

# High expression of TGF- $\beta$ 1 in the vaginal incisional margin predicts poor prognosis in patients with stage Ib-IIa cervical squamous cell carcinoma

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**Abstract** This study evaluated the relationship between altered cytoplasmic expression of TGF- $\beta$ 1 in tissues of the vaginal incisional margin and vaginal cancer recurrence in patients with stage Ib-IIa cervical squamous cell carcinoma (CSCC). This paper also discusses the prognostic value of TGF- $\beta$ 1 expression at these locations. We found that TGF- $\beta$ 1 expression in the vaginal margin had a close association with vaginal recurrence of stage Ib-IIa CSCC and was an independent prognostic marker of this disease.

**Keywords** TGF- $\beta$ 1 · Cervical squamous cell carcinoma (CSCC) · Vaginal incisional margin · Prognosis

## Introduction

The morbidity of cervical squamous cell carcinoma (CSCC) gradually increases every year [1]. Patients with stage Ib-IIa cancer account for approximately 60–70% of all patients with CSCC [2, 3] in recent years. Thus, improving the prognosis of patients with stage Ib-IIa CSCC will increase

the total survival rate for women with this disease. Several factors are related to the prognosis of CSCC, including uterine wall infiltration and lymph node metastases, which also serve as clinical and therapeutic prognostic indicators. However, not all patients with particular clinicopathological parameters or treatments have the same prognoses. These differences occur because many genes are involved in the growth and development of cancer, a fact that yields interpatient differences in patients with the same clinicopathological parameters. Thus, the inability to universally apply these clinicopathological parameters imposes restrictions on their use for predicting a patient's prognosis and guiding therapeutic measures. Since the advent of molecular medicine, molecular markers have gradually become the primary indicators for the prognosis of various diseases. A recent study indicated that the gene expression pattern at the incisional margin was closely related to the prognosis of patients with hepatocellular carcinoma [4]. It is clear that transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) has a close relationship with CSCC and is involved in carcinogenesis of the cervical epithelium after human papillomavirus infection [5, 6]. Although many studies have reported that TGF- $\beta$ 1 is highly expressed in CSCC [7, 8], the data of Hazelbag et al. [8] did not show any prognostic value of TGF- $\beta$ 1 expression for CSCC. Residual tumor at the vaginal margin is the most frequent cause and location of tumor recurrence. It is also the main factor impacting the prognosis of CSCC [9]. Still, whether the molecular mechanism of vaginal recurrence of CSCC is similar to that of local recurrence of hepatocellular carcinoma remains unknown. In this sense, the relationship between the expression of TGF- $\beta$ 1 in the incisional margin and the recurrence and prognosis of CSCC requires further investigation. This study was undertaken to evaluate both the relationship between the TGF- $\beta$ 1 expression at the incisional margin of

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excised CSCC tissues and vaginal recurrence by immunohistochemistry as well as to explore the role of this protein in the prognosis of stage Ib-IIa CSCC.

## Materials and methods

### Patients

This study was undertaken at the First Affiliated Hospital of Zhengzhou University after approval from the Medical Ethics Committee was obtained. We collect our cases by a random collection from a patient database which contain 2,109 patients with complete clinic pathological parameters. For the study, 51 patients who had undergone radical hysterectomy and pelvic lymphadenectomy as part of the treatment of stage Ib-IIa CSCC, 20 patients with chronic cervicitis and 20 patients with normal cervixes who received cervical biopsies at an outpatient clinic were selected from 2002 to 2005. All of these patients underwent surgery and treatment at the Department of Gynecological Oncology of the First Affiliated Hospital of Zhengzhou University between January 2002 and November 2005. All of the necessary informed consent was obtained. The average patient age at the time of surgery was  $46.3 \pm 13.6$  years (range: 30–71 years). None of the tumor patients received chemoradiotherapy or immunotherapy before surgery.

