Biomarkers in colorectal cancer

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PART II

Tumour immune interactions, HLA-G expression in colorectal cancer

Chapter 5

HLA-G and classical HLA class I expression in primary colorectal cancer and associated liver metastases

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ABSTRACT

De novo expression of HLA-G has been demonstrated in colorectal cancer. HLA-G, amongst others, inhibits natural killer cell function, contributing to host immune defense evasion. Another mechanism to escape anti-tumor immunity is loss of HLA class I. Therefore, we determined HLA-G and HLA class I expression on primary colorectal tumors and associated liver metastases, in order to get insight in the metastasizing process regarding escaping anti-tumor immunity. HLA-G expression was evaluated using three mAbs; 4H84, MEM-G/1 and MEM-G/2. In total 81 colorectal cancer patients were evaluated. Formalin-fixed paraffin-embedded tissue sections of primary tumors and associated liver metastases, were immunohistochemically stained. A concordance between expression or loss/downregulation in the primary tumor and associated liver metastasis regarding HLA class I expression was observed in 80% of the cases. In contrast with the hypothesis of escaping NK cell-killing, we demonstrated for each HLA-G detecting mAbs used in this study, that the majority of the primary tumors that positively stained for HLA-G did not express HLA-G in the associated liver metastasis. Furthermore, we revealed the existence of non-specific binding and in addition we found that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 mAbs were expressed differentially in colorectal tumor tissues.

INTRODUCTION

There is accumulating evidence which supports the notion that the immune system plays an important role in tumorigenesis. Currently, the concept of cancer immune-editing, resulting in an escape of the host defense immunity is widely accepted [1]. Therefore, evasion of immune surveillance is considered one of the emerging hallmarks of cancer [2]. Different mechanisms are used by tumor cells to differentiate towards cells with reduced immunogenicity. Understanding these complex mechanisms is a major challenge. Two important mechanisms in the escape from the host immune recognition and destruction is expression of the non-classical human leukocyte antigen-G (HLA-G) and complete loss or downregulation of classical HLA class I molecules.

HLA-G is rarely expressed in healthy tissues except in immune privileged sites, such as the placenta, where it is involved in the immune tolerance towards the maternal immune system [3, 4]. De novo expression of HLA-G has been reported in human malignant cells, including colorectal cancers [5-7]. A crucial role for the tumor-driven expression of the HLA-G molecule in escaping the hosts' immune surveillance is by direct interaction with inhibitory receptors on T lymphocytes and peripheral blood natural killer (NK) cells [5], summarized as an immune checkpoint inhibiting antitumor responses. This explains why expression of the HLA-G molecule on cancer cells is associated with higher tumor grade and poor prognosis [8].

A second well-known mechanism to escape anti-tumor immunity is downregulation or total loss of the classical HLA class I proteins. Tumor cells expressing HLA class I present tumor-associated antigens (TAA) on their cell surface and consequently are recognized and destructed by cytotoxic T-cells (CTL) [9]. Therefore, downregulation or total suppression of HLA class I results in inefficient TAA presentation and less recognition by CTL. In approximately 15% up to 75% of colon cancers the phenomena of downregulation or loss is observed [10-12]. Additionally, studies on the prognostic value of diminished expression of HLA class I in colon and rectal cancer patients, showed in general a worse overall survival in patients with loss or downregulation of HLA class I, compared with patients expressing HLA class I in tumor cells [13-15]. On the other hand, tumor cells with a complete loss of HLA class I, once they metastasize to the circulation, are targets for elimination by NK cells. Therefore, loss of HLA class I could also be related to a better patient survival. Especially, colorectal cancer patients with a microsatellite instable (MSI) type tumor and complete loss of HLA class I, were associated with a reduction of metastatic disease [16, 17].

In order to get insight in the immunogenic profile of metastasizing cells, we investigated

the expression of the immune-related tumor markers HLA-G and classical HLA class I in primary colorectal cancer and associated liver metastasis by immunohistochemistry. Furthermore, we investigate the correlation between synchronous and metachronous occurrence of the metastasis regarding HLA-G and HLA class I expression.

