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Original Contribution

Immunohistochemical staining of podoplanin is helpful in determining the microinvasion of cervical squamous cell carcinoma

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ABSTRACT

Cervical squamous cell carcinoma develops through a series of stages, including low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), microinvasive squamous cell carcinoma (MISCC), and invasive squamous cell carcinoma (ISCC). The difference between HSIL and MISCC is the appearance of microinvasion, which determines the treatment for patients. However, sometimes it is difficult to differentiate HSIL from MISCC in morphology, and no effective markers are available to help determine microinvasion. Here, we evaluated the expression patterns of podoplanin in cervical tissues by immunohistochemistry staining. Results showed that podoplanin was specifically expressed in a continuous or discontinuous linear pattern within the basal layer of cells from normal cervical squamous epithelium (NS) (100%, 96/96) and HSIL (81%, 57/70). However, its expression was completely absent in microinvasive lesions (0%, 72/72), and the location of podoplanin expression loss was consistent with that of microinvasive lesions. Thus, for HSIL with positive podoplanin expression, the sudden loss of podoplanin represents the occurrence of early invasion. Furthermore, podoplanin was expressed in 3.4% (4/118) of ISCC, and its expression was not correlated with the age of the patient, tumor size, differentiation, FIGO stage, depth of invasion, lymph node, or distant metastasis. The prognosis of patients with positive podoplanin was slightly better than those without it ($p > 0.05$). Therefore, we found that podoplanin, as a new specific marker for the basal layer cells of cervical squamous epithelium, could assist the diagnosis of microinvasion in cervical squamous cell carcinoma. The specific staining pattern of podoplanin provides the possibility of clinical application in the future.

1. Introduction

Cervical cancer is a common malignant tumor of the female reproductive system, which seriously threatens the health of women [1,2]. Among the pathological subtypes of cervical cancer, the most common is squamous cell carcinoma (SCC) [3]. It is well known that the carcinogenesis of SCC involves a series of stages, including low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), microinvasive squamous cell carcinoma (MISCC), and invasive squamous cell carcinoma (ISCC) [4]. Squamous intraepithelial lesions, including LSIL and HISL, refer to atypical cells confined to the epithelium without breaking through the basement membrane. Because the basement membrane is intact, even if atypical cells occupy the entire epithelium, patients only need to undergo conization with no possibility of vascular invasion. Once small clusters of the atypical cells destroy the basement membrane and invade the subepithelial stroma, the lesions develop into MISCC [5]. In regards to MISCC, the depth of infiltration does not exceed 5 mm. Patients with MISCC may have to undergo a hysterectomy because of the incidence of lymphatic and lymph node involvement as compared to HSIL [6].Thus, accurate assessment of microinvasion within the intraepithelial lesions is crucial for the guidance of treatment and prognosis of patients.

At present, pathologists mainly rely on the following morphological features to judge microinvasion: irregular or canine dentate-like margins, abundant eosinophilic or keratinized cytoplasm, clear chromatin or the occurrence of nucleoli, and desmoplastic reaction or

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Table 1
Podoplanin expression in cervical tissues.

Samples	Podoplanin-positive	Podoplanin-negative	Total
NS	96 (100%)	0 (0%)	96
NA	0 (0%)	91(100%)	91
HSIL	57 (81.4%)	13 (18.6%)	70
MISCC	0 (0%)	72(100%)	72
ISCC	4 (3.4%)	114 (96.6%)	118
Total	157	290	447

Note: normal cervical squamous epithelium, NS; normal cervical columnar epithelium, NA; high-grade intraepithelial lesions, HSIL; microinvasive squamous cell carcinoma, MISCC; invasive squamous cell carcinoma, ISCC.

inflammation in the adjacent stroma [7]. However, these diagnostic clues cannot solve all the problems in practical application. Studies have reported that the diagnostic consistency of MISCC is only about 50% due to the varying degrees of comprehension of the above diagnostic criteria. Those HSIL with extensive involvement of glands may be misdiagnosed as invasive lesions, and invasive squamous cell carcinoma may also be misdiagnosed as minimal invasive carcinoma [7]. Therefore, judgment of micro-infiltration is subjective, poorly reproducible, and challenging. It is meaningful to find new markers to assist in the accurate diagnosis of microinvasion.

