A novel tumor-based epithelial-to-mesenchymal transition score that associates with prognosis and metastasis in patients with stage II/III colorectal cancer

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Short Title: Tumour EMT as a predictive marker for micrometastasis in colorectal cancer

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Abbreviations: CRC – colorectal cancer; EMT – epithelial-to-mesenchymal transition; Zeb1 - Zinc-finger-enhancing-binding-protein-1; TMA – tissue microarray; CSS – cancer specific survival; CRP – C-reactive protein; mGPS – modified Glasgow prognostic score; TSP – Tumor stroma percentage; KM – Klintrup-Makinen; GMS – Glasgow microenvironment score; DAB - 3,3'-diaminobenzidine

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Novelty and Impact: In colorectal cancer, EMT has been described at the invasive margin, but it is also thought to occur at the tumor centre. We tested a novel tumor-based EMT score that stratified survival in stage II/III patients and associated with metastasis. This combined EMT score could be utilized to identify stage II/III patients at risk of micrometastases and who may benefit from standard adjuvant therapy.

ABSTRACT

It is increasingly appreciated that host factors within the tumor centre and microenvironment play a key role in dictating colorectal cancer (CRC) outcomes. As a result, the metastatic process has now been defined as a result of epithelial-mesenchymal transition (EMT). Establishment of the role of EMT within the tumor centre and its effect on the tumor microenvironment would be beneficial for prognosis and therapeutic intervention in CRC. The present study assessed five immunohistochemical EMT markers within the tumor centre on a 185 stage II/III CRC patient TMA.

In 185 patients with CRC; cytoplasmic snail (HR 1.94 95% CI 1.15-3.29, p=0.012), and a novel combined EMT score (HR 3.86 95% CI 2.17-6.86, p<0.001) were associated with decreased cancer-specific survival. The combined EMT score was also associated with increased tumor budding (p=0.046), and systemic inflammation (p=0.007), as well as decreased memory T-cells within the stroma (p=0.030) and at the invasive margin (p=0.035). Furthermore, the combined EMT score was associated with cancer specific survival independent of TNM-stage (HR 4.12 95% CI 2.30-7.39, p<0.001).

In conclusion**,** a novel combined EMT score stratifies patient's survival in stage II/III CRC and associates with key factors of tumor metastasis. Therefore, the combined EMT score could be used to identify patients at risk of micrometastases and who may benefit from standard adjuvant therapy, potentially in combination with EMT blockade.

INTRODUCTION

Colorectal cancer is the third most common cancer and second largest cause of cancer death in Europe and North America [1]. Even with advances in surgery and adjuvant treatment, five-year survival rates remain low at 60% [2]. While pathological staging of the tumor remains the gold standard prognostic marker for colorectal cancer, a subset of patients within the same disease stage will have a worse outcome. Identifying these patients with aggressive disease who may benefit from adjuvant therapy remains an important clinical need.

The metastatic process of tumor cells breaking away from the primary tumor, invading locally and migrating to distant sites has been defined as epithelial-mesenchymal transition (EMT). EMT is the process of de-differentiation of epithelial cells into mesenchymal cells [3], an important component in normal inflammatory physiology. EMT can be considered to be an example of the tumor exploiting a physiological mechanism, characterized by a loss of tumor cell polarity and adhesion, increased motility and evasion of apoptosis [4]. EMT is therefore thought to be one of the initiating and enabling steps in the invasion-metastasis cascade [4]. Several well-described molecular markers of EMT have been validated. Down-regulation of membrane E-cadherin is considered a hallmark of EMT [4, 5]. E-cadherin is a transmembrane protein that regulates cell-cell adhesion, and is now considered to have tumor suppressive activity by maintaining cells in a non-motile, quiescent state [6]. β-catenin, a prime effector in the Wnt pathway, is another integral component of EMT [5]. It links E-cadherin to the cytoskeleton by forming a complex and this is thought to support E-cadherin's role in cell-cell adhesion [7]. The loss of E-cadherin allows β-catenin to translocate into the nucleus and increase β-catenin-dependent gene transcription of pro-metastatic factors [8]. Further upstream, a complex of proteins

regulates EMT, which includes transcription factors Snail, Fascin and Zinc-finger-enhancing-binding-protein-1 (Zeb-1) [4, 5].

Alterations in these markers have been associated with the presence of lymphatic and distant metastases in colorectal cancer resulting in poorer patient survival [4, 5]. While the prognostic significance of the individual markers is well studied at the invasive margin, the significance of these markers within the tumor centre or as a combined EMT score to help predict the patients with aggressive disease has not been established. Furthermore, the interaction between EMT and components of the tumor microenvironment is still poorly understood [9]. Therefore, the present study aims to examine the prognostic value of these five EMT markers within the tumor centre and to construct a combined EMT prognostic score to help predict patients with aggressive disease. The study will also establish the relationship between this EMT score and clinicopathological factors to establish new therapeutic targets for these patients.

