Pathol. Oncol. Res. (2011) 17:909–915
DOI 10.1007/s12253-011-9402-y

RESEARCH

Ezrin is Associated with Gastric Cancer Progression and Prognosis

Li Li · Yuan-Yu Wang · Zhong-Sheng Zhao · Jie Ma

Received: 11 January 2011 / Accepted: 6 April 2011 / Published online: 30 June 2011

Abstract To investigat the clinical significance of Ezrin in the development and progression of gastric cancer. Immunohistochemistry was employed to analyze Ezrin expression in 436 clinicopathologically characterized gastric cancer cases. Ezrin protein levels were up-regulated in gastric cancer lesions compared with adjacent noncancerous tissues. Positive expression of Ezrin correlated with age, size of tumor, location of tumor, depth of invasion, vessel invasion, lymph node and distant metastasis and TNM stage. In stages I, II and III, the 5 year survival rate of patients with a high expression of Ezrin was significantly lower than those in patients with low expression. In stage IV, Ezrin expression did not correlate with the 5 year survival rate. Further multivariate analysis suggested that the depth of invasion, lymph node and distant metastasis, TNM stage, and up-regulation of Ezrin were independent prognostic indicators for the disease. Expression of Ezrin in gastric cancer is significantly associated with lymph node and distant metastasis, and poor prognosis. Ezrin protein could be useful markers to predict tumor progression and prognosis.

Keywords Gastric carcinoma · Ezrin · Progression · Prognosis

L. Li · Z.-S. Zhao (✉) · J. Ma
Department of Pathology, Zhejiang Provincial People's Hospital, Hangzhou 310014, People's Republic of China
e-mail: zhaozhongsheng50@126.com

Y.-Y. Wang
Gastrointestinal Surgery, Zhejiang Provincial People's Hospital, Hangzhou 310014, People's Republic of China

Introduction

Although the global incidence of gastric cancer has decreased in recent years, its mortality rate in China is the highest of all tumors and represents 25% of gastric cancer mortality worldwide. Despite recent advances in chemotherapy and surgical techniques, the overall 5-year survival rate in China is still only 40%. Most gastric cancer is diagnosed as stage III or IV, when the rate of lymph node metastasis is high (50–75%) [1]. Tumor invasion and metastasis is a very complicated and continuous process involving multiple steps, regulated at the molecular level by adhesion molecules, protein catabolic enzymes, cellular growth factors and various angiogenic factors. Therefore, it is of great clinical value to further understand the molecular mechanisms involved in the development and growth of gastric cancer and to identify valuable new diagnostic markers and novel therapeutic strategies.

Ezrin is a member of the Ezrin-Radixin-Moesin (ERM) family and is involved in a wide variety of cellular processes such as cell adhesion, cell survival, cell motility, and signal transduction [2–5], all of which are important for tumor development and progression. Ezrin is found in many malignant cell types including primary gastrointestinal stromal tumors, esophageal squamous cell carcinoma, advanced colorectal cancer and prostatic carcinoma [6–10]. Bal et al., examined Ezrin expression in 75 gastric carcinoma (53 intestinal types of adenocarcinoma, 22 diffuse types of carcinoma) tissues using immunohistochemistry and found that Ezrin immunostaining was positive in 43 cases (81.1%) of intestinal-type and 9 (40.9%) cases of diffuse-type adenocarcinomas [11]. In this study, we examined the expression of Ezrin in surgical specimens of gastric carcinoma to explore the possible

correlation between Ezrin expression and clinicopathological variables, and to determine its prognostic value.

