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Cell cycle regulators and apoptosis associated proteins in relation to proliferative activity and degree of apoptosis in HNPCC versus sporadic endometrial carcinoma

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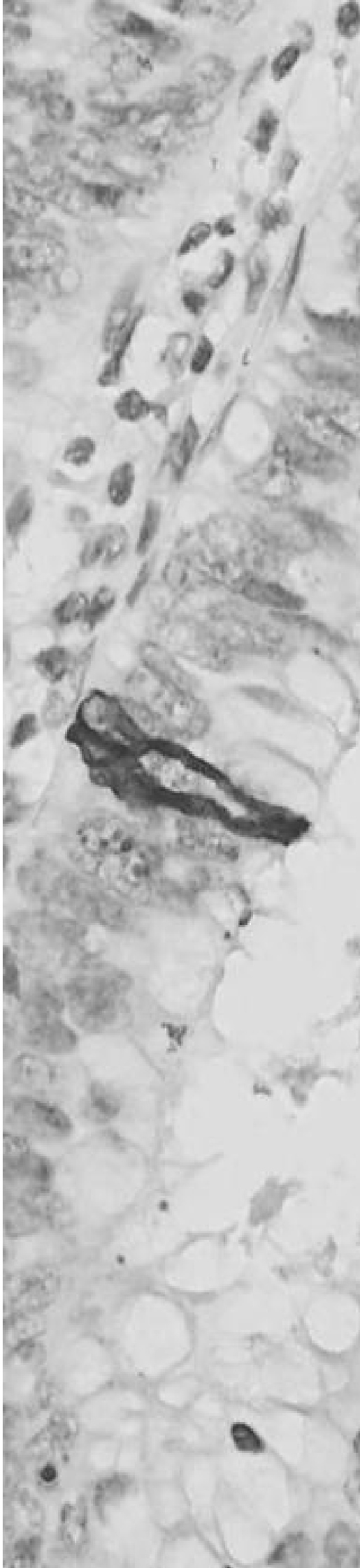

Abstract

Background **- Mismatch repair gene malfunction occurs early in the carcinogenesis of hereditary nonpolyposis colorectal cancers (HNPCCs) leading to an accelerated accumulation of mutations and possibly to change in expression of cell cycle proteins. There is strong evidence that tumorigenesis in HNPCCs differs from sporadic ones. HNPCC-related endometrial cancers are less well studied. Our aim was to compare expression of cell cycle and apoptosis-related proteins in relation to proliferation and apoptosis in HNPCC-related and sporadic endometrial cancers to identify differences in their carcinogenetic pathways.**

Methods and results **- 18 HNPCC-related endometrial cancers, each matched by tumor type, stage and grade with two sporadic endometrial cancers, were examined for proliferation, apoptosis and the expression of oestrogen and progesterone receptors, cyclin B1, D3 and E, p21, p27, bcl2, bax, p53 and cox-2. No differences in proliferation and apoptosis indices were detected between HNPCC and sporadic endometrial cancers. Cyclin B1 expression in HNPCC was significantly higher than in sporadic cancers. More HNPCC-related endometrial cancers had total loss of bax expression.**

Conclusions **- Apart from differences in cyclin B1 and bax expression, HNPCC-related and sporadic endometrial cancer are comparable. The subtle differences detected are consistent with the minor clinical diversity between HNPCC-related and sporadic endometrial cancers.**

In hereditary nonpolyposis colorectal cancer (HNPCC) germline mutations in DNA mismatch repair (MMR) genes, i.e. *MLH1, MSH2* and *MSH6*, lead to an increased risk of colorectal as well as extracolonic cancers. Endometrial cancer is the most frequently occurring extracolonic tumorand in genetically predisposed women the cumulative lifetime risk for endometrial cancer may exceed that of colorectal cancer. HNPCC endometrial cancers are on average diagnosed at an earlier age than nonhereditary endometrial cancers (further referred to as sporadic endometrial cancers) but have a prognosis similar to sporadic endometrial cancers.[1]

In HNPCC patients, loss of mismatch repair function will, among others, lead to mutations in genes with microsatellites (short tandem repeat sequences) in their coding regions. Mutations in these genes may lead to alternative pathways of carcinogenesis compared sporadic cases.[2] The consequences of MMR dysfunction on the pathogenesis of endometrial cancers, including proliferative activity and the rate of apoptosis, and the activity of proliferation- and apoptosis-regulating proteins have received little attention.

