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Expressions of survivin, P16^{INK4a}, COX-2, and Ki-67 in cervical cancer progression reveal the potential clinical application

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Summary

Purpose of investigation: To explore the significance of survivin, P16^{INK4a}, COX-2, and Ki-67 expressions for prediction of cervical cancer progression. *Materials and Methods:* A retrospective study was performed in 129 cases including 24 squamous carcinoma of the cervix (SCC), 70 cervical intraepithelial neoplasias (CIN), 15 cervical condyloma acuminatum (CCA), ten chronic cervicitis (CC), and ten normal cervix (NC). Protein expressions were evaluated using immunohistochemistry. *Results:* Survivin, P16^{INK4a}; COX-2, and Ki-67 were highly expressed in SCC and CIN compared with others. Their expression rates were gradually increased in CIN I, CIN II, CIN III, and SCC groups, showing 72.00%, 88.00%, 90.00%, and 95.83% for P16^{INK4a}, 68.00%, 84.00%, 95.00% and 100.00% for COX-2, 76.00%, 96.00%, 100.00%, and 100.00 for Ki-67, respectively. There were significant correlations between survivin and P16^{INK4a}, COX-2, Ki-67, as well as P16^{INK4a} and Ki-67. *Conclusion:* Survivin, P16^{INK4a}, COX-2 and Ki-67 play critical roles for development and progression of cervical cancer.

Key words: Cervical cancer; Survivin; P16^{INK4a}; Cyclooxygenase-2; Ki-67; Immunohistochemistry.

Introduction

Cervical cancer is a malignant neoplasm arising from cells originating in the cervix uteri. It is prevalent among women worldwide and performs a high mortality for a long time [1]. It is reported that there are 529,800 new cases all over the world, of which around 80% are diagnosed in developing countries [2, 3]. Cervical cancer has been reported to be largely associated with human papillomavirus (HPV). As reported, at least 93% of invasive cervical cancers were infected by HPV [4]. However, the exact mechanism is still controversial.

As is known, the progression of cervical cancer involves many oncogenes and cancer suppressor genes, which finally lead to abnormal tumor proliferation in cervix uteri [5, 6]. Of them, survivin as one of novel inhibitors of apoptosis proteins playing a key role in cervical intraepithelial neoplasia (CIN) and squamous cervical carcinoma (SCC) [7]. P16^{INK4a} and Ki-67 were indicated to be useful biomarkers of cervical neoplasia [8]. Additionally, as outlined in a previous study, cyclooxygenase-2 (COX-2) was reported to contribute to preventing epithelial malignancies [9].

Nowadays, the literatures clarifying the correlation of survivin, P16^{INK4a}, COX-2, and Ki-67 are relatively rare. Thus, survivin, P16^{INK4a}, COX-2, and Ki-67 were selected to assess their correlation in cervical cancer onset and progression in the present study. The authors aimed to explore the potentially predictive values of survivin, P16^{INK4a}, COX-2, and Ki-67 in the progression and development of cervical cancer.

Materials and Methods

Objects

This study was approved by the Ethics and Clinical Research Committee of the Faculty of Medicine of the present University.

Patients with cervical cancer were from Shanghai Fengxian District Central Hospital in China, ranging from 2005 to 2010. The total 129 patients contained 70 cases of cervical intraepithelial neoplasm (CIN) (CIN I: 25, CIN II: 25, CIN III: 20), 24 cases of squamous cell carcinoma (SCC), 15 cases of cervical condyloma acuminatum (CCA), and ten cases of chronic cervicitis (CC). Normal cervical (NC) tissues were separated from ten healthy individuals as control. The mean age of the total patients was 39.13 ± 7.07 years and the mean age of group CIN I, CIN II, CIN III, SCC, CCA, CC, and NC was 37.28 ± 5.64, 38.64 ± 6.15, 37.52 ± 6.41, 45.83 ± 7.19, 37.60 ± 3.33, 37.90 ± 5.80, and 36.60 ± 7.96 years, respectively. All cases did not undergo radiotherapy or chemotherapy before diagnosis.

