

Ezrin expression is related to poor prognosis in FIGO stage I endometrioid carcinomas

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As a cortical cytoskeletal protein, ezrin adapts the cytoplasmic tail of CD44 to the actin-based cytoskeleton and is functionally involved in migration and adhesion that are prerequisites for metastasis. To assess the importance of ezrin and its associated protein osteopontin for the progression of endometrioid carcinoma in FIGO stage I, we analyzed paraffin-embedded tissue from 164 patients by immunohistochemistry and correlated these data with clinicopathological parameters. Ezrin was expressed in normal proliferating endometrial glands, as was confirmed by quantitative PCR and immunohistochemistry. In endometrioid carcinoma, enhanced ezrin expression correlated with a reduced overall survival in univariate analysis ($P=0.041$). In contrast, no significant correlation was found for osteopontin. In multivariate survival analysis, among FIGO grade 3 and age, ezrin was still found to be an independent risk factor (relative risk 2.2, confidence interval 1.0–5.4, $P=0.047$). Hence, elevated ezrin expression is a new independent prognostic marker in FIGO stage I endometrioid carcinoma, and thus provides further evidence for an important role of ezrin in tumor progression.

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Endometrial carcinoma was responsible for an estimated number of 40 880 cases and 7310 deaths in the United States in 2005.¹ In Germany, approximately 11 250 new cases of endometrial cancer and 2536 cancer-related deaths were reported in 2003.² With advances in surgical treatment, disease-specific mortality declined over the past 30 years. However, diagnosis of endometrial carcinoma still imposes a substantial burden on patients. Prognosis is dependent on classical clinicopathological parameters like FIGO stage and grade as well as histological type (endometrioid vs nonendometrioid carcinomas). Nonetheless, even within a group of patients with a low risk of disease progression (endometrioid type, FIGO stage I) some patients suffer from early recurrence or disease-related death. Therefore, additional molecular factors seem to be needed to individualize both patient prognosis and

therapy. Because cell migration is an essential step in tumor progression, we have investigated the expression of two proteins involved in the regulation of cell motility. Ezrin, as a member of the ERM-family (ezrin, radixin, moesin), adapts CD44 to actin-based cytoskeleton, and therefore organizes the status of the membrane–cytoskeleton interactions.³ Recent studies suggest a metastasis-promoting role for ezrin in several tumor entities by facilitating tumor cell motility. These include pancreatic carcinoma,⁴ breast carcinoma,⁵ ovarian carcinoma,^{6,7} melanoma,⁸ and osteosarcoma.⁹ Furthermore, inhibition of ezrin by antisense oligonucleotides blocked invasiveness of endometrial adenocarcinoma cell lines.⁷ However, in endometrial carcinoma, only one study demonstrated an overexpression of ezrin in carcinoma compared to simple hyperplasia or normal endometrium, although its prognostic significance was not evaluated.¹⁰

Recently, other proteins that associate with ezrin and CD44 have been described. One of these is osteopontin, a mineral binding glycoprotein that under physiological conditions is secreted to the extracellular matrix by osteoblasts to provide an

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anchor to the bone matrix via $\alpha_v\beta_3$ integrin. At the cytoplasmic face of cell adhesion sites osteopontin is able to form a submembranal complex with ezrin and CD44.¹¹ Similar to ezrin, osteopontin seems to be involved in cell adhesion and migration.¹² Its expression has been investigated in a number of human tumors.¹³ In gynecological tumors including breast and ovarian carcinomas, the overexpression of intracellular osteopontin was associated with poor prognosis.^{14,15}

In order to gain further insights into the importance of ezrin and osteopontin for progression of endometrial cancer, we correlated the expression of both proteins with clinicopathological parameters as well as patients survival in a large set of FIGO stage I endometrioid carcinomas comprising 164 samples.

Materials and methods

Quantitative Polymerase Chain reaction

Tissue of seven endometrioid carcinomas and associated tumor-free endometrium was dissected from a pathologist during frozen section analysis, immediately quick-frozen and stored at -80°C until analysis. Before homogenizing, tissue samples were confirmed for tumor content by H&E section. Total RNA was extracted by RNeasy Kit (Qiagen) and reverse transcribed by MMLV-point mutant reverse transcriptase (Promega), SYBR Green quantitative PCR for ezrin (sense: 5'-gtttccccagttgtaatagtc-3', reverse: 5'-tccgtaattcaatcagtcctgc-3') or cyclophilin A (sense: 5'-gtcaaccacccgtgtctt-3', reverse: 5'-ctgctgtctttggacctgt-3') was performed using a ROX-supplemented $2 \times$ Taq Mix (Promega). The

relative amount of RNA was calculated based on the ΔC_T -method as described recently (Livak and Schmittgen, 2001). Cyclophilin A was used as an internal standard.

