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Rapid Communication

Immunohistochemical Detection of P-Glycoprotein in Endometrial Adenocarcinoma

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P-glycoprotein (Pgp) has emerged as the central mediator in classic multidrug resistance in model systems in vitro. High levels of Pgp also have been detected in many normal human tissues and tumors; and its role in clinical drug resistance is currently under investigation. Recently significant levels of Pgp were localized to gravid and secretory endometrium; and it was demonstrated that the combination of estrogen and progesterone is sufficient to induce high levels of both Pgp mRNA and Pgp in uterine secretory epithelium. These findings suggest that increased Pgp expression also may be present in hormone-responsive malignancies such as endometrial adenocarcinoma. To determine whether Pgp is expressed in endometrial adenocarcinoma, 36 endometrial adenocarcinomas (grade I [n = 17]; grade II [n = 6]; grade III [n = 13]) were investigated retrospectively by the avidin-biotin-complex immunohistochemical procedure using three murine monoclonal antibodies (MAb) MAb C219, MAb C494, and MAb JSB-1, which recognize spatially distinct cytoplasmic epitopes of Pgp. Seventy-two percent of the tumors showed positive immunostaining with at least one MAb; 67% showed immunostaining with MAb C219, 50% with MAb C494, and 62% with MAb JSB-1. Forty-six percent of tumors were immunoreactive to two and 29% to all three antibodies. Membranous and Golgi/paranuclear type staining patterns were observed. Overall the intensity of immunostaining varied from one sample to another for a given tumor type, and considerable heterogeneity of expression was commonly seen within a given tumor. Strong to moderate immunoreactivity was seen in diffusely infiltrating, adenosquamous, and serous papillary carcinomas. In general, immunoreactivity to MAb C494 was weaker than MAb C219 or MAb JSB-1. Adenomatous and non-neoplastic endometrium adjacent to the tumors displayed strong membranous immunostaining with MAb JSB-1. Endometrial capillaries showed weak-to-moderate immunostaining to all three antibodies. It is concluded that Pgp is commonly expressed in endometrial adenocarcinoma and may be a significant factor responsible for their drug-resistant nature subject to modulation by progesterone. (Am J Pathol 1991, 138:799–806)

Various molecular mechanisms underlie drug resistance in tumor cells.[1,2] Classic multidrug resistance (MDR) in human and animal tumor cell lines often is due to overexpression of a 170-kd, integral membrane glycoprotein, p-glycoprotein (Pgp),[3] which acts as an energy-dependent, unidirectional, transmembrane, drug-efflux pump decreasing the net intracellular accumulation of cytotoxic agents.[4,5] In humans, two closely related MDR genes (Mdr1 and Mdr2) with a high degree of nucleotide homology encode Pgp isoforms[6]; overexpression of both genes has been implicated in intrinsic and acquired clinical drug resistance to anticancer agents in specific tumor types (ie, sarcomas, leukemias, neuroblastoma, and ovarian, renal, and colon carcinomas).[7–12]

P-glycoprotein expression also has been identified in a variety of normal human tissues with diverse physio-

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Table 1. *Pathologic Classification, Grade, and Semiquantitative Scoring of Pgp Immunoreactivity in Human Endometrial Adenocarcinomas*

Case	Pathology/ grade	C219	C494	JSB-1	Immunoreactivity Type	(%)	Intensity
1	E/I	+			G/p	1+	1+
			+		G/p	2+	3+
				ND			
2	E/I	−				0	
			−			0	
				−		0	
3	E/I	−				0	
			−			0	
				−		0	
4	E/I	−				0	
			−			0	
				−		0	
5	E/I	+			m:G/p	1+	2+
			−			0	
				+	G/p	1+	2+
6	E/I	−				0	
			−			0	
				−		0	
7	E/I	+			G/p	1+	3+
			−			0	
				+	m;G/p	1+	3+
8	E/I	+			m;G/p	2+	3+
			−			0	
				+	m;G/p	3+	3+
9	E/I	−				0	
			−			0	
				−		0	
10	E/I	+			G/p	1+	2+
			−			0	
				+	G/p	1+	2+
11	E-Sq/I	+			m;G/p	2+	2+
			+		m;G/p	2+	2+
				ND			
12	E-Sq/I	+			m;G/p	1+	2+
			ND				
				ND			
13	E-Sq/I	−				0	
			−			0	
				−		0	
14	E-Sq/I	−				0	
			−			0	
				−		0	
15	E-DI/I	+			m;G/p	3+	2+
			+		m	2+	1+
				+	m	2+	2+
16	E-DI/I	+			G/p	3+	3+
			+		G/p	2+	1+
				ND			
17	E-DI/I	+			m;G/p	4+	3+
			+		m;G/p	4+	3+
				+	m;G/p	4+	3+
18	E/II	−				0	
			ND				
				ND			
19	E/II	+			G/p	1+	2+
			+		G/p	2+	3+
				ND			
20	E/II	+			G/p	3+	3+
			+		G/p	3+	2+
				ND			
21	E/II	+			m;G/p	4+	3+
			+		G/p	3+	2+
				+	m;G/p	3+	3+
22	E-CC/II	+			m;G/p	1+	2+

