Loss of Nuclear p16 Protein Expression Is Not Associated with Promoter Methylation but Defines a Subgroup of Aggressive Endometrial Carcinomas with Poor Prognosis[1]

Helga B. Salvesen,[2] Soma Das, and Lars A. Akslen

Department of Pathology, The Gade Institute [H. B. S., L. A. A.] and Department of Gynecology and Obstetrics [H. B. S.], Haukeland University Hospital, N-5021 Bergen, Norway, and Department of Human Genetics, The University of Chicago, Chicago, Illinois 60637 [H. B. S., S. D.]

ABSTRACT

The *p16^{INK4aa}* *(CDKN2)* tumor suppressor gene is altered in several tumor types, but the frequency and mechanism of inactivation are largely unknown for endometrial carcinomas. We therefore wanted to assess the pattern and prognostic impact of p16 protein expression and promoter region methylation in a population-based series of 316 endometrial carcinoma patients with long-term and complete follow-up. Nuclear staining of p16 protein was related to clinicopathological variables, tumor markers, patient survival, and the presence of promoter region methylation. Absent/minimal nuclear staining for p16 protein was found in 14% of the tumors. Methylation of the *p16* promoter region was found in only one tumor (0.7%) in a subset of 138 cases studied. This tumor lacked nuclear p16 protein expression as well. Loss of nuclear p16 staining was significantly associated with increased age, high FIGO (International Federation of Gynecology and Obstetrics) stage, serous papillary or clear cell histological types, high histological grade, aneuploidy, low estradiol and progesterone receptor concentrations, high expression of Ki-67, high intratumor microvessel density, and strong nuclear p53 protein expression. The 5-year survival was 47% for patients with absent/minimal nuclear p16 expression ($n = 39$) compared with 81% for patients with moderate/high nuclear p16 expression ($n = 247$; $P < 0.0001$). In Cox proportional hazards regression analysis, nuclear p16 expression showed an independent prognostic impact in addition to FIGO stage, age, Ki-67 expression, and microvessel density, with an adjusted hazard ratio of 2.9 (95% confidence interval, 1.3–6.5). The other variables lost their prognostic impact when nuclear p16 staining was added to the Cox model. In conclusion, loss of nuclear p16 protein expression was associated with aggressive endometrial carcinomas and high proliferative activity (Ki-67) and was found to represent a strong and independent prognostic indicator. Methylation of the promoter region seems to be an uncommon mechanism of *p16* inactivation in endometrial carcinoma.

INTRODUCTION

Alterations of the *p16^{INK4a}* *(CDKN2) g*ene, located within the commonly deleted chromosomal region 9p21, are probably involved in tumor development in several organs (1, 2). The p16 protein encoded by the *p16* gene has been identified as a tumor suppressor. It binds specifically to the cyclin-dependent kinase CDK4, inhibiting the catalytic activity of the CDK4-cyclin D complex, and thereby acts as a negative cell cycle regulator (3). A large body of emerging data has demonstrated the involvement of p16 in various tumor types, and the predominant mode of inactivation appears to be by homozygous deletions (1, 4, 5) and methylation (6, 7), whereas mutations of this gene appear to be less common (2, 8). As also evidenced by lack of p16 protein expression, *p16* inactivation has been reported for a wide range of tumors (9–12).

