Predictive Significance of the Alterations of p16INK4A, p14ARF, p53, and Proliferating Cell Nuclear Antigen Expression in the Progression of Cervical Cancer

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ABSTRACT

Purpose: **The purpose of this research was to evaluate the clinical significance of p16INK4A, p14ARF, p53, and proliferating cell nuclear antigen (PCNA) expression in tumor progression of cervical cancer.**

Design: **Seventeen patients (40 samples) with consecutive cervical lesions from normal squamous epithelium, inflammation of the cervix to cervical intraepithelial neoplasm (CIN) and invasive cervical squamous cell cancer (SCC), or from CIN to SCC were collected for this study. Expression of p16INK4A, p14ARF, p53, and PCNA were detected by immunohistochemistry on paraffin-embedded sections. Human papillomavirus DNA was detected simultaneously with PCR and typed according to its DNA sequence.**

Results: **p16INK4A overexpression was significantly higher in CIN (75%) and in SCC (75%) than in normal or inflammation of the cervix (12.5%; $P < 0.01$, $P < 0.05$, respectively). The positive rate of p14ARF expression was higher in SCC (83%) than in normal/inflammation of the cervix (25%; $P < 0.05$). PCNA expression was negative in normal or inflammation of the cervix, but an increased in expression was seen in 63.2% in CIN and 100% in SCC ($P < 0.01$, $P < 0.05$). When the time interval for disease progression from initial biopsy to CIN 3 or invasive cancer was compared with states of p16INK4A expression, cases stained positive for p16INK4A progressed within 64.2 months as compared with 122.3 months among those stained negatively ($P < 0.01$). Cases with increased p14ARF expression also had a short time interval for disease progression of 78.8 months as compared with 108.3 months in cases that were p14ARF negative. Cases with stable or decreased p53 expression had the shortest time interval for progression of 32.3 months in contrast to cases with no p53 expression (113.9 months). However, cases with increasing p53 expression progressed within 60.8 months.**

Conclusions: **Our results suggested that altered states of p16INK4A, p14ARF, p53, and PCNA may be valuable markers to predict the progression of cervical neoplasia.**

INTRODUCTION

Cervical cancer is the third most common cancer among women worldwide, and it is the principle cancer among women in most developing countries (1). The natural history of cervical intraepithelial neoplasia (CIN) is extremely variable. If left untreated, CIN may regress to normal, or persist or progress to invasive cancer. Several studies have shown that approximately one-third to one-half of cases of CIN 1 and CIN 2 spontaneously regress without treatment. Even cases of CIN 3 have been observed to regress spontaneously. The more severe the abnormality of the lesion, the less likely it is to regress. Women with CIN confer an increased risk for developing invasive cancer. The time taken from onset of CIN 3 to invasive cancer has been estimated to range from 1 to 30 years, reflecting biological diversity of the disease. The majority of cases follow the progressive stages, and the time taken estimated to invasive cancer is ~15 years.

It is not possible at present to predict which cases of CIN will progress and which will persist or regress. Regular cytological screening can afford good protection through detection of cervical abnormalities before the onset of invasion cancer. This has led to significant reduction of both incidence and mortality of cervical cancer (2). However, the extent of this reduction and the cost-effectiveness of current screening programs remain the subject of debate (3). For example, cytological screening relies on subjective, morphological evaluation. Histopathological diagnosis that directs treatment is also affected by high rates of disconcordance among pathologists (4, 5). An objective biomarker would allow unambiguous identification of truly dysplastic cells and/or predict disease progression.

The etiologic role of human papillomavirus (HPV) in cervical cancer has been strongly supported by vast experimental and epidemiological evidence (6–8). But not all of the infected individuals will develop cervical cancer. The HPV genome has been found to interact with host cell proteins disrupting some of the biological functions and causing imbalance in the expression of the viral oncoproteins E6 and E7. HPV proteins E6 and E7 increase degradation of p53 or inactivation of pRb tumor sup-

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pressor proteins respectively, allowing escape from p53 induced apoptosis and normal cell cycle control in G_1 (9).

p53 and pRb pathway are in part controlled by the *CDKN2A* (*INK4A/ARF*) locus that encodes two tumor suppressor proteins p16INK4A and p14ARF (10). The *CDKN2A* locus is often mutated, deleted, or methylated in most types of human cancers (10). p16INK4A is an element of the pRb pathway and can induce G_1 cell cycle arrest by inhibiting the phosphorylation of pRb by cyclin-dependent kinase 4 and cyclin-dependent kinase 6 (11), and p16INK4A also plays an important role in cellular senescence (12). Recent studies have shown that immunohistochemical staining for p16INK4A is a promising marker for dysplastic and malignant cervical epithelia (13–15).

