Virchows Arch (2001) 438:470–477
DOI 10.1007/s004280000361

ORIGINAL ARTICLE

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Endometrial carcinoma: association of steroid hormone receptor expression with low angiogenesis and bcl-2 expression

Received: 31 August 2000 / Accepted: 27 October 2000 / Published online: 8 March 2001

Abstract In endometrial tissues, malignant change may be accompanied by a loss of hormone dependence which is, usually, reflected in a parallel loss of oestrogen and progesterone receptors (ER and PR). In this study, the steroid receptor status of 164 endometrial carcinomas was related to intratumoural angiogenesis and the apoptotic proteins bcl-2 and p53. Relationships to conventional histopathological features and patient survival were also sought. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues. The mean follow-up was 55 months (range 19–167 months). Specific nuclear staining for ER and PR was detected in 35% and 32% of endometrial carcinomas, respectively, and was very commonly co-expressed (P<0.0001). The failure of demonstrating a steroid receptor complement in endometrial neoplasms was, in general terms, an adverse prognostic sign. Thus, ER or PR loss was significantly associated with non-endometrioid carcinomas (ER P=0.01; PR P=0.004) and with deep myometrial invasion (ER P<0.0002), high intratumoural angiogenesis (PR P<0.01) and the absence of bcl-2 expression (PR P<0.005). There was a trend for patients with ER or simultaneous ER/PR expression to have an improved survival, but this association did not reach the level of statistical significance. In multivariate analysis (all stages), tumour cell type (endometrioid versus non-endometrioid carcinomas) and stage of disease were the only variables associated with prognosis (P=0.01 and P<0.0001, respectively), with tumour cell type retaining its independent prognostic value and within stage-I endometrial carcinomas (P=0.02). It is suggested that the loss of steroid hormone receptors in endometrial carcinomas is associated with a more aggressive phenotype and the switching-on of angiogenic pathways.

Keywords Endometrial carcinoma · Steroid receptors · Angiogenesis · bcl-2, p53

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Introduction

Oestrogens and progesterone exert their action on endometrial cells through some intracellular molecules, called receptors, which bind the ovarian hormones with high affinity and specificity. During malignant transformation, the endometrial cells may lose some or all of their steroid hormone compliment [9, 32], and this phenomenon has been connected with partial or complete loss of hormone dependence. In many studies, such tumours were shown to exhibit a more aggressive phenotype, poorer prognosis [5, 9, 19, 20] and an ineffective response to endocrine therapy [5, 9, 10, 32, 42].

Relatively little information is at present available, however, with regard to the significance of oestrogen and progesterone receptor (ER and PR) status in endometrial carcinomas in relation to some of the newer, and more promising markers of tumour progression, namely neoangiogenesis and the anti-apoptotic proteins bcl-2 and mutant p53. Tumours cannot grow beyond a certain and clinically undetectable size unless new capillaries are formed (angiogenesis) [11]. Furthermore, tumour growth is dependent not only on the rate of cell proliferation but also on the rate of cell death. This is reflected in the expression of the apoptosis-related proteins bcl-2 and p53.

This study investigates the frequency of steroid hormone receptors in endometrial carcinomas and explores their possible relationship to angiogenesis and the aforementioned anti-apoptotic proteins. It also re-evaluates the ER and PR status in relation to conventional histopathological parameters and to patient survival.

Materials and methods

Materials

Formalin-fixed, paraffin-embedded specimens from 164 patients with endometrial adenocarcinoma were retrieved from the files of the Department of Pathology, Democritus University of Thrace. All patients had been treated surgically with total abdominal hysterectomy and bilateral salpingo-oophorectomy. Of these, 139 patients were stage I, and 25 patients were stage II/IIIa [International Federation of Gynecology and Obstetrics (FIGO)]. No lymph node sampling of the iliac nodes was performed, and N staging was based on pre-operative lymphangiography or pelvic and abdominal computed tomography (CT) scan. Histological typing and grading of the endometrial tumours (grade 1 vs grades 2 and 3) and the depth of myometrial invasion (<1/2 vs >1/2) were evaluated on haematoxylin and eosin-stained sections, according to the criteria of the World Health Organization [38]. Lymphatic vascular space invasion was recorded as being present if tumour cells were seen within a space with a definite and clearly identifiable endothelial lining.

