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# Tumor Heterogeneity Uncovered by HLA-G Isoforms Expression

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**Abstract**

The heterogeneity of cancer cells introduces significant challenges in designing effective therapeutic approaches. This tremendous flexibility is maintained via the expression of multiple isoforms mainly produced by alternative splicing, in a non-uniform distribution, constituting probably one cause of treatment failure. Characterizing isoforms heterogeneity might therefore allow for a better understanding of tumorigenesis and facilitate the development of more suitable therapeutic strategies. In this study we have explored the level of tumor heterogeneity by studying HLA-G as paradigm in clear cell renal cell carcinoma (ccRCC).

HLA-G is a non-classical HLA-class I molecule whose best known role is to inhibit the cytotoxic activity of natural killer and T-cells. This molecule was first described to play a crucial role in feto-maternal tolerance. HLA-G expression is not ubiquitous, in contrast to other HLA-class I molecules. Its physiological expression is mainly restricted to extravillous cytotrophoblasts. However, under pathological conditions, HLA-G expression can be induced in almost all tissues. Particularly, HLA-G has been found in most of the tumors analyzed, such as ccRCC, the most common human renal malignancy, in which a high incidence of HLA-G was described. More recently, we have reported novel HLA-G isoforms in addition to the seven already reported that include spliced forms having an extended 5'-region and lacking the transmembrane and/or alpha1 domains. In the present study we have extended our analysis by simultaneous assessment of immunohistochemistry (IHC) labeling and RT-PCR. Combining both methods was necessary since the specificity of the different antibodies have been questioned. The study of tumors retrieved from ccRCC patients reveal that the expression of HLA-G is highly variable among tumors and distinct areas of the same tumor. In particular, we have repeatedly noticed that ccRCC samples contain eosinophilic hyaline globules exhibiting glassy appearance with a clear surrounding halo. These hyaline globules represent a well-characterized morphologic and functional entity associated with a more aggressive behavior and seem to be directly linked to autophagy defects. Interestingly, we observed that hyaline globules are associated with enhanced expression of HLA-G. Moreover, the novel isoforms were also detected in placental trophoblast cells demonstrating that they did not arise from spontaneous mutations in the process of tumor formation. The absence of alpha1 domain from one of the membrane-bound isoforms prevents its detection by immunostaining since all antibodies recognize an epitope located into the alpha1 domain common to all HLA-G isoforms. Therefore, we also report here the development of a lentivirus-vector cellular model to specifically analyze the characteristics of this particular novel isoform. Collectively, our approach extends the portrait of HLA-G isoforms expression and provides data that might facilitate the development of more-effective personalized therapies.

**Keywords:**

HLA-G;

Immune-checkpoints;

Isoforms;

ccRCC;

Tumor heterogeneity.

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## Introduction

The heterogeneity of cancer cells introduces significant challenges in designing effective therapeutic approaches. Given this heterogeneity, a relevant number of patients has unsatisfactory responses or tumor recurrence following an initial clinical benefit. The expression of multiple isoforms in tumors, mainly produced by alternative splicing in a non-uniform distribution, might be in part, responsible for treatment failure. An accurate assessment of tumor heterogeneity based on the characterization of isoform diversity might therefore allow for a better understanding of tumorigenesis and facilitate the development of more effective therapeutic strategies.

Cancer immunotherapy has positively revolutionized outcomes and basic concepts of oncological treatments. Most of such breakthroughs are due to the discovery and therapeutic modulation of key immune-regulatory molecules (checkpoints) at the interface between tumor cells and immune effectors [1]. These immune-checkpoints (IC) have been broadly defined as cell-surface molecules that can transduce signals into effector cells to positively (stimulatory receptors) or negatively (inhibitory receptors) modulate signaling upon ligand binding. Tumor cells take advantage of these associations to escape destruction by the immune system. At present, antibody-based therapeutics targeting IC proved to improve prognosis and help establishing a more effective antitumor response.

Among IC, the interaction between HLA-G and its receptors, ILT2 and ILT4 (LILRB1/CD85j and LILRB2/CD85d) has lately arouses great interest [2]. HLA-G was originally described on trophoblast cells at the maternal-fetal interface where it plays a critical role in protecting fetal allograft from maternal immune rejection [3]. Its primary transcript is alternatively spliced, producing at least seven mRNAs encoding four membrane-bound (HLA-G1 to HLA-G4) and three soluble (HLA-G5 to HLA-G7). These isoforms display one, two or three extracellular domains. The soluble proteins have retained intron sequences that include stop signals that prevent the translation of the transmembrane and intracytoplasmic domains.

HLA-G has a broad immunoregulatory function that affects both innate and adaptive immunity. Via its interaction with the inhibitory receptors ILT2 and ILT4 which are differentially expressed by immune cells, HLA-G inhibits the cytolytic function of NK cells, the Ag-specific cytolytic function of cytotoxic T cells, the alloproliferative response of CD4+ T cells, and the maturation and function of dendritic cells. Unlike other IC, HLA-G is characterized by a restricted expression in normal tissues which would make HLA-G particularly pertinent as therapeutic target. Besides its limited distribution in healthy tissues, HLA-G expression can be ectopically induced under malignant transformation in tumor cells and/or in tumor-infiltrating immune cells [2].

