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Migfilin, α-parvin and β-parvin are differentially expressed in ovarian serous carcinoma effusions, primary tumors and solid metastases

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Abstract

Objective—To analyze the expression and clinical role of integrin-linked kinase (ILK), α-parvin, β-parvin and migfilin in advanced-stage serous ovarian carcinoma.

Methods—Expression of these 4 proteins was investigated in 205 effusions and in 94 patient-matched solid lesions (33 primary tumors, 61 solid metastases) using immunohistochemistry. Protein expression was analyzed for association with clinicopathologic parameters and survival.

Results—ILK, α-parvin, β-parvin and migfilin were expressed in tumor cells in 53%, 2%, 28% and 53% of effusions and 57%, 20%, 83% and 25% of solid lesions, respectively. Statistical analysis showed significantly higher α-parvin and β-parvin expression in primary carcinomas ($p=0.02$ and $p=0.001$, respectively) and solid metastases ($p=0.001$ and $p<0.001$, respectively), compared to effusions, with opposite findings for migfilin ($p=0.006$ and $p=0.008$ for primary carcinomas and solid metastases, respectively). ILK expression was comparable at all anatomic sites. β-parvin expression in effusions was associated with better response to chemotherapy at diagnosis ($p=0.014$), with no other significant association with clinicopathologic parameters or survival. Expression in primary tumors and solid metastases was similarly unrelated to clinicopathologic parameters or survival.

Conclusions—This study provides further evidence to our previous observations that the adhesion profile of ovarian serous carcinoma cells in effusions differs from their counterparts in primary carcinomas and solid metastases. β-parvin may be a novel marker of chemoresponse in metastatic ovarian carcinoma.

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Keywords

ovarian carcinoma; effusions; adhesion; tumor progression; survival

Introduction

Ovarian cancer is the most lethal gynecologic malignancy and one of the major causes of cancer-related death among women in the Western world. The disease is diagnosed at advanced stage (FIGO stage III-IV) in two-thirds of the patients, with a U.S. 5-year survival rate of 27% for this group [1]. In a recent Norwegian study, 5-year survival for stage IV disease was 16% [2]. With chemoresistance as major hindrance to long-term cure in ovarian cancer, identifying molecular markers that are candidates for targeted therapy is crucial for improving survival [3]. Investigation of such markers should take into account the fact that tumor progression is associated with molecular changes in cancer cells, underlining the need to study metastatic disease. In ovarian carcinoma (OC), molecular differences are especially pronounced between solid lesions and effusions, the latter representing growth in suspension as spheroid-like structures under anoikis-free conditions [4]. One aspect of tumor biology that appears to be particularly affected of the changing microenvironment in effusions compared to solid lesions is adhesion, as in the case of cadherins and their transcriptional regulators [5].

Integrins, heterodimeric transmembrane glycoproteins composed of α and β subunits that are involved in invasion, metastasis, angiogenesis, proliferation and apoptosis, are major regulators of interactions between tumor cells and the extracellular matrix (ECM) [6]. Integrins bind ECM proteins, including laminin, fibronectin, collagen, vitronectin, entactin, tenascin and fibrinogen, and connect to the actin cytoskeleton via integrin-linked kinase (ILK), which binds the cytoplasmic tail of β-subunits. ILK, regulator of AKT and glycogen synthase kinase-3β (GSK-3β) signaling, additionally binds PINCH and α-parvin or β-parvin to form a stable ILK-centered heterotrimer. Each protein in this complex binds in turn several molecular partners, including WT-1, EphA1, Rictor, AKT, paxillin and actin [7]. ILK, PINCH and parvin are necessary for embryogenesis [7,8] and have been shown to be expressed and related to poor patient outcome in several cancers [9,10]. Whether ILK is a true kinase or a pseudo-kinase is a controversial subject [7-9], with the latter view recently gaining support from advanced molecular studies [7,8].

Another protein involved in interactions between the cell membrane and the actin cytoskeleton is migfilin. Migfilin localizes to cell-cell adhesion sites with β-catenin, part of the E-cadherin adhesion complex. It is additionally linked to the actin cytoskeleton through binding of Mig-2, filamin and VASP, thereby regulating cell morphology and motility. It is further translocated to the nucleus in a process regulated by RNA splicing and Ca^{2+}, where it interacts in cardiomyocytes with the transcription factor CSX/NKX2-5 to promote differentiation [11].

