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Biomarkers in colorectal cancer

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Chapter 6

Promoter methylation and mRNA expression of *HLA-G* in relation to HLA-G protein expression in colorectal cancer

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ABSTRACT

Expression of human leukocyte antigen-G (HLA-G) is a suggested mechanism used by tumor cells to escape from host immune recognition and destruction. Advances in the field have made it evident that HLA-G is expressed in different types of malignancies including colorectal cancer (CRC). We analyzed HLA-G expression in 21 low passage CRC cell lines. The level of DNA methylation of the *HLA-G* gene and the presence of mRNA encoding HLA-G was measured. Moreover, HLA-G protein expression was determined by flow cytometry and immunohistochemistry (IHC). IHC was performed with three different monoclonal antibodies (mAbs) (4H84, MEM-G/1 and MEM-G/2). In addition, HLA-G protein expression was measured in matching primary tumor tissues. RNA analysis using RT-PCR followed by sequencing in 6 samples indicated strong homology of the PCR product with HLA-G3 in 5 samples. In accordance, in none of the cell lines, HLA-G1 expression was detected by flow-cytometry. Furthermore, no association between *HLA-G* DNA methylation patterns and *HLA-G* mRNA expression was observed. In addition, different immunohistochemical staining profiles among various anti-HLA-G mAbs were observed. In conclusion, the results of this study show that the HLA-G3 isoform was expressed in some of the CRC cell lines irrespective of the level of DNA methylation of *HLA-G*.

INTRODUCTION

Evasion of immune surveillance is considered one of the emerging hallmarks of cancer [1, 2]. Understanding the complex mechanisms used by tumor cells to differentiate towards cells with reduced immunogenicity is a major challenge. It is suggested that tumor cells can escape immune recognition and destruction by expression of human leukocyte antigen-G (HLA-G) and it provides to an explanation why expression of the HLA-G molecule is associated with poor patient prognosis [3-5]. HLA-G is a non-classical HLA class I (class Ib) molecule, expressed at immune-privileged sites such as the placenta, in extravillous trophoblast cells [6-8]. Alternative splicing of the primary transcript has been reported to result in seven different HLA-G protein isoforms; four membrane-bound (HLA-G1, G2, G3, G4) and three soluble (HLA-G5, G6, G7) isoforms [9]. Advances in the field have made it evident that HLA-G is also expressed in different types of malignancies in a *de novo* manner [10]**.** This tumor driven expression of HLA-G inhibits the function of several types of immune cells, among which T cells and natural killer (NK) cells, inhibits proliferation of immune cells, and can additionally induce expansion of an immunosuppressive T cell subset [11]. Therefore HLA-G expression in tumors was recently described as an immune checkpoint molecule [12]**.** Expression of HLA-G appears as a promising clinical prognostic factor in several types of cancer, including colorectal cancer (CRC) [13-16]. However, among and within different tumors types variances in HLA-G expression were observed [17]. For example, HLA-G expression has been reported by Zeestraten *et al.* in 20% of colon tumors, whereas Guo *et al.* reported levels up to 72% in CRC [15, 18]. Immunohistochemistry (IHC) is a widely accepted technique to detect HLA-G expression, although the obtained results are still controversial [17, 19]. It is important to note that most studies used a single type of monoclonal antibody (mAb), usually 4H84. However, in CRC we showed previously discrepant expression profiles among various types anti-HLA-G mAbs [20]. For that reason and because of the known cross reactivity of mAb 4H84, it is recommended to use multiple HLA-G specific mAbs [17, 19]. Furthermore, additional molecular biological and biochemical analyses will be required to evaluate HLA-G expression in cancer and to firmly validate HLA-G expression patterns.

Previously it has been shown that HLA-G transcription is regulated by epigenetic mechanisms, including by DNA methylation [21, 22]. Nevertheless, many of the established cell lines utilized in research have been in culture for decades and may present aberrant genetic and epigenetic characteristics. In the current study we therefore investigated *HLA-G* DNA methylation level in 21 recently established CRC cell lines never investigated before for HLA-G expression. Furthermore, the presence of HLA-G mRNA was measured. Membrane expression of the HLA-G protein was evaluated by flow cytometry and IHC. We used three different anti-HLA-G mAbs for analyzing

expression of HLA-G by IHC in the CRC cell lines and results were compared with paraffin-embedded tumor tissue of which the tumor cell lines were derived from.

MATERIALS AND METHODS

TUMOR CELL LINES

21 CRC cell lines were established from primary CRC tumors and colorectal liver metastasis at the Department of Pathology, LUMC (Table 1). The cell lines have been extensively characterized for several cancer related mutations by Boot et al. [23]. The CRC cell lines were cultured in RPMI-1640 (Gibco™,Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal calf serum (FCS). Dulbecco's Modified Eagle medium (DMEM)/F12 (Gibco™) with 10% FCS was used for culturing JVE222 and JVE371. DMEM (Gibco™) with 10% FCS was used to culture the choriocarcinoma cell line JEG-3, known for expressing all HLA-G isoforms.

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Table 1. Characteristic of the colorectal cancer cell lines. Cell line name, passage number of *in vitro* culture at RNA isolation, and tumor location and morphology.