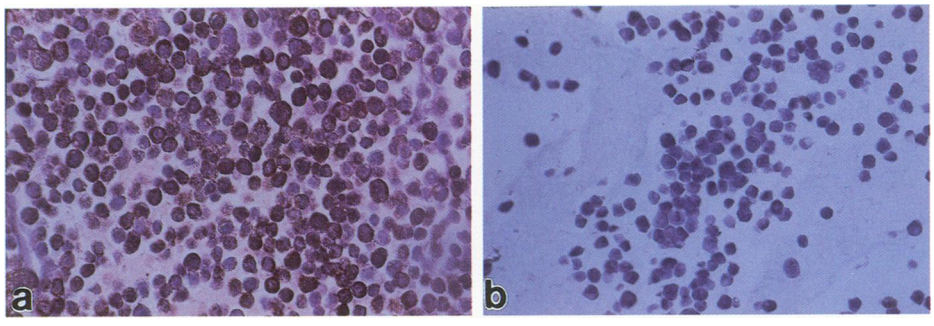
Table 1. (*Continued*).

Case	Pathology/ grade	C219	C494	JSB-1	Immunoreactivity Type	(%)	Intensity
			ND				
				ND			
23	E-CC/II	+			G/p	2+	3+
			+		m;G/p;	3+	3+
				ND			
24	E/III	+			m;G/p	3+	2+
			ND				
				ND			
25	E/III	−				0	
			+		G/p	2+	2+
					m;G/p	2+	2+
				ND			
26	CC/III	+			m;G/p	1+	2+
			ND				
				ND			
27	CC/III	−				0	
			ND				
				ND			
28	AS/III	+			G/p	2+	2+
			+		G/p	2+	1+
				+	G/p	2+	2+
29	AS/III	+			m;G/p	4+	3+
			+		G/p	3+	1+
				+	G/p	3+	1+
30	UPSC/III	+			m;G/p	4+	2+
			ND				
				ND			
31	UPSC/III	+			m;G/p	1+	2+
			−			0	
				+	m;G/p	3+	2+
32	UPSC/III	+			m;G/p	2+	2+
			ND				
				ND			
33	UPSC/III	−				0	
			+		G/p	2+	1+
				+	m;G/p	1+	2+
34	UPSC-CC/III	+			m;G/p	2+	2+
			+		m;G/p	3+	3+
				+	m;G/p	3+	3+
35	UPSC-SGC/III	−				0	
			−			0	
				−		0	
36	U-SGC/III	+			m;G/p	2+	1+
			−			0	
				ND			

E, endometrioid; E-Sq, endometrioid with squamous metaplasia; E-DI, endometrioid, diffusely infiltrating; E-CC, endometrioid with clear cell areas; CC, clear cell carcinoma; AS, adenosquamous; UPSC, uterine papillary serous carcinoma; UPSC-CC, combined uterine papillary serous carcinoma and clear cell carcinoma; UPSC-SGC, uterine papillary serous carcinoma with syncytitial giant cells; U-SGC, undifferentiated carcinoma with syncytitial giant cells; m, membranous; ND, not done; G/p, Golgi/paranuclear; %: 0%–10% (0), 11%–25% (1+), 26%–50% (2+), 51%–75% (3+), 76%–100% (4+); intensity: mild (1+), moderate (2+), strong (3+).

