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EMMPRIN is associated with S100A4 and predicts patient outcome in colorectal cancer

K Boye*[,1,2], JM Nesland[3,4], B Sandstad[5], M Haugland Haugen[1], GM Mælandsmo[1,6] and K Flatmark[1,7]

[1]Department of Tumour Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, PO Box 4953 Nydalen, NO-0424 Oslo, Norway; [2]Department of Oncology, The Norwegian Radium Hospital, Oslo University Hospital, PO Box 4953 Nydalen, NO-0424 Oslo, Norway; [3]Department of Pathology, The Norwegian Radium Hospital, Oslo University Hospital, PO Box 4953 Nydalen, NO-0424 Oslo, Norway; [4]Medical Faculty, University of Oslo, PO Box 1078 Blindern, NO-0316 Oslo, Norway; [5]Unit of Biostatistics and Epidemiology, The Norwegian Radium Hospital, Oslo University Hospital, PO Box 4953 Nydalen, NO-0424 Oslo, Norway; [6]Department of Pharmacy, Faculty of Health Sciences, University of Tromsø, NO-9037 Tromsø, Norway; [7]Department of Surgical Oncology, The Norwegian Radium Hospital, Oslo University Hospital, PO Box 4953 Nydalen, NO-0424 Oslo, Norway

BACKGROUND: Proteolytic enzymes and their regulators have important biological roles in colorectal cancer by stimulating invasion and metastasis, which makes these factors attractive as potential prognostic biomarkers.

METHODS: The expression of extracellular matrix metalloproteinase inducer (EMMPRIN) was characterised using immunohistochemistry in primary tumours from a cohort of 277 prospectively recruited colorectal cancer patients, and associations with expression of S100A4, clinicopathological parameters and patient outcome were investigated.

RESULTS: One hundred and ninety-eight samples (72%) displayed positive membrane staining of the tumour cells, whereas 10 cases (4%) were borderline positive. EMMPRIN expression was associated with shorter metastasis-free, disease-specific and overall survival in both univariate and multivariate analyses. The prognostic impact was largely confined to TNM stage III, and EMMPRIN-negative stage III patients had an excellent prognosis. Furthermore, EMMPRIN was significantly associated with expression of S100A4, and the combined expression of these biomarkers conferred an even poorer prognosis. However, there was no evidence of direct regulation between the two proteins in the colorectal cancer cell lines HCT116 and SW620 in siRNA knockdown experiments.

CONCLUSION: EMMPRIN is a promising prognostic biomarker in colorectal cancer, and our findings suggest that it could be used in the selection of stage III patients for adjuvant therapy.

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The vast majority of patients who die from colorectal cancer succumb to metastatic disease (Cunningham *et al*, 2010). Improved detection strategies have resulted in patients being diagnosed at potentially curable disease stages, but despite adequate surgery and adjuvant therapy 20–30% of potentially cured patients experience disease recurrence, mainly in the form of distant metastases (O'Connell *et al*, 2004). To initiate growth in a secondary organ, tumour cells must complete a number of biological events, including local invasion of host stroma, intravasation, survival in the circulation, extravasation and proliferation at the metastatic site (Valastyan and Weinberg, 2011). Protease activity is required for several of these steps, and matrix metalloproteinases (MMPs) are among the key enzymes responsible for protease activity during the metastatic process (Egeblad and Werb, 2002). Elevated expression of several MMPs has been associated with poor outcome in colorectal cancer, including MMP-1, -2, -7, -9, -13 and -14. (Murray *et al*, 1996; Curran *et al*, 2004; Hilska *et al*, 2007; van der Jagt *et al*, 2009). Correspondingly, proteins that promote MMP activity are often associated with a proteolytic and pro-invasive phenotype, and overexpression of such proteins in primary tumours of cancer patients thus often confers a poor prognosis (van der Jagt *et al*, 2009).

One MMP-associated candidate biomarker is the metastasis-promoting protein S100A4, and we recently demonstrated that nuclear expression of S100A4 was a robust prognostic factor in colorectal cancer (Boye *et al*, 2010). This small calcium-binding protein is involved in epithelial–mesenchymal transition and has been implicated in several steps of the metastatic cascade, including motility, invasion and angiogenesis (Boye and Mælandsmo, 2010). It stimulates the expression and activity of several MMPs (Bjørnland *et al*, 1999; Schmidt-Hansen *et al*, 2004; Saleem *et al*, 2006), and MMP activation is most likely critical for S100A4-induced metastasis, but the mechanisms involved in S100A4-mediated regulation of MMP activity and expression are largely unknown. Another key regulator of MMP activity in both stromal and tumour cells is the extracellular matrix metalloproteinase inducer (EMMPRIN/CD147/basigin) (Yan *et al*, 2005), a cell surface glycoprotein belonging to the immunoglobulin superfamily (Biswas *et al*, 1995). In experimental models, overexpression of EMMPRIN promotes tumour growth, invasion, angiogenesis and metastasis (Zucker *et al*, 2001; Tang *et al*, 2005). EMMPRIN is expressed in the majority of human tumour types, including colorectal cancer (Riethdorf *et al*, 2006; Li *et al*, 2009), and associations between EMMPRIN expression and poor

