

p16, p14, p53, and Cyclin D1 Expression and HPV Analysis in Small Cell Carcinomas of the Uterine Cervix

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Summary: Small cell carcinomas (SmCCs) of the uterine cervix are rare tumors. The knowledge regarding protein expression of several checkpoint candidates of cell cycle regulation is limited. Surgically treated SmCCs were selected from our files for immunohistochemical staining (neuroendocrine markers, p53, p16, p14, and cyclin D1). Polymerase chain reaction analysis, using general primers, was performed for human papillomavirus analysis. Nine of 677 tumors (1.3%) were classified as SmCCs after Grimelius staining (8/9 tumors positive) and immunohistochemical reaction against neurone-specific enolase, chromogranin A, synaptophysin (7/9 positive tumors), and CD 56 (8/9 positive tumors). All specimens were positive for at least two of the above. Two SmCCs were p53 positive and one case was p14 positive. Cyclin D1 staining was completely negative. All cases showed strong nuclear and/or cytoplasmic p16-immunostaining. Seven tumors represented human papillomavirus positivity for high-risk types. Four patients died of the tumor after a median time of 36.7 months (range, 15–56 months), representing a 5-year survival rate of 56%. The results suggest that p16 is up-regulated or accumulated in the SmCCs of the uterine cervix, probably caused by infection with human papillomavirus. p14 inactivation is of high prevalence in SmCCs and detection rate of p53 is similar to other histologic types of cervical carcinomas. **Key Words:** Small cell carcinoma—Cervix uteri—p16—p14—Human papillomavirus infection—Cyclin D1.

Small cell carcinomas (SmCCs) comprise a rare but aggressive subset of uterine cervical neoplasms with high rate of recurrence and poor overall survival (1–5).

In concordance with squamous cell and adenocarcinoma of the uterine cervix, cervical SmCCs are associated with high-risk human papillomavirus (HPV) (2,3,6,7).

It is well accepted that the oncoproteins encoded by E6 and E7 of high-risk HPV have the ability to bind host cell regulatory proteins, thus causing consecutive functional

alterations. E6 is known to bind specifically to the wild type p53 protein, an event that leads to the functional inactivation and rapid degradation through the ubiquitin pathway (6–8). Because of inactivation, the p53 protein is stored in affected cells, allowing immunohistochemical detection in cervical carcinoma (9).

Inactivation of the retinoblastoma (Rb) tumor-suppressor gene is realized by the binding of the E7 gene product (10). As a result of the loss of the tumor Rb suppressor function, a decrease in the p21 protein level and liberation of the transcriptional factor E2F-1 from the E2F-Rb complex may occur, allowing the activation of cyclin-dependent kinase (CDK) and transcriptional activation of target promoters (10). In several tumors, Rb inactivation is accompanied by cyclin D1 overexpression (11). It has been reported that aberrant expression of cyclin D1 is low in non-SmCCs of the uterine cervix (12,13), but, when it occurs, it is associated with poor prognostic outcome (14).

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The p16 protein, a CDKN2A gene product, is a tumor suppressor protein that inhibits CDK-4 and -6, which are capable of regulating the G1 checkpoint in the cell cycle (6,11,15). These CDKs phosphorylate pRb, which results in a conformational change and release of E2F from the pRb. Thus, functional inactivation of p16 or pRb allows the cells to enter the S phase after only a short stop at the G1 checkpoint. It has been shown that immunohistochemical expression of the p16 protein is associated with the presence of oncogenic HPV infections in preneoplastic lesions of the uterine cervix (12,13,16,17).

Alterations of INK4a-ARF locus at 9p21, encoding the growth suppressive genes *p14^{ARF}* and *p16^{INK4a}*, is one of the major hotspots for genetic alterations in a variety of human cancers (18), representing a convergence of the two major pathways of tumorigenesis: the Rb and p53 pathway (14). *p14^{ARF}* leads to the localization and sequestration of MDM2 in the nucleolar compartment, thereby blocking MDM2-mediated inhibition of p53, inducing G1 and G2 arrest (19,20).

In contrast to squamous cell and adenocarcinomas of the uterine cervix (9,13,14,17), there is limited experience regarding the expression of these proteins in SmCCs.

