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Decreased p21 Expression in HPV-18 Positive Cervical Carcinomas

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Abstract The aim of this study was to investigate the relationship between different human papillomaviruse (HPV) genotypes and the expression of p53, p21 and p27 in cervical carcinomas. A total of 103 cases of cervical carcinomas were assayed for expression of p53, p21 and p27 by immunohistochemistry. HPV typing was carried out by two polymerase chain reaction-based methods. Overall, HPV prevalence was 97.1% among the cervical carcinomas in this study. HPV-16 was detected in 66% of the tumors, HPV-18 in 7.8%, HPV-16/18 in 7.8% and other HPV types in 15.5%. The expression of p53 and p27 was not related to HPV genotype. However, in the HPV-18 positive cervical carcinomas, expression of p21 was significantly decreased or completely absent (P=0.019). Our results indicated that down-regulation of p21 was strongly associated with HPV-18 positive cervical carcinomas. The significantly lower expression of p21 protein in HPV-18 positive samples compared to HPV-18 negative cervical carcinomas supports the hypothesis that inactivation and degradation of p21 proteins by HPV-18 E7 may play an important role in the carcinogenesis of HPV-18 positive cervical neoplasia.

Keywords Human papillomavirus (HPV) · p21 · p53 · p27 · Cervical carcinoma

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Introduction

High-risk human papillomaviruses (HPVs) are considered to be the necessary agents in cervical carcinogenesis [1]. *In vitro* studies have showed that the oncoproteins E6 and E7 produced by high-risk HPVs are necessary for efficient malignant transformation of human cells [2]. These HPV oncoproteins can bind to cell cycle regulatory proteins and interfere with both the G1/S and G2/M cell cycle check-points [3, 4]. A range of regulatory proteins are involved in the normal cell cycle and these include cyclins, cyclin-dependent kinases and cyclin-dependent kinases inhibitors. Some of the cyclin-dependent kinases inhibitors are members of the Kip/Cip family and consists of three related proteins, p21, p27 and p57. $p21^{waf1/cip1}$ encodes a protein of M_r 21000 (p21), which binds to a number of cyclins and cyclin-dependent kinases complexes and this gives rise to cell cycle arrest; therefore an increase in p21 causes growth inhibition in the tumor tissues. This has been demonstrated *in vitro* in a previous study [5]. Furthermore, down-regulation of p27 expression has been reported to be a poor prognostic factor in patients with several different types of tumors, such as

epithelial ovarian cancer, colorectal cancer and gastric cancer [6–8].

The p27 gene was first identified as involved in G1 phase arrest and inactivation of p27 results in multiple organ hyperplasia, an increased body size, pituitary tumors and retinal dysplasia in p27 knockout mouse [9]. A limited number of studies have demonstrated that p27 expression is significantly lower in patients with cervical carcinoma, when this is compared with normal epithelium and dysplasia [10, 11].

Wild type p53 is an important transcription factor and is involved in the regulation of cell growth, DNA repair and apoptosis [12]. In response to DNA damage, p53 is activated and turns on the downstream gene $p21^{waf1/cip1}$ gene [13]. Inactivation of wild-type p53 results in a bypassing of normal growth arrest signals, which leads to the acquisition of numerous genetic alterations that contribute to malignant transformation [14].

Although HPV infection has been established as an important initial event in the tumorigenesis of cervical carcinoma, reports regarding any tendency towards malignant progression of infections involving different HPV types in cervical intraepithelial neoplasia (CIN) are conflicting. Some results seem to indicate that patients infected with HPV-18 undergo a rapid progression through the precancerous stage [15]. Other reports suggest that CIN containing HPV-16 is associated with the development of high-grade lesions and invasive carcinoma [16, 17]. More research is necessary to clarify the biological processes and pathological significance of specific HPV genotypes during cervical carcinogenesis.

We previously have demonstrated reduced p21 and p27 expression in invasive carcinomas and that this suggests that these two proteins may play a tumor-suppressor function in cervical carcinogenesis [18]. In addition, we found that there was significantly increased expression of p53 in advanced-stage cervical carcinomas, which implies that inactivation of p53 is associated with tumor progression [19]. *In vitro*, high-risk HPV E6 protein can bind to p53, which results in the rapid ubiquitin-dependent degradation of p53 [20]. Moreover, high-risk HPV E7 protein has been demonstrated to neutralize the inhibitory activities of the p21 and p27 [21, 22]. Nevertheless, a correlation between the specific HPV genotype and the expression of p53, p21and p27 in cervical carcinomas is still less clear.

