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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 (p16^{ink4a}) in combination with low level expression of a proliferation marker (ki-67) relate to T cell infiltrates in CRC, and whether p16^{ink4a}, Ki-67 and immune infiltrates have similar prognostic value.

Methods: Immunostaining of p16^{inka} and Ki-67 was performed within a CRC tissue microarray. Nuclear p16^{inka} 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 p16^{ink4a} was expressed in 63% and high proliferation (Ki-67 $>15\%$) in 61%. p16^{ink4a} 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 p16^{ink4a} 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 (p16^{ink4a}; $P=0.002$) were independently associated with poorer cancer survival.

Conclusion: Senescence, proliferation and immune cell infiltrates are independent prognostic factors in CRC. Although related to survival, p16^{ink4a}-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

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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; Mlecknik *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; Sinicrope *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 thought to be an evolutionary mechanism to mitigate tumour development. The presence of senescent cells within a tumour may be a favourable prognostic feature in cancer, as such cells would not have bypassed cellular damage checkpoints (Narita and Lowe, 2005). Senescent cells are associated with a proinflammatory secretome including interleukins (ILs) and other cytokines (IL-6, IL-8, MMP 1 and 3, CXCR, IGF1 and 2; Campisi, 2005; Cairney *et al*, 2012). Work in animal models has identified increased infiltrates of innate immune cells (NK cells and macrophages) and CD4+ lymphocytes drawn in by the proinflammatory secretome (Xue *et al*, 2007). This upregulation of intratumoural immune responses was thought to delay tumour progression. Therefore, one plausible explanation for the presence of high-grade lymphocytic infiltrates in colorectal tumours is that cellular senescence and the associated inflammatory secretome drive their presence.

To date, no single gold-standard biomarker for the detection and quantification of senescent cells in tissues has been developed. Biomarkers developed so far attempt to measure three main components related to senescence: cell cycle arrest (p16, p21, and Ki-67), chromatin (for example. HMGA1 and HMGA2) and the secretome (for example, IL-6, IL-8, MMP1 and so on; Cairney *et al*, 2012). Cell cycle markers of senescence include p16, p21 and Ki-67 expression. Of these markers, most work has utilised $p16^{ink4a}$ expression, which is thought to represent an established and reliable marker of cellular senescence in tumours as well as normal tissues (Campisi, d'Adda di, 2007; Collado *et al*, 2007; Schmit *et al,* 2007; Raabe *et al*, 2011). $P16^{ink4a}$ is a CDK inhibitor (Dimri *et al*, 1995; Campisi, 2005), the expression of which is upregulated in cellular senescence and ageing in normal tissues (Krishnamurthy *et al,* 2004; McGlynn *et al*, 2009). Importantly, $p16^{ink4a}$ expression has previously ben associated with improved survival in colorectal tumours (Esteller *et al*, 2001; Lyall *et al*, 2006; Mitomi *et al*, 2010) but the mechanisms through which $p16^{ink4a}$ expression influence cancer outcome are not yet established.

It is hypothesised that senescent tumour cells are associated with an immunogenic reaction in and around colorectal tumours. The aim of the present study was to establish whether markers of intratumoural senescence (evidence of nuclear $p16^{ink4a}$ expression and low Ki-67 labelling) are associated with increased immune cell infiltration representative of an immunogenic phenotype in human CRCs and to determine whether senescence, proliferation and immune cell infiltration were associated with long-term survival.

MATERIALS AND METHODS

Since 1997, all resectional CRC surgery performed at Glasgow Royal Infirmary (GRI) has been entered into a prospectively compiled and maintained database. For the present study, 230 patients undergoing surgery for stage I–III CRC between 1997–2005, and whose tumour biopsy was included in a CRC tissue microarray were studied. On the basis of the preoperative radiological staging and intraoperative findings, patients were considered to have undergone curative resection. Exclusion criteria were neoadjuvant treatment or death within 30 days of surgery. Local ethical committee approval was granted.

