High expression of EZR (ezrin) gene is correlated with the poor overall survival of breast cancer patients

Rongju Zhang1, Shaohui Zhang2, Rongge Xing1 & Qin Zhang3
1 Department of Pathology, Cangzhou Central Hospital, Changzhou, China
2 Department of Orthopaedics, Cangzhou Hospital of Integrated Traditional Chinese and Western Medicine of Hebei Province, Cangzhou, China
3 Department of Thyroid and Breast Surgery, Cangzhou Central Hospital, Changzhou, China

Keywords
Breast cancer; EZR gene; immunohistochemistry; prognosis.

Abstract
Background: To evaluate the EZR (ezrin) gene expression in breast cancer and correlation with the prognosis through bioinformatics analysis and immunohistochemistry assay.

Methods: EZR gene expression in breast cancer and corresponding normal breast tissue was compared in the TCGA database. Protein-protein interaction (PPI) network relevant EZR was established through the STRING database. The correlation between EZR expression and prognosis of breast cancer was analyzed by the log-rank analysis from the TCGA. Ezrin protein (coded by EZR) expression was also examined by immunohistochemistry assay in 120 breast cancer patients.

Results: EZR expression level in tumor tissue was significantly upregulated compared to that of normal breast tissue of breast cancer patients ($P < 0.05$). In the PPI analysis, there were 51 nodes and 455 edges in the network. The top 10 hub genes of the network were identified. High expression of EZR mRNA was correlated with poor overall survival (OS) of the breast cancer patients (HR = 1.40, $P = 0.038$). However, the disease-free survival (DFS) of breast cancer patients did not correlate with the EZR mRNA level (HR = 0.86, $P = 0.44$). The ezrin protein expression was positive with uniform brown-yellow granules in the cell membrane, cavity surface and cytoplasm of the breast cancer cells. Of the included 120 cancer samples, 98 cases were positive for ezrin expression and 22 were negative. No correlation was found between ezrin expression site and patients’ clinicopathological features.

Conclusion: EZR is upregulated in breast cancer and can be used as potential biomarker for overall survival.

Introduction

Breast cancer is known as one of the leading causes of malignant carcinoma-related death globally. In recent years, the incidence of breast cancer had gradually increased, surpassing cervical cancer and becoming the highest incidence of malignant tumors for women. It has been estimated that around 1.2 million breast cancer patients in the world are diagnosed each year. In China, the incidence of breast cancer is about 23/100000, accounting for 7–10% of all malignant tumors. However, the molecular mechanism of breast cancer remains unclear.

Studies have demonstrated that EZR gene is upregulated in malignant solid carcinoma and is correlated with patients' prognosis. However, correlation between EZR gene expression in breast cancer and its correlation with patients' survival is not fully understood. In recent years, with the development of cancer databases, more and more studies have been published in the aspects of deep data mining in the database through bioinformatics analysis. In the present work, we investigated the EZR gene expression in breast cancer, function enrichment and correlation with the prognosis through bioinformatics analysis and immunohistochemistry assay in order to provide a greater understanding of the EZR gene in breast cancer.
Methods

**EZR expression in breast cancer and corresponding normal tissue**

EZR expression in breast cancer and corresponding normal tissue was identified from The Cancer Genome Atlas (TCGA) database through UALCAN online data mining website (http://ualcan.path.uab.edu/index.html). EZR expression in pan-cancer and corresponding normal tissue was also evaluated.

**PPI network construction and hub genes identification**

The search tool for the retrieval of interacting genes (STRING) (http://string-db.org/cgi/input.pl) was used to construct the protein-protein network (PPI) of EZR and relevant genes to evaluate the correlation between EZR expression and breast cancer. To screen the stable correlation between EZR and relevant genes, the maximum number of interactors was restricted to no

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**Figure 1** EZR expression in breast cancer and other malignant tumors (a) EZR mRNA expression in pan-cancers and corresponding normal tissue; (b) scatter plot of EZR expression breast cancer and normal breast tissue; (c) EZR expression in breast cancer (stages I–X).

