The *in situ* local immune response, tumour senescence and proliferation in colorectal cancer

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**Background:** Immune cell infiltrates are important determinants of colorectal cancer (CRC) outcome. Their presence may be driven by tumour or host-specific factors. From previous studies in mice, senescence, a state of cell cycle arrest, may moderate tumour progression through upregulation of antitumour immune responses. The relationships between senescence and immune infiltrates have not previously been studied in humans. We explore whether a marker of senescence (p16ink4a) in combination with low level expression of a proliferation marker (ki-67) relate to T cell infiltrates in CRC, and whether p16ink4a, Ki-67 and immune infiltrates have similar prognostic value.

**Methods:** Immunostaining of p16ink4a and Ki-67 was performed within a CRC tissue microarray. Nuclear p16ink4a and Ki-67 were categorised as high/low. T-cell markers, CD3, CD45RO, CD8 and FOXP3 were scored separately as high/low grade in three areas of the tumour: the invasive margin (IM), tumour stroma and cancer cell nests (CCNs).

**Results:** Two hundred and thirty stage I–III cancers were studied. High nuclear p16ink4a was expressed in 63% and high proliferation (Ki-67 > 15%) in 61%. p16ink4a expression was associated with reduced CD45RO+ cells at the IM (P<0.05) and within the stroma (P<0.05) and reduced CD8+ cells at the IM (P<0.01). A low Ki-67 proliferative index was associated with reduced density of CD3+ cells in CCNs (P<0.01), reduced CD45RO+ cells at the IM (P<0.05) and within the CCNs (P<0.001), reduced FOXP3+ cells at the IM (P<0.001), within the stroma (P=0.001) and within CCNs (P<0.001) and reduced CD8+ cells at the IM (P<0.05) and within the CCNs (P<0.05). Tumours with both a low proliferative index and expression of p16ink4a demonstrated similar consistent relationships with reduced densities of T-cell infiltrates. On multivariate analysis, TNM stage (P<0.001), low CD3 cells at the IM (P=0.014), low CD8 cells at the IM (P=0.037), low proliferation (Ki-67; P=0.013) and low senescence (p16ink4a; P=0.002) were independently associated with poorer cancer survival.

**Conclusion:** Senescence, proliferation and immune cell infiltrates are independent prognostic factors in CRC. Although related to survival, p16ink4a-associated senescence is not associated with an upregulation of antitumour T-cell responses.

The role of immune cell infiltrates in determining outcome in colorectal cancer (CRC) is increasingly appreciated. Over 100 published studies report consistent relationships between improved cancer-specific survival and an increasing number, or density, of immune cells in and around colorectal tumours (Roxburgh and McMillan, 2012). The evidence is strongest for a generalised lymphocytic/inflammatory cell infiltrate at the invasive margin (IM), based on over 40 studies (Jass, 1987; Kilntrup et al, 2005; Roxburgh et al, 2009a,b; Richards et al, 2012a,b; Roxburgh and McMillan, 2012). Recently, several groups have attempted to...
characterise these immune reactions, most work focusing on T lymphocytes and their subsets (CD3+ , CD4+ , CD8+ , CD45RO+ and FOXP3+ ) and macrophages (CD68+ ; Naito et al., 1998; Pages et al., 2005; Galon et al., 2006; Roxburgh and McMillan, 2012). With the appreciation that density, type and location of intratumoural immune cells predict survival independent of stage (Galon et al., 2006), several validated scores of immune cell infiltrates have been developed including the immune score (based on C45RO+ and CD8+ ) and the Klintrup–Makinen score (Klintrup et al., 2005; Roxburgh et al., 2009a,b; Mlecnik et al., 2011); consequently, there are increasing calls for immune scoring as part of the routine prognostication in CRC (Galon et al., 2012).

To date, it is unclear whether intratumoural immune responses represent tumour- or host-specific phenomena. High-grade infiltrates are commoner in early-stage disease (low T stage, absence of lymphovascular invasion or nodal/distant metastases) degrading as tumours enlarge and disseminate (Roxburgh et al., 2009a,b; Mlecnik et al., 2011). Associations with molecular tumour characteristics have also been reported, namely microsatellite instability, intratumoural HLA expression, and CpG island methylation (Mlecnik et al., 2011). Microsatellite unstable tumours have an established clinical phenotype including right-sided location, moderate-well differentiation as well as the presence of a pronounced lymphocytic infiltrate (Michael-Robinson et al., 2001a,b). However, immune cell infiltrates are consistently reported to offer additional prognostic information, independent of stage, pathological characteristics and mismatch repair status, potentially reflecting the complex interplay between host immune responses and the tumour (Michael-Robinson et al., 2001a; Baker et al., 2007; Laghi et al., 2009; Sincrope et al., 2009; Deschoolmeester et al., 2010).

One potential tumour characteristic, that may in part account for immune cell infiltrates, is the presence of intratumoural cellular senescence and the ‘senescence-associated secretory phenotype’ (SASP). Cellular senescence, a state of stable cell cycle arrest, is associated inflammatory secretome drive their presence. Infiltrates in colorectal tumours is that cellular senescence and the ‘senescence-associated inflammatory secretome’ (for example, IL-6, IL-8, MMP1 and so on; Cairney et al., 2012). These scores are subsequently given a binary classification as either low-risk characteristics included tumour differentiation, serosal involvement, margin involvement, tumour perforation and venous invasion. The modified Glasgow Prognostic Score (mGPS), a measure of systemic inflammation based on serum C-reactive protein and albumin measured before surgery was also assessed (CRP < 10 mg l−1, albumin > 35 g dl−1 = mGPS 0; CRP > 10 mg l−1, albumin > 35 g dl−1 = mGPS 1; CRP > 10 mg l−1, albumin < 35 g dl−1 = mGPS 2; Roxburgh and McMillan, 2012).