*Correspondence: Dr K Boye, E-mail: kjetil.boye@rr-research.no
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prognosis have previously been shown in breast cancer (Reimers *et al*, 2004; Li *et al*, 2009), genitourinary carcinomas (Han *et al*, 2010) and non-small cell lung cancer (Sienel *et al*, 2008). In colorectal cancer, however, no large studies on patient outcome have been reported.

In the present work, associations between EMMPRIN expression, clinicopathological parameters and patient outcome were investigated in a cohort of prospectively recruited colorectal cancer patients undergoing curatively intended surgery. As EMMPRIN and S100A4 both modify the activity of a number of different MMPs, a reciprocal regulation could exist to induce a proteolytic and pro-invasive phenotype. To explore this possibility, expression data from this cohort were analysed for associations between expression of EMMPRIN and S100A4 in primary tumour samples, and functional studies were carried out in two colorectal cancer cell lines.

MATERIALS AND METHODS

Patient cohort

Between September 1998 and July 2000, 316 patients from five hospitals in the Oslo region were included in the study at the time of primary surgery for assumed or verified colorectal cancer (Flatmark *et al*, 2002). The study was approved by the Regional Ethics Committee (no. S-98080) and informed consent was obtained from the patients. Thirty-one patients were excluded for the following reasons: not invasive cancer (25), histology other than adenocarcinoma (5) and unknown stage of disease (1). The total study population thus consisted of 285 patients, and paraffin sections were available from 277 of these patients. For the prognostic studies, 43 patients were excluded due to distant metastases at the time of surgery (34), inadequate surgical margins (7) and preoperative chemoradiotherapy (2). The study population for the survival analyses thus included 242 patients in TNM stage I-III who had undergone curative surgery. The follow-up of the patient cohort has been described in detail previously (Boye *et al*, 2010). Briefly, patients were followed by physicians at the participating hospitals, and metastasis-free, disease-specific and overall survival was registered. In addition, survival data were obtained from the National Registry of Norway and updated by 1 October 2008. The cause of death was registered and classified as death from colorectal cancer, death of other causes or death of unknown cause. For overall survival, median follow-up of patients still alive was 9.1 years (range 8.2–10.0). Metastasis-free survival was defined as time to first metachronous distant metastasis, and patients without metastases were censored at time of death.

Immunohistochemistry

The primary tumour sections were re-evaluated by the study pathologist (JMN), and representative paraffin blocks for each tumour were identified for subsequent immunohistochemical analysis. Immunohistochemistry was performed using the biotin–streptavidin–peroxidase method (Supersensitive Immunodetection System, LP000-UL; Biogenex, San Ramon, CA, USA) and the Optimax Plus Automated Cell Staining System (Biogenex). After treatment with 1% hydrogen peroxide for 10 min to block endogenous peroxidase, the sections were incubated with goat polyclonal anti-EMMPRIN antibody (sc-9752; Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1 : 300, for 30 min at room temperature. The sections were then incubated with biotin-labelled secondary antibody (1 : 30) and streptavidin–peroxidase (1 : 30) for 20 min each. Slides were stained for 5 min with 0.05% 3,3′-diaminobenzidine tetrahydrochloride freshly prepared in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.024% hydrogen peroxide and then counterstained with haematoxylin, dehydrated, and mounted in Diatex. The dilutions were made with phosphate-buffered saline, pH 7.4, containing 1% bovine serum albumin. EMMPRIN-negative cell types in each section served as negative control, and in addition negative controls with omission of the primary antibody were performed. As positive controls, tissues with known expression of EMMPRIN were applied. Cytoplasmic and membrane staining were recorded as separate variables, and the number of EMMPRIN-positive tumour cells was semi-quantitatively estimated and graded from 0 to 5 (percentage of positive carcinoma cells in parentheses): 0 (0%), 1 (1–4%), 2 (5–9%), 3 (10–14%), 4 (15–49%) and 5 ($>$50%). For all statistical analyses, tumours in grade 2–5 were grouped as positive. Classifying the borderline positive tumours (1–4% positive tumour cells) as positive did not significantly affect the univariate survival analyses or the associations with other clinicopathological variables. Expression of EMMPRIN in stromal cells or neighbouring non-malignant epithelium was not systematically assessed.

