Caveolin-1 Expression in Ovarian Carcinoma Is MDR1 Independent

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Key Words: Serous effusions; Immunohistochemistry; mRNA in situ hybridization; Reverse transcription–polymerase chain reaction; Caveolin; Multidrug resistance

Abstract

We studied the role of caveolin-1 in tumor progression and prognosis in serous ovarian carcinoma and the association between caveolin-1 and MDR1 expression. The study involved immunohistochemical analysis for caveolin-1 and P-glycoprotein (P-gp) expression in 75 effusions and 90 solid lesions from ovarian and primary peritoneal carcinoma; in situ hybridization for MDR1 messenger RNA (mRNA) expression in 62 effusions and all 90 tumors; and reverse transcription–polymerase chain reaction (RT-PCR) for caveolin-1 mRNA expression in 23 effusions. Immunohistochemical analysis localized caveolin-1 to the cell membrane in 43 effusions and 24 tumors. P-gp membrane expression was detected in 14 effusions and 11 tumors; MDR1 mRNA, in 20 effusions and 30 tumors. Caveolin-1 mRNA was expressed in 19 effusions. Caveolin-1 protein expression showed no association with that of P-gp protein or MDR1 mRNA. The expression of all markers was similar in carcinoma cells in pleural and peritoneal effusions. Caveolin-1 is a novel diagnostic marker for effusions; expression is moderately elevated in tumor cells in effusions, possibly owing to altered signal transduction and metabolism in cancer cells at this site. Expression seems MDR1 independent.
carcinoma and ovarian carcinoma cell lines, compared with benign ovarian surface epithelium.16

Multidrug resistance (MDR), in either intrinsic or acquired form, occurs in a large number of solid tumors by at least 8 different mechanisms, making them unresponsive to chemotherapy.17,18 Expression of the \textit{MDR1} gene and its protein product, P-glycoprotein (P-gp), is one of the common mechanisms of MDR and has been previously documented in ovarian carcinoma.19-22 An interesting observation has been made of an association between caveolin-1 expression and the MDR phenotype in human cell lines of colon and breast carcinoma.23 In agreement with these findings, caveolin-1 expression was shown to be up-regulated in a paclitaxel-resistant lung carcinoma cell line (A549) and in the ovarian carcinoma cell line SKVLB1.24 The up-regulation in caveolin-1 levels occurred in a paclitaxel-inducible manner in A549 cells.24 As A549 cells expressed low levels of P-gp, while the reverse was true for the SKVLB1 cells, these findings, as well as those reported for colon and breast carcinomas, were postulated to occur in a P-gp-independent manner.23,24

Ovarian cancer is the leading cause of death due to gynecologic cancer in women in industrialized countries, with an incidence that seems to be increasing.25,26 Owing to their insidious development, two thirds of these tumors are diagnosed at stage III or IV. Abdominal discomfort, experienced by two thirds of the patients, reflects the accumulation of ascitic fluid that often contains malignant cells. The presence of ovarian carcinoma cells in peritoneal effusions has been attributed traditionally to direct shedding from the ovarian tumor surface. However, their phenotype and genotype have remained largely uncharacterized, owing to a lack of large comparative studies of primary tumors, effusions, and metastatic lesions. Davidson et al27-29 and Berner et al30 found several alterations in the expression of metastasis-associated molecules in ovarian carcinoma cells in effusions compared with primary tumors.

The aim of the present study was to evaluate the expression of caveolin-1 in effusions and corresponding primary and metastatic lesions of patients diagnosed with ovarian carcinoma and its prognostic role. In addition, the relationship between caveolin-1 and P-gp/MDR1 expression in our patient cohort was analyzed.

