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Original Article

Upregulation of fibronectin, vitronectin and claudin-7 in cervical cancer

Bin Zhang, Lihong Chen, Qiufang Bao, Xiu Zheng

Department of Gynaecology and Obstetrics, The 1st Affiliated Hospital of Fujian Medical University, Fuzhou 350005, Fujian Province, PR China

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Abstract: Objectives: To examine the protein expression pattern and clinical significance of cell adhesion-related molecules including fibronectin, vitronectin and claudin-7 in cervival cancer tissues. Methods Western blotting were carried out to detect the protein expression of the above three molecules in 40 pairs of cervical cancer tissues. The association of their protein expression levels with clinical characteristics (age, tumor size, tumor grade, LNM, vessel invasion) was statistically analyzed to reveal the clinical significance of these proteins. Results All these three molecules emerged as notably upregulation in our collected cervical cancer tissues. Specifically, the upregulation rate was 70% (28/40) for fibronectin, 82.5% (33/40) for vitronectin, and 87.5% (35/40) for claudin-7. Statistical analysis revealed that almost none of these three proteins correlated with any of our recorded clinicalpathological factors. Notably, vitronectin was expressed higher in CC patients younger than 55 years old. Conclusion: Our findings demonstrated that fibronectin, vitronectin and claudin-7 were all significantly upregulated in cervival cancer tissues, indicating their vital roles in cervical cancer cells. However, these three proteins seemed not correlated with any of the recording clinical characteristics, except that higher vitronection was negatively correlated with age. Efforts were still needed to annotate their clinical significance in future.

Keywords: Cervical cancer, fibronectin, vitronectin, caudin-7, clinical significance

Introduction

Currently, cancer of the cervix, namely cervical cancer (CC), is the first leading common kind of human malignance originated from the female reproductive system [1, 2]. Also, CC represents as the third most frequently diagnosed cancer and the fourth leading cause of cancer-related death in woman all around the world. Previous studies had found that persistent human papillomavirus (HPV) infection could serve as an important factor contributing to the initiation and development of CC, accounting for over 90% of cases with CC [3, 4]. It was also known that HPV alone could not sufficiently induce cervical transformation [5]. In other words, there existed other key factors involved in the malignant transformation process of CC. Frankly speaking, typical therapy methods including surgery, radiotherapy and platinum-based chemotherapy are in general efficient for this disease. Nevertheless, tumor recurrence might still occurred in some patients, with distant metastasis and finally bad outcomes [6, 7]. Hence, still much work are needed to understand the biological mechanisms underlying this disease for therapy improvements.

Cell adhesion is the process by which cells form contacts with each other or with their substratum through specialized protein complexes. Cell adhesion occurs from the action of transmembrane glycoproteins, called cell adhesion molecules. Fibronectin, vitronectin and claudin-7 are three independent examples of these cell adhesion-related proteins. Specifically, fibronectin is a glycoprotein emerged as a soluble dimeric form in plasma, and binds cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. Fibronectins are well known to be involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape and tumor metastasis [8, 9]. Vitronectin is a cell adhesion and spreading factor found in serum and tissues [10]. Claudin-7 is a member of the