D2-40, a monoclonal antibody against podoplanin, was found to be a sensitive and specific marker for lymphatic endothelial cells. Now it is widely used to identify tumor emboli in lymphatic vessels [8,9]. Studies have revealed that podoplanin can be a regulator of angiogenesis and lymphangiogenesis, both of which influence the growth, invasion, and metastasis of various solid tumors [10-12]. Moreover, podoplanin positivity was also found to be negatively correlated with vascular invasion and lymph node metastasis in cervical squamous cell carcinoma [13]. In 2017, Chen et al. first reported that podoplanin was a diagnostic marker that could be used to distinguish the early infiltration of esophageal squamous cell carcinoma [14]; however, it is not clear if it can be used in determining the early invasion of cervical cancer. In the present study, we assessed the podoplanin positivity in normal cervical squamous epithelium, HSIL, MISCC, and ISCC tissues by immunohistochemistry staining. We analyzed the value of podoplanin expression in the diagnosis of early infiltration in cervical cancer and clarified its relationship with both clinicopathological parameters and the prognosis of cervical cancer patients.

2. Materials and methods

2.1. Ethics statement

This study was approved by the ethics committee of the Medical School of Shandong University (ethic vote 201,301,050) in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before use of the materials.

2.2. Patient population

The paraffin-embedded specimens included 96 cases of normal cervix squamous epithelium (NS), 91 cases of normal cervix columnar epithelium (NC), 72 cases of MISCC, and 118 cases of ISCC. The MISCC tissues were collected from March 2005 to March 2013 in the Departments of Pathology at Qilu Hospital, Shandong Cancer Hospital, Shandong Provincial Hospital, and Jinan Maternal and Child Health Hospital. Other tissues were obtained from the Department of Pathology at Qilu hospital. Hematoxylin and eosin (HE) stained sections of all cases were blindly evaluated by two experienced pathologists. Diagnosis was made according to WHO classification of tumors. Cases

