Prognostic and predictive biomarkers in colorectal cancer. Towards precision medicine

Reimers, M.S.

PART ONE

Prognostic biomarkers in colorectal cancer

CHAPTER 2

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

Marlies S. Reimers*, Eliane C.M. Zeestraten*, Sepideh Saadatmand, Jan-Willem T. Dekker, Gerrit Jan Liefers, Peter J.van den Elsen, Cornelis J.H. van de Velde, Peter J.K. Kuppen

** Both authors contributed equally to this work*

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ABSTRACT

Background

Evasion of immune surveillance and suppression of the immune system are important hallmarks of tumor development in colon cancer. The goal of this study was to establish a tumor profile based on biomarkers that reflect a tumors' 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 immuno-histochemically stained for presence of Foxp3+ cells and tumor 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 3 tumor immune phenotypes: a, strong immune system tumor recognition; b, intermediate immune system tumor recognition; and c, poor immune system tumor 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 tumors that 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 to 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). Furthermore, the tumor immune phenotype was an independent predictor for OS and DFS (*p*=0.009 and 0.013 respectively).

Conclusions

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

INTRODUCTION

Historically, the immune system has been attributed an important role in controlling tumor growth and metastasis [1-4]. Evasion of immune surveillance and suppression of the immune system are two important traits cancer cells have to acquire during the process of tumorigenesis [5]. Research of the last century has indicated that the influence of the immune system on tumor cells, both in the tumor micro-environment as well as during the process of tumor metastasis, also contributes to tumor progression [6]. The cancer immune-editing hypothesis describes both the host-protective as well as the tumor-promoting actions the immune system might have on developing tumors, shaping tumor immunogenicity [7-13]. Tumors are thought to be 'edited' through a Darwinian selection process into poorly immunogenic tumor 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 tumor cell level contribute to this process. The first mechanism is downregulation of human leukocyte antigen (HLA) class I expression. Downregulation of HLA class I minimizes the level of tumor-associated antigen (TAA) expression by tumor cells and therefore their recognition and subsequently destruction by cytotoxic T-cells (CTL) [5;14-16]. The second mechanism is the ability of tumor 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 tumor cell escape from immune surveillance [17-20]. HLA-E is regularly expressed in various healthy tissues and correlated with HLA class I expression [21]. In contrast, HLA-G is rarely found in healthy tissues, but is frequently observed in tumors [19]. Thirdly, tumor cell immune reactivity can become suppressed by the attraction of immunosuppressive regulatory T cells (Tregs) into the tumor micro-environment [22;23]. Tregs are able to modulate the anti-tumor 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 [24-26]. Tregs and CTLs therefore show opposing actions in tumor immunity [27].

Previously, both the downregulation of HLA class I, presence of Tregs and HLA-E and -G expression have been shown to be of clinical relevance in several types of cancers [28-31]. In colorectal cancer (CRC), various studies have described the impact of the level of HLA class I tumor expression or the presence of Foxp3+ Tregs cells on patients with varying results [32-39]. In general, loss of HLA class I tumor expression seemed to result in a better prognosis [39;40]. 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 [33;34;37;38;41]. Studies on the prognostic value of HLA-E and

HLA-G showed that expression of these molecules correlated with poor prognosis and tumor progression [42-45].

Previous studies have shown a complex interaction between different immune markers, highlighting the need for combined marker analysis [29;41;46]. 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 tumor'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 tumor in the Leiden University Medical Center (LUMC) between 1991 and 2001. Of these patients tumor material, clinico-pathological data and information on the follow-up was 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. Tumor Node Metastasis (TNM) was defined by the Union for International Cancer Control (UICC) [47]. Tumor 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 tumor at the same time, and patients that received radio- or chemotherapy treatment prior to 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 recognize the heavy chains of HLA Class I, and were kindly provided by Prof. 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, HLA-A33) [46]. 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 [48;49]. 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 recognizes

denatured HLA-E [50;51], while 4H84 recognizes denatured HLA-G molecules and also binds to free heavy chains of classical HLA class I molecules [51-53].

