Biomarkers in colorectal cancer

Swets, M.

Chapter 7

HLA-G protein expression in colorectal cancer evaluated by immunohistochemistry and western blot analysis: its expression characteristics remain enigmatic

Marloes Swets, Anne Wouters, Daniëlle Krijgsman, Ronald L.P. van Vlierberghe, Arnoud Boot , Jaap D. van Eendenburg, Tom van Wezel, Hans Gelderblom, Cornelis J.H. van de Velde, Peter J. van den Elsen, Peter J.K Kuppen

Clin Immunol. 2018 Sep;194:80-86.

ABSTRACT

HLA-G protein expression could play a role in evasion of tumor immune surveillance. Accumulating evidence demonstrates that HLA-G is expressed in different types of malignancies, including colorectal cancer (CRC). The purpose of the current study was to further unravel whether HLA-G protein expression could play a role in immune evasion of CRC. Therefore, to firmly establish HLA-G protein expression, eight early passage human CRC cell lines and five human rectal cancer tissues were analyzed by western blot analysis. The results obtained by western blot analysis were compared with immunohistochemistry on tumor tissue sections of the same patient. Furthermore, multiple monoclonal antibodies (mAbs), 4H84, MEM-G/1 and 5A6G7, targeting HLA-G were used to unravel staining patterns. We showed that results obtained with immunohistochemistry did not correlate with protein expression detected by western blot analysis, using three different HLA-G targeting mAbs. Furthermore, with respect to the specificity of the mAbs employed, additional immune reactivity was detected using the mAbs MEM-G/1 and 5A6G7 in western blot analysis with K562 control cell lines overexpressing HLA-A2 or HLA-G, all tumor tissues and in two out of eight CRC cell lines. Based on the current study and our previously reported results, we conclude that claiming HLA-G plays a role in immune modulation of CRC seems premature, as results from anti-body based detection of HLA-G protein remain inconclusive. Until the time that detection of HLA-G is sensitive enough to detect all aspects of HLA-G expression in biological samples, rather than transfected cells or long time cultured cell lines, conclusions should be drawn with great care.

INTRODUCTION

In 2011, evasion of immune recognition was added as an additional hallmark to the well-known six hallmarks of cancer [1,2]. Currently, escaping the host's defense immunity, by the concept of cancer immune-editing, is widely accepted. To completely unravel the complex mechanisms that contribute to immune defense evasion of the host, is a major challenge. During the past decade the non-classical HLA class I molecule HLA-G, has been of interest as a possible immune tolerogenic molecule in cancer. As a result of its proposed immunosuppressive capabilities, HLA-G protein expression could play a role in tumor immune surveillance. Notably, altered HLA class I expression is a well-known mechanism in escaping anti-tumor immunity [3-6].

In non-pathological conditions the HLA-G protein was found to be expressed in immune privileged sites, for example extravillous trophoblast cells of the placenta. Its expression in these cells of the placenta plays an important role in establishing maternal-fetal immune tolerance [7-9]. The expression of HLA-G protein by fetal trophoblast cells has been proved by using different biochemical techniques together with many different anti-HLA-G monoclonal antibodies (mAbs) [10,11]. Though, despite the impressive expansion of knowledge regarding HLA-G, many questions remain unanswered [12].

HLA-G protein expression has been observed in a wide range of human tissues and in several malignancies, including colorectal cancer (CRC) [13-15]. This tumor driven, *de novo* expression of the HLA-G protein, which possibly could contribute to evasion of the host's immune surveillance, has been associated with a poor clinical outcome in patients with CRC, warranting further research into its expression patterns in cancer [13,16]. HLA-G is considered an immune checkpoint molecule, and as a consequence, *de novo* HLA-G tumor expression has been ascribed potential as a immunotherapy target [17,18]. However, among and within different tumor types discrepancies regarding HLA-G expression were reported. In CRC, HLA-G protein expression was found in 20% of the patients by *Zeestraten et al.* [19], whereas Guo *et al.*[20] reported HLA-G protein expression in 72% of their patient cohort. Notably, even higher percentages were described by *Kirana et al* [21], who observed HLA-G protein expression in 86% of their CRC cohort. Detecting HLA-G expression with the use of immunohistochemistry (IHC) is a widely used technique, but remains controversial [22-24]. Due to known cross-reactivity of mAb 4H84, it has been suggested to use multiple mAbs to evaluate HLA-G expression by IHC [22,23]. However, most studies based on IHC used a single mAb, usually 4H84, and therefore should be interpreted with caution. Moreover, different discrepant expression profiles in sequential CRC tissue sections have been observed [24,25]. Therefore, it seems

premature to conclude HLA-G is expressed in a pathological condition such as CRC. To firmly evaluate HLA-G expression in CRC additional molecular and biochemical analyses are required.

