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P16/MTS1 and pRB expression in endometrial carcinomas

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Abstract P16$^{MTS1/CDKN1}$ and the retinoblastoma protein Rb are both involved in negative regulation of G1/S progression in the mammalian cell cycle. Inactivation of one of these tumour suppressor genes is involved in many malignant tumours, and in some studies a negative correlation of p16 and Rb expression has been found. In order to study this interaction in endometrial carcinogenesis, we investigated 36 endometrial carcinomas, 11 cases of hyperplasia, 23 normal endometrial samples, and two uterine carcinoma cell lines by immunohistochemistry or RT-PCR. Rb was expressed in normal endometrial epithelium, hyperplasia, cell lines, and most carcinomas; negative immunostaining was only detected in 1 of 36 tumours. In contrast, p16 expression was weak in normal endometrium and increased in most cases of hyperplasia, but negative or minimally positive in 74% of the carcinomas and the Hec1B adenocarcinoma cell line, and there was no significant association with Rb immunostaining. Strikingly high p16 expression was found in foci of squamous metaplasia within hyperplastic or carcinomatous tissue. Deletion and mutation analysis of the p16 gene was performed in DNA from microdissected tumour samples and cell lines. No p16 deletion was found, and mutations were detected in only one tumour sample and Skut1B uterine mixed mesodermal tumour cells. Our data indicate that in spite of low or absent p16 expression, genetic alterations of the p16 and Rb tumour suppressor genes are rare in endometrial carcinogenesis.

Key words p16 · Rb · Uterus · Endometrium · MTS1 · Carcinoma

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Introduction

Progression through the mammalian cell cycle is controlled by complexes of cyclin-dependent kinases (cdk's) and their regulatory subunits, the cyclins. Cyclin-bound cdk's phosphorlyate and modulate important regulatory proteins, including the tumour suppressor protein RB, reducing its suppressive function upon the cell cycle and allowing progression to the S-phase. Cdk's are negatively regulated by cdk inhibitors, which include p15, p16, p18, p19, p21, p27, and p57 [6]. P16 (MTS1/INK4a) binds and regulates cyclin D/cdk4 or cyclin D/cdk6 complexes, the predominant cyclin/cdk's in mid/late G1 phase.

Observations that the INK4a locus on chromosome 9p21 is often deleted or mutated in human malignant tumours and tumour cell lines [4, 11] implied that p16 inactivation might be involved in the development of human malignancies. The tumour suppressor function of this gene was further emphasized by experiments with p16 knock-out mice, which develop spontaneous tumours at an early age and are highly sensitive to carcinogenesis [20].

In experimental systems, p16 expression is linked to the product of the retinoblastoma susceptibility gene, pRB, by feedback. pRB inactivation leads to increased p16 expression, whereas p16 overexpression results in decreased RB phosphorylation by cdk's and greater RB suppressor activity [21]. As both suppressor genes belong to a common pathway regulating the G1/S transition in the cell cycle [14], down-regulation of p16 and pRB should have similar effects and inactivation of both genes in tumours should be redundant. Negative correlations between pRB and p16 expression have been found in cell lines [3] and various tumours [12].

Homozygous deletion or mutation of the p16/INK4a gene leading to loss of the p16 suppressor function has been reported in many human tumour cell lines [11], but also in primary carcinomas of the pancreas [5], bladder [23], and oesophagus [16], in familial melanomas [10] and in other tumours. An alternative route of p16 inacti-

Table 1 Results of p16 immunohistochemistry (*IHC*) in paraffin-embedded endometrial tissue sections

Cases	*n*	p16 Immunostaining			
		Negative	Very weak/ focal	Weak	Moderate/ strong
IHC score		0	1–2	3–5	6–12
Normal glandular epithelium	23	14	4	3	2
proliferating	8	6	2	0	0
Secretory	10	7	2	1	0
Irregular	5	1	0	2	2
Hyperplasia	11	2	5	3	1
Carcinoma	35	5	21	6	3

vation is de novo methylation of CpG islands in the regulatory 5′ region of the gene [9]. In our study on p16 inactivation in ovarian carcinomas, loss of p16 protein expression was observed mainly in mucinous and endometrioid histological subtypes [15]. This observation led us to investigate the p16 and Rb status in endometrial carcinomas, which are histologically closely related to the endometrioid ovarian tumours. Rb and p16 protein expression in normal endometrium, hyperplasia and carcinoma was studied by immunohistochemistry, followed by deletion and mutation analysis of the gene. The results were correlated with p53 expression and various clinical and histological variables. For comparison, we investigated two uterine tumour cell lines, the Hec1B adenocarcinoma cell line and Skut1B cells derived from a mixed mesodermal tumour.