Human p14ARF (mouse p19ARF) is a cell cycle inhibitor that arrests cell growth in G_1-S and G_2-M in both p53-dependent and p53-independent manners (16). p14ARF is induced by activated oncogenes such as E2F1, and expression of p14ARF is also elevated if wild-type p53 function is lost due to mutation or enhanced degradation (16, 17). Inactivation of pRb results in release of E2F transcription factors that in turn leads to the expression of p14ARF that stabilizes p53 by inhibiting MDM2-mediated p53 degradation (18, 19). Sano *et al.* (20) considered that the mode of p14ARF overexpression in cervical neoplastic cells with HPV association differ from that in non-HPV-associated cancers of other organs. p14ARF overexpression is presumably attributable to the functional inactivation of the pRb and p53 proteins by HPV oncoproteins E6 and E7 (15, 16).

Proliferating cell nuclear antigen (PCNA) relates to cell proliferation specifically highlighting cells with active DNA replication, and it is universally used to evaluate cell proliferation by immunohistochemistry. However, it does not distinguish dysplastic or malignant proliferating cells from normal proliferating cells. p53 can inhibit cell proliferation by blocking entry into the S phase of the cell cycle and is also a master regulator of apoptosis. There is a good correlation between tumor-cell kinetics measured by PCNA expression and p53 gene status in several malignancies (21).

To understand the role of these markers in the progression of cervical lesions, a series of consecutive biopsies with progressing lesions from CIN to invasive cervical cancer were investigated. The findings on the expression of p16INK4A, p14ARF, p53, and PCNA on paraffin sections of serial consecutive biopsies of women who developed invasive cervical cancer were studied to evaluate the roles of these proteins during disease progression. These findings will also be correlated with HPV status and the time taken for development of invasive cancer of each individual case to additionally identify those markers that have predictive value for progression.

MATERIALS AND METHODS

Case Selection. Cervical cancer patients residing in northern Sweden were treated in Umeå University Hospital. Seventeen cases who had biopsies taken and were diagnosed previously with CIN, and whom later present with histopathological diagnosis of invasive cervix cancer, were identified from the pathological registry between 1982 and 2000 and were selected for this study. The mean age of these women was 38 years (range, 21–78 years). Each case had 2–3 consecutive biopsy samples that had normal squamous epithelium or inflammatory cervical tissue, and CIN or invasive squamous cell carcinoma (SCC). In total, there were 40 biopsy samples taken from the 17 cases. Among these, 8 biopsy samples (2 cases with normal squamous epithelium and 6 cases with inflammation of the cervix) had normal epithelium or inflammation of the cervix, 20 biopsy samples had CIN diagnosis (2 cases with CIN1, 6 cases with CIN2, and 12 cases with CIN3 or carcinoma *in situ*), and 12 biopsy samples had invasive SCC. The diagnosis of all of the cases was reviewed and reconfirmed by a pathologist. Ethical permission was approved by the Institutional Review Board of Umeå University.

Immunohistochemistry. All of the specimens were formalin-fixed and paraffin-embedded. Serial sections (4 μm thick) were cut, and the end section was stained with H&E to ensure that the lesion was still present in the serial sections. The sections were processed for immunohistochemical analysis as followed. Deparaffinization was carried out with xylene followed by rehydration through graded alcohols. Epitope retrieval was performed by heating the sections for 10 min in 10 mM citrate buffer (pH6.0) in the microwave oven. The sections were incubated in 0.75% hydrogen peroxide (H_2O_2) in methanol for 10 min to block endogenous activity. This is followed by blocking of nonspecific binding of primary antibodies to epitopes by a preincubation step with 5% normal goat serum for 30 min at room temperature. The primary antibodies were added and incubated at 4°C overnight. After washing thoroughly with PBS, the sections were incubated with secondary antibody conjugated to horseradish peroxidase for 30 min at room temperature and developed with 3-amino-9-ethylcarbazole + substrate-chromogen (DAKO Cytomation, Glostrup, Denmark). The sections were counterstained lightly with hematoxylin. Paraffin-embedded tissue sections containing colon cancer were used as positive controls for p16INK4A. Tissue sections of squamous cell cancer of lung were used as positive controls for PCNA and p53. Paraffin-embedded sections of HeLa cervical cancer cell lines grown on collagen-fibroblast matrix were used as positive controls for p14ARF. PBS (pH 7.4) was used in place of primary antibodies in negative controls. The images of the immunohistochemistry staining were photographed on Zeiss Axioskop 2 MOT microscope (Carl Zeiss, Jena, Germany) using Zeiss plan-Apochromat objectives (Carl Zeiss) and captured by high-resolution color digital camera AxioCam HR (Carl Zeiss). The following primary antibodies were used in this study, mouse monoclonal F-12 anti-p16INK4A (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal Ab-4 anti-p14ARF (Neomarkers, Fremont, CA), mouse monoclonal anti-p53 DO-7 (DAKO), and mouse monoclonal PC-10 anti-PCNA (Santa Cruz Biotechnology). Antibodies were diluted to 1:100 except for DO-7 that was diluted to 1:75. The secondary antibodies used were goat antimouse (Amersham Biosciences AB, Uppsala, Sweden) in 1:100 dilution and donkey antirabbit according to instructions in the commercial kit (DAKO). Immunohistochemistry scoring results were based on the staining intensity and the distribution of immunopositive cells, as described previously (20, 22). Staining intensity score: −, no expression; +, weak expression; ++, moderate expression; and +++, strong expression. Only distinct nuclear staining was considered positive for p53 and PCNA. Nuclear staining with or without cytoplas-