MATERIALS AND METHODS

Patient Cohort

274 patients were retrospectively identified from a prospectively collected and maintained database of CRC resections performed between 1997 and 2007 in Glasgow Royal Infirmary who had undergone an elective, potentially curative resection for stage II-III CRC and were contained within a previously constructed tissue microarray (TMA) with four cores per patient taken from differing pathologist-defined areas of the tumor centre. Patients who received neoadjuvant therapy or had died within 30 days of surgery were excluded. Ethical approval was obtained from the West of Scotland Research Ethics Committee.

Clinicopathological Characteristics

Tumors were staged using the fifth edition of the AJCC/UICC-TNM staging system [10]. Tumor differentiation was graded in accordance with Royal College of Pathologists [11]. The presence of venous invasion was assessed using Elastica staining. Differentiation, margin involvement, peritoneal involvement, and necrosis were taken from pathology reports issued following resection. Ki67 was already available for this cohort using a threshold of 50%. MMR status was assessed as previously described [12]. Patients were followed up for at least five years and date and cause of death were crosschecked with electronic case records. Cancer-specific survival (CSS) was measured from date of surgery until date of death from CRC.

Serum C-reactive protein (CRP) and albumin were recorded prospectively and measured within 30 days prior to surgery. The pre-operative systemic inflammatory response was defined using the modified Glasgow prognostic score (mGPS) as previously described [13].

Tumor Microenvironment Characteristics

Stromal infiltration was assessed using tumor stroma percentage (TSP) as previously described [14]. The local inflammatory cell infiltrate was assessed using the Klintrup-Makinen (KM) grade [15]. The Glasgow microenvironment score (GMS) was calculated as previously described [16]. Individual T-cell characterization was already available for this cohort.

Immunohistochemistry

An immunohistochemical analysis of 5 fully validated EMT markers was performed utilizing a previously constructed CRC patient TMA: (1) E-cadherin, (2) β-catenin, (3) Zeb-1, (4) Fascin and (5) Snail (Figure S1A).

TMAs were dewaxed with Histoclear and rehydrated in decreasing concentrations of alcohol. Antigen retrieval was then performed under pressure in citrate buffer, pH6.0 for 5 minutes (for E-cadherin, Zeb-1, fascin and snail) or in a water bath with EDTA buffer, pH8.0 at 96°C for 50 minutes (for β-catenin). Endogenous peroxide activity was blocked with 3% hydrogen peroxide solution for 20 minutes (for E-cadherin, Zeb-1, fascin and snail) and 0.5% hydrogen peroxide solution for 30-45 minutes (for β-catenin). TMAs were then incubated in 10% casein (Vector Laboratories) for 2 hours (E-cadherin) or 30 minutes (Zeb-1, fascin and snail) and 1% BSA for 30 minutes (for β-catenin). Primary E-cadherin antibody (1:500; BD Biosciences, 610182) and Zeb-1 (1:800; Sigma-Aldrich, HPA027524) were added at 4°C overnight or for 2 hours at room temperature for β-catenin (1:50; BD Biosciences, 610154), fascin (1:100; Atlas Antibodies, HPA005723) and snail (1:50; abcam, ab53519). TMAs were then incubated in envision (DAKO) for 30 minutes (E-cadherin, Fascin and Zeb-1) or 2 hours (β-catenin) or ImmPRESS anti-goat IgG for 30 minutes (snail).

Antibody visualization was performed with 3,3'-diaminobenzidine (DAB; Vector Laboratories) until colour developed. Slides were counterstained with haematoxylin and dehydrated in alcohol and Histoclear. Slides were then mounted with DPX.

Scoring Method

Stained TMA sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at x20 magnification and visualized on Slidepath Digital Image Hub (Leica Biosystems, Milton Keynes, UK). Assessment of EMT markers was performed by a single examiner blinded to clinical data at x20 magnification (total magnification x400) using the weighted histoscore. The weighted histoscore was calculated as follows: 0x% not stained + 1x% weakly stained + 2x% moderately stained + 3x% strongly stained. This gave a range of scores from 0 to 300 with nuclear, cytoplasmic and membrane tumor-specific staining scored separately. For all EMT markers the four TMA cores were scored separately to ensure reproducibility and an average score taken (Figure S1B). 10% of tumors were also co-scored by a co-investigator and the interclass correlation coefficient calculated to be <0.7 for all proteins.