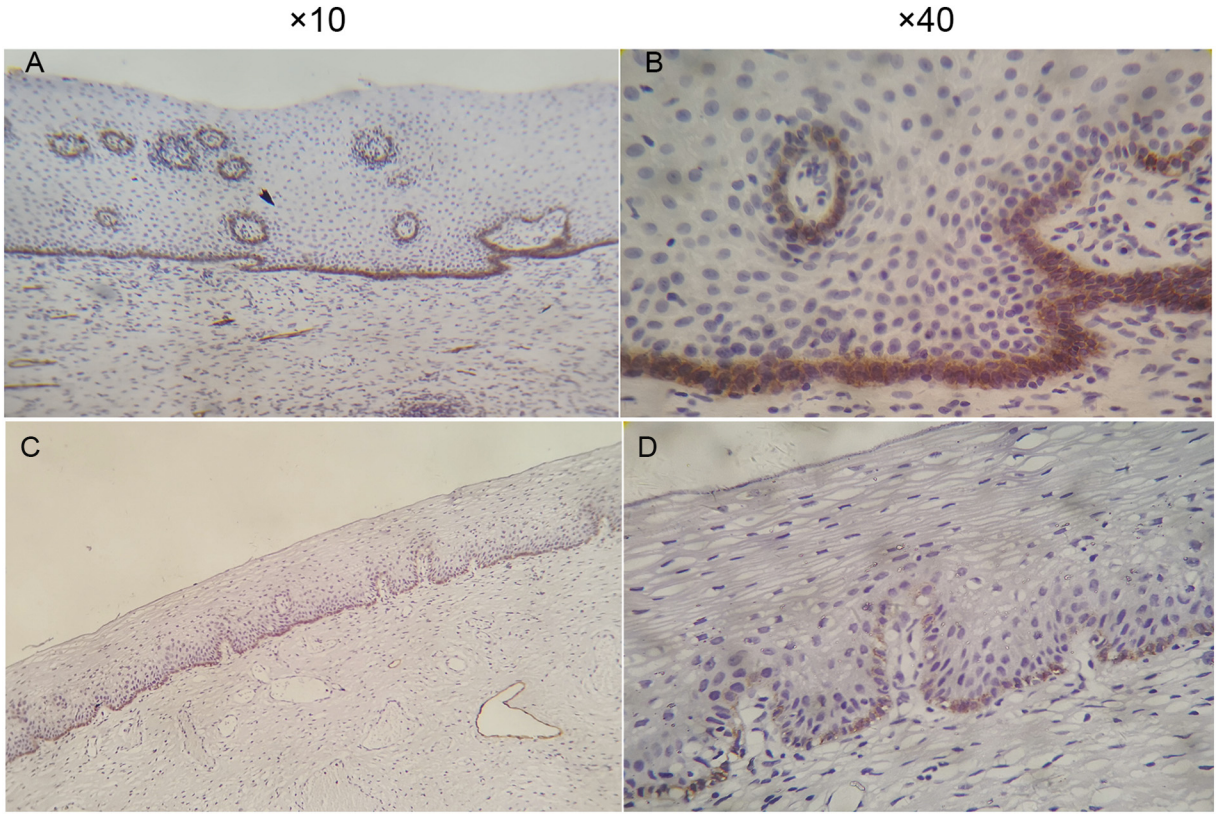

Fig. 1. Expression of podoplanin within the basal layer cells of normal cervical squamous epithelium in a continuous and discontinuous pattern. A–B. Representative figures showing continuous expression of podoplanin. C–D. Representative figures showing discontinuous expression of podoplanin. The lamina propria papillae projecting into the epithelium exhibit reduced signal of podoplanin protein.

