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ORIGINAL ARTICLE

Pathological characteristics and immunophenotype analysis of cervical intraepithelial neoplasia

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Abstract

Objective To explore the clinical pathological features and immunophenotypes of cervical intraepithelial neoplasia (CIN).

Methods The protein expression of p16, p53, Bcl-2, and c-erbB-2 in 59 cases of CIN, 20 cases of cervical squamous cell carcinoma, and 20 cases of normal cervical tissues were tested using immunohistochemistry staining.

Results The expression rates of p16, p53, Bcl-2, and c-erbB-2 in CIN tissues were 76.3% (45/59), 28.85 (17/59), 61.0% (36/59), and 40.0% (23/59), respectively. The expression rates of p16, p53, Bcl-2, and c-erbB-2 in cervical squamous cell carcinoma tissues were 60.0% (12/20), 60.0% (12/20), 75.0% (15/20), and 65.0% (13/20), respectively. The expression rates of p16, p53, Bcl-2, and c-erbB-2 in normal cervical tissues were 0.0% (0/20), 0.0% (0/20), 35.0% (7/20), 0.0% (0/20), respectively. In comparison to normal tissues, the differential expressions of p16, p53, and Bcl-2 in squamous cell carcinoma and CIN were statistically significant (P <0.001), whereas the difference between the squamous cell carcinoma and CIN was not significant (P >0.05). In comparison to normal tissues, the differential expressions of c-erbB-2 and p53 in squamous cell carcinoma and CIN were statistically significant (P >0.05). The differential expressions of Bcl-2, c-erbB-2, and p53 in CIN 3 were statistically significant in comparison to CIN 1 and CIN 2 (P <0.05).

Conclusion Overexpression of Bcl-2 occurs early in the development of cervical cancer, whereas p16 and c-erbB-2 overexpression are markers for cell malignancy. The expression of p53 is correlated with the development of cervical cancer.

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Key words: cervical intraepithelial neoplasia; clinical pathology; protein; immunohistochemistry

Cervical intraepithelial neoplasia (CIN) is a precancerous lesion of invasive cervical cancer. The progression from CIN to invasive carcinoma takes approximately five to 15 years, but not all cases of CIN will transform into invasive carcinoma. Among these, CIN grade I (CIN l) and CIN grade II (CIN 2) are pathologically unstable, in which 50% of the cases progress into remission or have no further changes, whereas 20% to 30% of these cases deteriorate [1–4]. CIN can be treated, and understanding the disease progression patterns and receiving timely and effective treatment could reduce its incidence rate. The biological patterns of CIN development are difficult to predict and are affected by observers' experiences. In this study, the protein expression of p16, p53, Bcl-2, and c-erbB2 in CIN l, CIN 2, and CIN grade III (CIN 3) were analyzed retrospectively using immunohistochemistry staining techniques. The results can serve as a reference for objective diagnosis of CIN development and its clinical treatment.

Materials and methods

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Clinical cases and specimens

Paraffin-embedded specimens of hysterectomy or uterine biopsy/cervical conization were collected at the 150th Central Hospital of People's Liberation Army between June 2003 and June 2014. Based on the guidelines of the World Health Organization (WHO) Pathology and Genetics of Tumours of the Breast and Female Genital

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Organs (2006) [5–6], there were 59 cases of CIN, 20 cases of cervical squamous cell carcinoma, and 20 cases of normal cervical tissues. Among the 59 cases of CIN, 12 cases were classified as CIN 1, 19 cases were classified as CIN 2, and 28 cases were classified as CIN 3. Among the 20 cases of cervical squamous carcinoma, 10 cases were of the keratinizing type and the remaining 10 cases were of the non-keratinizing type. The 20 normal cervical tissues were cervical intraepithelia from either uterine fibroids or ovarian cancer hysterectomy. Patients with CIN were between 21 and 58 years old, and the average was 34.2 ± 8.5 years old. Although most patients sought treatment due to an increase in vaginal secretions or contact bleeding, some patients sought treatment after discovering lesions during physical examination. In general, in comparison to normal cervical tissues, these CIN cervical tissues showed mild to severe erosion and tumors were clearly visible in particular cases. All patients were followed up with for six to 34 months. Among the 59 CIN cases followed up, two cases experienced recurrence, whereas 57 cases remained in remission.