### Materials and Methods

#### Serous Effusions

Seventy-five fresh, nonfixed peritoneal and pleural effusions (volume range, 20-2,000 mL) from 68 patients diagnosed with ovarian or primary peritoneal carcinoma (PPC) were submitted to the Division of Cytology, Department of Pathology, Norwegian Radium Hospital, Oslo, during the period January 1998 to April 2000. Effusion specimens, as well as relevant clinical data, were obtained from the Department of Gynecologic Oncology, Norwegian Radium Hospital. The specimen handling protocol was described previously.31 The diagnostic criteria used were according to established guidelines.32 The morphologic diagnostic protocol, as well as the staining procedure of cell-block sections using monoclonal antibodies directed against epithelial markers, has been described previously.31 The distribution of effusions according to tumor type and site is shown in Table 1.

#### Tumor Specimens

Ninety surgical specimens consisting of primary tumors (n = 30) and metastatic lesions (n = 60) from 37 of the 68 patients diagnosed with ovarian carcinoma or PPC were studied. Formalin-fixed, paraffin-embedded tissue blocks were obtained from archival material in the Department of Pathology, Norwegian Radium Hospital (33 patients), and from other hospitals in Norway (4 patients). Biopsy specimens from 1 site were available for 8 patients, from 2 sites for an additional 8 patients, from 3 sites for 18 patients, and from 4 sites for the remaining 3 patients. All tissue specimens underwent microscopic confirmation of diagnosis, tumor type, and histologic grade, following established criteria.33 Twenty nonneoplastic fallopian tubes and ovaries also were studied. The latter were obtained from patients with a gynecologic malignant neoplasm. The distribution of solid tumors according to tumor type and site is shown in Table 2.

#### Immunohistochemical Analysis

Formalin-fixed, paraffin-embedded sections were stained for caveolin-1 using a rabbit polyclonal antibody directed against the N-terminal domain of the protein (Santa Cruz Biotechnology, Santa Cruz, CA). Microwave pretreatment of 2 times for 5 minutes each in citrate buffer was used. Staining for P-gp was done using the JSB1 monoclonal antibody (Signet, Dedham, MA). Microwave
pretreatment 4 times for 5 minutes each in citrate buffer was used. Staining using both antibodies was performed using the biotin streptavidin peroxidase method (supersensitive immunodetection LP000-UL, BioGenex, San Ramon, CA) and Optimax Plus automated cell staining system (BioGenex). Negative controls consisted of sections that underwent a similar staining procedure, with the exclusion of primary antibody application, as well as sections stained using a nonrelevant antibody of the same isotype. Positive controls consisted of ovarian carcinoma specimens that showed immunoreactivity for the studied antigens in previous studies of ovarian carcinoma (see the “Discussion” section regarding caveolin-1).

**Interpretation of Immunohistochemical Results**

Caveolin-1 immunoreactivity was scored as cytoplasmic, membranous, or both. Staining was scored in carcinoma cells, mesothelial cells, and leukocytes. P-gp staining in cancer cells was scored as positive only if membranous or combined membranous and cytoplasmic staining was present. The presence and extent of staining were scored using the following scale: 0, no staining; 1, staining of 0% to 20% of tumor cells; and 2, staining of 21% to 100% of tumor cells. A minimum of 500 cells, when present, were evaluated.

**Oligonucleotide Probe**

A specific antisense oligonucleotide DNA probe for the messenger RNA (mRNA) transcript of MDR1 was obtained from Research Genetics (Huntsville, AL). The probe sequence (5’-3’) was as follows:

CAG ACA GCA GCT GAC AGT CCA AGA ACA GGA CT

A poly d(T)20 oligonucleotide (Research Genetics) was used to verify the integrity and lack of degradation of mRNA in each sample. The DNA probe was hyperbiotinylated. The stock dilution was diluted with probe diluent (Research Genetics) immediately before use. A working dilution of 1:200 was used. A specific sense oligonucleotide was used for the evaluation of nonspecific activity.

