Drug Resistance-Associated Marker Lrp for Prediction of Response to Chemotherapy and Prognoses in Advanced Ovarian Carcinoma

Miguel A. Izquierdo, Ate G. J. van der Zee, Jan B. Vermorken, Paul van der Valk, Jeroen A. M. Beliën, Giuseppe Giaccone, George L. Scheffer, Marcel J. Flens, Herbert M. Pinedo, Peter Kenemans, Chris J. L. M. Meijer, Elisabeth G. E. de Vries, Rik J. Scheper*

Background: Drug resistance is a major impediment to the successful treatment of ovarian carcinoma. None of the earlier-identified resistance mechanisms, such as overexpression of the MDR1 gene product, P-glycoprotein (Pgp), has been shown to be a major determinant of clinical response to chemotherapy and survival for ovarian cancer patients. The multidrug resistance-associated protein (Mrp) and the lung resistance protein (Lrp, also called the p110 major vault protein), are newly described proteins associated with multidrug resistance in vitro. **Purpose:** The aim of this retrospective study was to investigate the expression of Mrp and Lrp, in addition to Pgp, in advanced ovarian carcinoma and to determine whether such expression was predictive of response to chemotherapy and survival. **Methods:** Fifty-seven banked frozen specimens, previously collected and frozen at the time of diagnosis from an equal number of patients with International Federation of Gynecology and Obstetrics (FIGO) stage III or IV ovarian carcinoma, were immunostained for Pgp (with monoclonal antibodies [MAbs] MRK-16 and JSB-1), Mrp (with MAb MRPr1), and Lrp (with MAb LRP-56). All patients had received platinum- or alkylating-based chemotherapy after debulking surgery. Clinicopathologic parameters determined at diagnosis were retrospectively assessed for their relationship with Pgp, Mrp, and Lrp expression. Response to treatment and survival were compared between Pgp, Mrp, and Lrp expression groups. Qualitative variables were analyzed using Fisher's exact test or the chi-squared test. All reported *P* values are two-tailed. **Results:** Nine (16%), 39 (68%), and 44 (77%) of the 57 tumor specimens examined showed positive immunostaining for Pgp, Mrp, and Lrp, respectively. Positive immunostaining for these proteins was not associated with any other prognostic factor examined. No association was found between Pgp and Mrp expression and response to chemotherapy and survival. In contrast, patients with Lrp-positive tumors had poorer response to chemotherapy (*P* = .004) and shorter progression-free (*P* = .003) and overall (*P* = .007) survival than Lrp-negative patients. Multivariate analysis of Lrp expression, FIGO stage, residual tumor after initial surgery, tumor grade, and presence or absence of ascites showed that only Lrp status was independently related

to both progression-free survival and overall survival. **Conclusions:** Positive Lrp immunostaining in advanced ovarian carcinoma appears to be an indicator of poor response to standard chemotherapy (platinum or alkylating agents) and of adverse prognoses. **Implications:** The functional characterization of Lrp and related proteins may reveal new approaches to modulate Lrp-associated drug resistance. A large prospective study is warranted to confirm the prognostic value of Lrp. [J Natl Cancer Inst 87:1230-1237, 1995]

Epithelial ovarian carcinoma is the fourth highest cause of cancer death in women and the leading cause of gynecologic cancer mortality (1). Approximately 75% of women who have ovarian carcinoma present with surgically incurable advanced disease. Their treatment with platinum-based chemotherapy after cytoreductive surgery has improved response rate and survival, but the complete pathologic response and 5-year survival rates are still only 20%-40% and 10%-20%, respectively (2,3). Chemotherapy failure may be due to drug-resistant cancer cells that exist prior to or arise during treatment (4). Drug resistance observed in clinical ovarian carcinoma is broad and encompasses diverse, unrelated drugs (i.e., platinum compounds, alkylating agents, and natural products), suggesting concurrent operation of different mechanisms of resistance (4).

In vitro, resistance to platinum and alkylating agents includes decreased drug accumulation, enhanced detoxification, and increased DNA repair capacity (4). Multidrug resistance to natural products is linked to the overexpression of the MDR1 gene product [P-glycoprotein (Pgp)] (5) and alteration in

*Affiliations of authors: M. A. Izquierdo, P. van der Valk, J. A. M. Beliën, G. L. Scheffer, M. J. Flens, C. J. L. M. Meijer, R. J. Scheper (Department of Pathology), J. B. Vermorken, G. Giaccone, H. M. Pinedo (Department of Oncology), P. Kenemans (Department of Gynecology), Free University Hospital, Amsterdam, The Netherlands; A. G. J. van der Zee (Department of Gynecology), E. G. E. de Vries (Department of Medical Oncology), University Hospital, Groningen, The Netherlands.

Correspondence to: Prof. Rik J. Scheper, Ph.D., Department of Pathology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.

See "Notes" section following "References."

topoisomerase II activity (6). To date, most clinical studies of ovarian carcinoma have failed to demonstrate a role for either of these mechanisms as a major determinant of response to chemotherapy and survival (7-10).

