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Prognostic Significance of E-cadherin and Ezrin Immunohistochemical Expression in Prostate Cancer

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Objective. Abnormal expression of molecules connected with cell to cell adhesion, such as E-cadherin or ezrin, can be contributing factors for increased invasiveness and metastatic potential of cancer. We investigated E-cadherin and ezrin immunoreactivity in prostate carcinomas, and its relation with clinical parameters and patient outcome.

Methods. E-cadherin and ezrin expression was examined by immunohistochemical analysis of bioplate tissues from patients with prostate cancer. Normal appearing prostate epithelium was used as an internal control for each specimen. The relation between E-cadherin and ezrin staining, and other clinicopathological features (e.g. patient survival) were analyzed using Kaplan-Meier and Cox regression methods.

Results. In moderate differentiated to poorly differentiated tumors (Gleason scores 5-10), we observed a trend of increasing percentage of tumors with aberrant staining for E-cadherin ($P < 0.001$ by χ^2 for linear trend). A significant inverse correlation between ezrin expression and tumor differentiation was also found ($P < 0.002$ by χ^2 for linear trend).

The survival time of patients with aberrant staining of ezrin and E-cadherin was significantly shorter than that of patients with a normal staining pattern but in Cox multivariate survival analysis, only E-cadherin immunoreactivity had independent effect on survival ($P = 0.03$), when controlling for the other clinicopathological factors.

Conclusions. The aberrant or decreased expression of E-cadherin seems to be one of most promising markers of poor prognosis in localized prostate cancer. Our study also supports a role of ezrin in progression in human prostate cancers but additional studies are mandatory to provide further evidence for an important role of ezrin in prostate tumors progression.

Introduction

Prostate cancer is the most common malignant neoplasm diagnosed in the developed world and the second cause of death among men in Western countries.

The Gleason score is so far the best prognostic factor in prostate cancer. However, there is a considerable interest in finding the new prognostic indicators. It could help to avoid unnecessary treatment and develop more effective therapeutic strategy.

Metastatic potential and invasiveness of cancer cells depends on the loss of cell adhesion and on increasing cell motility. There is a variety of molecules playing a crucial role in cell to cell adhesion.

The cadherin transmembrane glycoprotein family is one of them. Extracellular domain of cadherins is calcium-dependent homotypic binding site, whereas intracellular domain is anchored to cytoskeleton by the complex of alpha-, beta- and gamma-catenin molecules.

E-cadherin is the prime mediator of intercellular adhesion in epithelial cell [30].

A lot of data indicate that prostate cancer can develop abnormalities in the expression of E-cadherin [9, 27]. There was shown, in many studies, that well-differentiated tumors (low Gleason score) retain normal (membrane) expression of E-cadherin, whereas in poorly-differentiated tumors (high Gleason score) expression is often decreased or aberrant (e.g. cytoplasmic). Abnormal or reduced expression of E-cadherin has been associated with advanced stage and poor clinical outcome [7, 9].

Ezrin is a member of ezrin-radixin-moesin (ERM) protein family which participates not only in maintaining of cell shape and polarity, but also in cell migration, growth and differentiation [13].

Moreover ezrin can signal cell survival through the PI 3-kinase/Akt pathway. Another function of this protein is to maintain the link between adhesive molecules (e.g. E-cadherin) and cytoskeleton actin [16].

Overexpression of ezrin has been detected in several human neoplasms, including osteosarcoma, astrocytoma, uveal malignant melanoma or endometrioid carcinoma [8, 34].

However, the expression of ezrin and its role in development and progression of prostate cancer has been evaluated in few researches only [29, 35].

In this study we investigated the prognostic value of E-cadherin and ezrin expression in prostate cancer according to microscopic and clinical data. We compared the expression of these markers between normal prostate epithelium and prostate cancer in the same tissue sample.

Material and Methods

Human PC Tissue

123 formalin-fixed, paraffin embedded archival biopsy specimens were obtained from patient with prostate cancer, during the years 2001-2004 (from Kalisz administrative unit). The specimens with complete survival data were selected to our study.

The histological grade according to Gleason's score and other microscopic features (e.g. perineural infiltration, vascular invasion or mitotic index) were assessed on routinely stained (H&E) sections.