**In Situ mRNA Hybridization**

Tissue sections (4 µm thick) of formalin-fixed, paraffin-embedded specimens were mounted on ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA). Sectioning was performed in RNase-free water. Slides were dewaxed and rehydrated using xylene (twice for 10 minutes each) and isopropyl alcohol (5 minutes). In situ mRNA hybridization (ISH) was carried out by using the microscope manual staining system (Fisher Scientific). Hybridization of the probe was carried out as previously described. A positive enzymatic reaction in this assay stained dark blue. Known positive controls were used in each hybridization reaction. These consisted of 2 cases for which positive hybridization was reproducible in pilot studies. Controls for endogenous alkaline phosphatase included treatment of the sample in the absence of the probe and use of chromogen alone.

**Evaluation of ISH Results**

Staining was scored in carcinoma and stromal cells. The staining extent was scored using a cutoff of 20%. Staining of 20% or fewer of tumor or stromal cells was scored as focal (score, 1), while staining of more than 20% of cells was interpreted as diffuse (score, 2). Evaluation was done without knowledge of the clinical outcome.

**Reverse Transcription–Polymerase Chain Reaction**

We analyzed 23 malignant effusions (16 of peritoneal and 7 of pleural origin) for the presence of caveolin-1 mRNA by reverse transcription–polymerase chain reaction (RT-PCR). Twenty-one effusions originated from patients with ovarian carcinoma and 2 from patients with PPC. All specimens contained a distinct population of carcinoma cells. RNA was isolated using the Tri-reagent kit (Sigma, St Louis, MO). Complementary DNA was transcribed from 0.5 µg of RNA. The following RNA transcripts were detected by amplification of the corresponding complementary DNA:

Caveolin-1: sense primer, 5’-GAG CGA GAA GCA AGT CTA GAC AGT GGA GTG GAC GAA GAT; antisense primer, 5’-ACA GAC GGT GTG GAC GAA GAT; product size, 361 base pairs

beta-actin: sense primer, 5’-GTA CCA CTG GCA TCG TGA TGG ACT; antisense primer, 5’-ATC CAC ACG GAG TAC TTG CGC TCA; product size, 410 base pairs

The cycle program consisted of 34 runs of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds,

| Table 2 | Tissue Distribution of the Studied Primary Tumors and Metastatic Lesions According to Histologic Type |
|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Histologic Type | Ovary | Omentum | Peritoneum | Intestine | Lymph Node | Other | Total |
| Serous | 25 | 22 | 5 | 7 | 4 | 11 | 74 |
| Combined | 2 | 0 | 2 | 2 | 0 | 1 | 7 |
| Primary peritoneal carcinoma | 3 | 3 | 2 | 1 | 0 | 0 | 9 |
| Total | 30 | 25 | 9 | 10 | 4 | 12 | 90 |
and elongation at 72°C for 2 minutes. The cycle program was preceded by an initial denaturation at 94°C for 5 minutes, followed by a final extension at 72°C for 10 minutes. PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide.

The 3T3-cav-1 cell line, transfected with the caveolin-1 gene and expressing large amounts of caveolin-1 mRNA, was used as control in all reactions. Band size was evaluated using the caveolin/beta-actin ratio. Weak signals were defined as a caveolin/beta-actin ratio of 0.01 or less. Strong signals were defined as a caveolin/beta-actin ratio of more than 0.01.

Statistical Analysis

Statistical analysis was performed applying the SPSS-PC package (version 9.0, 1999; SPSS, Chicago, IL). A probability of less than .05 was considered statistically significant. Comparative analyses of malignant cytologic specimens, primary tumors, and metastatic lesions were executed using the Wilcoxon signed rank test. In cases for which more than 1 metastatic lesion was available, the lesion showing the most intense staining was included in the statistical evaluation. Studies of the association between staining results in effusions and clinicopathologic parameters were executed using the chi-square test. Univariate survival analyses for effusion specimens were executed using the Kaplan-Meier method and the log-rank test. Membranous and cytoplasmic staining results were analyzed separately.