TMA production and immunohistochemistry

The histo-pathological characteristics of the tumor material from all patients included were determined by qualified pathologists according to current standards. Of the formalin-fixed paraffin-embedded (FFPE) tumor blocks of the primary tumors, sections were cut for haematoxylin and eosin staining. Based on microscopic inspection of the slides, histo-pathologically representative bulk tumor regions from each tumor block were identified and punched for preparation of tumor tissue microarray (TMA) blocks. From each donor block, three 0.6 mm diameter tissue cores were punched from the identified tumor areas and transferred into a receiver paraffin block using a custom-made precision instrument. Immuno-histochemical staining (IHC) for Foxp3+ cells, non-classical HLA-E and HLA-G, and classical HLA class I tumor expression was performed on 4 µm sections, which were cut from each receiver block and mounted on glass.

The sections were deparaffinized and rehydrated according to standard procedures. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen peroxide in PBS. For antigen retrieval, slides were boiled in 0.01 M EDTA buffer (pH 8) for 10 minutes at maximum power in a microwave oven. Sections were incubated overnight with anti-Foxp3+, -HLA-E or -HLA-G antibodies at pre-determined optimal dilution. After 30 minutes of incubation with Envision anti-mouse (K4001; DAKO Cytomation, Glostrup, Denmark), sections were visualized 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 hours with either HCA2 or HC10 antibodies at pre-determined optimal dilution, followed by incubation with Envision FLEX/HRP (DAKO). Sections were visualized 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 expression 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 tumor 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 [54]. 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 tumor 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 tumor cells of one of the two markers as HLA class 1 downregulation; and if expression in more than 5% of the tumor cells for each of the two markers this was denoted as HLA class I expression. For HLA-E and HLA-G, intensity of tumor staining (absent, weak, moderate or strong intensity) was determined. For HLA-E, absent and weak staining together versus moderate and strong staining together were used for the final analysis. For HLA-G, absent tumor staining was analyzed versus weak, moderate and strong tumor staining together, because HLA-G is normally not expressed on healthy tissues in comparison to HLA-E [19;21]. Quantification of the number of Foxp3+ cells was microscopically assessed in the entire tumor 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 2mm tumor-cores. Paraffin was dissolved in xylene, tissue was rehydrated in ethanol (100%/70%) and dried for 10 minutes 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 [55].

Statistical Analysis

Statistical analyses were performed using the statistical package SPSS (version 17.0 for Windows; SPSS Inc.). The Student's T-test and the Chi-squared test were used to evaluate associations between tumor expressions of HLA class I, and non-classical HLA-E and HLA-G and tumor 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 multivariable analysis for OS and DFS. Significant variables ($p<0.05$) in univariate analysis were included in multivariable 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 IHC results of 43 cases could not be analyzed. Representative images of HLA class 1 staining and frequencies of HLA class I expression in the different groups are shown in Figure 1 and 2. Patient characteristics and data on HLA class I expression are shown in

