Int J Clin Exp Pathol 2018;11(1):248-254
www.ijcep.com /ISSN:1936-2625/IJCEP0065456

Original Article

Detecting the location and significance of Ezrin protein expression in hepatocellular carcinoma

Yan Wang[1*], Shaochen Jiang[2*], Zhongliang Cao[3], Chunxiao Xie[5], Weidong Li[6], Yibing Ma[4], Jinhui Zhang[1], Lijuan Lin[1]

[1]Institute of Molecular Medicine, Medical College of Eastern Liaoning University, Dandong 118003, China; [2]Liaoning Coloproctological Hospital, The Third Affiliated Hospital of Liaoning University of Traditional Medicine, Shenyang 110000, China; Departments of [3]General Surgery, [4]Pathology, Dandong Center Hospital, Dandong 118002, China; Departments of [5]Emergency, [6]Anesthesiology, Dandong First Hospital, Dandong 118001, China. [*]Equal contributors.

Received September 12, 2017; Accepted December 7, 2017; Epub January 1, 2018; Published January 15, 2018

Abstract: To explore the characteristics of localization and prognostic implication of Ezrin expression in HCC, 92 cases of HCC meeting strict follow-up criteria were selected for immunohistochemical staining of Ezrin protein. Correlations between Ezrin expression and clinicopathological features of HCC were evaluated using Chi-square tests, survival rates were calculated using the Kaplan-Meier method, and the relationship between prognostic factors and patient overallsurvival was analyzed using Cox proportional hazard analysis. In results, Ezrin protein was mainly expressed in the inner side of the cell membrane of the adjacent non tumor tissues, and diffusely expressed in cytoplasm of HCC tissues. There was an obviously difference between the benign and malignant tissues about the location of Ezrin expression. Ezrin strong-expression rates were significantly higher in HCC samples compared with the adjacent non tumor tissues ($P<0.05$). The Ezrin strong-expression rate was closely related with the differentiation ($P<0.01$), AJCC stage and metastasis of HCC ($P<0.05$, respectively). Therefore, the sub-cellular localization of Ezrin protein in the liver cells will be changed in the process of the transformation from the benign to malignant, and Ezrin plays an important role in the progression of HCC.

Keywords: Ezrin, localization, survival, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer and the third leading cause of cancer-related death worldwide. More than 75% of these cases occur in the Asia-Pacific region and China alone accounts for 55% cases of HCC worldwide [1, 2]. It is mostly important to identify risk factors for understanding the molecular and cellular processes involved, and searching for possible new therapeutic molecular targets.

Ezrin is a member of the Ezrin-Radixin-Moesin (ERM) cytoskeleton-associated protein family and involved in a wide variety of cellular processes. It is a component of the cell-surface structures involved in cell adhesion to the extracellular matrix, and has also been implicated in membrane-cytoskeleton interactions [3, 4]. Ezrin has been suggested contributing to the tumor invasion and metastasis *in vitro* and *in vivo* [5-11]. It is likely to be correlated with tumor invasiveness, metastasis, and clinical prognosis in numerous human cancers, including HCC [8, 12-14]. Moreover, the subcellular location of Ezrin protein may be essential for this gene function [15-17], and the molecular characteristic is considered as a potential importance during tumor progression [18]. However, the clinical significance of this characteristic still remains to be clarified in human cancer. This study is conducted to investigate prognostic implication in HCC patients, and determine the potential role of the subcellular location of Ezrin during HCC progression.

Materials and methods

Clinical samples

92 cases of routinely processed and paraffin-embedded HCC meeting strict follow-up criteria were selected at random from patients under-

