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ORIGINAL ARTICLE

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Expression of cyclin-dependent kinase inhibitors $p16^{MTS1}$, $p21^{WAF1}$, and $p27^{KIP1}$ in HPV-positive and HPV-negative cervical adenocarcinomas

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Abstract Inactivation or down-regulation of the cell-cycle inhibitors $p16^{MTS1}$, $p21^{WAF1}$, and $p27^{KIP1}$ is involved in the carcinogenesis of various human tumors. In cervical squamous cell carcinomas that are associated with human papillomavirus (HPV) infection, the expression or function of these proteins is impaired by the action of viral oncoproteins E6 and E7. Comparably less is known about the role of these cyclin-dependent kinase inhibitors in cervical adenocarcinomas, 15–40% of which are HPV negative. Therefore, we studied the expression of $p16^{MTS1}$, $p21^{WAF1}$, and $p27^{KIP1}$ by immunohistochemistry in 60 cervical adenocarcinomas. HPV infection was determined by PCR, and HPV 16 and 18 E6/E7 oncogene expression was analyzed by RNA-RNA in situ hybridization. We found significant correlations of strong p16 expression with HPV 16/18 infection and HPV 16/18 E6/E7 oncogene expression (P=0.001). Moderate or strong p16 expression was also observed in 41% of HPV-negative carcinomas, indicating that HPV-independent mechanisms might also lead to p16 overexpression. In addition, stronger p21 and p27 expression was significantly associated with the detection of HPV 16 or 18 E6/E7 transcripts (P=0.015 and 0.030, respectively). Obviously, the tumor suppressor action of these proteins can be overcome in HPV-positive lesions. In contrast, absent or low p16, p21, and p27 immunostaining was observed in most HPV-negative cervical adenocarcinomas and might contribute to carcinogenesis in these tumors.

Keywords Cervical adenocarcinoma · HPV · p16 · p21 · p27

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Introduction

Our knowledge about the etiology of cervical cancer has dramatically increased during the last 20 years. It is now widely accepted that nearly 100% of cervical squamous cell carcinomas (SCC) are associated with certain high-risk human papillomavirus types, mainly HPV 16, 18, 31, 33, 35, 45 [34]. Malignant transformation of the cervical epithelial cells is associated with expression of the viral oncogenes E6 and E7 with dramatic implications for cell cycle regulation, differentiation, and genomic stability [32, 40].

Cervical adenocarcinomas represent 15–25% of all cervical carcinomas [10, 16]. In contrast to SCC, HPV18 is the most common HPV type in cervical adenocarcinomas, and 15–40% of these tumors are not associated with papillomavirus DNA [6, 21, 26]. The etiology of these HPV-negative cases is still unknown. Activation of oncogenes (i.e. K-ras) or inactivation of tumor-suppressor genes (i.e. p53) has only been detected in a small fraction of these carcinomas [11, 22].

Cyclin-dependent kinase (cdk) inhibitors are important negative regulators of cell cycle progression. After binding to cdk–cyclin complexes in the G1 phase of the cell cycle, they block the kinase activity of cdk's, preventing phosphorylation of members of the Rb gene family and transition to S phase [9, 37]. P16 (additional names: MTS1 for "multiple tumor suppressor 1" or CDK4I for "CDK4-inhibitor"), the product of the *ink4a* gene, specifically binds to cyclin D–cdk4/cdk6 complexes. Loss of this cell-cycle inhibitor by homozygous deletion of the gene, mutation or promoter hypermethylation is observed in several human tumors: many pancreatic carcinomas, bladder carcinomas, T-ALL, familial melanomas, etc. [27]. In contrast, strong p16 overexpression was observed in cervical squamous intraepithelial lesions (SIL) and squamous cell carcinomas which are associated with high-risk HPV types [28], indicating that the suppressor function of p16 can be overcome in the presence of viral oncogenes.

The cdk inhibitors p21/WAF1 and p27/KIP1 are able to bind in a less selective manner to various cdk–cyclin complexes [9]. Both cell-cycle inhibitors are bound and inactivated by HPV E7 proteins in experimental systems [39].

P21 is induced by p53 after DNA damage which leads to a delayed cell cycle progression and leaves some time for DNA repair processes [4]. In addition, activation of various intracellular signaling pathways, i.e. MAPK cascade or TGFß, can lead to enhanced p21 expression.

P27 expression is mainly regulated post-translationally by protein degradation via ubiquitin–proteasome complexes. Strong p27 expression is found in most normal epithelial cells, and p27 down-regulation is frequently observed in several human tumor types [3, 31].