Cell Line	Passage	Location	Tumor morphology
JVE015	p.15	Sigmoid	Unknown
JVE017	p.13	Cecum	Unknown
JVE044	p.13	Rectum	Unknown
JVE059	p.15	Colon	Adenocarcinoma
JVE103	p.13	Liver[1]	Adenocarcinoma
JVE109	p.21	Colon	Adenocarcinoma
JVE114	p.13	Liver[1]	Adenocarcinoma
JVE127	p.12	Colon	Mucinous adenocarcinoma
JVE187	p.13	Liver[1]	Adenocarcinoma
JVE192	p.13	Colon	Mucinous adenocarcinoma
JVE207	p.14	Colon	Adenocarcinoma
JVE222	p.19	Colon	Adenocarcinoma
JVE 241	p.8	Cecum	Mucinous adenocarcinoma
JVE253	p.6	Liver[1]	Mucinous adenocarcinoma
JVE367	p.7	Ileocecal junction	LNEC[2]
JVE371	p.7	Liver[1]	Adenocarcinoma
JVE528	p.8	Colon	Adenocarcinoma
JVE774	p.17	Rectum	Adenocarcinoma
KP283T	p.6	Liver[1]	Adenocarcinoma
KP363T	p.8	Colon	Adenocarcinoma
KP7038T	p.7	Colon	Adenocarcinoma

DNA PROMOTER METHYLATION

DNA isolation was performed with the Wizard genomic DNA purification kit (Promega, Madison, WI,USA) according to the manufacturer's instructions. Bisulfite conversion was performed with EZ DNA Methylation Gold kit (Zymo Research Corp, Orange, CA, USA) on genomic DNA, according to the manufacturer's instructions, using an input of 200ng of DNA. Primers (Biolegio, Nijmegen, the Netherlands) previously designed by Holling *et al.* [21], targeting the minimal promoter and part of CpG island-3 in exon 3, were used with an additional M13-tail (underlined) for sequencing purposes. BSHLA-GF1:*5'TGTAAAACGACGGCCAGTGATTTAGGGAGATATTGAGATAGAA-3'* and BSHLA-GR1:*5'CAGGAAACAGCTATGACCCACCTAATAAAAATAAAAACTAAAACC-3'* detecting the minimal promoter. BSHLA-GF3: *5'TGTAAAACGACGGCCAGTATATTTTTTAGTGGATGATTGGTTG-3'* and BSHLA-GR3: *5'CAGGAAACAGCTATGACCCCTCCAAATAAACTCTCCTTTATTC-3'*spanning a part of exon 3. PCR was performed in a final volume of 10 µl using AmpliTaq Gold polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (1 unit per reaction), 1X AmpliTaq Gold buffer, $MgCl_2$ (1.9mM), dNTPs (200µM each), Syto9 (0.4µM) and pooled primers (2.5µM) according to the manufacturer's instructions with 100 ng of genomic DNA of each of the 21 novel CRC cell lines. The following PCR protocol was executed: 5min 95°C, 15x (15sec 95°C, 30sec 59°C,with 0.6°C decrease every cycle, 20sec 72°C), 39x (10sec 95°C, 30sec 62°C, 20sec 72°C), 1min 60°C, 10sec 65°C, 5sec 95°C). The PCR product was analyzed using QIAxcel (QIAGEN, Hilden, Germany) followed by product purification with purifying plates (Thermo Fisher Scientific). Samples were sent for sequencing to Macrogen, Amsterdam, the Netherlands. In order to analyze CpG nucleotide methylation the Epigenetic Sequencing Methylation Software (ESME) was used. Methylation patterns were deduced from aligning the bisulfite-converted sequences of the CRC cell lines to the genomic sequence of the *HLA-G* gene (NG_029039.1). DNA methylation levels were quantified by calculation of the total methylation percentage of the CpG sites. The total methylation percentage was calculated as the sum of methylated CpG sites divided by the number of CpG sites. The methylation pattern of JEG-3 was used as a comparative parameter, not as a reference parameter.

RNA ISOLATION, cDNA SYNTHESIS

Trypsin/EDTA was used to harvest cultured JEG-3 cells for RNA isolation with the RNeasy mini-kit (QIAGEN, Limburg, Netherlands). DNase treatment with RNase-free DNase Set (QIAGEN) was performed according to the manufacturer's instructions. RNA (1µg) was converted to cDNA using iSCRIPT cDNA synthesis kit (Biorad, Hercules, Califrnia, USA). TRIzol Reagent (Life Technologies, Bleiswijk, the Netherlands) was used to isolate RNA from the 21 CRC cell lines. DNAse treatment was performed in suspension using rDNAse (Macherey Nagel GmbH & Co. KG, Düren, Germany). cDNA was synthesized using 1-2µg RNA, 100ng oligo-dT, 1mM dNTP's, 5U AMV-RT transcriptase and 10U

RNasin (#M5108 and # N2615)(Promega).

