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Ezrin Intracellular Cytoskeleton Marker is Over Expressed in Pancreatic Ductal Adenocarcinoma: A Prospective Cohort Study

Livia Petrusel[1], Ioana Rusu[2], Ramona Suharoschi[3], Andrada Seicean[1], Daniel Corneliu Leucuta[4] and Radu Seicean[5]*

[1]Department of Gastroenterology, Iuliu Hatieganu University of Medicine and Pharmacy, Romania

[2]Department of Pathology, Iuliu Hatieganu University of Medicine and Pharmacy, Romania

[3]Department of Food Science and Technology, University of Agricultural Sciences and Veterinary Medicine, Romania

[4]Department of Medical Informatics and Biostatistics, Iuliu Hatieganu University of Medicine and Pharmacy, Romania

[5]First Surgery Clinic, Iuliu Hatieganu University of Medicine and Pharmacy, Romania

Abstract

Background: Intracellular cytoskeleton in Pancreatic Ductal Adenocarcinoma (PDAC) might be a key factor in its poor outcome. Reliable biomarkers estimating the cytoskeleton involvement are lacking. Ezrin is involved in intracellular signaling and adhesion, by linking in the PI3K/Akt pathways.

Aim of the Study: To assess the significance of ezrin protein expression in PDAC related to the clinical stage and survival.

Methods: This prospective cohort study enrolled patients with proven adenocarcinoma and a matched group of controls without any malignancies. The plasma levels of ezrin were analyzed using western blotting and were correlated with the clinicopathological features and survival data. These results were validated by immunohistochemical analyses of the pancreatic tumor tissue of the patients included in the study and a supplementary group of surgically resected specimens from patients with a benign disease.

Results: The study comprised 51 patients with PDAC, 53 controls and a supplementary group of 13 normal pancreatic tissue samples. EZR was over expressed more frequently in the plasma of patients with PDAC than in the controls (80% vs. 32%, P<0.001). EZR was detected in the fine needle aspiration tumor tissue by immunohistochemistry and it was not significantly correlated with its plasma expression. The EZR protein expression was closely related to the advanced clinical stage (P=0.02), and the risk of metastasis was five times higher (P=0.048) and with no influence on survival.

Conclusion: Ezrin pathway as an intracellular cytoskeleton biomarker is related to the local spread and metastasis of PDAC, but not in the survival.

Keywords: Pancreatic adenocarcinoma; Ezrin; Biomarker; Cytoskeletal; Survival; Metastasis

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Correspondence:

Daniel Corneliu Leucuta, Department of Medical Informatics and Biostatistics, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, 6 Pasteur Street, Cluj-Napoca 400012, Romania, E-mail: Romania.danny.ldc@gmail.com

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Introduction

Pancreatic cancer is the fourth most common cause of cancer death. More than half (53%) of patients are diagnosed at an advanced tumor stage, with a 5-year survival rate less than 6% [1]. This is related to rapid metastatic potential and chemoresistance [2]. The stromal environment around the cell tumor, comprised by extracellular matrix, fibroblasts, endothelial cells and immune cells, plays a significant role in activating cell growth.

Ligands such as Ezrin (EZR) relate the cell membrane to the actin cytoskeleton through regulating adhesion molecules and signal transduction [3,4]. It participates in the regulation of cell shape, adhesion, motility, and apoptosis, in correlation with the invasion and metastasis in different cancers [5,6].

Ezrin is over expressed in many cancers, including Pancreatic Ductal Adenocarcinoma (PDAC) [7].

The level of anti-ezrin auto antibodies was significantly higher in pre-diagnostic serum samples from PDAC cases compared to matched controls [8]. Also, an increased level of ezrin expression has been found to be positively associated with malignancy and metastasis, being an indicator of a poor prognosis [9,10].

The aim of our study is to assess the significance of ezrin protein expression in PDAC related to the clinical stage and survival.