The first demonstration of the *in vivo* role of HLA-G as a tumor escape mechanism was shown in immunocompetent mice by direct injection of cells expressing or not the full length HLA-G1 or HLA-G5 protein. We demonstrate that xenograft tumors expressing these isoforms promoted tumor progression while control tumor cells not expressing HLA-G were rejected. Of note, there was no tumor development when HLA-G was blocked by a specific antibody providing the proof of concept for new antitumor therapeutic strategy based on anti-HLA-G antibodies [4, 5].

Over the past few years, we showed heterogeneous expression of HLA-G/ILT in tumor cells and in infiltrating CD8+ and CD4+ T cells of patients with clear cell renal cell carcinoma (ccRCC) [6]. In addition to the seven known HLA-G isoforms, we also revealed the presence of yet undescribed spliced HLA-G isoforms in tumors derived from these patients. These included transcripts that have an extended 5'-region, or may lack the transmembrane and/or α1 domains (HLA-GΔα1) [7]. It is likely that HLA-G expression in tumors might have been underestimated since currently available antibodies cannot detect the HLA-GΔα1 isoform.

Therefore, to consider HLA-G as a potential target for cancer therapy, a critical issue is to characterize and quantify the degree of functional specificity or overlap exhibited by the different isoforms. To this end, we have extended our analysis of HLA-G isoforms in ccRCC lesions and carried out a systematic analysis of placenta trophoblasts to assess whether the new isoforms arise as a consequence of spontaneous mutations in the process of tumor formation. In addition, the different isoforms were expressed in two different cell lines: the RCC7 cell line which derives from a tumor of a patient with ccRCC [8] and the erythroleukemic K562 cell line, which is commonly used as target cell for NK function studies [9] in order to derived human cellular ccRCC models and conduct structure-function studies.

## Marked subcellular heterogeneity of HLA-G isoforms distribution in ccRCC

To consider HLA-G as a potential target for cancer therapy, we ought to assess whether different HLA-G isoforms are expressed in tumor cells. We use as paradigm tumors derived from patients with ccRCC. All patients of this study underwent a radical nephrectomy for ccRCC as first therapeutic intervention in the urology department of Saint-Louis Hospital (Paris, France). We have analyzed different sections for each tumor with two different antibodies directed against HLA-G: 4H84, which recognizes an epitope located into the α1 domain common to all seven reported

HLA-G isoforms and the antibody 5A6G7 that only recognizes soluble HLA-G5 and HLA-G6 isoforms. This latter antibody targets the amino acids encoded by the retained intron 5 (previously known as intron 4 according to the IMGT/HLA nomenclature).

The immunostaining patterns revealed that even though all tumors expressed HLA-G in at least one area, this expression was distinct between and inside tumors. Four representative ccRCC tumors are illustrated in Figure 1. Tumors of patients 1 and 2 showed a strong immunostaining with 4H84 antibody in all regions. The staining was membranous and cytoplasmic. Noteworthy, an additional very strong staining of hyaline globules located in the cytoplasm of the tumor cells was also detected. In H&E staining, these eosinophilic hyaline globules exhibited a glassy appearance with a clear surrounding halo located in the cytoplasm of the tumor cells. The immunostaining pattern obtained with the 4H84 antibody was similar for tumors 1 and 2 whereas the profiles obtained with 5A6G7 were distinct. A moderate and fine granular intracytoplasmic stain was observed in patient 1's tumor whereas this staining was discrete in patient 2's tumor. Nevertheless, in these two cases, the hyaline globules weren't stained by this antibody. In tumor of patient 3, the expression of HLA-G evaluated by 4H84 antibody was noted in a small microscopic area of only one tumor region. Of note, this HLA-G positive area corresponded precisely to the tumor area containing intracytoplasmic hyaline globules. In addition, no stain was observed for 5A6G7 in the different regions of this tumor.

These results contrasted with the pattern observed for patient 4, which revealed a complete negative staining with antibody 4H84. The lack of labeling of tumor sections with this antibody normally accounts for the absence of HLA-G expression. However, a strong granular intracytoplasmic 5A6G7 immunostaining, particularly in hyaline globules, was observed in this patient's tumor. This was unexpected within the boundaries of our prevailing knowledge on the structure of the seven reported HLA-G isoforms since they all contain the α1 domain recognized by the 4H84 antibody.

