Immunohistochemical Studies of Human Papillomavirus Infection and Cell Proliferation and Cycle-associated Markers in Cervical Carcinomas

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Human papillomavirus (HPV) infection is suggested to be a risk factor for cervical carcinoma. Thirty-nine cervical carcinoma histological specimens obtained from patients treated in Lithuanian Oncology Center were analyzed for the presence of HPV infection and for cell proliferation and cycle-associated markers. Investigation was carried out by using immunohistochemical technique with the antibodies anti-BPV-1, SX118 and PC10 (DAKO A/S, Denmark). Screening of oncogenic infection has revealed that all 39 tumour samples obtained from patients with cervical carcinoma were infected with HPV. Epithelium cells of these carcinomas displayed a positive immunoreactivity with antibodies anti-BPV-1 against virus capside protein L1. Analysis of carcinomas processed for immunohistochemical examination by using monoclonal antibodies PC10 against proliferating cell nuclear antigen PCNA showed a strong intertumoral heterogeneity in PCNA expression level. The percentage of PCNA-positive cells ranged from 0% to 76.7%, the mean value was 43.5 ± 22.6%. 28/39 (71.8%) of cervical carcinomas were shown to have $p21^{WAF1/cip}$-positive malignant cells. Thus, immunohistochemical studies confirmed the presence of papillomavirus infection in all cervical carcinoma specimens and determined a majority of tumour samples to have a high proliferation activity.

Key words: human papilloma virus HPV, cervical carcinoma, immunohistochemistry, PCNA , p21

INTRODUCTION

It has been established that oncogenic viral infection contributes greatly to human carcinogenesis (1). Human papillomaviruses (HPVs) have been associated with a range of human malignancies, including anogenital malignancies, most cervical cancers, several laryngeal and oral carcinomas, the skin carcinomas of patients with epidermodysplasia verruciformis. It is now widely accepted that certain types of HPV are involved in cervical cancer (2). However, it is important to stress that HPV infection alone is insufficient for tumour development.

HPVs that infect the genital tract are classified as either high-risk or low-risk on the basis of their clinical associations. The high-risk HPV types, including HPV-16 and HPV-18, are commonly associated with lesions that can progress to high-grade cervical intraepithelial neoplasia and ultimately to cervical carcinoma. The availability of molecular methods for HPV detection has influenced significantly the development of epidemiology of cervical neoplasia as well as attempts directed to prevention of HPV-associated disease with a vaccine covering a broad range of areas linking to basic diagnostic and clinical aspects (3). The evolution in understanding of cervical carcinogenesis has been driven by a strong association between virus and disease and by a molecular data elucidating the mechanisms of HPV-mediated tumorigenesis.

Investigations carried out in many countries confirmed the presence of HPV in most cervical carcinomas and preinvasive lesions. Hybrid capture analysis performed on cervical smears obtained from Lithuanian women has shown a high percentage of the presence of viral infection (4).

The current study was undertaken to perform immunohistochemical screening of HPV infection in tumour histological specimens obtained from patients

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with cervical carcinoma and to evaluate expression of cell proliferation and cycle-associated markers: proliferating cell nuclear antigen PCNA and p21.

MATERIALS AND METHODS

Formalin-fixed and paraffin-embedded tumor specimens from 39 patients with cervical carcinoma treated in Lithuanian Oncology Center, Vilnius, dating from 2000 to 2001, were processsed for immunohistochemical investigation Selection criteria included no treatment before biopsy, availability and sufficient biopsy material.

The age of the patients at diagnosis ranged from 21 to 76 years, with an average of 50 years. Tumour series included 34 specimens of squamous cell carcinoma (SCC), 4 specimens of adenocarcinoma and 1 specimen of carcinoma *in situ*. Clinicopathological factors including age at diagnosis, the stage of disease, grade were abstracted from the pathology reports and medical record of each patient.

Immunohistochemical reactions were carried out by a streptavidin-biotin peroxidase technique (5). Serial sections from the same tissue blocks were cut for HPV, PCNA and $p21^{WAF1/cip}$ immunostainings. Paraffin-embedded tissue sections were mounted on precleaned bovine albumin coated slides. After this, tissue sections were de-waxed in xylene, taken through graded alcohol and then incubated for 10 min in 0.3% hydrogen peroxide in absolute methanol to block endogeneous peroxidase. After microwave retrieval of antigens in 10 mmol/l (pH 6.0) citrate buffer and washes in phosphate buffered saline (PBS), all the slices were treated with 1% normal horse serum in PBS and then incubated for 30 min with polyclonal antibodies anti-BPV-1 against virus capside protein L1 (Dako A/S, Denmark). Parallel sections were incubated 30 min with monoclonal antibodies PC10 against proliferating cell nuclear antigen PCNA or monoclonal antibodies SX118 against protein $p21^{WAF1/cip}$ (Dako A/S, Denmark).