Materials and Methods

Study design and setting

Data from patients diagnosed with pancreatic cancer between January 2016 and June 2017 were collected prospectively. Patients were enrolled from the "O. Fodor" Regional Institute of Gastroenterology and Hepatology in Cluj-Napoca, a tertiary regional referral hospital in Romania.

Participants

Subjects of the study group were at least 18 years old, with no previous history of any other cancer in the last five years.

The diagnosis of all pancreatic cancers was verified by histology after fine-needle aspiration biopsy during Endoscopic Ultrasonography (EUS) or surgery. All subjects gave informed consent before being interviewed. Patients with an unclear pathological diagnosis for pancreatic adenocarcinoma were excluded.

The subjects of the control groups were healthy people who were at least 18 years old, with no previous history of any cancer or other chronic diseases. For the most part, controls were matched to cases for sex and age (plus/minus five years).

The study was approved by the Ethics Committee of the hospital (No. 11387).

Data collection

We collected information regarding demographic data, diagnosis, staging, therapy and survival. Demographic data included age and gender of patients.

Cancer-related data included the date of diagnosis, extension of the disease, location of the primary tumor, histological type and the level of CA 19-9 at the time of diagnosis.

Diagnosis and staging of pancreatic cancer were based on imaging tests including Computer Tomography (CT) and Endoscopic Ultrasonography (EUS). A primary resectable tumor was distinguished between locally advanced and metastatic disease.

Survival was defined as the number of months between the date of diagnosis and date of death. The date of diagnosis was defined as the time from the first imaging modality (CT, MRI or EUS) giving the diagnosis of pancreatic cancer.

Blood sampling

Blood samples were collected at the time of diagnosis. Peripheral venous blood was collected into a tube containing Ethylenediaminetetraacetic Acid (EDTA) and was prepared by centrifugation at 5000 × g for 5 min. The plasma samples were stored at - 80°C until use.

The selected protein was quantified from plasma using western blot analyses.

Western blotting analysis

Protein concentration was determined using a protein assay kit - Quick Start™ Bradford Protein Assay (Bio Rad Laboratories, Inc.). A 60 µg of total protein from each plasma sample was loaded per lane onto a 5/12% polyacrylamide gel. Electrophoresis was performed at 100 mV and then the protein fractions were electrotransferred onto a nitrocellulose membrane at 100 mV for one h. The membranes were blocked during three h with 5% non-fat dry milk powder (Bio Rad Laboratories, Inc.) in Tris buffered saline containing 0.1% Tween 20 (TBS T), and 1% BSA under constant agitation at room temperature. Subsequently, the membranes were incubated overnight at 4°C with polyclonal anti-rabbit ezrin IgG antibody (HPA 021616, Sigma-Aldrich) diluted 1:250 in TBST, with nonfat dry milk powder.

For the loading control, a rabbit polyclonal antibody to GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) (cat. no. ab37168 AbCam) was used, at a concentration of 0.8 µg/ml in non-fat dry milk powder in TBS T.

Membranes were washed with TBST and incubated at room temperature for one h with a Horseradish Peroxidase (HRP) conjugated goat anti-rabbit IgG H.L antibody (cat. no. ab97051, Abcam) diluted 10,000 fold in TBST with an additional washing step performed prior to detection. For GAPDH determination, a HRP conjugated goat IgG anti rabbit IgG antibody (cat. no. ab97051, Abcam) diluted 10,000 fold in TBST was used.

Total ezrin expressions were normalized by dividing the ezrin units by those for GAPDH for each band.

The proteins were detected by the enhanced chemiluminescence system (Bio Rad Laboratories, Inc.). All immunoblottings were separately developed using a clarity western ECL substrate kit (Bio Rad Laboratories, Inc.), the membranes were exposed to the ChemiDoc imaging system (Bio Rad Laboratories, Inc.) and analyzed using Image Lab Software version 5.2.1 for Windows (Bio Rad Laboratories, Inc.).

Measurement and confirmation of the EZR protein levels are often performed with normalization against "housekeeping proteins", such as Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), to correct for protein loading and other factors, such as transfer efficiency.