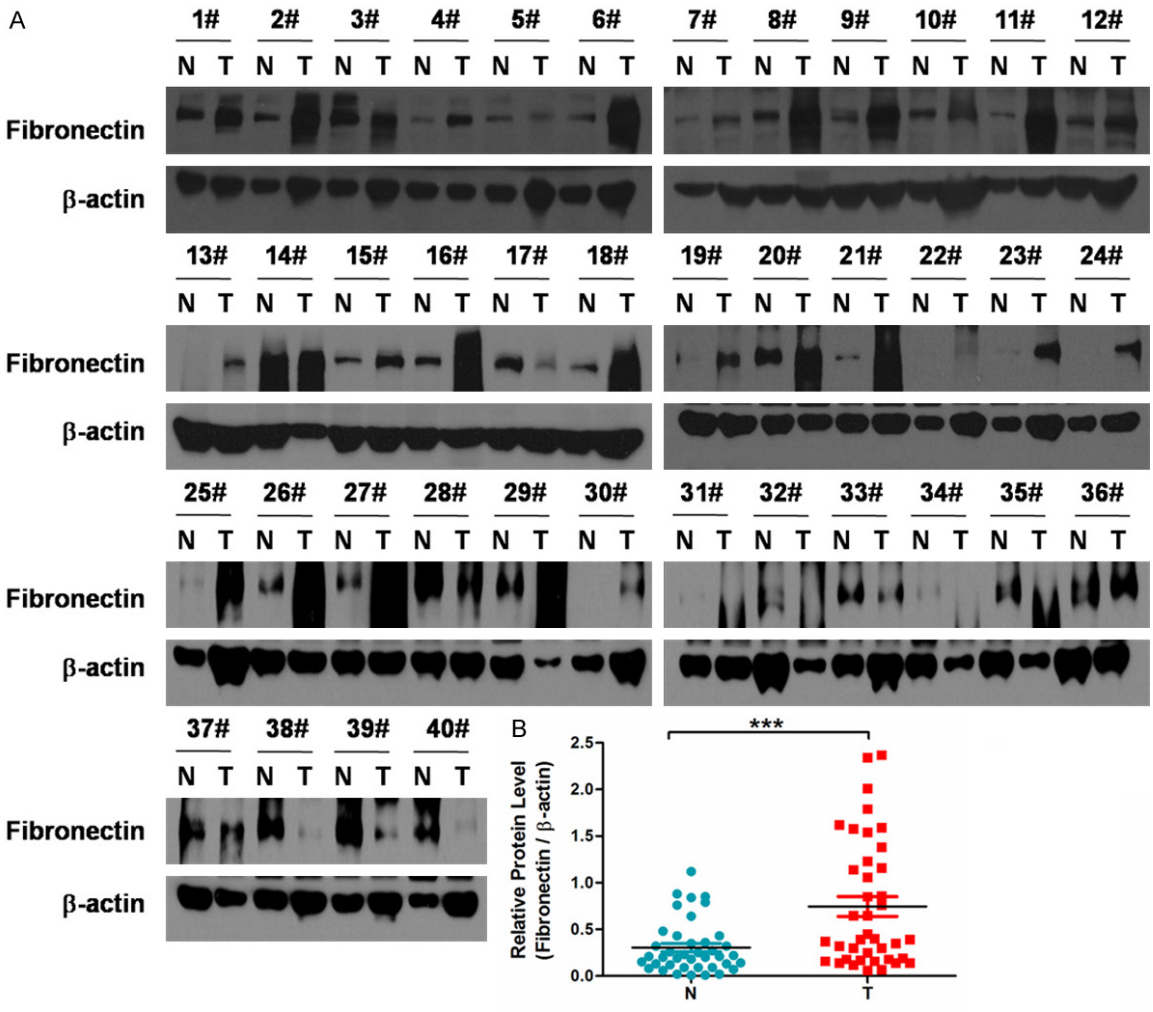

Figure 1. Expression of fibronectin is increased in human CC. A. Western blotting was performed to determine fibronectin protein levels of CC tissues and paired non-cancerous tissues. (N: non-cancerous tissues; T: cancer tissues; n=40). B. Quantitative results of the expression level of fibronectin in 40 paires of human CC samples by ImageJ software. (Normalized to β-actin; ***: $P<0.001$).

claudin family. Claudins are integral membrane proteins and components of tight junction strands, which play critical roles in maintaining cell polarity and signal transductions [11, 12]. Differential expression of these three molecules has been observed in multiple cancers [13-16], but there are few reports regarding their expression pattern in CC tissues. In this study, we mainly applied western blotting to investigate the expression and putative clinical significance of these three proteins in CC.