PCR, GEL ELECTROPHORESIS AND SEQUENCING

To detect the different HLA-G isoforms, HLA-G primers (Biolegio) G.257 (exon 2; 5'-'GGAAGAGGAGACACGGAACA-3') and G.1225 (3'-UT; 5'-TGAGACAGAGACGGAGACAT-3') were used, first described by Kirszenbaum *et al.* [24, 25]. For the RT-PCR, 2µL cDNA was used in combination with 1pmol primers in a total volume of 20 µL, containing 1x IQ SYBR Green Supermix (Biorad). The RT-PCR was performed using a CFX96 TOUCH™ (Biorad). The following PCR protocol was executed: 5min 95°C, 49x (30sec 95°C , 30sec 62°C, 1min 72°C). The final amplified PCR products were run on 1.5% agarose gel. The housekeeping gene GAPDH was used as an positive control [26]. The RT-PCR was performed multiple times. After gel electrophoresis the amplicons were extracted and column purified with a MinElute® PCR Purification kit (Qiagen). The PCR protocol was performed for a second time on the purified amplicons in order to increase the amplicon concentration, needed for sequencing. After column purification the samples were sent to the Leiden Genome Technology Center, LGTC (Leiden, the Netherlands) for Sanger sequencing. Alignment of the obtained sequences was performed using the BLAT-the BLAST-like alignment tool [27]. In addition, sequence alignments with HLA-G3 mRNA (ENST00000376815*)* was performed using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) software [28].

FLOW CYTOMETRY

The HLA-G1 targeting mAb MEM-G/9 (Exbio, Czech Republic) was used to assess cell surface HLA-G expression in the cell lines. The HLA class I targeting mAb W6/32 (Thermo Fisher Scientific) was used as a positive control. Controls, using the secondary antibodies only, were included for each cell line. Cells were thawed and washed in phosphate-*buffered* saline (PBS)/0.5% BSA twice before and after antibody incubations. Primary antibodies MEM-G/9, control antibody W6/32 and the secondary antibodies IgG1-FITC (PickCell Laboratories, the Netherlands) or IgG2a-FITC (PickCell) were incubated for 30 min on ice. IgG1-FITC and IgG2a-FITC are detecting MEM-G/9 and W6/32 respectively. All antibodies were used at predetermined optimal dilutions. The results were analyzed with FlowJo (Tree Star Inc, OR. USA), to compare the reactivity of targeting antibodies with the conjugated controls.

IMMUNOHISTOCHEMISTRY

HLA-G targeting mouse mAb 4H84 (Exbio, Czech Republic), MEM-G/1 (Abcam, Cambridge, UK, ab 7759) and MEM-G/2 (AbCam, ab26090), were used to assess HLA-G expression on formalin-fixed paraffin-embedded tissue sections. Cells from the CRC cell lines, were collected by careful scraping from the cell culture flasks, followed by

centrifugation. Conform standard procedures the collected cells underwent formalin fixation and were embedded in paraffin. Paraffin blocks with tumor tissue from which the cell lines were derived, were collected from the pathology archive. Tissue sections of 4 μm were cut from the paraffin blocks and processed for IHC. A standard IHC protocol using the Dako Envision+ (Dako, Glostrup, Denmark) was used. Briefly, after deparaffinization and rehydration endogenous peroxidase was blocked by incubating the sections in a 0.3% hydrogen peroxide solution for 20min (30% hydrogen peroxide, 100x diluted in water). Subsequently, antigen retrieval was performed as follows; slides for staining with mAbs MEM-G/1, MEM-G/2, were heated 10min at 95°C in pH low Target Retrieval Solution (Dako). Slides for staining with mAb 4H84 were heated 10min at 95°C in pH high Target Retrieval Solution (Dako). Sections were blocked with goat serum (5%) in PBS before incubation with MEM-G/1 and MEM-G/2. The sections were incubated, at pre-determined optimal dilutions, overnight with 4H84, MEM-G/1 or MEM-G/2. Next, sections were washed three times for 5min in PBS followed by 30min incubation with EnVision+ System-HRP anti mouse (Dako). The staining was completed by 10min incubation with 3,3′-diaminobenzidine (DAB)+ Substrate-Chromogen System (Dako). Finally, sections were washed twice in demineralized water, counterstained with haematoxylin, dehydrated and mounted in Pertex. Placenta tissue was used as a positive control for all three anti-HLA-G mAbs. Sections serving as negative controls underwent the entire protocol except primary antibody incubations. HLA-G expression was evaluated as positive or negative.

RESULTS

HLA-G PROMOTER METHYLATION

Transcription of *HLA-G* may be controlled by epigenetic mechanisms including DNA methylation**.** The methylation levels of 10 CpG dinucleotides in the minimal promoter and 15 CpG dinucleotides in CpG island-3 of *HLA-G* were analyzed in the early CRC cell lines**.** The methylation status was compared with the HLA-G expressing cell line JEG-3. The calculated JEG-3 methylation levels were 72% and 85% in the investigated CpG dinucleotides in the promoter and CpG Island-3, respectively. Compared with JEG-3, lower methylation levels in the minimal promoter were observed in the CRC cell lines JVE103, JVE192 and JVE371. In the CpG island-3 JVE059, JVE114, JVE192, JVE207, JVE222, JVE241, JVE253 JVE371, JVE774 KP283T, and KP7038T showed lower methylation levels compared with JEG-3 (Figure 1). Moreover, occasional cell lines were almost completely methylated in these locations e.g. JVE015 with 99% methylation in the minimal promoter and 96% in the CpG island-3 and JVE187 with 100% methylation

in the minimal promoter and 96% in the CpG island-3. In summary, high variance in methylation level was observed in the cell lines.