For protein expression, after densitometry, the Integrated Density Value (IDV) for each protein band (EZR) was determined and normalized levels of EZR calculated by dividing the IDV of a protein band by the IDV of the GAPDH (arbitrarily assigned a value of 100) within the same sample, thus quantifying the expression of the proteins as high or low-expressed.

Tissue samples

Tumor tissue samples from endoscopic ultrasound-fine needle aspiration and surgery were fixed with 10% formalin for pathology studies.

In addition, there was a supplementary group of 13 samples containing normal pancreatic tissues from patients who received partial pancreatectomy for benign tumors that were used as normal controls for the immunohistochemical interpretation.

Immunohistochemistry

The expression of s proteins was determined by immunohistochemistry. This analysis was performed with the pancreatic tumor and normal tissue samples fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 1-3 microns

depending on cell tissue density.

The primary antibody used was rabbit polyclonal to Anti-Ezrin IgG antibody (HPA 021616, Sigma-Aldrich) diluted 1:150. The BOND-III staining instrument (Leica Biosystems) and Bond Polymer Refine Detection Kit (Leica Biosystems) were used for the antibody.

All slides were scored by one pathologist (I.R.) who was blinded to all clinical data. Finally, the tissue slides were evaluated under a microscope. The intensity of staining was scored as negative, weak, moderate, or strong (score 0, 1, 2, or 3).

Statistical analysis

Qualitative data were expressed as counts and percentages. Continuous data were presented as means and standard deviations if they followed the normal distribution; otherwise it was presented as medians and quartiles. The differences between the two groups regarding qualitative data were examined with the Chi square test or the Fisher exact test, where appropriate. Continuous data that followed the normal distribution were evaluated with the t-test for independent samples or the Wilcoxon rank-sum test. We assessed the predictors involved in the prediction of metastases, in an attempt to explore the role of ezrin. We built univariate logistic regression models with known predictors of metastases, as well as with ezrin expression, and ezrin immunohistochemistry (both as a binary or ordinal variable). Next, we adjusted ezrin expression incrementally with important known predictors in several models, but maintained a reduced number of variables to avoid over fitting. For all the multivariate models, we checked for multicollinearity with the variance inflation factor. For all logistic regression models, we checked the goodness of fit and misspecification (Stukel test and Osius-Rojek test), and we presented the odds ratios along with 95% confidence intervals and p-values.

Associations between survival and different characteristics were explored using Cox proportional hazard regressions. Univariate models were built first, and then a multivariate model was created for ezrin expression (high-expressed *vs.* low-expressed), adjusted for characteristics known to influence survival: Age (years), T4 *vs.* T1-3, N1 *vs.* N0, and metastases (yes *vs.* no). The models were checked for assumptions and they were met (proportional hazard assumption checked by Schoenfeld residuals and a formal test, multicollinearity in a multivariable model using the variance inflation factor, and the correct functional form checked with penalized smoothing splines). For all Cox regressions the hazard ratio along with a 95% confidence interval was presented.

For all statistical tests we used the two-tailed p-values, and a level of significance of 0.05. All analyses were performed in the R environment for statistical computing and graphics (R Foundation for Statistical Computing, Vienna, Austria), version 3.6.1.

Results

Patients characteristics

There were 104 patients included in the study, 51 with adenocarcinoma and 53 healthy controls. Mean age of the population was 63.42 years (SD 11.24, range: 27 to 87 years). There were more males than females (61% *vs.* 39%) (Table 1). The controls had a higher BMI than PDAC patients (Table 1).

Expression of plasma and tissue EZR in pancreatic cancer

EZR in plasma was expressed in 41 (80.39%) PDAC patients compared to 17 (32.08%) controls (p<0.001) (Figure 1). Immunohistochemistry in PDAC tissue was performed in 37 (73%) patients and the expression was weak in 8 (21.62%), moderate in 6 (16.22%), strong in 20 (54.05%) and negative in 3 (8.11%) patients. The ezrin expression in the tissue from the supplementary controls was weak in 6 (46.2%), moderate in 6 (46.2%), and negative in 1 (7.7%) with a statistically significant difference compared to the PDAC tissue (p=0.001) (Figure 2).

