Original Article

Tumour invasiveness, the local and systemic environment and the basis of staging systems in colorectal cancer

Running Title: T stage and the local and systemic environment

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Abstract

Background

The present study aimed to examine the relationship between tumour invasiveness (T stage), the local and systemic environment and cancer specific survival (CSS) of patients with primary operable colorectal cancer.

Methods

The tumour microenvironment was examined using measures of the inflammatory infiltrate (Klintrup-Makinen (KM) grade and Immunoscore), tumour stroma percentage (TSP) and tumour budding. The systemic inflammatory environment was examined using modified Glasgow Prognostic Score (mGPS) and neutrophil:lymphocyte ratio (NLR). 5-year CSS was examined.

Results

331 patients were included. Increasing T stage was associated with colonic primary, N stage, poor differentiation, margin involvement and venous invasion ($P<0.05$). T stage was significantly associated with KM grade ($P=0.001$), Immunoscore ($P=0.016$), TSP ($P=0.006$), tumour budding ($P<0.001$), and elevated mGPS and NLR (both $P<0.05$).

In patients with T3 cancer, N stage stratified survival from 88% to 64%, whereas Immunoscore and budding stratified survival from 100% to 70% and from 91% to 56% respectively. The Glasgow Microenvironment Score, a score based on KM grade and TSP, stratified survival from 93% to 58%.

Conclusion

Although associated with increasing T stage, local and systemic tumour environment characteristics, and in particular Immunoscore, budding, TSP and mGPS, are stage-independent determinants of survival and may be utilised in the staging of patients with primary operable colorectal cancer.

Key words: Colorectal cancer, tumour microenvironment, inflammation, prognosis, staging

Introduction

The staging of patients with colorectal cancer is based on the Tumour, Node, Metastasis (TNM) classification as described by the Union for International Cancer Control/ American Joint Committee on Cancer (UICC/ AJCC). For patients without metastatic disease, prognosis is primarily determined by the depth of invasion of the primary tumour (T stage) as well as the presence of regional lymph node metastases (N stage). However, the use of TNM-based staging remains problematical, since increasing disease stage does not necessarily reflect a stepwise increase in risk of recurrence or death. For example, the survival of patients with Stage IIIa (T1/2, N1) colon cancer is superior to that of patients with stage IIb (T4, N0) disease (O'Connell *et al*, 2004).

Given that TNM criteria are suboptimal, there is increasing effort to refine colorectal cancer staging. One potential approach is to examine the molecular characteristics of the tumour, and various approaches ranging from assessment of gene expression profiles to more comprehensive molecular subtyping have been described (Guinney *et al*, 2015; Salazar *et al*, 2011). These have largely failed to translate from use as clinical research tools, with the practicalities of assays employed, differing methodologies, and high costs prohibiting routine clinical use (Church *et al*, 2012; Munro *et al*, 2005). Additionally, except for assessment of *KRAS* status and microsatellite instability (De Roock *et al*, 2010; Guastadisegni *et al*, 2010), the clinical utility of such characteristics as predictive markers of treatment response remain largely unknown.

A differing approach is assessment of the local and systemic tumour environment, encompassing the interface between tumour and host (Hanahan & Weinberg, 2011; McAllister & Weinberg, 2014). Loss of local anti-tumour immune responses (Bindea *et al*, 2013; Klintrup *et al*, 2005; Pages *et al*, 2009), expansion of the tumour-associated stroma

(Huijbers *et al*, 2013; Mesker *et al*, 2007), and the presence of tumour budding (Ueno *et al*, 2002), have all been identified as markers of poor prognosis. Such characteristics may be readily assessed utilising routinely available formalin-fixed paraffin embedded specimens and pathological techniques, and have been validated as stage independent predictors of survival. Similarly, the presence of an elevated systemic inflammatory response, as evidenced not only by circulating cytokines (Kantola *et al*, 2012), but also routinely measured inflammatory mediators (McMillan, 2013), is similarly associated with poorer survival.