Materials and Methods

Patients and Tissue Samples

Gastric cancer tissues were collected from the gastrectomy specimens of 436 patients (311 male, 125 female; median age = 60.0 years; range = 30–91 years) treated at the Department of Surgery, Zhejiang Provincial People's Hospital between January 1998 and January 2004. Tissues were formalin-fixed, paraffin-embedded, and clinically and histopathologically diagnosed in the Departments of Gastrointestinal Surgery and Pathology. All patients had follow-up records for over 5 years. The follow-up deadline was December 2008. The survival time was counted from the date of surgery to the follow-up deadline, or date of death (usually the result of carcinoma recurrence or metastasis). There were 55, 163, and 218 cases from the cardia, body, and antrum, respectively. According to the WHO histological classification of gastric carcinoma formulated in 2002, there were 326 tubular adenocarcinomas, 16 papillary adenocarcinomas, 29 mucinous adenocarcinomas, and 65 signet-ring cell carcinomas. Thirteen were highly differentiated adenocarcinomas, 128 were well- or moderately differentiated adenocarcinomas, 293 were poorly differentiated and two were undifferentiated adenocarcinomas. There were 61 cases with distant metastasis. Ninety cases were categorized as stage I, 104 as stage II, 173 as stage III and 69 as stage IV. Ninety-two noncancerous human gastric tissues were obtained from gastrectomy of adjacent gastric cancer margins (greater than 5 cm). Routine chemotherapy was given after surgery to patients with advanced-stage disease, but none of the patients received radiation treatment.

Tissue Microarrays

Blocks containing a total of 528 cases (436 cancer samples + 92 non-cancer tissue samples) were prepared as previously described [12]. Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded gastric tumors (donor blocks) using a trephine and arranged in recipient paraffin blocks (tissue array blocks). Because staining results obtained from different intra-tumoral areas in various tumors correlate well [13], a core was sampled in each case. An adequate case was defined as a tumor occupying >10% of the core area [14]. Each block contained more than three internal controls comprising non-neoplastic gastric mucosa. Sections (4 μm) were cut from each tissue array block, deparaffinized and dehydrated.

Immunohistochemistry

Immunohistochemical analysis was done to study altered protein expression in the 92 non-cancerous human gastric tissue controls and 436 human gastric cancer tissues. In brief, slides were baked at 60°C for 2 h, deparaffinized with xylene and rehydrated. The sections were submerged in EDTA antigenic retrieval buffer and microwaved for antigenic retrieval, after which they were treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, followed by incubation with 1% bovine serum albumin to block nonspecific binding. Sections were incubated with rabbit anti-Ezrin (ABCAM USA) overnight at 4°C. Normal goat serum was used as a negative control. After washing, tissue sections were treated with a secondary antibody. Tissue sections were then counterstained with hematoxylin, dehydrated, and mounted. Cytoplasmic and nuclear Ezrin was stained buffy-brown. The degree of immunostaining was reviewed and scored independently by two observers based on the proportion of positively stained tumor cells and the staining intensity [15, 16], Tumor cell proportion was scored as follows: 0 (≤5% positive tumor cells), 1 (6–25% positive tumor cells), 2 (26–50% positive tumor cells), and 3 (>51% positive tumor cells). Staining intensity was graded according to the following criteria: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown) and 3 (strong staining = brown). The staining index was calculated as the product of the staining intensity score and the proportion of positive tumor cells. Using this method of assessment we evaluated Ezrin expression in benign gastric epithelia and malignant lesions by determining the staining index with scores of 0, 1, 2, 3, 4, 6, or 9. The cut off value for high and low expression levels was chosen based on a measure of heterogeneity using the log-rank test with respect to overall survival: a staining index score of ≥4 was used to indicate high Ezrin expression and a staining index score of ≤3 was used to indicate low Ezrin expression.

Statistical Analysis

All statistical analyses were performed using SPSS 13.0 software. Measurement data were analyzed using the Student's *t* test, while categorical data were analyzed using χ^2 or Fisher exact tests. Survival curves were estimated using the Kaplan-Meier method and the log-rank test was used to compute differences between the curves. Multivariate analysis using the Cox proportional hazards regression model was performed to assess the prognostic value of protein expression levels. Correlation coefficients between protein expression and clinicopathological findings were estimated using the Pearson correlation method. Statistical significance was set at $P<0.05$.