Table 1: Patients characteristics.

		PDAC (n=51, 49%)	Control (n=53, 51%)	p value
Age (years), mean (SD)		64.53 (9.74)	62.35 (12.52)	0.5
Gender (female), n (%)		19 (37.25)	22 (41.51)	0.657
BMI (kg/m^2), mean (SD)		24.5 (4.2)	26.3 (4.77)	0.045
Weight status, n (%)	Underweight	7 (13.7)	2(3.8)	0.247
	Normal	13 (25)	10 (19)	
	Overweight	26 (51)	30 (56.6)	
	Obese	5 (9.8)	11 (20.8)	
Smoking, n (%)		19 (37.3)	26 (49.1)	0.3
Location				
Pancreatic head, n (%)		28 (46%)		
Pancreatic body, n (%)		16 (27%)		
Pancreatic isthmus, n (%)		10 (17%)		
Pancreatic tail, n (%)		5 (8%)		
Uncinated process, n (%)		1 (2%)		
New onset diabetes, n (%)		13 (25.49)	1 (1.89)	<0.001
Long-term diabetes, n (%)		8 (15.7)	14 (26.4)	0.2
Diabetes, n (%)		22 (43.1)	15 (28.3)	0.1
CA 19-9 (U/ml), median (IQR)		400 (332.05)	59.1 (242.17)	0.018
T stage, n (%)		1-2: 5 (9.8)		
		3: 26 (51)		
		4: 29 (56.9)		
Histological grade, n (%)		G1: 2/20 (10)		
		G2: 14/20 (70)		
		G3: 4/20 (20)		
N stage n (%)		45 (88.2)		
Metastasis n (%)		21 (41.2)		

PDAC: Pancreatic Ductal Adenocarcinomas; BMI: Body Mass Index; CA 19-9: Carbohydrate Antigen 19-9; SD: Standard Deviation; IQR: Interquartile Range; CI: Confidence Interval

EZR was detected in the tumor tissue by immunohistochemistry and it was not significantly correlated with its plasma expression (p=0.182).

Relationships of EZR expression with clinicopathological features in pancreatic cancer

The plasma level of EZR was highly expressed in advanced T tumor stage (29 patients- 70.7%) (p=0.02) and in non-smokers (12 patients- 29.3%) (p=0.028) (Table 2).

Risk of metastases in PDAC and EZR expression

Metastases were present in 21 of 51 patients with PDAC. In the univariate model, the over expressed EZR increased the risk of metastasis (odds ratio =5.15, p=0.046) (Table 3).

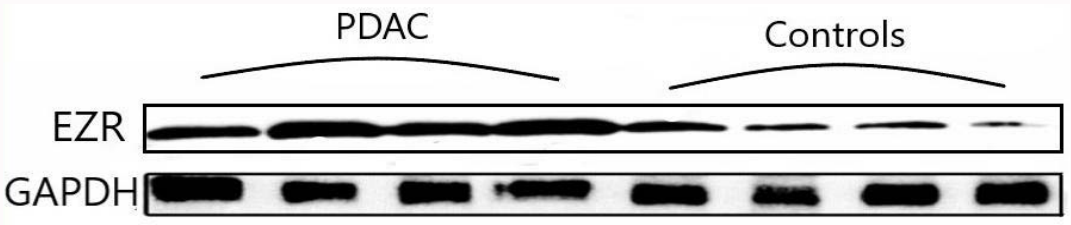

Figure 1: Western blot analyses of EZR in patients with PDAC and controls. GAPDH was used as an internal control.

