Ezrin expression combined with MSI status in prognostication of stage II colorectal cancer

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Abstract

Currently used factors predicting disease recurrence in stage II colorectal cancer patients are not optimal for risk stratification. Thus, new biomarkers are needed. In this study the applicability of ezrin protein expression together with MSI status and BRAF mutation status were tested in predicting disease outcome in stage II colorectal cancer. The study population consisted of 173 stage II colorectal cancer patients. Paraffin-embedded cancer tissue material from surgical specimens was used to construct tissue microarrays (TMAs) with next-generation technique. The TMA-slides were subjected to following immunohistochemical stainings: MLH1, MSH2, MSH6, PMS2, ezrin and anti-BRAF V600E antibody. The staining results were correlated with clinicopathological variables and survival. In categorical analysis, high ezrin protein expression correlated with poor disease-specific survival (p = 0.038). In univariate analysis patients having microsatellite instable / low ezrin expression tumors had a significantly longer disease-specific survival than patients having microsatellite stable / high ezrin expression tumors (p = 0.007). In multivariate survival analysis, the presence of BRAF mutation was associated to poor overall survival (p = 0.028, HR 3.29, 95% CI 1.14–9.54). High ezrin protein expression in patients with microsatellite stable tumors was linked to poor disease-specific survival (p = 0.01, HR 5.68, 95% CI 1.53–21.12). Ezrin protein expression is a promising biomarker in estimating the outcome of stage II colorectal cancer patients. When combined with microsatellite status its ability in predicting disease outcome is further improved.

Introduction

Five-year survival in stage II colorectal cancer (CRC) is 70–80% [1,2]. Unfavorable prognostic factors for stage II CRC include lymphovascular invasion, less than 12 examined lymph nodes, poor differentiation grade, tumor spreading to the peritoneum or adjacent tissue structures as well as tumor obstruction or perforation. [3,4]. These risk factors have been utilized in the
assessment of stage II colorectal cancer patients in need of postoperative adjuvant treatments. The benefit of chemotherapy in stage III colorectal cancer patients is apparent, but controversial in stage II colorectal cancer patients even with above-mentioned risk factors [3]. Consequently, there is a crucial need to discover new markers to better define those at highest danger of disease recurrence.

DNA mismatch repair competence is a feature associated with CRC outcome. Inactivation of genes responsible for mismatch repair competence cause microsatellite instability (MSI), which can be studied by expression of the gene products MLH1, MSH2, MSH6 and PMS2 or by PCR-based methods [6,7,8,9,10,11]. MSI is reported in about 15–20% of CRC [8,12]. MSI is linked to right-sided, poorly differentiated tumors with higher T stage and younger patient age [12]. Stage II CRCs with MSI, as demonstrated by immunohistochemistry of mismatch repair proteins, have a more favorable prognosis as compared to microsatellite stable (MSS) tumors [13,14,4]. Moreover, patients with defective mismatch repair (dMMR) stage II tumors do not seem to benefit from fluorouracil-based adjuvant chemotherapy [15,16]. MSI tumor can evolve in Lynch syndrome patients carrying a germ-line mutation in one MMR gene, or through sporadic events involving epigenetic silencing of the MLH1 gene [17].

BRAF gene encodes a protein kinase of the RAS/RAF/MEK-ERK signaling cascade, which is regulated by KRAS [18,19]. Previously, BRAF V600E mutation was shown to be an adverse prognostic factor for overall survival in stage II-III colon cancer [20]. MSS together with BRAF mutation is associated with poor prognosis in CRC [9,21]. On the contrary, MSI stage II tumors with BRAF V600E mutation are associated with a rather favorable prognosis [21]. Moreover, colorectal cancer with MSI phenotype and a concomitant BRAF mutation indicates a sporadic tumor, thus excluding Lynch syndrome [17,21].

Ezrin is a cytoskeleton-associated protein, which participates in cellular signaling, cell survival, proliferation and migration. Its association with malignant behavior has been suggested in several experimental models, and in several cancers strong ezrin expression correlates with inferior outcome. [22,23,24,25,26,27,28]. Our previous work has demonstrated the impact of ezrin expression on the outcome in metastatic CRC as well as in localized rectal cancer [27,28]. To our knowledge, the role of ezrin as a prognostic marker in stage II colorectal cancer has not been studied before.

