Significance of Cellular Distribution of Ezrin in Pancreatic Cystic Neoplasms and Ductal Adenocarcinoma

Ta-Sen Yeh, MD, PhD; Jeng-Hwei Tseng, MD; Nai-Jen Liu, MD; Tse-Ching Chen, MD, PhD; Yi-Yin Jan, MD; Miin-Fu Chen, MD

Hypothesis: Pancreatic cystic neoplasms and ductal adenocarcinoma manifest diverse clinical features and prognoses, which might be related to cellular distribution of ezrin modulated through various trophic molecules.

Design: Laboratory investigation and retrospective analysis.

Setting: Medical school–affiliated university hospital.

Patients and Methods: Patients with solid pseudo-papillary tumor (SPT) (n=12), mucinous cystic neoplasm (MCN) (n=18), intraductal papillary mucinous tumor (IPMT) (n=18), and ductal adenocarcinoma (PA) (n=73) of the pancreas were studied. Expression of epidermal growth factor (EGF) and its receptor (EGFR) and ezrin were determined using immunohistochemistry. Epidermal growth factor receptor and ezrin expression in sodium butyrate (SB)–treated PA cell line PANC-1 was determined using immunocytochemistry. Messenger RNA expression of ezrin in the PANC-1 cell line treated with SB was determined using reverse transcriptase–polymerase chain reaction. Multivariate analysis of survival of patients with PA was performed.

Results: None of 12 SPTs displayed synchronous expression of EGF and EGFR, while 4 of 6 borderline malignant and 8 of 8 malignant MCNs displayed membranous ezrin expression. Two of 4 borderline malignant and 11 of 11 malignant IPMTs displayed synchronous expression of EGF and EGFR, and all borderline malignant and malignant IPMTs displayed membranous ezrin expression. Less differentiated PA displayed EGF, EGFR, and membranous ezrin expression more frequently compared with more differentiated PA. Epidermal growth factor receptor expression of PANC-1 cells decreased in an SB dose dependent manner, in which PANC-1 cells became more differentiated and membranous ezrin expression of PANC-1 cells decreased correspondingly. Messenger RNA expression of ezrin in PANC-1 cells also decreased in an SB dose dependent manner. Patients with PA with membranous ezrin expression had a poorer prognosis compared with those without (P=.02).

Conclusions: Membranous translocation of ezrin might play a role during malignant transformation of SPT, MCN, IPMT, and PA, which are either dependent on (IPMT and PA) or independent of (SPT and MCN) the EGF-EGFR pathway. Membranous ezrin expression represents a prognostic factor for PA.

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PANCREATIC DUCTAL ADENOCARCINOMA (PA) is one of the leading causes of cancer death worldwide because of its rapid growth and propensity to invade adjacent organs and metastasize. On the other hand, cystic neoplasms of the pancreas are uncommon tumors that account for only 1% of pancreatic malignancies and are associated with a better prognosis. In 1978, Compagno and Oertel described the mucinous cystic neoplasm (MCN) with overt or latent malignancy, capable of progression to invasive mucinous cystadenocarcinoma. In 1986, a subtype of mucinous cystadenoma was called ductectatic cystic neoplasm. Since 1996, the former mucinous ductal ectasia has been separated from MCN and called intraductal papillary mucinous tumor (IPMT) of the pancreas. Both MCN and IPMT definitely progress from adenoma, borderline malignancy, to frank malignancy, if not treated quickly. Solid pseudopapillary tumor (SPT) of the pancreas is supposed to originate from pluripotential embryonal stem cells rather than from acinar cells. Solid pseudopapillary tumor is found strikingly in young female patients and is considered a low-grade malignancy. However, aggressive behavior, with an extension of the tumor...
into surrounding organs or vessels, has been sporadically observed. We have previously reported the expression of hormonal receptors, trefoil factor 1, and epidermal growth factor (EGF) and its receptor (EGFR) in pancreatic cystic neoplasms and PA, which elucidate their corresponding sex and age predilection and cell origin. Nevertheless, the downstream molecular events concerning their diverse potential for tumor invasion and metastasis remain unclear.