Immunohistochemistry is a widely accepted technique, although detecting HLA-G expression with immunohistochemistry is still controversial. For example, the reported level of HLA-G expression, detected by immunohistochemistry, in colorectal cancer differs in literature ranging from 20.3% by Zeestraten *et al. to* 72% by Guo *et al.* [15, 18]. These discrepancies are not solely observed in colorectal cancer. For example, HLA-G expression in melanoma cell lines was demonstrated by Paul *et al.,* whereas, no HLA-G expression was observed by Frumento *et al.* [19, 20]. These discrepancies in melanoma cell lines were attributed to the fact that different, not commercially available, monoclonal antibodies (mAbs) were used. Currently, several mAbs are commercially available, but many of these show cross reactivity. For instance the commercially available and widely used 4H84 mAb cross reacts with the ß2-microglubulin (ß2m) free classical HLA class I antigens [21]. For that reason it is recommended to use multiple HLA-G specific mAbs [21, 22]. Therefore, in this study we used three different antibodies to detect HLA-G expression and we compared the reactivity pattern of the different anti-HLA-G mAbs.

MATERIALS AND METHODS

PATIENTS AND TISSUE SAMPLES

The study cohort consisted of patients diagnosed with colorectal cancer between 1986 and 2001 who underwent a hepatic resection for metastatic colorectal cancer at the department of Surgery, Leiden University Medical Center. Sufficient formalin-fixed paraffin-embedded tumor tissue, of both primary tumor and associated liver metastasis, was available of 81 patients. Patient and tumor data were retrieved from patient's medical files and pathology reports. Patients were divided in two groups, based on having synchronous or metachronous metastasis. Synchronous metastasis is defined as liver metastases diagnosed before or during the resection of the primary tumor, metachronous metastasis were defined as metastases diagnoses after the resection of the primary tumor [23].

ANTIBODIES

HLA-G targeting mouse monoclonal antibodies 4H84 (Exbio, Czech Republic), MEM-G/1 (Abcam, ab 7759) and MEM-G/2 (AbCam, ab26090), recognizing all HLA-G

isoforms, were used to assess HLA-G expression on formalin-fixed paraffin-embedded tumor tissue sections. To visualize the classical HLA class I protein, mouse monoclonal antibodies HCA2 and HC10 were used. Both of these antibodies were kindly provided by Prof. Dr. J. Neefjes, NKI Amsterdam, the Netherlands. The mAb HCA2 recognizes all HLA-A chains (except HLA-A24), and some HLA-B, HLA-C, HLA-E, HLA-F and HLA-G chains [24, 25]. The mAb HC10 recognizes HLA class I heavy chains and reacts mostly with HLA-B and HLA-C and some HLA-A (HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, HLA-A33) [26].

IMMUNOHISTOCHEMISTRY

Paraffin blocks with tissue from the primary colorectal tumors and associated liver metastases were collected. Tissue sections of 4 µm were cut and processed for immunohistochemical staining. Briefly, after deparaffinization and rehydration endogenous peroxidase was blocked by incubating the sections in a 0.3% hydrogen peroxide solution for 20 min. Antigen retrieval was performed as follows; slides for staining with mAbs MEM-G/1, MEM-G/2, HCA2 and HC10, were heated 10 minutes at 95ºC in; pH low Target Retrieval Solution (Dako, Heverlee, Belgium). Slides for staining with mAb 4H84 were heated 10 minutes at 95ºC in; pH high Target Retrieval Solution (Dako, Heverlee, Belgium). The sections were incubated, in pre-determined optimal dilutions, overnight with 4H84 (dilution 1:500), MEM-G/1 (dilution 1:400), MEM-G/2 (dilution 1:400), HCA2 (dilution 1:800) and HC10 (dilution 1:500). Sections were washed three times for 5 minutes in phosphate *buffered* saline (PBS) followed by 30 minutes incubation with EnVision+ System-HRP anti mouse (DAKO, Glostrup, Denmark). The staining was completed by 10 minutes incubation with 3,3'-diaminobenzidine (DAB)+ Substrate-Chromogen System (DAKO, Glostrup, Denmark). Finally, sections were washed two times in demineralised water, counterstained with haematoxylin, dehydrated and mounted in Pertex.