Materials and methods

Patients and tissue samples

A total of 40 CC tissues and paired non-cancerous tissues were obtained from patients who underwent surgery in the 1st affiliated hospital of Fujina medical university between 2013 and 2015. Clinicopathological features were obtained from the patient database of the hospital. The tissue samples were immediately frozen in liquid nitrogen and stably stored at -80°C after surgical removal until use. The clinical stage was determined by the International League of Gynecology and Obstetrics (FIGO).

Western blotting

The paired tissues were crushed in liquid nitrogen and transferred to a tube with cold RIPA buffer (Beyotime, China). The lysate were incubated on ice for 0.5 h. The supernatants were resolved on 6% or 11% gradient SDS-PAGE and transferred NC membranes. Membranes were

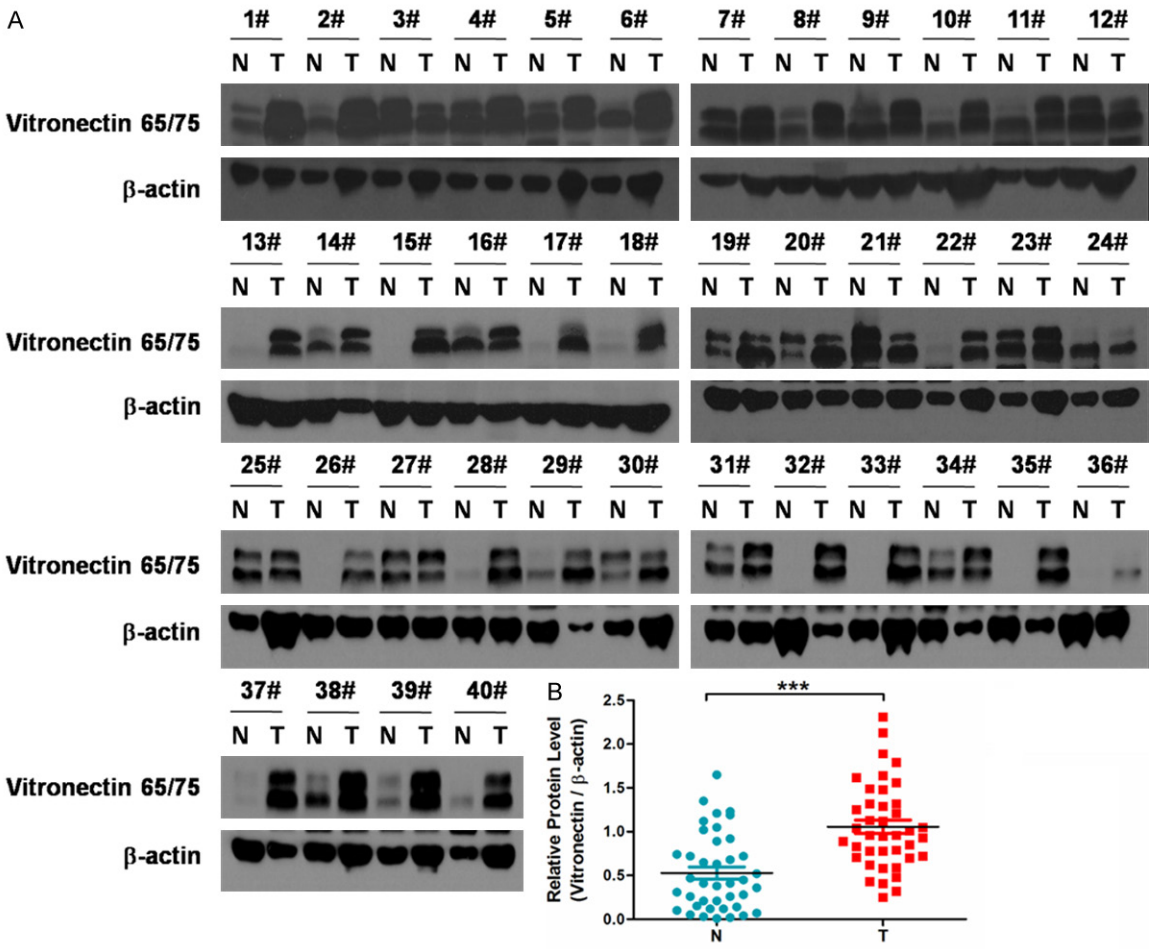

Figure 2. Expression of vitronectin is increased in human CC. A. Western blotting was performed to determine vitronectin protein levels of CC tissues and paired non-cancerous tissues. (N: non-cancerous tissues; T: cancer tissues; n=40). B. Quantitative results of the expression level of vitronectin in 40 paires of human CC samples by ImageJ software. (Normalized to β-actin; ***: $P<0.001$).