In this work, we utilized tumor tissue collection form consecutive stage II CRC patients, together with extensive clinical, disease outcome and follow-up data to search for tissue-based prognostic markers. We report the association of MSI status, BRAF mutation status and ezrin protein expression with clinicopathological variables and patient outcome. Our results suggest that combined MSI and ezrin analysis can stratify tumors according to their clinical behavior.

**Patients and methods**

**Study population**

We collected archived paraffin-embedded tumor material from consecutive stage II CRC patients operated in Turku University Hospital in 2005–2012. This study was approved by Chief Executive Officer of TYKS-SAPA, Hospital District of Southwest Finland (T52/2014). The use of tissue material was approved by Scientific Steering Group of Auria Biobank (AB15-8108, 25.5.2012). The study was conducted in accordance with the Declaration of Helsinki. The clinical data were retrieved and histological samples collected and analyzed with the endorsement of the National Authority for Medico-Legal Affairs (VALVIRA). The patient records were accessed anonymously.

In 2005–2012 a total of 232 stage II CRC patients were radically operated in our hospital. Computed tomography (CT) of the abdomen and chest x-ray or CT had been performed...
preoperatively to rule out distant metastases. We carefully checked the patient files, including surgery and pathology reports and excluded patients with verified lymph node or distant metastases, those who had been operated with palliative-intent surgery, and also patients with other than adenocarcinoma histology (e.g. neuroendocrine tumors). Only patients with stage II CRC were included in the current study. For tumor staging, TNM7 classification of malignant tumors [29] was used. From the original cohort \( n = 232 \), tumor material for MSI staining was available from 214 patients. For further BRAF and ezrin stainings, material was available from 173 patients. These patients \( n = 173 \) were included in statistical analyses.

**TMA construction**

Tissue microarrays (TMA) were constructed and analyzed using the next-generation TMA technique [30]. Shortly, the appropriate formalin-fixed paraffin-embedded (FFPE) tissue specimens were chosen based on clinical data and retrieved from the pathology archives. A representative hematoxylin-eosin (H&E) section containing areas of invasive carcinoma was selected from each tumor. New H&E slides were produced, scanned (Pannoramic P250, 3DHistech) and uploaded into the university digital microscopy web portal (casecenter.utu.fi). Each slide was viewed using Pannoramic Viewer software (3DHistech). Using the 1.2 mm diameter annotation tool, annotations of different colors corresponding to various histological areas were placed onto each digital slide. Two annotations were placed in the center of the tumor, two in the tumor front and two in the normal colonic epithelium. The corresponding tissue cores were then transferred into the TMA blocks using an automated TMA instrument (TMA Grandmaster, 3DHistech) by overlaying each annotated digital slide with the corresponding tissue specimen. One tissue core containing benign tissue was selected from each tumor to act as a control. The constructed TMA blocks were sectioned, stained, scanned and uploaded into the portal (casecenter.utu.fi) and each individual spot was scored by two pathologists (KS, JS). The resulting scores were combined with the clinical data for statistical analysis.

**Immunohistochemistry**

Immunohistochemical staining against MMR proteins is a useful screening method in research materials with paraffin-embedded TMA-samples. In contrast to PCR-based methods, it also readily provides information on the inactivated gene. Immunohistochemical stainings (IHC) were performed using standard procedures. Shortly, 3,5 μm sections were cut from the TMA blocks. They were stained with monoclonal antibodies against MLH1 (Clone G168-15BD Pharmingen, dilution: 1:5), MSH2 (Clone G219-1129, BD Pharmingen, dilution: 1:200) and MSH6 (Clone EP49, Epitomoc, dilution: 1:200). The signal was detected with UltraView Universal DAB Detection kit. For PMS2, Clone EPR3947 (Ventana/Roche, ready to use antibody) was used and the signal was detected with OptiView Universal DAB Detection Kit and amplification kit. To detect BRAF V600E mutation, BRAF RTU antibody (Clone VE1, Roche/Ventana) was used and the signal was detected with OptiView Universal DAB Detection kit. For ezrin staining, immunoglobulin G antibody to human ezrin (clone 3C12) [31] was used. All the stainings were performed with BenchMark XT (Ventana/Roche) using ultraVIEW Universal DAB Detection Kit (Ventana/Roche), except ezrin, which was done with LabVision immunoautomate (Thermo Fisher Scientific) using the Power Vision Plus poly HRP antimouse/rabbit/rat IgG detection kit.