Nonspecific protein binding was blocked with 5% goat serum before incubation with MEM-G/1 and MEM-G/2 for making a comparison between blocking and not using a block.

Placenta tissue served as an external positive control for staining with anti-HLA-G mAbs. Human tonsil tissue served as an external positive control for classical HLA class I staining. Normal epithelium, stromal cells or lymphoid cells served as an internal positive control for the HCA2 and HC10 antibodies. Negative controls consisted of slides that underwent the whole immunohistochemistry protocol without the use of the primary antibody.

EVALUATION OF IMMUNOHISTOCHEMISTRY

Microscopic analysis was performed by two independent observers in a blinded manner (M.S and M.H.K). The Cohen's Kappa coefficient ranged from 0.64 to 1.00 for al staining's, which indicates a substantial agreement between the observers.

Three different mAbs were used to evaluate HLA-G expression (4H84, MEM-G/1 and MEM-G/2) and the presence or absence of positive stained tumor cells was determined. The concordance between expression in the primary tumors and associated liver metastases was evaluated for each mAb separately.

For HCA2 and HC10 the percentage of tumor cells with a positive stained membrane were assessed. According to the International HLA and Immunogenetics Workshop [27], the HLA class I expression status was determined as follows; HLA class I expression is defined as 5% or more of the tumor cells stained positive for HCA2 and HC10. Loss of HLA class I expression is defined as less than 5% of the tumor cells stained positive for both HCA2 and HC10 and downregulation of HLA class I is defined as less than 5% of tumor cells expressing either of the markers.

STATISTICAL ANALYSIS

Statistical analyses were performed using the statistical package SPSS (version 20.0 for windows; SPSS Inc.). The Chi-square test and Cohen's Kappa coefficient were used to perform the statistical analysis.

RESULTS

PATIENTS

Tissue was collected from 81 patients with primary colorectal cancer and associated liver metastasis. Patient characteristics are shown in Table I. Of the 81 patients 38% (31/81) were diagnosed with synchronous metastases and 62% (50/81) were diagnosed with metachronous metastases.

EVALUATION OF THE REACTIVITY OF 4H84, MEM-G/1 AND MEM-G/2

To evaluate the reactivity of the different HLA-G antibodies used in tissue sections of colorectal tumors and liver metastases, we first investigated the effect of blocking with 5% goat serum. Figure 1 shows the different staining patterns for MEM-G/1 with and without the use of 5% goat serum. As shown in Figure 1A, a MEM-G/1 staining, positive stained tumor cells were observed without the use of 5% goat serum and a completely

negative tumor cells were observed with the use of a 5% goat serum block, revealing the existence of non-specific binding in colorectal tumor tissue. As shown in Figure 1B, in some cases positive stained tumor cells remain positive despite the use of a 5% goat serum. Correspondingly, for MEM-G/2 non-specific binding was observed in a same way as MEM-G/1 (data not shown). In contrast to MEM-G/1 and MEM-G/2 the staining pattern with 4H84 was not influenced by blocking with 5% goat serum (data not shown).

In contrast, as shown in Supplementary figure 1, all anti-HLA-G mAbs showed a similar reactivity pattern in placenta tissue. Blocking with 5% goat serum did not affect this staining pattern, revealing the specificity of these HLA-G antibodies in placenta.

Table 1. Patient characteristics of total patients population.

	n = 81	%
Sex		
Male	53	(65)
Female	28	(35)
Age median (years)	59.0	(±8.8)
Stage		
I	3	(4)
II	6	(7)
III	41	(51)
IV	31	(38)
Differentiation		
Good	4	(5)
Moderate	66	(82)
Poor	10	(12)
Missing	1	(1)
Location		
Colon	43	(53)
Rectosigmoid	9	(11)
Rectum	29	(36)
Metastasis		
Synchronous	31	(38)
Metachronous	50	(62)

Representative images of HLA-G staining with the three different HLA-G mAbs in sequential tissue sections of colorectal tumor tissue are shown in Figure 2. As demonstrated in this figure different staining patterns, regarding positive and negative stained tumor cells, were observed among the three different HLA-G mAbs. The colorectal tumor tissue in panel A stained negative for 4H84 and MEM-G/1, although it was positive for staining with MEM-G/2. The sequential tissue sections in panel B show tumor cells stained positive for 4H84 and negative for MEM-G/1 and MEM-G/2. The

sequential tumor tissue sections in panel C were positive stained for 4H84, MEM-G/1 and MEM-G/2 in the corresponding tumor cells. This suggests that the different epitopes of HLA-G detected by these mAbs are expressed differentially in colorectal tumor tissues.