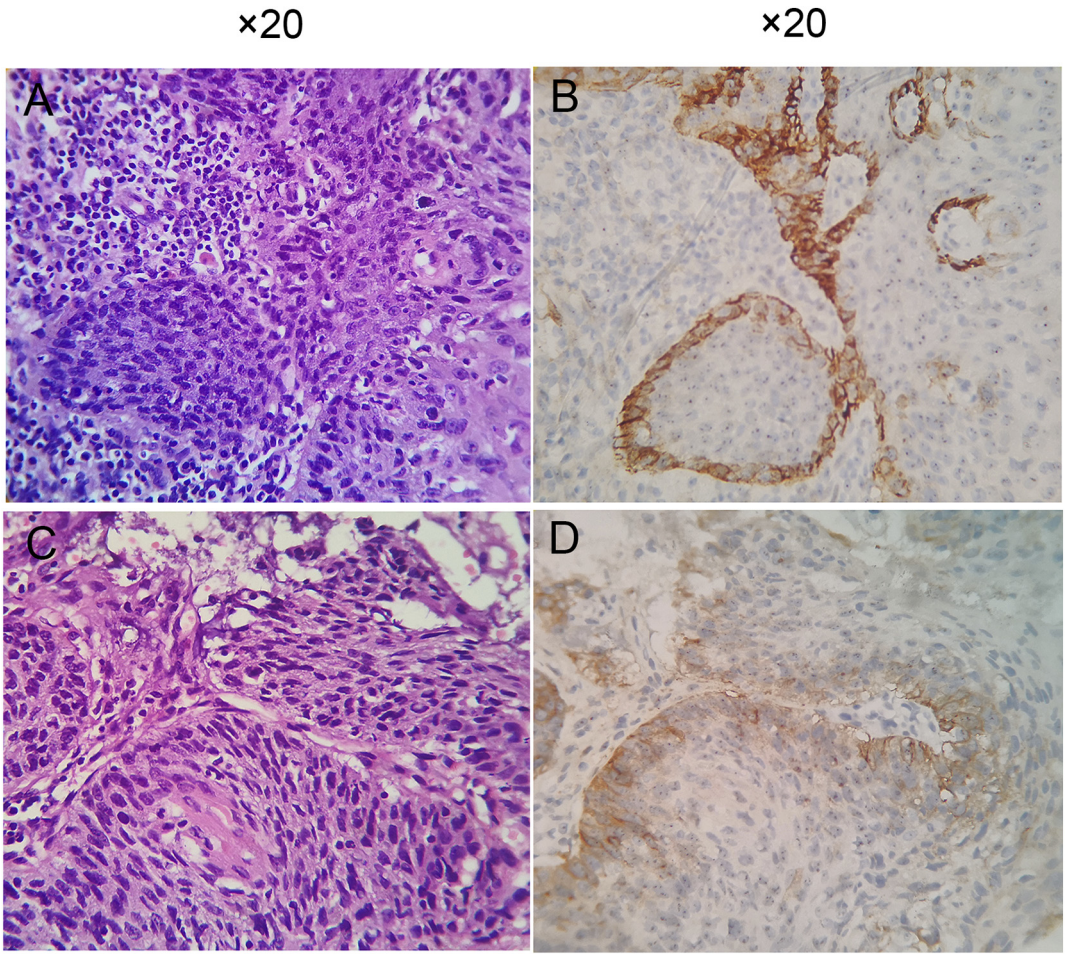

Fig. 2. Expression of podoplanin within the basal layer cells of high-grade squamous intraepithelial lesions (HSIL) in a continuous and discontinuous pattern. A. H&E staining of HSIL (×20); B. Immunohistochemistry (IHC) staining showed the continuous expression of podoplanin in HSIL (×20); C. H&E staining of HSIL (×20); D. IHC staining showed the discontinuous expression of podoplanin in HSIL (×20).

with inconsistent diagnosis were not enrolled in the study. Patient information were retrieved from the medical record room of each hospital.

2.3. Follow-up

Patients' follow-up was performed as previously described [15]. Patients were followed-up every 3 months until death or March 2018. Survival time, disease-free time, and development of metastases were recorded as survival data. The time period between the date of operation and death was recorded as the overall survival (OS) time. The time period between the date of surgery and recurrence or death was noted as the disease-free survival (DFS) time. Patients who were alive at the last follow-up were marked as censored observations.

2.4. Immunohistochemistry staining

Immunohistochemical staining was performed according to procedures previously described [16]. In brief, the slides were dewaxed, rehydrated, and immersed in EDTA buffer for antigen retrieval. Then, the sections were treated with 3% hydrogen peroxide, sealed with normal serum, and incubated with the primary antibody of podoplanin (ZM-0465, Ready-to-use type, Zhongshan Biotechnology Company, China) at 4 ˚C overnight. On the second day, Reagent 1 and Reagent 2 (PV-9000 kit, Zhongshan Biotechnology Company, China) were added to the slides separately. The slides were then stained with diaminobenzidine (DAB) and re-stained with hematoxylin. PBS buffer was used as a negative control. The staining results were evaluated by two pathologists in a blinded manner. The grading of staining intensity was as follows: 0 (no staining); 1+ (light yellow); 2+ (yellow brown); and 3+ (strong brown). Samples scored 0 to 1+ were considered negative, while others were positive.

2.5. Statistical analysis

SPSS 17.0 software (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. The chi-square test and Fisher's exact test were used to assess the relationship between podoplanin positivity and the clinicopathologic characteristics. The Kaplan-Meier method and log-rank test was used to analyze the survival of patients. $P < 0.05$ was considered to have statistical significance.

3. Results

3.1. Expression of podoplanin in normal cervical squamous epithelium

In order to assess the expression pattern of podoplanin in normal cervical epithelium, we used immunohistochemical staining to analyze podoplanin protein in normal cervical squamous epithelium and columnar epithelium. Our results showed that podoplanin was expressed in 100% of normal squamous epithelium (96/96), however it was not detected in columnar epithelium (0/91) (Table 1). Podoplanin protein was located in the membrane of the basal cellular layer of squamous epithelium in both a continuously and discontinuously linear pattern