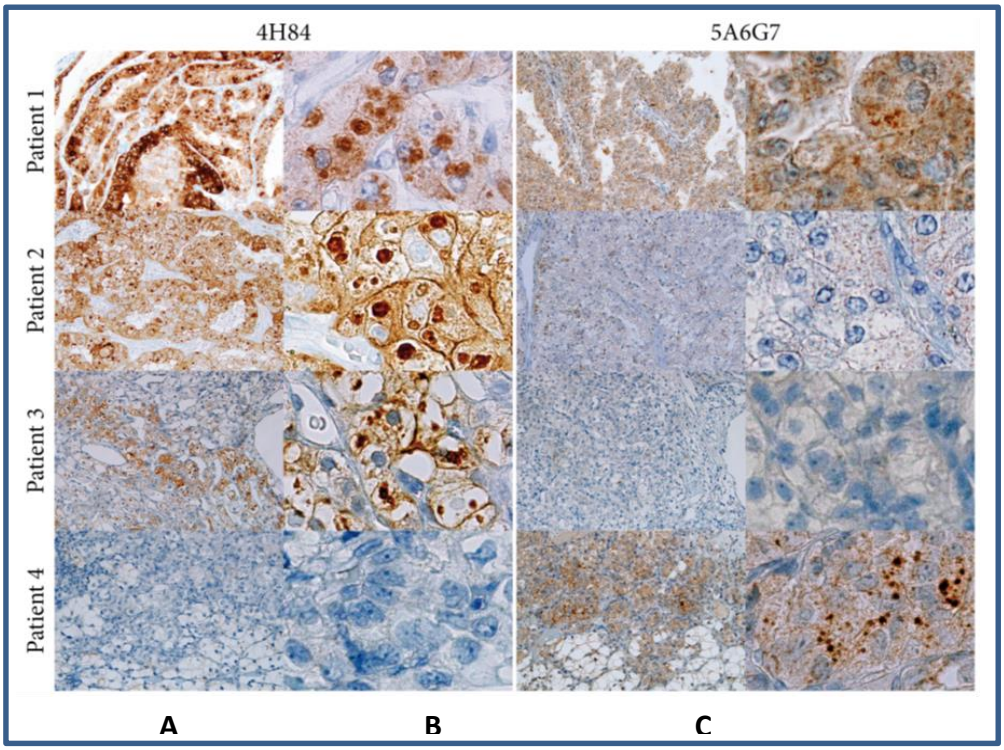


**Figure 1: Representative immunohistochemistry analysis of HLA-G expression probed with mAbs 4H84 and 5A6G7. A and C are overall primary ccRCC sections. B and D, specifically show hyaline globules.**

Together, the results of the immunohistochemical study clearly demonstrate intra- and inter-heterogeneity of HLA-G expression in ccRCC tumors and are consistent with the presence of several isoforms expressed in different subcellular regions of ccRCC patients' tumors.

## Novel HLA-G isoforms present in trophoblasts

To assess whether the new isoforms arise from spontaneous mutations in the process of tumor formation, we have performed a systematic analysis of HLA-G isoform diversity in placenta trophoblasts by RT-PCR. We have used specific combinations of primers (Figure 2) that allow distinguish the different isoforms. All primer sequences were described previously [7].

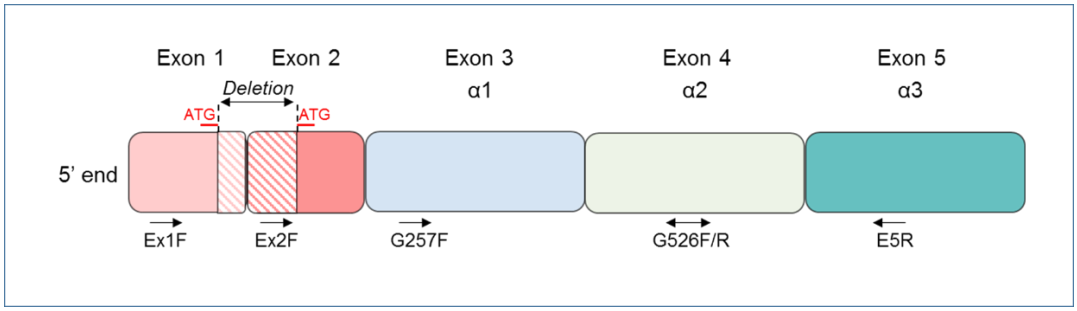


**Figure 2: Localization of primers used for the different RT-PCR strategies to amplify HLA-G isoforms.**

First, cDNA derived from RNA prepared from 14 throphoblasts were amplified by RT-PCR using primer Ex2F and G526R. Primer Ex2F is complementary to the region immediately upstream of the ATG located in exon 2, which was found in all reported HLA-G isoforms (Figure 2). The 3'-amplification primer G526R is located in exon 4 (the Ensembl nomenclature will be used throughout the whole text). The results of the RT-PCR reveal the presence of an amplified fragment of 560 bp in almost all trophoblasts analyzed. Trophoblasts 6 and 13, however, produce a band smaller than expected (Figure 3).

We next ought to determine whether transcripts containing the supplementary 5' exon 1 absent from the IMGT/HLA database can be detected in physiological conditions since no transcript initiated at exon 1 was previously reported. To this end, we use the primer named Ex1F, which binds a region complementary to the exon1 (schematically represented in Fig 2). If the extended 5'-region that is absent from the IMGT/HLA-G database is indeed transcribed, the amplification with Ex1F in combination with G526R will produce a band of 690bp. The results of the RT-PCR analysis have effectively revealed amplification products of 690 bp in 4 out of the 14 trophoblast samples (7, 11, 13, 14). Notably, for six other trophoblasts, we found at least three supplementary transcripts. This shows that trophoblasts might express one or different isoforms. The heterogeneity of transcripts starting in exon 1 was not reported before in trophoblasts.