Table 1 Patient's age, diagnosis, interval time, and expression of p16INK4A, p14ARF, p53, and PCNA[a]

Case no.	Age (year)	Diagnosis	Interval time (month)	HPV type	p16INK4A % Intensity	p14ARF % Intensity	p53 % Intensity	PCNA % Intensity
1a	29	CIN2		16	48 ++	−	−	−
1b	35	CIS	77	16	70 +	30 +	−	20 ++
1c	43	SCC	92	16	42 +++	60 +	5 +	52 +++
2a	34	CIN2		−	30 +	20 ++	−	−
2b	39	SCC	58	18	42 +	40 ++	−	20 +
3a	48	Inflam		45	−	25 ++	−	−
3b	58	CIS	129	HPV+	10 +	30 ++	−	48 ++
4a	37	CIN2		−	30 +	20 +	−	30 ++
4b	42	Condyloma	72	16	10 +	20 +	−	10 +
4c	44	CIN3	27	16	37 +	30 +	10 +	30 ++
5a	21	Inflam		33	−	−	−	−
5c	32	SCC	126	16	−	20 +	8 +	60 +++
6a	39	CIN3	31	−	−	−	−	
6e	50	SCC	137	16	−	−	−	55 ++
7a	31	CIN2		16	10 +	10 +	−	10 +
7b	35	SCC	55	16	20 +	30 ++	10 +	60 +++
8a	53	Inflam		33	20 ++	−	−	−
8b	58	SCC	67	18	−	20 +	6 +	30 ++
9a	33	CIN2		−	38 ++	15 +	−	20 ++
9b	44	CIS	135	18	20 ++	30 ++	−	43 +++
9c	44	SCC	2	18	25 +	60 ++	−	70 +++
10a	32	CIN3		18	25 ++	45 +	5 +	20 +
10b	34	SCC	22	18	60 +++	50 +	−	40 ++
11aII	40	CIN2–3		45	50 ++	−	−	20 ++
11c	43	Inflam	39	16	−	−	−	−
11d	57	SCC	173	31	40 +	−	−	45 +++
12a	47	Inflam		33	−	−	20 +	−
12b	53	CIN3	69	16	30 ++	20 +	−	10 ++
13a	23	Nor		−	−	−	15 +	−
13b	24	CIN3	14	33	50 +	10 +	30 +	28 ++
13c	25	SCC	12	33	60 +	20 ++	30 +	70 ++
14a	29	CIN1		16, 18	29 ++	ND	ND	Nod
14b	38	CIS	104	16	−	10 +	−	25 ++
17a	41	CIN2		16	20 +	30 +	5 +	10 +
17b	42	CIS	6	16	40 ++	10 +	−	20 ++
17c	42	SCC	10	16	62 +++	20 +	25 ++	30 +++
18a	35	CIN1		−	20 ++	10 +	−	28 +
18c	37	CIN2–3	35	18	50 ++	60 +	60 ++	51 ++
19a	78	Nor		−	−	−	−	−
19b	84	SCC	68	45	60 ++	30 +	10 +	48 +++

PCNA, proliferating cell nuclear antigen; Nor, normal epithelium of the cervix; inflam, inflammation of the cervix; CIN, cervical intraepithelial neoplasm; CIS, carcinoma *in situ*; SCC, squamous cell cancer; ND, not done (no section); interval time, the time between consecutive biopsies of the same patient; HPV+, HPV DNA positive, but could not be typed by DNA sequence; +, weak staining; ++, moderate staining; +++, strong staining.

mic staining was considered positive for p16INK4A and p14ARF. Positive staining is considered when the percentage of positive cells was ≥20% for p16INK4A, p14ARF, and PCNA, and >5% in the case of p53 according to data published previously (20–24). This criterion was applied in all analyses except for Table 1, which shows the actual data.