Two new drug resistance-related proteins have been described (11-14). The multidrug resistance-associated protein (Mrp) is a member of the ABC-transporter superfamily that confers resistance to a similar, but not identical, spectrum of drugs as Pgp (11-14). Mrp is mainly localized in the plasma membrane, and it may cause resistance by extrusion of drugs from the cell (13). To date, little is known about the expression of Mrp in human cancers. The lung resistance protein (called Lrp because it was first identified in the lung cancer multidrug-resistant cell line SW-1 573/2R120) is also linked to multidrug resistance in cancer cell lines (15). Reversal of resistance in 2R120 cells results in a parallel Lrp decrease, further supporting the close association of Lrp with resistance in these cells (15). Lrp expression in 155 untreated tumors comprising 26 tumor types showed that Lrp is more frequently detected in chemoresistant tumor types than in chemosensitive cancers, thus suggesting its potential clinical use as a drug-resistance marker (16). We recently cloned and sequenced the LRP gene and identified its product Lrp as the human p110 major vault protein (17). Vaults are novel cellular organelles first described in 1986 by Kedersha and Rome (18), which are thought to mediate intracellular (nucleocytoplasmic) transport of a wide variety of substrates (19). The aim of this retrospective study was to investigate the expression of Mrp and Lrp, in addition to Pgp, in advanced ovarian carcinomas and to determine whether such expression was predictive of response to chemotherapy and survival.

Patients and Methods

We studied the expression of Pgp, Mrp, and Lrp in banked frozen specimens from advanced ovarian carcinoma patients and retrospectively associated this expression with clinicopathologic characteristics obtained from the clinical records.

Frozen Sample Selection

After obtaining informed consent in accordance with institutional guidelines, tumor specimens were collected by standard procedures as part of routine clinical practice. Freshly frozen samples of ovarian specimens from 57 women undergoing initial surgery for advanced ovarian carcinoma [stage III or IV according to the International Federation of Gynecology and Obstetrics (FIGO) classification (20)] were obtained from diagnostic histopathology laboratories at the Free University Hospital, Amsterdam, The Netherlands, and the University Hospital, Groningen, The Netherlands, during the period 1984-1993 (52 of them were obtained after 1987). The samples for study were selected in sequence only for availability of frozen material. No known systematic bias resulted from the strategy used at our tissue banks to freeze tumors. The size of the tumor specimen has been recognized as a potential source of bias in a frozen bank. However, this bias may not be a significant factor in our group of tumors, since the majority of advanced ovarian carcinoma patients present with bulky disease. All frozen samples had been stored in liquid nitrogen until use. Diagnosis was based on conventional morphologic examination of paraffin-embedded specimens, and tumors were classified according to the World Health Organization (WHO) classification (21). Carcinomas were classified as well-differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III) (22).

Clinicopathologic Characteristics of Patients

Table 1 shows the clinicopathologic characteristics of the 57 patients included in this study. The median age at diagnosis was 66 years (range, 29-84

years). The patients included had no other form of cancer. All patients were primarily treated with debulking surgery and postoperative chemotherapy, which consisted of a platinum-based regimen in 50 patients (in five of these patients an anthracycline was added) and a single alkylating agent in seven patients (Table 1). Response to induction chemotherapy was assessed by second-look surgery or clinical and/or radiographic evaluation according to WHO criteria (23) after five to six and 10 to 12 cycles of platinum regimen and alkylating agent, respectively. Thirty patients underwent second-look surgery: seven achieved complete response, 13 partial response, and 10 no response. Twenty-seven patients did not undergo second-look surgery because it was considered not indicated (because of clinically evident lack of response to chemotherapy in most cases) or was refused. Eight of these 27 patients had no clinically evaluable disease after initial surgery and were, therefore, considered not assessable for response. One patient achieved clinical complete response, four patients had partial response, and 14 had no response. Overall, of 49 patients assessable for response to chemotherapy, eight (16%) achieved a complete response, 17 (35%) achieved a partial response, and 24 (49%) had no response. Twenty (80%) of 25 of the actual responses were assessed by direct inspection at second-look laparotomy and were pathologically confirmed. Patients with incomplete response to induction chemotherapy and patients with recurrent tumors were treated with a variety of second-line chemotherapy regimens. Follow-up for the 57 patients included in the survival analysis was updated in May 1994 (median follow-up was 18 months; range, 1-111 months). At that time, 38 patients had died of ovarian carcinoma and 19 were alive (median follow-up for the last group was 31 months; range, 7-111 months).

Monoclonal Antibodies

Pgp expression was studied with two MDR1 gene-specific murine monoclonal antibodies (MAbs): MRK-16 (IgG2 [immunoglobulin G2]), which recognizes an external epitope of Pgp, was supplied by T. Tsuruo (24), and JSB-1 (IgG1), which reacts against an internal epitope of Pgp, was produced in our laboratory (25). Both MAbs have been well characterized and extensively utilized in the literature and are considered to yield better results in cryostat sections than in paraffin-embedded sections (26). For immunochemical detection of Mrp, the rat MAb MRPr1 (IgG2a) was developed in our laboratory (27). MRPr1 was raised after immunization with a fusion protein containing a segment of 168 amino acids in the amino-proximal one half of the Mrp protein and has been well characterized by protein blot analysis, immunocytochemical studies (including specific detection of Mrp in SI cells transfected with a full-length complementary DNA of the MRP gene), and immunohistochemical studies. MRPr1 does not cross-react with human Pgp (27). For the investigation of Lrp expression, we used the murine MAb LRP-56 (IgG2b) that was obtained in our laboratory after immunization with the non-Pgp multidrug-resistant human lung cancer cell line SW-1573/2R120 (15). LRP-56 specifically recognizes a 110-kd protein (Lrp) overexpressed in a number of non-Pgp multidrug-resistant cancer cell lines of different histogenetic origins (15). Both MRPr1 and LRP-56 display better immunoreactivity in cryostat sections than in paraffin-embedded sections.

