

**Keywords:** ovarian cancer; E-Cadherin fragments; Calpain-mediated proteolysis; tumour dissemination; peritoneal progression

# E-Cadherin fragments as potential mediators for peritoneal metastasis in advanced epithelial ovarian cancer

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**Background:** Peritoneal dissemination and retroperitoneal lymph node involvement are main routes for tumour spread of epithelial ovarian cancer (EOC), possibly determined by the intercellular connecting protein E-Cadherin (E-Cad) and its fragments.

**Methods:** Tumour tissue of 105 advanced EOC patients was evaluated for protein expression of E-Cad,  $\beta$ -Catenin and Calpain by western blotting and immunohistochemistry. Expression patterns were compared between tumours with solely intraperitoneal (pT3c, pN0;  $n = 41$ ) and tumours with retroperitoneal metastases (pT1a-3c, pN1;  $n = 64$ ). Lysates of the EOC cell line SKOV3 and tumour tissue from the intraperitoneal group were tested for E-Cad expression following Calpain treatment.

**Results:** E-Cad full-length (E-Cad-FL, 120 kDa) and two major fragments at 85 kDa (E-Cad-85) and 23 kDa (E-Cad-23) were detected by western blotting. E-Cad-85 expression was significantly higher in tumours with solely intraperitoneal metastases and correlated strongly with E-Cad-23 and the protease Calpain. Calpain-mediated cleavage was identified as a potential mechanism to generate E-Cad-85 from E-Cad-FL by treating lysates from SKOV3 cells and tumour tissue with this enzyme. Increased cytoplasmic localisation of  $\beta$ -Catenin in tumours with high E-Cad-85 expression corroborates that E-Cad-85 loses the binding site for  $\beta$ -Catenin after fragmentation, enabling tumour cluster formation and peritoneal dissemination.

**Conclusions:** Calpain-mediated E-Cad fragmentation appears to promote intraperitoneal EOC progression. Understanding these mechanisms might eventually lead to new tailored subtype-specific diagnostic and therapeutic interventions.

Epithelial ovarian cancer (EOC) represents the most lethal gynaecological malignancy with 239 000 cases and 152 000 deaths per year worldwide (Ferlay *et al*, 2014). About two-thirds of the patients are diagnosed in already advanced tumour stage (Nossov *et al*, 2008) with poor prognosis and a 5-year survival rate between 30% and 40% (Allemani *et al*, 2014). Major steps in ovarian cancer progression are peritoneal tumour dissemination and/or retroperitoneal lymph node involvement while distant metastases are rarely seen at first diagnosis (Prat and Oncology FCoG, 2014;

Gasimli *et al*, 2015). Over the past years, rising evidence has shown that distinct modes for progression may have prognostic implications for the patients. This has been taken into account in the most recent FIGO (International Federation of Gynecology and Obstetrics) staging classification for cancer of the ovary, fallopian tube and peritoneum published in 2014 (Prat and Oncology FCoG, 2014). In this revised classification, patients with retroperitoneal lymph node metastases and otherwise intrapelvic disease (FIGO stage IIIA1) are now distinguished from patients with macroscopic

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Received 12 July 2015; revised 21 September 2015; accepted 19 November 2015; published online 12 January 2016

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peritoneal extrapelvic disease (FIGO stage IIIB/C) that translated into significantly improved prognosis for FIGO stage IIIA1 in first explorative retrospective analyses (Pereira *et al*, 2015).

The molecular mechanisms underlying these different patterns of progression are poorly understood. E-Cadherin (E-Cad), a well-known intercellular connecting protein maintaining the integrity of the conjunct cell–cell structure via homologue dimers, influences cellular adherence. Indispensable for E-Cad-mediated cellular function is  $\beta$ -Catenin enabling the connection to the cytoskeleton (Pokutta and Weis, 2007). For several adenocarcinomas, decreased E-Cad expression is correlated with an aggressive tumour type and poor survival (Faleiro-Rodrigues *et al*, 2004). In contrast to that, E-Cad expression was described to be overexpressed during transformation from normal ovarian surface epithelium to EOC (Dyck *et al*, 1996; Sundfeldt, 2003), although the role of E-Cad in metastases of EOC is inconclusive (Sundfeldt, 2003; Imai *et al*, 2004; Takai *et al*, 2014). Recent tumour biological investigations in breast cancer cell lines could demonstrate that E-Cad is processed by several proteases, including Calpain, resulting in fragments of different molecular size. These fragments exhibit divergent functions, including generation of tumour cell spheroids (Ye *et al*, 2013). As spheroid genesis and formation of lymphovascular emboli may promote tumour progression, this could represent a functional role for E-Cad in metastasis formation of ovarian cancer patients.

Therefore, it was hypothesised that E-Cad and its fragments may determine the route of tumour progression in ovarian cancer patients. Western blotting analyses, immunohistochemistry and calpain cleavage experiments were carried out to quantify the expression of E-Cad and its fragments in EOC with exclusive peritoneal tumour spread compared with tumours with retroperitoneal lymph node involvement. With the results, we aimed to better characterise their tumour growth pattern and identify possible new targets for therapeutic interventions.

## MATERIALS AND METHODS

**Patients.** A total of 105 patients with available tumour tissue from primary advanced EOC treated at the University Medical Centre Hamburg-Eppendorf between 1995 and 2012 were included in the study. All patients underwent primary debulking surgery according to German guidelines (Wagner *et al*, 2013). Patient cohorts were classified with regard to their mode of tumour progression. A total of 41 patients had solely intraperitoneal tumour dissemination without lymph node involvement and a corresponding tumour stage pT3c, pN0 (intraperitoneal cohort). In contrast, 64 patients were diagnosed with lymph node metastases in tumour stage pT1a-3c, pN1 (retroperitoneal cohort). Of these, 42 patients had positive lymph nodes with concurrent significant intraperitoneal tumour load (pT3c, pN1, two-sided group) and 22 patients with lymph node metastasis but only small peritoneal or pelvic tumour dissemination (pT1a-3b, pN1, predominant retroperitoneal group).