The role of p16, p21, and p27 in the etiology of glandular cervical carcinomas has not been well analyzed. In a study without HPV analysis, Lu et al. [18] found enhanced expression of these cell-cycle inhibitors in most cervical adenocarcinomas relative to normal endocervical glands. However, to enhance our understanding of the role of these proteins in cervical cancer, the papillomavirus infections which influence their expression or function should be considered. To compare the expression of p16, p21, and p27 with the HPV status in cervical adenocarcinomas, we performed immunohistochemistry in 60 tumors. All cases were analyzed for HPV 16 and 18 infection by PCR, and HPV 16 and 18 E6/E7 oncogene expression was studied by RNA-RNA in situ hybridization.

Materials and methods

Tissue samples

We studied paraffin-embedded tissue samples from 60 patients (mean age 47 years; range 27–89 years) with cervical adenocarcinomas selected from the files of the Department of Gynecopathology, University Clinics Hamburg, and the Division of Women's and Perinatal Pathology at Brigham and Women's Hospital (Boston, Mass.). All cases were re-classified by two pathologists independently according to the most recent (1994) WHO criteria, and histological subtyping was performed. The pathologists classified 45 tumors as FIGO stage I (IA: 7 cases, stage IB: 38 cases), 8 cases as stage II, and 5 carcinomas as stage III or IV. In 2 cases, staging data were not available. The histological subtypes of the tumors included in the study are shown in Table 2. All tissues had been routinely fixed in 4% buffered formalin and paraffin embedded.

Detection of HPV infection and HPV 16/18 E6/E7 oncogene expression

HPV detection was performed by PCR after DNA extraction from paraffin sections as described with general primers MY09/MY11 [19] and type-specific primers for HPV 16, HPV 18, HPV 6/11 [26], and HPV 31 [20]. In order to analyze HPV 16 and 18 E6/E7 expression in the tumors, in situ hybridization with ^{35}S-labeled RNA probes was performed, and the results were evaluated as described before [25, 26]. For statistical analysis, all HPV 16 and 18 E6/E7 RNA-positive cases were combined and compared with those tumors without detectable HPV 16/18 oncogene expression.

Immunohistochemistry

P16 IHC was performed on paraffin-embedded tissue sections after microwave pretreatment with the monoclonal antibody G175-405 (Pharmingen, Hamburg, Germany; 1:100) in an automated immunohistochemistry system (Ventana Medical Systems, Strasbourg, France). Bound primary antibodies were detected using an amplification kit and a detection kit (both Ventana Medical Systems). Expression of p21 and p27 was investigated manually with the monoclonal antibodies NCL-p27 (Novocastra; 1:50), and SXM30 (Pharmingen, Hamburg, Germany; 1:150) after microwave pretreatment of the tissue sections.

Immunoreactivity was evaluated by examination of staining intensity (0: no staining; 1: weak staining; 2–3: intermediate and strong staining intensity) and percentage of positive tumor cells (0: no positive cells; 1: <10%; 2: 11–50%; 3: 51–80%; 4: >80% positive tumor cells) by two independent persons. After multiplication of both values, the IHC results were graded from 0 (no reactivity in tumor cells) to 12 (more than 80% positive tumor cells with strong staining intensity in the majority of the cells). For p16, both nuclear and cytoplasmic immunoreactivity was evaluated, whereas for p21 and p27, only nuclear staining was observed.

Statistics

Correlations of the data were examined by Chi-square statistics and Fisher's exact test using the SPSS 10.0 software. *P*-values of 0.05 or less were considered statistically significant.

Results

Sixty cases of cervical adenocarcinoma were included in the study. By PCR, 17 tumors (28%) were HPV-negative, 22 carcinomas (37%) harbored HPV 18 DNA, 15 cases (25%) were positive for HPV 16, whereas in 6 (10%) tumors, HPV 16 and 18 DNA could be detected. HPV 6/11 and HPV 31 DNA were detected in none of the tumors. In addition, none of the carcinomas was general primer-positive, but negative for HPV 16 and HPV 18.

RNA-RNA in situ hybridization with ^{35}S-labeled probes from the E6/E7 region of the HPV 16 and HPV 18 genomes was performed in 53 tumors. These results have partly been described before [26]. In 20 cases (38%), no E6/E7 mRNA could be detected. There were 24 carcinomas (45%) that were positive for HPV 18 mRNA, and 9 cases harboring HPV 16 mRNA (17%). In 4 tumors infected with both HPV 16 and HPV 18 only HPV 18 transcripts (*n*=4) could be detected. HPV infection and E6/E7 oncogene expression results are summarized in Table 1, and an example of HPV 16 E6/E7 ISH is shown in Fig. 1A.