The current study was undertaken to explore differences in carcinogenic pathways between HNPCC-related and sporadic endometrial cancer by evaluating proliferation and apoptotic indices and the immunohistochemical expression of proliferation- and apoptosis-regulating proteins. We investigated proliferation-stimulating proteins, cyclin B1, D3 and E, which regulate the cell cycle at the G_2/M, G_0/S and G_1/S phase. Moreover, *cyclin B1* and *E* have been shown to be major proliferation regulating genes in sporadic endometrial cancers.[3] We determined the expression of p53, the product of a tumor suppressor gene, as it is an important gene in the apoptotic route. P53 blocks cell cycle progression from G_1 into S phase by G_1 arrest through transcriptional regulation of the cyclin-dependent kinase inhibitor p21. P53 overexpression in sporadic endometrial cancer is related to high-grade morphology or a non-endometrioid morphology.[4,5] Whether *p53* mutations leading to p53 overexpression play a role in HNPCC endometrial cancer is not known. P27 is another cyclin-dependent kinase inhibitor downstream of p21 that also ultimately leads to cell cycle arrest. A second protein regulated by p53 is bcl-2, which appears to function as an associated heterodimer of bcl-2-bax that inhibits apoptosis. Bax, alternatively, promotes apoptosis. It is vulnerable to mutations in case of MMR dysfunction as the *bax* gene contains a nucleotide repeat sequence.[6] *Cyclooxygenase-2* (cox-2) has gained increasing attention as it is a potential target for chemoprevention. Cox-2 expression is elevated in several malignant tumors and probably has

a role in programmed cell death.[7] Finally, given that the presence of oestrogen receptors (ER) and progesterone receptors (PR) has been linked to better prognosis and to the carcinogenesis of endometrial cancers, the expression of ER and PR were also determined.[8]

Materials and methods

Paraffin-embedded tissue blocks of HNPCC-related and sporadic (control group) endometrial cancers were retrieved from the archives of the Department of Pathology of the University Medical Center Groningen. Women fulfilling the Amsterdam Criteria [9] and/or having a known germline mutation in one of the DNA mismatch repair genes were included in the HNPCC group. The control group consisted of endometrial cancers from women without a family history of endometrial cancer or colorectal cancer. Tumors were classified according to histological type, stage and grade following the current recommendations of the International Federation of Gynaecology and Obstetrics (FIGO).[10] Each HNPCC-related endometrial cancer was matched with two sporadic endometrial cancers of the same tumor type and FIGO stage and grade. It was not possible to match the HNPCC and sporadic tumors by age at diagnosis. Endometrial cancers from patients with a history of hormone replacement therapy were excluded from the study.

Immunohistochemistry

A slide of each endometrial cancer was stained in one run per antibody. If staining was not adequate in slides, these specific slides with inclusion of a well-stained slide from the first batch as reference were stained again. As negative control, the primary antibody was substituted with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS).

Table 1 summarizes the 11 primary antibodies, the companies from which they were purchased, the dilution at which they were used and the corresponding technique for antigen retrieval. Serial 3µm thick sections were cut from paraffin blocks, fixed onto coated slides and deparaffinized. For the high-pressure cooker antigen retrieval method, slides were immersed in 200µl blocking reagent (Boehringer Mannheim, Germany) and underwent 3 sessions of 5 minutes at 115°C. The endogenous peroxidase activity was quenched by incubation with 30% H_2O_2 in PBS for 30 minutes. Subsequently, the primary antibody diluted in PBS-1% BSA was

applied for one hour at room temperature. In the microwave antigen retrieval method deparaffinized, rehydrated sections were submersed either in preheated 10mM citrate buffer (pH 6.0) or in 1mM EDTA (pH 8.0), as noted in *table 1*, and heated for 8 minutes at 700 watts in a microwave. After cooling at room temperature for 7 minutes the sections were thoroughly rinsed with PBS for 5 minutes and the primary antibody was applied, diluted in PBS-1% BSA as described in *table 1*, for one hour at room temperature. In both the high-pressure cooker and the microwave retrieval method, the sections were consecutively incubated with secondary antibody, i.e. rabbit antimouse peroxidase (diluted 1:50 in PBS-1% BSA) and tertiary antibody, i.e. goat antirabbit peroxidase (diluted 1:50 in PBS-1% BSA) for 30 minutes. The peroxidase activity was visualized with diaminobenzidine. The sections were counterstained with haematoxylin.

Apoptosis was quantified on haematoxylin and eosin-stained sections using light microscopy with an eyepiece grid. Apoptotic cells were morphologically identified using the criteria as described by others.[11,12] This approach has been shown to have the same yield as techniques such as TUNEL.[13] Apoptotic bodies shed into the gland lumen or in necrotic areas were not counted.