blocked for 1 h at room temperature in 5% milk in PBST and incubated with anti-fibronectin antibody (1:500, Santa Cruz), anti-vitronectin antibody (1:1000, Santa Cruz), anti-claudin-7 antibody (1:500, Santa Cruz) or anti-β-actin antibody (1:6000, SIGMA) at 4°C overnight. The membranes were then incubated with appropriate HRP-conjugated secondary antibody at optimized concentration. The densitometry of immunoblotting results was measured by using ImageJ software.

Statistical analysis

All data were analyzed by SPSS program v21.0 (Illinois, USA). The Paired t-test or Non parametric test (Wilcoxon Signed Rank Test) were performed to evaluate the protein expression difference between cancer tissues and paired non-cancerous tissues. The Fisher's exact test was performed to investigate the association of protein expression level with clinical parameters. A *P*-value of less than 0.05 was considered statistically significant.

Results

Upregulation of fibronectin, vitronectin and claudin-7 proteins in CC tissues

We firstly collected 40 pairs of CC tissues matched with adjacent normal tissues from the clinic. Proteins were extracted from these tissues following the conventional procedures described in the Method section. The expression of fibronectin, vitronectin and claudin-7

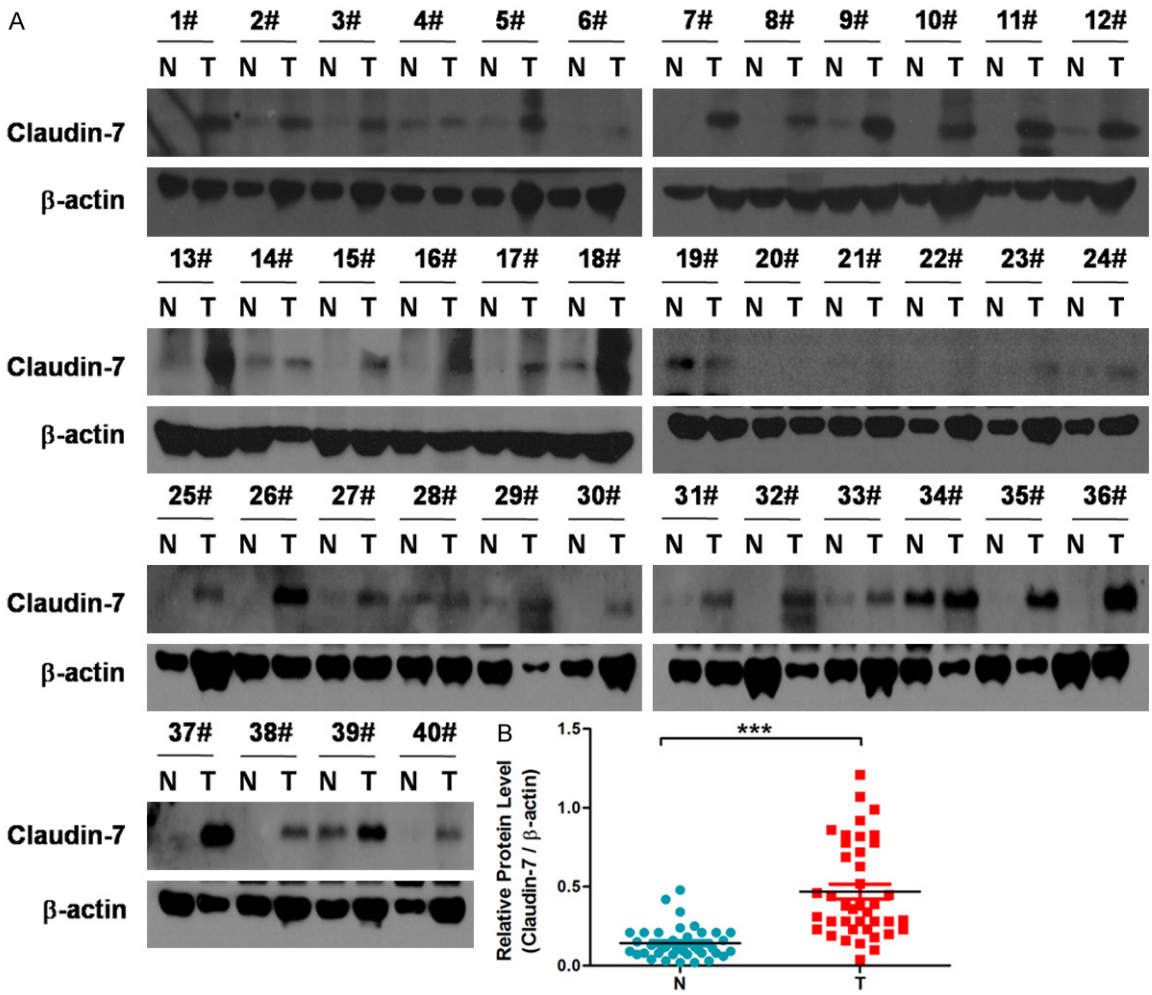

Figure 3. Expression of claudin-7 is increased in human CC. A. Western blotting was performed to determine claudin-7 protein levels of CC tissues and paired non-cancerous tissues. (N: non-cancerous tissues; T: cancer tissues; n=40). B. Quantitative results of the expression level of claudin-7 in 40 paires of human CC samples by ImageJ software. (Normalized to β-actin; ***: $P<0.001$).

were analyzed by using western blotting with indicated antibodies (**Figures 1A**, **2A**, **3A**). β-actin were as the loading controls of these blottings. The quantitative data were graphed in **Figures 1B**, **2B**, **3B**, respectively. Significant upregulation of these three proteins were observed ($P<0.001$). Specifically, the upregulation