Table 1 Comparison of human papillomavirus (*HPV*) infection as detected by PCR and HPV 16 or HPV 18 E6/E7 expression as detected by RNA-RNA in situ hybridization (*ISH*)

	HPV-DNA (PCR)			
	Negative	HPV 16	HPV 18	HPV 16+18
n	(17)	(15)	(22)	(6)
E6/E7-RNA (ISH)				
Negative	12	3	3	2
HPV 16	–	9	–	–
HPV 18	2	–	18	4
ISH not done	3	3	1	–

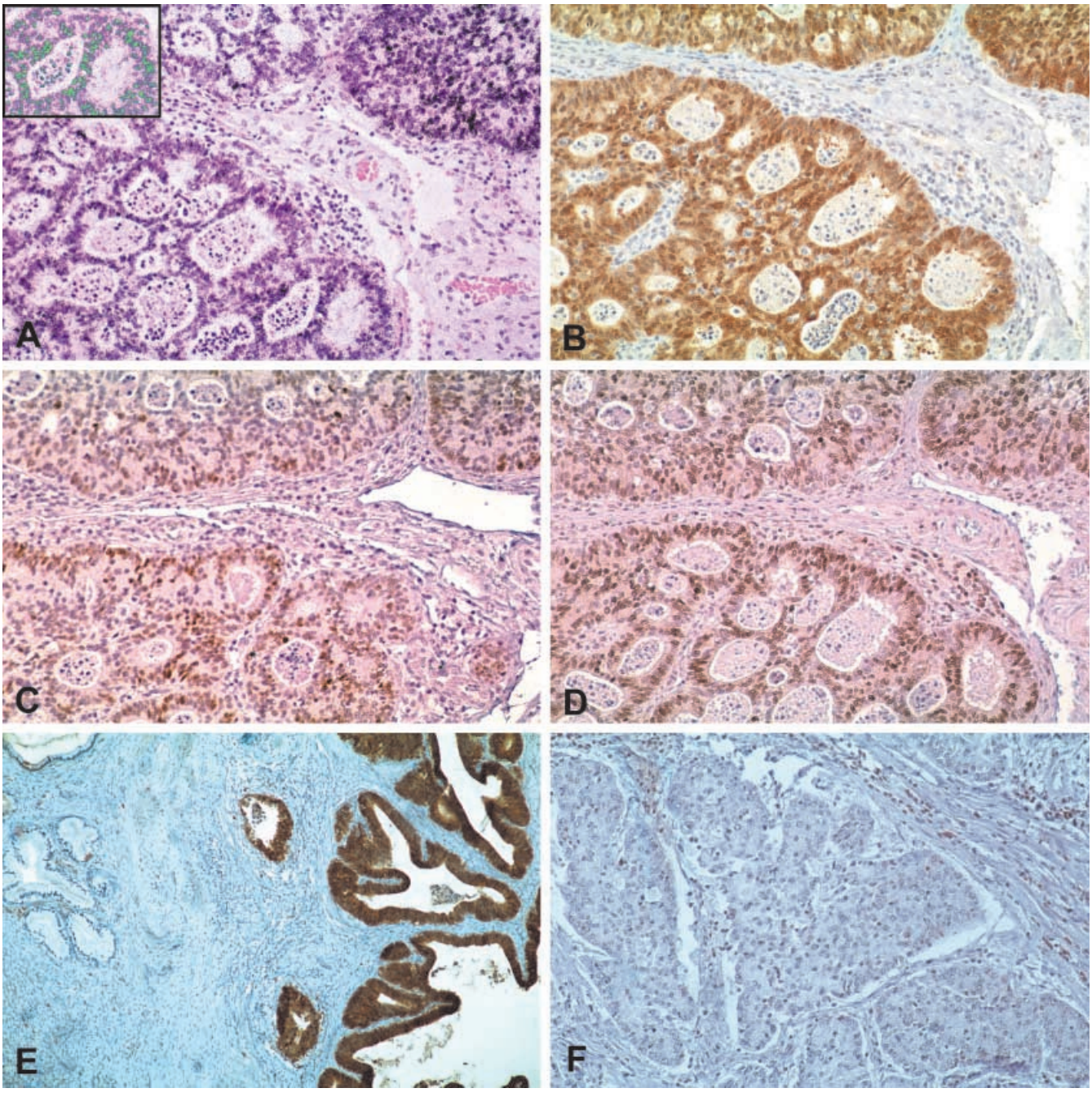

Fig. 1 **A** HPV E6/E7 expression and **B**, **E** p16, **C** p21, and **D**, **F** p27 immunostaining in cervical adenocarcinomas. **A**–**D** Well-differentiated adenocarcinoma. ×400 **A** HPV 16 E6/E7 oncogene expression shown by *black* grains over carcinoma cells. In the insert region, hybridization signals were labeled *green*. **B** Strong nuclear and cytoplasmic p16 expression in tumor cells, weak nuclear expression in single fibroblasts. **C** Moderate nuclear p21 expression in tumor cells. **D** Strong nuclear p27 expression in tumor cells and surrounding fibroblasts. **E** Well-differentiated cervical adenocarcinoma with strong nuclear and cytoplasmic p16 immunoreactivity in tumor cells. On the *left*, a normal endocervical gland without detectable p16 staining is surrounded by several weakly positive fibroblasts. ×200 **F** Adenosquamous cervical carcinoma showing very weak p27 expression in tumor cells and p27-positive nuclei in several fibroblasts. ×400