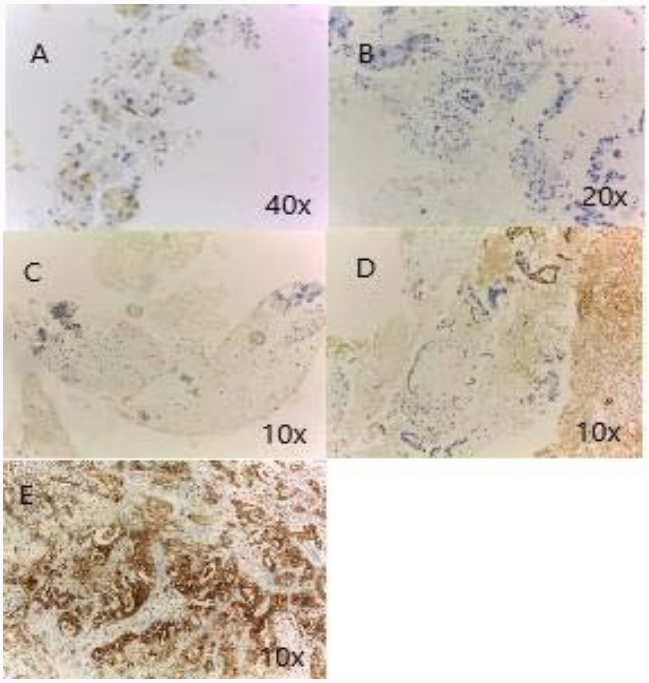

Figure 2: Immunohistochemical staining of Ezrin in PDAC tumor and normal pancreatic tissue. (A) Negative Ezrin staining in normal pancreatic tissue. (B) Negative Ezrin staining in pancreatic tumor tissue. (C) Weak Ezrin expression in pancreatic tumor tissue. (D) Moderate Ezrin expression in pancreatic tumor tissue. (E) Strong Ezrin expression in pancreatic tumor tissue.

In the multivariate model, from the possible risk factors, the plasma EZR association with lymph nodes was significant for metastasis (p=0.048) (Table 3).

Survival in PDAC patients

Although the univariate analysis was a significant factor influencing survival, it lost the independent role in the multivariate Cox proportional survival analysis in favor of age (HR=1.07, p=0.005) and metastasis (HR=2.06, p=0.042) (Table 4).

Discussion

In the present study, we found that the plasmatic level of ezrin was correlated with the clinicopathological status and advanced age of the patients, but not with the prognosis of patients with PDAC.

Serum EZR level in PDAC was higher compared with controls (80% *vs.* 32%) (p<0.001) (Figure 1) and the immunohistochemistry was positive in 73% of patients, most of whom had a high level of expression. The discordance between the plasma level and the tissue immunohistochemistry might be explained by the use of fine needle aspiration samples for tissue analysis when inconclusive results or lower expression compared to surgical specimens may occur,

Table 2: Protein plasma expression in adenocarcinoma patients and interaction with clinic- biological parameters.

		Ezrin		
Protein expression		Low (n=10)	High (n=41)	P
Age (years), mean (SD)		58.9 (9.32)	65.9 (9.45)	0.04
BMI (kg/m^2), mean (SD)		25.16 (4.05)	24.33 (4.27)	0.5
CA 19-9 (U/ml), median (IQR)		400 (349.18)	400 (336.4)	0.8
Gender (female), n (%)		2 (20)	17 (41.5)	0.287
Stage (III-IV *vs.* I-II), n (%)		3 (30)	29 (70.7)	0.02
Histological grade, n (%)	G1	0/6 (0)	2/14 (14.3)	0.5
	G2	5/6 (83.3)	9/14 (64.3)	
	G3	1/6 (16.7)	3/14 (21.4)	
Metastasis, n (%)		2 (20)	19 (46.3)	0.167
Tumor size >= 3 cm, n (%)		6 (60)	32 (72)	0.253
Smoking, n (%)		7 (70)	12 (29.3)	0.028
Yes		3(30)	29 (70.7)	
No				
New onset diabetes, n (%)		4 (40)	9 (22)	0.253
Long-term diabetes, n (%)		0 (0)	8 (19.5)	0.329
Diabetes, n (%)		4 (40)	18 (43.9)	1
IHC, n (%)		1/8 (12.5)	2/29 (6.9)	0.1
		0/8 (0)	8/29 (27.6)	
		3/8 (37.5)	3/29 (10.3)	
		4/8 (50)	16/29 (55.2)	