Immunohistochemistry

Immunostaining was performed on acetone-fixed (for 10 minutes) cryostat sections. Slides were preincubated with normal rabbit serum for 15 minutes and then incubated with MRK-16 1:200, JSB-1 1:100, MRPr1 1:50, or LRP-56 1:500 for 1 hour. For Pgp and Mrp detection, an avidin-biotin complex procedure was used in which rabbit anti-mouse biotin conjugate (1:500 for 30 minutes; Zymed Laboratories Inc., South San Francisco, CA) or rabbit anti-rat biotin conjugate (1:100 for 30 minutes; Dako Corp., Glostrup, Denmark) incubation, and horseradish-streptavidin (1:500 for 1 hour; Zymed Laboratories Inc.) incubation were the second and third steps, respectively. For Lrp detection, a single-step indirect immunoperoxidase method was used with affinity-purified rabbit anti-mouse IgG conjugated to horseradish-peroxidase (1:25 for 30 minutes; Dako Corp.). The selection of the immunoperoxidase reaction conditions used for each MAb was based on previous optimization of the immunohistochemical protocols. Staining with an irrelevant mouse IgG or an isotype-matched rat IgG were routinely performed as a negative control procedure for the murine and rat MAbs, respectively. Slides were counterstained with hematoxylin. Diaminobenzidine (Sigma Chemical Co., St. Louis, MO) was used as a chromogen.

Cytospin preparations of tumor cell lines (squamous carcinoma KB-3-1, small-cell lung cancer GLC4, and non-small-cell lung cancer SW-1 573) and

Table 1. Patient characteristics of 57 advanced ovarian carcinoma patients treated with chemotherapy according to Pgp, Mrp, and Lrp expression*

	Total No. of patients	Pgp expression, No. of patients (%†)		Mrp expression, No. of patients (%†)		Lrp expression, No. of patients (%†)	
		Negative	Positive	Negative	Positive	Negative	Positive
No. of patients (%‡)	57	48 (84)	9 (16)	18 (32)	39 (68)	13 (23)	44 (77)
Age, y							
≤60	22	19 (40)	3 (33)	7 (39)	15 (38)	4 (31)	18 (41)
>60	35	29 (60)	6 (67)	11 (61)	24 (62)	9 (69)	26 (59)
Performance status§							
0/1	20	15 (68)	5 (83)	4 (57)	16 (76)	4 (57)	16 (76)
2/3	8	7 (32)	1 (17)	3 (43)	5 (24)	3 (43)	5 (24)
FIGO stage							
III	43	36 (75)	7 (78)	12 (67)	31 (79)	9 (69)	34 (77)
IV	14	12 (25)	2 (22)	6 (33)	8 (21)	4 (31)	10 (23)
Residual tumor							
≤2 cm	24	19 (40)	5 (56)	6 (33)	18 (46)	7 (54)	17 (39)
>2 cm	33	29 (60)	4 (44)	12 (67)	21 (54)	6 (46)	27 (61)
Histology							
Serous	40	33 (69)	7 (78)	12 (67)	28 (72)	8 (62)	32 (73)
Mucinous	5	4 (8)	1 (11)	2 (11)	3 (8)	0	5 (11)
Undifferentiated carcinoma	8	8 (17)	0	3 (17)	5 (12)	3 (23)	5 (11)
Endometrioid	4	3 (6)	1 (11)	1 (5)	3 (8)	2 (15)	2 (5)
Grade							
I or II	21	17 (35)	4 (44)	7 (39)	14 (36)	2 (15)	19 (43)
III	36	31 (65)	5 (56)	11 (61)	25 (64)	11 (85)	25 (57)
Ascites							
Present	37	32 (67)	5 (56)	12 (67)	25 (64)	7 (54)	30 (68)
Absent	20	16 (33)	4 (44)	6 (33)	14 (36)	6 (46)	14 (32)
Chemotherapy							
Platinum-based	50	41 (85)	9 (100)	17 (94)	33 (85)	12 (92)	38 (86)
Cis/Car + Cy		38	9	16	31	12	35
Cis + Car		3	0	1	2	0	3
Alkylating	7	7 (15)	0	1 (6)	6 (15)	1 (8)	6 (14)
Melphalan		6	0	0	6	1	5
Chlorambucil		1	0	1	0	0	1

*All comparisons (chi-squared or Fisher's exact test) between Pgp, Mrp, and Lrp expression groups with the different variables were not statistically significant ($P>.05$).

†Percentage within each expression group.

‡Percentage of the total.

§Eastern Cooperative Oncology Group; data available for 28 patients.

||Cis = cisplatin; Car = carboplatin; Cy = cyclophosphamide. Includes an anthracycline in four Pgp-negative patients, one Pgp-positive patient, two Mrp-negative patients, three Mrp-positive patients, and five Lrp-positive patients.

their corresponding multidrug-resistant sublines overexpressing Pgp (KB-8-5) (28), Mrp (GLC4/ADR) (29), and Lrp (2R120) (30), respectively, served as controls for the immunohistochemical assays. The sensitivity of our assays allowed the detection of the low levels of Pgp, Mrp, and Lrp present in KB-8-5, GLC4, and SW-1573 cells, respectively (15,27,28).