Electronic colposcopy

The electronic colposcope test was performed by full-time physicians. Before test, the patients with vaginitis were excluded. In the preliminary observation, the cervix secretion of every individual was wiped off with normal saline cotton ball to observe the cervical characters including morphology, size, pathological feature, color, and luster. Then the patients were treated with 3% acetic acid to inspect the vessels by a green filter. After that, patients were conducted to receive the iodine test, followed by being

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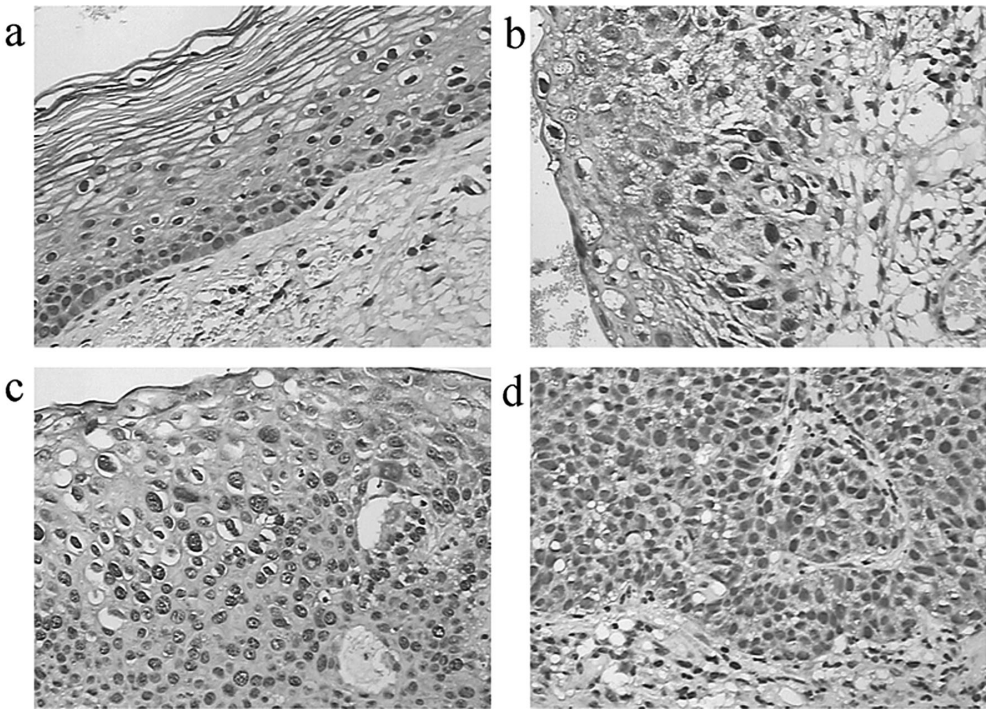

Figure 1. — Positive staining for survivin in CIN and SCC. a: CIN I (I H C ×200), b: CIN II (I H C ×200), c: CIN III (I H C ×200), d: SCC (I H C ×200).

graded using Reid colposcopic scores. For the patients with abnormal or suspicious image areas, about four to five biopsy samples were obtained, fixed in 10% neutral formalin solution, and then sent to pathology department for examination. The endocervical curettage was carried out if necessary and the image of abnormal areas were collected for further investigation.

Hematoxylin/eosin (HE) staining

The tissue specimens from normal and disease cervix uteri were embedded in paraffin. Four-μm serial sections were cut and dried at 60°C overnight. After dewaxing by dimethylbenzene, sections were stained with HE.

Immunohistochemistry

The immunohistochemical procedure was performed according to the method previously reported [10]. The primary antibodies used in this assay as followed: mouse monoclonal antibody p16 ^{INK4A} (1:100 dilution), mouse monoclonal antibody Survivin (M-068) (1:100 dilution), mouse monoclonal antibody COX-2 (M-0715) (1:100 dilution), and mouse monoclonal antibody Ki-67 (M-0693) (1:100 dilution). The sections from the same sample were divided into two groups. The negative control group had phosphate buffered saline (PBS) added instead of primary antibodies. Then the sections were stained with HE for one minute and observed by microscope.

Positive results analysis

The positive cell gathered region of each sample was analyzed randomly by light microscope under high power fields. The total of 1,000 cells were observed. The percentage of positive cells was calculated as following:

Positive expression rate (%) = the score of immunoreactivity was in the light of two criterions. One criterion was in accordance with the degree of positive cells coloring and the immunoreactivity was divided into four grades: 0 (negative- no stained cells), 1 (weak positive- pale-yellow cells), 2 (moderate positive –Brown cells), and 3 (strong positive- Sepia cells). Another criterion was based on the proportion of positive cells and scored as follows: 0 (< 5%), 1 (5% ~ 25%), 2 (26% ~ 50%), 3 (51% ~ 75%), and 4 (> 75%). The final results were cumulative by the two criterion scores and defined as 0: negative (-), 1~4: weak positive (+), 5~8: moderate positive (++), 9~12: intense positive (+++). P16^{INK4a} and survivin positively expressed cells were defined as the cells with brown particles in nucleus or cytoplasm, while COX-2 and Ki-67 positive cells were defined as the cells with brown particles only in cytoplasm and nucleus, respectively.