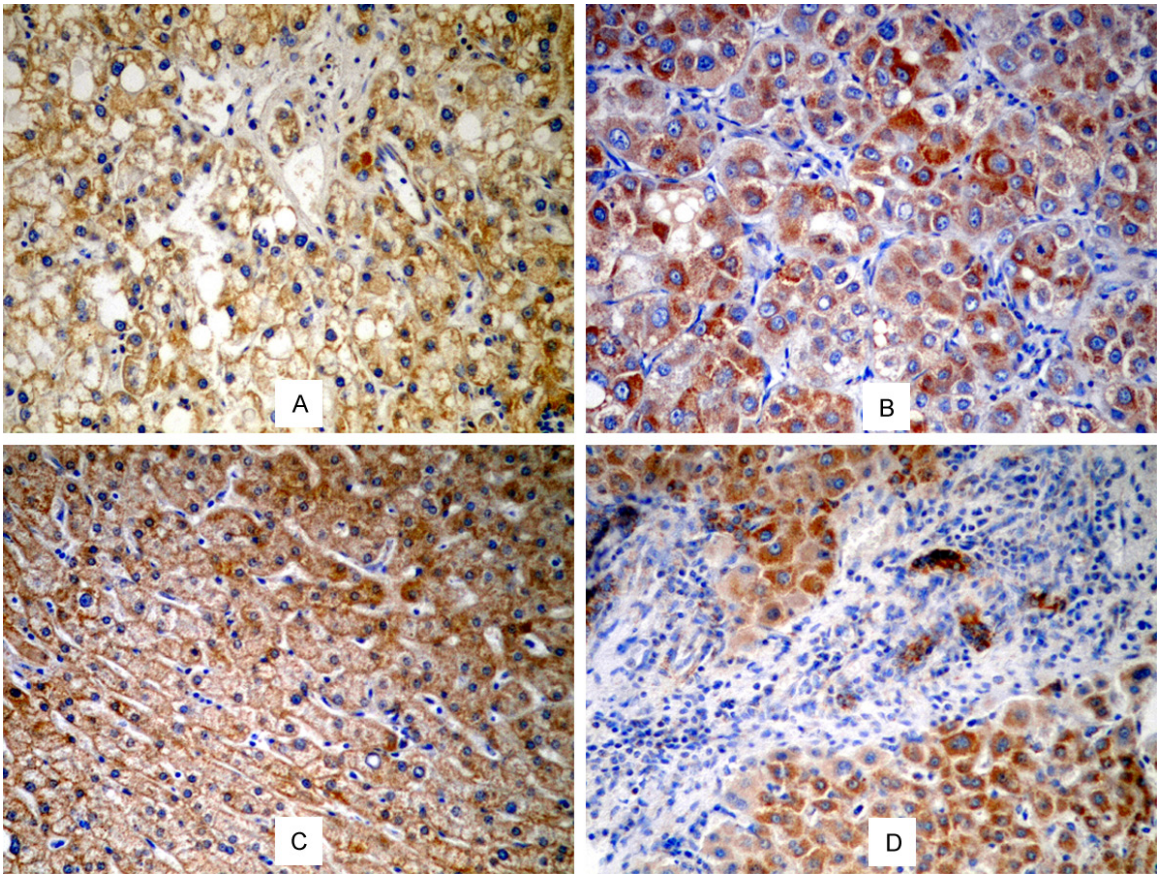

Figure 1. The expression models of Ezrin in HCC tissues (in the inner side of the membrane A, and in cytoplasm B), and the adjacent benign liver tissues (in the inner side of the membrane C, and in cytoplasm D). (Immunoperoxidase stain, ×200).

going surgery between 2011 and 2013 at the Department of Pathology and Tumor Tissue Bank, Eastern Liaoning University. The male to female ratio was 80:12. Patients' ages ranged between 23 and 90 years, with a mean age of 55.3 years. These cases were diagnosed as primary HCC by postoperative pathology, which showed 17 cases of well differentiation, 65 of moderate differentiation, 10 of low differentiation; 25 of tumor size ≤3 cm, 67 of tumor size >3 cm; 11 cases of being accompanied by vascular, lymph nodes, intrahepatic, and extrahepatic metastasis (1 or multiple); 66 cases of stage I, II according to the latest standard of AJCC, 26 of stage III, IV; 92 cases of the adjacent benign liver tissues including 51 cases with cirrhosis were also enrolled. Follow-up was started from surgery, and ended on the 15th May, 2016.