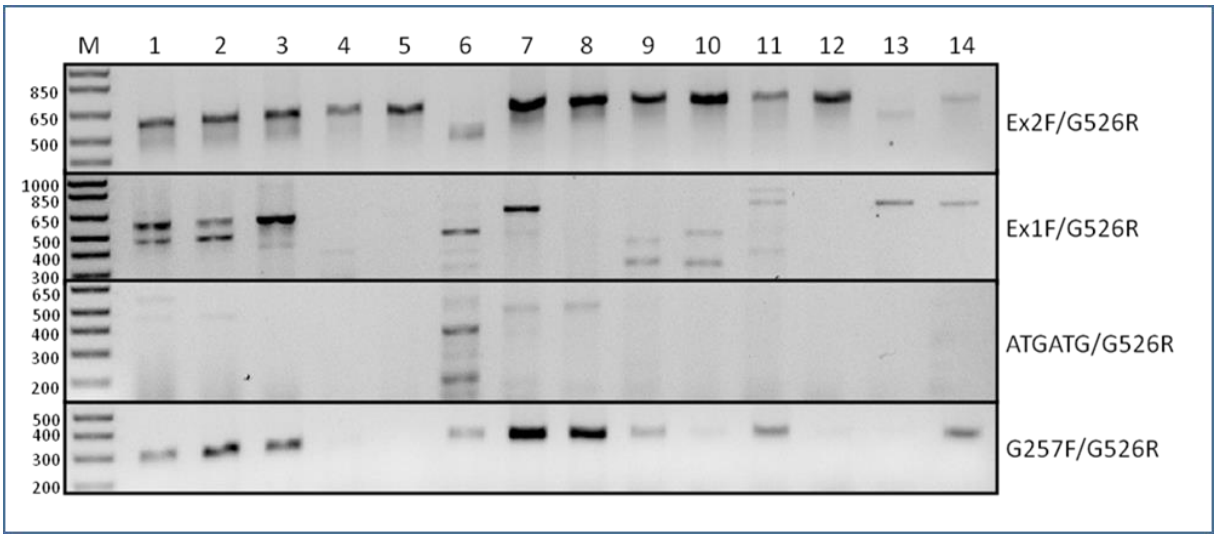


**Figure 3: Expression of HLA-G isoforms in 14 placenta trophoblasts. RNA were subjected to RT-PCR using specific primers whose locations were shown in Figure 2. M: 100-bp size marker.**

To assess for the presence of the novel isoform HLA-G1L that results from the deletion of a 106 bp fragment 5' of the ATG situated in exon 2, we designed a specific primer, named ATGATG, complementary to the region, surrounding the area between the two ATG, that has been deleted (Fig 2). This deletion reduces the distance between the ATG located at the end of exon1 and exon 2's ATG from 118 bp to 12 bp. Resulting from this deletion, the ATG present at the end of exon 1 becomes in frame with the ATG located in exon 2, which was previously considered as the only suitable translation initiation site. This primer located therefore at the junction of exon 1 and exon 2, when used in combination with G526R, only allows to amplify HLA-G1-L transcripts, which have deleted the 106 bp-sequence. In contrast, the Ex1F primer cannot amplify HLA-G1L since the complementary region is deleted in this isoform (Fig 2). The results of this analysis showed a 560 bp-amplification fragment (Fig 3), consistent with the presence of HLA-G1L in 4 out of 14 trophoblasts (1, 2, 7 and 8). The RT-PCR profile of trophoblast 6 suggests that other undescribed isoforms might also exist that have spliced out supplementary exons.

Finally, we amplified the cDNA from the fourteen trophoblasts with the well-known G257F and G526R primers currently used for the amplification of HLA-G1 transcripts. These primers amplify a region of 290 bp that contains the epitope recognized by the 4H84 antibody. The results of the RT-PCR amplification (Figure 3) reveal that 4 out of 14 trophoblasts (4, 5, 12 and 13) are not amplified with this couple of primers. This is consistent with the presence of an isoform that lacks the exon encoding the α1 domain.

**Table 1: Summary of HLA-G isoforms expressed in placenta trophoblasts.**

| *Trophoblastes* | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *13* | *14* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ex2F/G526R | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Ex1F/G526R | + | + | + | - | - | + | + | - | + | + | + | - | + | + |
| ATGATG/G526R | + | + | - | - | - | + | + | + | - | - | - | - | - | - |
| G257F/G526R | + | + | + | - | - | + | + | + | + | - | + | - | - | + |

Altogether, the RT-PCR analysis, summarized in Table 1, shows that the novel HLA-G isoforms that we have previously found in tumors of patients with ccRCC are also expressed in trophoblasts. These isoforms are not mutually exclusive and may co-exist in physiological and pathological conditions. In addition, the survey of the 14 trophoblasts further indicates that several HLA-G isoforms are yet to be discovered.

## Cellular models expressing different HLA-G isoforms.

To analyze the expression and function of the different isoforms we have engineered lentivirus encoding HLA-G1, HLA-G1L and HLA-GΔα1. The cDNA corresponding to the three isoforms were introduced into plasmid pWPXL (Addgene plasmid # 12257). Since HLA-GΔα1 cannot be detected with available antibodies directed against HLA-G nor other technique, we inserted in frame with the HLA-GΔα1 cDNA, the sequence encoding the peptide DYKDDDDK at the 3'end of the molecule. This peptide constitutes a tag that can be detected by a highly specific anti-tag antibody and allows tracking the protein in any cell. These plasmids were used to further obtain lentivirus specifically expressing each isoform.