Immunohistochemistry

Paraffin sections were mounted onto SuperFrost slides, deparaffinized, then treated in a microwave oven in a solution of citrate buffer, pH 6.0 for 20 minutes and transferred to distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in distilled water for 5 minutes, and then sections were rinsed with Tris-buffered saline (TBS, DakoCytomation, Denmark) and incubated with mouse anti-human E-cadherin antibody (Dako Cytomation; clone: NCH-38), dilution: 1:100, monoclonal mouse anti-human Ezrin antibody (Upstate), dilution: 1:200. Afterwards EnVision+System-HRP (DakoCytomation, Denmark) prepared according to the instructions of the manufacturer were used. Visualisation was performed by incubating the sections in a solution of 3,3'-diaminobenzidine (DakoCytomation, Denmark). After washing, the sections were counter-stained with hematoxylin and coverslipped. For each antibody and for each sample a negative control was processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

Microscopic evaluation of immunostaining

Localization and intensity of E-cadherin and ezrin tissue expression was scored independently by two patholo-

gists blinded to result of grading and clinical outcome. Routine semiquantitative method was used to assess expression across the whole section. In 16 cases with discrepancy a consensus score was reached using a multiheaded microscope.

The intensity of E-cadherin and ezrin staining in prostate cancer was assessed as normal, decreased or increased in comparison with surrounding normal prostate epithelium.

In our study we also evaluate the pattern of E-cadherin staining. The staining uniformly positive pattern with strictly membranous expression in more than 70 % of cells was regarded as normal. Membranous staining in less than 70 % of cells or cytoplasmic staining was recognized as aberrant expression.

Positive apical membranous ezrin staining in more than 50 % of cells was regarded as normal expression pattern. Apical membranous ezrin binding in less than 50 % of cells or cytoplasmic staining was recognized as aberrant expression. Intensity of ezrin staining was evaluated in the same way as E-cadherin. The lack of E-cadherin and ezrin expression was recognized when less than 10 % of tumour cells showed membranous staining.

Statistical analysis

Prostate cancer-specific survival was calculated from the date of diagnosis to the date of death or last follow-up, and data for patients who died from causes other than prostate cancer were censored at the time of death. 2 by k Chi square test with trend was employed to test for compatibility between dichotomized values of ezrin and E-cadherin expression and values of other histological and clinical parameters. Cancer-specific survival was estimated using the Kaplan–Meier method. Differences in survival distributions were evaluated using a log-rank test. Multivariate survival analysis based on the Cox's proportional hazard model was used to test the independence of these parameters in the prediction of overall survival. $P < 0.05$ was considered significant.

Results

E-cadherin and ezrin expression

Uniform expression of E-cadherin and ezrin was localized to the cell membrane particularly at the intercellular junction in benign prostatic epithelium. Normal expression pattern of E-cadherin was shown in 84 (68%) of tumors. Aberrant E-cadherin expression pattern was evident in

39 of 123 (32%) tumors. 46 (37%) of tumors revealed anti-E-cadherin expression with intensity comparable to the surrounding normal prostatic epithelium. Decreased intensity of E-cadherin expression was found in 76 (62%) cases. None of tumors showed increased intensity of E-cadherin expression. Lack of staining was found in one case.

Normal ezrin expression pattern was evident in 80 (65%) of tumors. Abnormal ezrin expression pattern was revealed in 43 of 123 (35%) tumors. 64 (52%) of tumors were showed ezrin expression with intensity comparable to the surrounding normal prostate epithelium. Decreased intensity of ezrin expression was revealed in 16 (13%) cases. 43 of 123 (35%) tumors showed increased intensity of E-cadherin expression.

The positive correlation between E-cadherin and ezrin expression pattern in prostate cancer was highly significant. We also revealed negative correlation between intensity of E-cadherin and ezrin expression in cancerous tissue. Decreased intensity expression of E-cadherin was characteristic for cancer cells while increased ezrin expression were recognized in these specimens.

Correlation between E-cadherin and ezrin expression and Gleason score

In 68% of prostate cancer cases the staining pattern of E-cadherin was similar to this observed in nonmalignant prostatic tissue (Table 1). However, in moderate differentiated to poorly differentiated tumors (Gleason scores 5-10), we observed a trend of increasing percentage of tumors with aberrant staining for E-cadherin. Most of moderately differentiated tumors (Gleason score 5 and 7), exhibited the normal pattern of E-cadherin staining however, 8 of 79 (10%) of these tumors exhibited aberrant staining.