Ezrin, a member of the ezrin/radixin/moesin family of proteins, is a cytosolic molecule that cross-links the plasma membrane to actin filaments. Ezrin/radixin/moesin has functions related to cell motility, signal transduction, cell-cell and cell-matrix recognition, and invasion. The binding of ezrin to cell-surface adhesion molecules, such as CD43, CD44, intercellular adhesion molecule-1, intercellular adhesion molecule-2, and phosphatidylinositol 4, 5-biphosphate, furthermore raises the possibility that it is involved in cell migration and metastasis. Recently, many studies have indicated that ezrin/radixin/moesin proteins are up-regulated in various kinds of tumors.

In the present study, we investigated the role of ezrin expression during malignant transformation and disease progression in pancreatic cystic neoplasms and PA, as well the interaction with the EGF-EGFR signaling pathway. To further elucidate the relationship of differentiation of PA and ezrin cellular distribution, we conducted an in vitro study in which sodium butyrate (SB), a differentiating agent, was used to treat a poorly differentiated pancreatic cell line, PANC-1. This in vitro study provided a reversal model of dedifferentiation of pancreatic carcinoma.

### METHODS

Patients with SPT (n=12), MCN (n=18), IPMT (n=18), and PA (n=73), who had undergone curative resection from January 1993 to December 2003 in Chang Gung Memorial Hospital, Taipei, Taiwan, were recruited for this study. All 12 patients with SPT were women with a mean age of 34 years (range, 18-47 years). Four patients underwent a Whipple operation, whereas the remaining 8 patients underwent distal pancreatectomy. Among the 18 patients with MCN, there were 5 men and 13 women with a mean age of 45 years (range, 19-70 years). Six patients underwent a Whipple operation, and the remaining 12 patients underwent distal pancreatectomy. For those 18 patients with IPMT, there were 14 men and 4 women with a mean age of 68 years (range, 34-76 years). Whipple operation, nearly total pancreatectomy, and distal pancreatectomy were performed in 8, 4, and 6 patients, respectively. We histologically divided MCNs and IPMTs into benign (adenoma), borderline malignant, and malignant (carcinoma in situ and invasive carcinoma) based on World Health Organization criteria. Among the 73 patients with PA, there were 45 men and 28 women with a mean age of 60 ± 9 years (range, 37-81 years). Sixty-five of the 72 patients with PA underwent a Whipple operation and the remaining 7 underwent distal pancreatectomy. Histological grading based on World Health Organization classifications revealed 19 well-differentiated, 37 moderately differentiated, and 17 poorly differentiated carcinomas. If there was intratumor heterogeneity, the higher grade was assigned. All surgical specimens had been fixed with formalin, embedded in paraffin, and stored in the archives of the pathology department until use.

### CULTURED TUMOR CELL LINE

The PA cell line used in this study was PANC-1, derived from a human, poorly differentiated carcinoma. The A431 human epidermoid cell line acted as the positive control of EGFR and ezrin expression. All cells were grown in Dulbecco modified Eagle medium (DMEM/F-12) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 20 µg/mL of gentamicin. The cells were treated using 0, 1, 3, and 5 mM SB (Sigma Chemical Co, St Louis, Mo), respectively, for 5 days.