HPV Detection and Typing. HPV DNA was detected by PCR. Genomic DNA was extracted from the paraffin-embedded tissue sections. In brief, tissue blocks were cut into five, 4-μm thick sections with microtome. An empty paraffin block was sectioned in between tissue blocks to avoid cross-contamination. All of the tissue sections were deparaffinized twice in xylene, dehydrated in graded alcohols, and the tissue pellet was incubated in digestion buffer, which contained 100 mM NaCl, 10 mM Tris-Cl, 25 mM EDTA, 5% SDS (pH 8.0), 10 μl of 20 mg/ml proteinase K, and digestion was carried out at 55°C to 60°C for a minimum of 2 h. Proteinase K was inactivated by incubation at 98°C for 10 min. After cooling, 1 μl of each sample was used for PCR. HPV was detected by nested PCR using MY09/11and GP5+/6+ primer sets, targeting the HPV L1 open reading frame. The sequences were as follows: MY09, 5′ CGTCCMARRGGAWACTGATC 3′; MY11, 5′GCMCAGGGWCATAAYAATGG3′ (M:A or C, R: A or G, W: A or T, Y: C or T); GP5+, 5′TTTGTTACTGTGGTAGATACTAC3′; and GP6+, ′GAAAAAATAAACTGTAAATCATATTC3′. The PCR products of MY09/MY11 were 450 bp long and of GP5+/6 +, 150 bp long. The 50 μl PCR reaction volume contains 1 × AmpliTaq PCR buffer, 10 μM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP, dTTP, 5 μl of 2% BSA, and 1.5 units of TaqDNA polymerase (Promega, Madison, WI). The PCR reaction mixture was preincubated at 96°C for 1 min to denature the genomic DNA. This was followed by 40 cycles starting with denaturation step for 30 s at 94°C and annealing at 50°C for MY09/11 or 40°C for GP5+/6+ for 30 s, and extension

Table 2 Expression of p16INK4A, p14ARF, p53, and PCNA[a] in cervical neoplasia

		p16INK4A+		p14ARF+		p53+		PCNA+	
Lesions	*n* (total)	*n*	%	*n*	%	*n*	%	*n*	%
Nor/inflam	8	1	12.5	2	25.0	2	25.0	0	
CIN/CIS[b]	20	15	75.0[c]	10	52.6	5	26.3	12	63.2[c]
SCC	12	9	75.0[d]	10	83.3[c]	7	58.3	12	100.0[d]

[a] PCNA, proliferating cell nuclear antigen; Nor/inflam, normal epithelium or inflammation of the cervix; CIN, cervical intraepithelial neoplasm; CIS, carcinoma *in situ*; SCC, squamous cell carcinoma.
[b] 19 samples of CIN were detected for p14ARF, p53, and PCNA.
[c] Compared with group nor/inflam, $P < 0.01$.
[d] Compared with group nor/inflam, $P < 0.05$.

at 72°C for 45 s. A final extension at 72°C for 5 min was carried out. The two-step nested PCR was performed first with the MY09/MY11 primers, and 1 μl of the 450 bp PCR products was added to the second PCR mixture containing the GP5+/6+ primers. Ten μl of each PCR products were run on 2% agarose gel and stained with ethidium bromide. PCR targeting human S14 gene was used to evaluate the DNA quality for PCR reaction. Positive HPV PCR products were purified with Qiaquick PCR purification kit (Qiagen, GmbH, Germany). HPV typing was obtained from direct sequencing of purified PCR products using ABI 377 (Applied Biosystems, Foster City, CA). HPV amplimeres that could not be typed by direct sequencing were subjected to pyrosequencing (25). Positives controls included DNA extracted from CaSki cervical cancer cell line. Blanks containing no DNA were included to check for contamination.

Statistical Analysis. The data were analyzed with the SPSS program. Statistical analysis was based on χ^2 test with Yates' correction. An additional two-tailed Fisher's exact test was used only when the number of samples in any well of a given statistical table was ≤5. The *t* test was used to analyze quantitative data. It was considered a significant difference when $P < 0.05$.