Results

Serous Effusions

Cancer cells were detected in all 75 malignant effusion specimens. An additional population of reactive mesothelial and/or inflammatory cells was found in 50 cases.

Immunohistochemical Results

Staining at the cell membrane generally was confined to a small cell population using both antibodies Table 3. Image 1C, and Image 1H. Membrane immunoreactivity for caveolin-1 had a dot-like quality, in agreement with the distribution of caveolae at this site. Focal cytoplasmic immunoreactivity for caveolin-1 was detected in mesothelial cells in only 4 specimens, while membranous staining was absent from both mesothelial and inflammatory cells. The reactive cell population showed no expression of P-gp. Ovarian carcinoma cells in pleural (n = 22) and peritoneal (n = 53) effusions showed a comparable immunoreactivity profile.

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A positive signal using a poly d(T) probe was detected in all cases (data not shown). The presence of MDR1 mRNA, as that of P-gp, was seen infrequently (Table 3). When detected, it was limited to a small population of carcinoma cells, with the exception of 1 specimen Image 1D. As with immunohistochemical results, MDR1 mRNA expression showed no correlation with effusion site.

Table 3
Caveolin-1, P-gp, and MDR1 Results in Carcinoma Cells in the Entire Patient Cohort*

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**Tissue Sections**

**Immunohistochemical Results**

The distribution of immunoreactivity in primary tumors and metastatic lesions is detailed in Table 3. As in effusion specimens, caveolin-1 was expressed in the cytoplasm, the cell membrane, or both and was of a dot-like quality and a more focal distribution at the cell membrane. Staining for caveolin-1 was detected in endothelial cells of all vessel sizes in all cases, providing an internal control of staining adequacy. Concomitant intense staining of peritumoral stromal cells additionally was found in some tumors, irrespective of biopsy site. Combined membrane and cytoplasmic immunoreactivity was seen in 17 (85%) of 20 nonneoplastic lesions. The latter was diffuse in 11 of the 17 immunoreactive cases. No significant differences were seen between solid metastases from different sites. P-gp staining was focal and limited to malignant cells. The expression pattern was comparable in primary tumors and metastases from a given patient. Consequently, no significant differences were seen between solid metastases from different sites. P-gp staining was focal and limited to malignant cells.
differences were found when expression in primary tumors was compared with that in respective metastatic lesions. Positive controls showed consistent immunoreactivity for the antigens studied. Staining of both epithelial and endothelial cells was seen in benign and malignant control specimens for caveolin-1. Both types of negative controls showed no staining.

**In Situ mRNA Hybridization**

A positive signal using a poly d(T) probe was detected in all cases (data not shown). ISH results for MDR1 mRNA are shown in Table 3 and in Image 1E and Image 1F. As for immunohistochemical results, primary tumors and metastases showed comparable expression.

**Comparison of Cancer Cells in Effusion Specimens and Tissue Sections**

Immunohistochemical results for the total study cohort showed an up-regulation in the number of cases showing caveolin-1 membrane immunoreactivity from 24 (27%) of 90 solid lesions to 43 (57%) of 75 effusions. A similar trend was observed for cytoplasmic staining, from 40 (44%) of 90 to 52 (69%) of 75 caveolin-1–positive specimens (Table 3). P-gp expression was similarly elevated in effusions (14/75 [19%]) compared with solid tumors (11/90 [12%]). However, in the statistical evaluation, when only cases with corresponding effusion and solid lesions were evaluated, these findings failed to reach statistical significance ($P > .05$) Table 4. MDR1

**Image 1 (cont)**

**E**, MDR1 mRNA expression in carcinoma cells in a primary serous ovarian carcinoma. All cells are labeled (NBT-BCIP, ×400). **F**, MDR1–negative primary ovarian carcinoma. Cells are stained by nuclear fast red, used as counterstain (nuclear fast red counterstain, ×400). **G**, P-gp–positive ovarian carcinoma cells in an ovarian metastasis to omentum (P-gp, ×400). **H**, P-gp–positive ovarian carcinoma cells in a peritoneal effusion (P-gp, ×400).
mRNA expression was similar in effusions and solid tumors when the entire material was evaluated (effusions, 20/62 [32%]; tumors, 30/90 [33%]). This also was reflected in the comparison of paired lesions from the same patient (Table 4).

