Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients

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Background: Evasion of immune surveillance and suppression of the immune system are important hallmarks of tumour development in colon cancer. The goal of this study was to establish a tumour profile based on biomarkers that reflect a tumour's immune susceptibility status and to determine their relation to patient outcome.

Methods: The study population consisted of 285 stage I-IV colon cancer patients of which a tissue micro array (TMA) was available. Sections were immunohistochemically stained for the presence of Foxp3+ cells and tumour expression of HLA Class I (HLA-A, -B, -C) and non-classical HLA-E and HLA-G. All markers were combined for further analyses, resulting in three tumour immune phenotypes: strong immune system tumour recognition, intermediate immune system tumour recognition and poor immune system tumour recognition.

Results: Loss of HLA class I expression was significantly related to a better OS (P-value 0.005) and DFS (P-value 0.008). Patients with tumours who showed neither HLA class I nor HLA-E or -G expression (phenotype a) had a significant better OS and DFS (P-value <0.001 and 0.001, respectively) compared with phenotype b (OS HR: 4.7, 95% CI: 1.2–19.0, P = 0.001) or c (OS HR: 8.2, 95% CI: 2.0–34.2, P = 0.0001). Further, the tumour immune phenotype was an independent predictor for OS and DFS (P-value 0.009 and 0.013, respectively).

Conclusion: Tumours showing absence of HLA class I, HLA-E and HLA-G expressions were related to a better OS and DFS. By combining the expression status of several immune-related biomarkers, three tumour immune phenotypes were created that related to patient outcome. These immune phenotypes represented significant, independent, clinical prognostic profiles in colon cancer.

Historically, the immune system has been attributed an important role in controlling tumour growth and metastasis (Halvorsen and Seim, 1989a; Naito et al, 1998; Murphy et al, 2000a; Menon et al, 2004a). Evasion of immune surveillance and suppression of the immune system are two important traits cancer cells have to acquire during the process of tumorigenesis (Cavallo et al, 2011). Research of the last century has indicated that the influence of the immune system on tumour cells, both in the tumour micro-environment as well as during the process of tumour metastasis, also contributes to tumour progression (Schreiber et al, 2011). The cancer immune-editing hypothesis describes both the host-protective as well as the tumour-promoting actions the immune system might have on developing tumours, shaping tumour immunogenicity (Dunn et al, 2002, 2004a, b, 2006; Smyth et al,

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2006; Swann and Smyth, 2007; Vesely et al, 2011). Tumours are thought to be 'edited' through a Darwinian selection process into poorly immunogenic tumour cell variants invisible to the immune system and able to grow progressively. Immune editing might therefore have substantial effects on patient's prognosis.

Several mechanisms taking place at the tumour cell level contribute to this process. The first mechanism is downregulation of human leukocyte antigen (HLA) class I expression. Downregulation of HLA class I minimises the level of tumour-associated antigen (TAA) expression by tumour cells and therefore their recognition and subsequently destruction by cytotoxic T cells (CTL) (Halvorsen and Seim, 1989b; Murphy et al, 2000b; Menon et al, 2004b; Cavallo et al, 2011). The second mechanism is the ability of tumour cells to regulate the expression of non-classical HLA class I molecules (HLA-E and HLA-G) on the cell surface. Expression of these markers has been found to inhibit natural killer (NK) cell recognition in the blood stream and therefore results in further tumour cell escape from immune surveillance (Khong and Restifo, 2002; Marin et al, 2003; Wischhusen et al, 2007; Zilberman et al, 2012). HLA-E is regularly expressed in various healthy tissues and correlated with HLA class I expression (Palmisano et al, 2005). In contrast, HLA-G is rarely found in healthy tissues but is frequently observed in tumours (Wischhusen et al, 2007). Third, tumour cell immune reactivity can become suppressed by the attraction of immunosuppressive regulatory T cells (Tregs) into the tumour micro-environment (Zitvogel et al, 2006; Nosho et al, 2010). Tregs are able to modulate the antitumour immune response, as they suppress the activity of CTL through direct cell-to-cell contact or via the release of cytokines like transforming growth factor-β (Needham et al, 2006; Zou, 2006; Curiel, 2007). Tregs and CTLs therefore show opposing actions in tumour immunity (Liu et al, 2011).

Previously, both the downregulation of HLA class I, presence of Tregs and HLA-E and -G expressions have been shown to be of clinical relevance in several types of cancers (Curiel et al, 2004; Hiraoka et al, 2006; Gao et al, 2007; de Kruijf et al, 2010b). In colorectal cancer (CRC), various studies have described the impact of the level of HLA class I tumour expression or the presence of Foxp3 + Treg cells on patients with varying results (Moller et al, 1991; Menon et al, 2002; Loddenkemper et al, 2006; Watson et al, 2006; Benevolo et al, 2007; Salama et al, 2009; Deng et al, 2010; Suzuki et al, 2010). In general, loss of HLA class I tumour expression seemed to result in a better prognosis (Sandel et al, 2005; Watson et al, 2006). The presence of high levels of Foxp3 + cells in CRC patients was related to a worse prognosis in some studies, although this relation could not always be established in CRC patients (Loddenkemper et al, 2006; Salama et al, 2009; Deng et al, 2010; Suzuki et al, 2010; Zeestraten et al, 2011). Studies on the prognostic value of HLA-E and HLA-G showed that expression of these molecules correlated with poor prognosis and tumour progression (Ye et al, 2007; Yie et al, 2007a, b, c).

Previous studies have shown a complex interaction between different immune markers, highlighting the need for combined marker analysis (de Kruijf et al, 2010a, b; Zeestraten et al, 2011). The purpose of this study was to investigate the prognostic value of the immune-related biomarkers, HLA Class I, HLA-E and -G and Foxp3 +, to establish distinct patterns that reflect a tumour's immune-escape mechanism by combining these markers and to relate these patterns to clinical outcome.

