Signal transduction and activator of transcription-3 (STAT3) in patients with colorectal cancer: associations with the phenotypic features of the tumour and host

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Translational relevance

The presence of a conspicuous local inflammatory reaction is associated with improved survival of patients with colorectal cancer independent of stage, whereas the presence of elevated systemic inflammatory responses, as measured by acute phase proteins, is associated with decreased survival. One potential candidate pathway linking these responses is the Janus kinase/ signal transduction and activator of transcriprition-3 (JAK/STAT3) pathway, with increasing evidence that this is a potential therapeutic target in cancer. In the present study, increased expression and nuclear localisation of STAT3 was associated with aberrant local and systemic inflammatory responses in patients undergoing resection of stage I-III colorectal cancer, and was associated with poorer survival. In addition to suggesting a role for JAK/STAT3 inhibitors in restoring host anti-tumour immune responses, the results of the present study further support the rationale for stratifying patients by host inflammatory responses in future trials of such agents.

Abstract

Purpose

In patients with colorectal cancer (CRC), a high-density local inflammatory infiltrate response is associated with improved survival, whereas elevated systemic inflammatory responses are associated with poor survival. One potential unifying mechanism is the IL-6/JAK/STAT3 pathway. The present study examines the relationship between tumour total STAT3 and phosphorylated $STAT3_{Tyr705}$ (pSTAT3) expression, host inflammatory responses and survival in patients undergoing resection of stage I-III CRC.

Experimental Design

Immunohistochemical assessment of STAT3/pSTAT3 expression was performed using a tissue microarray and tumour cell expression divided into tertiles using the weighted histoscore. The relationship between STAT3/pSTAT3 expression and local inflammatory ($CD3^+$, $CD8^+$, $CD45R0^+$, $FOXP3^+$ T-cell density and Klintrup-Mäkinen grade) and systemic inflammatory responses and cancer-specific survival were examined.

Results

196 patients were included in the analysis. Cytoplasmic and nuclear STAT3 expression strongly correlated ($r=0.363$, $P<0.001$); nuclear STAT3 and pSTAT3 expression weakly correlated ($r=0.130$, $P=0.068$). Cytoplasmic STAT3 was inversely associated with the density of CD3+ ($P=0.012$), CD8+ ($P=0.003$) and FOXP3+ T-lymphocytes ($P=0.002$) within the cancer cell nests and was associated with an elevated systemic inflammatory response as measured by modified Glasgow Prognostic Score (mGPS2: 19% vs. 4%, $P=0.004$).

The combination of nuclear STAT3/pSTAT3 stratified five-year survival from 81% to 62% (*P*=0.012), however was not associated with survival independent of venous invasion, tumour perforation or tumour budding.

Conclusion

In patients undergoing CRC resection, STAT3 expression was associated with adverse host inflammatory responses and reduced survival. Up-regulation of tumour STAT3 may be an important mechanism whereby the tumour deregulates local and systemic inflammatory responses.

INTRODUCTION

Colorectal cancer is the second most common cause of cancer death in Europe. Despite improved outcomes over the past decades, survival still remains poor, with 5-year survival of around 50% across all disease stages (1). Indeed, it is clear that the present TNM-based staging of colorectal cancer is suboptimal, with a need to identify characteristics pertaining to both the tumour and the host which may not only guide prognosis, but also the need for existing and novel adjuvant therapies.

One such characteristic is tumour-associated inflammation, which is now undisputed as impacting on both the development and progression of cancer (2). In patients with colorectal cancer, for example, host local and systemic inflammatory responses are important determinants of disease progression, and their assessment is now accepted as holding independent prognostic value. To date, over 100 studies have examined the role of the local inflammatory cell infiltrate in determining outcome in patients with colorectal cancer (3), with a consistent, stage-independent decrease in disease recurrence and increase in survival observed in association with the presence of a conspicuous inflammatory cell infiltrate (3, 4).

In contrast, an elevated systemic inflammatory response is associated with increased risk of recurrence and reduced survival across a number of cancers including colorectal cancer (5). Up-regulation of the systemic inflammatory response, as characterised by dysregulation of circulating pro-inflammatory cytokines, acute phase proteins such as C-reactive protein (CRP) and albumin (6), and myeloid cells (7, 8), propagates a systemic environment which favours tumour growth and metastasis (9). Furthermore, routine assessment of the systemic inflammatory response utilising routinely available biomarkers,

such as acute phase proteins and components of the differential white cell count, informs prognosis complimentary to current TNM-based staging (8, 10).

One potential mechanism linking the local and systemic inflammatory responses is activation of the Janus-activated kinase/ signal transduction and activator of transcription-3 (JAK/STAT3) pathway by interleukin-6 (IL-6). Circulating IL-6 is commonly elevated in a number of cancers, including colorectal cancer (11-13), and is the predominant stimulus for the hepatic synthesis of acute phase proteins, including CRP (6). Cancer-associated fibroblasts and inflammatory cells contribute to high levels of IL-6 within the tumour microenvironment (14, 15), with subsequent tumour cell activation of the soluble IL-6 receptor/ glycoprotein 130 complex (16). Interleukin-6 trans-signalling regulates JAK activity within the tumour cell to promote phosphorylation of the tyrosine 705 residue of STAT3. Phosphorylated STAT3 (pSTAT3) translocates to the nucleus where it is a key transcription factor for numerous T_h2-type cytokines, including IL-6 (12, 14), which promote a pro-tumour, immunosuppressive environment and attenuate host anti-tumour immune responses (15, 17). Indeed, given its role in not only de-regulation of the host anti-tumour immune response, but also in orchestrating numerous pro-oncogenic processes (15, 18, 19), it is not surprising that STAT3 expression and activation has previously been associated with reduced survival in a number of gastrointestinal cancers, including colorectal cancer (20).

We hypothesise that the host systemic and local inflammatory responses in patients with colorectal cancer may be linked by STAT3. As such, the aim of the present study was to examine the relationship between tumour cell STAT3 expression, host local and systemic inflammatory responses and survival in a cohort of patients undergoing potentially curative, elective resection of stage I-III colorectal cancer.