In endometrial carcinomas, *p16* alterations seem to be rare, although a few studies have been published. None of these studies, however, has focused on prognostic relevance of *p16* alterations. Wong *et al.* (13) found homozygous deletions in only 1 of 41 tumors (2%). In the recent report by Nakashima *et al.* (14), homozygous deletions were found in 1 of 38 tumors (3%), and *p16* gene mutations were found in 2 of 38 tumors (5%). Peiffer *et al.* (15) found *p16* gene mutations in 2 of 34 tumors (6%), whereas Hatta *et al.* (16) did not find any alterations in 15 endometrial carcinomas studied. In the recent report by Milde-Langosch *et al.* (17), one point mutation and no deletions were found in 36 tumors (3%), despite negative or minimal nuclear staining for p16 protein in 74% of the tumors. Another previous immunohistochemical study of p16 protein expression in endometrial carcinomas showed that 66% of the cases were negative for nuclear staining (18), whereas Nakashima *et al.* (14) found negative nuclear staining in 19% of the tumors in their immunohistochemical study. The recent study of Nakashima *et al.* (14) reported no cases with *p16* promoter methylation in the 26 cases studied. With this background, the aim of our study was to assess the pattern and prognostic impact of p16 protein expression and *p16* promoter methylation in a population-based, large series of endometrial carcinomas and to correlate the findings with data on clinicopathological variables and other tumor markers.

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[2] To whom requests for reprints should be addressed, at Department of Gynecology and Obstetrics, Haukeland University Hospital, N-5021 Bergen, Norway. Phone: 47-55-97-42-00; Fax: 47-55-97-49-68; E-mail: hesa@haukeland.no.

PATIENTS AND METHODS

All patients diagnosed with endometrial carcinoma in Hordaland County, Norway during the 10-year period from 1981 through 1990 have been studied. Hordaland County has approximately 400,000 inhabitants, representing about 10% of the total Norwegian population, and has an age-adjusted incidence rate of endometrial cancer similar to that of the total Norwegian population (19). Data on different patient characteristics, treatment, and follow-up were collected for all 316 of the patients diagnosed during this period. The patients were staged retrospectively according to the 1988 FIGO[3] criteria, and all microscopic slides were reclassified and graded by one pathologist (L. A. A.) according to the 1994 WHO criteria (20). The distribution of patient characteristics and the treatment protocol for this period have been reported previously (20–22). Twelve cases were excluded due to changed diagnosis at reclassification. In five cases, the diagnosis was based on a cytological examination only, with no histological specimens available. Paraffin blocks from 286 of the 299 (96%) remaining patients were available for further investigation. Additional frozen tumor tissue was available from 138 of these patients for molecular studies.

To investigate the potential selection bias in the patient population with frozen tumor tissue available, patient age, FIGO stage, histological type and grade, treatment, and survival were compared for these patients ($n = 138$) with those of the rest of the patients from Hordaland County treated for endometrial carcinoma during the same period from whom fresh tumor tissue was not available ($n = 161$). Curative surgical treatment was more often possible in the group with fresh tumor tissue available (92%) than in the rest of the population (77%; $P < 0.001$, χ^2 test). This is in accordance with the current practice in the area; women ineligible for curative treatment due to either advanced age or serious intercurrent or extensive disease are less often referred to the University Hospital, where fresh tissue was collected during primary surgery. With this exception, there were no significant differences in the other patient characteristics. Among the patients treated for cure, no significant difference in survival was found for the patients with fresh tumor tissue available compared with the rest of the patients.

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded specimens. H&E-stained sections were used to select the most cellular part of the tumor and the area with lowest architectural differentiation in the case of heterogeneity. Five-μm sections from selected areas were used for immunohistochemistry, and the staining procedures and results for microvessel density counts and Ki-67, p53, and p21 expression have been described previously (20, 22, 23).