Immunohistochemical staining and criterion

Immunohistochemical analysis was performed using a DAKO LSAB kit (DAKO A/S, Glostrup, Denmark). Briefly, to eliminate endogenous peroxidase activity, 4 μm thick tissue sections were deparaffinized, rehydrated and incubated with 3% H_2O_2 in methanol for 15 minites at room temperature. The antigen was retrieved at 95°C for 20 minites by placing the slides in 0.01 M sodium citrate buffer (pH 6.0). The slides were then incubated with Ezrin antibody (1:150, BD, Inc., USA) at 4°C overnight. After incubation with biotinylated secondary antibody at room temperature for 30 minutes, the slides were incubated with streptavidin-peroxidase complex at room temperature for 30 min. Immunostaining was developed using 3,3'-diaminobenzidine, and Mayer's hematoxylin was used for counterstaining. Tonsil sections were used as positive controls, and mouse IgG as isotope controls. For negative controls, positive tissue sections were processed in the same manner but primary antibody (anti-mouse Ezrin) was omitted.

Immunoreactivity was evaluated independently by two researchers who were blinded to

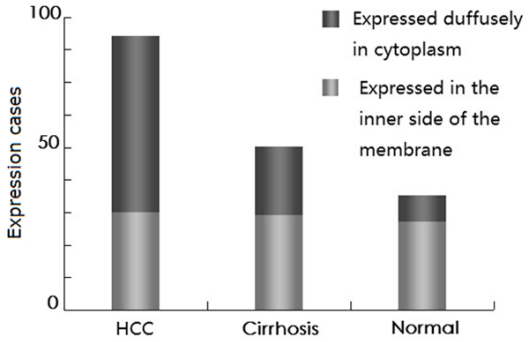

Figure 2. The cellular localization of Ezrin protein Ezrin in different liver tissues [HCC *vs.* liver cirrhosis, x^2=6.317, *P*=0.012; Liver cirrhosis *vs.* normal liver tissues, x^2=0.835, *P*=0.363; HCC *vs.* benign liver tissues (normal tissue + liver cirrhosis), x^2=15.385, *P*<0.001].

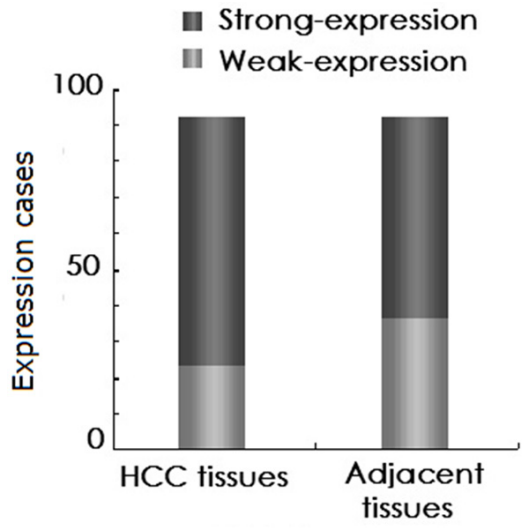

Figure 3. Ezrin protein expression in HCC tissues and adjacent normal tissues (x^2=4.216, *P*=0.041).

patient outcome. The evaluation was based on the staining intensity and extent of staining [19]: Staining intensity for Ezrin was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Staining extent was scored as 0 (0%), 1 (<10%), 2 (10-50%), 3 (51-80%), and 4 (>80%), depending on the percentage of positive-stained cells. Final score was derived by multiplying the two: 0-5 as a weak-expression, 6 points or more as a strong-expression. Additionally, the Ezrin showed expressed in the inner side of membrane or in cytoplasm.

Statistical analysis

Statistical analyses were performed using SPSS 19.0. χ^2 tests and Fisher's exact tests were applied in data analysis. The overall survival rates after tumor removal were calculated using the Kaplan-Meier method, and differences in survival curves were analyzed using log rank tests. Multivariate survival analysis was performed on all significant characteristics measured by univariate survival analysis with the Cox proportional hazard regression model. A *P*-value of <0.05 was considered statistically significant.