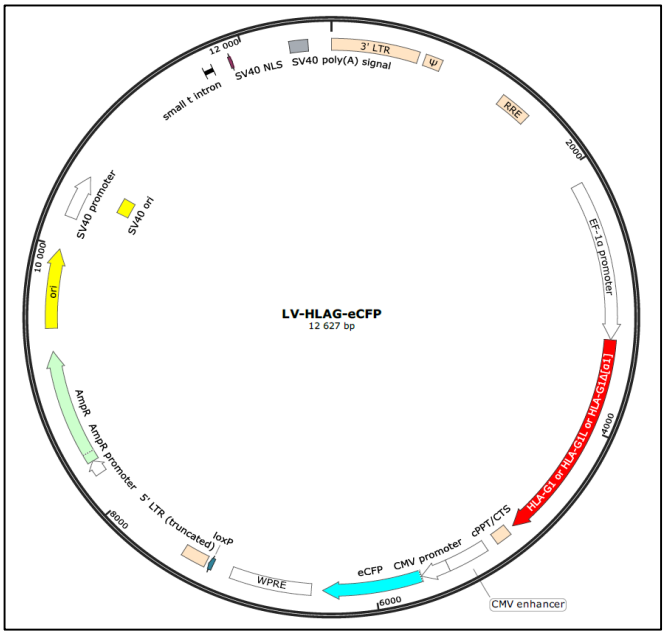


**Figure 4: Schematic representation of lentivirus carrying the HLA-G isoforms**

In these constructs, the expression of all HLA-G isoforms is directed by the EF-1α promoter. The constructs also include a variant of the green fluorescent protein (GFP), named "enhanced Cyan Fluorescent Protein (eCFP), which expression is under the control of the CMV promoter (Figure 4). These plasmids were used to produce lentivirus WPXL ΔU3 SIN, VSV-G envelope, at the Plateforme Vecteurs Viraux et Transfert de Gènes (VVTG), SFR Necker, US 24, UMS 3633, Paris. The lentivirus were successfully introduced in K562 and ccRCC7cell lines to generate valuable models to specifically analyze the function of individual HLA-G isoforms and obtain ccRCC cellular models expressing different HLA-G isoformes.

To verify the HLA-G expression in these cells, RNA transcripts were analyzed by RT-PCR (Figure 5). First, actin primers were used as control for RNA integrity. Then HLA-G isoforms were assessed using primers EX1-526R; G257-G526R ATGATG-526R and G526F-E5R, which specifically detect the isoforms described in the previous section.

A specific amplification was obtained with primers G257F and G526R for HLA-G1 and HLA-G1L. As expected, no amplification was detected for HLA-GΔα1 since primer G257F is located in the exon encoding the alpha 1 domain which is absent from HLA-GΔα1. As expected, the amplification with primers ATGATG/G526R reveals only the expression of HLA-G1L. Finally, amplification with primers G526F-E5R allows identifying the three isoforms. No HLA-G expression was found in wild type cells under these conditions. The same expression profiles were obtained for K562 or RCC7 cell lines.

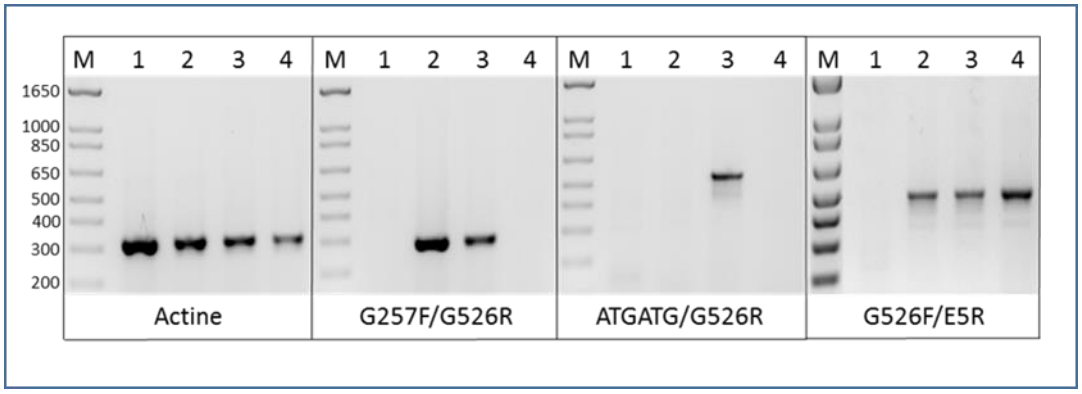


**Figure 5: RT-PCR analysis of HLA-G transduced cells. Line 1:WT, line 2: HLA-G1, line 3: HLA-G1L and line 4: HLA-G Δα1. M: 100-bp size marker.**

## HLA-G1, HLA-G1L and HLA-GΔα1 are addressed at the cell-surface.

To determine whether the transcripts encoding the three HLA-G isoforms: HLA-G1, HLA-G1L and HLA-GΔα1 are translated into proteins, we carried out both Western blot and flow cytometry analysis of HLA-G-transduced cell lines. The corresponding wild type (WT) cell lines were used as controls. Western blot experiments were first conducted using the 4H84 mAb, which specifically detects denatured HLA-G via the α1 domain epitope. As expected we found that HLA-G1 and HLA-G1L transcripts were translated into a 39- to 40-kDa protein, in both K562 and RCC7 cell lines (Fig. 6). As predictable, the HLA-GΔα1 protein missing the α1 domain could not be detected in any cell line with 4H84 mAb (Fig. 6).