The slides were examined and scored independently by two observers without knowledge of clinical information about the patients. By prior agreement, each of the four MABs used in the study samples were scored as positive if more than 10% of the tumor cells were stained (for a specimen to be considered Pgp positive this percentage of staining was required for both MRK-16 and JSB-1). The 10% cutoff level was prespecified for the following reasons. 1) It has been shown that a small percentage of cells positive for multidrug resistance-related proteins (i.e., Pgp) can have clinical significance (31). 2) In previous optimization of our immunohistochemical protocols, 10% positive tumor cells was considered the lowest level of expression that could be most consistently detected in cryostat sections. 3) Experience with LRP-56 staining in 155 cancer specimens suggested that a 10% cutoff may distinguish two groups of Lrp-expressing tumors (16). The same cutoff was used for all three multidrug resistance-related proteins, since they share the same biologic background (i.e., relationship with multidrug resistance in vitro). The kappa test of reliability between the two observers was 0.553 for Pgp, 0.763 for Mrp, and 0.854 for Lrp, indicating fair to good agreement for Pgp and strong agreement for Mrp and Lrp. All specimens

in which both observers disagreed (eight for Pgp, six for Mrp, and three for Lrp) were restained. In addition, since the evaluation of immunoreactivity with MRK-16 and JSB-1 was found to be particularly difficult because of the presence of some background staining with the avidin-biotin complex method, we repeated the study of Pgp expression in 42 selected specimens, using a sensitive double alkaline phosphatase monoclonal antialkaline phosphatase (APAAP) method (32). Both observers looked together at all the slides available for each of the restained specimens (including the initial ones) and reached an agreement about the final evaluation.

Statistical Analysis

Data analysis was performed using the BMDP statistical software package (BMDP Statistical Software, Inc., Los Angeles, CA) (33). The kappa test of reliability was used to determine the consistency between the two observers in evaluating the slides (34). Clinicopathologic parameters determined at diagnosis were retrospectively assessed for their relationship with Pgp, Mrp, and Lrp expression. Treatment response rates were compared between Pgp, Mrp, and Lrp expression groups. Qualitative variables were analyzed using Fisher's exact two-tailed test or the chi-squared test, as appropriate (35). Progression-free and overall survival were measured in months from the month of surgery to a documented relapse or death, respectively. Actuarial survival curves were es-

estimated using the Kaplan–Meier method (36), and differences in survival between subgroups were compared with the logrank test (Mantel–Cox) (37). The nonparametric 95% confidence intervals of the median progression-free and overall survival were calculated according to the Brookmeyer–Crowley technique (38). Nonparametric confidence intervals have been shown to be more conservative than parametric intervals (38). Multivariate analysis was performed with a Cox proportional hazards stepwise regression model (39); the limits of the *P* values to enter and remove the variables into or from the model were .10 and .15, respectively. Hypotheses were evaluated at a significance level of .05. Two-sided statistical tests were used in all analyses.

Results

Expression of Pgp, Mrp, and Lrp in Ovarian Carcinoma

Nine (16%) of the 57 patients were Pgp positive, 39 (68%) were Mrp positive, and 44 (77%) were Lrp positive (Table 1). There was no statistical difference in prognostic factors between tumors depending on the expression of multidrug resistance-related proteins (for all comparisons *P* > .05; Table 1). However, small significant differences may be below the power of detection of our analysis.

Pgp immunoreactivity was cytoplasmic and, in some cases, it was also localized to the plasma membrane. Mrp staining was predominantly observed at the cell membrane, but some cytoplasmic staining was also present (Fig. 1, A). The intensity of the reactions varied from weak to moderate (more similar to GLC4 than to GLC4/ADR cells), suggesting that, in ovarian carcinoma, the cellular level of Mrp is relatively low or modest. In normal epithelial ovarian cells, Mrp could not be detected using the same procedure (Flens MJ, Zaman GJ, van der Valk P, et al.: unpublished observation). Lrp expression was detected as characteristic granular cytoplasmic staining, which was generally strong. (Fig. 1, B).

The expression of all three multidrug resistance-related proteins was heterogeneous. However, whereas the number of Pgp-positive cancer cells was more variable and, in general, less than 50%, Mrp- and Lrp-positive cases usually showed immunoreactivity in the majority of cancer cells. Stromal cells, such as macrophages, were occasionally positive for Pgp and Mrp but more frequently showed strong immunoreactivity for Lrp. In agreement with this observation, Lrp-related vaults have been reported to be abundant in macrophages (19).

Expression of Pgp, Mrp, and Lrp and Response to Chemotherapy

There was no statistically significant association between Pgp and Mrp expression and response to induction chemotherapy in 49 patients in whom response was assessable (Table 2). Nevertheless, none of the nine Pgp-positive patients achieved a complete response, whereas 20% did so in the Pgp-negative group (not significant).

Interestingly, patients with Lrp-positive tumors had a significantly inferior response to chemotherapy (8% complete, 36% partial, and 56% no response) compared with patients with Lrp-negative tumors (50% complete, 30% partial, and 20% no response) (*P* = .004 by the chi-squared test for the three categories of response; *P* = .006 by Fisher's exact test comparing the rates of complete response). When only platinum-treated

Fig. 1. Immunohistochemical staining of Mrp and Lrp proteins in ovarian carcinoma, using cryostat sections. **A)** Mrp-positive tumor stained with MAb MRPr1. Note the distinct plasma membrane staining plus a weaker cytoplasmic staining, as is commonly observed in multidrug-resistant cell lines. Compared with most ovarian carcinomas, this specimen showed relatively intense immunoreactivity (immunoperoxidase stain, original magnification $\times 40$). **B)** Lrp-positive tumor stained with MAb LRP-56. Note the cytoplasmic granular staining that is also characteristic of Lrp-overexpressing multidrug-resistant cell lines (immunoperoxidase stain, original magnification $\times 40$).

Table 2. Relationship between Pgp, Mrp, and Lrp expression and response to chemotherapy (n = 49)*

	No. of patients	No. of patients (%)			<i>P</i>
		Complete response	Partial response	No response	
Pgp					
Negative	40	8 (20)	13 (32)	19 (48)	.33†
Positive	9	0	4 (44)	5 (56)	.32‡
Mrp					
Negative	16	3 (19)	5 (31)	8 (50)	.69†
Positive	33	5 (15)	12 (36)	16 (49)	.67‡
Lrp					
Negative	10	5 (50)	3 (30)	2 (20)	.004†
Positive	39	3 (8)	14 (36)	22 (56)	.006‡

*Eight of 57 patients were not assessable for response (eight in the Pgp-negative group, two and six in the Mrp-negative and Mrp-positive groups, and three and five in the Lrp-negative and Lrp-positive groups).