Results

Ezrin Expression in Gastric Cancer and non-cancer Mucosa

Ezrin was detected in 11/92 (11.2%) human non-tumor mucosae, and all samples expressed protein at low levels. Ezrin was detected in 258/436 (59.2%) human gastric cancer cases. High expression was detected in 236 (54.1%) tumors and low expression in 22 (5.1%). Ezrin was mainly localized in the cytoplasm, and a few was localized in nuclei of primary cancer cells (Fig. 1).

Overexpression of Ezrin and Clinico-Pathological Features

Positive expression of Ezrin correlated with age, tumor size, location, differentiation stage, depth of invasion, vessel invasion, lymph node and distant metastasis, and TNM stage ($P<0.05$) (Table 1). Ezrin expression did not correlate with gender or histological type ($P>0.05$) (Table 1). Possible prognostic factors for gastric carcinoma were analyzed by Cox regression analysis. This showed that the depth of invasion ($P=0.015$), lymph node ($P=0.012$) and distant metastasis ($P=0.009$), TNM stage ($P=0.008$), and

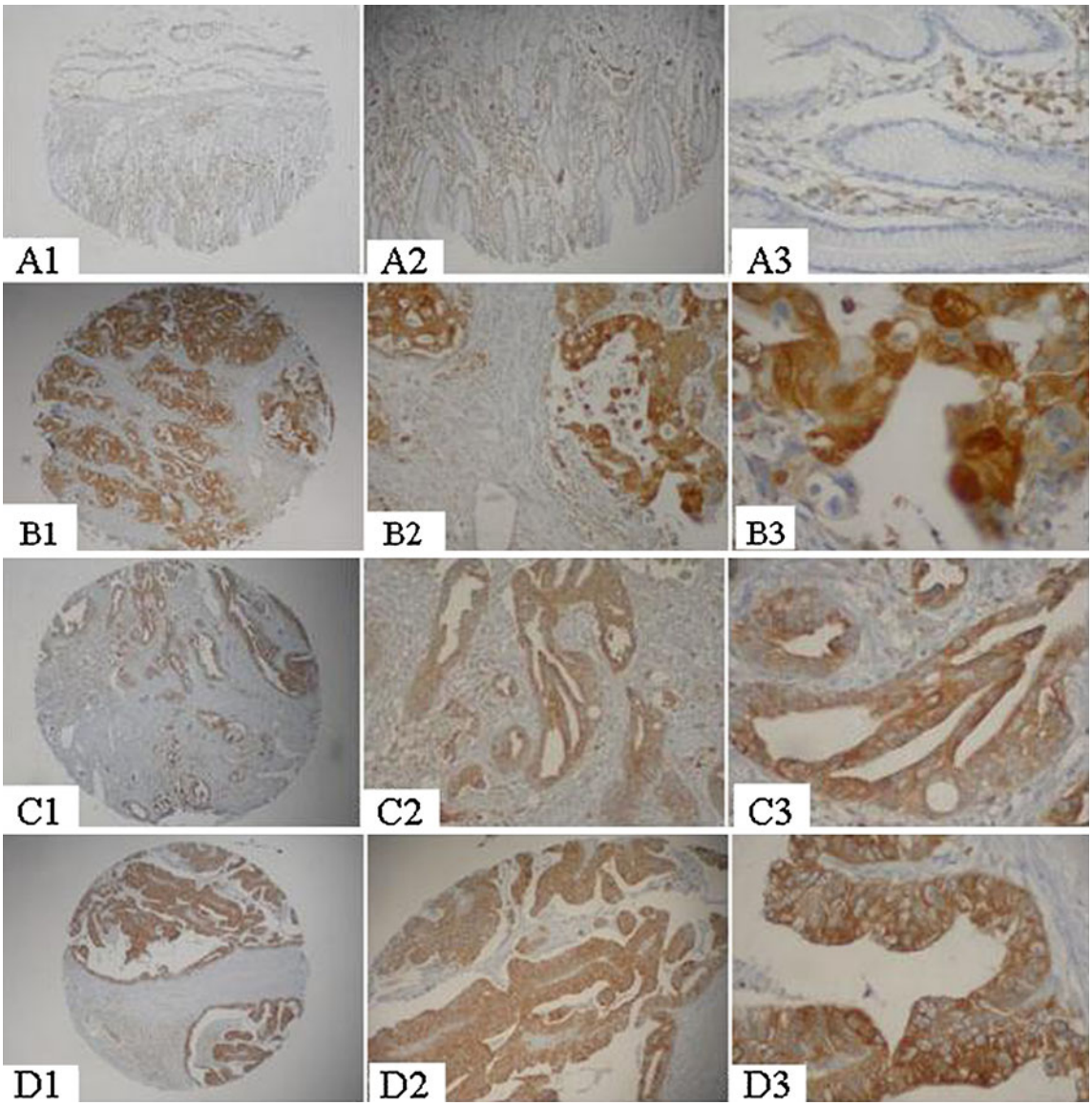

Fig. 1 Immunohistochemical staining for Ezrin in gastric cancer lesions and noncancerous tissues. A1-3 Ezrin negative in noncancerous tissues, magnification ×40, ×100, and ×400, respectively. B1-3 Ezrin was highly expressed in cytoplasm and nuclei of poorly differentiated adenocarcinoma, magnification ×40, ×100, and ×400, respectively. C1-3 Ezrin was highly expressed in moderately differentiated adenocarcinoma, magnification ×40, ×100, and ×400, respectively. D1-3 Ezrin was highly expressed in papillary adenocarcinoma, magnification ×40, ×100, and ×400, respectively

Table 1 Relationship of Ezrin expression with pathological parameters of tumor