Statistical Analysis

Histoscores were split into high and low expression using the median for each marker at each location. SPSS (version 22) was used for statistical analysis. Pearson's $\chi 2$ test assessed associations between EMT markers, clinicopathological features and the tumor microenvironment. Kaplan-Meier curves and log-rank analysis compared EMT markers and CSS. HRs and confidence intervals (CI) were calculated from univariate cox regression survival analysis. Multivariate cox regression survival analysis using a backward conditional elimination model and a significance threshold of $p<0.05$ was

performed to identify independent prognostic biomarkers. The study is reported in line with the REMARK guidelines [17] and significance was set as $p<0.05$.

RESULTS

A total of 185 patients were studied that underwent a potentially curative resection for stage II-III CRC and had a valid score for all five EMT markers within the tumor centre. The patient characteristics for the cohort are shown in Table S1. The median follow-up for patients was 11.4 years (range 6.8-16.0) with 62 cancer deaths and 50 non-cancer deaths.

Firstly, the cohort was assessed for associations with CSS (Table 1). E-cadherin and fascin did not associate with CSS at any cellular location. Membrane beta-catenin trended towards associations with improved CSS (HR 0.62 95% CI 0.37-1.02, p=0.053, figure 1A). Conversely, zeb1 trended towards associations with decreased CSS in the cytoplasm (HR 1.57 95% CI 0.94-2.60, p=0.080, figure 1B). However, cytoplasmic snail significantly associated with decreased survival (HR 1.94 95% CI 1.15-3.29, p=0.012, figure 1C). Next, the five markers were analyzed for correlations to assess if they are working together (Table S2). Strong correlations were seen between membrane e-cadherin and nuclear beta-catenin as expected ($p<0.001$). Cytoplasmic snail and zeb1 also correlated with membrane e-cadherin (p=0.001 and p=0.009) and nuclear beta catenin (p=0.010 and p=0.042). Cytoplasmic zeb1 also strongly correlate with cytoplasmic fascin (p=0.005).

Therefore, membrane e-cadherin, nuclear beta-catenin and cytoplasmic fascin, snail and zeb1 were combined to create a combined EMT score. Patients with high membrane e-cadherin and low expression of all other markers were grouped as absent EMT, patients with low membrane e-cadherin or high expression of any of the other markers were grouped as low EMT and patients with low membrane e-cadherin and high expression of all other markers were grouped as high EMT. A high EMT score

was significantly associated with decreased CSS (HR 3.86 95% CI 2.17-6.86, p<0.001, Figure 1D). As MMR deficient tumors are known to lack aggressive invasive features associated with EMT and therefore could be a confounding factor; these patients were removed from the cohort. However, no significant effect on the combined EMT scores association with CSS was observed (HR 3.79 95% CI 2.02-7.11, p<0.001) therefore all future analysis was performed utilizing the full cohort.

Cytoplasmic snail and the combined EMT score were then assessed for associations with clinicopathological factors as shown in Table 2. Patients with high cytoplasmic snail were more likely to have received adjuvant therapy (p=0.007) and trended towards having a high mGPS (p=0.060). Similarly, patients with a high EMT score were more likely to have increased tumor budding (p=0.046) and an increased mGPS (p=0.007). A trend towards increased venous invasion (p=0.055) and decreased local inflammation was also seen (p=0.157). A high combined EMT score also showed increased lymph node metastasis levels, which was not seen for absent or low EMT scores (Figure 2A).

To assess if the trend towards decreased inflammation was dependent on a specific lymphocyte population, associations were assessed as shown in Table 3. Cytoplasmic snail associated with decreased T regulatory cells within the stroma (p=0.006) and tumor centre (p=0.006). Similarly, the combined EMT score associated with decreased stromal T-regulatory cells (p=0.049) but also marginal memory T-cells (p=0.035) and stromal memory T-cells (p=0.030). When assessed for expression for each score, only patients with a high EMT score had decreased levels of CD8+ cytotoxic (Figure 2B), CD45RO+ memory (Figure 2C) and Fox3P+ regulatory (Figure 2D) T-cells.

Cytoplasmic snail and the combined EMT score were then taken forwards into multivariate analysis with common clinicopathological factors (Table 4). Under univariate analysis (n=185), TNM-stage ($p<0.001$), venous invasion ($p<0.001$), Ki67 proliferation index ($p=0.001$), TSP ($P=0.005$), tumor budding ($p<0.001$), GMS ($p=0.007$), mGPS ($p=0.011$), cytoplasmic snail ($p=0.014$), and the combined EMT score ($p<0.001$) were associated with for CSS. The combined EMT score also associated with CSS independent of TNM-stage (HR 4.12 95% CI 2.30-7.39, $p<0.001$).

DISCUSSION

The results of the present study show that both cytoplasmic snail and a novel combined EMT score stratify patients with stage II/III CRC by survival, with high expression having the worst prognosis. Furthermore, the combined EMT score is associated with increased tumor budding, invasion into blood vessels, and lymph node invasion suggesting it is a key factor in tumor metastasis. Therefore, blockade of EMT in stage II/III patients may be a potential therapeutic route to inhibit metastatic spread, allowing surgical resection of the primary tumor to be a curative procedure.