Therefore, we studied the immunohistochemical expression of the p53, p14, and p16 proteins and cyclin D1 in cervical SmCCs in correlation to HPV analysis.

MATERIALS AND METHODS

Tumor Specimens

A total of 677 surgically treated carcinomas of the uterine cervix, histopathologically staged from pT1b1 to pT2b, were available from the "Wertheim-Archive" of the Department of Pathology, Division of Gynecologic Pathology (21). Eleven cases showed small cell differentiation on initial staining. These cases were selected for clinical and immunohistochemical analysis.

The follow-up information were obtained from the clinical files.

HPV Analysis

DNA was extracted from paraffin blocks using the method as previously described (22). Briefly, formalin-fixed, paraffin-embedded tissue sections were digested overnight with 2 mg/mL proteinase K (Biometra, Göttingen, Germany) at 55°C. After digestion, extraction with phenol (chloroform/isoamyl alcohol [25:24:1]) in two steps and afterward with chloroform/isoamyl alcohol (24:1) was performed. The liquid phase was precipitated with 2 volumes of 100% ethanol and 1/10 volume of 3 mol/L sodium acetate. Finally, the DNA

pellet was dissolved in Tris/EDTA buffer and measured with a spectrophotometer.

All samples were subjected to polymerase chain reaction amplification using the general consensus primers GP5+ and GP6+ using the primer sequences and amplicon sizes as described by Jacobs et al. (23), using a protocol with minor modifications as published recently (24).

Immunohistochemical Analysis

After reevaluation of all 677 cases, tumors with small cell appearance on H&E-stained slides were selected for Grimelius staining and immunohistochemical analysis.

To determine the neuroendocrine differentiation, immunohistochemical staining included synaptophysin (catalog no. M0776, 1:50; DakoCytomation, Glostrup, Denmark), chromogranin (catalog no. MU126-UC, 1:150; BioGenex, San Ramon, CA), S100 (catalog no. MU058-UC, 1:250; BioGenex), neuron-specific enolase (catalog no. M0873, 1:300; DakoCytomation), CD99 (catalog no. M3601, 1:50; DakoCytomation), and CD 56 (catalog no. CD56-186, prediluted; Ventana Medical, Illkirch, France).

The p53 staining analysis was performed as previously published (9). Using a monoclonal antibody (DO-7; catalog no. M7001, 1:50; DakoCytomation), cases were stated as positive when a minimum of 10% of the nuclei showed positive nuclear staining, regardless of staining intensity.

For p16 and p14 immunostaining, the following antibodies were used: p16 (p16INK4a research kit; catalog no. OA 315, 1:100; DakoCytomation) and p14 (polyclonal; rabbit, catalog no. ZF 14, 1:100; Zymed Laboratories, South San Francisco, CA). The immunohistochemical analysis for p16 and p14 was performed as described recently (25).

Immunostaining for cyclin D1 was performed using a monoclonal antibody (catalog no. AM29, 1:100; Zymed Laboratories). According to reported analysis in cervical carcinoma (14), cases were stated as positive when a minimum of 10% of the nuclei showed positive nuclear staining, regardless of staining intensity.

Sections known to stain positively were included in each batch, and negative controls were also performed by replacing the primary antibody with mouse or goat ascites fluid (Sigma-Aldrich Biochemicals, St. Louis, MO).

RESULTS

Of the 677 cases in which the H&E-stained slides were reevaluated histologically, 78.9% were squamous cell

and 5.6% adenocarcinoma and 11 tumors showed small-cell histology; 14.2% of the carcinomas represented tumors of other histologic type (e.g., adenoid basal cell carcinoma or unclassified carcinomas).

Two cases that were initially grouped into SmCCs were excluded from the study because of negative results with Grimelius staining or neuroendocrine markers. Therefore, 9 of 677 carcinomas of the uterine cervix (1.3%) represented small cell differentiation (Fig. 1).

Eight of nine tumors showed positive Grimelius staining, seven of nine represented positive staining results with antibodies against neurone specific enolase, chromogranin A, or synaptophysin, and eight of nine tumors were positive for CD 56. Staining results for CD99 were completely negative.

Almost all cervical carcinomas were positive for HPV analysis (Table 1). Only one tumor (Table 1, case 6) was negative, and one tumor represented low-risk HPV infection (case 1).