Stage-I patients with myometrial invasion to a depth of less than one-half of the myometrial thickness were treated with surgery alone. Abdominal radiotherapy was given to stage-I patients with deep myometrial invasion and to those of stage II/IIIa. This consisted of external beam 6MV X-ray (44 Gy box technique followed by 6 Gy with shielding of the midline pelvic area) and vaginal intracavitary booster radiotherapy (25 Gy to 0.5 cm from the vaginal mucosa; selectron Cs 137 medium dose rate after loading system). None of the patients in the series had been irradiated or exposed to progestins or hormone replacement therapy prior to hysterectomy. The follow-up of the patients ranged from 19 months to 167 months with a mean of 55 months.

Immunohistochemistry

Sections were cut at 3 μm and stained immunohistochemically with the following techniques: a standard streptavidin-biotin method for ER and PR; the alkaline phosphatase/anti-alkaline phosphatase (APAAP) method for microvessel staining and the avidin-biotin peroxidase complex (ABC) procedure to demonstrate bcl-2 and p53 proteins [6, 15, 29]. Antibody reactivity was detected with 3,3′-diaminobenzidine as chromogen. The slides were counterstained with Mayer's haematoxylin. Pretreatment was performed by microwave heating or enzyme digestion. Details of the primary antibodies, the working dilutions and the antigen retrieval methods are given in Table 1. Known positive controls were included in each staining run. Negative controls comprised sections of cases under investigation, treated in the same way except that the primary antibody was substituted by tris-buffered saline.

Interpretation of immunohistochemistry

Positivity was indicated as a distinct brown nuclear staining for ER, PR, endothelial cells and p53 and as cytoplasmic staining for bcl-2. There is no consensus strategy on the evaluation of ER or PR in immunohistochemical sections and values above 5–10% of neoplastic cells expressing these receptors have been considered positive [22, 37]. In the current study, steroid receptor status was classified as either negative (<5% positive tumour nuclei), positive (5–40% positive tumour nuclei) or strongly positive (41–100% positive tumour nuclei). All positive tumour nuclei were counted, irrespective of the intensity of staining.

Microvessel counting was used for assessing tumour angiogenesis. Three areas of high vascular density ("hot spots") were selected at low power (×40 and ×100). Counts were performed with a Leitz microscope at ×250 magnification, corresponding to 0.384 mm^2 field size, and the average value of these three areas was taken as the "microvessel score". Only blood vessels with a well-defined lumen or a linear vessel shape were taken into account. The 33% and 66% percentiles were used to group cases into three categories of microvessel density (MVD): low (<15), medium (15–30) and high (>30). A cut-off value of 10% was used to qualify a positive staining for bcl-2 and p53.

Statistical analysis

Statistical analysis and graphs were performed using the Instat 3.1 package and graphpad prism 2.01 package (GraphPad, San Diego, Calif.). A Fisher's exact test was used for testing relationships between categorical tumour variables, as appropriate. Non-parametric analysis was used to assess intra- and inter-observer variability of immunohistochemistry appraisal. Survival curves were plotted using the method of Kaplan and Meier, and the log-rank test was used to determine statistical differences between life tables. A Cox proportional hazard model was used to assess the effect of tumour variables on overall survival. The end points were the overall survival from the day of surgery. A P value less than 0.05 was considered significant.

Results

Steroid receptor status

The percentage of tumour cells showing nuclear ER or PR expression ranged from 0% to almost 100% (median 0%). Inter-observer variability was minimal (P<0.0001, r=0.93). As indicated in Table 2, 57 cases (35%) were

Table 1 Details of the antibodies, dilutions, and antigen retrieval methods used in the present study. *MW* microwave heating (650 watts, 3×3 min in citrate buffer, pH 6.0); *PR* progesterone receptor; *ER* oestrogen receptor