In a previous study, we evaluated HLA-G expression in 21 low passage CRC cell lines and in the corresponding primary tumor tissues [25]. Flow-cytometry, RT-PCR and IHC with 3 different mAbs was performed. Of the known HLA-G isoforms, only mRNA showing strong homology with HLA-G3 was detected, albeit at very low levels and in only 5 out of 21 CRC cell lines. In accordance with the RT-PCR results, HLA-G1 was not detected by flow-cytometry performed on the same CRC cell lines. Moreover, IHC of the CRC tissue matching the cell lines showed different staining patterns with the different anti-HLA-G mAbs.

The purpose of the current study was to further unravel whether HLA-G protein expression plays a role in immune evasion of CRC. Therefore, early passage CRC cell lines and CRC tissues were analyzed using western blot for HLA-G protein expression. In addition, the results obtained by western blot analysis were compared with IHC on corresponding frozen tumor tissue sections. Furthermore, multiple mAbs targeting HLA-G were used to unravel binding patterns. We showed that results obtained with IHC did not correlate with protein expression detected by western-blot analysis, using three different HLA-G targeting mAbs.

7

MATERIALS AND METHODS

CELL AND CULTURE CONDITIONS

The myelogenous leukemia cell line (K562), was transfected with a single HLA heavy chain i.e. HLA-G1 or HLA-A2, in pLNCX (Ampicillin and Neomycin resistant), and named K562-G1 and K562-A2 respectively. K562 wild type (K562-WT), transfected with empty pLNCX vector, was used as a negative control. The transfected cell lines were previously described and were a kind gift of Y.M. Zoet from the Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands [26]. All K562 cell lines were cultured in RPMI-1640 (Gibco™,Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal calf serum (FCS) and 2-mM L-Glutamine. The transfected K562 cells were cultured with G418 (neomycin derivative, final concentration: 200 µg/ml; Invitrogen, Groningen, The Netherlands). The HLA-G expressing choriocarcinoma cell line JEG-3, was cultured in DMEM (Gibco™) with 10% FCS.

Early passage CRC cell lines were established from primary CRC tumors and colorectal liver metastasis at the department of Pathology, Leiden University Medical Center, Leiden, The Netherlands. The cell lines have been characterized for cancer-related mutations by Boot et al. [27]. These CRC cell lines were previously investigated for the presence of mRNA encoding the HLA-G protein [25]. Four cell lines (JVE114, JVE192, JVE222 and JVE528) were previously identified to express mRNA encoding the HLA-G3 protein, albeit at very low level. Furthermore, four CRC cell lines negative for mRNA encoding HLA-G protein were selected as well (JVE044, JVE103, JVE207, JVE241). The selected early passage CRC cell lines were cultured in Dulbecco's Modified Eagle medium (DMEM)/F12 (Gibco™) with 10% FCS. Culturing conditions were the same for all the cell lines (37^0C, 5% CO_2).

ANTIBODIES

HLA-G targeting mouse mAb 4H84 (Exbio, Czech Republic) and MEM-G/1 (Abcam, Cambridge, UK, ab 7759), both recognizing all HLA-G isoforms (according to the manufacturer), were used to assess HLA-G expression on frozen tumor tissue sections. Furthermore, the mAb clone 5A6G7 (Sigma-Aldrich, Saint Louis, USA) detecting soluble HLA-G (sHLA-G) was used. To visualize HLA-class I mAb HCA2 was used (kindly provided by Prof. Dr. J. Neefjes, Leiden University Medical Center, Leiden**,** the Netherlands). The HCA2 mAb recognizes all HLA-A chains (except HLA-A24), some HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G chains [28,29]. For protein loading control in western blot analysis the mAb Anti-alpha-Tubulin (Abcam, ab7291) was used.

CELL AND TISSUE LYSIS, PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

For preparation of protein extracts, 30 sections of 10µm frozen rectal cancer tissues were crushed with a mortar under ice cold conditions and lysed with NP-40 lysis buffer (50 mM Tris-base pH=7.8, 150 mM NaCl, 5mM ethylenediaminetetraacetic acid, 0.5% NP-40, 1x HALT™ Protease & phosphatase inhibitor Cocktail, EDTA-free, Thermo scientific). Cell lines were washed three times with ice cold PBS. Next, cell pellets were collected and lysed with NP-40 lysis buffer, 100 µl per 1×10^6 cells/ml, for 30 min. After centrifugation at 12.000 rpm at 4°C for 20 min, supernatants were collected and protein concentration was determined using the Pierce™ BCA protein assay (ThermoFisher Scientific, Waltham, MA USA). All samples were heated for 5 min at 95°C in Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, California, USA) before loading. Cell lysate aliquots were separated in 12% SDS-PAGE (mini-PROTEAN® TGX™ precast gel, Bio-Rad). Proteins were electro-blotted onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad) and blocked by incubation with TBST ((20mM Tris (pH=7.6), 150mM NaCl and 0.2% Tween-20)) containing 5% non-fat dry milk for 1hr at room temperature. After blocking, membranes were washed in TBST three times for 5

