

Immunohistochemical Analysis for Expression of Calpain 1, Calpain 2 and Calpastatin in Endometrial Cancer

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Abstract. Background: Calpains (CAPN) are intracellular, non-lysosomal cytoplasmic cysteine endopeptidases and they are expressed ubiquitously. Their endogenous specific inhibitor is calpastatin. When calcium is present, calpastatin and calpain attach to each other, inhibiting the protease. The calpain system plays an important role in many processes including apoptosis, necrosis, ischaemia and exocytosis. The role of calpains in pathogenesis or further tumour progression has been proved in related studies. This study focused on the expression of the enzymes calpain 1, calpain 2 and the inhibitor calpastatin in normal and malignant endometrial tissue. Materials and Methods: Immunohistochemical stainings were performed on paraffin slices and staining intensity, percentage of positive cells and international ratio score were evaluated. Results and conclusion: The endometrial carcinoma showed a higher expression of calpastatin than benign endometrial tissue.

Endometrial cancer. Endometrial cancer (EC) is the fourth most common malignant disease in women in Germany, with 11,370 new cases diagnosed per year and 2,700 deaths attributed to this disease every year. (1, 2). The large international variation in incidence rates indicates that much of the risk is modifiable. Most of the major known risk factors for EC contribute to prolonged and excessive exposure of the endometrium to oestrogens unopposed by progesterone, as occurs with unopposed postmenopausal

oestrogen therapy and obesity. Whereas obesity may place a patient at increased risk for medical comorbidities, including diabetes, cardiovascular diseases, and osteoarthritis, it is also a major risk factor for EC. A recent study reported that 68% of women with early-stage EC are obese (3).

There are the two major types of EC: Type I or endometrioid adenocarcinoma and type II nonendometrioid ECs. Type I EC represents about 80% of all endometrial malignancies and occurs mostly in perimenopausal and early postmenopausal women (4). It is related to oestrogen exposure and is frequently associated with endometrial hyperplasia. Type II nonendometrioid ECs (usually serous or clear cell carcinoma) develop more often after the menopause. They are unrelated to oestrogen stimulation and endometrial hyperplasia, occasionally arising in endometrial polyps, or from precancerous lesions that develop in atrophic endometria. Type I and Type II EC have different malignancy potentials. Type I is characterized as a low-grade malignancy (5). Five-year overall survival amounts to 82%. Type II is correlated with low differentiation (G3) and is characterized as a high-grade malignancy. Five-year overall survival amounts to only 58% (6).

Calpain and calpastatin. Calpains are calcium-regulated, non-lysosomal neutral cysteine proteases (7). The best characterized calpains are the ubiquitous isozymes, termed m-calpain (also: calcium activated neutral proteases CAPN 1 or calpain 1) and μ -calpains (also: CAPN 2 or calpain 2) (8). These are heterodimers consisting of a unique 80 kDa large subunit (calpain 1 and 2) and a common 28 kDa small subunit (9). These have been identified, requiring micro- and millimolar Ca^{2+} concentrations, respectively, for their *in vitro* activity. *In vitro* analysis has shown that the Ca^{2+} concentration required for optimal activity is 200-1000 μM for calpain-1 and 5-50 μM for calpain-2 (10). Physiological

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roles of calpains include cell motility and attachment, differentiation, signal transduction including cell survival pathways, membrane fusion, cell cycle progression, regulation of gene expression, and long term potentiation. Many calpain substrates are similar to, or overlap with those of caspase 3, consistent with the hypothesis that calpains may also function as death-related proteases.

More than 50 endogenous and exogenous inhibitors of the calpain have been described. Several classes of inhibitors, including peptidyl epoxide, aldehyde, and ketoamide inhibitors are in the process of evaluation (11, 12). The specific endogenous inhibitor of calpain activity is calpastatin, which binds specifically to both isoforms of calpain in a substrate-competitive manner and is ubiquitously present in most cell types, similarly to calpain (8, 13). The intracellular level of calpastatin correlates directly with calpain activation, and the affinity of calpastatin for the activated forms of the calpains is greater than its affinity for the proenzyme (12). Calpains modify a variety of proteins including cytoskeletal proteins, membrane proteins, membrane proteins (growth factor receptor, adhesion molecules, and ion transporters), enzymes (kinases, phospholipases, and phosphatases), as well as cytokines and various transcription factors by selective and very limited proteolytic degradation, suggesting that its function is to modulate protein structure and activity rather than to catabolize proteins (14-16).