MATERIALS AND METHODS

Patients who between 1997 and 2007 had undergone elective, potentially curative resection for stage I-III colorectal cancer in a single surgical unit in Glasgow Royal Infirmary, and who were included in a previously constructed tissue microarray (TMA) were included. Resection was considered curative on the basis of pre-operative computed tomography and intra-operative findings. Patients were excluded on the following criteria: emergency resection, resection with palliative intent, resection for IBD-associated colorectal cancer, known familial cancer syndrome, neoadjuvant therapy, underlying inflammatory condition, or death within 30 days of surgery. Local ethical approval was obtained from the West of Scotland Research Ethics Committee and tissue for analysis of STAT3 expression was obtained from the National Health Service Greater Glasgow & Clyde Tissue Biorepository

Patient demographics were collected prospectively. Tumours were staged using AJCC/UICC-TNM 5th edition, consistent with current Royal College of Pathologist guidelines (21). The presence of venous invasion was assessed routinely using elastica staining. Following surgery, patients were discussed at local colorectal cancer multidisciplinary meetings, where patients with stage III or high-risk stage II disease were considered for 5-fluorouracil-based chemotherapy according to treatment protocols at the time. Patients were followed up for a minimum of five years according to local guidelines at the time. Date and cause of death were crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 31st March 2014 that served as the censor date. Cancer-specific survival was measured from date of surgery until date of death from colorectal cancer.

Serum CRP, albumin and differential white cell count were measured within 30 days prior to surgery and recorded prospectively. Pre-operative systemic inflammatory responses were defined using the modified Glasgow Prognostic Score (mGPS), the neutrophil: lymphocyte ratio (NLR) and the neutrophil: platelet score (NPS). The mGPS was calculated as previously described (10); patients with CRP ≤10mg/L were allocated a score of 0, patients with CRP >10mg/L and albumin ≥35g/L were allocated a score of 1, and patients with CRP >10mg/L and albumin <35g/L were allocated a score of 2. On the basis of previously published thresholds, NLR>5 was considered elevated (7). The NPS was calculated as previously described (22); patients with a platelet count<400x10^9/L and neutrophil count<7. 5x10^9/L were allocated a score of 0, either a neutrophil count>7.5x10^9/L or platelet count>400x10^9/L a score of 1, and those with both an elevated neutrophil and platelet count a score of 2.

Assessment of tumour microenvironment

The tumour-associated stroma, the generalised local inflammatory cell infiltrate and tumour budding have previously been characterised in this cohort using full haematoxylin & eosin (H&E)-stained sections of the deepest point of invasion according to previously published methodology (23-26). The tumour-associated stroma was assessed using tumour stroma percentage (TSP) and graded as either low (≤50%) or high (>50%) (25). The local inflammatory cell infiltrate was assessed using the Klintrup-Mäkinen (KM) grade and graded as either low-grade (no increase or mild/patchy increase in inflammatory cells at invasive margin) or high-grade (prominent band-like inflammatory reaction or florid cup-like inflammatory reaction at invasive margin with destruction of cancer cell islands) (27). Tumour budding was examined using the 10-high powered field method as previously described Tumour budding was examined using the 10-high powered field method as

previously described (26). Budding was considered high grade if greater than 20 buds (tumour cells with less than five nuclei or single tumour cells) were identified per 10-high powered fields.

Immunohistochemistry for $CD3^+$ (mature), $CD8^+$ (cytotoxic), $CD45R0^+$ (memory) and $FOXP3^+$ (regulatory) T-lymphocytes was performed using formalin fixed paraffin embedded full sections of the deepest point of invasion as previously described (27). Cellular epitopes were identified using the following antibodies: CD3 (Vector Labs, code VP-RM01, 1:100 dilution), CD8 (DakoCytomation, code M7103, 1:100 dilution), CD45R0 (DakoCytomation, code M0742, 1:150 dilution) and FOXP3 (Abcam, code 20034, 1:200 dilution). T-lymphocyte density at the invasive margin or within the cancer cell nests was semi-quantitatively graded using a four-point scale (absent/ low/ moderate/ high). For the purposes of statistical analysis, density was subsequently graded as low (absent/ low) or high (moderate/ high). Investigators blinded to clinicopathological data and outcomes performed all assessments, with independent scoring of 10% of cases by two investigators to ensure consistency.

Assessment of STAT3 expression

Immunohistochemical assessment of tumour cell STAT3 activity was performed using a previously constructed colorectal cancer TMA consisting of four 0.6mm cores per patient (28). The TMA was constructed from formalin fixed paraffin embedded tissue blocks corresponding to the full sections utilised for assessment of the tumour microenvironment. In addition to activation of STAT3 by phosphorylation of the tyrosine 705 residue by IL-6/JAK activation, activation may also occur through phosphorylation of the serine 727 residue in response to MAPK activation (29). As the present study hypothesised an association between

the systemic and local inflammatory response via IL-6/JAK/STAT3 activation, only total STAT3 expression and phosphorylated STAT3$_{Tyr705}$ (pSTAT3) expression was measured. Sections were dewaxed in xylene before being rehydrated using graded alcohols. Antigen retrieval was performed using a citrate buffer at 96^{o}C for 20 minutes for STAT3, and using a Tris-EDTA buffer at high pressure in a microwave for 5 minutes for pSTAT3. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 10 minutes before rinsing in water. Casein and 5% horse serum in TBS were applied for 20 minutes at room temperature as a blocking agent for STAT3 and pSTAT3 respectively. Sections were then incubated overnight at 4^{o}C with the primary antibody (STAT3: product code 9132, Cell Signaling Technologies; pSTAT3: product code 9131, Cell Signaling Technologies) at a concentration of 1:100 and 1:50 for STAT3 and pSTAT3 respectively before washing in TBS for ten minutes. Envision (Dako) was then added to the sections for 30 minutes at room temperature before washing in TBS for ten minutes. DAB substrate was added for five minutes until colour developed before washing in running water for ten minutes. Slides were counterstained in haematoxylin for 60 seconds and blued with Scotts' tap water before dehydration through graded alcohols. Cover slips were applied using distrene, plasticizer, xylene.

Sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at x20 magnification and visualization was carried out using Slidepath Digital Image Hub (Slidepath, Leica Biosystems, Milton Keynes, UK). Assessment of STAT3 and pSTAT3 expression within the cancer cell cytoplasm and nucleus was performed at x20 magnification by a single examiner (J.H.P) blinded to clinical using the weighted histoscore (30). To ensure reproducibility of scoring, 15% of tumours were co-scored by a second investigator (J.C.); the intraclass correlation coefficient was 0.826 and 0.837 respectively. For the purposes of the present study, cytoplasmic STAT3 expression was

considered representative of total STAT3 expression whereas nuclear STAT3 and pSTAT3 expression were considered representative of STAT3 transcriptional activity.