Methods

Pathological specimens were fixed in 10% buffered formalin, and processed and embedded in paraffin for subsequent hematoxylin and eosin (H&E) staining and reaction with immunohistochemical markers. Immunohistochemical staining was carried out using the En Vision two-step system. The primary antibodies used were p16 (6H12), p53 (DO-7), Bcl-2 (8C8), and c-erbB-2 (EP3), all of which were purchased from Fuzhou Maixin Biotech. Co. Ltd. Immunohistochemical staining was carried out according to routine procedures. 3,3'-diaminobenzidine (DAB) was used for staining, and phosphate buffered saline (PBS) was employed as a negative control for the primary antibody.

Observations

Positively stained cellular cytoplasm and/or membrane and nucleus appeared as brownish yellow under the microscope. p16 was localized in the nucleus/cytoplasm, Bcl-2 was localized in the cytoplasm/membrane, p53 was localized in the nucleus, and c-erbB-2 was localized in the cell membrane. For each specimen, 10 high-magnification views (400×) were randomly selected, and 200 cells were counted for each view. Samples showing an average of 5% to 25% positive results were considered weakly positive (+), an average of 26% to 75% were considered moderately positive (++), and an average of >75% were considered strongly positive (+++). Cytoplasm/membrane/nucleus that did not appear brownish yellow, or cells showing an average of <5% positive results were considered negative (–).

Statistical analysis

SPSS 12.0 was used for data analysis and Chi-square test and Fisher's exact test were used to compare groups. Values of P <0.05 were considered as statistically significant.

Results

Clinical manifestations and pathological features

Among the 59 cases of CIN, 48 patients sought treatment due to an increase in vaginal secretions or contact bleeding, whereas 11 patients sought treatment after discovering lesions during physical examination. At the tissue morphology level, 2/3 of CIN 1 epithelia were mature cells and cells from the superficial layer showed heterogeneity (Fig. 1). Half of CIN 2 epithelia were mature cells and cells from the upper and middle layers showed significant nuclear heterogeneity (Fig. 2). No mature cells were seen for the CIN 3 epithelia and heterogeneity was seen for the entire epithelial layer (Fig. 3).

The expression of p16, p53, Bcl-2, and c-erbB-2 proteins in CIN

p16 was localized in the nucleus/cytoplasm (Fig. 3). The differential expressions of p16 in CIN, squamous cell carcinoma, and normal cervical tissue were significant (P <0.05), although there was no significant difference between its expression in CIN 3 in comparison to CIN 1 and CIN 2 (P >0.05). p53 protein was localized in the nucleus (Fig. 3) and showed diffuse, flaky, and spotty distribution in squamous cell. The differential expressions of p53 in CIN, squamous cell carcinoma, and normal cervical tissue were significant (P <0.05). In addition, its expression in CIN 3 was significantly higher than that in CIN 1 and CIN 2(P <0.05). Bcl-2 protein was distributed in the basal layer of the upper epithelia of normal squamous tissues but showed diffuse, flaky, and spotty distribution in squamous cell carcinoma. The expression of Bcl-2 was higher in CIN and squamous cell carcinoma than in normal cervical tissue (P <0.05). However, there was no significant difference between Bcl-2 expression in squamous cell carcinoma and CIN (P >0.05). Bcl-2 expression in CIN 3 was significantly different from that in CIN 1 and CIN 2 (P <0.05). The expression of Bcl-2 was also higher in non-keratinizing squamous cell carcinoma than in keratinizing squamous cell carcinoma (P <0.05). c-erbB-2 protein was localized in the plasma membrane (Fig. 3). The differential expressions of c-erbB-2 in CIN, squamous cell carcinoma, and normal cervical tissue were significant (P <0.05), with higher expression in CIN 3 than in CIN 1 and CIN 2 (P <0.05) (Table 1).