We have previously reported that combined assessment of the tumour inflammatory cell infiltrate (utilising both generalised inflammatory cell infiltrate and $CD3^+$ and $CD8^+$ T-lymphocyte subsets) and the tumour-associated stroma (using tumour stroma percentage (TSP)), hold independent and complimentary prognostic value in patients with colorectal cancer (Park *et al*, 2015a; Park *et al*, 2015b). Furthermore, the addition of tumour budding further stratifies survival independent of these two characteristics (van Wyk *et al*, 2016). As such, assessment of these measures, in addition to the systemic inflammatory response, provides the opportunity to utilise characteristics of both the tumour and the host to determine prognosis.

Although the presence of adverse local and systemic characteristics has been previously reported to be associated with increasing T stage, it is of interest that they retain independent prognostic value (Pages *et al*, 2005; Park *et al*, 2015b; Park *et al*, 2016b; van Wyk *et al*, 2016). Therefore, given the routine reporting of T stage, it would be of interest to examine their prognostic value relative to present TNM-based staging. As such, the aim of the present study was to examine the interrelationships between T stage, components of the

local and systemic environment, and survival of patients undergoing potentially curative resection of primary operable colorectal cancer.

Patients and Methods

Patients were identified from a prospectively maintained database of colorectal cancer resections in a single surgical unit in Glasgow Royal Infirmary. For the present study, patients who on the basis of pre-operative computed tomography and intra-operative findings were considered to have undergone potentially curative, elective resection of stage I-III colorectal adenocarcinoma between January 1997 and May 2008 were included. Exclusion criteria included emergency, localised or palliative resection, pre-operative chemoradiotherapy and death within 30 days of operation. Study approval was granted by the West of Scotland Research Ethics Committee.

Patients were staged according to the 5th edition of TNM criteria as is current practice in the United Kingdom (Loughrey *et al*, 2014). Tumours were categorised as either proximal (caecum, ascending colon, hepatic flexure and transverse colon), distal (splenic flexure, descending colon, sigmoid) or rectal (rectosigmoid and rectum) on the basis of operative and pathological reports. The presence of venous invasion was identified routinely using routine elastica staining.

Patients were followed up for a minimum of five years. Patients were discussed following surgery at multi-disciplinary meetings comprised of clinicians with a specialist interest in colorectal cancer, where those with stage III and high-risk stage II disease were considered for adjuvant, 5-fluoruracil-based chemotherapy according to contemporary treatment protocols. Cause and date of death were crosschecked with the cancer registration system and Registrar General (Scotland), with records complete until 31st March 2014 which acted as the censor date. Cancer-specific survival was measured from date of surgery until date of death from recurrent or metastatic colorectal cancer, and overall survival was measured until date of death from any cause.

Assessment of mismatch repair status

Mismatch repair (MMR) status was performed for a subgroup of patients who had tissue included in a tissue microarray (TMA) as previously described (Park *et al*, 2016a). Briefly, TMA sections were stained for MLH1, MSH6, MSH2 and PMS2. In accordance with UK NEQAS (Arends *et al*, 2008), tumours were considered MMR competent if tumour epithelial nuclear staining was positive, and MMR deficient if tumour epithelial staining was negative with positive staining of intratumoural lymphocytes.

Assessment of the tumour microenvironment

The generalised inflammatory cell infiltrate was examined using the Klintrup-Mäkinen (K-M) grade as previously described (Klintrup *et al*, 2005). Briefly, using H&E-stained sections of the deepest point of tumour invasion, the density of the generalised inflammatory cell infiltrate was graded as low-grade (no increase or mild, patchy increase in inflammatory cells) or high-grade (prominent inflammatory reaction, forming a band at the invasive margin, or florid cup-like infiltrate at the invasive edge with frequent destruction of cancer cell islands). The adaptive, T-lymphocytic infiltrate was examined as previously described (Richards *et al*, 2014). Briefly, full sections of the deepest point of invasion were stained for mature ($CD3^+$) and cytotoxic ($CD8^+$) T-lymphocytes and the density of each cell type within intraepithelial compartment and invasive margin semi-quantitatively graded as either high or low. The Immunoscore, a quantitative assessment of $CD3^+$ and $CD8^+$ density in both regions, has previously been reported utilising automated digital pathology (Galon *et al*, 2014). Manual semi-quantitative assessment has been shown to correlate strongly with automated assessment, whilst allowing for increased discrimination of non-specific background staining (De Smedt *et al*, 2015). As such, a semi-quantitative Immunoscore was

utilised, calculated from the number of compartments with a high density of immune cells, ranging from Im0 (all regions low density) to Im4 (all regions high density). On the basis of previous work, patients were stratified into three prognostic groups: Im0/1 (low density), Im 2/3 (moderate density) and Im4 (high density) (Park *et al*, 2015a).

