Original Article

# High ezrin immunoreactivity is an indicator of poor survival in squamous cell carcinoma of uterine cervix

**Tahany Mohamed Shams, Hanaa Omar Badr-El-Din[1], Iman Loay Hussein Abulkheir[2]**

Department of Pathology, Suez Canal University, Ismailia, [1]Department of Obstetrics and Gynecology, Al-Azhar University, [2]Department of Pathology, National Cancer Institute, Cairo University, Egypt

## ABSTRACT

**Context:** Ezrin, the membrane-linking protein, is highly expressed in several types of human cancers, and correlations between its immunoreactivity and histopathological data as well as patient outcome have previously been shown. **Aims:** The aim of our study was to investigate the expression of ezrin in cases of cervical squamous cell carcinoma, then correlate the data with survival and other clinicopathological variables. **Materials and Methods:** We carried out immunohistochemical analysis of ezrin in 64 cases of cervical cancer and correlated it to clinicopathologic variables and disease outcome. **Results:** Ezrin was identified in 50 (78.1%) of 64 studied cervical cancer cases, which was classified as weak, moderate and marked in 16%, 28% and 56%, respectively. In contrast to the predominantly membranous immunoreactivity in normal cervical epithelium, it was cytoplasmic in cancer cells. Ezrin expression significantly correlated with lymph node metastasis and development of distant metastasis ($P<0.001$ and 0.0006, respectively), as well as with tumor grade and stage ($P=0.02$ and 0.05, respectively), but not with other variables such as age, tumor size, and histological type. Regarding outcome, high ezrin expression was associated with short disease-specific survival (DSS) and disease-free survival (DFS) and poor metastasis survival (MS); ($P<0.001$). Ezrin expression significantly predicted both the 5-year MS, DSS, and DFS, recording $P$ value $<0.001$ in each. **Conclusions:** Our results suggested that ezrin expression may represent an effective prognostic marker and a potential target for treatment of invasion and metastasis in cervical cancer.

**Keywords:** Cervical cancer, clinicopathological, ezrin, immunohistochemistry, patient survival, squamous cell carcinoma

## INTRODUCTION

Cervical cancer (CC) is one of the most common cancers affecting women worldwide. It was responsible for an estimated of 12,200 cases and 4210 deaths in the United States in 2010.[1] It is one of the most common gynecologic cancers in the developing world; 80% of cases occur in low-income regions. Approximately 230,000 women die from cervical cancer annually; over 190,000 of them are from developing countries.[2] In years 2003 and 2004, 169 cases of cervical cancer were referred to National Cancer Institute, representing 36.58% of cancers affecting female genital tract.[3]

Prognosis of cervical cancer is dependent on classical clinicopathological parameters such as tumor grade, stage of disease, vascular invasion and lymphatic invasion.[4] Nonetheless, even within a group of patients with a low risk of disease progression (well differentiated, FIGO stage I), some patients suffer from early recurrence or disease-related death. Therefore, additional molecular factors seem to be needed to individualize both patient prognosis and therapy.

Ezrin is a membrane cytoskeleton cross-linker protein that is a member of the ERM (ezrin/radixin/moesin) family.[5] It regulates the determination and maintenance of cell shape, cell adhesion to the extracellular matrix, cell-cell interaction, receptor tyrosine-kinase signaling, signal transduction pathway and interactions with the Akt-mediated cellular apoptotic machinery. Ezrin plays an important role in cell motility, cell polarity, cytokinesis, phagocytosis, and stabilization of intercellular junctions; and participates in membrane trafficking pathways and integration of

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**Address for correspondence:** Dr. Tahany Shams, Department of Pathology, Suez Canal University, Ismailia, Egypt. E-mail: tahanishams@hotmail.com

membrane transport with signaling pathways.[6]

Ezrin binds adhesion molecules such as CD43, CD44, ICAM-1, and ICAM-2, which are implicated in cell migration and metastasis. Several studies suggest a metastasis-promoting role for ezrin in several tumor entities by facilitating tumor cell motility. These include pancreatic carcinoma,[7] breast carcinoma,[8] ovarian carcinoma,[9] melanoma,[10] and osteosarcoma.[11] Furthermore, inhibition of ezrin by antisense oligonucleotides blocked invasiveness of endometrial adenocarcinoma cell lines.[12] However, study of the expression of ezrin in cervical carcinoma and its prognostic significance is very limited.