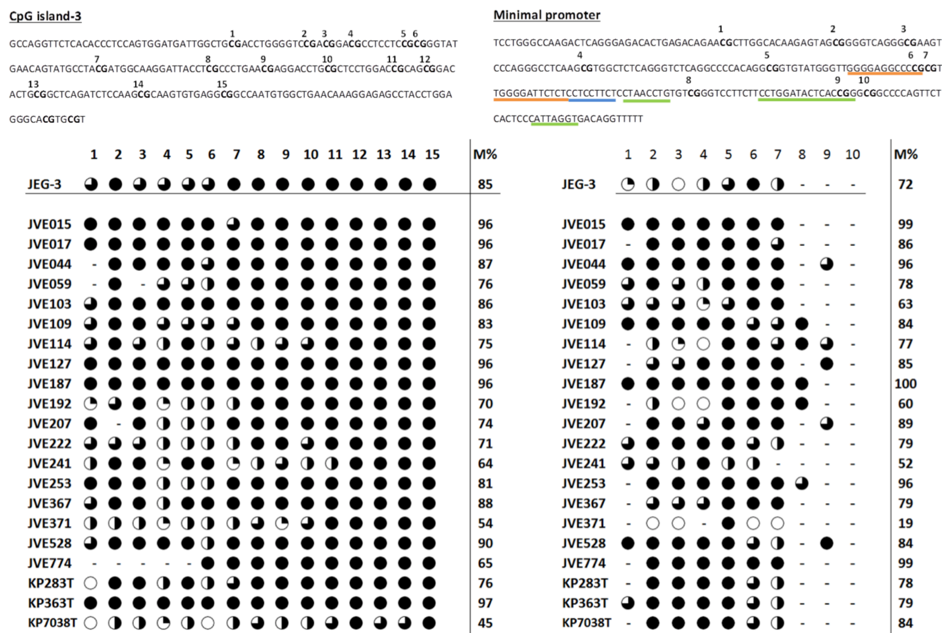


Figure 1: Methylation percentage for the minimal promoter and CpG island 3 in the 21 colorectal cancer cell lines and JEG-3. CpG nucleotides in the minimal promoter (1-10) and in the CpG island 3 (1-15). Regions in the minimal promoter resembling the Enh A (orange), ISRE (blue), S, X1, X2 and Y (green) are underlined. Circles indicate percentage of methylation in quartiles. M% reflects the percentage of methylation. Several of the later CpG dinucleotides in the minimal promoter could not be determined due to polymerase slippage in T rich areas. Therefore, an analysis of the reversed sequence was not possible.

mRNA TRANSCRIPTION OF HLA-G

The presence of HLA-G isoform transcripts was investigated by real time polymerase chain reaction (RT-PCR) of the respective cDNAs followed by Sanger sequencing. The sizes of visible amplicons detected with gel electrophoresis were compared to those obtained with the positive control JEG-3. The results of these analyses showed single amplicons, corresponding in size with detected amplicons in JEG-3, mainly HLA-G3. However, smaller unknown product bands were detected as well (Figure 2A). Furthermore, variability in presence of positive amplicons in the CRC cell lines was

observed among the RT-PCR performed. JEG-3 was positive in each RT-PCR performed and displayed amplicons comparable in size to isoform G5, G1, G6, G2/G4 and G3 (Figure 2A). The positive control GAPDH yielded a product in each PCR performed (Figure 2B). As a consequence of low amplicon concentration it was not possible to sequence each positive sample. In total, 6 purified amplicons were sequenced (Table 2).

Table 2. HLA-G mRNA expression per cell line. The table shows the cell lines positive after performing the RT-PCR and DNA sequencing. Due to low amplicon concentration the results obtained by Sanger sequencing of the visible band in cell line JVE187 showed very low signal peaks. Consequently, the obtained sequence was not reliable and is indicated as "unknown". Cell line JVE371 does show homology with HLA-G, however it was not possible to determine the isoform and is therefore indicated as "unknown".

Cell Line	Positive PCR[1]	Sequence corresponds to HLA-G[2]	HLA-G isoform
JVE015			
JVE017	yes		
JVE044			
JVE059			
JVE103			
JVE109			
JVE114	yes	yes	G3
JVE127	yes		
JVE187	yes	unknown	
JVE192	yes	yes	G3
JVE207			
JVE222	yes	yes	G3
JVE241			
JVE253	yes		
JVE367	yes		
JVE371	yes	yes	unknown
JVE528	yes	yes	G3
JVE774	yes		
KP283T	yes		
KP363T			
KP7038T	yes		

The amplicons obtained from the CRC cell lines JVE114, JVE192, JVE222 and JVE528 showed a strong homology with HLA-G3, when compared with the HLA-G3 mRNA sequence (ENST00000376815) (Figure 2C) and the coding sequence in order to evaluate mutations. In cell line JVE114 three single nucleotide polymorphisms (SNPs) were observed in the mRNA sequence. One SNP was located in exon 5 and the other

two were located in the 3'-UTR (indicated with red, figure 2C). Furthermore, in JVE114 a deletion in the first part of exon 7 was observed (represented by the empty box in figure 2C). In cell line JVE192 a SNP was observed in exon 5 and multiple known 3'-UTR variants were observed (indicated with red, figure 2C). Furthermore, an insertion of 14 base pairs was located in exon 7 (insertions are indicated by a green line in figure 2C). This insertion was originating from intron 6. Furthermore in JVE192 three single nucleotide insertions were observed in the 3'-UTR of exon 7 (indicated in green, figure 2C). However, these insertions were not observed in the coding sequence, since they were located in the 3'-UTR. In cell line JVE222 a "COSMIC" mutation (Catalogue Of Somatic Mutations In Cancer) was observed in exon 5 [29]. In the 3'-UTR one known and one unknown 3'-UTR variant was observed (indicated in red, figure 2C). Cell line JVE528 has three known SNPs. Furthermore, a deletion of exon 7 was observed in JVE528. JVE371 showed homology with HLA-G. However, in the results obtained by Sanger sequencing a substantial amount of non-matching and unknown base pairs were observed, which is most likely due to low amplicon concentrations. Therefore, we were not able to draw conclusion regarding the HLA-G isoform in JVE371. Also, most likely due to low amplicon concentration, the results obtained by Sanger sequencing of the visible band in cell line JVE187 showed very low signal peaks. Consequently, the obtained sequence was not reliable.