Assessment of mismatch repair status

Mismatch repair (MMR) protein deficiency was determined using the TMA utilised for STAT3 assessment. Sections were stained for MLH1, MSH6, MSH2 and PMS2 (product codes: M3640, M3646, M3639 and M3647, respectively; Dako UK Ltd, Cambridgeshire, UK) as described previously (31). Mismatch repair status was determined according to UK NEQAS guidelines (32), using appendix and normal colon as positive controls and intra-tumoural lymphocytes as internal positive controls. Tumours were considered MMR competent if tumour cell nuclear expression was positive with positive immune cell expression, and MMR deficient if tumour nuclear expression was absent with normal immune cell expression.

Statistical analysis

For the purpose of statistical analysis, patients were divided into tertiles (low/ moderate/ high) on the basis of cytoplasmic and nuclear STAT3 and pSTAT3 expression as measured by h-score. The relationship between clinicopathological characteristics and cytoplasmic and nuclear STAT3 expression was examined using the Chi-square test for linear trend. The relationship between STAT3 expression and five-year cancer-specific survival was examined using Kaplan-Meier log-rank analysis and displayed as percentage surviving (standard error). The relationship between STAT3 expression, clinicopathological characteristics and cancer-specific survival was examined using Cox proportional hazards regression; variables with a $P \leq 0.1$ on univariate analysis were entered into a multivariate model using a backwards conditional model to calculate hazard ratios (HR) and 95%

confidence intervals (CI). Given the number of comparisons performed, a *P*-value ≤0.01 was considered statistically significant for Chi-square analysis, with a *P*-value ≤0.05 considered statistically significant for survival analysis. All analyses were performed using SPSS version 22.0 (IBM SPSS).

RESULTS

A total of 196 patients who underwent elective, potentially curative resection of stage I-III colorectal cancer were included. Clinicopathological characteristics are displayed in Table 1. Almost two thirds of patients were older than 65 at time of surgery and 52% were male. Pathological assessment confirmed Stage I disease in 16 patients (8%), stage II disease in 94 patients (48%) and stage III disease in 86 patients (44%). Fifty-four patients (28%) received adjuvant therapy; 1 patient with stage I disease, 14 patients with stage II disease and 39 patients with stage III disease received adjuvant therapy. Mismatch repair deficiency was identified in 27 patients (14%).

Expression of STAT3 was observed in both the cytoplasm and nucleus, whereas pSTAT3 expression was only observed in the nucleus. The h-score range for cytoplasmic and nuclear STAT3 expression ranged from 0-168 and from 0-130 respectively. Nuclear pSTAT3 h-score for the cohort ranged from 5-205. Cytoplasmic expression of STAT3 was associated with nuclear expression of STAT3 (Spearman's r=0.363, P<0.001) but not pSTAT3 (r=0.111, P=0.121). Nuclear STAT3 expression was not significantly associated with nuclear pSTAT3 expression (r=0.130, P=0.068). Normal, non-cancer epithelium expression of STAT3 was available for 10 patients. Although this precluded meaningful statistical analysis, it was of interest that 7 patients showed similar or higher expression of cytoplasmic STAT3, nuclear STAT3 and nuclear pSTAT3 in normal tissue compared to cancer tissue. The remaining three patients showed heterogeneous expression of each of the studied markers.

The relationship between STAT3 and pSTAT3 expression tertiles and clinicopathological characteristics is displayed in Table 1. Cytoplasmic expression of STAT3

was not associated with any clinicopathological characteristics. Although failing to reach statistical significance ($P \leq 0.01$), nuclear STAT3 expression showed an inverse association with use of adjuvant chemotherapy ($P=0.038$), whereas pSTAT3 expression was associated with younger age were younger ($P=0.026$) and an increased prevalence of lymph node positive disease (low pSTAT3 expression – 35% vs. high pSTAT3 expression – 52%, $P=0.039$).

The relationship between STAT3 and pSTAT3 expression and components of the tumour microenvironment is displayed in Table 2. Cytoplasmic STAT3 expression was inversely associated with the cancer cell nest density of $CD8^+$ and $FOXP3^+$ (both $P<0.01$) T-lymphocytes and showed a trend towards a similar relationship with $CD3^+$ density ($P=0.012$) but was not associated with TSP, tumour budding or the local inflammatory cell density at the invasive margin as measured by Klintrup-Mäkinen grade or T-lymphocyte density. Nuclear expression of STAT3 showed no statistically significant association with characteristics of the tumour microenvironment, however a lower density of $CD8^+$ ($P=0.039$) and $CD3^+$ (P=0.055) T-lymphocytes was identified in patients with nuclear STAT3 expression. There were no statistically significant associations between nuclear pSTAT3 expression and tumour microenvironment characteristics; patients with high nuclear pSTAT3 expression however were observed to have a lower density of $CD45R0^+$ T-lymphocytes ($P=0.037$) and more frequent high-grade tumour budding ($P=0.022$).

When analysis was restricted to patients with MMR competent colorectal cancer (Supplementary Table 1), the observed relationship between cytoplasmic STAT3 and cancer cell nest density of $CD3^+$ ($P=0.061$) $CD8^+$ ($P<0.05$) and $FOXP3^+$ ($P<0.01$) T-lymphocytes remained. Nuclear STAT3 was no longer associated with $CD8^+$ density within cancer cell nests but was associated with $CD3^+$ density within the invasive margin ($P<0.05$). Nuclear

pSTAT3 expression again showed a non-significant trend towards low cancer cell nest density of $CD45R0^+$ T-lymphocytes. Although the small number of patients limited statistical power, when analysis was restricted to patients with MMR deficient colorectal cancer, the relationship between cytoplasmic STAT3 expression and cancer cell nest density of $CD3^+$ ($P<0.05$) and $CD8^+$ ($P<0.01$) T-cells, and nuclear STAT3 expression and cancer cell nest density of $CD8^+$ T-cells ($P<0.05$) remained. Nuclear pSTAT3 expression, however, was not associated with T-lymphocyte density of patients with MMR deficient colorectal cancer.

The relationship between STAT3 and pSTAT3 expression and systemic inflammatory responses is displayed in Table 3. Cytoplasmic STAT3 expression was associated with the systemic inflammatory response as measured by mGPS; this was predominantly due to an increase in the number of patients with mGPS=2 (high expression – 19% vs. low expression 4%, $P=0.004$). Neither cytoplasmic nor nuclear STAT3 expression were associated with the systemic inflammatory response as measured by circulating platelets or components of the differential white cell count. Nuclear pSTAT3 expression was not associated with any measures of the systemic inflammatory response.

