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Original contribution

The relationship between the Glasgow Microenvironment Score and markers of epithelial-mesenchymal transition in TNM II-III colorectal cancer☆

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Summary Recently published work on the Glasgow Microenvironment Score (GMS) demonstrated its relevance as a biomarker in TNM II-III colorectal cancer (CRC). Epithelial-mesenchymal transition (EMT) markers in CRC have also shown promise as prognostic biomarkers. This study aimed to assess the relationship between GMS and markers of EMT in stage II-III CRC. A previously constructed tissue microarray of CRC tumors resected between 2000 and 2007 from the Western Infirmary, Stobhill, and Gartnavel General Hospitals in Glasgow was used. Immunohistochemistry was performed for 5 markers of EMT: E-cadherin, β-catenin, Fascin, Snail, and Zeb1. Two-hundred and thirty-eight TNM II-III CRC with valid scores for all EMT markers and GMS were assessed. The prognostic significance of markers of EMT in this cohort and relationships between GMS and markers of EMT were determined. High cytoplasmic and nuclear β-catenin and membrane Zeb-1 were significant for worse cancer-specific survival (hazard ratio [HR] 1.67, 95% confidence interval [CI] 1.01−2.76, $P < .05$; HR 2.22, 95% CI 1.24−3.97, $P < .01$; and HR 2.00, 95% CI 1.07−3.77, $P = .03$, respectively). GMS

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0 was associated with low membrane Fascin ($P = .03$), whereas membrane and cytoplasmic Fascin were observed to be highest in GMS 1, but lower in GMS 2. Nuclear β-catenin was lowest in GMS 0, but highest in GMS 2 ($P = .03$), in keeping with its role in facilitating EMT. Novel associations were demonstrated between GMS categories and markers of EMT, particularly β-catenin and Fascin, which require further investigation in independent cohorts.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and was responsible for the second highest cancer-related death rate in 2018 [1]. Despite the increase in knowledge and understanding of the pathophysiological processes underlying CRC, the TNM staging system on which clinical management is based does not fully account for prognosis [2]. One of the reasons for this is that TNM does not give an account of host factors such as systemic inflammation [3] or the local antitumor inflammatory response [4]. One scoring system based on assessment of the tumor microenvironment on routinely used hematoxylin & eosin (H&E)-stained slides is the Glasgow Microenvironment Score (GMS). The GMS combines an assessment of the inflammatory cell infiltrate at the tumor's invasive margin (Klintrup-Mäkinen [KM] grade) with the quantity of connective tissue, stroma, within the tumor (tumor stromal percentage [TSP]), a marker found to denote poor prognosis related to a mesenchymal phenotype [5,6]. The GMS was recently validated in 2 large independent data sets, including 2912 TNM II-III CRCs from a randomized trial data set (TransScot). GMS was able to stratify both data sets into 3 distinct groups: GMS 0, high KM, had a good prognosis; GMS 1, low KM, but low TSP had an intermediate prognosis that varied with TNM stage; and GMS 2, low KM and high TSP had a poor prognosis, even in early-stage disease. Furthermore, GMS 0 was found to select those receiving FOLFOX chemotherapy for better outcomes compared with those receiving CAPOX chemotherapy, whereas GMS 2 tumors were found to have a poor response to standard chemotherapy [7].

Previous work in CRC disease biomarkers has also shown that markers of epithelial-mesenchymal transition (EMT) have prognostic value [8]. The phenomenon of EMT in its truest form is an embryological process essential for organogenesis [9], whereas in the development of epithelial cancer metastases, it is thought to represent a process in which epithelial cells become less well-differentiated, losing cell-cell adhesion molecules (eg, cadherins) and becoming more motile [9,10]. It is believed that the cancer EMT process (henceforth referred to as EMT) gives rise to circulating tumor cells [11]. The cells that survive in the bloodstream and go on to form metastases in distant organs will be pluripotent cancer stem cells, enabling them to establish new tumors in distant sites [12].

There are several validated markers of EMT. E-cadherin is a cell surface protein functioning closely with the actin cytoskeleton that is involved in cell-cell adhesion, the loss of which is a marker of dedifferentiation [13]. β-Catenin, a member of the catenin family, which links cadherins to the actin cytoskeleton, is also a transcription factor and may be released when not linking E-cadherin to the cell membrane, although the process that drives β-catenin from the cell cytoplasm to the nucleus is unclear [14]. β-Catenin is one of the proteins in the Wnt pathway and in embryological development is involved in both EMT and stem cell formation [14]. The presence of nuclear β-catenin also reduces transcription of E-cadherin [14]. Both higher nuclear β-catenin and lower membrane E-cadherin have been observed in tumor buds, a mesenchymal phenotype believed to be associated with EMT [15]. Both Snail and Zinc finger-E-box binding homeobox 1 (Zeb-1) are transcriptional factors that promote a mesenchymal phenotype and reduce the expression of membrane E-cadherin [16]. Fascin is a downstream target of β-catenin and is usually responsible for bundling of actin cytoskeleton but is upregulated in epithelial cancers [17] and results in increased cell motility and migration [18].