min and then probed with the mAbs overnight at 4°C. Subsequently, the membranes were washed with TBST and incubated for 1hr at room temperature with rabbit-anti mouse-HRP (Dako, Glostrup, Denmark) in TBST containing 5% non-fat dry milk. Finally, membranes were washed three times and developed with Clarity™ Western ECL substrate (Bio-Rad), followed by imaging with ChemiDoc™ MP (Bio-Rad).

IMMUNOHISTOCHEMISTRY

To assess HLA-G expression on frozen tissue sections, HLA-G targeting mouse mAbs 4H84 and MEM-G/1 were used. Furthermore, mAb 5A6G7 recognizing sHLA-G and HCA-2 was used to detect HLA class I. Tissue sections of 4 µm were cut from the fresh frozen tumor tissues. A standard IHC protocol using the Envision+ (Dako) was performed. Briefly, slides were fixed in acetone for 10 min and washed twice with Phosphate Buffered Saline (PBS). Followed by blocking with BLOXALL (Vector laboratories, California, USA) for 10 min. Thereafter, tissue sections were washed two times for 5 min with PBS. Nonspecific protein binding was blocked with 5% normal goat serum (Dako) for 20 min. The sections were incubated, at pre-determined optimal dilutions, for 1hr at room temperature with the primary antibody (4H84, MEM-G/1, 5A6G7 or HCA-2). Incubation was followed by washing the tissue sections, three times for 5 min in PBS followed by 30 min incubation with EnVision+ System-HRP anti-mouse (Dako). The staining was completed by 10 min incubation with 3,3'-diaminobenzidine (DAB)+ Substrate-Chromogen System (Dako). Next, sections were washed twice in demineralized water and counterstained with Haematoxylin. Finally, sections were dehydrated and mounted in Pertex**.** For anti-HLA-G mAbs, placenta tissue was used as a positive control. Sections serving as negative controls underwent the entire protocol except primary antibody incubations. Expression of HLA-G was evaluated as positive or negative.

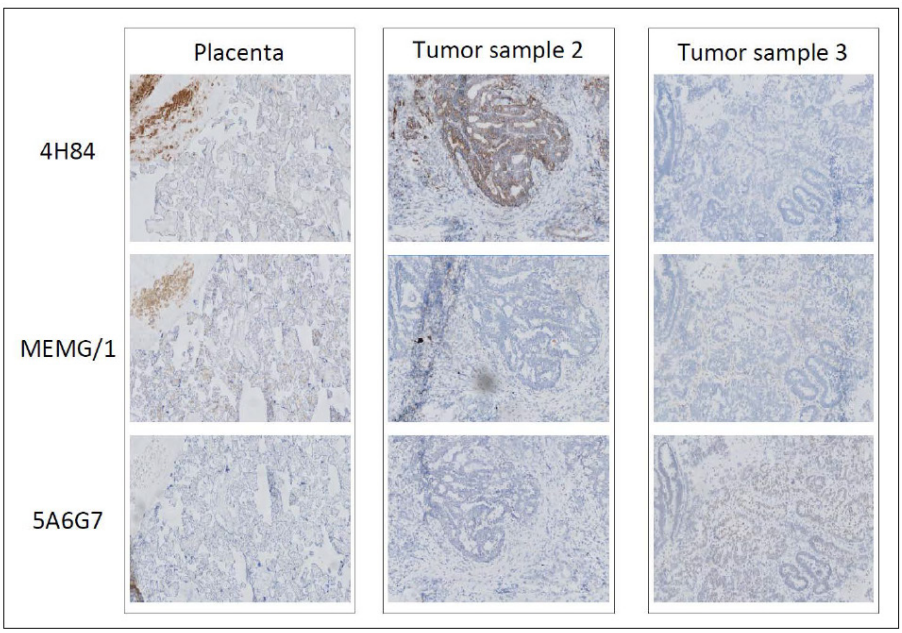
RESULTS

IMMUNOHISTOCHEMISTRY OF TUMOR TISSUE SECTIONS

To evaluate HLA-G expression in fresh-frozen tissue sections by IHC, two mAbs detecting all HLA-G isoforms were used, namely 4H84 and MEM-G/1. Furthermore, the mAb 5A6G7 was used, detecting soluble HLA-G5/HLA-G6 by recognizing the retrained intron 4 (according to the manufacturer). Since sequential tissue sections were used, variation due to tissue heterogeneity was not expected. Representative images of the staining patterns are shown in Figure 1. In line with previous reported results [24,25], both 4H84 and MEM-G/1 (detecting all HLA-G isoforms) showed a similar positive reactivity

pattern in placenta tissue (Figure 1). In contrast, tumor tissue sections stained with both 4H84 and MEM-G/1 showed varying staining patterns dependent on the mAb used. For example, tumor sample 2 was positive for 4H84, but no reactivity with MEM-G/1 was observed. Tumor sample 3 did not reveal any reactivity with 4H84 or MEMG/1.