p16 Expression. For estimation of p16 protein expression, the sections were incubated overnight at room temperature with polyclonal antibody SC-468 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:250 in PBS. In every run, positive control sections known to express p16 were included. In addition, nuclei in stromal cells were used as internal positive controls. Negative controls obtained by omitting the primary antibody were also included in every run. Nuclear p16 staining was recorded by a semiquantitative and subjective grading, considering both the intensity of staining and the proportion of tumor cells in the selected section showing an unequivocal positive reaction as described previously for p53 (20, 23). Intensity was recorded as 0 (no staining) to 3 (strong staining) whereas the percentage of nuclear staining was recorded as 0 (no tumor cells positive), 1 (positive staining in <10% of the tumor cells), 2 (positive staining in 10–50% of the tumor cells), and 3 (positive staining in >50% of the tumor cells). A staining index was calculated as the product of staining intensity and staining area. The tumors were categorized as moderate/high expressing (staining index ≥ 4) or absent/minimal expressing (staining index < 4) tumors. An equivalent staining index was also estimated for the cytoplasmic staining by scoring the cytoplasmic staining intensity and area. On the basis of previous studies (24), we especially wanted to see whether clearly reduced nuclear p16 expression was of prognostic importance. We therefore compared the group with absent or minimal nuclear staining (staining index < 4) with the rest (staining index ≥ 4). However, additional survival analyses categorizing the nuclear staining into smaller groups according to each level of the index showed a similar survival for the subgroups with staining indices < 4, which differed from that of the subgroups with staining indices ≥ 4, further justifying this cutoff value.

To investigate the observer reproducibility of the p16 staining reading, a subset of the sections was investigated two times by the same observer (H. B. S.) and by two different observers (H. B. S. and L. A. A.). The exact intraobserver agreement for nuclear staining index ≥ 4 *versus* nuclear staining index < 4 was 96% ($\kappa = 0.89$). The corresponding exact interobserver agreement was 93% ($\kappa = 0.83$). In addition to using internal and external positive controls as described above, the immunohistochemical staining method was further validated by repeating the staining procedure for 25 sections. This gave an 88% exact agreement for the nuclear staining index (≥ 4 *versus* <4, $\kappa = 0.75$).

In a subset of this population-based series, DNA ploidy, S-phase fraction, steroid receptor concentration, and *p16* methylation status were assessed on frozen tissue specimens taken during the primary operation from a site considered macroscopically to be representative for the tumor. These samples were divided into three parts: (*a*) one for receptor analyses (estradiol/progesterone receptors); (*b*) one for flow cytometric and molecular studies; and (*c*) one for routine histological examination to confirm that the tissue contained at least 50% tumor cells. Technical details regarding measurements of DNA ploidy, S-phase fraction, and steroid receptor concentration have been presented previously (22, 25).

DNA Isolation. DNA was isolated from the 138 frozen tumors by digestion with proteinase K in STE and 10% SDS, followed by a standard phenol-chloroform extraction and ethanol precipitation.

Methylation Analysis. The methylation status of the *p16* gene in the tumors was determined by M-PCR as described by Herman *et al.* (26). Briefly, genomic tumor DNA was treated

[3] The abbreviations used are: FIGO, International Federation of Gynecology and Obstetrics; M-PCR, methylation-specific PCR; MVD_{mean}, mean intratumoral microvessel density; PH, proportional hazards.

Table 1 PCR primers used for *M*-PCR

The p16-M1-3 sets are specific for the methylated version of the *p16* promoter region, whereas the p16-U1 set is specific for the unmethylated version. Sequence differences between the primers designed for the treated DNA strand and the untreated DNA strand are shown in bold. Differences between the methylated and unmethylated sequences are underlined.

Primer set	Forward primer, 5′ to 3′	Reverse primer, 5′ to 3′	Size (bp)	Annealing temperature (°C)	Genomic position[a]
p16-M1	**TTATT**AGAGGGTGGGGCGGA**T**CGC	GACCCCG**AA**CCGCG**A**CCGT**AA**	150	65	−104
p16-M2	GTGCG**TT**CGGCGG**T**TGCGGA	CCACCT**AA**ATCG**A**CCTCCGACCG	210	66	−80
p16-M3	GTGCG**TT**CGGCGG**T**TGCGGA	GACCCCG**AA**CCGCG**A**CCGT**AA**	126	66	−80
p16-U1	GAGGGG**T**TGG**TT**GGT**TATT**AG	CCACCT**AA**ATC**A**A**A**CCTCC**A**ACC**A**	248	60	−118

[a] Genomic position is the location of the 5′-end of the forward primer from the start codon of the *p16* gene; GenBank accession number, X94154.