Statistical analysis

All the data were analyzed by SPSS 18.0 software. Count data were analyzed with rates or proportions. Significant differences between different groups were evaluated using chi-square. The difference and correlation of graded scores were analyzed by nonparametric Wilcoxon test and Spearman rank correlation analysis, respectively. The statistically significant difference was defined as $p < 0.05$.

Results

Survivin expression in cervical tissue

All normal cervical tissues showed negative expression for survivin. Most of the koilocytes in CCA group showed survivin positive expression. Figure 1 shows cell nuclear staining and cytoplasm staining for survivin, especially in nuclear staining of the koilocytes in groups of CIN II, CIN III, and SCC group.

As shown in Table 1, there was statistically significance of survivin expression rate in NC, CC, CCA, CIN, and SCC group ($p < 0.05$). The positive staining for survivin among CIN I, CIN II, CIN III, and SCC groups were not statisti-

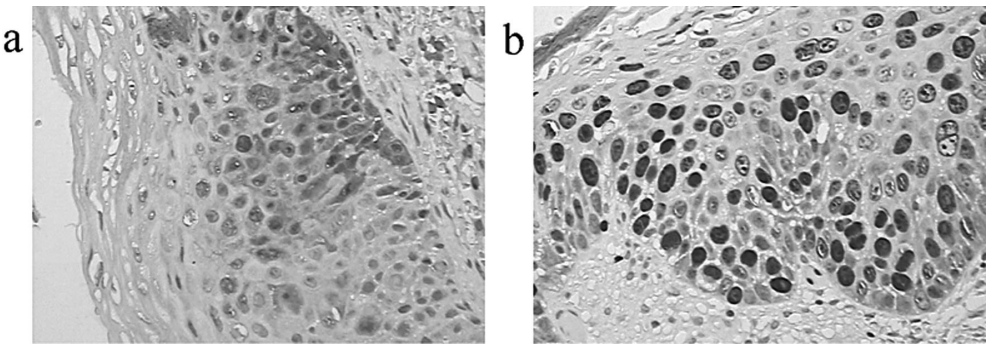

Figure 2. — Positive staining for P16^{INK4a} in CIN II and CIN III. a: CIN II (I H C ×200), b: CIN III (I H C ×200).

Table 1. — *Survivin expression in cervical carcinoma of each group.*

Group	n	Survivin				Positive
		-	+	++	+++	Rate (%)
NC	10	10	0	0	0	0.00
CC	10	8	2	0	0	20.00
CCA	15	9	5	1	0	40.00
CIN I	25	5	14	6	0	80.00
CIN II	25	2	5	14	4	92.00
CIN III	20	3	5	8	4	85.00
SCC	24	5	5	9	5	79.17

Table 2. — *P16^{INK4a} expression in cervical carcinoma of each group.*

Group	n	P16^{INK4a}				Positive
		-	+	++	+++	Rate (%)
NC	10	10	0	0	0	0.00
CC	10	9	1	0	0	10.00
CCA	15	10	3	2	0	33.33
CIN I	25	7	11	5	2	72.00
CIN II	25	3	8	10	4	88.00
CIN III	20	2	3	9	6	90.00
SCC	24	1	2	8	13	95.83

cally significant ($p > 0.05$). The Spearman rank correlation coefficient (r = 0.486, $p = 0.000$) showed that graded expression of survivin in each group were positively correlated. The degree difference of survivin expression between CIN I and CIN II, CIN III, and SCC groups were statistically significant (z = -3.323, $p = 0.001$; z = -2.265, $p = 0.023$; z = -2.009, $p = 0.045$).

P16^{INK4a} expression in cervical tissue

P16^{INK4a} was not stained in the cervical tissues. P16^{INK4a} was expressed in both cytoplasm and nuclear. P16^{INK4a} was stained in the lower 2/3 of epithelium cells in CIN II and CIN III group, which correspond with the histopathology results (Figure 2). The staining trend of P16^{INK4a} was similar with survivin from CIN to SCC (Figure 1). CIN and SCC group showed strong P16^{INK4a} staining in squamous koilocytes almost accompanied with HPV infection.