Statistical analysis

Associations between EMMPRIN staining and clinicopathological variables were tested using two-tailed Fisher's exact test or linear-by-linear association χ^2 test. Univariate survival analysis was performed according to the Kaplan–Meier method, and survival was compared using the log-rank test. Multivariate analysis was conducted using the Cox proportional hazards regression model with backward, stepwise elimination of variables. Survival was measured from date of surgery until death for overall and disease-specific survival, and from date of surgery until diagnosis of distant metastasis for metastasis-free survival. Data analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). P-values <0.05 were considered statistically significant.

Cell culture and treatment

The human colorectal cancer cell lines HCT116 and SW620 were purchased from the American Type Culture Collection (Rockville, MD, USA). All cell cultures were routinely tested for Mycoplasma infection, and the identity of the cell lines was verified by STR profiling using Powerplex 16 (Promega, Madison, WI, USA). Cells were cultivated in RPMI 1640 (BioWhittaker, Lonza Verviers, Belgium), supplemented with 8.5% heat-inactivated fetal calf serum (PAA, New Bedford, MA, USA), 20 mM Hepes (BioWhittaker) and 2 mM Glutamax (Gibco, Invitrogen, Carlsbad, CA, USA). For experiments with extracellular S100A4, cells were seeded at $1.2 \times 10^5\,cm^{-2}$ in T25-flasks and allowed to attach overnight. Fresh cell culture medium with or without $10\,\mu g\,ml^{-1}$ human recombinant S100A4 was added, and the cells were further incubated for 24 or 48 h before being harvested by scraping in ice-cold PBS. Production of recombinant S100A4 has been described previously (Berge *et al*, 2011).

siRNA transfection

Cells were seeded at $2.0 \times 10^4\,cm^{-2}$ in T25-flasks and allowed to attach overnight. siRNA constructs were mixed with Lipofectamine 2000 (Invitrogen) for 20 min at room temperature in Opti-MEM I and added to the cell cultures at a final concentration of 50 nM. After incubation for 24 h cell culture medium with the transfection mixture was removed and replaced with fresh cell culture medium. Cells were incubated for another 48 h and then harvested by scraping in ice-cold PBS. siRNA constructs used were as follows: Silencer Select Negative Control no. 2 siRNA (Ambion/Applied Biosystems, Austin, TX, USA), S100A4 siRNA (Boe *et al*, 2007) and Silencer Pre-designed siRNA ID215973 (siEMMPRIN1), ID147251 (siEMMPRIN2) and ID10372 (siEMMPRIN3) (Ambion).

Protein isolation and western blotting

Cell lysates were prepared as described previously (Grotterød *et al*, 2010). Total protein lysates were separated on 4–12% NuPAGE Novex Bis-Tris Gels (Invitrogen) in MES buffer and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). As a transfer and loading control, membranes were stained with 0.1% amidoblack. Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk was used for all incubations. After 1 h blockage of non-specific binding sites, membranes were incubated for 1 h at room temperature or overnight at 4°C with mouse anti-S100A4 22.3 diluted 1 : 1000 (Flatmark *et al*, 2004), goat anti-EMMPRIN diluted 1 : 500 (sc-9753; Santa Cruz Biotechnology) or mouse anti-α tubulin (1 : 1000; Calbiochem, Darmstadt, Germany). After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidise-conjugated secondary antibody (DAKO, Glostrup, Denmark) diluted 1 : 5000. Signals were visualised using Super Signal West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA, USA) and analysed using G:BOX (Syngene, Frederick, MD, USA) with the GeneSnap software (Syngene).

RESULTS

EMMPRIN protein expression in primary colorectal adenocarcinomas

The expression of EMMPRIN was analysed by immunohistochemistry in primary tumours from a prospectively recruited cohort of 277 colorectal cancer patients. In tumour cells, staining was observed both in the cytoplasm and on the cell membrane (Figure 1). Varying degrees of membrane expression was detected in 208 samples (75%), of which 10 cases (4%) were borderline positive (<5% positive tumour cells) and 250 samples (91%) displayed cytoplasmic staining (Table 1). As expected, a highly significant association between EMMPRIN expression in the cytoplasm and the membrane was observed ($P<0.001$). As the presently known biological function of EMMPRIN is mainly confined to the cell membrane, only membrane staining was considered positive and used for further analyses.