Study Population

Paraffin-embedded tissue samples from a total of 164 patients with FIGO stage I endometrioid carcinoma, which were diagnosed at the Institute of Pathology, Martin-Luther-University Halle-Wittenberg, Halle, Germany, between 1998 and 2002 were used for this study. Furthermore, five proliferative and two secretory endometrium samples were examined. The study was approved by local ethical committee. Patient's age and FIGO stage were retrieved from clinical and pathological files (Table 1). All histological slides were re-evaluated by two pathologists (MK & SH). Histology was classified according to WHO and grading was assessed according to the FIGO system. Maximal primary surgery was given if patients with pT1aG1/G2 or pT1bG1 tumors had an abdominal hysterectomy and bilateral salpingo-oophorectomy. Tumors with pT1bG2 or pT1c were only expected to be treated maximal if sampling of lymph nodes was carried out. Thus, 118 (72%) of patients has a maximal primary surgery. Follow-up for this cohort was updated to October 2005 by inquiry from the tumor and local population registry. Complete data with a median follow-up time were available for 164 (100%) patients with a median follow-up time of 57.4 months (range 0.13–93.4 months). The median age was 68.9 years. A total of 27 (16.5%) patients died of disease. Four (2.4%) patients died of other

Table 1 Correlation of clinicopathological parameters of 164 patients with ezrin and osteopontin expression

Characteristic	Ezrin weak	Ezrin strong	All cases	P-value	Osteopontin weak	Osteopontin strong	All cases	P-value
All cases	81	83	164		84	80	164	
<i>Age at diagnosis</i>								
≤68.9 years	42	40	82	0.63	39	43	82	0.34
>68.9 years	39	43	82		45	37	82	
<i>pT</i>								
1a	23	7	30	0.03	16	14	30	0.36
1b	43	51	94		44	50	94	
1c	15	25	40		24	16	40	
<i>Maximal surgery</i>								
Yes	62	56	118	0.19	58	60	118	0.39
No	19	27	46		26	20	46	
<i>FIGO grade</i>								
1	62	64	126	0.33	65	61	126	0.75
2	16	12	28		15	13	28	
3	3	7	10		4	6	10	
<i>Osteopontin expression</i>								
Weak	44	40	84	0.43				
Strong	37	43	80					

cause and were censored for survival analysis, 5-year survival rate was 82.6%.

Immunohistochemistry

Immunohistochemistry was performed using standard procedures as recently described.⁶ For detection of ezrin and osteopontin we used mouse monoclonal antibodies clone 3C12 from Sigma (Deisenhofen, Germany) and clone OP3N from Novocastra (Newcastle, UK). To retrieve antigens, slides were treated with sodium citrate buffer, pH 6, in a microwave oven for 4 × 5 min. Primary antibodies were diluted 1:5.000 for ezrin and 1:50 for osteopontin in PBS and incubated at 37°C for 30 min. After washing with PBS, a biotinylated secondary antibody followed by enhancing streptavidin—horseradish peroxidase was applied. Red color was developed by aminoethylcarbazol (AEC, Zytomed, Berlin, Germany). To minimize daily variation, the slides were immunostained together in three runs. As positive control for ezrin, colonic mucosa showed a faint apical staining and additional expression in scattered lymphocytes. Predecidualized endometrial stroma cells served as positive control for osteopontin.

Evaluation of Immunohistochemical Staining

Tumor staining was independently examined by two investigators (MK and TL) blinded to the clinical data. Each tumor was represented by one tissue slide. Since staining intensity did not differ significantly, percentage of positive tumor cells was estimated. In cases where the score differed more than 5%, consensus was achieved at a multiheaded microscope. Moreover, the subcellular localization of staining (predominantly cytoplasmic, pure apical, or mixed) as well as the stromal staining of osteopontin was recorded.

