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Ezrin Expression as a Prognostic Marker in Colorectal Adenocarcinoma

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Abstract Ezrin protein acts in the regulation of cytoskeletal and directly influences survival and tumor progression; there is an increase in its expression in metastatic cells and tissues in several types of cancer including colorectal cancer. 250 Patients with colorectal cancer submitted to surgery from 1995 to 2002. Protein expression was carried through by *Tissue Micro Array* immunohistochemical tests of paraffined neoplasic tissues and associated with clinical variables. Differentiation degree, lymph node invasion, metastasis at diagnosis, and palliative surgery were associated to a higher expression of the protein and survival. Higher expression of the Ezrin correlates with tumor aggressiveness and worse prognosis for colorectal cancer.

Keywords Colorectal cancer · Ezrin · Biological marker · Prognostic · Adenocarcinoma

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Introduction

Cytoskeleton is a three-dimensional network of proteins distributed through cells cytoplasm, and it is involved in the movement, support, resistance, phagocytosis, cytokinesis, junctions, and conformational changes [1, 2].

Recent biochemical studies showed that each filaments class has a specific protein organization that determines cytoskeleton organization and function [2].

Ezrin protein, a member of the ezrin-radixin-moesin (ERM) family, is an important molecule linking the cytoskeleton to the membrane. These are proteins related to important cytoskeleton regulation functions, such as apical joint remodeling [3], with a direct influence in cellular survival and evidences of regulation in tumor progression [4].

ERM proteins are specialized cell-membrane components linked to actin cytoskeleton, and are associated to adhesion molecules such as CD43, CD44, ICAM-1 and ICAM-2, among others [5] through the amino-terminal grouping to actin filaments by the carboxyl-terminal group [6]. They are also associated to signal receptors for growth factors [7], Rho GTPases regulation [8], intracellular adhesion and communication with extracellular matrix [9, 10], all connected to metastases [7, 11, 12]. Ezrin expresses in a great variety of tissues, and its co-location may depend on cell type and stimulus affecting cells [13]. There are evidences suggesting that ezrin subcellular distribution is significantly correlated with tumorigenesis [14]. The ezrin protein is regulated by an intramolecular association between an amino-terminal grouping and a carboxyl-terminal grouping from protein to protein that promotes its binding to specific cell locations [15]. Protein activation takes place through phosphorylation of threonine 558 in the carboxyl-terminal. This activation produces in cell mem-

branes several adhesion molecules and ionic channels in the amino-terminal region causing actin-F polymerization through carboxyl-terminal [16]. The signal for ezrin production depends on the presence of tyrosine kinase. Growth factors stimulate cells and produce phosphorylation of primary ezrin and of two residues of tyrosine which are important in ezrin function regulation. Ezrin phosphorylation and of two residues of tyrosine is important for the formation of microtubules and cellular mobility [7].

The analysis of genic expression comparing cells and tissues in metastatic and no metastatic situations reveals important increases in ezrin expression in several types of tumors in human, including osteosarcomas, melanomas, prostate, pancreas, lung and endometrial carcinoma [6, 11, 17–22]. These studies also point out that the increase of the expression of this protein is associated to malignant transformations, increase of cell proliferation, cell survival and growth of metastatic mobility or make tumor cells respond to growth factors, adhesion molecules and other signaling modalities [11]. The association between ezrin immunoreactivity and malignity is stronger than other markers such as Ki-67/MIB-1 [23]. For uveal melanoma, ezrin immunoreactivity was significantly associated with an increase in microvascular density and a worse prognosis for this tumor [24, 25]. It is known that high microvascular density is correlated to angiogenesis, associated with hematogenic metastasis processes [26]. Vasculogenesis is connected to channels and sinusoids formation, and when there is an increase of ezrin expression, its association to actin filaments increases tubulogenesis [7, 26].

Adhesion relations among cells, associated to cadherins and integrins mediating interactions with the extracellular matrix may important in metastatic progression [27].