Tumour stroma percentage (TSP), tumour necrosis, and tumour budding were all examined using H&E-stained sections of the invasive margin as previously described (Mesker *et al*, 2007; Pollheimer *et al*, 2010; van Wyk *et al*, 2016). Briefly, excluding necrosis and mucin deposits, TSP was calculated as low (<50% of tumour area) or high (>50% of tumour area). Tumour necrosis was graded as low (absent or <10% of tumour area) or high (>10% of tumour area). To assess tumour budding, the number of tumour buds (tumour cells with up to five nuclei or single tumour cells) in 10 high-power fields was counted. On the basis of previous work, a budding count greater than 20 was considered high-grade (van Wyk *et al*, 2016).

Assessment of the systemic inflammatory response

Pre-operative serum C-reactive protein (CRP), albumin and differential white cell count were measured within 30 days prior to surgery as routine and recorded prospectively. The systemic inflammatory response was measured using the modified Glasgow Prognostic Score (mGPS) and neutrophil:lymphocyte ratio (NLR) as previously described (Guthrie *et al*, 2013; McMillan, 2013). Patients with CRP ≤10mg/l were given a score of 0, patients with CRP>10mg/l a score of 1, and patients with CRP>10mg/L and albumin <35g/L a score of 2. On the basis of previous literature review, NLR>5 was considered elevated (Guthrie *et al*, 2013).

Statistical Analysis

The relationship between T stage and characteristics of the local and systemic environments was examined using the χ^2-test for linear trend. Their relationship and cancer-specific and overall survival was examined using Kaplan-Meier log-rank analysis to calculated five-year survival (standard error (SE)). Variables associated with survival were entered into a Cox proportional hazards regression analysis, using a backwards-conditional method. A p-value$\leq$0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 22.0 (IBM SPSS, IL, USA).

Results

A total of 331 patients were included. Two thirds of patients were 65 or older at time of surgery and 52% were male. Thirty percent of patients underwent resection of rectal cancer. Eighty-two patients received adjuvant therapy. The majority of patients (90%) had a tumour breaching through muscularis propria, with 208 and 90 patients with T3 and T4 tumours respectively. Of the remaining patients, eight had a T1 tumour and 25 had a T2 tumour.

The relationship between T stage and clinicopathological characteristics is displayed in Table 1. T stage was associated with colonic primary ($P<0.001$), N stage ($P<0.01$), margin involvement, venous invasion (both $P<0.001$), and poor differentiation ($P<0.05$). In addition, T stage was associated with adjuvant chemotherapy ($P<0.05$) but not age or sex. Mismatch repair status was available for 209 patients, and was not associated with increasing T stage.

The relationship between T stage and the local and systemic environment was examined (Table 2). T stage was associated with high-grade necrosis, infiltrative invasive margin, high-grade tumour budding, low K-M grade (all $P\leq0.001$) and high TSP ($P<0.01$). Furthermore, increasing T stage was associated with lower Immunoscore and elevated systemic inflammatory responses as measured by mGPS and NLR (all $P<0.05$). Certain characteristics appeared to become more prevalent earlier than others; there was a statistically significant increase in the number of patients with high grade necrosis and low K-M grade observed in the shift from T2 to T3 (Bonferroni-corrected $P<0.05$), whereas the proportion of patients with an infiltrative margin, high grade budding and high TSP showed a statistically greater increase between T3 to T4 ($P<0.05$). Although an elevated mGPS and NLR showed a greater stepwise increase between T3 to T4, this did not reach statistical significance.

The relationship between tumour site, T stage and the local and systemic environment was examined (Supplementary Table 1). In patients with cancer of the right colon, increasing T stage was associated with tumour budding and TSP (both $P<0.01$) and showed a trend towards an association with necrosis ($P=0.054$) and an infiltrative margin ($P=0.081$). In patients with cancer of the left colon, increasing T stage was associated with necrosis ($P<0.01$), an infiltrative margin ($P<0.05$) and tumour budding ($P<0.001$). In patients with rectal cancer, increasing T stage was associated with an infiltrative margin, weak KM grade (both $P\leq0.001$) and showed a trend towards weak Immunoscore ($P=0.096$).