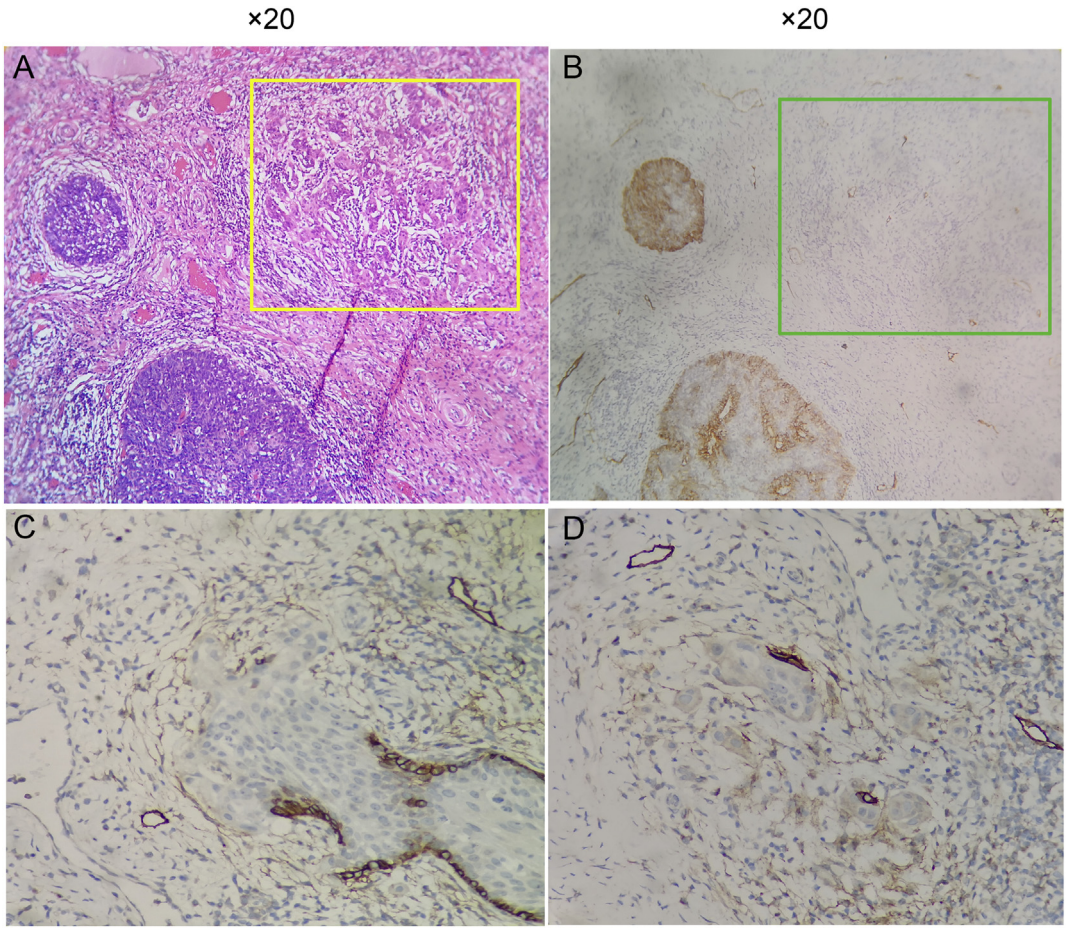

Fig. 3. Microinvasion showed loss of podoplanin expression as compared with high-grade squamous intraepithelial lesions (HSIL) in microinvasive squamous cell carcinoma (MISCC) cases. A. H&E staining of a MISCC case with HSIL (×20). The yellow box shows the microinvasion. B. IHC staining of podoplanin protein in the MISCC case with HSIL (×20). The green box shows that podoplanin expression was deficient in the micro-infiltrated area, while the surrounding HSIL showed strong expression of podoplanin. C & D. IHC staining of podoplanin protein in another MISCC case with HSIL (×20; ×20). Panel C shows that strong podoplanin expression in HSIL turned to complete loss during microinvasion. Panel D shows the complete loss of podoplanin expression during microinvasion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 1), and its staining intensity ranged from 1+ to 3+. It is noteworthy that the positive intensity of podoplanin in the papillary region of lamina propria was lower than that in other basal cells (Fig. 1). No significant non-specific staining was found in the stroma, aside from the lymphatic vessels. These results suggested that podoplanin has the potential to be used as a histological marker for the basal layer cells from the cervical squamous epithelium.

3.2. Expression of podoplanin in high-grade squamous intraepithelial lesions (HSIL)

MISCC develops from HSIL, and as such, the pathological changes of HSIL was able to be easily found around the microinvasive focus. To verify the expression of podoplanin in HSIL, HE sections of 117 cases of MISCC were collected. After blind review by two pathologists, 72 cases of MISCC with consensual diagnosis were used for immunohistochemical detection. Our results showed that HSIL was found in 70 of 72 cases, and podoplanin was detected in 81% (57/70) (Fig. 2), but lost in 19% (13/70) of HSIL (data not shown). Among the cases with podoplanin signal, the protein was expressed in a continuously linear pattern (66.7%, 38/57) (Fig. 2B) or discontinuously linear pattern (33.3%, 19/57) (Fig. 2D) (Table 1) within the membrane of the basal cell layer. No significant non-specific staining was found in the stroma, aside from the lymphatic vessels.