Some studies connect the reduction of ezrin protein expression to the reduction of lung metastases of rabdomiosarcoma, osteosarcoma [11] and breast cancer [4], suggesting that the expression of this protein has a regulating effect in cancer malignancy. On the other hand, the reduction of its expression has been associated to unfavorable characteristics of some types of tumor and survival vines [28]. Meantime, it is known that the levels of this protein can vary in accordance with its location inside the cell [28].

The aim of this study is to verify ezrin protein expression, through immunohistochemical tests, in colorectal cancer, and correlate findings to clinical and pathological characteristics and survival.

Materials and Methods

The inclusion criteria were patients with colorectal cancer, treated at A.C. Camargo Hospital from 1995 to 2002, with no previous treatment. Clinical information was obtained through a retrospective study of medical records by the same research group with the same protocol. Samples of tumor tissue, which led to the construction of the TMA were raised from the existing files in the field of pathology at the A.C. Camargo Hospital, where they had to be preserved and fixed in paraffin blocks.

The collection of material and information on the patient data were approved by the Ethics and Research Hospital AC Camargo and were registered in the regulator of the Brazilian government (SISNEP) which sets guidelines for the conduct of scientific research in this country.

TMA Construction

Of the evaluated cases, 253 cases that met the inclusion criteria were selected and submitted to *Tissue Micro Array* (TMA) technique with the construction of six TMA blocks with samples of colorectal adenocarcinomas prepared for immunohistochemistry, and the first *core* of each block was a normal liver fragment used as reference. After TMA blocks preparation, 3 μm thick cuts were obtained, put in slides with special markers for the technique (Instrumedics Inc). TMA was built using the Manual Tissue Arrayer I, from Beecher Instruments Inc.

Immunohistochemistry

Ezrin protein immunohistochemical reaction was carried through in duplicate for each TMA block, and each evaluated patient presented four different colors from the same tumor to be stained. Ezrin-specific monoclonal antibody Ezrin/p81/80 K/Cytovillin Ab-1 (3 C12) Cat.#MS-661-R7 (LABVISION CORPORATION—NEOMARKERS) were used. Slides were previously treated with 3-aminopropiltrietoxi-silano (Sigma, A-3648, USA) and left for 24 h in a 60°C stove. Histological cuts were deparaffinized in xylol at 60°C for 20 min and, then, to xylol at room temperature for other 20 min. Slides were subsequently prepared by successive passages in ethanol (100%, 95% and 70%) at 30 s intervals, being afterwards washed in running water. Slides were submitted to antigenic recuperation by heat using a (Eterna®, Nigro) pressure cooker and a 10 mM pH 6.0 solution. Next, endogenous peroxidase blocking was done with a 3% hydrogen peroxide solution (10 vol peroxide) with four changes at 5-minute intervals, followed by washings with PBS—*phosphate buffered saline*—10 mm and pH 7,2 for 5 min. Slides were incubated with ezrin protein primary monoclonal antibody previously described, diluted in PBS buffer containing bovine albumin (BSA) at 1% (SIGMA, A9647, USES) and sodium azide (NaN_3) at 0.1% for 18 h at 4°C, in a wet chamber. After incubation, slides were washed

in PBS buffer with three exchanges at 3-minute intervals. Slide incubation with the secondary antibody, biotynylated C-reagent (Biotinylated goat anti-mouse/rabbit Ig) of the kit StreptABComplex/HRP Duet (mouse/rabbit) (Dako A/S, K492, Denmark), in the preestablished 1:200, diluted in PBS, for 30 min at 37°C and subsequently washed in PBS buffer with three exchanges at every 3 min. Then a reagent (Streptavidin) incubation was done in the preestablished 1:200, diluted in PBS for 30 min at 37°C and subsequently washed in buffer PBS with three exchanges of 3 min each. The slides were then incubated in a 3.3' solution substrate diaminobenzidine tetrahydrochloride (DAB) 60 mg% (Sigma, D-5637 USA), 1 mL dimetilsulphoxide (DMSO), 1 mL peroxide 6% 100 mL PBS, for 5 min at 37°C sheltered from light. After the observation of the development of the brownish precipitate, the slides were washed in running water and distilled water for 3 min and counter-stained with Harris Hematoxylin de Harris (Merck) for 1 min. Slide dehydration was then done in ethanol and xylol. The reactions were accompanied by a positive control in tissue known to be positive for the antibody tested, and by a negative control carried out by the omission of the primary antibody.