Clinical parameters	Ezrin			
	low	high	$t/\chi^2/r$	P
Age(yrs)	56.61±10.99	61.12±12.66	3.93	0.001
Gender			0.851	0.356
Male	147(47.3%)	164(52.7%)		
Female	53(42.4%)	72(57.6%)		
Location			5.639	0.06
Proximal	19(34.5%)	36(65.5%)		
Middle	70(42.9%)	93(57.1%)		
Distal	111(50.9%)	107(49.1%)		
Size			21.168	0.0001
<5 cm	141(55.1%)	115(44.9%)		
≥5 cm	59(32.8%)	121(67.2%)		
Lauren classification			136.2	0.0001
Intestinal	163(73.1%)	60(26.9%)		
Diffuse	37(17.4%)	176(82.6%)		
Histology			1.521	0.677
Papillary adenocarcinoma	9(56.2%)	7(43.8%)		
Tubular adenocarcinoma	149(45.7%)	177(54.3%)		
Mucinous adenocarcinoma	11(37.9%)	18(62.1%)		
Signet-ring cell carcinoma	31(47.7%)	34(52.3%)		
Histologic differentiation			8.156	0.043
Well	11(84.6%)	2(15.4%)		
Moderately	58(45.3%)	70(54.7%)		
Poorly	130(44.4%)	163(55.6%)		
Others	1(50.0%)	1(50.0%)		
Invasion depth			78.012	0.0001
T1	51(89.5%)	6(10.5%)		
T2	64(58.7%)	45(41.3%)		
T3	82(33.6%)	162(66.4%)		
T4	3(11.5%)	23(88.5%)		
TNM Stages			157.2	0.0001
I	78(86.7%)	12(13.3%)		
II	73(70.2%)	31(29.8%)		
III	45(26.0%)	128(74.0%)		
IV	4(5.8%)	65(94.2%)		
Vessel invasion			39.596	0.0001
No	145(54.1%)	123(45.9%)		
Yes	7(10.8%)	58(89.2%)		
Lymphatic metastasis			113.6	0.0001
No	130(78.3%)	36(21.7%)		
Yes	70(25.9%)	200(74.1%)		
Regional lymph nodes			123.4	0.0001
PN0	130(78.3%)	36(21.7%)		
PN1	44(32.4%)	92(67.6%)		
PN2	25(25.3%)	74(74.7%)		
PN3	1(2.9%)	34(97.1%)		
Distant metastasis			44.148	0.0001
No	196(52.3%)	179(47.7%)		
Yes	4(6.6%)	57(93.4%)		

the level of Ezrin expression (P=0.008) were all independent prognostic factors in patients with gastric carcinoma. However, the location of the tumor, tumor size, histological type, differentiation, and vessel invasion had no prognostic value.