Detailed patient characteristics are presented in Table 1. All patients gave written informed consent to access their tissue and review their clinical records according to our investigational review board and ethics committee guidelines (no. 200814). Clinical parameters were retrieved from a detailed institutional database providing information on clinicopathological factors, histological classifications and therapeutic procedures. Clinical outcome of all patients was followed from date of first diagnosis until the end of 2014.

**Tissue samples.** Tissue samples were obtained intraoperatively and immediately stored in liquid nitrogen as fresh frozen samples. The histological characteristics of each sample were evaluated on cryo-cut and haematoxylin–eosin-stained sections. The tissue was tailored if necessary to obtain at least 70% invasive tumour

cells in the sample used for protein extraction. Explorative western blotting analyses of different localisations from the same ovarian cancer were performed in parallel for a total of 10 tumour samples to test intratumour variability. In these investigations of the neighbouring tumour probes, no significant differences in terms of presence and intensity of the E-Cad-85 band was noted, confirming representativity of our results (Supplementary Figure S1).

**Protein extractions.** As described previously (Mahner *et al*, 2008), samples of approximately 100 mg were cut from the tissue and pulverised using a micro-dismembrator (Braun-Melsungen, Melsungen, Germany) for 2 min and 45 s at 200 r.p.m. Proteins were lysed in ice-cold sample buffer (50 mM Tris pH 6.8, 1% sodium dodecyl sulphate (SDS), 10% sucrose and  $10 \mu\text{l ml}^{-1}$  protease inhibitor cocktail (Sigma, Taufkirchen, Germany)). The protein concentration was determined following standard protocols and using bovine serum albumin protein standards.

**Western blotting analysis.** For western blotting analysis, equal amounts of protein lysates (20  $\mu\text{g}$ ) were analysed per well. The proteins were separated on a 10% SDS gel (11% Glycerin, aqua dest., 10% SDS, Tris and 10% Acrylamid) and transferred to polyvinylidene difluoride membranes (Merck Millipore KGaA, Darmstadt, Germany). Later, the membranes were blocked with 5% nonfat dry milk (Bio-Rad Laboratories, Hercules, CA, USA) in TBST (TBS: 50 mM Tris, 150 mM NaCl, pH 7.4 with 10% TWEEN 20), washed in TBST and incubated with the primary antibody. The monoclonal antibodies for E-Cad (24E10 no. 3195, 1:2000),  $\beta$ -Catenin (D10A8 no. 8480, 1:10 000) and the polyclonal anti-Calpain antibody (M-type no. 2539 1:1500; all purchased from Cell Signaling Technology, Leiden, The Netherlands) were diluted in 5% BSA in TBST and incubated at 4 °C overnight. After incubation, membranes were washed in TBST and incubated with the corresponding secondary antibody (anti rabbit sc 2054, Santa Cruz Biotechnology, Heidelberg, Germany) in 1.5% nonfat dry milk in TBST for 1 h at room temperature. Finally, after washing in TBST, the detection was carried out with the SuperSignal West Pico chemoluminescent kit (Thermo Scientific, Rockford, IL, USA) to visualise the protein expression on FUJI-super RX medical X-ray films (FUJI, Tokyo, Japan).

Band intensities were quantified by densitometry (GS-700 Imaging Densitometer, Bio-Rad, Munich, Germany). Protein lysates from the breast cancer cell line MCF7 were used as positive controls for E-Cad, from the ovarian cell line SKOV3 for  $\beta$ -Catenin and from the cervical cancer cell line HELA for Calpain. All detected bands of the investigated proteins except for the E-Cad fragment of 23 kDa (E-Cad-23) were standardised with the positive controls, which were defined as 100%. Protein expression levels were normalized to GAPDH (Santa Cruz FL 335; 1:5000). E-Cad-23 was analysed semi-quantitatively and assigned to four categories (no expression = 0, weak = 1, moderate = 2, strong = 3, very strong = 4) by two investigators.

**Immunohistochemistry.** Paraffin sections of tumour samples of 4–6  $\mu\text{m}$  were deparaffinised in xylene and hydrated in a descending series of alcohol. After washing in aqua dest, the antigens were demasked over 20 min in 90 °C heated citrate buffer (10 mM). Between two additional washing steps, the endogenous peroxidase was blocked with 0.5%  $\text{H}_2\text{O}_2$ . Later, sections were incubated at 4 °C overnight with the E-Cad (1:2000) or the  $\beta$ -Catenin (1:100) antibody, diluted 1:500 in antibody diluent (S0809, DAKO, Hamburg, Germany). Control sections were incubated simultaneously with an ISO-type control from DAKO (Rabbit IgG, No. X0903) at 4 °C overnight. After washing in TBS (5 mM Tris, 15 mM NaCl, pH 7.4), the sections were incubated with the secondary antibody (DAKO ABC Elite Kit PK-6100) for 1 h at room temperature. After additional washing in TBS, E-Cad was visualised on tissue sections with the Peroxidase Substrate Kit