The median follow-up of survivors was 143 months (range 101-204) with 57 cancer-associated deaths and 64 non-cancer deaths. For the purposes of survival analysis, low and moderate expression of each marker was combined to form one group (low expression). The relationship between cytoplasmic STAT3, nuclear STAT3 and nuclear pSTAT3 and cancer-specific survival is displayed in Figure 1 and in Table 4. High nuclear STAT3 expression was associated with poorer cancer-specific survival ($P<0.05$). High expression of both cytoplasmic STAT3 expression and nuclear pSTAT3 expression showed a non-significant trend towards poorer survival ($P=0.068$ and $P=0.116$ respectively).

To examine the relationship between expression and activation of STAT3 and survival, the cumulative prognostic value of cytoplasmic STAT3, nuclear STAT3 and nuclear pSTAT3 was examined with respect to five-year cancer-specific survival (Table 4). Three models were examined: model 1 (cytoplasmic STAT3/ nuclear STAT3) stratified survival from 81% (low expression of both) to 63% (high expression of both) (P=0.022), model 2 (cytoplasmic STAT3/ nuclear pSTAT3) stratified survival from 81% to 54% (P=0.018), and model 3 (nuclear STAT3/ nuclear pSTAT3) stratified survival from 81% to 62% (P=0.012). When the three models were entered into a multivariate model using a backwards conditional method, only model 3 (nuclear STAT3/ nuclear pSTAT3) remained independently associated with cancer-specific survival (HR 1.63, 95%CI 1.14-2.34 P=0.008, Figure 1).

The relationship between this prognostic model and cancer-specific survival was examined on multivariate analysis. As the prognostic value of the Klintrup-Mäkinen grade has previously been shown to be similar to assessment of individual T-lymphocyte subsets (24), only Klintrup-Mäkinen grade was entered into the multivariable model. On multivariate survival analysis (Table 5), combined nuclear STAT3/ pSTAT3 expression was not associated with cancer-specific survival (P=0.220), whereas venous invasion (HR 2.89, P=0.001), tumour perforation (HR 8.30, P<0.01), NPS (HR 1.69, P<0.05) and tumour budding (HR 4.12 P<0.00) were all independently associated with survival. Low Klintrup-Mäkinen grade (HR 2.14, P=0.060) and elevated mGPS (HR 1.52, P=0.060) showed a trend towards poorer survival, however failed to reach statistical significance.

The prognostic value of combined nuclear STAT3/pSTAT3 expression as stratified by T stage and N stage was examined (Supplementary Figure 1). Nuclear STAT3/pSTAT3 expression was associated with reduced survival of patients with T1-2 colorectal cancer (P<0.001) but was not associated with survival of patients with T3-4 colorectal cancer

(*P*=0.192). Furthermore, nuclear STAT3/pSTAT3 expression stratified survival of patients with lymph node positive (*P*=0.001) but not lymph node negative disease (*P*=0.516).

DISCUSSION

In the present study of patients undergoing elective, potentially curative colorectal cancer resection, STAT3 was not associated with clinicopathological characteristics of the tumour but was associated with adverse host inflammatory responses. In particular, increased tumour cell STAT3 expression was associated with down-regulation of the local inflammatory cell infiltrate.

Although in keeping with previous clinical studies of colorectal and pancreatic adenocarcinoma (33, 34), the present study is to our knowledge the first to examine the relationship between tumour STAT3 expression and the density of the local adaptive immune infiltrate as evidenced by T-lymphocytes in the clinical context of patients with gastrointestinal cancer. Whereas previous studies found a decrease in the density of the generalised inflammatory cell infiltrate or tumour-infiltrating lymphocytes using H&E-based assessments (33, 34), the present study utilised immunohistochemistry and found a decrease in the density of tumour-associated T-lymphocyte populations. Indeed, this would suggest a direct effect of STAT3 activation on adaptive, T-lymphocyte-mediated anti-tumour immunity. Furthermore, the relationship between STAT3 expression and the local inflammatory cell infiltrate would appear to be independent of MMR status.

Although assessment of cytoplasmic STAT3 expression was significantly associated with the density of T-lymphocytes, it was of interest that the K-M grade, an assessment of the generalised inflammatory cell infiltrate, did not differ with STAT3 expression. This may reflect the ability of STAT3 to simultaneously suppress anti-tumour immune responses whilst promoting pro-tumour immunity (17, 35). Whereas anti-tumour, adaptive, T_h1-polarised immune responses are down-regulated (36, 37), STAT3-dependent transcription and release

of T_h2-type cytokines favours recruitment of tumour-promoting tumour-associated macrophages and myeloid-derived cells (17). Furthermore, STAT3 activation may additionally favour the differentiation of naïve T-lymphocytes into tumour-promoting lymphocytic subsets (17). Consistent with such a hypothesis, Morikawa and colleagues found that although intratumoural lymphocyte density decreased, the density of the peritumoural inflammatory cell infiltrate increased with increasing STAT3 activity in a cohort of patients with stage I-IV colorectal cancer (33). Furthermore, it has been shown in some tumours, such as ependymomas, that STAT3 immunosuppression is mediated by up-regulation of myeloid-derived cell activity, with a subsequent deleterious effect on T-lymphocytic, anti-tumour activity (38). As such, future studies of STAT3 activation in patients with gastrointestinal cancers should also consider the nature and density of local innate immune responses.

Of interest, pSTAT3 expression was associated with high-grade tumour budding. The presence of tumour buds is a phenotypic characteristic of epithelial-mesenchymal transitioning (EMT), a vital step in tumour cell dissemination which requires immune cell evasion (39). The present results may suggest that STAT3 activation is one mechanism by which tumour buds evade host anti-tumour immune responses. However, it is also recognised that STAT3 activation promotes tumour cell stemness and is an upstream activator of EMT (40, 41), which may explain the present associations.

Although failing to reach statistical significance, the density of tumour-associated stroma, as measured by TSP, appeared to be associated with pSTAT3 expression. Given that an increase in TSP primarily reflects an increased population of cancer-associated fibroblasts within the tumour microenvironment, this would further support the importance of IL-6 secretion by fibroblasts in the activation of the JAK/STAT3 pathway in tumour cells (14, 15).

Indeed, the present results suggest that the JAK/STAT3 pathway may be an important mechanism by which the tumour influences the composition of the tumour microenvironment and deregulates host anti-tumour immune responses.