3.3. Expression of podoplanin in cervical microinvasive squamous cell carcinoma (MISCC)

In order to detect the changes of podoplanin expression during the progression of HSIL to MISCC, we compared the differences of podoplanin expression between small invasive lesions and their surrounding HSIL regions. Our results showed that the expression of podoplanin was lost when HSIL had microinvasive lesions, regardless of the size of the invasion and whether it was connected with the original basal cells (Fig. 3) (Table 1). The podoplanin-missing location and areas were consistent with that of microinvasion. In HSIL-negative cases, the expression of podoplanin was still negative in the small infiltration area (data not shown). No significant non-specific staining was found in the stroma, aside from the lymphatic vessels.

3.4. Expression of podoplanin in cervical invasive squamous cell carcinoma (ISCC) and its association with clinicopathological parameters and prognosis of patients

In order to assess podoplanin expression in cervical invasive squamous cell carcinoma, immunohistochemical staining was used to detect its expression in 118 specimens. Our results showed that the majority of ISCC cases (114/118; 96.6%) had a complete loss of podoplanin expression (Fig. 4A & B), while four cases exhibited weak-to-moderate expression (Table 1). The expression pattern was similar to that of HSIL,

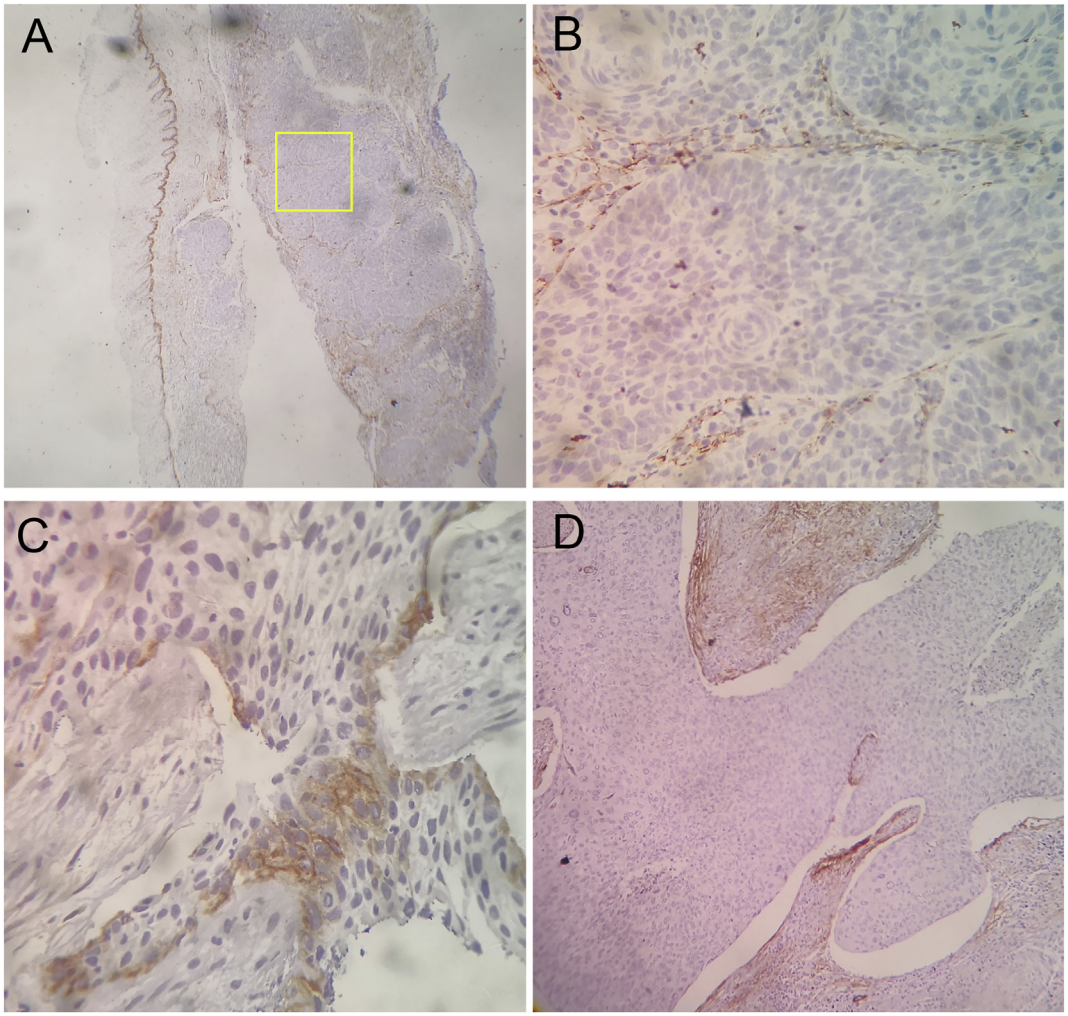

Fig. 4. Podoplanin expression in invasive squamous cell carcinoma (ISCC) and the adjacent stroma. A. In invasive cervical cancer, the normal squamous epithelium showed a continuous linear expression of podoplanin, while invasive cervical cancer showed no expression of podoplanin (yellow box) (×10). B. Enlargement of the yellow box sections of panel A (×40). C. Representative figure of ISCC cases showing podoplanin expression predominantly within the basal tumor cell layer (×40). D. Representative figure of ISCC cases showing weak-to-moderate cytoplasmic expression within the stroma surrounding the tumor cells, which were podoplanin negative. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

predominantly within the basal tumor cell layer with enhanced staining pattern (Fig. 4C). In addition, most cases of ISCC had no podoplanin staining within the stroma; however, 20 cases showed weak-to-moderate cytoplasmic expression within the stroma surrounding the tumor cells, which had negative podoplanin expression (Fig. 4D).

We next analyzed the relationship between podoplanin expression and the clinicopathological parameters of the patients. Among 118 cases, 98 of them with complete pathological information were enrolled. Our results showed that podoplanin positivity, not only in tumor cells but also in the stroma, was not correlated with the patient's age, tumor size, tumor differentiation, depth of invasion, FIGO stage, lymphatic vascular invasion, lymph node, or distant metastasis (Table 2). K-M (Kaplan-Meier) survival analysis showed that among 98 cases, patients with podoplanin expression had better overall and disease-free survival than those without it; however, the differences were not significant (data not shown).