Routinely assessed pathological data were taken from reports issued by the GRI Department of Pathology at the time of surgery in accordance with the RCPath data set for colorectal cancer report (Williams *et al*, 2007). In addition, TNM stage (5th edition), other high-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; $CRP > 10\,mg\,l^{-1}$, allbumin $> 35\,g\,dl^{-1}$ = 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), 'focal' (<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; $CD8^+$ (DakoCytomation,

Glostrup, Denmark; code M7103, 1/100 dilution), CD3$^+$ (Vector Laboratories, Burlingame, CA, USA; code VP-RM01, 1/100 dilution), CD45R0$^+$ (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 haemotoxylin, 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 CD45R0$^+$ 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 p16^{ink4a} 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-mm^2 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 p16^{ink4a} and Ki-67. Before p16^{ink4a} antibody staining of TMAs, the antibody was validated using a

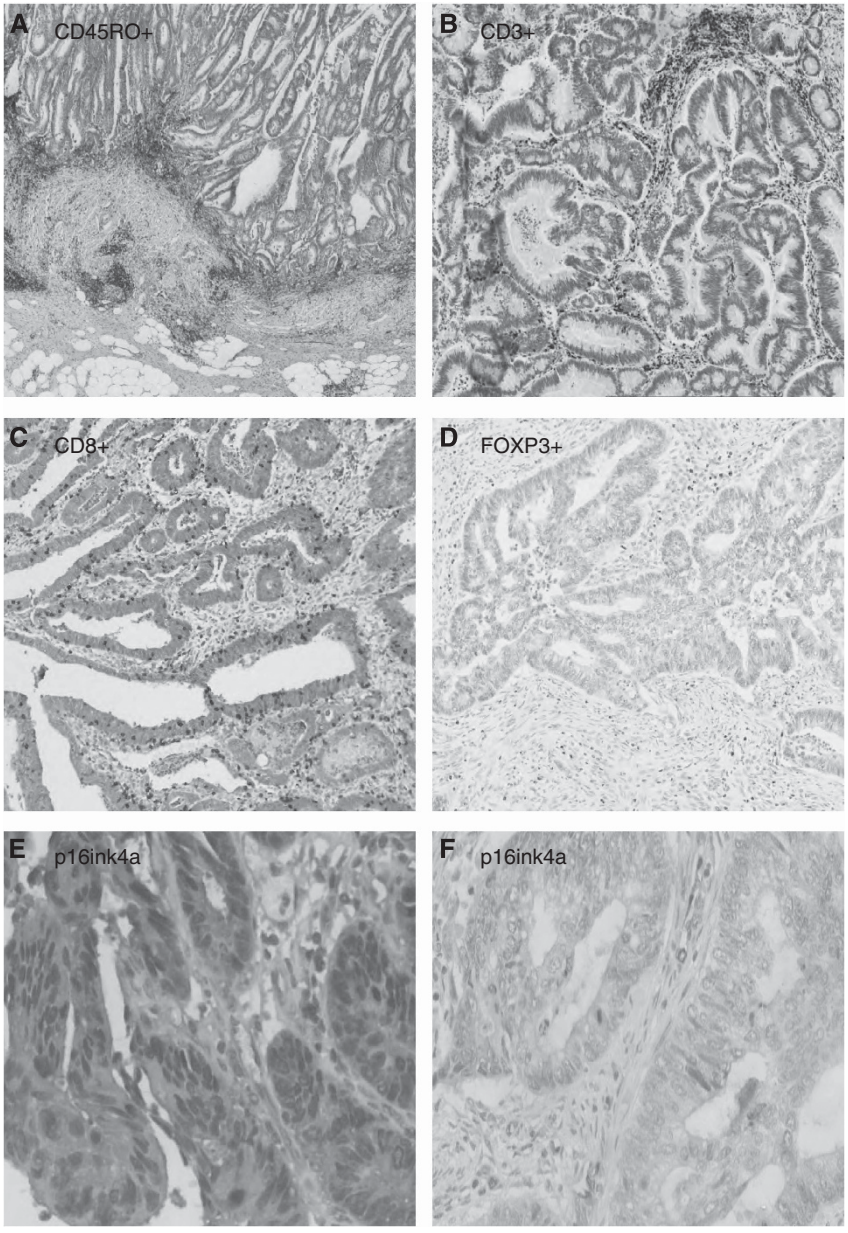

Figure 1. Examples of immunohistochemistry for immune cell infiltrates and p16^{ink4a} in colorectal tumours. (**A**) High-density CD45RO+ staining at IM at 45× magnification, (**B**) High-density CD3+ staining within ST at 40× magnification. (**C**) High-density CD8+ staining within CCN at 115× magnification. (**D**) Low-density FOXP3+ staining within ST at 115× magnification. (**E**) High-density nuclear p16^{ink4a} staining at 180× magnification (**F**) Low-density nuclear p16^{ink4a} staining at 200× magnification.