Multiple studies have elucidated the clinical value of individual EMT markers at the invasive margin of CRC [18]. Decreased membrane e-cadherin expression and increased nuclear beta-catenin expression have consistently been described as prognostic markers in colorectal cancer [19, 20]**.** Furthermore, Bhangu et al. reported that reduced e-cadherin and increased nuclear beta-catenin expression at the invasive margin can predict patients who will not respond to neoadjuvant therapy [21]. Hao et al. also reported that reduced expression of membrane beta-catenin was associated with colorectal adenoma-carcinoma progression [22]. Furthermore, the association between reduced membrane beta-catenin expression and poor survival has been reported widely [23-26]. However, increasingly, studies have disputed that EMT only occurs at this site.

Kroepil et al. reported similar expression levels of e-cadherin and snail at the tumor centre and invasive margin [27]. Kahlert et al. reported raised expressions of zeb-2 in both tumor centre and invasive margin compared with normal colorectal tissue and both were correlated with outcome [28]. Several other studies using TMAs in which the cores are taken from non-selected areas of the tumor, have also reported consistent

relationships between markers of EMT (beta-catenin, e-cadherin, zeb-1, snail and fascin-1) and clinical outcome [29-31]. This is in agreement with the present study that shows high expression of cytoplasmic snail is associated with decreased survival in a TMA constructed from the centre of the tumor confirming that EMT marker alterations are observable in the tumor centre and can be used to predict prognosis. Furthermore, snail has value as a therapeutic target, with the snail inhibitor MRX34, undergoing a phase I trial in advanced cancers. The trial not only showed that the drug was tolerable but that it also had anti-tumor activity via EMT inhibition in hepatocellular carcinoma patients, suggesting it may be a suitable therapeutic for other EMT-related cancers [32].

The value of EMT markers for prognosis and therapeutic intervention is further strengthened by the results of our combined EMT score utilizing five markers of EMT from the tumor centre. The combined EMT score strongly stratified patient survival and was independent of TNM-stage. The prognostic power of the combined score was far superior to that of any of the individual markers, suggesting the pathway needs to be assessed as a whole to fully elucidate its prognostic role in CRC. To the authors knowledge, this is the first combined EMT score tested for CRC. Kim et al. did report a combined score for EMT using nine markers in gastric cancer with similar associations with survival [33]. However, as the current combined EMT score only uses five markers it may be more readily translated to the clinical setting. The combined EMT score also associated with well-established markers of metastasis. Furthermore, associations were also seen with increased systemic inflammation along with a trend towards decreased local inflammation suggesting the tumor microenvironment may play a role in regulating EMT.

There has been limited studies showing that the tumor microenvironment interacts with the EMT process [9]. It has been shown that tumor-associated macrophages promote the invasion-metastasis cascade [9]. However, associations between EMT and the local inflammatory response have not been reported. In the present study increased EMT associates with decreased memory and regulatory T-cells at the margin and within the stroma. This decrease in regulatory T-cells was also seen for cytoplasmic snail. This suggests that local inflammation may antagonize EMT within the tumor-centre, therefore as inflammation decreases EMT can increase to promote lymph node metastasis as seen in patients with a high EMT (Figure 2).

In conclusion, the findings of the present study indicate that elucidating the presence of EMT markers together within the combined EMT score may allow selection of poor prognosis patients within stage II/III tumors and provides evidence that the local immune responses may regulate EMT at the tumor centre. The limitations of this study include a modest sample size and the use of tissue micro-arrays of the tumor core without normal tissue controls. Despite this, the results show that EMT within the tumor centre and this can be utilized as a prognostic score for patients with CRC that associates with markers of metastatic spread. Therefore, the EMT score could be used to identify patients at risk of micrometastases and who may benefit from standard adjuvant therapy, potentially combined with an EMT inhibitor.

EMT inhibitors are still at an early stage with only a few phase II clinical trials underway in advanced solid tumors. The humanized monoclonal antibody, AB-16B5, an inhibitor of the EMT inducer secretory clusterin, is currently in a randomized phase II clinical trial in advanced solid tumors (NCT02412462). Similarly, MK-0646 a novel humanized IGF1R monoclonal antibody is being evaluated in combination

with gemcitabine in a phase II trial for pancreatic cancer (NCT00769483). In CRC no clinical trials are currently active, however translational research has shown that metformin has promise as an inhibitor of EMT with anti-cancer activity [34]. This lack of CRC clinical trials suggests that a biomarker such as the combined EMT score is needed to stratify CRC patients for EMT inhibitor clinical trials +/- adjuvant therapy.

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REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;**136**: E359-86.