Thus, regulated proteolytic modification of proteins by calpain may transduce a cellular signal or induce cellular functions such as membrane fusion and cell proliferation. It is evident that non-physiological calpain activation and proteolytic degradation of calpastatin play roles in cataract development, various neurodegenerative diseases, muscle dystrophy, and pathological apoptosis (17). A number of pathological conditions have been associated with disturbances of the calpain system. They include type II diabetes, cataracts, Duchenne's muscular dystrophy, Parkinson's disease, Alzheimer's disease, rheumatoid arthritis, ischaemia, stroke and brain trauma, various platelet syndromes, hyper-tension, liver dysfunction, and some types of cancer such as liver, kidney, and pancreas (18-23).

It is speculated that the calpain system may play a role in the development of EC. However, no study has been reported about the relationship between the alteration of calpain and calpastatin in women with EC.

In this study, the alteration of calpain and calpastatin in women with physiological endometrial tissue and EC was investigated in comparison to tumour size and grading by detection of calpain 1 and calpain 2 and calpastatin to show a potential link between the calpain system and EC.

Materials and Methods

This study group involved 47 patients, 29 of them with EC, 18 with benign endometrial tissue. The patients were all operated on in the Department of Gynecology and Obstetrics, University Homburg/Saar.

Table I. Age of patients

Tissue	Average	Min.	Max.
Endometrial cancer	64 years	39 years	84 years
Benign endometrial tissue	50 years	33 years	73 years

The ages of the patients with EC are presented in Table I.

Concerning the grading of the patients with EC there were three patients with highly differentiated tumors, 18 of them with intermediate differentiation and seven with low differentiation.

All the patients enrolled in this study were diagnosed objectively by a combination of gynaecological examination, ultrasonography, and endometrial biopsy. Initial examination of pathology consisted of routine haematoxylin and eosin (H&E) staining. Ten adjacent 4 µm sections were cut from each paraffin block.

For representation of calpain 1 and calpastatin, monoclonal antibodies were used. These were obtained from ascites fluid of mice. In contrast, calpain 2 was shown with polyclonal antibodies, which were derived from antiserum of rabbits.

Secondary antibodies. When the primary antibody is used from rabbits (anti-calpain 2) there must be a secondary antibody which may be obtained from the goat, which is directed against the first animal. Without using a different animal there will be non-specific background reactions. Since anti-calpastatin, and anti-calpain 1 used were both of the mouse, the secondary antibody was made in rabbit for this study.

Streptavidin-biotin method. This method is based on the ability of the glycoprotein streptavidin to bind the vitamin biotin strongly to itself. Avidin has four binding sites for biotin. Necessary are a primary antibody, a secondary antibody, streptavidin and chromogen. The primary antibody is specific against the antigen. The secondary antibody binds biotin and the primary antibody. Streptavidin binds the biotin, which is bounded at the secondary antibody. Chromogen is able to show the enzyme peroxidase and thus of the antigen becomes visible.

Immunoreactive score. To perform immunohistochemical staining for the presence of the enzymes calpain 1 and 2 and calpastatin, the international ratio score (IRS) according to Remmele and Stegner was used. The number of stained cells was counted in a representative part of the preparation. The percentage of positive cells (PP) was converted into a PP score, which ranged between 0 and 4. The staining intensity (SI) of positive cells was evaluated and also expressed as a score which ranged between 0 and 3. The IRS score was then calculated by multiplying the PP score with the SI score. Thus, a total IRS between 0 and 12 was obtained.

Statistical analysis. The data of the expressions of calpain 1, calpain 2, and calpastatin protein are given as mean±standard deviation (SD). The difference between the groups was compared by using Chi-squared test with Statistical Package for the Social Science software windows version 13.0. Differences were considered statistically significant when $p < 0.05$.