**Evaluation of immunohistochemical stainings**

All IHC stainings were separately evaluated by two observers (KS and JS), blinded to clinical data. For MLH1, MSH2, MSH6, PMS2 and ezrin, inflammatory cells of the stroma were used...
as positive controls. For analyses of MSI (MLH1, MSH2, MSH6 and PMS2) also the cores from normal colonic epithelium were used as positive controls. As a positive control in evaluating the BRAF-stainings, we used BRAF V600E mutation-positive cancer tissue obtained from a CRC patient who did not belong to this study cohort. These IHC stainings were evaluated dichotomously as positive or negative. For ezrin protein expression, cytoplasmic staining was recorded [27, 28]. Four staining categories were used: 0 for negative staining, 1 for weak staining (distinguishable from the background staining), 2 for moderate staining and 3 for strong staining (corresponding to immunoreactivity in lymphocytes) [27]. In addition, a category of non-evaluable was used for all stainings. For statistical purposes a dichotomous grading, ezrin low (negative or weak staining) and ezrin high (moderate or strong staining) was used.

Statistical analysis

Statistical analyses were performed with IBM SPSS version 23 with standard packages. Clinical data were analyzed in correlation with histological, immunohistochemical and mutational analysis data using χ² or Fisher’s exact-test for discrete variables and one-way ANOVA for continuous variables. Overall survival (OS), disease free survival (DFS) and disease-specific survival (DSS) were calculated using Kaplan-Meier curves. Survival was analyzed with respect to (stratified to) different biomarkers using log-rank test. For multivariate analyses, the following variables were used: tumor grade, tumor-side, obstruction, perforation, vascular invasion, BRAF mutated/wild type, ezrin low/high and MSS/MSI combinations were included. Multivariate Cox proportional hazard regression model was used to adjust the survival curves for covariates and to obtain estimates on hazard ratios. All p-values were two-sided, and values less than 0.05 were considered statistically significant.

Results

General aspects of clinical patient characteristics

Altogether 173 patients were included in this study. The tumor was located in the proximal colon in 70 (40%), transverse colon in 19 (11%), descending colon in 8 (5%), sigmoid colon in 44 (25%) and rectum/rectosigmoid in 32 (19%) patients. There were 30 (17%) T4-tumors included in the study. Vascular invasion was reported in 32 (18%) patients and preoperative bowel obstruction in 26 (15%) of patients. Adjuvant fluorouracil-based chemotherapy had been given to 51 (30%) patients. The median follow-up time was 57 months. At the latest follow-up data collection time point in September 2016, 116 patients (67%) were alive without CRC, 3 (2%) alive with CRC, 17 dead of CRC, 18 (10%) dead of other cancers and 19 (11%) dead of other causes than cancer. The clinical characteristics of the patients are shown in Table 1.

General aspects of MSI staining

The results of the MSS/MSI analysis in relation to clinicopathological variables are shown in Table 2. Overall, 136 (79%) of the tumors were MSS and 37 (21%) were MSI high. MSI was significantly more common in the right-sided tumors (n = 30; 34%), as compared with the left-sided tumors (n = 7; 8%) (Pearson’s chi-square test, p = 0.0001). MSI was infrequent in well-differentiated tumors (1/39, 3%), but common in tumors with poor differentiation grade (15/39, 40%). Ten out of 22 (45%) mucinous cancers presented MSI. MSI status in relation to clinic-pathological variables is presented in Table 2.
General aspects of ezrin staining

The results of the ezrin stainings in relation to clinicopathological parameters are shown in Table 3. Generally, in 135 (78%) tumors, ezrin staining intensity was scored as low, and in 38

Table 1. The clinicopathological variables of the patient population included in the MSI, BRAF and Ezrin analyses (n = 173). NA = not available, R0 = microscopically radical surgery, R1 macroscopically radical surgery, R2 macroscopically non-radical surgery.