**Clinicopathologic Data**

Patient age ranged from 35 to 79 years (mean, 61 years). With the exception of 1 patient who had stage IIc disease, all patients had stage III (34 patients) or stage IV (33 patients) disease at diagnosis. The extent of residual disease after primary operation ranged from 1 to 5 cm in largest diameter. Three patients had well-differentiated, 28 had moderately differentiated, and 37 had poorly differentiated tumors. No association was observed between caveolin-1, P-gp, or MDR1 mRNA expression in effusions and patient age, disease stage, tumor grade, or the extent of residual disease.

**Discussion**

Ovarian carcinoma shows a remarkably typical pattern for tumor progression, centering on a consistent predilection...
for the peritoneal cavity and its bordering organs, far more often with than without the presence of tumor cells in the ascitic fluid. Not uncommonly, this phenomenon precedes or is concomitant with the appearance of a malignant pleural effusion. The study of adhesion molecules and other molecules participating in the metastatic process therefore is central to the understanding of this disease. Davidson et al recently reported up-regulated expression of the carbohydrate antigens Tn and sialyl Tn, mRNA and protein levels of the enzyme matrix metalloproteinase-2, and E-cadherin complex proteins, as did Berner et al for the adhesion molecule CD44s in ovarian carcinoma cells in effusions compared with primary tumors. In addition, Davidson et al found down-regulation in mRNA expression of tissue inhibitor of metalloproteinase-2 and angiogenic genes in that cohort. These findings may be triggered by altered expression of signal transduction or regulatory molecules.

Caveolin-1 expression in ovarian carcinoma was studied by Hurlstone et al. The authors found that transcriptional silencing through methylation of the caveolin-1 promoter was not a feature of various carcinoma cell lines and in 26 primary ovarian tumors. By using RT-PCR and Western blot analysis for further characterization of the cell lines, they found low mRNA and protein expression in some, including 4 of ovarian origin. Conversely, down-regulation of caveolin-1 expression in 6 ovarian carcinoma cell lines and 8 clinical specimens, compared with 2 specimens of normal ovarian surface epithelium, was reported by Bagnoli and coworkers. To date, however, no data are available regarding the in vivo expression of caveolin-1 protein and mRNA in ovarian carcinoma cells in effusions or the differences between caveolin-1 expression at this site and that in solid tumors.

Our findings for solid tumors are in agreement with those reported by Bagnoli et al, as caveolin-1 expression in solid tumors was markedly reduced compared with that in benign ovarian or tubal epithelium. However, the low protein and mRNA expression reported for cell lines and solid tumors does not seem to characterize the majority of our effusions. Rather, up-regulated expression of caveolin-1 was observed in tumor cells at this site, at both membranous and cytoplasmic localization, compared with both primary tumors and metastases (40%, 33%, and 21% of cases, respectively, for membrane expression). Although failing to reach statistical significance in the aforementioned comparison of corresponding lesions from the same patient, this finding became all the more apparent when the entire study cohort was included in the analysis (57%, 33%, and 23% of cases, respectively). In view of the association of caveolin-1 with integrin-mediated adhesion, this may reflect an altered adhesion phenotype in metastatic cells in effusions, with possible implications for tumor-mesothelium interactions, as mesothelial cells have been shown to express integrin receptors.

Tyrosine phosphorylation of caveolin-1 at its tyrosine-14 residue was shown to be involved in epidermal growth factor–stimulated migration of src- and abl-transformed cells. Caveolin-1 expression thus may offer carcinoma cells in effusion an advantage in migration along the peritoneal surface and the subsequent establishment of metastases. Unlike its expression in peritumoral stromal cells and endothelial cells in solid lesions, the detection of caveolin-1 in reactive mesothelium and leukocytes was a rare event. Furthermore, it was never seen at the cell membrane of these cells. Thus, although of moderate sensitivity, caveolin-1 is also a novel marker for the diagnosis of ovarian (and possibly other) adenocarcinomas in effusions.