Two cases represented positive nuclear immunostaining for p53 of about 15 and 18% of the cells, respectively.

All tumors showed strong intracytoplasmatic and nuclear immunoreactivity against p16 within the small tumor cells (Fig. 2).

Only one case represented positive nuclear staining for p14. Staining against cyclin D1 was completely negative in all cases.

One patient was lost during follow-up. Four of the remaining eight patients died of the disease after a mean time of 36.7 months (range, 15–56 months) after surgery (Table 1), representing a 5-year survival rate of 56%. On autopsy, distant metastases were found at the lungs, the liver, brain, bones, and mesenterial and para-aortic lymph nodes.

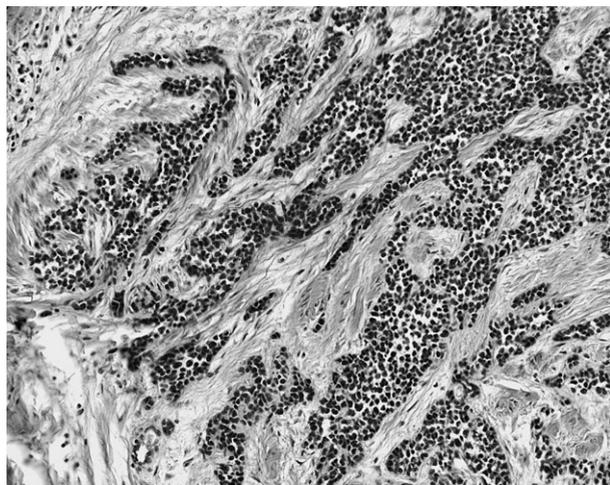


FIG. 1. Well-defined trabecular pattern in an SmCC of the uterine cervix (H&E staining, $\times 105$).

DISCUSSION

Albores-Saavedra et al. (26) first reported the occurrence of SmCCs of the uterine cervix. The frequency of these tumors range between 0.5 and 5% (5,27). This is in accordance with our results of 1.3% (9/677 cases).

SmCC is a highly aggressive tumor with early recurrence, irrespective of initial treatment (1,5), and a very low 5-year survival rate of 14 to 29% (2,3,5). In our study, 4 patients died of the disease after a median time of 36.5 months (range, 15–56 months). In concordance with the literature (1,28–30), the aggressive course of the disease is characterized by the development of widespread hematogenous metastases. Therefore, each case that is suspicious for small cell differentiation on H&E staining should further be examined immunohistochemically, using a panel of neuroendocrine markers (31).

About two thirds of non-SmCCs of the cervix showed p53 expression, but only a small subset of these tumors represent mutations of the *p53* gene (9,32). p53 protein expression has been reported in 35.5% of SmCCs (range, 21.4–50%) (32,33), which is in concordance with our results. The results regarding a mutation of the *p53* gene in SmCCs of the uterine cervix is contrary in the literature (32). However, further studies using microdissection are necessary.

The association of HPV infection and the development of cervical carcinoma is well known and ranges between 53 and 85% (2,3,7,34). Except two cases in our study, all tumors represented HPV infection with high-risk types (Table 1).

Recent reports mentioned that p16, a tumor suppressor protein, is upregulated by E7-encoded genes of high-risk HPV in cervical carcinomas and precancerous lesions (16,17). In 2 previous reports in the literature, SmCCs of the uterine cervix were positive for p16 in 100% and 91%, respectively (7,34). As in squamous cell and adenocarcinomas, p16 was expressed in all cases of our study. This suggests that p16 is up-regulated or accumulated in the SmCCs of the uterine cervix, caused by infection of high-risk HPV.

In contrast to other carcinomas, mutational inactivation of the *p16* gene seems to be a rare event in cervical cancer (32,35). However, Wistuba et al. (6) reported that the allelic loss of 9p21, encoding for the p16 protein, was the second most frequent deletion (43%) in neuroendocrine tumors of the uterine cervix. According to our results, p16 is present in all tumor cells, indicating an unmethylated, undeleted, and unmutated genetic status.