### IMMUNOHISTOCHEMICAL STAININGS FOR EGF, EGFR, AND EZRIN

Formalin-fixed, paraffin-embedded tissues were cut into 4-µm sections and mounted on glutamate-coated slides. A modification of the avidin-biotin-peroxidase complex immunohistochemical method was performed. Slides were heated at 60°C for 60 minutes, then deparaffinized in xylene, and rehydrated in graded alcohols. Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxidase in methanol, and slides were rehydrated and washed in phosphate-buffered saline (PBS) (pH 7.4) for 15 minutes. Sections were then blocked with 10% normal goat serum in PBS with 1% bovine serum albumin for 15 minutes. The blocking serum was decanted and various primary antibodies (EGF, 0.1 mg/mL; DAKO, Glostrup, Denmark; EGFR, dilution, 1:200; DAKO; and ezrin, dilution, 1:100; Zymed Laboratories, San Francisco, Calif) were applied, respectively, in PBS with 1% bovine serum albumin for 16 hours at 4°C. Slides were washed in PBS with 1% Tween 20 for 10 minutes. After 3 PBS rinses, biotinylated goat antirabbit immunoglobulin (Vector Laboratories, Burlingame, Calif) at a dilution of 1:500 was applied for 30 minutes at room temperature. Following another PBS rinse, Avidin DH–biotinylated horseradish peroxidase complex (Vector Laboratories) was applied for 30 minutes. After a final PBS rinse, the tissue sections were reacted with 0.06% diaminobenzidine (Sigma Chemical Co) for 5 minutes, rinsed, counterstained with hematoxylin, dehydrated with graded alcohols, cleared in xylene, and coverslipped with Permount.

### IMMUNOCYTOCHEMICAL STAINING FOR EGFR AND EZRIN EXPRESSION OF SB-TREATED PANC-1 CELLS

Cells grown on 8-chamber slides were fixed with 4% paraformaldehyde for 1 hour. Phosphate-buffered saline containing 0.05% Tween 20, 2% normal goat serum, and 2 mM levamisole hydrochloride was used as the diluent for the antibody. Cells were incubated with mouse monoclonal antibody to EGFR (dilution, 1:100; DAKO) and ezrin (dilution, 1:100; Zymed Laboratories), respectively, overnight at 4°C. Subsequently, cells were incubated with biotinylated horse antimouse IgG (Vector Laboratories) at a 1:200 dilution for 1 hour at room temperature. Bound antibodies were visualized according to standard protocols for the avidin-biotin–alkaline phosphatase complex method.

### REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ANALYSIS OF THE EZRIN MESSNER RNA IN SB-TREATED PANC-1 CELL LINE

Total cellular RNA was extracted from the cell line, and 3 µg of total RNA was dissolved in 8 µL of pancreatic ribonuclease
(RNase)-free water. One microliter of 0.01 absorbance unit of random hexanucleotide primer, 1 µL of 200-U/µL of Moloney murine leukemia virus reverse transcriptase, 4 µL of Moloney murine virus reverse transcriptase buffer, 0.1 µL of RNase inhibitor, 0.1M dithiothreitol, and 5µM deoxyribonucleotide triphosphate mixture were added to each tube. Reverse transcriptase was performed at 37°C for 60 minutes. Five microliters each of reverse transcriptase product were amplified by means of polymerase chain reaction. Complementary DNA was amplified by 0.5 U of Taq DNA polymerase in a 20-µL reaction mixture containing 10mM tromethamine hydrochloric acid (pH 8.3), 50mM potassium chloride, 1.5mM magnesium chloride, 0.2mM deoxynucleotides, and 40pM each primer. The primers for ezrin were as follows: sense, 5’-ATCTTTG-GCTTGAGTGGGA-3’; antisense, 5’-CGATGGGCTTGAGACAA-3’. The polymerase chain reaction profile consisted of a 7-minute initial denaturation at 94°C, 1-minute annealing at 60°C, 3-minute extension at 72°C, and finally a 10-minute extension at 72°C. Five microliters each of the polymerase chain reaction products was run on a 1% agarose gel and visualized using ethidium bromide staining. Internal control was performed using G3PDH.

Quantification of EGF, EGFR and ezrin detected by immunohistochemistry or immunocytochemistry was performed using a scale of negative, weakly positive, moderately positive, and strongly positive, taking into account the percentage of stained cells and the intensity of staining. For simplicity of analysis, those with scales of moderately positive and strongly positive were considered immunopositive, whereas those with scales of negative and weakly positive were considered immunonegative.