RESULTS

Immunohistochemistry staining for the expression of p16INK4A, p14ARF, p53, and PCNA, and HPV PCR analyses were performed on 17 cases (40 samples). The results are presented in Table 1. Subsequently, the levels of p16INK4A, p14ARF, p53, and PCNA expression were compared among normal or inflammatory tissue, CIN lesions, and SCC. The positive rate for p16INK4A expression between CIN and SCC were 75% (15 of 20) and 75% (9 of 12), respectively, whereas normal and inflammatory tissue had a positive rate of 12.5% (1 of 8; $P < 0.01$ and $P < 0.05$, respectively). Increased p14ARF expression was also observed to be significant with disease progression (25% in the normal or inflammatory group, 53% in CIN, and 83% in SCC). p14ARF expression was significantly different between normal or inflammatory disease of the cervix (25%; 2 of 8) and SCC (83.3%; 10 of 12; $P < 0.05$). p53 expression increased gradually with progression of lesions, but its positive rate was not significantly different between premalignant and malignant lesions. PCNA expression was not detected in any normal or inflammatory tissue. However, its expression was high in CIN (63%; 12 of 19; $P < 0.01$) and in SCC (100%; 12 of 12; $P < 0.05$; Table 2).

S14 PCR results indicated that all of the samples contained DNA amplifiable by PCR. To analyze the role of HPV infection in the progression of cervical cancer, all of the samples were tested for presence of HPV DNA and its types. The positive rates of HPV DNA were 75% (6 of 8) among normal or inflammatory tissue, 80% (16 of 20) in CIN, and 100% (12 of 12) in SCC. The prevalence of HPV types were as follows, HPV 16 (40%), HPV 18 (20%), HPV 33 (13%), HPV 45 (8%), and HPV 31 (5%). The most common HPV infections among cervical cancers, namely HPV 16 and HPV 18, were found in 33% (2 of 6) in the group with normal or inflammatory diagnosis, 81% (13 of 16) with CIN, and 75% (9 of 12) with SCC. In one biopsy, the HPV type could not be determined by direct sequencing or pyrosequencing (case 3b). In 8 of 17 cases HPV persistence of the same HPV type was observed, and in 6 cases the HPV types were different in the consecutive biopsies of these cases.

To analyze the relationship between HPV infection, and the expression of p16INK4A, p14ARF, p53, and PCNA, the 17 cases were divided into two groups according to HPV status alternation during disease progression, that is, those with HPV DNA negative to positive and those with HPV DNA persistence. Six cases had status HPV DNA negative to positive and 11 cases had persistent infection. The expression of p16INK4A and p14ARF during disease progression were similar between these two groups (67% *versus* 64% and 67% *versus* 73%, respectively). p53 expression during disease progression was higher in the group HPV DNA negative to positive (67%; 4 of 6) than in the group with HPV DNA persistence (27%; 3 of 11) but the difference was not significant ($P > 0.05$). PCNA expression during disease progression was high among those with HPV DNA negative to positive (83%; 5 of 6) and in those with HPV DNA persistence (100%; 11 of 11). However, the difference was also not significant ($P > 0.05$). Thus, p16INK4A, p14ARF, and PCNA expression were up-regulated according to change in HPV status during progression, whereas p53 expression was down-regulated.

The 40 samples were divided into five groups according to disease progression status to evaluate the alternations of p16INK4A, p14ARF, p53, and PCNA expression during progression as depicted in Table 3. The time taken for progression from normal or inflammatory to CIN was significantly shorter for CIN (59.8 ± 44.9 months) than of normal or inflammatory disease of the cervix to SCC (108.5 ± 44.2 months; $P < 0.05$). The interval time between progression from CIN 1 or CIN 2 to

Table 3 Alterations of p16INK4A, p14ARF, p53, and PCNA[a] expression and interval time of cervical lesion progression

			p16INK4A ↑		p14ARF ↑		p53 ↑		PCNA ↑	
Progression	Case *n*	Interval time (month)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Nor/inflam to CIN	4	59.8 ± 44.9	3	(75.0)	3	(75.0)	1	(25.0)	3	(75.0)
Nor/inflam to SCC	4	108.5 ± 44.2	2	(50.0)	3	(75.0)	1	(25.0)	4	(100)
CIN1–2 to CIN3/CIS	4	71.4 ± 46.3	4	(80.0)	2	(40.0)	1	(20.0)	4	(80.0)
CIN2–3 to SCC	9	32.3 ± 28.9	6	(66.6)	7	(77.8)	3	(33.3)	8	(88.9)
CIN to nor/inflam	2	55.0 ± 16.0	0		0		0		0	

[a] PCNA, proliferating cell nuclear antigen; ↑, increased expression; Nor/inflam, normal epithelium or inflammation of the cervix; CIN, cervical intraepithelial neoplasm, CIS, carcinoma *in situ*; SCC, squamous cell cancer.

CIN 3 or carcinoma *in situ* (71.4 ± 46.3 months) and for CIN regression (55.0 ± 16.0 months) was rather similar. However, the time for disease progression from CIN 2 or CIN 3 to SCC took 32.3 ± 28.9 months. Fig. 1 shows immunohistochemical staining of p16INK4A, p14ARF, p53, and PCNA in the consecutive biopsies of case 13 with normal squamous epithelium adjacent to CIN 2, carcinoma *in situ*, and SCC diagnoses. Progressing lesions showed increased p16INK4A, p14ARF, p53, and PCNA expression. In regressing lesions; the p16INK4A and PCNA expression were distinct in biopsies with CIN diagnoses but were negative in the corresponding normal epithelium (Fig. 2, 11aII to 11c).