Table 2 Nuclear p16 expression related to age, FIGO stage, histological type, histological grade, DNA index, S-phase fraction, estradiol receptors, progesterone receptors, nuclear Ki-67 expression, MVD_{mean}, and nuclear p53 and p21 expression in a population-based study of endometrial carcinoma patients

		Nuclear p16 expression		
Variable	Categories	Index < 4 *n* (%)	Index ≥ 4 *n* (%)	*P*[a]
Age (yr)	≤65.0	13 (9.3)	127 (90.7)	0.04
	>65.0	26 (17.8)	120 (82.2)	
FIGO stage[b]	I	22 (10.4)	189 (89.6)	0.014
	II	4 (21.1)	15 (78.9)	
	III	8 (19.0)	34 (81.0)	
	IV	5 (38.5)	8 (61.5)	
Histological type	Endometrioid	17 (8.2)	190 (91.8)	<0.001
	Adenoacanthoma/adenosquamous	9 (18.0)	41 (82.0)	
	Serous papillary/clear cell	13 (44.8)	16 (55.2)	
Histological grade	High differentiation (grade 1)	0	64 (100.0)	<0.001
	Moderate differentiation (grade 2)	20 (12.3)	143 (87.7)	
	Poor differentiation (grade 3)	19 (32.2)	40 (67.8)	
DNA index[c]	≤1.00	6 (7.9)	70 (92.1)	0.001
	>1.00	9 (36.0)	16 (64.0)	
S-phase fraction[c,d]	≤15%	4 (8.3)	44 (91.7)	NS[e]
	>15%	3 (18.8)	13 (81.3)	
Estradiol receptors[c]	≤30 fmol/mg protein	18 (31.6)	39 (68.4)	0.001
	>30 fmol/mg protein	9 (9.5)	86 (90.5)	
Progesterone receptors[c]	≤60 fmol/mg protein	16 (38.1)	26 (61.9)	<0.001
	>60 fmol/mg protein	11 (10.1)	98 (89.9)	
Ki-67 expression[c,d,f]	≤35%	10 (6.7)	139 (93.3)	<0.001
	>35%	14 (29.8)	33 (70.2)	
MVD_{mean}[c,d,g]	≤90	14 (9.3)	136 (90.7)	0.025
	>90	10 (21.7)	36 (78.3)	
p53 expression[c]	Index ≤ 4	11 (7.1)	144 (92.9)	<0.001
	Index > 4	13 (31.7)	28 (68.3)	
p21 expression[c,d,f]	≤9%	10 (19.6)	41 (80.4)	0.06
	>9%	14 (9.7)	131 (90.3)	

[a] χ^2 test.
[b] Missing FIGO stage for case.
[c] Information available about the DNA index in 101 cases. S-phase fraction in 64 cases, estradiol receptors in 152 cases, progesterone receptors in 151 cases, Ki-67 expression, MVD_{mean}, p53, and p21 expression in 196.
[d] Upper quartile was used as the cut point for S-phase fraction, Ki-67 expression, and MVD_{mean}, and lower quartile was used for p21 expression.
[e] NS, nonsignificant.
[f] Proportion of positive nuclei.
[g] MVD_{mean} in counts/mm^2.

with sodium bisulfite, which converts all unmethylated cytosine residues to uracil, which is then converted to thymidine in the subsequent PCR. Two primer sets were used to amplify the region of interest: (*a*) one specific for the unmethylated sequence where CpG sites are modified by bisulfite treatment (p16-U1); and (*b*) one specific for the methylated sequence where CpG sites remain unmodified by bisulfite treatment (p16-M1; Table 1). We used the same primer sequences described by Herman *et al.* (26) for p16-M1 and the p16-U1 reverse primer, which localize to regions in and around the transcription start site of the *p16* gene, a region previously reported to be correlated with loss of gene expression (7, 26). All of the tumors were investigated with the p16-M1 primer set at least twice.