In contrast, the majority (86%) of poorly differentiated and undifferentiated tumors (Gleason score 8-10) had aberrant E-cadherin expression. Substantial fractions of tumor samples in these latter groups showed decreased of E-cadherin staining (86%). The decreasing and aber-

TABLE 1

Pattern of E-cadherin (*ezrin*) expression and tumor grade

	No.	Normal	Aberrant
All cases	123	84 (80)	39 (43)
Gleason score			
2-4	0	0 (0)	0 (0)
5-7	87	79(78)	8 (10)
8-10	36	5 (2)	31 (34)

TABLE 2

Intensity of E-cadherin (*ezrin*) expression and tumor grade

	No.	Absent	Decreased	Normal	Increased
All cases	123	1 (0)	76 (16)	46 (64)	0 (43)
Gleason score					
2-4	0	0 (0)	0 (0)	0 (0)	0 (0)
5-7	87	0 (0)	45 (16)	42 (61)	0 (10)
8-10	36	1 (0)	31 (0)	4 (3)	0 (33)

rant expression of E-cadherin with increasing Gleason score was highly statistically significant ($P < 0.001$ by Chi square test). A significant inverse correlation between ezrin expression and tumor differentiation was also found (Table 1 and 2). The majority (90%) of moderately differentiated (Gleason score, 5 to7) tumors showed normal ezrin expression. In contrast, 94 % of poorly differentiated (Gleason score, 8 to10) tumors showed aberrant ezrin expression ($P < 0.001$ by Chi square test).

Conversely than E-cadherin immunostaining, the intensity of ezrin expression was increased in most (92%) of poorly differentiated tumors compared to normal prostate tissue ($P < 0,002$ by Chi square test).

Correlation between E-cadherin and ezrin expression and patient survival

The aberrant staining of E-cadherin and ezrin was significantly related to patient survival. The survival time of patients with aberrant staining of ezrin and E-cadherin was significantly shorter than that of patients with a normal staining pattern. We observed that 60 % of cases with patient death showed aberrant expression of E-cadherin. Similarly the aberrant pattern of ezrin expression was detected in 64 % of cases with patient decease (Table 3 and 4).

These results were combined to analyze the effect on survival of each marker alone and in combination. Univariate survival analysis showed that aberrant E-cadherin expression in tumour cells was positively correlated with poor patient survival (Hazard Ratio = 4.30187, Exact Fisher - 95% CI = 1.786028 to 10.828609, two sided $P = 0.0004$) (Fig. 1). In log-rank test survival analysis, ezrin aberrant immunoreactivity also had an adverse effect on survival (Hazard Ratio = 5.042298 Exact Fisher - 95% CI = 2.07284 to 13.088308, two sided $P < 0.0001$) (Fig. 2). However, localization of ezrin stain had no independent significance on survival, when controlling for the Gleason score and E-cadherin expression in Cox multivariate survival analysis.

TABLE 3

Pattern of ezrin expression and patient survival

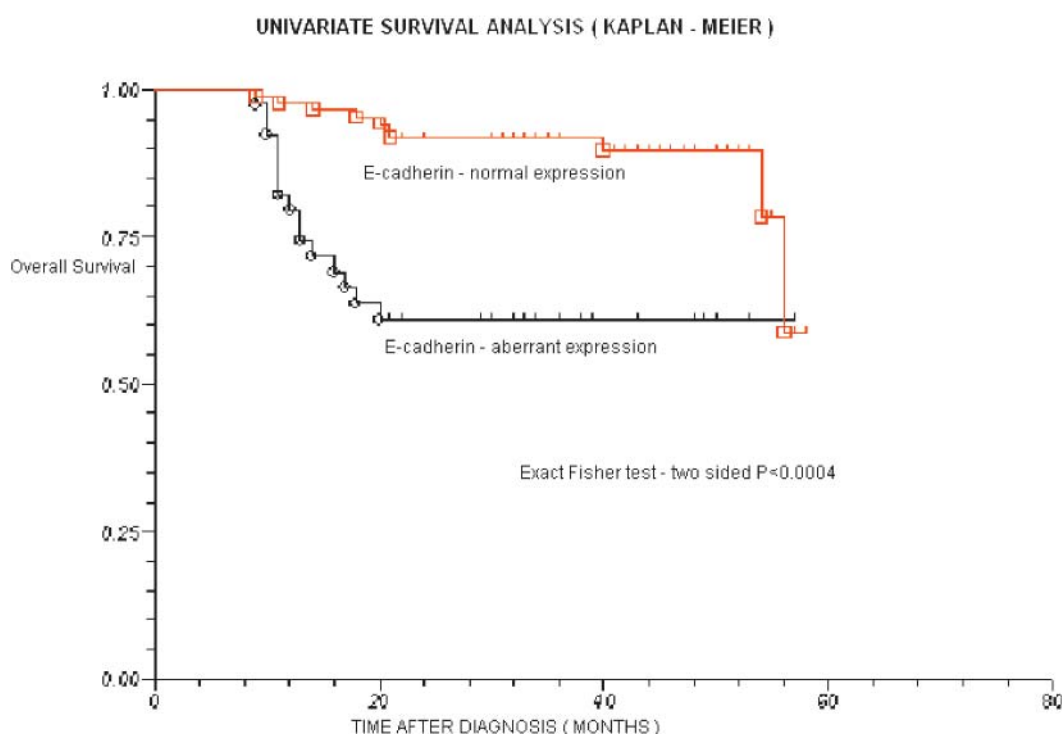
	No.	Normal	Aberrant
All cases	123	80	43
Death	25 (20%)	9 (11%)	16 (37%)
Live	98 (80%)	71 (89%)	27 (63%)