Statistical Analysis

For statistical analysis, the cutoff level for grouping in weak or strong expression was defined at the median percentage score that was 15% of tumor cells for ezrin and 10% of tumor cells for osteopontin. Statistical correlation between clinicopathological factors and expression of ezrin and osteopontin was assessed by using χ^2 test for trends. The probability of differences in overall survival as a function of time was determined by Kaplan–Meier test, with probing for significance by a log-rank test. Multivariate regression analysis based on the Cox's proportional hazard model was used to test the independence of these parameters in the prediction of overall survival. Generally, *P*-values smaller than 0.05 were considered to show a significant

difference. For all statistical procedures SPSS v12.0 software (SPSS GmbH, Munich, Germany) was used.

Results

Ezrin Protein Expression in Endometrial Tissue

Immunoreactivity of ezrin was detected in all phases of normal cycling endometrium. As shown in Figure 1a, proliferative endometrium showed an apical membranous staining with an additional faint cytoplasmic signal that was pronounced at the cell basis.

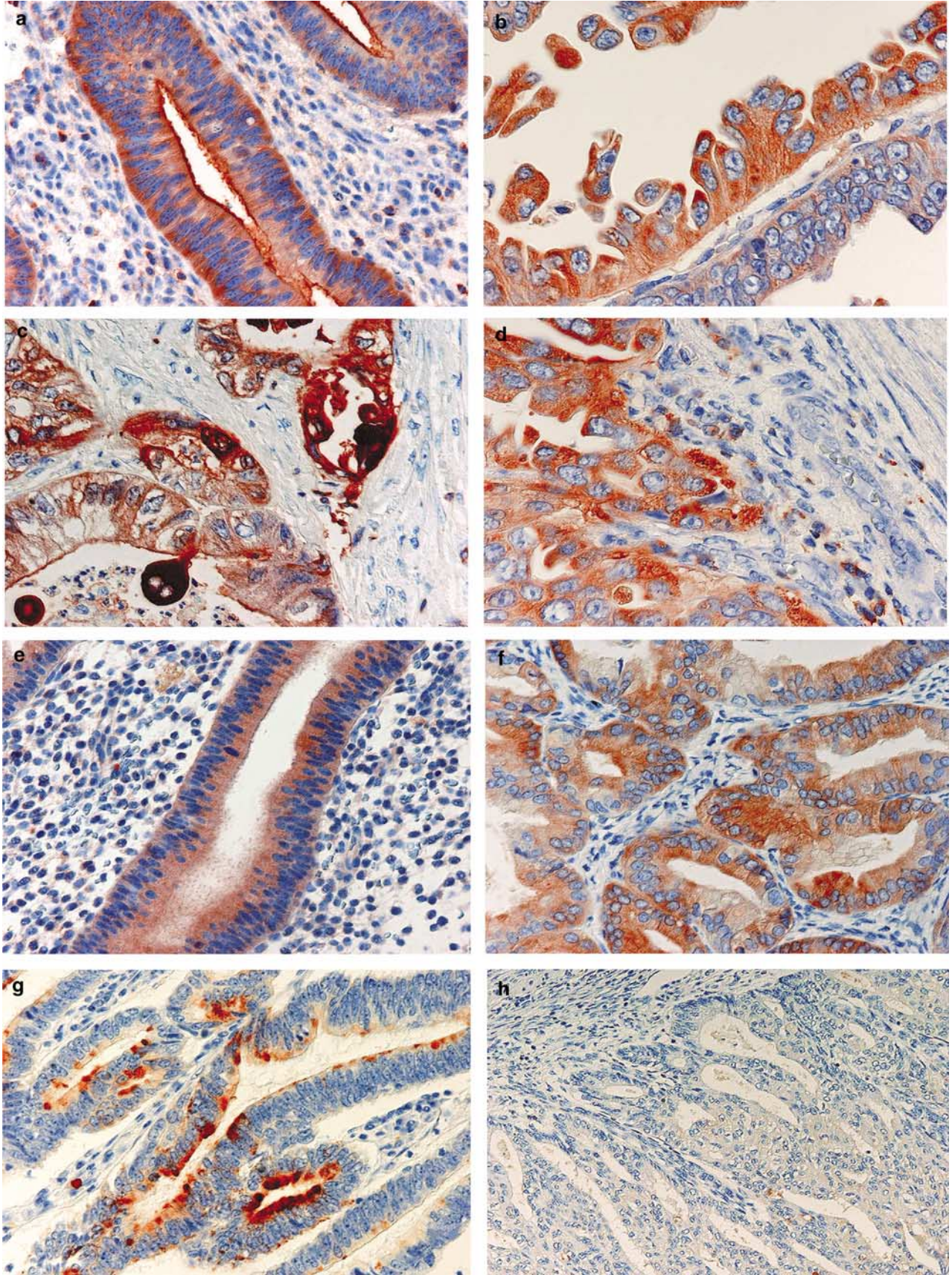
In endometrioid carcinomas, ezrin was at least focally expressed in 93% of cases. Only 7% of cases were completely negative. Expression was quite heterogeneous with a wide range from only a few to nearly 100% of positive tumors cells. The cutoff value for weak and strong expressing cases was specified at the median percentage score of positive tumor cells resulting in a value of 15%. The cellular expression pattern in carcinomas was predominantly cytoplasmic (Figure 1b–d). Interestingly, tumor cells with an intensive cytoplasmic staining seemed to lose their cell-to-cell contacts. This was either observed in free floating cell groups that were detached from papillae (Figure 1c) or in cells at the tumor–host interface (Figure 1c and d). Except for scattered lymphocytes, tumor stroma was always negative for ezrin staining.