MATERIALS AND METHODS

Study population. The patient population comprised a consecutive series of 470 colorectal cancer patients all treated with surgery for their primary tumour in the Leiden University Medical Center (LUMC) between 1991 and 2001. Of these patients' tumour materials, clinico-pathological data and information on the follow-up were collected in retrospect. This research was performed according to the code of conduct for responsible use. Mucinous differentiation was defined as fully ($>50\%$), partly (0–50%) or no mucinous differentiation. Tumour-node-metastasis (TNM) was defined by the Union for International Cancer Control (UICC) (Schiffmann et al, 2013). Tumour differentiation was defined as good, moderate or poor, as described in the pathology report. Patients with rectal cancer, patients with a history of cancer other than basal cell carcinoma or cervical carcinoma in situ, patients with more than one colon tumour at the same time and patients who received radio- or chemotherapy treatment before resection were excluded from the analysis ($n = 185$ in total). The study cohort therefore consisted of 285 colon cancer patients.

Antibodies. The mouse monoclonal antibodies HCA2 and HC10 were used, which recognise the heavy chains of HLA Class I, and were kindly provided by Professor Dr. J. Neefjes. The reactivity spectrum of HCA2 comprises all HLA-A chains (except HLA-A24), as well as some HLA-B, HLA-C, HLA-E, HLA-F and HLA-G chains. HC10 reacts with HLA-B and HLA-C heavy chains and some HLA-A (HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32 and HLA-A33) (de Kruijf et al, 2010a). The mouse antibodies against human Foxp3 (ab20034 clone 236A/E7; Abcam) were used for Treg identification. The reactivity spectrum of Foxp3 is composed of regulatory T cells and may include small numbers of CD8 + cells but is generally considered to be the best single marker for Treg identification (Hill et al, 2007; Generali et al, 2009). For HLA-E and HLA-G identification, mouse monoclonal antibodies against HLA-E (ab2216 clone MEM-E/02: AbCam, UK) and HLA-G (4H84: Exbio, Czech Republic) were used. MEM-E/02 recognises denatured HLA-E (Menier et al, 2003; Lo et al, 2008), whereas 4H84 recognises denatured HLA-G molecules and also binds to free heavy chains of classical HLA class I molecules (Menier et al, 2003; Polakova et al, 2004; Zhao et al, 2012).

TMA production and immunohistochemistry. The histopathological characteristics of the tumour material from all patients included were determined by qualified pathologists according to the current standards. Of the formalin-fixed paraffin-embedded (FFPE) tumour blocks of the primary tumours, sections were cut for haematoxylin and eosin staining. On the basis of microscopic inspection of the slides, histopathologically representative bulk tumour regions from each tumour block were identified and punched for preparation of tumour tissue microarray (TMA) blocks. From each donor block, three 0.6 mm diameter tissue cores were punched from the identified tumour areas and transferred into a receiver paraffin block using a custom-made precision instrument. Immunohistochemical staining (IHC) for Foxp3 + cells, non-classical HLA-E and HLA-G and classical HLA class I tumour expression was performed on $4\,\mu$m-thick sections, which were cut from each receiver block and mounted on glass.

The sections were deparaffinised and rehydrated according to the standard procedures. Endogenous peroxidase was blocked for 20 min in 0.3% hydrogen peroxide in PBS. For antigen retrieval, slides were boiled in 0.01 M EDTA buffer (pH 8) for 10 min at maximum power in a microwave oven. Sections were incubated overnight with anti-Foxp3 +, -HLA-E or -HLA-G antibodies at predetermined optimal dilution. After 30 min of incubation with Envision anti-mouse (K4001; DAKO Cytomation, Glostrup, Denmark), sections were visualised using diaminobenzidine solution (DAB +). Tissue sections were counterstained with haematoxylin, dehydrated and finally mounted in pertex. The IHC for HCA2 and HC10 was performed using the Autostainer Link 48 (DAKO). For antigen retrieval, Envision TM Target Retrieval Solution (DAKO), pH low, was used. The sections were incubated for 18 h with either HCA2 or HC10 antibodies at predetermined

optimal dilution, followed by incubation with Envision FLEX/HRP (DAKO). Sections were visualised using DAB + liquid solution (DAKO). Finally these slides were counterstained with haematoxylin as well, dehydrated and finally mounted in pertex. All slides were stained simultaneously to avoid interassay variation. For each patient, normal epithelium, stromal cells or lymphoid cells served as internal positive control for HLA class I antibody reactivity. Placenta tissue slides served as positive control for HLA-E and HLA-G staining. Slides from human tonsil tissue served as positive control for Foxp3 + staining. Negative controls were tissue slides that did undergo the whole immunohistochemical staining without primary antibody.

Evaluation of immunohistochemistry. Microscopic analysis of HCA2, HC10, HLA-E and HLA-G expressions and presence of Foxp3 + cells was performed by two independent observers in a blinded manner (M.S.R.: 100% of the cohort, E.C.M.Z. 30% of the cohort). The Cohen's Kappa was > 0.75 for all stainings, indicating substantial agreement between the two observers. The scores of the three 0.6 mm punches were averaged. For HCA2 and HC10, the percentage of tumour cells with membranous staining was assessed. HLA class I expression status was determined according to the standard set by the International HLA and Immunogenetics Workshop (Chew *et al*, 2007). HCA2 and HC10 expression percentages were divided into two categories: 0–5% of the tumor cells show expression and 5–100% show expression. If < 5% of the tumour cells showed expression for each of the two markers, this was determined to represent loss of HLA class I expression; if expression in < 5% of the tumour cells of one of the two markers, this was determined as HLA class 1 downregulation; and if expression in > 5% of the tumour cells for each of the two markers, this was denoted as HLA class I expression. For HLA-E and HLA-G, intensity of tumour staining (absent, weak, moderate or strong intensity) was determined. For HLA-E, an absence of staining and weak staining together *vs* moderate and strong staining together were used for the final analysis. For HLA-G, an absence of tumour staining was analysed *vs* weak, moderate and strong tumour staining together, because HLA-G is normally not expressed on healthy tissues in comparison with HLA-E (Palmisano *et al*, 2005; Wischhusen *et al*, 2007). Quantification of the number of Foxp3 + cells was microscopically assessed in the entire tumour punches of the TMA, and the absolute number of positive cells was used for the analysis.