†By chi-squared test comparing the three categories of response.

‡By Fisher's exact test comparing the rate of complete response.

patients were analyzed, the relationship remained significant ($P = .01$ in both types of comparisons).

Expression of Pgp, Mrp, and Lrp and Survival

All 57 patients, including those in whom response to chemotherapy was not assessable, were analyzed for survival. The median progression-free survival and overall survival for the whole group were 11 months and 20 months, respectively; values were within the expected range for a group of unselected advanced ovarian carcinoma patients. The univariate survival analysis according to different clinicopathologic features is summarized in Table 3. No association was found between Pgp or Mrp expression and progression-free survival or overall survival. In contrast, patients with Lrp-positive tumors had a significantly shorter progression-free and overall survival than did patients with Lrp-negative tumors. The median progression-free survival for the Lrp-positive and Lrp-negative groups was 9 months and 28 months, respectively ($P = .003$), and the median overall survival was 15 and 42 months, respectively ($P = .007$) (Fig. 2). These values remained significant when only the 50 platinum-treated patients were included ($P = .01$ and $P = .03$ for progression-free and overall survival, respectively).

Although the number of patients is relatively small and the results must be interpreted with caution, we performed a multivariate analysis to determine if the prognostic value of Lrp dis-

appeared when other prognostic factors were considered. Multivariate analysis of Lrp expression, FIGO stage, residual tumor after initial surgery, tumor grade, and presence or absence of ascites showed that only Lrp was independently related to both progression-free survival and overall survival. No other factor made an additional contribution to the model. After step number one, the global chi-squared test for Lrp for progression-free and overall survival was 8.26 ($P = .004$) and 6.81 ($P = .009$), respectively.

Discussion

This study shows that in contrast to Pgp, two newly described multidrug resistance-related proteins, Mrp and Lrp, are frequently expressed in untreated advanced ovarian carcinoma, denoting their potential as contributors to and/or markers of clinical drug resistance in this disease.

The retrospective analysis of the clinical data demonstrated that only the pretreatment status of Lrp was an independent prognostic factor of response to chemotherapy and survival. The lack of independent significance of other known prognostic factors may be due not only to the strong impact of the expression of Lrp on prognosis but also to the relatively small number of patients. A prospective study with a larger number of patients is warranted to confirm our finding.

Table 3. Univariate analysis of clinicopathologic parameters and multidrug resistance-associated proteins in 57 patients with advanced ovarian carcinoma*

	No. of patients	No. of mo (95% CI)†	$P‡$	No. of mo (95% CI)†	$P‡$
		progression-free survival		overall survival	
Age, y			.87		.54
≤60	22	10 (6-16)		26 (11-36)	
>60	35	11 (7-16)		15 (7-16)	
FIGO stage			.05		.03
III	43	11 (9-16)		22 (14-30)	
IV	14	5 (4-8)		8 (7-§)	
Residual tumor			.07		.04
≤2 cm	24	15 (11-19)		30 (18-§)	
>2 cm	33	8 (6-11)		12 (9-20)	
Histology			.92		.94
Serous	40	11 (8-16)		20 (11-27)	
Other	17	11 (6-21)		18 (8-§)	
Grade			.18		.02
I or II	21	16 (8-19)		27 (16-42)	
III	36	9 (6-11)		12 (8-14)	
Ascites			.04		.06
Present	37	16 (6-15)		15 (10-26)	
Absent	20	18 (11-19)		30 (18-35)	
Pgp			.77		.70
Negative	48	11 (8-16)		12 (8-25)	
Positive	9	11 (6-12)		20 (14-27)	
Mrp			.45		.67
Negative	18	11 (5-28)		15 (8-35)	
Positive	39	11 (8-15)		20 (11-27)	
Lrp			.003		.007
Negative	13	28 (18-§)		42 (35-§)	
Positive	44	9 (6-11)		5 (11-18)	

*Performance status is not included because data for only 28 patients were available.

†Data are median survival in months (95% Brookmeyer-Crowley confidence intervals).

‡ P value of the logrank test (Mantel-Cox).

§Upper confidence limit cannot be obtained because the confidence interval contains the last observed event or because of extensive censoring (38).

Fig. 2. Progression-free and overall survival according to Lrp expression in 57 patients with FIGO stage III or IV ovarian carcinoma treated with postsurgery chemotherapy. At the end of the study, five (38%) of 13 Lrp-negative (LRP neg.) and five (11%) of 44 Lrp-positive (LRP pos.) patients were free of progression, whereas eight (62%) of 13 Lrp-negative and 11 (25%) of 44 Lrp-positive patients were alive (median follow-up for Lrp-negative and Lrp-positive patients was 34 months and 13 months, respectively).

The predictive value of Lrp is striking because most Lrp-overexpressing multidrug-resistant cancer cell lines do not show cross-resistance to platinum or alkylating agents (15). However, other drug-selected cell lines exist that, as commonly observed in the clinical setting, are cross-resistant to a wide variety of drugs for which Pgp and Mrp do not have a role (40,41). It has been shown that Lrp is overexpressed in cross-resistant 8226/MR myeloma cells (41). Furthermore, in 61 drug-unselected cancer cell lines, Lrp expression was associated not only with resistance to doxorubicin, but also to carboplatin, cisplatin, and melphalan (Izquierdo MA, Shoemaker RH, Flens MJ, et al.: manuscript submitted for publication). These data suggest frequent coexpression of Lrp with other drug resistance genes (i.e., genes involved in resistance to platinum and alkylating agents), resulting in a broad spectrum of drug resistance. This coexpression may also be the case in ovarian carcinoma. Thus, besides potentially contributing to resistance to certain drugs, Lrp expression seems to be at least a pleiotropic marker

of resistance. Alternatively, Lrp may be associated with biologically more aggressive tumors, although our clinicopathologic data in ovarian carcinoma do not support this possibility. Further evidence of the prognostic value of Lrp through its relationship with chemoresistance was obtained in a prospective study (42) that included 93 acute myeloid leukemia patients, which showed that Lrp was an independent prognostic factor for response to chemotherapy.