Whereas ILK has been the topic of several studies of OC or ovarian surface epithelium (OSE) [12-19], parvin and migfilin expression has not been previously studied in this tumor. Our group previously identified *PINCH2* as a gene that is overexpressed in malignant mesothelioma, native cancer of the serosal cavities, compared to serous OC based on gene expression array analysis [20]. While *PINCH2* mRNA levels by qPCR were higher in mesotheliomas compared to OC and breast carcinoma, PINCH-2 protein expression by flow cytometry was frequent in effusions from all 3 cancers [21]. This suggested that molecular partners of PINCH-2, i.e. ILK and parvins, may be expressed and have a clinical role in OC. Migfilin was deemed to be of interest in view of the unique cadherin-related adhesion

profile of OC in effusions [5]. The present study analyzed the expression of ILK, α-parvin, β-parvin and migfilin in OC at various anatomic sites, with focus on malignant effusions.

Materials and methods

Patients and material

Specimens and clinical data were obtained from the Department of Gynecologic Oncology, Norwegian Radium Hospital. Informed consent was obtained according to Norwegian and international guidelines. Study approval was given by the Regional Committee for Medical Research Ethics in Norway. Clinicopathologic data are detailed in Table 1. The Johns Hopkins grading system, classifying serous OC as low- or high-grade, was used [22].

Effusions—Fresh non-fixed malignant peritoneal (n=170) and pleural (n=35) effusions were obtained from 205 patients diagnosed with serous carcinoma, including 171 diagnosed with OC, 25 with primary peritoneal carcinoma, and 9 with tubal carcinoma. Due to their closely-linked histogenesis and phenotype, all are referred to as serous OC henceforth. The majority of patients (n=124) underwent primary surgery, 63 received neoadjuvant chemotherapy with interval debulking surgery and 14 patients only received chemotherapy. Data were unavailable for 4 patients. One-hundred-and-twenty-six samples were obtained prior to chemotherapy administration, and 76 were obtained post-chemotherapy, at interval debulking surgery or at recurrent disease. Data regarding chemotherapy status were unavailable for 3 patients. The majority of patients (n=188) received platinum-based therapy, of whom 156 received platinum/paclitaxel combination. Effusions were submitted for routine diagnostic purposes during 1998-2005 and were processed immediately after tapping. Cell blocks were prepared using the Thrombin clot method. Remaining material, when available, was fresh-frozen. Diagnoses were established using morphology and immunohistochemistry (IHC) [4].

Surgical specimens—Tissue microarray (TMA) slides containing 33 patient-matched primary serous OC and 61 solid metastases from a total of 44 patients were additionally studied. Metastases were from the omentum (n=37), peritoneum (n=12), intestine (n=7) or other intra-abdominal sites (n=5). An average of 2 punches (2 mm in diameter) was available from each tumor.

IHC

Formalin-fixed paraffin-embedded sections were analyzed for protein expression of ILK, α-parvin, β-parvin and migfilin using the EnVision system (Dako, Glostrup, Denmark) using IgG mouse monoclonal antibodies raised in the laboratory of Prof. Wu, as previously described [23-26].

Slides were deparaffinized and rehydrated prior to pretreatment to unmask epitopes and subsequently treated with 0.03% H_2O_2 for 5 minutes to block endogenous peroxidase. Sections were then incubated with the above-mentioned antibodies, all applied at 1:100 dilution, with antigen retrieval in citrate buffer. A secondary antibody was applied for an additional 30 minutes. Visualization was achieved using 3‘3-diaminobenzidine tetrahydrochloride substrate (DAB) and hematoxylin counterstaining. Positive controls consisted of serous OC that was shown to express the protein in a pilot study. Negative controls consisted of sections that underwent similar staining procedures with nonrelevant mouse IgG (Sigma-Aldrich, St Louis MO).

Staining extent was scored by a surgical pathologist experienced in cytopathology and gynecopathology (BD) on a scale of 0-4, as follows: 0= no staining, 1=1-5%, 2=6-25%,

3=26-75%, 4=76-100% stained tumor cells. All specimens contained at least 100 tumor cells.