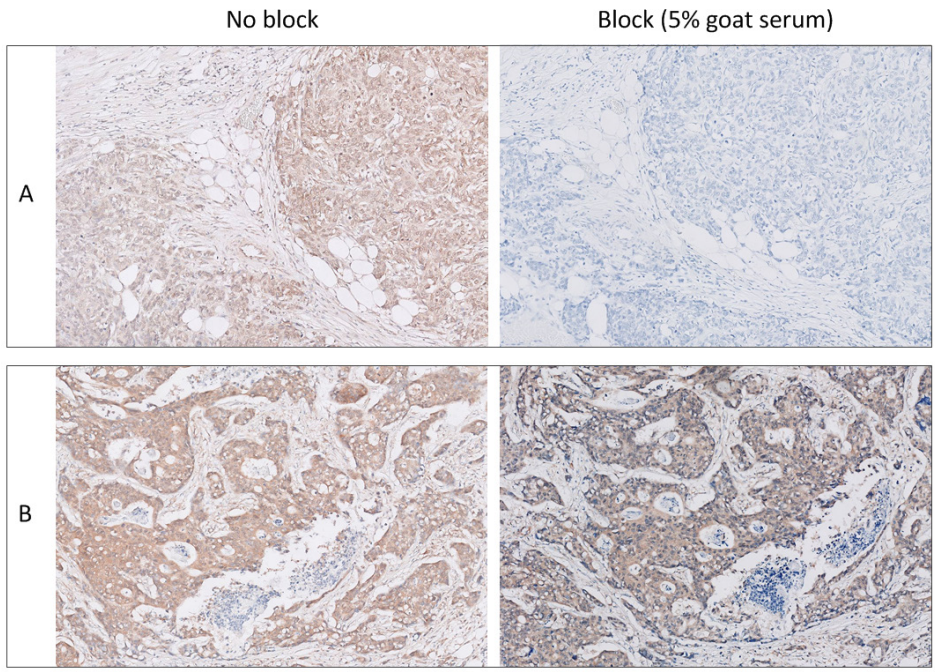

Figure 1. Representative images of MEM-G/1 staining with and without the use of 5% goat serum. **A.** In the left panel MEM-G/1 positive stained colorectal tumor section without the use of 5% goat serum. In the right panel a sequential tissue section negatively stained with MEM-G/1, with the use of 5% goat serum. **B.** In the left panel MEM-G/1 positive stained colorectal tumor section without the use of 5% goat serum. In the right panel a sequential tissue section also positive stained with MEM-G/1, with the use of 5% goat serum.

HLA-G AND HLA CLASS I EXPRESSION IN PRIMARY TUMOR AND ASSOCIATED LIVER METASTASIS

HLA-G expression was analyzed for each antibody separately, as a consequence of the previously suggested differentially expressed epitopes of HLA-G in colorectal cancer tissues detected by 4H84, MEM-G/1 and MEM-G/2 mAbs. For the analysis of the results data from tissues sections blocked with 5% goat serum were used, in order to avoid inclusion of false positive stained tissue sections. Due to loss of material during the staining procedure the total number of tissue sections evaluated is not reaching 81.

Positive staining for HLA-G in the primary tumor was observed in 29% for staining with 4H84, 6% for staining with MEM-G/1 and 10% for staining with MEM-G/2 (Table 2). In the liver metastases 30%, 4% and 0% showed positivity for staining with 4H84, MEM-G/1 and MEM-G/2 respectively (Table 2). The analysis for primary tumor and associated liver metastasis sets included 77, 76 and 76 sets for 4H84, MEM-G/1 and MEM-G/2, respectively (Table 3).