Ezrin mRNA Expression in Endometrial Tissue

To investigate expression of ezrin mRNA, we analyzed seven endometrioid carcinomas as well as associated tissue free of tumor by quantitative PCR. For each sample, ezrin expression level was compared between normal (100%) and tumor tissue. Additionally, we controlled tumor samples for the ratio of epithelial vs stromal component by verifying a corresponding H&E section. Differences in the tumor/stroma ratio were adjusted to 100%. Two of the analyzed endometrioid carcinomas showed a decrease of ezrin mRNA, while a slight increase of ezrin expression was observed in three cases and an up to four-fold overexpression in two cases (Figure 2). This suggests that endometrioid carcinomas can be divided in two groups: one with increased and another with retained or reduced ezrin mRNA concentrations.

Osteopontin Expression in Endometrial Tissue

Osteopontin is also expressed in normal cycling endometrium. Proliferating and secretory endometrium showed a diffuse cytoplasmic signal in epithelial cells (Figure 1e). Predecidualized stroma cells served as internal control showing strong cytoplasmic expression whereas other stromal components were negative.



Most of endometrioid carcinomas expressed osteopontin whereas 29% of cases were completely negative (Figure 1h). The expression was also heterogeneous. The median percentage score to separate strong and weak expressing cases was set to 10% of tumor cells positive for osteopontin. The cellular localization in endometrioid carcinomas was either a diffuse or patchy cytoplasmic staining (Figure 1f and g). Stromal osteopontin was of negligible amounts.

Ezrin and Osteopontin Expression in FIGO I Endometrioid Carcinomas: Univariate Correlation with Overall Survival

In univariate survival analysis, a significant correlation of strong ezrin expression with overall survival was found (Figure 3, $P=0.041$). Strong ezrin expression was also correlated with pT stage. However, no correlation was observed between ezrin expression and age, FIGO grade, maximal surgery, or osteopontin expression (Table 1). The 5-year survival rate was decreased for patients with strong ezrin-expressing tumors from 89 to 76% (Table 2). Other clinicopathological parameters, such as age, pT stage, maximal surgery and FIGO grade were also significantly associated with overall survival (Table 2). In contrast, patients with strong osteopontin expression had a more favorable outcome (Figure 3), even though this difference was not significant.

Ezrin Expression in Endometrial Carcinomas: Multivariate Correlation with Overall Survival

For multivariate analysis, all factors with significance in univariate analysis were included in the