As the ability to identify the process of EMT in CRC will indicate which tumors may metastasize, a simple yet robust means of identifying such tumors is essential. In a previous study, our research group showed that a combination of these 5 markers was associated with survival in a cohort of patients with CRC [8].

The aims of the present study were to assess the prognostic role of markers of EMT in an independent cohort and the relationship between the GMS and markers of EMT. In particular, it was hypothesized that tumors with high immune infiltrates (GMS 0) would have lower expression of EMT markers, whereas those with a mesenchymal phenotype (GMS 2) may have a higher expression of EMT markers [6,7].

2. Methods

2.1. Patients

Two-hundred and thirty-eight TNM II-III CRC specimens were identified retrospectively from Glasgow hospitals (Stobhill Hospital, the Western Infirmary, and Gartnavel General). All patients had undergone surgery

with curative intent between 2000 and 2007. Those who had endoscopic or palliative procedures and those with involved surgical margins (R1) were excluded, as were those who died within 30 days of surgery and those who received neoadjuvant chemoradiotherapy. These specimens were all part of a tissue microarray (TMA) that had previously been constructed with 4 cores per patient taken from representative areas of the tumor [19]. The West of Scotland Research Ethics Committee provided ethical approval for the research. The primary end point was cancer-specific survival (CSS), defined as the time from surgery to death from CRC. Survival data were available until the July 1, 2020.

2.2. Clinicopathological characteristics

Pathological characteristics, including TNM, tumor differentiation, peritoneal invasion, and tumor perforation, were recorded from pathology reports, and clinical characteristics were recorded from clinical case notes. The fifth TNM staging edition was used, consistent with the Royal College of Pathologists reporting guidelines in place at the time of surgery. H&E-stained sections were used to assess venous invasion and either intramural or extramural invasion was considered present. Tumor budding [20] and DNA mismatch repair (MMR) status [21] were already available for this cohort. The Petersen index was used to assess clinical risk as in clinical practice indicating low- or high-risk stage II CRC [22]: venous invasion and peritoneal involvement were assigned a score of 1, whereas tumor perforation was assigned a score of 2. TNM II disease with Petersen index of 2 or higher, or TNM III disease was considered high-risk. Peritumoral inflammatory scores (KM grade) and TSP scores were already available [20]. These were combined as the GMS as previously described [7]. In brief, high KM and any TSP scored GMS 0; low KM with low TSP scored GMS 1 and low KM with high TSP scored GMS 2. The modified Glasgow Prognostic Score (mGPS) was calculated using serum C-reactive protein (CRP) and albumin levels obtained in the 30 days before surgery or at the time of admission as previously described [23].

2.3. Immunohistochemistry

Immunohistochemical analysis of 5 markers of EMT was performed on a previously constructed TMA [19], using the same method described previously [8]. This method involved dewaxing of TMAs, performed using Histoclear, before rehydration using decreasing concentrations of alcohol. Antigen retrieval was then performed as follows. For E-Cadherin, Fascin, Snail, and Zeb-1, a citrate buffer was used under pressure at pH 6.0 for 5 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min. TMAs were subsequently incubated in 10% casein (Vector Laboratories) for 30 min (E-cadherin) or 2 h (Zeb-1, Fascin, and Snail). Primary E-cadherin antibody (1:500; BD Biosciences, 610,182) and Zeb-1 (1:800, Sigma−Aldrich, HPA027524) were added at 4°C overnight or for 2 h at room temperature for Fascin (1:100; Atlas Antibodies, HPA005723), and Snail (1:50; Abcam, ab53519). Following this, TMAs were incubated in envision (DAKO) for 30 min for E-cadherin, Fascin, and Zeb-1; or ImmPRESS anti-goat IgG for 30 min (Snail). For β-catenin, a water bath was used under pressure at pH8.0 at 96°C for 50 min. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide for 30 min. TMAs were subsequently incubated in 1% BSA for 30 min. Primary β-catenin antibody (1:50; BD Biosciences, 610,154) was added for 2 h at room temperature. Following this, TMAs were incubated in envision (DAKO) for 2 h. Antibody visualization was achieved using 3,3′-diaminobenzidine (DAB; Vector Laboratories) until color developed. All slides were counterstained with hematoxylin, then dehydrated in alcohol and Histoclear before mounting with DPX.