The aim of our study was to investigate the possible role(s) of these regulatory proteins in tumorigenesis among type-specific HPV positive cervical carcinomas. To clarify this, 103 patients used in a previous report were HPV genotyped for inclusion in this retrospective study. Our findings demonstrated that samples with HPV-18 infection showed decreased p21 expression.

Materials and Methods

Patients

A total of 103 cases of cervical carcinomas, diagnosed at Shin Kong Wu Ho-Su Memorial Hospital (Taipei, Taiwan) from 1993 through to 1996, were investigated. The patients were staged according to criteria of the International Federation of Gynecology and Obstetrics. Of the 103 cervical carcinomas, 67 patients were classified as stage I, 28 patients as stage II and eight patients as stage III or IV. All slides were reclassified and graded by one pathologist according to World Health Organization criteria; there were 95 squamous cell carcinomas, five adenocarcinomas, and three adenosquamous cell carcinomas. Of the 103 cervical carcinomas, seven patients had grade 1, 59 patients had grade 2, and 37 patients had grade 3 differentiation. The median age at diagnosis was 49 years old (range, 28 years to 80 years). In addition, 20 normal cervical tissues from patients with benign uterine leiomyoma were used as controls. The protocol for the research project has been reviewed and approved by the Institute Review Board of our institution. Clinicopathological variables including the patient's age, stage of disease and histological diagnosis were abstracted from the medical records.

Tissue Samples and DNA Preparation

Tissue sections from same paraffin-embedded blocks used for the expression of cell cycle proteins were processed for the extraction of HPV DNA. Depending on the size of the biopsy, one to three 5-μm paraffin sections were deparaffined in xylene and rehydrated in graded ethanol. DNA extracts from the samples were prepared using a High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany). The DNA extracts were stored at −20°C until amplification.

HPV DNA Detection and Typing

Detection and typing of HPV DNA by PCR amplification was performed with two different primer sets made up of the E7 type specific primer set and L1 general primer set (MY11/GP6+ and GP5+/GP6+) as previously described [23].

Briefly, the HPV detection and typing method was based on TS-PCR amplification followed by detection of the PCR products in a standard microtiter plate format using a standardized PCR/enzyme-linked immunoadsorbent assay kit (Roche Diagnostics). HPV TS primers were selected to amplify approximately 100 base pairs (bp) in the E7 open reading frame of HPV types 16, 18, 31, 33, 45, 52 and 58 according to the original report by Walboomers et al. with modifications.[1] DNA amplification and labeling was per-

formed in a 50-μl PCR reaction volume. The conditions and number of denaturation-annealing-extension cycles varied with each set of primers. To exclude false-negative results, the 110-bp sequence of the β-globin gene was amplified using the PC03 and PC04 primers. Oligonucleotide probes specific for HPV types 16, 18, 31, 33, 45, 52 and 58 were biotinylated and used in the hybridization reaction. Amplified products, labeled with digoxigenin during the amplification reaction, were hybridized separately with the TS probes and with biotinylated probe for the human β-globin gene sequence. Each reaction volume was transferred to a streptavidin-coated microtiter plate well (Roche Diagnostics) and incubated at 45°C for 3 h. The colorimetric reaction was read using a spectrophotometer at an optical density (OD) of 405 nanometers. Results were expressed as net absorbance after the absorbance of the buffer blank was subtracted and the index value was calculated as the OD of the sample/cutoff. An index value >1 was considered to be positive. We used two times the mean OD value of the PCR-negative controls as a cutoff point.