Primary antibody	Dilution/incubation time	Antigen retrieval	Specificity	Source
1D5	1:20 (75 min[a])	MW	ER	Immunon – Shandon, Pa.
1A6	1:20 (75 min[a])	MW	PR	Immunon –
JC70 (CD31)	1:50 (30 min[a])	Prot.[c]	Endothelium	Dako, Denmark
DO-7	1:30 (Overnight[b])	MW	p53	Dako
124	1:80 (Overnight[b])	MW	bcl-2	Dako

[a] At room temperature [b] At 4°C [c] Protease-type XXIV for 20 min

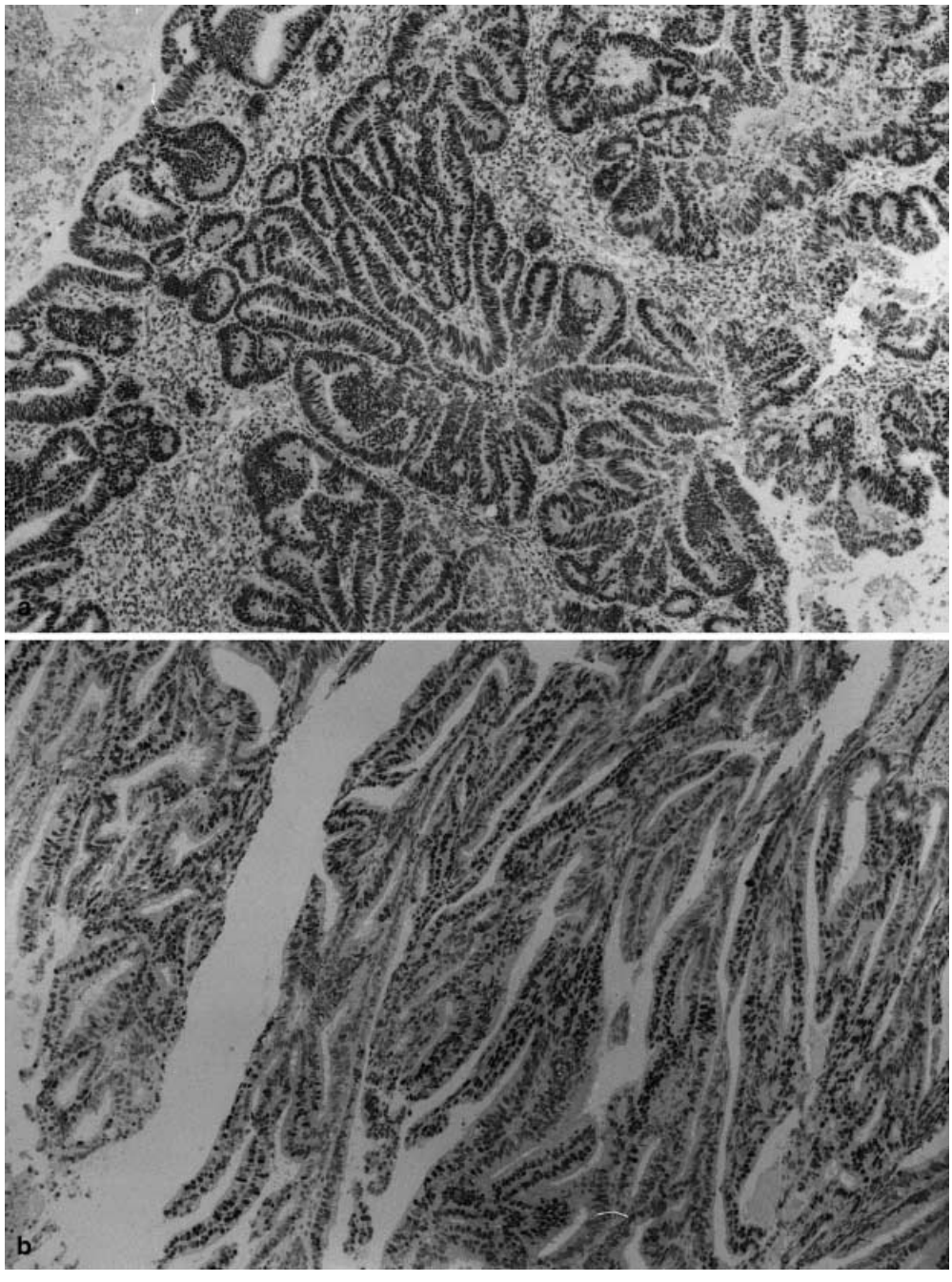

Fig. 1 **a** Endometrial carcinoma. Tumour nuclei stained strongly positive for the oestrogen receptor [ER; monoclonal antibody (mAb) 1D5]. Streptavidin-biotin method with Mayer's haematoxylin counterstained (×250 magnification). **b** Endometrial carcinoma. Tumour nuclei stained strongly positive for the progesterone receptor (PR; mAb 1A6). Streptavidin-biotin method with Mayer's haematoxylin counterstained (×250 magnification)

Table 2 Endometrial carcinoma (*n*=164). Oestrogen and progesterone receptor status, according to the proportion of tumour nuclei showing positive staining: negative (<5%), positive+ (5–40%) or strongly positive++ (41–100%)

	Oestrogen receptors			
	Negative	Positive+	Positive++	*P* value
Progesterone receptors				
Negative	96 (58.5%)	13 (7.9%)	3 (1.8%)	<0.0001
Positive+	7 (4.3%)	12 (7.3%)	12 (7.3%)	
Positive++	4 (2.5%)	7 (4.3%)	10 (6.1%)	

ER positive, and 52 cases (32%) were PR positive. The median percentage of tumour cells with positive ER and PR expression was 60% and 30%, respectively. Using these cut-off points, we found that 32 of 164 cases (19%) were weakly positive for ER, and 25 of 164 cases (15%) were strongly positive for this receptor. The respective figures for PR positivity were: weakly positive 31 of 164 cases (19%), and strongly positive 21 of 164 cases (13%). There was a significant association of ER with PR expression ($P<0.0001$). Figure 1a, b shows representative cases of strongly positive nuclear ER and PR expression.

Association of steroid receptor status with histopathological parameters