To determine whether the proteins were found at the cell surface, flow cytometry experiments were further carried out using the K562 cell line model because it does not express any HLA class I molecule in the WT form and allows to specifically analyzing individual HLA-G isoforms. The results showed that both HLA-G1 and HLA-G1L were detected at the cell surface of K562 transduced cells (Figure 7). Indeed, both HLA-G1 and HLA-G1L were stained by the MEM-G/9 antibody which recognizes a conformational epitope located in the α1 domain, dependent upon the association with the β2m.

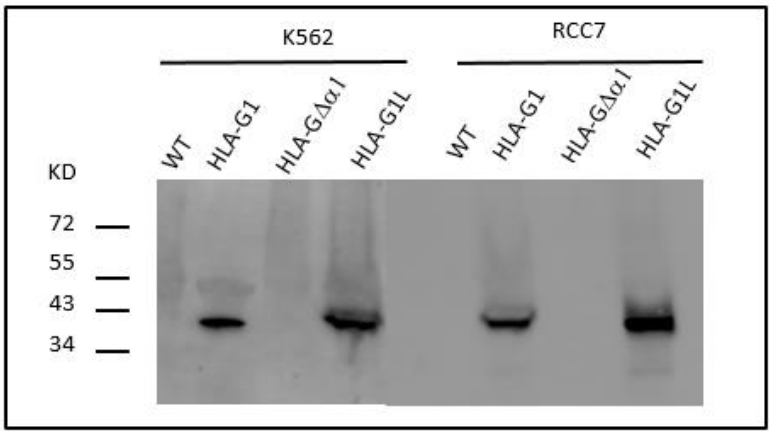


**Figure 6: Western blot analysis of K562 and RCC7 cell lines**

**Western blot analysis of lysates from K562 and RCC7 cell lines was conducted using the 4H84 mAb under reducing conditions. Briefly, total protein aliquots from the corresponding cell line lysate were separated on SDS-PAGE gels (mini-protean TGX stain-free precast Gels, Bio-Rad). The gels were blotted onto nitrocellulose membranes (Transblot turbo transfer pack, Bio-Rad). The membranes were then probed with the 4H84 mAb (Exbio) at a 1:1000 dilution, and then with peroxidase-conjugated sheep anti-mouse IgG Ab (Sigma) at 1:10,000. Finally, the membranes were stained with enhanced chemiluminescence reagent (Amersham). Numbers at the *left* of each figure refer to *M*r in kilodaltons. One of three representative experiments is shown.**

Since this, or any other commercially available anti-HLA-G antibodies (i.e., 87G, MEM-G/9, G233, 01G, 4H84) directed against the HLA-G-α1 domain, the HLA-GΔα1 protein could not be detected. Therefore, we tested a panel of anti-HLA class I antibodies kindly provided by Soldano Ferrone (Harvard Medical School, Boston, USA) to determine whether one of this could detect another domain of the HLA-G protein. We found that one antibody, TP123, was able to detect the novel HLA-GΔα1 protein transduced into the K562 cell line as well as HLA-G1 and HLA-G1L (Fig. 7A). All three proteins were detected at the cell surface. This observation constitutes the first demonstration that such truncated HLA-G isoform missing the α1 domain can be translated into protein and can reach the cell surface.

To determine whether the β2m-associated protein could also be directed to the membrane in the absence of the α1 domain, we have used the B1G6 mAb that recognizes specifically the β2microglobuline (Fig. 7A). The results show that the β2microglobuline was found at the surface of cells expressing HLA-G1 and HLA-G1L whereas this protein could not be detected at the surface of K562 cells expressing HLA-GΔα1 protein (Fig. 7A).

To further study the function of the HLA-GΔα1 protein in cells expressing other HLA class I molecules and purify those that specifically express HLAGda1, we have analyzed the eCFP fluorescence and used the antibody directed against the Tag to detect the expression of this isoform. The α-tubulin was used as positive intracellular marker. The results of the cytometric analysis reported in Figure 6B clearly show the fluorescence emitted by the eCFP and the positive stain with the tag antibody, which further demonstrates the expression of the HLA-GΔα1 /Tag protein. The results are therefore consistent with the use of this model in future strategies.

## Discussion

Despite recent breakthroughs in immuno-oncology and the use of therapeutic antibodies targeting checkpoint molecules, only a portion of patients respond to these therapeutic antibodies. This was attributed in part to the heterogeneity of cancer cells, which results at least from the expression of several tumor relevant protein isoforms, mainly produced by alternative splicing in a non-uniform distribution.

Alternative splicing imparts distinct functions by allowing single genes to produce several distinct protein isoforms. These isoforms have prominent role in many biological processes [10], and may alter the function, cellular localization, and stability of the corresponding RNA or protein [11, 12] A large majority of human genes uses alternative isoforms. In some cases, the functions of two isoforms of the same protein can have opposing effects on a cellular process (cell survival versus death, anti- or pro-angiogenic, etc.). Despite the enormous body of experimental knowledge accumulated on different aspects of gene expression, there still remain many fundamental unanswered questions regarding the biology and functional significance of the different isoforms of a particular gene.