After washes in PBS, this step was followed by an application of secondary biotinylated anti-rabbit antibody or anti-mouse antibody and streptavidin conjugated with peroxidase. Between incubations the sections were washed 3 times in PBS. Chromogenic development was obtained using 3.3'-diaminobenzidine (DAB) with 0.03% hydrogen peroxide. Negative controls were performed, omitting primary antibodies. Finally, sections were counterstained with haematoxylin, cleared, mounted and examined under a 40x objective.

Scoring of PCNA and $p21^{WAF1/cip}$ immunostainings was performed from all section areas. For evaluation of PCNA scores, in each case, 1000 tumor cells were counted from all areas, ensuring that the whole section was scanned. All the reactive nuclei were scored as positive, regardless of the intensity of staining, and the fraction of positive cells was determined. In every case 3 sections were scored.

The p21 immunostainings which were confined to cell nuclei were assessed in a semiquantitative way. The proportion of positive cells relative to the amount of tumour cells was classified as follows: 0, no staining, +, positivity in a few scattered cells; ++, more than a few scattered cells but ≤50% of cells were positive; +++, >50% of cells were positive. The pattern of the distribution of p21-positive cells in a carcinoma area was characterized as scattered, confined or mixed type. All data were analyzed with Microsoft Excell programme.

RESULTS AND DISCUSSION

All 39 biopsies were evaluable as regards the HPV infection. The presence of viral infection was evidenced by a strong nuclear expression of viral infection marker – papillomavirus capside protein L1 in all histological specimens of cervical carcinomas following immunohistochemistry with polyclonal antibodies anti-BPV-1. In all of the 39 cervical carcinoma samples papillomavirus immunostaining was positive. Representative viral infection marker immunostainings are shown in Figure. In many cases, a great majority of epithelium cells of carcinomas displayed strong and mild positive immunoreactivity with antibodies anti-BPV-1 against virus capside protein L1.

Diagnostic methods of HPV infection include morphological methods such as cytological, pathological and electron microscopical diagnoses; immunohistochemistry (IH), DNA hybridization methods such as Southern blot hybridization (SBH) and Dot blot hybridization (DBH) and a combination of the two methods such as *in situ* hybridization, *in situ* polymerase chain reaction (*in situ* PCR) and polymerase chain reaction – *in situ* hybridization (PCR-

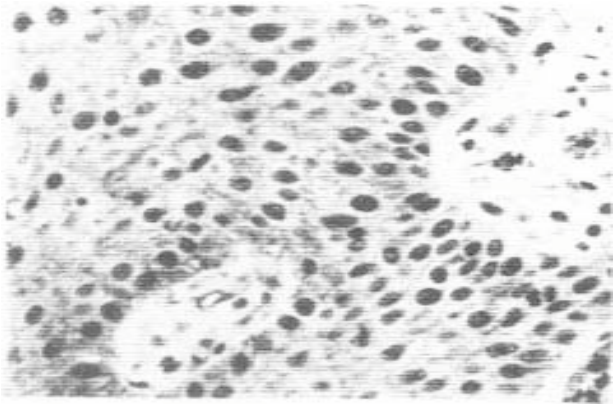

Figure. Nuclear expression of viral infection marker: presence of papillomavirus capside protein L1 in cervical squamous cell carcinoma by immunohistochemistry with polyclonal antibodies anti-BPV1. Hematoxylin counterstain

ISH) (6). The advantages, disadvantages and detection rates of the various diagnostic methods are well established. Methods, including immunohistochemistry, enabling visual localization of the detected viral proteins or DNA in cells or tissues, are very important for cytopathologists and histopathologists. Besides that, immunohistochemistry provides advantage for establishment of wide preliminar screening of series of tumour specimens for evaluating viral status by revealing viral markers.

Over 110 types of HPV are known, of which 30 types have been detected in specimens from the cervix. These have been classified into a low risk group, a high risk group and an intermediate risk group according to their association with malignant transformation. Therefore, diagnosis of cervical HPV infection and typing are extremely important. Immunohistochemical detection of viral infection in cervical carcinomas may include tumours infected with the predominating – high or intermediate risk group HPVs, as well tumours infected with rare types of HPV. Therefore, a further investigation designed for HPV typing may be valuable for the next step of analysis.

The activity of tumour cell proliferation evaluated according to the percentage of PCNA-positive cells strongly differed between individual cervical carcinomas (Table 1). The lowest PCNA positivity index was 0% and the highest 76.7%, the mean value being 43.5 ± 22.6%; 4 of 39 (10.3%) carcinomas displayed negative PCNA staining. Carcinomas with undetectable PCNA expression included 1 case of carcinoma *in situ,* 2 cases of adenocarcinoma and 1 case of squamous cell carcinoma.