Slides were read in a common optical microscope by only one pathologist. When the protein was not expressed we considered it negative and gave it score 0, but when cytoplasmatic expression was observed, results regarding intensity were distributed in: 1 = weak positivity, 2 = moderate positivity and 3 = strong positivity. The result of the expression for each patient was carried out multiplying each *core* analyzed of the same case and dividing results by the number of viable *colors*, that is, with the presence of tumor.

Statistical Analysis

Statistical analysis was done through the SPSS program for Windows, version 10.0, SPSS Inc. To check the association between protein expression and clinical and anatomopathological characteristics, we used chi-square test or Fisher's exact test. The calculation of the estimate of global survival was carried out by Kaplan-Meier technique, and the comparisons of survival curves regarding the studied variables, by logrank test. Multivariate analysis was done by Logistic Regression. For all analyses, $p<0.05$ values were considered significant. Due to the similarities of immunohistochemical results and to make viable some statistical calculations, it was necessary to group some categories, and groups I and II were built, according to immunohistochemical results, as is described below.

Results

Immunohistochemistry

Ezrin expression was evaluated in 253 cases having duplicate specimens of colorectal adenocarcinomas arranged in duplicate in six *Tissue Micro Arrays* (TMA). From 253 cases considered, 3 cases were excluded for lack of data, thus 250

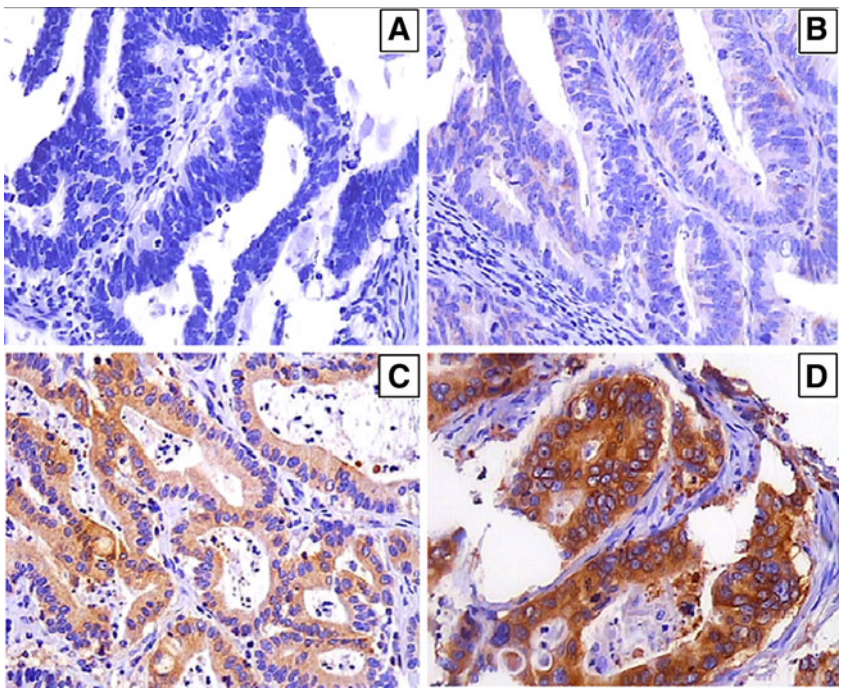

Fig. 1 Images of Ezrin Immunoreactivity in Colorectal Adenocarcinoma: **a** negative staining in malignant cells; **b** weak cytoplasmic positivity; **c** moderate cytoplasmic positivity; and **d** strong cytoplasmic positivity

Table 1 Characteristics of the selected sample and correlation with ezrin expression through univariate analysis