rate was 70% (28/40) for fibronectin, 82.5% (33/40) for vitronectin, and 87.5% (35/40) for claudin-7. These results strongly demonstrated that all these three molecules might act as potent oncogenes in CC. Our data was in line with the previous findings addressing the importance of these molecules in cell adhesion and/or motility processes. Overall, our results indicated that fibronectin, vitronectin and claudin-7 are upregulated in CC tissues.

Correlation of fibronectin, vitronectin and claudin-7 proteins with the recorded CC clinicopathological factors

To further understanding the clinical significance of these proteins, we investigated the correlation of fibronectin, vitronectin and claudin-7 with the clinicopathological factors of CC by Fisher's exact test. Recorded clinical parameters including age, tumor size, FIGO stage, tumor grade, LNM, vessel invasion and histology were provided by the pathologist (**Table 1**). According to our quantified data from the western blottings, we classified these 40 pairs of CC tissues into two groups: increased (T/N>1.5 folds) and non-increased (T/N≤1.5 folds). Statistical analysis revealed that almost

Table 1. The CC patients' parameters for primary culture

Total case number	*n*=40
Median age (Range)	52.45 (29-73)
Age	
<50	15
≥50	25
Tumor size	
<4 cm	21
≥4 cm	19
FIGO stage	
I	11
II	29
Tumor grade	
I+II	22
III+IV	18
LNM	
+	10
-	30
Vessel invasion	
+	4
-	36
Histology	
Squamous cell cancer	38
Adenocarcinoma	2

none of these three proteins correlated with any of our recorded clinicalpathological factors. However, vitronectin was observed to be expressed higher in CC patients younger than 50 years old (**Table 2**). These results suggested that vitronectin might serve as a diagnosis biomarker for CC in people below 50 years old, although much epidemiological survey is still needed to verify this conclusion. Similarly, the clinical significance of fibronectin and claudin-7 seemed minor, but the truth would come out after surveying in a large sample containing more detailed clinical features.