The present study found that increased tumour STAT3 expression was associated with elevated systemic inflammatory responses as measured using the mGPS, a cumulative score based on serum CRP and albumin concentrations. Such routinely measured biomarkers of the systemic inflammatory response represent only "the tip of a far larger iceberg" of cancer-associated systemic inflammation, whereby circulating cytokines, growth factors and myeloid-derived cells promote cancer progression and dissemination (9). One such cytokine, IL-6, is commonly elevated in colorectal cancer (11, 13), and is the main determinant of hepatic synthesis of CRP and responsible for the acute phase reduction in hepatic albumin synthesis (6). Given the importance of IL-6 as both an activator of the JAK/STAT3 pathway and as an end product of its activation, the present results are perhaps not surprising, and suggest that STAT3 activation may play a role in the systemic inflammatory response in colorectal cancer.

However, although STAT3 expression was associated with an elevated mGPS, it was not associated with components of the differential white cell count. This is in keeping with previous work from Guthrie and colleagues, whereby serum IL-6 concentration correlated strongly with the mGPS but not the NLR in patients with colorectal cancer (13). However, other groups have found contradictory results, with a positive association between serum IL-6 concentrations and the NLR in patients with colorectal cancer. This disparity may be explained by differences in the groups studied; whereas the patients in the present study and that of Guthrie and colleagues were undergoing potentially curative resection of stage I-III colorectal cancer, the groups studied by Kantola and Chen included patients with stage I-IV

colorectal cancer at varying stages of treatment. Taken together, it would appear that, at least in patients with non-metastatic colorectal cancer, the effects of the IL-6/JAK/STAT3 pathway on the cancer-associated systemic inflammatory response may not be entirely modulated by an effect on circulating innate and adaptive immune cells.

Of interest, only total cytoplasmic STAT3 expression was associated with the systemic inflammatory response as measured by mGPS. The reason for this is not clear, however may represent the dynamic nature of JAK/STAT3 activation and translocation. Although activation of the IL-6 receptor leads to rapid accumulation of STAT3, mechanistic studies have shown that less than 30% of total cytoplasmic STAT3 translocates to the nucleus on cytokine stimulation (42). Furthermore, STAT3 also exhibits transcription-independent activity within the cytoplasm without nuclear translocation (42, 43). Another plausible hypothesis is that rather than being directly causative, the presently observed associations between the mGPS and tumour cell STAT3 expression may represent separate down-stream events of a common precursor, such as elevated systemic IL-6 concentrations. Finally, given the lack of a consistent relationship across different measures of the systemic inflammatory response, the present results may simply represent a Type-I statistical error. Indeed, rather than the tumour itself, other end organs, such as liver or skeletal muscle, may be the predominant drivers of the systemic inflammatory response in such patients (44). As such, the present observations should be regarded as hypothesis-generating, and remain to be further investigated by mechanistic and clinical studies.

Consistent with previous reports in patients with gastrointestinal cancers (20), the present study found that increased tumour cell STAT3 expression and activity was associated with reduced survival. The pleiotropic nature of STAT3 activation is reflected in the fact that combined assessment of total nuclear STAT3 and pSTAT3 held greater prognostic value than

either measure alone. Whereas the present study investigated IL-6/JAK-mediated activation of STAT3 by phosphorylation of tyrosine residue 705, mitogen-activated protein kinase-dependent activation results in phosphorylation of the serine 727 residue, with differing results on transcriptional activity (29). Furthermore, STAT3 may also undergo nuclear import without phosphorylation (45). In addition to its role in mediating host immune responses, STAT3 activation plays an integral role in many key tumour cell pathways, including proliferation, epithelial-mesenchymal transitioning and promotion of cancer cell stemness (40). Indeed, the heterogeneity of upstream activation of STAT3 is reflected in the present results, whereby 74 patients showed discordant expression of nuclear STAT3 and pSTAT3. As such, further investigation of these other upstream pathways, and their relationship to the present results is required. Furthermore, rather than targeting upstream activation of STAT3, future therapeutic strategies may benefit from targeting STAT3 itself and its subsequent activation.

Nuclear STAT3/pSTAT3 expression showed differential prognostic value according to T stage and N stage. Although potentially reflecting the limited statistical power of the present study for subgroup analysis, the present results could suggest that STAT3 activation and expression may have a differential effect dependent on disease stage and invasiveness. In addition, assessment of the local and systemic environment held greater prognostic value than nuclear STAT3/pSTAT3 expression. Rather than being defined by one mechanism such as the JAK/STAT3 pathway, characteristics within the tumour microenvironment and the systemic inflammatory response are likely to be multifactorial in origin. Therefore, it might be anticipated that such phenotypic characteristics would be of greater prognostic value than a single signal transduction pathway. Indeed, it would be of considerable interest to examine and compare other signal transduction pathways associated with inflammation, such as the NF-κB pathway (46, 47), in future studies.

The present study provides further clinical evidence of the role of the IL-6/JAK/STAT3 pathway in the amelioration of host anti-tumour immune responses, and raises two interesting points that remain to be investigated. Firstly, it would suggest a role for inhibitors of the IL-6/JAK/STAT3 pathway in restoring anti-tumour immune responses in patients with colorectal cancer (48, 49). Secondly, it would support the hypothesis that routine markers of the systemic inflammatory response, and in particular the mGPS, may aid in the identification and selection of patients likely to benefit from such targeted therapies (50). In keeping with such a scheme, one recent clinical trial of a JAK inhibitor in patients with metastatic pancreatic cancer found an increase in overall survival only in those patients with an elevated CRP or mGPS (51). Therefore, it is clear that markers of the host inflammatory response should be incorporated into future studies of agents targeting the IL-6/JAK/STAT3 pathway in cancer.

Given the increasing appreciation of distinct molecular subtypes of colorectal cancer (52), the results of the present study are perhaps limited by the lack of molecular characterisation of the tumours studied. Although not associated with MMR status in the present cohort, the relationship between STAT3 and other characteristics, such as *KRAS* and *BRAF* status, would be of interest. However, a previous comprehensive study by Morikawa and colleagues found no association between STAT3, a number of molecular characteristics and survival in a cohort of over 700 patients (33). Furthermore, it has also been suggested that STAT3 may have a role in not only induction of *KRAS* mutated tumours (53), but also in conferring chemoresistance in patients with *KRAS* wild-type tumours (54). Indeed, this would suggest that STAT3 is independent of such characteristics. A further limitation is the relatively small sample size, precluding meaningful subgroup analysis. Analysis was restricted to a previously constructed TMA, and only patients who had complete staining for both STAT3 and pSTAT3 were included. However, post-hoc power calculation shows that

the present study has adequate power to examine the relationship between STAT3 and the local and systemic environment. For example, post-hoc analysis suggests that the present study holds 84% power to determine a difference in cancer cell nest $CD8^+$ T-lymphocyte density between those with low and high cytoplasmic STAT3 expression.