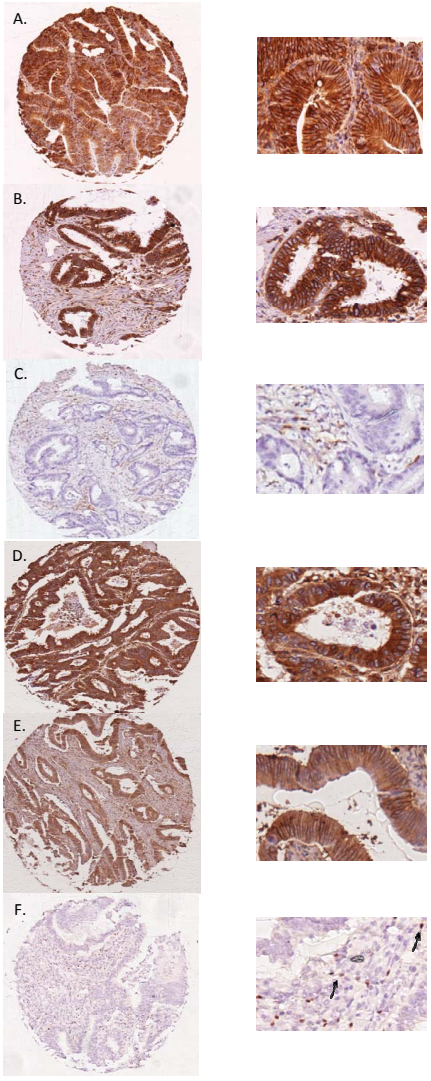


Figure 1: Representative images of HLA class 1, HLA-E, HLA-G and Foxp3+ staining.
Representative images of immunohistochemical stainings for HLA Class I expression (HCA2 and HC10), HLA-E and HLA-G expression and presence of FOXP3+ on the left side with magnifications on the right side, performed according to standard protocols (details in *Material and Methods*).
(A) HCA2-positive tumor (B) HC10-positive tumor (C) HC10- negative tumor with positive internal control (D) HLA-E-positive tumor (E) HLA-G- positive tumor and (F) Presence of Foxp3+ cells as indicated by the arrows.

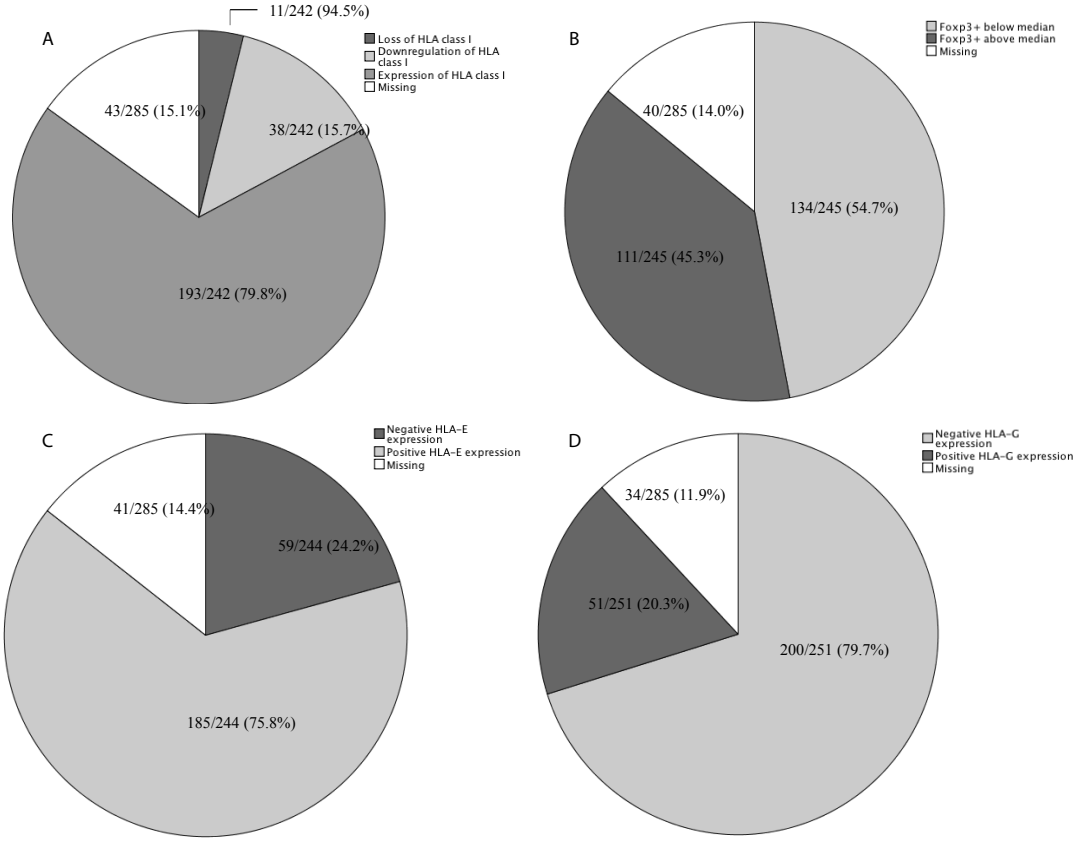


Figure 2: Frequencies of HLA class I tumor expression, Foxp3+ tumor infiltration and HLA-E and –G tumor expression.