**Figure 2** Protein-protein network analysis of EZR in breast cancer.
more than 50 and minimum required interaction score of 0.4. Ten hub genes were identified by Cytoscape (Cytoscape_v3.6.1, https://cytoscape.org/).

### Gene ontology (Go) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment

The biological function and KEGG pathway\textsuperscript{10–12} of EZR and relevant genes in the network was enriched. For GO enrichment,\textsuperscript{13} there was three aspects including biological process (BP), cellular component (CC) and molecular function (MF). The enrichment was demonstrated by the bubble chart which shows the gene ratio, Q-value, gene count and gene description.

### Survival analysis

According to the median expression level of EZR in breast cancer tissues, the patients were divided into high and low expression groups. Survival analysis (Kaplan-Meier curve) and log-rank test were used to calculate the difference of total survival (OS) and disease-free progression survival (DFS) between high and low expression groups through the log-rank analysis by the online data analysis tool GEPIA. GEPIA is a newly developed interactive web server.

![Figure 3 Hub gene identification of the protein-protein network relevant to EZR in breast cancer.](image)

**Table 1** Biological process enrichment of EZR and relevant genes

| Gene ratio | Q-value | Count | Description                                      |
|-----------|---------|-------|--------------------------------------------------|
| 45/5163   | 8.76E-17| 45    | Cellular component organization                   |
| 25/961    | 1.87E-16| 25    | Regulation of anatomical structure morphogenesis  |
| 32/2198   | 1.87E-15| 32    | Cell surface receptor signaling pathway           |
| 42/4738   | 2.29E-15| 42    | Signal transduction                               |
| 23/876    | 3.28E-15| 23    | Response to organonitrogen compound               |
| 27/1427   | 3.90E-15| 27    | Response to oxygen-containing compound            |
| 33/2524   | 4.59E-15| 33    | Regulation of localization                        |
| 46/6507   | 1.13E-14| 46    | Multicellular organismal process                  |
| 43/5459   | 2.13E-14| 43    | Positive regulation of biological process         |
| 45/6212   | 2.13E-14| 45    | Cellular response to stimulus                     |

![Figure 4 Go enrichment of EZR and relevant genes in the aspects of biological process.](image)
for analyzing the RNA sequencing expression data of 9736 tumors and 8587 normal samples from the TCGA and the GTEx projects, using a standard processing pipeline. GEPIA provides customizable functions such as tumor/normal differential expression analysis, profiling according to cancer types or pathological stages, patient survival analysis, similar gene detection, correlation analysis and dimensionality reduction analysis.

**Immunohistochemistry assay**

A total of 120 cancer tissue samples were included in the present work which was approved by the Ethical Committee of Cangzhou Central Hospital. All patients gave their signed informed consent. Ezrin protein (coded by EZR) expression was examined by immunohistochemistry assay in 120 breast cancer patient and ezrin expression and patients’ clinical characteristics were analyzed.

**Results**

**EZR expression in breast cancer**

EZR mRNA expression level in tumor tissue was significantly upregulated compared to that of normal breast tissue of breast cancer patients ($P < 0.05$). However, EZR mRNA expression level was not significantly different among different clinical stages (Fig 1).

**PPI analyses and hub genes identification**

To further investigate the role of the EZR gene in the development of breast cancer, we constructed the protein-protein network by STRING to identify the interaction between the relevant genes. There were 51 nodes and 455 edges in the network (Fig 2). The top 10 hub genes of the network were further screened through Cytoscape (Fig 3).