Determination of microsatellite stability status. DNA was extracted from 2 mm tumour cores. Paraffin was dissolved in xylene, tissue was rehydrated in ethanol (100%/70%) and dried for 10 min at 37 °C. Nucleospin 96 Tissue kit (Machery-Nagel, Düren, Germany) was used for DNA extraction according to the manufacturer's protocol.

MSS status was tested using the MSI Analysis System Version 1.2 (Promega, Mannheim, Germany) and interpreted by an experienced pathologist, as described previously (Zeestraten *et al*, 2012).

Statistical analysis. Statistical analyses were performed using the statistical package SPSS (version 17.0 for Windows; IBM, Armonk, NY, USA). The Student's *t*-test and the χ^2-test were used to evaluate associations between tumour expressions of HLA class I, and non-classical HLA-E and HLA-G and tumour infiltration of Foxp3 + cells and various clinico-pathological variables. Overall survival (OS) was defined as time of surgery until death and disease-free survival (DFS) as time of surgery until death or relapse of disease, whichever came first. The Kaplan–Meier method was used for calculation of survival probabilities and the log-rank test for comparison of survival curves between these three phenotypes. Cox regression was used for univariate and multivariate analysis

for OS and DFS. Significant variables ($P < 0.05$) in univariate analysis were included in multivariate analysis.

RESULTS

HLA class I expression. Microscopic quantification of HLA class I expression was performed on 242 patients as, due to staining artifacts and loss of material during the staining procedure, the
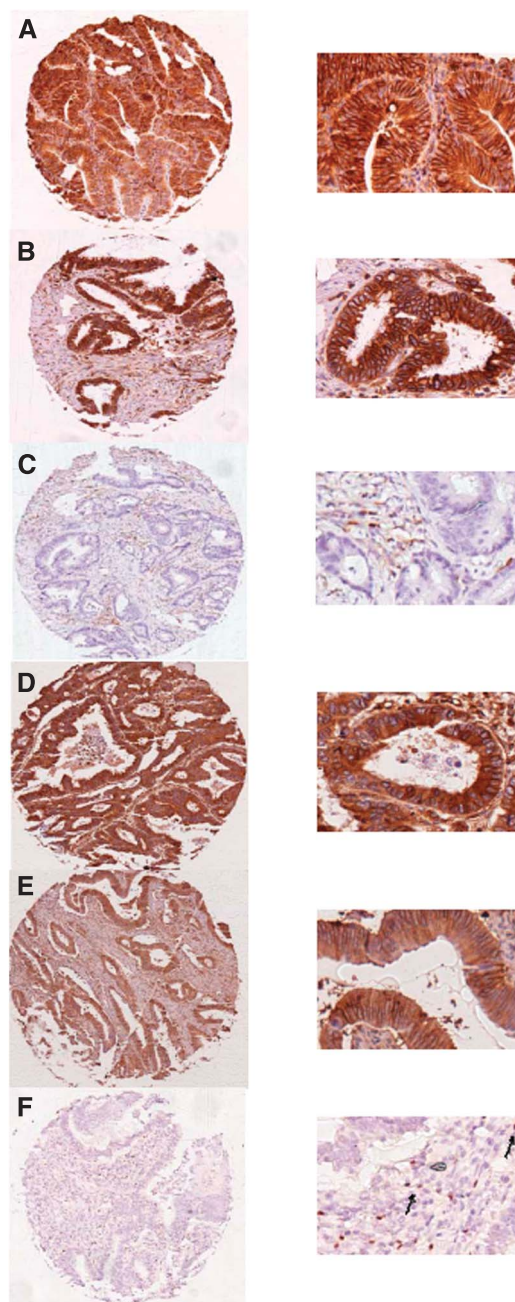
Figure 1. Representative images of HLA class I, HLA-E, HLA-G and Foxp3 + staining. Representative images of immuno-histochemical stainings for HLA Class I expression (HCA2 and HC10), HLA-E and HLA-G expression and presence of FOXP3 + on the left side with magnifications on the right side, performed according to standard protocols (details in *Material and Methods*). (**A**) HCA2-positive tumour (**B**) HC10-positive tumour (**C**) HC10-negative tumour with positive internal control (**D**) HLA-E-positive tumour (**E**) HLA-G- positive tumour and (**F**) Presence of Foxp3 + cells as indicated by the arrows.

IHC results of 43 cases could not be analysed. Representative images of HLA class 1 staining and frequencies of HLA class I expression in the different groups are shown in Figures 1 and 2. Patient characteristics and data on HLA class I expression are shown in Table 1. As HCA2 also reacts with some HLA-G chains (de Kruijf *et al*, 2010a), we examined the relationship between HCA-2 reactivity and HLA-G expression and found no correlation ($P = 0.348$).

Patients whose tumours showed loss of HLA class I had a significantly better OS and DFS (logrank *P*-value 0.005 and 0.008) compared with patients with tumours with HLA class I down-regulation or expression (Figure 3). The hazard ratios (HRs) for OS and DFS for HLA class I tumour expression are shown in Tables 2a and 2b.