2.4. Immunohistochemistry scoring

After staining, all slides were scanned using Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at 20× magnification and visualized on NDP viewer (NanoZoomer Digital Pathology software, Hamamatsu Photonics K.K.). EMT marker staining was assessed by one researcher (PGA), blinded to clinicopathological data, using a weighted histoscore. The weighted histoscore is a well-established method used to quantify expression of the protein of interest. Following immunohistochemical staining, expression within each cellular compartment (membrane, cytoplasm, and nucleus) is scored separately by manual assessment of the proportion of the compartment stained at each density of staining (strong, moderate, weak, or negative). These proportions are then multiplied as follows: (% tumor no staining × 0) + (% tumor weak staining ×1) + (% tumor moderate staining × 2) + (% tumor strong staining ×3), giving a range of scores between 0 and 300 for each marker per cellular compartment. Since each score was calculated in up to 4 cores, an average of the scored cores was taken as the final value. One hundred ninety-two cores were co-scored by a second assessor (JE) with excellent correlation (ICC (intraclass correlation coefficient) >0.88 for all markers and loci) (Fig. 1).

2.5. Statistical analysis

Data for EMT markers were dichotomized into high and low scores using a data-derived threshold for each score at each cellular location according to CSS using RStudio (R Studio, MA) (Supplementary Table S1). Missing data were excluded from the analysis. All other data analysis was performed using SPSS version 27.0 (IBM SPSS). Univariate Cox regression analysis was used to calculate hazard ratios (HRs) with 95% confidence intervals (CIs) for CSS. When testing for associations between categorical variables,

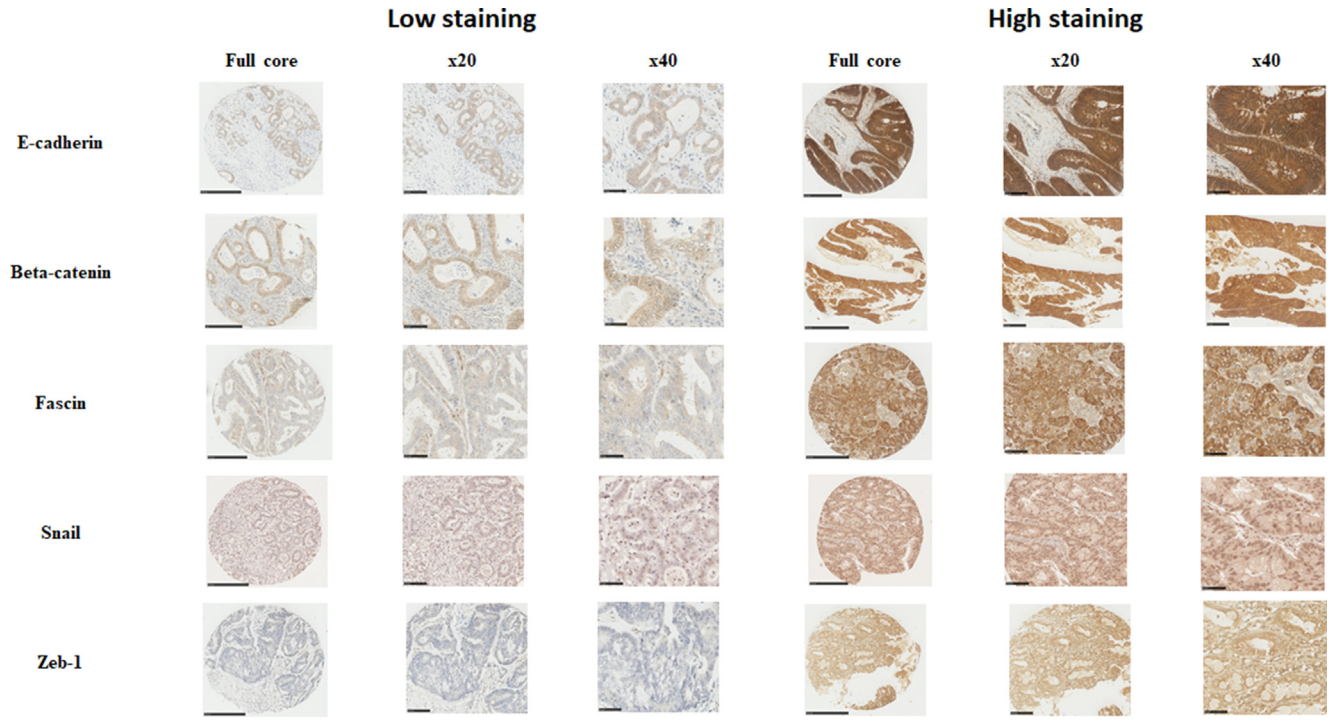


Fig. 1 Immunohistochemical (IHC) staining for 5 epithelial-mesenchymal transition (EMT) markers. Representative images of low and high IHC staining for E-cadherin, β-catenin, Fascin, Snail, and Zeb1 in full core (bar = 250 μm), × 20 (bar = 100 μm), and × 40 magnification (bar = 50 μm).

Pearson's chi-squared test was used. Where there were fewer than n = 6 events in any cell, chi-squared analysis was not performed. REMARK guidelines [24] were followed in reporting this study. $P < .05$ was considered to be statistically significant.