Discussion

Cervical cancer (CC) is the third most common gynecologic cancer in women worldwide. Right now CC screening has been globally and widely appreciated, but there are still lots of patients suffer from advanced diseases, especially in developing countries including China. Additionally, the molecular mechanisms that governing pathogenesis and progression of CC is complicated and till now poorly understood. Cell adhesion-related molecules are known to play vital roles in cancer progression, especially for cancer metastasis. Facilitated by cell-cell interactions between tumor cells and the endothelium in distant tissues, then adhesion of tumor cells, metastasis occur at the advance stages of multiple cancers, including CC, which induces bad outcome among these patients with distant metastasis. Inhibition of the adhesion process also emerges as a therapeutically effective target for attenuation of cancer metastasis.

Here in our study, we mainly analyzed the protein expression of cell adhesion-related fibronectin, vitronectin and claudin-7 in CC tissues. We surprisingly found that all these proteins exhibited as significant increase in CC tissues compared with that of matched non-cancerous tissues. There are three main problems required to be resolved in future: 1) We noticed that for vitronectin, we had detected two separate bands of different molecular weights (65 or 75 kD), which might be produced via alternative RNA splicing. We calculated the total amounts of this protein in each tissue. However, whether RNA splicing-mediated differential vitronectin isoforms expression contributed for CC progression remained unclear. Further study considering this post-transcriptional regulation of vitronectin might provide more informative clues for understanding the differential splicing and functions of this protein. 2) We also feel sorry to study the protein expression of these three molecules just by western blotting. Indeed, immunohistochemistry could better reveal the expression extent and location information of these proteins in vivo. 3) Most importantly, we did not found significant clinical value of these three proteins in CC patients. It seemed that fibronectin, vitronectin and claudin-7 were not correlated with any of the recording clinical characteristics, except that higher vitronectin was negatively correlated with age. The internal reasons might be our small scale and incompleted records of our clinical features.

Taken together, our results demonstrated that fibronectin, vitronectin and claudin-7 were upregulated in CC tissues at the protein level. Higher vitronectin levels were more observed in ages minor than 50 years old. Further study on the biological functions of these proteins will

Table 2. Correlation between protein expression and the clinicopathological features of patients with CC

Clinical characteristics	Fibronectin		Vitronectin		Claudin-7	
	Increased (n=28)	Non-increased (n=12)	Increased (n=33)	Non-increased (n=7)	Increased (n=35)	Non-increased (n=5)
Age						
<50	11	4	15	0	13	2
≥50	17	8	18	7	22	3
		P=1.000		*P*=0.0328*		*P*=1.000
Tumor size						
<4 cm	15	6	19	2	19	2
≥4 cm	13	6	14	5	16	3
		P=1.000		*P*=0.2258		*P*=0.6544
FIGO stage						
I	9	2	9	2	11	0
II	19	10	24	5	24	5
		P=0.4507		*P*=1.000		*P*=0.2975
Tumor grade						
I+II	17	5	19	3	18	4
III+IV	11	7	14	4	17	1
		P=0.3154		*P*=0.6798		*P*=0.3555
LNM						
+	6	4	10	0	9	1
-	22	8	23	7	26	4
		P=0.4507		*P*=0.1612		*P*=1.000
Vessel invasion						
+	2	2	3	1	4	0
-	26	10	30	6	31	5
		P=0.5698		*P*=0.5522		*P*=1.000

*Vitronectin was expressed higher in CC patients younger than 50 years old P<0.05.

benefit for understanding the consequences of their abnormal elevation in CC.

Disclosure of conflict of interest

None.

Address correspondence to: Bin Zhang, Department of Gynaecology and Obstetrics, The 1st Affiliated Hospital of Fujian Medical University, Fuzhou 350005, Fujian Province, PR China. Tel: 136-00856618; E-mail: zhangbin_fujian@yahoo.com

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