The differences of P16^{INK4a} expression rates in NC, CC, CCA, CIN and SCC were statistically significant ($p < 0.05$). There were no statistically significance of the positive expression rates among CIN I, CIN II, CIN III, and SCC groups ($p > 0.05$). The correlation analysis of group levels expression, Spearman rank correlation coefficient r = 0.706, $p = 0.001$, were positively correlated. The comparisons of survivin expression levels among CIN I, CIN II, CIN III, and SCC were statistically significant (z = -2.015, $p = 0.0044$, z = -2.919, $p = 0.004$, z = -4.241, and $p = 0.001$). There was a significant difference between CIN II group and SCC group (z = -3.004, $p = 0.003$, Table 2).

COX-2 expression in cervical tissue

COX-2 was localized in cytoplasm and mainly expressed in cancer and atypical hyperplasia cells. Results showed that it could be observed in regenerately vascular endothelial cells of cervical tissue (Figure 3).

The differences of COX-2 expression rates among NC, CC, CCA, CIN, and SCC group were statistically significant ($p < 0.05$). Compared analysis of COX-2 expression rate in CIN I, CIN II, CIN III, and SCC group, only CIN I with CIN III group ($p = 0.030$, $p = 0.004$), and CIN I with SCC group were significantly different, while the other groups had no statistically differences ($p > 0.05$). The correlation analysis of group levels expression, in which the Spearman rank correlation coefficient (r = 0.728 and $p = 0.001$) were positively correlated (Table 3). Compared with CIN II, CIN III, and SCC group, the difference in the degree of positive COX-2 expression of CIN I group had statistical significance (z = -2.015, $p = 0.0044$; z = -2.919, $p = 0.004$; z = -4.241, $p = 0.001$), but the statistically significant differences were not tested between CIN II and CIN III, CIN III, and SCC group ($p > 0.05$). Compared CIN (CIN I, CIN II, CIN III) group with the SCC group, the difference was significant (z = -2.373, $p = 0.018$, Table 3).

Ki-67 expression in cervical tissue

The normal cervical epithelium, cervical glandular epithelium and metaplasia showed scattered staining particle of Ki-67 around cell nucleus. Ki-67 was positively expressed in nucleus of the cervical warts koilocytes. In cervical intraepithelial lesions and SCC tissues, Ki-67 staining