3. Results

There was a total of 502 patients undergoing potentially curative resection of stage II-III CRC that also had a valid sample for assessment of one or more EMT markers and GMS, but only 238 tumors had scores for all 5 markers of EMT. Clinicopathological characteristics are given for patients with full scores available versus patients with missing scores in Supplementary Table S2. There were no significant differences in any clinicopathological characteristics between these 2 groups. For those patients with scores available, 57% of patients were younger than 75 years, whereas 38% were node-positive. Fifty-three percent had low-risk disease, whereas 47% had high-risk disease. The medians, range and split into high and low for each marker are presented in Supplementary Table S1. Median follow-up for survivors was 140 months (interquartile range: 120−175 months). There were 156 deaths, of which 61 were CRC related.

Univariate CSS was assessed for each EMT marker (Table 1; Fig. 2). E-cadherin, Fascin, and Snail did not associate with survival at any cellular locus. Cytoplasmic and nuclear β-catenin were significant for worse CSS (HR 1.67, 95% CI 1.01−2.76, $P < .05$, and HR 2.22, 95% CI 1.24−3.97, $P < .01$, respectively). Membrane Zeb-1 was also significant for worse CSS (HR 2.00, 95% CI 1.07−3.77, $P = .03$).

In terms of associations between EMT markers and clinicopathological variables, these are given in Supplementary Table S3. In brief, E-cadherin did not associate with any clinicopathological variables.

Nuclear β-catenin was more likely to be found in rectal tumors ($P = .03$), cytoplasmic and nuclear β-catenin were associated with well/moderate differentiation ($P = .03$ and $P < .01$), β-catenin at any cellular location was associated with MMR proficiency (all $P < .001$), membrane β-catenin was associated with lower peritoneal involvement ($P = .04$).

Nuclear Fascin was associated with poorer differentiation ($P = .03$), whereas membrane Fascin was associated with greater peritoneal involvement ($P = .03$). Snail did not associate with any clinicopathological variables. Cytoplasmic Zeb-1 was associated with lower venous invasion ($P = .04$).

There was no association between any EMT marker and tumor budding or lymph node status.

In a previous study, a combined EMT score was constructed [8], which divided patients into 3 groups as follows: absent EMT described high membrane E-cadherin with all other markers low; low EMT was marked by low membrane E-cadherin or high individual markers; high EMT was marked by low membrane E-cadherin and all

Table 1 Cancer-specific survival in stage II-III colorectal cancer for individual EMT markers (N= 238).

Clinicopathological characteristics		Cancer-specific survival		
	N (%)[a]	Events (CSS)	Univariate HR (95% CI)	*P*
E-Cadherin				
Membrane low	28 (12)	3		
Membrane high	210 (88)	58	2.80 (0.88−8.94)	.08
Cytoplasm low	140 (59)	30		
Cytoplasm High	98 (41)	31	1.40 (0.85−2.32)	.19
Nucleus low	220 (92)	57		
Nucleus high	18 (8)	4	0.71 (0.26−1.94)	.50
β-Catenin				
Membrane low	28 (12)	3		
Membrane high	210 (88)	58	2.92 (0.92−9.34)	.07
Cytoplasm low	146 (61)	30		
Cytoplasm high	92 (39)	31	1.67 (1.01−2.76)	**.046**
Nucleus low	96 (40)	15		
Nucleus high	142 (60)	46	2.22 (1.24−3.97)	**.007**
Fascin				
Membrane low	171 (72)	40		
Membrane high	67 (28)	21	1.62 (0.96−2.75)	.07
Cytoplasm low	31 (13)	5		
Cytoplasm high	207 (87)	56	1.91 (0.76−4.76)	.17
Nucleus low	159 (67)	37		
Nucleus high	79 (33)	24	1.53 (0.92−2.57)	.10
Snail				
Membrane low	46 (19)	16		
Membrane high	192 (81)	45	0.60 (0.34−1.07)	.08
Cytoplasm low	56 (24)	18		
Cytoplasm high	182 (76)	43	0.72 (0.42−1.26)	.25
Nucleus low	55 (23)	11		
Nucleus high	183 (77)	50	1.46 (0.76−2.80)	.26
Zeb1				
Membrane low	74 (31)	12		
Membrane high	164 (69)	49	2.00 (1.07−3.77)	**.03**
Cytoplasm low	195 (82)	53		
Cytoplasm high	43 (18)	8	0.64 (0.31−1.35)	.24
Nucleus low	26 (11)	5		
Nucleus high	212 (89)	56	1.60 (0.64−3.98)	.32
EMT score (original)				
Absent EMT	7 (3)	1	1.94 (0.27−14.00)	.51
Low EMT	231 (97)	60	REF	1.0
High EMT	0 (0)	–	–	–