**Table 1. Patient characteristics according to the pattern of tumour dissemination**

	Overall cohort	Intraperitoneal group	Retroperitoneal group	P-value
No. of patients	105 (100%)	41 (39.1%)	64 (60.9%)	
Age at diagnosis (years)				0.42 <sup>a</sup>
Median	62	62	61	
Range	34–84	42–84	34–84	
Tumour stage				<b>0.018<sup>b</sup></b>
pT1c	3 (2.9%)	0 (0%)	3 (4.7%)	
pT2b	2 (1.9%)	0 (0%)	2 (3.1%)	
pT2c	2 (1.9%)	0 (0%)	2 (3.1%)	
pT3a	2 (1.9%)	0 (0%)	2 (3.1%)	
pT3b	14 (13.3%)	1 (2.4%)	13 (20.3%)	
pT3c	81 (77.1%)	40 (97.6%)	41 (64.1%)	
Unknown	1 (0.9%)	0 (0%)	1 (1.6%)	
Lymph node status				<b>&lt;0.001<sup>b</sup></b>
N0	41 (39.1%)	41 (100%)	0 (0%)	
N1	64 (60.9%)	0 (0%)	64 (100%)	<b>0.02<sup>a</sup></b>
<b>Number of resected lymph nodes</b>				
Median	41	33.5	44	
Range	0–97	1–71	1–97	
Mode of tumour dissemination				<b>&lt;0.001<sup>b</sup></b>
Solely intraperitoneal group (pT3c, pN0)	41 (39.1%)	41 (100%)	0 (0%)	
Predominant retroperitoneal group (pT1a-3b, pN1)	22 (20.9%)	0 (0%)	22 (34.4%)	
Two-sided group (pT3c, pN1)	42 (40.0%)	0 (0%)	42 (65.6%)	
Grading				0.53 <sup>c</sup>
G1	4 (3.8%)	1 (2.4%)	3 (4.7%)	
G2	27 (25.7%)	12 (29.3%)	15 (23.4%)	
G3	74 (70.5%)	28 (68.3%)	46 (71.9%)	
Histological subtype				0.51 <sup>b</sup>
Serous	90 (85.7%)	38 (92.7%)	52 (81.2%)	
Endometrioid	4 (3.8%)	1 (2.4%)	3 (4.7%)	
Clear cell	3 (2.9%)	1 (2.4%)	2 (3.1%)	
Mixed differentiation	6 (5.7%)	1 (2.4%)	5 (7.8%)	
NOS	2 (1.9%)	0 (0%)	2 (3.1%)	
<b>Surgical procedures</b>				
Large bowel resection	66 (62.9%)	24 (58.5%)	42 (65.6%)	0.70 <sup>b</sup>
Small bowel resection	9 (8.6%)	2 (4.9%)	7 (10.9%)	0.80 <sup>b</sup>
<b>Upper abdomen interventions</b>				
Liver	30 (28.6%)	13 (31.7%)	17 (26.6%)	0.66 <sup>b</sup>
Spleen	19 (18.1%)	9 (21.9%)	10 (15.6%)	0.45 <sup>b</sup>
Pancreas	3 (2.9%)	1 (2.4%)	2 (3.1%)	1.00 <sup>b</sup>
Postoperative residual tumour				0.33 <sup>b</sup>
Microscopic	82 (78.1%)	30 (73.2%)	52 (81.2%)	
Macroscopic	22 (20.9%)	11 (26.8%)	11 (17.2%)	
Unknown	1 (0.9%)	0 (0%)	1 (1.6%)	
Preoperative CA 125 level (kU l <sup>-1</sup> )				0.65 <sup>a</sup>
Median	364	416.5	347.5	
Range	14–13 089	14–8703	31–13 089	
Postoperative CA 125 level (kU l <sup>-1</sup> )				0.14 <sup>a</sup>
Median	103	100	106	
Range	11–1267	11–872	25–1267	

Abbreviations: FIGO = International Federation of Gynecology and Obstetrics; NOS = not otherwise specified. Overview of the clinical characteristics from all included patients with epithelial ovarian cancer (n = 105). A total of 41 patients with solely intraperitoneal tumour dissemination as opposed to 64 patients with retroperitoneal tumour involvement. Statistically significant P-values are printed in bold.

<sup>a</sup>Analysis of variance analysis.

<sup>b</sup>Pearson Chi-square test.

<sup>c</sup>Kruskal–Wallis test.

(DAB, SK-4100; DAKO), while cell nuclei were stained by haematoxylin (51275L, Sigma-Aldrich, St Louis, MO, USA). Finally, the sections were dehydrated in an ascending alcohol series and xylol.

E-Cad as well as  $\beta$ -Catenin expression and their localisation was analysed by two independent investigators evaluating scores for staining intensity (SI, 0 = negative, 1 = weak, 2 = slightly

positive, 3 = strong expression) and percentage of positive cells (PP, 1 = < 50%, 2 =  $\geq$  50%). To objectify cytoplasmic expression, SI was applied while membranous expression was the product of SI multiplied by PP.

**Calpain assay.** The ovarian cancer cell line SKOV3 was cultivated in McCoy's 5A Medium (GE Healthcare, PAA Laboratories,

Pasching, Austria) with 10% foetal bovine serum (PAA Laboratories) till 90% confluency. After washing in PBS, the cells were scratched with a cell scraper and homogenised in extraction buffer (50 mM HEPES (pH 7.5), 1 mM EGTA, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM Mercaptoethanol and 1  $\mu$ l protease inhibitor cocktail without Leupeptin (P8849, Sigma-Aldrich) per 100  $\mu$ l extraction-buffer). The solution was stored 30 min on ice before centrifugation at 1000 g for 10 min at 4 °C. Equal amounts of protein lysates were incubated with different treatments for 60 min at 30 °C: without adjunct, with 1 mM CaCl<sub>2</sub>, with 1 mM CaCl<sub>2</sub> and 0.3 U (Units)  $\mu$ -Calpain (C6108, Sigma-Aldrich), and with 1 mM CaCl<sub>2</sub>, 0.3 U  $\mu$ -Calpain and 10 mM Calpeptin (CAS 117591-20-5, Calbiochem, Darmstadt, Germany). Treatment was stopped by addition of 2  $\times$  sample buffer (62.5 mM Tris-HCL (pH 6.8), 4% SDS, 10% glycerol) and 10-min incubation at 100 °C. In addition to SKOV3, three exemplary lysates of tumour tissue from the intraperitoneal group (T5213, T6362, T6570) were treated in the same way. After treatment, the protein lysates were investigated in western blotting analysis with the same monoclonal E-Cad antibody (24E10).