Tumour necrosis was graded in this cohort, scored semiquantitatively by two observers as ‘absent’ (none), ‘local’ (< 10% of tumour area), ‘moderate’ (10–30%) or ‘extensive’ (> 30%) according to published methodology (Pollheimer et al., 2010; Richards et al., 2012a,b). An assessment of inflammatory cell infiltrate at the IM using H&E slides (the Klintrup–Makinen score) has also been performed in all cancers within this cohort. The methods for inflammatory infiltrate scoring are described elsewhere (Klintrup et al., 2005; Roxburgh et al., 2009a,b). Briefly, the generalised inflammatory cell infiltrate at the margin is graded according to a 4-point score: 0 indicates there was no increase in inflammatory cells at the tumour’s IM; 1 denotes a mild/patchy increase in inflammatory cells; 2 denotes a prominent inflammatory reaction forming a band at the IM; 3 denotes a florid cup-like inflammatory infiltrate at the invasive edge with destruction of cancer cell islands. These scores are subsequently given a binary classification as either low grade (scores 0 and 1) or high grade (scores 2 and 3).

Immunohistochemistry

Full section analysis for immune cell infiltrates. Archived paraffin-embedded blocks of the central tumour were retrieved. One block, representative of the point of deepest tumour invasion, was chosen. Consecutive 4 μm sections were cut and mounted on silanised slides before being dewaxed in xylene and rehydrated using graded alcohol washes. Heat-induced antigen retrieval was performed by microwaving under pressure using a citrate or Tris/EDTA buffer before endogenous peroxidase activity was blocked (5% normal goat serum in TRIS-buffered saline (TBS)), and the following primary antibodies were applied according to manufacturer’s instructions; CD8+ (DakOCTymation,
Glostrup, Denmark; code M7103, 1/100 dilution), CD3⁺ (Vector Laboratories, Burlingame, CA, USA; code VP-RM01, 1/100 dilution), CD45RO⁺ (DakoCytomation, code M0742, 1/150 dilution) and FOXP3⁺ (Abcam, Cambridge, UK; code 20034, 1/200 dilution). Sections were washed with TBS, incubated with Dako Envision, washed again and had 3,3'-diaminobenzidine (DAB) applied. Finally, sections were washed with water, counter-stained with haematoxylin, dehydrated and mounted.

Evaluation of T-cell density was undertaken blinded to outcome. Density was graded semiquantitatively as absent, weak, moderate or strong in three separate tumour compartments: (1) IM, (2) tumour stroma (ST) and (3) cancer cell nests (CCN). Examples of immune cell staining in various tumour locations are shown in Figures 1A–D. To confirm consistency of grading, 100 cases were scored independently by two investigators (CHR and CSR).

In addition to assessing individual T-cell subtypes, a previously proposed ‘immune score’ was applied: the Galon Immune Score, a composite immunohistochemistry-based score, which grades CD45RO⁺ and CD8⁺ infiltration in both the margin and central tumour (Mlecnik et al., 2011). The scoring groups the tumours based on high or low density of each cell type in each area HiHiHiHi to LoLoLoLo for CD45RO⁺ and CD8⁺ at the IM and tumour centre (Mlecnik et al, 2011).

TMA construction. In addition to full section analysis described above, CRC tissue microarrays (TMA) were used to assess p16INK4a and Ki-67 expression. In brief, a trained pathologist with a specialist gastrointestinal interest identified tumour-rich area within each specimen and four 0.6-mm² tumour cores were used to construct the TMA (Tovey et al, 2005). TMA sections (2.5 μm) were cut and mounted on slides coated with aminopropyltriethoxysilane.

Immunohistochemistry for p16INK4a and Ki-67. Before p16INK4a antibody staining of TMAs, the antibody was validated using a
p16<sup>ink4a</sup> blocking peptide competitor assay on tissue sections, in the presence of p16<sup>ink4a</sup> antibody. Specificity of the p16<sup>ink4a</sup> antibody was evidenced by an absence of staining in the presence of the blocking peptide.

Paraffin-embedded TMAcs were baked, dewaxed, and rehydrated followed by antigen retrieval in Tris/EDTA (pH 8.0 for p16<sup>ink4a</sup> and pH 6.0 for Ki-67) with boiling for 5 min in a microwave under pressure. After cooling, endogenous peroxidase was quenched by incubating sections in 3% hydrogen peroxide for 10 min. Slides were blocked for 1 h at 25 °C in 1× casein (Vector Laboratories, SP-5020) diluted in TBS buffer. Slides were then incubated at 4 °C overnight with the primary antibody: 1 in 300 dilution of p16<sup>ink4a</sup> (Santa Cruz sc-468; Santa Cruz Biotechnology, Dallas, TX, USA) antibody in Dako diluent (Dako, Glostrup, Denmark, S0809), and 1 in 50 dilution of Ki-67 antibody (Monoclonal mouse anti-human, Ki-67 antigen, Clone MIB-1, CodeM7240) with a negative (no primary antibody) slide control. Slides were then washed and incubated with Dako REAL Envision HRP Rabbit/Mouse (Dako, Glostrup, Denmark, S0809), and in 1 with a negative (no primary antibody) block control. Slides were then washed and incubated with Dako REAL Envision HRP Rabbit/Mouse (Dako, Glostrup, Denmark, S0809), and in 1 with a negative (no primary antibody) block control. Slides were then washed and incubated with Dako REAL Envision HRP Rabbit/Mouse (Dako, Glostrup, Denmark, S0809), and in 1 with a negative (no primary antibody) block control. Slides were then washed and incubated with Dako REAL Envision HRP Rabbit/Mouse (Dako, Glostrup, Denmark, S0809), and in 1 with a negative (no primary antibody) block control.