STATISTICAL ANALYSIS

Continuous variables are presented as medians and ranges. Kaplan-Meier curves and log-rank tests were performed to compare survival discrepancy between groups. Cox proportional hazards modeling was used to calculate hazard ratios while adjusting for multiple potential confounding variables. Statistical significance was set at P<.05.

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Table 1. Expression of EGF, EGFR, and Ezrin in Pancreatic Cystic Neoplasms and Ductal Adenocarcinoma

<table>
<thead>
<tr>
<th>Disease Entity</th>
<th>EGF</th>
<th>EGFR</th>
<th>Cytoplasmic</th>
<th>Membranous Translocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPT Benign (n = 9)</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Malignant (n = 3)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>MCN Benign (n = 4)</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Borderline malignant (n = 6)</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Malignant (n = 2)</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>IPMT Benign (n = 3)</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Borderline malignant (n = 4)</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Malignant (n = 11)</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>PA Well differentiated (n = 19)</td>
<td>7</td>
<td>0</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Moderately differentiated (n = 37)</td>
<td>22</td>
<td>18</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td>Poorly differentiated (n = 17)</td>
<td>15</td>
<td>12</td>
<td>4</td>
<td>11</td>
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</tbody>
</table>

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IPMT, intraductal papillary mucinous tumor of the pancreas; MCN, mucinous cystic neoplasm; PA, pancreatic ductal adenocarcinoma; SPT, solid pseudopapillary tumor of the pancreas.

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RESULTS

EXPRESSION AND CELLULAR DISTRIBUTION OF EGF, EGFR, AND EZRIN

The expression and cellular distribution of EGF, EGFR, and ezrin in SPTs, MCNs, IPMTs, and PA, respectively, are summarized in Table 1. Using immunohistochemistry, EGF expression was cytoplasmic staining and EGFR was membranous staining. Ezrin expression was divided into 2 patterns of cellular distribution. One was cytoplasmic staining, and the other was membranous staining (membranous translocation). All 12 SPTs displayed EGF expression in the absence of concomitant EGFR expression. Two of 9 benign SPTs displayed cytoplasmic ezrin expression, while all 3 malignant SPTs displayed membranous ezrin expression (Figure 1A). Of 18 MCNs, 1 displayed synchronous expression of EGF and EGFR. All 4 benign MCNs displayed cytoplasmic ezrin expression (Figure 1B); in contrast, 4 of 6 borderline malignant and 8 of 8 malignant MCNs displayed membranous ezrin expression (Figure 1C and D). All 3 benign IPMTs displayed EGF expression in the absence of EGFR expression, and all of them had cytoplasmic ezrin expression. In contrast, 2 of 4 borderline malignant and 11 of 11 malignant IPMTs displayed synchronous expression of EGF and EGFR, of which all had membranous ezrin expression (Figure 1E, F, and G). None of 19 well-differentiated PAs displayed synchronous expression of EGF and EGFR, and all of them had cytoplasmic ezrin expression (Figure 1H). Eighteen of 37 moderately differentiated PAs displayed synchronous expression of EGF and EGFR, and all of them had cytoplasmic ezrin expression (Figure 1I). Eighteen of 37 moderately differentiated PAs displayed synchronous expression of EGF and EGFR, of which 16 had membranous ezrin expression. The remaining 21 moderately differentiated PAs revealed cytoplasmic ezrin expression. Finally, 12 of 17 poorly differentiated PAs displayed synchronous expres-
SION OF EGF AND EGFR, OF WHICH 11 HAD MEMBRANOUS EZRIN EXPRESSION (FIGURE 1I).

