis and TNM stage of ESCC (*P* < 0.05). Positive expression rates of Caveolin-1 in various differentiated ESCC were 47.62% (well differentiated), 70.91% (moderate differentiated), 94.11% (poor differentiated) respectively (*P* = 0.000); those in ESCC with lymph node metastasis or not were 84.62% and 46.88% (*P* = 0.000); those in ESCC with liver metastasis or not were 95.24% and 68.54% (*P* = 0.013); those were 87.23% and 63.49% in III-IV stage ESCC and I-II stage ESCC respectively (*P* = 0.005). Caveolin-1 expression was not effected by gender, age of patients, tumor size or depth of invasion (*P* > 0.05).

Expression of MMP-2 and its relationship with the clinicopathological parameters of ESCC

MMP-2 were not stained or canary yellow in the cytoplasm of normal esophageal mucosa epithelium cells, but were focal or diffuse brownish yellow particles in the cytoplasm of ESCC cells with different size and color (**Figure 3A**). MMP-2 expression in ESCC tissues significantly higher than that in normal esophageal mucosa tissues (84.55% vs 26.67%; χ^2 = 4.744, *P* = 0.029) (**Figure 3B** and **3C**). Positive rates of MMP-2 expression in well differentiated ESCC, moderate differentiated ESCC and poor differentiated ESCC were significantly different (61.9%, 97.27% and 94.12% ; *P* = 0.004); those in ESCC with lymph node metastasis or not, ESCC with liver metastasis or not, III-IV stage ESCC and I-II stage ESCC were also obviously different (96.51% vs 56.25%, *P* = 0.000; 100% vs 80.90%, *P* = 0.029; 93.62% vs 77.78%, *P* = 0.023, respectively). On the con-

Table 3. Relationship between the expression of MMP-2 in ESCC and the clinicopathological characteristics

Group	n	Positive (n)	Positive (%)	χ^2	P
Gender				0.697	0.404
Male	61	50	81.97		
Female	49	43	87.76		
Age				0.218	0.641
< 60	51	44	86.27		
≥ 60	59	49	83.05		
Tumor size				1.158	0.282
< 5 cm	58	47	81.03		
≥ 5 cm	52	46	88.46		
Differentiation				10.936	0.004
Well	21	13	61.90		
Moderate	55	48	87.27		
Poor	34	32	94.12		
Depth of invasion				0.071	0.790
T1-T2	42	36	85.71		
T3-T4	68	57	83.82		
Lymph node metastasis				27.652	0.000
Yes	78	75	96.15		
No	32	18	56.25		
TNM stage				5.168	0.023
I-II	63	49	77.78		
III-IV	47	44	93.62		
Liver metastasis				4.745	0.029
Yes	21	21	100.00		
No	89	72	80.90		

trary, the positive expression rates of MMP-2 had no obvious difference between different genders, ages, tumor sizes or depth of invasion ($P > 0.05$) (**Table 3**).

Expression of β-catenin and its relationship with the clinicopathological parameters of ESCC

Normal expression of β-catenin was observed in more than 70% of the cells located in cytomembrane, otherwise, it will be defined as lack of membrane expression. Positive expression of β-catenin was observed in more than 10% of tumor cells located in the cytoplasm or nucleus and defined as ectopic expression. In total 110 ESCC tissues, 91 cases (82.73%) were ectopic expression cytomembrane were mostly stained in normal esophageal mucosa epithelial tissues and only 3 cases (20.00%) were ectopic expression. Ectopic expression of β-catenin in ESCC tissues were obviously higher than that in normal esophageal mucosa epithelial tissues ($\chi^2 = 5.33$, $P < 0.05$) (**Figure 4**). The ectopic expression of β-catenin was more pronounced as it was less differentiated; and the positive rates were 52.38% (poor differentiated ESCC), 85.45% (moderate differentiated ESCC), 97.06% (well differentiated ESCC) respectively, there was a significant difference in differentiation pattern ($P = 0.000$). The differences of β-catenin ectopic expression also were significant between ESCC with lymph node metastasis or not (94.87% vs 53.13%, $P = 0.000$) and ESCC with liver metastasis or not (100% vs 78.65%, $P = 0.020$). Ectopic expression of β-catenin in III-IV stage ESCC was 95.74%, which higher than that in I-II stage ESCC (73.02%, $P = 0.002$). But ectopic expressions of β-catenin in ESCC tissues were not changed apparently with the gender, age, tumor size or depth of invasion (**Table 4**).