Evaluable p16 results were obtained in 58 cervical adenocarcinomas. In 50% of the cases (n=29), strong p16 immunoreactivity (IHC 8–12) in tumor cells was observed. In all p16-positive cases, both nuclear and cytoplasmic staining were present in varying proportions (Fig. 1B, E). Negative IHC results were obtained in only 8 cases (14%), among them mainly endometrioid and mucinous tumors (Table 2), whereas weak p16 staining was shown in 21 carcinomas (36%). Some carcinomas displayed strong heterogeneity with respect to staining intensity and the percentage of p16-positive tumor cells. Nuclear staining was observed in some stromal fibroblasts, whereas normal glandular or squamous epithelia were always p16 negative. In cases with positive p16 re-

Table 2 P16 expression in cervical adenocarcinomas: associations with HPV infection as detected by PCR, HPV 16/18 E6/E7 expression, and histological type

	n	p16 (IHC score)			
		0–2	3–7	8–12	
HPV-DNA (*n*=58)					
Negative	17	10	5	2	
HPV 16	13	1	4	8	
HPV 18	22	1	2	19	
HPV 16+18	6	1	5	–	*P*<0.001
E6/E7-mRNA (*n*=50)					
Negative	19	7	8	4	
Positive	31	2	6	23	*P*=0.001
Histological type					
Endometrioid	15	3	7	5	
Mucinous	21	8	3	10	
Clear cell	2	1	1	–	
Adenosquamous	9	1	5	3	
Serous-papillary	4	–	–	4	
Mixed differentiation	5	–	–	5	
Intestinal	1	–	–	1	
Undifferentiated	1	–	–	1	

Table 3 P21 and p27 expression in cervical adenocarcinomas: associations with HPV infection as detected by PCR, HPV 16/18 E6/E7 expression, and histological type

	n	p21 (IHC score)				p 27 (IHC score)		
		0	1–3	4–8		0–5	6–12	
HPV-DNA (*n*=58)								
Negative	17	8	6	3		8	9	
HPV 16	15	3	4	8		2	13	
HPV 18	22	9	7	6		4	18	
HPV 16+18	6	4	1	1	n.s.	3	3	n.s.
E6/E7-mRNA (*n*=52)								
Negative	20	11	7	2		10	10	
Positive	32	8	9	15	*P*=0.015	6	26	*P*=0.030
Histological type								
Endometrioid	16	5	3	8		2	14	
Mucinous	22	10	8	4		9	13	
Clear cell	2	2	–	–		1	1	
Adenosquamous	9	6	1	2		4	5	
Serous-papillary	4	–	3	1		–	4	
Mixed differentiation	5	1	2	2		–	5	
Intestinal	1	–	1	–		–	1	
Undifferentiated	1	–	–	1		1	–	

sults, normal glands and neoplastic cells were clearly distinguishable by their different p16 immunoreactivity (Fig. 1E).

P16 expression was significantly associated with both HPV 16/18 infection and HPV 16/18 oncogene expression (Table 2). Among the tumors with minimal or absent p16 staining, 10 of 13 were HPV negative according to PCR, and 7 of 9 had no detectable E6/E7 expression. In contrast, strong p16 expression was associated with HPV infection (mostly HPV 18) and HPV 16/18 E6/E7 oncogene expression. No correlations of p16 expression with age, stage, or histological subtype were found.

P21/WAF1 expression showed an exclusively nuclear pattern with 1–90% positive tumor cells in 60% (36/60) of the cases, whereas 24 tumors (40%) were p21 negative (Table 3, Fig. 1C). Interestingly, mucinous, clear-cell, and adenosquamous carcinomas were most often p21 negative, although the low numbers of cases in the histological subgroups do not allow statistical analysis. Normal glandular epithelia showed weak p21 staining in 10–30% of the cells. In squamous epithelia, moderate p21 immunoreactivity in the suprabasal epithelial layers was observed as described by others [38], and positive nuclei were only sporadically found in stromal cells.

For statistical analysis, p21-negative carcinomas, tumors with weak p21 expression (IHC score 1–3) and cases with higher p21 expression (IHC 4–12) were combined. No correlation of p21 expression with age, stage, histological subtype, or HPV infection as detected by PCR was observed. In contrast, strong p21 immunore-

activity was significantly associated with HPV 16 or 18 E6/E7 oncogene expression (P=0.015; Table 3).