Scoring Method

Without knowledge of the clinical data, two authors (FR and HH) scored all stained slides. The immunoreactivity of each antibody was scored in the same area of the carcinoma. MMR protein expression was scored as positive (present) or negative (absent). Proliferation was quantified as labelling index, i.e. the percentage of the positively stained nuclei. Using an eye-piece grid, the apoptotic index was quantified by counting the number of apoptotic cells crossing the consecutive horizontal lines per total number of counted epithelial cells on the horizontal lines in 10 high power fields (HPF= 400 x magnification). Estrogen and progesterone receptors, cyclin B1, D3 and E, p21 and cox-2 were scored quantitatively as a labelling index. The intensity of p27, bax, and bcl-2 staining was heterogeneous within a tumor. These assessments were therefore quantified with a weighted score by multiplying the intensity (1= weak staining, 2= moderate, 3= strong) by the percentage of stained cells. A cut-off level of 50 % of cells strongly staining for p53 was used to define overexpression. In our laboratory, this cut-off level correlates well to p53 somatic mutations in endometrial cancer (data not published).

Statistics

Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, Munich, Germany) for Windows. The statistical difference between HNPCC and sporadic endometrial cancers for each protein was calculated using the nonparametric Mann-Whitney U-test. The relations between each of the investigated parameters were analyzed using Pearson's correlation coefficient. For both tests, $p<0.05$ was considered significant.

Table 1. Antibodies and antigen retrieval methods used for immunohistochemistry.

Protein	Antigen retrieval	Clone	Company	Dilution
ER	Microwave EDTA	6F11	Novocastra, Newcastle, UK	1:400
PR	Microwave EDTA	1A6	Novocastra, Newcastle, UK	1:400
Ki-67	High-pressure cooker	MIB-1	Immunotech, Westbrook, ME	1:400
Cyclin B1	High-pressure cooker	7A9	Novocastra, Newcastle, UK	1:200
Cyclin D3	Microwave EDTA	DCS-22	Novocastra, Newcastle, UK	1:50
Cyclin E	Microwave EDTA	13A3	Novocastra, Newcastle, UK	1:10
P21	High-pressure cooker	WAF1(Ab-1)	Oncogene, Darmstadt, Germany	1:50
P27	High-pressure cooker	1B4	Novocastra, Newcastle, UK	1:50
Bax	Microwave citrate	B-9	St Cruz Biotechnology, St Cruz, CA	1:200
Bcl-2	High-pressure cooker	MAb 124	DAKO, Glostrup, Denmark	1:50
P53	High-pressure cooker	B-p53-12-1	Biogenex, San Ramon, CA	1:400
Cox-2	Microwave EDTA	33	Transduction Lab., Lexington, KY	1:50
MLH1	High-pressure cooker	G168-728	PharMingen, San Diego, USA	1:500
MSH2	High-pressure cooker	Ab-2	Calbiochem, San Diego, USA	1:100
MSH6	High-pressure cooker	44	Transduction Lab., Lexington, KY	1:200

Results

Patient characteristics

A total of 18 HNPCC-related endometrial cancers could be retrieved from the archive and were included in the study. Six women had a germline mutation in *hMLH1*, two women had a mutation in *hMSH2* and two in *hMSH6*. The remaining eight women belonged to families fulfilling the Amsterdam criteria. Either no mutational study had been done in these patients or a mutation had not yet been found. One woman belonged to an HNPCC-family in which the mutation (in MLH1) was known but the patient chose not to be genetically tested. Three women were postmenopausal, one was in the climacteric phase and the remaining 14 women were premenopausal at time of diagnosis. The average age at diagnosis was 45 years. In the

control group, the average age at diagnosis was 65 years. Twenty-six of these women were postmenopausal, one was in the climacteric phase and nine were premenopausal at diagnosis.

Histology

Fifteen HNPCC-related endometrial cancers were endometrioid adenocarcinomas, one was a pure clear cell endometrial cancer and two were mixed clear cell and endometrioid adenocarcinomas (clear cell component <50%). Data concerning stage and grade of the HNPCC carcinomas are summarized in *table 2*. Two sporadic endometrial cancers were matched to each HNPCC endometrial cancer for tumor type, stage and grade.

Table 2. Tumortype, stage and grade of the HNPCC endometrial cancers.

Tumour stage & grade	**n**	**Tumour stage & grade**	**n**
IA GI	3	IIIA GIII	1
IB GI	4	IIIC GIII	1
IB GII	2	III A (clear cell)	3
IIA GII	1		
IIB GrII	2		
IIB GIII	1		

Imunnohistochemistry

Mismatch repair

In proven DNA mismatch repair gene mutation carriers, the endometrial tumors showed loss of the MMR protein expression corresponding to the mutated gene. Three of the eight HNPCC-related endometrial cancers from women fulfilling the Amsterdam criteria but from whom the genetic status was not known stained positive for all three MMR proteins, four showed loss of MLH1 expression and one of MSH2 expression. All sporadic endometrial cancers expressed the three MMR proteins.

