1 | INTRODUCTION

Poorly differentiated clusters (PDC, Figure 1A), which are defined as solid cancer nests lacking gland formation and comprising ≥5 cancer cells, are histologic features observed dominantly at the leading edges of primary colorectal cancers (CRC).\(^1\) Quantification of PDC at the invasive fronts of CRC revealed that PDC are highly relevant to long-term survival in patients with curative surgery\(^2\) and to nodal involvement in early invasive CRC. Later, a formula for PDC grading (G1-G3) was established based on the number of PDC.\(^2\,\,^3\) Using this formula, multiple studies have shown that PDC grading is a robust parameter for the prognosis of primary CRC regardless of stage.\(^3\,\,^10\)

Abstract

Multicellular structures, such as tumor buddings and poorly differentiated clusters (PDC), exist at the invasive front of colorectal cancers (CRC). Although it has been reported that CRC with PDC showed frequent lymph node metastases with a worse prognosis, the molecular markers of PDC that are responsible for prognosis have not been identified. We here noticed for the first time that Ezrin, a regulator of the actin cytoskeleton, is expressed in the corner cells of PDC. We then aimed to verify whether heterogeneous Ezrin expression in PDC predicts the prognosis of CRC patients. We immunohistochemically analyzed Ezrin expression in PDC of 184 patients with completely resected stages I-III CRC. We established the Ezrin corner score (ECS), which quantifies the tendency of Ezrin-positive cells to accumulate at the corners of PDC. On the basis of ECS values, 2 indices, the mean ECS and the number of PDC with high ECS, were obtained. Both indices were significantly higher in CRC with lymphatic invasion, higher PDC grade, and presence of micropapillary (MP) PDC. The mean ECS-high group showed shorter recurrence-free survival than the mean ECS-low group but without significance. The other index, the number of ECS-high PDC, was significantly associated with recurrence-free survival. These results suggest that Ezrin is involved in PDC progression and lymphatic invasion, and that ECS may be a marker for aggressive PDC.

KEYWORDS
colorectal cancer, Ezrin, micropapillary, poorly differentiated clusters, prognosis
In a recent multicenter study analyzing 3243 CRC, PDC grading predicted the prognosis, such as recurrence-free survival, better than other methods: TNM staging and the American Joint Committee on Cancer grading system. PDC are now investigated in the biopsy specimens, chemotherapy-resistant and radiotherapy-resistant CRC, and metastasized CRC in the liver. Recently, PDC in CRC were also reported to share biological and morphological similarities with micropapillary (MP) patterns. MP patterns are characterized by small cell clusters without fibrovascular cores; the clusters are surrounded by empty lacunar spaces (Figure 1B). MP features in CRC showed a high likelihood of nodal involvement and of distant metastases.

Despite the importance of PDC and MP features for prognosis, the functions and molecular mechanisms generating and regulating them are not fully understood. Recent progress in live imaging suggests that cancer cells invade not only as single cells but also as cell clusters. Ezrin is a member of the Ezrin-radixin-moesin (ERM) protein family, which links the actin cytoskeleton to the plasma membrane. Several lines of evidence suggest relationships among Ezrin expression, nodal involvement and prognosis. Even though the authors detected Ezrin expression mainly in the cytoplasm in CRC specimens, Ezrin expression in PDC and MP components has not been analyzed.

In this report, we focused on the heterogeneous immunostaining pattern of Ezrin in PDC and micropapillary PDC (MP PDC). We attempted to quantify this heterogeneity using a method called the Ezrin corner score (ECS). To investigate the prognostic impact of the unique Ezrin expression pattern in PDC, we analyzed a series of CRC with PDC, using the ECS.

**2 | MATERIALS AND METHODS**

### 2.1 Patients and pathological sections

Table 1 summarizes the clinicopathologic information used in this study. In brief, the study included 195 consecutive patients with stages I-III CRC that had been completely resected by surgery between 2009 and 2012 at Kanazawa Medical University Hospital. Patients who had received preoperative therapy or who had been diagnosed with carcinoma in situ were excluded. Information about patient age, sex, tumor location, WHO histologic grade, pT, pN tumor grade, lymphatic and venous invasion, follow-up data, and survival status was obtained from the hospital medical records and pathological reports. Tumor stage was coded according to the 8th edition of the American Joint Committee on Cancer (AJCC).