The relationship between the local and systemic tumour environment and five-year survival was examined (Table 3). The median follow-up of survivors was 134 months (interquartile range 108-170 months) with 96 cancer deaths and 105 non-cancer deaths. Five-year cancer-specific survival of the whole cohort was 77%. N stage, character of margin, budding, K-M grade, TSP, Immunoscore and mGPS all stratified five-year cancer-specific survival (all $P<0.001$), whereas tumour necrosis, the NLR and MMR status did not. On multivariate analysis (Table 4), controlling for age, adjuvant chemotherapy, T stage and venous invasion, tumour budding, Immunoscore and mGPS remained independently associated with survival whereas N stage, character of margin and K-M grade did not; TSP showed a non-significant association with survival (HR 1.64, $P=0.084$).

Five-year overall survival was 65% (Table 3). N stage, necrosis, budding, K-M grade, TSP, Immunoscore and mGPS (all $P<0.05$), but not MMR status, character of margin or NLR stratified five-year overall survival. On multivariate analysis (Table 4), controlling for age, adjuvant therapy, T stage and venous invasion, tumour budding, TSP and mGPS remained independently associated with survival, whereas N stage, necrosis and K-M grade

did not; Immunoscore showed a non-significant association with improved survival (HR 0.77, *P*=0.053).

As tumour budding, TSP, Immunoscore and mGPS appeared to be consistently associated with both cancer-specific and overall survival, the relationship between these characteristics, tumour site and survival was examined (Table 5). Tumour budding and mGPS were associated with both cancer-specific and overall survival across all tumour sites. Tumour stroma percentage showed an association with cancer-specific across all tumour sites, but only appeared to stratify overall survival of patients with right and left colonic cancer but not rectal cancer. Immunoscore was associated with cancer-specific and overall survival of patients with right-sided and rectal cancers; although appearing to stratify cancer-specific and overall survival of patients with left colonic cancers, this did not reach statistical significance.

Discussion

The results of the present study confirm the relationship between tumour invasion and the presence of adverse characteristics within the local and systemic environment. Such characteristics, namely tumour budding, TSP, Immunoscore and the mGPS, appeared to have greater prognostic value than evaluation of N stage in patients with primary operable colorectal cancer.

Advancing T stage correlated significantly with the presence of an increasingly tumour-supportive microenvironment as evidenced by loss of host immune responses, expansion of the tumour-associated stroma and the presence of tumour budding. This is consistent with previous work, whereby such adverse characteristics become more prevalent with increasing tumour size and depth of invasion (Bindea *et al*, 2013; Park *et al*, 2015b; Vayrynen *et al*, 2016). It was of interest however, that the progression of each of these characteristics appeared to occur in a stepwise manner, with the proportion of some appearing to increase at an earlier T stage than others. For example, attenuation of the generalised local inflammatory cell infiltrate appeared to occur at a relatively early stage (between T2 and T3), whereas the presence of tumour budding and increasing TSP appeared to occur later, with a clear stepwise change evident between T3 and T4 tumours.

Although based on observational data, the present results potentially inform our understanding of the nature of the tumour microenvironment and its development in patients with colorectal cancer. Loss of adaptive, anti-tumour immune responses, or 'immune escape' may be the initial precipitant allowing sustained tumour growth and invasion (Mlecnik *et al*, 2011), with other adverse tumour microenvironment characteristics developing further downstream in the presence of "pro-tumour" local and systemic immune responses

(McAllister & Weinberg, 2014). Certainly, it is recognised that the immune microenvironment evolves in tandem with stage progression, favouring the development of a more pro-tumour "immunome" as T stage increases (Bindea *et al*, 2013). As this progresses and anti-tumour immunity is degraded, it may allow the development of further pro-tumour microenvironment characteristics, such as recruitment and activation of tumour-associated fibroblasts (Chrysanthopoulou *et al*, 2014), and budding (Koelzer *et al*, 2015).