4. Discussion

Since the treatment options of patients with *in situ* carcinoma and early invasive carcinoma are completely different, pathologists are often asked to determine whether early infiltration exists in the diagnosis of malignant tumors. However, in many cases, the observation of HE slices alone does not lead to a definite diagnosis [17]. Therefore, researchers have explored many immunohistochemical indicators of the basement membrane, such as collagen IV, fibronectin, and laminin, to assist pathologists in diagnosis [18,19]. However, these indicators target the extracellular matrix or interstitial components, which inevitably leads to varying degrees of non-specific staining. In addition, the newborn basement membrane has been found to deposit around tumor nests [20-22]. Therefore, microinvasion cannot be visualized by evaluating the continuity of the basement membrane. For some human tissues, such as the breast duct, salivary duct, and prostate gland, p63 can be used to label myoepithelium and basal cells, which can help pathologists to effectively determine microinvasion [18]. However, p63 is expressed within the basal and parabasal cells of normal cervical squamous epithelium and cervical cancer cells, and as such, this index cannot be used to determine minimal invasion in cervical cancer. At present, there is still no specific marker to indicate the early infiltration of cervical cancer. Therefore, efforts in this field are still worthwhile.

In the present study, we demonstrated that podoplanin was specifically located within the basal cells of cervical squamous epithelium and lymphatic endothelial cells with a high specificity of staining. This result was consistent with previous studies [9]. It is well known that the basal cells of normal squamous epithelium contain stem cells with a high proliferative activity, which gradually differentiate into the upper

Table 2
Association of clinicopathological parameters with podoplanin expression.

Characteristics	No. of patients	Podoplanin-positive	Podoplanin-negative	*p* value
Age, years				$P = 0.275$
≤40	32	4(12.5%)	28(87.5%)	
> 40	66	4(6%)	62(94%)	
Tumor size				$P = 0.115$
< 4 cm	62	3(4.8%)	58(95.2%)	
≥4 cm	36	5(13.9%)	31(86.1%)	
Differentiation grade				$P = 0.193$
High/moderate	40	5(12.5%)	35(87.5%)	
Low	58	3(5.2%)	55(94.8%)	
FIGO staging (range)				$P = 0.348$
IA2 to IB1	72	7(9.7%)	65(90.3%)	
IB2 to II A	26	1(4%)	25(96%)	
The depth of invasion				$P = 0.417$
< 1/2	25	3(12%)	22(88%)	
≥1/2	73	5(6.8%)	68(93.2%)	
LVSI				$P = 0.494$
No	93	8(8.6%)	85(91.4%)	
Yes	5	0(0%)	5(100%)	
LN metastasis				$P = 0.972$
No	74	6(8%)	68(92%)	
Yes	24	2(8.3%)	22(91.7%)	