logic functions. Immunohistochemical studies demonstrated specific localization of Pgp to the apical surfaces of secretory epithelial cells, implicating a broader role for Pgp as a normal transporter protein.[13] In 1988, Arceci et al[14] demonstrated increased Pgp expression in the endometrial glands of the gravid mouse uterus; they suggested that Pgp gene expression may be controlled by pregnancy-associated changes and also may be involved in the uteroplacental transport of localized substrates necessary for the maintenance of pregnancy and fetal maturation. More recently they showed that the combination of estrogen and progesterone is sufficient to increase both MDR mRNA and Pgp in mouse uterine secretory epithelium,[15] and that progesterone can interact differentially with Pgp isoforms in gravid mouse endometrium and MDR tumor cells, reversing drug resistance in the latter.[16,17]

These data prompted us to investigate, using immunohistochemical methods, MDR gene expression in endometrial adenocarcinomas. We made the observation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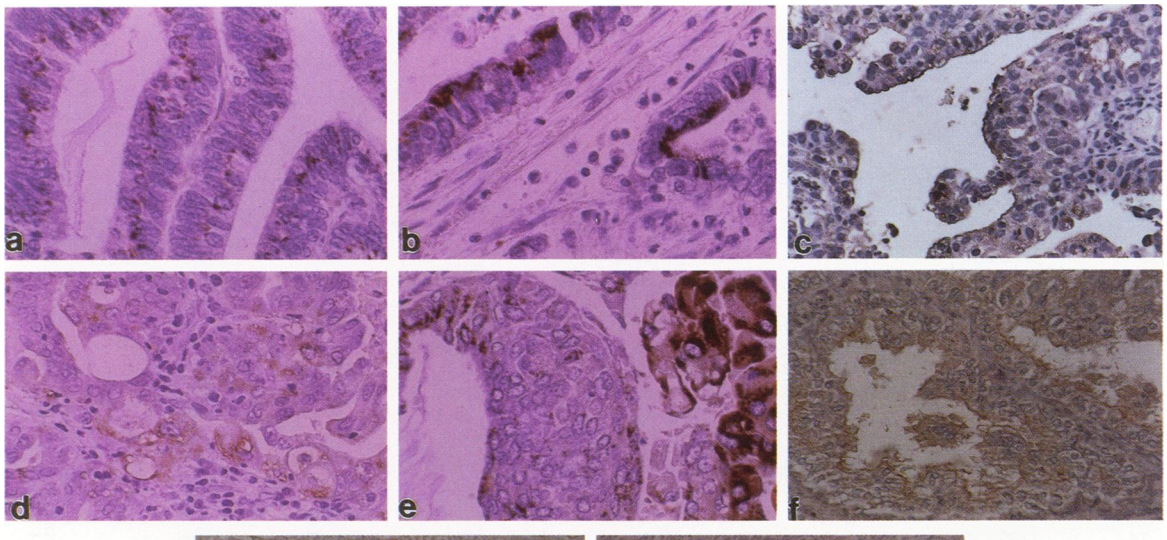
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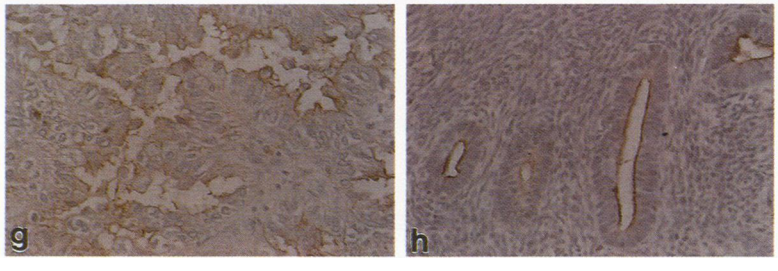
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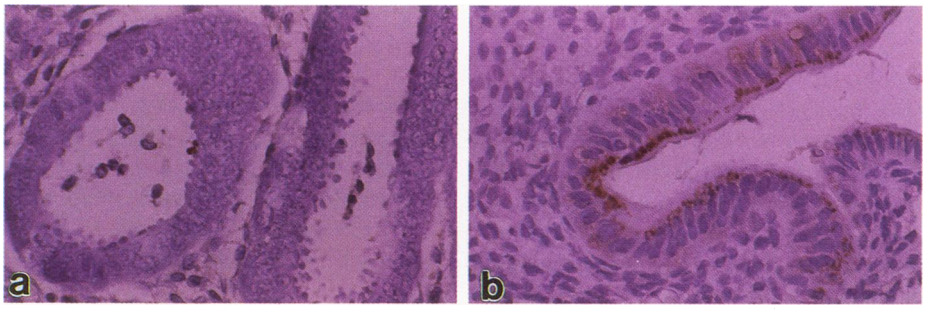
a
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that Pgp is expressed in endometrial adenocarcinoma, adjacent adenomatous and non-neoplastic endometrium, and in endometrial capillaries. These findings raise the possibility that Pgp plays a role in drug resistance of uterine adenocarcinoma and may be subject to modulation by progesterone.