Brooks et al. (36) reported that p14^{ARF} mRNA was clearly elevated in CIN III and squamous cell carcinomas relative to matched normal cervical epithelium. There

TABLE 1. Age, stage distribution, follow-up information, and results of immunostaining and HPV analysis in SmCC of the uterine cervix

No.	Age	Tumor stage	p14	P16	p53	Cyclin D1	HPV genotypes	Follow-up
1	57	pT1b1, pN0	Neg.	100%	Neg.	Neg.	HPV 3	DOD, 4.2 years
2	57	pT1b1, pN0	Neg.	100%	Neg.	Neg.	HPV 16	NED, 4.2 years
3	58	pT2a, pN0	25%	100%	Neg.	Neg.	HPV 16	DOD, 4.8 years
4	25	pT1b1, pN0	Neg.	100%	Neg.	Neg..	HPV 18	NED, 12.6 years
5	49	pT1b1, pN1	Neg.	100%	18%	Neg.	HPV 90	DOD, 1.3 years
6	61	pT2b, pN0	Neg.	100%	Neg.	Neg.	Negative	NED, 8.5 years
7	38	pT1b1, pN0	Neg.	100%	15%	Neg..	HPV 16	NED, 9.7 years
8	24	pT1b1, pN0	Neg.	100%	Neg.	Neg.	HPV 68	DOD, 2.2 years
9	57	pT1b1, pN0	Neg.	100%	Neg.	Neg.	HPV 16	Lost

DOD, dead of disease; NED, no evidence of disease.

was simultaneous deregulation of both p73 and p14^{ARF} in the majority of cases with corresponding immunohistochemical overexpression of p73 and p14, respectively.

Data for p14, however, at least in SmCCs, are lacking in the literature. Whereas p16 was unaffected, we were able to detect p14 immunoreactivity only in one of our cases. Our results indicate that p14 inactivation is of high prevalence in SmCCs. In neuroendocrine tumors of the gastrointestinal tract, the genetic locus of p14, 9p21 is altered in a high percentage of cases (37). Further studies are therefore mandatory to look for different inactivation pathways of p14 in SmCCs.

It has been reported that cyclin D1 is overexpressed only in a minority of cervical carcinomas (3%) (13) and that cyclin D1 overexpression is associated with poorly differentiated tumors (38). These results are in concordance with our negative staining results in SmCCs of the uterine cervix and indicate that cyclin D1 might play a role in tumor progression rather than tumorigenesis of non-SmCCs of the cervix uteri but not in SmCCs (12).

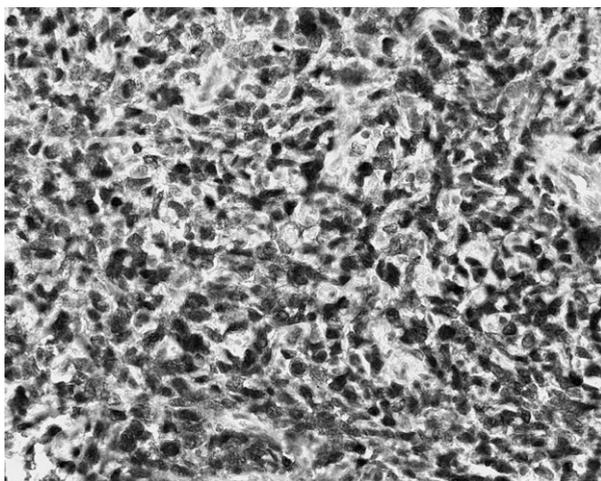


FIG. 2. SmCC of the uterine cervix with strong intracytoplasmic and intranuclear staining with p16 (magnification, $\times 215$).

Both *p53* and *p16* genes are checkpoint candidates regulating cell cycle. Earlier studies have shown that fibroblasts expressing the HPV oncoproteins E6 and E7 contain high levels of p16 (39). Interestingly, these cells continue to proliferate, suggesting that HPV oncoproteins could override the growth arrest function of the *p16* as well as the *p53* gene (39). In contrast to cell lines of different nongynecologic tumors, adenovirally transferred p16^{INK4a} and p53 induced no apoptotic tumor cell death in the Rb-deficient cell line C33A, derived from cervical carcinoma (40). This suggests that a complex network of coregulation of different tumor-suppressor genes exists in cervical carcinoma, which is altered by HPV infection.

Available data are consistent with the fact that SmCCs of the uterine cervix show similar alterations of the *p16* and *p53* genes as non-SmCCs, caused by the infection with high-risk HPV.

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