For second method, the modified primer set MY11/GP6+ was used for the first PCR to amplify the corresponding part of the HPV L1 gene and then the nested-PCR primers GP5+/GP6+ were used to obtain the final DNA fragment. Each amplification reaction was performed in a total volume of 50 μl containing PCR Master Mixture (PE Applied Biosystems, Foster City, CA). HPV typing analysis was done by autosequencing. Briefly, all products of the PCR were purified using a presequencing kit and then sequenced using GP5+ primer and a DNA sequencing kit (Big-Dye™ Terminator Cycle Sequencing Version 2.0, PE Applied Biosystems). Finally, the sequencing products were purified using ethanol precipitation and analyzed on an ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems). Sequence alignments were obtained using returned results from the GeneBank on-line BLAST server (available from URL: http://www.ncbi.nlm.nih.gov/BLAST/).

Immunohistochemical Analysis

Immunostaining of the p53, p21 and p27 proteins was performed using the avidin-biotin immunoperoxidase method (Super Sensitive Immunodetection System, QP900-9 L, BioGenex, San Romon, CA) and the Optimax Plus Automated Cell Staining System (BioGenex). Sections (5 μm) of formalin-fixed paraffin-embedded tissue were prepared, air-dried at room temperature overnight, incubated at 55°C for 1 h, deparaffinized in xylene and finally rehydrated using graded alcohol concentrations. Antigen retrieval was performed by boiling for 10 min in 0.1 M citrate buffer (pH 6) in a microwave oven (600 W) and then endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase for 10 min. As the next step, the tissue sections were incubated with p53 antibody (clone 1801, 1:100, 100 ug/ml, BioGenex, San Romon, CA), p21 antibody (clone EA 10, 1:40, 2.5 ug/ml, Oncogene Science, Cambridge, MA) and p27 antibody (clone DCS 72, 1:100, 1.0 μg/ml, Oncogene Science, Cambridge, MA) at room temperature. Next, the tissue sections were incubated with a biotinylated secondary antibody and staining was achieved by the avidin-biotin peroxidase complex technique with visualization using diaminobenzidine as the chromogen; the sections were also counterstained with hematoxylin. Negative controls were prepared by replacing the primary antibody with normal serum. Sections taken from patients with breast cancer, which are known to stain positively for p53 antibody and sections of normal human colon, which are known to stain positively for p21 antibody, were used as the positive controls. Infiltrating mature lymphocytes that showed positive p27 immunostaining were used as the internal positive control for p27 antibody.

The results of immunostaining were recorded independently by two investigators. For p53, p21 and p27 immunoreactivity, only a distinct brown nuclear staining was scored as positive. For each section, the total number of tumor cell nuclei in each field was identified with a total of at least 1,000 cells per sample being counted at ×400 magnification. Any discrepancies in staining interpretations were resolved by shared observation using a multi-headed scope until a consensus was reached. The fraction of positive tumor cells was analyzed using a continuous scale, but for the statistical calculation, the cutoffs for p53, p21 and p27 were based on the median values of these markers. Therefore, p53, p21 and p27 immunoreactivity was considered low if the percentage of positive cells was below the median value and it was considered high if the percentage of positive cells was equal to or greater than the median value.

Statistical Analysis

The SPSS-Win 10.0 program package (SPSS Inc., Chicago, IL) was used in a personal computer for the basic statistical calculations. The relationship between the type-specific rate of HPV and protein expression were evaluated using contingency tables, which were analyzed further using the χ^2 or Fisher exact test. A value of $P<0.05$ was considered statistically significant. All P values were two sided.

Results

Prevalence and Genotype of HPV DNA

Of the 103 cases, 100 (97.1%) were positive for HPV DNA. In the current study, HPV positivity was defined as

detection of HPV DNA by either one or both of the PCR methods. The most prevalent type was HPV-16 and this type was found in 68 (66.0%) of the 103 cases. The prevalence of other types were as follows: HPV-18: 7.8%, HPV-16/18: 7.8% and the other HPV types: 15.5%. The prevalence of other types were as follows: HPV-31: 5.8%, HPV-33: 4.9%, HPV-45: 1.0%, HPV-52: 1.0%, HPV-58: 1.9%, and HPV-67: 1.3%. HPV types 16/18 co-infection was detected in eight cases.

p53 Protein Expression

Among the normal epithelium, no nuclear immunostaining for p53 was detected. p53 overexpression was displayed in 38 (36.9%) of the 103 invasive carcinomas, with a mean value of 4.8±9.1%. p53 immunoreactivity was observed exclusively in the nucleus. For patients with invasive carcinoma, p53 staining was absent in 65 of the 103 (63.1%) and the median value of p53 expression in the tumors of invasive carcinoma was 0% (range, 0–41.1%).