Associations between EMMPRIN expression and clinicopathological parameters

The clinical and histopathological baseline data of the study cohort are presented in Table 2. Mean age at the time of surgery was 70 years (range 21–98 years). Sixty-eight percent of the tumours were localised in the colon and 32% in the rectum. The majority of patients were in early disease stages, with 19% in TNM stage I, 40% in stage II, 30% in stage III and 12% in stage IV. There were no statistically significant associations between EMMPRIN expression and any of the clinical or histopathological parameters (Supplementary Table 1).

EMMPRIN expression and patient outcome

For the survival analyses, only patients in TNM stage I-III that had undergone R0 resections were included, resulting in a study population of 242 patients. The clinicopathological data and outcome parameters of this cohort have been described previously (Boye *et al*, 2010). Using a cut-off value of 5% positive tumour cells, EMMPRIN expression was a highly significant predictor of metastasis-free survival (Figure 2A), and the 5-year metastasis-free survival rate of patients with EMMPRIN-negative tumours was 87%, compared with 63% for patients with EMMPRIN-positive tumours. EMMPRIN expression was also associated with disease-specific survival (Figure 2B), whereas the association with overall survival was not statistically significant (Figure 2C). To determine whether the relationship between EMMPRIN expression and patient outcome was influenced by other clinical and histopathological parameters, multivariate Cox regression analysis was performed. Variables included in the multivariate analysis were EMMPRIN, age, gender, TNM stage, differentiation, tumour localisation, lymphocyte infiltration, vascular invasion and perineural invasion. Remarkably, EMMPRIN expression was the most significant predictor of metastasis-free survival (Table 3). EMMPRIN was also an independent prognostic factor for disease-specific survival ($P=0.04$; hazard ratio 2.3; 95% confidence interval (CI) 1.0–5.0; data not shown), but not statistically significant for overall survival ($P=0.07$; hazard ratio 1.5; 95% CI 1.0–2.5; data not shown).

Prognostic impact of EMMPRIN expression in TNM stage III

To investigate the prognostic impact of EMMPRIN expression in the separate disease stages, patients were stratified according to TNM stage, and univariate survival analyses were performed. In TNM stage III, EMMPRIN was strongly associated with patient outcome (Figure 2F), whereas no prognostic significance was observed in TNM stage I and II (Figures 2D and E). In fact, only 2 of 21 (9.5%)

Table 1 Immunohistochemical expression of EMMPRIN

	Membrane staining Number[a] (%)	Cytoplasmic staining Number[a] (%)
0	68 (25)	26 (9)
1	10 (4)	0 (0)
2	19 (7)	11 (4)
3	41 (15)	33 (12)
4	70 (25)	67 (24)
5	68 (25)	139 (50)
ND	1	1

Abbreviations: EMMPRIN = extracellular matrix metalloproteinase inducer; ND = not determined. [a]A total of 277 samples were examined.

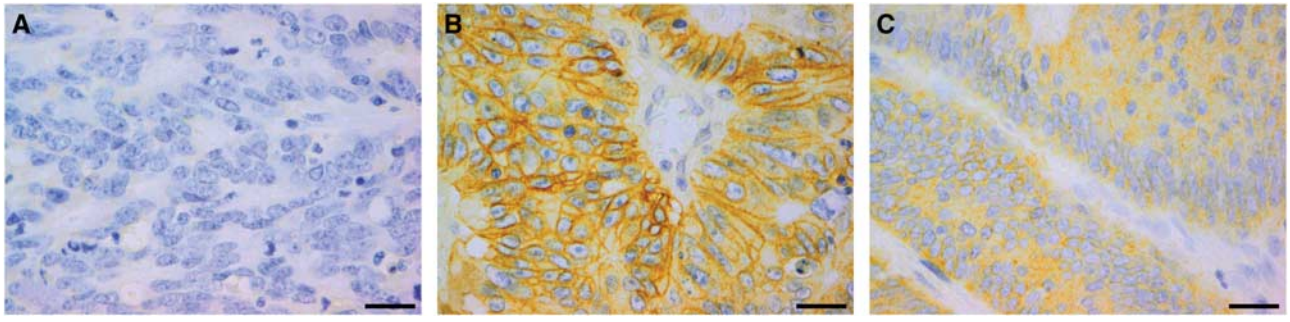

Figure 1 Representative photomicrographs of immunohistochemical staining of colorectal cancer specimens with anti-EMMPRIN antibody. (**A**) No immunoreactivity. (**B**) Strong cytoplasmic and membrane staining (score 5). (**C**) Strong cytoplasmic staining (score 5) and no membrane staining (score 0). Scale bar = 20 μm.