In order to gain further insights into the expression of ezrin and its importance in progression of cervical cancer, we studied the expression of this protein in a set of cervical cancers, then correlated expression of ezrin with clinicopathological parameters as well as patient survival including disease-specific survival (DSS), disease-free survival (DFS), and metastasis survival (MS).

## MATERIALS AND METHODS

### Patients and Clinical Parameters

A total of 64 female patients diagnosed as cervical squamous cell carcinoma (SCC) were included in this study between January 2001 and December 2005. Patients were diagnosed, treated and followed up at the National Cancer Institute, Cairo University, and the Departments of Obstetrics and Gynecology at Al Zahraa university hospital in Al-Azhar univershty for girls-only and Suez Canal University Hospital.

Paraffin-embedded archival tissue blocks concerning these cases were obtained from the Departments of Pathology, National Cancer Institute, Cairo University; Faculties of Medicine, Suez Canal and Al-Azhar Universities. All hematoxylin and eosin-stained sections were reviewed, the quality of the material was checked, and the best sections from each specimen were selected. Patients' medical records were reviewed to obtain all available clinicopathological characteristics and follow-up data. The clinical staging of cervical carcinoma was established according to International Federation of Gynecology and Obstetrics (FIGO) staging system.[13] The histological classification of cervical carcinoma was based on the World Health Organization (WHO) classification.[2]

Patients with stage I–IV cervical SCC were treated surgically with radical hysterectomy plus bilateral pelvic and para-aortic lymph node dissection or anterior pelvic exenteration. All patients were initially diagnosed by Pap smear, visual inspection with acetic acid and colposcopy, then punch biopsy or conization to confirm the diagnosis of invasive SCC. The standard preoperative preparation protocol has included hematological and biochemical evaluation, chest radiograph, an electrocardiogram, and abdominopelvic ultrasound. Additional radiological studies including intravenous pyelography, computed tomography, magnetic resonance imaging, rectosigmoidoscopy or cystoscopy were performed when clinically indicated.

This study also included 10 patients with normal cervices who underwent total abdominal hysterectomy for causes other than cervical neoplasia, which were confirmed by histopathologic examination of the cervix.

### Technique of immunostaining

One 4-μm section from each submitted paraffin block was first stained with hematoxylin and eosin in order to verify the diagnosis and the presence of adequate tissue for immunohistochemical staining. Serial sections (4-μm) were prepared from the selected blocks and float mounted on adhesive-coated glass slides for immunostaining. Slides were de-waxed with xylene and gradually rehydrated. Endogenous peroxidase activity was blocked in (0.3% hydrogen peroxidase for 15 min), and then antigen retrieval was achieved by using microwave heating (three times of 10 min; 10 mM citrate buffer, pH 6.0). For detection of ezrin, we used mouse monoclonal antibody clone 3C12 from Sigma (Deisenhofen, Germany). Primary antibody was diluted 1:500 for ezrin in phosphate-buffered saline (PBS). The primary antibody for this protein was incubated overnight at 4°C. The standard avidin-biotin peroxidase complex technique was carried out by using the universal ABC peroxidase kit (ultra-vision detection system, Anti-polyvalent, ready to use, LAB VISION, New York, United State, USA). The sections were incubated with secondary antibody for 15 min, and then the detection of bound antibody was accomplished using the ABC reagent for 20 min; each step was followed by PBS wash. Finally, 0.1% solution of diaminobenzidine (DAB) was used for color development and Mayer's hematoxylin was used for counterstaining. Negative controls were obtained by omitting the primary antibody. Colonic adenocarcinoma and intratumoral scattered lymphocytes were used as positive control.

### Evaluation of immunostaining

All slides were scored independently by two investigators, blinded to patient outcome, by means of light microscopy. Each tumor was represented by one tissue slide. Staining intensity and percentage of positive tumor cells were estimated in each case. In cases where the score differed more than 5%, consensus was achieved at a multi-headed microscope. Cytoplasmic staining intensity of ezrin protein was evaluated.