Correlation between caveolin-1 expression, MMP-2 expression and β-catenin expression in ESCC tissues

In total 110 ESCC tissues, 76 cases were Caveolin-1 positive and MMP-2 positive, 12 cases were Caveolin-1 negative and MMP-2 negative; correlation analysis using Spearman showed a significant positive correlation between Caveolin-1 expression and MMP-2 expression ($r = 0.429$, $P = 0.000$) (**Table 5**). There were 77 cases of β-catenin positive expression in 81 cases of Caveolin-1 positive expression and 15 cases of β-catenin negative expression in 29 cases of Caveolin-1 negative expression, which also showed a significant positive correlation ($r = 0.545$, $P = 0.000$) (**Table 6**). There also was a significant positive correlation between MMP-2 expression and β-catenin expression in ESCC tissues ($r = 0.469$, $P = 0.000$): 84 cases were β-catenin positive and MMP-2 positive, 10 cases were β-catenin negative and MMP-2 negative (**Table 7**).

Discussion

Caveolin-1 inhibits the Ras/ERK and MAPK/ERK kinase systems, mediated the excessive

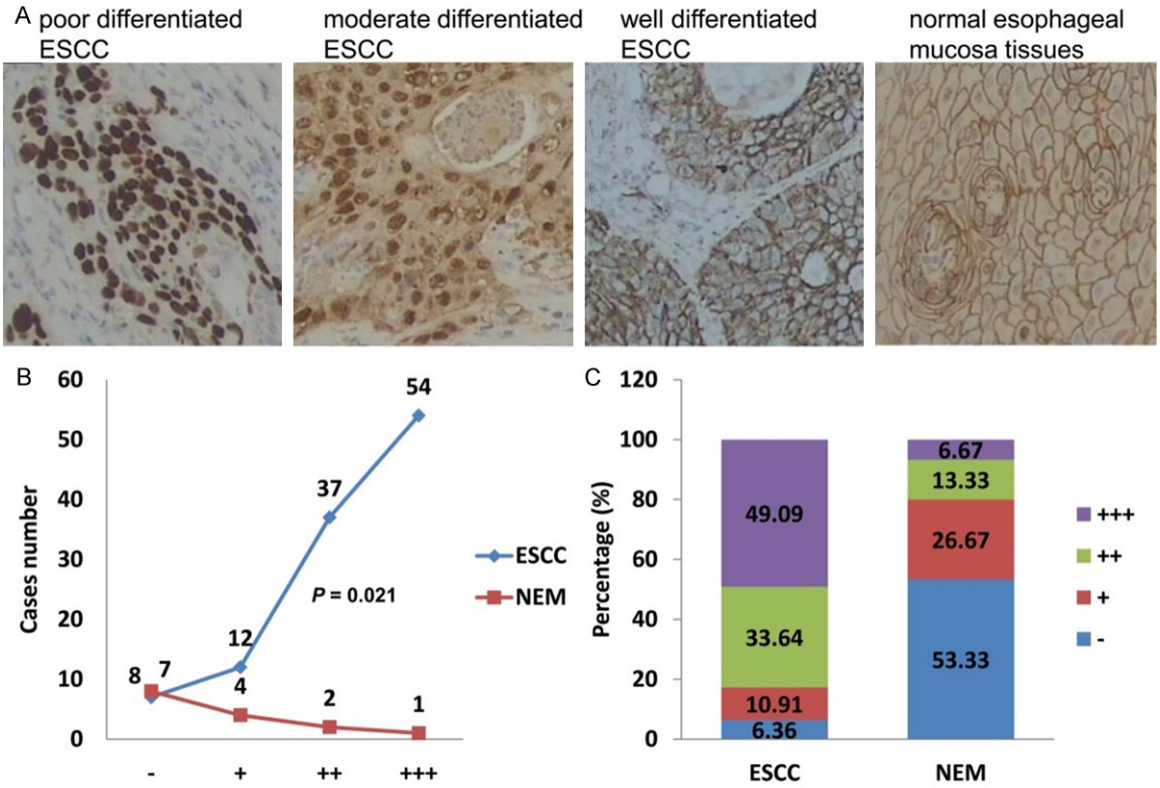

Figure 4. Expression of β-catenin in ESCC tissues and normal esophageal mucosa tissues. A: Immunohistochemical results of β-catenin expression in ESCC tissues; B: Cases numbers of positive expression (++, +++) and negative expression (–, +) in ESCC tissues and normal esophageal mucosa tissues (χ^2 = 5.333, P = 0.021); C: Percentage of positive expression (++, +++) and negative expression (-, +) in ESCC tissues and normal esophageal mucosa tissues (NEM).

proliferation and apoptosis of cells to promote cancer cells [22]. Early research has reported that Caveolin-1 promotes the activity of p53 protein and contributes to abnormal cell escaping from immune surveillance, which is more conductive to survive and proliferate for abnormal cells [23]. The results in this study showed significant high positive rate of Caveolin-1 expression in ESCC tissues than in normal esophageal mucosa epithelial tissues (73.64% vs 33.33%, $P < 0.05$), which suggested that high expression of Caveolin-1 might be associated with the occurrence and development of