PRESENCE OF HLA-G PROTEIN

To measure the presence of HLA-G protein, we performed flow cytometry as well as IHC. Cell surface expression of HLA-G1 was investigated by using mAb MEM-G/9 in flow cytometry. As expected, HLA-G1 was expressed on the JEG-3 cell line, used as a positive control. JEG3 cells were also reactive with W6/32 (pan beta-2 microglobulin-associated HLA class I). Although, the majority of the CRC cell lines were positive for W6/32 (Figure 3A) albeit in varying levels, they were all negative for HLA-G1 surface expression compared with the positive control JEG-3 (Figure 3B). These observations therefore reveal that the HLA-G1 isoform was not expressed in the CRC cell lines.

The CRC cell lines were embedded in paraffin (16 out of 21). We were able to collect associated paraffin-embedded tumor tissue of 18 patients. To evaluate HLA-G expression in the paraffin-embedded tissues by IHC, three different anti-HLA-G mAbs were used (4H84, MEM-G/1 and MEM-G/2. To avoid variation due to tissue heterogeneity, sequential sections were used in IHC using the different mAbs. An overview of the staining results and representative images of HLA-G staining with the three different mAbs are shown in Figure 4. As demonstrated in this figure, different staining patterns, regarding positive and negative stained tumor cells were observed between the three different HLA-G mAbs. For example, the paraffin-embedded cell line

KP283T was positive for 4H84 and MEM-G/1, but was negative for MEM-G/2. Cell line JVE253 very intensely stained with 4H84, but was negative for MEM-G/1 and MEM-G/2 (Figure 4B). No concordance was observed between positive stained tumor sections and those from the CRC cell lines derived from these tumors. In contrast, all anti-HLA-G mAbs showed a similar positive reactivity pattern in JEG-3 (Figure 4B) and in placenta tissue (data not shown).

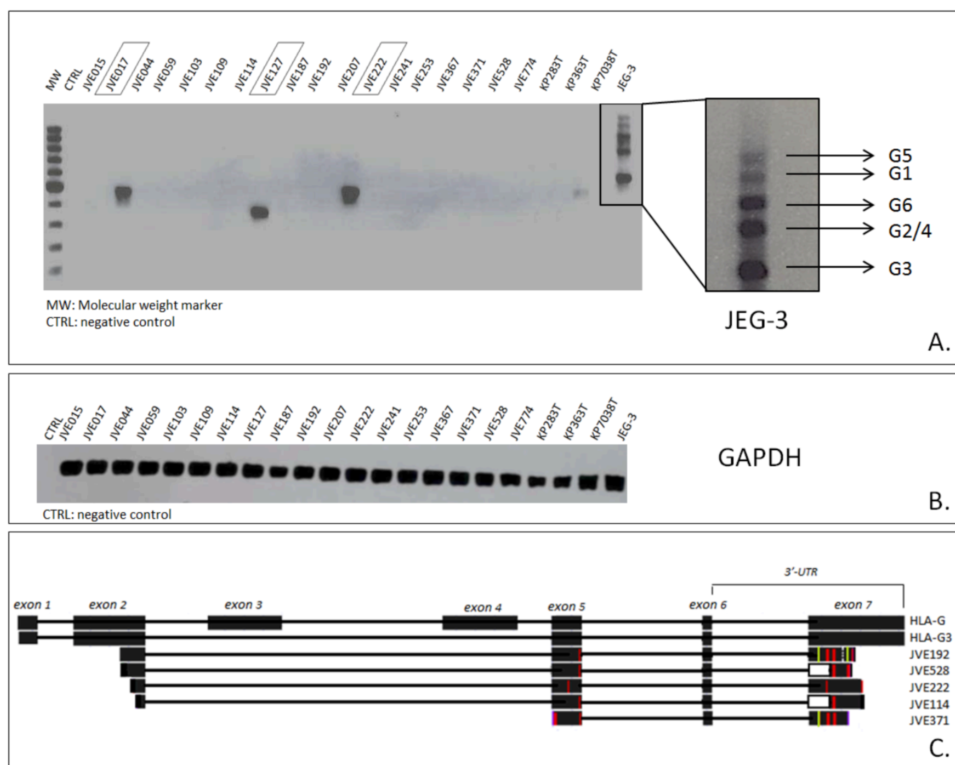


Figure 2. HLA-G, mRNA expression. A. The upper panel shows the results of a PCR performed with primer G.257 and with primer G.1225. As shown all different isoforms were detected in JEG-3. Furthermore, JVE017, JVE127 and JVE222 are showing positive amplicons. **B.** Panel B shows the results of a PCR performed with primers designed for the housekeeping gene GAPDH in the CRC cell lines. **C .** Alignment of the sequenced amplicons of the positive cell lines with HLA-G (ENST00000428701) and HLA-G3 (ENST00000376815) mRNA sequence. The amplicons of the cell lines JVE192, JVE528, JVE222, JVE 114 showed a strong homology to the HLA-G3 mRNA sequence. JVE371 displayed less homology to the HLA-G3 mRNA sequence. Red indicates a SNP at this position between the HLA-G3 sequence and the amplicons (two or more SNPs almost adjacent are appearing as a “bold” red line). Green indicates an insertion. Purple indicates that the obtained sequence extends beyond the end of the alignment. The empty box indicates a deletion.