2. Oliphant R, Nicholson GA, Horgan PG, Molloy RG, McMillan DC, Morrison DS, West of Scotland Colorectal Cancer Managed Clinical N. Deprivation and colorectal cancer surgery: longer-term survival inequalities are due to differential postoperative mortality between socioeconomic groups. *Ann Surg Oncol* 2013;**20**: 2132-9.

3. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009;**9**: 265-73.

4. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009;**119**: 1420-8.

5. Zlobec I, Lugli A. Epithelial mesenchymal transition and tumor budding in aggressive colorectal cancer: tumor budding as oncotarget. *Oncotarget* 2010;**1**: 651-61.

6. Berx G, van Roy F. Involvement of members of the cadherin superfamily in cancer. *Cold Spring Harb Perspect Biol* 2009;**1**: a003129.

7. Jeanes A, Gottardi CJ, Yap AS. Cadherins and cancer: how does cadherin dysfunction promote tumor progression? *Oncogene* 2008;**27**: 6920-9.

8. Brembeck FH, Rosario M, Birchmeier W. Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. *Curr Opin Genet Dev* 2006;**16**: 51-9.

9. Jing Y, Han Z, Zhang S, Liu Y, Wei L. Epithelial-Mesenchymal Transition in tumor microenvironment. *Cell Biosci* 2011;**1**: 29.

10. Fleming ID, American Joint Committee on Cancer., American Cancer Society., American College of Surgeons. *AJCC cancer staging manual*, 5th ed / ed. Philadelphia: Lippincott-Raven, 1997. xv, 294 p.

11. Quirke P, Morris E. Reporting colorectal cancer. *Histopathology* 2007;**50**: 103-12.

12. Park JH, Powell AG, Roxburgh CS, Horgan PG, McMillan DC, Edwards J. Mismatch repair status in patients with primary operable colorectal cancer: associations with the local and systemic tumour environment. *Br J Cancer* 2016;**114**: 562-70.

13. Park JH, Watt DG, Roxburgh CS, Horgan PG, McMillan DC. Colorectal Cancer, Systemic Inflammation, and Outcome: Staging the Tumor and Staging the Host. *Annals of Surgery* 2015.

14. Park JH, Richards CH, McMillan DC, Horgan PG, Roxburgh CS. The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer. *Ann Oncol* 2014;**25**: 644-51.

15. Richards CH, Roxburgh CS, Powell AG, Foulis AK, Horgan PG, McMillan DC. The clinical utility of the local inflammatory response in colorectal cancer. *Eur J Cancer* 2014;**50**: 309-19.

16. Park JH, McMillan DC, Powell AG, Richards CH, Horgan PG, Edwards J, Roxburgh CS. Evaluation of a Tumor Microenvironment-Based Prognostic Score in Primary Operable Colorectal Cancer. *Clin Cancer Res* 2014.

17. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, Statistics Subcommittee of the NCIEWGoCD. REporting recommendations for tumour MARKer prognostic studies (REMARK). *Eur J Cancer* 2005;**41**: 1690-6.

18. Lourenco FC, Munro J, Brown J, Cordero J, Stefanatos R, Strathdee K, Orange C, Feller SM, Sansom OJ, Vidal M, Murray GI, Olson MF. Reduced LIMK2 expression

in colorectal cancer reflects its role in limiting stem cell proliferation. *Gut* 2014;**63**: 480-93.

19. He X, Chen Z, Jia M, Zhao X. Downregulated E-cadherin expression indicates worse prognosis in Asian patients with colorectal cancer: evidence from meta-analysis. *PLoS One* 2013;**8**: e70858.

20. Chen Z, He X, Jia M, Liu Y, Qu D, Wu D, Wu P, Ni C, Zhang Z, Ye J, Xu J, Huang J. beta-catenin overexpression in the nucleus predicts progress disease and unfavourable survival in colorectal cancer: a meta-analysis. *PLoS One* 2013;**8**: e63854.

21. Bhangu A, Wood G, Brown G, Darzi A, Tekkis P, Goldin R. The role of epithelial mesenchymal transition and resistance to neoadjuvant therapy in locally advanced rectal cancer. *Colorectal Dis* 2014;**16**: O133-43.

22. Hao X, Tomlinson I, Ilyas M, Palazzo JP, Talbot IC. Reciprocity between membranous and nuclear expression of beta-catenin in colorectal tumours. *Virchows Arch* 1997;**431**: 167-72.

23. Lugli A, Zlobec I, Minoo P, Baker K, Tornillo L, Terracciano L, Jass JR. Prognostic significance of the wnt signalling pathway molecules APC, beta-catenin and E-cadherin in colorectal cancer: a tissue microarray-based analysis. *Histopathology* 2007;**50**: 453-64.