Table 2. Frequencies of 4H84, MEMG/1 and MEM-G/2 positive and negative stained tumor cells in tissue sections of primary colorectal cancer and liver metastases. For MEMG/1 and MEM-G/2 the effect of blocking with 5% goat serum are shown. Due to loss of material during the staining procedure the total number of tissue sections evaluated is not reaching 81.

	Colorectal tumor		Liver metastasis	
	Positive	Negative	Positive	Negative
4H84	23 (29%)	57 (71%)	23 (30%)	55 (71%)
MEM-G\1				
Block –	28 (35%)	53 (65%)	10 (13%)	68 (87%)
Block +	5 (6%)	74 (94%)	3 (4%)	75 (96%)
MEM-G\2				
Block –	15 (19%)	66 (82%)	0 (0%)	78 (100%)
Block +	8 (10%)	71 (90%)	0 (0%)	78 (100%)

A concordance between expression or no expression in the primary tumor and associated liver metastasis was observed in 77% regarding staining with 4H84. In addition, 9 out of 22 of the primary tumors were positively stained for 4H84 and negatively stained in the associated liver metastasis, which accounts for 12% of the primary tumor and liver metastases sets. For MEM-G/1, in 91% of the primary tumor and associated liver metastasis sets, no HLA-G expression was detected in either of the tissues. Furthermore, 5% of the primary tumor and liver metastasis sets were positive for MEM-G/1 in the primary tumor and negative for MEM-G/1 in the liver metastasis. Compared to MEM-G/1, a similar pattern was observed for the staining with MEM-G/2, all primary tumors that stained positive for MEM-G/2 were negative in the associated liver metastases. Furthermore, no significant differences were observed between HLA-G expression in primary tumor regarding synchronous or metachronous onset of metastases (4H84 p=0.140, MEM-G/1 p=0.633, MEM-G/2 p=0.139).

The analysis of HLA class I expression included 80 primary tumors en 77 associated liver metastasis. Due to loss of material during the staining procedure the results of 5 sets of primary tumor and associated liver metastasis could not be analyzed. Representative images of HLA class I expression and loss of expression are shown in Figure 3. The

majority of the primary tumors (72%) and liver metastases (64%) were expressing HLA class I. A concordance between the expression or loss/downregulation in the primary tumor and associated liver metastasis regarding HLA class I expression was observed in 80% of the cases (Table 3). Furthermore, no significant difference (p=0.698) was observed between the HLA class I expression profiles and the synchronous or metachronous onset of liver metastases.

Table 3. Cross tables comparing expression of HLA class I and HLA-G in primary colorectal tumors (primary CRC) and associated liver metastases. For each mAbs detecting HLA-G (4H84, MEM-G/1 and MEM-G2) a separate cross table is shown. For MEM-G/1 and MEM-G/2 cross tables with and without blocking with 5% goat serum are shown.

		Liver metastases
HLA Class I	Expression	Loss + Downregulation
Primary CRC		
Expression	44 (58%)	11 (15%)
Loss + Downregulation	4 (5%)	17 (22%)

		Liver metastases
4H84	Positive	Negative
Primary CRC		
Positive	13 (17%)	9 (12%)
Negative	9 (12%)	46 (60%)

		Liver metastases
MEM-G/1	Positive	Negative
Primary CRC		
Positive	1 (1%)	4 (5%)
Negative	2 (3%)	69 (91%)

		Liver metastases
MEM-G/2	Positive	Negative
Primary CRC		
Positive	0 (0%)	8 (11%)
Negative	0 (0%)	68 (90%)

		Liver metastases
MEM-G/1 (No block)	Positive	Negative
Primary CRC		
Positive	6 (8%)	21 (27%)
Negative	4 (5%)	47 (60%)

		Liver metastases
MEM-G/2 (No block)	Positive	Negative
Primary CRC		
Positive	0 (0%)	15 (19%)
Negative	0 (0%)	63 (81%)