Foxp3 + cells. The number of Foxp3 + cells could be evaluated only in 245 patients, because, due to staining artefacts and loss of material during the staining procedure, the IHC results of 40 cases could not be analysed. The mean number of positive cells per tumour punch was 19 with a median of 12.0. In 4.1% ($n = 10$) of the patients, no Foxp3 + cells were present. Representative images of Foxp3 + staining are shown in Figure 1. Patients with expression of HLA class I showed significant higher levels of Foxp3 + cells at borderline significance in their tumour punches compared with HLA class I downregulation or loss: a mean of 21 Foxp3 + cells in the expression group *vs* a mean of

12 and 14 positive cells in the downregulation group and in the loss of HLA class I group, respectively, $P = 0.07$. Patients with stage 1 tumours showed significantly higher levels of Foxp3 + cells compared with patients with stage 2, stage 3 and stage 4 tumours: mean level of Foxp3 + cells in stage 1 tumours was 38 compared with 13, 17 and 20 for the stage 2, 3 and 4 tumours, $P = <0.001$. For further analysis, Foxp3 + was categorised as below *vs* above median, based on the median due to the skewness in the spread of the data. Frequencies are shown in Figure 2. The presence of Foxp3 + cells in the tumour micro-environment was not related to OS (logrank *P*-value 0.114) or DFS (logrank *P*-value 0.155).

HLA-E and HLA-G. Representative images for HLA-E and HLA-G and frequencies in the different groups are shown in Figures 1 and 2. HLA-E and HLA-G were not related to OS and DFS (logrank *P*-values for OS 0.809 and 0.239, respectively, logrank *P*-values for DFS 0.876 and 0.117, respectively). None of the clinico-pathological characteristics were significantly related to tumour expression of HLA-E or HLA-G (data not shown).

A combined variable of HLA-E and HLA-G scores was created (cEG). Expression was considered positive when both HLA-E and HLA-G were expressed (HLA-E+/-G+ further denoted as cEG +) and negative when either HLA-E or HLA-G was not expressed (HLA-E+/-G- or HLA-E-/-G+ or HLA-E-/-G- further denoted as cEG-). Positive cEG was found in 14.7% (42 of 244) of tumours. Patient characteristics and data on the combined variable
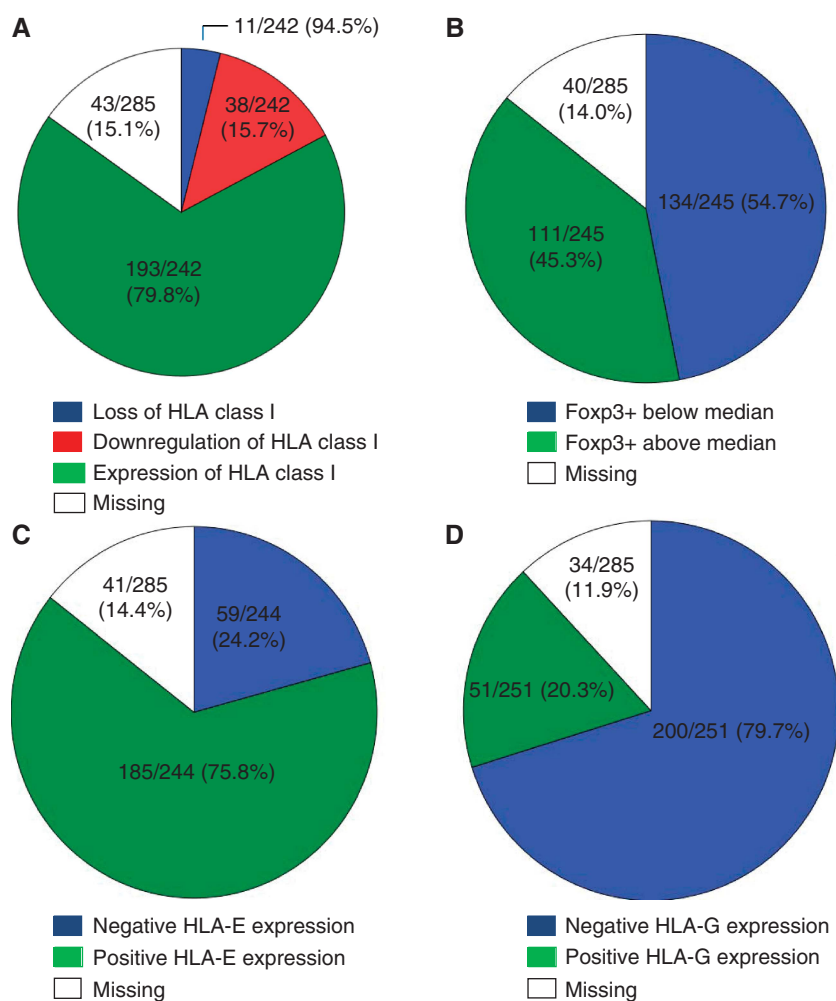

Figure 2. Frequencies of HLA class I tumour expression, Foxp3 + tumour infiltration and HLA-E and -G tumour expression. Pie-charts indicating the frequencies of all stainings including missings due to staining artifacts and loss during the staining procedure. Details about group composition and scoring methods are written in *Material and Methods*. (**A**) Frequency of HLA class I tumour expression. (**B**) Frequency of Foxp3 + tumour cell infiltration. (**C**) Frequencies of HLA-E tumour expression. (**D**) Frequency of HLA-G tumour expression.

Table 1. Patient characteristics of the total colon cancer cohort and stratified for HLA class I, HLA-EG and Foxp3+ expression