p21 Protein Expression

In the normal epithelial control samples, p21 expression was seen in the nuclei of the parabasal cells and the majority of cells in the basal layers showed no immunostaining. The median value of p21 expression in the tumors of invasive carcinoma was 6.9% (range, 0–41.1%). For patients with invasive carcinoma, p21 staining was absent in 29 of the 103 (28.2%).

p27 Protein Expression

p27 expression was identified in all the samples of normal epithelium. In the normal epithelial control samples, p27 expression was seen in the nuclei of intermediate and superficial cells. The 103 cervical cancer cases displayed a wide range of expression of p27 (0–65.5%). Moreover, 57 (55.3%) of the 103 patients with invasive carcinoma had no p27 staining and the median value of p27 expression was 0%. The expression of the three proteins was not associated with the histological type and histological grade.

HPV Genotype in Association with p53, p21 and p27 Expression

For further analysis only the HPV positive cervical carcinomas were included. The relationship between the HPV genotype and expression of these three proteins is shown in Table 1. Our results revealed that p21 expression was significant less widespread and was frequently absent in HPV-18 positive cervical carcinomas (p=0.019) (Fig. 1). Seven (87.5%) of the 8 HPV-18 and 6 (75.0%) of the 8 HPV-16/18 positive cervical carcinoma patients showed low p21 protein expression. However, there was no significant association between HPV type and p53 or p27 expression in the cervical tumors.

Discussion

To our knowledge, this is the first study to examine a possible association between specific HPV genotype and the expression of p53, p21 and p27 in cervical carcinomas. We found that there was significant decrease in the expression of p21 in HPV-18 positive cervical carcinomas. However, the expression of p53 and p27 was not related to HPV type in cervical carcinomas.

Persistent infection with high-risk or intermediate-risk HPV types has been shown to be required for the development of invasive carcinoma. However, different HPV genotypes have been reported to have different oncogenic potentials [24]. The molecular mechanisms responsible for malignant transformation and tumor progression in cervical neoplasms by different HPV type are still unclear. Up to now, there have been only few reports describing any association of p53, p21 and p27 expression with either the presence or absence of HPV infection or

Table 1 The correlation between HPV genotypes and p53, p21, and p27 expression

	No.	No. of cases (%) with positive p53 expression	*P*	No. of cases (%) with low p21 expression	*P*	No. of cases (%) with low p27 expression	*P*
HPV-16	68	30 (44.1)	0.156	35 (51.5)	0.019	40 (58.8)	0.773
HPV-18	8	1 (12.5)		7 (87.5)		4 (50.0)	
HPV-16/18	8	1 (12.5)		6 (75.0)		3 (37.5)	
HPV-16/18 (-)	16	4 (25.0)		5 (31.3)		8 (50.0)	
HPV- negative	3	1 (33.3)		0 (0)		2 (66.7)	
Total	103	37 (35.9)		53 (53.0)		57 (55.3)	