TABLE 4

Pattern of E-cadherin expression and patient survival

	No.	Normal	Aberrant
All cases	123	84	39
Death	25 (20%)	10 (12%)	15 (38%)
Live	98 (80%)	74 (88%)	24 (62%)

Fig. 1.



In Cox multivariate survival analysis, Gleason score ($P = 0.0118$) and E-cadherin expression intensity ($P = 0.0366$) had independent significance of survival. The ezrin expression intensity and localization and the E-cadherin aberrant staining did not have independent significance to survival when adjusted for the clinicopathological variables (Table 5).

Discussion

Most prostate cancer deaths are due to metastatic disease. Aspects of the molecular and cellular biology underlying the metastatic process are well described in the literature. The metastatic cascade is composed of a number of separate steps. Abnormalities are very important factors of metastatic disease in the prostate cancer. These abnormalities extend to intercellular adhesion molecules like E-cadherin and related molecules, e.g. ezrin [20]

The importance of biological function of E-cadherin and its role in human cancer was extensively studied. Reduction or loss of E-cadherin expression were well documented in tumors from various organs, including colon, stomach, pancreas, esophagus, breast, bladder and oral cavity [30]. There was also strong evidence indicating a pivotal role of abnormal expression of E-cadherin in progression of prostate cancer [7].

Altered ezrin expression contributes to many changes on the cell surface and intracellular signaling cascade that confer the metastatic capability on tumor cells [15]. Therefore, it is conceivable that ezrin overexpression and/or deregulation could contribute to the metastatic behavior of tumors [36].

In this study we evaluated the expression of E-cadherin and ezrin and its correlation with Gleason score and patient survival with prostate cancer.