**Statistical analysis.** The expression levels of E-Cad and  $\beta$ -Catenin bands were compared between the different cohorts by ANOVA analysis, as well as *post hoc* with the Least Significant Difference Test. Fragments of E-Cad were correlated by Spearman Rho among themselves and with the protease Calpain, as well as with  $\beta$ -Catenin. Correlation coefficients were assessed as significantly strong according to the notes on medical statistics (Bland and Altman, 1994). Expression scores from immunohistochemical analyses were compared by Student's *t*-test. Kaplan–Meier estimates and log-rank test were carried out to illustrate and compare progression-free survival (PFS) and overall survival (OS). In this context, patients were categorised in tertiles according to their levels of E-Cad-85 expression. The associated hazard ratios were determined by Cox regression. *P*-values  $\leq 0.05$  were considered to be statistically significant. All statistical analyses were carried out with SPSS Statistics version 22 for Windows (IBM, Armonk, NY, USA). Figures were edited with Stata 13.1 (StataCorp, College Station, TX, USA, 2014).

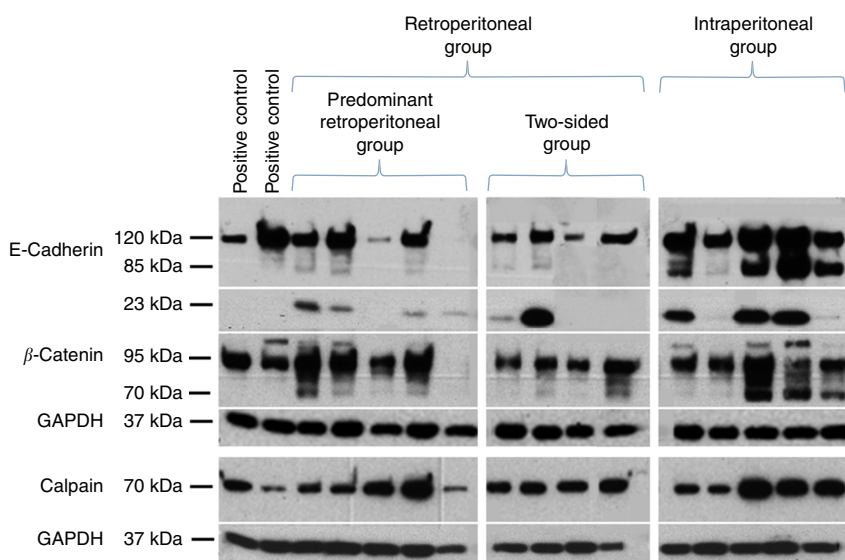
## RESULTS

A total of 105 patients were included in this study, 41 (39.1%) with only intraperitoneal and 64 (61.0%) with retroperitoneal tumour growth. Patient characteristics were well balanced between both groups except for the obvious difference in tumour stage as well as significantly more resected lymph nodes in patients with retroperitoneal tumour dissemination (median 44 vs 34 lymph nodes, *P* = 0.02; Table 1).

**Ovarian cancer with solely peritoneal metastases exhibits high levels of E-Cad fragments.** In western blotting analysis, full-length E-Cad (E-Cad FL) at 120 kDa was detected in almost all investigated tumour samples. No significant differences between the solely intraperitoneal group compared with tumours with retroperitoneal involvement were noted (median 1.57 vs 1.20, *P* = 0.21; Figures 1 and 2A).

In addition to the expected band at 120 kDa, further signals for fragments between 70 and 100 kDa were detected by the anti-E-Cad monoclonal antibody. Of these, the 85-kDa fragment (E-Cad-85) exhibited the strongest intensity, while fragments at 100 kDa were only very weak and not reliably detectable (Figure 1). E-Cad-85 showed a significantly higher expression in the group with exclusive peritoneal metastases compared with the group with retroperitoneal metastasis (median 0.48 vs 0.31, *P* = 0.01; Figure 2B). An additional signal at 23 kDa (E-Cad-23) could be detected representing a further, seemingly cytoplasmic fragment of E-Cad according to the cytoplasmic binding site of the E-Cad antibody 24E10 (Figure 1). Expression differences between the intraperitoneal and the retroperitoneal group of E-Cad-23 were non-significant (median 2.00 vs 1.00, *P* = 0.07; Figure 2C).

Within the overall cohort, E-Cad-FL expression correlated significantly with E-Cad-85 (*r* = 0.80; *P* < 0.0001) and E-Cad-23 (*r* = 0.44; *P* < 0.0001, Table 2A, Figure 1) with comparable interactions in tumours with solely peritoneal metastases (E-Cad-FL and E-Cad-85 *r* = 0.79; *P* < 0.0001, E-Cad-FL and E-Cad-23 *r* = 0.49; *P* = 0.001, Table 2B) as well as in tumours with



**Figure 1.** Expression of E-Cadherin,  $\beta$ -Catenin and Calpain according to the mode of tumour progression. Western blotting analysis showing representative expression of E-Cadherin (E-Cad-85 and E-Cad-23),  $\beta$ -Catenin and Calpain in the different types of tumour dissemination. Protein lysates from the breast cancer cell line MCF7 were used as positive controls for E-Cadherin, from the ovarian cell line SKOV3 for  $\beta$ -Catenin and from the cervical cancer cell line HELA for Calpain. Equal amounts of protein lysate (20  $\mu$ g) were loaded per well. As a loading control, GAPDH protein expression was detected.