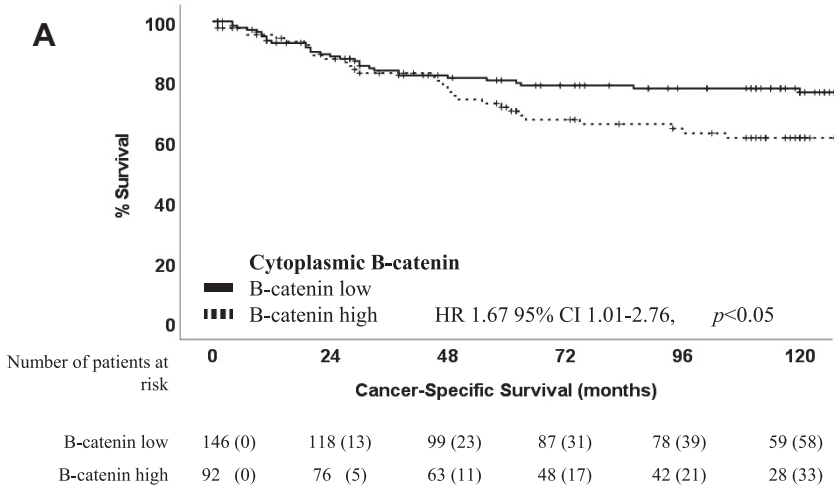
Bold indicates significant result.

Abbreviations: CI, confidence interval; CSS, cancer-specific survival; EMT, epithelial-mesenchymal transition; HR, hazard ratio.

[a] Percentages rounded to the nearest whole number and may not total 100%.

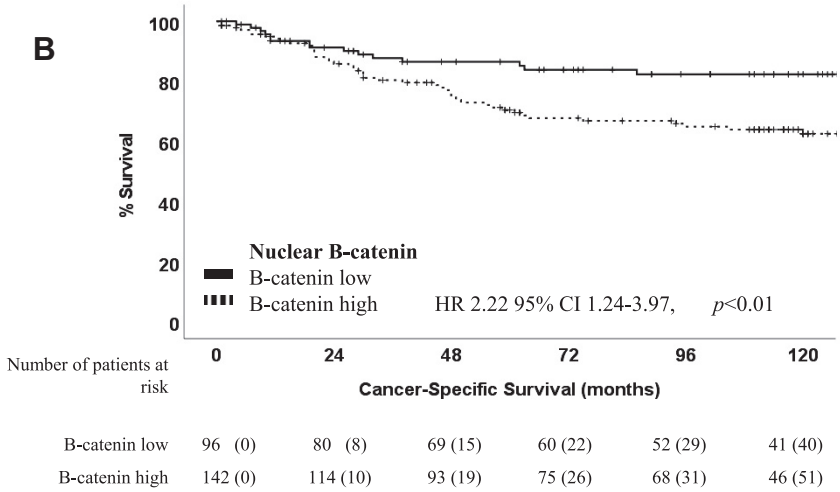
other markers high. However, owing to differences in staining between the original study and the present study, the data thresholds from the original study could not be used. New thresholds were thus generated using R Studio as described (Supplementary Table S1). Once these thresholds were applied to the present cohort, there were no tumors identified as having "high EMT" and only 7 with absent EMT (Table 1). The combined EMT score was therefore not used in this study.

Associations between individual EMT markers and GMS were subsequently assessed (Table 2). Nuclear β-catenin was the only EMT marker with a significant association with GMS as a whole ($P = .03$). For GMS 2 tumors, 68% had high nuclear β-catenin versus 47% for GMS 0, in keeping with EMT as a key process in mesenchymal tumors. However, GMS 0, 1, and 2 are not associated linearly but are in fact separate entities categorized by phenotypic tumor subtype. Therefore, the phenotypic elements that comprise GMS (ie, KM and TSP) were assessed individually for associations with markers of EMT (Table 3). The analysis for high KM versus low KM revealed that membrane β-catenin was significantly lower in high KM ($P = .03$). Nuclear β-catenin



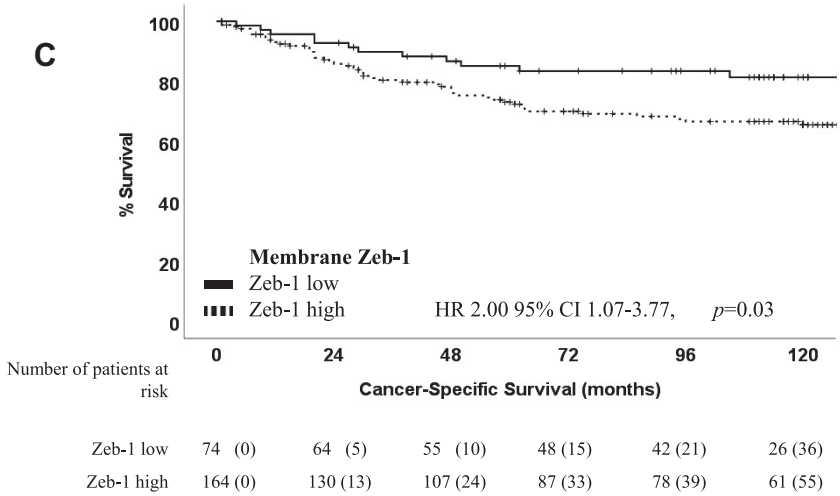
Number of patients at risk

B-catenin low	146 (0)	118 (13)	99 (23)	87 (31)	78 (39)	59 (58)
B-catenin high	92 (0)	76 (5)	63 (11)	48 (17)	42 (21)	28 (33)