**GO and KEGG analysis**

EZR and network relevant genes were mainly enriched in the cellular component organization, regulation of anatomical structure morphogenesis, cell surface receptor signaling pathway and etc for the biological process, Fig 4, Table 1. For cellular component, the genes enriched in cell periphery, plasma membrane, plasma membrane bounded cell projection (Fig 5, Table 2) For molecular function, the EZR and network relevant genes were mainly enriched in protein binding, signaling receptor binding, and cell adhesion molecule binding (Fig 6, Table 3). KEGG pathway analysis indicated that the

| Table 2: Cellular component enrichment of EZR and relevant genes |
|---------------------------------------------------------------|
| Gene ratio | Count | Q-value | Description |
|------------|-------|---------|-------------|
| 46/5254    | 46    | 1.09E-18| Cell periphery |
| 43/5159    | 43    | 1.44E-15| Plasma membrane |
| 29/1900    | 29    | 1.04E-14| Plasma membrane bounded cell projection |
| 30/2651    | 30    | 4.21E-12| Plasma membrane part |
| 14/371     | 14    | 3.79E-11| Cell leading edge |
| 13/300     | 13    | 4.15E-11| Membrane raft |
| 20/1061    | 20    | 4.15E-11| Plasma membrane region |
| 14/432     | 14    | 1.50E-10| Actin cytoskeleton |
| 9/96       | 9     | 1.83E-10| Filopodium |
| 10/164     | 10    | 5.71E-10| Ruffle |

Figure 5: Go enrichment of EZR and relevant genes in the aspects of cellular component.
leukocyte transendothelial migration, proteoglycans in cancer, regulation of actin cytoskeleton were included in the KEGG pathway (Fig 7, Table 4).

Survival analysis

High expression of EZR mRNA was correlated with poor overall survival of the breast cancer patients (HR = 1.40, \( P = 0.038 \)), Fig 8a. However, the disease-free survival (DFS) of breast cancer patients was not correlated with the EZR mRNA level (HR = 0.86, \( P = 0.44 \)), Fig 8b. Subgroup analysis showed that the overall survival of HER2+ non-luminal breast cancer was significantly decreased in the EZR high expression group compared to the low expression group (HR = 3.7, \( P = 0.038 \)) (Fig 9).

EZR expression and clinicopathological features

The ezrin (coded by EZR gene) expression was positive with uniform brown-yellow granules in the cell membrane, cavity surface and cytoplasm of the cancer cell in breast cancer patients (Fig 10). Of the 120 cancer samples, ezrin expression was positive in 98 cases and negative in 22 cases. No correlation was found between ezrin expression site and patients’ clinicopathological features.

Discussion

Ezrin protein (coded by EZR) is a member of the ERM (ezrin-radixin-moesin) protein family.\(^{13}\) Its function is to connect cell surface receptors, especially adhesion molecules and actin cytoskeleton.\(^{14,15}\) Ezrin is mainly expressed at the top of cell surface and participates in maintaining the polarity of epithelial cells.\(^{16-18}\) Recent studies have found that ezrin participates in cells and bases interaction by regulating adhesion molecules and signal transduction pathways which may play an important role in the invasion and metastasis of cancer cells.\(^{19,20}\)

The role of ezrin is to change the infiltration potential of cancer cells, which can be divided into two aspects: (i) Forming complex with calyx glycoprotein to play an anti-adhesion role thereby reducing the adhesion between cancer cells, and (ii) activating calyx glycoprotein to participate in the remodeling of cytoskeleton, forming pseudopodia and enhancing the migrational ability of cells. Studies have shown that ezrin expression in tumors was significantly increased compared to corresponding normal tissues.\(^{21,22}\) At the same time,
time, the location of ezrin in tumor cells also changed when compared to normal cells. In normal cells, ezrin was mainly expressed in actin-rich microvilli, pseudopodia and other surface structures. However, in tumor cells, ezrin expression was positive in cytoplasm and cell membrane.\cite{23} A study in pleomorphic adenoma of the salivary gland showed that ezrin not only enhanced cell proliferation, but also promoted malignant transformation of pleomorphic adenoma.\cite{24} The expression level of ezrin in hepatocellular carcinoma cell lines was consistent with its invasive ability, and transfection of antisense oligonucleotides could significantly inhibit the invasive ability of cells.\cite{25} In addition, ezrin was also associated with metastasis of primary osteosarcoma according to the previous study.\cite{26} Approximately 55.7\% of nonmetastatic osteosarcoma cases express ezrin, and the prognosis of ezrin-positive expression osteosarcoma cases was poor compared