Variables	Categories	N	(%)	G I*	G II*	X^2	p
Age	Up to 50 years	50	20,0	45	5	0,208	0,648
	50 years or more	200	80,0	184	16		
Sex	Female	146	58,4	133	8	0,116	0,734
	Male	104	41,6	96	13		
Tumor location	Right colon	43	17.2	37	6	4.177	0.383
	Transverse colon	20	8.0	17	3		
	Left colon	21	8.4	19	2		
	Sigmoid Colon	56	22.4	53	3		
	Rectum	110	44,0	103	7		
Vascular Invasion	No	217	86.8	198	19	0.123	0.725
	Yes	33	13.2	31	2		
Lymphatic Invasion	No	172	68,8	161	11	3,212	0,073
	Yes	78	31,2	68	10		
Perineural Invasion	No	193	77,2	177	16	0,036	0,849
	Yes	57	22,8	52	5		
Anatomo-Pathological Margins	Free	245	98,0	225	20	1,653	0,199
	Compromised	5	2,0	4	1		
Degree of Differentiation	Well	56	22,3	54	2	23,769	<0,001
	Moderately	183	73,1	169	14		
	Poorly	11	4.4	6	5		
Character of Surgery	Curative	216	86.4	201	15	7.776	0.005
	Palliative	34	13.6	28	6		
T Stage	T1 and T2	64	25.6	62	2	2.465	0.116
	T3 and T4	186	74.4	167	19		
N Stage	N0	132	52.8	129	3	13.520	<0.001
	N1 and N2	118	47.2	100	18		
M Stage	M0	204	81.6	193	11	14.534	<0.001
	M1	46	18.4	36	10		
Stage	I and II	122	48,8	120	2	13.241	<0.001
	III and IV	128	51.2	109	19		

*G I: Group I—negative and weak positive and G II: Group II—moderate and strong positive

cases were statistically analyzed and divided according to ezrin immunohistochemical results in four different expression categories (Fig. 1). Tumors with no ezrin expression (80.4%), with a weak positivity (11.2%), a moderate positivity (6.4%) and a strong positivity (2.0%) were then divided in two groups for analysis: group I—negative and weak positivity and I group II—moderate and strong positivity.

Clinicopathological Characteristics and Correlation with Ezrin Using Univariate Analysis

Clinical and anatomophatological characteristics and ezrin expression are summarized in Table 1. As Table 1 shows, poorly differentiated tumors with positive lymph nodes and metastases at diagnosis present a higher expression of the protein. More often than not, patients submitted to palliative surgery had tumors with strong ezrin expression.

Correlation with Ezrin (Multivariate Analysis)

Multivariate analysis correlating ezrin with clinicopathological characteristics that showed a significant correlation in univariate analysis is presented in Table 2. Factors associated with a moderate/strong expression of ezrin were

Table 2 Clinicopathological characteristics and correlation with ezrin expression through multivariate analysis

Variables	Categories	Odds ratio	CI 95%	p
Degree of differentiation	Well/Moderated	1	3.2–71.7	0.024
	Poorly	15.2		
N Stage	N0	1	1.9–42.3	0.005
	N1/N2	9.7		
M Stage	M0	1	2.9–9.7	<0.001
	M1	5.3		

differentiation (OR 15.2), lymph node status (OR 9.7), and metastasis at diagnosis (OR 5.3).

Analysis of Survival and Correlation with Ezrin

5-year global survival curve is presented in Fig. 2. Mean survival time for Group I patients was 141.87 months (CI 95% 126.61–157.14) whereas patients of Group II the average was 33.16 months (CI 95% 20.58–45.74). Group I five-year survival rate was about 72.4%, with 49 deaths from 230 cases, while for Group II the rate was of 60%, with 8 deaths in 20 cases.

Discussion

Wald et al. [29] demonstrated that colonic ezrin is physiologically definite in normal tissue and showed its expression in colorectal cancer. In normal colonic tissue ezrin is located in the cell-cell adhesion region and has a weak presence in epithelium basal portion, whereas in colonic adenocarcinoma and colonic tumor lineage it is co-located in malignant cells cytoplasm [30].