| Variable                                      | n (%)  |
|-----------------------------------------------|--------|
| Gender                                        |        |
| Female                                        | 92 (53)|
| Male                                          | 81 (47)|
| Age                                           |        |
| <70 years                                     | 66 (38)|
| >70 years                                     | 107 (62)|
| Postoperative stage                           |        |
| T3N0                                          | 143 (83)|
| T4aN0                                         | 17 (10)|
| T4bN0                                         | 13 (7)|
| Tumor side                                    |        |
| Right                                         | 89 (51)|
| Left                                          | 84 (48)|
| Tumor grade (analyzed from surgical specimens)|        |
| G1                                            | 19 (11)|
| G2                                            | 114 (66)|
| G3                                            | 40 (23)|
| Histology                                     |        |
| Conventional adenocarcinoma                   | 151 (87)|
| Mucinous adenocarcinoma                       | 22 (13)|
| Vascular invasion                             |        |
| Yes                                           | 32 (18)|
| No                                            | 131 (76)|
| NA                                            | 10 (6)|
| Lymph node count                              |        |
| ≥12 lymph nodes examined                      | 138 (80)|
| <12 lymph nodes examined                      | 35 (20)|
| Radicatly                                     |        |
| R0                                            | 162 (94)|
| R1                                            | 8 (5)|
| R2                                            | 3 (2)|
| Preoperative obstruction                      |        |
| Yes                                           | 26 (15)|
| No                                            | 147 (85)|
| Tumor perforation                             |        |
| Yes                                           | 15 (9)|
| No                                            | 157 (91)|
| NA                                            | 1 (0)|
| Adjuvant chemotherapy                         |        |
| Yes                                           | 51 (30)|
| No                                            | 121 (69)|
| NA                                            | 1 (0)|

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Table 2. MSS/MSI status in relation to clinicopathological variables (n = 173). NA = not available, R0 = microscopically radical surgery, R1 = macroscopically radical surgery, R2 = macroscopically non-radical surgery, CRC = colorectal cancer.

| Variable                  | MSS        | MSI high    | Significance (p) |
|---------------------------|------------|-------------|------------------|
|                           | n (%)      | n (%)       |                  |
| Gender                    |            |             | 0.266            |
| Female                    | 69 (51)    | 23 (62)     |                  |
| Male                      | 67 (49)    | 14 (38)     |                  |
| Age                       |            |             | 0.707            |
| Under 70 years            | 53 (39)    | 13 (35)     |                  |
| Over 70 years             | 83 (61)    | 24 (65)     |                  |
| Postoperative stage       |            |             | 0.253            |
| T3N0                      | 115 (85)   | 28 (76)     |                  |
| T4aN0                     | 13 (10)    | 4 (11)      |                  |
| T4bN0                     | 8 (6)      | 5 (13)      |                  |
| Tumor side                |            |             | 0.0001           |
| Right                     | 59 (43)    | 30 (81)     |                  |
| Left                      | 77 (57)    | 7 (19)      |                  |
| Tumor grade               |            |             | 0.010            |
| G1                        | 18 (13)    | 1 (3)       |                  |
| G2                        | 93 (68)    | 21 (57)     |                  |
| G3                        | 25 (18)    | 15 (40)     |                  |
| Histology                 |            |             | 0.009            |
| Conventional adenocarcinoma | 124 (91)  | 27 (73)     |                  |
| Mucinous adenocarcinoma   | 12 (9)     | 10 (27)     |                  |
| Vascular invasion         |            |             | 0.383            |
| Yes                       | 28 (21)    | 4 (11)      |                  |
| No                        | 101 (74)   | 30 (81)     |                  |
| NA                        | 7 (5)      | 3 (8)       |                  |
| Lymph node count          |            |             | 0.646            |
| 12 or more examined       | 107 (78)   | 31 (84)     |                  |
| Less than 12 examined     | 29 (21)    | 6 (16)      |                  |
| Radicability of surgery   |            |             | 0.446            |
| R0                        | 128 (94)   | 34 (92)     |                  |
| R1                        | 5 (4)      | 3 (8)       |                  |
| R2                        | 3 (2)      | 0 (0)       |                  |
| Preoperative obstruction   |            |             | 0.604            |
| Yes                       | 22 (16)    | 4 (11)      |                  |
| No                        | 114 (84)   | 33 (89)     |                  |
| Tumor perforation         |            |             | 0.797            |
| Yes                       | 11 (8)     | 4 (11)      |                  |
| No                        | 124 (91)   | 33 (89)     |                  |
| NA*                       | 1 (1)      | 0 (0)       |                  |
| Adjuvant chemotherapy     |            |             | 0.218            |
| Yes                       | 37 (27)    | 14 (38)     |                  |
| No                        | 99 (73)    | 22 (59)     |                  |
| NA*                       | 0 (0)      | 1 (3)       |                  |

