Expression of P-Akt, NFκB and their correlation with human papillomavirus infection in cervical carcinoma

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Summary

Purpose: To investigate the expression of P-Akt and NFκB and their correlation with human papillomavirus (HPV) infection in cervical carcinoma. *Material and Methods:* Expression of P-Akt and NFκB was detected by an immunohistochemical SP technique with HPV DNA detetion by PCR in 26 cases of cervical carcinoma tisuues, 18 cases of cervical intraepithelial neoplasia tisuues (CINI / n = 5, CINII / n = 3, CINIII / n = 10) and 19 cases of chronic cervicitis tissues. The different expressions of P-Akt and NFκB were compared in different pathological types of cervical carcinoma (cervical squamous cell carcinoma, cervical adenocarcinoma), different pathological grading (high, medium, poorly differentiated) and different clinical stage (FIGO I to IV). The relationships between P-Akt and NFκB, respectively, with HPV infection in cervical carcinoma were analyzed. *Results:* The positive expression rate of P-Akt in chronic cervicitis tissues, CIN and cervical carcinoma tisuues was 21.05%, 66.67%, and 92.31%, respectively. There was no obvious difference in the expression of P-Akt in cervical carcinoma in different pathological types or in pathological grading and no obvious difference in different clinical stages. The positive expression rate of NFκB in chronic cervicitis tissues, CIN and cervical carcinoma tissues was 10.52%, 72.22% and 96.15%, respectively; there was no statistically significant difference among the groups for different pathological types and there was no obvious difference in different pathological grading or different clinical stage. There was an obviously positive correlation between P-Akt and NFκB expression rate and degree of disease ($r = 0.998$, $p < 0.05$). Cervical carcinoma and CIN cases totaled 44; the positive expression rate of P-Akt was 87.55% in 32 cases of positive HPV-DNA of the 44 cases, and the positive expression rate of P-Akt was only 16.70% in 12 cases of negative HPV-DNA of the 44 cases. The positive expression rate of NFκB was obviously higher in the HPV DNA positive than in the HPV-DNA negative cases. There was a statistically significant difference among the groups ($p < 0.05$). *Conclusion:* The positive expression rate of P-Akt and NFκB was closely related with cervical disease extent, and closely related with HPV infection in cervical carcinoma. This study suggests that P-Akt and NFκB more probably play an important role in the occurrence of cervical carcinoma.

Key words: Cervical carcinoma; P-Akt; NFκB; Human papilloma virus.

Introduction

Cervical carcinoma is the second most common malignant neoplasm among woman around the world [1]. At present, its morbidity and mortality are still high and there has been a younger trend in our country in the last 20 years [2]. Although human papillomavirus (HPV) infection is known to be a major risk factor for cervical carcinoma, its specific pathogenesis is still not very clear. The ubiquitously expressed serine/threonine kinase Akt and the transcription factor of the nuclear factor (NF)-κB family are both involved in cell proliferation and apoptosis. Akt is a serine/threonine protein kinase in the PI3K/Akt signal transduction pathway at the hub site. The phosphorylation of protein kinase B(P-Akt) is the activated form of Akt. The NFκB family controls expression of genes which promote cell growth, survival, and neoplastic transformation by a phosphatidylinositol 3 (PI3)-kinase to the Akt/protein kinase B (PkB) pathway and this signaling pathway has been shown to activate NFκB [3]. Furthermore, the activation of Akt and/or NF-κB has been suggested to be associated with occurrence and development of human tumors. In this study the expression of P-Akt and NFκB was detected by the immunohistochemical SP technique with HPV DNA detection by PCR in cervical carcinoma tissues to explore its role in the development of cervical carcinoma, and the relationship to HPV infection.