The immunoreactivity of the proliferation- and apoptosis-regulating proteins was heterogeneous in most HNPCC and sporadic tumors. *Figure 1* illustrates several immunohistochemical results in one HNPCC-related endometrial tumor. The mean immunoreactivity for each protein is noted in *table 3*. Estrogen and progesterone receptor staining in HNPCC-related endometrial cancers was not significantly different from that in sporadic endometrial cancers. A wide range in Mib-1 labelling indices was observed in HNPCC-related and sporadic endometrial cancer: 5-80% and 5-70%, respectively. A trend towards higher proliferation in HNPCC-related endometrial cancers compared to sporadic endometrial cancers was observed (p=0.084). The immunoreactivity of proliferation-regulating proteins, cyclin D3 and E, and cyclin-dependent kinase inhibitors, p21 and p27, was similar in HNPCC-related and sporadic endometrial cancers. However, cyclin B1 expression was significantly higher in HNPCC-related than in sporadic endometrial cancers.

The apoptotic index ranged from 1-22% in HNPCC-related and 1-32% in sporadic endometrial cancers. The apoptotic index in HNPCC-related endometrial cancers was not significantly different from that in sporadic cancers. The apoptosis-regulating proteins, bax and bcl-2, were expressed in a similar number of cells in HNPCC-related as in sporadic endometrial cancers. However, total loss of bax expression was observed in three HNPCC-related cancers while all sporadic cancers expressed bax. Bax expression in HNPCC-related and sporadic endometrial cancer is significantly different when using this classification method, presence or absence of bax expression (p=0.013). The ratio bax:bcl-2 in HNPCC-related was not significantly different from that in sporadic endometrial cancers (mean bax:bcl-2, 46:1 vs. 29:1, p=0.868, respectively). No significant difference was observed in cox-2 and p53 expression between HNPCC-related and sporadic endometrial cancers. P53 overexpression was seen in two (11%) of the 18 HNPCC-related endometrial cancers. One of these cancers had pure clear cell histology, the other was a mixed clear cell and endometrioid tumour. In the control group, 11 (31%) tumours, five clear cell and six endometrioid (five grade III and one grade I), overexpressed p53.

Table 3. Immunohistochemistry results of the HNPCC and sporadic endometrial cancers.

Staining categories	†		**p-value**
	HNPCC	Sporadic	
Estrogen receptor (%)	45 ± 10	38 ± 15	n.s.
Progesterone receptor (%)	28 ± 9	36 ± 6	n.s.
Mib-1 labelling index (%)	35 ± 6	25 ± 3	n.s.
Cyclin B1 (%)	27 ± 6	12 ± 2	p=0.038
Cycin D3 (%)	20 ± 7	9 ± 3	n.s.
Cyclin E (%)	39 ± 7	27 ± 5	n.s.
P21 (%)	11 ± 4	13 ± 3	n.s.
P27 (%)	48 ± 9	38 ± 6	n.s.
Apoptotic index (%)	4 ± 1	3 ± 1	n.s.
Bax (%)	144 ± 26	190 ± 15	n.s.
Bcl-2 (%)	67 ± 21	81 ± 15	n.s.
Cox-2 (%)	33 ± 8	36 ± 6	n.s.
P53 (n)	2/18	11/36	n.s.

Correlation

In table 4 the correlation between various immunohistochemical results are noted.

In HNPCC-related neoplasms, poor tumor differentiation (high grade) was correlated with higher stage (p=0.025). PR immunoreactivity was inversely correlated with the grade of the HNPCC-related endometrial cancers (p=0.002). Absent ER staining (p=0.039) and p53 overexpression (p<0.001) were correlated with clear cell histology. ER immunoreactivity was related to bcl-2 expression (p=0.018) and inversely related to p53 expression (p=0.022). Neither the Mib-1 labelling index nor the apoptotic index was related to the grade or stage of the HNPCC-related endometrial cancers. Cyclin B1 expression was related to stage (p=0.026), grade (p=0.021) and a higher Mib-1 labelling index (p=0.003). The degrees of expression of the apoptosis-regulating proteins were not correlated with the apoptotic index.

Table 4A.

	Stage	Grade	Histo	PR	ER	MIB	B1	D3	E	P21	P27	Apop	Bax	Bcl2	Cox	P53
Stage	-	0.025	-0.668	-0.180	-0.805	0.281	**0.026**	-0.459	0.538	-0.302	-0.474	0.657	-0.700	-0.125	0.757	1.00
Grade		-	**0.035**	**-0.002**	-0.129	*0.063*	**0.021**	-0.711	0.109	-0.458	0.313	0.432	*-0.077*	-0.148	*0.065*	0.106
Histo			-	-0.166	**-0.039**	0.512	-0.668	-0.545	0.195	0.882	0.344	0.214	-0.856	0.160	0.203	**<.001**
PR				-	0.204	-0.952	-0.292	0.206	-0.704	0.424	-0.432	-0.152	0.345	0.656	0.739	-0.279
ER					-	-0.567	-0.750	-0.824	-0.215	-0.677	0.837	-0.142	-0.938	**0.018**	0.963	**-0.022**
MIB						-	**0.003**	0.184	0.476	0.121	*-0.074*	0.547	-0.270	-0.208	*0.885*	-0.680
B1							-	0.224	0.664	-0.558	-0.258	0.231	-0.352	-0.236	-0.893	-0.311
D3								-	-0.481	*0.059*	0.901	0.689	-0.359	-0.979	-0.864	-0.823
E									-	0.783	0.766	0.254	-0.968	-0.682	*0.059*	*0.096*
P21										-	-0.371	0.236	0.858	0.388	-0.519	0.992
P27											-	0.235	0.822	0.579	*0.063*	*0.067*
Apop												-	0.187	-0.885	-0.258	0.951
Bax													-	0.622	-0.482	0.939
Bcl2														-	0.708	0.272
Cox															-	*0.066*
P53																-