### Sample selection and grouping

Paraffin-embedded tissues were obtained from patients with CSCC ( $n = 51$ ), chronic cervicitis ( $n = 20$ ) and normal cervical tissue ( $n = 20$ ). In the cancer group, there were nine cases with tumor diameter greater than 4 cm and 42 cases below 4 cm; 20 cases infiltrated more than two-thirds of the cervix, and the other 31 cases infiltrated to a lesser extent; 19 cases had a vaginal recurrence, and 32 cases did not; and 25 cases had lymph node metastases, while 26 did not. Immunohistochemical staining was used to detect the expression of TGF- $\beta$ 1. All patient care of the study participants was carried out at the First Affiliated Hospital of Zhengzhou University. The mean follow-up time was 51 months (range: 16–92 months). According to the FIGO 2000 staging standard, patients with CSCC were classified as stage Ib ( $n = 16$ ) or stage IIa ( $n = 35$ ) which were verified after surgery. According to the FIGO 2000 staging standard, patients with CSCC were classified after surgery as stage Ib ( $n = 16$ ) or stage IIa ( $n = 35$ ). A negative surgical margin and thorough pelvic lymphadenectomy was considered a complete resection [10]. The therapy options were selected based on the Vujkov protocol [10]. Clinicopathological information was obtained from the subjects' medical records. All cases were diagnosed by two pathologists. Tissue samples were taken

from the carcinoma and the incisional margin of cancer patients, the cervix of chronic cervicitis patients and the normal cervix of healthy women. These samples were fixed in 10% buffered formalin, processed and embedded in paraffin. All of the cases selected by us in our study should be negative in margin (no carcinoma and CIN were found in the vaginal incisal margin). According to the diagnostic standard of primary carcinoma of vagina by FIGO: no carcinoma was found in cervical and vulva; carcinoma in vagina was found 5 years later from the surgery treatment of invasive cervical carcinoma, 2 years later from the surgery treatment of cervical carcinoma in situ and 10 years later from the radiotherapy of invasive cervical carcinoma. All of the patients with vaginal recurrence we selected were found within 5 years from the surgery treatment.

### Immunohistochemical staining

According to the manufacturer's protocol, a standard streptavidin-biotin-peroxidase method was used. Sections were cut at a thickness of 4  $\mu$ m and dried overnight at 60°C. After deparaffinization, the sections were rehydrated in graded dilutions of alcohol and incubated in 0.03% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were heated in a microwave oven to 98°C for 15 min for antigen retrieval and were washed in PBS three times. To avoid background staining, the sections were then pre-incubated with 5% w/v purified bovine serum albumin (BSA) (Dade Behring, Liederbach, Germany) diluted in Tris buffer, pH 7.6, for 10 min. The sections were incubated with primary antibody overnight at 4°C. The primary specific goat polyclonal antibody against human TGF- $\beta$ 1 (sc-31609, Santa Cruz) was diluted 1:50 (38 ng/ml) in 1% w/v purified BSA (Dade Behring) in Tris buffer, pH 7.6. After incubation for 60 min, the sections were then incubated with a streptavidin-peroxidase conjugate for 30 min at room temperature and were washed with PBS ( $3 \times 10$  min). The color was developed with DAB plus chromogen for 10 min. The sections were rinsed in water, counterstained with Mayer's Hematoxylin for 3 min and finally dehydrated, mounted and coverslipped with Pertex. Washes with 5 mM Tris buffer, pH 7.6, with NaCl 0.9% w/v and Tween 0.1% v/v (TBS) were used between all steps in the procedure. After heating, all steps were performed at room temperature in a humidity chamber to avoid air-drying of the sections. The positive control was a breast carcinoma tissue section. Negative control staining was obtained by omitting the primary antibody.