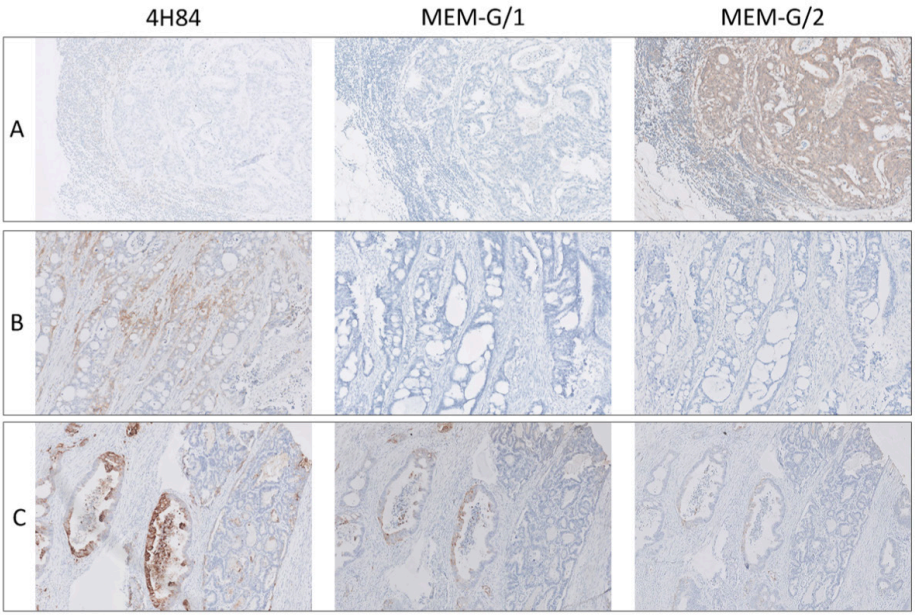

Figure 2. Different reactivity patterns among the three mAbs (4H84, MEM-G/1 and MEM-G/2) in sequential tissue sections of colorectal tumors. For the staining with MEM-G/1 and MEM-G/2 blocking with 5% goat serum was used.

A. Sequentially sections of colorectal tumor tissue. Negative stained for 4H84 and MEM-G/1. Positive stained for MEM-G/2. **B.** Sequentially sections of colorectal tumor tissue. Positive stained for 4H84 and negative stained for MEM-G/1 and MEM-G/2. **C.** Three sequentially tissue sections positive stained for 4H84, MEM-G/1 and MEM-G/2.