	Total population (n = 285)	HLA class 1 Loss (n = 11)	HLA class 1 downregulation (n = 38)	HLA class 1 expression (n = 193)	HLA-EG absence (n = 202)	HLA-EG presence (n = 42)	Foxp3+ absence (n = 134)	Foxp3+ presence (n = 111)
Gender (%)								
Male	137 (48.1)	6 (54.5)	18 (47.4)	99 (51.3)	103 (51.0)	17 (41.5)	63 (47.0)	57 (51.4)
Female	148 (51.9)	5 (45.5)	20 (52.6)	94 (48.3)	99 (49.0)	25 (58.5)	71 (53.0)	54 (48.6)
Age in years (%)								
Below 50	32 (11.3)	3 (27.3)	1 (2.6)	23 (12.0)	24 (12.0)	3 (7.1)	13 (9.7)	14 (12.8)
Above 50	251 (88.7)	8 (72.9)	37 (97.4)	168 (88.0)	176 (88.0)	39 (92.9)	121 (90.3)	95 (87.2)
T stage (%)								<0.001
1	17 (6.0)	0	2 (5.3)	12 (6.3)	11 (5.5)	3 (7.1)	3 (2.3)	11 (9.9)
2	37 (13.0)	1 (9.1)	1 (2.6)	25 (13.0)	24 (11.9)	4 (9.5)	7 (5.3)	21 (18.9)
3	193 (68.0)	8 (72.7)	26 (68.4)	135 (70.3)	143 (71.1)	27 (64.3)	104 (78.2)	67 (60.4)
4	37 (13.0)	2 (18.2)	9 (23.7)	20 (10.4)	23 (11.4)	8 (19.0)	19 (14.3)	12 (10.8)
Differentiation (%)								
Moderate	145 (64.2)	4 (44.4)	16 (64.0)	104 (65.4)	104 (64.2)	20 (64.5)	69 (64.5)	56 (64.4)
Poor	23 (10.2)	0 (0)	3 (12.0)	18 (11.3)	15 (9.3)	6 (19.4)	11 (10.3)	10 (11.5)
Good	58 (25.7)	5 (55.6)	6 (24.0)	37 (23.3)	43 (26.5)	5 (16.1)	27 (25.2)	21 (24.1)
Mucinous aspect (%)								
No	233 (83.5)	7 (63.6)	28 (77.8)	165 (86.8)	164 (83.2)	36 (85.7)	105 (80.2)	96 (88.1)
Fully	33 (11.8)	4 (36.4)	6 (16.7)	15 (7.9)	24 (12.2)	3 (7.1)	18 (13.7)	9 (8.3)
Partly	13 (4.6)	0 (0)	2 (5.6)	10 (5.3)	9 (4.6)	3 (7.1)	8 (6.1)	4 (3.7)
Microsatellite stability (%)								
MSS	168 (84.8)	6 (66.7)	25 (86.2)	132 (87.4)	136 (86.6)	29 (85.3)	84 (81.6)	81 (92.0)
MSI	30 (15.2)	3 (33.3)	4 (13.8)	19 (12.6)	21 (13.4)	5 (14.7)	19 (18.4)	7 (8.0)

Abbreviations: MSI = Microsatellite instability; MSS = microsatellite stability. This table shows the patient characteristics of the entire colon cancer cohort (n = 285) and stratified according to HLA class 1, HLA-EG and Foxp3+ staining. Only T stage was significantly related to Foxp3+. Note: HLA-EG is a combination of HLA-E and HLA-G (explained in the material and methods section).

HLA-E and -G expression can be found in Table 1. None of the clinico-pathological variables shown in Table 1 were significantly related to tumour expression of cEG. cEG was not significantly related to OS (logrank P-value 0.245) and DFS (logrank P-value 0.100).

Multivariate analysis for single immune markers. Both for OS and DFS, a univariate analysis was performed for the following parameters: sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumour grade, adjuvant therapy and microsatellite status. In the univariate analysis for OS, age (P-value <0.001), TNM status (P-value <0.001) and HLA class I expression status (P-value 0.011) were significant predictors of survival. The same was true for the univariate analysis for DFS with a P-value of <0.001 for age and TNM status and a P-value of 0.02 for HLA class I expression. Therefore, all three were included in the multivariate analysis. In this analysis, age and TNM stage remained significant for both OS and DFS (OS and DFS P-values all <0.001): HLA class I was a borderline independent significant predictor for OS (P-value 0.08) (Tables 2a and 2b).

Analysis of tumour immune phenotypes. Except for HLA class I, none of the tumour immune markers showed a significant correlation with patients' clinical outcome. The interaction between tumour cells and immune cells, however, is complex and multifaceted. Therefore, we hypothesised that analysis of combined tumour immune markers; describing a tumour's immune phenotype may better reflect outcome of the interaction between tumour cells and the immune system. We combined all of

the data into one combined variable. The Kaplan–Meier curves performed with this combined variable indeed revealed three distinct patterns in relation to patient outcome (Figures 4 and 5). The entire population could be divided into three phenotypes:

(a) Strong immune system tumour recognition: patients with tumours that showed loss of HLA class I expression, presence of Foxp3+ cells in the tumour micro-environment and negative cEG expression (n = 11).

(b) Intermediate immune system tumour recognition: patients with tumours that showed downregulation of HLA class I expression and negative cEG expression but were found to have Foxp3+ cells in the tumour micro-environment or patients with tumours that showed normal HLA class I expression irrespective of cEG expression and the presence of Foxp3+ cells (n = 184).

(c) Poor immune system tumour recognition: patients with tumours showing normal or downregulated HLA class I and no presence of Foxp3+ cells irrespective of their cEG expression (n = 460).

These three phenotypes showed significant differences for OS (logrank P-value <0.001) and DFS (logrank P-value 0.001). The HRs of the three phenotypes for OS and DFS are shown in Tables 2a and 2b.