Statistical analysis

Statistical analysis was performed applying the SPSS-PC package (Version 18.0, Chicago IL). Probability of <0.05 was considered statistically significant. Analysis of the association between ILK, α-parvin, β-parvin and migfilin protein expression and anatomic site for patient-matched specimens was executed using the Wilcoxon Signed Ranks test. Analyses of the association between protein expression and clinicopathologic parameters were undertaken using the Mann-Whitney U test. Clinicopathologic parameters for the effusion cohort were grouped as follows: age: ≤60 vs. >60 years; effusion site: peritoneal vs. pleural; grade: low vs. high; FIGO stage: III vs. IV; chemotherapy status: pre- vs. post-chemotherapy; residual disease volume (≤1 vs. >1 cm); response to chemotherapy: complete vs. partial response/stable disease/progression. For primary carcinomas and solid metastases, protein expression was studied for association with age, FIGO stage and residual disease volume. The association with histological grade was not studied in view of the small number of patients with low-grade tumors.

Progression-free and overall survival (PFS, OS) were calculated from the date of the last chemotherapy treatment/diagnosis to the date of recurrence/death or last follow-up, respectively. Univariate survival analyses of PFS and OS were executed using the Kaplan-Meier method and log-rank test. Platinum resistance was defined as PFS ≤6 months according to guidelines published by the Gynecologic Oncology Group (GOG) [27] and progressive disease or recurrence was evaluated by the RECIST criteria [28]. Expression categories in survival analyses were clustered as low vs. high based on IHC score of 0-2 vs. 3-4.

Results

α-parvin, β-parvin and migfilin are differentially expressed in primary serous OC, solid metastases and effusions

IHC analysis of 205 effusions showed ILK, α-parvin, β-parvin and migfilin expression in tumor cells in 109/205 (53%), 4/205 (2%), 58/205 (28%) and 108/204 (53%; one failed test) of effusions, respectively (Figure 1). ILK and parvins were absent from reactive mesothelial cells, while migfilin was expressed in this population in 39 specimens. Staining of 94 patient-matched primary tumors and solid metastases demonstrated ILK, α-parvin, β-parvin and migfilin tumor expression in 54/94 (57%), 19/94 (20%), 78/94 (83%) and 23/93 (25%; one failed test) specimens, respectively (Figure 1; Table 2).

Statistical analysis of patient-matched specimens showed significantly higher α-parvin and β-parvin expression in primary carcinomas (p=0.02 and p=0.001, respectively) and solid metastases (p=0.001 and p<0.001, respectively) compared to effusions, with opposite findings for migfilin (p=0.006 and p=0.008 for primary carcinomas and solid metastases, respectively). ILK expression was comparable at all anatomic sites.

β-parvin expression in effusions is associated with better response to chemotherapy at diagnosis

ILK, α-parvin, β-parvin and migfilin expression in effusions and solid specimens was studied for association with clinicopathologic parameters. β-parvin expression in effusions was associated with better response to chemotherapy at diagnosis (p=0.014), with no other significant association with clinicopathologic parameters for any of the proteins (p>0.05; data not shown). The association between β-parvin and chemotherapy response retained its

significance, though more weakly, in comparison of patients who had complete or partial response vs. stable disease or progression (p=0.043), with no significant differences between effusions from patients with complete vs. partial response (p>0.05). Expression in primary tumors and solid metastases was unrelated to clinicopathologic parameters (p>0.05).

ILK, α-parvin, β-parvin and migfilin expression is unrelated to survival in serous OC

For OC patients with effusions (n=205), median PFS was 6 months (range=0-103 months), with 49 patients never achieving a progression-free period. Median OS was 26 months (range=1-117 months). At last follow up, 194 patients were dead of disease, 6 were alive with disease, and 4 were disease-free. One patient died of treatment complications. No association was seen between expression of the 4 studied proteins in effusions and PFS or OS (p>0.05). This was observed both using the cut-off usually applied in our studies (25%) and when comparing positive to negative specimens (0% vs. all other). The clinical parameters associated with OS in this cohort were histological grade (p=0.004), FIGO stage (p=0.017), and residual disease volume (p=0.001), with a trend for association with age (p=0.065). In Cox multivariate analysis in which these 4 parameters, as well as migfilin expression (p=0.178 in univariate analysis, trend for shorter OS), were included, grade (p=0.009) and residual tumor volume (p=0.034) were independent predictors of OS.