IN VITRO STUDY

The cellular morphologic characteristics of the PANC-1 cell line treated with SB (0, 1, 3, and 5mM) appeared more differentiated in a dose dependent manner. The EGFR expression of SB-treated PANC-1 cells decreased in a dose dependent manner (EGFR expression was strongly positive in 0mM SB, weakly positive in 1mM SB, and negative in 3mM and 5mM SB), and membranous ezrin expression of PANC-1 cells decreased correspondingly and shifted to a cytoplasmic pattern (Figure 2). Messenger RNA expression of ezrin in the PANC-1 cell line, detected using reverse transcriptase–polymerase chain reaction, also decreased in an SB dose dependent manner (Figure 3).

LONG-TERM SURVIVAL

At the time of this writing, there was neither disease-related mortality nor recurrence in 12 patients with SPT following the operation. All 4 patients with benign MCN and 6 patients with borderline malignant MCN were still alive without disease, while the 3-year and 5-year survival rates of 8 patients with malignant MCN were 74% and 48%, respectively. All 3 patients with benign IPMT and 4 patients with borderline malignant IPMT were still alive. The 3-year and 5-year survival rates of 11 patients with malignant IPMT were 69% and 46%, respectively. The 3-year and 5-year survival rates for patients with PA were 18% and 14%, respectively. Based on univariate analysis, lymph node invasion (P = .01), serum carcinoembryonic antigen level (P = .04), and membranous ezrin expression (P = .02) emerged as significant prognostic factors for PA (Table 2) (Figure 4). Multivariate analy-
sis confirmed that lymph node invasion (odds ratio, 3.67 [95% confidence interval, 2.38-6.13]; \(P = .03\)) and membranous ezrin expression (odds ratio, 2.73 [95% confidence interval, 1.65-5.37]; \(P = .03\)) were independent prognostic factors for PA.

**COMMENT**

In the present study, there was no disease-related death of patients with SPT, benign MCN, and benign IPMT following the operation, while the 5-year survival rates of patients with malignant MCN, malignant IPMT, and PA were a disappointing 48%, 46%, and 14%, respectively. Reviewing the surgical reports in the literature worldwide, overall survival rates were generally better in patients with MCN and IPMT compared with those treated for PA, but this difference was no longer significant in the subgroups of patients with malignant MCN and IPMT with stage II or stage III tumors.\(^1\),\(^2\),\(^6\),\(^20\) The apparent better prognosis in patients with malignant MCN and IPMT compared with PA may be attributable to the high prevalence of in situ or invasive stage I diseases, which are diagnosed earlier because of overt symptoms. However, when the tumor is locally advanced (peripancreatic extension or lymph node involvement), the prognosis is as poor as for PA. Therefore, it is of paramount significance to elucidate the molecular mechanism driving the cellular invasion and metastatic cascade of these potentially curable diseases.

Ezrin is reported to be a substrate for tyrosine kinase (tyrosine 145 and 353 are phosphorylated).\(^21\) On receipt of appropriate activators, such as IL-11, phosphatidylinositol 4, 5-biphosphate, and phosphorylation of moesin (Thr\(^{558}\)), ezrin translocates from cytoplasm to juxtamembrane by disruption of its intramolecular binding.\(^17\),\(^21\) Recently, it was reported that binding of EGFR causes a phosphorylation cascade in which ezrin is im-
portant because it links the cytoskeleton to the membrane. On the contrary, Kondo et al have shown that during Fas ligand–induced apoptosis, ezrin translocated from the microvilli to the cytoplasm, concomitant with dephosphorylation and microvillar breakdown. IL-2, IL-8, IL-10, and insulin-like growth factor I also have an inhibitory effect on ezrin expression of the colon cancer cell line HT29. Thus, the net effect of activators and inhibitors of ezrin expression of the colon cancer cell line HT29. Therefore, we investigated the interaction of ezrin expression and the EGF-EGFR signaling pathway in various pancreatic cystic neoplasms and PA. According to our data, membranous expression of ezrin was correlated with the synchronous expression of EGF and EGFR during malignant transformation in IPMT and dedifferentiation in PA; however, this correlation was not observed in SPT and MCN. Although all 12 patients with SPT had strong expression of the EGF ligand, expression of EGFR was absent in all of them. We suggest that there were other trophic molecules, other than the EGF-EGF signaling pathway, to stimulate membranous translocation of SPT and MCN during their malignant transformation, which merits further investigation.