Subgroup analysis found that the relationship between T stage and local and systemic environment characteristics was not consistent across tumour sites. In patients with right-sided tumours, increasing T stage was associated with increasing TSP but not loss of the inflammatory cell infiltrate; conversely, the opposite was found in patients with rectal cancer. This may reflect the molecular heterogeneity of tumour arising from different sites (Birkenkamp-Demtroder *et al*, 2005), with tumour microenvironment characteristics, such as necrosis, mesenchymal and inflammatory cell infiltration being associated with distinct molecular characteristics (Guinney *et al*, 2015; Vayrynen *et al*, 2016). Consistent with this, in the present study MMR deficiency was identified in 30% of right-sided cancers compared to only 6% of rectal cancers ($P<0.001$).

Subsequent revisions of the TNM staging system have introduced significant changes to pathological definitions, particularly with respect to nodal stage and often with little supporting evidence (Quirke *et al*, 2007). Such changes have led to concern regarding the potential "upstaging of patients" (Nagtegaal *et al*, 2011; Ueno *et al*, 2012). Given that the criteria for T stage remains relatively standardised and largely unchanged since first described by Dukes (Dukes, 1932), it presents an attractive and logical foundation to base disease staging upon. It has previously been proposed that staging should be weighted more towards T stage, with less reliance on the presence of nodal involvement as a defining factor

for high-risk disease (Gunderson *et al*, 2010; Li *et al*, 2016). However, although associated with increasing T stage, when controlling for T stage, N stage and venous invasion, assessment of local and systemic environment characteristics were independently associated with survival. Indeed, such characteristics may further stratify T stage in terms of survival. For example, the presence of budding, an expanded stroma and loss of the local immune response may identify patients with T1/2 tumours with poorer survival. If this were proven to be the case then such characteristics may aid, for example, in the decision between polypectomy rather than formal segmental resection in patients with polyp cancers.

In addition to MMR status, numerous other molecular characteristics have been confirmed to hold prognostic value in patients with colorectal cancer (Guinney *et al*, 2015; Sinicrope *et al*, 2015). However, these are not uniformly employed in routine clinical practice and remain costly. Therefore, it was of interest that assessment of the local and systemic environment was of greater prognostic value than MMR status. Furthermore, prognostic utility appeared consistent across different tumour sites, suggesting that molecular heterogeneity may not confound the present results. This further supports results of previous studies, whereby assessment of local and systemic inflammatory profiles, tumour-associated stroma and tumour budding have been shown to hold prognostic value independent of both MMR status (Huijbers *et al*, 2013; Park *et al*, 2016a; Rozek *et al*, 2016), and more extensive molecular characterisation (Ogino *et al*, 2009). Indeed, the relatively simple methodologies employed in the present study, and their reliance on routine pathological specimens, would make them attractive candidates for widespread clinical use.

The relatively small number of patients with T1/2 disease limits the present study. Indeed, validation in a larger cohort, encompassing patient with earlier stage disease is warranted. Furthermore, it was not possible to examine the predictive value of local and systemic environment characteristics with respect to the use of adjuvant chemotherapy.

Whether the tumour and host factors examined in the present study may be utilised in such a manner would be of considerable interest. In conclusion, the local and systemic environment, although associated with increasing T stage, have independent prognostic value. In particular, the Immunoscore, tumour budding, TSP and the mGPS may be effectively employed in the staging of patients with primary operable colorectal cancer.

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Conflict of interest statement

The authors have declared no conflicts of interest.

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Table 1. The relationship between T stage and clinicopathological characteristics of patients undergoing elective, primary resection of stage I-III colorectal cancer.