Multivariate analysis for tumour immune phenotypes. Again, both for OS and DFS, a univariate analysis was performed for the
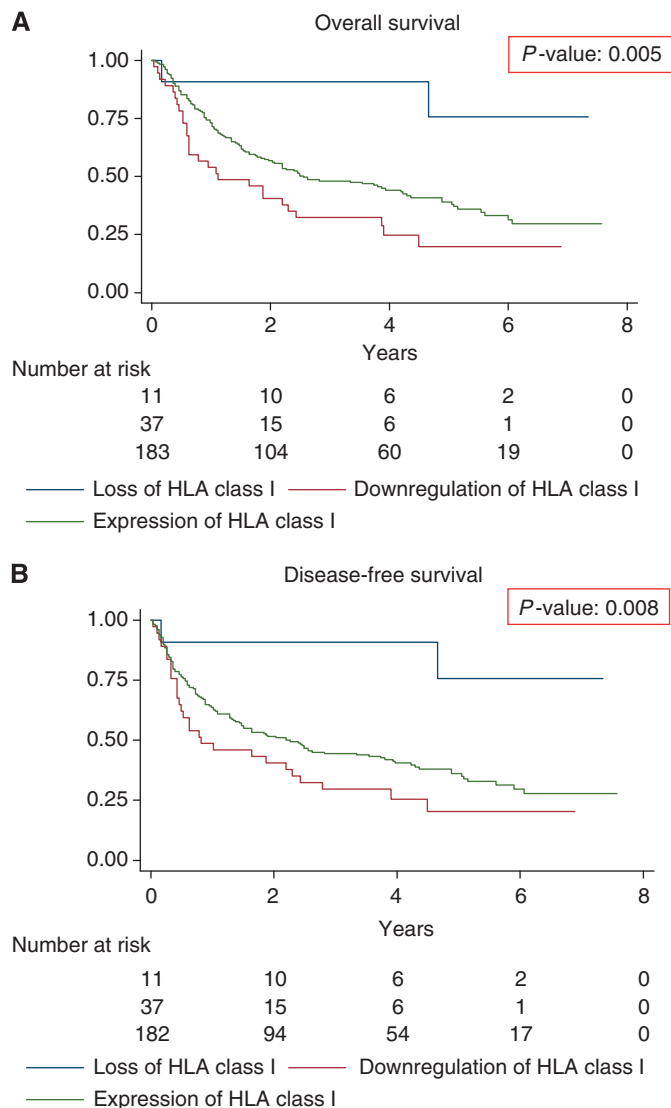
Figure 3. Survival curves stratified for HLA class I tumour expression in colon cancer. (A) Kaplan–Meier curve for OS in the study population of 285 colon cancer patients stratified for HLA class I tumour expression status. **(B)** Kaplan–Meier curve for DFS in the study population of 285 CRC patients again stratified for HLA class I tumour expression.

following parameters: sex, age, TNM stage, tumour immune phenotype, mucinous differentiation, tumour grade, adjuvant therapy and microsatellite status. In univariate analysis, next to age and TNM status, the tumour immune phenotype was a significant predictor for OS (P-value 0.001) and DFS (P-value 0.002). Therefore, all these three parameters were included in multivariate analysis. The tumour immune phenotype was an independent significant predictor for both OS (P-value 0.009) and DFS (P-value 0.013), and HRs are shown in Tables 2a and 2b.

DISCUSSION

Tumour–immune interactions may be important for the prognosis of cancer patients (Khong and Restifo, 2002). In this study, we showed that, by combining the immune-related markers HLA class I, HLA-E, HLA-G and Foxp3 +, we were able to determine three distinct patterns in survival, which might represent how immune surveillance controls tumour growth and metastasis.

The first marker of tumour immunogenicity used was the level of HLA class I expression of cancer cells. Our results are comparable with the results of other studies that were able to determine a prognostic effect of the HLA class I status in colon cancer (Menon et al, 2002; Watson et al, 2006). Watson et al (2006) showed that tumours with downregulation of HLA class I had a worse survival comparable with our results. In contrast, Menon et al (2002) showed a survival benefit in patients with downregulated HLA-A tumours. However, when HLA-A and HLA-B/C were combined, statistical significance was lost. Further, patients with expression of HLA class I were related to a better survival in the study by Watson et al (2006), whereas our study showed an improved survival in patients with loss of HLA class I expression. Possible explanations for these differences might be a different definition for HLA class I expression, differences in staining techniques and scoring or a different patient cohort, especially regarding the number of tumours showing microsatellite instability (MSI), which is associated with loss of HLA class I and a better prognosis (Dierssen et al, 2007; Mouradov et al, 2013). In our study, 33% of the tumours with loss of HLA class I showed the MSI phenotype, in comparison with

Table 2a. Univariate and multivariate analyses of overall survival in the different immune markers and in the tumour immune phenotypes

	Univariate analysis			Multivariate analysis[a]		
	HR	95% CI	P-value	HR	95% CI	P-value
HLA class I			0.011			0.08
Loss	1.0	1.7–30.8		1.0	1.0–18.5	
Downregulation	7.3	1.2–20.0		4.3	0.7–12.6	
Expression	4.9			3.1		
Foxp3 +			0.116			
Below median	1.0	0.6–1.1				
Above median	0.8					
HLA-E			0.810			
Negative	1.0	0.7–1.4				
Positive	1.0					
HLA-G			0.242			
Negative	1.0	0.9–1.8				
Positive	1.2					
HLA-EG			0.248			
Negative	1.0	0.8–1.9				
Positive	1.3					
Immune phenotypes			0.001			0.009
Phenotype a	1.0	1.2–19.0		1.0	0.7–11.9	
Phenotype b	4.7	2.0–34.2		2.9	1.1–20.2	
Phenotype c	8.2			4.8		

[a]Corrected for sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumour grade, adjuvant therapy and microsatellite status. Only significant variables in univariate analysis are corrected in multivariate analysis. Note: HLA-EG is a combination of HLA-E and HLA-G (as explained in the results section).

Table 2b. Univariate and multivariate analyses of disease-free survival (DFS) in the different immune markers and in the tumour immune phenotypes

|	Univariate analysis			Multivariate analysis[a]		
	HR	95% CI	P-value	HR	95% CI	P-value
HLA class I			0.021			0.104
Loss	1.00	1.7–30.1		1.00	1.1–19.7	
Downregulation	7.2	1.3–21.8		4.6	0.9–15.0	
Expression	5.4			3.7		
Foxp3 +			0.159			
Below median	1.00	0.6–1.1				
Above median	0.8					
HLA-E			0.877			
Negative	1.00	0.7–1.4				
Positive	0.97					
HLA-G			0.121			
Negative	1.00	0.9–1.9				
Positive	1.3					
HLA-EG			0.104			
Negative	1.00	0.9–2.1				
Positive	1.4					
Immune phenotypes			0.002			0.013
Phenotype a	1.00	1.3–20.7		1.00	0.8–14.2	
Phenotype b	5.1	2.0–34.9		3.5	1.3–22.7	
Phenotype c	8.4			5.4		

[a]Corrected for sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumour grade, adjuvant therapy and microsatellite status. Only significant variables in univariate analysis are corrected in multivariate analysis. Note: HLA-EG is a combination of HLA-E and HLA-G (as explained in the results section).