The consistent differences between ovarian carcinoma cells in effusions and in primary tumors raise serious doubts about the validity of the oversimplified shedding hypothesis. No convincing evidence is present to date to support any similarities between carcinoma cells at these two locations, as would be implied from a passive process of shedding. As shown in the aforementioned consecutive studies of our patient cohort, the cells in effusions possess entirely different features on both the phenotypic and genotypic levels. These differences may represent the acquisition of a metastatic phenotype that is a prerequisite for tumor progression. Alternatively, they may be induced by the altered environment and interactions with resident mesothelial cells of the peritoneal cavity. Further support of our hypothesis comes from our observations that the cells in peritoneal and pleural effusions have a remarkably similar expression pattern on both protein and mRNA levels. Our findings regarding caveolin-1 expression are in full agreement with these previous results.

Of note, unlike the cytoplasmic pattern of immunoreactivity reported in prostatic carcinomas, localization to the cell membrane, previously not reported for human carcinomas, as well as the cytoplasm, was seen in the present study. Tyrosine-14 phosphorylated caveolin-1 was localized to both cell membrane and the cytoplasm in v-src–expressing rat fibroblasts. The presence of caveolin-1 in the cytoplasm was hypothesized to represent adhesion and fusion of membrane-derived caveolae. Alternatively, it may represent synthesized protein localized to the Golgi complex. The similar colocalization of caveolin-1 in our specimens suggests the presence of functional protein in ovarian carcinomas.

Expression of the MDR1 gene product P-gp has been shown to be a central pathway in the acquisition of resistance to chemotherapy in both solid and hematologic tumors and is one of the common mechanisms of MDR. The association between caveolin-1 expression and the MDR phenotype in human cell lines of colon and breast carcinoma is of
interest in view of the role of caveolin-1 in the mediation of growth-promoting signals. However, the report by Yang and coworkers suggested the involvement of a P-gp–independent pathway in the association between caveolin-1 and the MDR phenotype. Our results are in agreement with these findings, as both P-gp and MDR1 mRNA expression showed no association with caveolin-1 expression. This finding was observed in both solid lesions and effusions.

Caveolin-1 protein expression did not show an association with established prognostic factors in ovarian carcinoma, such as patient age, tumor grade, disease stage, or the extent of residual disease. Interestingly, cytoplasmic expression was detected more often in well-differentiated and moderately differentiated tumors, associating caveolin-1 expression with a better-developed epithelial phenotype. These findings may further support the role of this protein in adhesion, as ovarian carcinoma cells tend to preserve their glandular architecture in grade I and II tumors, often losing it in grade III carcinomas. In a study of solid tumors in a cohort of advanced-stage ovarian carcinomas, followed up for up to 20 years, Davidson et al failed to find correlation between caveolin-1 expression and survival. Studies of early-stage ovarian carcinomas may reveal a more central prognostic role for this protein. This would be in agreement with the prognostic significance attributed to caveolin-1 expression in localized (early-stage) prostate cancer.

Caveolin-1 expression is reduced in solid lesions of advanced-stage ovarian carcinoma but seems to be elevated in tumor cells in effusions, while absent from benign reactive cells at this site. It thus is a novel promising diagnostic marker for effusions. Caveolin-1 up-regulation in effusions suggests alteration in signal transduction and/or adhesion in tumor cells at this site. Caveolin-1 expression is independent of those of MDR1 mRNA and its protein product, P-gp. The absence of difference in expression patterns in tumor cells in the peritoneal and pleural cavities provides further support for our hypothesis that ovarian carcinoma cells at these sites are of similar nature.

References