		T1	T2	T3	T4	
		***N*=8 (%)**	***N*=25 (%)**	***N*=208 (%)**	***N*=90 (%)**	***P***
Host characteristics						
Age						0.713
	<65	1 (13)	9 (36)	69 (33)	33 (37)	
	65-74	5 (62)	8 (32)	70 (34)	27 (30)	
	>75	2 (25)	8 (32)	69 (33)	30 (33)	
Sex						0.533
	Female	5 (62)	16 (64)	93 (45)	46 (51)	
	Male	3 (38)	9 (36)	115 (55)	44 (49)	
Adjuvant therapy (330)						0.030
	No	6 (75)	23 (92)	159 (76)	60 (67)	
	Yes	2 (25)	2 (8)	49 (24)	29 (33)	
Tumour characteristics						
Tumour site						<0.001
	Right colon	0 (0)	7 (28)	78 (38)	47 (52)	
	Left colon	2 (25)	5 (20)	67 (32)	26 (29)	
	Rectum	6 (75)	13 (52)	63 (30)	17 (19)	
N stage						0.002
	0	5 (62)	20 (80)	139 (67)	45 (50)	
	1	3 (38)	4 (16)	56 (27)	32 (36)	
	2	0 (0)	1 (4)	13 (6)	13 (14)	
Tumour differentiation						0.016
	Well/ mod	7 (87)	24 (96)	189 (91)	72 (80)	
	Poor	1 (13)	1 (4)	19 (9)	18 (20)	
Margin involvement						<0.001
	Absent	8 (100)	25 (100)	205 (99)	72 (80)	
	Present	0 (0)	0 (0)	3 (1)	18 (20)	
Venous invasion						<0.001
	Absent	8 (100)	23 (92)	140 (67)	45 (50)	
	Present	0 (0)	2 (8)	68 (33)	45 (50)	
Mismatch repair status (209)						0.161
	Competent	7 (87)	15 (88)	110 (87)	44 (77)	
	Deficient	1 (13)	2 (12)	17 (13)	13 (23)	

(*n*) denotes number of cases when patients missing

Table 2. The relationship between T stage, the tumour microenvironment and systemic environment of patients undergoing elective, primary resection of stage I-III colorectal cancer.

		T1 (%)	T2 (%)	T3 (%)	T4 (%)	
Tumour microenvironment		***N*=8 (%)**	**N=25 (%)**	***N*=208 (%)**	***N*=90 (%)**	***P***
Necrosis (297)						<0.001
	Absent	7 (87)	19 (90)	106 (56)	37 (46)	
	Present	1 (13)	2 (10)	82 (44)	43 (54)	
Invasive margin (312)						<0.001
	Expansile	7 (87)	18 (82)	119 (60)	34 (40)	
	Infiltrative	1 (13)	4 (18)	78 (40)	51 (60)	
Tumour budding (302)						<0.001
	Low	5 (71)	18 (78)	146 (74)	33 (43)	
	High	2 (29)	5 (22)	50 (26)	43 (57)	
Klintrup-Mäkinen grade (307)						0.001
	Strong	5 (62)	14 (67)	63 (32)	21 (26)	
	Weak	3 (38)	7 (33)	133 (68)	61 (74)	
Tumour stroma percentage (331)						0.006
	Low	7 (87)	19 (76)	168 (81)	56 (62)	
	High	1 (13)	6 (24)	40 (19)	34 (38)	
Immunoscore (226)						0.016
	0-1	2 (29)	8 (42)	68 (49)	37 (61)	
	2-3	2 (29)	7 (37)	54 (39)	17 (28)	
	4	3 (42)	4 (21)	17 (12)	7 (12)	
Systemic environment						
mGPS (330)						0.031
	0	6 (75)	16 (64)	127 (61)	45 (51)	
	1	2 (25)	8 (32)	51 (25)	29 (33)	
	2	0 (0)	1 (4)	30 (14)	15 (17)	
NLR (225)						0.033
	≤5	6 (86)	17 (85)	115 (82)	39 (67)	
	>5	1 (14)	3 (15)	25 (18)	19 (33)	

(*n*) denotes number included when patients missing. mGPS – modified Glasgow Prognostic Score, NLR – neutrophil:lymphocyte ratio

Table 3. The relationship between tumour, microenvironment and systemic environment characteristics of patients undergoing elective, primary resection of stage I-III colorectal cancer and five-year cancer-specific and overall survival.