Two expert pathologists with abundant experience evaluating immunohistochemically stained tissues immediately evaluated the expression of TGF- $\beta$ 1. A semi-quantitative scoring system was used to evaluate the IHC staining. The evaluation of IHC grade was performed by

referring to the study of Watari et al. [11]. A staining index was obtained by multiplying the score related to the percentage of positive cells (0: <20% positive staining; 1: 20–80%; 2: >80%) and the staining intensity score (0: negative staining; 1: weak; 2: moderate; 3: strong). Immunostaining of all slides was evaluated by specialists who were blinded to the clinical data. Scores of 0–1 were defined as low expression (including negative staining), a score of 2 indicated moderate expression, and scores  $\geq 3$  indicated high expression. The samples were divided into either the negative group (not stained at all) or the positive group based on the staining of TGF- $\beta 1$  various tissues.

### Statistical analysis

The Pearson's  $\chi^2$  test was used to compare the expression rates of TGF- $\beta 1$  among the three groups and to compare of demographics and clinicopathological differences among the groups. The contingency coefficient was used to measure the correlation among the vaginal recurrence, the history of HPV infection and the expression of TGF- $\beta 1$  at the incisional margin. Overall survival was estimated using Kaplan–Meier curves, and the log-rank test was used to compare the differences between survival curves. The Cox regression was used to calculate hazard ratios and 95% confidence intervals. All of the statistical analyses were carried out with SPSS 13.0, and a two-sided  $P < 0.05$  was considered significant.

## Results

### Expression of TGF- $\beta 1$ in different tissues of the cervix

The positive immunostaining of TGF- $\beta 1$  appears as cytoplasmic, granular brown-colored staining, as shown in Fig. 1. The frequency of cytoplasmic TGF- $\beta 1$  expression in CSCC (70.6%) was significantly higher than in chronic

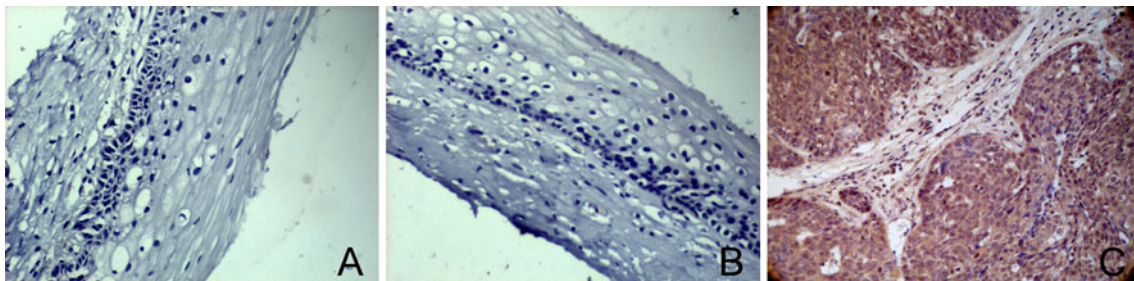
**Table 1** Expression of TGF- $\beta 1$  in different epithelial cells of the cervix

Epithelial of cervix	Positive cases	Negative cases	Positive rate of TGF- $\beta 1$ expression (%)	$\chi^2$	<i>P</i>
Normal	2	18	11.1	21.96	<0.0001
Chronic cervicitis	3	17	17.6		
Squamous carcinoma	31	20	70.6		

cervicitis (17.6%) and normal cervical tissues (11.1%) ( $P < 0.05$ , Table 1; Fig. 1a–c).