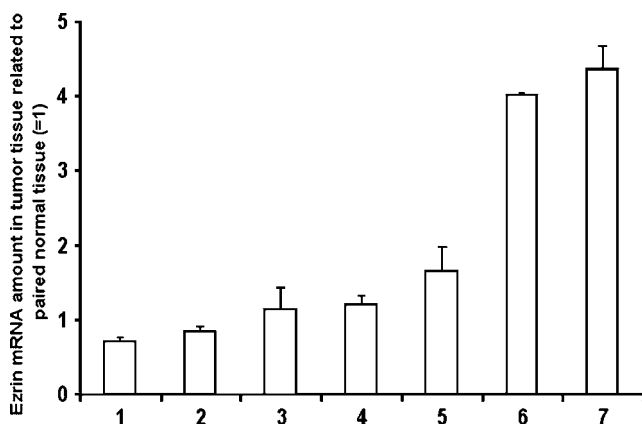


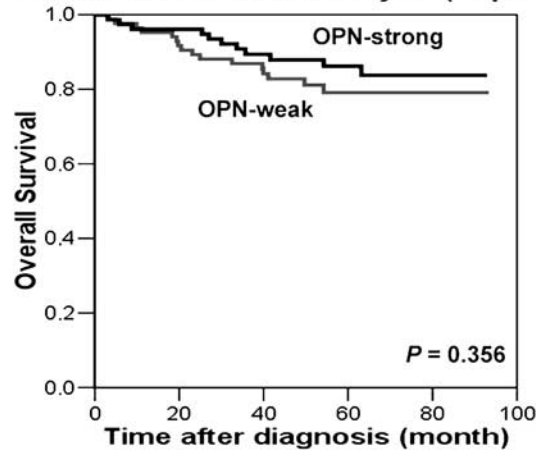
Figure 2 Expression of ezrin mRNA in seven endometrioid carcinomas. Each sample was normalized to cyclophilin A, to the relative tumor/stroma ratio of the sample and to tumor-free associated endometrium in percent.

multivariate analysis using the Cox's hazard regression model. Multivariate risk factors in decreasing order were FIGO grade 3, age ≥ 68.9 and strong ezrin expression (Table 3).

Discussion

Positioned at the submembranal—cytoskeletal interface, ezrin may be a nexus in metastatic progression with a central role in the regulation of invasion. Ezrin is physiologically expressed in a variety of epithelial tissue including intestine, lung, and kidney. Within these tissues it is found in the apical region of microvilli presenting cells suggesting an involvement in defining cell polarity. However, besides its localization to microvilli, ezrin is also

a Univariate Survival Analysis (Kaplan-Meier)



b Univariate Survival Analysis (Kaplan-Meier)

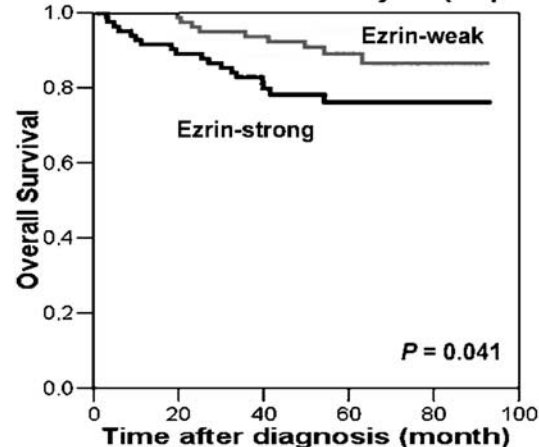


Figure 3 Univariate survival analysis (Kaplan-Meier). (a) Correlation of weak and strong ezrin expression with overall survival. (b) Correlation of weak and strong osteopontin expression with overall survival.

Figure 1 Ezrin (left) and osteopontin (right) expression by immunohistochemistry. (a) Proliferative endometrium with ezrin expression. (b-d) Endometrioid carcinoma with strong ezrin expression. (c and d) Note: cells with intense cytoplasmic ezrin expression are either detached or located at the tumor-host interface. (e) Proliferative endometrium with osteopontin expression. (f and g) Endometrioid carcinoma with strong osteopontin expression. (h) Endometrioid carcinoma negative for osteopontin.

Table 2 Univariate survival analysis (Kaplan–Meier): 5-year survival rate of 164 patients with FIGO I endometrioid carcinomas according to clinicopathological factors and ezrin or osteopontin expression

	Cases	Events	5 year survival rate in %	Standard error	Log-rank test (P-value)
All cases	164	27	83	4	
<i>Age at diagnosis</i>					
≤68.9 years	82	6	92	3	0.0017
>68.9 years	82	21	73	5	
<i>pT</i>					
1a	30		100		0.0015
1b	94	15	83	4	
1c	40	12	68	8	
<i>Maximal surgery</i>					
Yes	118	14	88	3	0.0047
No	46	13	66	8	
<i>FIGO grade</i>					
Grade 1	126	14	81	3	<0.0001
Grade 2	28	7	71	10	
Grade 3	10	6	33	16	
<i>Ezrin expression</i>					
Weak	81	9	89	4	0.041
Strong	83	18	76	5	
<i>Osteopontin expression</i>					
Weak	84	16	79	5	0.356
Strong	80	11	86	4	

Table 3 Multivariate survival analysis (Cox's regression model) for overall survival of 164 patients with FIGO I endometrioid carcinomas