14% and 13% for HLA class I downregulation and expression. Results from Menon et al (2002) showed that 50% of the tumours with loss of HLA class I had the MSI phenotype. Unfortunately, Watson et al (2006) did not mention microsatellite status of their study cohort.

As hypothesised, loss of HLA class I expression in tumour cells could also be related to a better patient survival because such cells, once they metastasise to the bloodstream, are eliminated by NK cell attacks (Menon et al, 2002; Hokland and Kuppen, 2005; Watson et al, 2006). Tumours with loss of HLA class I have also shown to have significantly higher NK cell infiltration (Menon et al, 2004b). More interestingly, the tumours showing loss of HLA class I in our cohort were also the ones that showed to be negative for HLA-E and -G expression (phenotype a). Absence of the HLA-E and -G expression makes them even more susceptible to NK cell elimination (Khong and Restifo, 2002; Marin et al, 2003; Wischhusen et al, 2007; Zilberman et al, 2012). Further, this is also confirmed by CRC tumours with the loss of HLA class I expression, which do not metastasise to the liver (Tikidzhieva et al, 2012).

The presence of the third marker Foxp3 + is thought to represent the inhibition of host-protective antitumour responses. When stimulated, they inhibit the function of CTL (Schreiber et al, 2011). Although the exact mechanism by which these cells are drawn into the tumour micro-environment remains unexplained, their immunosuppressive effect has been proven with a high density of tumour-infiltrating Foxp3 + cells found to be associated with an unfavourable prognosis in a wide range of human carcinomas, including breast and lung cancer (Bates et al, 2006; Petersen et al, 2006). However, in colon cancer, different results are reported as well (Salama et al, 2009; Suzuki et al, 2010). One possible explanation for these opposite results might be a different micro-environment of colon cancer, which is colonised with many gastro-intestinal bacteria, triggering the production of pro-inflammatory cytokines causing tumour-enhancing effects. Instead of the specificity of infiltrating T cells for tumour antigens, T cells in colon cancer could be more specific for the microflora and suppress inflammation and immune responses from bacterial invasion, resulting in an antitumourigenic effect, which could explain the better prognosis of patients with tumours with a strong Foxp3 + infiltration (Ladoire et al, 2011). We were not able to demonstrate differences in disease outcome for Foxp3 + tumour infiltration supporting this latter hypothesis, but we did see differences in Foxp3 + infiltration if we combined them with HLA class I expression and with HLA-E and -G expression, especially in patients who have retained their HLA class I expression. Patients with normal HLA class I expression and absence of Foxp3 + cell infiltration showed a worse patient outcome. We hypothesise that the tumours of these patients have had a minimal CTL attack because the HLA class I expression is preserved, indicating no selective outgrowth of HLA class I-negative or downregulated tumours directed by CTL. As CTL and Foxp3 + cells show opposing actions (Liu et al, 2011) and CTLs are supposed to be absent in these tumours, Foxp3 + cell infiltration might not be necessary. These tumours could therefore progress aggressively as immune surveillance is poor. In contrary, tumours with HLA class I expression, which were able to attract Foxp3 + cells, showed a slightly better prognosis. In this case, Foxp3 + cell infiltration might indicate CTL activity, resulting in suppression of tumour growth.
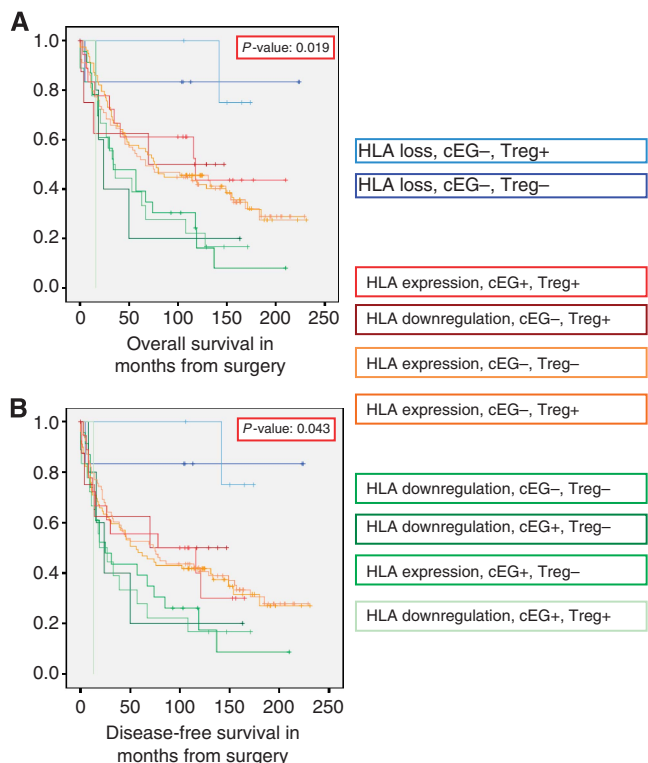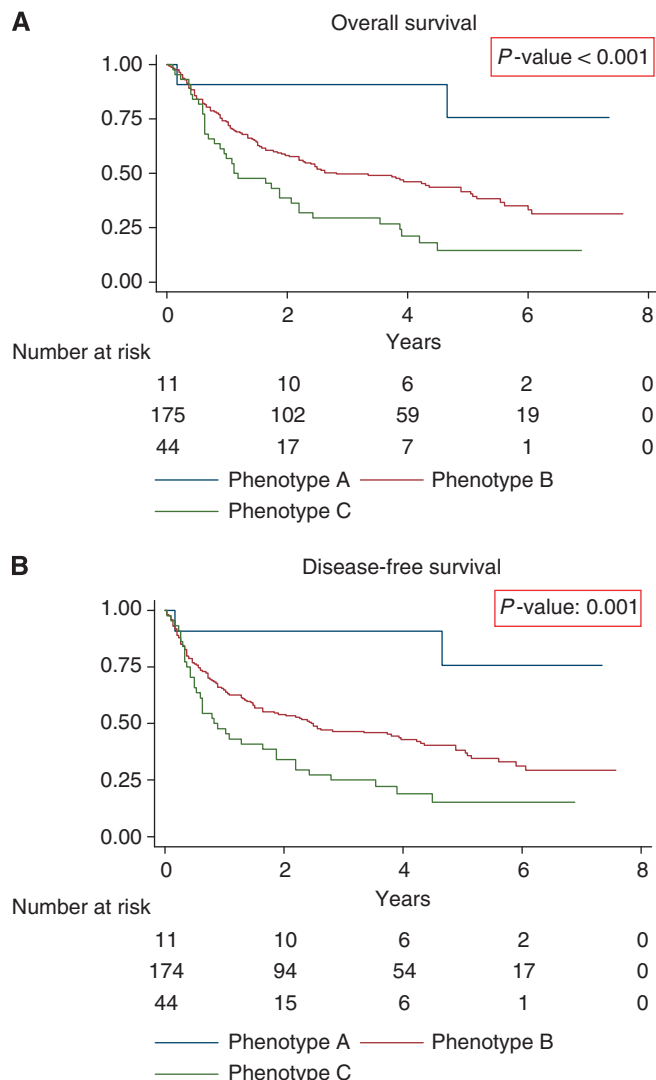
Figure 4. Survival curves stratified for combined tumour expression of HLA class I, HLA-E, HLA-G and Foxp3+ in colon cancer. (**A**) Kaplan–Meier curve for OS in the study population of 285 colon cancer patients stratified for all the different combinations between tumour expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which three distinct patterns could be distinguished as shown in Figure 5 (**B**) Kaplan–Meier curve for DFS in the study population of 285 colon cancer patients stratified for all the different combinations between tumour expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which three distinct patterns could be distinguished as shown in Figure 5.