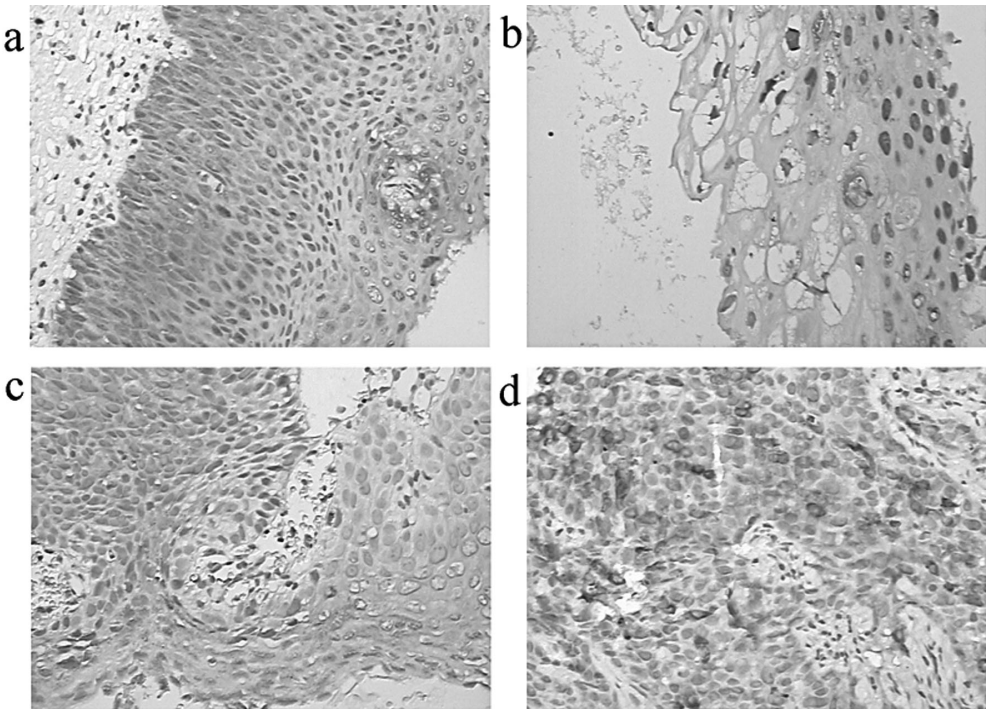

Figure 3. — Positive staining for COX-2 in CIN and SCC. a: CIN I (I H C ×200), b: CIN II (I H C ×200), c: CIN III (I H C ×200), d: SCC (I H C ×100).

Table 3. — *COX-2 expression in each group of cervical carcinoma.*

Group	n	COX-2				Positive
		-	+	++	+++	Rate (%)
NC	10	10	0	0	0	0.00
CC	10	9	1	0	0	10.00
CCA	15	13	2	0	0	13.33
CIN I	25	8	12	5	0	68.00
CIN II	25	4	5	13	3	84.00
CIN III	20	1	4	8	7	95.00
SCC	24	0	8	7	9	100.00

Table 4. — *Ki-67 expression in each group of cervical carcinoma.*

Group	n	Ki-67				Positive
		-	+	++	+++	Rate (%)
NC	10	9	1	0	0	10.00
CC	10	7	2	1	0	30.00
CCA	15	8	5	2	0	46.66
CIN I	25	6	12	6	1	76.00
CIN II	25	1	7	13	4	96.00
CIN III	20	0	0	9	11	100.00
SCC	24	0	0	6	18	100.00

degree enhanced and the distribution was extended ranging from the upper 2/3 of cervical epithelium to the full-thickness (Figure 4).

There were statistically significant differences of Ki-67 positive expression rates between NC and CCA group ($p < 0.05$), while compared with CCA and CC group; the difference was not significant ($p > 0.05$). The statistically significant difference of the positive expression rates were shown between CIN I, CIN II, CIN III, SCC and NC, CC, CCA groups ($p < 0.05$). Through correlation analysis of graded Ki-67 expression, the correlation coefficient was calculated as r = 0.817, $p = 0.001$, which showed the data were positively correlated. The comparisons of Ki-67 graded expression among CIN I, CIN II, CIN III, and SCC group were statistically significant (z = -2.998, $p = 0.003$; z = -3.322, $p = 0.001$; z = -5.595, $p = 0.001$; z = -3.322, $p = 0.001$; z = -4.364, $p = 0.001$) except the comparison between CIN III and SCC group (z = -10378, $p = 0.168$) and there was a significant difference between CIN group and SCC group (z = -20373, $p = 0.018$, Table 4).

The correlation analysis of survivin, P16^{INK4a}, COX-2 and Ki-67 expression in CIN and SCC group

As shown in Table 5, the correlations were significant between survivin and P16^{INK4a}, COX-2, Ki-67 expression ($p < 0.05$). There was statistical significant between P16INK4a and Ki-67 expression ($p < 0.001$). The expression of COX-2 was relevant with P16INK4a and Ki-67 expression, but the expression differences were not significant statistically ($p > 0.05$).

Discussion

The development of cervical cancer is a long-term process and the pathogeny seems to be diversified. Of this, the imbalance of the cell proliferation and apoptosis is one of the