(Continued)
High ezrin expression was more common in MSI tumors (19/37, 51%) than in MSS tumors (19/134, 14%) (Pearson’s chi-square test, p = 0.0001). There were no statistically significant differences in ezrin intensity according to clinicopathological variables, except for disease outcome (see below). Ezrin staining in relation to clinic-pathological variables is presented in Table 3.

**BRAF staining**

BRAF staining was available from 171 patients. Of the tumors, 146 (85%) were BRAF wild type and 25 (15%) BRAF V600E mutated. The BRAF mutated tumors predominantly presented with MSI (21/25, 84%), whereas BRAF wild type tumors were mostly MSS (130/146, 89%). Of the BRAF wild type tumors only 25/146 (17%) showed high ezrin IHC, while 13/25 (52%) of BRAF mutant tumors were ezrin high (Pearson’s chi-square test, p = 0.0001). Combinatorial analysis of the three variables showed that BRAF wild type tumors were predominantly MSS / low ezrin (112/146, 77%), whereas 12/25 (48%) of the BRAF mutated tumors were MSI / high ezrin (Fisher’s exact test, p = 0.0001). Follow-up data according to BRAF status is presented in Table 4.

**Clinical correlations**

The clinical correlations of the MSI status and ezrin staining are shown in Tables 2 and 3. Altogether, high ezrin staining correlated with inverse DSS (Fisher’s exact test, p = 0.038). On the other, MSI status as a single variable did not correlate with survival. In categorical analysis of 5-year disease-specific survival time, 11 out of 18 (61%) patients with MSI / low ezrin were alive compared to only 4 out of 18 (21%) patients with MSS / high ezrin (Fisher’s exact test, p = 0.040). In univariate analysis, patients whose tumors were MSI / low ezrin tended to have the best OS probability, and those with MSI / high ezrin the worst, but the difference was not statistically significant (log-rank test, p = 0.235). Patients with MSI / low ezrin tumors had the longest DSS and those with MSS / high ezrin tumors had the shortest (log-rank test, p = 0.007). An example of staining patterns of patients belonging to groups of best and worst DSS are presented in Figs 1 and 2, respectively. Patients with MSI / low ezrin had the longest DFS and those with MSI / high ezrin had the shortest, but the difference was no statistically significant (log-rank test, p = 0.069). The survival curves are presented in Fig 3 and the results of univariate survival analysis in S1 Table.

A summary of the multivariate analyses results is presented in Table 5. This table shows T4bN0 tumors to be associated with inferior OS (Cox model, HR 2.86, 95% CI [1.06–7.74], p = 0.038) and DFS (Cox model, HR 8.05, 95% CI [2.31–28.01], p = 0.001). Likewise, perforation was linked to inferior OS (Cox model, HR 3.8, 95% CI [1.57–9.17], p = 0.003), DSS (Cox model, HR
Table 3. Ezrin expression in relation to clinicopathological variables (n = 173). CRC = colorectal cancer.