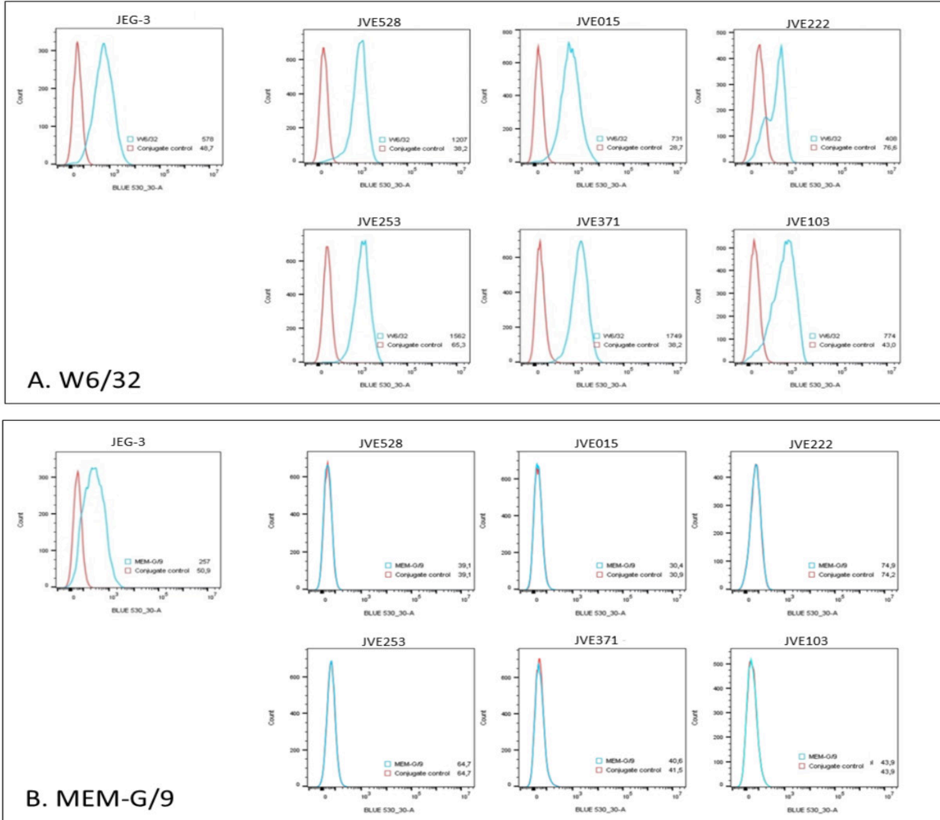
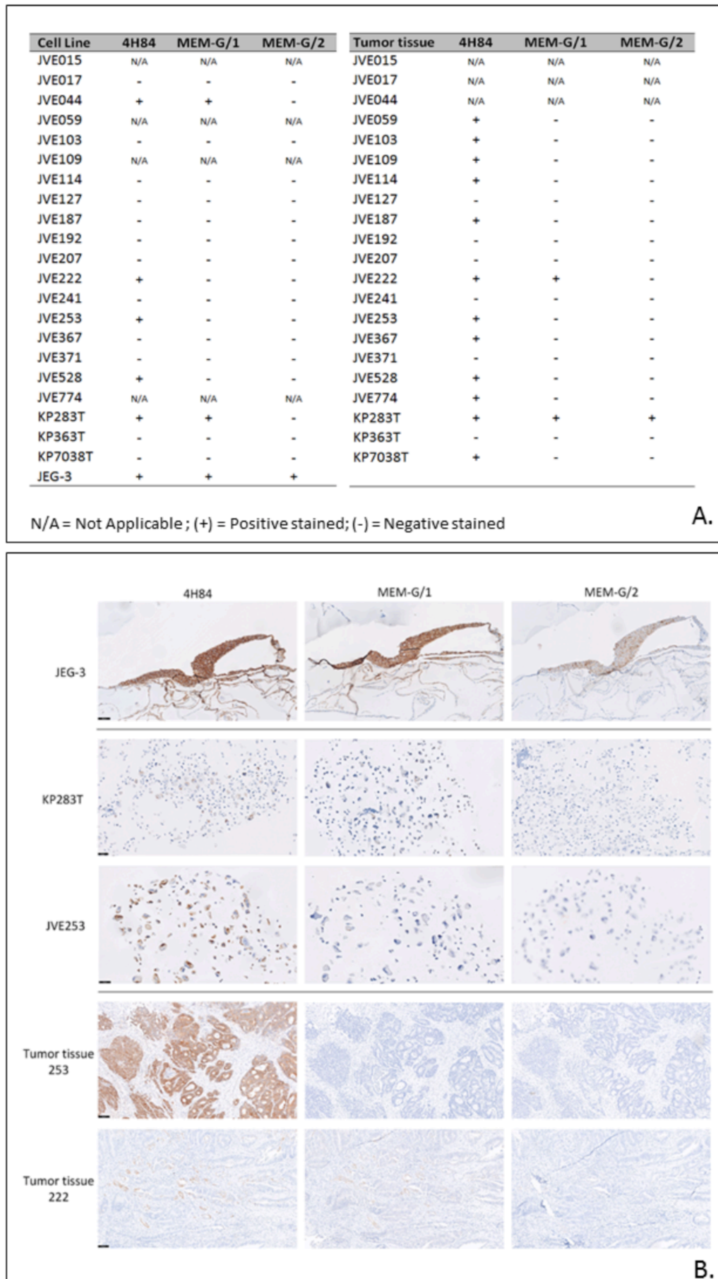