Materials and methods

We studied paraffin-embedded endometrial tumour tissue from 35 patients (mean age 65.4 years, range 41–87 years) treated at the Hamburg University Hospital in the period 1984–1996. Histologically, 31 tumours displayed endometrioid differentiation. In addition, 3 serous-papillary tumours and 1 undifferentiated carcinoma were investigated. Six tumours were FIGO stage Ia, 18 tumours, stage Ib, 5 cases stage Ic, and 6 tumours, stage II.

We also investigated 23 samples of normal functional endometrium and hyperplasia from 11 patients (6 cases of simple hyperplasia without atypia, 4 of complex hyperplasia with atypia, 1 of hyperplasia with secretory transformation). Most of the carcinomas had been characterized before with respect to p53 tumour suppressor gene expression [19]. The uterine tumour cell lines Hec1B and Skut1B were purchased from ATCC and cultivated as described elsewhere [2].

P16 immunohistochemistry (IHC) was performed on paraffin-embedded tissue sections with the monoclonal antibody G175-405 (Pharmingen, Hamburg, Germany) as described elsewhere [15]. The nuclear immunoreactivity was evaluated by examination of staining intensity (0, no nuclear staining; 1, weak staining; 2–3, intermediate and strong staining intensity), and percentage of positive tumour cells (0, no positive cells; 1, <10%; 2, 11–50%; 3, 51–80%; 4, >80% positive tumour cells). After multiplication of both values, the IHC results were graded from 0 (no reactivity in tumour cell nuclei) to 12 (more than 80% positive tumour cells, with strong staining intensity in most of the cells). Expression of RB was investigated with the monoclonal antibody G3-245 (Pharmingen) diluted 1:1000, after microwave pretreatment of the tissue sections.

In order to study the p16 and RB expression in cell lines, cDNA was tested with the primers RB05/RB06 [24] spanning a 560-bp fragment encompassing the RB exons 18–23, and the primers p16-RF (GGAGCAGCATGGAGCCG) and p16-RR (CAAGGTCCACGGGCAGA) spanning exon 1 and part of exon 2 of the p16 gene. For comparison, cDNAs of the mammary carcinoma cell lines T47D and MDA-MB 231 (purchased from the ATCC) with hypermethylation and deletion of the p16 gene, respectively, and of the EFE148 cell line (gift of Dr. Fritz Hölzel, Hamburg) were analysed in the same way.

In order to investigate the p16 status of the tumour cells, contamination with stromal cells had to be reduced to minimum. Tumour cell clusters were selected by microdissection, followed by proteolytic treatment as already described [27]. For controls, nontumour tissue from the same patients was processed in the same way.

For deletion analysis a 101-bp p16 fragment and, as internal control, a 125-bp fragment of the GAPDH (glyceraldehyde phosphate dehydrogenase) gene were co-amplified in the same reactions as described [18]. PCR products were separated in 3% agarose gels. A deletion should be characterized by missing or strong reduction of the 101-bp band. As a control, DNA from the mammary carcinoma cell line MDA-MB 231, which harbours a homozygous p16 deletion, was used.