The loss of ER and PR expression from endometrial carcinomas was associated with deep myometrial invasion, a difference which was statistically significant only in the case of ER loss (ER P=0.0002; PR P=0.06). Further, ER and PR loss tended to be more frequent in non-endometrioid carcinomas (mainly serous papillary and clear cell carcinomas) than in endometrioid neoplasms. However, this difference reached the level of statistical significance only after excluding a subordinate endometrioid component from the immunohistochemical evaluation of mixed carcinomas having such a component (ER P=0.01; PR P=0.004). No association was established between the absence of ER or PR and the histological grade of the tumour, the lymph-vascular space invasion or the FIGO stage of disease (Table 3).

Association of steroid receptor status with angiogenesis, bcl-2 and p53 expression

Cytoplasmic bcl-2 reactivity and nuclear p53 accumulation was noted in 16 of 164 cases (9.7%) and 55 of 164 cases (33%), respectively. MVD was low in 52 of 164 cases, medium in 62 of 164 cases and high in 50 of 164 cases.

The association of steroid receptor status with MVD, bcl-2 and p53 expression is presented in Table 4. Bcl-2 expression was strongly associated with PR but not ER expression (P=0.005). Similarly, PR but not ER expression was significantly associated with low MVD (P<0.01). No association was found between ER or PR expression and nuclear p53 accumulation.

Survival analysis

Overall survival analysis showed that neither ER nor PR expression was significantly associated with prognosis (Fig. 2a, b). There was, however, a trend for endometrial carcinomas with strong ER expression to have a somewhat better prognosis (P=0.16). Similarly, the combined analysis of the ER and PR status showed a trend for endometrial neoplasms expressing both receptors to be associated with a more favourable prognosis (Fig. 2c; P=0.12). None of the patients in the series with simultaneous strong ER and PR expression died during the course of this investigation (Fig. 2d; P=0.17). Analysis within the stage-I cases, as for the steroid receptor expression, did not reveal any subgroup of patients with significantly different survival.