Comparison of expression of p16, p53, Bcl-2, and c-erbB-2 proteins in CIN and

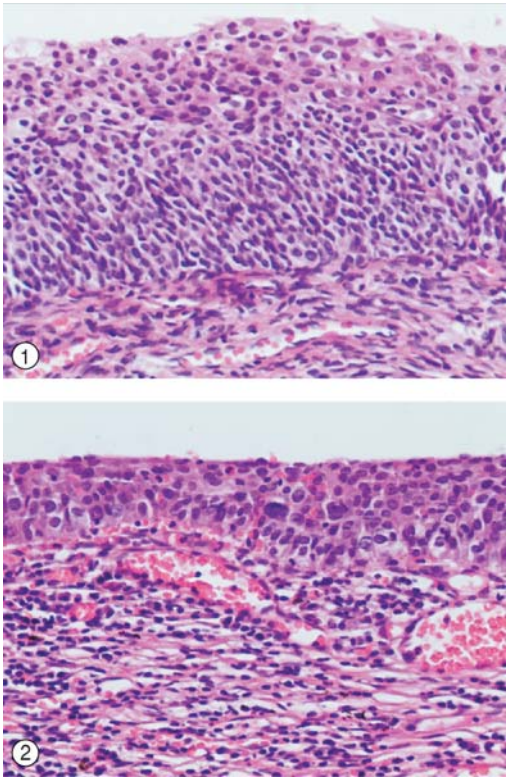

Fig. 1 2/3 of the epithelium was mature in CIN 1, with cells on the superficial layer showing heterogeneity
Fig. 2 1/2 of the epithelium was mature in CIN 2, with cells on the superficial layer showing heterogeneity

cervical squamous cell carcinoma

The expression of p53 was similar to that of c-erbB-2, with their differential expressions in squamous cell carcinoma, CIN, and normal cervical tissue being significantly different (P <0.05). The expression of p53 in CIN 3 was significantly higher than in CIN 1 and CIN 2 (P <0.05). In cervical squamous carcinoma, the expression of Bcl-2 was opposite to that of p53. The expressions of Bcl-2 and c-erbB-2, Bcl-2 and p53, and c-erbB-2 and p53 in squamous cell carcinoma showed no significant differences (P >0.05). However, the expressions of Bcl-2 and c-erbB-2 as well as of Bcl-2 and p53 in CIN showed significant differences (P <0.05). The expressions of Bcl-2 and p53 in CIN 3 showed significant differences (P <0.05) (Table 2).

Discussion

In clinical practice, CIN diagnosis is often affected by the observers' experience, such as confusion stemming from cervical immature squamous metaplasia, repairing of squamous epithelia, and aging squamous epithelia, which could lead to under-diagnosis or misdiagnosis. Previous studies have shown that activation of oncogenes, mutations in tumor suppressor genes, and over-expression of anti-apoptotic genes were related to the development of cervical cancer [7–8]. Histologically, cervical cancer progresses from squamous metaplasia, dysplasia, in situ carcinoma, and finally to an invasive cancer. p16 is a tumor suppressor gene encoding the p16 protein, which is directly involved in the regulation of the cell cycle. Mutations, deletion, or methylation of the p16 gene can result in functional changes and eventually lead to tumorigenesis. This study found that p16 protein level was closely correlated with CIN, particularly CIN 3, and cervical squamous cell carcinoma. For lesions stemming from aging cervical intraepithelial and basal layer hyperplasia and CIN 3, p16 can be used for diagnosis of CIN 3 and cervical squamous cell carcinoma. In this study, the expression rate of p16 in CIN 1 was 59%, but there were negative cases in CIN 2 to CIN 3. Therefore, p16 staining alone cannot serve as a precise diagnosis of CIN lesions. Results from this study also showed that the combination of p16, p53, and Bcl-2 expression can be applied to determine the CIN grade. Patients with low-grade CIN lesions with high expression of these proteins should be followed up with and closely monitored.