		5-yr CSS % (SE)	*P*	5-yr OS % (SE)	*P*
All		77 (2)	-	65 (3)	-
N stage			<0.001		0.011
	N0	86 (2)		74 (3)	
	N1	64 (5)		56 (5)	
	N2	46 (10)		33 (9)	
Mismatch repair status			0.100		0.551
	Deficient	88 (6)		79 (7)	
	Competent	73 (3)		62 (4)	
Necrosis			0.130		0.001
	Absent	80 (3)		75 (3)	
	Present	72 (4)		53 (4)	
Margin			<0.001		0.269
	Expansile	82 (3)		69 (3)	
	Infiltrative	69 (4)		60 (4)	
Tumour budding			<0.001		<0.001
	Low	90 (2)		75 (3)	
	High	54 (5)		49 (5)	
Klintrup-Mäkinen grade			<0.001		0.004
	Strong	90 (3)		78 (4)	
	Weak	70 (3)		59 (3)	
Tumour stroma percentage			<0.001		0.015
	Low	81 (3)		69 (3)	
	High	64 (6)		53 (6)	
Immunoscore			<0.001		<0.001
	4	96 (3)		84 (7)	
	2-3	87 (4)		75 (5)	
	0-1	62 (5)		51 (5)	
Modified Glasgow Prognostic Score			<0.001		<0.001
	0	83 (3)		75 (3)	
	1	72 (5)		57 (5)	
	2	57 (8)		39 (7)	
Neutrophil: Lymphocyte Ratio			0.362		0.080
	≤5	79 (3)		70 (3)	
	>5	73 (7)		56 (7)	

CSS – cancer-specific survival, OS – overall survival

Table 4. The relationship between N stage, the tumour microenvironment and systemic environment and cancer-specific and overall survival of patients undergoing elective, primary resection of stage I-III colorectal cancer.

	Multivariate survival analysis (HR, 95%CI)			
Characteristic	**Cancer-specific survival**	***P***	**Overall survival**	***P***
N stage (0/ 1/ 2)	1.04 (0.71-1.52)	0.836	1.10 (0.83-1.47)	0.509
Necrosis (Absent/ present)	-	-	1.40 (0.97-2.02)	0.074
Margin (Expansile/ infiltrative)	1.29 (0.73-2.27)	0.388	-	-
Budding (Absent/ present)	2.80 (1.58-4.94)	<0.001	1.56 (1.07-2.27)	0.021
Klintrup-Mäkinen grade (Strong/ weak)	1.18 (0.58-2.41)	0.650	1.20 (0.78-1.83)	0.406
Tumour stroma percentage (Low/ high)	1.64 (0.94-2.88)	0.084	1.89 (1.25-2.84)	0.002
Immunoscore (0-1/ 2-3/ 4)	0.41 (0.25-0.67)	<0.001	0.77 (0.59-1.00)	0.053
mGPS (0/ 1/ 2)	1.55 (1.08-2.23)	0.017	1.46 (1.14-1.88)	0.003

mGPS- modified Glasgow Prognostic Score. Multivariate analysis performed controlling for age, tumour site, adjuvant therapy use, T stage and venous invasion.

Table 5. The relationship between local and systemic environment characteristics, tumour site, and five-year cancer-specific and overall survival of patients undergoing elective, primary resection of stage I-III colorectal cancer.

		5-year cancer-specific survival (SE)						5-year overall survival (SE)					
		Right	***P***	**Left**	***P***	**Rectal**	***P***	**Right**	***P***	**Left**	***P***	**Rectal**	***P***
Tumour budding			<0.001		0.001		<0.001		0.010		0.052		0.005
	Low	91 (3)		90 (4)		91 (4)		76 (5)		74 (6)		74 (6)	
	High	50 (8)		68 (9)		43 (10)		47 (8)		66 (9)		36 (9)	
Tumour stroma percentage			0.029		0.037		0.063		0.026		0.133		0.617
	Low	80 (4)		82 (4)		78 (5)		73 (4)		70 (5)		63 (6)	
	High	60 (9)		67 (10)		66 (9)		47 (9)		57 (10)		57 (9)	
Immunoscore			0.003		0.191		0.002		0.046		0.289		0.005
	4	100 (0)		87 (12)		100 (0)		92 (7)		75 (15)		80 (13)	
	2-3	83 (8)		85 (7)		92 (5)		76 (9)		71 (9)		78 (8)	
	0-1	61 (7)		67 (9)		58 (9)		50 (7)		61 (9)		45 (8)	
Modified Glasgow Prognostic Score			0.001		0.061		0.052		0.001		<0.001		0.115
	0	83 (5)		85 (5)		81 (5)		79 (5)		79 (5)		68 (6)	
	1	81 (6)		60 (10)		68 (11)		67 (7)		43 (9)		55 (11)	
	2	48 (11)		86 (13)		53 (15)		35 (9)		50 (18)		42 (14)	

Supplementary Table 1. The relationship between tumour site, T stage, the tumour microenvironment and systemic environment of patients undergoing elective, primary resection of stage I-III colorectal cancer.