Results

The sub-cellular localization of Ezrin protein in different liver tissues

In 92 HCC cases, 8 cases were absolutely not expressed (0 score), 54 cases were expressed in cytoplasm, and 30 cases were expressed in the inner side of the cell membrane. In 51 cases of liver cirrhosis tissues, 29 cases were expressed in the inner side of the membrane and 22 cases were expressed in cytoplasm. In 41 cases of normal liver tissues, 6 cases were absolutely not expressed (0 score), 27 cases were expressed in the inner side of the cell membrane, and 8 cases were expressed in cytoplasm. There was statistical significance between the cellular localization of Ezrin protein in HCC and benign tissue (*P*<0.001, **Figures 1**, **2**).

Ezrin protein expression rate in different tissues

Ezrin strong-expression rate was 75.0% (69/92) in HCC tissues, which was significantly higher than in the adjacent tissues (60.9%, 56/92) (*P*=0.041) (**Figure 3**). The Ezrin strong-expression rate was closely related with the differentiation (*P*=0.005), metastasis (*P*<0.042), and AJCC stage (*P*=0.017), but not associated with other factors (*P*>0.05) (**Table 1**).

Survival analysis

To further confirm the role of Ezrin strong-expression in HCC progression, we analyzed overall survival rates of 92 HCC cases using the Kaplan-Meier method. We found that HCC patients with Ezrin strong-expression had a lower overall survival rate (*log-rank* =14.034, *P*<0.001) than those patients with Ezrin weak-expression (**Figure 4**).

Moreover, we also analyzed the survival significance of Ezrin expression in cytoplasm and in

Table 1. Correlation between Ezrin expression and clinicopathological factors in 92 patients with HCC

Parameters	No.	Ezrin		*P*-value
		Weak-expression	Strong-expression	
Gender				0.477
Male	80	21	59	
Female	12	2	10	
Age (years)				0.188
≥55	45	14	31	
<55	47	9	38	
Tumor size				0.686
≤3 cm	25	7	18	
>3 cm	67	16	51	
Differentiation				0.005**
High	17	10	7	
Moderate	65	11	54	
Low	10	2	8	
Metastasis				0.042*
Yes	11	0	11	
No	81	23	58	
AJCC stage				0.017*
I-II	66	21	45	
III-IV	26	2	24	
Cirrhosis				0.278
Yes	51	15	36	
No	41	8	33	

Statistical analyses were performed using Pearson Chi-Square test. *P<0.05, **P<0.01.

the inner side of the membrane respectively. The results showed that the HCC patients with strong-expression had lower overall survival rate than the patients with weak-expression in cytoplasm (*log-rank* =11.509, P=0.001). However, overall survival rates were not correlated to Ezrin expression status in the inner side of the membrane (*log-rank* =2.114, P=0.146) (**Figure 5**).

Univariate analysis showed that AJCC stage, metastasis, and Ezrin expression were associated with the overall survival rate. Therefore, multivariate survival analysis was performed using the Cox proportional hazards model for all the significant variables found with univariate survival analysis. The results suggested that Ezrin strong-expression also emerged as a significant independent prognostic factor in prognosis of HCC (RR: 2.400, 95% CI: 1.429-4.029, P<0.001), as well as AJCC stage, and metastasis. (**Table 2**).

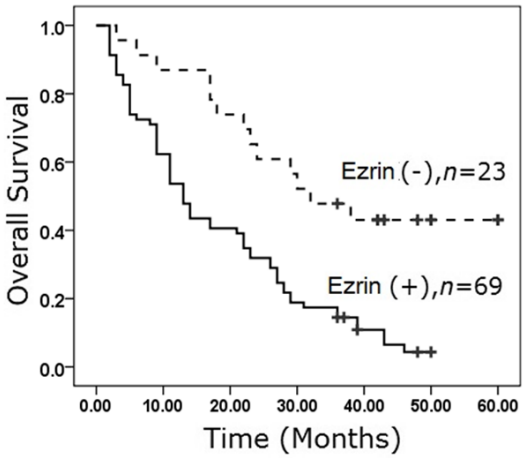

Figure 4. Kaplan-Meier analysis of overall survival rates in 92 HCC patients in relation to Ezrin protein expression (*log-rank* =14.034, P<0.001). (-, weak-expression; +, strong-expression).