Bcl-2 plays an important role in the regulation of apoptosis [9]. It can extend cellular lifespan and prevent apoptosis. This study found that the expression of Bcl-

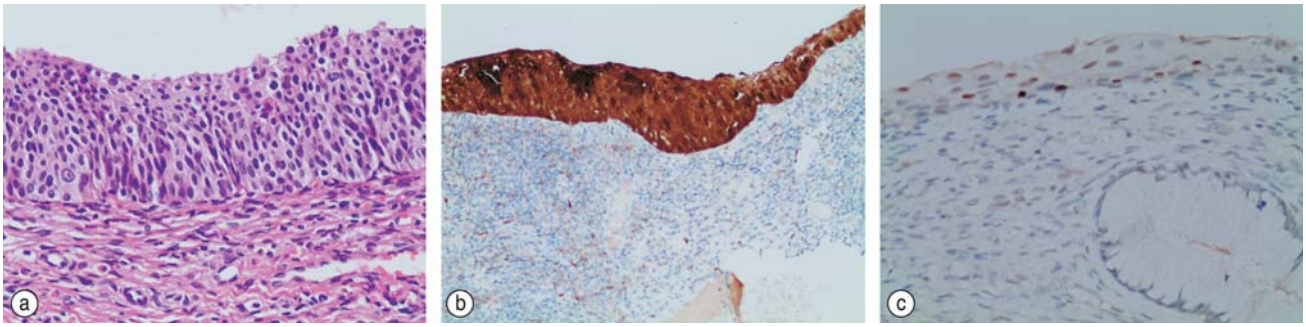

Fig. 3 (a) No mature epithelium in CIN 3, with the entire epithelial layer showing heterogeneity; (b) p16 expression in CIN 3; (c) p53 expression in CIN 2

2 protein was increased from normal cervix to CIN and cervical squamous cell carcinoma tissues. Similarly, the expression of Bcl-2 gradually increased with the aggravation of CIN lesions. The higher expression of Bcl-2 in CIN 3 than in CIN 1 and CIN 2 was significant, whereas there was no significant difference in Bcl-2 expression between cervical squamous cell carcinoma and CIN. This suggests that Bcl-2 overexpression is correlated with the development of cervical cancer, and Bcl-2 overexpression is an early event in the development of cervical cancer. One possible mechanism for this observation is that Bcl-2 overexpression may inhibit cell apoptosis. Apoptosis inhibition is conducive not only to continuous cell proliferation but also to further accumulation of abnormal genes and induction of genome instability, which further promotes transformation of cells that is beneficial for the development of tumors. Furthermore, this study also found that the expression of Bcl-2 protein decreased with increasing CIN grades. This result suggests that Bcl-2 might inhibit cellular apoptosis in the early stages of cervical cancer to promote cell transformation and cancer development. When cancer cells are at low differentiation levels, the inhibitory function of Bcl-2 protein on cell apoptosis decreases, the Bcl-2 expression reduces, and at this stage, tumor development is likely to be associated with other genes. In addition, the differential expression of Bcl-2 in keratinizing and non-keratinizing types was significant, indicating that Bcl-2 expression was related to tissue types.

The *c-erbB-2* gene is located on chromosome 17q21 and encodes a 185-kDa transmembrane glycoprotein with tyrosine kinase activity involved in signal transduction. When *c-erbB-2* is highly expressed, the c-erbB-2 protein serves as a "cancerous" protein receptor on the membrane. Extracellular signals can activate multiple signal transduction pathways, including activation of oncogenes such as c-ras, c-src, and c-myc via c-erbB-2. Such activation promotes cell transformation and cancer development, and c-erbB-2 activation plays a central role in this cascade of event [10–12]. Results from this study show that the expression rate of c-erbB-2 increased from normal cervical tissues to CIN to cervical squamous cell carcinoma, and the differential expression was significant. The expression of c-erbB-2 was also similar with that of Bcl-2, with its expression level increasing with increasing grades of CIN lesions. The expression of c-erbB-2 was significantly higher in CIN 3 than in CIN 1 and CIN 2. Thus, c-erbB-2 is also closely associated with the development of cervical cancer. Its expression in cancer cells makes c-erbB-2 one of the cellular markers of malignant transformation. The study found that there was no correlation between c-erbB-2 expression and histological types, pathological grades, clinical stages and metastasis, suggesting the lack of a significant relationship between c-erbB-2 over-expression and clinical prognosis.