Nuclear p27 expression was observed in all analyzed carcinomas with varying staining intensities and different proportions of positive tumor cells (Fig. 1D, F). Weak immunostaining was observed mainly in mucinous and adenosquamous carcinomas. Normal endocervical glandular epithelial cells and most stromal fibroblasts and endothelial cells were p27 positive. In squamous epithelia, negative or only weak p27 staining was found in basal cells and the first suprabasal cell layer, but strong p27 expression was detected in the outer epithelial layers as described [38].

For statistical analysis, carcinomas with p27 expression in less than 50% of tumor cells and an IHC score of <6 (n=17; 28%) were combined and compared with those with >50% positive tumor cells (n=43; 72%). No significant correlation of p27 expression with age, stage, histological subtype, or HPV DNA detection results was found (Table 3). However, in a similar way to p21, reduced p27 expression was significantly more often detected in carcinomas without detectable HPV oncogene expression (50%) than in HPV 16/18 E6/E7-positive tumors (19%).

Discussion

In contrast to cervical squamous cell carcinomas, only 60–85% of the cervical adenocarcinomas are associated with papillomavirus DNA [6, 21, 26], and the viral load is often very low in glandular lesions. The percentage of 72% HPV-positive cases detected in our study after PCR with general primers MY09/MY11 and primers for HPV 16, 18, 6/11, and 31 is lower than that of a recent study in which a highly sensitive short-fragment PCR was used, where 83% HPV-positive invasive carcinomas were found, among them 4% HPV45- and 1% HPV35-positive tumors [24]. These HPV types should have been detectable by general-primer PCR, but this method is not as sensitive as type-specific PCR protocols, resulting in smaller PCR products. Therefore, we cannot exclude the possibility that single carcinomas that are weakly positive for HPV 45, 35, 52, etc. may have escaped our approach, although HPV 45 is less prevalent in Europe than in North America [1].

The mechanisms of carcinogenesis in the HPV-negative cervical adenocarcinomas are still largely unknown. Cyclin-dependent kinase inhibitors (CDKIs) play an important part in the regulation of cell cycle progression and differentiation [9]. Inactivation of CDKIs is involved in the etiology of various malignant tumors, i.e. breast cancer, pancreatic carcinomas etc. [2, 3, 4, 5, 17, 27]. In cervical carcinoma cells, cell-cycle regulation is disrupted by viral oncoproteins in various ways, i.e. binding and inactivation of p53 by HPV E6 proteins and binding of Rb, p21, and p27 by E7 oncoproteins [32, 39, 40]. If this is a necessary condition for malignant transformation in HPV-associated tumors, inactivation of cell-cycle-regulatory proteins by HPV-independent mechanisms may be involved in the etiology of the HPV-negative group of cervical adenocarcinomas. Therefore, the aim of our present study was to compare the expression of CDKIs with the HPV status in cervical adenocarcinomas, and to ascertain whether CDKIs p16, p21, and p27 might be involved in carcinogenesis in HPV-negative cervical tumors.

Functional inactivation of p16/MTS1/Ink4a by homozygous deletion, somatic mutation, or promoter hypermethylation is involved in the etiology of many human tumors [27]. In cervical carcinomas (mostly SCC), p16 mutations have not been found [13, 36] and homozygous deletions were absent [13] or detected in only 5% of the carcinomas [36]. Instead, cervical squamous intraepithelial lesions (SIL) and carcinomas which are associated with high-risk HPV types show a striking overexpression of p16 [28]. A possible explanation of this phenomenon might be the functional inactivation of the retinoblastoma protein Rb which is able to act as a repressor of p16 transcription [27]. The release of p16 expression from Rb repression in HPV-infected cervical dysplastic cells might lead to strong p16 overexpression, but obviously displays no cell-cycle-inhibitory effect because Rb has already been blocked by HPV E7 oncoproteins. Therefore, additional inactivation of cdk4 by p16, which would normally keep the Rb protein in its active, hypophosphorylated form, probably has no influence on cell proliferation.

Detectable p16 expression has been described in 58% of cervical adenocarcinomas in a prior study [18]. The higher percentage of p16-positive tumors found by us is probably due to the highly sensitive immunohistochemistry protocol used in this study. P16 expression and HPV status in cervical adenocarcinomas have only been analyzed within the scope of larger studies on cervical carcinomas. In an immunohistochemical analysis of 44 cervical carcinomas, among them 5 adenocarcinomas, Kim et al. [14] found a positive association of p16 staining with HPV infection, but significantly weaker immunoreactivity in adenocarcinomas compared with squamous tumors. Similarly, Sano et al. studied 54 cervical carcinomas including 9 adenocarcinomas and 6 adenosquamous tumors and found negative p16 and HPV results in 4 adenocarcinomas and 1 adenosquamous carcinoma, but in none of the squamous cell carcinomas [28].