Number of patients at risk

B-catenin low	96 (0)	80 (8)	69 (15)	60 (22)	52 (29)	41 (40)
B-catenin high	142 (0)	114 (10)	93 (19)	75 (26)	68 (31)	46 (51)



Number of patients at risk

Zeb-1 low	74 (0)	64 (5)	55 (10)	48 (15)	42 (21)	26 (36)
Zeb-1 high	164 (0)	130 (13)	107 (24)	87 (33)	78 (39)	61 (55)

Fig. 2 Cancer-specific survival for (A) cytoplasmic and (B) nuclear β-catenin, and (C) membrane Zeb-1, in stage II-III CRC (n = 238).

Table 2 Associations of EMT markers with GMS in stage I-III colorectal cancer (N= 238).

	GMS category						
	0 (n = 61) N (%)[a]		1 (n = 133) N (%)		2 (n = 44) N (%)		*Pearson* χ^2
E-Cadherin							
Membrane low	9	(15)	12	(9)	7	(16)	1.00
Membrane high	52	(85)	121	(91)	37	(84)	
Cytoplasm low	38	(62)	76	(57)	26	(59)	0.69
Cytoplasm high	23	(38)	57	(43)	18	(41)	
Nucleus low	55	(90)	122	(92)	43	(98)	—[b]
Nucleus high	6	(10)	11	(8)	1	(2)	
β-Catenin							
Membrane low	12	(20)	10	(8)	6	(14)	0.22
Membrane high	49	(80)	123	(92)	38	(86)	
Cytoplasm low	36	(59)	81	(61)	29	(66)	0.49
Cytoplasm high	25	(41)	52	(39)	15	(34)	
Nucleus low	32	(53)	50	(38)	14	(32)	**0.03**
Nucleus high	29	(47)	83	(62)	30	(68)	
Fascin							
Membrane low	50	(82)	89	(67)	32	(73)	0.21
Membrane high	11	(18)	44	(33)	12	(27)	
Cytoplasm low	10	(16)	12	(9)	9	(21)	0.72
Cytoplasm high	51	(84)	121	(91)	35	(79)	
Nucleus low	43	(71)	87	(65)	29	(66)	0.58
Nucleus high	18	(29)	46	(35)	15	(34)	
Snail							
Membrane low	11	(18)	23	(17)	12	(27)	0.29
Membrane high	50	(82)	110	(83)	32	(73)	
Cytoplasm low	17	(28)	27	(20)	12	(27)	0.82
Cytoplasm high	44	(72)	106	(80)	32	(73)	
Nucleus low	13	(21)	28	(21)	14	(32)	0.25
Nucleus high	48	(79)	105	(79)	30	(68)	
Zeb1							
Membrane low	17	(28)	43	(32)	14	(32)	0.63
Membrane high	44	(72)	90	(68)	30	(68)	
Cytoplasm low	47	(77)	109	(82)	39	(89)	—[b]
Cytoplasm high	14	(23)	24	(18)	5	(11)	
Nucleus low	9	(15)	14	(11)	3	(7)	—[b]
Nucleus high	52	(85)	119	(89)	41	(93)	

Bold indicates significant result.

Abbreviations: EMT, epithelial-mesenchymal transition; GMS, Glasgow Microenvironment Score.

[a] Percentages rounded to the nearest whole number and may not total 100%.

[b] For cells where n < 6, *Pearson* χ^2 analysis was not performed.

was again demonstrated as significantly lower in high KM ($P = .03$). Membrane Fascin was also significantly lower in high KM ($P = .04$). Membrane Fascin was highest in GMS 1 and slightly lower in GMS 2, hence why there was no linear association with GMS, as a whole (Table 2). There were no other associations between GMS categories and EMT markers, neither were there any further associations between TSP and markers of EMT.

4. Discussion

The results from the present study once again demonstrate the association between β-catenin and survival in stage II-III CRC. Both cytoplasmic and nuclear β-catenin were associated with poor survival outcomes. Whereas in the present study, the higher expression of cytoplasmic and nuclear β-catenin was associated with worse survival outcomes, Roseweir et al. [8] found the loss of membrane β-catenin to have the same effect.

Furthermore, the presence of membrane Zeb-1 was found to be significant for CSS. Others have found the presence of Zeb-1 to be associated with a process known as "vasculogenic mimicry," the ability of cells to express endothelial cell markers, which is thought to be a feature of EMT [25]. In the present study, when Zeb-1 localized to the cytoplasm, this was associated with lower venous invasion.

Table 3 Associations of EMT markers according to pathological phenotype in stage II-III colorectal cancer (N= 238).