The mean percentage of positive tumor cells was determined in at least 10 randomly selected fields at 400-fold magnification and the cutoff level for positive cases was 10%. The intensity of immunoreactivity was initially scored as a four-category variable in comparison to positive control: negative (immunoreactivity 0), weak (immunoreactivity 1), intermediate (immunoreactivity 2) and marked (immunoreactivity 3). Because tumors showed heterogeneous staining, the dominant pattern was used for scoring.[14]

### Statistical analysis used

The data were collected, tabulated and processed using SPSS version 16. Frequency tables were analyzed using the $X^2$ test, with likelihood ratio or Fisher exact test for significance between variables in the same category. Univariate survival analyses for DSS (disease-specific survival), DFS (disease-free survival) and MS (metastasis survival) were made, and survival curves were formatted according to Kaplan-Meier method with probing for significance by a log-rank test. DSS was measured since the diagnosis of cervical cancer to the patient's death or when the patient was last seen alive; (all deaths because of other causes not related to cervical cancer were excluded). MS was defined as the period from the diagnosis of recurrent disease until death or until last visit when seen alive. DFS was measured as the time from the diagnosis to the appearance of recurrent disease. To calculate relative risk (RR) with 95% confidence interval (CI), we categorized our results concerning ezrin expression into two groups where (0 and 1) (negative and weak) and (2 and 3) (intermediate and marked) as group I and group II, respectively.

ANOVA was used for deriving the 95% CI for each category. Also, multivariate regression analysis based on the Cox's proportional hazard model was used to test the independence of ezrin expression in the prediction of DSS, DFS and MS. Tests were considered significant when *P* values were ≤0.05 and highly significant when *P* value ≤0.01

## RESULTS

### Clinico-pathological data of studied cases [Table 1]

Patients were selected in this retrospective study based on their diagnosis, specimens, clinical data and follow-up availability. Of these 64 cases; the age at diagnosis of the studied group ranged from 28 to 75 years (mean age was 50.9 years). Regarding FIGO stage of tumor, 5/64 (7.8%) cases were of stage I, 21/64 (32.8%) were of stage II, 10/64 (15.6%) were of stage III, and 28/64 (43.8%) were of stage IV. Regarding tumor size, 34 patients had tumor sizes <4 cm, while the remaining 30 cases had tumors ≥4 cm. With respect to histological type, 31 patients had keratinizing SCC while non-keratinized type was diagnosed in 33 cases. Well-differentiated tumors were detected in 7 cases (10.9%), while tumors showed moderate and poor grade of differentiation in 40 (62.5%) and 17 (26.6%) cases, respectively. Pelvic and/or para-aortic lymph node metastases (N) were reported as N0 in 24 (37.5%) and N+ in 40 (62.5%) of cases. Development of distant metastasis (M) was reported as (M0) in 36 (56.2%)

**Table 1: Expression of ezrin according to clinicopathological characteristics of the cervical cancer patients**

| Variable | Ezrin positive | | Ezrin negative | | Total | *P* value* |
|---|---|---|---|---|---|---|
| | No. | (%) | No. | (%) | | |
| Patients | 50 | 78.1 | 14 | 21.9 | 64 | - |
| Age | | | | | | |
| <50 years | 30 | 78.9 | 8 | 21.1 | 38 | 0.85 |
| ≥50 years | 20 | 76.9 | 6 | 23.1 | 26 | |
| Tumor size | | | | | | |
| <4 cm | 28 | 82.4 | 6 | 17.6 | 34 | 0.38 |
| ≥4 cm | 22 | 73.3 | 8 | 26.7 | 30 | |
| Histological type | | | | | | |
| Keratinizing SCC*** | 26 | 83.9 | 5 | 16.1 | 31 | 0.28 |
| Non-Keratinizing SCC | 24 | 72.7 | 9 | 27.3 | 33 | |
| Tumor grade | | | | | | |
| Grade I | 335 | 42.9 | 4 | 57.1 | 7 | 0.02* |
| Grade II | 12 | 87.5 | 5 | 12.5 | 40 | |
| Grade III | | 70.6 | 5 | 29.4 | 17 | |
| FIGO stage | | | | | | 0.05* |
| Stage I | 1 | 20.0 | 4 | 80.0 | 5 | |
| Stage II, III, IV | 49 | 98.1 | 10 | 16.9 | 59 | |
| Primary nodal status (N) | | | | | | |
| No | 10 | 41.7 | 14 | 58.3 | 24 | <0.001** |
| N+ | 40 | 100 | 0 | 0 | 40 | |
| Metastasis (M) | | | | | | |
| M0 | 22 | 61.1 | 14 | 38.9 | 36 | 0.0006** |
| M+ | 28 | 100 | 0 | 0 | 28 | |

**P* value ≤0.05 is statistically significant, ***P* value ≤0.01 is statistically highly significant, ***SCC; Squamous cell carcinoma

and M1 in 28 (43.8%) of cases.