Intratumoural p16<sup>ink4a</sup> expression and a low Ki-67 proliferative index were employed as markers of senescence in this study. Relationships between intratumoural p16<sup>ink4a</sup> expression, Ki-67 proliferative index and their combination with immune cell infiltrates are shown in Table 2. High nuclear p16<sup>ink4a</sup> expression was associated with reduced CD45RO+ cells at the IM (P < 0.05) and within the stroma (P < 0.05) and reduced CD8+ cells at the IM (P < 0.01). A low Ki-67 proliferative index was associated with reduced density of CD3+ cells in CCN (P < 0.01), reduced CD45RO+ cells at the IM (P < 0.05) and within the CCN (P < 0.001), reduced FOXP3+ cells at the IM (P < 0.001), within the stroma (P = 0.001) and within CCN (P < 0.001), and reduced CD8+ cells at the IM (P < 0.05) and within the CCN (P < 0.05). A lower proliferative index was associated with a low-grade Klintup–Makinen inflammatory cell infiltrate (P < 0.001) and a low Galon–Pages Immune Score (P < 0.005; Table 2). Figure 2 shows the relationships between increasing Klintup–Makinen category (absent/weak/moderate and strong) and p16<sup>ink4a</sup>expression and Ki-67 labelling.

When p16<sup>ink4a</sup> and Ki-67 were combined as markers of senescence, similar and more consistent relationships were observed (Table 2). Intratumoural senescence was associated with reduced infiltrates of CD3+ cells at the IM (P < 0.05) and within CCN (P < 0.005), reduced CD45RO+ cells at the IM (P < 0.05) and within CCN (P < 0.005), reduced FOXP3+ cells at the IM (P < 0.001), within the stroma (P < 0.001) and within CCN (P < 0.001), reduced CD8+ cells at the IM (P < 0.001), within the stroma (P < 0.001) and within CCN (P < 0.001). Senescence was associated with a low-grade Klintup–Makinen inflammatory cell infiltrate (P < 0.001) and a low Galon–Pages Immune Score (P < 0.001; Table 2).

Median follow-up for survivors was 115 months (minimum 73 months), during which there were 117 deaths, of which 78 were from cancer. Univariate and multivariate analysis for cancer-specific survival is shown in Table 3. On univariate analysis, the following features were significantly associated with poorer cancer-specific survival: presence of a systemic inflammatory response (mGPS) P < 0.001, increasing TNM stage (P < 0.001), and tumour necrosis (P < 0.05). Lower counts of immune cells at the IM also significantly related to poorer cancer-specific survival: CD3+ cells (P < 0.001), FOXP3+ cells (P < 0.01) and CD8+ cells (P < 0.005) were related to poorer cancer-specific survival (Table 3). Survival curves
demonstrating relationships between margin CD3⁺, Ki-67 and nuclear p16ink4a expression and cancer-specific survival are shown in Figure 3.

On multivariate analysis of significant variables, the following were independently associated with poorer cancer-specific survival: systemic inflammation mGPS (HR 1.41 (95% CI: 1.00–1.99) P = 0.053), TNM stage (HR: 2.70 (95% CI: 1.69–4.32) P < 0.001), reduced margin CD3⁺ count (HR 2.37 (95% CI 1.19–4.73) P = 0.014), reduced margin CD8⁺ count (HR: 2.16 (95% CI: 1.05–4.44) P = 0.037), lower tumour proliferation (Ki-67; HR: 1.89 (95% CI: 1.15–3.11) P = 0.013) and reduced p16ink4a expression (HR: 2.22 (95% CI: 1.33–3.72) P = 0.002; Table 4).

Table 4 shows the relationships between tumour immune cell infiltrates and survival (log-rank P-value) in tumours with both high and low p16ink4a and according to Ki-67 expression. Increasing densities of immune cell infiltrates of CD3⁺, CD45RO⁺ and CD8⁺, in addition to scores such as the Klintrup–Makinen and Galon–Pages immune scores, consistently related to improved survival in tumours with high and low levels of p16ink4a and high and low Ki-67 expression. It is noteworthy that increasing levels of T-regulatory cells (FOXP3⁺) at the margin, ST or CCN were associated with outcome in patients whose tumours had low p16ink4a expression but not in patients with high p16ink4a expression. Similarly, survival relationships were not observed with FOXP3⁺ expression in low Ki-67 tumours.