24. Salim T, Sjolander A, Sand-Dejmek J. Nuclear expression of glycogen synthase kinase-3beta and lack of membranous beta-catenin is correlated with poor survival in colon cancer. *Int J Cancer* 2013;**133**: 807-15.

25. Resnick MB, Routhier J, Konkin T, Sabo E, Pricolo VE. Epidermal growth factor receptor, c-MET, beta-catenin, and p53 expression as prognostic indicators in stage II colon cancer: a tissue microarray study. *Clin Cancer Res* 2004;**10**: 3069-75.

26. Matsuoka T, Mitomi H, Fukui N, Kanazawa H, Saito T, Hayashi T, Yao T. Cluster analysis of claudin-1 and -4, E-cadherin, and beta-catenin expression in colorectal cancers. *J Surg Oncol* 2011;**103**: 674-86.

27. Kroepil F, Fluegen G, Vallbohmer D, Baldus SE, Dizdar L, Raffel AM, Hafner D, Stoecklein NH, Knoefel WT. Snail1 expression in colorectal cancer and its correlation with clinical and pathological parameters. *BMC Cancer* 2013;**13**: 145.

28. Kahlert C, Lahes S, Radhakrishnan P, Dutta S, Mogler C, Herpel E, Brand K, Steinert G, Schneider M, Mollenhauer M, Reissfelder C, Klupp F, et al. Overexpression of ZEB2 at the invasion front of colorectal cancer is an independent prognostic marker and regulates tumor invasion in vitro. *Clin Cancer Res* 2011;**17**: 7654-63.

29. Kang H, Min BS, Lee KY, Kim NK, Kim SN, Choi J, Kim H. Loss of E-cadherin and MUC2 expressions correlated with poor survival in patients with stages II and III colorectal carcinoma. *Ann Surg Oncol* 2011;**18**: 711-9.

30. Roy HK, Smyrk TC, Koetsier J, Victor TA, Wali RK. The transcriptional repressor SNAIL is overexpressed in human colon cancer. *Dig Dis Sci* 2005;**50**: 42-6.

31. Oh SY, Kim YB, Suh KW, Paek OJ, Moon HY. Prognostic impact of fascin-1 expression is more significant in advanced colorectal cancer. *J Surg Res* 2012;**172**: 102-8.

32. Beg MS, Brenner AJ, Sachdev J, Borad M, Kang YK, Stoudemire J, Smith S, Bader AG, Kim S, Hong DS. Phase I study of MRX34, a liposomal miR-34a mimic, administered twice weekly in patients with advanced solid tumors. *Invest New Drugs* 2017;**35**: 180-8.

33. Kim MA, Lee HS, Lee HE, Kim JH, Yang HK, Kim WH. Prognostic importance of epithelial-mesenchymal transition-related protein expression in gastric carcinoma. *Histopathology* 2009;**54**: 442-51.

34. Wang Y, Wu Z, Hu L. Epithelial-Mesenchymal Transition Phenotype, Metformin, and Survival for Colorectal Cancer Patients with Diabetes Mellitus II. *Gastroenterol Res Pract* 2017;**2017**: 2520581.

Table 1. EMT markers and cancer specific survival in patients undergoing elective, potentially curative resection of stage II/III colorectal cancer (n=185)

	Nuclear			Cytoplasmic			Membrane/total		
	N (%)	**10yr CSS**	***P***	***N (%)***	**10yr CSS**	***P***	***N (%)***	**10yr CSS**	***P***
E cadherin			0.592			0.833			0.583
Low expression	94	61 (6)		87	68 (5)		85	65 (5)	
High expression	91	68 (5)		98	62 (5)		100	65 (5)	
Beta catenin			0.289			0.460			0.053
Low expression	88	68 (5)		90	61 (6)		69	56 (6)	
High expression	97	62 (5)		95	68 (5)		116	60 (5)	
Fascin			0.861			0.675			0.521
Low expression	172	65 (4)		133	66 (4)		125	68 (5)	
High expression	13	67 (13)		52	61 (7)		60	59 (7)	
Snail			0.876			0.012			0.270
Low expression	97	65 (5)		88	76 (5)		95	70 (5)	
High expression	88	64 (5)		97	55 (6)		90	60 (6)	
Zeb1			0.360			0.080			0.268
Low expression	108	62 (5)		91	71 (5)		91	70 (5)	
High expression	77	69 (6)		94	59 (5)		94	60 (6)	
Combined EMT Score									<0.001
Absent	-	-	-	-	-	-	12	100 (7)	
Low							160	65 (4)	
High							13	31 (13)	

Table 2. Relationship between EMT markers, clinicopathological characteristics and inflammatory responses in patients undergoing elective, potentially curative resection of stage II/III colorectal cancer (n=185).