Right colon *N*=132		**T1**	**T2**	**T3**	**T3**	
Tumour microenvironment		***N*=0**	**N=7**	***N*=78**	***N*=47**	***P***
Necrosis (119)						0.054
	Absent	-	4	42	20	
	Present	-	0	30	23	
Invasive margin						0.081
(121)	**Expansile**	-	4	44	21	
	Infiltrative	-	1	28	23	
Tumour budding						0.002
(123)	**Low**	-	6	53	21	
	High	-	0	21	22	
Klintrup-						0.625
Mäkinen grade	**Strong**	-	1	24	12	
(120)	**Weak**	-	3	48	32	
Tumour stroma						0.001
percentage (132)	**Low**	-	7	66	29	
	High	-	0	12	18	
Immunoscore (84)						0.194
	0-1	-	2	25	19	
	2-3	-	0	15	10	
	4	-	2	8	3	
Systemic environment						
mGPS (132)						0.415
	0	-	5	39	22	
	1	-	1	24	15	
	2	-	1	15	10	
NLR (89)						0.229
	≤5	-	3	42	21	
	>5	-	1	11	11	

mGPS – modified Glasgow Prognostic Score, NLR – neutrophil:lymphocyte ratio

Left colon *N*=100		**T1**	**T2**	**T3**	**T3**	
Tumour microenvironment		***N*=2**	**N=5**	***N*=67**	***N*=26**	***P***
Necrosis (93)						0.006
	Absent	2	5	34	9	
	Present	0	0	28	15	
Invasive margin						0.020
(99)	**Expansile**	1	4	42	8	
	Infiltrative	1	1	25	17	
Tumour budding						<0.001
(90)	**Low**	2	4	50	5	
	High	0	1	12	16	
Klintrup-						0.354
Mäkinen grade	**Strong**	1	3	25	8	
(98)	**Weak**	1	2	42	16	
Tumour stroma						0.224
percentage (100)	**Low**	2	3	55	17	
	High	0	2	12	9	
Immunoscore (67)						0.196
	0-1	1	1	18	11	
	2-3	1	1	21	5	
	4	0	1	6	1	
Systemic environment						
mGPS (99)						0.309
	0	2	2	46	13	
	1	0	3	15	10	
	2	0	0	6	2	
NLR (59)						0.555
	≤5	1	5	33	10	
	>5	1	0	5	4	

mGPS – modified Glasgow Prognostic Score, NLR – neutrophil:lymphocyte ratio

Rectum *N*=99		**T1**	**T2**	**T3**	**T3**	
Tumour microenvironment		***N*=6**	**N=13**	***N*=63**	***N*=17**	***P***
Necrosis (85)						0.126
	Absent	5	10	30	8	
	Present	1	2	24	5	
Invasive margin						0.001
(92)	**Expansile**	6	10	33	5	
	Infiltrative	0	2	25	11	
Tumour budding						0.962
(89)	**Low**	3	8	43	7	
	High	2	4	17	5	
Klintrup-						<0.001
Mäkinen grade	**Strong**	4	10	14	1	
(89)	**Weak**	2	2	43	13	
Tumour stroma						0.324
percentage (99)	**Low**	5	9	47	10	
	High	1	4	16	7	
Immunoscore (75)						0.096
	0-1	1	5	25	7	
	2-3	1	6	18	2	
	4	3	1	3	3	
Systemic environment						
mGPS (99)						0.266
	0	4	9	42	10	
	1	2	4	12	4	
	2	0	0	9	3	
NLR (77)						0.146
	≤5	5	9	40	8	
	>5	0	2	9	4	

mGPS – modified Glasgow Prognostic Score, NLR – neutrophil:lymphocyte ratio