Figure 5. Survival curves stratified for immune phenotypes in colon cancer. (**A**) Kaplan–Meier curve for OS in the study population of 285 colon cancer patients stratified for all the different combinations between tumour expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs), based on which three immune phenotypes could be distinguished. See results section for explanation of the phenotypes. (**B**) Kaplan–Meier curve for DFS in the study population of 285 colon cancer patients stratified for all the different combinations between tumour expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs), based on which three immune phenotypes could be distinguished. See results section for explanation of the phenotypes.

Therefore, in our opinion, the clinical relevance of the studies by Watson *et al* (2006) and several others does not provide an optimal perspective on prognosis (Menon *et al*, 2002) because expression of a single immune marker is not sufficient for the selection of high-risk colon cancer patients or treatment allocation. As shown by our results and previous studies, immune markers are related to each other (Galon *et al*, 2006; de Kruijf *et al*, 2010a, b; Angell and Galon, 2013).

When all markers were combined, patients showing the worst prognosis were patients with HLA class I downregulation, negative or positive cEG expression and absence of Foxp3+ cells denoted as phenotype c. We hypothesise that these poor immune system recognised tumours were able to elicit only a minimal CTL attack because they partly preserved HLA class I expression and subsequently attracted little to no Foxp3+ cells in their tumour micro-environment. Furthermore, these tumours showed a positive expression of HLA-E and -G, further escaping immune surveillance through inhibition of NK-cell recognition (Khong and Restifo, 2002; Marin *et al*, 2003; Wischhusen *et al*, 2007; Zilberman *et al*, 2012). These tumour cells can therefore quickly progress to the bloodstream and might eventually metastasise.

It is important to realise that what we are evaluating is just a 'snapshot' of the ongoing process of cancer immune editing in the patient's primary tumour at time of resection. Still, from a clinical point of view, at the patient's bedside this is usually the only data

available based on which clinical decision making has to take place, and these data can actually be of clinical value to, for example, the allocation of adjuvant therapy as opposed by de Kruijf *et al* (2010b) in breast cancer and other studies (Ghiringhelli *et al*, 2004; Ladoire *et al*, 2008;).

Our study does have a few limitations. Not all combinations between HLA class I, HLA-E and -G and Foxp3+ were present in our cohort. There was no representation of tumours with loss of HLA class I, which were HLA-E and -G positive. Therefore, we were not able to investigate the prognosis of these tumours, but we hypothesise that these tumours have a worse prognosis as these tumours might escape NK-cell attack. Although there is a physiological correlation between HLA-E and HLA class I

molecules, this has been found to be disturbed in tumours, suggesting further escape from immune recognition through upregulation of HLA-E (de Palmisano et al, 2005; Kruijf et al, 2010a). To truly investigate these tumours, our study has to be validated in a bigger cohort. Second, the antibodies we used for HLA class I detection only detected the heavy chain but not the trimeric complex consisting of β2-microglobuline heavy chain and antigen (Perosa et al, 2003). Therefore, we should be careful using the term total loss of HLA class I. Third, we did not investigate the role of NK cells in patients with loss or downregulation of HLA class I, possibly explaining the positive prognostic effect of patients with loss of HLA class I expression. However, NK-cell infiltration at the tumour site is scarce, indicating that tumour staining for NK cells might be minimally informative (Sandel et al, 2005).

In conclusion we were able to identify local immune escape mechanisms of colon cancer, where the presence of Foxp3 + cell infiltration favors a better prognosis, indicating CTL activity. HLA-E and -G expressions might have a pivotal role in distant immune escape mechanisms; in case of loss or downregulation of HLA class I, HLA-E and -G expressions determine distant metastases and prognosis of colon cancer patients. Furthermore, we were able to determine three distinct survival patterns in colon cancer patients based on immune surveillance. In the future, these findings might contribute to better treatment allocation and maybe even the development of new cancer immuno-therapies.

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