In this study, ezrin protein expression increase in malignant epithelial cells cytoplasm is connected to tumor histological degree of differentiation, which can imply varied cell activation mechanisms, such as transduction of growth factors and adhesion molecules that induce survival and migration capacity. As cell migration is essential for tumor progression, to investigate proteins linked to cell regulation and motility such as ezrin may help to understand how tumor cells develop metastatic ability, as shown in previous studies [5, 9, 13, 29]. Increased ezrin expression in the primary tumor may directly contribute to metastatic process [11, 23] and thus to a worse prognosis [19, 31, 33, 34].

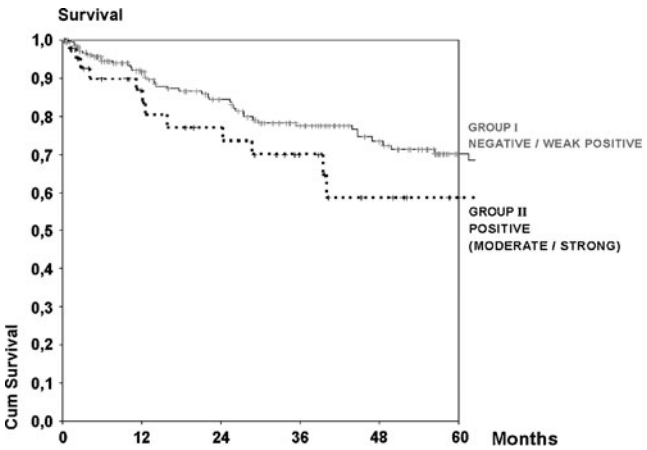

Fig. 2 Calculation of the estimate of global survival by Kaplan-Meier method. Comparisons among survival curves of Group I and Group II by *logrank* test ($p<0{,}001$)

Recently, Elzagheid et al. [32] suggest that ezrin may play a role in colorectal cancer progression and that ezrin expression might provide clinically valuable information in predicting the biological behavior of colorectal cancer. In our study, ezrin expression was higher in tumors with lymph nodes invasion, distant metastasis and also in poorly differentiated tumors, as confirmed by univariate and multivariate analyses. Besides, survival analysis shows a strong correlation between protein expression and prognosis. Patients having ezrin expression of moderate to strong intensity at immunohistochemistry had a lesser fiver-year global survival rate when compared to patients with a negative or low-intensity protein expression. Similar results are verify by Wang et al. [33] in that ezrin expression is higher in colorectal cancer tissues than in normal colorectal mucosa tissues, and the high level of ezrin expression is closely related to the colorectal cancer invasion and metastasis process.

Bal et al. [35] showed that gastric adenocarcinomas infected by *H. pylori* presented had a higher ezrin expression in neoplastic cells. *H. pylori* is said to have an important role in carcinogenesis of gastric carcinomas of the intestinal type [36].

A precise explanation of the mechanism by which the ezrin protein contributes to tumor dissemination is still not well established, but it is known that when activated, ezrin interacts with the membrane protein and with cytoskeleton actin and may affect cell processes of migration, invasion, adhesion and survival, which are important for the establishment of cancer progression [37]. Ohtani et al. [20] showed that ezrin transcription is necessary for invasion and consequent tumor progression. Our study suggests that this may really have a strong influence in the disease progression, but it cannot be taken as the only determinant, since multiple factors are involved in cancer dissemination. Nevertheless, our results corroborate other reports on the importance of ezrin expression in these processes.

The increase of cytoplasmic ezrin expression has a strong correlation to colorectal cancer and with the main clinicopathological characteristics that show the possibility of metastatic disease. This is probably due to its direct or indirect contribution in tumor progression through growth factors, adhesion molecules of and other factors.

Conclusions

We may conclude that a higher cytoplasmatic ezrin expression correlates with higher tumor aggressiveness and a worse prognosis in colorectal cancer patients. Thus

ezrin protein may be an important target for anti-metastatic therapies and its expression is an important prognostic factor for the disease.

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Conflict of interest The senior author, Marcelo Patara, certify that the authors of the article don't have any commercial association that might pose a conflict of interest in connection with the submitted article.

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