| Variable                     | Ezrin low | Ezrin high | Significance (p) |
|------------------------------|-----------|------------|------------------|
|                              | n (%)     | n (%)      |                  |
| **Gender**                   |           |            |                  |
| Female                       | 60 (44)   | 21 (55)    | 0.272            |
| Male                         | 75 (56)   | 17 (45)    |                  |
| **Age**                      |           |            |                  |
| Under 70 years               | 56 (41)   | 10 (26)    | 0.130            |
| Over 70 years                | 79 (58)   | 28 (74)    |                  |
| **Postoperative stage**      |           |            | 0.634            |
| T3N0                         | 113 (84)  | 30 (79)    |                  |
| T4aN0                        | 13 (10)   | 4 (10)     |                  |
| T4bN0                        | 9 (7)     | 4 (10)     |                  |
| **Tumor side**               |           |            | 0.141            |
| Right                        | 65 (48)   | 24 (63)    |                  |
| Left                         | 70 (52)   | 14 (37)    |                  |
| **Tumor grade**              |           |            | 0.119            |
| G1                           | 14 (10)   | 5 (13)     |                  |
| G2                           | 94 (70)   | 20 (53)    |                  |
| G3                           | 27 (20)   | 13 (34)    |                  |
| **Histology**                |           |            | 0.099            |
| Conventional adenocarcinoma  | 121 (90)  | 30 (79)    |                  |
| Mucinous adenocarcinoma      | 14 (10)   | 8 (21)     |                  |
| **Vascular invasion**        |           |            | 0.677            |
| Yes                          | 26 (19)   | 6 (16)     |                  |
| No                           | 100 (74)  | 31 (82)    |                  |
| NA                           | 9 (7)     | 1 (3)      |                  |
| **Lymph node count**         |           |            | 1.000            |
| 12 or more examined          | 108 (80)  | 30 (79)    |                  |
| Less than 12 examined        | 27 (20)   | 8 (21)     |                  |
| **Radicality of surgery**    |           |            | 0.568            |
| R0                           | 127 (94)  | 35 (92)    |                  |
| R1                           | 6 (4)     | 2 (5)      |                  |
| R2                           | 1 (1)     | 1 (3)      |                  |
| **Preoperative obstruction**  |           |            | 0.607            |
| Yes                          | 19 (14)   | 7 (18)     |                  |
| No                           | 116 (86)  | 31 (82)    |                  |
| **Tumor perforation**        |           |            | 0.476            |
| Yes                          | 10 (7)    | 5 (13)     |                  |
| No                           | 124 (92)  | 33 (87)    |                  |
| NA*                          | 1 (1)     | 0 (0)      |                  |
| **Adjuvant chemotherapy**    |           |            | 1.000            |
| Yes                          | 40 (30)   | 11 (29)    |                  |
| No                           | 95 (70)   | 26 (68)    |                  |
| NA*                          | 0 (0)     | 1 (3)      |                  |
| **MSI status**               |           |            | 0.001            |
| MSS                          | 117 (87)  | 19 (50)    |                  |
| MSI                          | 18 (13)   | 19 (50)    |                  |

(Continued)
model, HR 5.44, 95% CI [95% CI 1.3–22.75], p = 0.02), as well as DFS (Cox model, HR 4.87 95% CI [1.38–17.23]; p = 0.014). Moreover, the presence of BRAF mutation was associated to shortened OS (Cox model, HR 3.29, 95%CI [1.14–9.54], p = 0.028). High ezrin expression together with MSS were linked to shorter DSS (Cox model, HR 5.68, 95%CI [1.53–21.12], p = 0.01).

### Discussion

Stage II CRC patients possess a treatment challenge, because current diagnostic methods do not enable their accurate risk stratification. The purpose of this study was to test, whether analysis of ezrin, a promising prognostic marker, together with microsatellite instability and BRAF mutation status could be used for prognostication. Indeed, our results show ezrin as an independent prognostic marker for disease-specific survival in stage II CRC, and indicate this correlation to be further strengthened by concomitant microsatellite instability testing.

Previous studies by others and us have indicated an association between ezrin expression and CRC outcome. The earlier studies have been carried out with mixed cohorts, including various disease stages, which can lead to inaccurate conclusions. However, we are not aware of any studies that would have specifically focused on ezrin expression in stage II CRC. The current results indicate that tumors with high ezrin expression possess adverse biological features already at a stage, when the cancer has not yet disseminated. Importantly, as demonstrated by this study, these features are not associated with tumor location, histological grade, vascular invasion or other outcome-related clinicopathological features. Our results do not clarify the mechanism, by which ezrin may be linked with oncogenic properties. One explanation is that ezrin expression provides an advantage for the disseminating cells early on during metastatic