The molecular characterization of Lrp as the human major vault protein has constituted the first step to clarify its actual contribution to drug resistance. Cytoplasmic vaults were originally isolated from vesicle fractions (18) and, concordantly, LRP-56 staining is primarily vesicular (15). Vaults ($\approx 5\%$ of the total) have also been localized to the nuclear pore complexes (43), which control bidirectional nucleocytoplasmic transport of a wide variety of substrates (44). Vaults are thought to comprise transporter units for nuclear pore complexes, thus implicating Lrp vaults in nucleocytoplasmic transport processes (19,43). Lrp vaults may regulate the vesicular and nucleocytoplasmic transport of drugs. In support of this view, entrapment of drugs in exocytotic vesicles and decreased nuclear to cytoplasmic drug ratios were reported in Lrp-overexpressing multidrug-resistant cells (45,46).

Lack of an association between Pgp and Mrp expression with response to chemotherapy and survival is consistent with the inability of the corresponding genes to confer resistance to cisplatin in transfection experiments (5,12-14). However, our study lacks the power to detect a small effect of the expression of Pgp or Mrp. A larger study will be needed to exclude this possibility. Interestingly, none of the few Pgp-positive patients achieved complete remission, and Pgp appeared to add significance to Lrp in predicting complete response (data not shown; a larger number of patients will be needed to confirm this interaction). Similarly, MDR1 messenger RNA expression detected by polymerase chain reaction was found to be associated with the response to platinum-based chemotherapy in ovarian carcinoma (47). This apparent relationship may be due to MDR1 association with more aggressive tumors or to coexpression with other drug resistance genes (47). However, other studies (9,10) failed to show such a link, and since the relationship is based on a relatively small number of patients, it needs confirmation. Furthermore, the low Pgp expression rate in untreated ovarian carcinoma lessens any potential role in resistance to primary chemotherapy (9,10).

For platinum and alkylating agents, reduced glutathione (GSH)-conjugation has been described (4). Mrp can mediate the transport of certain GSH-conjugates (i.e., leukotriene C4), and it might cause drug resistance by exporting GSH-drug conjugates out of the cells (48,49). Our results do not support such a role for Mrp in clinical resistance to platinum and alkylating agents in ovarian carcinoma. Whether Mrp contributes to resistance to natural products, as suggested by its high expression rate, needs investigation in groups of patients treated with such drugs. A role for Mrp in resistance to taxol would have great impact because of the increasing incorporation of this drug in first-line treatment of ovarian carcinoma. However, *in vitro* studies (13,14) suggest that Mrp confers no resistance or only low resistance to Taxol.

In conclusion, in this study, the expression of the novel multi-drug resistance-related protein Lrp, but not of Pgp and Mrp, was a strong and independent prognostic factor to predict tumor response to standard chemotherapy and survival in advanced ovarian carcinoma patients. If confirmed in a large prospective study, the prognostic information provided by Lrp might be incorporated as a stratification factor in clinical trials of postoperative chemotherapy and might help in selecting patients for alternative therapeutic strategies. The ongoing functional characterization of Lrp vaults will clarify the implication of Lrp-vaults in a potential novel mechanism of drug resistance. This clarification may lead to innovative approaches to prevent the emergence of drug resistance or to reverse the phenotype when it occurs.