Written informed consent was obtained from all patients enrolled in the study at the time of surgery. The study was approved by the research ethics committee of Kanazawa Medical University Hospital.

The H&E-stained slides of the CRC representing the deepest invasive front (ranging from 1 to 5 slides per case) were re-reviewed by AA. For further analysis, we selected the 1 slide from each patient in which PDC were most intensively occurring. The corresponding formalin-fixed paraffin-embedded (FFPE) block was processed as described below (immunohistochemistry [IHC]).

### 2.2 Poorly differentiated cluster grading and definition of micropapillary poorly differentiated clusters

For each case, the PDC grade was assessed as previously described. In brief: we identified the area with the highest number of PDC. Under a ×20 objective lens, tumors with <5, 5-9 and ≥10 counts of PDC were classified as PDC grade 1 (G1), grade 2 (G2) and grade 3 (G3), respectively.
We defined PDC showing reversed polarity with empty spaces around the clusters as PDC with micropapillary (MP) features (Figure 1B). Reversed polarity was certified by villin immunohistochemistry. When PDC with MP features occupied all the ×20 objective lens field, we designated the corresponding case as MP PDC-positive.

### 2.3 Immunohistochemistry

Formalin-fixed paraffin-embedded blocks were each cut to a thickness of 4 μm. For villin IHC, sections were treated with Bond Epitope Retrieval Solution 2 (Leica Microsystems K.K.) diluted with PBS at a 1:5 ratio. Furthermore, a villin antibody (Clone 1D2C3, Dako) and an automated staining instrument, Bond Max (Leica), were used according to the manufacturers’ protocol. For Ezrin IHC, sections were boiled in a water bath for 30 minutes at 95-98°C in 10 mmol/L citrate buffer (pH 6.0), then left for 30 minutes at room temperature. After treatment with 3% H₂O₂ to block endogenous peroxidase activity, the sections were incubated with the primary antibody against Ezrin (Clone 3C12, Thermo Fisher Scientific K.K.), which was diluted 1:400 in SignalStain Antibody Diluent (Cell Signaling Technology Japan, K.K.) overnight at 4°C. To visualize the bound antibody, an EnVision Dual Link System-HRP (Dako) and a DAB substrate kit were used.

### 2.4 Ezrin corner score

The tendencies of Ezrin-positive cells to accumulate at the corners of the clusters were quantified using the ECS (Figure 2A and its legend). First, the areas of the MP PDC were selected. If there were no MP PDC in the case, then the area with the highest number of PDC was used. The ECS of all the PDC in the defined area was calculated as in the formula. As shown in Figure 2B, each PDC (a–q for Case I and a–k for Case II) had its own ECS. To judge the Ezrin positivity, we focused on the relative expression within a cluster. For example, when several cells in a cluster showed apparently higher expression than other cells in the same cluster, only these cells with higher expression were designated as Ezrin-positive. When cells in a cluster showed homogenous intensity with subtle difference, all the cells were designated as Ezrin positive. These criteria did not include the intensity of Ezrin positivity among clusters.

Using the ECS value of each PDC, we further evaluated the clinicopathological impact of ECS in 2 different ways (Figure 2B). One is the “mean ECS,” the mean value of all ECS in the defined area. The other is “the number of ECS-high PDC,” the number of PDC whose ECS values are higher than 1.45 (the value based on the receiver operating characteristic [ROC] curve for recurrence-free survival [RFS]).

### 2.5 Statistical analysis

The differences in the characteristics of cases with and without PDC were analyzed using the χ² test or Fisher’s exact test. The association between the 2 ECS indices and clinicopathological characteristics were analyzed using the Mann-Whitney test or the Kruskal-Wallis test. RFS was estimated using Kaplan-Meier analysis, and the data were compared using the log-rank test. \( P < 0.05 \) was considered statistically significant. All analyses were performed using Prism6 software (GraphPad).