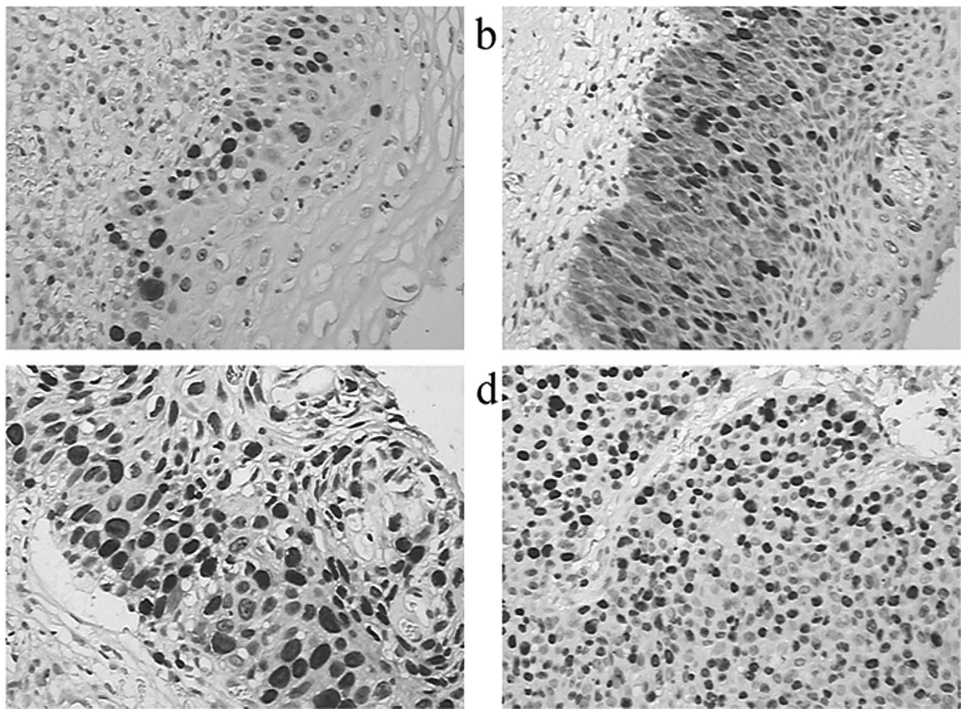

Figure 4. — Positive staining for Ki-67 in CIN and SCC. a: CIN I (I H C×200), b: CIN II (I H C×200), c: CIN III (I H C×200), d: SCC (I H C×100).

Table 5. — C*orrelation analysis of Survivin, COX-2, Ki-67 and P16^{INK4a}*.

	Survivin		COX-2		Ki-67	
	r	*p*	r	*p*	r	*p*
Survivin	-	-	0.330	0.001	0.319	0.002
P16^{INK4a}	0.246	0.017	0.197	0.058	0.356	0.000
Ki-67	-	-	0.212	0.245	-	-

main factors leading to cervical cancer. The cell factors, affecting cellular proliferation and apoptosis, were considered to be functional in the formation and development of tumor, such as survivin, P16^{INK4a}, COX-2, and Ki-67. In this paper, the authors applied HE and immunohistochemical staining to explore the relationship of these factors with cervical cancer.

Survivin, a member of apoptosis inhibitor family, is recently known to be relevant to cancer onset and progression [12]. Survivin inhibits apoptosis and ensuring normal cell division by regulating G2/M phase of cell-cycle. Overexpressed survivin sequesters physiologically relevant caspase on microtubules to default apoptosis [13]. There were evidences that the expression of survivin was elevated in CIN and SCC tissues along with tumor progression [14, 15]. Survivin was considered to be an independent prognostic predictor for the development and prognosis of cervical cancer [16]. As shown in the present result, survivin was not expressed in NC group, but there was an increasing expression in cells from CC, CCA to CIN and SCC. Survivin expression in CIN II, CIN III, and SCC was significantly higher than that in normal tissues. In addition, the survivin staining was observed in cytoplasm of all groups except for NC. CIN II, CIN III and SCC group showed positive staining in both cell nucleus and cytoplasm. It indicates that in the early-stage cervical cancer, survivin mainly expressed in cytoplasm of immature squamous cells. As the cervical cancer developed, survivin was gradually expressed in cell nucleus. The nucleus expression of survivin might be the biomarker for the prediction of cervical cancer.

P16^{INK4a}, a tumor suppressor protein, plays a key role in preventing cell proliferation by specifically inhibiting cyclin D-dependent kinases [17]. Previous studies reported that P16^{INK4a} was increasingly expressed in HPV infected cervical cells to regulate the increasing expression of viral oncogenes [18]. A strong expression of P16^{INK4a} was detected in all the cervical cancer cases with a high-risk HPV positive tying [19]. The degree of P16^{INK4a} expression in cervical tissue had a indicative effect on the severity of cervical neoplasia ($p < 0.001$) [8]. These conclusions are consistent with the present results. Therefore, P16^{INK4a} can be regarded as a specific marker in HPV-induced cervical neoplasia, which can help to identify the severity of diseases [20]. COX-2 which is responsible for prostaglandins synthesis, is prevalent in normal cells but COX-2 expression can be upregulated in tumor formation. The elevated expression of COX-2 directly leads to an increasing level of prostaglandins. It is reported that prostaglandins may possess multiple function in cancer development [21, 22], for instance prostaglandin E2 up-regulate apoptosis protein Bcl-2 to promote tumor

growth [23]. COX-2 may enhance tumor invasion and metastasis by suppressing cell apoptosis [24]. As outlined in previous studies, COX-2 expressed in cervical cancer cells but it was undetectable in normal cervical tissues [9]. The similar result was found in the present work that there was no COX-2 expression in NC. The degree of COX-2 staining had positive correlation with degree of cervical neoplasia from CIN to SCC ($r = 0.460$). In the progression of cervical cancer, COX-2 expression was a common molecule event.