Table 1. Clinicopathological characteristics including immune cell infiltrates, senescence and proliferation and 5-year cancer-specific survival rates in patients undergoing potentially curative resection for colorectal cancer (n = 230)

| Clinicopathological characteristic | N (%) |
|-----------------------------------|-------|
| **Age**                           |       |
| <65                               | 76 (33) |
| 65–75                             | 78 (34) |
| >75                               | 76 (33) |
| **Sex**                           |       |
| Female                            | 107 (46) |
| Male                              | 123 (54) |
| **Site**                          |       |
| Colon                             | 149 (65) |
| Rectum                            | 81 (35) |
| **Adjuvant chemotherapy**         |       |
| No                                | 159 (69) |
| Yes                               | 71 (31) |
| **mGlasgow Prognostic Score**     |       |
| 0                                 | 128 (54) |
| 1                                 | 77 (33) |
| 2                                 | 25 (11) |
| **Stage**                         |       |
| TNM I                             | 17 (7) |
| TNM II                            | 108 (47) |
| TNM III                           | 105 (46) |
| **T stage**                       |       |
| 1                                 | 7 (2) |
| 2                                 | 18 (8) |
| 3                                 | 135 (59) |
| 4                                 | 70 (30) |
| **N stage**                       |       |
| 0                                 | 125 (54) |
| 1                                 | 80 (35) |
| 2                                 | 25 (11) |
| **Differentiation**               |       |
| Mod/Well                          | 201 (87) |
| Poor                              | 29 (13) |
| **Venous invasion**               |       |
| No                                | 143 (62) |
| Yes                               | 87 (38) |
| **Serosal involvement**           |       |
| No                                | 160 (70) |
| Yes                               | 70 (30) |
| **Margin involvement**            |       |
| No                                | 212 (92) |
| Yes                               | 18 (8) |
| **Tumour perforation**            |       |
| No                                | 221 (96) |
| Yes                               | 9 (4) |

Table 1. (Continued)

| Clinicopathological characteristic | N (%) |
|-----------------------------------|-------|
| **Tumour necrosis**               |       |
| Absent                            | 15 (7) |
| Focal                             | 122 (53) |
| Moderate                          | 63 (28) |
| Extensive                         | 28 (12) |
| **Klintrup–Makinen infiltrate**   |       |
| Weak                              | 154 (67) |
| Strong                            | 75 (33) |
| **CD3 infiltrate margin**         |       |
| Weak                              | 126 (58) |
| Strong                            | 92 (42) |
| **CD45RO infiltrate margin**      |       |
| Weak                              | 120 (55) |
| Strong                            | 99 (45) |
| **FOXP3 infiltrate margin**       |       |
| Weak                              | 125 (57) |
| Strong                            | 93 (43) |
| **CD8 infiltrate margin**         |       |
| Weak                              | 134 (61) |
| Strong                            | 85 (39) |
| **Tumour proliferation (Ki-67)**  |       |
| High                              | 141 (61) |
| Low                               | 89 (39) |
| **P16ink4a expression**           |       |
| High                              | 145 (63) |
| Low                               | 85 (37) |

Abbreviation: TNM = tumour, nodes and metastases.

Table 4 shows the relationships between tumour immune cell infiltrates and survival (log-rank P-value) in tumours with both high and low p16ink4a and according to Ki-67 expression. Increasing densities of immune cell infiltrates of CD3⁺, CD45RO⁺ and CD8⁺, in addition to scores such as the Klintrup–Makinen and Galon–Pages immune scores, consistently related to improved survival in tumours with high and low levels of p16ink4a and high and low Ki-67 expression. It is noteworthy that increasing levels of T-regulatory cells (FOXP3⁺) at the margin, ST or CCN were associated with outcome in patients whose tumours had low p16ink4a expression but not in patients with high p16ink4a expression. Similarly, survival relationships were not observed with FOXP3⁺ expression in low Ki-67 tumours.


DISCUSSION

The present study reports for the first time that in a large cohort of curative CRC patients with mature follow-up, immune cell infiltrates and intratumoural senescence (measured with p16ink4a and Ki-67 labelling) are stage-independent prognostic features. It was hypothesised that based on tumour p16ink4a expression and Ki-67 proliferative index, strong relationships would be observed between markers of senescence and increased density of immune cell infiltrates, potentially drawn in by a proinflammatory SASP. Further, we hypothesised that p16ink4a expression would at least partly explain the associations between immune cell infiltrates and survival in CRC. Although p16ink4a expression was related to improved cancer-specific survival, the present study’s results suggest that this is unlikely to be mediated via immune interactions involving tumour-infiltrating T lymphocytes (TILs; CD3+, CD8+, CD45RO+ and FOXP3+). If anything, higher p16ink4a expression was associated with lower density of immune cell infiltrate. 

Table 2. The relationships between intratumoural nuclear P16ink4a expression and Ki-67 expression with type, density and location of immune cell infiltrate