p16^{ink4a} blocking peptide competitor assay on tissue sections, in the presence of p16^{ink4a} antibody. Specificity of the p16^{ink4a} antibody was evident by an absence of staining in the presence of the blocking peptide.

Paraffin-embedded TMAs were baked, dewaxed, and rehydrated followed by antigen retrieval in Tris/EDTA (pH 8.0 for p16^{ink4a} 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 antibodiy: 1 in 300 dilution of p16^{ink4a} (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, K5007) for 1 h at 25 °C before developing with DAB (Vector Laboratories, SK-4100). Slides were washed and counterstained with haematoxylin before being dehydrated and mounted. Scoring of the nuclear p16^{ink4a} was performed using the weighted histoscore method. Histoscoring is a widely applied and validated method of obtaining reliable and reproducible assessments of scoring immunohistochemical stains in heterogeneous tissues (Kirkegaard *et al*, 2006). The percentage of positive nuclei within tumour tissue was multiplied by the strength of staining: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Only tumour tissue was scored. On the basis of the distribution of histoscores in the whole cohort, the median p16 histoscore was 19.5. Therefore, nuclear p16 expression was considered high if >20. For Ki-67 scoring, 108 specimens were co-scored by two observers blinded to outcome (AP and LM). The ICCC was 0.94. For p16^{ink4a} scoring, 65 specimens were co-scored by two observers blinded to outcome. The ICCC was 0.72. In addition to ICCC to ensure correlation between observers, bland Altman plots were performed to ensure there was no bias. Figures 1E and F show both strong and weak nuclear staining for p16^{ink4a}.

In order to calculate the Ki-67 proliferation index, all tumour cells within each core was scored providing the total number of positive and negative cells and allowing calculation of the percentage positive cells. The mean representative score was taken across all cores. The threshold of 15% was taken based on previously published work (Mohammed *et al,* 2012).

Statistical analysis. Information on date and cause of death was derived from data linkage with cancer registry data performed by the West of Scotland Cancer Surveillance Unit. Date and cause of death was cross-checked with information from clinical notes where possible.

All variables were grouped according to standard or previously published thresholds. Associations were examined using χ^2-tests for linear trend as appropriate. Death records were complete until 1 December 2011, which served as the censor date. Univariate survival analyses were performed using the Kaplan–Meier survival curves with log-rank tests. Multivariate analyses were performed using Cox proportional hazards regression with a backwards method. The proportionality assumptions for age, mGPS, TNM and necrosis were explored using log–log plots and were found to be satisfactory. A *P*-value of <0.05 was required to enter the multivariate regression model. Statistical analyses were performed using SPSS version 19.0 (SPSS, Chicago, IL, USA).

RESULTS

Two hundred and thirty patients were included who underwent curative resection for CRC between January 1997 and December 2005. Clinicopathological characteristics are described in Table 1. Most were $\geqslant 65$ years (67%), male (54%), and had colonic tumours (65%). Adjuvant chemotherapy was administered to 31%. On pathological analysis, most were stage I/II (54%), moderate/well differentiated (87%), with presence of venous invasion (52%), absence of serosal invasion (70%), absence of tumour perforation (96%) with uninvolved surgical margins (92%). Most had absent or weak scores for necrosis (60%; Table 1).

Tumour immune cell infiltrates were categorised as low-grade Klintrup–Makinen inflammatory cell infiltrates (67%) and weak/absent infiltrates of IM CD3+ cells (58%), CD45RO+ cells (55%), FOXP3+ cells (57%) and CD8+ cells (61%; Table 1). Most were classed highly proliferative by Ki-67 staining (61%) and the most expressed p16^{ink4a} (63%; Table 1). A weak association was observed between p16^{ink4a} expression and low tumour proliferation ($P=0.087$).