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 tumor expression; (B) Frequency of Foxp3+ tumor cell infiltration; (C) Frequencies of HLA-E tumor expression; (D) Frequency of HLA-G tumor expression.

Table I. Since HCA2 also reacts with some HLA-G chains [46], we examined the relationship between HCA-2 reactivity and HLA-G expression and found no correlation (p=0.348).

Patients whose tumors showed loss of HLA class I had a significantly better OS and DFS (logrank p-value 0.005 and 0.008) compared to patients with tumors with HLA class I downregulation or expression (Figure 3). The Hazard Ratios (HRs) for OS and DFS for HLA class I tumor expression are shown in Table IIa and IIb.

Table I: 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)

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+. Abbreviations. MSS; microsatellite stability, MSI; Microsatellite instability.
Note: HLA-EG is a combination of HLA-E and HLA-G (explained in the material and methods section).

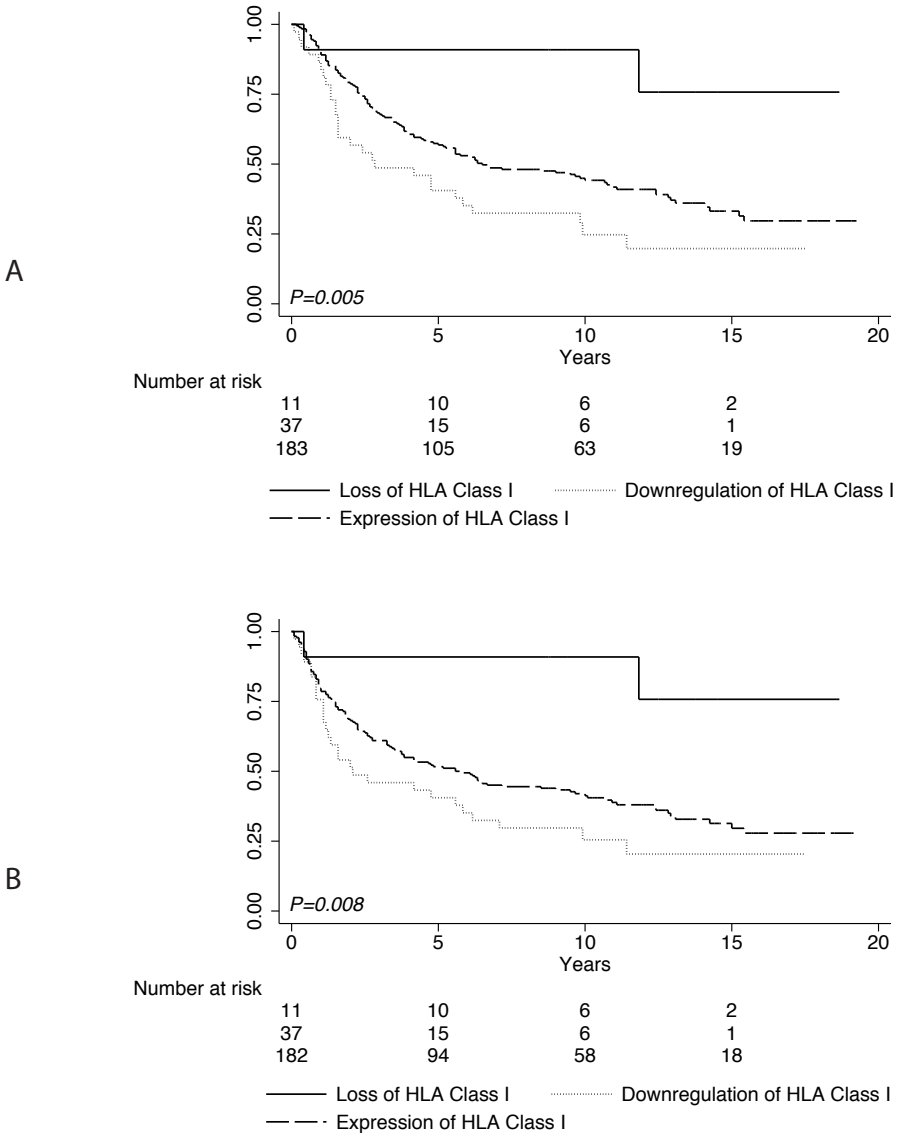


Figure 3: Survival curves stratified for HLA class I tumor expression in colon cancer.
A) Kaplan Meier curve for OS in the study population of 285 colon cancer patients stratified for HLA class I tumor expression status. B) Kaplan Meier curve for DFS in the study population of 285 colon cancer patients again stratified for HLA Class I tumor expression.

Foxp3+ cells

The number of Foxp3+ cells could be evaluated in 245 patients, because, due to staining artifacts and loss of material during the staining procedure, the IHC results of 40 cases could not be analyzed. The mean number of positive cells per tumor 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 borderline significantly higher levels of Foxp3+ cells in their tumor punches compared to HLA class I downregulation or loss: mean in expression

group 21 vs. a mean of 12 and 14 positive cells in the downregulation group and loss of HLA class I group respectively; p-value 0.07. Patients with stage 1 tumors showed significantly higher levels of Foxp3+ cells compared to patients with stage 2, stage 3 and stage 4 tumors: mean level of Foxp3+ cells in stage 1 tumors was 38 compared to 