Like survivin, Ki-67 is another cell-cycle regulatory protein which has been found to be involved in cervical carcinoma [25]. Ki-67 expressed in all active phases of normal cell cycle except for G_0 [26]. The Ki-67 expression is defined as a proliferation marker for assessment of cervical cancer [27-29]. It was reported that the detection of Ki-67 protein showed highest accuracy in recognizing cervical cancer [30] and progression prediction in CIN [31]. Moreover, another research showed that the expression of Ki-67 detected in low grade squamous intraepithelial lesion (LGSIL), high grade squamous intraepithelial lesion (HGSIL), and SCC cases were 25%, 68%, and 65.5%, respectively [32]. The change of Ki-67 expression correlates with the severity of the lesion, which is helpful for diagnosis in cervical cancer development [32]. In the present study, Ki-67 staining in CIN I, CIN II, CIN III, and SCC was 76%, 96%, 100%, and 100%, respectively. CIN and SCC showed stronger staining than NC, CC, and CCA. CIN I to III had an increasing trend in Ki-67 expression but there was no statistical significance between CIN III and SCC ($p > 0.05$). Therefore the present authors speculated that with the increase of level of CIN, cell proliferation activity gradually enhanced and Ki-67 index was the crucial value in cervical cancer progression.

The development and progression of cervical cancer is characterized for deregulated cell cycle and abnormal cell proliferation [33, 34]. Potential biomarkers, $P16^{INK4a}$, Ki-67, and survivin and COX-2 were all associated with cervical lesions in different degrees and could predict the progression of cervical cancer [35]. The dysregulation of survivin, $P16^{INK4a}$, COX-2, and Ki-67 expression increased the risk of cervical cancer. In the present study, the correlation of survivin expression with $P16^{INK4a}$, Ki-67, and COX-2 were statistically significance ($p < 0.05$). Survivin has multiple bio-functions in cell cycle, proliferation and apoptosis [36-38]. $P16^{INK4a}$ and COX-2 were considered to induce cell cycle arrest leading to apoptosis in cancer [39, 40]. Ki-67 was detected to be only in proliferating cells and strictly associated with cell cycle [41]. Survivin might control apoptosis with interaction with $P16^{INK4a}$, COX-2, and Ki-67. Survivin competitively interacted with the Cdk4/$P16^{INK4a}$ for initiating cell cycle entry [37]. Survivin had positive correlation with COX-2 in endometrial carcinoma. It was speculated that survivin and COX-2 enhanced each other's function in the same molecule pathway [42]. Survivin and Ki-67 simultaneously present in melanomas [43] suggested that survivin might have positive interaction with Ki-67.

The correlation of $P16^{INK4a}$ and Ki-67 was significant ($r = 0.356$, $p < 0.001$). As a previous study reported, the degree of $P16^{INK4a}$ and Ki-67 expression showed close association with severity of cervical neoplasia ($p < 0.001$) [8]. Detection of both $P16^{INK4a}$ and Ki-67 can help to improve the diagnostic accuracy of HSIL and SCC [44]. A large amount of $P16^{INK4a}$ positive cells in CIN I showed Ki-67 expression and CIN II, CIN III and carcinoma cases also showed co-expression of Ki-67 and $P16^{INK4a}$ in different degrees [45]. All these implied that the Ki-69 and $P16^{INK4a}$ might have synergistic effect on cell cycle and proliferation in cervical cancer progression.

Overall, the progression and development of cervical cancer involved multi-factors and pathways. Potent evidence indicated that survivin, $P16^{INK4a}$, Ki-67, and COX-2 played essential roles in cervical cancer progression. Although the mechanism of the interaction for survivin, $P16^{INK4a}$, Ki-67, and COX-2 was still unclear, these factors showed strong correlation with the lesion progression. So the present authors speculated that the comprehensive assessment of survivin, $P16^{INK4a}$, Ki-67, and COX-2 might have potential predictive value in lesion progression of cervical cancer.

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