### II-Immunohistochemical Expression of Ezrin in cervical tissue

The normal epithelium of the cervix showed predominantly membranous staining intensity with faint cytoplasmic staining [Figure 1a]. In cervical cancer, negative ezrin staining was detected in 14 cases (21.9%). [Figure 1b]. Ezrin was expressed in 50 cases (78.1 %): 8 (16%) showed weak (1), 14 (28%) had intermediate (2), and 28 (56%) had marked (3) ezrin expression as shown in Figure 1c, 1d, and 1e, respectively.

We observed heterogenous pattern of staining in cancer cases; the groups of cells were usually weakly stained but the staining became more intense at invasive margin of the tumor and at poorly differentiated areas where tumor cells lose their cell-to-cell contact.

### III-The Association of Ezrin expression with clinicopathological data

Relations between ezrin expression and various clinicopathologic variables of cancer cases are listed in Table 1. The statistical evaluation of ezrin expression according to age, tumor size, and histological type showed no significant correlation (*P*=0.85, 0.38 and 0.28, respectively), while significant correlation was obtained with tumor grade, *P* value 0.02. When we classified the FIGO stage into two groups, there was statistically significant correlation between ezrin expression and FIGO stage (*P*=0.05), where 1/5 (20%) of patients with stage I demonstrated ezrin expression, while 98% of patients with stages II–IV were positive for ezrin expression. Patients with lymph node metastases demonstrated significantly higher rates of ezrin expression as compared with patients without lymph node metastases (100% vs. 41.7% respectively, *P*<0.001). Moreover, all cases (100%) who developed metastatic recurrence (M1) showed ezrin expression in comparison to 22/36 (61.1%) of M0 cases, recording statistically significant relation (*P*=0.0006).

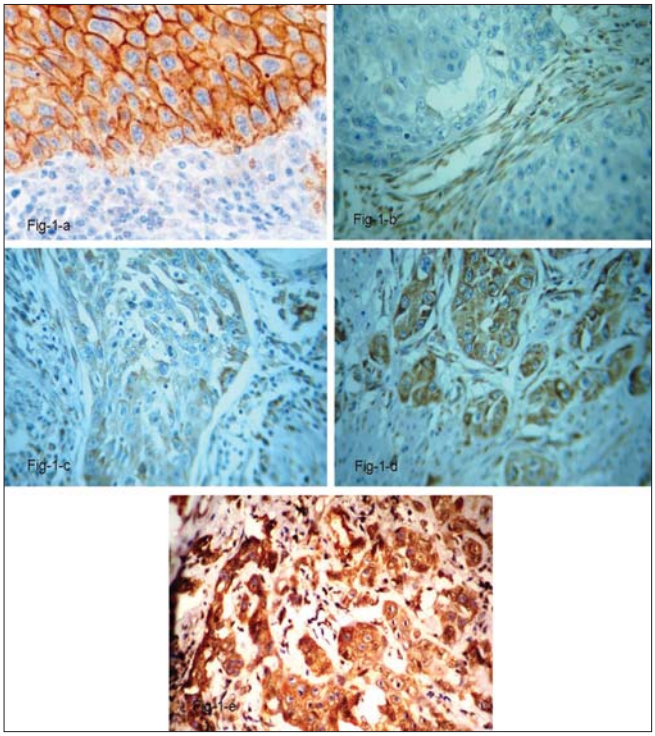


**Figure 1:** (a) Normal cervical squamous epithelium showed membranous ezrin expression (× 400). (b) Negative ezrin immunoreactivity (0) (× 200), (c) Weak ezrin immunoreactivity (1) (× 400), (d) Intermediate ezrin reactivity (2) (× 100), (e) Marked ezrin immunoreactivity (3) (× 200)

### IV-Correlation of Ezrin expression and overall survival

Patients were enrolled into this retrospective study between January 2001 and December 2005 and followed up until July 2009 for a median of 35.9 months (range 6.8–130.7 months). For survival analysis, we dichotomized grading of ezrin expression into two groups (negative/weak) and (moderate/marked).