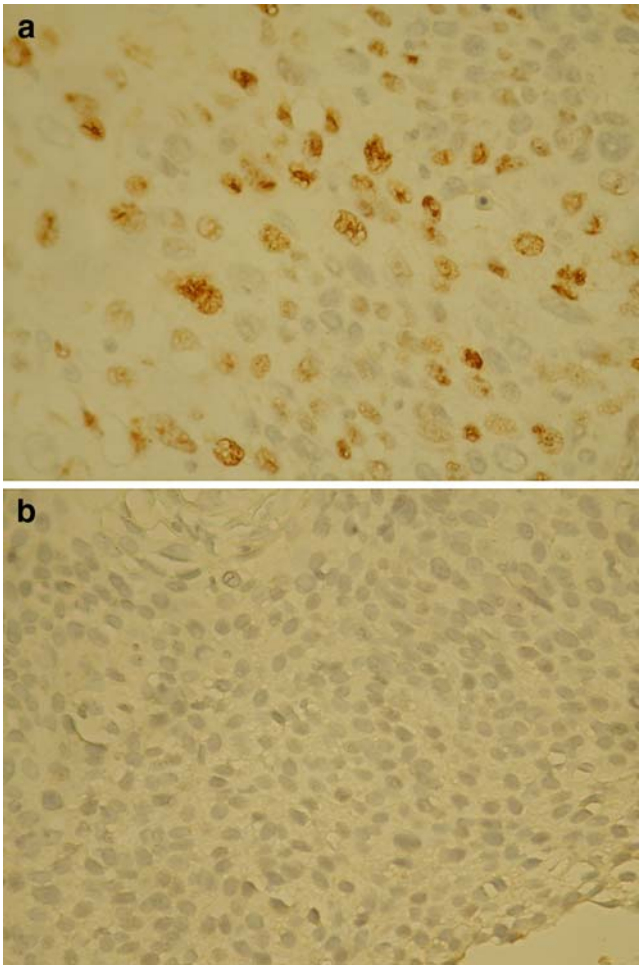

Fig. 1 Normal p21 immunostaining of HPV-16 positive tumor cells is shown in (**a**) and in (**b**), the presence of reduced p21 expression can be noted in HPV-18 positive tumor cells of cervical carcinomas. Original magnification ×400 (**a**, **b**)

with infection by high risk/low risk HPV types. A study of cervical intraepithelial neoplasia showed that there was no correlation between HPV type and expression of p21 and p53 [25]. Our observations in this study have revealed that p21 expression was decreased in HPV-18 positive cervical carcinomas; however, changes in p53 expression were not specifically associated with any particular HPV genotype (s). The present results are supported by Cavuslu et al., who showed the presence of detectable p53 protein to be independent of HPV infection in cervical cancer [26]. It has been proposed that expression of the p21 gene is regulated by two pathways, namely, by p53-dependent and p53-independent mechanisms [27, 28]. Our results imply that the inactivation of p21 may result from *in vivo* mechanisms, which are independent of p53 during tumorigenesis in the HPV-18 positive cervix. Although the mechanisms involved in p21 regulation are not fully understood, a study by Jones et al. showed that the HPV E7 oncoprotein may induce S phase in differentiated cells by interacting with p21 and abrogating p21-mediated inhibition of cyclin A and E-associated kinase activities [29]. Additionally, it has been proved that endogenous Notch1 is required for induction of p21 expression in differentiating primary keratinocytes, and activated Notch1 causes growth suppression by inducing p21 expression [30]. These findings suggest that HPV E7 oncoprotein may regulate the p21 through Notch signaling in lesions infected by HPV-18, rather than by the abrogation of p53 function. With HPV-16/18 co-infection, p21 expression was also reduced, which would seem to indicate that expression of the p21 gene was impaired in HPV-18 multiply infected cells. This finding indicates that p21 protein may be the principal cellular target oncoprotein found in HPV-18 positive cervical carcinomas.

A previous study revealed that the highest increase in p21 expression was seen in high-grade cervical dysplasia and invasive cervical carcinomas [31]. However, Nadal et al. reported that high p21 expression was noted in squamous cell differentiation of laryngeal carcinomas, whereas poorly differentiated carcinomas had low p21 protein expression [32]. In our previous study, p21 was abundantly expressed in precancerous lesions but was down regulated in the microinvasive carcinoma and further down regulated in the invasive carcinoma [19]. This paradoxical increase in p21 expression might be related to the role of p21 in cell differentiation.

Previous reports have shown that HPV-18 is preferentially associated with adenocarcinomas of uterine cervix [33]. We observed p21 expression was lower level in HPV-18 positive carcinomas but it was not associated with histological type. This finding suggests that loss of p21 molecule may play a major role in the malignant transformation of HPV-18 positive carcinomas, irrespective of histological types.

In summary, we have demonstrated that HPV-18 infection decreases the integrity of cell checkpoints by the inactivation of p21 protein and further showed expression of p53 and p27 not to be related to HPV type in cervical carcinomas. Although this needs to be substantiated in a larger number of tumors, p21 protein might therefore serve as a potential biomarker for HPV-18 positive cervical carcinomas. Moreover, further investigation of the mechanisms involved in p21 expression could provide further insights into the molecular pathology of cervical carcinogenesis and might also be helpful when designing alternative therapies for the treatment of HPV type-specific cervical carcinomas.

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