	Cytoplasmic Snail			Combined EMT Score			
	Low (n=88)	High (n=97)	*P*	Absent (n=12)	Low (n=160)	High (n=13)	*P*
Age			0.524				0.287
<65	30 (34)	39 (40)		2 (17)	62 (39)	5 (39)	
>65	58 (66)	58 (60)		10 (83)	98 (61)	8 (61)	
Sex			0.546				0.856
Female	42 (48)	42 (43)		6 (50)	72 (45)	6 (46)	
Male	46 (52)	55 (57)		6 (50)	88 (55)	7 (54)	
Adjuvant			0.007				0.890
No	68 (77)	57 (59)		7 (58)	110 (69)	8 (62)	
Yes	20 (23)	40 (41)		5 (42)	50 (31)	6 (38)	
Tumor site			0.193				0.545
Colon (right-side)	40 (45)	37 (38)		5 (42)	68 (42)	4 (31)	
Colon (left-side)	21 (24)	35 (36)		2 (16)	48 (30)	6 (46)	
Rectum	27 (31)	25 (26)		5 (42)	44 (28)	3 (23)	
TNM-stage			0.812				0.838
II	46 (52)	49 (51)		7 (58)	81 (51)	7 (54)	
III	42 (48)	48 (49)		5 (42)	79 (49)	6 (46)	
Differentiation			0.413				0.122
Mod/well	78 (89)	82 (84)		12 (100)	136 (85)	12 (92)	
Poor	10 (11)	15 (16)		0 (0)	24 (15)	1 (8)	
Venous invasion			0.306				0.055
Absent	59 (67)	58 (60)		10 (83)	101 (63)	6 (46)	
Present	29 (33)	39 (40)		2 (17)	59 (37)	7 (54)	
Margin involvement			0.270				0.371
No	81 (92)	93 (96)		11 (92)	150 (94)	13 (100)	
Yes	7 (8)	4 (4)		1 (8)	10 (6)	0 (0)	
Peritoneal involvement			0.984				0.771
No	60 (68)	66 (68)		9 (75)	108 (68)	9 (69)	
Yes	28 (32)	31 (32)		3 (25)	52 (32)	4 (31)	
Mismatch repair status			0.211				0.618
Competent	77 (89)	79 (83)		11 (92)	134 (86)	11 (85)	
Deficient	9 (11)	16 (17)		1 (8)	22 (14)	2 (15)	
Proliferation Index			0.552				0.210
Low	35 (41)	35 (37)		8 (67)	57 (37)	5 (42)	
High	50 (59)	60 (63)		4 (33)	99 (63)	7 (58)	
Necrosis			0.252				0.413
Low	45 (51)	56 (60)		9 (75)	85 (54)	7 (58)	
High	43 (49)	38 (40)		3 (25)	73 (46)	5 (42)	
Tumor stroma percentage			0.891				0.352
Low	58 (71)	60 (70)		9 (82)	102 (70)	7 (64)	
High	24 (29)	26 (30)		2 (18)	44 (30)	4 (36)	
Tumor budding			0.376				0.046
Low	49 (60)	59 (66)		9 (82)	94 (63)	5 (42)	
High	33 (40)	30 (34)		2 (18)	54 (37)	7 (58)	
Klintrup-Makinen grade			0.924				0.157
Strong	29 (33)	31 (32)		5 (42)	53 (33)	2 (15)	
Weak	59 (67)	65 (68)		7 (58)	106 (67)	11 (85)	
GMS			0.973				0.151
0	29 (35)	31 (35)		5 (46)	53 (36)	2 (18)	
1	36 (43)	38 (43)		4 (36)	65 (44)	5 (46)	
2	18 (22)	19 (22)		2 (18)	31 (20)	4 (36)	
mGPS			0.060				0.007
0	53 (60)	47 (48)		10 (83)	84 (52)	6 (46)	
1	28 (32)	35 (36)		2 (17)	59 (37)	2 (15)	
2	7 (8)	15 (16)		0 (0)	17 (11)	5 (39)	

Table 3. Relationship between EMT markers and local inflammatory inflitrate in patients undergoing elective, potentially curative resection of stage II/III colorectal cancer (n=185).