### TABLE 1 Clinicopathological characteristics of cases without PDC and with 1 or more PDC

|                      | Cases without PDC | Cases with 1 or more PDC | \( P \)  |
|----------------------|------------------|--------------------------|--------|
| Number of cases      | 11               | 184                      | 1.0000 |
| Age                  |                  |                          |        |
| Below median (<72)   | 5 (45.5%)        | 87 (47.3%)               | 1.0000 |
| Above median         | 6 (54.5%)        | 97 (52.7%)               |        |
| Sex                  |                  |                          |        |
| Male                 | 6 (54.5%)        | 98 (53.3%)               | 1.0000 |
| Female               | 5 (45.5%)        | 86 (46.7%)               |        |
| Tumor location       |                  |                          |        |
| Right colon          | 6 (54.5%)        | 78 (42.4%)               | 0.3462 |
| Left colon           | 5 (45.5%)        | 77 (41.8%)               |        |
| Rectum               | 0 (0%)           | 29 (15.8%)               |        |
| WHO histologic grade |                  |                          |        |
| G1                   | 6 (54.5%)        | 64 (34.8%)               | 0.3957 |
| G2                   | 5 (45.5%)        | 117 (63.6%)              |        |
| G3                   | 0 (0%)           | 3 (1.6%)                 |        |
| pT                   |                  |                          |        |
| 1,2                  | 4 (36.4%)        | 48 (26.1%)               | 0.5982 |
| 3                    | 5 (45.4%)        | 112 (60.9%)              |        |
| 4                    | 2 (18.2%)        | 24 (13.0%)               |        |
| pN                   |                  |                          |        |
| 0                    | 10 (90.9%)       | 118 (64.1%)              | 0.1824 |
| 1                    | 1 (9.1%)         | 48 (26.1%)               |        |
| 2                    | 0 (0%)           | 18 (9.8%)                |        |
| Lymphatic invasion   |                  |                          |        |
| Absent               | 7 (63.6%)        | 54 (29.3%)               | 0.0379 |
| Present              | 4 (36.4%)        | 130 (70.7%)              |        |
| Venous invasion      |                  |                          |        |
| Absent               | 4 (36.4%)        | 35 (19.0%)               | 0.2350 |
| Present              | 7 (63.6%)        | 149 (81.0%)              |        |
| Postoperative chemotherapy | 11 (100%)       | 89 (48.4%)               | 0.0008 |
| Yes                  | 0 (0%)           | 95 (51.6%)               |        |
| Outcome              |                  |                          |        |
| Unrelapsed           | 11 (100%)        | 156 (84.8%)              | 0.3705 |
| Relapsed             | 0 (0%)           | 28 (15.2%)               |        |

PDC, poorly differentiated cluster.
3.2 | Heterogeneity of Ezrin expression in poorly differentiated clusters

We stained CRC sections with Ezrin antibody and found that Ezrin showed heterogeneous staining patterns in PDC, especially in MP PDC (Figure 3A,B). Ezrin expression within individual PDC varied from negative to high, showing a mosaic pattern. Cells with strong Ezrin expression tended to localize at the corners of each cluster. In some cases, Ezrin-positive cells were observed in the pedunculated cells and tumor buds (Figure 3C,D). To confirm the MP components, the reverse polarity was examined by villin IHC (Figure 3E,F). As reported previously,24 villin was localized to the apical membrane in the gland structures (Figure 3E), and villin expression was lost in non–MP PDC (Figure 3E). In contrast, villin was localized to the membrane of MP PDC facing the interstitial tissues (Figure 3F, arrows).