Separate survival analyses for patients with pre- and post-chemotherapy effusions similarly showed no prognostic role for the 4 studied proteins, although the association between migfilin expression and shorter OS became stronger for patients with post-chemotherapy effusions (p=0.088; OS=27 vs. 35 months for expression in >25% vs. ≤25% of tumor cells, respectively).

Survival analysis for patients with primary carcinomas and solid metastases (the latter scored as average expression for patients with >1 metastasis) similarly showed no association with OS or PFS (p>0.05).

Analysis of the association between ILK, β-parvin and migfilin expression and previously-studied proteins involved in adhesion and intracellular signaling

In view of the role of the studied proteins in adhesion and intracellular signaling, we investigated their potential association with markers related to these processes that have been previously studied in our effusion material, including proteins analyzed by proteomics (cadherin, FAK, paxillin, pyk, EpCAM, p-ERK, p-AKT) [29], PINCH-2 by flow cytometry [21] and proteins analyzed using IHC, including claudin-1, -3, -4 and -7 [30], the anti-apoptotic proteins XIAP and Survivin [31], p-AKT and p-mTOR [32], and NFkB-p65 [33]. α-parvin was excluded from this analysis in view of its almost universal absence from OC effusions.

ILK expression was negatively related to p-AKT, p-ERK and P-cadherin by proteomics (p=0.014, p=0.031 and p=0.035, respectively) and positively related to cytoplasmic survivin and NFkB p65 expression by IHC (p=0.041 and p=0.001, respectively). β-parvin expression was positively related to cytoplasmic Survivin, NFkB-p65 and p-AKT by IHC (p=0.045, p=0.014 and p=0.03, respectively). Migfilin expression was positively related to claudin-1, cytoplasmic NFkB-p65 and p-mTOR by IHC (p=0.004, p=0.021 and p=0.013, respectively). The observations which remained significant after Bonferroni correction for multiple comparisons were between ILK and NFkB-p65 expression and between migfilin and claudin-1 by IHC.

Discussion

Integrins are central to the metastatic capabilities of multiple cancers, including OC. Studies of both experimental models and patient material have shown that integrins mediate OC attachment to the peritoneal mesothelium and invasion of mesothelial monolayers [34-36], and our group reported on high expression of integrin subunits αV, β1 and β6 on OC cells in effusions [37,38]. E-cadherin and its catenin partners are overexpressed in effusions compared to primary OC and solid metastases, concomitant to reduced expression of its transcriptional suppressors Snail and Slug [39,40]. The present study analyzed the expression of proteins related to these adhesion systems, with focus on malignant effusions.

ILK was previously reported to mediate proliferation initiated by integrin in OC cells *in vitro* [12]. Another group showed higher ILK expression in clinical OC specimens compared to normal ovaries and benign ovarian tumors, with highest expression in high-grade tumors. ILK expression in OC cell lines was increased upon exposure to peritoneal fluid [13], and ILK expression in OSE was associated with EMT [16]. ILK was further shown to be involved in EMT triggered by endothelin-A, receptor of endothelin-1, with resulting effect on motility, invasion and matrix metalloproteinase activation [15,17]. Association between ILK and AKT, as well as the β-catenin signaling pathway protein GSK-3β was shown in several of these studies. In another study, ILK was shown to mediate transforming growth factor-β1 (TGFβ1)-regulation of the urinary-type plasminogen activator (uPA) system [18]. To the best of our knowledge, however, neither the dynamic expression of ILK along tumor progression in OC, nor its prognostic role, has been studied to date.

We found comparable expression of ILK in primary OC, malignant effusions and solid metastases, ranging from 53-60% of tumors. ILK expression was unrelated to any clinicopathologic parameters or to survival at all anatomic locations. While the number of primary carcinomas and solid metastases analyzed was relatively small, these data suggest that ILK has a biological rather than prognostic role in serous OC. The association between ILK and cytoplasmic survivin and NFkB p65 expression is of interest, especially with respect to the latter one, which was statistically stronger and remained significant after correction for multiple analyses. The negative association with p-AKT, p-ERK and P-cadherin levels by proteomics should be interpreted cautiously as these are 2 different methods which may be difficult to compare.