Table 2 Baseline clinical and histopathological data of the study cohort

	Patients[a]	
Parameter	**Number**	**%**
Gender		
Female	130	47
Male	147	53
TNM stage		
I	52	19
II	111	40
III	82	30
IV	32	12
pT		
1	8	3
2	51	19
3	179	65
4	37	13
ND	2	
pN		
0	166	60
1	64	23
2	45	16
ND	2	
Differentiation		
Well	7	3
Intermediate	229	83
Poor	40	14
ND	1	
Tumour localisation		
Colon	188	68
Rectum	89	32
Lymphocyte infiltration		
High	32	12
Intermediate	175	64
Low	65	24
ND	5	
Vascular invasion		
Present	62	23
Absent	212	77
ND	3	
Perineural invasion		
Present	29	11
Absent	245	89
ND	3	
Perinodal growth[b]		
Present	66	61
Absent	43	39

Abbreviations: ND = not determined; pN stage = pathological nodal stage; pT stage = pathological tumour stage; TNM stage = tumour node metastasis stage. [a]A total of 277 patients were examined. [b]Perinodal growth was assessed in node positive patients only.

EMMPRIN-negative stage III patients developed distant metastasis, whereas 28 of 55 (51%) of EMMPRIN-positive stage III patients did. For the TNM stage III patients, there were no associations between EMMPRIN expression and pN status ($P = 0.29$), perinodal growth ($P = 0.80$) or administration of adjuvant chemotherapy ($P = 0.61$), indicating that the prognostic impact of EMMPRIN in this patient group is not confounded by other variables.

EMMPRIN and S100A4

We have previously demonstrated that nuclear expression of the metastasis-associated protein S100A4 was a prognostic biomarker in the same patient cohort (Boye *et al*, 2010), and when the expression of the two biomarkers was combined, an even more pronounced survival difference was observed (Figure 3A). For EMMPRIN- and S100A4-negative patients, the estimated 5-year metastasis-free survival rate was 86%, compared with 36% for patients with EMMPRIN- and S100A4-positive tumours ($P < 0.0001$). Estimated 10-year overall survival rate was 68% and 24%, respectively ($P = 0.001$; data not shown). Combined expression of both proteins was also significantly associated with poor metastasis-free survival in multivariate analysis ($P = 0.002$, hazard ratio 5.8; 95% CI 1.9–17.5; data not shown).

Interestingly, EMMPRIN expression was significantly associated with cytoplasmic expression of S100A4 ($P < 0.001$). Seventy-one percent of EMMPRIN-positive cases were S100A4-positive, whereas only 47% of EMMPRIN-negative tumours expressed S100A4 (Table 4). These results could indicate a direct regulation between S100A4 and EMMPRIN, and to investigate this hypothesis we downregulated the expression of both proteins using siRNA transfection in two colorectal carcinoma cell lines. Figure 3 shows that siRNA against EMMPRIN (B) and S100A4 (C) suppressed expression of the target protein. However, no regulation of S100A4 was observed in siEMMPRIN-transfected cells, and no regulation of EMMPRIN was seen in siS100A4-transfected cells. These data are further substantiated by investigations in HCT116 and SW620 cells stably transduced with shRNA against S100A4, where no changes in EMMPRIN expression levels were observed (data not shown). As S100A4 is present in the extracellular space, we also investigated whether treatment with recombinant S100A4 could affect EMMPRIN expression levels. Figure 3D clearly demonstrates that extracellular S100A4 did not alter EMMPRIN expression significantly. Taken together, these results show that expression of S100A4 and EMMPRIN is strongly associated in tumours from colorectal cancer patients, but no direct regulation between these proteins was evident in the two cell lines investigated.

DISCUSSION

In colorectal cancer there is a need for improved disease classification algorithms to predict outcome and to make optimal therapeutic decisions. Molecular biomarkers hold great promise to fulfil this need, and numerous investigations have been performed over the last years to identify such novel prognostic and predictive factors. Prognostic biomarkers could be identified through several approaches, of which high-throughput methods such as gene expression arrays or proteomics have been frequently employed. Such unbiased approaches have several advantages, but are generally hampered by the lack of functional data to understand the mechanisms involved. We have undertaken a biology-based approach, where we have investigated several proteins directly implicated in the metastatic process using immunhistochemistry, and analysed associations between expression data, clinicopathological parameters and patient outcome (Boye *et al*, 2010; Ingebrigtsen *et al*, 2012; Haugen *et al*, in preparation).

