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Comparison of benign and malignant endometrial lesions for their p53 state, using immunohistochemistry and temperature-gradient gel electrophoresis

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Abstract The aim of this study was to evaluate the presence and distribution of p53 alterations in pure endometrioid adenocarcinomas (n=120) of different grades and stages, as opposed to normal endometrium (n=13) and various risk groups of hyperplasia (n=39). All samples were initially analysed by immunohistochemistry with the monoclonal antibody Ab-6. Normal endometria were negative. With increasing degrees of malignancy, the number of cases with p53 accumulation rose and ranged from 9% to 18% in hyperplasia, through 25% in low-grade carcinomas (G1), to 69% in high-grade carcinomas (G3). This increase was also seen when comparing tumours by stage. Of carcinomas in stage IA, only 17% showed p53 immunostaining, in contrast with 72% in stage IC. Of this material, 34 carcinomas and 8 hyperplasias were analysed for p53 mutations in exons 5–8 by means of polymerase chain reaction and temperature-gradient gel electrophoresis (TGGE). In none of 5 hyperplasia and 6 of 12 carcinomas showing p53 accumulation by immunohistochemistry, p53 mutations were detected by TGGE. In contrast, 4 of 22 carcinomas harboured mutant p53 but were negative by immunohistochemistry. Immunohistochemical and molecular investigations revealed that p53 alterations are related to the standard prognostic markers of endometrial cancer, i.e. grading and staging. TGGE, an indirect screening procedure for p53 mutations, is used to detect the type of p53 alteration and may provide additional insight into the complex figure of p53 abnormalities in the development and progression of malignant endometrial lesions.

Key words p53 · Endometrioid carcinoma · Endometrial hyperplasia · Temperature-gradient gel electrophoresis · Immunohistochemistry

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Introduction

Wild-type p53 plays an important role in the regulation of cell growth and differentiation. After DNA damage, p53 expression is needed to arrest the cell cycle in G1 transiently, enabling cellular repair mechanisms to act. Mutation of one p53 allele is found in a wide variety of malignant human tumours, especially carcinomas of the respiratory and digestive tract, breast, bladder and liver, as well as sarcomas [9, 22]. In the course of cancer progression, mutation is frequently followed by a loss of the wild-type counterpart. In consequence, the natural suppressor action of the p53 protein is abrogated or even replaced by damaging activity [7]. The mutant p53 protein may have a prolonged half-life and it is then easily detectable by immunohistochemistry [20]. However, p53 accumulation and function may also be up- and/or downregulated by heterologous control mechanisms, for instance by the generation of p53-HPV-E6 complexes, and their accelerated degradation via the ubiquitin pathway [27].

Previous studies on endometrial carcinomas, particularly on the most common tumour type, endometrioid carcinoma, differ from the reported frequency of p53 alteration, and its correlation with grading and staging [2, 8, 10, 11, 23]. The available data are even more conflicting when looking at p53 in normal and hyperplastic endometrium [1, 2, 28].

To evaluate the impact of p53 alterations in malignant endometrial lesions we analysed archival material fitting the most recent WHO and FIGO criteria comprising normal endometria, endometrial hyperplasias and pure endometrioid carcinomas of different grades and stages for p53 protein accumulation using the p53 antibody AB-6 in immunohistochemistry. One half of the cases were examined for p53 alterations by means of temperature-gradient gel electrophoresis (TGGE).

Materials and methods

Samples were obtained from 172 patients admitted to the Clinic of Obstetrics and Gynaecology, University of Hamburg, Germany

over the last decades. We analysed 13 normal endometria, 39 endometrial hyperplasias and 120 endometrioid carcinomas. Most tissues derived from hysterectomy specimens. Hyperplasias and carcinomas were classified by WHO criteria [19]; carcinomas were staged according to the 1988 proposals of the International Federation of Gynaecology and Obstetrics (FIGO) [6].