Discussion

Ezrin was first identified as an important metastatic regulator in rhabdomyosarcoma and osteosarcoma. *Zeng* [20] found that Ezrin had localization alteration in esophago squamous-cell carcinoma, which suggested that Ezrin subcellular localization played an important role in cell adhesion. Ezrin's FERM structural domain could combine with cell C-termination adhesion molecule CD44, which integrate the hyaluronic-acid in the matrix, then hyaluronic-acid-CD44-ERM-actin cytoskeleton compound formed, which promote cell matrix adhesion [21]. But activation Ezrin could destroy the E-cadherin mediated cell-cell junction, decrease cells adhesion [22]. *Arslan* et al. [23] found that Ezrin was concentrated at the apical (luminal) membrane surface with a weak cytoplasmic distribution in normal breast epithelial cells. In early stage IA breast tumors, Ezrin intracellular distribution localized with a strong perinuclear pattern and weak cytoplasmic and membrane staining. In stage IIB tumors, Ezrin acquired a greater level of diffuse cytoplasmic staining and retained a weaker perinuclear and membrane distribution. In late stage IIIB large tumors, Ezrin was almost exclusively confined to a diffuse cytoplasmic distribution with little evidence for membrane association or perinuclear localization in virtually all of the breast tumors.

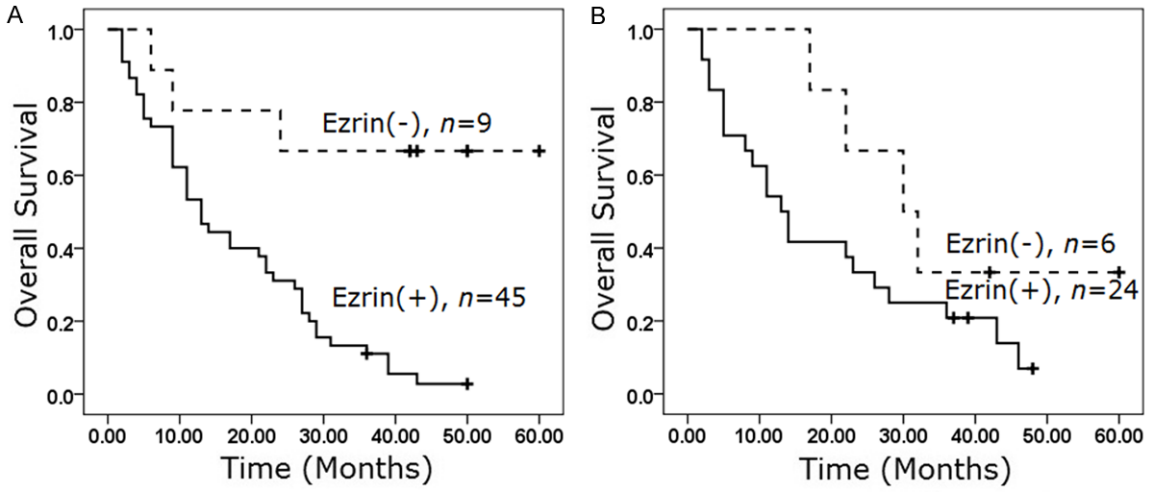

Figure 5. Kaplan-Meier analysis of overall survival rates in 54 HCC patients with Ezrin expressing in the cytoplasm and 30 HCC patients wiht Ezrin expressing in the inner side of the membrane (in the cytoplasm A, *log-rank* =11.509, *P*=0.001; in the inner side of the membrane B, *log-rank* =2.114, *P*=0.146). (-, weak-expression; +, strong-expression).