The relationships between intratumoural p16^{ink4a} expression, Ki-67 labelling and clinicopathological characteristics in CRC are shown in Supplementary Table 1. p16^{ink4a} expression was not related to any patient or tumour characteristics, including age, sex, systemic inflammatory response (mGPS), TNM stage or venous invasion. Similarly, a high Ki-67 index did not relate to any patient or tumour characteristic. A weak association was observed between low tumour proliferation and surgical margin involvement ($P=0.042$; Supplementary Table 1).

Intratumoural p16^{ink4a} expression and a low Ki-67 proliferative index were employed as markers of senescence in this study. Relationships between intratumoural p16^{ink4a} expression, Ki-67 proliferative index and their combination with immune cell infiltrates are shown in Table 2. High nuclear p16^{ink4a} 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 Klintrup–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 Klintrup–Makinen category (absent/weak/moderate and strong) and p16^{ink4a}expresson and Ki-67 labelling.

When p16^{ink4a} 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 ($P<0.005$) and within CCN ($P<0.005$), reduced FOXP3+ cells at the IM ($P<0.001$), within the stroma ($P<0.05$) and within CCN ($P=0.001$), reduced CD8+ cells at the IM ($P=0.001$), within the stroma ($P=0.001$) and within CCN ($P<0.01$). Senescence was associated with a low-grade Klintrup–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 113 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+ ($P<0.01$), CD8+ ($P<0.001$) and a lower Klintrup–Makinen Score ($P<0.005$). Lower Ki-67 expression ($P<0.001$) and reduced p16^{ink4a} expression ($P<0.01$) were related to poorer cancer -specific survival (Table 3). Survival curves

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 (56)
1	77 (33)
2	25 (11)
Stage	
TNM I	17 (7)
TNM II	108 (47)
TNM III	105 (46)
T stage	
I	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)
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.

demonstrating relationships between margin CD3+, Ki-67 and nuclear $p16^{ink4a}$ 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 $p16^{ink4a}$ 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 $p16^{ink4a}$ 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 $p16^{ink4a}$ 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 $p16^{ink4a}$ expression but not in patients with high $p16^{ink4a}$ expression. Similarly, survival relationships were not observed with FOXP3+ expression in low Ki-67 tumours.

Table 2. The relationships between intratumoural nuclear P16^{ink4a}expression and Ki-67 expression with type, density and location of immune cell infiltrate

	P16^{ink4a} expression			Ki-67 proliferation index			P16^{ink4a}/Ki-67 combined		
Immune cell infiltrate	**Low nuclear P16^{ink4a} exp N = 85**	**High nuclear P16^{ink4a} exp N = 145**	***P*-value**	**Ki-67 (high) N = 141**	**Ki-67 (low) N = 89**	***P*-value**	**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.

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 p16^{ink4a} and Ki-67 labelling) are stage-independent prognostic features. It was hypothesised that based on tumour p16^{ink4a} 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 p16^{ink4a} expression would at least partly explain the associations between immune cell infiltrates and survival in CRC. Although p16^{ink4a} 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 p16^{ink4a}