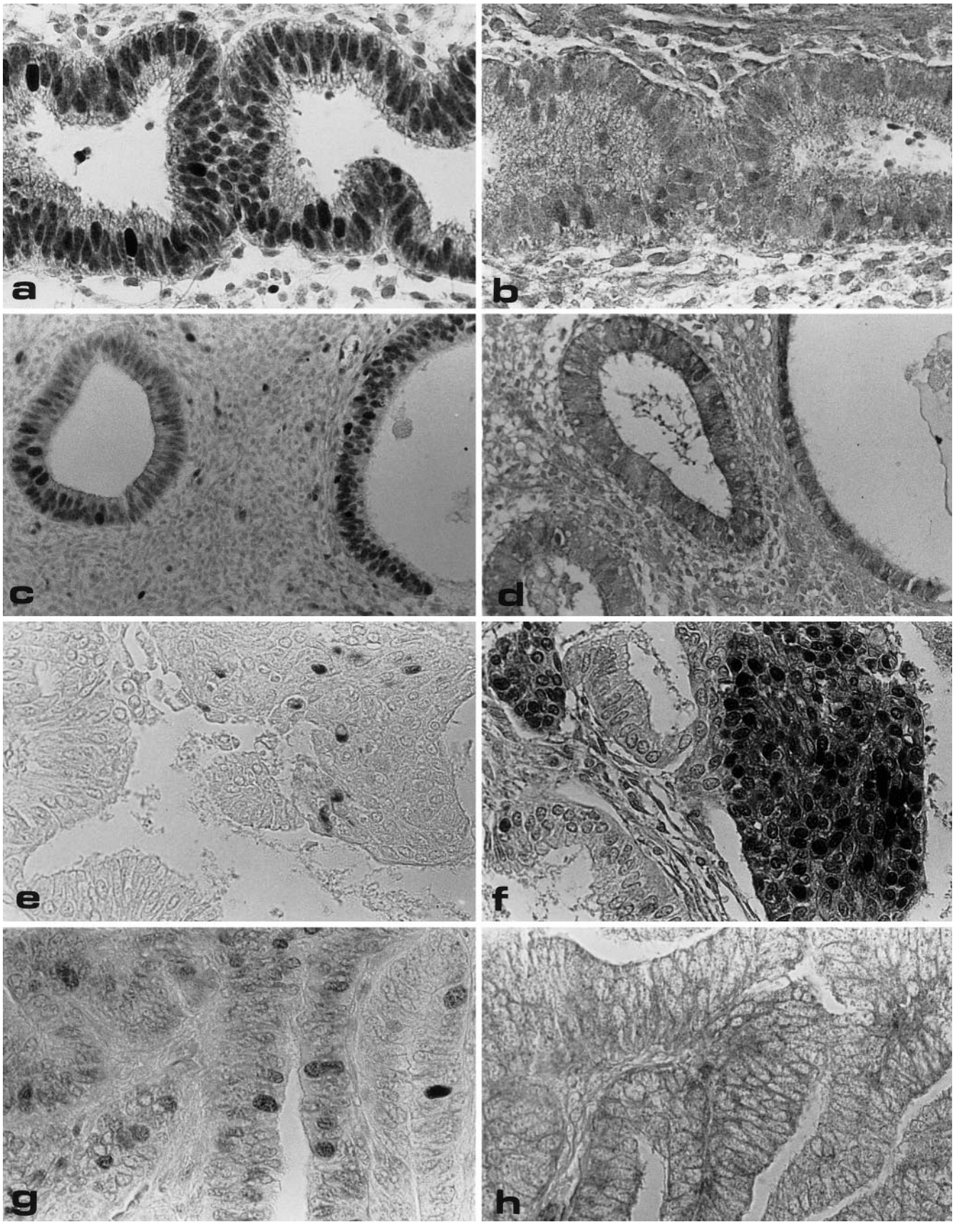
P16 mutations in exons 1 and 2 were investigated in carcinomas and cell lines by SSCP analysis in horizontal MDE gels as described elsewhere [15]. In cases showing aberrant band patterns, PCR and SSCP were repeated using nontumour tissue from the same patient as a control. For sequence analysis of suggestive cases, silver-stained bands were cut out with sterile scalpel blades, eluted in 0.1 ml distilled water, reamplified and analysed by nonradioactive sequencing and automatic sequence analysis as described elsewhere.

The association of p16 and Rb expression with different histological and clinical parameters was evaluated using the χ^2 test.