In conclusion, the results of the present study suggest a relationship between tumour cell STAT3 expression and the host inflammatory response, and may be one potential mechanism whereby the tumour promotes a local and systemic environment amenable to tumour growth and dissemination. Further studies are required to confirm such a relationship, and whether therapeutic targeting of the IL-6/JAK/STAT3 may be utilised in the treatment of patients with colorectal cancer and elevated systemic inflammatory responses.

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Table 1 The relationship between tumour cell STAT3 and pSTAT3 expression and clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

			Cytoplasmic STAT3 h-score				Nuclear STAT3 h-score				Nuclear pSTAT3 h-score			
		All *n*-196 (%)	Low (0-20) *n*=76 (%)	Mod (21-65) *n*=56 (%)	High (66-168) *n*=64 (%)	*P*	Low (0-15) *n*=75 (%)	Mod (16-30) *n*=66 (%)	High (30-130) *n*=55 (%)	*P*	Low (5-80) *n*= 72 (%)	Mod (81-105) *n*=61 (%)	High (106-205) *n*=63 (%)	*P*
Host characteristics														
Age						0.571				0.199				0.026
	<65	72 (37)	23 (30)	22 (39)	27 (42)		29 (39)	25 (37)	18 (33)		19 (26)	26 (43)	27 (43)	
	65-74	61 (31)	29 (38)	18 (32)	14 (22)		25 (33)	22 (33)	14 (26)		23 (32)	19 (31)	19 (30)	
	>75	63 (32)	24 (32)	16 (29)	23 (36)		21 (28)	10 (29)	23 (41)		30 (42)	16 (26)	17 (27)	
Sex						0.833				0.647				0.906
	Female	94 (48)	40 (53)	21 (37)	33 (52)		35 (47)	31 (47)	28 (51)		38 (53)	23 (38)	33 (52)	
	Male	102 (52)	36 (47)	35 (63)	31 (48)		40 (53)	35 (53)	27 (49)		34 (47)	38 (62)	30 (48)	
Adjuvant therapy						0.532				0.038				0.389
	No	142 (72)	55 (72)	44 (79)	43 (67)		48 (64)	50 (76)	44 (80)		56 (78)	41 (67)	45 (71)	
	Yes	54 (28)	21 (28)	12 (21)	21 (33)		27 (36)	16 (24)	11 (20)		16 (22)	20 (33)	18 (29)	
Tumour characteristics														
Tumour location						0.375				0.242				0.860
	Colon	130 (66)	48 (63)	37 (66)	45 (70)		47 (63)	43 (65)	40 (73)		49 (68)	37 (61)	44 (70)	
	Rectum	66 (34)	28 (37)	19 (34)	19 (30)		28 (37)	23 (35)	15 (27)		23 (32)	24 (39)	19 (30)	
T stage						0.288				0.480				0.694
	1-2	25 (13)	10 (13)	9 (16)	6 (9)		10 (13)	10 (15)	5 (9)		10 (14)	8 (13)	7 (11)	
	3	121 (61)	49 (65)	34 (61)	38 (59)		46 (61)	41 (62)	34 (62)		43 (60)	39 (64)	39 (62)	
	4	50 (26)	17 (22)	13 (23)	20 (31)		19 (25)	15 (23)	16 (29)		19 (26)	14 (23)	17 (27)	
N stage						0.183				0.470				0.039
	0	110 (56)	47 (61)	30 (53)	33 (51)		34 (45)	46 (70)	30 (54)		47 (65)	33 (54)	30 (48)	
	1	68 (35)	24 (32)	20 (36)	24 (38)		34 (45)	16 (24)	18 (33)		21 (29)	21 (34)	26 (41)	
	2	18 (9)	5 (7)	6 (11)	7 (11)		7 (10)	4 (6)	7 (13)		4 (6)	7 (12)	7 (11)	
TNM stage						0.211				0.494				0.051
	I	16 (8)	6 (8)	7 (13)	3 (5)		6 (8)	8 (12)	2 (4)		7 (10)	5 (8)	4 (6)	
	II	94 (48)	41 (54)	23 (41)	30 (47)		28 (37)	38 (58)	28 (50)		40 (55)	28 (46)	26 (41)	
	III	86 (44)	29 (38)	26 (46)	31 (48)		41 (55)	20 (30)	25 (46)		25 (35)	28 (46)	33 (52)	
Tumour differentiation						0.530				0.108				0.174
	Mod/well	174 (89)	69 (91)	49 (87)	56 (87)		63 (84)	60 (91)	51 (93)		60 (83)	57 (93)	57 (91)	
	Poor	22 (11)	7 (9)	7 (13)	8 (13)		12 (16)	6 (9)	4 (7)		12 (17)	4 (7)	6 (10)	
Venous invasion						0.465				0.337				0.271
	No	129 (66)	51 (67)	39 (70)	39 (61)		46 (61)	45 (68)	38 (69)		51 (71)	39 (64)	39 (62)	
	Yes	67 (34)	25 (33)	17 (30)	25 (39)		29 (39)	21 (32)	17 (31)		21 (29)	22 (36)	24 (38)	
Margin involvement						0.856				0.649				0.562
	No	187 (95)	72 (95)	54 (96)	61 (95)		70 (93)	65 (98)	52 (94)		70 (97)	57 (93)	60 (95)	
	Yes	9 (5)	4 (5)	2 (4)	3 (5)		5 (7)	1 (2)	3 (6)		2 (3)	4 (7)	3 (5)	
Peritoneal involvement						0.423				0.794				0.787
	No	144 (3)	57 (75)	43 (77)	44 (69)		55 (73)	50 (76)	39 (71)		53 (74)	46 (75)	45 (71)	
	Yes	52 (27)	19 (25)	13 (23)	20 (31)		20 (27)	16 (24)	16 (29)		19 (26)	15 (25)	18 (29)	
Tumour perforation						0.652				0.799				0.087
	No	192 (98)	74 (97)	55 (98)	63 (98)		73 (97)	66 (100)	53 (96)		69 (96)	60 (98)	63 (100)	
	Yes	4 (2)	2 (3)	1 (2)	1 (2)		2 (3)	0 (0)	2 (4)		3 (4)	1 (2)	0 (0)	
Mismatch repair status						0.741				0.406				0.491
	Competent	169 (86)	65 (85)	48 (86)	56 (87)		62 (83)	59 (89)	48 (87)		61 (85)	52 (85)	56 (89)	
	Deficient	27 (14)	11 (15)	8 (14)	8 (13)		13 (17)	7 (11)	7 (13)		11 (15)	9 (15)	7 (11)	