Correlation between Ezrin Expression and Patient Prognosis

For patients with stage I, II or III disease, the 5-year survival rate for those with high Ezrin expression were significantly lower than in patients with low expression. For stage I, the cumulative 5-year survival rate was 94.9% in the low expression group, but only 75.0% in the high expression group (P=0.001, Fig. 2); for stage II, the cumulative 5-year survival rate was 71.2% in the low expression group, but only 54.8% in the high expression group (P=0.0001, Fig. 3); and for stage III, the cumulative 5-year survival rate was 62.0% in the low expression group, but only 22.7% in the high expression group (P=0.0001, Fig. 4). For stage IV the expression of Ezrin did not correlate with the 5-year survival rate (3.1% in the low expression group and 0 in the high expression group; P=0.439).

Discussion

Surgical resection remains the primary curative treatment option for gastric cancer, with 5-year survival rates of 58–78% and 34% reported for stage I and II disease, respectively. Despite this, the overall 5-year survival rate for all patients remains poor, ranging from 15% to 38% [17]. The prognosis for patients who have undergone curative resection remains poor because of the high rate of local recurrence and early lymph node and systemic metastases. Thus, identification of reliable molecular prognostic markers is important in gastric cancer, and their measurement in serum or small biopsy samples should provide important prognostic information.

In this study, immunohistochemistry was used to analyze the levels of Ezrin expression in 436 clinicopathologically characterized gastric cancer cases. The results showed that Ezrin was up-regulated in gastric cancer tissues compared with normal gastric tissues and correlated significantly with prognosis. In addition, high levels of Ezrin expression in gastric cancer lesions were associated with age, tumor size, location, depth of invasion, vessel invasion, lymph node and distant metastasis and TNM stage. Previous studies show that Ezrin is expressed in 66% gastrointestinal stromal tumors (GISTs) and is significantly associated with non-gastric locations [6]. Positive Ezrin expression was significantly higher in esophageal squamous cell carcinoma (ESCC) than in para-cancer normal squamous epithelium,

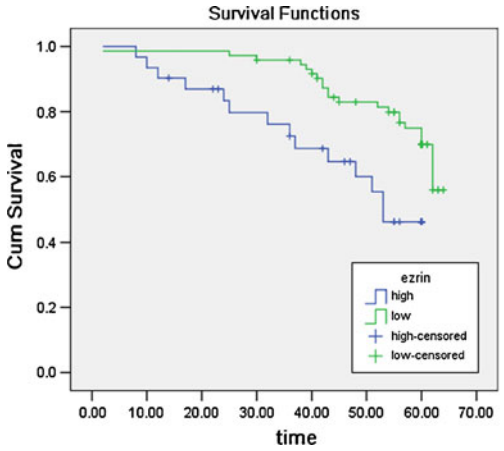

Fig. 3 Kaplan-Meier curves with univariate analyses (log-rank) for patients with low Ezrin expression versus high Ezrin expression tumors in all gastric cancer in stage II

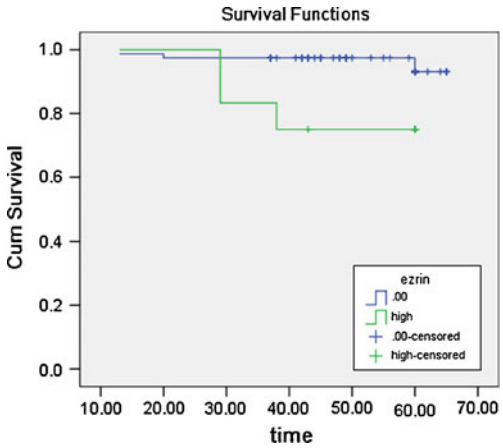

Fig. 2 Kaplan-Meier curves with univariate analyses (log-rank) for patients with low Ezrin expression versus high Ezrin expression tumors in all gastric cancer in stage I