	Relative risk	95% confidence interval	P-value
<i>FIGO grade</i>			
G1	1.00		
G2	2.34	0.87–6.25	0.090
G3	6.93	2.35–20.37	<0.001
<i>Age</i>			
≤68.9 years	1.00		
>68.9 years	3.28	1.29–8.44	0.013
<i>Ezrin expression</i>			
Weak	1.00		
Strong	2.22	1.00–5.37	0.047
<i>pT</i>			
1a, 1b	1.00		
1c	1.50	0.55–4.10	0.423
<i>Maximal surgery</i>			
Yes	1.00		
No	1.03	0.36–2.92	0.951

targeted to the leading edge of spreading cells suggesting an essential role in controlling cell motility. To date, several binding partners for ezrin

have been identified. The N-terminal binding domain of ezrin mediates membrane attachment by binding the cytoplasmic tail of CD44, CD43, or intercellular adhesion molecules. On the other end, ezrin via its C-terminal domain associates with F-actin and contributes to microfilament organization.

Recently, by use of similar methods, we analyzed ezrin expression in ovarian carcinomas and found a strong correlation with poor prognosis that was independent from other known risk factors as residual tumor burden or FIGO stage.⁶ In endometrial carcinoma, two studies postulated a putative role for ezrin in cancer progression.^{7,10} However, to our knowledge, this is the first comprehensive description of ezrin expression in a large set of FIGO I endometrioid carcinomas showing its prognostic value. We found that patients suffering from ezrin-rich endometrioid carcinoma were prone to a poorer prognosis regardless of FIGO grade and pT stage as well as age of patient and with an estimated 2.2 relative risk of death ($P=0.047$).

Apparently, the cellular localization of ezrin shifted when comparing normal endometrium and endometrial carcinomas from an apical membranous to a predominantly cytoplasmic distribution. Cytoplasmic staining was even accentuated in carcinoma cells losing their cell–cell contacts. This was observed in both, free floating cells detached from micropapillae or in cells at the tumor–host interface. It is well known that ezrin is exchanged between cytoplasm and membrane. Cytoplasmic ezrin exists in a 'closed' conformation based on intra- or intermolecular interactions between the N- and C-terminus. Threonine and tyrosine phosphorylation induces an 'open' conformation. In this state ezrin is localized towards the membrane where it modulates F-actin dynamics and tethers the microfilament system to the cytoplasmic face of cell adhesion sites.¹⁶ Disruption of actin filaments and a decrease in focal adhesion are common features of epithelial–mesenchymal transition which is associated with the onset of invasion. Besides a structural role, ezrin may as well act as a signaling or scaffold molecule. Recently, it was revealed that ezrin is involved in modulating signalling pathways acting through Rho¹⁷ and phosphatidylinositol 3-kinase/Akt.¹⁸

However, when comparing ezrin levels in endometrial carcinoma with tumor-free endometrium, no straightforward overexpression in carcinomas was detectable. Rather, ezrin expression intensity in normal endometrial epithelium was within the same range as in a subset of carcinomas. Regardless, loss of ezrin expression apparently rendered the tumor less aggressive, as revealed by a better prognosis.

Osteopontin is reported to be an integral part of the CD44–ezrin complex and it is known to associate with poor prognosis of breast and ovarian carcinomas.^{14,15} However, the present study shows no impact on overall survival in endometrial carcinomas. In the contrary, there is a trend that

strong osteopontin expression correlates with a better prognosis. This finding is supported by a recent study which indicates that osteopontin expression, in contrast to other cancers, was not correlated with tumor stage in endometrial carcinomas.¹³ Like ezrin, osteopontin is not upregulated in endometrial carcinomas compared to normal tissue, and localization of osteopontin was within the cytoplasm of tumor cells. Extracellular osteopontin was of negligible amounts suggesting that stromal cells are not a significant source of osteopontin in endometrial carcinomas as it also has been shown in ovarian carcinomas.¹⁵ Although immunohistochemical studies could not replace functional investigations, the lack of correlation between ezrin and osteopontin expression suggests that the documented interaction of ezrin and osteopontin¹¹ may not be of high importance for the role of ezrin in endometrial carcinoma.

In conclusion, our study revealed a rather high predictive value for tumor-related death of strong ezrin expression in patients with low-stage endometrial carcinomas. This finding warrants further elucidation in prospective trials to evaluate whether it is useful for therapeutic decisions or not.

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