References

- (1) Boring CC, Squires TS, Tong T: Cancer statistics, 1993. *CA Cancer J Clin* 43:7-26, 1993
- (2) Neijt JP, ten Bokkel Huinink WW, van der Burg ME, et al: Long-term survival in ovarian cancer. Mature data from The Netherlands Joint Study Group for Ovarian Cancer. *Eur J Cancer* 27:1367-1372, 1991
- (3) Ozols RF: Treatment of ovarian cancer: current status. *Semin Oncol* 21:1-9, 1994
- (4) Johnson SW, Ozols RF, Hamilton TC: Mechanisms of drug resistance in ovarian cancer. *Cancer* 71(2 Suppl):644-649, 1993
- (5) Goldstein LJ, Pastan I, Gottesman MM: Multidrug resistance in human cancer. *Crit Rev Oncol Hematol* 12:243-253, 1992
- (6) Beck WT, Danks MK: Mechanisms of resistance to drugs that inhibit DNA topoisomerases. *Semin Cancer Biol* 2:235-244, 1991
- (7) van der Zee AG, van Ommen B, Meijer C, et al: Glutathione S-transferase activity and isoenzyme composition in benign ovarian tumours, untreated malignant ovarian tumours, and malignant ovarian tumours after platinum/cyclophosphamide chemotherapy. *Br J Cancer* 66:930-936, 1992
- (8) Bissett D, McLaughlin K, Kelland LR, et al: Cisplatin-DNA damage recognition proteins in human tumour extracts. *Br J Cancer* 67:742-748, 1993
- (9) Arao S, Suwa H, Mandai M, et al: Expression of multidrug resistance gene and localization of P-glycoprotein in human primary ovarian cancer. *Cancer Res* 54:1355-1359, 1994
- (10) van der Zee AG, Hollema H, Suurmeijer AJ, et al: Value of P-glycoprotein, glutathione S-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas. *J Clin Oncol* 13:70-78, 1995
- (11) Cole SP, Bhardwaj G, Gerlach JH, et al: Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line [see comment citation in Medline]. *Science* 258:1650-1654, 1992
- (12) Grant CE, Valdimarsson G, Hipfner DR, et al: Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res* 54:357-361, 1994
- (13) Zaman GJ, Flens MJ, van Leusden MR, et al: The human multidrug resistance-associated protein (MRP) is a plasma membrane drug efflux pump. *Proc Natl Acad Sci U S A* 91:8822-8826, 1994
- (14) Cole SP, Sparks KE, Fraser K, et al: Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* 54:5902-5910, 1994
- (15) Scheper RJ, Broxterman HJ, Scheffer GL, et al: Overexpression of a M(r) 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. *Cancer Res* 53:1475-1479, 1993
- (16) Izquierdo MA, van der Valk P, Scheffer GL, et al: Distribution in normal human tissues and tumors of a 110 kD protein associated with non P-glycoprotein multidrug resistance. *Proc Am Assoc Cancer Res* 34:Abstr 1849, 1993
- (17) Scheffer GL, Wijngaard PL, Glens MJ, et al: The drug resistance related protein LRP is the human major vault protein. *Nature Med* 1:578-582, 1995
- (18) Kedersha NL, Rome LH: Isolation and characterization of a novel ribonucleoprotein particle: large structures contain a single species of small RNA. *J Cell Biol* 103:699-709, 1986
- (19) Rome L, Kedersha N, Chugani D: Unlocking vaults: organelles in search of a function. *Trends Cell Biol* 1:47-50, 1991
- (20) International Federation of Gynecology and Obstetrics: Changes in definitions of clinical staging for cancer of the cervix and ovary. *Am J Obstet Gynecol* 156:263-264, 1987
- (21) Serov SF, Scully RE, Sobin LH: Histological Typing of Ovarian Tumours. Geneva: WHO, 1973, pp 17-18
- (22) Sorbe B, Frankendal B, Veress B: Importance of histologic grade in the prognosis of epithelial ovarian carcinoma. *Obstet Gynecol* 59:576-582, 1982
- (23) World Health Organization: WHO Handbook on Reporting Results of Cancer Treatment. Geneva: WHO, 1979
- (24) Hamada H, Tsuruo T: Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells revealed by monoclonal antibodies. *Proc Natl Acad Sci U S A* 83:7785-7789, 1986
- (25) Scheper RJ, Bulte JW, Brakkee JG, et al: Monoclonal antibody JSB-1 detects a highly conserved epitope on the P-glycoprotein associated with multi-drug-resistance. *Int J Cancer* 42:389-394, 1988
- (26) Weinstein RS, Kuszak JR, Kluskens LF, et al: P-glycoproteins in pathology: the multidrug resistance gene family in humans. *Hum Pathol* 21:34-48, 1990
- (27) Flens MJ, Izquierdo MA, Scheffer GL, et al: Immunochemical detection of the multidrug resistance-associated MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res* 54:4557-4563, 1994
- (28) Akiyama SI, Fojo A, Hanover JA, et al: Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. *Somat Cell Mol Genet* 11:117-126, 1985
- (29) Zijlstra JG, de Vries EG, Mulder NH: Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Res* 47:1780-1784, 1987
- (30) Kuiper CM, Broxterman HJ, Baas F, et al: Drug transport variants without P-glycoprotein overexpression from a human squamous lung cancer cell line after selection with doxorubicin. *J Cell Pharmacol* 1:35-41, 1990
- (31) Chan HS, Haddad G, Thorner PS, et al: P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma [see comment citation in Medline]. *N Engl J Med* 325:1608-1614, 1991
- (32) Cordell JL, Falini B, Erber WN, et al: Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J Histochem Cytochem* 32:219-229, 1984
- (33) Dixon WJ, ed: BMDP Statistical Software Manual. Los Angeles: Univ Calif Press, 1990
- (34) Fleiss JL: Statistical Methods for Rates and Proportions. New York: John Wiley & Sons, 1981
- (35) Daly LE, Bourke GJ, McGilvray J: Interpretation and Uses of Medical Statistics, 4th ed. Oxford: Blackwell, 1991
- (36) Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 53:457-481, 1958
- (37) Mantel N: Evaluation of survival data and two new rank order statistics arising in its considerations. *Cancer Chemother Rep* 50:163-170, 1966
- (38) Brookmeyer R, Crowley J: A confidence interval for the median survival time. *Biometrics* 38:29-41, 1982
- (39) Cox DR, Oakes D: Analysis of Survival Data. London: Chapman & Hall, 1984
- (40) Hamaguchi K, Godwin AK, Yakushiji M, et al: Cross-resistance to diverse drugs is associated with primary cisplatin resistance in ovarian cancer cell lines. *Cancer Res* 53:5225-5232, 1993
- (41) Futscher BW, Abbaszadegan MR, Domann F, et al: Analysis of MRP mRNA in mitoxantrone-selected, multidrug-resistant human tumor cells. *Biochem Pharmacol* 47:1601-1606, 1994
- (42) List AF, Spier CS, Abbaszadegan M, et al: Non-P-glycoprotein (PGP) mediated multidrug resistance (MDR): identification of a novel drug resistance phenotype with prognostic relevance in acute myeloid leukemia (AML). *Blood* 82:abstr 443, 1993
- (43) Chugani DC, Rome LH, Kedersha NL: Evidence that vault ribonucleoprotein particles localize to the nuclear pore complex. *J Cell Sci* 106(Pt 1):23-29, 1993
- (44) Rout MP, Wentz SR: Pores for thought: nuclear pore complex proteins. *Trends Cell Biol* 4:357-365, 1994
- (45) Schuurhuis GJ, Broxterman HJ, de Lange JH, et al: Early multidrug resistance, defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein. *Br J Cancer* 64:857-861, 1991
- (46) Dietel M, Arps H, Lage H, et al: Membrane vesicle formation due to acquired mitoxantrone resistance in human gastric carcinoma cell line EPG85-257. *Cancer Res* 50:6100-6106, 1990
- (47) Holzmayer TA, Hilsenbeck S, Von Hoff DD, et al: Clinical correlates of MDR1 (P-glycoprotein) gene expression in ovarian and small-cell lung carcinomas [see comment citation in Medline]. *J Natl Cancer Inst* 84:1486-1491, 1992