Results

The results of p16 immunohistochemistry in normal endometrium, hyperplasias and carcinomas are shown in Table 1. Only nuclear staining was taken into account, although cytoplasmic p16 staining was observed in most of the normal or hyperplastic endometrial samples and carcinomas. Nuclear staining for p16/MTS1 was ob-

Fig. 1a–b Proliferative endometrium showing **a** strong nuclear labelling for Rb and **b** moderate staining for p16. ×400 **c**, **d** Simple hyperplasia with **c** strong nuclear staining for Rb and **d** weak cytoplasmic and nuclear staining for p16. ×200 **e**, **f** Complex hyperplasia with squamous metaplasia showing **e** Rb expression in only a few metaplastic cells and **f** p16 labelling in most metaplastic, but few columnar cells. ×400 **g**, **h** Endometrial carcinoma with **g** moderate Rb expression and **h** only weak cytoplasmic staining for p16. ×400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served in only 9 of 23 samples (39%) of normal endometrium, among them 2 of the 8 from the proliferative phase, 3/10 from the secretory phase and 4 of the 5 samples with irregular proliferation. In addition, 9 of 11 (82%) cases of endometrial hyperplasia displayed detectable nuclear immunostaining (Fig. 1d). In two of these cases and in one carcinoma, focal regions of squamous metaplasia were observed. Strikingly, these cell clusters exhibited strong nuclear and cytoplasmic p16 immunoreactivity, whereas the surrounding cells with glandular differentiation were negative or weakly positive (Fig. 1f).

Negative p16 results or only weak and focal nuclear staining were also observed in 26/35 (74%) carcinomas (Fig. 1h). In some cases, there was marked heterogeneity between tumour areas with respect to p16 reactivity. Reduced protein expression was found predominantly in endometrioid and highly differentiated carcinomas, but the number of undifferentiated and nonendometrioid tumours was too low for statistical analysis. There was no significant association with tumour stage or p53 status (Table 3).

In contrast to p16, RB immunohistochemistry revealed only nuclear reactivity. RB protein expression was observed in 17 of 22 (77%) normal endometrial samples, 10 of the 11 (91%) cases of hyperplasia, and 35 of the 36 (97%) carcinomas (Fig. 1a, c, e, g, Table 2). In normal endometria, the strongest staining intensity was found in the proliferating phase, although there were wide variations between the samples. RB expression in carcinomas was not associated with histological grade, invasiveness (FIGO stage), or p53 status of the tumours (not shown). In addition, there was no inverse correlation of p16 and RB expression as reported by others (Table 3).

In the uterine carcinoma cell lines, p16 and RB expression were studied by RT-PCR. RB mRNA was strongly expressed in both cell lines, whereas p16 expression was low in Skut1B and absent in Hec1B cells (Fig. 2d, e).

Analysis of microdissected tumour cells from 34 tumours and of the two uterine carcinoma cell lines for p16 gene inactivation failed to detect any homozygous deletion (Fig. 2a). SSCP analysis and subsequent direct sequencing of aberrant bands revealed sequence abnormalities in 3 cases and one cell line (Table 4, Fig. 2b). In 2 tumours, the frequent polymorphism in codon 140 leading to substitution of alanine by threonine was found. One case harboured a silent mutation in codon 76 and a heterozygous mutation in codon 82. In 1 tumour, aberrant band patterns in SSCP gels indicated a somatic mutation in exon 1, but these bands could not be successfully reamplified and sequenced. In Skut 1B, two bands were already visible in agarose gels after amplification of exon 1 and surrounding sequences (Fig. 2c). Sequence analysis of the eluted and reamplified bands revealed a

Table 2 Rb immunostaining in paraffin-embedded endometrial tissue

	n	RB immunostaining		
		Negative	Weak/focal	Moderate/ strong
Normal endometrium	22	5	8	9
Proliferating	8	1	2	5
Secretory	9	1	4	4
Irregular	5	3	2	0
Hyperplasia	11	1	5	5
Carcinoma	36	1	8	27

Table 3 Correlation of p16 immunoreactivity with clinical and histological parameters and expression of the tumour suppressor genes p53 and RB