13, 17 and 20 for the stage 2, 3 and 4 tumors, *p*-value <0.001. For further analysis Foxp3+ was categorized 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 tumor 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 Figure 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 tumor 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 tumors. Patient characteristics and data on the combined variable HLA-E and -G expression can be found in Table I. None of the clinico-pathological variables shown in Table I were significantly related to tumor expression of cEG. cEG was not significantly related to OS (logrank *p*-value 0.245) and DFS (logrank *p*-value 0.100).

Multivariable analysis

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, tumor 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 multivariable 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) (Table IIa and IIb).

Table IIa: Univariate and multivariable analyses of Overall Survival (OS) in the different immune markers and in the tumor immune phenotypes

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

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

Table IIb: Univariate and multivariable analyses of Disease Free Survival (DFS) in the different immune markers and in the tumor immune phenotypes.

	Univariate analysis			Multivariable analysis*		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
HLA class I			0.021			0.104
Loss	1.00			1.00		
Downregulation	7.2	1.7-30.1		4.6	1.1-19.7	
Expression	5.4	1.3-21.8		3.7	0.9-15.0	
Foxp3+			0.159			
Below median	1.00					
Above median	0.8	0.6-1.1				

Table IIb: Univariate and multivariable analyses of Disease Free Survival (DFS) in the different immune markers and in the tumor immune phenotypes. *Continued*

	Univariate analysis			Multivariable analysis*		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
HLA-E			0.877			
Negative	1.00					
Positive	0.97	0.7-1.4				
HLA-G			0.121			
Negative	1.00					
Positive	1.3	0.9-1.9				
HLA-EG			0.104			
Negative	1.00					
Positive	1.4	0.9-2.1				
Immune phenotypes			0.002			0.013
Phenotype a	1.00			1.00		
Phenotype b	5.1	1.3-20.7		3.5	0.8-14.2	
Phenotype c	8.4	2.0-34.9		5.4	1.3-22.7	