3.3 | Associations between Ezrin corner score and clinicopathological characteristics

To assess the tendencies of Ezrin-positive cells to accumulate at the corners of the clusters, we established a quantification method with a simplified formula and called it the ECS (Figure 2A and its legend). As described in the Materials and Methods, we further obtained 2 indices, “mean ECS” and “the number of ECS-high poorly differentiated carcinomas (PDC).” The relationships between the 2 ECS indices and various clinicopathological characteristics were statistically examined (Table 2). The mean ECS was significantly linked to lymphatic invasion (P = 0.0094), higher PDC grade (P = 0.0001), and the presence of MP PDC (P = 0.0001).

Table 1 summarizes the clinicopathological information on cases with and without PDC. Among the 195 cases in this study, 11 did not contain any PDC. None of 11 cases received postoperative chemotherapies, while 95 cases (51.6%) with PDC received postoperative chemotherapies. During the study period, 28 cases experienced a recurrence, all of which were PDC positive. To investigate Ezrin expression in PDC, 11 cases which did not contain any PDC were excluded from further analyses. The median follow-up period of PDC positive cases was 60.8 months (range, 0.5 months to 8.6 years).

3.4 | Patients with MP PDC

Table 1 shows the clinicopathological characteristics of patients with MP PDC. The number of patients with MP PDC tended to be higher in the group with higher mean ECS (Figure 4A) and the number of ECS-high PDC (Figure 4B). The relapsed cases with MP PDC (red triangles), non-MP PDC-G3 (orange), -G2 (green) and -G1 (black) are indicated at the bottom of each graph. In Figure 4A, we could not detect particular distribution of relapsed cases. However, by focusing on the relapsed PDC G3 cases including MP PDC positive cases (orange and red triangles), we noticed that...
they located on the left side of the graph (the reddish area). Similar to this, in Figure 4B, it appeared that the relapsed cases with PDC G3 and MP PDC (orange and red triangles) located on the left side of the graphs (the reddish area). In fact, the relapsed cases out of the cases with the higher (≥4, the reddish area; see Subsection 3.4) and lower (≤3, the yellow area) numbers of ECS-high PDC lesion were 33.4% (9/27 cases) and 12.1% (19/157 cases), respectively. This result suggested an importance of 2 indices, “mean ECS” and “the number of ECS-high PDC” for relapse, and led us to examine the prognoses of the patients according to the ECS indices.

3.4 | Survival analysis

Cases were subgrouped by PDC grade (Figure 5A), the presence of MP PDC (Figure 5B), the mean ECS index (Figure 5C) and the number of ECS-high PDC (Figure 5D). The relationship between each of these factors and RFS was examined. Higher PDC grades and the presence of MP PDC were significantly associated with shorter RFS ($P = 0.0066$ and $P = 0.0014$, respectively). The mean ECS-high group also showed shorter RFS, but the difference was not significant ($P = 0.2851$). According to the ROC curve for RFS, the number of ECS-high PDC was divided into ≥4 and ≤3 groups. Interestingly, the cases with ≥4 ECS-high PDC were significantly associated with shorter RFS ($P = 0.0067$).

4 | DISCUSSION

In this study, we established a novel method of ECS to quantify the heterogeneity of Ezrin expression in PDC. Based on the ECS, we
further obtained 2 indices (the mean ECS and the number of ECS-high PDC) and found that both were significantly higher in CRC with lymphatic invasion, higher PDC grade, and MP PDC. It should be noted that the cases with high numbers of ECS-high PDC showed shorter RFS of completely resected CRC. In addition to the PDC grading and the presence of MP PDC, the increased number of ECS-high PDC can be a marker for predicting poor RFS.

Several lines of evidence indicate the importance of Ezrin expression in CRC. A proteomic approach found that Ezrin expression was significantly higher in CRC tissues than in adjacent normal colonic mucosa. High Ezrin expression levels were significantly associated with tumor progression and poor prognosis, as well as with independent predictors of lymph node metastasis. A meta-analysis showed that Ezrin expression is significantly associated with tumor grade, TNM stage and lymph node metastasis in CRC. Intense cytoplasmic Ezrin immunoreactivity predicts poor survival in CRC. In addition to these Ezrin functions, we here propose a novel role of Ezrin in the progression of PDC to MP PDC in CRC (Figure 6). In this model, MP PDC may serve as a platform for converting the migration manner from collective-cell to single-cell invasion and for migrating cells to the extracellular matrix with Ezrin expression. Our hypothesis is supported by previous findings that Ezrin overexpression leads to increased cell scattering upon HGF stimulation and that Ezrin knockdown in cultured cells derived from colon cancer inhibited cell migration and invasion.