Materials and Methods

Tumors

The material studied comprised 36 hysterectomy specimens. Tumor blocks fixed in 10% neutral formalin and embedded in paraffin were evaluated by two pathologists (CA Axiotis, MJ Merino) independently for diagnosis (Table 1). Five-micron–thick sections from one to two representative areas of tumor were used for immunostaining.

Reagents

Three murine monoclonal antibodies (MAb) specific for Pgp, C219, C494 (provided by Centocor, Malvern, PA) and JSB-1 (Sanbio bv, Uden, The Netherlands) were used for Pgp immunohistochemical staining. They recognize spatially distinct epitopes on the cytoplasmic domain of Pgp and have been extensively characterized.[18–20] Avidin and biotinylated peroxidase (Vectastain kit, PK 4002) and biotinylated horse anti-mouse IgG antibodies were obtained from Vector Laboratories (Burlingame, CA). Purified mouse IgG (cat. 6011-0080) was obtained from Organon Technika (West Chester, PA).

Immunostaining

Five-micron–thick tumor sections were immunostained according to the ABC procedure.[21] Briefly, the slides were incubated with 2% normal horse serum (20 minutes), with the MAbs (18 hours, at 4°C), and with biotinylated horse anti-mouse antibody (30 minutes). This was followed by an incubation in avidin-biotinylated peroxidase (for 45 minutes); they were developed in a mixture of 3-3′ diaminobenzidine tetrahydrochloride (Lipshaw, Detroit, MI) and H_2O_2. All other incubations were performed at room temperature. Endogenous peroxidase activity was blocked by exposing the tissue sections in 0.3% H_2O_2 in absolute methanol for 30 minutes. Dilutions were 10 μ/ml for MAbs C219 and JSB-1, and 30 μ/ml for MAb C494; and 1/200 for the biotinylated horse IgG. Tween 20, 0.1%, in 50 mmol/l (millimolar) TRIS-buffered saline (TBS)-0.5% bovine serum albumin, pH 7.5, was used for all washes and for antibody dilutions.

Controls

Sections from formalin-fixed, paraffin-embedded cell blocks of breast carcinoma cell line MCF-7 and its doxorubicin (Adriamycin, Adria Laboratories, Columbus, OH)-resistant phenotype (Adr^r) MCF-7 (provided by Dr. K. Cowan); and acetone-fixed cell preparations from a drug-sensitive human acute lymphoblastic leukemia cell line CCRF-CEM (ATCC cat. CCL 119) and its drug-resistant derivative CEM-VLB_{100} (P-glycoCHECK™ control slides cat. 800-680, Centocor, Malvern, PA) were used to demonstrate the specificity of Pgp immunostaining. Negative controls for immunostaining were performed by substituting the MAbs to Pgp with TBS and purified mouse IgG at the same protein concentration as the primary MAbs.