The 17 cases in this study can be subdivided into two groups of which 12 cases progressed to SCC and 5 cases progressed to CIN 3 or carcinoma *in situ*. The time interval between the first biopsy and the last biopsy were 91 months and 82 months, respectively. When the time interval for disease progression was compared with altered states of p16INK4A, p14ARF, p53, and PCNA expression (increase ↑, stable → or decrease ↓, − negative), increased p16INK4A expression had

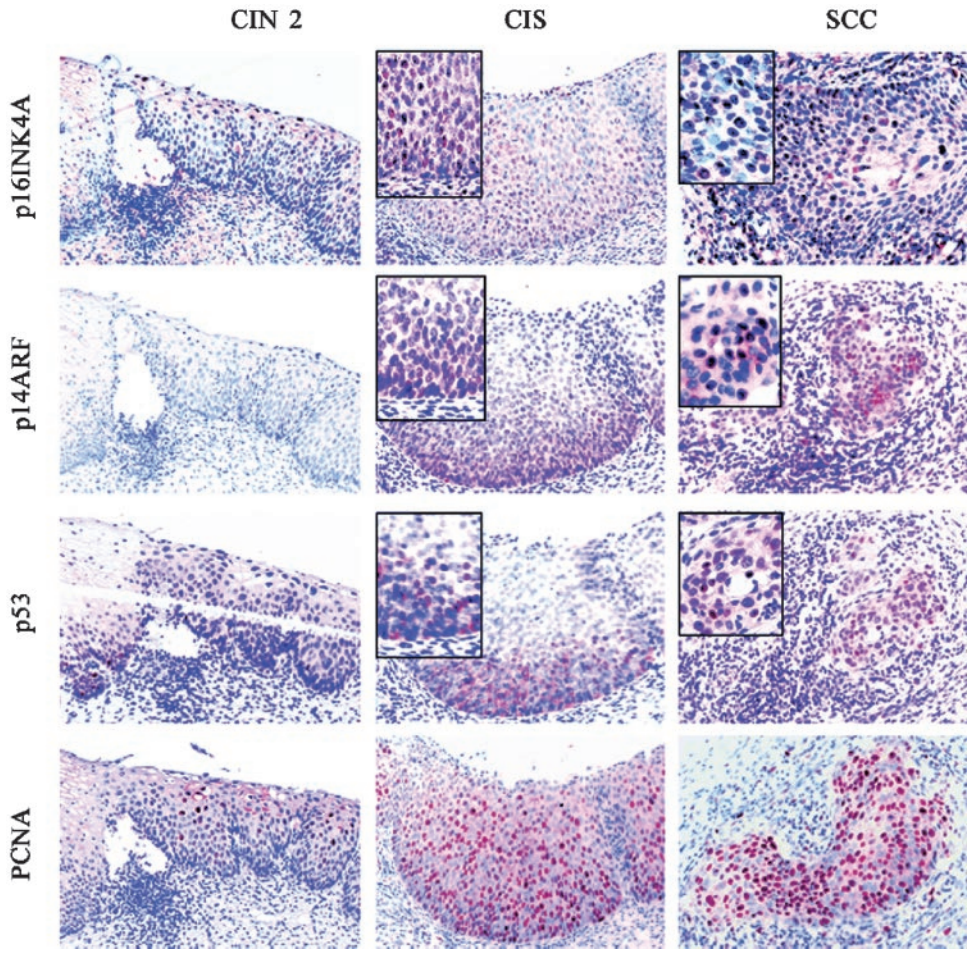

Fig. 1 Expression patterns of p16INK4A, p14ARF, p53, and proliferating cell nuclear antigen (PCNA) in three consecutive biopsies of cervical cancer progression: cervical intraepithelial neoplasm (CIN) 2, carcinoma *in situ* (CIS), and invasive cancer of case 13a, 13b, and 13c taken at (original magnification ×20). Biopsies 13b and 13c had human papillomavirus 33 whereas 13a was human papillomavirus DNA negative. The squamous epithelium of 13a adjacent to CIN 2 stained weakly with p16INK4A, whereas CIS and squamous cell cancer (SCC) staining intensity was slightly increased. p14ARF expression was negative in normal epithelium and in CIN 2, but positive in CIS and SCC. p53 stained positive in the basal layer of normal epithelium and in CIN 2, but was slight increased in CIS and SCC. PCNA expression increased throughout tumor progression. p14ARF-and p53-positive cells were confined to the lower layer of CIS, whereas p16INK4A- and PCNA-positive cells were located throughout the entire epithelium of CIS.