	Immune phenotype (KM)					Mesenchymal phenotype (TSP)				
	KM high (n = 61) N (%)[a]		KM low (n = 177) N (%)		*Pearson* χ^2	TSP low (n = 183) N (%)		TSP high (n = 55) N (%)		*Pearson* χ^2
E-Cadherin										
Membrane low	9	(11)	19	(15)	0.40	21	(12)	7	(13)	0.80
Membrane high	52	(89)	158	(85)		162	(89)	48	(87)	
Cytoplasm low	38	(62)	102	(58)	0.52	107	(59)	33	(60)	0.84
Cytoplasm high	23	(38)	75	(42)		76	(42)	22	(40)	
Nucleus low	55	(90)	165	(93)	0.44	166	(91)	54	(98)	–[b]
Nucleus high	6	(10)	12	(7)		17	(9)	1	(2)	
β-Catenin										
Membrane low	12	(20)	16	(9)	**0.03**	22	(12)	6	(11)	0.82
Membrane high	49	(80)	161	(91)		161	(88)	49	(89)	
Cytoplasm low	36	(59)	110	(62)	0.67	113	(62)	33	(60)	0.82
Cytoplasm high	25	(41)	67	(38)		70	(38)	22	(40)	
Nucleus low	32	(53)	64	(36)	**0.03**	79	(43)	17	(31)	0.10
Nucleus high	29	(47)	113	(64)		104	(57)	38	(69)	
Fascin										
Membrane low	50	(82)	121	(68)	**0.04**	132	(72)	39	(71)	0.86
Membrane high	11	(18)	56	(32)		51	(28)	16	(29)	
Cytoplasm low	10	(16)	21	(12)	0.37	22	(12)	9	(16)	0.40
Cytoplasm high	51	(84)	156	(88)		161	(88)	46	(84)	
Nucleus low	43	(71)	116	(66)	0.89	126	(69)	33	(60)	0.22
Nucleus high	18	(29)	61	(35)		57	(31)	22	(40)	
Snail										
Membrane low	11	(18)	35	(20)	0.77	31	(17)	15	(27)	0.09
Membrane high	50	(82)	142	(80)		152	(83)	40	(40)	
Cytoplasm low	17	(28)	39	(22)	0.36	41	(22)	15	(27)	0.46
Cytoplasm high	44	(72)	138	(78)		142	(78)	40	(73)	
Nucleus low	13	(21)	42	(24)	0.70	40	(22)	15	(27)	0.41
Nucleus high	48	(79)	135	(76)		143	(78)	40	(73)	
Zeb1										
Membrane low	17	(28)	57	(32)	0.53	56	(31)	18	(33)	0.77
Membrane high	44	(72)	120	(68)		127	(69)	37	(67)	
Cytoplasm low	47	(77)	148	(84)	0.25	148	(81)	47	(86)	0.44
Cytoplasm high	14	(23)	29	(16)		35	(19)	8	(14)	
Nucleus low	9	(15)	17	(10)	0.27	23	(13)	3	(6)	–[b]
Nucleus high	52	(85)	160	(90)		160	(87)	52	(95)	

Bold indicates significant result.

Abbreviations: EMT, epithelial-mesenchymal transition; KM, Klintrup-Mäkinen; TSP, tumor stromal percentage.

[a] Percentages rounded to the nearest whole number and may not total 100%.

[b] For cells where n < 6, *Pearson* χ^2 analysis was not performed.

In a murine model, Kudo et al. [26] found that CRP suppressed Zeb-1 on colon cancer tumor cells. CRP is one of the main markers of the mGPS, which identifies individuals with systemic inflammation and is a known poor prognostic indicator [23]. However, in the present study, there was no association between mGPS and Zeb-1 expression, nor between serum CRP and Zeb-1 expression at any cellular locus (data not shown). The presence of Zeb-1, when assessed by real-time PCR in CRCs, has also previously been demonstrated to indicate worse survival, independent of other clinicopathological features on multivariate analysis [27].

The aforementioned combined EMT score was not able to split the patients adequately into the 3 different stages of EMT as in the original study [8] and the reasons for this are unclear. Different data thresholds were necessary and this may have had an influence. The reason that different thresholds were required in this study is that the specimens stained in the previous study had globally lower weighted histoscores. As the staining technique was standardized and the antibodies used were the same antibodies from the same suppliers, it is unclear why this was the case. It is possible that batch-to-batch variability of antibodies or a difference in production by the company, e.g., of the

secondary DAB stain, caused this change in staining density. Therefore, it is not possible to say whether the thresholds set in the present study will be applicable to future studies. Perhaps the inability for the original combined EMT score to apply is due to differences in the patient cohorts studied, although they were of similar TNM stage. It may be that the difference lies in the patients with missing scores although the numbers for the original study and the present study are similar and the clinicopathological variables for patients with missing scores were not statistically different from those with a complete set of scores available.