Figure 3: HLA-G expression measured by flow cytometry. Membrane expression of HLA-G measured by flow cytometry with mAb MEM-G/9. The mAb W6/32 recognizing HLA class I was used as a control. **A.** Representative histograms of flow cytometry performed on JEG-3 and CRC cell lines with mAb W6/32. JEG-3 and all CRC cell lines reacted positive with this antibody. **B.** Representative histograms of flow cytometry performed on JEG-3 and colorectal cancer cell lines with mAb MEM-G/9. Only JEG-3 was positive, while all CRC cell lines were negative



Cell Line	4H84	MEM-G/1	MEM-G/2
JVE015	N/A	N/A	N/A
JVE017	-	-	-
JVE044	+	+	-
JVE059	N/A	N/A	N/A
JVE103	-	-	-
JVE109	N/A	N/A	N/A
JVE114	-	-	-
JVE127	-	-	-
JVE187	-	-	-
JVE192	-	-	-
JVE207	-	-	-
JVE222	+	-	-
JVE241	-	-	-
JVE253	+	-	-
JVE367	-	-	-
JVE371	-	-	-
JVE528	+	-	-
JVE774	N/A	N/A	N/A
KP283T	+	+	-
KP363T	-	-	-
KP7038T	-	-	-
JEG-3	+	+	+

Tumor tissue	4H84	MEM-G/1	MEM-G/2
JVE015	N/A	N/A	N/A
JVE017	N/A	N/A	N/A
JVE044	N/A	N/A	N/A
JVE059	+	-	-
JVE103	+	-	-
JVE109	+	-	-
JVE114	+	-	-
JVE127	-	-	-
JVE187	+	-	-
JVE192	-	-	-
JVE207	-	-	-
JVE222	+	+	-
JVE241	-	-	-
JVE253	+	-	-
JVE367	+	-	-
JVE371	-	-	-
JVE528	+	-	-
JVE774	+	-	-
KP283T	+	+	+
KP363T	-	-	-
KP7038T	+	-	-

N/A = Not Applicable ; (+) = Positive stained; (-) = Negative stained

A.

Figure 4. Reactivity patterns among three anti-HLA-G mAbs (4H84, MEM-G/1 and MEM-G/2) in sequential tissue sections of the cell lines and colorectal tumors**.** For the staining with MEM-G/1 and MEM-G/2 blocking with 5% goat serum was used. **A.** Overview of CRC cell lines showing positive or negative stained sections, for the three mAbs**,** (+) indicates positive staining, (–) indicates negative staining. **B.** Overview of CRC cell lines showing positive or negative stained

sections, for the three mAbs, (+) indicates positive staining (–) indicates negative staining. C. Representative images of different reactivity patterns. JEG-3 showed positive staining for all three mAbs. Cell line KP 2883T showed positive staining for 4H84 and MEM-G/1 and was negative for MEM-G/2. Cell line JVE 253 showed positive staining for 4H84 and was negative for MEM-G/1 and MEM-G/2. Sequential tissue sections of tumor tissue associated with JVE 253 showed positive staining for 4H84 and was negative for MEM-G/1 and MEM-G/2. Sequential tissue sections of tumor tissue associated with JVE 22 showed positive staining for 4H84 and MEM-G/1 and was negative for MEM-G/2.

DISCUSSION

HLA-G has been of interest for the last decades as a possible immune tolerogenic molecule in several malignancies such as CRC. As a consequence HLA-G is considered as a potential target for immunotherapy strategies [30]. However, the results regarding HLA-G expression in cancer are controversial. Therefore, careful evaluation of HLA-G expression is necessary. In the current literature, several factors have been suggested to be essential for HLA-G expression such as the epigenetic status, miRNAs, tissue specific activators and polymorphisms [12]. It is generally accepted that long term culturing of cells can lead to changes in transcription and expression of specific genes. It was proposed that low passage CRC cell lines demonstrate a closer resemblance to the primary tumor source regarding HLA-G expression. Therefore a number of recently developed CRC cell lines, which were never studied before in the context of HLA-G, were analyzed for *HLA-G* DNA methylation of the minimal promoter and the CpG island-3, the presence of mRNA encoding HLA-G and expression of the HLA-G protein.