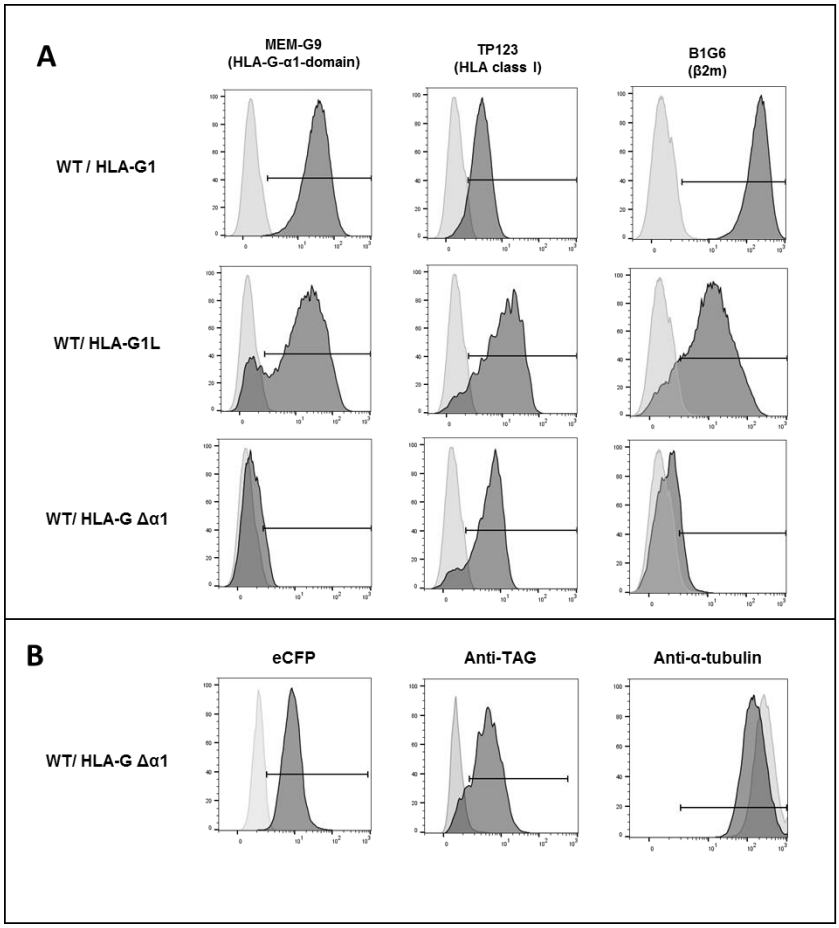


**Figure 7 : Flow cytometry analysis of transduced K562 cell lines.**

**Panel (A) Membrane staining shows HLA-G cell surface expression level on K562 transduced cell lines (dark histogram) compared to K562 WT cell line (light histogram). The following antibodies were used: the anti-HLA-G MEM-G9-PE conjugated (Exbio) (left column), the anti-HLA class I TP123, kindly provided by S. Ferrone (USA) (middle column), the anti-β2-microglobulin B1G6 (Exbio) (right column). (B) Intracellular staining of K562 WT (lighter histogram) compared to K562 transduced by the HLA-GΔα1 lentiviral construct (dark histogram) shows the expression level of (left) the lentiviral construct through eCFP fluorescence, (middle) the HLA-GΔα1 protein using anti-TAG-APC conjugated antibody (Miltenyi Biotec) and, (right) the-α-tubulin protein (Sigma) as positive control for cell permeabilization. When the first antibody used was not conjugated to fluochrome, cells were secondly labelled with PE-conjugated $F(ab')_2$ goat anti-mouse IgG (Beckman Coulter). Only viable cells were analyzed in these experiments. Acquisition was made on a MACSQuant 10 flow cytometer (Miltenyi biotec); analysis was performed using the MACSQuantify software (Miltenyi biotec) and Flowjo software. One of three representative experiments is shown.**

In this study, by combining immunohistochemistry with transcripts and proteins analysis we have provided an overview of structurally different HLA-G isoforms expressed in tumors from patients with ccRCC. Our molecular survey has not only revealed a high intra- and inter-tumor heterogeneity regarding HLA-G expression, but also highlighted distinct subcellular locations and, for the first time, we observed that hyaline globules are associated with expression of particular HLA-G isoforms. The hyaline globules represent a well-characterized morphologic and functional entity. They constitute a very uncommon aspect of tumor cells associated with aggressive behavior and seem to be directly linked to autophagy defects [13].

Autophagy, although beneficial to normal cells, helps malignant cells to adapt to an unfavorable environment, and hence, promotes tumor cell survival. This decreases the effectiveness of chemotherapy by enhancing cancer cell metabolism inhibiting thus its cell death pathway. Recent studies have shown that autophagy significantly controls immune responses by modulating the functions of immune cells and the production of cytokines. In fact, the induction of autophagy may also benefit tumor cell escape from immune surveillance and result in intrinsic resistance to immunotherapy [14]. Conversely, some cytokines and immune cells have a prominent effect on the function of autophagy. Thus, opposite properties have been reported where autophagy may attenuate or enhance the effects of immunotherapies [15].

Immunomodulatory molecules, such as indoleamine 2,3 dioxygenase(IDO), CTLA-4 and PD-1 have been involved in tumor immune tolerance through autophagy pathways. Blocking the PD-L1/PD1 axis via antibodies such as anti-PD1 or anti-PD-L1 triggers autophagy in tumor cells [16]. Therefore, understanding the synergistic interaction between autophagy and IC, in particular with each isoform of HLA-G, is important for developing tumor immunotherapy strategies.