α-parvin and β-parvin have not been previously studied in OC. The present study shows that β-parvin is the more frequently expressed family member at all anatomic sites affected by serous OC. Expression of both parvins was infrequent in effusions compared to primary carcinomas and solid metastases, suggesting that their biological role may be dependent on interactions with the peri-tumoral stroma, in which ECM proteins are abundant. β-parvin was recently reported to suppress growth of breast carcinoma cells *in vitro* and *in vivo* [41], as well as to have reduced expression in urothelial carcinomas compared to normal urothelium [42], and one may speculate that loss of parvin expression by OC cells, particularly in effusions, may have a growth-promoting effect. This would concur with the association between β-parvin expression in OC cells in effusions and better response to chemotherapy at diagnosis. The positive association between β-parvin expression and the presence of cytoplasmic survivin, NFkB-p65 and p-AKT by IHC in OC effusions would argue against a tumor-suppressive role, as these 3 proteins promote cell survival. However, this association was weak and lost when corrected for multiple comparisons. The lack of correlation between parvin expression and clinicopathologic parameters is in agreement with the data of Papachristou for chondrosarcomas [23], though not with the report by Wu, in which β-parvin loss was significantly associated with poor disease-specific survival [42].

Migfilin was the only protein among the 4 analyzed in the present study which was overexpressed in effusions. OC cells in effusions are growing in tri-dimensional clusters which acquired the ability to escape cell death related to loss of cell-ECM contacts (anoikis) and survive in anchorage-independent conditions. Intriguingly, migfilin was shown to mediate resistance to anoikis through Src activation in experimental models [43], suggesting that its role in intracellular signaling and interactions with the cytoskeleton may mediate survival in OC effusions. Data suggesting that migfilin may contribute to a more aggressive tumor phenotype are available from several studies. Migfilin was reported to be associated with higher histological grade in leiomyosarcoma [44] and its expression was associated with poor OS in chondrosarcoma [23]. Migfilin was recently reported to be associated with migration and invasion via epidermal growth factor (EGF)-mediated phospholipase Cγ (PLCγ) and STAT3 signaling. In a series of 217 gliomas, migfilin was associated with tumor grade and poor survival [45]. In contrast, migfilin expression in esophageal carcinoma was associated with lymph node-negative status and reduced motility through GSK-3β-mediated degradation of β-catenin [46]. In our cohort, migfilin was not significantly associated with clinicopathologic parameters or survival, although a trend for shorter OS was observed in patients with post-chemotherapy effusions, which may be of interest to analyze in a larger series. In agreement with this, migfilin expression was positively related to that of claudin-1, NFkB-p65 and p-mTOR by IHC, all previously shown to be markers of poor survival in our cohort [30,32,33].

Of note, this patient group is somewhat special, as almost all patients were dead of disease at last follow-up. This reflects the fact that specimens included in this study were almost exclusively from patients diagnosed with FIGO stage IIIc or IV disease, who were either referred to the Norwegian Radium Hospital for primary operation with heavy tumor burden or referred with disease recurrence after primary operation at other hospitals, where debulking has not always been as aggressive as at our institution. Despite complete response to chemotherapy at diagnosis, these tumors recur and become chemoresistant, as reflected in the dismal outcome of this cohort.

In conclusion, we present the first analysis of the expression and clinical role of α-parvin, β-parvin and migfilin in OC, as well as the first report analyzing the clinical role of ILK in this cancer. Our data document preferential expression of β-parvin over α-parvin, as well as anatomic site-specific expression of α-parvin, β-parvin and migfilin, characterized by overexpression of migfilin and reduced expression of parvins in effusions. ILK and the 2 parvins appear to be tumor-specific, whereas migfilin is expressed in reactive mesothelial cells, suggesting it may have a role in suppressing anoikis in more than one cell class, i.e. both tumor cells and reactive mesothelium, within the serosal cavities. The clinical role of migfilin in disease recurrence post-chemotherapy specimens and its potential role as a therapeutic target may be worthy of future investigation.