\begin{table}[h]
\centering
\caption{KEGG pathway enrichment of \textit{EZR} and relevant genes}
\begin{tabular}{llll}
\hline
Gene ratio & Count & Q-value & Description \\
\hline
15/112 & 15 & 2.52E-19 & Leukocyte transendothelial migration \\
16/195 & 16 & 7.12E-18 & Proteoglycans in cancer \\
16/205 & 16 & 1.01E-17 & Regulation of actin cytoskeleton \\
14/167 & 14 & 7.15E-16 & Tight junction \\
12/197 & 12 & 5.25E-12 & Focal adhesion \\
11/181 & 11 & 5.11E-11 & Chemokine signaling pathway \\
8/72 & 8 & 6.45E-10 & Bacterial invasion of epithelial cells \\
10/173 & 10 & 6.84E-10 & Axon guidance \\
9/133 & 9 & 1.69E-09 & Fluid shear stress and atherosclerosis \\
13/515 & 13 & 9.61E-09 & Pathways in cancer \\
\hline
\end{tabular}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{KEGG_enrichment}
\caption{KEGG enrichment of \textit{EZR} and relevant genes.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Survival_curve}
\caption{Survival curve for \textit{EZR} high and low expression group of breast cancer (\textbf{a}) Overall survival comparison between \textit{EZR} high (---) and low (---) expression group; (\textbf{b}) disease-free survival comparison between \textit{EZR} high (---) and low (---) expression groups.}
\end{figure}
Detection of ezrin expression in colorectal cancer showed that the expression of ezrin in colorectal cancer was significantly higher than that in normal colorectal tissues, and the expression of ezrin was correlated with the degree of differentiation, lymph node metastasis and Dukes stage.27

Figure 9 Survival curve for EZR high and low expression group of subgroup breast cancer (a) Overall survival of triple negative breast cancer; (b) disease-free survival of triple negative breast cancer; (c) overall survival of HER2+ non-luminal breast cancer; (d) disease-free survival of HER2+ non-luminal breast cancer; (e) overall survival of luminal A breast cancer; (f) disease-free survival of luminal A breast cancer; (g) overall survival of luminal B breast cancer; (h) disease-free survival of luminal A breast cancer.

Figure 10 Ezrin expression in breast cancer examined by immunohistochemistry assay. (a) Ezrin expression was localized in cancer cell membrane ×100; (b) Ezrin expression was negative in the cell membrane ×100; (c) Ezrin expression was positive in the cytoplasm of breast cancer cells ×100; (d) Ezrin expression was negative in the cytoplasm of breast cancer cells ×100.
In our present work, we have investigated the EZR gene expression in breast cancer, biological function, pathway and correlation with the prognosis through bioinformatics analysis and immunohistochemistry assay. We found that the EZR gene was upregulated in cancer tissue compared to normal breast tissue in breast cancer patients. This may indicate that EZR could play an important role in the development of breast cancer. Furthermore, we investigated the ezrin protein coded by EZR gene in 120 samples of breast cancer tissue by immunohistochemistry assay and found that the ezrin positive expression in 98 cases was present in 81.7% of all the cases. The EZR and relevant genes were mainly enriched in signaling receptor binding, cell adhesion molecule binding, leukocyte transendothelial migration, proteoglycans in cancer, regulation of actin cytoskeleton pathway. Survival analysis of the present work showed that high expression of EZR mRNA was correlated with poor overall survival of the breast cancer patients. However, the disease-free survival was not associated with the EZR expression level.

In conclusion, EZR mRNA was upregulated in breast cancer which was in accordance with its coding protein (ezrin) expression pattern detected by immunohistochemistry assay. The upregulated EZR mRNA and protein can be used as potential biomarker for overall survival in the future.

Disclosure
The authors confirm that there is no conflict of interest.

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