ESCC. The significant positive correlations were found between expression of Caveolin-1 and differentiation of ESCC cells, lymph node metastasis, TNM stage, liver metastasis ($P < 0.05$), indicating that Caveolin-1 might be related to the infiltration, invasion, distant metastasis and other malignant biological behaviors of ESCC, and these results were supported by Chu Guangmin's research [24].

Among the MMPs family, MMP-2 is one of the members that most closely associated with malignant tumors, which is capable of degrading type I, III, IV, V, VII, X, XI collagens [25], inducing ECM degradation and allows cancer cells to migrate out of the primary tumor to form metastases. MMP-2 plays an important role in the process of occurrence, progression and metastasis of multiple malignant tumors (such as breast cancer, gastric cancer, colorectal cancer, ovarian cancer and endometrial cancer) [26]. In addition, MMP-2 also responsible for release of vascular endothelial growth factor which is stored in extracellular matrix and induces tumor angiogenesis to provide the necessary nutrition foundation for the infiltration and proliferation of tumor [27]. In this study, the positive rates of MMP-2 expression in ESCC, ESCC with lymph node metastasis and ESCC with liver metastasis were 84.55%, 96.15% and 100.00% respectively, significantly higher than of those in normal esophageal mucosa epithelial tissues(26.67%, $P < 0.05$), ESCC

Table 4. Relationship between the expression of β-catenin in ESCC and the clinicopathological characteristics

Group	n	Positive (n)	Positive (%)	χ^2	P
Gender				0.055	0.814
Male	61	50	81.97		
Female	49	41	83.67		
Age				0.837	0.360
< 60	51	44	86.27		
≥ 60	59	47	79.66		
Tumor size				1.040	0.308
< 5 cm	58	50	89.66		
≥ 5 cm	52	41	78.85		
Differentiation				18.707	0.000
Well	21	11	52.38		
Moderate	55	47	85.45		
Poor	34	33	97.06		
Depth of invasion				0.017	0.895
T1-T2	42	35	83.33		
T3-T4	68	56	82.35		
Lymph node metastasis				27.675	0.000
Yes	78	74	94.87		
No	32	17	53.13		
TNM stage				9.732	0.002
I-II	63	46	73.02		
III-IV	47	45	95.74		
Liver metastasis				5.419	0.020
Yes	21	21	100.00		
No	89	70	78.65		