Table 4B.

	Stage	Grade	Histo	PR	ER	MIB	B1	D3	E	P21	P27	Apop	Bax	Bcl2	Cox	P53
Stage	-	**0.025**	**0.001**	-0.471	0.166	**0.043**	0.990	0.719	0.675	*0.059*	-0.159	0.758	0.733	-0.580	0.162	**0.006**
Grade		-	**0.003**	**-0.044**	**-0.026**	**0.038**	0.007	-0.307	**0.025**	*0.071*	-0.078	0.589	-0.564	-0.209	*0.063*	**<0.001**
Histo			-	-0.644	0.254	-0.128	0.959	-0.631	0.781	*0.056*	-0.431	0.254	-0.956	**-0.039**	**0.036**	**0.001**
PR				-	**0.001**	-0.553	-0.502	*-0.082*	**-0.004**	-0.337	-0.830	-0.145	**0.001**	**0.031**	*0.052*	*-0.075*
ER					-	0.163	-0.983	-0.171	-0.824	0.589	-0.481	-0.258	**0.008**	**0.027**	-0.965	**-0.001**
MIB						-	-0.401	0.212	**0.028**	0.960	-0.251	0.365	-0.888	-0.235	*0.061*	0.801
B1							-	-0.581	0.092	**0.023**	-0.947	-0.689	-0.972	0.437	*0.057*	0.402
D3								-	**0.007**	0.588	0.988	-0.129	*-0.225*	-0.129	0.524	-0.664
E									-	**0.042**	0.835	0.825	0.609	0.503	**0.003**	**0.047**
P21										-	0.635	0.521	**0.014**	**0.042**	**0.043**	*0.077*
P27											-	0.156	0.842	**0.006**	0.183	-0.538
Apop												-	0.142	-0.097	0.094	0.386
Bax													-	**0.004**	0.753	0.493
Bcl2														-	*0.068*	0.632
Cox															-	0.168
P53																-

In sporadic endometrial cancers high stage was correlated with a high Mib-1 labelling index (p=0.043) and p53 overexpression (p=0.004). PR immunoreactivity was inversely correlated with tumor grade (p=0.044), while Mib-1 labelling index (p=0.038), cyclin B1 expression (p=0.007), cyclin E expression (p=0.025) and overexpression of p53 (p<0.001) were positively correlated with grade in sporadic endometrial cancers. Only cyclin E expression (p=0.035) was positively correlated with the Mib-1 labelling index. The apoptotic index was not correlated with stage or grade in sporadic endometrial cancers. The apoptosis-regulating proteins did not correlate with the apoptotic index. However, an increased bax:bcl-2 ratio was related to an increased apoptotic index (p=0.030). Bax and bcl-2 expression were both positively correlated with ER (p=0.008, p=0.027, respectively) and PR (p=0.001, p=0.031, respectively) immunoreactivity and with p21 expression (p=0.014, p=0.042, respectively). Cox-2 expression in sporadic endometrial cancers was positively correlated with cyclin B1 (p=0.042) and cyclin E (p=0.003) expression but not with the Mib-1 labelling index. Clear cell histology was correlated with high cox-2 expression (p=0.036) and overexpression of p53 (p=0.003).

Discussion

Endometrial cancer is the most common extracolonic tumor in HNPCC but little is known about its carcinogenesis and if it differs from non-hereditary endometrial cancers. The present study reports proliferation and apoptosis in relation to a number of proliferation- and apoptosis-regulating proteins in HNPCC-related and sporadic endometrial cancers. In contrast to a similar immunohistochemical study of colorectal lesions [14], immunohistochemistry demonstrated few differences between HNPCC-related and sporadic endometrial cancers. Apparently, the differences in carcinogenesis of HNPCC-related and sporadic endometrial cancers are subtle.