- (48) Jedlitschky G, Leier I, Buchholz U, et al: ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. *Cancer Res* 54:4833-4836, 1994
- (49) Müller M, Meijer C, Zaman GJ, et al: Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc Natl Acad Sci U S A* 91:13033-13037, 1994

Notes

Supported by fellowships from the European Society of Medical Oncology (M. A. Izquierdo) and the European Cancer Center (M. A. Izquierdo) and by a grant from the Dutch Cancer Society (R. J. Scheper).

Manuscript received January 11, 1995; revised April 21, 1995; accepted June 9, 1995.

Vascular Permeability Factor (Vascular Endothelial Growth Factor) Expression and Angiogenesis in Cervical Neoplasia

*Anthony J. Guidi, Graziella Abu-Jawdeh, Brygida Berse, Robert W. Jackman, Kathi Tognazzi, Harold F. Dvorak, Lawrence F. Brown**

Background: Angiogenesis is a critical factor in the progression of solid tumors, including cervical cancers. The mechanisms responsible for angiogenesis in cervical neoplasia, however, are not well defined. **Purpose:** Our goal was to determine the relationship between angiogenesis and the expression of the angiogenic cytokine vascular permeability factor (VPF), also known as vascular endothelial growth factor, and its receptors in cervical neoplasia. **Methods:** Sixty-six cervical biopsy specimens were evaluated; among these, 16 samples were designated as benign, 17 as low-grade squamous intraepithelial lesions, 18 as high-grade squamous intraepithelial lesions, and 15 as invasive squamous cell carcinomas. Histologic sections immunostained for factor VIII-related antigen were evaluated quantitatively for microvessel density and for the presence of epithelial-stromal vascular cuffing. Sections were also evaluated for VPF messenger RNA (mRNA) expression by in situ hybridization. **Results:** VPF mRNA expression, epithelial-stromal vascular cuffing, and microvessel density counts were significantly increased in invasive carcinoma and in high-grade intraepithelial lesions as compared with low-grade intraepithelial lesions and benign squamous epithelium. Vascular cuffing and increased microvessel density counts were also significantly associated with increased VPF mRNA expression. **Conclusions:** These observations suggest that VPF is an important angiogenic factor in cervical neoplasia. [*J Natl Cancer Inst* 87:1237-1245, 1995]

Angiogenesis is associated with high-grade cervical dysplasia and with invasive squamous cell carcinoma of the cervix (1-12), and altered vascular patterns have proved useful in the colposcopic detection of these lesions (1-5). In addition, increased microvessel density has been associated with improved response to radiation therapy and chemotherapy in patients with invasive squamous cell carcinoma of the cervix (13,14), and tumor vessels have been suggested as potential targets of antineoplastic

therapies (15-17). Although angiogenesis is an important factor in the progression, detection, and treatment of cervical neoplasms, the mechanisms responsible for inducing angiogenesis in cervical neoplasia are not well defined.

Vascular permeability factor (VPF), also known as vascular endothelial growth factor, is a 34- to 42-kd glycoprotein that is expressed in a variety of human tumors (15,18-23) and is widely considered to be an important angiogenic factor (24). VPF acts as a potent inducer of microvessel permeability (25,26) and is also a selective endothelial cell mitogen (27-32). VPF binds to at least two specific tyrosine kinase receptors, flt-1 and KDR; both are associated with the surface of endothelial cells (33,34). Inhibition of either VPF or the VPF receptor flk-1 (the murine equivalent of KDR) has been shown to diminish tumor angiogenesis and to suppress tumor growth in vivo (35,36).

The purpose of this study was to examine the relationship between angiogenesis and the expression of VPF and VPF receptor messenger RNAs (mRNAs) in benign and neoplastic cervical tissues.

Materials and Methods

Surgical pathology reports of cervical biopsies performed at the Beth Israel Hospital, Boston, MA, between September 1993 and March 1994 were reviewed to identify cases with low-grade squamous intraepithelial lesions (flat condyloma with or without low-grade dysplasia) or high-grade squamous intraepithelial lesions (high-grade dysplasia or carcinoma in situ) as well as biopsy specimens with no morphologic evidence of dysplasia or human papillomavirus infection. Reports from November 1991 to July 1994 were screened for consecutive cervical biopsy specimens showing invasive squamous cell carcinoma. Hematoxylin-eosin-stained sections were reviewed (three levels from each biopsy), and 72 biopsy specimens with residual diagnostic tissue after level sectioning were randomly selected for independent review by three pathologists (A. J.

**Affiliation of authors:* Department of Pathology, Beth Israel Hospital and Harvard Medical School, Boston MA.

Correspondence to: Lawrence F. Brown, M.D., Beth Israel Hospital, Department of Pathology, 330 Brookline Ave., Boston, MA 02215.

See "Notes" section following "References."