	Cytoplasmic Snail			Combined EMT Score			
	Low (n=88)	High (n=97)	*P*	Absent (n=12)	Low (n=160)	High (n=13)	*P*
CD3+ lymphocytes - Margin			0.359				0.221
Low	44 (54)	55 (61)		4 (40)	87 (58)	8 (67)	
High	37 (46)	35 (39)		6 (60)	62 (42)	4 (33)	
CD3+ lymphocytes - Stroma			0.173				0.144
Low	37 (44)	51 (54)		5 (45)	74 (47)	9 (75)	
High	48 (56)	44 (46)		6 (55)	83 (53)	3 (25)	
CD3+ lymphocytes - Centre			0.186				0.142
Low	52 (61)	57 (71)		6 (55)	103 (66)	10 (83)	
High	33 (39)	28 (29)		5 (45)	54 (34)	2 (17)	
Cytotoxic T-cells - Margin			0.436				0.099
Low	47 (59)	60 (65)		4 (40)	94 (62)	9 (75)	
High	33 (41)	33 (35)		6 (60)	57 (38)	3 (25)	
Cytotoxic T-cells - Stroma			0.254				0.223
Low	57 (70)	74 (77)		6 (60)	115 (74)	10 (83)	
High	25 (30)	22 (23)		4 (40)	41 (26)	2 (17)	
Cytotoxic T-cells - Centre			0.149				0.459
Low	55 (66)	73 (76)		7 (70)	111 (71)	10 (83)	
High	28 (34)	23 (24)		3 (30)	46 (29)	2 (17)	
Memory T-cells - Margin			0.315				0.035
Low	45 (53)	55 (60)		7 (58)	83 (54)	10 (91)	
High	40 (47)	36 (40)		5 (42)	70 (46)	1 (9)	
Memory T-cells - Stroma			0.121				0.030
Low	34 (39)	48 (51)		6 (50)	67 (42)	9 (82)	
High	53 (61)	47 (49)		6 (50)	92 (58)	2 (18)	
Memory T-cells - Centre			0.384				0.215
Low	59 (68)	70 (74)		8 (67)	111 (70)	10 (91)	
High	28 (32)	25 (26)		4 (33)	48 (30)	1 (9)	
Tregs - Margin			0.070				0.251
Low	49 (58)	63 (71)		8 (67)	94 (62)	10 (91)	
High	36 (42)	26 (29)		4 (33)	57 (38)	1 (9)	
Tregs - Stroma			0.006				0.049
Low	44 (51)	65 (71)		6 (50)	93 (60)	10 (91)	
High	43 (49)	27 (29)		6 (50)	63 (40)	1 (9)	
Tregs - Centre			0.006				0.141
Low	37 (43)	58 (63)		6 (50)	80 (51)	9 (82)	
High	50 (57)	34 (37)		6 (50)	76 (49)	2 (18)	

Table 4. Clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage II/III colorectal cancer and cancer-specific survival (n=185)

	Univariate HR (95% CI)	*P*
Clinicopathological Characteristics		
Age (<65/>65)	1.23 (0.91-1.66)	0.182
Sex (Female/Male)	1.16 (0.70-1.93)	0.562
Adjuvant Therapy (No/Yes)	0.90 (0.52-1.54)	0.696
Tumor Site (Colon (right)/colon (left)/Rectum)	0.88 (0.64-1.20)	0.402
BRAF status (WT/mutant)	0.86 (0.47-1.56)	0.620
TNM-Stage (II/III)	2.50 (1.48-4.24)	0.001
Differentiation (Moderate or well/Poor)	1.04 (0.49-2.18)	0.924
Venous Invasion (Absent/Present)	2.74 (1.65-4.54)	<0.001
Margin Involvement (No/Yes)	1.36 (0.50-3.77)	0.546
Peritoneal Involvement (No/Yes)	1.58 (0.95-2.62)	0.077
Necrosis (Low/High)	1.00 (0.60-1.67)	0.999
Mismatch Repair Status (Competent/Deficient)	1.50 (0.78-2.87)	0.229
Ki67 proliferation Index (Low/High)	0.40 (0.24-0.67)	0.001
Tumor Stroma Percentage (<50%/>50%)	2.16 (1.26-3.71)	0.005
Tumor budding (yes/no)	2.73 (1.61-4.64)	<0.001
Inflammatory Characteristics		
Klintrup-Makinen Grade (Strong/Weak)	1.62 (0.91-2.90)	0.104
GMS (0/1/2)	1.65 (1.15-2.37)	0.007
mGPS (0/1/2)	1.55 (1.10-2.17)	0.011
NLR (<5/>5)	0.76 (0.36-1.62)	0.477
EMT markers		
Cytoplasmic Snail	1.94 (1.15-3.29)	0.014
Combined EMT score	3.86 (2.17-6.86)	<0.001

FIGURE LEGENDS

Figure 1. EMT markers are associated with poor prognosis in patients with Stage II/II Colorectal cancer (n=185). (A-D) Kaplan Meier curves showing associations between cancer-specific survival and tumor-based markers of EMT: (A) membrane beta-catenin, (B) cytoplasmic snail, (C) cytoplasmic Zeb1 and (D) the combined EMT score.

Figure 2. A high combined EMT score is associated with increased metastasis and decrease lymphocytes (n=185). (A) Amount of lymph node metastasis for each EMT score. (B-D) Level of (B) CD8+ T-cells, (C) memory T-cells and (D) regulatory T0cells for each EMT score.