The endometrial cancers included in our study are representative for those diagnosed in HNPCC patients. The stage and grade distribution of the HNPCC-related endometrial cancers are in accordance with the HNPCC endometrial cancers reported by Boks *et al.* [1] However, the series of Boks *et al.* did not include clear cell endometrial cancers [1], whereas we found one pure clear cell and two mixed endometrioid/clear cell endometrial cancers. Even though no HNPCC-related clear cell endometrial cancers have been described in the literature, seven

microsatellite unstable sporadic clear cell and mixed endometrioid and clear cell carcinomas have been described.[15-17] Approximately 10% of clear cell carcinomas have been found to demonstrate the mutator phenotype, microsatellite instability.[17] The percentage of clear cell endometrial cancers in our HNPCC population is similar to the incidence of clear cell endometrial cancers found in the general population in the northern part of the Netherlands (Schaapveld, Comprehensive Cancer Center North Netherlands, unpublished). Thus tumor type does not discern HNPCC-related endometrial cancers from sporadic ones.

A tendency towards an increased proliferation rate was observed in HNPCC-related endometrial cancers in comparison with sporadic endometrial cancers. HNPCC-related endometrial cancers differed from sporadic endometrial cancers by increased cyclin B1 expression. The increased cyclin B1 expression possibly indicates that more cells were in the G_2 phase of the cell cycle. Having passed the G_1 phase, the cell may become refractory to external stimuli, such as growth inhibitors. In accordance with the above-mentioned theory, we found no inverse correlation between p21 or p27 expression and the Mib-1 labeling index. Cyclin B1, however, correlated with the Mib-1 labeling index, supporting the concept that cyclin B1 is a major cell cycle proliferation regulator in HNPCC-related endometrial cancers.

The immunohistochemical apoptotic profile of HNPCC-related endometrial cancers did not differ from that of sporadic endometrial cancers. No significant differences were found in the apoptotic index or expression of apoptosis regulators. However, when a dual scoring method (presence versus absence of staining) was applied, bax expression was significantly different between HNPCC-related and sporadic endometrial cancers. The finding of total loss of bax expression in three HNPCC-related endometrial cancers while all sporadic endometrial cancers expressed bax to a certain degree, points to *bax* as a mutation-susceptible gene in HNPCC. Vassileva *et al.* concluded that *bax* is an early mutational target in the development of microsatellite unstable endometrial cancers[18], while de Leeuw *et al.* demonstrated instability of the *bax* gene in 22% of HNPCC-related endometrial cancers.[19] The *bax* gene contains a repeat sequence and is thus vulnerable to mutation caused by MMR dysfunction. Even though *bax* seems to be a target gene of the microsatellite phenotype in endometrial cancers, the carcinogenic consequences are disputable. The altered bax expression had a limited functional role as the apoptotic index was not influenced. Apparently the loss of

expression of bax, which theoretically should result in a decrease in apoptosis, was compensated by other apoptosis-inducing pathway(s).

In general, the immunohistochemical profile of the sporadic endometrial cancers was in accordance with previous studies.[3,19-31] In the present study, the mean Mib-1 labelling index was 25%, which is slightly lower than that reported by others, 30-40%.[11,31-33] The manner of selection of the control group could explain this slight discrepancy in Mib-1 labelling index. The Mib-1 labelling index in the sporadic endometrial cancers seemed to be predominantly regulated by cyclin E. Previously cyclin B1 and E have been reported to be involved in proliferation regulation in endometrial cancer.[3] Cyclin E pushes the cell through the cell cycle from late G_1 phase to DNA synthesis. On the other hand, p21 expression results in cell cycle arrest in G_1 phase. Accumulation of p21 is relatively common in endometrial cancers [20,22] as also seen in our study but p21 expression was not found to be inversely correlated to proliferation. Michieli *et al.* observed that at least two pathways can lead to increased p21 expression; a p53-independent pathway, triggered by growth factors and associated with cell growth, and a p53-dependent pathway, elicited by DNA damage and resulting in growth arrest.[34] As in most studies, we found no relation between p53 and p21.[20,22] The more abundant cyclin E may neutralize the possible proliferation inhibiting effects of p21. Cox-2, which is expressed in most endometrial cancers, especially in clear cell carcinomas, was correlated to increased cyclin E expression and tends to correlate positively with Mib-1 labelling indices. The observed cox-2 immunoreactivity offers perspectives for chemoprevention with non-steroidal anti-inflammatory drugs of HNPCC-related as well as sporadic endometrial cancers.