| Immune cell infiltrate | P16ink4a expression | Ki-67 proliferation index | P16ink4a/Ki-67 combined |
|------------------------|---------------------|--------------------------|-------------------------|
|                        | Low nuclear P16ink4a exp N = 85 | High nuclear P16ink4a exp N = 145 | Ki-67 (high) N = 141 | Ki-67 (low) N = 89 | P16 low/Ki-67 high N = 180 | P16 high/Ki-67 low N = 50 | P-value |
| CD3 margin             |                      |                          |                        |                        |                        |                        |          |
| Low/high               | 42/37               | 84/55                    | 0.297                  | 71/62                  | 55/30                  | 0.100                  | 91/79    | 35/13    | 0.017    |
| CD3 stroma             |                      |                          |                        |                        |                        |                        |          |
| Low/high               | 37/48               | 76/66                    | 0.146                  | 66/73                  | 47/41                  | 0.385                  | 83/95    | 30/19    | 0.071    |
| CD3 cancer cell nests  |                      |                          |                        |                        |                        |                        |          |
| Low/high               | 53/32               | 100/42                   | 0.210                  | 83/56                  | 70/18                  | 0.002                  | 111/67   | 42/7     | 0.002    |
| CD45RO margin          |                      |                          |                        |                        |                        |                        |          |
| Low/high               | 36/44               | 84/55                    | 0.027                  | 65/76                  | 55/32                  | 0.042                  | 83/86    | 37/13    | 0.002    |
| CD45RO stroma          |                      |                          |                        |                        |                        |                        |          |
| Low/high               | 31/54               | 74/68                    | 0.022                  | 64/74                  | 41/48                  | 0.964                  | 97/80    | 25/25    | 0.548    |
| CD45RO cancer cell nests |                  |                          |                        |                        |                        |                        |          |
| Low/high               | 55/28               | 109/33                   | 0.111                  | 89/49                  | 77/12                  | <0.001                 | 122/55   | 45/5     | 0.003    |
| FOXP3 margin           |                      |                          |                        |                        |                        |                        |          |
| Low/high               | 40/37               | 85/56                    | 0.235                  | 63/69                  | 62/24                  | <0.001                 | 85/84    | 40/9     | <0.001   |
| FOXP3 stroma           |                      |                          |                        |                        |                        |                        |          |
| Absent/weak            | 40/37               | 85/56                    | 0.382                  | 69/66                  | 64/23                  | 0.001                  | 96/77    | 37/12    | 0.012    |
| FOXP3 cancer cell nests |                  |                          |                        |                        |                        |                        |          |
| Absent/weak            | 43/37               | 67/75                    | 0.349                  | 51/84                  | 59/28                  | <0.001                 | 75/98    | 35/14    | 0.001    |
| CD8 margin             |                      |                          |                        |                        |                        |                        |          |
| Low/high               | 39/40               | 95/45                    | 0.007                  | 73/60                  | 61/25                  | 0.018                  | 76/94    | 40/9     | 0.001    |
| CD8 stroma             |                      |                          |                        |                        |                        |                        |          |
| Low/high               | 60/22               | 107/34                   | 0.653                  | 98/39                  | 70/16                  | 0.097                  | 124/50   | 44/5     | 0.008    |
| CD8 cancer cell nests  |                      |                          |                        |                        |                        |                        |          |
| Low/high               | 61/21               | 105/37                   | 0.942                  | 95/43                  | 72/14                  | 0.013                  | 123/52   | 44/5     | 0.006    |
| KM inflammatory infiltrate |                  |                          |                        |                        |                        |                        |          |
| Weak/strong            | 28/56               | 47/98                    | 0.887                  | 58/82                  | 17/72                  | <0.001                 | 110/69   | 44/6     | <0.001   |
| Galon–Pages immune score |                |                          |                        |                        |                        |                        |          |
| 0/1/2                  | 42/21/14            | 88/31/16                 | 0.104                  | 68/36/24               | 62/16/6                | 0.002                  | 90/44/21 | 40/8/1   | <0.001   |

Cells shaded in red indicate significant (P<0.05) and weakly significant (P<0.1) associations with lower density of immune cell infiltrate. The full colour version of this table is available at British Journal of Cancer online.
expression alone and in combination with low Ki-67 labelling was associated with reduced density of several T-cell markers at the margin, within the stroma and within the CCN (Table 2). Therefore, we would conclude that the method by which the presence of intratumoral senescence slows cancer progression in CRC does not appear to relate to overt upregulation of antitumour effector T-cell immune responses.

These results provide further evidence that the in situ immune response has a key role in determining CRC outcome independent of tumour stage and makers of senescence.

The fact that no significant relationship between p16ink4a and immune cell infiltrates invalidates the present study’s hypothesis. Further, tumours with high levels of senescence should demonstrate a low proliferative index. The fact that immune cell infiltrates demonstrated a strong relationship with proliferative tumours further strengthens the argument that the presence of tumour lymphocytic infiltrates exist independently of senescent cells. Data from murine models previously reported that intratumoral senescence was associated with generation of an antitumour immune response represented by the presence of CD4+ cells, macrophages and natural killer cells (Xue et al, 2007). The majority of published evidence on the prognostic value of immune cell infiltrates in human CRC has focused on T cells, particularly effector T cells (for example, CD8+ cytotoxic T cells). We chose a panel of T-cell markers representative of the most validated and studied adaptive immune cells, CD3+ (generic T-cell marker), memory T cells (CD45RO), T-regulatory cells (FOXP3+), and cytotoxic T cells (CD8+). Although we cannot say p16ink4a expression did not relate to innate (macrophages or NK cell-predominant infiltrates) or Th2-type adaptive (CD4+ infiltrates) intratumoral immune responses, we can conclude that p16ink4a expression does not appear to relate to a Th1-type response represented by higher densities of intratumoral effector CD8+ infiltrates.