To confirm the findings with this initial primer set and to

allow the analysis of additional CpG sites in the promoter region, two additional primer sets specific for the methylated sequence, p16-M2 and p16-M3, were designed (Table 1). The M-PCR was repeated with these two primer sets for all 26 cases with fresh tumor tissue available showing absent or minimal nuclear p16 protein expression and for an equal number of tumors showing nuclear p16 protein expression.

The PCRs were carried out in a 25-μl volume containing 1× PCR buffer II (Perkin-Elmer, Foster City, CA), 2.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, 0.5 μM of each PCR primer, 0.5 unit of AmpliTaq Gold (Perkin-Elmer), and approximately 40 ng of bisufite-modified DNA. Amplification was carried out in a Perkin-Elmer model 9600 thermocycler at 95°C for 10 min and cycled at 94°C for 45 s, 30 s at the annealing temperature listed in Table 1, and 30 s at 72°C (40 cycles for primer sets U1, M1, and M2 and 35 cycles for primer set M3), followed by a 10-min extension at 72°C. The positive control (C2) for the methylated primer sets consisted of lymphocyte DNA treated *in vitro* with excess *Sss*I methyltransferase (New England Biolabs, Beverly, MA), which generated DNA that was completely methylated at all CpG sites. The positive control (C1) for the unmethylated primer set consisted of lymphocyte DNA from normal individuals; however, because the tumors also contain normal cells, amplification of the unmethylated sequence is expected to be present for all tumors. Reactions containing untreated DNA (C3) and no DNA (C4) were included as negative controls for all primer sets. The amplification products were separated on a 3% agarose gel and visualized by ethidium bromide staining and UV illumination.

Follow-Up. The median follow-up period for the survivors was 9 years (range, 4–15 years). None of the patients was lost due to insufficient follow-up data. Information about survival was obtained from medical records and correspondence with the primary physician. The data were cross-checked with information from the Cancer Registry of Norway, which is matched against the Register of Deaths at Statistics Norway.

Statistics. Comparisons of groups were performed using the χ^2 test. Reproducibility was assessed using κ statistics. Univariate survival analyses of time to death due to endometrial carcinoma (cause-specific death) were performed using the product-limit procedure (Kaplan-Meier method), with the time of the primary operation as the entry date. Patients who died of other causes were censored at the date of death. The Mantel-Cox test was used to compare the survival curves for groups of patients defined by categories of each variable. The variables with significant impact on survival in univariate analyses ($P \leq 0.10$) were further examined by log-minus-log plot to decide how these variables should be incorporated in the multivariate Cox PH regression model. Hazard ratios were estimated as a measure of effect. Tests for interactions were carried out for the variables with independent impact on survival in the multivariate analysis. Data were analyzed using the SPSS software package (SPSS Inc., Chicago, IL).

The research has been approved by the Norwegian Data Inspectorate and the Institutional Review Board at the University of Chicago (Protocol 9457).

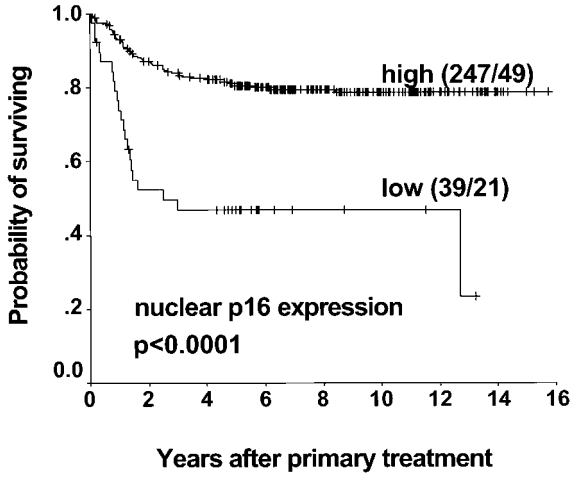

Fig. 1 Nuclear p16 expression influenced survival significantly (Mantel-Cox test) in this population-based study of patients with endometrial carcinoma. Survival curves are estimated according to the Kaplan-Meier method, with death due to endometrial carcinoma as end point.