Correlation between the expressions of calpain 1, calpain 2 and calpastatin. Spearman's rank correlation coefficient (r) was calculated for correlations in the percentage of positive-reacting cells

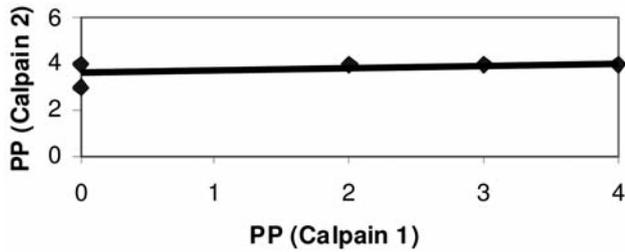


Figure 1. Correlation between PP score for calpain 1 and calpain 2 in endometrial cancer (n=29).

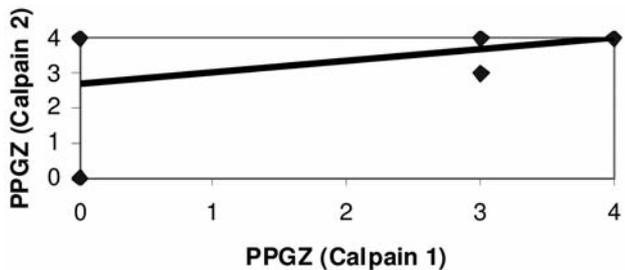


Figure 2. Correlation between the expression of calpain 1 and calpain 2 in normal endometrial tissue (n=18).

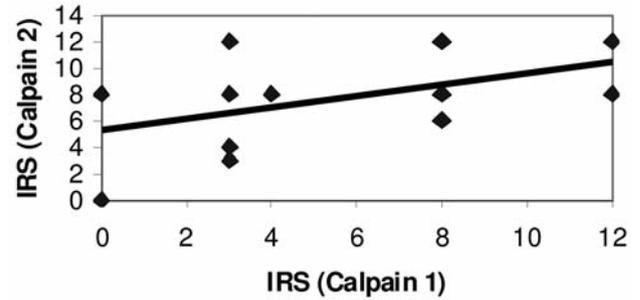


Figure 3. Correlation between IRS for calpain 1 and calpain 2 in endometrial tissue (n=18).

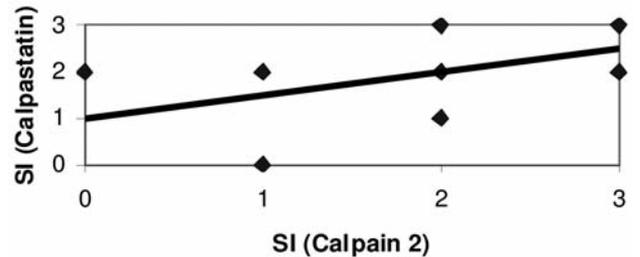


Figure 4. Correlation between the expression of calpain 2 and calpastatin in endometrial tissue (n=18).

(PPGZ) and IRS. There was considered to be a strong correlation with values of r greater than 0.7, a moderate correlation at values from 0.4 to 0.7 and a weak correlation with values less than 0.4.

Results

Expression and analysis. After the expression of calpain 1, calpain 2 and calpastatin in the 29 ECs and 18 benign endometrial tissue was assessed immunohistochemically, samples were evaluated by light microscopy. For each specimen, the SI, PP and IRS were determined.

The number of specimens, for which distant metastasis was given in the histopathological report was too low for statistical analysis.

For EC, incidence tables were drawn up regarding their tumour stage (pT), the degree of malignancy (G) and the metastasis in lymph nodes (pN).

Correlation between histopathological data of the tumours and expression of calpain 1, calpain 2 and calpastatin. Tumour characteristics were compared to tumour stage (pT), lymph node status (pN) and malignancy (G) of endometrial carcinoma, with immunohistochemical characteristics as colour index (SI), number of positive cells (PPGZ/PP) and IRS. The Chi-square test and the Mann-Whitney test were performed for the tumour stage (pT) and grading (G). The stage of EC did not correlate with the expression of the enzymes calpain 1 and 2, and calpastatin.

Correlation between tumour stage (pT) and expression of calpain 1, calpain 2 and calpastatin. The Chi-square test and the Mann-Whitney test showed no statistical significances.

Correlation between pN and expression of calpain 1, calpain 2 and calpastatin. All tissue samples were assigned to the lymph node status N0, so it was not possible to make a statement about a correlation with the expression.