In this study, we report for the first time in a large cohort of prospectively recruited patients that expression of the MMP-inducer EMMPRIN is associated with poor outcome in colorectal cancer. The 5-year metastasis-free survival rate for patients with EMMPRIN-negative tumours was 87% compared with 63% for EMMPRIN-positive patients, and EMMPRIN was also a predictor of metastasis-free survival in multivariate analysis. Furthermore, 49 of the 55 patients that developed distant metastasis had EMMPRIN-positive tumours, resulting in a sensitivity for prediction of metastatic disease of 89%. The specificity, however, was rather low (32%). By combining EMMPRIN expression with nuclear expression of S100A4, a previously identified prognostic biomarker in this patient cohort, we observed that the prognosis for patients with expression of both biomarkers was very poor.

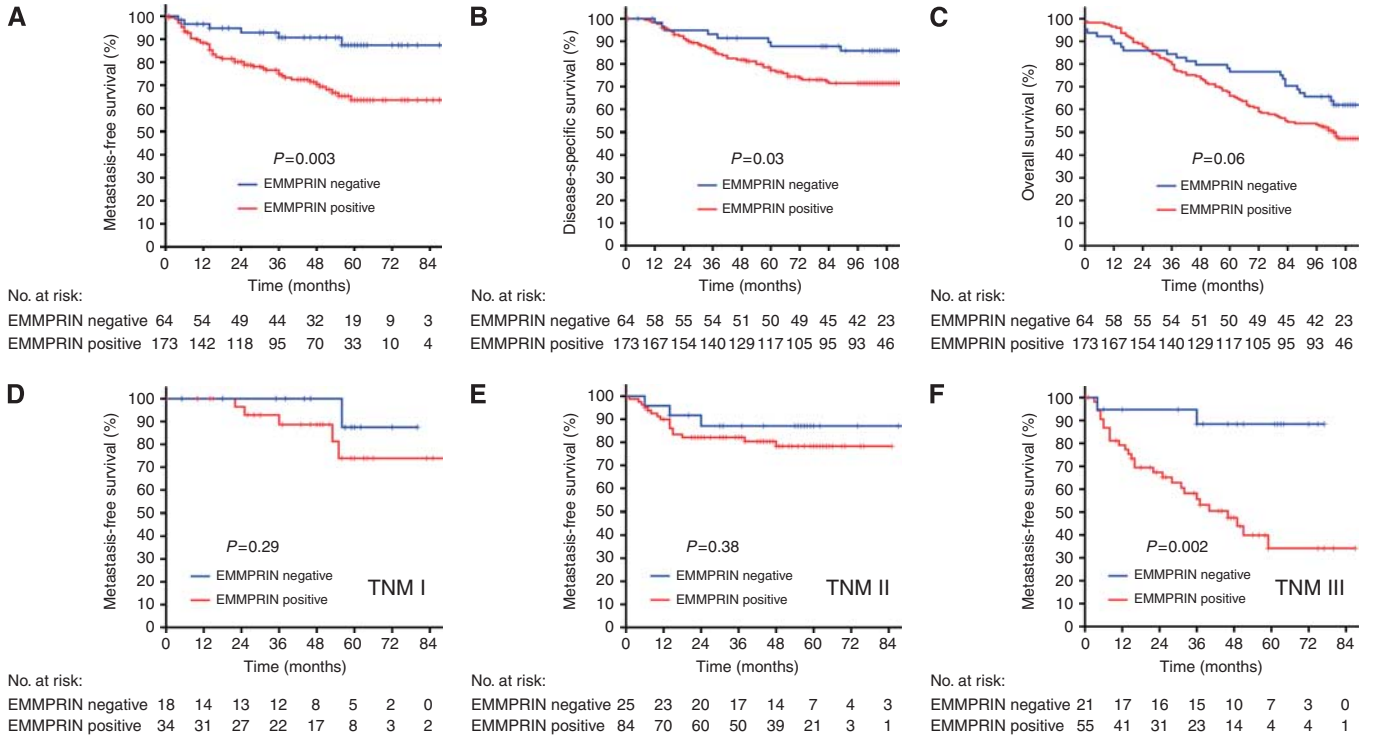

Figure 2 Kaplan–Meier survival plots depicting metastasis-free (**A**), disease-specific (**B**) and overall survival (**C**) based on EMMPRIN expression. (**D**–**F**) Kaplan–Meier survival plots depicting metastasis-free survival stratified according to TNM stage and based on expression of EMMPRIN.