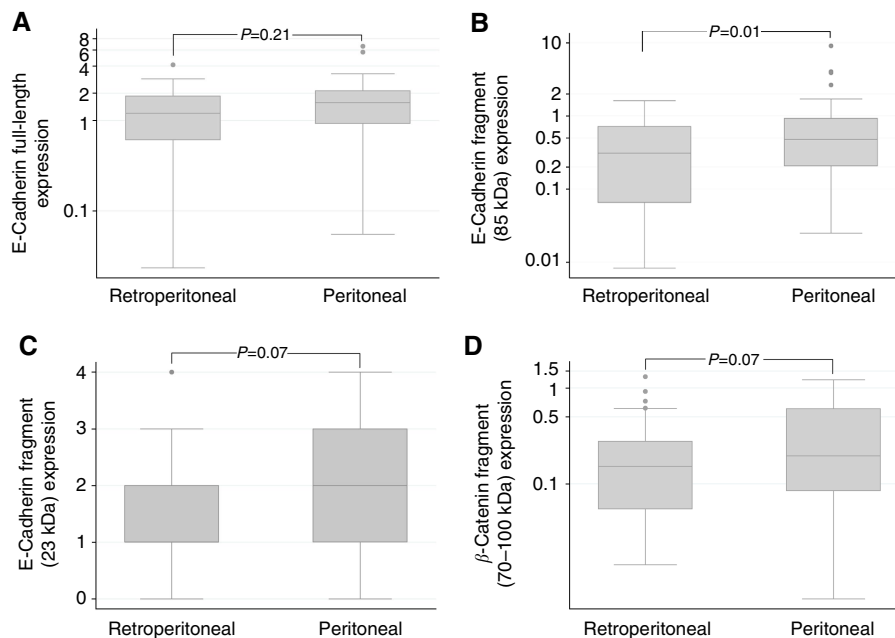


Figure 2. Quantitative expression of E-Cad-FL, E-Cad-85, E-Cad-23 and  $\beta$ -Catenin fragments according to the mode of tumour progression. (A) E-Cad-FL was expressed in all investigated tumour samples but without significant differences between the two groups ( $P=0.21$ ). (B) E-Cad-85 with significantly higher expression in the group of solely intraperitoneal metastases compared with retroperitoneal metastases ( $P=0.01$ ). (C, D) E-Cad-23 and  $\beta$ -Catenin (70–100 kDa) did not exhibit significant differences in the expression values between both groups (each with  $P=0.07$ ).

**Table 2A. Correlation of the protein expression among each other in the overall cohort ( $n = 105$ )**

	E-Cad-85	E-Cad-23	Calpain	$\beta$ -Catenin
E-Cad-85	—	$r=0.47$ $P<0.0001$	$r=0.30$ $P=0.002$	$r=0.17$ $P=0.09$
E-Cad-23	$r=0.47$ $P<0.0001$	—	$r=0.20$ $P=0.04$	$r=0.24$ $P=0.01$
Calpain	$r=0.30$ $P=0.002$	$r=0.20$ $P=0.04$	—	$r=0.35$ $P=0.0003$
E-Cad-FL	$r=0.80$ $P<0.0001$	$r=0.44$ $P<0.0001$	$r=0.24$ $P=0.016$	$r=0.32$ $P=0.001$

$P$ =P-value; correlation coefficient:  $r$ =Spearman Rho. Bold values indicate statistically significant P-values.

**Table 2B. Correlation of the protein expression among each other in the group of solely intraperitoneal metastases ( $n = 41$ )**

	E-Cad-85	E-Cad-23	Calpain	$\beta$ -Catenin
E-Cad-85	—	$r=0.56$ $P=0.0001$	$r=0.48$ $P=0.002$	$r=0.21$ $P=0.19$
E-Cad-23	$r=0.56$ $P=0.0001$	—	$r=0.31$ $P=0.048$	$r=0.28$ $P=0.08$
Calpain	$r=0.48$ $P=0.002$	$r=0.31$ $P=0.048$	—	$r=0.19$ $P=0.24$
E-Cad-FL	$r=0.79$ $P<0.0001$	$r=0.49$ $P=0.001$	$r=0.51$ $P=0.001$	$r=0.21$ $P=0.20$

$P$ =P-value; correlation coefficient:  $r$ =Spearman Rho. Bold values indicate statistically significant P-values.

retroperitoneal involvement (E-Cad-FL and E-Cad-85  $r=0.80$   $P<0.001$ , E-Cad-FL and E-Cad-23  $r=0.37$ ;  $P=0.003$ , Table 2C).

A significant correlation between E-Cad-23 and E-Cad-85 could be demonstrated in the overall cohort ( $r=0.47$ ;  $P<0.0001$ , Table 2A, Figure 1) being even stronger in the cohort of solely peritoneal metastases ( $r=0.56$ ;  $P<0.0001$ , Table 2B, Figure 1) and weaker in tumours with retroperitoneal metastases ( $r=0.40$ ;  $P=0.001$ , Table 2C).

For  $\beta$ -Catenin, fragments between 70 and 90 kDa in addition to full-length  $\beta$ -Catenin were detected. Yet, the expression values were not statistically different between the two groups (median 0.20 vs 0.15,  $P=0.07$ ; Figure 2D). Accordingly,  $\beta$ -Catenin did not correlate with E-Cad-85 ( $\beta$ -Catenin and E-Cad-85  $r=0.17$   $P=0.09$ ) and only weakly with E-Cad-23 in the overall cohort ( $\beta$ -Catenin and E-Cad-23  $r=0.24$ ;  $P=0.01$ ; Table 2A).