BMI: Body Mass Index; CA 19-9: Carbohydrate Antigen 19-9; IHC: Immunohistochemistry; SD: Standard Deviation; IQR: Interquartile Range; CI: Confidence Interval

as proved for other molecules [11]. Results from the literature are conflicting, some authors suggesting its involvement in early diagnosis [8], while others reported negative findings in the plasma of patients with a risk for pancreatic cancer [12] or with PDAC [13].

Similar to our findings, a higher positivity of tissue EZR expression in PDAC (82.1%) compared with the adjacent non-tumor tissues (37.8%) (P=0.01) and normal pancreas tissue (19.0%) was proved in a study of 106 patients with PDAC [5]. However, for our group the discrepancy between cancer and controls was more evident for strong IHC expression (54% *vs.* 0%).

A high level of EZR in plasma was more frequently observed in elderly patients (65.9 *vs.* 58.9 years old for the low level of EZR) and in patients with tumor stage III to IV (70% with high expression compared to 30% for low expression EZR). Other authors reported that the positive expression of EZR correlated with more clinic-demographic factors such as age, tumor size, location, differentiation

Table 3: The risk factors associated with metastasis.

Univariate analysis	OR unadjusted	95% CI	*P* value
Age	0.99	0.94-1.05	0.819
Age >50 yr	0.54	0.06-4.82	0.557
Sex (male *vs.* female)	0.96	0.31-3.1	0.944
Tumor size ≥ 3 cm	3.24	0.97-12.96	0.069
N1	3.75	0.46-62.2	0.314
T4	1.96	0.67-5.93	0.226
EZR expression (high-expressed *vs.* low-expressed)			
Plasma level (high *vs.* low)	5.15	1.22-35.53	0.046
IHC Tissue level (strong *vs.* negative)	1.8	0.15-42.7	0.653
Multivariate analysis - OR for Ezrin			
Tumor size >= 3 cm + Ezrin plasma expression	4.02	(0.89-28.56)	0.099
Tumor size >= 3 cm + N1 + Ezrin plasma expression	4.11	(0.91-29.28)	0.095
T4 *vs.* T1,2,3 + Ezrin plasma expression	4.57	(1.03-32.61)	0.071
N1 *vs.* N0 + Ezrin plasma expression	5.09	(1.2-35.31)	0.048
T4 + N1 + Ezrin plasma expression	4.69	(1.04-33.66)	0.068

OR: Odds Ratio; CI: Confidence Interval; IHC: Immunohistochemistry

Table 4: The hazard ratio associated with survival.

	Univariate analyses			Multivariate analyses		
	HR	(95% CI)	p	HR	(95% CI)	p
Age (years)	1.1	1.02-1.1	0	1.1	1.02-1.12	0.005
Age>50 years	2.7	0.64-11.1	0.18			
Sex (male vs. female)	0.8	0.45-1.57	0.59			
N1	1.3	0.47- 3.71	0.6	0.7	0.19-2.45	0.562
Tumor size >=3cm	2.1	1-4.48	0.05			
T4	1.9	1.04-3.55	0.04	1.4	0.34-5.54	0.663
Metastases	2.1	1.15-3.94	0.02	2.1	1.03-4.12	0.042
Ezrin expression (high-expressed vs. low-expressed)	3.1	1.19-7.95	0.02	1.2	0.4-3.45	0.772

HR: Hazard Ratio; CI: Confidence Interval; *: Adjusted for age; N1 *vs.* N0; tumor size >= 3 cm, and metastases

stage, depth of invasion, vessel invasion, lymph node, distant metastasis, and TNM stage [14,15].