With respect to sHLA-G, no reactivity was observed with 5A6G7 in placental tissue. Reactivity for 5A6G7 was however observed in tumor sample 3. The results of all IHC analyses are summarized in Figure 1, panel B.



B.

	Immunohistochemistry		
	4H84	MEM-G/1	5A6G7
Tumor 1	-	-	-
Tumor 2	+	-	-
Tumor 3	-	-	+
Tumor 4	-	-	-
Tumor 5	+	-	+

Figure 1. Representative images of immunohistochemistry with 4H84, MEM-G/1 and 5A6G7.
A. In the left panel sequential placenta tissue sections positively stained with 4H84 and MEM-G/1 which showed the same reactivity pattern. Placenta tissue sections stained negative for 5A6G7. Sequential tissue sections of tumor sample 2 was positive for 4H84, negative for MEM-G/1 (both detecting all HLA-G isoforms) and negative for 5A6G7. The right panel, showing sequential tumor tissue section of tumor sample 3, was negative for 4H84 and MEM-G/1 and showed positive staining with 5A6G7. **B.** Summary of the immunohistochemistry analysis.

WESTERN BLOT ANALYSIS OF CRC CELL LINES

Low levels of HLA-G expression in tumors have been reported [25,30]. Therefore, a dilution experiment was performed to establish the lowest HLA-G expression level that we were able to detect by western blot analysis with 4H84 and MEM-G/1. The HLA-G overexpression cell line K562-G1 was serially diluted with the HLA negative cell line K562-WT. As shown in Figure 2, we were able to detect HLA-G expression as low as 5% of HLA-G expressing cells in a mixture for 4H84 and 10% for MEM-G/1. Therefore, K562-G1-10% was used as a positive control, in addition to K562-G1-100%. Next, we investigated HLA-G expression in the selected early passage CRC cell lines which were found to express very low levels of mRNA encoding HLA-G3 (JVE114, JVE192, JVE222 and JVE528) and four CRC cell lines, lacking expression of any HLA-G isoform (JVE044, JVE103, JVE207, JVE241) [25].

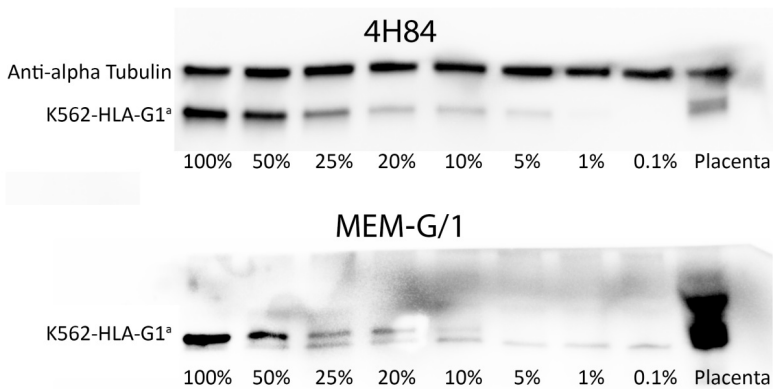


Figure 2. Dilution experiment. Representative image of the dilution experiment, to establish the lowest HLA-G expression level which we were able to detect by western blot analysis with 4H84 and MEM-G/1. The HLA-G overexpression cell line K562-G1 was diluted with the HLA-G negative cell line K562-WT. As shown in this figure we were able to detect HLA-G expression as low as 5% for 4H84 and 10% for MEM-G/1. For MEM-G/1 a smaller, non-specific, band was detected as well, visible in every dilution.

As shown in Figure 3A, all CRC cell lines clearly showed expression of HLA-A2 molecules, when western blot analysis was performed with mAb HCA-2. HCA-2 is also known for reacting with HLA-G. As shown in Figure 3A a clear positive band at approximately 35 kDa, corresponding with the molecular weight of HLA-G, was observed for the positive control cell line K562-G1. Furthermore, a clear positive band, corresponding with the size of HLA-A2, was observed in control cell line K562-A2 at approximately 39 kDa. No HLA-G expression was detected in any of the CRC cell lines using mAb HCA-2.