The association of TGF- $\beta 1$  expression in CSCC and at the incisional margin with the clinicopathological parameters of patients with stage Ib-IIa CSCC

The age, tumor diameter, infiltration depth, vaginal recurrence, history of HPV infection and evidence of lymph node metastasis of each patient with CSCC are summarized in Table 2. TGF- $\beta 1$  expression was observed in 29.4% (15/51) of patients at the incisional margin and 70.6% (31/51) in the carcinoma itself (Fig. 2). TGF- $\beta 1$  expression in CSCC and incisional margin tissues were both closely associated with a history of HPV infection, infiltration depth, and lymph node metastasis in patients with stage Ib-IIa CSCC ( $P$  all  $< 0.05$ ); however, TGF- $\beta 1$  expression showed no relation to patient age or tumor diameter ( $P$  all  $> 0.05$ ). Patients that had a history of HPV infection, deeper infiltration and lymph node metastases tended to have more positive cases of TGF- $\beta 1$  expression in CSCC and incisional margin tissues. Further, expression of TGF- $\beta 1$  exhibited a positive correlation with a history of HPV



**Fig. 1** The positive immunostaining of TGF- $\beta 1$  appears as cytoplasmic, granular brown-colored staining



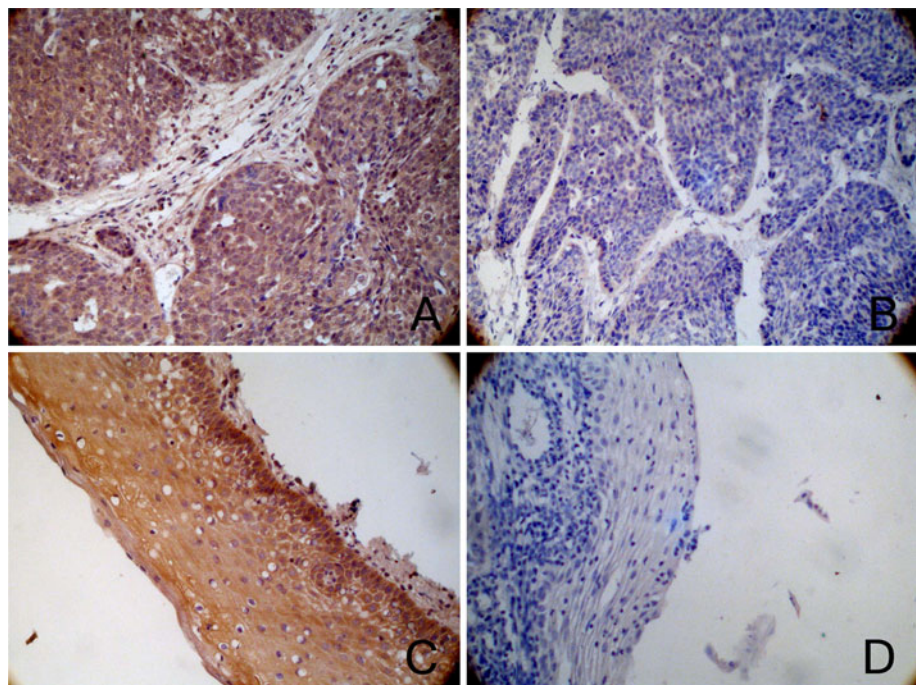
**Table 2** Association of TGF- $\beta$ 1 expression in CSCC and in the incisional margin with clinicopathological parameters in stage Ib-IIa CSCC

	Expression of TGF- $\beta$ 1 in incisional margin			Expression of TGF- $\beta$ 1 in CSCC tissue		
	Negative	Positive	<i>P</i>	Negative	Positive	<i>P</i>
<b>Age</b>						
≤40	22	11	0.4053	11	22	0.2440
>40	14	4		9	9	
<b>Tumor diameter (cm)</b>						
≤4	28	14	0.1843	16	26	0.7233
>4	8	1		4	5	
<b>Infiltration depth</b>						
≤2/3	18	2	0.0145	12	8	0.0146
>2/3	18	13		8	23	
<b>History of HPV infection</b>						
No	18	1	0.0035	12	7	0.0070
Yes	18	14		8	24	
<b>Vaginal recurrence</b>						
No	25	5	0.0170	14	16	0.1927
Yes	11	10		6	15	
<b>Lymph node metastasis</b>						
No	23	3	0.0043	15	11	0.0058
Yes	13	12		5	20	

infection and vaginal recurrence in cancer group (contingency coefficients: 0.37,  $\chi^2 = 8.06$ ,  $P = 0.0045$ ; 0.38,  $\chi^2 = 8.51$ ,  $P = 0.0035$ ; 50.32,  $\chi^2 = 5.70$ ,  $P = 0.0170$ ).