Table 2. Univariate and multivariate analysis of clinicopathological factors for the overall survival of 92 patients with HCC

Parameters	B	SE	Wald	RR	95% CI		*P*
					Lower	Upper	
Univariate analysis							
Gender	0.011	0.021	0.303	1.012	0.971	1.054	0.582
Age	0.010	0.013	0.512	0.990	0.965	1.017	0.474
Tumer size	-0.004	0.015	0.055	0.996	0.967	1.027	0.814
Differentiation	-0.006	0.022	0.066	0.994	0.952	1.038	0.797
Metastasis	-0.487	0.180	7.313	0.614	0.432	0.875	0.007**
AJCC stage	-0.060	0.020	8.743	0.942	0.905	0.980	0.003**
Ezrin	0.871	0.259	11.325	2.389	1.439	3.968	0.001**
Multivariate analysis							
Metastasis	2.411	0.444	29.457	11.148	4.667	26.628	0.000**
AJCC stage	0.999	0.260	14.743	2.714	1.630	4.519	0.000**
Ezrin	0.875	0.264	10.966	2.400	1.429	4.029	0.001**

**$P<0.01$.

However, Ezrin localization was found to change distinctly with tumor stage. However, the distribution characteristics of Ezrin in HCC have not yet been reported. This is the first study to our knowledge to report the significance of abnormal localization of Ezrin protein in HCC. Here we observed that Ezrin showed cytoplasmic expression in 54 cases of HCC (54/92), 22 cases of cirrhosis tissues (22/51), and 8 cases of normal liver tissues (8/41). However, the membranous staining pattern of Ezrin protein was found in 30 HCCs (30/92), 29 cirrhosis tissues (29/51) and 27 normal liver tissues (27/41). These results indicated that the location change of Ezrin in HCC lead to reduce the connections between tumor cells and hyaluronic acid, which induced the tumor to get more ability of infection.

Accumulating evidences showed that Ezrin protein expression was markedly increased in a variety of human cancers compared with their non-malignant tissue counterparts. Ezrin expression also has been found to be positively related to the degree of malignancy in many tumors, and its expression has also been linked to poor survival in several cancers, including carcinomas of the breast [24], endometrium [25] and in melanomas [26] and soft tissue sarcomas [27]. A recent study also demonstrated that high Ezrin expression could promote processes involved in tumorigenesis including cell proliferation, colony formation and migration, which may preliminarily explain the reason why Ezrin predicts poor prognosis in pancreatic cancer [28], ovarian cancers [29] and osteosarcomas [30]. In present study, Ezrin strong-expressed in 75.0% of HCC tissues, which obviously higher than that seen in adjacent benign tissues (60.9%).

Additionally, to test the levels of Ezrin might function as a prognostic factor and could be associated with tumor progression in HCC, we examined Ezrin expression and the clinicopathological features of HCC and found that Ezrin expression was significantly correlated to differentiation, AJCC stage and metastasis. With regard to survival, moreover, we found that HCC patients with Ezrin strong-expression had a lower overall survival rate than patients with Ezrin weak-expression. Meanwhile, HCC patients with cytoplasmic strong-expression of Ezrin had lower overall survival rate than those with cytoplasmic weak-expression of Ezrin, indicating that cytoplasmic Ezrin expression potentially played an important role in the progression of HCC. Finally, multivariate survival analysis demonstrated that Ezrin expression emerged as a significantly independent hazard factor for overall survival in HCC, along with AJCC stage and metastases.

In conclusion, Ezrin is a potential effective predictor of poor prognosis of HCC patients, especially for those with cytoplasmic expression. Determination of Ezrin expression levels and location may help to identify high-risk HCC patients and thus aid the selection of appropriate therapies.

Acknowledgements

Supported by grants from the Special Fund of the Central Government (2017), Projects of Science Research of Education Department in Liaoning Province (L2015189), and Liaoning Science & Technology Project (201520554).

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Lijuan Lin and Jinhui Zhang, Institute of Molecular Medicine, Medical College of Eastern Liaoning University, Dandong 118000, China. E-mail: linlijuan3066@163.com (LJL); 13942599899@163.com (JHZ)

References

[1] Morikawa K, Shimazaki T, Takeda R, Izumi T, Umumura M, Sakamoto N. Hepatitis B: progress in understanding chronicity, the innate immune response, and cccDNA protection. Ann Transl Med 2016; 4: 337.