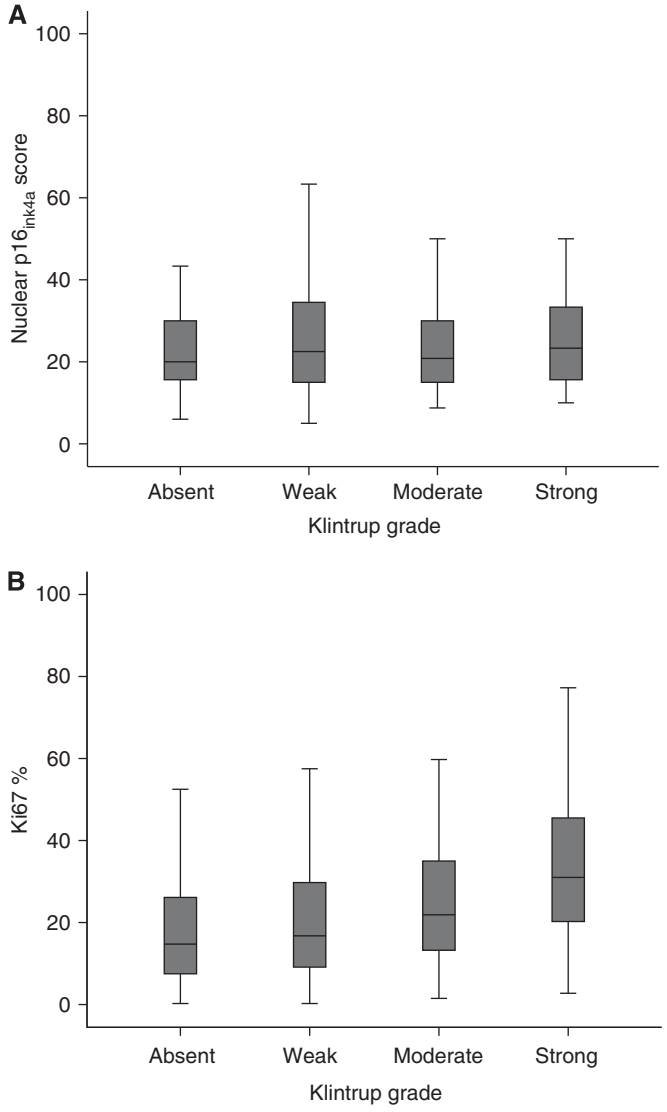

Figure 2. (**A**) The relationship between nuclear p16^{ink4a} expression and peritumoural inflammation measured with the Klintrup score $P=0.782$. (**B**) The relationship between tumour proliferation (Ki-67%) and increasing peritumoural inflammation measured with the Klintrup score $P<0.001$.

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 (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
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 P16^{ink4a} expression				
High/low	1.85 (1.18–2.89)	0.007	2.22 (1.33–3.72)	0.002

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 intratumoural 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 intratumoural 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 p16^{ink4a} expression did not relate to innate (macrophages or NK cell-predominant infiltrates) or Th2-type adaptive (CD4+ infiltrates) intratumoural immune responses, we can conclude that p16^{ink4a} expression does not appear to relate to a Th1-type response represented by higher densities of intratumoural effector CD8+ infiltrates.

P16^{ink4a} and Ki-67 are considered part of a panel of potential biomarkers of senescence. Other biomarkers include markers of