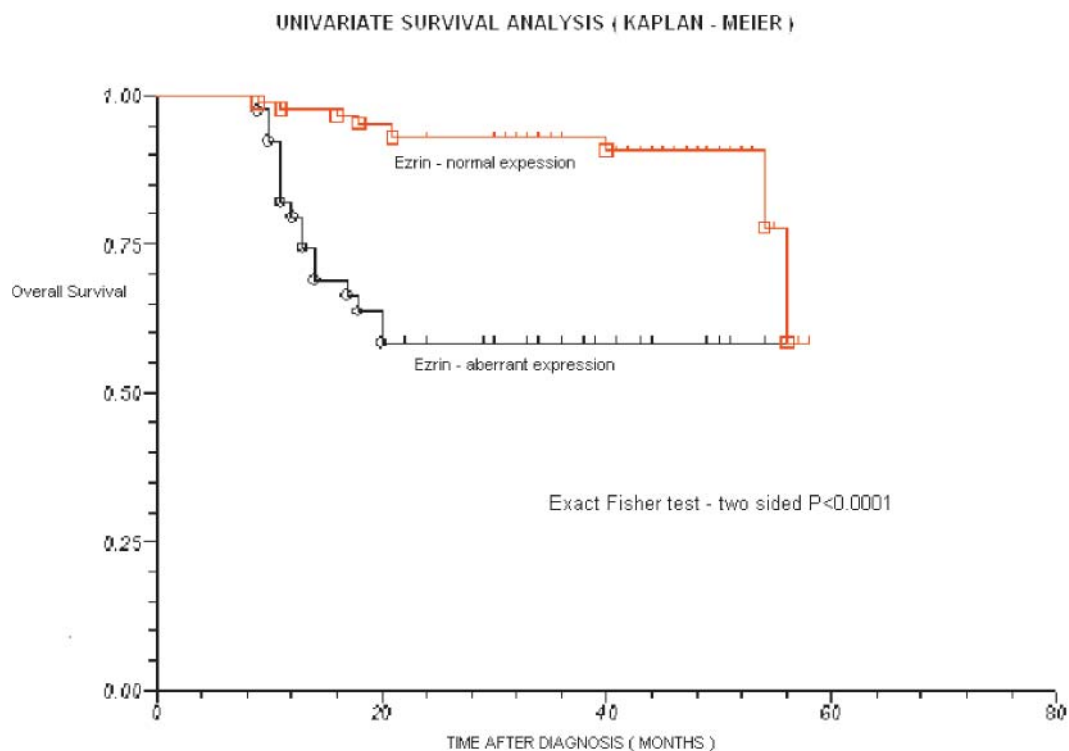
In the present study, almost every poorly differentiated tumor (Gleason 8-10) showed abnormal or decrease E-cad-

TABLE 5

Multivariate survival analysis (Cox proportional hazards regression)

	Risk Ratio	95% CI	P-value
AGE	0.94863	0.886327 to 1.015313	0.1281
GLEASON SCORE	3.186258	1.292702 to 7.853503	0.0118
PIN	1.328031	0.734029 to 2.402722	0.3483
PERINEURAL INVASION	2.731883	0.717385 to 10.403314	0.1407
ANGIOINVASION	0.663153	0.186582 to 2.356985	0.5255
INFLAMMATORY REACTION	1.379993	0.544292 to 3.49882	0.4974
NECROSIS	1.720549	0.306711 to 9.651724	0.5374
MITOTIC INDEX	0.984094	0.802151 to 1.207305	0.8778
CANCER VOLUME	0.992558	0.966717 to 1.019089	0.5789
MUSCLES INVASION	0.78003	0.242682 to 2.507173	0.6767
E-CADHERIN EXPRESSION			
Aberrant	0.23626	0.049263 to 1.133083	0.0713
Weak	5.65331	1.11417 to 28.68496	0.0366
EZRIN EXPRESSION			
Aberrant	0.504188	0.086564 to 2.936628	0.4462
Strong	1.032056	0.197326 to 5.397864	0.9702

Fig. 2.



herin expression compared to the normal prostate epithelium, which was also correlated to short average patient survival. These findings are comparable to the results of other studies [32, 34]. Umbas et al. evaluated immunohistochemically 89 prostate cancer specimens using specific antibodies raised against E-cadherin. The results were related to histological grade, tumor stage, presence of metastasis and survival. Patients with prostate cancer with low expression of E-cadherin have a shorter average survival time than patients with high level of E-cadherin expression [33]. De Marzo et al. found that reduced level of E-cadherin expression was correlated with advanced Gleason score and advanced pathologic stage (pTNM) in prostate cancer [10].

The prognostic value of reduce level of E-cadherin expression in prostate cancer was also suggested by Richmond [26]. Moreover, Rhodes et al. revealed that the low E-cadherin immunostaining was related to clinical recurrence [25].

However, Assikis et al. did not confirm these findings. They evaluated 16 clinically androgen-independent prostate cancers, characterized by regional progression without metastases and showed no evidence of E-cadherin down-regulation [2].

The mechanisms leading to dysfunction of E-cadherin mediated adhesion in human cancer are still unclear. It is well-known that E-cadherin gene can be considered as an invasion suppressor gene [6, 18, 24].

Therefore, some authors suggested that loss of heterozygosity of 16q22 (locus of E-cadherin gene) could be one of the step associated with the loss of suppressive function of this protein. This type of mutation was also detected frequently in metastasizing malignancies derived from the liver, prostate and breast [5].