Materials and Methods

Study

This study was conducted in the Department of Gynecology at the First Affiliated Hospital of He'nan University of Science and Technology. Patients who underwent surgery for cervical disease in the period from September 2009 through April 2010 were enrolled. The research project was approved by the Institution Research Ethics Committee and all patients signed an informed consent. The mean age of the patients was 38.73 years with a range from 28 to 64 years. Cervical biopsy pathological examination confirmed that highest level at the final pathological diagnosis of the patients; there were 26 cases of cervical carcinoma tissues, 18 cases of cervical intraepithelial neoplasia tisuues (CINI/n = 5, CINII/n = 3, CINIII/n = 10) and 19 cases of chronic cervicitis tissues. Three months elapsed where none of the patients were treated by radiotherapy, chemotherapy or any other special therapy until this study was initiated.

HPV DNA detection

HPV DNA of all samples was detected by PCR. Cervical cells were collected as a sample from each patient. The HPV GenoArray Test Panel included: 1) high-risk: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68; 2) low-risk: HPV 6, 11, 42, 43, 44, CP830. The HPV GenoArray DNA testing

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method makes use of both DNA amplification and Hybribio's proprietary flow-through hybridization technology (US patents 5,741,647 & 6,020,187) to genotype the samples with specific DNA probes. Extracted samples of DNA amplified the amount of DNA through PCR amplification. Based on the principle of double-stranded DNA complementary, the flow-through hybridization technology works by directing the flow of the targeted molecules towards the specific probe pre-fixed on low-density gene chips and hence enables rapid hybridization to occur and finally specific hybridization results. HPV testing: A total of 24 hybrid membrane points, including biotin and Ic for the normal control point. Other points appeared as blue-purple dots, compared with the corresponding HPV genotype - positive or negative.

Immunohistochemical SP technique

Representative specimens were fixed in 10% dehydrated formalin. All tumor specimens were fixed in formalin and embedded in paraffin. All the above-mentioned samples were obtained from each patient, and were classified according to the World Health Organization (WHO) classification (2003). Hematoxylin and eosin stained slides were reviewed to confirm histological diagnoses. Representative specimens were selected for immunohistochemistry-SP. Immunoperoxidase staining for P-Akt and NFκB was performed in 4.0-μm-thick tissue sections from all specimens. The BioGenex Automatic Staining System (Santa Cruz, CA) was used. In brief, tissue sections were deparaffinized, rehydrated, and soaked in 0.6% hydrogen peroxide for 30 min in order to block endogenous peroxidase activity. Microwave antigen retrieval in citrate buffer with pH 6.0 (Santa Cruz, CA) for 25 min followed. Tissue sections were incubated with the polyclonal rabbit anti-P-Akt1/2/3 antibody (Santa Cruz, CA) at a dilution of 1:50 and the polyclonal rabbit anti-NFκB/p50 antibody (Maixin, China) respectively, for 30 min. Incubation with a peroxidase-streptavidin conjugate for 20 min followed. Diaminobenzidine tetrahydrochloride was then used as a chromogen and sections were counterstained with hematoxylin, dehydrated and mounted. Tissue sections from testicle tissue with strong membranous and cytoplasmic staining for P-Akt and NF-κB were used as a positive control. For evaluation of immunohistochemical data a scoring system was used, as described previously [4]. In brief, staining intensity was characterized using the following scale: colorless (0 point), pale yellow (1 point), brown (2 points), nigger-brown (3 points); the percentage of stained cells varied between: smaller than 5% (0), 5 ~ 25% (1 point), 26% ~ 50% (2 points), greater than 50% (3 points), Staining intensity score with the number of positive cells multiplied by the criteria: 0 to 2 points as negative, or more than 3 points as positive.

Statistical analysis

Statistical analysis of experimental data was carried out by SPSS17.0 software of which the level of significance was $p < 0.05$. The chi-square test, Fisher exact test and Pearson correlation test were used for statistical analysis.