In our larger cohort of cervical adenocarcinomas, we found strong positive correlations of p16 expression and HPV infection status or HPV 16/18 E6/E7 expression, respectively. Interestingly, p16 expression was significantly associated with both HPV infection and HPV oncogene expression, even though in 20% of the HPV DNA-positive carcinomas, no E6/E7 mRNA was detected by in situ hybridization. This indicates that, in addition to the disruption of Rb control by E7 oncoproteins, other as yet unknown mechanisms might lead to p16 upregulation after HPV infection. In a model system with HPV-immortalized or transformed ectocervical and endocervical epithelial cells, Nakao et al. [23] found a

dramatic enhancement of p16 mRNA and protein expression after immortalization by HPV 16 or HPV 18. This overexpression was not further increased after malignant transformation of the cells. In in situ hybridization studies, strong E6/E7 expression was only observed in severe dysplasia or cervical carcinomas, but not in weak dysplasia [7], whereas p16 expression has already been shown in early stages of oncogenic progression [23]. Further studies will be necessary to elucidate the mechanism of p16 up-regulation after infection of cervical cells with high-risk HPV types.

In contrast to the tumors with strong p16 expression, which are associated with HPV infection in 93% of cases, 31% of the carcinomas showing moderate p16 immunostaining (IHC score 3–7) were negative for HPV DNA. In addition, moderate or strong immunostaining was observed in 41% of the HPV DNA-negative carcinomas. These results indicate that HPV-independent mechanisms can lead to p16 overexpression in these tumors, i.e. Rb inactivation or accumulation of the relatively stable p16 proteins in strongly proliferating cells.

In HPV-negative cervical carcinomas, the control of the G1 restriction point by the Rb protein is not influenced by viral oncoproteins. Therefore, weak or absent p16 expression in 10 out of 17 tumors may contribute to enhanced cell cycle progression in these cases.

Expression of p21/Waf1 was significantly associated with HPV oncogene expression but not with HPV infection, indicating that E6/E7 proteins may be involved in the regulation of this cell-cycle inhibitor. In cervical squamous carcinomas, Skomedal et al. [29] found p21 overexpression in 96% of the tumors, and in a study conducted in Sweden and Ireland on 40 cervical adenocarcinomas [30], p21 expression was detected in 85% of the tumors, in comparison with 60% p21 immunostaining found by us. These discrepancies might be explained by different IHC protocols or differences in evaluation of IHC results.

Mutations of the p21 gene are rare in human carcinomas and have not been described in cervical cancer. P21 protein expression is activated by the tumor suppressor protein p53, which is bound and blocked by HPV 16/18 E6 oncoproteins. Therefore, reduced p21 expression might be expected in tumors with E6/E7 expression. On the other hand, the HPV E7 oncoprotein leads to a stabilization and increased protein levels of p21/WAF1 in human fibroblasts or keratinocytes [12, 33]. The positive correlation of p21 and HPV oncogene expression found in our study is in accordance with this p53-independent p21 regulation. Zehbe et al. [38] reported that the cell-cycle-inhibitory function of p21 can be overcome in HPV-positive cervical carcinoma cells, probably through direct interaction of p21 with E7.

In contrast to the tumors with HPV 16/18 oncogene expression, which were p21-positive in 75% of cases, 55% of the HPV E6/E7-negative tumors had no detectable p21 protein. A possible reason might be p53 mutations, which are reported in 8% [22] to 32% [11] of cervical adenocarcinomas, mainly in HPV-negative tumors. Alternatively, p53-independent down-regulation of p21 may contribute to proliferation in these tumors. Werness et al. [35] reported that p21 expression in cervical carcinomas is influenced by various transactivators, such as TGFß, p73 and c/EBPß [8].

Similar to p21, p27/Kip1 expression was significantly associated with HPV E6/E7 expression, but not with HPV infection. P27 down-regulation was observed in 50% of the tumors without E6/E7 expression, but in only 19% of the E6/E7-positive carcinomas. The molecular mechanisms leading to these differences are still unknown. It was previously shown that HPV oncogenes can overrun the cell-cycle-inhibitory effects of p27, probably by direct interaction of E7 and p27 proteins [38]. In HPV-negative adenocarcinomas, down-regulation of p27 protein levels may be involved in enhanced cell-cycle progression and carcinogenesis.