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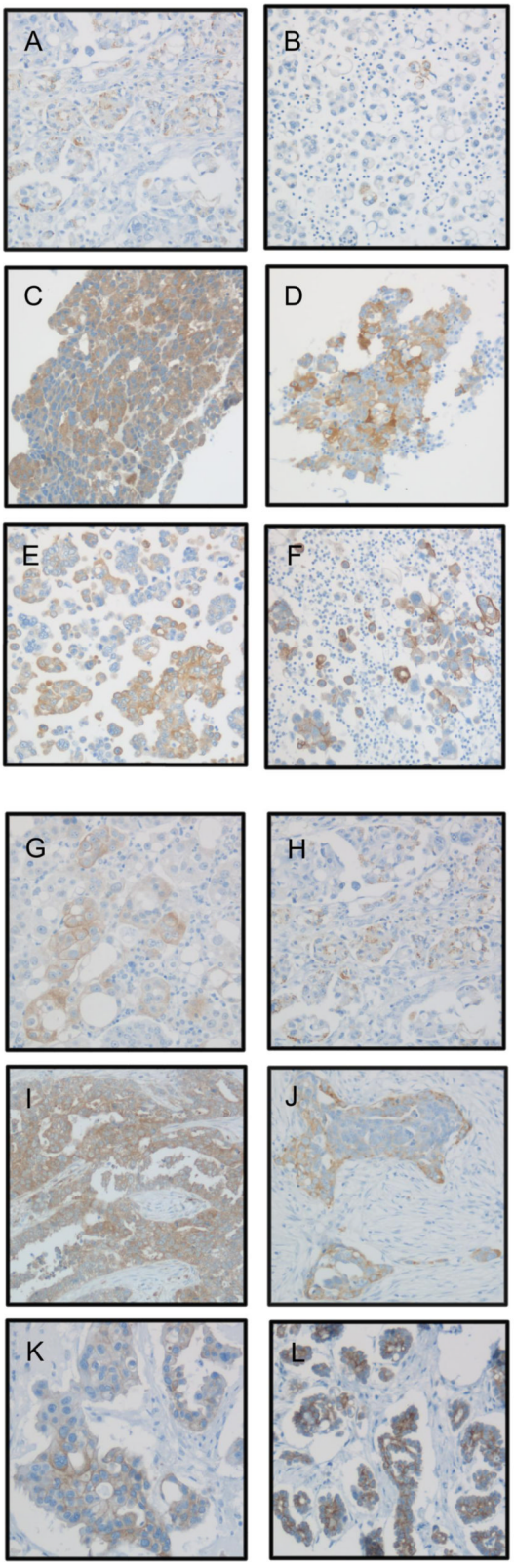
Highlights

- α-parvin, β-parvin and migfilin are differentially expressed at various anatomic sites in serous ovarian carcinoma, while ILK expression is unaltered.
- Higher β-parvin expression is associated with better response to chemotherapy at diagnosis
- ILK, α-parvin, β-parvin and migfilin protein expression is unrelated to overall or progression-free survival in serous ovarian carcinoma