This suggests again a role of EZR in tumor invasion in PDAC, especially based on the contribution of stroma because EZR, a member of the Ezrin-Radixin-Moesin (ERM) family participates in multiple cellular processes as a cytoskeleton protein [16-18]. Previous studies sustain its role in tumor cell migration, morphogenesis, adhesion, apoptosis, cancer stem cell differentiation chemoresistance [19-22]. Although initially it was considered that EZR could interact with podocalyxin for enabling the transition of cancer cells from a non-polarized, rounded cell morphology, to an invasive extravasation-competent shape of pancreatic cancer cells (15c) [23], this was not confirmed by a subsequent study [24]. It is believed that surface membrane EZR may be involved in several signaling pathways. It binds adhesion molecules such as CD43, CD44, intercellular adhesion molecule-1 and 2 [25-27] and works downstream of cell surface receptors through the activation of Rho and PI3K/Akt signaling pathways [28,29]. Other findings related to tumor invasion in PDAC sustained that EZR links to cortactin at the level of an acting- binding domain, which modifies the cytoskeleton of cancer cells as an adaptive process to the substrate from the stromal environment in PDAC [30].

Besides its presence in pancreatic cancer, EZR is also over expressed in other cancers, colorectal [4], scuamous esophageal [31], endometrial [32], ovarian [21], breast [20], melanoma [10] and even in PanIN-type precancerous lesions [7,33,34]. Also, other activated pathways by EZR have been proved in colon cancer based on the interaction with neural cell adhesion molecule L1 and regulation of the NF-κB signaling pathway [35], while depletion of EZR down-regulated the Mitogen-Activated Protein Kinase (MAPK) and transforming growth factor-β pathways in esophageal squamous cell carcinoma [36].

Also, it is known that EZR is a substrate for tyrosine kinase, serving in intracellular signal transduction related to cell migration and metastasis [27,28,37]. Its role in metastasis is sustained by recent in vitro experiments [38]; also we found a direct relationship between the plasma level and the risk of metastasis in our patients (Table 3). The mechanism of metastasis was reported as an activation of the ERK/MAPK pathway in osteosarcoma [39], or increasing Akt and ERK1/2 activity in breast and cervical carcinoma [40,41].

In order to check how ezrin predicts metastasis, we built several logistic regression models including ezrin IHC and ezrin expression. The univariate models with ezrin IHC were not statistically significant, for both the binary as well as the ordinal variants of the variable. Since ezrin plasma expression in the univariate model had statistically

significant very high odds to predict metastasis (Table 3), and also this relation holds after adjustment for the N stage, EZR expression might be an important independent predictor of metastasis. Nevertheless, adjusting for other important predictors of metastasis, the relation lost its statistical significance (although close to the limit of significance), thus, this hypothesis should be further explored in larger studies. Our study could not control for more confounders due to the risk of over fitting.

The univariate analysis showed a significant influence on the survival of EZR expressions, but its independent value was lost in the multivariate analysis in favor of the presence of metastasis (p=0.042) and advanced age (p=0.005) (Table 4), so probably there is a relation between the EZR expression and metastasis at the microscopic level which influences the survival rate. Limited data on prognostic PDAC and EZR expression exists; some (group of 69 patients) are similar with our results [8], others showed a significant hazard ratio for the survival of 2.16 (group of 106 patients) [5].

There are several limitations to our study. First, there were a limited number of patients included which can influence the lack of association between the plasma and tissue expression. Second, the clinicopathological associations were made based on the expression of plasma proteins rather than in tissue. Third, the immunohistochemistry for patients was done on fine-needle aspiration samples which can lead to inexact results compared to the surgical specimens and this was the reason for avoiding the analysis based on these results.

Further larger studies are therefore warranted to explore the mechanism of EZR function and to investigate its potential as therapeutic targets in PDAC progression.

In conclusion, the Ezrin pathway could be a potential effective biomarker related to the local spread of PDAC and metastasis, and it might be a novel therapeutic marker for differentiating benign pancreatic nodules from adenocarcinoma.

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