In contrast to our results, Kim et al. [15] found lower p27 expression in HPV-positive than in HPV-negative tumors in a study on 45 cervical carcinomas including 4 adenocarcinomas. Yet, in this study HPV was only detected by HPV 16 and HPV 18 PCR, which resulted in 31% HPV-negative cases. Given the fact that nearly 100% of cervical SCC are associated with HPV according to various studies [34], the supposed HPV-negative cases probably harbored additional HPV types, such as HPV 31, 33, 35, 45, and 51, and the differences in p27 expression in this study may reflect variations in tumors with different HPV types.

In conclusion, our study has demonstrated high levels of the cdk inhibitors p16, p21, and p27 in cervical adenocarcinomas with detectable HPV E6/E7 protein expression. Obviously, the anti-proliferative effect of these proteins can be overcome by the viral oncogenes in these lesions. Interestingly, negative immunostaining or only low protein levels of p16, p21, and p27 were detected in most HPV E6/E7-negative carcinomas suggesting that down-regulation of these cell-cycle inhibitors may be involved in the etiology of this group of cervical adenocarcinomas.

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References

1. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV (1995) Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J Natl Cancer Inst 87:796–802
2. Caffo O, Doglioni C, Veronese S, Bonzanini M, Marchetti A, Buttitta F, Fina P, Leek R, Morelli L, Dalla Palma P, Harris AL, Barbareschi M (1996) Prognostic value of $p21^{WAF1p}$ and p53 expression in breast carcinoma: an immunohistochemical study in 261 patients with long-term follow-up. Clin Cancer Res 2:1591–1599