For the detection of HLA-G with mAbs 4H84 and MEM-G/1, K562-G1 served as a positive control and HLA-G1 protein expression was clearly present (Figure 3A). The JEG-3 choriocarcinoma cell line (originating from trophoblast cells), known for expression of soluble HLA-G, was used as a positive control for western blot analysis using mAb 5A6G7. The staining pattern revealed a clear positive band at approximately 33kDa corresponding in size with HLA-G6 (Figure 3B). As shown in Figure 3A and B, western blot analysis using 4H84, MEM-G/1 or 5A6G7, did not detect any HLA-G isoforms in CRC cell lines corresponding in size with those expressed in the control cell lines K562-G1 or JEG-3.

Notably, western blot analysis using MEM-G/1, all K562 control cell lines and also in some CRC cell lines showed additional immune reactivity at a molecular weight of approximately 32kDa, (indicated by the (*) in Figure 3A). Additional immune reactivity ranging from 36-175kDa was detected with 5A6G7 as well (Figure 3B).

WESTERN BLOT ANALYSIS WITH RECTAL CANCER TISSUE

Figure 4 shows the western blot analysis for rectal cancer protein lysates, derived from 5 frozen primary tumor tissues. As shown in Figure 4A, tumor tissue sections of all these 5 tumors stained positive for HCA-2. When using the mAb 4H84 no clear detectable HLA-G encoding proteins could be detected in these rectal tumor tissue samples. However, some reactivity with an estimated molecular weight of 37kDa in tumor 2, 32kDa in tumor 4 and 5 and 41kDa in all tumors was visible indicated by (*) (when exposure time was increased, bands became more visible; data not shown). The size of these additional proteins did not correspond with any of the known HLA-G isoforms. Importantly, the observed immune reactions in the western blot analysis did not correspond with the IHC staining patterns in matching tumor tissues (Figures 1 and 4). Similarly, in the western blot analysis using MEM-G1, none of the rectal tumor tissue samples revealed expression of proteins corresponding in size with HLA-G.

Like for the 4H84 mAb, the western blot analysis using MEM-G/1 also revealed additional immune reactivity with a molecular weight of approximately 32kDa and 48kDa in all controle cell lines and in all CRC tissues.

For western blot analysis using 5A6G7 all tumor tissue samples did not show immune reactivity corresponding in size to those detected in JEG-3 (Figure 4B). More importantly, intense additional immune reactivity was observed with proteins with a molecular weight varying from 36-175kDa in the K562 control cell lines. In all tumor tissues addition immune reactivity at approximately 44kDa were detected.

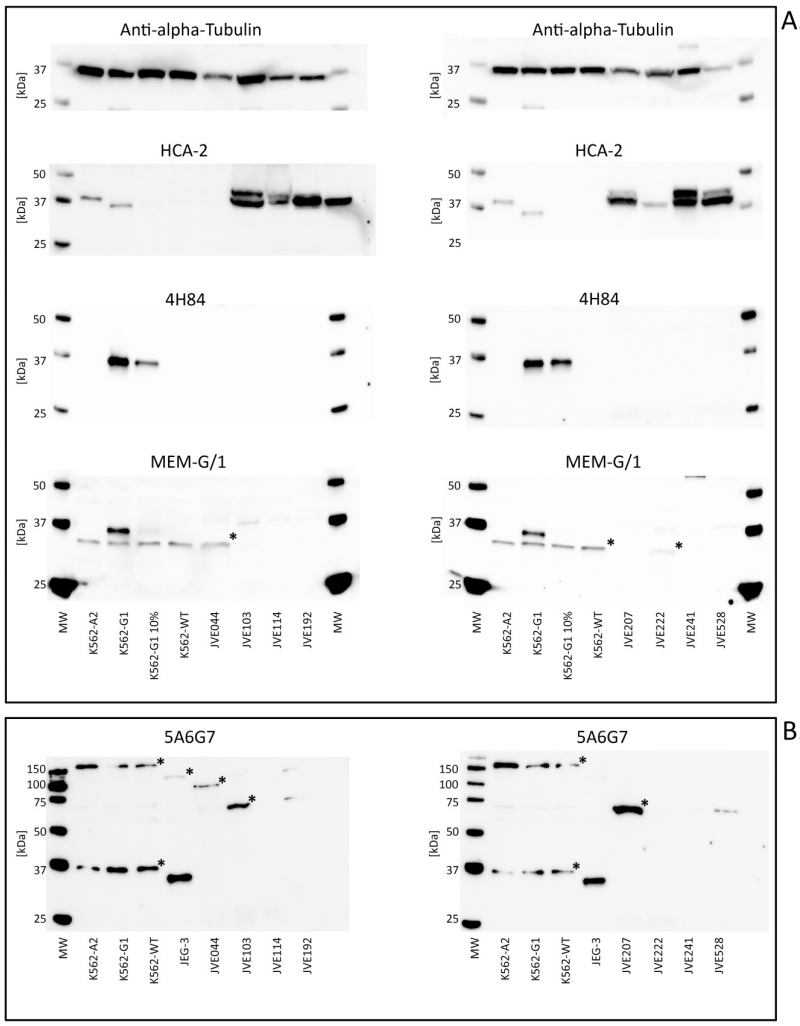


Figure 3. Western blot analysis for colorectal cancer cell lines protein lysate.