[2] Li W, Wang Y, Gao W, Zheng J. HCC with tumor thrombus entering the right atrium and inferior vena cava treated by percutaneous ablation. BMC Surg 2017; 17: 21.

[3] Wang A, Lu C, Ning Z, Gao W, Xie Y, Zhang N, Liang J, Abbasi FS, Yan Q, Liu J. Tumor-associated macrophages promote Ezrin phosphorylation-mediated epithelial-mesenchymal transition in lung adenocarcinoma through FUT4/LeY up-regulation. Oncotarget 2017; 8: 28247-28259.

[4] Kawaguchi K, Yoshida S, Hatano R, Asano S. Pathophysiological roles of Ezrin/Radixin/Moesin proteins. Biol Pharm Bull 2017; 40: 381-390.

[5] Biri-Kovács B, Kiss B, Vadászi H, Gógl G, Pálfy G, Török G, Homolya L, Bodor A, Nyitray L. Ezrin interacts with S100A4 via both its N- and C-terminal domains. PLoS One 2017; 12: e0177489.

[6] He J, Ma G, Qian J, Zhu Y, Liang M, Yao N, Ding Q, Chen L, Liu X, Xia T, Wang S. Interaction between ezrin and cortactin in promoting epithelial to mesenchymal transition in breast cancer cells. Med Sci Monit 2017; 23: 1583-1596.

[7] Li L, Liu M, Lin JB, Zhu Y, Liang M, Yao N, Ding Q, Chen L, Liu X, Xia T, Wang S. Diagnostic value of autoantibodies against ezrin in esophageal squamous cell carcinoma. Dis Markers 2017; 2017: 2534648.

[8] Xie JJ, Xu LY, Wu ZY, Zhao Q, Xu XE, Wu JY, Huang Q, Li EM. Prognostic implication of ezrin expression in esophageal squamous cell carcinoma. J Surg Oncol 2011; 104: 538-543.

[9] Fan LL, Chen DF, Lan CH, Liu KY, Fang DC. Knockdown of ezrin via RNA interference suppresses Helicobacter pylori-enhanced invasion of gastric cancer cells. Cancer Biol Ther 2011; 11: 746-752.

[10] Yu Y, Zeng P, Xiong J, Liu Z, Berger SL, Merlino G. Epigenetic drugs can stimulate metastasis through enhanced expression of the pro-metastatic Ezrin gene. PLoS One 2010; 5: e12710.

[11] Chuan YC, Iglesias-Gato D, Fernandez-Perez L, Cedazo-Minguez A, Pang ST, Norstedt G, Pousette A, Flores-Morales A. Ezrin mediates c-Myc actions in prostate cancer cell invasion. Oncogene 2010; 29: 1531-1542.

[12] Korkeila EA, Sundström J, Pyrhönen S, Syrjänen K. Carbonic anhydrase IX, hypoxia-inducible factor-1α, ezrin and glucose transporter-1 as predictors of disease outcome in rectal cancer: multivariate Cox survival models following data reduction by principal component analysis of the clinicopathological predictors. Anticancer Res 2011; 31: 4529-4535.

[13] Zhou L, He J, Zhang Y. MicroRNA-22 expression in hepatocellular carcinoma and its correlation with ezrin protein. J Int Med Res 2013; 41: 1009-1016.