P16ink4a and Ki-67 are considered part of a panel of potential biomarkers of senescence. Other biomarkers include markers of

| Table 3. Univariate and multivariate analysis of clinicopathological characteristics for cancer-specific survival in stage I–III colorectal cancer (n = 230): Cox regression analysis |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                 | Hazard ratio    | P-value         | Hazard ratio    | P-value         |
|                                 | (95% CI)        |                 | (95% CI)        |                 |
| Age                             |                 |                 |                 |                 |
| < 65/ 65–75/ > 75              | 1.21 (0.92–1.60) | 0.174           |                 |                 |
| Sex                             |                 |                 |                 |                 |
| Male/female                     | 1.06 (0.67–1.66) | 0.809           |                 |                 |
| Site                            |                 |                 |                 |                 |
| Colon/rectum                    | 1.35 (0.86–2.13) | 0.198           |                 |                 |
| mGlasgow Prognostic Score       |                 |                 |                 |                 |
| 0/1/2                           | 1.74 (1.28–2.37) | <0.001          | 1.41 (1.00–1.99) | 0.053           |
| TNM stage                       |                 |                 |                 |                 |
| I/II/III                        | 2.27 (1.50–3.44) | <0.001          | 2.70 (1.69–4.32) | <0.001          |
| Necrosis                        |                 |                 |                 |                 |
| Absent/weak/moderate/strong     | 1.36 (1.03–1.81) | 0.033           | 0.730           |                 |
| CD3 margin                      |                 |                 |                 |                 |
| High/low                        | 3.36 (1.93–5.87) | <0.001          | 2.37 (1.19–4.73) | 0.014           |
| CD45RO margin                   |                 |                 |                 |                 |
| High/low                        | 2.68 (1.60–4.50) | <0.001          | 0.569           |                 |
| CD8 margin                      |                 |                 |                 |                 |
| High/low                        | 3.30 (1.87–5.83) | <0.001          | 2.16 (1.05–4.44) | 0.037           |
| FOXP3 margin                    |                 |                 |                 |                 |
| High/low                        | 2.42 (1.25–4.72) | 0.009           | 0.813           |                 |
| Klintrup–Makinen score          |                 |                 |                 |                 |
| Strong/weak                     | 2.79 (1.53–5.06) | 0.001           | 0.400           |                 |
| Ki-67                            |                 |                 |                 |                 |
| Low/high                        | 2.32 (1.48–3.64) | <0.001          | 1.89 (1.15–3.11) | 0.013           |
| Nuclear P16ink4a expression     |                 |                 |                 |                 |
| High/low                        | 1.85 (1.18–2.89) | 0.007           | 2.22 (1.33–3.72) | 0.002           |
the senescence-associated secretome including the proinflammatory cytokine IL-6. Although no measure of intratumoural cytokines were available here, we included serum measures of systemic inflammation (mGPS based on C-reactive protein and albumin). Interleukin-6 is responsible for 90% of the hepatic production of C-reactive protein (Gabay and Kushner, 1999). No significant relationships were seen between p16ink4a expression, C-reactive protein and albumin. It is possible that the IL-6 produced in the presence of the SASP does not represent a systemic phenomenon and may have more subtle intratumoural effects. Further, the presence of a systemic inflammatory response is known to persist independent of local intratumoural inflammation (Roxburgh et al, 2009a, b).

The present study also demonstrated significant relationships between T-cell infiltrates and highly proliferative tumours. The reasons for such observations have not been fully elucidated, but previous studies have demonstrated associations between microsatellite instability and higher proliferative index (Michael-Robinson et al, 2001). Highly proliferative tumours were associated with improved survival in this cohort; this relationship may be partly explained by increased immunogenicity of these tumours drawing in immune reactions. Unfortunately, at present no measure of microsatellite instability is available in this cohort, but the high Ki-67 labelling may be a surrogate measure for genomic instability in part explaining these relationships with immune cell infiltrates and improved survival. Alternatively, one may construct the hypothesis that an altered tumour secretome overrides any effect the SASP has. Highly proliferative tumour cells may then produce factors modulating immune activity, based on altered tumour cell metabolism and consequently the tumour cell secretome (Sebastián et al, 2012).

The role of p16ink4a expression has previously been examined in CRC using immunohistochemistry (Zhao et al, 2003; Cui et al, 2004; Gonzalez-Quevedo et al, 2004; Lyall et al, 2006; Wassermann et al, 2009; Shima et al, 2011). To date, published data on loss of p16ink4a expression is variable, reported between 8–91% in stage I–IV disease. Many studies had a low number of cancers studied (n = 32–117). The largest study (n = 802) reported that p16ink4a was expressed in 75%, more comparable with the current study (63%) (Shima et al, 2011). This study by Shima et al (2011) found
that p16INK4A loss was associated with poorer overall survival (HR: 1.30, 95% CI: 1.03–1.63, P = 0.026), also comparable with the present study’s findings for poorer cancer-specific survival (univariate analysis, HR: 1.85, 95% CI: 1.18–2.90, P = 0.007). However, the study by Shim a et al (2011) reported that the prognostic value of p16INK4A was lost on multivariate analysis; age and tumour grade were the principal confounders. In the present study of 230 patients, p16INK4A expression was an independent prognostic factor when age and grade (differentiation) were considered.

There is an increasing literature on the role of immune scoring in CRC prognostication with calls for the introduction of these stage-independent prognostic biomarkers into routine clinical practice in addition to utilisation within clinical trials of cancer therapeutics for stratification purposes (Galon et al, 2012). The basis or driving force for the immune cell infiltrates is largely unclear. Some have suggested that this response is representative of the local in situ host immune reaction against the tumour and once lost, or evaded, the tumour can grow and metastasise or ‘escape’ (Mlecnik et al, 2011; Roxburgh and McMillan, 2012). This is in keeping with previous reports that suggest the response is lost with increasing tumour size or T and N stage (Roxburgh et al, 2009a; Mlecnik et al, 2011). It is probable that intratumoural factors have a partial role in determining the specific densities of immune cell infiltrates including microsclast tissue, CpG island methylation and intratumoural HLA expression (Mlecnik et al, 2011), but at this stage these features do not yet seem to explain fully their presence.