Expression and cellular patterns of ezrin in SPT, MCN, IPMT, and PA are of clinical interest and provide insight into tumorigenesis and metastasis potential. According to our data, 2 of 9 histologically benign SPTs showed cytoplasmic ezrin expression, whereas all 3 malignant SPTs displayed membranous ezrin expression. Again, all benign MCNs and IPMTs displayed cytoplasmic ezrin expression, whereas borderline malignant and frank malignant MCNs and IPMTs consistently displayed membranous ezrin expression. Finally, less differentiated PA displayed membranous ezrin expression more frequently compared with more differentiated PA. Akisawa et al showed that ezrin is expressed by a number of pancreatic carcinoma cell lines at different levels among the different cell lines. According to their study, the highly metastatic pancreatic cancer cell lines S2-VP10 and S2-CP9, derived from lung colonies of nude mice following tail-vein injection of the parent pancreatic cancer cell line SUIT-2, had stronger ezrin expression compared with that of SUIT-2. To verify the redistribution of ezrin during dedifferentiation observed in our patients with PA, we designed an in vitro study in which a poorly differentiated pancreatic carcinoma cell line was treated with a differentiating agent, during which cellular expression of EGFR and ezrin were investigated. Sodium butyrate is a short-chain fatty acid naturally present in gastrointestinal tracts. Sodium butyrate inhibits histone deacetylation, leading to changes in chromosomal structure and gene expression. Clinical trials using SB as a differentiating agent have been conducted in patients with hematopoietic malignancies, colon cancer, and hepatoma. Based on the present results, expression of EGFR in PANC-1 cells decreased in an SB dose dependent manner during which PANC-1 appeared dedifferentiated and membranous ezrin expression of PANC-1 cells decreased correspondingly and shifted to a cytoplasmic pattern. This in vitro study confirmed our clinical observation that membranous translocation of ezrin occurs during dedifferentiation of PA. Taken together, we propose that increased expression and membranous translocation of ezrin play an important role during malignant transformation and disease progression of SPT, MCN, IPMT, and PA. Since surgical approaches have reached a plateau with reasonable mortality and morbidity, the present focus of interest is identifying prognostically relevant subgroups by improving pathological examination with molecular markers, as well as searching for novel therapeutic strategy. Many conventional prognostic factors for PA have been proposed, including tumor site, size, stage, residual tumor, DNA ploidy, and Ki-67 labeling index. Based on our multivariate analysis, lymph node invasion and membranous ezrin expression emerged as independent prognostic factors for PA. Furthermore, ezrin membranous translocation of PA appeared to be heavily dependent on the EGF-EGFR signaling pathway based on our data, membranous expression of ezrin was correlated with the synchronous expression of EGF and EGFR during malignant transformation in IPMT and dedifferentiation in PA; however, this correlation was not observed in SPT and MCN. Although all 12 patients with SPT had strong expression of the EGF ligand, expression of EGFR was absent in all of them. We suggest that there were other trophic molecules, other than the EGF-EGF signaling pathway, to stimulate membranous translocation of SPT and MCN during their malignant transformation, which merits further investigation.

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on our clinical observation and in vitro study. Accordingly, it is rational to use target therapy, such as an EGFR inhibitor,29,30 to block membranous translocation of ezrin and deter disease progression.

In conclusion, membranous translocation of ezrin might play a role during malignant transformation and disease progression of SPT, MCN, IPMT, and PA, which are either dependent on (IPMT and PA) or independent of (SPT and MCN) the EGF-EGFR signaling pathway. Membranous ezrin expression represents a prognostic factor for PA.

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