The apoptotic index observed in the sporadic endometrial cancers was in accordance with previously documented apoptotic indices.[11] In the present study, one third of the sporadic tumors overexpressed p53. In accordance with other studies, P53 overexpression strongly correlates with the histological type (non-endometrioid versus endometrioid) and among endometrioid carcinoma with grade and stage. [4,5] P53 overexpression has previously also been related to hormone receptor negative tumors and poor survival.[31] Our results support the former relation. We found no direct correlation between the apoptosis-regulating proteins and the apoptotic index, leading us to speculate on other apoptosis-regulating genes which play a larger role in apoptosis regulation in endometrial cancers. A possible candidate is PTEN, an

essential lipid phosphate that is a negative regulator of anti-apoptosis pathways and has been found to be mutated in many endometrial cancers. [35]

The subtle differences found between HNPCC-related and sporadic endometrial cancers are in accordance with the clinical study of Boks *et al.,* who found no difference in survival between HNPCC-related and sporadic tumors.[1] A number of factors limited the possibility of detecting elucid differences if present. First, the present study is restricted by the relatively small number of HNPCC-related endometrial cancers of *MLH1*, *MSH2* and *MSH6* mutation carriers. Not all HNPCC-related endometrial cancers were from proven mutation carriers or had a known microsatellite status. Secondly, HNPCC-related endometrial cancers have a lower mutational rate than HNPCC-related colorectal tumors, which may explain why clear differences can be found by immunohistochemistry between HNPCC-related and sporadic colorectal tumors, but not between the two groups of endometrial cancers.[6] Furthermore, in HNPCC-related endometrial cancers, the microsatellite pattern has been shown to be very heterogeneous within and between tumours.[36] Intra- and intertumoral heterogeneity of most immunohistochemical reactivity was also observed in the HNPCC-related endometrial cancers, which may reflect diverse microsatellite patterns. Due to the wide range in immunoreactivity observed for each protein, a larger group of tumors may be necessary to detect statistical differences.

In conclusion, despite the underlying differences in pathogenesis, dysfunctional and functional mismatch repair genes, the carcinogenic pathway of HNPCC-related endometrial cancers differs only in a subtle manner (i.e. higher cyclin B1 expression and more often total loss of bax expression) from sporadic endometrial cancers. The subtle differences detected are consistent with the minor clinical disparity between HNPCC-related and sporadic endometrial cancers.

References

1. Boks DE, Trujillo AP, Voogd AC, Morreau H, Kenter GG, Vasen HF. Survival analysis of endometrial carcinoma associated with hereditary nonpolyposis colorectal cancer. *Int J Cancer* 2002;102;198-200.

2. Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, Boyd J. Genetic instability of microsatellites in endometrial carcinoma. *Cancer Res* 1993;53;5100-5103.

3. Milde-Langosch K, Bamberger AM, Goemann C *et al.* Expression of cell-cycle regulatory proteins in endometrial carcinomas: correlations with hormone receptor status and clinicopathologic parameters. *J Cancer Res Clin Oncol* 2001;127;537-544.

4. Lax SF, Kendall B, Tashiro H, Slebos RJ, Hedrick L. The frequency of p53, K-ras mutations, and microsatellite instability differs in uterine endometrioid and serous carcinoma: evidence of distinct molecular genetic pathways. *Cancer* 2000;88;814-24.

5. Sherman ME, Bur ME, Kurman RJ. p53 in endometrial cancer and its putative precursors: evidence for diverse pathways of tumorigenesis. *Hum Pathol* 1995;26;1268-74.

6. Duval A, Reperant M, Compoint A *et al.* Target Gene Mutation Profile Differs between Gastrointestinal and Endometrial Tumors with Mismatch Repair Deficiency. *Cancer Res* 2002;62;1609-1612.

7. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994;107;1183-1188.

8. Fukuda K, Mori M, Uchiyama M, Iwai K, Iwasaka T, Sugimori H. Prognostic significance of progesterone receptor immunohistochemistry in endometrial carcinoma. *Gynecol Oncol* 1998; 69;220-225.

9. Vasen HF, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology* 1999;116;1453-1456.

10. Scully RE, Bonfiglio TA, Kurman RJ, Silverberg SG, Wilkinson K. *International histological classification and typing of female genital tract tumours.* New York: Springer-Verlag 1994.

11. Ioffe OB, Papadimitriou JC, Drachenberg CB. Correlation of proliferation indices, apoptosis, and related oncogene expression (bcl-2 and c-erbB-2) and p53 in proliferative, hyperplastic, and malignant endometrium. *Hum Pathol* 1998;29;1150-1159.

12. Mourits MJ, Hollema H, De Vries EG, Ten Hoor KA, Willemse PH, van der Zee AG. Apoptosis and apoptosis-associated parameters in relation to tamoxifen exposure in postmenopausal endometrium. *Hum Pathol* 2002;33;341-346.

13. Drachenberg CB, Ioffe OB, Papadimitriou JC. Progressive increase of apoptosis in prostatic intraepithelial neoplasia and carcinoma: comparison between in situ end-labeling of fragmented DNA and detection by routine hematoxylin-eosin staining. *Arch Pathol Lab Med* 1997;121;54-58.

14. Edmonston TB, Cuesta KH, Burkholder S *et al.* Colorectal carcinomas with high microsatellite instability: defining a distinct immunologic and molecular entity with respect to prognostic markers. *Hum Pathol* 2000;31;1506-1514.