Whilst at some point in the future, EMT may be integrated into clinical practice. However, currently, there is no evidence that any EMT markers have been studied in the context of clinical trials and therefore, significant further work is required to investigate whether these findings are replicable in larger cohorts and in the context of clinical trial data before any further conclusions regarding clinical applicability can be drawn.

The relationship between individual EMT markers and GMS was assessed. GMS 0, the CRC phenotype characterized by high peritumoral inflammation (KM), was found to be associated with lower membrane Fascin expression. Fascin overexpression has been implicated in chronic inflammation-related CRC carcinogenesis [28]. Conversely, high peritumoral inflammation in the context of CRC as a whole is known to be a good prognostic indicator [4] and appears to be protective against mesenchymal phenotype [6,29]. Therefore, the presence of lower membrane Fascin expression in GMS 0 may be reflective of a less aggressive phenotype than the other 2 GMS categories. Furthermore, membrane and nuclear expression of β-catenin was significantly lower in GMS 0. The loss of membrane β-catenin is believed to occur simultaneously with the loss of membrane E-cadherin and is one of the hallmarks of EMT [14]. The data demonstrate that this group, while categorized by the protective feature of higher peritumoral inflammation, has a lower membrane expression of β-catenin than tumors with lower peritumoral inflammation, a feature that Roseweir et al. [8] found to predict worse outcome. Briede et al. [30] recently published a study on a Latvian CRC cohort finding no association with peritumoral inflammation and E-cadherin, but did not assess any other markers of EMT. Zlobec et al. [31], on the other hand, found peritumoral inflammation to be protective against the otherwise negative feature of E-cadherin loss. These studies used E-cadherin as a primary marker of EMT. No other studies were identified assessing peritumoral inflammation in the context of other markers of EMT. It is unclear why there would be lower expression of membrane β-catenin in this group. However, it may be that, although not directly linked, the higher peritumoral inflammation is protective despite the loss of membrane β-catenin in this subgroup.

GMS 1 defines a CRC phenotype with neither high peritumoral inflammation nor high TSP. This phenotype was previously demonstrated to have an intermediate survival outcome compared with the other 2 GMS categories [6,7]. GMS 1 tumors were observed to have the highest membrane and cytoplasmic Fascin. Owing to Fascin's role in bundling the cell's actin cytoskeleton and the greater motility of cells with higher levels of Fascin [18], this may indicate that some of these tumors already have features of EMT, although they do not display the phenotype of higher TSP.

In GMS 2, the CRC phenotype characterized by high TSP and worse survival, there were greater numbers of tumors with high nuclear β-catenin, in keeping with a mesenchymal phenotype. However, cytoplasmic Fascin levels were lower in this group and the role of cytoplasmic Fascin is therefore unclear. Perhaps the role of Fascin in the EMT process is in facilitating transition to the mesenchymal phenotype and it may play a lesser role once this phenotype has been attained. This feature also requires further investigation in independent cohorts.

In conclusion, the present study confirms the prognostic significance of markers of EMT in CRC that have been identified in previous studies, in particular β-catenin and membrane Zeb-1. Furthermore, markers of EMT have been demonstrated to associate with individual GMS categories in a manner not previously identified. Specifically: nuclear β-catenin levels increased with increasing GMS category; membrane Fascin levels similarly were lowest in GMS 0 and highest in GMS 1, which may indicate an early role in transition to the mesenchymal phenotype that is less pronounced after this phenotypic appearance has been achieved. These findings warrant further investigation in independent patient cohorts.

Author contributions

PGA, AAMM, AKR, DCM, PGH, CSDR, CT, JE, and JHP, conceptualization; PGA, AAMM, AKR, KAFP, HCvW, JQ, JE, and JHP, data curation; PGA and JE, formal analysis; fundingAKR, PGH, DCM, CSDR, CT, JHP, and JE, funding acquisition; PGA, AAMM, KAFP, HCvW, NJ, JQ, JHP, and JE, investigation; PGA, AAMM, AKR, KAFP, DCM, CSDR, JQ, JE, and JHP, methodology; PGH, DCM, CSDR, CT, and JE, resources; DCM, PGH, AKR, CT, JQ, JHP, and JE, supervision; PGA, AAMM, AKR, JHP, and JE, validation; PGA and AAMM, writing: original draft; and PGA, AAMM, AKR, NJ, KAFP, HCvW, PGH, DCM, CSDR, JQ, CT, JE, and JHP, writing: review and editing.

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Ethics approval and consent to participate

All patients provided written informed consent. The study complied with the Declaration of Helsinki and was approved by the West Glasgow Research Ethics Committee (REC No. 16/WS0207; 07/S0703/136).

Consent for publication

All authors consent to publication of this research/data.

Data availability

The data sets that formed the basis of this article are contained in the University of Glasgow's MVLS institute and are continually being updated with ongoing research. They contain patient-sensitive information and therefore cannot be made available on a public repository.

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Appendix A. Supplementary data

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