As shown in panel **A**, all cell lines were similar in expressing Anti-alpha-Tubulin, used as a loading control. All CRC cell lines were expressing HLA-A2. Furthermore, HLA-G was clearly detected by HCA2 in K562-G1, illustrated by the difference in molecular weight of the detected protein. No bands corresponding in size with HLA-G1 were detected with HCA-2 in the CRC cell lines. For the detection of HLA-G with 4H84 and MEM-G/1, cell line K562-G1 served as a positive control and a clear expression of the HLA-G protein at approximately 35kDa was shown. In all CRC cell lines, HLA-G expression was absent using mAb 4H84. For mAb MEM-G/1, K562-G1 showed a clear positive band. In all CRC cell lines HLA-G expression was absent, using mAb MEM-G/1. In all K562 control cell lines and in JVE044 and JVE222 additional immune reactivity with a molecular weight of approximately 32kDa, was detected indicated by (*).

In the panel **B** the results obtained with mAb 5A6G7 are shown. JEG-3 was clearly positive, whereas

all CRC cell lines sHLA-G expression was absent. Additional immune reactivity was detected for 5A6G7 for proteins with a varying molecular weight, indicated by (*).

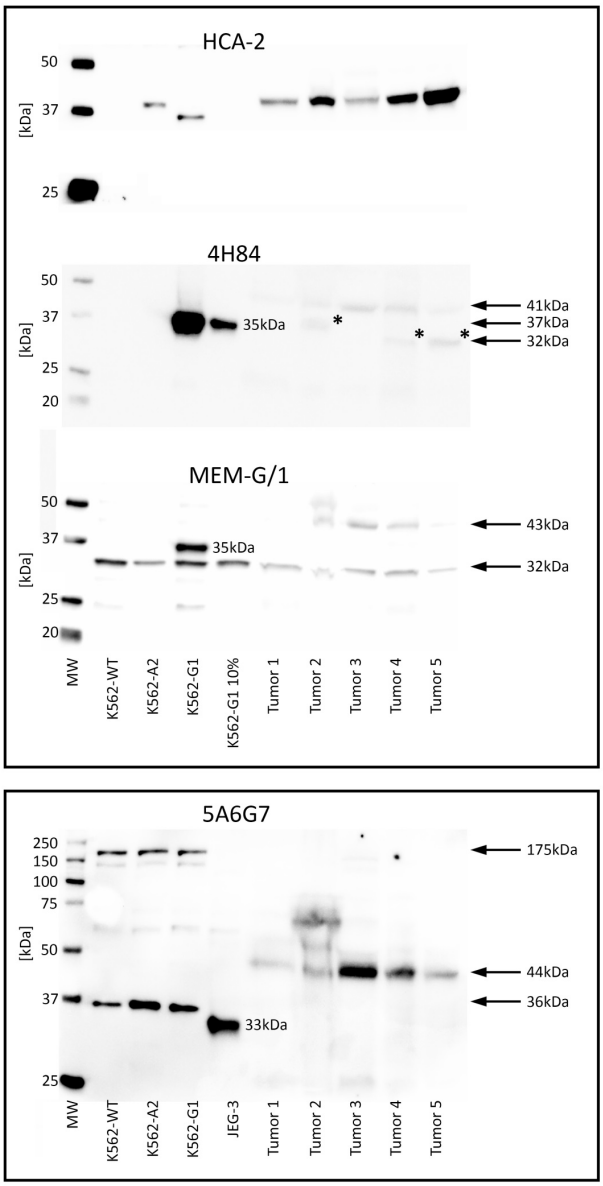


Figure 4. Western blot analysis for rectal cancer tissue protein lysate.

The figure demonstrates western blot analysis for rectal cancer protein lysates, derived from frozen primary tumor tissue. As shown in panel **A.** all tumor tissue sections were positive for mAb HCA-2. For mAb 4H84 positive bands were detected in K562-G1 and K562-G1 10%. No clear visible bands were detectable in the tissue samples compared with positive control K562-G1 and K562-G1 10%.