We analyzed the methylation status of the HLA-G gene at two separate locations. In comparison with HLA-G expressing JEG-3 cells, lower methylation levels were observed in the minimal promoter in 4 out of 21 of the CRC cell lines. Likewise, lower methylation percentages in CpG island-3 were also observed in 12 out of 21 CRC cell lines. More importantly, no correlation between mRNA expression and methylation levels of the *HLA-G* gene was observed. This observation is in line with the observation by *Holling et al.*, they analyzed the methylation pattern of the minimal promoter and a CpG rich area in exon 3 of the *HLA-G* gene in the HLA-G positive cell line JEG-3 and the HLA-G negative cell line JAR [21]. No significant difference in DNA methylation was observed in this study. Therefore, HLA-G methylation of these areas alone cannot predict HLA-G expression. These data suggest the involvement of other mechanisms in regulating transcription of the *HLA-G* gene, such as specific histone modifications

or miRNA [31, 32]. In this respect, especially miR-148/152 family members have proven to target the 3'-region of HLA-G resulting in downregulation of HLA-G expression [33]. Furthermore, members of the miR-148/152 family are regulated by DNA methylation of CpG islands and are thought to contribute to the regulation of expression of DNA methylation transferase 1 (DNMT1) [34]. Therefore, demethylating agents, used in cancer treatments, could influence the mechanism of action of these miRNAs.

After performing a RT-PCR multiple times followed by gel electrophoresis 13 out of 21 cell lines yielded a positive signal. We were able to sequence amplicons obtained from 6 cell lines. Four out of 6 of these sequenced amplicons displayed a strong homology with HLA-G3. The minor sequence variations in de coding region observed with HLA-G3 did not yield a stop codon and to our knowledge were corresponding with known single nucleotide polymorphisms. Furthermore, the observed deletions and insertions were located in the 3'-UTR. These observations therefore support the notion that these CRC cell lines potentially express functional HLA-G3. Furthermore, in one cell line a high degree of not matching nucleotides was observed suggesting that this cell line expresses a non-functional HLA-G3 protein. In a number of cases unknown mutations were found. In order to firmly establish the nature of these novel mutations a comparison with genomic DNA obtained from normal tissue of these CRC patients would be necessary. Unfortunately, normal tissue of these patients was not available.

Corresponding with the results obtained by RT-PCR, no HLA-G1 membrane expression was detected with flow cytometry in all CRC cell lines investigated. Some cell lines were positive for HLA-G protein expression visualized with IHC. For example JVE222, that proved to be transcribing HLA-G3, had low methylation levels compared to JEG-3 and showed to be positive in IHC for 4H84. The associated tumor tissue section was positive for 4H84 and MEM/G1. In contrast, cell line JVE044 which was in none of the tests positive for mRNA expression and highly methylated, showed positive IHC staining with 4H84 and MEM-G/1. Comparing HLA-G mRNA expression, methylation status and protein expression, the results did not correspond. Regarding IHC, no concordance was observed between positive stained cell lines and the corresponding tumor sections. This observation of no concordance between tumor tissue sections and tumor derived cells lines could be explained by the fact that tumors are heterogeneous in nature. Together with the notion that only a small number of cells within tumor sections were positive stained for HLA-G, makes it feasible that positive stained tumor sections could be negative for HLA-G in the associated cell line. More importantly, we observed discrepant expression profiles among various anti-HLA-G mAbs used in sequential tissue sections. This suggests that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 are expressed differentially in colorectal tumor tissues as we

described previously [20]. In contrast, for all anti-HLA-G mAbs tested, positive staining patterns were observed in both JEG-3 and placenta tissue. This reveals the HLA-G specificity of these mAbs in placenta and the choriocarcinoma derived cell line JEG-3. However, in tumor cells and tissues conclusions based on immunohistochemistry alone 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 [17, 19, 20]**.** Furthermore, it has been reported in HLA-G cancer research, that only low levels of HLA-G expression are present in tumors [35]. Low expression of HLA-G could be an explanation of our findings regarding the mRNA expression of HLA-G. Except for HLA-G3, the levels could have been below our detection level. This notion indicated that some HLA-G isoforms might be more prominent in some specific tumor types. For example, in pregnancy disorders it is suggested that smaller HLA-G isoforms are expressed as a substitute in situations in which HLA-G1 is altered [36]. Perhaps, such substitution mechanism could be extrapolated to tumor situations. In tumor cells that do not express HLA class I, a well-known mechanism to escape anti-tumor immunity, expression of smaller HLA-G isoforms might still occur [18, 37]. In this study HLA-G3 mRNA was found in CRC cell lines. This HLA-G isoform only contains the α1 domain. The α1 domain, common to all HLA-G isoforms, is suggested to interact with the KIR2DL4 receptor and thereby inhibiting NK cell function [38, 39]. However, contradictory evidence for the HLA-G/KIR2DL4 interaction has been published as well [40, 41]. Therefore, the functional consequences of the HLA-G3 expression in CRC remains to be elucidated.
In conclusion, HLA-G has been proposed as an interesting and promising protein in cancer research**.** However, based on the results of our study it is evident that methods utilized in this field are not selective enough to detect all aspects of HLA-G expression in CRC.

In the results of this study no strong association between *HLA-G* DNA methylation patterns and HLA-G expression was observed. Furthermore, we argue that HLA-G might be expressed differently in specific tumors types or some HLA-G isoforms might be more prominent in some specific tumor types. For CRC it could be HLA-G3. Many of the present studies mainly aim at investigating G1 and G5 isoforms. However, the discovery of a null allele (G*01:05N), resulting in aberrant synthesis of the G1, G5 and G2 isoforms, urges for more research regarding the importance of other isoforms [42]. Therefore, to make further steps ahead, a shifted study focus towards all isoforms of HLA-G in conjunction with increasing sensitivity of the methods available is a prerequisite.

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