Mutational inactivation of E-cadherin function has been observed in subset of gastric end colon carcinomas [3, 11]. Alternatively, the reduction of dosage of the gene due to allelic loss may result in the reduced expression of E-cadherin to a level below a critical threshold, which by itself could be sufficient to impair functioning of E-cadherin. Evidence for this hypothesis comes from the study of Vlemminckx et al, who showed that a reduction, not elimination, of E-cadherin expression was sufficient to induce the invasiveness of kidney carcinoma cells [37].

The other explanation of adhesion abnormalities mediated by E-cadherin is an impaired function of E-cadherin related molecules (catenins) anchored this protein to the cytoskeleton. Morton et al. found that, impaired E-cadherin function could be explained by homozygous deletion of α -catenin gene in the prostate cancer cell line [4, 22]. Increased tyrosine phosphorylation of β -catenin also can lead to dysfunction of E-cadherin-mediated adhesion [16]. This

phenomenon might explain the discrepancy between the normal E-cadherin expression in primary tumors and the presence of metastasis in the same time.

Identically, as in case of E-cadherin expression, we revealed a significant correlation between level of ezrin expression and Gleason score and time of survival in prostate cancer.

The intensity of ezrin expression was stronger in tumors with high Gleason score and it also inversely correlated with patient survival. Additionally aberrant expression pattern in ezrin immunostaining was also correlated with poor differentiation of cancer and patient higher mortality.

The exact mechanism by which ezrin contributes to tumor progression and dissemination is not fully understood [12, 17]. When ezrin and other ezrin-radixin-moesin proteins are activated by phosphorylation, they interact with membrane proteins and with the cytoskeleton actin and can thus affect processes such as migration, invasion, adhesion, and survival of the cell. All these processes are important for establishment and progression of cancer [31].

Recent studies have suggested a role for ezrin in the behavior of several nonepithelial tumor types. Immunohistochemical analysis demonstrated a significant correlation between ezrin immunoreactivity (IR) and the histological grade of astrocytomas [14]. Normal astrocytes and grade II astrocytomas showed weak ezrin IR, while the staining was increased in anaplastic astrocytomas and was strongest in malignant glioblastomas. The association between ezrin IR and malignancy was stronger than the correlation between the proliferation marker Ki-67/MIB-1 and malignancy. In uveal melanomas, an increased ezrin IR was associated with increased mortality [19]. In that study, strong ezrin IR correlated with high microvascular density, a known risk factor, but not with tumor size or melanoma cell type. Analysis of genes upregulated in metastatic murine osteosarcomas demonstrated a threefold increase of ezrin in tumors with high metastatic potential in comparison with tumors of low metastatic capacity [18].

So far, very little is known concerning ezrin expression in epithelial tumors. Cultured malignant cell lines almost invariably express ezrin abundantly. This may truly reflect the situation in vivo, as indicated by our study. Several studies have associated ezrin with features of epithelial tumors malignancy.

The switch of ezrin localization from the apical membrane to whole membrane or to the cytoplasm was correlated with dedifferentiation, and adverse features in invasive breast tumors. Ezrin expression in normal breast epithelium was localized at the apical, but not lateral, cell surface, whereas, in most breast tumor cases it was localized in the cytoplasm. There were significant positive associations

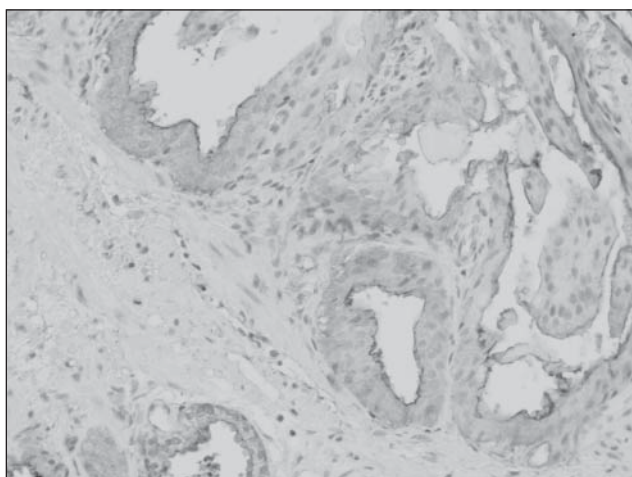


Fig. 3. Ezrin expression in normal prostate epithelium (10x).