Correlation between grading (G) and expression of calpain 1, calpain 2 and calpastatin. The Chi-square test did not determine any statistical significance between the grading and enzyme expression.

Comparison of enzyme expression from benign tissues with carcinoma tissues. Calpain 1. Two of the endometrial specimens (benign and malignant) showed no immunoreactivity for calpain 1. The Chi-square test did not show any statistical significance. On average, the immunoreactivities of the malignant and benign endometrial tissues were the same.

Calpain 2. A benign endometrial tissue was negative for calpain 2. The Chi-square test and the Mann-Whitney test did not reveal statistically significant differences. ECs showed slightly higher immunoreactivity compared to benign tissue.

Calpastatin. All specimens, except for one benign endometrial tissue, showed immunoreactivity for calpastatin. A relationship between the number of counted positive cells

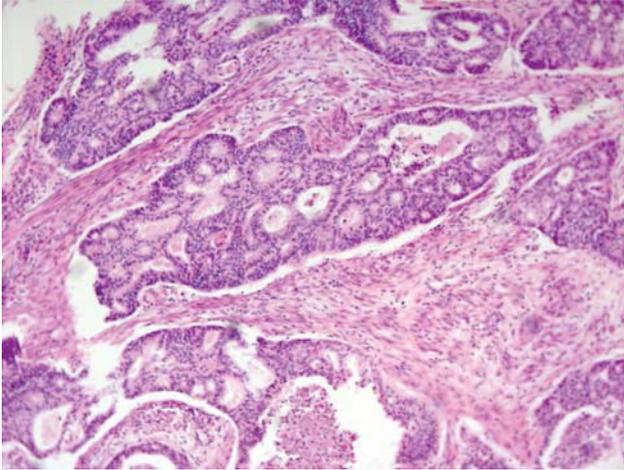


Figure 5. EC, endometrioid, G2.

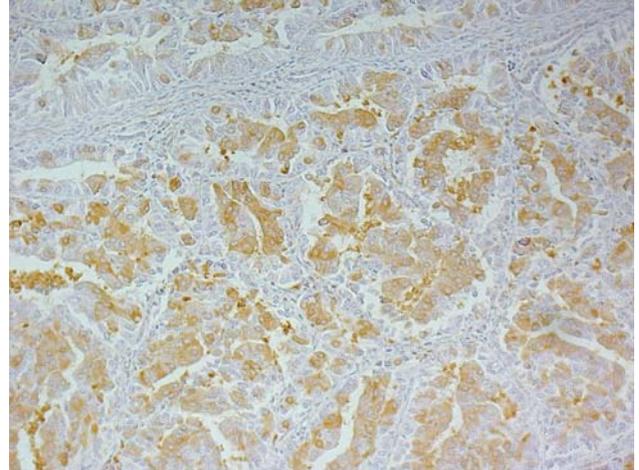


Figure 8. EC, endometrioid, G2, immunohistochem. presentation of calpstatin.

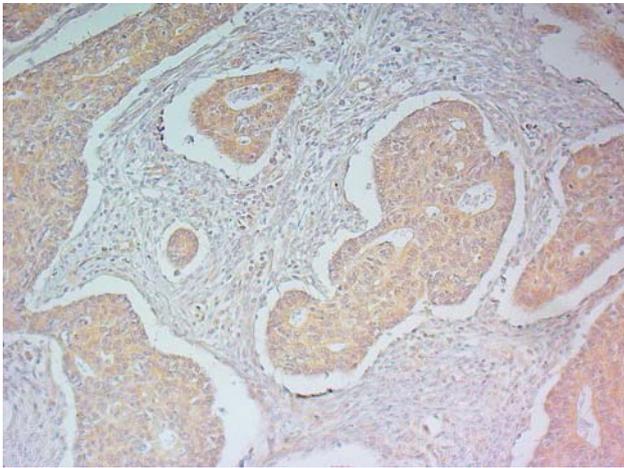


Figure 6. EC, endometrioid, G2, immunohistochem. presentation of calpain 1.