RESULTS

Thirty-nine cases (14%) had absent or minimal nuclear expression of p16 protein (staining index < 4), whereas 247 cases (86%) had moderate or high nuclear expression of p16 protein (index ≥ 4). Absent or minimal nuclear p16 protein expression was associated with increased patient age at primary operation, high FIGO stage, serous papillary or clear cell histological types, high histological grade, aneuploidy, low estradiol and progesterone receptor concentrations, high expression of Ki-67, high microvessel density, and strong nuclear p53 protein expression, as shown in Table 2. The mean Ki-67 expression was 40% positive nuclei, and the MVD_{mean} was 97 microvessels/mm^2 among the tumors with absent/minimal nuclear expression of p16 protein (staining index < 4), compared with 23% and 71 microvessels/mm^2 among the tumors with moderate/high nuclear expression of p16 protein (index ≥ 4).

In univariate survival analysis, the 5-year survival was 47% for patients with absent/minimal nuclear p16 expression ($n = 39$) compared with 81% for patients with moderate/high nuclear expression of p16 ($n = 247$; $P < 0.0001$), as illustrated in Fig. 1. Nuclear p16 expression was studied further in Cox PH regression analysis, together with age at primary operation, FIGO stage, histological type, histological grade, DNA ploidy, S-phase fraction, progesterone receptor concentration, microvessel density, Ki-67, p53, and p21 expression (all with $P \leq 0.10$ in univariate analysis). As shown in Table 3, nuclear p16 expression had an independent prognostic impact in addition to FIGO stage, patient age, Ki-67 expression, and microvessel density. The other variables lost their prognostic impact when nuclear p16 expression was added to the Cox model.

Cytoplasmic staining and nuclear p16 staining were highly correlated ($P < 0.001$). Absent/minimal cytoplasmic expression of p16 protein (staining index < 4) was seen in 108 of the 286 cases (38%). Compared with nuclear p16 staining, the cytoplasmic staining showed correlations with age, FIGO stage, histological type and grade, ploidy, estradiol receptor concentration,

Table 3 Unadjusted and adjusted hazard ratios (HRs) based on the Cox proportional hazard regression model for clinicopathological variables, nuclear Ki-67 expression, MVD_{mean}, and nuclear p16 expression in a population-based study of endometrial carcinoma patients (n = number of patients)

Variable	Categories	n	Unadjusted HR[a]	95% CI[b]	P[c]	Adjusted HR[d]	95% CI	P[c]
Age at primary operation[e]		195	1.05	1.0–1.1	0.002	1.04	1.0–1.1	0.007
FIGO stage	I/II	159	1		<0.0001	1		<0.0001
	III/IV	36	9.7	5.2–17.9		7.8	4.0–15.2	
Ki-67 expression[f]	≤35%	149	1		<0.0001	1		0.01
	>35%	46	4.2	2.3–7.8		2.6	1.3–5.3	
MVD_{mean}[g]	≤90	149	1		0.0002	1		0.03
	>90	46	3.0	1.6–5.6		2.1	1.1–4.1	
Nuclear p16 expression	Index < 4	24	6.6	3.5–12.5	<0.0001	2.9	1.3–6.5	0.01
	Index ≥ 4	171	1			1		

[a] Analyses based on patients with complete information for all variables (n = 195).
[b] CI, confidence interval.
[c] Likelihood ratio test.
[d] Hazard ratio adjusted for variables with $P < 0.10$ in the Cox PH regression model.
[e] Continuous variable with hazard ratio given per year.
[f] Proportion of positive nuclei.
[g] MVD_{mean} in counts/mm^2.