Table 2 The relationship between tumour cell STAT3 and pSTAT3 expression and tumour microenvironment of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		Cytoplasmic STAT3 h-score				Nuclear STAT3 h-score				Nuclear pSTAT3 h-score			
		Low (0-20) *n*=76 (%)	**Mod (21-65) *n*=56 (%)**	**High (66-168) *n*=64 (%)**	***P***	**Low (0-15) *n*=75 (%)**	**Mod (16-30) *n*=66 (%)**	**High (30-130) *n*=55 (%)**	***P***	**Low (5-80) *n*= 72 (%)**	**Mod (81-105) *n*=61 (%)**	**High (106-205) *n*=63 (%)**	***P***
Klintrup-Makinen grade					0.208				0.657				0.582
	Weak	28 (37)	20 (36)	17 (27)		25 (33)	24 (36)	16 (29)		26 (36)	19 (31)	20 (32)	
	Strong	48 (63)	36 (64)	47 (73)		50 (67)	42 (64)	30 (71)		46 (64)	42 (69)	43 (68)	
Tumour stroma					0.241				0.794				0.090
percentage (195)	**Low**	59 (78)	43 (78)	44 (69)		56 (75)	51 (77)	39 (72)		55 (78)	50 (82)	40 (64)	
	High	17 (22)	12 (22)	20 (31)		19 (25)	15 (23)	15 (28)		16 (22)	11 (18)	22 (36)	
Tumour budding (182)					0.834				0.822				0.022
	Low	45 (64)	40 (74)	38 (65)		45 (63)	44 (76)	34 (64)		49 (75)	41 (71)	33 (56)	
	High	25 (36)	14 (26)	20 (35)		26 (37)	14 (24)	19 (36)		16 (25)	17 (29)	26 (44)	
CD3$^+$ margin density					0.332				0.055				0.648
(184)	**Low**	36 (49)	30 (60)	35 (57)		37 (51)	28 (46)	36 (71)		34 (54)	31 (52)	36 (58)	
	High	37 (51)	20 (40)	26 (43)		35 (49)	33 (54)	15 (29)		29 (46)	28 (48)	26 (42)	
CD3$^+$ cancer cell nest					0.012				0.262				0.150
density (192)	**Low**	38 (51)	42 (79)	45 (70)		47 (64)	38 (59)	40 (74)		43 (62)	35 (58)	47 (75)	
	High	37 (49)	11 (21)	19 (30)		27 (37)	26 (41)	14 (26)		26 (38)	25 (42)	16 (25)	
CD8$^+$ margin density					0.630				0.177				0.806
(184)	**Low**	41 (59)	34 (64)	33 (54)		38 (53)	37 (61)	33 (65)		38 (59)	33 (55)	37 (62)	
	High	29 (41)	19 (36)	28 (46)		34 (47)	25 (39)	18 (35)		26 (41)	27 (45)	23 (38)	
CD8$^+$ cancer cell nest					0.003				0.039				0.730
density (190)	**Low**	41 (57)	45 (83)	51 (80)		47 (63)	47 (76)	43 (80)		50 (72)	41 (68)	46 (75)	
	High	31 (43)	9 (17)	13 (20)		27 (37)	15 (24)	11 (20)		19 (28)	19 (32)	15 (25)	
CD45R0$^+$ margin density					0.960				0.089				0.282
(186)	**Low**	38 (52)	27 (51)	31 (52)		33 (47)	31 (48)	32 (63)		32 (48)	29 (50)	37 (57)	
	High	35 (48)	26 (49)	29 (48)		38 (54)	33 (52)	19 (37)		38 (52)	29 (50)	26 (43)	
CD45R0$^+$ cancer cell					0.408				0.268				0.037
density (192)	**Low**	48 (64)	43 (80)	44 (70)		48 (67)	46 (70)	41 (76)		46 (64)	39 (67)	50 (81)	
	High	27 (36)	11 (20)	19 (30)		24 (33)	20 (30)	13 (24)		26 (36)	19 (33)	12 (19)	
FOXP3$^+$ margin density					0.180				0.373				0.466
(186)	**Low**	37 (51)	29 (56)	38 (62)		39 (53)	34 (54)	31 (62)		40 (60)	32 (54)	32 (53)	
	High	36 (49)	23 (44)	23 (38)		34 (47)	29 (46)	19 (38)		27 (40)	27 (46)	28 (47)	
FOXP3$^+$ cancer cell nest					0.002				0.807				0.181
density (188)	**Low**	26 (36)	26 (49)	39 (63)		39 (53)	25 (39)	27 (53)		38 (56)	26 (44)	27 (44)	
	High	47 (64)	27 (51)	23 (37)		34 (47)	39 (61)	24 (47)		30 (44)	33 (56)	34 (56)	