15. Tibiletti MG, Furlan D, Taborelli M *et al.* Microsatellite instability in endometrial cancer: relation to histological subtypes. *Gynecol Oncol* 1999;73;247-252.

16. Duggan BD, Felix JC, Muderspach LI, Tourgeman D, Zheng J, Shibata D. Microsatellite instability in sporadic endometrial carcinoma. *J Natl Cancer Inst* 1994;86;1216-1221.

17. Basil JB, Goodfellow PJ, Rader JS, Mutch DG, Herzog TJ. Clinical significance of microsatellite instability in endometrial carcinoma. *Cancer* 2000;89;1758-1764.

18. Vassileva V, Millar A, Briollais L, Chapman W, Bapat B. Genes involved in DNA repair are mutational targets in endometrial cancers with microsatellite instability. *Cancer Res* 2002;62;4095-4099.

19. de Leeuw WJ, Dierssen J, Vasen HF *et al.* Prediction of a mismatch repair gene defect by microsatellite instability and immunohistochemical analysis in endometrial tumours from HNPCC patients. *J Pathol* 2000;192;328-335.

20. Ito K, Sasano H, Matsunaga G *et al.* Correlations between p21 expression and clinicopathological findings, p53 gene and protein alterations, and survival in patients with endometrial carcinoma. *J Pathol* 1997;183;318-324.

21. Lukas J, Groshen S, Saffari B *et al.* WAF1/Cip1 gene polymorphism and expression in carcinomas of the breast, ovary, and endometrium. *Am J Pathol* 1997;150;167-175.

22. Backe J, Gassel AM, Hauber K *et al.* p53 protein in endometrial cancer is related to proliferative activity and prognosis but not to expression of p21 protein. *Int J Gynecol Pathol* 1997;16;361-368.

23. Burton JL, Stewart RL, Heatley MK, Royds JA, Wells M. p53 expression, p21 expression and the apoptotic index in endometrioid endometrial adenocarcinoma. *Histopathology* 1999;35;221-229.

24. Taskin M, Lallas TA, Barber HR, Shevchuk MM. bcl-2 and p53 in endometrial adenocarcinoma. *Mod Pathol* 1997;10;728-734.

25. Henderson GS, Brown KA, Perkins SL, Abbott TM, Clayton F. bcl-2 is down-regulated in atypical endometrial hyperplasia and adenocarcinoma. *Mod Pathol* 1996;9;430-438.

26. Stewart RL, Royds JA, Burton JL, Heatley MK, Wells M. Direct sequencing of the p53 gene shows absence of mutations in endometrioid endometrial adenocarcinomas expressing p53 protein. *Histopathology* 1998;33;440-445.

27. Caduff RF, Johnston CM, Svoboda-Newman SM, Poy EL, Merajver SD, Frank TS. Clinical and pathological significance of microsatellite instability in sporadic endometrial carcinoma. *Am J Pathol* 1996;148;1671-1678.

28. Berchuck A. Biomarkers in the endometrium. *J Cell Biochem Suppl* 1995;23;174-178.

29. Kounelis S, Kapranos N, Kouri E, Coppola D, Papadaki H, Jones MW. Immunohistochemical profile of endometrial adenocarcinoma: a study of 61 cases and review of the literature. *Mod Pathol* 2000;13;379-388.

30. Lax SF, Pizer ES, Ronnett BM, Kurman RJ. Clear cell carcinoma of the endometrium is characterized by a distinctive profile of p53, Ki-67, estrogen, and progesterone receptor expression. *Hum Pathol* 1998;29;551-8.

31. Yamauchi N, Sakamoto A, Uozaki H, Iihara K, Machinami R. Immunohistochemical analysis of endometrial adenocarcinoma for bcl-2 and p53 in relation to expression of sex steroid receptor and proliferative activity. *Int J Gynecol Pathol* 1996;15;202-208.

32. Kuwashima Y, Uehara T, Kishi K, Shiromizu K, Matsuzawa M, Takayama S. Proliferative and apoptotic status in endometrial adenocarcinoma. *Int J Gynecol Pathol* 1995;14;45-49.

33. Geisler JP, Geisler HE, Miller GA, Wiemann MC, Zhou Z, Crabtree W. MIB-1 in endometrial carcinoma: prognostic significance with 5-year follow-up. *Gynecol Oncol* 1999;75;432-436.

34. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res* 1994;54;3391-3395.

35. Myers MP, Pass I, Batty IH *et al.* The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc Natl Acad Sci U S A* 1998;95;13513-13518.

36. Kuismanen SA, Moisio AL, Schweizer P *et al.* Endometrial and colorectal tumors from patients with hereditary nonpolyposis colon cancer display different patterns of microsatellite instability. *Am J Pathol* 2002;160;1953-1958.