As shown in Table 2, high ezrin expression was significantly related to DSS. The DSS was longer in patients with negative/weak ezrin expression compared with moderate/marked ezrin expression (mean month was 60.1±25.2 months versus 24.5±8.9 months, respectively) with significant *P* value of <0.001. Moreover, patients with moderate/marked ezrin expression had a mean of 20.0±8.5 months as regard metastasis survival compared with mean of 59.0±23.2 months in patients with negative/weak expression (p<0.001). This result indicates that high ezrin expression is an indicator of poor metastasis survival. Regarding DFS, patients with moderate/marked ezrin expression had a mean of 18±9.8 months compared with mean of 54.5±25.1 months in patients with negative/weak expression (p<0.001).

In univariate analysis (Kaplan–Meier), ezrin expression significantly predicted 5-year MS [Figure 2], DSS [Figure 3] and DFS [Figure 4], recording *P* value <0.001 in each.

**Table 2: Comparison between ezrin expression and survival in cervical cancer**

| Ezrin<br>Follow-up | Ezrin negative/ weak (- /1) | Ezrin intermediate/ marked (2/3) | *P*-value* |
|---|---|---|---|
| Disease specific survival (DSS) (Mean months ± SD) | 60.1 ± 25.2 months | 24.5 ± 8.9 months | <0.001 |
| Metastasis survival (MS) (Mean months ± SD) | 59.0 ± 23.2 months | 20.0 ± 8.5 months | <0.001 |
| Disease free survival (DFS) (Mean months ± SD) | 54 ± 25.1 months | 18.0 ± 9.8 months | <0.001 |

*(ANOVA); *P* value ≤0.01 is statistically highly significant

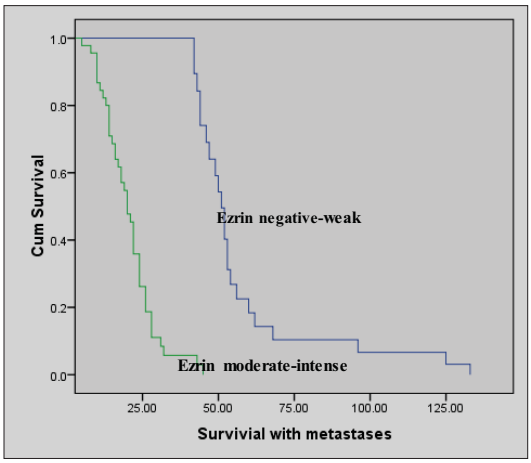


**Figure 2:** The 5-year metastasis survival (MS) (cumulative survival) and its relation to the ezrin expression. The tumors were grouped into two groups: negative and weak (-/+) and moderate and intense (++/+++)

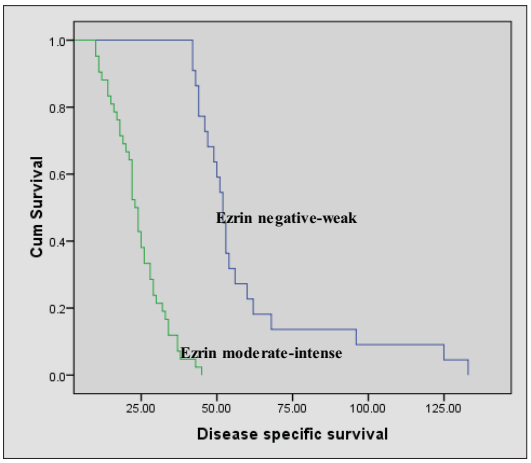


**Figure 3:** The 5-year disease-specific survival (DSS) (cumulative survival) in relation to the ezrin expression. The tumors were grouped into two groups: negative and weak (-/+) and moderate and intense (++/+++)