Topological analysis of the distribution of PCNA-positive cells in different layers of 35 cervical carcinomas revealed some characteristic features. In 17 cases, including 2 cases of adenocarcinoma and 15 of SCC, PCNA-positive cells were distributed to a similar extent in the upper and middle layers of the epithelium. Only a few scattered PCNA-positive cells were located in the lower layers. In 4 cases of SCC, the presence of PCNA expression was notable in the upper layers of the epithelium, while a lack of PCNA expression was confined to cells of the middle and lower layers. In 14 cases, PCNA-expressing cells were scattered in higher proportions in the middle than in the upper layers of the epithelium.

Methods for asssessing cell proliferation in routinely fixed tissues are of great interest in histopathology, because they preserve tissue architecture and allow retrospective studies. One of these is immunohistochemical study of proliferating nuclear antigen PCNA (cyclin), a protein which is involved in DNA synthesis and can be revealed in routinely processed tissues by using monoclonal antibodies PC10 (7, 8). Currently, cell proliferation associated markers are considered to show a strong prognostic significance for certain cancers, including cervical carcinoma (9–11). Analysis of our data on PCNA study shows that no correlation between PCNA and clinicopathological features presented in Table 1 was established. Further investigation based on patients' follow-up and survival results could be useful for elucidating the prognostic significance of PCNA.

Table 1. **Proliferation indices in 39 cervical carcinomas according to clinicopathological features**

Variable	n	PCNA-positive cells, the percentage	
		M ± SD	range
All series	39	43.5 ± 22.6	0 – 76.7
Age, years:			
≤45	19	44.7 ± 22.8	0–76.2
46–70	16	39.8 ± 24.3	0–76.7
>70	4	52.8 ± 13.9	40.2–72.7
Tumour histological type:			
Carcinoma *in situ*	1	0	0
SCC	34	47.02 ± 21.03	0–76.7
Adenocarcinoma	4	24.9 ± 19.6	0–47.9
pT category			
pT0	1	0	0
pT1	4	28.02 ± 30.4	0–65.2
pT2	14	43.6 ± 24.4	0–76.2
pT3	20	48.8 ± 17.	8.5–76.7
pN category			
N0	7	31.3 ± 29.2	0–72.7
N1	1	0	0
NX	31	48.2 ± 17.8	6.4–76.7
M			
M0	38	43.1 ± 22.7	0–76.7
M1	1	61.6	61.6
FIGO stage			
Stage 0	1	0	0
Stage I	3	37.4 ± 29.5	6.4–65.2
Stage II	13	43.5 ± 23.9	0–76.2
Stage III	21	45.6 ± 20.5	0–76.7
Stage IV	1	61.6	61.6
Grading			
G1	2	23.9 ± 33.8	0–47.9
G2	7	46.2 ± 18.2	25.7–76.7
G3	24	43.5 ± 23.5	0–76.2
G4	3	44.7 ± 32.9	8.5–72.7
GX	3	49.2 ± 14.8	33.8–63.5

After immunohistochemical examination with SX118 monoclonal antibody, when the cases were simply categorized as p21-positive or negative, in 28 of the 39 carcinoma samples (71.8%) p21 immunoreactivity was demonstrated. The results of p21 immunostainings are summarized in Table 2. Positivity of SX118 monoclonal antibody was confined to the neoplastic cells, in a proportion and extent that varied from case to case. The p21-positivity was mainly nuclear. The immunostaining showed some gradation in the intensity from nucleus to nucleus within the same section. Mainly, the extent of staining was assessed as +; thus the antigen was demonstrated in few cells in the biopsies, and the corresponding p21 score was low. In the majority of p21-immunoreactive cases (18/28; 64.3%), the extent was assessed as +, while in in the remaining positive cases (10/28; 35.7%) the extent was considered ++. According to the results obtained from semiquantitative assessment of tumour specimens, no specimen with extent +++ was found. The immunostaining pattern was characterized also by distribution of p21-positive cells in tumour sections of every case. The distribution of p21-positive cells was determined as scattered or confined or mixed type. Topological analysis of the distribution of p21-positive cells according to the layers of epithelium has shown that p21-positive cells tended to locate mainly in the upper and middle layers of the epithelium.