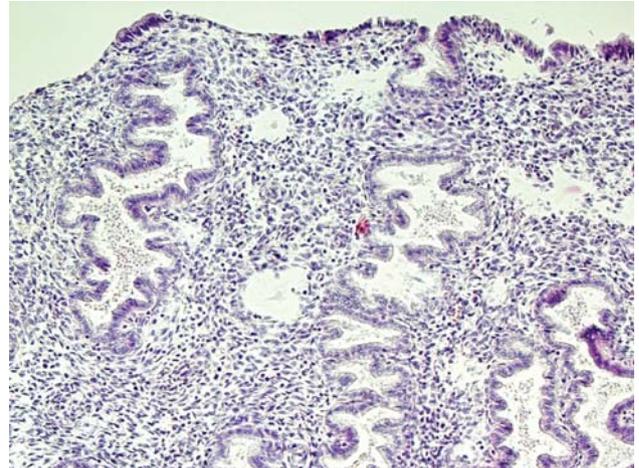


Figure 9. Endometrial tissue.

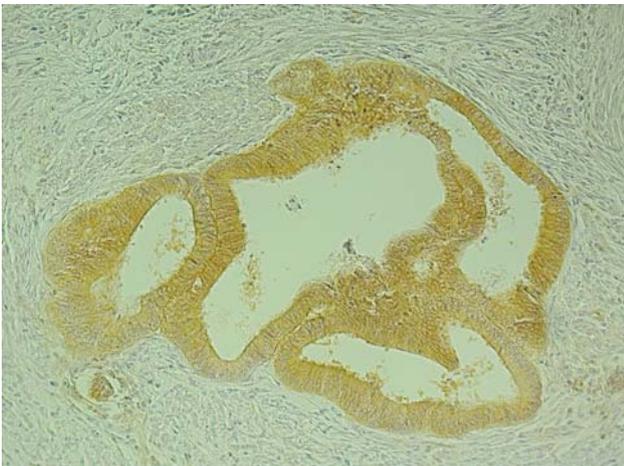


Figure 7. EC, endometrioid, G2, immunohistochem. presentation of calpain 2.

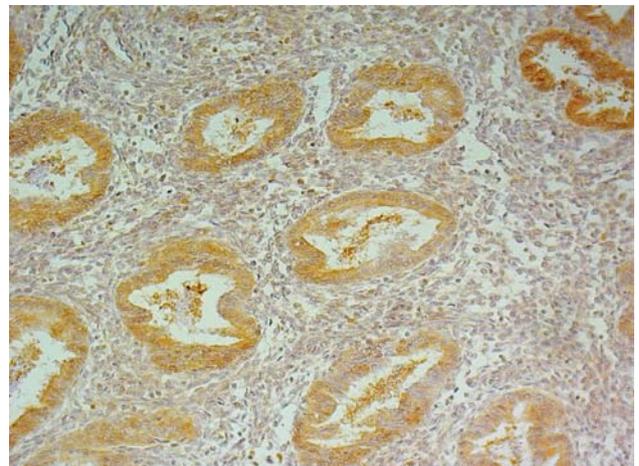


Figure 10. Endometrial tissue, immunohistochem. presentation of calpain 1.

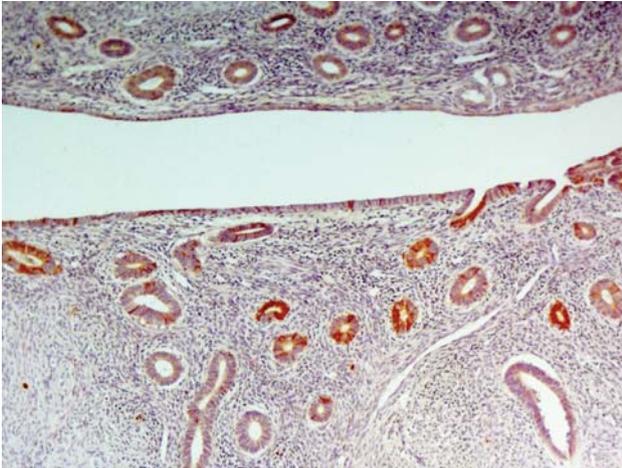


Figure 11. Endometrial tissue, immunohistochem. presentation of calpain 2.