Multivariate analysis

In multivariate regression analysis in which ER, PR status and the histopathological parameters of histological type, histological grade, depth of invasion, vascular invasion and stage of disease were included, only tumour cell type (endometrioid versus non-endometrioid carcinomas) and stage of disease were independent variables defining prognosis (P=0.01, t ratio 2.45 for tumour cell type and P<0.0001, t ratio 5.03 for stage). Multivariate

Table 3 Endometrial carcinoma (n=164). Relationship of oestrogen and progesterone receptor status to histopathological characteristics. P values refer to receptor negative versus weak/positive groups

Parameter	Oestrogen receptors				Progesterone receptors			
	Negative	Positive+	Positive++	P value	Negative	Positive+	Positive++	P value
Histological type								
Endometrioid	82	28	20	0.01	87	26	17	0.004
Non-endometrioid[a]	29	3	2		31	2	1	
Histological grade								
G1	77	26	19	0.35	81	23	18	0.44
G2-G3	30	6	6		31	8	3	
Depth of myometrial invasion								
<50%	47	17	19	0.0002	51	19	13	0.06
>50%	60	15	6		61	12	8	
Lymph–vascular space invasion								
No	91	28	23	0.81	95	28	19	0.46
Yes	16	4	2		17	3	2	
Stage of disease								
I	89	28	22	0.49	94	26	19	0.81
II/III	18	4	3		18	5	2	

[a] Mixed carcinomas having a subordinate endometrial component were evaluated immunohistochemically only on their predominant non-endometrioid cell component

Table 4 Endometrial carcinoma (n=164). Relationship of oestrogen and progesterone receptor status to microvessel density (MVD) and to apoptosis related proteins bcl-2 and p53

Parameter	Oestrogen receptors				Progesterone receptors			
	Neg	Pos+	Pos++	*P* value	Neg	Pos+	Pos++	*P* value
MVD								
Low	31	10	11	0.88(*)	27	14	11	0.002(*)
Medium	39	14	9	0.12(**)	47	8	4	0.01(**)
High	37	8	5		38	6	6	
bcl-2								
Negative	76	22	11	0.11	81	21	7	0.005
Positive	31	10	14		31	10	14	
p53								
Negative	96	29	23	1	101	27	20	1
Positive	11	3	2		11	4	1	

P values refer to receptor negative vs. weak/positive groups
* Low vs medium MVD
** Low vs high MVD