In summary, the present study highlights the prognostic value of intratumoural senescence. Importantly, the presence of senescence determined by p16INK4A expression does not appear to mediate its effect on oncological outcome through TILs. On the other hand, high tumour proliferation is strongly associated with the presence of a local immune response. However, TILs and the in situ local inflammatory response remain strong prognostic factors independent of these intratumoural characteristics. p16INK4A expression and tumour proliferation as markers of intratumoural senescence do not explain the presence of TILs in CRCs.

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REFERENCES

Baker K, Zlobec I, Tornillo L, Terracciano L, Jass JR, Lugli A (2007) Differential significance of tumour infiltrating lymphocytes in sporadic mismatch repair deficient versus proficient colorectal cancers: a potential role for dysregulation of the transforming growth factor-beta pathway. Eur J Cancer 43(3): 624–631.

Cairney CJ, Bilsland AE, Evans TR, Roffey J, Bennett DC, Narita M, Torrance CJ, Keith WN (2012) Cancer cell senescence: a new frontier in drug development. Drug Discov Today 17(5–6): 269–276.

Campisi J (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 120(4): 513–522.

Campisi J, d’Adda di Fagagna (2007) Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8(9): 729–740.

Collado M, Blasco MA, Serrano M (2007) Cellular senescence in cancer and aging. Cell 130(2): 223–233.

Cui X, Shirai Y, Yokoyama N, Hirano S, Hatakeyama K (2004) Aberrant expression of pRb and p16(INK4) alone or in combination, indicates poor outcome after resection in patients with colorectal carcinoma. Hum Pathol 35(10): 1189–1195.

Deschoomweeter V, Baay M, Van Marck E, Weyer J, Vermeulen P, Lardon F, Vermorken JB (2010) Tumor infiltrating lymphocytes: an intriguing player in the survival of colorectal cancer patients. BMC Immunol 11: 19.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linkens M, Rubelj I, Pereira-Smith O (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci US A 92(20): 9363–9367.

Esteller M, Gonzalez S, Risques RA, Marcuello E, Mangues R, Germa JR, Hertman JG, Capella G, Peinado MA (2001) K-ras and p16 alterations confer poor prognosis in human colorectal cancer. J Clin Oncol 19(2): 299–304.

Gabay C, Kushner I (1999) Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 340(6): 448–454.

Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lageorge-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pagès F (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313(5795): 1960–1964.

Galon J, Franck P, Marincola FM, Angell HK, Thurin M, Lugli A, Zlobec I, Berger A, Bifulco C, Botti G, Tatangelo F, Britten CM, Kreiter S, Chouchane L, Delrio P, Arndt H, Asslaber M, Maio M, Masucci GV, Milhm M, Vidal-Vanaclocha H, Allison JP, Gnjatic S, Hakansson L, Huber C, Singha-Sajua H, Ottemosser C, Zwierzina H, Lugli L, Grizzli F, Ohashi PS, Shaw PA, Clarke BA, Wouters BG, Kawakami Y, Hazama S, Okuno K, Wang E, O’Donnell-Tormey J, Lageorge C, Pawelec G, Nishimura MI, Hawksin R, Lapointe R, Lundqvist A, Kheifl SN, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel BS, Shukla SN, Palmqvist R, Nagtegaal ID, Wang Y, D’Arrigo C, Kopetz S, Sicinsac FE, Trinchieri G, Gajewski TF, Ascieto PA, Fort SA (2012) Cancer classification using the Immunoscore: a worldwide task force. J Transl Med 10(1): 205.

Gonzalez-Quevedo R, Garcia-Aranda C, Moran A, De Juan C, Sanchez-Pernaute A, Torres A, Diaz-Rubio E, Balibrea JL, Benito M, Iniesta P (2004) Differential impact of p16 inactivation by promoter methylation in non-small cell lung and colorectal cancer: clinical implications. Int J Oncol 24(2): 349–355.

Kass JR (1987) The pathological classification of colorectal cancer. Ann Acad Med Singapore 16(3): 469–473.

Kirkegaard T, Edwards J, Tovey S, McGlynn LM, Krishna SN, Mukherjee R, Tam L, Munro AF, Dunne B, Bartlett JM (2006) Observer variation in immunohistochemical analysis of protein expression, time for a change? Histopathology 48(7): 787–794.

Klintrup M, Makinen JM, Kauppila S, Vare PO, Melkko J, Tuomiainen H, Tuppuranen M, Mäkäjä I, Kalmarten TJ, Mäkinen MJ (2005) Inflammation and prognosis in colorectal cancer. Eur J Cancer 41(17): 2645–2654.

Kranichmuthy J, Torricc C, Ramsey MR, Kovalev GI, Al-Ragaiy K, Su L, Sharpless NE (2004) Inktaka/Arf expression is a biomarker of aging. J Clin Invest 114(9): 1299–1307.

Lagli L, Bianchi P, Miranda E, Balladore E, Pacetti V, Grizzi F, Allavena P, Torri V, Repici A, Santoro A, Mantovani A, Roncalli M, Malesci A (2009) CD3 + cells at the invasive margin of deeply invading (pT3–T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study. Lancet Oncol 10(9): 877–884.