[14] Okamura D, Ohtsuka M, Kimura F, Shimizu H, Yoshidome H, Kato A, Miyazaki M. Ezrin expression is associated with hepatocellular carcinoma possibly derived from progenitor cells and early recurrence after surgical resection. Mod Pathol 2008; 21: 847-855.
[15] Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. Nat Rev Mol Cell Biol 2002; 3: 586-599.
[16] Reczek D, Berryman M, Bretscher A. Identification of EBP50: a PDZ-containing phosphoprotein that associates with members of the ezrin-radixin-moesin family. J Cell Biol 1997; 139: 169-179.
[17] Morales FC, Takahashi Y, Kreimann EL, Georgescu MM. Ezrin-radixin-moesin (ERM)-binding phosphoprotein 50 organizes ERM proteins at the apical membrane of polarized epithelia. Proc Natl Acad Sci U S A 2004; 101: 17705-17710.
[18] Schlecht NF, Brandwein-Gensler M, Smith RV, Kawachi N, Broughel D, Lin J, Keller CE, Reynolds PA, Gunn-Moore FJ, Harris T, Childs G, Belbin TJ, Prystowsky MB. Cytoplasmic ezrin and moesin correlate with poor survival in head and neck squamous cell carcinoma. Head Neck Pathol 2012; 6: 232-243.
[19] Köbel M, Weichert W, Crüwell K, Schmitt WD, Lautenschläger C, Hauptmann S. Epithelial hyaluronic acid and CD44v6 are mutually involved in invasion of colorectal adenocarcinomas and linked to patient prognosis. Virchows Arch 2004; 445: 456-464.
[20] Zeng H, Xu L, Xiao D, Zhang H, Wu X, Zheng R, Li Q, Niu Y, Shen Z, Li E. Altered expression of ezrin in esophageal squamous cell carcinoma. J Histochem Cytochem 2006; 54: 889-896.
[21] Zhang J, Dong J, Yang Z, Ma X, Zhang J, Li M, Chen Y, Ding Y, Li K, Zhang Z. Expression of ezrin, CD44, and VEGF in giant cell tumor of bone and its significance. World J Surg Oncol 2015; 13: 168.
[22] Briggs JW, Ren L, Nguyen R, Chakrabarti K, Cassavaugh J, Rahim S, Bulut G, Zhou M, Veenstra TD, Chen Q, Wei JS, Khan J, Uren A, Khanna C. The ezrin metastatic phenotype is associated with the initiation of protein translation. Neoplasia 2012; 14: 297-310.
[23] Arslan AA, Silvera D, Arju R, Giashuddin S, Belitskaya-Levy I, Formenti SC, Schneider RJ. Atypical ezrin localization as a marker of locally advanced breast cancer. Breast Cancer Res Treat 2012; 134: 981-988.
[24] Sizemore S, Cicek M, Sizemore N, Ng KP, Casey G. Podocalyxin increases the aggressive phenotype of breast and prostate cancer cells in vitro through its interaction with ezrin. Cancer Res 2007; 67: 6183-6191.
[25] Stemmer-Rachamimov AO, Wiederhold T, Nielsen GP, James M, Pinney-Michalowski D, Roy JE, Cohen WA, Ramesh V, Louis DN. NHE-RF, a merlin-interacting protein, is primarily expressed in luminal epithelia, proliferative endometrium, and estrogen receptor-positive breast carcinomas. Am J Pathol 2001; 158: 57-62.
[26] Riecken LB, Zoch A, Wiehl U, Reichert S, Scholl I, Cui Y, Ziemer M, Anderegg U, Hagel C, Morrison H. CPI-17 drives oncogenic Ras signaling in human melanomas via Ezrin-Radixin-Moesin family proteins. Oncotarget 2016; 7: 78242-78254.
[27] Hanlon A, Stasko T, Christiansen D, Cyrus N, Galan A. LN2, CD10, and Ezrin do not distinguish between atypical fibroxanthoma and undifferentiated pleomorphic sarcoma or predict clinical outcome. Dermatol Surg 2017; 43: 431-436.
[28] Sun Y, Wu J, Cai H, Wang S, Liu Q, Blot WJ, Shu XO, Cai Q. A prospective study of autoantibodies to Ezrin and pancreatic cancer risk. Cancer Causes Control 2016; 27: 831-835.
[29] Horwitz V, Davidson B, Stern D, Tropé CG, Tavor Re'em T, Reich R. Ezrin is associated with disease progression in ovarian carcinoma. PLoS One 2016; 11: e0162502.
[30] Hlavaty J, Wolfesberger B, Hauck M, Obermayer-Pietsch B, Fuchs-Baumgartinger A, Miller I, Walter I. Ezrin and moesin expression in canine and feline osteosarcoma. Histol Histopathol 2017; 32: 805-816.