However, some reactivity was visible in tumor sample 2 (37kDa), tumor sample 4 (32kDa), and tumor sample 5 (32kDa), indicated by (*), (when the exposure time was adjusted bands became clearer, data not shown). Furthermore, for all tumor samples immune reactivity was observed at an estimated molecular weight of 41kDa. Using MEM-G/1, a clear positive band was detected in K562-G1 at approximately 35kDa. In none of the tumor tissue samples the HLA-G protein expression could be detected with MEM-G/1. Notably, using MEM-G/1, in all control cell lines and in all rectal cancer additional immune reactivity with a molecular weight of approximately 32kDa was detected. In the tumor samples a protein of approximately 43kDa was detected as well.
Using mAb 5A6G7, panel **B,** JEG-3 was clearly positive at approximately 33kDa, while all rectal tumor tissue samples did not show positive staining of proteins corresponding in size with JEG-3. More importantly, additional immune reactivity was detected for 5A6G7 with proteins with a varying molecular weight.

Similar results were seen in the tumor samples. For example IHC of tumor sample 2 showed intensive staining using mAb 4H84 while negative for MEM-G/1 and 5A6G7, but western blot, showed no clear bands corresponding with HLA-G. Likewise, tumor sample 3 stained positive with IHC using mAb 5A6G7, but no bands were detected corresponding in size with sHLA-G detected in JEG-3.

DISCUSSION

In the present study, HLA-G protein expression in CRC was studied in more detail than in any of the previous studies. The results of our studies show that in early-passage CRC cell lines and primary rectal tumor tissues, no clear HLA-G protein expression could be detected, using mAbs 4H84, MEM-G/1 or 5A6G7 in western blot analysis. The size of the immune reactive proteins in the rectal tumors did not correspond with the size of the HLA-G protein observed in K562-G1 or in JEG-3. In contrast with the results obtained by western blot analysis, strong positive IHC staining patterns in matching tumor tissue were observed.

However, no concordance was observed between the results obtained by western blot analysis and IHC. Additionally, in sequential fresh frozen tissue sections stained with various HLA-G detecting mAbs, discrepant expression profiles were observed. This suggests non-specific binding. However, similar analyses with placenta tissue using 4H84 and MEMG/1 showed that the results of the IHC and the western blot analysis were consistent. This shows the specificity of these mAbs for HLA-G expression in placenta

tissue, but not for rectal cancer tissue. Furthermore, in all K562 control cell lines, in all tumor tissues and in two out of eight CRC cell lines additional immunoreactivity with a varying molecular weight was detected using MEM-G1 and 5A6G7 in western blot analysis. Consequently, it could be debated whether these mAbs are in fact detecting HLA-G in some tumor tissue samples. In summary, for CRC cell lines and rectal tumor tissues results obtained by western blot analysis did not correspond with the results obtained by IHC. This reveals the existence of non-specific binding, which could result in false positive recognition of HLA-G expression in CRC. As a consequence this may lead to an over-estimation of HLA-G expression in cancer, especially in studies using solely IHC.

The possibility that *de novo* expression of the HLA-G protein by tumor cells is present to escape immunosurveillance is fascinating, but considerable controversy exists. In a study by *Real et al.* [31] analyzing 50 solid tumors and 31 tumor cell lines of different origin, no HLA-G protein expression was detected, despite the presence of mRNA encoding HLA-G, although at several orders of magnitude lower than mRNA encoding classical HLA class I. They proposed that HLA-G protein expression could be under very strong post-translational control. The study by *Real et al*. corresponds with our previous findings of extremely low levels of mRNA encoding the HLA-G3 protein in some of the JVE cell lines [25], while no corresponding HLA-G protein expression could be detected by western blot analysis in this study. These findings suggests that the role of HLA-G protein expression in escaping immunosurveillance by NK cells in colorectal cancer, if any, will only be minor. We therefore feel that caution should be taken in literature describing solely IHC experiments performed with one mAb only, as the noted cross-reactivity with proteins other than HLA-G, may lead to false interpretation of HLA-G protein expression [23,32]. Accordingly, it could be proposed that conclusions based on IHC alone should be drawn very carefully.

Based on the current study in combination with our previous reported results [25], we conclude that the role of HLA-G as immune modulator in CRC is premature. HLA-G is considered as an immune checkpoint molecule and studied as a potential target for immunotherapy. Until the time that detection of HLA-G is selective enough to detect all aspects of HLA-G expression in biological samples, rather than transfected cells or long time cultured cell lines, therapeutic applications involving HLA-G will remain enigmatic.