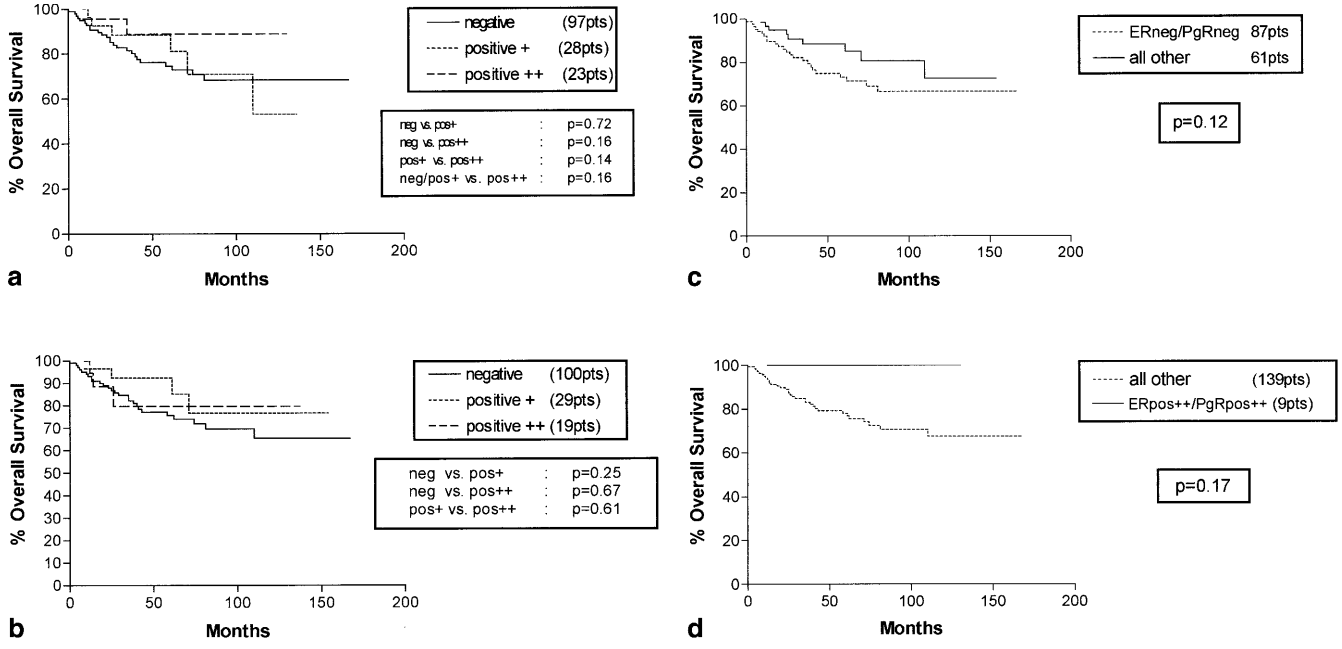

Fig. 2 Kaplan–Meier overall survival curves stratified for the oestrogen receptor (ER; **a**), and the progesterone receptor (PR; **b**), ER/PR negative cases versus one or both steroid receptor positive cases (**c**) and strongly positive ER/PR cases versus all other combinations (**d**)

analysis within stage-I endometrial carcinomas failed to show any significant relationship between steroid receptor status and prognosis. Tumour cell type was the only variable independently related to survival (P=0.02, t ratio 2.3). Further analysis within stage-I endometrial carcinomas of the endometrioid cell type did not show any association between steroid hormone receptor status or histological variables and prognosis.

Discussion

Endometrial carcinoma is a hormone-dependent tumour [23, 42] and, therefore, the detection of ER and PR receptors in endometrial glandular tumour cells is not unexpected. Not all malignant endometrial tissues, however, retain their ER and PR complement, as a variable number of them lose, either partially or completely, their steroid hormone receptors during malignant transformation. The reported incidence of ER and PR in endometrial carcinomas varies in several series from 35% to 70% [9, 13, 32, 44, 46], and our results are at the lower end of this range, sharing, in accordance with other studies [5, 9, 20], a frequent co-expression of both receptors.

In most series, the lack of ER and PR expression by endometrial tumour cells has been a marker of aggressive tumour behaviour and, indeed, many investigators connected the loss of hormone dependence with non-endometrioid-type carcinomas [4, 22] or with poorly differentiated endometrial tumours and advanced stage of disease [5, 10, 12, 13, 20, 25, 42]. In this series, the receptor status was unrelated to tumour differentiation or the stage of disease, but it was certainly related to the non-endometrioid carcinomas and deep myometrial invasion, particularly ER loss. The former, which was revealed after excluding a subordinate endometrioid component from the evaluation of mixed carcinomas having such a component, was proven to be an independent

prognostic factor in multivariate analysis. With regard to myometrial invasion, Kauppila, Morris, Fukuda and their associates reported results comparable to ours [13, 20, 25]. Moreover, Kauppila and colleagues found that the simultaneous presence of ER and PR is associated with a favourable survival for stage-I and -II endometrial tumours [20], and others extended this observation to include recurrent or advanced stage endometrial disease [5, 7, 9]. Others found that ER [5, 13] or PR alone [10] is a better indicator of prognosis, and still others failed to attach any prognostic significance in the receptor status of endometrial malignancies [12, 25]. In an earlier study, with a 10-year follow-up, we found a significant relationship between a high PR or ER/PR content and an improved disease-free survival in patients with endometrial carcinoma, but these results were based on the detection of cytoplasmic, rather than nuclear, receptors [41]. In the current work, based on the detection of nuclear receptors, neither ER nor PR expression, alone or in combination, was significantly associated with prognosis, although there was a propensity towards carcinomas with a strong ER or ER/PR expression having an improved outcome. These discrepancies can probably be explained by variations in methodology, mainly differences in the specificity of antibodies and the dilutions used [28, 45]. They may also reflect a small number of cases, a short follow-up after surgery and the lack of a defined criterion of positivity.