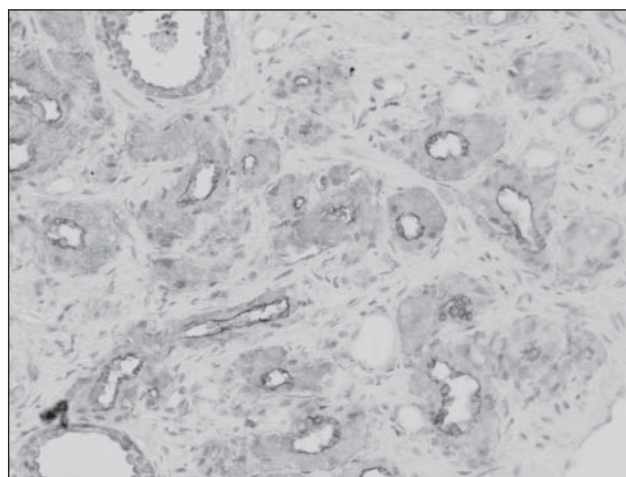


Fig. 4. Ezrin expression in prostate adenocarcinoma (**normal**) (10 x).

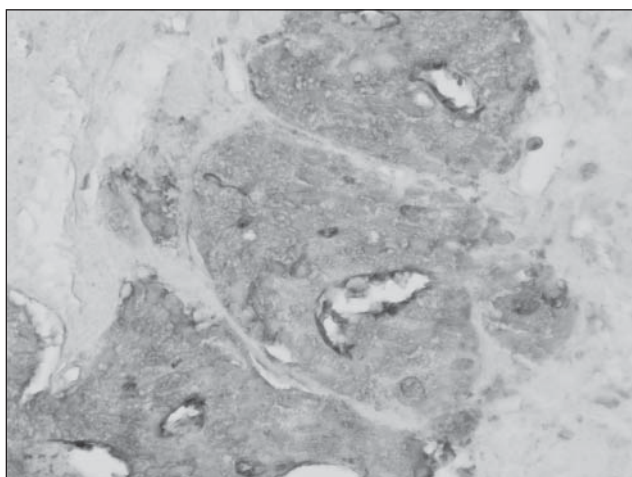


Fig. 5. Ezrin expression in prostate adenocarcinoma (**aberrant**) (20x).

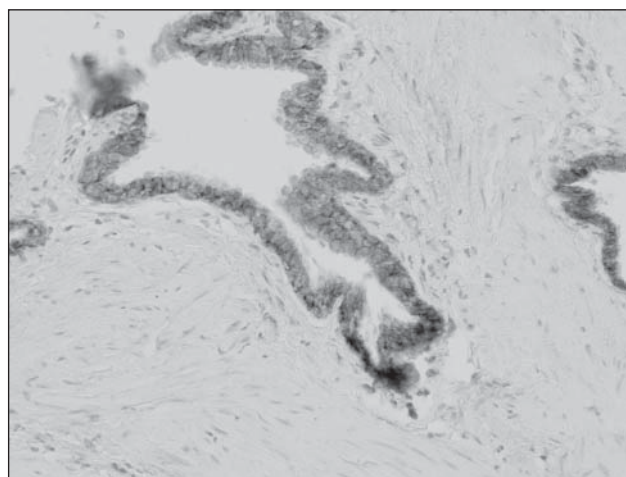


Fig. 6. E-cadherin expression in normal prostate epithelium (10x).

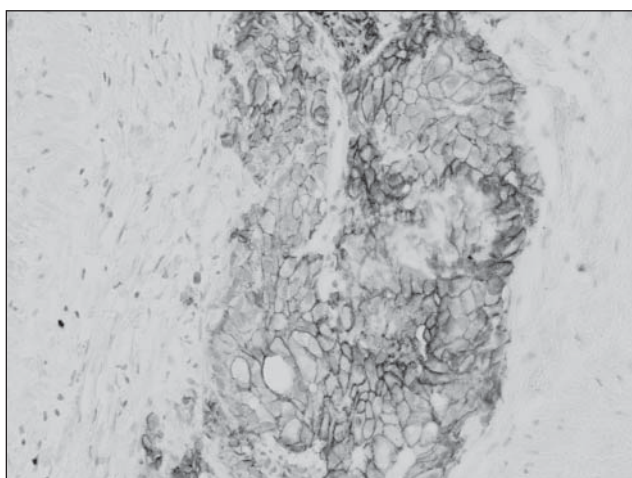


Fig. 7. E-cadherin expression in prostate adenocarcinoma (**normal**) (20x).