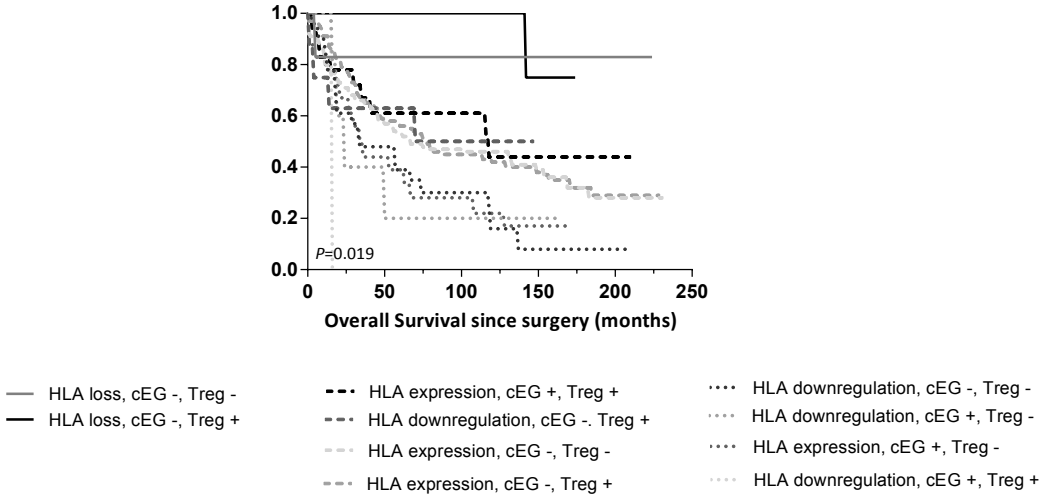
**Corrected for sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumor grade, adjuvant therapy and microsatellite status. Only significant variables in univariate analysis are corrected in multivariable analysis. Note: HLA-EG is a combination of HLA-E and HLA-G (as explained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

Analysis of tumor immune phenotypes

Except for HLA class I, none of the tumor immune markers showed a significant correlation with patients' clinical outcome. The interaction between tumor cells and immune cells, however, is complex and multifaceted. Therefore, we hypothesized that analysis of combined tumor immune markers; describing a tumor's immune phenotype may better reflect outcome of the interaction between tumor 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 3 distinct patterns in relation to patient outcome (Figure 4 and 5). The entire population could be divided in 3 phenotypes:

a) Strong immune system tumor recognition: Patients with tumors that showed loss of HLA class I expression, presence of Foxp3+ cells in the tumor micro-environment, and negative cEG expression (n=11).
b) Intermediate immune system tumor recognition: Patients with tumors that showed downregulation of HLA Class I expression and negative cEG expression, but were found to have Foxp3+ cells in the tumor micro-environment or patients with tumors that showed normal HLA class I expression irrespective of cEG expression and the presence of Foxp3+ cells (n=184).

A



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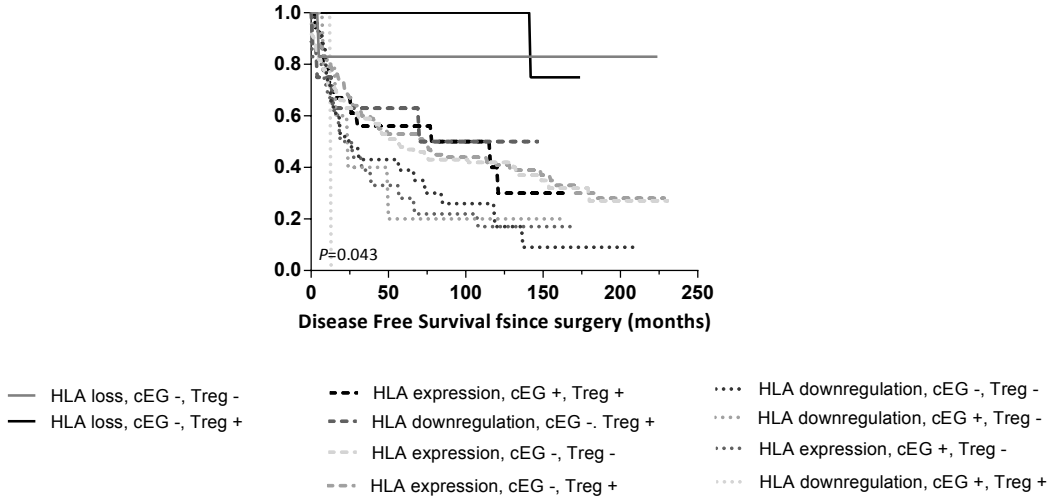
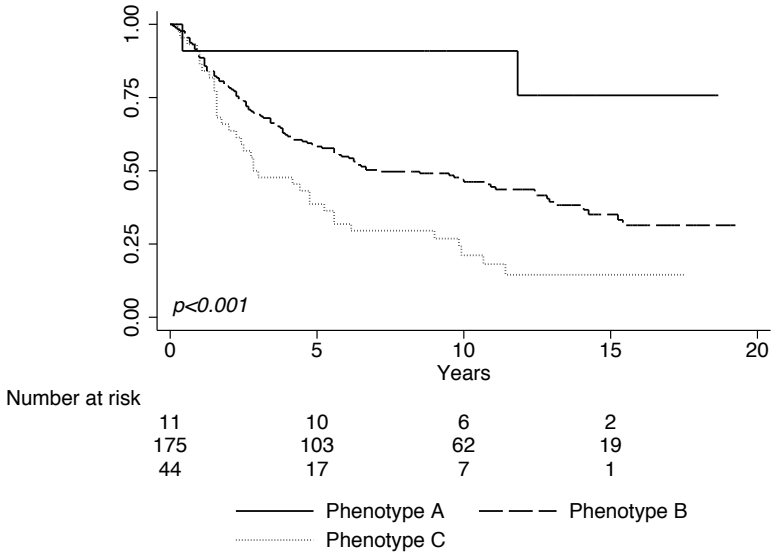