Table 3. (Continued)

| Variable                  | Ezrin low | Ezrin high | Significance (p) |
|---------------------------|-----------|------------|------------------|
|                           | n (%)     | n (%)      |                  |
| **BRAF status**           |           |            | 0.001            |
| BRAF WT                   | 121 (91)  | 25 (66)    |                  |
| BRAF mutated              | 12 (9)    | 13 (34)    |                  |
| **Disease-specific outcome** |         |            | 0.038            |
| Alive without CRC         | 93 (69)   | 23 (61)    |                  |
| Alive with CRC            | 3 (2)     | 0 (0)      |                  |
| Dead of CRC               | 8 (6)     | 9 (24)     |                  |
| Dead of other cancer      | 16 (12)   | 2 (5)      |                  |
| Dead of other causes      | 11 (8)    | 4 (11)     |                  |
| Dead cause unspecified    | 4 (3)     | 0 (0)      |                  |

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Table 4. BRAF status in relation to ezrin and MSS/MSI (n = 171).

| Variable                  | Ezrin low MSS | Ezrin low MSI | Ezrin high MSS | Ezrin high MSI | Significance (p) |
|---------------------------|---------------|---------------|----------------|----------------|------------------|
|                           | n (%)         | n (%)         | n (%)          | n (%)          | p = 0.0001       |
| **BRAF mutated**          |               |               |                |                |                  |
| n (%)                     | 3 (3)         | 9 (50)        | 1 (5)          | 12 (63)        |                  |
| **BRAF wild type**        |               |               |                |                |                  |
| n (%)                     | 112 (97)      | 9 (50)        | 18 (95)        | 7 (37)         |                  |

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seeding. Indeed, some previous studies have indicated a role for ezrin in this process [32,33]. Interestingly, ezrin turned out to be a stronger DSS predictor than any of the clinipathological factors, apart from tumor perforation.

Ezrin expression is linked to the activity of several oncogenic signaling cascades. Ezrin can act both as a regulator and/or a down-stream target in several signaling pathways, including
Src, Akt-PI3K and PKA, and these associations have been suggested to be of importance in ezrin’s oncogenic properties [34,35,36]. Here, we found that ezrin expression correlated with BRAF mutation status; high ezrin immunoreactivity being significantly more common in BRAF V600E than BRAF wild-type tumors. This is a novel finding, there are no previous reports that would have linked ezrin with BRAF. Even if the specific mechanism of the connection between these two genes is unknown, the association of both high ezrin expression and BRAF mutation with the activity of several oncogenic signaling pathways might partly explain this interesting finding.

In this study, MSI status alone did not correlate with survival, although the superior prognosis of patients with MSI CRC over MSS tumors has been demonstrated earlier in many