Note: FIGO staging, The International Federation of Gynecology and Obstetrics Staging; LN, lymph node; LVSI, Lymph Vascular Space Invasion. $P < 0.05$ was regarded as statistically significant.

cell layers [19]. The specific expression of podoplanin within basal cells suggests that it tends to be expressed in primitive and immature cells. Consistently, podoplanin was found to be expressed in some primordial cells and their corresponding tumors, such as embryonic spermatogonia and seminoma [9,23]. Podoplanin has also been reported as a marker of cells with stem-cell-like properties, facilitating the formation of colonies *in vitro* [24,25].

Previous studies have shown that a majority of LSIL cases expressed podoplanin within basal cells. During the progression of LSIL to HSIL, the expression of podoplanin gradually absent [26]. In accordance with this, we found that 19% of HSIL in our study showed complete loss of podoplanin expression. This suggests that misdiagnosis would be unavoidable if we directly determined microinvasion based on the absence of podoplanin. However, HSIL cases with positive podoplanin, the continuous or discontinuous linear pattern of podoplanin were completely absent once microinvasion occurred. The absence of podoplanin matched with the appearance of micro-infiltration perfectly. Ideally, no significant non-specific staining was found in the stroma, aside from the lymphatic vessels. This observation was similar in patients with esophageal cancer [14]. Therefore, podoplanin has the potential to assist in the diagnosis of microinvasion of cervical cancer as a valuable indicator.

It has been reported that podoplanin could bind to a cell's cytoskeleton physically, activate RhoA protein, interact with ezrin/radixin/moesin (ERM) proteins, and engage in the epithelial mesenchymal transition (EMT). Thus, podoplanin can promote the migration and invasion ability *in vitro* and lymph node metastasis *in vivo* [27]. However, EMT has been shown to play an essential role in the invasion of single cells or small groups of cells during the early stages of cancer metastasis [28], but a minor role in massive invasion with collective cell invasion [29,30]. Similarly, in our study podoplanin expression was defective in MISCC cases and increased in invasive cervical cancer tissues. Previously, podoplanin expression was found to be associated with poor prognosis, especially in glioblastomas and squamous cell carcinoma [31-33]. However, in our study, patients with positive expression of podoplanin had better survival than those with negative expression of podoplanin. The data were consistent with a previous observation [13]. Therefore, we speculated that the role of podoplanin in cervical cancer was unique and differed from other tumors. In HSIL, the deletion of podoplanin expression might initiate EMT, which enables cells to acquire a greater invasive ability, thus destroying the basement membrane and forming micro-infiltration. However, the mechanism behind this phenomenon needs further investigation.

We further detected the expression of podoplanin within the stroma of cervical lesions. For NS, HSIL, and MISCC cases, no significant non-specific staining was found in the stroma even if lymphocyte infiltration occurred. The specific staining pattern of podoplanin ensured its utilization in the determination of microinvasion and the accuracy of pathological diagnosis. However, 20 of 118 ISCC cases showed weak-to-moderate cytoplasmic expression within the stroma surrounding the tumor cells. This result was consistent with previous reports that showed that podoplanin was a marker of cancer-associated fibroblasts (CAFs) in a variety of malignancies [30,32]. These podoplanin-positive fibroblasts could prevent immune cells from going deeper in the tumor parenchyma, in addition to inhibiting T-cell proliferation [33]. The non-specific staining of podoplanin protein indicated that it is not suitable for judging infiltration in invasive cervical cancer.

In this study, we systematically examined the expression of podoplanin in cervical tissues and evaluated its diagnostic role in the determination of early invasion in cervical squamous cell carcinoma. Our results indicated that for HSIL lesions with positive podoplanin expression, the complete loss of podoplanin represented the occurrence of early invasion. Thus, this marker can be used to assist pathologists in determining microinvasion. Considering the simple operation procedure, specific staining pattern, and the ease of evaluating the results, podoplanin has the potential to be used in clinicopathological diagnosis in the future.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

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