Figure 4: Survival curves stratified for combined tumor expression of HLA class I, HLA-E, HLA-G and Foxp3+ in colon cancer.

A) Kaplan Meier curve for OS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 distinct patters 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 tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 distinct patterns could be distinguished, as shown in Figure 5.

A



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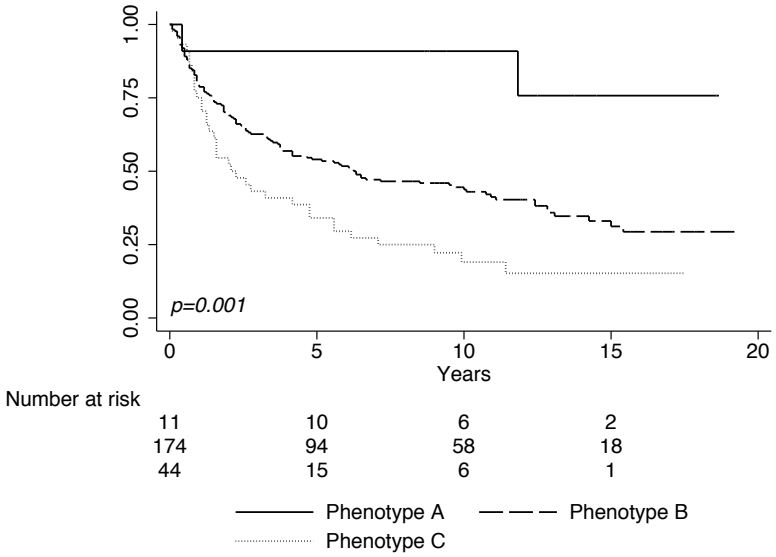

Figure 5: Survival curves stratified for immune phenotypes in colon cancer.
A) Kaplan Meier curve for OS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 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 tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 immune phenotypes could be distinguished. See *Results* section for explanation of the phenotypes.

c) Poor immune system tumor recognition: Patients with tumors 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 Table IIa and IIb.

Multivariable analysis

Again, both for OS and DFS a univariate analysis was performed for the following parameters: sex, age, TNM stage, tumor immune phenotype, mucinous differentiation, tumor grade, adjuvant therapy, and microsatellite status. In univariate analysis, next to age and TNM status, the tumor immune phenotype was a significant predictor for OS (*p*-value 0.001) and DFS (*p*-value 0.002). Therefore all three these parameters were included in multivariable analysis. The tumor 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 table IIa and IIb.