Table 5. Correlation of Caveolin-1 expression and MMP-2 expression in ESCC

Caveolin-1	MMP-2		Total	r	P
	+	-			
+	76	5	81	0.429	0.000
-	17	12	29		
Total	93	17	110		

+, positive; -, negative.

without lymph node metastasis (56.25%, $P < 0.05$), ESCC without liver metastasis (80.9%, $P < 0.05$) accordingly. Furthermore, the MMP-2 expression also had an association with differentiation and TNM stage of ESCC ($P < 0.05$); these results suggest that MMP-2 is related with ESCC occurrence, development, invasion and metastasis [28].

The functions of β-catenin mainly is regulating intercellular adhesion and playing an important role in Wnt signaling pathway. β-catenin could combine with E-cadherin to mediate the connection between same type intercellular adhesion, and this adhesion would be weaken when the complex dissociated because of β-catenin phosphorylation, promoted the invasion and metastasis of tumor [29]. Abnormality in the Wnt signal pathway could inhibit the β-catenin phosphorylation, it would induce β-catenin accumulating in cytoplasm and promoted the tumorigenesis [30]. Our results showed that positive rate of β-catenin expression in ESCC was remarkably higher than in normal esophageal mucosa epithelial tissues (82.73% vs 20.00%, $P < 0.05$), and those in ESCC with lymph node metastasis and ESCC with liver metastasis were clearly higher than in ESCC without lymph node metastasis and ESCC without liver metastasis (94.87% vs 53.13%, $P < 0.05$; 100% vs 78.65%, $P < 0.05$) respectively. Correlation analyses also revealed the positive correlation between differentiation of TNM stage of ESCC and β-catenin expression ($P < 0.05$). These also suggest β-catenin is related with ESCC occurrence, development, invasion and metastasis [28].

In addition, correlation analyses among Caveolin-1, MMP-2 and β-catenin showed Caveolin-1 expression in ESCC was positive related to the expressions of MMP-2 and β-catenin. Caveolin-1 might promote the activation of downstream protein MMP-2 through inhibiting Ras/ERK, MAPK/ERK kinase systems to induce ECM degradation and neovascularization, promoting the infiltration and metastasis of ESCC [28]. An earlier study suggested that Caveolin-1 was located in cell membrane through specifically combining with β-catenin, then β-catenin would be dissociated from the complex by Wnt signals and transduced signals to regulate the cell growth, differentiation and anti-apoptosis. Furthermore, Caveolin-1 could inhibit combination of β-catenin and E-cadherin and promote the down-regulation of E-cadherin, in this way weaken the adhesion between cancer cells and neighboring cells, which enhanced the metastasis and infiltration of tumor cells [31].

Table 6. Correlation of Caveolin-1 expression and β-catenin expression in ESCC

Caveolin-1	β-catenin		Total	r	P
	+	-			
+	77	4	81	0.545	0.000
-	14	15	29		
Total	91	19	110		

+, positive; -, negative.

Table 7. Correlation of MMP-2 expression and β-catenin expression in ESCC

MMP-2	β-catenin		Total	r	P
	+	-			
+	84	9	93	0.469	0.000
-	7	10	17		
Total	91	19	110		

+, positive; -, negative.

In general, Caveolin-1, MMP-2 and β-catenin proteins were highly expressed in ESCC tissues, and the expressions of these proteins were positive correlated with differentiation of ESCC cells, lymph node metastasis, TNM stage, and liver metastasis. Furthermore, Caveolin-1 expression was significant positive correlated with MMP-2 expression and β-catenin expression in ESCC. Caveolin-1 may mediate the abnormal expressions of MMP-2 and β-catenin to induce and promote the invasion and metastasis of ESCC. This study illustrates the high correlation between Caveolin-1 and occurrence, development, infiltration and metastasis of ESCC from different sides, provides new ideas and potential therapeutic targets for diagnosis, treatment and metastasis monitoring of ESCC.

Disclosure of conflict of interest

None.

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