Lyall MS, Dundas SR, Carunan S, Murray GI (2006) Profiling markers of prognosis in colorectal cancer. Clin Cancer Res 12(4): 1184–1191.

Michael-Robinson JM, Bierner-Huttman A, Purdie DM, Walsh MD, Simms LA, Borden KG, Young JP, Leggett BA, Jass JR, Radford-Smith GL (2001a) Tumour infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status. Gut 48(3): 360–366.

Michael-Robinson JM, Reid LE, Purdie DM, Biemer-Huttman AE, Walsh MD, Pandey N, Simms LA, Young JP, Leggett BA, Jass JR, Radford-Smith GL (2001b) Proliferation, apoptosis, and survival in high-level microsatellite instability sporadic colorectal cancer. Clin Cancer Res 7(8): 2347–2356.

McGlynn LM, Stevenson K, Lamb K, Zino S, Brown M, Prina A, Kingsmore D, Shili PG (2009) Cellular senescence in pretransplant renal biopsies predicts postoperative organ function. Aging Cell 8(1): 45–51.

Mitomi H, Fukui N, Tanaka N, Kanawaza H, Saito T, Matsuoka T, Yao T (2010) Ablation p16INK4A methylation is a frequent event in colorectal
cancers: prognostic value and relation to mRNA expression and immunoreactivity. J Cancer Res Clin Oncol 136(2): 323–331.
Mlecník B, Tosolini M, Kirilovsky A, Berger A, Binde A, Meachi T, Brunel P, Trajanoski Z, Fridman WH, Páges F, Galon J (2011) Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol 29(6): 610–618.
Mohammed ZM, McMillan DC, Elsberger B, Going JJ, Orange C, Mallon E, Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H (1998) Immunoreactivity of Ki-67 in human colorectal cancer. Cancer Res 58(16): 3491–3494.
Narita M, Lowe SW (2005) Senescence comes of age. Nat Med 11(9): 920–922.
Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molder R, Mlecník B, Kirilovsky A, Nilsson M, Damotte D, Meachi T, Brunel P, Cugnenc PH, Trajanoski Z, Fridman WH, Galon J (2005) Efector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 353(25): 2654–2666.
Pollheimer MJ, Kornprat P, Lindtner RA, Harbaum L, Schlemmer A, Rehak P, Langner C (2010) Tumor necrosis is a new promising prognostic factor in colorectal cancer. Hum Pathol 41(12): 1749–1757.
Raabe EH, Lin KS, Kim JM, Meeker A, Mao XG, Nikihaq G, Maciavsky J, Kahler U, Jain D, Bar E, Cohen KJ, Eberhart CG (2011) BRAF activation induces transformation and then senescence in human neural stem cells: a pilocytic astrocytoma model. Clin Cancer Res 17(11): 3590–3599.
Richards CH, Flegg KM, Roxburgh CS, Going JJ, Mohammed Z, Horgan PG, McMillan DC (2012a) The relationships between cellular components of the peritumoral inflammatory response, clinicopathological characteristics and survival in patients with primary operable colorectal cancer. Br J Cancer 106(12): 2010–2015.
Richards CH, Roxburgh CS, Anderson JH, McKee RF, Foulis AK, Horgan PG, McMillan DC (2012b) Prognostic value of tumour necrosis and host inflammatory responses in colorectal cancer. Br J Surg 99(2): 287–294.
Roxburgh CS, McMillan DC (2012) The role of the in situ local inflammatory response in predicting recurrence and survival in patients with primary operable colorectal cancer. Cancer Treat Rev 38(5): 451–466.
Roxburgh CS, Salmond JM, Horgan PG, Oien KA, McMillan DC (2009a) Tumour inflammatory infiltrate predicts survival following curative resection for node-negative colorectal cancer. Eur J Cancer 45(12): 2138–2145.

Roxburgh CS, Salmond JM, Horgan PG, Oien KA, McMillan DC (2009b) Comparison of the prognostic value of inflammation-based pathologic and biochemical criteria in patients undergoing potentially curative resection for colorectal cancer. Ann Surg 249(5): 788–793.
Sebastian C, Zwaans BM, Silberman DM, Gynrek M, Goren A, Zhong L, Ram O, Truelove J, Guimaraes AR, Toiber D, Cosentino C, Greenson JK, MacDonald AL, McGlynn L, Maxwell F, Edwards J, Giacosa S, Guccione E, Weissleder R, Bernstein BE, Regev A, Shils PG, Lombard DB, Mostoslavsky R (2012) The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. Cell 151(6): 1185–1199.
Shima K, Nosho K, Baba Y, Cantor M, Meyerhardt JA, Giovannucci EL, Fuchs CS, Ogino S (2011) Prognostic significance of CDKN2A (p16) promoter methylation and loss of expression in 902 colorectal cancers: Cohort study and literature review. Int J Cancer 128(5): 1080–1094.
Schmitt CA (2007) Cellular senescence and cancer treatment. Biochim Biophys Acta 1775(1): 5–20.
Sinecrope FA, Rego RL, Ansell SM, Knutson KL, Foster NR, Sargent DJ (2009) Intraepithelial effector (CD3+/regulatory (FoxP3+) T-cell ratio predicts a clinical outcome of human colon carcinoma. Gastroenterology 137(4): 1270–1279.
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