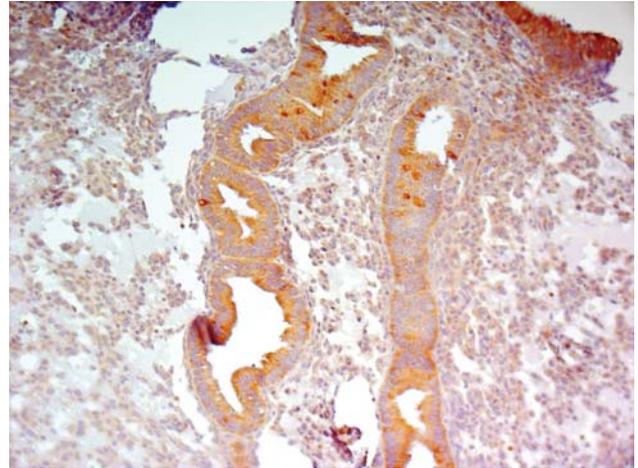


Figure 12. Endometrial tissue, immunohistochem. presentation of calpastatin.

and the type of the endometrial tissue could be shown ($p=0.037$). The endometrial immunoreactive scores on average were higher than those of benign tissue.

Correlation between the expression of calpain 1 and calpain 2. The relationship of the proportion of positive cells was assigned a p -value of 0.002 in EC (Figure 1) and 0.015 in normal tissue (Figure 2). Spearman's rank correlation coefficient (0.488) yielded a moderate correlation between the IRS values of calpain 1 and 2 of endometrial tissue (Figure 3). The p -value was 0.040. The correlation between IRS for calpain 1 and calpain 2 was statistically significant ($p=0.024$) (Figure 3).

Correlation between the expression of calpain 2 and calpastatin. The Chi-square test gave a statistically significant p -value of 0.039 with regard to the staining intensity of endometrial tissue (Figure 4). There were no significant correlations evident from the Spearman rank correlation coefficient.

Correlation between the expression of calpain 1 and calpastatin. A statistically significant correlation in the number of positive cells was found for EC using the Chi-square test ($p=0.025$). The Spearman rank correlation showed a moderate correlation between the IRS values of EC for calpain 1 in comparison with the percentage of cells positive for calpastatin ($p=0.016$, $r=0.442$). Calpain 1 and calpastatin correlated moderately ($p=0.001$, $r=0.579$). The benign endometrial tissues showed a p -value of 0.032, but only a moderate correlation ($r=0.508$) compared with the IRS values for calpain 1 and calpastatin together.

EC and benign endometrial tissue. Figure 5 shows an intermediate grading Type I EC without special staining. In

Figures 6 to 8, intermediate grading of Type I EC with staining for calpain 1 (Figure 6), calpain 2 (Figure 7) and calpastatin (Figure 8) is shown.

Figure 9 shows benign endometrial tissue. Figure 10 to 12 are correlating to Figure 6 to 8 and show stained benign endometrial tissue. They present calpain 1 (Figure 10), calpain 2 (Figure 11) and calpastatin (Figure 12) staining.

Discussion

This work deals with of the relationship between the expression of calpain 1, calpain 2 and calpastatin in benign and malignant endometrial tissue. A possible correlation was shown between the expression levels of these enzymes to each other.

A regulatory role of calpain has been shown in various tumours (prostate cancer (23), liver (18) renal cell carcinoma (20), brain (19, 21) and skin (22)).

For example in human glioblastomas, cell death has been associated with calcium and calpain activation (24) and results of another study suggest a degradation of androgen receptor which is intrinsic to the induction of apoptosis in prostate cancer cells, where androgen receptor breakdown was attenuated by calpain inhibitors (25).

In another study it was shown that calpain 6 was overexpressed in lyomyosarcoma compared with normal myometrium. A marginally significant association between tumour subtype and staining intensity was shown (26).

According to these studies, calpains have not yet been described as risk factors for the development of endometrial carcinoma. The goal of this study was determine the importance of calpain 1, 2 and their inhibitor, calpastatin, in EC.

A study examined the expression of calpain in human renal cell carcinomas in correlation with the lymph node status and histological type of tumour (20). That study found decreased calpain 1 expression with increasing grading of the tumour. Furthermore, it appeared that for patients with clear cell carcinomas, which showed no lymph node metastasis, there was a quantitatively lower calpain 1-mRNA expression than in patients with a pre-existing lymph node involvement. It can be assumed that this increased calpain level led to a dispersion of tumour cells in the lymph nodes. This study pointed to calpain 1 as a prognostic factor for clear cell carcinomas (20).