Cell lines with known mutations in the p53 gene used as positive controls for the TGGE analysis were BXPC-3 (exon 6 [12]), CEM (exon 5 and 7 [4]), Capan-1 (exon 5 [12]) and A 431 (exon 8 [3]). SV 80 was used as a cell line with wild-type sequence in the p53 gene (personal communication, Dr. W. Deppert, Pette-Institut, Department of Tumour Virology, Hamburg).

For immunohistochemistry deparaffinized tissue sections were washed in phosphate-buffered saline and preincubated in 0.5% blocking serum for 20 min. The slides were incubated overnight at 4° C using the mouse monoclonal p53 antibody Ab-6 (Oncogene Science, Dianova, Hamburg, Germany) diluted at 1:150 in 1% bovine serum albumin (Sigma, Deisenhofen, Germany). This AB-6 antibody is specific for an epitope located near the amino end of p53 between aa 37 and 45. For its detection, biotinylated rabbit anti-mouse secondary antibody (1:100; Amersham, Braunschweig, Germany) and streptavidine-alkaline phosphatase conjugate (1:1000; Gibco-BRL, Eggenstein, Germany) were employed, each applied for 1 h at 37° C. After colour development with NBT/BCIP (NBT: 0.3 mg/ml; BCIP: 0.2 mg/ml; Gibco-BRL, Eggenstein, Germany) for 15 min in the dark, the slides were mounted in glycerine gelatine without counterstaining.

Samples were considered positive for p53 when any of the epithelial/tumour cells revealed nuclear labelling. The proportion of nuclear staining in the tissue was roughly estimated. All cases were grouped into four grades: no positive cell nuclei in the tissue; up to 25% positive nuclei; 26–50% positive nuclei; 50–100% positive nuclei. Staining evaluation was independently performed by two pathologists. Questionable cases were reviewed in conference and discrepancies were resolved by consensus.

Genomic DNA was extracted from cell nuclei of frozen endometrial tissues by standard methods [5] using caesium chloride and guanidinium isothiocyanate, proteinase K/phenol-chloroform extraction and ethanol precipitation. DNA derived from 8-µm paraffin sections was extracted by boiling in the presence of chelating resin (Sigma; [15]). Of the aqueous phase, we used 5–10 µl for polymerase chain reaction (PCR). DNA derived from 8-µm cryostat sections was isolated using 200 µg/ml (final concentration) proteinase K (Merck, Darmstadt, Germany) for digestion.

Fifty-five samples were screened for the presence of p53 gene mutations by TGGE. For DNA amplification, we used GC-clamped, exon-specific primers binding to adjacent intron regions [13]. For the analysis of genomic DNA by TGGE, we amplified exons 5–8 of the p53 gene separately, as described previously [26]. The PCR products were purified by phenol/chloroform/isoamylalcohol extraction (25:24:1) and precipitation with ethanol. The pellets were redissolved in running buffer which consists of 20 mM MOPS, 1 mM EDTA, pH 8.0.

The TGGE of the purified amplification products was performed in a TGGE system from Qiagen (Hilden, Germany), using horizontal 8% polyacrylamide gels in 8 M urea, 20 M MOPS, 1 mM EDTA, 2% glycerol. The electrophoresis conditions for exons 5–8 of the p53 gene were used as previously described [26]. Finally, the bands of the screened PCR products were visualized by silver staining according to the manufacturer's instructions.

Results

The age of all patients ranged from 33 to 90 years with a mean of 59.0 years. The median age of the patients with endometrial carcinoma was 65.0 years.

Normal endometria consisting of proliferative (5), secretory (2), menopausal (1 and atrophic endometrium (5) did not show any immunohistochemical reaction. Most cases of hyperplasia were negative, with immunostaining noticed only focally in 5 instances (Fig. 1a). Although positive cases appeared in all groups of hyperplasia, reactions prevailed in complex and atypical complex samples (Table 1). In addition, we examined and compared hyperplasias within and adjacent to carcinomas (see below). Again, only 2 cases showed p53 staining, which was focal and restricted to atypical complex hyperplasia next to the carcinoma.