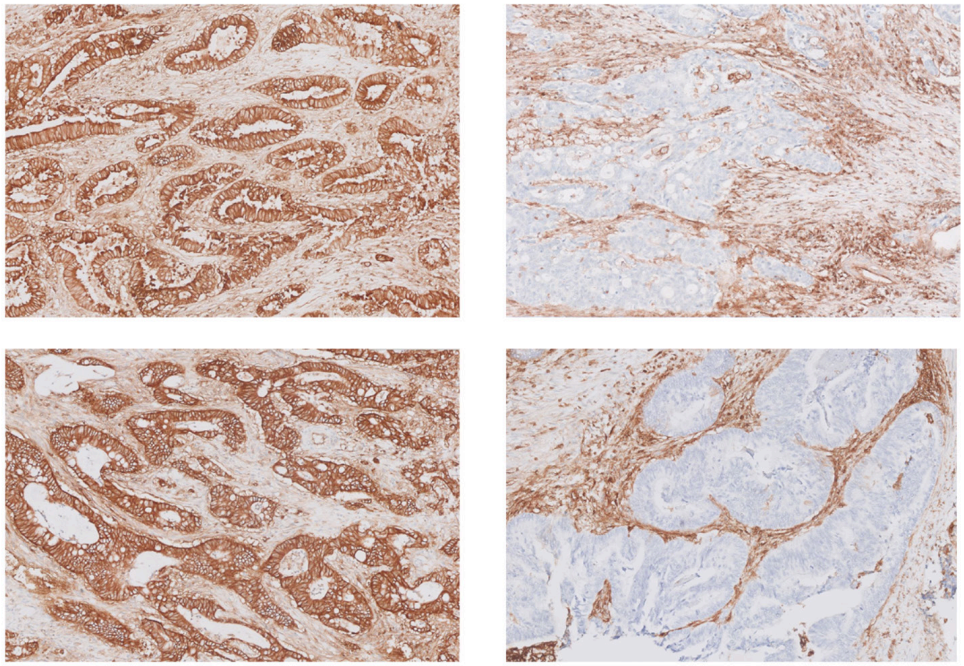

Figure 3. Representative images of immunohistochemical HLA class I staining. HLA class I expression is defined as 5% or more of the tumor cells stained positive for HCA2 and HC10. Loss of HLA class I expression is defined as less than 5% of the tumor cells stained positive for both HCA2 and HC10. Downregulation of HLA class I is defined as less than 5% of tumor cells expressing either of the markers. **A.** HCA2 positive tumor **B.** HCA2 negative tumor **C.** HC10 positive tumor **D.** HC10 negative tumor

DISCUSSION

HLA-G is thought to act as an immune checkpoint molecule; de novo expression of HLA-G on tumor cells results in activation of immune response inhibitory signalling. Therefore, HLA-G is considered as a potential target for optimization of current cancer immunotherapy strategies [28]. In previous research no significant differences in overall survival associated with HLA-G expression on tumor cells was observed in patients with colon and rectal cancer [13, 15]. In contrast, Guo *et al* did show a worse patient survival regarding HLA-G expression [18]. In our study, the included patients were all experiencing metastatic disease and their clinical condition allowed resection of their liver metastases. Therefore, it was not possible to calculate survival outcomes. We were able to perform analyses regarding synchronous or metachronous onset of liver

metastases. No significant difference between synchronous or metachronous onset of liver metastasis was observed regarding HLA-G expression.

Previous studies showed a worse survival rate in patients not expressing HLA class I on the cell surface of their tumors. Furthermore, an even more unfavorable prognosis was observed when combining HLA class I downregulation with HLA-G expression in colorectal cancer patients, which supports the hypothesis of an immune escape advantage for tumor cells with diminished HLA class I expression [13-15]. In contrast, we found that the majority of the tumor cells within the liver metastases did express HLA class I. Therefore, HLA class I loss may be an advantage in the hosts immune defense evasion, but not absolutely required for formation of metastases.

The precise immune phenotype of metastasizing cells is difficult to reveal, due to the heterogeneity of tumors. Studying only one immune marker is not sufficient to understand the complete immune escape mechanism. Immune markers are related to each other and combining markers could give more insight in the complex mechanism of evasion of the host's immune surveillance. As hypothesized, cells lacking HLA class I protein expression on their cell surface are a NK cell target in the circulation. Besides, cells expressing HLA-G are supposed not to be lysed by NK cells in the circulation. In contrast with the hypothesis of escaping lysis by NK cells, we demonstrated for each HLA-G detecting mAbs used in this study, that the majority of the primary tumors positive stained for HLA-G do not express HLA-G in the associated liver metastasis. This suggests that HLA-G is not a major contributor to the metastatic process in the circulation. It would have been interesting to combine HLA-G expression and HLA class I loss. Nevertheless, our data suggest that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 are expressed differentially in colorectal tumor tissues. The 4H84 mAb is a widely used mAb to detect HLA-G. However, cross reactivity with ß2m free classical HLA class I molecules on activated leukocytes has been demonstrated [21]. This could results in false recognition of HLA-G expression in pathologies that are recognized by leucocyte infiltration such as colorectal cancer. Consequently, it is recommended not to rely on an analysis with solely 4H84 but to detect HLA-G with a number of different mAbs [21, 22]. Accordingly, we decided to stain the tissues with three different mAbs targeting HLA-G. It should be noted that to the best of our knowledge a consensus about interpreting the results of different antibodies is not available. Furthermore, we revealed the existence of non-specific binding and in addition our data suggest that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 mAbs are expressed differentially in colorectal tumor tissues. However we did reveal the specificity of these HLA-G antibodies in placenta.

In literature, the reported level of HLA-G expression, detected by immunohistochemistry, in colorectal cancer and other cancer types differs extensively [15, 18, 22]. Besides the variation in reported HLA-G expression, the proportion of positive stained cells in a tumor is highly variable as well [22]. We suggested that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 mAbs are expressed differentially in colorectal tumor tissues. Additionally, HLA-G might be expressed differently in specific tumors or perhaps some HLA-G isoforms might be more prominent in some specific tumor types. G*enomic instability* is a characteristic of almost all human cancers causing mutations in the HLA-G gene, which could be an explanation for the different staining patterns.