an average time interval of 64.2 months among cases as compared with cases that were negative (122.3 months; $P < 0.01$). Cases with increased p14ARF expression had a time interval of 78.8 months between the two groups as compared with 108.3 months among cases that were p14ARF negative ($P > 0.05$). Cases negative for p53 expression had a long interval time of 113.9 months, whereas those with increasing expression had a time interval of 60.8 months. However, cases with stable or decreasing p53 expression had the shortest time for progression (32.3 months; $P < 0.05$; Fig. 3). Thus, lesions with increased p16INK4A, and stable or decreased p53 expression are significant for tumor progression.

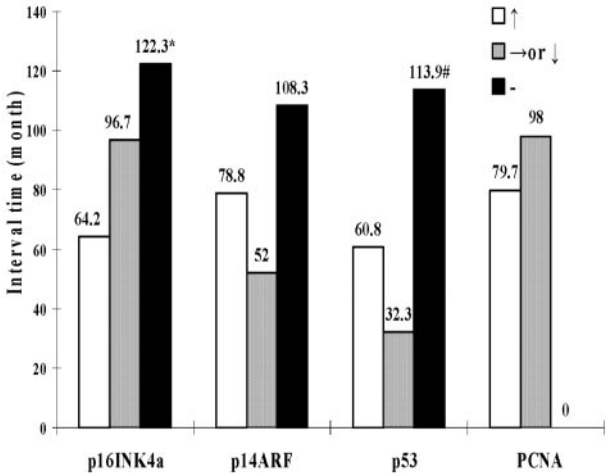

Fig. 3 The time interval between diagnoses of biopsies during disease progression in relation to expression alterations of p16INK4A, p14ARF, p53, and proliferating cell nuclear antigen (PCNA). ↑, increased expression during disease progression. → or ↓, stable or decreased expression during disease progression. −, negative expression. ∗ compared with p16INK4A expression increased, $P < 0.01$; # compared with p53 expression decreased, $P < 0.05$.

DISCUSSION

Approximately 13,000 new cases of cervical cancer and 4,000 cervical cancer-related deaths occurred in 2002 in the United States (26). Cervical cancer remains one of the leading causes of cancer death in women worldwide (27). Cytological screening to reduce cervical cancer incidence had been successful among nations with organized screening; however, still a proportion of women developed invasive cervical cancer (28). HPV is an important causal factor but still other genetic and epigenetic factors may be involved in tumor progression (29). Most of the early lesions of the cervix are heterogeneous in its biological nature. Additional biomarkers may be essential to assist in identifying which cases will progress or regress (30).

This group of women represented the high-risk women who developed invasive cancer despite earlier intervention to prevent disease progression. All of the cases had high-risk HPV types detected, of which 50% had persisting infection of the same HPV type, whereas 35% had different HPV types among the different consecutive biopsies.

It is known that HPV infection causes a number of changes in gene or protein expression within the infected host cells. E6 and E7, products of HPV infection, can bind to and inactivate the p53 and pRb, respectively (9). Because expression of the cyclin-dependent kinase inhibitor gene p16INK4A is under negative feedback control of functional Rb protein, overexpression of p16INK4A ultimately occurs in cells infected by high-risk HPV (9). A number of studies showed p16INK4A expression only in CIN and cervical cancer recently, but not in normal squamous epithelium. Furthermore, cases that had been positive for high-risk HPV types were also strongly reactive for p16INK4A expression, and cases that were positive for low-risk HPV types were negative for p16INK4A (13–15, 20–23). Thus, p16INK4A could be an important marker for distinguishing between benign immature squamous metaplasia from high-grade CIN. However, in this study, we detected p16INK4A in some cases with normal or inflammatory lesions, CIN and SCC. Notably, in those cases, p16INK4A expression in normal squamous epithelium was seen particularly in the basal cell layer, and its positive rate was lower (12.5%) as compared with CIN (75%) or SCC (75%). In CIN, cells with positive expression were seen throughout the dysplastic epithelium, whereas in SCC, the positive cells might be either focal or evenly distributed within the tumor tissue, as seen in Fig. 1. In our study, the positive rate of p16INK4A expression did not have any significant correlation with presence of HPV infection in the individual biopsies. It is possible that not all of the HPV classified in this group possess the same potential for cell cycle disruption or altered gene expression that leads to p16 up-regulation. But p16INK4A overexpression during disease progression was high in both HPV DNA negative to positive (67%) cases and in those with HPV DNA persistence (64%). However, a positive correlation was seen when the time interval between disease states was analyzed. Cases with increased p16INK4A expression will develop cervix cancer at a shorter time than those that lack the expression. This suggested that there might be other yet unknown mechanisms of p16INK4A regulation than HPV infection.