Group of endometrial carcinomas	*n*	p16 Expression	
		Negative/ focal	Moderate/ strong
Histological type			
Endometrioid	31	25 (81%)	6 (19%)
Nonendometrioid	4	1 (25%)	3 (75%)
Grading			
G1	18	15 (83%)	3 (17%)
G2	12	9 (75%)	3 (25%)
G3	5	2 (40%)	3 (60%)
Clinical stage			
Ia	6	4 (67%)	2 (33%)
Ib	18	15 (83%)	3 (17%)
Ic	5	3 (60%)	2 (40%)
II	6	3 (50%)	3 (50%)
RB expression			
Negative/weak	9	8 (89%)	1 (11%)
Moderate/strong	26	18 (69%)	8 (31%)
p53 Expression[a]			
Negative/weak	27	20 (74%)	7 (26%)
Moderate/strong	7	5 (71%)	2 (29%)

[a] p53 Expression studies have been published elsewhere [19]

Table 4 Results of direct sequencing in cases with abnormal SSCP band patterns

Case	Codon	Nucleotide change	Amino acid change
7	140	gcg-acg	ala-thr
49	140	gcg-acg	ala-thr
19	76	gac-gat	asp-asp
	82	ttc-ctc	phe-leu
Skut 1B	5′ Untranslated region	Deletion of 23 bp (nt 76–98[a]) (AGAGCAGGCAGCGGGCGGCGGGG)	No change

[a] Nucleotide numbers refer to the genomic sequence, GenBank no. U12818

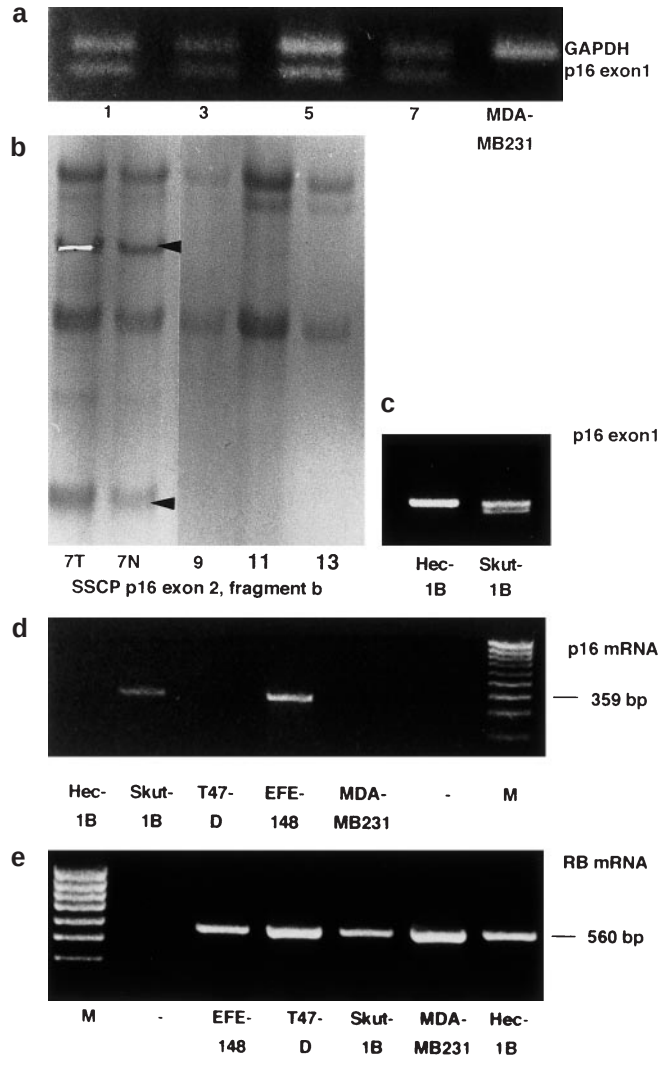

Fig. 2 **a** Deletion analysis of the p16 gene by comparative PCR after microdissection of tumor cells, showing the known deletion in the control cell line, but in none of the tumor samples (*1–7*). **b** SSCP analysis of the second fragment of p16 exon 2 after microdissection. Additional bands in one tumour (7T) and the corresponding normal tissue (7N), and wild-type configuration in samples 9, 11, and 13. * This band was excised and further analysed by re-amplification and direct sequencing (table 4). **c** PCR amplification of p16 exon 1 and surrounding sequences. Two bands were obtained with DNA from the Skut1B cell line suggesting a deletion in one allele. **D**, **E** Analysis of p16 and Rb expression in Skut1B and Hec1B cells by RT-PCR. For control, the mammary carcinoma cell lines MDA-MB 231 (p16 deletion) and T47D (p16 promoter methylation) were used, as well as the uterine carcinoma cell line EFE148 (gift of F. Hölzel)

wild-type sequence in one allele, and a 23-bp deletion in the 5′-uncoding region in the other (Table 4), which abolished one Sp1-binding site. Comparative sequencing of Hec1B DNA revealed a wild-type sequence in p16 exons 1 and 2.