Table 3 Multivariate Cox regression analysis of metastasis-free survival[a]

	P-value	Hazard Ratio	95% CI
EMMPRIN	0.008		
0–1			
2–5		3.3	1.4–7.8
Lymphocyte infiltration	0.01		
High			
Intermediate		3.2	0.7–13.4
Low		5.8	1.3–25.5
TNM stage	0.02		
I			
II		1.5	0.6–3.8
III		2.9	1.2–7.4
Tumour localisation	0.06		
Colon			
Rectum		1.8	0.9–3.3
Differentiation	0.08		
Well			
Intermediate		0.6	0.08–4.7
Poor		1.6	0.2–13.4
Vascular invasion	0.08		
Absent			
Present		1.7	0.9–3.3

Abbreviation: EMMPRIN = extracellular matrix metalloproteinase inducer. [a]All parameters included in the final model are shown.

Still, the clinical impact of the combined expression is rather modest compared with EMMPRIN expression alone, with a sensitivity of 93% and a specificity of 21% for expression of at least one biomarker, and 40% and 14%, respectively, for expression of both proteins. Taken together, the major clinical significance of our findings seems to be that EMMPRIN-negative patients rarely experienced disease recurrence. In particular, EMMPRIN-negative stage III patients had a remarkably good prognosis, and only 2 of 21 patients in this group developed distant metastasis. Stage III patients are routinely offered adjuvant chemotherapy to reduce the risk of disease progression, even though ~40% are cured by surgery alone (Laurie *et al*, 1989; Moertel *et al*, 1990). Thus, the discovery of a biomarker to identify low-risk stage III disease would spare these patients the morbidity and mortality associated with adjuvant chemotherapy, and our results indicate that EMMPRIN expression, if validated in future studies, could be used in the selection of patients for adjuvant treatment.

Several previous investigations have examined the expression of EMMPRIN in colorectal cancer (Jin *et al*, 2006; Riethdorf *et al*, 2006; Buergy *et al*, 2009; Zheng *et al*, 2011). In most reports, the majority of cases show some degree of EMMPRIN immunoreactivity, both in the cytoplasm and at the cell membrane, but different scoring systems preclude direct comparisons. To our knowledge, only two studies have explored the relationship between EMMPRIN expression and patient outcome (Buergy *et al*, 2009; Jung *et al*, 2011). Buergy *et al* (2009) found that a relative increase in EMMPRIN expression in the tumour compared with normal epithelium was associated with poor disease-specific survival in a cohort of 40 colorectal cancer patients from all disease stages. In contrast, EMMPRIN expression was not a prognostic factor in a retrospective study including 210 patients (Jung *et al*, 2011). However, overall survival was the only outcome measure reported, and the lack of complete follow-up data in addition to the retrospective recruitment of cases might explain the discrepant results.

In addition to its expression in the majority of primary tumours, EMMPRIN is frequently expressed in disseminated tumour cells (DTCs) isolated from bone marrow from breast, prostate and lung cancer patients (Klein *et al*, 2002; Reimers *et al*, 2004). In a panel of 38 colorectal cancer patients, EMMPRIN-positive tumour cells in