The p21 protein represents suppressor gene products which possess inhibitory effects on cyclin-cyclin-dependent kinase complexes that regulate key proteins involved in cell cycle transitions (12). It is accepted that cell cycle arrest or apoptosis is mediated by wilde type p53-induced transcription of the p21WAF1 gene resulting in p21 protein translation. The cyclin-dependent kinase inhibitor p21 WAF1/CIP1 was initially identified as a downstream effector of the cell cycle arrest function of p53. p21 WAF1/CIP1 gene expression is directly upregulated by the wild-type but not mutant p53 at the transcriptional level (13). However, p21 WAF1/CIP1 gene is also regulated by p53-independent factors, including growth-promoting factors and differentiation-

Table 2. **Immunohistochemical staining of p21 in 39 cevical carcinomas and tumour characteristics**

	nr: negative/positive	Positive immunohistochemical staining:					
		extent			pattern		
		+	++	+++	scattered	confined	mixed
Total	11/28						
Histology:							
Carcinoma *in situ*	1/0	0	0	0	0	0	0
SCC	8/26	16	10	0	20	3	3
Adenocarcinoma	2/2	2	0	0	2	0	0
pT category							
pT0	1/0	0	0	0	0	0	0
pT1	3/1	1	0	0	1	0	0
pT2	4/9	6	3	0	7	1	1
pT3	3/18	11	7	0	15	2	1
pN category							
N0	4/2	1	1	0	2	0	0
N1	1/0	0	0	0	0	0	0
NX	6/26	17	9	0	20	3	3
M							
M0	11/27	17	10	0	21	3	3
M1	0/1	1	0	0	1	0	0
FIGO stage							
Stage 0	1/0	0	0	0	0	0	0
Stage I	2/1	1	0	0	1	0	0
Stage II	5/8	5	3	0	6	1	1
Stage III	3/18	11	7	0	14	2	2
Stage IV	0/1	1	0	0	1	0	0
Grading							
G1	0/1	1	0	0	1	0	0
G2	2/6	4	2	0	4	1	1
G3	8/16	12	4	0	14	1	1
G4	1/2	0	2	0	1	0	1
GX	0/3	1	2	0	2	1	0

associated transcription factors. On the other hand, p21 WAF1/CIP1 has been shown to be associated with senescence and terminal differentiation (14).

Shirakawa et al. (15) found that in the normal stratified squamous epithelium of the esophagus, only the third to the fifth layers of cells express the cyclin-dependent kinase inhibitor p21 WAF1/CIP1. Using immunohistochemical staining, they found that p21-expressing cells shifted to the upper layers of the epithelium with the progression of dysplasia. Based on these findings, they concluded that p21 plays a critical role in the differentiation process.

Thus, findings of our study designed for evaluating the presence of HPV infection in cervical carcinoma samples and revealing markers associated with cell proliferation and cycle demonstrated the presence of HPV infection in all cervical carcinoma specimens and determined a majority of tumour samples to exhibit a high proliferation activity. Further investigation is needed for other tumour markers, particularly oncoproteins, and for virus genotyping.

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ŽMOGAUS PAPILOMOS VIRUSO IR SU LĄSTELĖS PROLIFERACIJA BEI CIKLU SUSIJUSIŲ BIOŽYMENŲ IMUNOHISTOCHEMINIAI TYRIMAI GIMDOS KAKLELIO KARCINOMOSE

S a n t r a u k a

Žmogaus papilomos viruso (ŽPV) infekcija laikoma gimdos kaklelio vėžio rizikos veiksniu. Tirti 39 gimdos kaklelio karcinomų histologiniai bandiniai, paimti iš ligonių, gydytų Lietuvos onkologijos centre. Navikuose imunohistochemiškai analizuotas ŽPV infekcijos buvimas ir su ląstelės proliferacija bei ciklu susiję biožymenys, tam tikslui panaudojant antikūnus anti-BPV-1, SX118 ir PC10 (DAKO A/S, Danija). Onkogeninės infekcijos ieška parodė, kad visi 39 navikų bandiniai, paimti iš ligonių, sergančių gimdos kaklelio vėžiu, buvo infekuoti ŽPV. Karcinomų epitelio ląstelės teigiamai reagavo su antikūnais anti-BPV-1, specifiniais viruso kapsidiniam baltymui L1. Analizuojant karcinomų epitelio ląstelių proliferacinį aktyvumą, įvertintą pagal PCNA-teigiamų navikinių ląstelių dalį, nustatyta, kad tirtų navikų grupėje šio žymens ekspresijos rodiklis svyravo nuo 0 iki 76,7%, vidurkis 43,5 ± 22,6%. 68,4% karcinomų histologiniuose bandiniuose tarp navikinių ląstelių stebėta imunohistocheminė baltymo p21 $^{WAF1/Cip}$ hiperekspresija. Taigi, imunohistocheminiai tyrimai parodė, kad visos gimdos kaklelio karcinomos buvo infekuotos ŽPV ir dauguma jų pasižymėjo dideliu proliferaciniu aktyvumu.

Raktažodžiai: žmogaus papilomos virusas, ŽPV, gimdos kaklelio vėžys, imunohistochemija, PCNA, p21