Discussion

Comparison of normal endometrium, hyperplasia and endometrial carcinomas indicates that the tumour suppressor gene pRB is expressed in nearly all hyperplastic and neoplastic endometrial lesions and most normal endometrial tissue samples. For p16, the results are more complex. The strongest expression was found in endometrial hyperplasia, whereas most carcinomas exhibited only weak immunoreactivity in few cells or were p16 negative.

There is still controversy about the meaning of cytoplasmic staining in p16 immunohistochemistry. Geradts et al. [7] found cytoplasmic p16 reactivity in p16-negative cultured cells, suggesting a nonspecific artifact. In contrast, cytoplasmic p16 staining was interpreted as a “resting” status of the protein by Shiozawa et al. [22]. Although the latter possibility cannot be excluded, we took into account only nuclear staining in our interpretation of staining results.

Overexpression of tumour suppressor genes might be one mechanism by which the cell overcomes or reduces enhanced proliferation. In immortalized fibroblasts, p16 overexpression can induce senescence of the cells [1]. Interestingly, the highest p16 expression in our endometrial samples was found in highly differentiated squamous cells within hyperplastic or carcinomatous tissue. In the same areas, RB expression varied from negative to strong, indicating that p16 and RB expression are not always mutually exclusive.

Loss of the cell cycle regulatory pathway leading to control of G1/S transition by p16 or pRB deletion, mutation or reduced transcription is involved in carcinogenesis in various tumour types and might also be involved in the aetiology of endometrial carcinomas. Indeed, most of our cases exhibited only focal and weak p16 immunoreactivity or were negative on IHC. In an IHC study on 41 endometrial carcinomas, as many as 66% were negative for $p16^{INK4}$ [22]. The discrepancy between this and our results might derive from different interpretation of weak or focal nuclear immunoreactivity. Our negative p16 results in most normal endometrial tissues are in accordance with those of Shiozawa et al. [22], who, however, reported negative nuclear immunostaining in hyperplasia.

To seek the reasons for the negative immunoreactivity, we studied p16 deletion and mutations in exons 1 and 2, comprising 93% of the coding sequence of the p16 gene. In former studies on p16 gene alterations in uterine carcinomas [8, 25] homozygous deletion was detected in only 1 of 41 endometrial carcinomas, whereas mutations in exon 2 of the p16 gene were not found. Our results corroborate that deletion or mutation of the gene are rare events in endometrial cancer. The reason for the reduced p16 expression is therefore still uncertain: hypermethylation of the gene might be involved in abrogation of this cell-cycle-inhibitory mechanism.

In contrast to p16 results, pRB expression was detected in 35 of 36 carcinomas, which is in accordance with prior reports [13, 17]. Analysis of the retinoblastoma

gene in endometrical carcinoma cell lines and endometrial tumour tissue has shown that Rb mutation or loss of heterozygosity are rare events in these tumours [26]. The high RB expression in most of the carcinomas could lead to down-regulation of p16 expression and might be one reason for the weak immunoreactivity with p16/MTS1 antibodies. Nonetheless, the fact that there is no significant negative correlation of the two suppressor proteins suggests that other regulatory pathways may also be involved in the regulation of p16 expression in these tumours.

In agreement with the results for endometrial tumour tissue, loss of p16 expression with concomitant high Rb expression was also found in the endometrial adenocarcinoma cell line Hec 1B in the presence of an intact wild-type p16 gene. In contrast, p16 RNA expression was found in the uterine mixed mesodermal tumour cell line Skut 1B, which harbours a 23-bp deletion leading to loss of one Sp1 site in the promoter region. Although no mutation was found in the second allele, this deletion may have contributed to the decreased p16 expression in the cells.

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