#### Expression of Calpain correlates with E-Cad-85 and E-Cad-23.

As E-Cad-85 expression values differed significantly among tumours with different metastatic patterns, we were interested in the formation and functional significance of this fragment. Calpain is an intracellular protease, which cleaves E-Cad in the area of the

**Table 2C. Correlation of the protein expression among each other in the group of retroperitoneal involvement ( $n = 64$ )**

	E-Cad-85	E-Cad-23	Calpain	$\beta$ -Catenin
E-Cad-85	—	$r=0.40$ $P=0.001$	$r=0.12$ $P=0.37$	$r=0.07$ $P=0.60$
E-Cad-23	$r=0.40$ $P=0.001$	—	$r=0.08$ $P=0.55$	$r=0.19$ $P=0.14$
Calpain	$r=0.12$ $P=0.37$	$r=0.08$ $P=0.55$	—	$r=0.40$ $P=0.002$
E-Cad-FL	$r=0.80$ $P<0.001$	$r=0.37$ $P=0.003$	$r=-0.01$ $P=0.99$	$r=0.33$ $P=0.008$

$P$ =P-value; correlation coefficient:  $r$ =Spearman Rho. Bold values indicate statistically significant P-values.

amino-acid Pro780 at the cytoplasmic domain, leading to two fragments of 85 and 20–25 kDa (Supplementary Figure S2). Based on the hypothesis that the 85-kDa fragment found in our tumours is generated by calpain activity, we performed western blotting

analysis of our total cohort using an anti-calpain antibody. Within the overall cohort, Calpain expression correlated significantly with E-Cad-85 fragments ( $r=0.30$ ;  $P=0.002$ , Table 2A, Figure 1), which was more pronounced in the cohort of solely peritoneal metastases ( $r=0.48$ ;  $P=0.002$ , Table 2B, Figure 1). In addition, Calpain expression correlated with E-Cad-23 levels in the total cohort ( $r=0.20$ ;  $P=0.04$ ) and tumours showing exclusively intraperitoneal spread ( $r=0.31$ ;  $P=0.048$ , Tables 2A and 2B), which might link cleavage by Calpain with these E-Cad fragments. Contrarily, this correlation was not present in tumours with retroperitoneal involvement (E-Cad-85 and Calpain  $r=0.12$ ,  $P=0.37$ ; E-Cad-23 and Calpain  $r=0.08$ ,  $P=0.55$ , Table 2C).

**Evaluation of possible functional implications of the E-Cad-85 fragment.** Human E-Cad consists of an extracellular part, which exhibits five domains, a trans-membranous and a highly conserved cytoplasmic domain. Via the cytoplasmic domain, E-Cad is linked to the two connection proteins p120 catenin (p120ctn) and  $\beta$ -catenin establishing the intracellular anchoring. These protein complexes coordinate the organisation of the cell in basal and apical orientation guaranteeing a stable configuration of the cell-cell contact (Supplementary Figure S2; Pokutta and Weis, 2007).

The applied E-Cad antibody (24E10) detects E-Cad intracellularly in the area of the amino-acid sequence Pro780 of E-Cad (Supplementary Figure S2, labelled as 24E10-binding site), which is concomitantly the cleavage domain of Calpain. Supplementary Figure S2 illustrates our hypothesised position of the E-Cad-85 fragment in relation to the cell membrane leading to a large extracellular region of the protein with the 24E10-binding site intracellularly at the proximal end of the fragment. In this distribution, E-Cad-85 is characterised by the loss of the cytoplasmic-binding domain to  $\beta$ -Catenin, so that E-Cad-85 is not able to bind to the cytoskeleton. Consequently, a stable configuration of the conjunct cell-cell structure cannot be guaranteed.

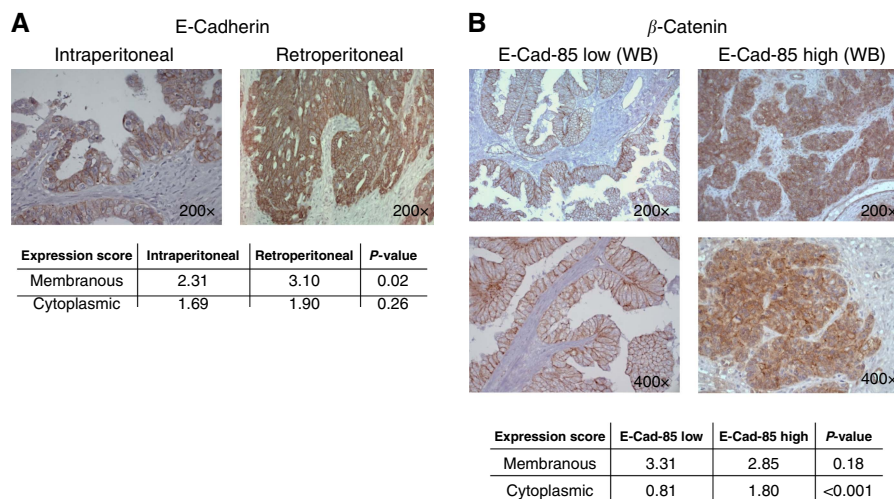
To test this biological hypothesis, paraffin-embedded sections of tumour samples were evaluated by immunohistochemistry for E-Cad and  $\beta$ -Catenin ( $n=40$ ; solely intraperitoneal  $n=15$ , retroperitoneal  $n=25$ ). A strong membranous E-Cad signal and a positive but weaker cytosolic expression was detected in all the investigated tumour sections. Membranous expression was significantly higher in patients with retroperitoneal involvement compared with patients with solely intraperitoneal tumour