Table 1 Alterations of p53 in endometrial hyperplasias by immunohistochemistry

Hyperplasia	Total (100%)	Alteration[a]
Total	39	5 (13)
Simple	11	1 (9)
Complex	17	2 (12)
Atypical complex	11	2 (18)

[a] Percentage in parentheses

Table 2 Correlation of p53 alterations with grading and staging in endometrioid carcinomas

Carcinomas	Total (100%)	Alteration[a]
Total	120	51 (42)
Grade		
G1	44	11 (25)
G2	41	16 (39)
G3	35	24 (69)
Stage (FIGO)		
IA	35	5 (17)
IB	23	5 (22)
IC	36	26 (72)
IIA	0	–
IIB	4	3 (75)
IIC–IVB	0	–
Unknown	22	14 (64)

[a] Percentage in parentheses

Of the 120 endometrial carcinomas, 51 cases (42%) were positive (Table 2, Fig. 1b). Up to 25% positive nuclei were found in 74%, 26–50% positive nuclei in 18%, and 50–100% positive nuclei in 8% of carcinomas. Circumscribed papillary and/or villoglandular areas within some endometrioid carcinomas showed no difference in immunostaining. Squamous metaplasia was negative.

Immunohistochemical results were clearly correlated with grading and staging of carcinomas as demonstrated in Table 2. Only 11 of 44 (25%) highly differentiated tumours (G I) were positive, in contrast to 16 of 41 (39%) moderately differentiated carcinomas, and 24 of 35 (69%) poorly differentiated carcinomas (G III). Tumour stage was known for 98 of the 120 carcinomas. As shown in Table 2, p53 immunoreactions were more frequent in stage IC than in stage IA and IB. Of 36 carcinomas in stage IC, 26 (72%) were positive, compared with only 17% and 22% in stage IA and IB.

Normal endometria and all hyperplasias showed wild-type p53 patterns on TGGE. In contrast, 10 of 34 carcinomas harboured mutant p53 as indicated by electropho-