DISCUSSION

Tumor-immune interactions may be important for the prognosis of cancer patients [17]. 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 tumor growth and metastasis.

The first marker of tumor-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 [35;39]. Watson *et al.* showed that tumors with downregulation of HLA class I had a worse survival comparable with our results [39]. In contrast, Menon *et al.* showed a survival benefit in patients with downregulated HLA-A tumors [35]. However, when HLA-A and HLA-B/C were combined, statistical significance was lost. Furthermore, patients with expression of HLA class I were related to a better survival in the study by Watson *et al.,* 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 tumors showing microsatellite instability (MSI), which is associated with loss of HLA class I and a better prognosis [56;57]. In our study, 33% of the tumors with loss of HLA class I showed the MSI phenotype, in comparison to 14% and 13% for HLA class I downregulation and expression. Results from Menon *et al.* showed that 50%

of the tumors with loss of HLA class I had the MSI phenotype. Unfortunately, *Watson et al.* did not mention microsatellite status of their study cohort.

As hypothesized, loss of HLA class I expression in tumor cells could also be related to a better patient survival because such cells, once they metastasize to the bloodstream, are eliminated by NK cell attacks [35;39;58]. Tumors with loss of HLA class I have also shown to have significantly higher NK cell infiltration [15]. More interestingly, the tumors 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 [17-20]. Furthermore, this is also confirmed by CRC tumors with loss of HLA class I expression who do not metastasize to the liver [59].

The presence of the third marker Foxp3+ is thought to represent the inhibition of host-protective antitumor responses. When stimulated, they inhibit the function of CTL [6]. Although the exact mechanism by which these cells are drawn into the tumor micro-environment remains unexplained, their immunosuppressive effect has been proven with a high density of tumor-infiltrating Foxp3+ cells found to be associated with an unfavorable prognosis in a wide range of human carcinomas, including breast and lung cancer [60;61]. However, in colon cancer different results are reported as well [37;38]. One possible explanation for these opposite results might be a different micro-environment of colon cancer, which is colonized with many gastro-intestinal bacteria, triggering the production of pro-inflammatory cytokines causing tumor-enhancing effects. Instead of the specificity of infiltrating T-cells for tumor-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 anti-tumorigenic effect, which could explain the better prognosis of patients with tumors with a strong Foxp3+ infiltration [62]. We were not able to demonstrate differences in disease outcome for Foxp3+ tumor 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 hypothesize that the tumors 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 tumors directed by CTL. Since CTL and Foxp3+ cells show opposing actions [27] and CTLs are supposed to be absent in these tumors, Foxp3+ cell infiltration might not be necessary. These tumors could therefore progress aggressively as immune surveillance is poor. In contrary, tumors 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 tumor growth.

Therefore, in our opinion, the clinical relevance of the studies by Watson *et al.* and several others does not provide an optimal perspective on prognosis [35;39], because ex-

pression 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 [29;46;63;64].

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 hypothesize that these poor immune system recognized tumors 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 tumor micro-environment. Furthermore, these tumors showed a positive expression of HLA-E and -G, further escaping immune surveillance through inhibition of NK cell recognition [17-20]. These tumor cells can therefore quickly progress to the bloodstream and might eventually metastasize.

It is important to realize that what we are evaluating is just a 'snapshot' of the ongoing process of cancer immuno-editing in the patient's primary tumor 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.* in breast cancer and other studies [29;65;66].

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 tumors with loss of HLA class I, which were HLA-E and -G positive. Therefore we were not able to investigate the prognosis of these tumors, but we hypothesize that these tumors have a worse prognosis as these tumors 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 tumors, suggesting further escape from immune recognition through upregulation of HLA-E [21;46]. To truly investigate these tumors, 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 [67]. Therefore we should be careful using the term total loss of HLA class I. Third, we did not investigated 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 tumor site is scarce, indicating that tumor staining for NK cells might be minimally informative [40].

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 expression might play a pivotal role in distant immune escape mechanisms, where in case of loss or downregulation of HLA class I, HLA-E and -G expression determines 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 immunotherapies.

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