Results

Table 1 and Figure 1 present the expression of P-Akt. The positive expression of P-Akt was mainly located in the cytoplasm, while a small part was located in the nucleus; it was cytoplasm-nucleus-type expression in cervical carcinoma cells. According to the staining intensity of expression, the color was brown to light yellow. The positive expression rate of P-Akt in chronic cervicitis tissues, CIN tissues and cervical carcinoma tisuues was 21.05%, 66.67%, and 92.31%, respectively ($p < 0.05$). There was no obvious difference among the expressions of P-Akt in cervical carcinoma in different pathological types and in pathological grading, and no obvious difference among different clinical stages.

Table 1. — *Expression of P-Akt in different cervical tissues*.

Characteristics	Case (n)	P-Akt		
		Positive	Positive rate (%)	*p* value
Histological type				
Chronic cervicitis	19	4	21.05	< 0.05
CIN	18	12	66.67	
Cervical carcinoma	26	24	92.31	
Squamous carcinoma	20	19	95.00	ns
Adenocarcinoma	6	5	83.33	
Grade				
High G1	19	17	89.47	ns
Low G2	7	7	100.00	
FIGO stage				
Stage I-II	21	19	90.48	ns
Stage III-IV	5	5	100.00	

ns = non significant.

Table 2. — *Expression of NFκB in different cervical tissues*.

Characteristics	Case (n)	NFκB		
		Positive	Positive rate (%)	*p* value
Histological type				
Chronic cervicitis	19	2	10.52	< 0.05
CIN	18	13	72.22	
Cervical carcinoma	26	26	96.15	
Squamous carcinoma	20	20	100.00	ns
Adenocarcinoma	6	5	83.33	
Grade				
High G1	19	18	94.74	ns
Low G2	7	7	100.00	
FIGO stage				
Stage I-II	21	20	95.25	ns
Stage III-IV	5	5	100.00	

ns = non significant.

Table 2 and Figure 2 present the expression of NFκB. NFκB proteins were located in the cervical nucleus and cytoplasm (brown nucleus or/and brownish yellow cytoplasm). It was regarded as strong expression when the nucleus was brown or/and cytoplasm was brownish yellow. The positive expression rate of NFκB in chronic cervicitis tissues, CIN and cervical carcinoma tissues was 10.52%, 72.22% and 96.15%, respectively. There was no statistically significant difference among the groups in different pathological types, and there was no obvious difference in different pathological grading or in different clinical stage.

With the increase of cervical histology and expression of NFκB and P-Akt the color deepened. The results showed low expression in cervicitis, increased expression in CIN, and the strongest expression in cervical carcinoma. The Pearson correlation method used for statistical analysis showed that P-Akt and NFκB expression in cervical lesions had a linear correlation (Pearson correlation index r = 0.998; $p < 0.05$).

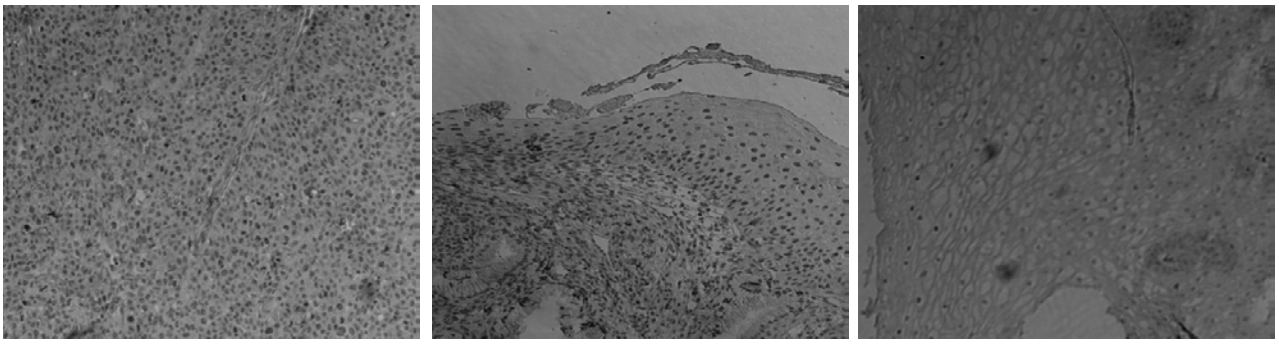

Figure 1.— Positive expression of P-Akt in cervical carcinoma tissue (A), CIN I tissue (B), chronic cervicitis tissue (C) (original magnification SP × 200).