Quantitation

Cases with membranous (m) and/or Golgi/paranuclear (G/p) staining in more than 10% of the tumor cells were considered positive. Criteria for membranous immunoreactivity included staining of the cell membrane and/or the membrane of intracytoplasmic lumina (vacuoles). P-glycoprotein immunostaining was semiquantitatively expressed as follows: percentage of tumor cells staining: 0, 0%–10%; 1+, 11%–25%; 2+, 26%–50%; 3+, 51%–75%; 4+, 76%–100%; and intensity of immuno-

Figure 1. *(Top)* **a:** *Pgp immunoreactivity in (Adr^r) MCF-7 cells with MAb C219 (×250).* **b:** *Lack of immunoreactivity in wild type MCF-7 cells with MAb C219 (×250); chromogen DAB, hematoxylin counterstain).*
Figure 2. *(Middle)* **a:** *Moderate (2+) G/p immunostaining in adenocarcinoma, grade I with MAb C219 (×400).* **b:** *Strong (3+) G/p immunostaining in adenocarcinoma, diffusely infiltrating, grade I with MAb C219 (×400).* **c:** *Moderate (2+) membranous immunostaining in adenocarcinoma, grade II with MAb C219 (×400).* **d:** *Mild (1+) membranous immunostaining in adenocarcinoma, grade III with MAb C219 (×400).* **e:** *Strong (3+) membranous and G/p immunostaining in adenosquamous carcinoma, grade III with MAb C219 (×400).* **f:** *Moderate (2+) membranous immunostaining in combined papillary serous/clear cell carcinoma, grade III with MAb C494 (×400).* **g:** *Moderate (2+) membranous immunostaining in combined papillary serous/clear cell carcinoma, grade III with MAb JSB-1 (×400).* **h:** *Strong (3+) membranous immunostaining in non-neoplastic proliferative type endometrial glands adjacent to papillary serous carcinoma with MAb JSB-1 (×400); chromogen DAB, hematoxylin counterstain).*
Figure 3. *(Bottom)* **a:** *No immunostaining in early proliferative endometrium with MAb C219 (×400).* **b:** *Strong (3+) G/p immunostaining in mid-secretory endometrium with MAb C219 (×400; chromogen DAB, hematoxylin counterstain).*

staining: 1+, weak staining; 2+, moderate staining; 3+, strong staining.

Results

Specificity of Pgp Immunostaining

Preparations of (Adr^r) MCF-7 and CEM-VLB$_{100}$ cells stained strongly with the anti-Pgp antisera. Sections incubated with TBS and purified mouse IgG did not show a reaction product (Figure 1). Specific Pgp immunostaining also was observed in normal human adrenal, colon, and kidney.

Pgp Immunoreactivity in Endometrial Carcinoma

Formalin-fixed, paraffin-embedded sections of 36 endometrial carcinomas listed in Table 1 were evaluated for Pgp-specific immunostaining. Figure 2 shows a representative selection of the results obtained. P-glycoprotein commonly is expressed in endometrial carcinoma regardless of histologic grade or subtype. Seventy-two percent of the tumors (26 of 36), 59% (10 of 17) grade I, 83% (5 of 6) grade II, and 85% (11 of 13) grade III, showed positive immunostaining to at least one MAb. Sixty-seven percent (24 of 36) showed immunostaining with MAb C219; 50% (14 of 28) with MAb C494; and 62% (13 of 21) with MAb JSB-1. Forty-six percent (13 of 28) were immunoreactive to two and 29% (6 of 21) to all three antibodies. Both m and G/p staining were observed. Diffuse cytoplasmic staining also was seen in some cases. The intensity of immunostaining varied from one sample to another for a given tumor type; considerable heterogeneity of expression commonly was seen within a given tumor. Grade I tumors showed focal, weak-to-moderate Pgp immunostaining, except for the diffuse infiltrating subtype, an aggressive variant of well-differentiated endometrial adenocarcinoma,[22] which displayed moderate-to-strong Pgp staining in most tumor cells. Adenosquamous, serous papillary, and clear cell carcinomas, high-grade lesions associated with a particularly poor prognosis, showed moderate-to-strong immunoreactivity in most cases. The type, distribution, and intensity of immunostaining is shown semiquantitatively in Table 1. In general, the intensity of MAb C494 immunostaining was weaker than that of MAb C219 or MAb JSB-1.