A particularly important finding of the present study was the frequent association of PR with bcl-2 positive carcinomas. A number of recent studies have afforded results directly comparable to those found in our series [23, 33, 39, 43, 46] and connected PR-positive, bcl-2-positive endometrial neoplasms with favourable histopathological features, i.e. low histological grade, superficial myometrial invasion, early stage of disease [34, 43, 46] and with a more favourable clinical outcome [39]. We have also reported recently a trend for bcl-2-positive endometrial carcinomas of being of low grade [14] but, more interestingly, we have associated these neoplasms with low vascular density [15] which, in turn, is the mirror image of improved survival [15].

It is intriguing how bcl-2, a protein which typically inhibits apoptosis, should be associated with favourable histological features and a good prognosis. It has to be emphasised, however, that in some studies, bcl-2 has been implicated in lowering growth in cancer cell lines [30] and increasing Ki-67-negative cancer cells [21]. Furthermore, Yamauchi et al. suggested that bcl-2 expression in endometrial carcinomas is regulated in a hormone-dependent manner [46], a function which has been, apparently, endowed with from the normal endometrium. Indeed, the bcl-2 protein, when expressed in normal endometrial tissues, appears to be under hormonal control [43], with the highest levels occurring during the proliferative phase of the menstrual cycle [17]. At this phase, the highest ER and PR levels are presumed to occur [1, 26, 40]. This was convincingly demonstrated in an experimental study by Critchley et al., where the administration of anti-progestins inhibited the progesterone-induced decline in steroid receptor levels in the normal endometrial glands [8]. This resulted in persistence of the proliferating endometrium, which was accompanied by bcl-2 secretion [8]. In another experimental study involving hormone-dependent tumours, such as the breast, incubation of cancer cell lines with oestradiol resulted in a direct upregulation of the bcl-2 protein [47] and the bcl-2/bax ratio [24], which offers a significant growth advantage. A downregulation of the bcl-2 protein by the anti-oestrogen tamoxifen has also been reported [3]. It appears, therefore, that malignant endometrial cells that retain the PR complement and concurrently express the bcl-2 protein have a phenotype that tends to be closer to that of the normal proliferative endometrium. Progressive loss of these features may be linked to the appearance of dedifferentiated cell clones, reflected as deep local invasiveness and metastatic behaviour. Thus, in this context, Runowitz et al. showed that metastatic endometrial disease is much less frequently PR-positive than primary malignant disease [32]. We showed, as others have [10, 25], that PR-negative primary endometrial tumours are characterised by local aggressiveness

Another equally, if not more important, finding that emerged from the present work was the relationship between PR status and angiogenesis. Low MVD has been associated with an increased survival in patients with endometrial carcinoma [15, 35] and, in this study, PR-positive endometrial carcinomas were significantly associated with low MVD. Interestingly, as already mentioned, bcl-2 expression is also significantly associated with decreased angiogenic activity [15]. These findings lend strong support to the hypothesis that the loss of both PR and bcl-2 protein expression may be an important step towards activation of pathways related to invasion and metastasis, i.e. angiogenesis. By analogy, PR-negative endometrial carcinomas have also been reported to be inversely associated with activation of the c-erbB-2 gene product [2, 44], a membrane glycoprotein which disrupts the cell–cell adhesion system, facilitating tumour growth and metastasis [18, 27].

With regard to the role of mutant p53 protein in endometrial carcinomas, this remains contradictory, while its relationship to steroid hormone receptor status is elusive. Thus, in some studies, nuclear p53 accumulation was inversely related to receptor positivity [8, 23, 34] and was more often detected in high-grade and stage endometrial disease [23, 43], with prominent proliferative activity, frequent lymph node metastases [34, 46] and a poor prognosis [31, 36, 39]. In other investigations, however, p53 overexpression was proven not to be an independent prognostic factor in endometrial carcinomas [14, 15, 16]. We found that nuclear p53 accumulation is infrequent in early endometrial carcinomas and is not related to poor prognosis [14, 15], although the lack of any association with ER/PR and bcl-2 expression noted in the present study should not be consistent with an improved clinical course.

It is concluded that the steroid receptor complement is retained in approximately one-third of endometrial carci-

nomas, and the loss of hormonal dependency is associated with loss of bcl-2 expression, local aggressiveness and the switching-on of angiogenic pathways. Further studies are on-going to further elucidate the mechanisms of tumour progression in endometrial carcinomas.

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