### Table 5. Summary of the results in multivariate analysis.

| Variable (n) | Overall survival | Disease-specific survival | Disease-free survival |
|--------------|------------------|---------------------------|----------------------|
|              | HR | 95% CI | p value | HR | 95% CI | p value | HR | 95% CI | p value |
| **Stage**    |     |        |         |     |        |         |     |        |         |
| T3N0 (143)   |     |        |         |     |        |         |     |        |         |
| T4aN0 (17)   | 1.76 | 0.64–4.83 | 0.275 | 3.40 | 0.72–15.98 | 0.121 | 3.04 | 0.82–11.33 | 0.097 |
| T4bN0 (13)   | 2.86 | 1.06–7.74 | **0.038** | 4.58 | 0.89–23.62 | 0.069 | 8.05 | 2.31–28.01 | **0.001** |
| **Grade**    |     |        |         |     |        |         |     |        |         |
| 1 (19)       |     |        |         |     |        |         |     |        |         |
| Grade 2 (114) | 0.50 | 0.20–1.29 | 0.153 | 0.93 | 0.13–6.56 | 0.946 | 0.82 | 0.13–5.21 | 0.838 |
| Grade 3 (40) | 0.53 | 0.18–1.53 | 0.241 | 0.68 | 0.08–6.12 | 0.732 | 1.27 | 0.18–8.87 | 0.809 |
| Right colon (89) | 1.35 | 0.69–2.65 | 0.378 | 1.27 | 0.37–4.36 | 0.702 | 1.04 | 0.39–2.83 | 0.933 |
| Vascular invasion (19) | 1.57 | 0.78–3.18 | 0.210 | 3.36 | 0.98–11.57 | 0.055 | 3.62 | 1.26–10.37 | **0.017** |
| Perforation (10) | 3.80 | 1.57–9.17 | **0.003** | 5.44 | 1.3–22.75 | **0.002** | 4.87 | 1.38–17.23 | **0.014** |
| Preop. obstruction (19) | 0.71 | 0.27–1.85 | 0.479 | 1.32 | 0.31–5.65 | 0.71 | 1.53 | 0.45–5.21 | 0.499 |
| **BRAF** mutation (12) | 3.29 | 1.14–9.54 | **0.028** | 1.41 | 0.20–9.90 | 0.728 | 1.00 | 0.20–5.07 | 0.997 |
| **Ezrin low MSS** (177) |     |        |         |     |        |         |     |        |         |
| Ezrin low MSS (18) | 0.34 | 0.10–1.15 | 0.083 | 0.00 | 0.00–0.00 | 0.986 | 0.78 | 0.09–6.66 | 0.824 |
| Ezrin high MSS (19) | 0.98 | 0.37–2.64 | 0.975 | 5.68 | 1.53–21.12 | **0.01** | 2.76 | 0.76–1.01 | 0.124 |
| Ezrin high MSS (19) | 0.76 | 0.26–2.21 | 0.619 | 3.19 | 0.61–16.74 | 0.17 | 3.01 | 0.78–11.66 | 0.110 |

[https://doi.org/10.1371/journal.pone.0185436.t005](https://doi.org/10.1371/journal.pone.0185436.t005)
studies [37,38,13,39,21]. However, the combination of ezrin expression with MSI status stratified the patients to prognostic groups, in which patients with MSS and high ezrin expression had the shortest DSS and patients with MSI and low ezrin expression had the best DSS (log-rank test, \( p = 0.007 \)). This correlation is of interest as high ezrin expression was significantly more infrequent in MSS tumors than MSI tumors. Why the prognostic role of ezrin is especially pronounced in MSS tumors awaits further studies.

With this university hospital area based cohort we could confirm earlier findings related to microsatellite instability and BRAF mutation status. Mucinous histology and poor differentiation grade were associated with MSI, which is in accordance with MSI high phenotype [40]. In the current study, about a fifth of the tumors were MSI high, and MSI high tumors were significantly more commonly right-sided, as reported previously for stage II tumors [37,13]. Sidedness in itself, however does not justify patient selection for possible adjuvant therapy in stage II CRC [41].

In the current study, 84% of BRAF mutated tumors were MSI, whereas most BRAF wild type tumors were MSS. BRAF mutation was also significantly linked to overall survival, the HR for mortality being 3.29 (95%CI [1.14–9.54], \( p = 0.028 \)). Similar results have also been reported in previous studies, showing BRAF mutation to associate with increased mortality due to CRC [21,42]. There is evidence that MSI phenotype may compensate the poor prognostic effect of BRAF mutation [43], but this issue remains controversial [44]. BRAF mutation is also reported to rule out Lynch syndrome [17], which may be of help to the clinicians in counseling the patients and their families.

At the time-point the patients were treated, MSI-status was not routinely tested among stage II patients. Altogether, 37% of the patients had received adjuvant chemotherapy, according to possible high-risk factors including preoperative obstruction or perforation, vascular invasion, poor differentiation grade and T4-stage and depending on their overall health, general health and patient preference. Patients with MSI tumors are reported not to gain benefit from fluorouracil-based adjuvant chemotherapy [13,45]. This concerns especially stage II colorectal cancer patients, while there are conflicting results concerning stage III patients [46].

In conclusion, our study found a correlation between ezrin expression and DSS in stage II CRC, and this correlation was further strengthened by microsatellite instability analysis. Of the different tumor categories, DSS was longest in patients presenting with MSI / low ezrin tumors and shortest in MSS / high ezrin tumors. These results imply that ezrin staining can provide important prognostic information for estimating stage II patients’ individual risk of disease recurrence and progression.

**Supporting information**

S1 Table. Univariate analysis survival data according to clinicopathological parameters and biomarkers.

(DOCX)

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