3. Catzavelos C, Bhattacharya N, Ung YC, Wilson JA, Roncari L, Sandhu C, Shaw P, Yeger H, Morava-Protzner I, Kapusta L, Franssen E, Pritchard KI, Slingerland JM (1997) Decreased levels of cell-cycle inhibitor p27kip1 protein: prognostic implications in primary breast cancer. Nat Med 3:227–230
4. Chen YQ, Cipriano SC, Arenkiel JM, Miller FR (1995) Tumor suppression by $p21^{WAF1}$. Cancer Research 55:4536–4539
5. Clurman BE, Roberts JM (1996) Cell cycle and cancer. J Natl Cancer Inst 87:1499–1501
6. Duggan MA, McGregor SE, Benoit JL, Inoue M, Nation JG, Stuart GCE (1995) The human papillomavirus status of invasive cervical carcinoma: a clinicopathological and outcome analysis. Hum Pathol 26:319–325
7. Dürst M, Glitz D, Schneider A, zur Hausen H (1992) Human papillomavirus type 16 (HPV 16) gene expression and DNA replication in cervical neoplasia: analysis by in situ hybridization. Virology 189:132–140
8. Elbendary A, Berchuck A, Davis P, Havrilevsky L, Bast RC, Iglehart D, Marks JR (1994) Transforming growth factor β1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells. Cell Growth Differ 5:1301–1307
9. Elledge SJ, Winston J, Harper JW (1996) A question of balance: the role of cyclin-kinase inhibitors in development and tumorigenesis. Trends Cell Biol 6:388–392
10. Hopkins MP, Morley GW (1991) A comparison of adenocarcinoma and squamous cell carcinoma of the cervix. Obstet Gynecol 77:912–917
11. Jiko K, Tsuda H, Sato S, Hirohashi S (1994) Pathogenetic significance of p53 and c-Ki-ras gene mutations and human papillomavirus DNA integration in adenocarcinoma of the uterine cervix and uterine isthmus. Int J Cancer 59:601–606
12. Jones DL, Thompson DA, Suh-Burgmann E, Grace M, Munger K (1999) Expression of the HPV E7 oncoprotein mimics but does not evoke a p53-dependent cellular DNA damage response pathway. Virology 258:406–414
13. Kim JW, Namkoong SE, Ryu SW, Kim HS, Shin JW, Lee JM, Kim DH, Kim IK (1998) Absence of $p15^{INK4B}$ and $p16^{INK4A}$ gene alterations in primary cervical carcinoma tissues and cell lines with human papillomavirus infection. Gynecol Oncol 70:75–79
14. Kim YT, Cho NH, Park SW, Kim JW (1998) Underexpression of cyclin-dependent kinase (CDK) inhibitors in cervical carcinoma. Gynecol Oncol 71:38–45
15. Kim YT, Choi KE, Cho NH, Ko JH, Yang WI, Kim JW, Lee SH (2000) Expression of cyclin E and p27(KIP1) in cervical carcinoma. Cancer Lett 153:41–50
16. Kudo R (1992) Cervical adenocarcinoma. In: Sasano N (ed) Current topics in pathology, vol 85. Springer, Berlin Heidelberg New York, pp 81–111
17. Lloyd RV, Jin L, Qian X, Kulig E (1997) Aberrant p27 kip1 expression in endocrine and other tumors. Am J Pathol 150: 401–407
18. Lu X, Toki T, Konishi I, Nikaido T, Fujii S (1998) Expression of $p21^{WAF1/CIP1}$ in adenocarcinoma of the uterine cervix. Cancer 82:2409–2417
19. Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM (1989) Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells 7:209–213
20. Milde-Langosch K, Becker G, Löning T (1991) Human papillomavirus and c-myc/c-erbB2 in uterine and vulvar lesions. Virchows Arch [A] 419:479–485
21. Milde-Langosch K, Schreiber C, Becker G, Löning T, Stegner H-E (1993) Human papillomavirus detection in cervical adenocarcinomas by polymerase chain reaction. Hum Pathol 24:590–594
22. Milde-Langosch K, Albrecht K, Joram S, Schlechte H, Giessing M, Löning T (1995) Presence and persistence of HPV infection and p53 mutation in cancer of the cervix uteri and the vulva. Int J Cancer 63:639–645
23. Nakao Y, Yang X, Yokoyama M, Ferenczy A, Tang S-C, Pater MM, Pater A (1997) Induction of p16 during immortalization by HPV 16 and 18 and not during malignant transformation. Br J Cancer 75:1410–1416
24. Pirog EC, Kleter B, Olgac S, Bobkiewicz P, Lindeman J, Quint WGV, Richart RM, Isacson C (2000) Prevalence of human papillomavirus DNA in different histological subtypes of cervical adenocarcinoma. Am J Pathol157:1055–1062
25. Riethdorf L, Riethdorf S, Gützlaff K, Prall F, Löning T (1996) Differential expression of the monocyte chemoattractant protein-1 in human papillomavirus-16-infected squamous intraepithelial lesions and squamous cell carcinomas of the cervix uteri. Am J Pathol 149:1469–1475
26. Riethdorf S, Riethdorf L, Milde-Langosch K, Park TW, Löning T (2000) Differences in HPV 16 and HPV 18 E6/E7 oncogene expression between in situ and invasive adenocarcinoma of the cervix uteri. Virchows Arch 437: 491-500
27. Ruas M, Peters G (1998) The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1378:F115-F177
28. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajiama T (1998) Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. Am J Pathol 153:1741–1748
29. Skomedal H, Kristensen GB, Lie AK, Holm R (1999) Aberrant expression of the cell cycle associated proteins TP53, MDM2, p21, p27, cdk4, cyclin D1, RB, and EGFR in cervical carcinoma. Gynecol Oncol 73:223–228
30. Skyldberg BM, Murray E, Lambkin H, Kelehan P, Auer GU (1999) Adenocarcinoma of the uterine cervix in Ireland and Sweden: human papillomavirus infection and biologic alterations. Mod Pathol 12:675–682
31. Steeg PS, Abrams JS (1997) Cancer prognosis: past, present and p27. Nat Med 3:152–154
32. Stoler MH (2000) Human papillomavirus and cervical neoplasia: a model for carcinogenesis. Int J Gynecol Pathol 19: 16–28
33. Stoppler H, Stoppler MC, Johnson E, Simbulan-Rosenthal CM, Smulson ME, Iyer S, Rosenthal DS, Schlegel R (1998) The E7 protein of human papillomavirus type 16 sensitizes primary human keratinocytes to apoptosis. Oncogene 17: 1207–1214
34. Walboomers JMM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJF, Peto J, Meijer CJLM, Munoz N (1999) Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189: 12–19
35. Werness BA, Wang H-Q, Chance J, Goldstein DJ (1997) P53-independent expression of $p21^{waf1/cip1}$ in preinvasive and invasive squamous neoplasms of the uterine cervix. Mod Pathol 10:578–584
36. Wong YF, Chung TKH, Cheung TH, Nobori T, Yim SF, Lai KWH, Yu AL, Diccianni MB, Li TZ, Chang AMZ (1997) $P16^{INK4}$ and p15INK4B alterations in primary gynecologic malignancies. Gynecol Oncol 65:319–324
37. Xiong Y (1996) Why are there so many CDK inhibitors? Biochim Biophys Acta 1288:O1-O5
38. Zehbe I, Rätsch A, Alunni-Fabbroni M, Burzlaff A, Bakos E, Dürst M, Wilander E, Tommasino M (1999) Overriding of cyclin-dependent kinase inhibitors by high and low risk human papillomavirus types: evidence for an in vivo role in cervical lesions. Oncogene 18:2201–2211
39. Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, Jansen-Dürr P (1996) Inactivation of the cdk inhibitor p27kip1 by the human papillomavirus type 16 E7 oncoprotein. Oncogene 13:2323–2330
40. Zur Hausen H (1994) Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. Curr Top Microbiol Immunol 186:131–156