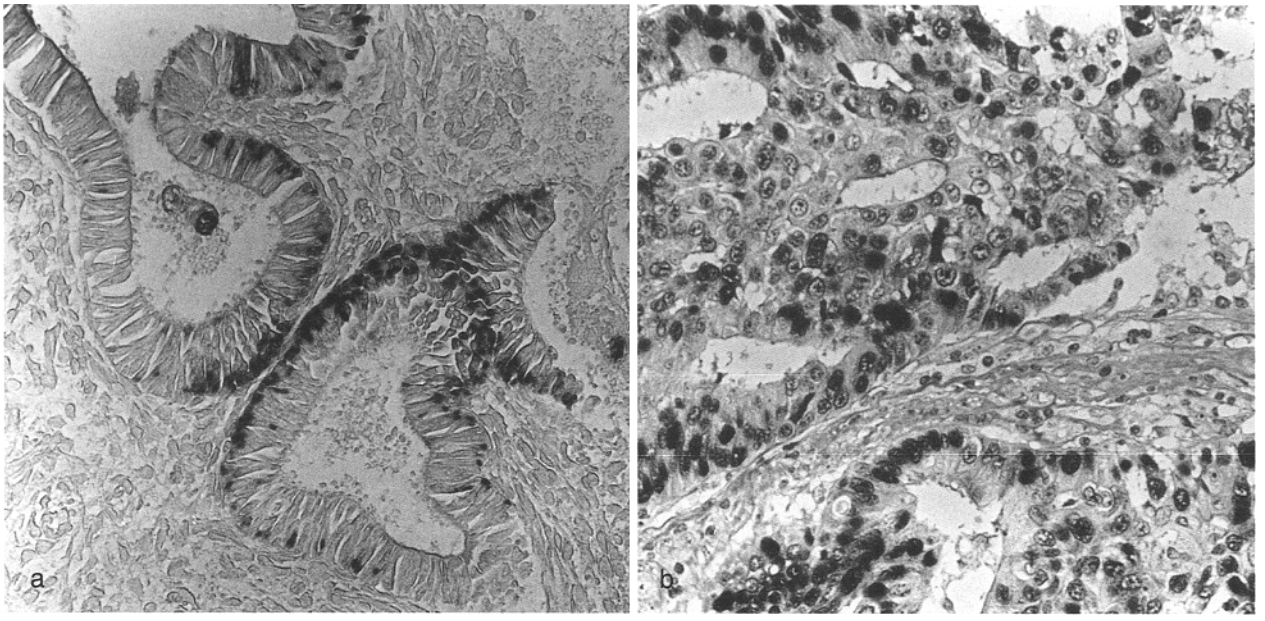

Fig. 1a, b Immunohistochemical detection of p53. Focal nuclear labelling in a case of endometrial hyperplasia (**a**), disseminated reaction in atypical glandular formations of an endometrioid carcinoma (**b**). ×200

Table 3 Alterations of p53 in 34 endometrioid carcinomas – a comparison of immunohistochemistry (*IH*) and temperature-gradient electrophoresis (*TGGE*)

	Total	Bandshift in TGGE	No bandshift in TGGE
IH positive	12	6	6
IH negative	22	4	18

retic mobility shifts and/or homo-/heteroduplex formation. Among them, 6 cases were immunohistochemically positive, and 4 negative (Table 3). Mutations clustered in exon 6 (7 cases) and exon 8 (3 cases). None of the mutations appeared in exons 5 and 7. Figure 2 shows an example of a TGGE run with typical band shift.

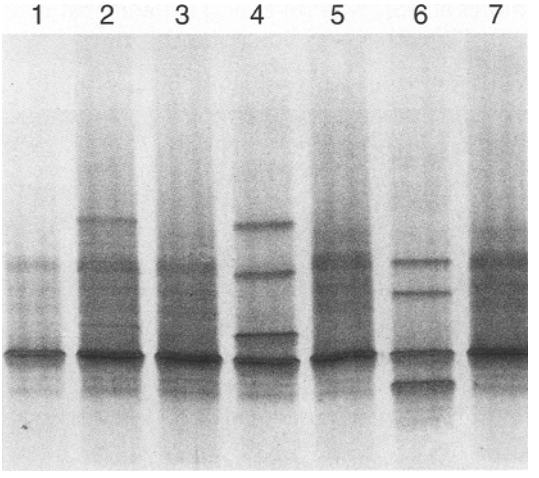

Fig. 2 P53 mutations in endometrioid carcinomas. TGGE analysis of exon 6. In cases with mutant p53 two homoduplexes (*lower bands*) and two heteroduplexes (*upper bands*) are visible (*lanes 2, 4, 6*). Wild-type configuration is shown in the other samples (*lanes 1, 3, 5, 7*)

Discussion

The aim of the present study was to analyse normal and hyperplastic endometrial mucosa as well as endometrial carcinomas of pure endometrioid type for p53 alterations, and to evaluate the relationship between the p53 state and standard predictive markers of malignancy (risk groups of hyperplasia) and grading and staging of carcinomas. Immunohistochemistry and TGGE were used for detection of p53 alterations.

While normal endometria were immunohistochemically negative and always harboured wild-type p53, as shown by other authors [1, 2], some focal staining was found in hyperplasia (Table 1) and adjacent to carcinoma. However, no p53 mutation was detected, which may be due to other regulatory mechanisms leading to p53 accumulation. With a high background of normal cells mutated cells may escape detection by TGGE. Yu et al. [28] observed focal p53 immunostaining in hyperplasias, yet did not correlate their figures to the grade of hyperplasia. In the more detailed contribution of Berchuck et al. [2], staining was confined to atypical hyperplasia (2 of 13 cases). In our material, p53 immunostaining occurs predominantly, but not exclusively, in atypical hyperplasia.