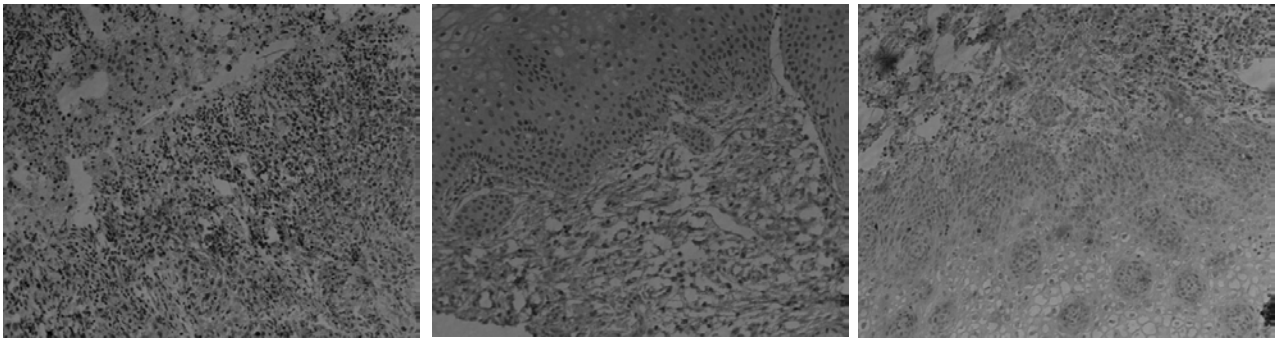

Figure 2. — Positive expression of NFκB in cervical carcinoma tissue (A), CIN II tissue (B), chronic cervicitis tissue (C) (original magnification SP × 200).

Table 3. — *Expression of P-Akt and NFκB associated with HPV infection in cervical carcinoma tissue and CIN tissue*.

	Case (n)	P-Akt		NF-κB	
		Positive	Positive rate (%)	Positive	Positive rate (%)
HPV (positive)	32	28	87.55 (28/32)	27	84.38 (27/32)
HPV (negative)	12	2	16.70 (2/12)	4	33.33 (4/13)
		$\chi^2 = 16.78$, $p < 0.05$		$\chi^2 = 9.65$, $p < 0.05$	

Table 3 shows the 44 cervical carcinoma and CIN cases. The positive expression rate of P-Akt was 87.55% in 32 cases of positive HPV-DNA of the 44 cases, and the positive expression rate of P-Akt was only 16.70% in 12 cases of negative HPV-DNA of the 44 cases ($\chi^2 = 16.78$, $p < 0.05$). The positive expression rate of NFκB was obviously higher in the HPV-DNA positive than in the HPV-DNA negative cases. There was a statistically significant difference among the groups ($\chi^2 = 9.65$, $p < 0.05$).