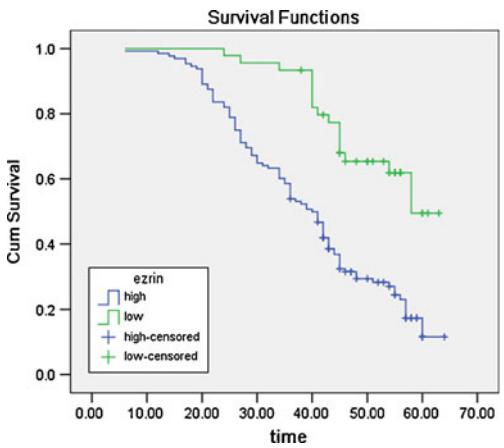

Fig. 4 Kaplan-Meier curves with univariate analyses (log-rank) for patients with low Ezrin expression versus high Ezrin expression tumors in all gastric cancer in stage III

and was related to invasiveness and lymph node metastasis of ESCC. The activation of Ezrin was shown to increase the tumorigenesis and metastasis of ESCC [7]. Elzagheid et al., showed that Ezrin expression was more intense in colon carcinomas than in rectal carcinomas, and may play a role in colorectal cancer progression. Also, Ezrin expression levels might provide clinically valuable information for predicting the biological behavior of colorectal cancer [8]. Ezrin expression was moderate or strong in 70% of prostate cancers, but negative (or only weakly positive) in benign epithelium. Expression also correlated with Gleason scores and seminal vesicle invasion, but not with extra-prostatic extension or margin status [9]. Bal et al., examined Ezrin expression in 75 gastric carcinoma tissues (53 intestinal-type adenocarcinomas and 22 diffuse-type carcinomas) using immunohistochemistry and found that there was no correlation between Ezrin expression and TNM stage or histological grade [11]. However, our results suggest that Ezrin expression in gastric cancer lesions is closely associated with TNM stage. This discrepancy may be due to the small numbers used their study.

The inhibition of Ezrin expression clearly inhibits the migration and invasion of the human gastric cancer cell line, SGC-7901, and increases both cell adhesion and sensitivity to camptothecin-induced apoptosis [18]. Overexpression of Ezrin also promoted cell protrusion, microvillus formation, anchorage-independent growth, motility and invasion in the pancreatic cancer cell line, MiaPaCa-2. Overexpression activated Erk1/2 in MiaPaCa-2 cells, which might be partially related to alterations in cell morphology and invasion [19]. c-Myc induces cell invasion and anchorage-independent growth by regulating Ezrin protein expression in the presence of androgens, as the activity of the Ezrin promoter is controlled by androgens through c-Myc [20].

In patients with stage I, II and III disease, the 5-year survival rate for those with high Ezrin expression was significantly lower than that of patients with low expression. However, for stage IV, expression did not correlate with the 5-year survival rate. Multivariate analysis suggested that the depth of invasion, lymph node and distant metastasis, TNM stage, and Ezrin up-regulation were independent prognostic indicators for disease. It is known that Ezrin expression correlates with a reduction in disease-free survival and is an independent predictor of poor outcome [6]. Elzagheid et al., also showed that the level of Ezrin expression is associated with adverse outcomes. Univariate survival analysis revealed that dichotomized (negative/weak versus moderate/strong) expression of Ezrin is a significant predictor of both disease-free survival and 5-year rates of metastases; however, this was not supported by multivariate (Cox) analysis [8]. Ezrin is also expressed by the majority of prostate cancers and correlates with a poor prognosis [9] and the survival time of prostate cancer patients with aberrant staining for Ezrin is also significantly shorter than that of patients with normal staining patterns as assessed by Cox multivariate survival analysis [10].

The results of our study suggest that overexpression of Ezrin in gastric cancer tissues might play an important role in the progression and metastases of the disease and that Ezrin may be a useful prognostic and survival indicator.

Acknowledgments Work was supported by Zhejiang Provincial Department of Science and Technology Research Foundation (2008C33040).

Declaration of Interest Statement The authors have no conflicts of interest to declare

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