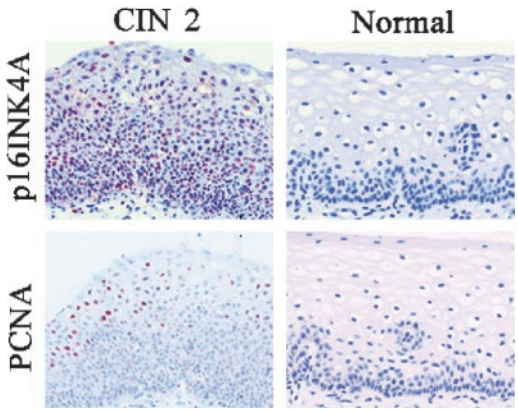

Fig. 2 p16INK4A and proliferating cell nuclear antigen (PCNA) expression in regressing lesions from cervical intraepithelial neoplasm (CIN) to inflammation of the cervix in two biopsies which were human papillomavirus (HPV) DNA positive (HPV45 and HPV16, respectively). The expression of p16INK4A and PCNA decreased with disease regression. Original magnification ×20.

p14ARF activates a p53 response that manifests itself as elevated levels of MDM2 and p21, and cell cycle arrest in both

G_1 and G_2-M (19). Expression of p14ARF is increased upon oncogenic activation or inactivation of the p53 and/or retinoblastoma pathways. Sano *et al.* (20) reported that p14ARF was expressed in almost all cervical cancers, thus, p14ARF emerged as another surrogate marker for cervical neoplasia after p16INK4A. Notably, p14ARF expression was also found among cervical cancers negative for HPV. In this study, increased p14ARF expression was correlated to increasing severity of the disease. HPV-negative biopsies with normal or inflammatory diagnoses were p14ARF negative. Two of 4 samples of CIN that were HPV DNA negative had p14ARF expression; hence, the expression of p14ARF was not always correlated to HPV status change nor to the severity of the disease, it was more correlated to disease progression. Increased p14ARF expression also had a shorter interval time (78.8 months) than those with negative expression (108.3 months).

Mutations in the *p53* gene are the most frequent mutations encountered in human tumors (21, 31). In cervical cancer, p53 expression rates varied between different studies from a small percentage to 62% (21, 23, 32). Most authors correlate the presence of p53 as a consequence of p53 mutation *i.e.,* protein accumulation due to failure of p53 to transactivate its own negative regulator MDM2. The immunohistochemistry staining is usually unable to distinguish wild-type p53 from mutated p53. HPV oncoprotein E6 binds to and inhibits the function of p53 protein by enhanced E6-mediated proteolysis. Degradation of p53 by HPV E6 could therefore result in low expression of p53 in cervical lesions, but the relationship between HPV and p53 immunohistochemistry staining in cervical lesions has thus far been controversial (21, 33, 34). The net outcome with regards to p53 protein levels could therefore be a complex issue, depending on the expression of HPV E6 and E7 oncoproteins, other cellular and viral cofactors, and the control of p53 by proteins other than MDM2. We found that p53 expression increased gradually with progression of lesions and was up-regulated in HPV DNA negative to positive status (57%), as compared with 30% of cases with HPV persistent infection. Neither was there any significant correlation to the time taken for progression between different pathological states.

PCNA is a good marker for cell proliferation. Its expression is strongly related to the mitotic index. Tjalma *et al.* (23) found that PCNA expression was associated with the presence of oncogenic HPV. But its expression cannot be ascertained if it is due to the effect of HPV infection only. In the current study, PCNA overexpression was not found in normal or inflammatory biopsies irrespective of its HPV status, but the positive rate of PCNA expression increased with progression of disease. Thus, the role of PCNA is more suitable as a marker for proliferation activity than as a marker for cancer progression.

Cervical cancer is preceded by a long period of premalignant disease with increasing morphological atypia and the potential for progression to malignancy. On average, cervical cancer takes at least a decade to develop. In this study, the interval time was $\sim$ 9.8 months from normal or inflammation of the cervix to CIN, 108.5 months from normal/inflammatory to SCC, 32.3 months from CIN to SCC. Our results showed that increased p16INK4A, p14ARF, and decreased or stable p53 expression are associated with disease progression. The times taken for progression were shorter among those stained positive for p16INK4A or p14ARF, and those with altered p53 expression. This finding suggests that alterations of p16INK4A, p14ARF, and p53 expression are valuable markers for prediction of progression of cervical disease.

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