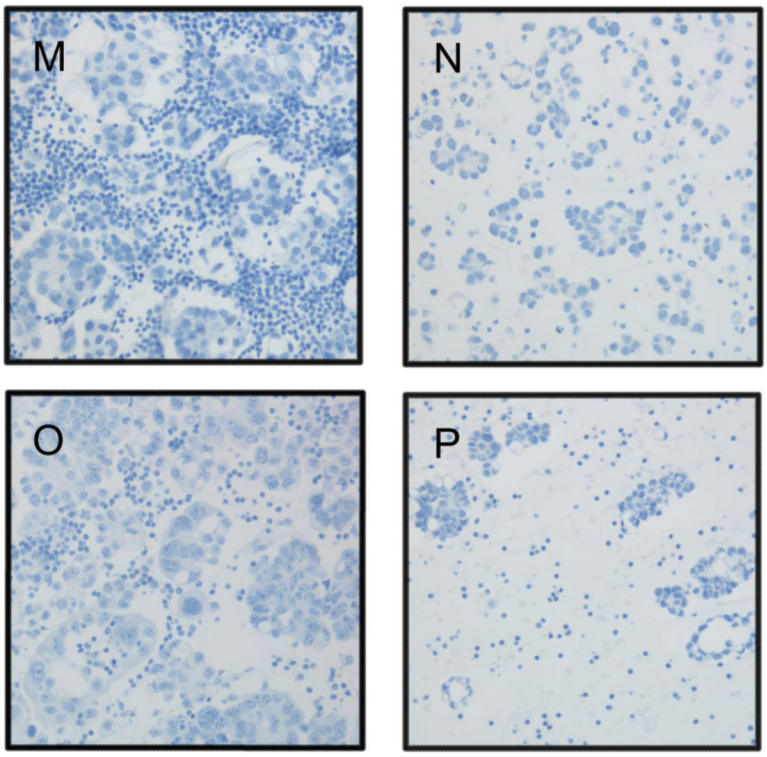


Figure 1.

Examples of α-parvin, β-parvin, migfilin and ILK immunostaining in primary and metastatic serous ovarian carcinoma.

(A-G) Effusions: (A-B) Tumor cells express α-parvin in >25% of cells (A) and more focally (B); (C) β-parvin expression in all carcinoma cells in another effusion; (D-E) Migfilin expression in 100% of tumor cells (D) and in >25% of carcinoma cells in two effusions; (F-G) Two ILK-positive effusions.

(H-L) solid tumors: (H) α-parvin staining in carcinoma cells in a primary ovarian carcinoma; (I) β-parvin in a primary ovarian carcinoma; (J) Migfilin in an omental metastasis; (K-L) ILK expression in 2 omental metastases.

(M-P): Negative controls for α-parvin (M), β-parvin (N), migfilin (O) and ILK (P).

Table 1

Clinicopathologic parameters of the effusion cohort (n=205)

Parameter		Number of patients
Age		**34-88 years (mean=62)**
Histological grade	Low	14
	High	169
	NA [a]	22
FIGO stage	IIIc	127
	IV	76
	NA	2
Residual disease	≤1 cm	78
	>1 cm	96
	NA [b]	31
Chemoresponse at diagnosis	Complete response	112
	Partial response	30
	Stable disease	13
	Progressive disease	31
	NA	19

[a]NA= non available, including effusions from inoperable patients where biopsy was too small for grading and patients operated on in other hospitals, for which the primary tumor could not be accessed for assessment of grade.

[b]Including 14 patients who only received chemotherapy, and 17 patients with missing information.

Table 2

ILK, α-parvin, β-parvin and migfilin protein expression

Protein	Tumor site	Staining extent (percentage of stained cells)					Total
		0%	1-5%	6-25%	26-75%	76-100%	
ILK	Effusion	96 (47%)	34 (17%)	31 (15%)	32 (15%)	12 (6%)	**205**
	Primary	13 (40%)	11 (33%)	3 (9%)	3 (9%)	3 (9%)	**33**
	Metastasis	27 (45%)	9 (15%)	10 (16%)	10 (16%)	5 (8%)	**61**
α-parvin [a]	Effusion	201 (98%)	2 (1%)	1 (0.5%)	0 (0%)	1 (0.5%)	**205**
	Primary	27 (82%)	0 (0%)	1 (3%)	5 (15%)	0 (0%)	**33**
	Metastasis	48 (78%)	4 (7%)	3 (5%)	4 (7%)	2 (3%)	**61**
β-parvin [a]	Effusion	147 (72%)	15 (7%)	13 (6%)	12 (6%)	18 (9%)	**205**
	Primary	4 (12%)	3 (9%)	3 (9%)	8 (24%)	15 (46%)	**33**
	Metastasis	12 (20%)	2 (3%)	8 (13%)	14 (23%)	25 (41%)	**61**
Migfilin [b]	Effusion	96 (47%)	37 (18%)	27 (13%)	28 (14%)	16 (8%)	**204** [c]
	Primary	25 (76%)	8 (24%)	0 (0%)	0 (0%)	0 (0%)	**33**
	Metastasis	45 (74%)	11 (18%)	2 (4%)	1 (2%)	1 (2%)	**60** [c]

[a] Significantly higher α-parvin and β-parvin expression in primary carcinomas (p=0.02 and p=0.001, respectively) and solid metastases (p=0.001 and p<0.001, respectively) compared to effusions.

[b] Significantly higher migfilin expression in effusions compared to primary carcinomas and solid metastases (p=0.006 and p=0.008, respectively).

[c] One failed reaction