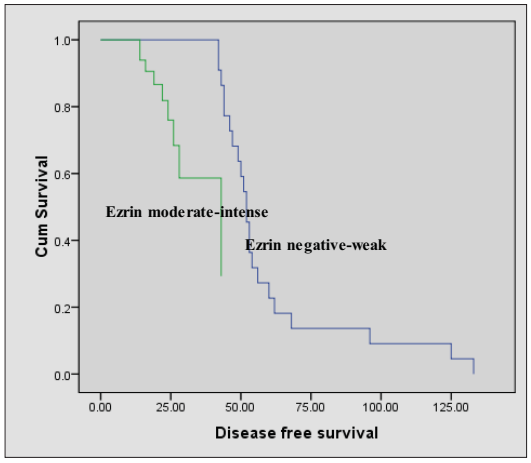


**Figure 4:** The 5-year disease-free survival (DFS) (cumulative survival) in relation to the ezrin expression. The tumors were grouped into two groups: negative and weak (-/+) and moderate and intense (++/+++)

As shown in Table 3, linear regression analysis for predictors of DSS, DFS and MS, including age, tumor grade, tumor stage, tumor size, tumor type and ezrin expression, tumor stage was proved to be significant predictor for DSS ($P$=0.043) as well as ezrin expression ($P$=0.003). Also, ezrin expression was significant predictor for MS and DFS ($P$<0.001).

For multivariate survival analysis for DSS, all variables including age, tumor grade, stage, tumor type, tumor size and ezrin expression were included in the multivariate analysis using Cox's regression model. Multivariate risk factors were tumor grade (Rr = 0.567, $P$ value = 0.017) and ezrin expression (Rr = 34.2, $P$ value <0.001) as shown in Table 4.

As regard DFS, multivariate analysis revealed that only ezrin expression was an independent risk factor (Rr = 28.688, $P$ value <0.001) as shown in Table 5.

## DISCUSSION

Cervical cancer is still one of the prime concerns in public health, mainly in the developing world, and it is considered as one of the most frequent female genital tract carcinomas as well as one of the leading causes of female cancer-related death. [2]

Ezrin is essential for cell-to-cell adhesion and is also necessary for several signaling pathways; that is, MAPK (mitogen-activated protein kinase), Akt, CD44, Rho kinase and HGF, which are identified as tumor metastasis-associated proteins.[11,15,16]

It is reported that ezrin is physiologically expressed in a variety of epithelial tissues including intestine, lung, and kidney. Within these tissues, it is found in the apical region of microvilli presenting cells, suggesting an involvement in defining cell polarity. However, besides its localization to microvilli, ezrin is also targeted to the leading edge of spreading cells, suggesting an essential role in controlling cell motility. To date, several binding partners for ezrin have been identified. The N-terminal binding domain of ezrin mediates membrane attachment by binding the cytoplasmic tail of CD44, CD 43 or intercellular adhesion molecules. On the other end, ezrin via its C-terminal domain associates with F-actin and contributes to microfilament organization.[8]

We aimed in this study to examine ezrin expression in cervical carcinoma and its correlation with clinicopathological parameters and clinical outcome.

Interestingly, we observed shifting of cellular localization of ezrin when comparing normal cervical epithelium and cervical carcinomas. We detected predominantly

**Table 3: Linear regression analysis for predictors of DSS, MS, and DFS**

| Variable | $R^2$ | *P*-value | Significant predictor (*P*-value) |
|---|---|---|---|
| DSS* | 0.241 | 0.012 | Tumor stage (0.043)<br>Ezrin (0.003) |
| MS** | 0.267 | 0.005 | Ezrin (<0.001) |
| DFS*** | 0.231 | 0.015 | Ezrin (<0.001) |

Predictors (age, tumor grade, tumor stage, tumor size, tumor type, ezrin stain), * DSS: disease-specific survival ** MS: metastasis survival ***DFS: disease-free survival

**Table 5: Multivariate regression analysis for disease-free survival (DFS) (Cox's regression model)**

| Item | Relative risk | 95% CI | *P*-value |
|---|---|---|---|
| Age | 1 | 0.983–1.023 | 0.799 |
| Tumor grade | 0.695 | 0.437–1.104 | 0.123 |
| Tumor stage | 0.875 | 0.661–1.159 | 0.352 |
| Tumor size | 0.894 | 0.520–1.537 | 0.684 |
| Tumor type | 0.626 | 0.363–1.081 | 0.093 |
| Ezrin expression | 28.688 | 9.828–50.7 | <0.001 |

membranous ezrin expression in sections of normal cervical epithelium, while ezrin expression was predominantly cytoplasmic in cases of cervical carcinoma. Ezrin was expressed in 50/64 cases (78.1%) of cervical carcinoma; the staining intensity was weak (1) in 8 (16%), intermediate (2) in 14 (28%) and marked (3) in 28 (56%). Cytoplasmic staining was even accentuated in carcinoma cells losing their cell–cell contacts.