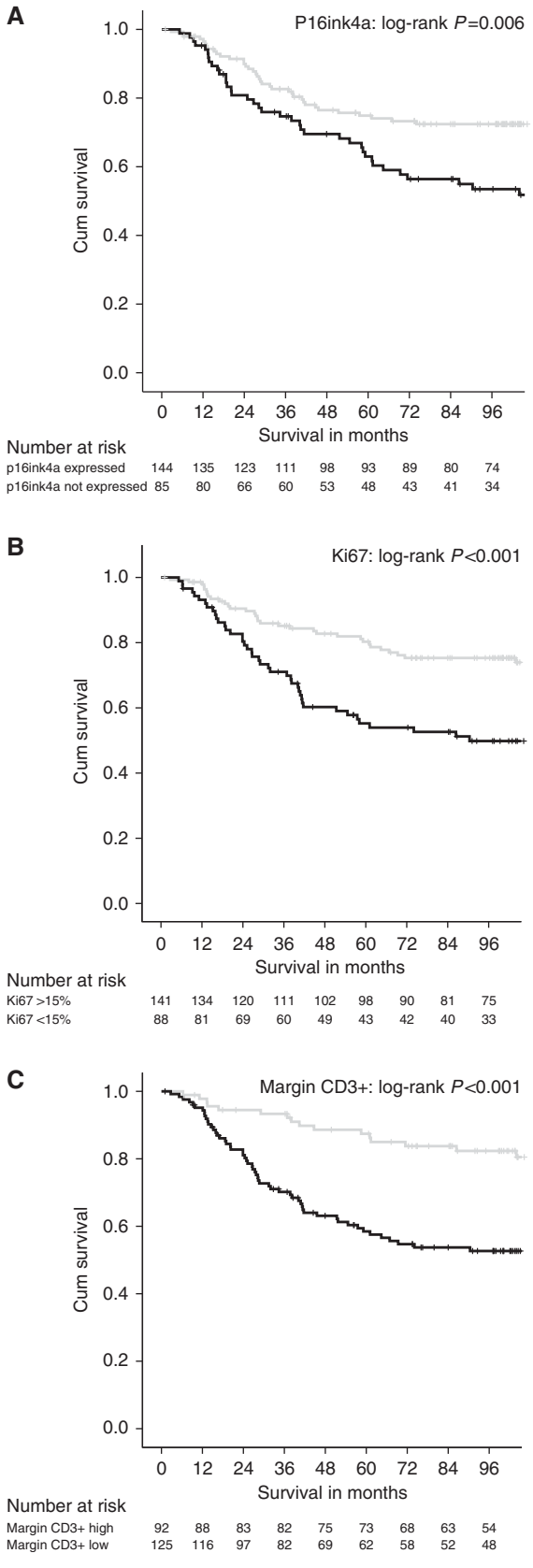

Figure 3. (**A**) The relationship between nuclear P16^{ink4a} expression and cancer-specific survival in stage I–III CRC ($n=230$). $P=0.006$. (**B**) The relationship between tumour proliferative activity (Ki-67) and survival in stage I–III CRC ($n=230$). $P<0.001$. (**C**) The relationship between Margin CD3+ density (low/high) and cancer-specific survival in stage I–III CRC ($n=230$). $P<0.001$.

Table 4. The relationships between immune cell infiltrates and cancer-specific survival (log-rank *P*-value) depending on senescence measured with P16^{ink4a} expression and proliferation (Ki-67) in patients undergoing curative resection for colorectal cancer ($n=230$)

	P16^{ink4a} low	P16^{ink4a} high	Ki-67 low	Ki-67 high
CD3 margin Hi/Lo	0.008	<0.001	0.003	0.003
CD3 stroma Hi/Lo	0.005	<0.001	0.058	<0.001
CD3 CCN Hi/Lo	0.001	0.001	0.021	0.002
CD45RO margin Hi/Lo	0.006	<0.001	0.049	0.003
CD45RO stroma Hi/Lo	0.067	0.003	0.013	0.080
CD45RO CCN Hi/Lo	0.017	<0.001	0.011	0.046
CD8 margin Hi/Lo	<0.001	0.001	0.002	0.013
CD8 stroma Hi/Lo	0.011	0.216	0.169	0.050
CD8 CCN Hi/Lo	0.004	0.001	<0.001	0.035
FOXP3 margin Hi/Lo	0.011	0.112	0.084	0.370
FOXP3 stroma Hi/Lo	<0.001	0.320	0.203	0.019
FOXP3 CCN Hi/Lo	<0.001	0.557	0.623	0.003
KM score Hi/Lo	0.005	0.019	0.026	0.050
Galon–Pages immune				
Score HiHi/Hi/Lo/LoLo	0.002	<0.001	0.001	0.018

Abbreviations: CCN = cancer cell nests; HI = high; KM = Klintrup–Makinen Score; Lo = low.

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 p16^{ink4a} 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 p16^{ink4a} 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 p16^{ink4a} 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 p16^{ink4a} was expressed in 75%, more comparable with the current study (63%) (Shima *et al*, 2011). This study by Shima *et al* (2011) found

that $p16^{ink4a}$ 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 Shima *et al* (2011) reported that the prognostic value of $p16^{ink4a}$ was lost on multivariate analysis; age and tumour grade were the principal confounders. In the present study of 230 patients, $p16^{ink4a}$ 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,b; Mlecnik *et al,* 2011). It is probable that intratumoural factors have a partial role in determining the specific densities of immune cell infiltrates including microsatellite instability, 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 $p16^{ink4a}$ 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. $p16^{ink4a}$ expression and low tumour proliferation as markers of intratumoural senescence do not explain the presence of TILs in CRCs.

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