dissemination (mean membranous expression score 2.31 vs. 3.10,  $P=0.02$ , Figure 3A).  $\beta$ -Catenin was evaluated with regard to localisation and staining intensity in two groups comparing patients with high E-Cad-85 expression (above median) in western blotting to patients with low E-Cad-85 (below median) expression values. In patients with high E-Cad-85 following suspected E-Cad fragmentation, cytoplasmic  $\beta$ -Catenin expression was significantly higher compared with patients with low E-Cad-85 levels (mean cytoplasmic expression score 1.80 vs. 0.80,  $P<0.001$ , Figure 3B) while membranous staining was not statistically different between both groups (mean membranous expression score 2.85 vs. 3.31,  $P=0.18$ , Figure 3B).

To evaluate a possible prognostic significance of E-Cad-85, we stratified the overall cohort expression in tertiles according to their E-Cad-85 expression levels resulting in three groups with low ( $n=72$ ), moderate ( $n=27$ ) and high ( $n=4$ ) E-Cad-85 expression. Patients with both low and moderate E-Cad-85 expression had a median PFS of 23 months, respectively, which was significantly longer compared with patients with high E-Cad-85 expression and a PFS of 8 months (Supplementary Figure S3A; moderate vs high,  $P=0.003$ ; low vs high,  $P=0.007$ ). In accordance, median OS of patients with low and moderate E-Cad-85 expression was significantly better with 50 and 53 months, respectively, compared with 17 months in that of patients with high E-Cad-85 expression (Supplementary Figure S3B; moderate vs high,  $P=0.04$ ; low vs high,  $P=0.001$ ).

#### Cleavage of E-Cad-FL to E-Cad-85 fragments by Calpain in lysates of SKOV3 cells and ovarian tumour tissue.

In western blotting analysis of SKOV3 ovarian tumour cells, E-Cad FL was almost exclusively detected. Following Calpain treatment of the SKOV3 proteins, a strong E-Cad-85 fragment appeared in addition to E-Cad FL. This Calpain cleavage could be inhibited by addition of the Calpain inhibitor Calpeptin (Figure 4A). In three exemplary lysates of tumour tissue from the intraperitoneal group (Figure 4B: T5213, Figure 4C: T6362, Figure 4D: T6570), the same trend could be confirmed, showing increased E-Cad-85 and decreased E-Cad FL levels after calpain cleavage, which was partly inhibited by calpeptin. These results correspond to the observed correlation of the Calpain expression with E-Cad fragments in EOC as shown in western blotting analysis.



**Figure 3.** Immunohistochemistry for E-Cad and  $\beta$ -Catenin expression pattern in ovarian cancer tissue samples. (A) Membranous E-Cad expression was significantly higher in tumour samples from patients of the retroperitoneal group compared with patients of the intraperitoneal group, while cytoplasmic E-Cad expression did not differ. (B) Cytoplasmic expression of  $\beta$ -Catenin was significantly higher in tumour tissue from patients with high E-Cad-85 compared with patients with low E-Cad-85 expression levels that were previously estimated by western blotting. No significant difference in membranous  $\beta$ -Catenin expression was noted.

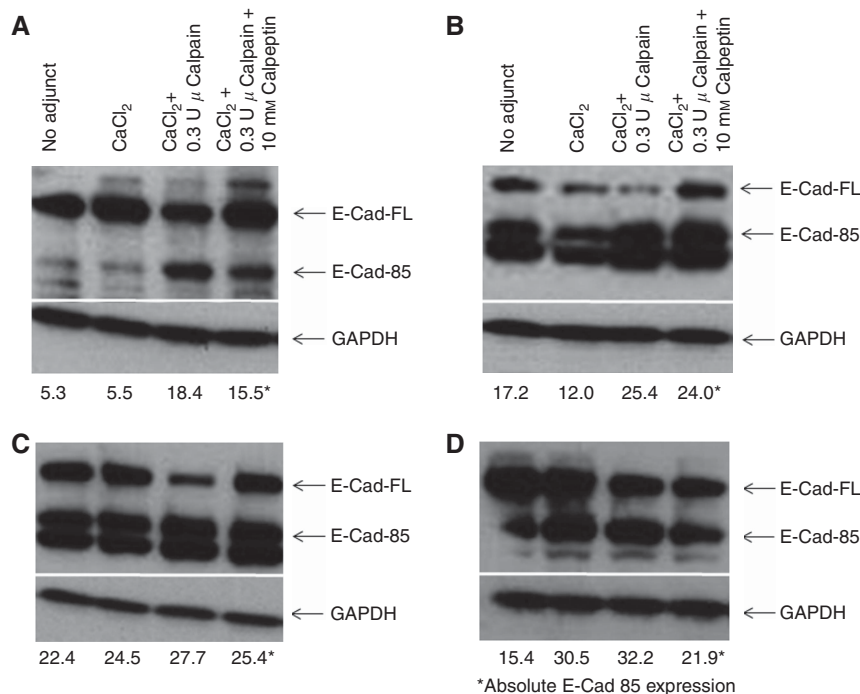


Figure 4. Expression of E-Cad-FL and E-Cad-85 following treatment with Calpain and Calpeptin in SKOV3-WT cells and tumour tissue lysates. (A) SKOV3-WT ovarian tumour cells exhibited E-Cad-85 in addition to E-Cad-FL after Calpain treatment, while E-Cad-85 expression was decreased following addition of Calpeptin. (B–D) Treatment of three tumour tissue lysates ((B) T5213, (C) T6362 and (D) T6570) revealed the same trend with Calpain-promoted increased expression of E-Cad-85 in contrast to reduced E-Cad fragmentation following Calpeptin treatment.