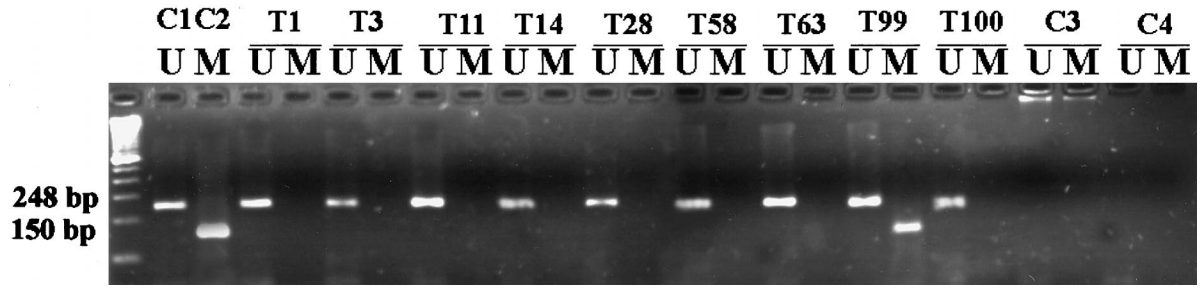

Fig. 2 M-PCR with bisulfite-treated samples. *Lanes U*, reactions using p16-U1 primers specific for the unmethylated CpG sites; *Lanes M*, reactions using p16-M1 primers specific for the methylated CpG sites. *C1* is the positive control for the unmethylated primer set, whereas *C2* is the positive control for the methylated primer set. Because the tumors also contain normal cells, amplification of the unmethylated sequence is seen for all tumors, whereas amplification of the methylated sequence is seen for only one tumor (*T99*). Reactions containing untreated DNA (*C3*) and no DNA (*C4*) were included as negative controls for both primer sets. A 100-bp DNA ladder (Life Technologies, Inc., Gaithersburg, MD) was used for sizing.

and p53 expression that were generally weaker but still significant. However, progesterone receptor concentrations, Ki-67 expression, and microvessel density were not significantly correlated with cytoplasmic p16 staining. In univariate survival analyses, cytoplasmic p16 staining influenced survival significantly ($P = 0.005$), but it had no independent prognostic impact in Cox PH regression analysis.

Of the 286 cases studied for p16 protein expression, fresh tumor tissue was available and used for M-PCR in 138 cases. This group of patients included 26 patients (19%) with absent or minimal nuclear expression of p16 protein (staining index < 4), whereas 112 patients (81%) had moderate or high p16 expression (index ≥ 4). Only one tumor (0.7%) was found to be methylated in the *p16* promoter region (Fig. 2). This result was confirmed with three different primer sets. This tumor lacked nuclear p16 protein expression as well.

DISCUSSION

Absent or minimal nuclear expression of p16 protein was found in only 14% of the endometrial carcinomas in our population-based series, clearly lower than what was reported in two smaller previous studies (17, 18), but in line with the recent report by Nakashima *et al.* (14). Shiozawa *et al.* (18) and Milde-Langosch *et al.* (17) found that 66% and 74% of their tumors, respectively, revealed absent or minimal staining. This contrast could be due to differences in technical methods, criteria for positivity, and patient selection. Cytoplasmic staining, which is controversial, can make it more difficult to examine true nuclear expression of the protein. It has previously been pointed out by Geradts *et al.* (24) that the p16 protein might be damaged by microwave treatment. The microwave antigene retrieval used by Shiozawa *et al.* (18) may have influenced the level of positivity and could explain the higher number of negative cases in their study. Furthermore, the differences between various primary antibodies and dilutions and incubation times and temperatures are also important. In the study of Milde-Langosch *et al.* (17), incubation with the primary antibody was at 4°C; in our study, room temperature was used, which may also explain some of the difference in the level of positivity.