REFERENCES

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.
3. Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother 2004;53:904-10.
4. Atkins D, Breuckmann A, Schmahl GE, et al. MHC class I antigen processing pathway defects, ras mutations and disease stage in colorectal carcinoma. Int J Cancer 2004;109:265-73.
5. Momburg F, Degener T, Bacchus E, Moldenhauer G, Hammerling GJ, Moller P. Loss of HLA-A,B,C and de novo expression of HLA-D in colorectal cancer. Int J Cancer 1986;37:179-84.
6. Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? Semin Cancer Biol 2007;17:459-68.
7. Ellis SA, Palmer MS, McMichael AJ. Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule. J Immunol 1990;144:731-5.
8. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA-G, expressed in human trophoblasts. Science 1990;248:220-3.
9. Rouas-Freiss N, Goncalves RM, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. Proc Natl Acad Sci U S A 1997;94:11520-5.
10. Blaschitz A, Juch H, Volz A, et al. The soluble pool of HLA-G produced by human trophoblasts does not include detectable levels of the intron 4-containing HLA-G5 and HLA-G6 isoforms. Mol Hum Reprod 2005;11:699-710.
11. Loke YW, King A, Burrows T, et al. Evaluation of trophoblast HLA-G antigen with a specific monoclonal antibody. Tissue Antigens 1997;50:135-46.
12. Ferreira LM, Meissner TB, Tilburgs T, Strominger JL. HLA-G: At the Interface of Maternal-Fetal Tolerance. Trends Immunol 2017;38:272-86.
13. de Kruijf EM, Sajet A, van Nes JG, et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. J Immunol 2010;185:7452-9.
14. Paul P, Rouas-Freiss N, Khalil-Daher I, et al. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. Proc Natl Acad Sci U S A 1998;95:4510-5.
15. Rouas-Freiss N, Moreau P, Menier C, LeMaoult J, Carosella ED. Expression of tolerogenic HLA-G molecules in cancer prevents antitumor responses. Semin Cancer Biol 2007;17:413-21.
16. Reimers MS, Engels CC, Putter H, et al. Prognostic value of HLA class I, HLA-E, HLA-G and Tregs in rectal cancer: a retrospective cohort study. BMC Cancer 2014;14:486.
17. Amiot L, Ferrone S, Grosse-Wilde H, Seliger B. Biology of HLA-G in cancer: a candidate molecule for therapeutic intervention? Cell Mol Life Sci 2011;68:417-31.
18. Carosella ED, Rouas-Freiss N, Roux DT, Moreau P, LeMaoult J. HLA-G: An Immune Checkpoint Molecule. Adv Immunol 2015;127:33-144.

19. Zeestraten EC, Reimers MS, Saadatmand S, et al. Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients. Br J Cancer 2014;110:459-68.
20. Guo ZY, Lv YG, Wang L, et al. Predictive value of HLA-G and HLA-E in the prognosis of colorectal cancer patients. Cell Immunol 2015;293:10-6.
21. Kirana C, Ruszkiewicz A, Stubbs RS, et al. Soluble HLA-G is a differential prognostic marker in sequential colorectal cancer disease stages. Int J Cancer 2017;140:2577-86.
22. Apps R, Gardner L, Moffett A. A critical look at HLA-G. Trends Immunol 2008;29:313-21.
23. Polakova K, Kuba D, Russ G. The 4H84 monoclonal antibody detecting beta2m free nonclassical HLA-G molecules also binds to free heavy chains of classical HLA class I antigens present on activated lymphocytes. Hum Immunol 2004;65:157-62.
24. Swets M, Konig MH, Zaalberg A, et al. HLA-G and classical HLA class I expression in primary colorectal cancer and associated liver metastases. Hum Immunol 2016;77:773-9.
25. Swets M, Seneby L, Boot A, et al. Promoter methylation and mRNA expression of HLA-G in relation to HLA-G protein expression in colorectal cancer. Hum Immunol 2016;77:764-72.
26. Zoet YM, Eijsink C, Kardol MJ, et al. The single antigen expressing lines (SALs) concept: an excellent tool for screening for HLA-specific antibodies. Hum Immunol 2005;66:519-25.
27. Boot A, van EJ, Crobach S, et al. Characterization of novel low passage primary and metastatic colorectal cancer cell lines. Oncotarget 2016.
28. Seitz C, Uchanska-Ziegler B, Zank A, Ziegler A. The monoclonal antibody HCA2 recognises a broadly shared epitope on selected classical as well as several non-classical HLA class I molecules. Mol Immunol 1998;35:819-27.
29. Sernee MF, Ploegh HL, Schust DJ. Why certain antibodies cross-react with HLA-A and HLA-G: epitope mapping of two common MHC class I reagents. Mol Immunol 1998;35:177-88.
30. Real LM, Cabrera T, Collado A, et al. Expression of HLA G in human tumors is not a frequent event. Int J Cancer 1999;81:512-8.
31. !! INVALID CITATION !!! [29].
32. Zhao L, Teklemariam T, Hantash BM. Reassessment of HLA-G isoform specificity of MEM-G/9 and 4H84 monoclonal antibodies. Tissue Antigens 2012;80:231-8.