One may ask whether Ezrin expression is observed preferentially in tumor budding because Ezrin was expressed in cells being extruded from MP PDC (Figure 3C,D). Elzagheid et al. (2008) mentioned that the Ezrin staining is markedly more intense in the small groups and single cancer cells scattered within the stroma, but they did not examine the impact of these Ezrin expressing cells on clinical outcomes. Because the number of tumors budding at the invasive front of CRC is associated with poor outcomes as well as higher tumor stage and lymph node involvement, it will be important to analyze Ezrin expression in tumor budding and to investigate its biological significance in tumor budding. Unfortunately, our quantification method for ECS is not applicable for tumor budding because it was difficult to define the corner of a small cluster.

There are limitations related to the definition of indices based on ECS. Because the number of ECS-high PDC increased as the number of total PDC increased, the total number of PDC (ie, the PDC grade) affects the number of ECS-high PDC in many cases. However, not all cases followed this pattern. For example, the PDC G1 or G2 with high mean ECS (black and green triangles in reddish area; Figure 4A) and PDC G2 with increased numbers of ECS-high PDC (a green triangle in reddish area; Figure 4B) showed relapse. In such cases, the number of PDC was not predictive of the prognosis.

### Table 2: Association of Ezrin Corner Score with clinicopathological characteristics

|                        | Mean ECS | P     | Number of ECS-high (>1.45) PDC | P     |
|------------------------|----------|-------|---------------------------------|-------|
| **pT**                 |          |       |                                 |       |
| 1, 2 (n = 48)          | 1.12     | 0.9737| 1.27                            | 0.1732|
| 3 (n = 112)            | 1.18     |       | 1.91                            |       |
| 4 (n = 24)             | 1.19     |       | 1.83                            |       |
| **pN**                 |          |       |                                 |       |
| 0 (n = 118)            | 1.13     | 0.5029| 1.31                            | 0.0009|
| 1 (n = 48)             | 1.23     |       | 2.42                            |       |
| 2 (n = 18)             | 1.19     |       | 2.72                            |       |
| **Lymphatic invasion** |          |       |                                 |       |
| Absent (n = 53)        | 0.99     | 0.0094| 0.98                            | 0.0039|
| Present (n = 131)      | 1.24     |       | 2.05                            |       |
| **Venous invasion**    |          |       |                                 |       |
| Absent (n = 35)        | 1.10     | 0.4461| 1.31                            | 0.095 |
| Present (n = 149)      | 1.18     |       | 1.83                            |       |
| **PDC grade**          |          |       |                                 |       |
| G1 (n = 93)            | 0.97     | <0.0001| 0.56                           | <0.0001|
| G2 (n = 47)            | 1.24     |       | 1.96                            |       |
| G3 (n = 44)            | 1.49     |       | 3.98                            |       |
| **MP PDC**             |          |       |                                 |       |
| Absent (n = 158)       | 1.07     | <0.0001| 1.17                           | <0.0001|
| Present (n = 26)       | 1.74     |       | 5.15                            |       |

Data are presented as the mean value of each measurement.

ECS, Ezrin corner score; MP, micropapillary; PDC, poorly differentiated cluster.
AIKAWA et al. propose that ECS is a candidate parameter for the extraction of metastatic-prone PDC, which cannot be achieved by observing H&E images. The higher mean ECS index, which is not influenced by the number of PDC, also showed frequent lymphatic invasion and a tendency toward worse RFS. Because we can calculate the ECS of the cases with 1 PDC, it is interesting to collect the cases with fewer PDC but with metastasis. The drawback of using mean ECS is that the ECS of individual PDC affects this index of cases with small numbers of PDC.