Some of the negative cases in this study and/or the variable immunostaining in any given case may be due partially or totally to inherent technical problems associated with immunohistochemistry performed on paraffin-embedded material (ie, type and length of fixation, processing, masked epitopes, and so on). In addition, because cross-reactivities beyond Pgp have been reported for the MAbs used,[23] molecular confirmation of MDR gene expression and Western blots on parallel specimens would have been significant adjuncts to exclude false immunohistochemical false-positive results; however, given that MAbs C219, C494, and JSB-1 recognize different epitopes and have different cross-reactivities, concordant immunoreactivity with two or all of these MAbs argues strongly for the presence of Pgp; and we believe that using a panel of anti-Pgp MAbs is a useful strategy in detecting true Pgp expression in clinical samples.[24]

Pgp Immunoreactivity in Normal Endometrium

In the course of studying Pgp immunostaining in endometrial adenocarcinoma, we observed that some adenomatous and proliferative endometrium adjacent to the tumors displayed strong membranous staining for MAb JSB-1. Endometrial capillaries showed weak-to-moderate immunostaining with all three antibodies. We also demonstrated in other studies strong m and G/p immunostaining in secretory and gravid human endometrium, implying a normal role for Pgp in the trafficking of localized substrates required for the maintenance of pregnancy regulated by progesterone.[25] Figure 3 illustrates Pgp immunostaining in non-neoplastic endometrium.

Discussion

Monoclonal antibodies C219 and JSB-1 recognize distinct, highly conserved cytoplasmic epitopes found in all Pgp isoforms and represent universal probes for Pgp expression; C494 is gene specific and distinguishes a sequence conserved only in the MDR 1 isoform.[19] Our findings suggest widespread distribution of the MDR 1 gene product in endometrial adenocarcinomas consistent with previous results in normal human and murine endometrium.[25,26] The present immunohistochemical data distinguishes two staining patterns: 1) luminal, plasma membrane; and 2) dense, focal, paranuclear. This second pattern is thought to represent Golgi apparatus immunostaining.[27] Recently high levels of Golgi-Pgp have been coexpressed with another Golgi-processed, membrane-expressed system, the ABH(O) antigen system, in normal and malignant colonic epithelium, suggesting a possible glycosyl transferase function for

Pgp.[28] These observations raise the possibility that Golgi-Pgp also may be involved in the processing of epithelial blood group carbohydrate antigens in normal and neoplastic endometrium, given the remarkable similarity in expression of ABH(O) isoantigens in uterus and distal colon and their respective adenocarcinomas.[29] Diffuse, cytoplasmic Pgp staining was observed in a number of our cases. This pattern has been described in normal secretory epithelia and may represent early Pgp within minute vesicles in transit from the Golgi apparatus to the lumenal membrane.[28] Lack of Golgi immunostaining in adenomatous and proliferative endometrium does not necessarily rule out Golgi Pgp, but rather may be due to dispersed and poorly developed Golgi and secretory apparatus unresolved by light microscopy. Secretory and gravid endometrium manifest prominent Golgi staining that parallels the marked development of the Golgi apparatus at this stage of the cycle.

The function and regulation of Pgp in the endometrium and in endometrial adenocarcinoma is poorly understood. Recent data suggest that the normal role of Pgp in the uterus may be to transport localized substrates necessary for the maintenance of pregnancy and fetal development; and that this may be regulated by progesterone.[15,17] Progesterone is known to interact differentially with Pgp in gravid mouse endometrium and MDR tumor cells, increasing drug accumulation and sensitivity to antineoplastic agents in the later.[16] Our results raise the possibility that MDR gene expression is hormonally regulated in endometrial adenocarcinoma, a tumor known to be responsive to progestins,[30] and that progesterone may be helpful in reversing resistance and maintaining drug sensitive tumor cells.

Our immunohistochemical data do not distinguish between possible post-translational modifications of Pgp. In the mouse, Pgp in endometrial and multidrug-resistant cells have been shown to undergo differential N-linked glycosylation.[31] It remains unclear if differences in glycosylation alter the capacity of Pgp to transport endogenous compounds or chemotherapeutic agents; however it could account for the lack of recognition of Golgi immunoreactivity by MAbs MRK16, HYB-241, and HYB-612, which bind to extracellular domains and may recognize only more completely glycosylated forms of the protein. In addition, capillaries in the endometrium stained for Pgp, implicating a possible role for Pgp in the entry of chemotherapeutic agents to the endometrial anatomic compartment, as previously suggested for brain, testis, and skin.[32]

In conclusion, our results demonstrate widespread Pgp immunoreactivity in endometrial adenocarcinoma. Further studies are needed to elucidate its function and regulation in both normal and neoplastic endometrium.

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