Table 3 The relationship between tumour cell STAT3 and pSTAT3 expression and systemic inflammatory responses of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		Cytoplasmic STAT3 h-score				Nuclear STAT3 h-score				Nuclear pSTAT3 h-score			
		Low (0-20) *n*=76(%)	**Mod (21-65) *n*=56(%)**	**High (66-168) *n*=64(%)**	***P***	**Low (0-15) *n*=75(%)**	**Mod (16-30) *n*=66(%)**	**High (30-130) *n*=55(%)**	***P***	**Low (5-80) *n*= 72(%)**	**Mod (81-105) *n*=61(%)**	**High (106-205) *n*=63(%)**	***P***
Modified Glasgow Prognostic Score					0.004				0.244				0.651
	0	53 (70)	33 (59)	33 (51)		46 (61)	42 (64)	31 (56)		44 (61)	36 (59)	39 (62)	
	1	20 (26)	18 (32)	19 (30)		23 (31)	20 (30)	14 (26)		20 (28)	17 (28)	20 (32)	
	2	3 (4)	5 (9)	12 (19)		6 (8)	4 (6)	10 (18)		8 (11)	8 (13)	4 (6)	
Neutrophil count (195)					0.515				0.676				0.470
	≤7.5x10^9/L	67 (88)	47 (85)	54 (84)		63 (85)	60 (91)	45 (82)		60 (85)	52 (85)	56 (89)	
	>7.5x10^9/L	9 (12)	8 (15)	10 (16)		11 (15)	6 (9)	10 (18)		11 (16)	9 (15)	7 (11)	
Lymphocyte count (195)					0.942				0.174				0.209
	>4x10^9/L	76 (100)	54 (98)	64 (100)		74 (100)	66 (100)	54 (98)		71 (100)	61 (100)	62 (98)	
	≤4x10^9/L	0 (0)	1 (2)	0 (0)		0 (0)	0 (0)	1 (2)		0 (0)	0 (0)	1 (2)	
Platelet count (176)					0.557				0.587				0.895
	≤400x10^9/L	58 (87)	44 (86)	48 (83)		55 (85)	49 (83)	46 (88)		57 (85)	44 (85)	49 (86)	
	>400x10^9/L	9 (13)	7 (14)	10 (17)		10 (15)	10 (17)	6 (12)		10 (15)	8 (15)	8 (14)	
Neutrophil:lymphocyte ratio (195)					0.350				0.131				0.352
	≤5	62 (82)	45 (82)	48 (75)		61 (82)	55 (83)	39 (71)		56 (79)	45 (74)	54 (86)	
	>5	14 (18)	10 (18)	16 (25)		13 (18)	11 (17)	16 (29)		15 (21)	16 (26)	9 (14)	
Neutrophil:platelet score (176)					0.441				0.831				0.602
	0	52 (78)	40 (78)	40 (69)		47 (72)	46 (78)	39 (75)		49 (73)	39 (75)	44 (77)	
	1	13 (19)	7 (14)	17 (29)		16 (25)	10 (17)	11 (21)		15 (22)	11 (21)	11 (19)	
	2	2 (3)	5 (8)	1 (2)		2 (3)	3 (5)	2 (4)		3 (5)	2 (4)	2 (4)	

Table 4 Relationship between tumour cell STAT3 and pSTAT3 expression and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

	N	**5-year CSS % (SE)**	**Univariate HR (95% CI)**	***P***	**Multivariate HR (95% CI)**	**P**
Cytoplasmic STAT3				0.072		-
Low-mod expression	132	81 (3)	-		-	
High expression	64	67 (6)	1.62 (0.96-2.65)		-	
Nuclear STAT3				0.018		-
Low-mod expression	141	78 (4)	-		-	
High expression	55	70 (6)	1.89 (1.12-3.22)		-	
Nuclear pSTAT3				0.119		-
Low-mod expression	133	80 (4)	-		-	
High expression	63	69 (6)	1.52 (0.90-2.57)		-	
Combined cytoplasmic STAT3/ nuclear STAT3 (Model 1)				0.009		0.221
Both low-mod	106	81 (4)				
One high	61	73 (6)	1.56 (1.20-2.17)		-	
Both high	29	63 (9)				
Combined cytoplasmic STAT3/ nuclear pSTAT3 (Model 2)				0.024		0.526
Both low-mod	95	80 (4)				
One high	75	79 (5)	1.50 (1.06-2.13)		-	
Both high	26	54 (10)				
Combined nuclear STAT3/ nuclear pSTAT3 (Model 3)				0.008		0.008
Both low-mod	100	81 (4)				
One high	74	74 (5)	1.63 (1.14-2.34)		1.63 (1.14-2.34)	
Both high	22	62 (11)				

Table 5 Relationship between combined tumour cell nuclear STAT3/ pSTAT3 expression, clinicopathological characteristics and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

	Cancer-specific survival			
Clinicopathological characteristics	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	1.00 (0.73-1.37)	0.986	-	-
Sex (Female/ male)	1.43 (0.84-2.44)	0.188	-	-
Adjuvant therapy (No/ yes)	1.43 (0.83-2.47)	0.196	-	-
Tumour site (Colon/ rectum)	0.99 (0.57-1.74)	0.983	-	-
TNM stage (I /II /III)	2.16 (1.35-3.48)	0.001	-	0.416
Tumour differentiation (Mod-well/ poor)	1.18 (0.51-2.75)	0.700	-	-
Venous invasion (No/ yes)	3.35 (1.97-5.70)	<0.001	2.89 (1.59-5.28)	0.001
Margin involvement (No/ yes)	2.82 (1.12-7.09)	0.028	-	0.612
Peritoneal involvement (No/ yes)	2.45 (1.45-4.13)	0.001	-	0.650
Tumour perforation (No/ yes)	4.34 (1.04-18.11)	0.044	8.30 (1.84-37.43)	0.006
Modified Glasgow Prognostic Score (0/ 1/ 2)	1.43 (0.99-2.08)	0.057	1.52 (0.98-2.35)	0.060
NPS (0/ 1/ 2)	1.72 (1.13-2.62)	0.012	1.69 (1.07-2.67)	0.025
NLR (<5/ >5)	1.13 (0.60-2.13)	0.715	-	-
Mismatch repair status (Competent/ deficient)	1.37 (0.69-2.71)	0.370	-	-
Klintrup-Makinen grade (High/ low)	2.33 (1.20-4.49)	0.012	2.14 (0.97-4.71)	0.060
Tumour stroma percentage (Low/ high)	2.52 (1.48-4.30)	0.001	-	0.180
Tumour budding (Low/ high)	3.92 (2.25-6.85)	<0.001	4.12 (2.20-7.71)	<0.001
Nuclear STAT3/ nuclear pSTAT3 (Both low-mod/ one high/ both high)	1.63 (1.14-2.34)	0.008	1.28 (0.86-1.89)	0.220

Figure 1 The relationship between tumour cell STAT3 expression and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer (Kaplan-Meier log-rank analysis): (a) cytoplasmic STAT3 expression (P=0.068), (b) nuclear STAT3 expression (P=0.012), (c) nuclear pSTAT3 expression (P=0.116), and (d) combined nuclear STAT3/pSTAT3 expression (P=0.012)

Supplementary Figure 1 The relationship between tumour cell STAT3 expression and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer (Kaplan-Meier log-rank analysis): (a) Stage T1-2 (P<0.001), (b) Stage T3-4 (P=0.192), (c) Node negative (P=0.516), and (d) Node positive (P=0.001).