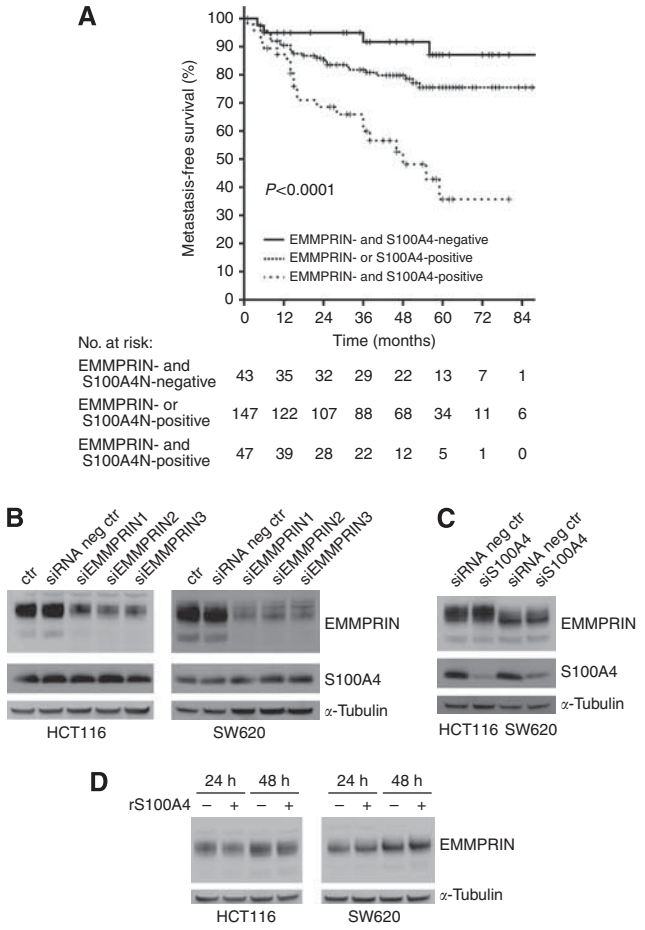

Figure 3 (**A**) Kaplan–Meier survival plot depicting metastasis-free survival based on expression of EMMPRIN and/or S100A4 as indicated. (**B**) Immunoblot of total cell lysates from HCT116 and SW620 control cells (lane 1 in each panel), cells transfected with siRNA negative control (lane 2) or siRNA against EMMPRIN (lanes 3–5). Membranes were stained with anti-EMMPRIN and anti-S100A4. α-Tubulin was used as a loading control. (**C**) Immunoblot of total cell lysates from HCT116 and SW620 cells transfected with siRNA negative control (lanes 1 and 3) or siRNA against S100A4 (lanes 2 and 4). Membranes were stained with anti-EMMPRIN and anti-S100A4. α-Tubulin was used as a loading control. The differences in molecular weight of EMMPRIN are due to glycosylation. (**D**) Immunoblot of total cell lysates from HCT116 and SW620 cells treated with 10 $\mu g\,ml^{-1}$ recombinant S100A4 for 24 or 48 h as indicated. Membranes were stained with anti-EMMPRIN and α-tubulin was used as a loading control. All results shown are representative of at least three independent experiments.

the bone marrow were identified in 11 cases, and interestingly, EMMPRIN-expressing cells were found in four of five patients with synchronous metastases (Buergy *et al*, 2009). In our patient cohort, the presence of DTCs analysed by immunocytochemistry (using an anti-cytokeratin antibody) and immunomagnetic selection (using an anti-EpCAM antibody) was an adverse prognostic factor (Flatmark *et al*, 2011). As both EMMPRIN expression in the primary tumour, the presence of DTCs and EMMPRIN expression in DTCs seem to be associated with metastatic disease in colorectal cancer, one might speculate that DTCs would be detected more frequently in patients with EMMPRIN-expressing primary tumours. However, no association was found between EMMPRIN expression and the presence of tumour cells in bone marrow in our cohort (DTCs were detected in 29% and 27% of EMMPRIN-negative and -positive patients, respectively; data not shown).

Table 4 Immunohistochemical expression of EMMPRIN and S100A4[a]

	S100A4[b]	
	Negative	**Positive**
EMMPRIN		
Negative	41 (53%)	37 (47%)
Positive	57 (29%)	141 (71%)

Abbreviation: EMMPRIN = extracellular matrix metalloproteinase inducer. [a]The number (and percentages) of patients within each category is shown. [b]Expression of cytoplasmic S100A4.

Similar findings have been reported for breast cancer (Reimers *et al*, 2004), suggesting that the DTC detection methods employed are not able to identify all relevant DTCs. Indeed, DTCs were found in only 21 of the 55 patients that developed metastatic disease in our study.

As both EMMPRIN and S100A4 stimulate MMP activity, we investigated the associations between expression of these metastasis-related proteins in the primary tumour samples. Interestingly, a highly significant association was observed, and nearly three quarters of the EMMPRIN-positive tumours were also S100A4-positive. These results suggest that a reciprocal regulation between S100A4 and EMMPRIN could exist. Further supporting this hypothesis are previous findings that EMMPRIN positively regulates the Wnt/β-catenin pathway (Sidhu *et al*, 2010), providing a possible mechanism for direct regulation of S100A4 (Stein *et al*, 2006). Similarly, EMMPRIN expression could be regulated by the S100A4 receptor RAGE (receptor for advanced glycation end products) (Bao *et al*, 2010) and the NF-κB signalling pathway (Hagemann *et al*, 2005), which is also activated by S100A4 (Boye *et al*, 2008). However, there was no evidence of any direct regulation between EMMPRIN and S100A4 in the two colorectal cancer cell lines investigated. These findings could indicate that the expression of both proteins is regulated in the same manner, that is, controlled by the same signalling pathways or transcription factors, resulting in their presence in tumour cells of a proteolytic and pro-invasive phenotype, associated with a dismal prognosis.

In conclusion, EMMPRIN expression in primary colorectal adenocarcinomas was a robust prognostic factor in this patient cohort, and patients in all disease stages with EMMPRIN-negative tumours had an excellent prognosis. Future studies should focus on validation of EMMPRIN as a biomarker to select stage III patients for adjuvant chemotherapy.

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Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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