It is known that p53 (a tumour suppressor protein), a substrate of calpains, plays an important role in the pathogenesis of various tumours (27). Inhibitors of calpains have a stabilising effect on p53 and can increase p53 levels and reduce tumour growth. This was demonstrated in 2001 by Benetti *et al.* who showed that the use of calpain inhibitor I and II in MCF-7 (breast cancer) and RKO cells (colon cancer) resulted in an increase in endogenous p53 levels by calpain inhibitors which inhibited β 1- and β 3-integrin-mediated cell migration through a strengthening of the cytoskeleton binding (28). The inhibitors stabilise the cell adhesion and increase the binding force. Therefore, the use of calpain inhibitors can be considered as a method to treat pathological cell migration (such as tumour metastasis). Inhibition of calpain inhibits the motility of fibroblasts and myofibroblasts (29).

Comparison of enzyme expression of normal tissue to carcinoma tissue. For calpain 1 and calpain 2, there were no statistically significant correlations in benign endometrial tissue or in EC. The endometrial tissue showed 89% positive cytoplasmic immunoreactivity for calpain 1, 2 and calpastatin. Three of the benign and one malignant endometrial tissue did not show any immunoreactivity.

In endometrial tissue, the number of positive cells correlated statistically significantly with the type of the tissue (mean PP calpastatin endometrial carcinoma: 83.17, mean PP calpastatin benign endometrium: 71.27, $p=0.037$). In relation to the endometrial tissue, the number of positive cells for calpastatin was statistically significant in the Chi-square test. The international ratio score of the malignant tissue was slightly higher than that of the benign tissue, but showed no statistical significance.

Correlation between the expressions of calpain 1, calpain 2 and Calpastatin. The malignant endometrial tissue showed significant correlations in the Chi-square test, positively reacting cells on the proportion between calpain 1 and calpain 2 ((PP (C1)×PP (C2): $p=0.002$) and between calpain 1 and calpastatin (PPZZ (C1)×PPZZ (CA): $p=0.025$). Higher levels of calpain 1 were correlated with higher levels of calpain 2 or calpastatin. Benign tissue samples resulted in Chi-square statistical significances for the staining intensity

in comparison of calpain 2 with calpastatin (SI (C2)×SI (CA): $p=0.039$) and also for the percentage of positively reacting cells in comparison calpain 1 with calpain 2 (PPZZ (C1)×PPZZ (C2): $p=0.015$).

Spearman's rank correlation coefficient showed moderate correlations. Regarding the p -values there were statistical significances for the endometrial carcinoma. A higher percentage of positive cells by calpain 1 was correlated with an increased percentage of calpastatin positive cells (PPGZ (C1)×PPGZ (CA): $p=0.001$, $r=0.579$). In benign endometrial tissue, increased IRS of calpain 1 correlated with increased IRS of calpain 2 (IRS (C1)×IRS (C2): $p=0.040$, $r=0.488$). Higher IRS for calpain 1 were associated with higher IRS for calpastatin (IRS (C1)×IRS (CA): $p=0.032$, $r=0.508$).

Spearman's rank correlation coefficient showed a moderate correlation ($0.4 > r > 0.7$) in all above mentioned cases. Thus, the results are inconclusive regarding the interactions in the calpain system.

Finally, it was possible to determine the expression of the enzymes calpain 1, calpain 2 and calpastatin with an immunohistochemical method in endometrial carcinomas and in the corresponding benign tissues. There was an increased expression of calpastatin in ECs. Comparing the expression of calpain 1, 2 and calpastatin with each other only moderate correlations were found.

These data suggest an important role of the enzymes calpain 1 and calpain 2 and calpastatin in progression and malignancy of tumours. It is well known that the calpain system with calpain 1 and 2 and its antagonist calpastatin are involved in various processes, for example, apoptosis and cell migration. It was shown by Norberg *et al.* that processing of apoptosis-inducing factor, which is essential for its function during apoptosis, is mediated by a calpain located in the mitochondrial intermembrane space (30).

The precise function of the calpain system is still not known. Further studies are needed to verify the role of calpains in EC and other tumours.

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