Ezrin is exchanged between cytoplasm and membrane; cytoplasmic ezrin exists in a "closed" conformation based on intra- or inter-molecular interactions between the N- and C-terminus. Threonine and tyrosine phosphorylation induces an "open" conformation. In this state, ezrin is localized towards the membrane where it modulates F-actin dynamics and tethers the microfilament system to the cytoplasmic face of cell adhesion sites.[5] Disruption of actin filaments and a decrease in focal adhesion are common features of epithelial–mesenchymal transition, which is associated with the onset of invasion. Besides a structural role, ezrin may as well act as a signaling or scaffold molecule. It was revealed that ezrin is involved in modulating signaling pathways acting through Rho [17] and phosphatidylinositol 3-kinase/Akt.[6]

These findings suggested that the change of ezrin location might have a key role in the progression of cervical cancer. Ferrari *et al*., 2008,[18] reported that the 3-year probability of disease-free survival was 80% for patients having osteosarcoma with only cytoplasmic immunostaining and 54% for patients with cytoplasmic and membranous immunostaining ($P<0.02$). They also suggested that the pattern of ezrin staining can identify patients with different risks of relapse.

**Table 4: Multivariate regression analysis for disease-specific survival (DSS) (Cox's regression model)**

| Item | Relative risk (Rr) | 95% confidence interval (CI) | *P*-value |
|---|---|---|---|
| Age | 0.999 | 0.978-1.02 | 0.939 |
| Tumor grade | 0.567 | 0.355-0.905 | 0.017* |
| Tumor stage | 0.833 | 0.628-1.105 | 0.204 |
| Tumor size | 0.945 | 0.559-1.599 | 0.833 |
| Tumor Type | 0.602 | 0.35-1.033 | 0.065 |
| Ezrin expression | 34.2 | 11.4-50.1 | <0.001** |

*$P$ value ≤0.05 is statistically significant, ** $P$ value ≤0.01 is statistically highly significant

We detected statistically significant correlation between ezrin expression and FIGO stage and tumor grade along with our results. Tan *et al*., 2011,[19] reported that ezrin expression was significantly higher in the cancerous tissues of patients with advanced cervical cancer as high-stage and high-grade than in those of patients with early-stage and grade cervical cancer ($P<0.05$).

In our study, ezrin expression was found to increase with lymph node metastasis and development of distant metastasis; in both instances the correlation was statistically significant. The same finding was shared by Tan *et al*., 2011,[19] who reported that ezrin was expressed at significantly higher levels in lymph node metastasis-positive patients than in lymph node metastasis-negative patients ($P<0.05$). Our results are in agreement with others that reported the association between ezrin expression and metastatic potential in different cancers.[11,20,21]

The mechanism by which ezrin links to increased metastasis of different neoplasms is still open; one of the possibilities is related to oncogenic non-receptor tyrosine kinase c-Src,[22] which has been linked to deregulation of cell-cell adhesion and actin cytoskeleton and induction of an invasive phenotype. It has been shown to be overexpressed in human neoplasms.[8,23-25] Ezrin is one of the downstream targets of c-Src[26,27] and it has been suggested to cause deregulation of cell-cell contacts and dissemination of cancer cells sharing in metastatic potentiality.[8]

We also detected significant correlation between ezrin immunoreactivity and as well as DSS, DFSMS. Patients with negative/weak ezrin expression had longer DSS, DFS, and MS compared to patients with moderate/marked ezrin expression.

## CONCLUSION

In the present study, we showed that ezrin expression was correlated with important prognostic parameters of cervical carcinoma, including FIGO stage, lymph node metastasis, as well as distant metastasis. These results strongly pointed

to the relation between ezrin expression and disease progression in cervical carcinoma.

Moreover, high expression of ezrin was associated with poor patient outcome, reflected in short DSS, short DFS and poor MS, which points to the prognostic role of ezrin.

Finally, we strongly suggest that ezrin is an effective prognostic marker, as high ezrin expression is proved to be a reliable marker, which can predict 5-year DSS, DFS and MS in cervical carcinoma. Moreover, ezrin could represent potential target for treatment of invasion and metastasis in cervical cancer.

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