Based on the results of this study, we have to conclude that it is not possible to compare the patterns of reactivity or combine the results to one "HLA-G expression value" due to the impressive different patterns observed with the antibodies used. For that reason, future addition of biochemical analyses will be necessary to evaluate the binding patterns of the three HLA-G mAbs. However, in the current study this was not feasible as our analyses were performed on paraffin-embedded tissue sections obtained from the pathology achieve and only a limited amount of tumor tissue from these patients was available. This might be *considered as a limitation of our study*.

We have to realize that immunohistochemistry for HLA-G is extremely difficult to interpret. In line with the results in this study, conclusions based on immunohistochemistry have to be drawn very carefully. Especially literature based on one anti-HLA-G mAb should be interpreted with caution as the noted cross-reactivity and existence of non-specific binding may lead to an over-estimation of HLA-G expression in cancer.

In conclusion, the non-classical HLA class I molecule HLA-G is an interesting and promising protein in cancer research and is considered as an attractive candidate molecule for therapeutic intervention. However, an emerging need for standardization of the procedures to detect HLA-G, especially in paraffin sections, is warranted. It is therefore crucial to completely unravel their binding domains and cross reactivity patterns. Until then, it is difficult to compare different staining patterns and draw conclusions related to HLA-G expression in cancer pathologies.

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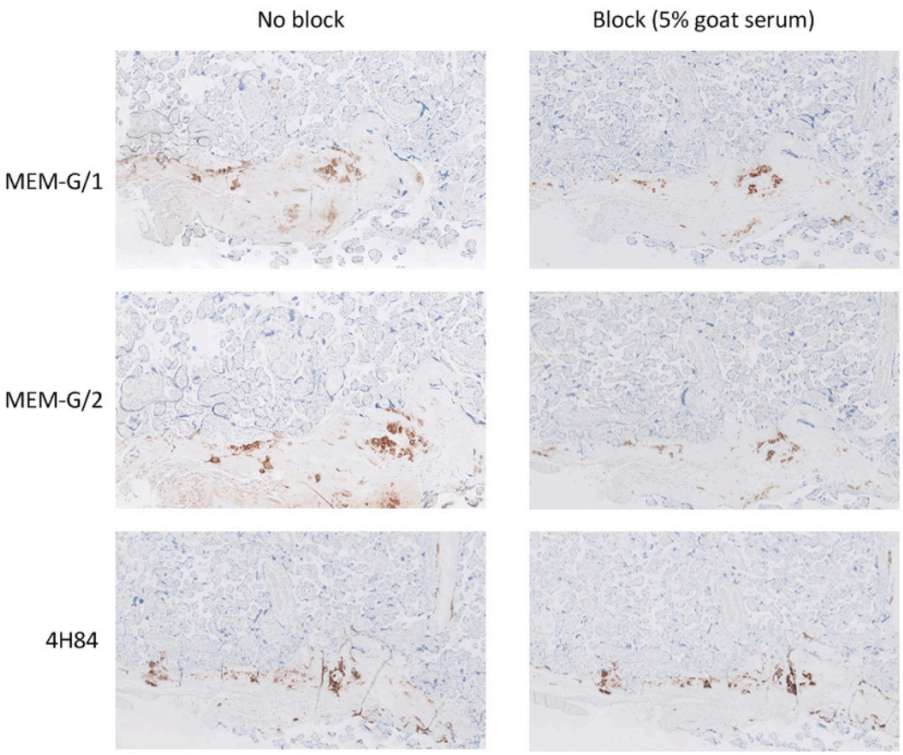
SUPPLEMENTARY FILE



Supplementary figure 1. Images of sequential tissue sections of placenta tissue, stained with MEM-G/1, MEM-G/2 and 4H84 with and without the use of 5% goat serum. As shown all three mAbs have a same reactivity pattern in placenta, in the presence of blocking serum (5% goat serum).