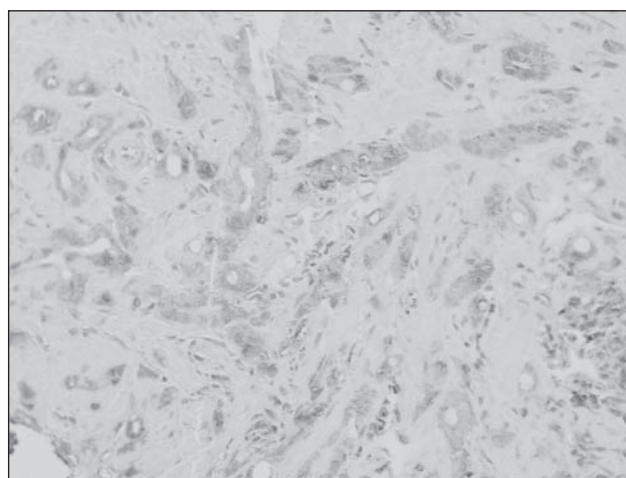


Fig. 8. E-cadherin expression in prostate adenocarcinoma (**aberrant**) (20x).

between cytoplasmic ezrin localization and adverse tumor characteristics such as high grade, high level of Ki-67 expression, hormonal-receptor negativity and lymph-node metastases. On the other hand apical ezrin staining was associated with favorable clinicopathological features and lymph node-negative tumors [28].

In a study with uterine endometrioid adenocarcinoma, the ezrin protein level and cellular location of this protein was compared with normal endometrium and endometrial hyperplasia by Ohtani et al. Protein level was found to be significantly higher in cancerous than normal tissues but in contrast to our study it was revealed that in non-tumour tissues, ezrin expression was mainly seen in the cytosolic fraction, whereas it was detected in both membrane and cytosolic fractions in tumour tissues [23].

Akisawa et al. investigated expression of ezrin in pancreatic adenocarcinoma cell lines of different metastatic potential. Among 16 pancreatic adenocarcinoma cell lines, several cell lines showed strong expression of ezrin. Two cell lines with high metastatic potential showed very high levels of ezrin mRNA and protein [1].

In contrast with our study, negative or weak ezrin immunoreactivity in serous ovarian carcinoma correlates with poor patient outcome [21]. Also in human colon cancer, ezrin was found to be expressed at a lower level compared with normal tissues. However, the most striking finding of this study is the difference of ezrin location in cells. The cell membrane expression is characteristic for normal colon mucosa but became cytosolic in tumour tissues [16].

All these studies raise the possibility that altered ezrin expression could modulate the behavior of malignant epithelial neoplasms. Our results are in concordance with studies showing a gradual increase in ezrin expression following tumor dedifferentiation and poor clinical outcome. However, there is one study in which ezrin expression would have been systematically analyzed or correlated with prognostic and clinical parameters in prostate carcinomas [35]. Unfortunately, no reasonable explanation of ezrin overexpression in high-grade prostate cancer was given in this study. Therefore, the reason for the increased ezrin expression in dedifferentiated prostatic carcinomas is currently not known.

It is a common feature for malignant cells to gain DNA mutations during dedifferentiation. In our opinion, this may be the one reason for up-regulating ezrin gene expression in high-grade prostate cancer. There is another possible explanation for a specific correlation between ezrin expression and tumor grade in prostate malignancies. Ezrin is a morphogenic protein and functionally active ezrin is involved in the maintenance of epithelial cell polarity and in tubulogenesis [36]. Therefore, one can speculate

that overexpression of mutated ezrin protein could result in impaired cell polarity and, thus, higher histological grade. However, in study performed by See-Tong et al, FISH analysis of the ezrin gene was unremarkable, suggesting a lack of mutations and an increase in the copy number of the gene in prostate cancer [29]. Thereby it cannot account for the increased ezrin expression in this neoplasm. This discrepancy indicates that additional molecular studies on a larger series of prostate cancer specimens are warranted to explain our presumption.

Conclusions

The results of the current study suggest that E-cadherin is involved in prostate cancer tumorigenesis and progression. The aberrant or decreased expression of E-cadherin seems to be one of most promising markers of poor prognosis in localized prostate cancer. Our study also supports a role of ezrin in progression in human prostate cancers. Additional studies are mandatory to validate ezrin as a marker of cancer progression and thus it maybe as a potential target for cancer therapy in the future.

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