Discussion

The ubiquitously expressed serine-threonine kinase Akt and the transcription factor NFκB are both involved in cell proliferation and apoptosis. Furthermore, the activation of Akt or NF-kappaB has been suggested to be associated more with human tumors [5]. Akt is known to be involved in the PI3-kinase/AKT signaling pathway; activated Akt (P-Akt) modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression and cellular growth. In recent years, it has been shown that the PI3K/Akt signalling pathway components are frequently altered in human carcinomas [6]. Amplification of chromosome arm 3q is the most consistent aberration in cervical carcinoma, and it is implicated in the progression of dysplastic uterine cervical cells into invasive carcinoma. The results of comparative genomic hybridization show that the 3q26.3 amplification was the most consistent chromosomal aberration in primary tissues of cervical carcinoma, and a positive correlation between an increased copy number of *PIK3CA* (detected by competitive PCR) and 3q26.3 amplification was found in tumor tissues and in cervical carcinoma cell lines [7-9]. In this study, the expression of P-Akt in CIN and cervical carcinoma rates were respectively, 66.67% (12/18) and 92.31% (24/26), significantly higher than that in the chronic cervicitis expression rate 21.05% (4/19). The experiments also suggest that the overexpression of P-Akt was significantly correlated with advanced stage disease, but did not reach statistical significance.

PI3K/Akt activity was analyzed by phosphatidylinositol trisphosphate production and phosphorylated Akt (p-Akt) expression, and also increased NFκB activity [10]. The larger NFκB family of proteins is composed of two

subfamilies: the NFκB (p50) proteins and the RelA (p65) proteins. It be able to regulate gene expression in a variety of protein molecules. Under normal circumstances, it exists in the cytoplasm in a non-active state. When there is an infectious virus or other factors, the NFκB protein is highly expressed in the tumor, with regulating gene transcription and expression, inhibits tumor cell apoptosis and promotes tumor development [11-13]. This experiment showed that with increased levels of cervical pathology, the extent of NFκB expression was enhanced, and was positively correlated with expression of P-Akt, indicating the incidence of cervical carcinoma development. NFκB and P-Akt may form a network for each other.

HPV infection is the most important factor, and an important initiation factor has been recognized in cervical carcinoma. HPV DNA was detected in more than 90% of patients with cervical carcinoma.The most common HPV types in cervical carcinoma were HPV type 16, 58 and 18. HPV infected patients have a higher risk of developing cervical carcinoma, which is 75.79 times more than non-infected people [14]. The E5 oncoprotein of HPV 16 plays an important role in early cervical carcinogenesis. Vascular endothelial growth factor (VEGF) plays a central role in switching on the angiogenic phenotype during early cervical carcinogenesis. E5-mediated epidermal growth factor receptor (EGFR) activation was accompanied by P-Akt and ERK1/2, which are also involved in VEGF expression. Furthermore, the mRNA stability of VEGF was not affected by E5, but VEGF promoter activity could be modulated by inhibitors of the EGFR, MEK-ERK1/2 and PI3K/Akt pathways in E5-expressing cells. Kim *et al's* results suggest that HPV 16 E5 increases VEGF expression by activating EGFR, MEK/ERK1/2 and PI3K/Akt [15]. The early gene product E7 from high-risk HPV is considered the major transforming protein expressed by the virus. Menges *et al's* data provide evidence linking inactivation of the retinoblastoma gene by E7 in the up-regulation of AKT activity during cervical cancer progression [16]. Western immunoblotting experiments demonstrated that high levels of NFκB precursors p100 and p105 proteins not only in HPV16+ cervical carcinoma-derived keratinocytes but also in keratinocytes stably transfected by HPV16 E6 or E7 oncogenes. Moreover, p100 and p105 proteins were predominantly cytoplasmic and nuclear in keratinocytes expressing E7 and E6, respectively. A predominantly cytoplasmic localization of E7 protein was also detected in all keratinocytes expressing E7. Havard *et al's* results suggest that HPV16 E6 and E7 proteins modulate the expression and the subcellular localization of p100 and p105 NFκB precursors [17]. In this study we showed that the positive expression rate of P-Akt and NFκB is closely related with cervical disease extent, and closely related with HPV infection in cervical diseases.

We conclude that HPV causes cervical carcinoma most probably by activating signaling pathways PI3K/Akt/NFκB. NFκB and P-Akt may be involved and play an important role in the occurrence of cervical carcinoma. A better understanding of this result can help to fully exploit the potential benefits of cervical cancer diagnosis, treatment and prevention.

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