The survival analysis of the stage Ib-IIa CSCC patients and the significance of TGF- $\beta$ 1 in the prognosis of stage Ib-IIa CSCC

Univariate survival analysis demonstrated that infiltration depth, history of HPV infection, vaginal recurrence, lymph node metastasis and TGF- $\beta$ 1 expression at the incisional margin were significant prognostic indicators. However, multivariate Cox analysis showed that after the revision of the influence of the lymph node metastasis, vaginal recurrence, HPV infection and infiltration depth on the TGF- $\beta$ 1 expression at the incisional margin, only of TGF- $\beta$ 1 expression at the incisional margin and vaginal recurrence were independent risk factors that influenced the prognosis of patients with stage Ib-IIa CSCC ( $P = 0.0274$ ,  $P = 0.0200$ , respectively; Table 3). Patients positive for expression of TGF- $\beta$ 1 in the incisional margin had a significantly higher risk of death than the negative group (HR: 4.635, 95% CI: 1.187–18.106). Still, the expression of TGF- $\beta$ 1 in CSCC tissue itself displayed no prognostic value for patients with stage Ib-IIa CSCC ( $P = 0.3446$ ). Of the 51 patients with stage Ib-IIa CSCC, the overall survival of patients with tumors that stained positive for TGF- $\beta$ 1 who had vaginal recurrence was significantly different from that of patients with tumors that stained negative for TGF- $\beta$ 1 and did not have vaginal recurrence ( $P < 0.05$ ). Additionally, Kaplan–Meier survival curves confirmed that TGF- $\beta$ 1 positivity at the incisional margin in stage Ib-IIa CSCC was associated with an overall decreased survival time compared to patients who were TGF- $\beta$ 1 negative at

**Fig. 2** TGF- $\beta$ 1 expression

**Table 3** Results of multivariate analysis of prognostic factors

	Parameter estimation	Standard error	$\chi^2$	<i>P</i>	HR	95% CI
Infiltration depth	-0.37429	1.13893	0.1080	0.7424	0.688	0.074–6.411
History of HPV infection	0.88493	1.02997	0.7382	0.3902	2.423	0.322–18.240
Vaginal recurrence	1.63254	0.70164	5.4137	0.0200	5.117	1.293–20.241
Lymph node metastasis	0.76390	0.98610	0.6001	0.4385	2.147	0.311–14.830
Expression of TGF- $\beta$ 1 in incisional margin	1.53364	0.69522	4.8663	0.0274	4.635	1.187–18.106

the incisional margin (Fig. 3a). Nevertheless, whether or not TGF- $\beta$ 1 was expressed in CSCC tissue was no related to any difference in survival probability (Fig. 3b). In other words, patients who were TGF- $\beta$ 1 negative at the incisional margin had longer overall survival times than those who were TGF- $\beta$ 1 positive (median survival time: 88 months vs. 72 months). Similarly, the mortality of patients with vaginal recurrence was 5.117 times higher than those without vaginal recurrence. Because the death rate of the patients with vaginal recurrence was less than 50%, we used the mortality risk to represent the relationship between the patients with or without vaginal recurrence and overall survival status.