## DISCUSSION

In this study of 105 patients with advanced ovarian cancer, we could observe a potential role for Calpain-mediated E-Cad fragments in promotion of intraperitoneal tumour progression.

For most tumour entities, decreased expression of adhesion proteins is associated with a more invasive growth pattern, lower histological differentiation and metastatic disease (Faleiro-Rodrigues *et al*, 2004). In terms of ovarian cancer, conflicting results regarding E-Cad have been published. E-Cad is initially upregulated when normal ovarian surface cells are transformed to ovarian cancer cells. During this process, mesothelial-like, N-Cadherin-positive cells transform to epithelial, E-Cad-positive tumour cells (Sundfeldt, 2003). Over the following steps towards metastatic EOC, a transient impact of E-Cad has been proposed, mainly based on immunohistochemical investigations (Sundfeldt, 2003; Imai *et al*, 2004; Takai *et al*, 2014). In the present study, E-Cad expression was detected in EOC with solely intraperitoneal tumour dissemination as well as in EOC with retroperitoneal involvement. At first glance, this was not anticipated as these tumours are characterised by agile, independent tumour cells and cell clusters and not by strong cell–cell interactions within the tumour. However, based on our western blotting results for different E-Cad fragments we hypothesised an important role of E-Cad-85 in defining the mode of tumour progression. Accounting for the cytoplasmic-binding site of the E-Cad antibody, the calpain cleavage site and the size of the fragment, E-Cad-85 might miss most of the intracellular domain, including the  $\beta$ -catenin-binding site (Supplementary Figure S2).

In 2013, Ye *et al* (2013) described a comparable distribution of E-Cad fragments in the breast cancer cell line MARY-X. Their experiments revealed that Calpain-generated E-Cad fragments at 100 kDa are spread all over the tumour cells instead of being localised in adherent junctions of cell–cell interactions. This non-directional distribution enables the formation of tumour-cell

clusters and E-Cad-positive spheroids potentially favouring metastases (Ye *et al*, 2013). E-Cad-85 seems to be further processed losing the N-terminus but retaining the ability to peritoneal progression in tumour environment, possibly by spheroid genesis as previously described for the E-Cad fragment at 100 kDa.

Calpain was identified as an intracellular protease that accurately cleaves E-Cadherin in the area of the amino-acid sequence Pro780 (Rios-Doria *et al*, 2003) and its expression correlated significantly with the E-Cad-85 and E-Cad-23 fragments. Following Calpain treatment *in vitro*, stronger E-Cad-85 fragments appeared in SKOV3 lysates. The same trend in lysates of exemplary tumour tissue from the intraperitoneal group corroborates the assumption that Calpain cleaves E-Cad FL to its fragments in EOC. E-Cad-85 therefore loses the binding site to  $\beta$ -Catenin enabling tumour cluster formation. This possible mechanism could be supported by immunohistochemical analyses for  $\beta$ -Catenin. In these experiments, cytoplasmic expression of  $\beta$ -Catenin was more pronounced in tumours with high E-Cad-85 expression seemingly attributed to the released connection between membranous E-Cad-85 and  $\beta$ -Catenin following E-Cad cleavage. Accordingly, Salehin *et al* (2011) observed decreased Calpain expression levels with increasing lymph node status in immunohistochemical investigations.

From our present results, it can be proposed that intraperitoneal metastases are promoted by Calpain-generated E-Cad-85. A different example of posttranslational E-Cad modification influencing tumour growth in ovarian cancer has been previously shown (Symowicz *et al*, 2007). In these experiments, exogenous MMP-9 catalysed E-Cad ectodomain shedding, which enhanced survival and migration of tumour cells by enabling multicellular aggregates in ascites (Symowicz *et al*, 2007). Based on these results and our present data, posttranslational changes might explain the divergent roles of E-Cad in benign conditions such as acute pancreatitis (Mayerle *et al*, 2005) as well as in inflammatory breast cancer (Ye *et al*, 2013), prostate cancer (Rios-Doria *et al*, 2003) and in EOC (Symowicz *et al*, 2007).



To date, E-Cad function has been investigated in early and advanced EOC but without accounting for different modes of metastases. Progression of advanced EOC has been explained by epithelial–mesenchymal transition (Strauss *et al*, 2011), which, however, conflicts with the described increased E-Cad expression in metastases (Imai *et al*, 2004). For peritoneal tumour spread, the formation of spheroids seems to be beneficial as these cell clusters can passively be carried by the peritoneal fluid and may overcome apoptosis and anoikis (Lengyel, 2010; Carduner *et al*, 2014). Our hypothesis attributes an important role to E-Cad-85 fragments for EOC progression and enables further understanding of the molecular process of peritoneal metastasis. In addition to quantitative analysis, we were able to draw functional conclusions from the expression of E-Cad, its fragments and their relation to  $\beta$ -Catenin as well as Calpain regarding the mode of tumour progression. These promising results are, however, based on western blotting and immunohistochemical investigations of retrospectively analysed fresh frozen tumour samples. Although the number of analysed samples from 105 patients is considerably large, it is important to highlight the explorative analytical approach. Although the elucidated negative prognostic impact of E-Cad-85 might represent a link to the observed worse prognosis of EOC with peritoneal tumour spread compared with tumours solely lymph node metastases, future studies with larger, ideally prospectively collected cohorts will be necessary to substantiate our results. Investigating the protease Calpain and E-Cad fragments as potential therapeutic targets might also be of high clinical interest as understanding the mechanisms for EOC tumour progression will open diagnostic and therapeutic interventions tailored for specific tumour types.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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