p53 is one of the most actively studied genes. The protein product of the tumor suppressor gene *p53*, wild-type p53 (wt p53) is responsible for maintaining genomic stability, inhibition of cancer, regulation of cell differentiation, and senescence. Immunohistochemical staining revealed that the p53 protein was usually inactivated. Reasons for p53 inactivation include point mutations, loss of heterozygosity, or the creation of stable p53 complexes by fusion with HPV E6 protein and other viral oncoproteins. Inactivated p53 proteins not only are unable to inhibit cancer development but also are able to promote cell transformation and prevent the function of wt p53, which in turn leads to the accumulation of mutations in DNA and finally cancer [7]. Results from this study showed that the expression of p53 in normal cervical tissue, CIN, and

Table 1 Comparison of 79 cases of cervicitis and intraepithelial neoplasia

Samples	*n*	p16		p53		Bcl-2		c-erbB-2	
		n (%)	*P* value	*n* (%)	*P* value	*n* (%)	*P* value	*n* (%)	*P* value
CIN	59	45 (76.3)	0.067	17 (28.8)	0.000	36 (61.0)	0.020	23 (40.0)	0.005
CIN 1	12	7 (58.3)		0 (0.0)		3 (25.0)		2 (16.7)	
CIN 2	19	13 (68.4)		2 (10.5)		9 (47.4)		4 (21.1)	
CIN 3	28	25 (89.3)		15 (53.6)		20 (71.4)		17 (60.7)	
Carcinoma	20	12 (60.0)	0.161	12 (60.0)	0.012	15 (75.0)	0.259	13 (65.0)	0.043
Control	20	0 (0.0)		0 (0.0)		7 (35.0)		0 (0.0)	

Table 2 Expression of p16, p53, Bcl-2, and c-erbB-2 proteins in CIN and cervical carcinoma tissues

	CIN			CIN 3			Cervical carcinoma		
	+	–	*P* value	+	–	*P* value	+	–	*P* value
p16	45	14	0.074	25	34	0.328	12	8	1.000
p53	17	42	0.000	15	13	0.168	12	8	1.000
Bcl-2	36	23	0.017	20	8	0.397	15	5	0.311
c-erbB-2	23	36	0.243	17	11	0.110	13	7	0.490

cervical squamous cell carcinoma was similar with that of c-erbB-2, suggesting that p53 protein overexpression is also correlated with the development of cervical cancer. However, the expression of p53 in CIN 3 and invasive cervical cancer was not significantly different. This result is consistent with studies in the literature that reported p53 expression as an early event in the cervical cancer development. It was found that p53 expression was higher as cells became less differentiated. The expression of p53 in keratinizing cases was significantly different from that in non-keratinizing cases, indicating that p53 is correlated with histological types. Tumor cells with high expression of p53 have higher invasive capability, and given that p53-positive tumors correlated with metastasis, patients with high p53 expressing tumors were prone to metastasis. The expression of p53 in clinical stage I tumors was significantly lower than in clinical stage II and III tumors, suggesting that p53 overexpression was correlated with cancer invasion and metastasis.

This study also found that the expressions of Bcl-2 and c-erbB-2, Bcl-2 and p53, as well as c-erbB-2 and p53 in cervical cancer tissues were not significantly different, but the expressions of Bcl-2 and c-erbB-2 as well as Bcl-2 and p53 in CIN tissues were significantly different, indicating that the expression of Bcl-2 and p53 proteins in CIN groups were significantly correlated. This might be due to the loss of wt p53 function leading to down-regulation of the Bcl-2 expression, which further leads to apoptosis inhibition. In contrast, high expression of Bcl-2 also could suppress p53-induced apoptosis, which could easily lead to the formation of cancerous cells and increase the probability of cervical cancer. There was no statistical significance between c-erbB-2 and p53, suggesting that c-erbB-2 amplification and overexpression as well as mutations in *p53* in the development of cervical cancer may not be co-existing.

For diagnosis of CIN lesions, the expressions of p16, p53, Bcl-2, and c-erbB-2 should be monitored. Close monitoring and follow-ups should be carried out as required to prevent over-treating patients.

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