To further understand the role of HLA-G isoforms in cancer, we asked whether the subset of alternative splicing events observed in tumors may represent independent oncogenic mutations that could be relevant to explain the functional transformations in cancer. Here, we clearly demonstrate that the different splicing events are also found in trophoblasts since similar expression patterns were obtained in both cell types. Trophoblasts, as tumor cells, can also express one or several isoforms simultaneously. The analysis of expression profiles also reveal that other isoforms are yet to be identified.

The novel HLA-G isoforms encoded mainly by transcripts initiated in exon 1, may have or not a deletion of 106 bp 5'of the ATG located in exon 2. The identification of these transcripts may result in a modification of the promoter localization, the length of the 5'-untranslated region and the transcription/translation initiation sites and therefore the regulation of the expression of HLA-G and its cellular fate. In particular, the HLA-G1L isoform might exhibit an extended N-terminal end if the translation initiation occurs at the ATG located in the first exon. The presence of five supplementary amino acids (MKTPR) may have extremely important physiological consequences. In fact it might modify the transport and targeting of the molecule to different cellular compartments of the cell, and hence, its function.

Although the HLA-G1L transcript displays an extended peptide signal, the mature processed HLA-G1L protein is expected to be similar to the HLA-G1 isoform, constituted by three extracellular domains α1, α2, α3, a transmembrane part and a short cytoplasmic tail. Here we demonstrate that HLA-G1L and HLA-G1 are translated into a 39/40 KDa cell surface proteins that can be associated with the β2m. Accordingly, we may expect that this isoform, like HLA-G1, interacts with both ILT2 and ILT4 receptors and may thus affect the immune function of NK, T, B, dendritic cells, monocytes, and neutrophils, which all express one or both receptors [17]. Even though the extracellular domains of ILTs bind to the α3 domain of the HLA class I molecules, the highest affinity is for that of HLA-G [18]. Structural analysis showed that ILT4 could recognize both HLA-G/β2m associated forms and free HLA-G heavy chains, whereas ILT2 only recognized HLA-G associated with β2m. Of note, HLA-G isoforms can be presented as homo- and hetero-multimers resulting from intermolecular disulphide bonds between Cys42 or Cys147 located in the α1 or α2 domain, respectively. These multimeric forms are of particular importance since they exhibit higher affinity for the ILT receptors and thus constitute the biologically active forms [19, 20].

The HLA-GΔα1 isoform, whose transcript lacks the exon coding for the α1 domain, is a totally novel HLA-G isoform since all reported HLA-G isoforms have in common the α1 domain. Our present results demonstrate for the first time the cell-surface expression of such HLA-G lacking the α1 domain isoform. This isoform has several features which are distinct from the other HLA-G molecules. First, this isoform, although presenting the α3 domain, does not associate with the β2m. The association between β2m and the heavy chain of HLA class I molecules is dependent on the conformational structure adopted by all 3 extracellular domains. Another specific feature of this novel HLA-G isoform is related to the absence of the peptide binding groove classically exhibited by HLA class I molecules due to the assembly of both α1 and α2 domains. Accordingly, the HLA-GΔα1 isoform may not be able to present peptides to T cells. Regarding interaction with ILT receptor, the HLA-GΔα1 isoform may not be able to interact with ILT2 because of the absence of β2m-associated forms, while binding to ILT4 may be effective. Homodimers of the HLA-GΔα1 isoform may be formed through disulfide bonds engaging the Cys 147 present in the α2 domain. Thus, the Cys147- Cys147 homodimers that might be formed in the α1-deleted α2α3 molecules may affect the specificity or modulate the affinity of such HLA-G isoforms for their receptors. Recently, the group of Maenaka reported that HLA-G2, which lack the α2-domain, naturally forms a β2-microglobulin-free and non-disulfide-linked homodimer having an overall structure that resembles that of the HLA class II heterodimer [21].

Based on these particularities, whether the HLA-GΔα1 isoform exhibits specific functions and interacts with receptors distinct from those well-known for the other HLA-G isoforms, remains to be explored.

The HLA-GΔα1 isoform cannot be recognized by the currently available HLA-G antibodies which all target the α1 domain and/or are dependent to the association with β2m. In particular, this isoform would not be recognized by the 4H84 mAb, largely used in immunohistochemical studies of cancer lesions. Consequently, HLA-G expression in cancer patients may be underestimated and novel antibodies aimed at recognizing all HLA-G isoforms are now required. In this regard, a recent study in colorectal cancer patients showed that different structural HLA-G molecules, in particular an α1 deleted isoform, may have different clinical significance [22]. The unexpected level of complexity emerging with the discovery of these unannotated transcripts and the molecules that might encode, strengthen the idea that the use of current commercial antibodies may bias diagnostic, signifying that a large campaign to develop new antibodies is ineluctable.

In conclusion, our molecular survey revealed a layer of complexity whereby a single gene can produce distinct proteins, which in turn may have functional consequences in cancer cells. The still unrecognized transcripts that might encode novel HLA-G proteins may have far-reaching implications. Accordingly, the cellular models expressing independently each isoform, that we have produced, might help defining whether the different proteins might play specific or overlapping functional roles in physiological and pathological conditions. Uncovering isoforms that are expressed in tumor cells might assuredly open new perspectives in the cancer research field, in particular, for use in diagnosis and therapeutic strategy design.

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