Regarding clinical application, it is not practical to calculate ECS for routine diagnosis because the process of quantification is extremely time-consuming. It is therefore assumed that PDC grading without IHC is suitable for predicting RFS for routine diagnosis, even though PDC grading and the number of ECS-high PDC showed similar impacts on RFS. In most histopathological studies, IHC reactivity has been evaluated according to the intensity or positivity from 1 or several sections, and no study has focused on the heterogeneity of Ezrin expression among clusters in the same CRC or among cells in the individual clusters. We would like to emphasize that the quantification in our study is necessary to understand the mechanistic insights of individual PDC, even though it is a laborious process with several limitations. Although single-cell sequencing has brought about huge progress in the research field, the use of the technology in formalin-fixed pathological sections in the hospital is not imminent. We believe ECS is at present the best way to observe all cases with 1 or more PDC in the investigation.

It is plausible that factors other than Ezrin expression are required for cancer cells to obtain their full capacity for progression. For example, patients with mutations in the KRAS oncogene exhibited higher PDC grading. PI3K and RAF mutations were found in PDC. It has also been reported that LCAM1 is expressed at higher levels in high-grade PDC. The nuclear localization of β-catenin, the lower expression of Ki-67 and the cytosolic localization of E-cadherin were observed in PDC. Frequent mutations in KRAS and BRAF were observed in CRC with MP features. Because our method of quantifying Ezrin heterogeneity in clusters can be applied to different kinds of proteins, IHC examination of other proteins in PDC will shed light on the mechanisms by which PDC progress and

FIGURE 4 Ezrin corner score (ECS) of 184 colorectal cancers arranged in order of mean ECS (A) and the number of ECS-high poorly differentiated clusters (PDC) (B). Each dot corresponds to an individual PDC, and PDC in each case is vertically aligned. ECS of individual PDC are colored as follows. Micropapillary (MP) PDC, red; MP PDC absent PDC G3 cases, orange; PDC G2, green; PDC G1, black. Filled circles and open squares denote PDC of unrelapsed and relapsed cases, respectively. Relapsed cases are also indicated by triangles at the bottom. The cases were separated by the lines of value 1.45 in A, and 4 in B. Reddish and yellow color in graphs highlight higher and lower areas, respectively.
invade, and will help in the search for better markers for predicting prognosis.

Our study here focused on CRC patients in stages I-III with curative operation and revealed the association between histopathological features and prognosis. Several studies examined the prognosis of patients in stages I-IV and addressed the worse overall survival of MP-component-positive CRC.18,33 Many factors in those studies, such as remaining or metastasized cancers, may have contributed to the death of patients. Because our study focused on cases with the complete resection of CRC in stages I-III, we concluded that tumors with MP patterns have a high potential for recurrence as well as higher PDC grades. Our results are similar to those in previous reports11,34; that is, higher PDC grades were associated with shorter RFS. In addition to this, our analysis is the first to show that the presence of MP PDC was significantly associated with shorter RFS.

In conclusion, we established a method to quantify the tendencies of Ezrin-positive cells at the corners of clusters. Using the parameter of Ezrin heterogeneity in PDC, we found that Ezrin-positive cells at the corner might be responsible for PDC progression and lymphatic invasion. Even though we believe that ECS is a marker for aggressive PDC, this method must be further improved before it can be put into practical use.

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CONFLICTS OF INTEREST
The authors declare that there are no conflicts of interest for this study.

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FIGURE 5 Recurrence-free survival of completely resected stages I-III colorectal cancers. Cases were subgrouped by poorly differentiated cluster (PDC) grade (A), presence of micropapillary PDC (B), mean Ezrin corner score (ECS) (high or low, C) and the number of ECS-high PDC (≤3 or ≥4, D). The numbers at the bottom show significances (log-rank test)

FIGURE 6 Summary of the Ezrin expression and poorly differentiated cluster (PDC) progression. Brown color indicates Ezrin expressing cells
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