With respect to endometrial carcinoma, the published detection rate of p53 alterations shows a broad range of 9–60% (Table 4). Our cumulative frequency of p53 alterations obtained by immunohistochemistry and TGGE is

Table 4 p53 alterations in endometrial carcinomas (? unknown)

Reference	Year	Methods	Histological type			
			Total (%)	Endometrioid	Serous	Clear cell
Yu et al. [28]	1993	IH	? (47)	?	?	?
Lukes et al. [21]	1994	IH	21/100[a] (21)	?/87	?/6	?/1
Inoue et al. [10]	1994	IH	37/88 (42)	31/82 (38)	5/5 (100)	1/1 (100)
Prat et al. [24]	1994	IH	6/10 (60)	–	6/10	–
Ito et al. [11]	1994	IH	47/221 (21)	47/221 (21)	–	–
Khalifa et al. [14]	1994	IH	6/69 (9)	0/45	5/16 (31)	1/8 (12)
Nielsen et al. [23]	1994	IH	36/109 (33)	36/109 (33)	–	–
Berchuk et al. [2]	1994	IH and PCR/SSCP	22/107 (20)	?	?	?
Enomoto et al. [8]	1995	PCR/SSCP sequencing	5/38 (13)	?	?	?
Present study		IH PCR/TGGE	51/120 (42) 10/34 (29)	51/120 (42) 10/34 (29)	–	–

[a] Including 6 cases of adenosquamous carcinoma

in accordance with most previous reports [10, 23, 25, 28], yet differs from the detection rates published by Enomoto et al. [8], Khalifa et al. [14] and Prat et al. [24] (Table 4). This discrepancy may result from:

1. Selection for tumour types with lower or higher mutation rates (low-grade carcinomas [8, 14] or serious-papillary and other high-grade carcinomas [24]).

2. Type and specificity of primary antibodies used, and/or level of detection methods (pretreatment protocols).

3. Nonsense mutations, splice mutations, and deletions/insertions resulting in frameshift errors, some missense or silent mutations; non-detectable by current p53 immunohistochemistry. In carcinomas, these account for up to one-fifth of p53 mutations [18].

We found a clear correlation between the frequency of p53 accumulation and the malignant potential of the tumours (Table 2). The detection rate went up from 25% in low-grade carcinomas (G1) to 69% in high-grade lesions (G3). This relationship is maintained when tumours are compared by stage. From carcinomas in stage IA, only 17% showed p53 immunostaining, in contrast to 72% in stage IC. These results are in agreement with the data described for ovarian, vulvar and cervical tumours [10, 13, 16, 17, 22]. Interestingly, Nielsen and Nyholm [23] found a dependence of p53 immunoreactions on mitotic activity and nuclear grade in endometrial carcinomas, rather than on grading by architectural criteria alone, or staging.

The use of PCR/TGGE as an additional method to detect p53 alterations was another important aspect of our examinations. In previous studies, it was demonstrated that TGGE is very efficient in the detection of p53 mutations [13, 26]. As shown in Table 3, in 50% (6/12) of immunohistochemically positive cases analysed by TGGE band shifts were found. Furthermore, 18% (4/22) of immunohistochemically negative cases were positive in TGGE. Further sequencing analysis of PCR products from TGGE-positive cases is in progress to obtain insight into the complex figure of p53 mutations, and their functional implications.

The power of p53 alterations to predict malignancy needs to be elucidated in curettage specimens, in order to classify risky hyperplasia better, and to assist in the grading of carcinomas. By modern technical means and adequate follow-up, cases with more or less favourable prognosis may be segregated in the future.

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