## Discussion

The significance of TGF- $\beta$ 1 expression in different epithelial locations of the cervix

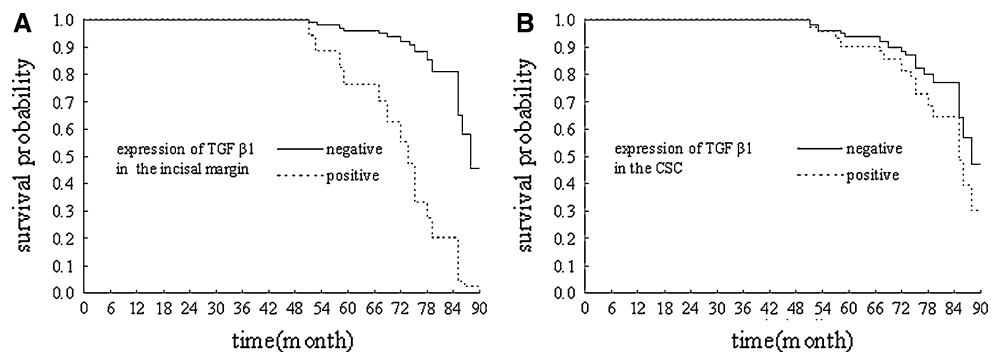
A series of studies elucidated the dual roles of TGF- $\beta$ 1 in the carcinogenesis of cervical epithelium [6, 7]: suppression of immortality and proliferation of cervical epithelium secondary to E6 and E7 protein expression after HPV infection [12] versus increased IL10 secreted by CSCC cells after HPV infection resulting in decreased antigen presentation by Langerhans cells and decreased cytotoxic T-cell proliferation. As a result, the cellular immunity of the body is weakened, and the virus can escape immunological surveillance, which ultimately supports the carcinogenesis of cervical epithelium [7]. No matter which mechanism

TGF- $\beta$ 1 acts through, many studies support that high TGF- $\beta$ 1 expression of in CSCC tissue is a reactive change [6, 7]. Baritaki et al. [5] also proposed that the upregulation of TGF- $\beta$ 1 in CSCC tissues was caused by HPV infection. Although the present study did not propose a mechanism by which of TGF- $\beta$ 1 could be elevated in CSCC, our data provides powerful histological evidence in support of the findings of Baritaki et al. The different expression of TGF- $\beta$ 1 in carcinoma, cervicitis and normal tissue of cervix also indicate that TGF- $\beta$ 1 might involves in the carcinogenesis of the cervical carcinoma.

The association of abnormal TGF- $\beta$ 1 expression in CSCC and incisional margin tissues in patients with stage Ib-IIa CSCC with metastasis and vaginal recurrence

The epithelial-mesenchymal transition (EMT) is one of the prerequisite conditions needed for a cancer cell to gain the properties of invasiveness and metastasis [13]. TGF- $\beta$ 1 had been verified as a key gene involved in the EMT [14]. The close relationship between (1) the expression of TGF- $\beta$ 1 in CSCC and at the incisional margin and (2) a series of invasive and metastatic indexes (lymph node metastasis and depth of infiltration) found in the present study suggests probable mechanisms of CSCC metastasis. Thus, patients with CSCC that had incisional margins positive for TGF- $\beta$ 1 may have residual tissue capable of achieving the EMT and subsequent metastatic potential. Yi et al. [15] found that the cervical carcinoma cell line Siha gained the characteristics of the EMT after the concentration of TGF- $\beta$ 1

**Fig. 3** The significance of TGF- $\beta$ 1 in the prognosis of stage Ib-IIa CSCC



was increased in the media; this result provides powerful support for our conclusion about the probable role of TGF- $\beta$ 1 in CSCC metastasis. In addition, the authors' data showed that the expression of TGF- $\beta$ 1 at the incisional margin was positively correlated with prior HPV infection and vaginal recurrence. These data indicate that after thorough resection of the carcinoma, carcinogenesis might occur in the squamous cells of the remaining vaginal tissue that is TGF- $\beta$ 1 positive; this TGF- $\beta$ 1 positivity was a result of HPV infection. Furthermore, we did not find a relationship between TGF- $\beta$ 1 expression in CSCC tissue itself and vaginal recurrence, which suggests that vaginal recurrence might be a direct result of TGF- $\beta$ 1 positivity at the incisional margin.