Because hypermethylation of *p16* has been documented in a number of other tumor types and appears to be a common

mode of inactivation of this gene (6, 7), we wanted to determine the role of *p16* promoter hypermethylation in this group of endometrial carcinoma patients, particularly in the group with loss of protein expression. One recent but smaller report found no hypermethylation of the *p16* promoter region in 26 endometrial carcinomas studied with a methylation-sensitive restriction enzyme method (14). Our finding that only 1 of the 138 cases studied showed hypermethylation for the *p16* promoter region (0.7%) supports that this is not the predominant mechanism of *p16* inactivation in these tumors. It is possible that deletions and/or mutations of the *p16* gene may play a more significant role in those tumors with minimal or low protein expression, but the incidence of deletions and mutations in our group of endometrial carcinoma tumors remains to be determined. However, previous studies of *p16* alterations in endometrial carcinoma appear to indicate that deletions and mutations of this gene are rare (13, 15–17). Therefore, to date, the mechanism of *p16* inactivation, as indicated by a lack of p16 protein expression, remains largely unknown for endometrial carcinomas.

Loss of p16 protein expression was significantly related to a subgroup of aggressive endometrial carcinomas and poor prognosis. Thus, absent or minimal nuclear staining was associated with increased age at treatment, higher FIGO stage, serous papillary or clear cell histological types, high histological grade, and aneuploid tumors. Furthermore, there were strong correlations with increased tumor cell proliferation, as measured by the expression of Ki-67, supporting the role of intact p16 protein as a cell cycle inhibitor (27). Interestingly, lack of nuclear p16 expression was also significantly associated with increased intratumor angiogenesis, as measured by factor VIII-related microvessel density. However, the reason for this is not clear. Finally, there was a statistically significant association between altered nuclear p16 and p53 expression. In 13 patients (7%), alterations were indicated in both of these major tumor suppressor pathways. This has also been indicated in studies of other tumors (28, 29). In 27% of the cases, alterations were present in either the p16 or the p53 systems, whereas 73% of the tumors showed no apparent alterations, as judged by immunohistochemical expression of the protein products.

In endometrial carcinoma, the prognostic impact of traditional clinicopathological variables is used to determine whether hysterectomy alone is likely to be curative (30, 31). In addition, a number of studies have identified aneuploidy and high S-phase fraction as negative prognostic factors (25, 32). Although markers for angiogenesis, proliferative activity, and selected cell cycle regulators have recently been found to add prognostic information (20, 22, 23, 32, 33), there is still a need for more specific and even better prognostic markers to avoid overtreatment of low-risk groups and to ensure that patients with highly aggressive tumors receive adequate postoperative treatment (30, 34). The prognostic impact of alterations of the *p16* system, which represents a major tumor suppressor pathway, has not previously been addressed in endometrial carcinomas. Survival analysis showed that lack of nuclear p16 protein expression was significantly associated with a markedly increased risk of disease-related death. Even in multivariate analysis including the traditional clinicopathological variables as well as markers for cell cycle regulation, proliferation, and angiogenesis, nuclear p16 protein expression had a strong and independent prognostic impact. When nuclear p16 expression was included in the Cox model, age, FIGO stage, Ki-67 expression, and intratumor microvessel density still had an independent prognostic impact, whereas p53 expression, for example, lost its importance.

In conclusion, we have shown that lack of nuclear p16 protein expression in endometrial carcinoma, as an indication of *p16* inactivation, is associated with a subset of aggressive tumors and has an independent prognostic impact. The mechanism of *p16* inactivation is not clear, but promoter region methylation seems to be very rare (only 0.7% in our present series). Because the immunohistochemical data indicate a higher frequency of inactivation, other genetic alterations or nongenetic mechanisms for inactivation should be further studied.

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Helga B. Salvesen, Soma Das and Lars A. Akslen

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