The prognostic value of TGF- $\beta$ 1 expression in CSCC and at the incisional margin in patients with stage Ib-IIa CSCC

Several factors, such as infiltration depth, vaginal recurrence and lymph node metastasis, can influence the prognosis of stage Ib-IIa CSCC [16]. However, individual differences in molecular expression limit the prognostic values of these clinicopathological indexes for patients with CSCC. Hoshida et al. [4] found that the abnormal expression of a series of sixteen genes at the incisional margin of resected hepatocellular carcinoma (HCC) had a strong correlation with HCC recurrence. They also concluded that the mechanism responsible for HCC recurrence might be abnormal gene expression, which could result in a “new born” tumor. The data of Hazelbag et al. [8] illustrated that, although the expression of TGF- $\beta$ 1 in CSCC was related to many clinicopathological parameters, its prognostic value in CSCC remains weak. In contrast to the results of the Hazelbag study, the multivariate model used in the present study confirmed that TGF- $\beta$ 1 expression is an independent prognostic marker for patients with stage Ib-IIa CSCC. Collectively, TGF- $\beta$ 1 positivity at the incisional margin of resected CSCC might represent residual “seeds” for tumor recurrence even after a thorough resection and standard adjuvant therapy. This supposition suggests that blocking TGF- $\beta$ 1 expression in patients with incisional margins positive for TGF- $\beta$ 1 expression and with a history of HPV infection could prolong the disease-free and, perhaps, overall survival of these patients. Another study [17] showed that vaginal recurrence and lymph node metastasis were independent prognostic factors for stage Ib-IIb CSCC; however, our study found that lymph node metastasis was not an independent prognostic marker for this malignancy. This discrepancy in findings strikingly highlights that lymph node metastasis mainly influences the prognosis of stage IIb CSCC only.

Moreover, the results of the present study revealed that abnormal TGF- $\beta$ 1 expression in CSCC had no prognostic value, while TGF- $\beta$ 1 expression at the incisional margin was closely correlated with stage Ib-IIa CSCC prognosis. This finding also suggested that, after resection, it may be beneficial to prescribe chemoradiotherapy based on differential TGF- $\beta$ 1 expression at the incisional margin. The data of Yang et al. [18] confirmed the relationship between TGF- $\beta$ 1 expression and tumor sensitivity to chemoradiotherapy, yet another explanation for the differing prognostic value of TGF- $\beta$ 1 expression at the incisional margin and within CSCC tissue itself.

Although little report indicate that expression of TGF-beta1 in carcinoma have poor prognostic value in some solid tumors, and that Hazelbag's [8] data showed it also have poor prognostic value in CSCC, our discovery undoubtedly be a well supplement for the forgoing research.

The present study is the first to report the powerful prognostic value of TGF- $\beta$ 1 expression at the incisional margin of patients with resected stage Ib-IIa CSCC. The Kaplan–Meier curve showed that TGF- $\beta$ 1 positivity at the incisional margin was associated with shortened overall survival of these patients. This result indicates that, although these patients are technically all at the same stage in terms of infiltration depth and lymph node metastasis, women with TGF- $\beta$ 1-positive incisional margins may warrant special attention due to a higher risk of earlier recurrence and poor prognosis. The reason of expression of TGF-beta1 in CSCC has no prognostic value and the molecular mechanism of the expression of TGF-beta1 in vaginal incisal margin has significantly prognostic value are next step of our work. Of cause, our present data probably imply that this might because that the expression of TGF-beta1 in carcinoma has no relevant with the independent prognostic marker—vaginal recurrence while the expression of TGF-beta1 in vaginal incisal margin was relevant to the vaginal recurrence. Herein, we provide not only a potential prognostic marker and a target for controlling the recurrence, but we also illustrate the importance of the molecular staging.

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